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RESEARCH TECHNIQUES AND METHODS FOR THE DETECTION AND PREVENTION OF CARCINOGENESIS IN THE INDUSTRIAL WORKER

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UNIVERSITY OF LOUISVILLE
HEALTH SCIENCES CENTER
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REPORT FOR THE
CHEMICAL MANUFACTURERS ASSOCIATION
(FORMERLY MANUFACTURING CHEMISTS ASSOCIATION)

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RESEARCH TECHNIQUES AND METHODS FOR THE DETECTION AND PREVENTION OF CARCINOGENESIS IN THE INDUSTRIAL WORKER

Program Final Report

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program and the human medical surveillance program which were ongoing concomitantly during the research periods of this study. This has led to a large body of literature which has changed the medical approach to the screening, identification and verification of occupationally-related chemical injury.

Although the contractual funding of this grant was only three years, each year was funded separately with varying time intervals between the funding from year to year so that almost six years have evolved between the initiation of these studies and this final report. This report, therefore, summarizes the work accomplished during this six-year interval and the related work which is still ongoing.

The investigators' reports vary in the degree of detail depending upon whether their work is in the process of being published or has already been published. Work not yet published is described in greater detail; published work is accompanied by a copy of the actual article(s) attached in the Appendix.

The investigators wish to express their appreciation to the B.F. Goodrich Company and especially to the entire management of its Bells Lane, Louisville plant. The continued cooperation and help of the plant managers, Philip Lawrence, Gabriel Le Febvre, Edward L. Beeler and especially Raymond Pruitt, Personnel Manager, along with Dr. John Creech, Jr., and the medical department staff, requires special acknowledgement.

The investigators also wish to thank their laboratory and research staff for their devoted work, and to thank Dr. Harold Boyer, Vice President for Health Affairs, and Dr. Joseph X. Musacchia, Dean of the Graduate School, for their administrative efforts, and especially, Mrs. Vicky Strong for her patient secretarial support.

A special thanks is given to Dr. Kathleen O'Connell without whose help and support completion of the periodic and final reports would have been much more difficult.

Finally, and very importantly, the participation and cooperation of the entire work force of the B.F. Goodrich Bells Lane Plant, and their representative unions--the Distillery Workers Union, the International Brotherhood of Electrical Workers #369, the International Association of Machinist Workers #681, and Pipefitters Union Local #522--is acknowledged with appreciation. This work would have been impossible without the active involvement and help of the workers. The objectives of this task were all directed toward improving and maintaining the best possible work environment.

Again, a thank you to all who have contributed to this effort.

INTRODUCTION

In 1974 Dr. John L. Creech, Jr., a surgeon and the plant physician for B.F. Goodrich's Chemical Plant on Bells Lane in Louisville, Kentucky, identified the increased occurrence of hepatic angiosarcoma in workers involved in polyvinyl chloride manufacturing. This discovery led to the implementation of an industrial cancer control, detection and prevention program at the Louisville B.F. Goodrich Plant involving approximately 1,800 workers: 1,200 were active employees and 600 were previously employed individuals. The University of Louisville, in cooperation with the B.F. Goodrich Company, developed and implemented the program and has continued to follow this cohort of workers clinically and epidemiologically.

A proposal submitted to the Chemical Manufacturers Association, formerly the Manufacturing Chemists Association, to foster scientific research in better technical methods for the detection and prevention of chemical injury and carcinogenesis in industrial workers was awarded in 1976. These investigations covered nine general areas of interest and involved both animal and human studies. The studies included: (A) the use of the human immunological system for the detection of injury from vinyl chloride and other chemicals, (B) the assessment of hepatic biochemical enzymatic systems to detect and characterize vinyl chloride and other chemical injury, (C) the use of tissue and urinary glycosaminoglycan changes for early detection and diagnosis of chemical injury including cancer development, (D) the histological evaluation of liver tissue from chemical workers and the assessment of its collagen content as an indicator of chronic latent injury, (E) synthesis and analysis of vinyl chloride intermediate and end products to further understand its biological metabolism and removal, (F) the identification and assessment of cell assays to determine the carcinogenic potential of industrial chemicals, (G) the evaluation of tissue antigenic systems for detecting chemically-induced carcinogenesis, (H) exploratory studies for the use of isolated human liver cells to determine individual ability to detoxify chemicals, and (I) the further assessment of vinyl chloride metabolism by the use of whole body autoradiographic technique.

Each of these areas of study were directed toward four major objectives: (1) to better characterize and understand the pathogenesis of chemically-induced injury, especially vinyl chloride injury, (2) to characterize the mechanisms of chemical injury and the body's biological defense to this injury using animal studies, (3) to evaluate and assess old and new biochemical parameters for the detection and verification of chemical injury in humans, and (4) to develop new techniques that could be applied to the problem of identifying occupationally-related chemical injury for purposes of prevention.

Many of the original objectives of the individual studies have been changed or modified because of developments and discoveries in both the animal

SUMMARIES OF RESEARCH PROGRAMS

evidence suggested that the tumor antigens found were distinctive for each histological type of tumor. Studies were undertaken to determine whether angiosarcoma tumor antigens would provide specific immune reactions that could be utilized as a specific test for vinyl chloride-induced tumor development. Lymphocytes from normal and liver diseased individuals were isolated and then grown in the presence of a liver reagent prepared from either normal individuals or individuals who had angiosarcoma. Reactivity of lymphocytes was assessed by determining the incorporation of ^{3}H-thymidine into stimulated cultures as compared to unstimulated cultures. A comparison study was performed between vinyl chloride plant workers and nonchemical plant workers utilizing reactions of lymphocytes to normal and angiosarcoma liver reagents. The lymphocytic response was also studied in vinyl chloride-exposed and non-vinyl chloride-exposed workers with and without evidence of liver disease. Initially, specific reactivity was seen only among vinyl chloride workers. Although these inital results were provocative, subsequent studies with large control populations demonstrated similar reactivities. The lack of further occurrences of angiosarcoma in the cohort worker population prevented further characterization of the tumor antigen and restudy of the earlier findings.

A4. Study of Histocompatibility Leukocyte Antigen (HLA) Frequencies in Chemically-Exposed Workers

This study involved the search for HLA tissue types which may identify individuals susceptible to chemically-induced injury. In 1976 Mulvihill suggested genotyping as a means of screening potential employees for antigen subtypes which may predispose them to neoplastic development after occupational exposure. It was prompted by the suggested increased occurrence of HLA-B27 antigen in workers with occupational asbestosis. The HLA frequencies in polyvinyl chloride manufacturing workers were compiled to determine if any increased genotype occurrences were associated with angiosarcoma or other chemically-related liver disease. Nine hundred individuals were eligible for this study: 538 individuals were HLA tissue typed and 30 different HLA-A, HLA-B, and HLA-C antigens were assessed. Preliminary comparisons were made with the HLA frequencies in vinyl chloride workers with and without liver disease, and later in those with liver disease of chemical vs. nonchemical origin. HLA frequencies were also analyzed in those individuals with and without biochemical evidence of liver injury. HLA-B15 was found to occur with a greater frequency among chemical workers with liver disease than in "control" and "normal" populations. The HLA-B15 occurrence was greater among those with chemical liver injury, although it was not statistically significant. Two other HLA markers occurred with unusual lower frequency.

A5. Identification of the Endothelial Cell as the Cell of Origin For Vinyl Chloride Angiosarcoma of the Liver

There has been considerable disagreement as to the cell of origin for liver angiosarcoma, a nonparenchymal cell malignancy of the liver. This is of

PROGRAM A

STUDIES OF HUMAN IMMUNOLOGICAL SYSTEMS IN THE DETECTION OF VINYL CHLORIDE AND OTHER CHEMICAL INJURY; Investigators - H. P. Fortwengler, Jr., and C. H. Tamburro

A1. Evaluation of Immunocompetence of Humans Chronically Exposed to Vinyl Chloride

The scientific literature is replete with the demonstrations of immunodepression as a manifestation of medical disease, especially cancer. Lymphocytes (T cells) can be cytotoxic to human tumor cells and are often found decreased or poorly functioning in cancer patients resulting in various degrees of immunodepression. The immunocompetence of a cohort of chemical workers was studied to determine if there was any evidence that (a) varying degrees of prolonged exposure to vinyl monomers was associated with immunological suppression, (b) the immune response was impaired in workers with exposure-related liver injury including angiosarcoma, and (c) exposure caused changes in immunological function which were identifiable in the pre-cancerous stages. The immunocompetence of 75 employees with demonstrated liver disease and 225 individuals without clinical or laboratory evidence of disease was studied. No significant clinical or laboratory evidence of impaired immunocompetence was found in the immunological parameters studied, nor were differences seen among the various subpopulations of workers.

A2. Assessment of the Leukocyte Adherence Inhibition Test for the Detection of Angiosarcoma of the Liver

Halliday et al. (1974) and Thompson (1976) had reported the development of a leukocyte adherence inhibition test which had identified the clinical stages in colon cancer. Similar findings were reported in individuals with hepatic cancer. This study was undertaken to determine the feasibility of applying this newly developed technique to screen vinyl chloride-exposed workers for angiosarcoma of the liver. It was assessed in a number of chemical and nonchemical workers who had cancer. This test system lacked sufficient reproducibility to be considered for clinical use.

A3. Assessment of Vinyl Chloride-Induced Angiosarcoma Antigen for the Detection of Latent Angiosarcoma in Exposed Workers

New antigens arise from tumors formed as a response to carcinogens. Their presence in methylcholanthrene-induced sarcomas was discovered by Foley in 1953. This discovery in mice was verified and extended by Prehn and Main (1957) to conclude that there were antigens particular to and specific for tumor tissue. Subsequent evidence of tumor antigen was found in humans by the Hellstroms, Vankey, Halliday, Maluish, Thompson, and others. The majority of

studies were conducted in sequential experiments exposing Sprague-Dawley rats to 10,000 ppm vinyl chloride for a period of up to 300 exposure hours. These studies demonstrate various adaptive changes in liver parenchymal cells that were not identifiable by conventional clinical laboratory test (CCLT). These data illustrate the limited usefulness of CCLT in identifying early liver cell changes even during high levels of exposure.

B2. Morphological Alterations in Livers of Rats Exposed to Vinyl Chloride

Sequential morphological assessments were performed in the rats from both chronic exposure experiments utilizing light and electron microscopy. Light microscopic findings included an increase in liver cell polyploidy, double nucleated cells, and areas of focal hepatocellular hyperplasia without evidence of cellular injury or increased fibrosis. Electron microscopic changes included proliferation of smooth endoplasmic reticulum without evidence of other subcellular organelles, or increased collagen deposit in the Space of Disse.

B3. Alterations of Oxidizing and Detoxifying Systems of Rat Liver After Prolonged Exposure to Vinyl Chloride

A second long-term study, based partly on the results from the B1 study, was conducted to determine the adaptive capability of the detoxifying systems of liver cells. Glutathione reductase activity, glutathione content, glutathione epoxide-S-transferase (GEST; Glutathione transferase E), and glutathione aralkyl-S-transferase (GAST; glutathione transferases A&B) were measured in rats exposed to vinyl chloride and to chloroethanol, a vinyl chloride metabolite. A third set of studies was conducted to compare these detoxifying enzymes in rats exposed to vinyl chloride and excessive alcohol consumption. These studies illustrated the adaptability of liver cells to prolonged exposure to xenobiotics. Liver cells showed increases in reduced glutathione and glutathione reductase activity and subsequent increases in GEST and GAST, reflecting a progressive increase in the liver cells' capacity for detoxification of vinyl chloride's toxic intermediates. Concomitant alcohol consumption appears to interfere with this detoxifying capability and appears to place the hepatocyte at greater risk of malignant transformation.

B4. Oxidative and Detoxifying Ability of the Liver Mesenchymal vs. Parenchymal Cells in the Metabolism of Xenobiotics

Another aspect of the prolonged vinyl chloride exposure studies was to determine the oxidative and detoxifying capability of various liver cells. In collaboration with Dr. Feldhoff, the oxidative and detoxifying ability of the liver mesenchymal versus parenchymal cells was studied by determining P450, glutathione reductase, GEST, and GAST in liver hepatocytes and endothelial cells.

great importance since the identification of the type of cell responsible for malignant transformation could provide the basis for a better understanding of chemically-induced carcinogenesis. This was particularly relevant to vinyl chloride-induced liver cancer since vinyl chloride is predominantly metabolized and detoxified by hepatocytes and produces a malignancy of the liver that is nonparenchymal in origin. Considerable controversy arose as to whether the cell of origin was a macrophage (Kupffer cell), a fibroblast, or an endothelial vascular lining cell. The recent identification of Factor VIII production by endothelial lining cells led us to determine whether liver angiosarcoma tumor contained increased amounts of coagulation Factor VIII. Factor VIII is a large protein, often called antihemophilic factor, found differentially in endothelial cells, platelets, and megakaryocytes. The studies described herein demonstrated Factor VIII presence in endothelial lining cells of arterial and venous vessels in normal liver, as well as in the cells of angiosarcoma. Histological verfication of the cell type was determined by light microscopy of the same tissue sections of the tumor. This verified the endothelial cell as the cell of origin in angiosarcoma.

PROGRAM B

STUDY OF HEPATIC BIOCHEMICAL AND ENZYMATIC SYSTEMS FOR THE IDENTIFICATION OF VINYL CHLORIDE CHEMICAL INJURY AND CANCER DEVELOPMENT: ANIMAL AND HUMAN STUDIES; Investigators - J. T. Du, M. T. Tseng, and C. H. Tamburro

B1. Characterization of Hepatic Enzyme Changes in Rats With Prolonged Vinyl Chloride Exposure: Decreased Glucose-6-Phosphatase Activity

Considerable concern was initially raised concerning the best technique for screening individuals with chemical exposure. At present, federal testing requirements recommend enzyme studies related to liver parenchymal cells. However, clinical evidence indicated that not only were the hepatocytes injured but so were Kupffer and endothelial cells, and that the endothelial cell or Kupffer cell was the malignant cell of origin. In addition, studies to determine the sequential enzymatic changes which occurred with prolonged continuous vinyl chloride exposure similar to that seen with the worker population were needed. Most studies at this time had been conducted with short term exposures. The early animal studies included assessment of the standard clinical, biochemical, and enzymatic studies and later explored the enzymatic changes occurring in various subcellular organelles. These included (a) markers for microsomal enzymes related to the metabolism of vinyl chloride, P-450, NADPH-cytochrome reductase, and mixed function oxidase, (b) cytosolic enzymes related to glutathione metabolism and glutathione content, (c) mitochondrial marker cytochrome-C-oxidase, (d) cytosolic enzymes glucose-6-phosphatase, glucose-6-phosphate dehydrogenase related to pentose phosphate pathway, and (e) nucleic acid synthesis. In addition, protein synthesis was evaluated by in-vitro incorporation of ^{3}H-leucine. These

hold promise as a potential detector of early liver injury, whether chemical or not. Bile acids have the advantage of being natural biological substances which may be taken orally for screening clearance studies.

PROGRAM C

STUDY OF GLYCOSAMINOGLYCAN CHANGES IN THE EARLY DETECTION OF HEPATIC FIBROTIC INJURY IN CHEMICAL EXPOSURE AND HEPATIC CANCER DEVELOPMENT; Investigators - C. E. Kupchella and R. Warick

ANIMAL STUDIES:

C1. The Study of Glycosaminoglycan Changes in Livers of Animals Bearing Metastasizing Hepatomas

C2. Significance of Tissue Glycosaminoglycan Elevations in Hepatic Necrosis and Regeneration

Animal studies to determine the sequence of GAG changes in experimentally-induced fibrotic liver injury, and the relationship between GAG patterns in tumor tissue and in urine were studied in animals with fast vs. slow growing, metastasizing vs. nonmetastasizing, chemically-induced and transplantable liver cells tumors.

The experimental animal studies identified heparan sulfate (a type of GAG) elevation in hepatic tissue undergoing fibrotic changes and that the increased levels were reflected in the urine. Both heparan sulfate and hyaluronic acid levels were 3-4 times higher in experimentally transplanted liver tumors than in normal controls. Urinary excretion reflects both tumor GAG composition and size. Livers from animals bearing metastasizing hepatomas had a 10-fold great concentration of nonsulfated neutral uronic acid positive material than animals bearing nonmetastasizing hepatomas. Finally, hepatic necrosis was shown to be accompanied by significant tissue GAG elevation; hepatic regeneration was not.

HUMAN STUDIES:

C3. Characterization of Glycosaminoglycan Patterns in Humans with Angiosarcoma

C4. Evaluation of Glycosaminoglycan Analysis of Urines of Chemically-Exposed Workers

The mesenchymal cells were shown to be capable of oxidizing xenobiotics which require P-450 and mixed function oxidases, although this capability was less than that of the parenchymal cells by a 70 to 1 ratio. GEST and GAST activity were also present but in the ratio of 1 to 65, and 1 to 500, respectively. These results demonstrate that the nonhepatocytic liver cells have the capability to activate vinyl chloride and other similar xenobiotics, although to a lesser degree than hepatocytes. However, their ability to detoxify vinyl chloride metabolites is significantly less and may contribute to the reason for nonmesenchymal cell malignant transformation.

B5. Effectiveness of Indocyanine Green (ICG) Clearances in the Detection of Liver Injury

A chemically-exposed worker cohort of almost 1,000 individuals were medically screened to determine the effectiveness of ICG clearances versus standard biochemical studies of the liver to identify latent hepatic injury. Alanine aminotransferase (ALT/SGPT), aspartic aminotransferase (AST/SGOT), gamma glutamyl transpeptidase (GGPT), alkaline phosphatase (AP), and total bilirubin (TB) were assessed in contrast to 3 different dose levels of ICG. Positive predictive values as well as the specificity and sensitivity of each of the screening tests were determined in "normal" and "abnormal" worker populations as well as in those with hepatic disease and nonhepatic disease. Chemical and nonchemical liver injury were also determined in analysis of their relationship of work histories to the biochemical abnormalities. These studies demonstrated that ALT/SGPT was the screening test with the highest sensitivity and specificity among federally-required studies. ICG clearance, even at the low dose, clearly remained the best overall screening test for the detection of subclinical hepatic disease.

B6. Assessment of Bile Acids vs. Indocyanine Green (ICG) Clearances in The Detection of Liver Injury Due to Chemical Exposure in The Human Population

Fasting serum bile acid levels and ICG clearances were performed for 400 employees with varying degrees of chemical exposure as well as biochemical injury related to vinyl chloride exposure. Bile acid levels, ICG clearances and standard enzymatic biochemical tests were assessed regarding their ability to identify normal individuals, individuals with liver disease of nonchemical origin, and individuals with liver disease with chemical origin, correctly. These more sensitive studies were to identify early chemical injury; six other enzyme studies were also conducted regarding more specific enzymes for hepatocellular injury, such as sorbitol dehydrogenase. One thousand seven hundred and sixty human seras taken from 900 exposed workers were assessed and the results compared to the vinyl chloride exposure.

Fasting serum bile acids demonstrated a high sensitivity separating normal versus abnormal individuals with early or latent liver dysfunction, particularly chemically-induced types of injury. Fasting serum bile acids

periportal, and pericentral areas in 4 different anatomical locations of the human liver of 4 different age groups. These studies demonstrated that the collagen content of the liver varies with age, increasing after the fourth decade. The collagen distribution shows an increased desposition in the perisinusoidal and midzonal regions with age. The computerized morphometric readout demonstrated that the collagen estimates vary from 2-6% of the total liver globules. (This does not include capsular fibrosis or collagen support tissue about major vessels.)

D4. Light Microscopic Assessment of Liver Tissue Obtained from Vinyl Chloride Workers Characterizing Various Histological Lesions

Light microscopic assessment of liver tissue obtained from vinyl chloride and nonvinyl chloride workers illustrated that focal hepatocellular hyperplasia and focal mixed hyperplasia, sinusoidal dilitation and focal areas of increased reticulum characterized chemical injury could be identified in a prospective blind study and that those individuals with these identified lesions had the highest correlation with their vinyl chloride accumulative exposure ranked months; this correlation was not seen with other chemicals within their exposure environment.

PROGRAM E

STUDIES OF VINYL MONOMER CHEMICALS AND THEIR METABOLITES USING CHEMICAL STRUCTURE AND SYNTHESIS IN THE DETERMINATION OF TOXICITY OF THESE AGENTS; Investigator - J. L. Wong

E1. Synthesis, Purification, and Utilization of Vinyl Chloride Metabolites in Mutagenicity and Carcinogenicity Studies

E2. Detoxification Studies of Vinyl Chloride and Its Metabolites

E3. Vinyl Chloride Metabolite Detection--Chloroacetic Acid

In order to further elucidate the intermediate metabolism of vinyl chloride, especially with regard to the carcinogenic potential of its intermediate metabolites--chlorooxirane and chloroacetaldehyde--laboratory synthesis of these two putative metabolites was performed. The materials were utilized to further study the detoxification of these agents and their effect on bacterial systems. Additional studies were conducted to determine the ability to identify chloroacetic acid by mass spectrometry and gas chromatography.

These studies illustrated that although chlorooxirane and chloroacetaldehyde eventually give the same final product, the rate of reaction and the

C5. Characterization of Glycosaminoglycan Patterns in the Identification of Chemical and Nonchemical Injury of the Liver

The study of glycosaminoglycans, glycoproteins involved in wound healing and scar formation, as potential indicators of chemical injury, fibrosis, and cancer development was conducted in a large cohort of exposed workers. Analysis of GAG in tissue and urine was performed in individuals with angiosarcoma and heptocellular tumors, in individuals with chemical and nonchemical liver injury, and in individuals with chemical liver abnormalities and in normal controls. The studies indicated that human hepatic angiosarcoma and fibrotic liver disease are accompanied by elevated tissue GAGs, that the tumor tissue is different from the adjacent fibrotic areas of the liver, and that patients with angiosarcoma and primary hepatocellular carcinoma (hepatoma) have characteristic urinary GAG patterns. These patterns are not found in "normal" individuals. Urinary GAG determinations give a better indication of liver disease than ultrasound or radioisotopic scanning, and although they are not as sensitive as transaminases or ICG clearances, fractionated GAG analysis appears to differentiate between active and inactive liver disease.

PROGRAM D

HISTOLOGIC AND MORPHOMETRIC ANALYSIS: A MEANS OF ASSESSING HEPATIC INJURY IN CHEMICAL WORKERS; Investigators - G. H. Barrows, G. R. Schrodt, and C. H. Tamburro

D1. Systematic Assessment of Histological Lesions Characteristic of Vinyl Chloride or Vinyl Monomer Injury

D2. Computer-Assisted Morphometric Analysis as a Means of Determining Collagen Content

D3. Development and Assessment of Morphometric Method of Analysis of Collagen Content from Liver Biopsies - Normal Occurrence with Age

Vinyl monomer injury has been associated with various hepatic histological abnormalities. These have included subcapsular, portal, and perisinusoidal fibrosis, as well as hyperplasia of the hepatocytes and nonparenchymal (sinusoidal) cells. The ability to assess light microscopic determinations of fibrosis (liver tissue collagen content) using computer-assisted morphometric analysis quantitatively, was delineated. The methodologies determined the normal presence of collagen and the effect of age. Prospective analysis to determine the ability of specific histological findings to correctly identify vinyl monomer exposure was also done.

Both manual and computer-assisted morphometric assessment of normal collagen content identified the distribution between perisinusoidal,

PROGRAM G

THE STUDY OF LIVER TISSUE ANTIGENS AND ANTIBODIES IN THE DETECTION OF VINYL CHLORIDE INJURY; Investigator - E. Espinosa

G1. The Study of Tissue Antigens From Liver Tumors, Angiosarcoma, and Hepatomas

G2. Circulating Antigens and Autoantibodies in Vinyl Chloride-Associated Liver Disease

The approach to the use of tissue antigens produced by neoplasms and identified in serum, either as the antigens or autoantibodies, were studied in angiosarcoma and in chemically-induced liver cancers. Several normal tissue antigens, a tumor-associated protein antigen, and a glycoprotein liver antigen absent in individuals with angiosarcoma were identified and characterized. The finding of a missing antigen in vinyl chloride-related angiosarcoma stimulated studies of antigenic deletion in chemically-induced hepatoma and cultured human liver carcinoma cells. Two liver antigens were found to be absent. One of these antigens was shown to be normally present in other tissues (kidney and spleen) in addition to the liver. The second antigen was detected only in the liver and was found to be unrelated to the liver-specific F antigen. Liver-specific F antigen was also studied as a possible sensitive indicator of chemically-induced liver tumor. F antigen appeared to be absent in fast growing hepatoma 7777 and undetectable or very low in the slow growing hepatomas. The level of the F antigen did not appear to correlate with the rate of growth of these tumors; however, an increased concentration of F antigen appears related to those tumors with the highest metastatic characteristics. Studies of cultured human cancer carcinoma cells also noted a deficiency in liver-specific F antigen similar to that of the experimental hepatoma 7777. These data support the clinical observation that tissue antigens appear to be more useful in treatment and follow-up care, while antigenic deletions may prove more important for screening and early detection.

PROGRAM H

USE OF ISOLATED MAMMALIAN LIVER CELLS FOR THE STUDY OF CHEMICAL MONOMER METABOLISM; Investigator - R. C. Feldhoff

H1. Isolation of Mammalian Liver Cells for the Study of the Metabolism of Chemical Monomers

H2. The *In Vitro* Study of Albumin Synthesis by Liver from Human Biopsies

intermediates in the two reactions are different. Chlorooxirane conjugates instantaneously with the sulfhydral compounds, while chloroacetaldehyde takes about 2 1/2 hours for a comparative reaction. Although both routes are converged to yield the cysteine-S-acetaldehyde conjugate, the reaction rates are vastly different, one taking minutes, the other hours to complete. Chloroacetic acid is identifiable by mass spectrometry in liquid solutions, solutions as small as 0.02M (2 mg/ml), while gas chromatography sensitivity was limited to concentrations of 0.2 to 2 mg/ml H_2O (200 to 2,000 ppm). Use of a porous polymer solid support and formic acid is expected to improve sensitivity by 100-fold. Combination of GC-MS techniques appear to be the most promising approaches to the detection of end metabolic products and biological tissue.

PROGRAM F

ASSESSMENT OF ASSAYS FOR THE CARCINOGENIC POTENTIAL OF INDUSTRIAL CHEMICALS USING PROKARYOTIC AND EUKARYOTIC SYSTEMS; Investigators - U.N. Streips and G. Sonnenfeld

F1. Further Development of Bacterial Systems for Testing Carcinogenicity and Mutagenicity of Chemical Agents Used in the Manufacturing of Vinyl Chloride and Synthetic Rubber

Application of mutagenic assays for the determination of mutagenic potential of a list of environmental chemicals was conducted using improved modifications of their bacterial system. In addition, a new screening technique for carcinogenesis was developed involving the inhibition of interferon induction. This was applied to several carcinogens and mutagens and compared to the effectiveness of the standard viral bacteriological assays. These studies have demonstrated improved techniques for rapid screening of large numbers of chemical formulations being utilized within an environmental region.

F2. Preliminary Studies on the Use of Interferon Induction as an Indicator of Mutagenicity and Carcinogenicity of Chemicals

The new method assessing the mutagenic/carcinogenic capabilities of chemicals using an interferon assay is reported as well as related research regarding the controlled process of cell division in bacteria and how these findings relate to mammalian cells.

Interferon induction inhibition in contrast to the Ames bacterial assay demonstrated the ability to separate more accurately agents with more carcinogenic potential related to human occurrences.

nonvolatile metabolites of vinyl chloride retained in the thymus after 24 hours suggested that there was covalent binding of these metabolites to the molecules in the thymus. Possible interactions of the thymus may reflect a dual mechanism of carcinogenic action, one related to tissue damage in the liver, the other to a suppressed immune surveillance system.

Presently, the rat has provided the most useful means of studying hepatic metabolism in mammalian cells. The intact liver, however, consists of at least four cell types which differ in their metabolic and functional characteristics. These studies were directed toward the development of clinically utilizable techniques to study human liver biopsy material in relationship to its ability to synthesize, retain, and secrete protein.

Improved techniques were also utilized for isolation of hepatic parenchymal and nonparenchymal cells so that nearly homogeneous populations of cells could be studied for their oxidizing and detoxifying capabilities. Preliminary results of this late addition to our research techniques have proven encouraging in the adaptability of highly sophisticated laboratory techniques to the study of human tissue in an in vitro system. It has demonstrated that animal and human tissue obtained by a clinical biopsy technique are viable in an in vitro system, being able to perform normal functions relative to albumin synthesis. The technique for the study of this tissue in an in vitro system may be applicable to human tissue from individuals with varying degrees and types of cellular injury. This technique would provide the ability to determine the oxidizing and detoxifying capability of liver cells to various xenobiotics in a quantitative fashion. Although very futuristic in its approach, it appears accomplishable since the present developed methodology is clinically applicable.

PROGRAM I

THE STUDY OF TISSUE DISPOSITION OF INDUSTRIAL CHEMICALS: THE VINYL CHLORIDE EXAMPLE; Investigators - W. J. Waddell and C. Marlowe

II. The Use of Whole Body Autoradiography in Specific Tissues. Localization of Accumulated and Retained Industrial Chemicals and Their Metabolites

The need for methods which more accurately identify the sites of localization of chemical agents and their metabolites has obvious importance. The use of whole-body autoradiography to identify the location of radioactively tagged chemicals was studied with regard to ^{14}C-vinyl chloride. Whole-body saggital sections of mice exposed for three hours in ^{14}C-vinyl chloride demonstrated that the highest levels of radioactivity in mice sacrificed at 20 minutes and one hour after removal from the vinyl chloride environment were observed in the liver, pancreas, kidney, intestinal contents, urine and bile. Concentrations of metabolites in the organs of excretion decreased continually over a 24-hour period. After nine hours removal from the vinyl chloride environment, Harder's gland, epithelium of the esophagus and intestine, and sublingual glands retained the highest levels of radioactivity, while moderate concentrations were seen in the liver, kidney, and intestinal contents. At 24 hours, the primary organs of retention were the thymus, Harder's gland, liver, and esophagus and intestinal epithelium. The high concentration of

RESEARCH PROGRAMS AND RESULTS

Research Results

An evaluation of immunocompetence was carried on in individuals with documented chemical hepatic injury and in those without biochemical or clinical evidence of liver injury. A second comparison was conducted between workers with high versus low exposure to vinyl chloride, utilizing our cumulative exposure rank months (CERM) ranking. Tests for immunocompetence included enumeration of lymphocytes, determination of certain lymphocyte subpopulations, and response of lymphocyte to nonspecific antigens as a test of lymphocytic function.

Results of lymphocyte response to stimulation with nonspecific antigens Phytohemagglutinin (PHA), Concanavallin A (Con-A), and Pokeweed mitogen (PWM) are shown in Figure 1.

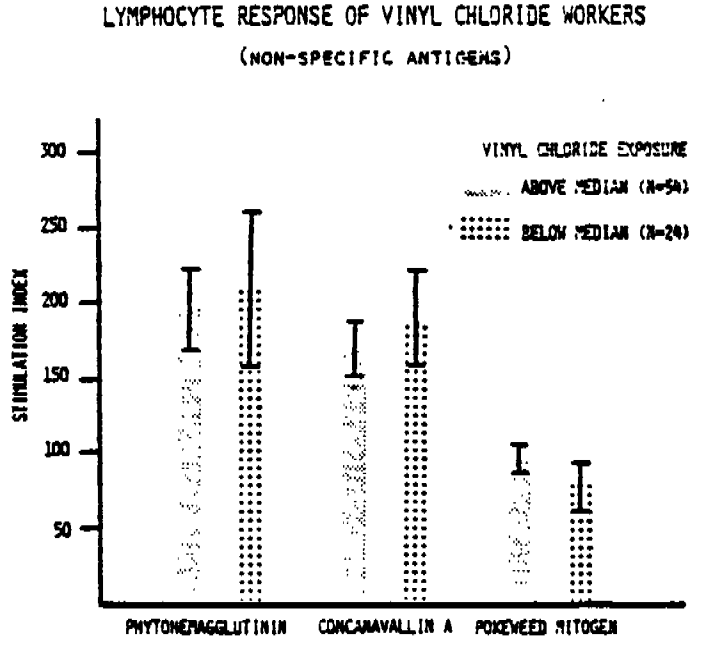

FIGURE 1

Response of the other immune parameters in high and low vinyl chloride exposure are shown in Table 1. No statistically significant difference was found between workers with high and low vinyl chloride exposure, or between unexposed invididuals and chemical workers (Figure 2).

PROGRAM A

STUDIES OF HUMAN IMMUMOLOGICAL SYSTEMS IN THE DETECTION OF VINYL CHLORIDE AND OTHER CHEMICAL INJURIES; Investigators - H.P. Fortwengler, Jr., and C. H. Tamburro.

A1. Evaluation of Immunocompetence of Workers Chronically Exposed to Vinyl Chloride

Background

The study of the human immunological systems as a means of detecting chemical injury was initiated to determine whether or not the body's ability to recognize substances that were foreign would make specific responses that could be identified clinically. The immune response can be divided loosely into two types: antibody formation and cell-mediated responses. Our initial studies were conducted to determine whether the mediators of immunity, i.e., lymphocytes, could be identified as having any immune defects which could be related to chemical exposure and be an early signal of an impaired immune system which might interfere with the body's normal defenses. These antibody forming cells constitute the humoral part of the human immune response. The lymphocytes are divided into at least two classes or types called B cells and T cells. The B cell lymphocytes are responsible for the synthesis and secretion of antibody molecules, while the T cells are responsible for helping B cells. In addition, the T cells are also able to carry out a whole series of reactions in their own right. Generally these reactions involve tissue destruction, and since antibodies are usually not involved, they are called cell-mediated reactions. There has been recent thinking that the body's defense against tumors is a result of a tumor's having a unique antigen which is therefore recognized as foreign. The immune system constantly responds against these new antigens, and in this manner, there is an ongoing immune surveillance against tumors. Failure to react with the new antigen results in cancers, according to this theory.

Objective

1. To determine the immune competence of chemical workers with prolonged exposure to vinyl chloride monomers

2. To compare their immune response to those workers who had developed liver injury, including angiosarcoma.

No statistical differences were seen in these same immune parameters between workers with or without liver injury (Tables 2 and 3).

TABLE 2

IMMUNE PARAMETERS OF VC WORKERS WITH AND WITHOUT LIVER DISEASE

TEST	LIVER DISEASE	NO LIVER DISEASE
PHYTOHEMAGGLUTININ STIMULATION	258*±35** (n=25)	178±35 (n=48)
CONCANAVALLIN A STIMULATION	195±36 (n=25)	162±29 (n=48)
POKEWEED MITOGEN STIMULATION	100±12 (n=25)	89±13 (n=43)
STREPTOCOCCAL ANTIGEN STIMULATION	208±42 (n=24)	181±32 (n=49)
STREPTOLYSIN O STIMULATION	8±2 (n=25)	16±6 (n=49)
PPD STIMULATION	18±6 (n=25)	30±9 (n=45)
VARIDASE STIMULATION	36±9 (n=25)	31±5 (n=50)
CANDIDA STIMULATION	14±5 (n=25)	7±1 (n=49)

*STIMULATION INDEX
**SEM

TABLE 3

IMMUNE PARAMETERS OF VC WORKERS WITH AND WITHOUT LIVER DISEASE

TEST	LIVER DISEASE	NO LIVER DISEASE
WHITE BLOOD CELL COUNT	6900±500* (n=25)	6650±380 (n=50)
LYMPHOCYTE COUNT (PERCENT)	36±2 (n=25)	38±2 (n=48)
ABSOLUTE LYMPHOCYTE COUNT	2350±200 (n=25)	2300±200 (n=48)
T-CELL ROSETTES AT 4°C (PERCENT)	67±3 (n=25)	52±2 (n=49)
T-CELL ROSETTES AT 4°C (ABSOLUTE)	1600±100 (n=25)	1500±100 (n=49)
T-CELL ROSETTES AT 37°C (PERCENT)	55±2 (n=25)	52±2 (n=47)
T-CELL ROSETTES AT 37°C (ABSOLUTE)	1300±100 (n=25)	1200±100 (n=47)

*SEM

Tables 4 and 5 show the results of these immunological parameters examined in old and young workers. Older individuals demonstrated significant differences in their total white count ($P<.01$), percentage of lymphocytes ($P<.001$), and lymphocyte reactivity to Streptolysin O ($P<.05$) and PPD (tuberculin) ($P<.05$) stimulation. We believe these findings to be age related with no relationship to the presence of liver disease or exposure to vinyl chloride. No other statistically significant differences were found between the various groups. There is no evidence from this data that immunological abnormalities result from chronic exposure to various levels of vinyl chloride.

TABLE 1

IMMUNE PARAMETERS OF VC WORKERS ACCORDING TO EXPOSURE

TEST	ABOVE MEDIAN EXPOSURE * ±**	BELOW MEDIAN EXPOSURE	STATISTICAL SIGNIFICANCE
STREPTOCOCCAL ANTIGEN STIMULATION	191 ±31 (n=52)	186±34 (n=24)	NONE
STREPTOLYSIN O STIMULATION	8±2 (n=53)	24±12 (n=24)	NONE
PPD STIMULATION	29±8 (n=49)	18±6 (n=23)	NONE
VARIDASE STIMULATION	29±5 (n=54)	41±12 (n=23)	NONE
CANDIDA STIMULATION	11±3 (n=53)	6±2 (n=23)	NONE
ABSOLUTE LYMPHOCYTE COUNT	2355±132 (n=52)	2316±148 (n=24)	NONE
T-CELL ROSETTES AT 4°C (ABSOLUTE)	1503±101 (n=50)	1524±132 (n=24)	NONE
T-CELL ROSETTES AT 33°C (ABSOLUTE)	1227±80 (n=51)	1260±123 (n=24)	NONE

*STIMULATION INDEX
**SEM

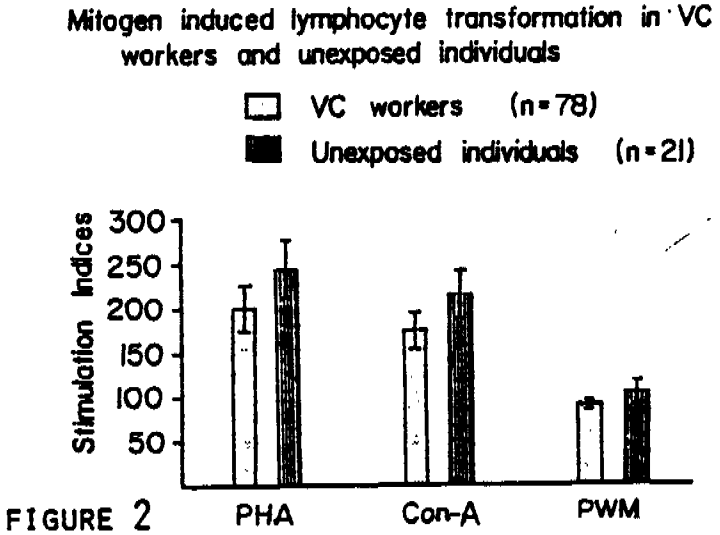

FIGURE 2

Lymphocytic reaction to normal and angiosarcoma liver reagents were studied in workers with high and low exposure (above or below the median exposures for the plant) to vinyl chloride (Figure 3). No statistical difference was noted between the reactivities of these two groups.

LYMPHOCYTE RESPONSE OF VINYL CHLORIDE WORKERS

(TISSUE ANTIGENS)

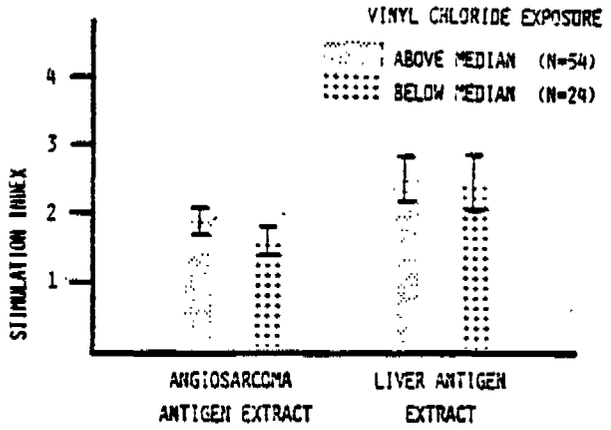

FIGURE 3

Similar studies in workers with and without liver disease also failed to show significant differences in their lymphocytic reactivity to tissue reagents (Table 8A).

TABLE 8A

LYMPHOCYTE REACTIVITY TO NORMAL AND ANGIOSARCOMA TUMOR TISSUES IN VINYL CHLORIDE WORKERS WITH AND WITHOUT LIVER DISEASE

	LIVER	
	NORMAL TISSUE	TUMOR TISSUE
LIVER DISEASE	2.4 ± 0.3*	1.5 ± 0.2
NORMAL	2.6 ± 0.4	1.8 ± 0.2

*STIMULATION INDEX ± SEM

TABLE 4

IMMUNE PARAMETERS OF VC WORKERS
OLD VERSES YOUNG

TEST	OLD	YOUNG
PHYTOHEMAGGLUTININ STIMULATION	140*±20** (N=37)	210±32 (N=40)
CONCANAVALLIN A STIMULATION	160±25 (N=37)	188±25 (N=40)
POKEWEED MITOGEN STIMULATION	90±15 (N=37)	90±20 (N=40)
STREPTOCOCCAL ANTIGEN STIMULATION	135±35 (N=38)	195±35 (N=38)
STREPTOLYSIN O STIMULATION	5±2 (N=38)	21±7 P<.05 (N=38)
PPD STIMULATION	36±12 (N=39)	13±3 P<.05 (N=35)
VARIDASE STIMULATION	25±6 (N=38)	39±9 (N=39)
CANDIDA STIMULATION	9±3 (N=37)	9±3 (N=39)

*STIMULATION INDEX
**SEM

TABLE 5

IMMUNE PARAMETERS OF VC WORKERS
OLD VERSES YOUNG

TEST	OLD	YOUNG	
WHITE BLOOD CELL COUNT	7150±400* (N=38)	6150±300 (N=40)	P<.01
LYMPHOCYTE COUNT (PERCENT)	33±2 (N=36)	41±2 (N=40)	P<.001
ABSOLUTE LYMPHOCYTE COUNT	2300±200 (N=36)	2400±100 (N=40)	
T-CELL ROSETTES AT 4°C (PERCENT)	64±2 (N=36)	64±2 (N=40)	
T-CELL ROSETTES AT 4°C (ABSOLUTE)	1400±150 (N=34)	1600±100 (N=40)	
T-CELL ROSETTES AT 33°C (PERCENT)	52±2 (N=36)	53±2 (N=40)	
T-CELL ROSETTES AT 33°C (ABSOLUTE)	1100±100 (N=34)	1300±100 (N=40)	

*SEM

A2. Assessment of the Leukocyte Adherence Inhibition Test for the Detection of Angiosarcoma of the Liver

Background

Halliday et al. (1974) reported the development of a leukocyte adherence inhibition test which identified the preclinical stages of colon cancer. Similar findings were reported in patients with liver cancer—the primary hepatocellular type. The need for a more specific screening test, especially for those who might be developing angiosarcoma that was not clinically detectable, led us to assess this test in our cohort population.

Objective

1. To verify the studies of Halliday and Maluish regarding the use of the leukocyte adherence inhibition test in the identification of carcinomas of the colon and its possible adaptation to vinyl chloride-induced angiosarcoma.

target antigens, immune response, tumor virus susceptibility, liver cyclic acenosine monophosphate levels, hybrid resistance, and T-cell: B-cell interactions. HLA antigens are found on almost all cells of the body except red blood cells. The major histocompatibility complexes in man are identified by letters (A, B, C, D) and numbers (A10, B12, C3). An increased incidence of certain HLA antigens had been shown to be associated with susceptibility to certain diseases. Mulvihill (1976) proposed that screening for these genetic markers to identify abnormal genotypes in potential employees should be done so that individuals who might be predisposed to neoplasia after occupational exposure would be separated from those from normal genotypes. The first occupational disease correlation reported was HLA-B27 antigen which was found with increased frequency in workers suspected of having occupational asbestosis. The possibility that the histocompatible complex could be used to identify individuals at increased risk of developing chemical injury led to its study in vinyl chloride workers.

Objective

1. To determine the HLA frequency in vinyl chloride workers

2. To compare the HLA frequency in vinyl chloride workers with and without liver disease.

   In order to deal with the statistical problem of multiple comparisons due to the large number of antigens to be studied, potential markers for liver disease were identified for study: A-9, B-15 and B-17.

Research Results

HLA determinations were done using the Terasaki microdroplet lymphocyte cytotoxicity test. HLA typing was performed on 538 of our chemical cohort population. Tissue typing included 11 HLA-A antigens, 15 HLA-B antigens and 4 HLA-C antigens. Frequency distributions were determined in our "standard" (healthy population) and compared with the "normal" groups studied by Scott et al. (1977) and the World Health Organization. The comparison of these frequencies are found in Tables 9 and 10.

A preliminary analysis of these HLA frequencies included the comparison of a subcohort population consisting of individuals identified through the medical screening program as having liver disease. These individuals had been transferred from the main chemical plant to an ancillary plant making wooden shipping pallets, referred to subsequently as "Pallet Plant Cohort".

As seen in Table 10 this cohort had increased frequency of HLA-B15 (12 percent-normal population versus 28 percent-Pallet Plant Cohort).

On the chance that non-specific reactions might be obscuring the specific reactions, workers with vinyl chloride-suspected disease (working in a non-vinyl chloride environment--Pallet Plant) were compared to workers without known liver disease (working at the Main Plant).

TABLE 8B

IN VITRO LYMPHOCYTE STIMULATION BY ANGIOSARCOMATOUS LIVER EXTRACT

SUBJECTS	NUMBER TESTED	NUMBER OF SUBJECTS REACTIVE AGAINST:		
		NORMAL LIVER ONLY	NORMAL & ANGIO	ANGIO ONLY
NON-PALLET PLANT WORKERS	16	1	1	1
PALLET PLANT WORKERS	18	1	0	2*
TOTAL	34	2	1	3

*ONE INDIVIDUAL HAD ANGIOSARCOMA

At first, this appeared to be a specific reaction to the angiosarcoma tumor extract, i.e., negative reactions to normal liver with positive reactions to tumor antigen. Initially, this type of reactivity occurred only among the vinyl chloride workers. Subsequent studies among additional nonchemical workers demonstrated similar reactivities to these liver reagents. Although these results were provocative, the lack of further occurrences of angiosarcoma in our worker population prevented us from further characterizing this (these) possible angiosarcoma antigen(s) and having the opporunity to verify these observations.

A4. Study of Human Leukocyte Antigen (HLA) Frequencies in Chemically-Exposed Workers

Background

The major histocompatibility complex of the immune system, HLA, is composed of multiallelic genes located on one region of chromosome 6 in the human and on chromosome 17 in the mouse. The genes in this region control a wide variety of surface antigens and lymphoid functions. This major histocompatibility complex is intimately related to the understanding of transplantations, graft versus host reactions, interactions of B and T Cells, genetic control of immune responses and several other phenomena in the immunological system. Certain genetic traits appear to be controlled in animals by the genes of this complex. These include transplantation antigens, cell-mediated lympholysis,

Table 11 illustrates the frequency of HLA-B15 among the entire worker cohort at the main chemical plant. This cohort was subdivided into those with liver disease versus normal individuals based on medical screening studies and liver biopsy. HLA-B15 occurred in 13 percent of the normal group versus 17 percent of the liver diseased group.

TABLE 11

DISTRIBUTION OF HLA-B15 ANTIGEN AMONG
CHEMICAL WORKERS WITH AND WITHOUT LIVER DISEASE
(MAIN PLANT COHORT)
CLINICAL DIAGNOSIS

N=509

B15	NORMAL	LIVER DISEASE
+	64	3
-	427	15
TOTAL	491	18
(%)	(13)	(17)

To determine if this increased occurrence of HLA-B15 was due to chemically-related liver injury and not due to incidental (non-occupational) liver disease, both the pallet plant and the main plant cohorts with liver disease were subclassified into those with chemical liver injury (CLI), and non-chemical liver disease (LD). The frequency of HLA-B15 in these cohorts is shown in Tables 12 and 13.

Table 14 illustrates the occurrence of HLA-B15 in the entire plant cohort subclassified into those without liver disease (NORMAL), those with liver disease, all types (BOTH). Those with liver disease are further subclassified into those with chemical liver injury (CLI) and those with liver disease, non-chemical in origin (LD).

TABLE 9

HLA-A FREQUENCIES

HLA-A ANTIGENS	HEALTHY CONTROLS	WHO 1974 WORKSHOP	PALLET PLANT(BPG)	NON PALLET PLANT(BPG)
A1	34	32	46	30
A2	51	49	57	52
A3	23	22	14	21
A9	16	17	11	23
A10	7	13	18	6
A11	13	8	7	11
A28	7	11	18	12
A29	9	7	0	8
AW30	ND**	5	4	6
AW31	ND	7	4	6
AW32	ND	8	7	7
Blank	ND	ND	14	19
N	900	503	28	519
Total Frequency	160%	175%	200%	200%

**NOT DONE

TABLE 10

HLA-B FREQUENCIES

HLA-B ANTIGENS	HEALTHY CONTROLS	WHO 1975 WORKSHOP	PALLET PLANT/BPG	NON PALLET PLANT/BPG
B5	10	11	11	9
B7	31	33	18	28
B8	27	20	21	20
B12	30	24	31	31
B13	3	6	0	5
B14	5	11	11	8
B15	10	7	29	12
BW16	ND	12	7	4
B17	6	7	14	12
B18	8	8	4	6
BW21	ND	4	4	4
BW22	2	5	7	5
B27	7	8	7	11
BW35	5	17	11	17
B40	11	12	18	16
Blank	ND	ND	18	12
N	903	503	28	519
Total Frequency	135%	176%	201%	201%

A5. Identification of the Endothelial Cell as the Cell of Origin for Vinyl Chloride-Induced Angiosarcoma of the Liver

Background

A search for evidence of vinyl chloride-induced tumor antigen has led to the finding that these tumors have an increased concentration of antigenic coagulation Factor VIII. Factor VIII is a known marker for the vascular lining cells of the endothelial type. Further experiments in animals indicated that the normal endothelial cell found lining the liver sinusoids has little if any Factor VIII fluorescence. Endothelial cells found lining larger vessels on the other hand demonstrated a striking fluorescence, as did experimentally transplanted mouse angiosarcomas. Conversely, mouse hepatic Kupffer cells, a second type of hepatic lining cell distinguished in histological cross section by engorgement with carbon particles, failed to demonstrate positive fluorescence. An increase in antigenic Factor VIII in hepatic angiosarcoma endothelial cells as compared to the normal, led us to believe that these cells have increased production or storage capacity for antigenic Factor VIII.

Objective

1. To study the Factor VIII fluorescence in human angiosarcomas, primary hepatocellular carcinomas, and normal livers.

Research Results

Examination of angiosarcoma liver demonstrated a significant fluorescence due to Factor VIII content in the malignant cells of the liver sinusoids. An intermittent pattern of fluorescence of the sinusoidal lining cells was seen. These cells were histologically identified as angiosarcomatous on hematoxylin and eosin (H & E) staining of adjacent tissue. This type fluorescence was not seen in individuals with primary hepatocellular carinomas and a different pattern was seen in normal livers. These cells and their fluorescent pattern are illustrated in Figures 4 and 5. Figure 4 illustrates the H & E stained cells and Figure 5 their Factor VIII fluorescence.

TABLE 12

DISTRIBUTION OF HLA-B15 ANTIGEN AMONG WORKERS WITH AND WITHOUT CHEMICAL RELATED LIVER DISEASE (PALLET PLANT COHORT)

CLINICAL DIAGNOSIS

N=29

B15	NORMAL	CLI	LD
+	1	4	3
-	3	9	9
TOTAL	4	13	12
(%)	(25)	(31)	(25)

TABLE 13

DISTRIBUTION OF HLA-B15 ANTIGEN AMONG WORKERS WITH AND WITHOUT CHEMICAL RELATED LIVER DISEASE (MAIN PLANT COHORT)

CLINICAL DIAGNOSIS

N=526

B15	NORMAL	CLI	LD
+	65	0	3
-	430	2	13
TOTAL	495	15	16
(%)	(15)	(0)	(19)

TABLE 14

OCCURRENCE OF HLA-B15 AMONG CHEMICAL WORKERS WITH AND WITHOUT CHEMICAL LIVER INJURY

CLINICAL DIAGNOSIS

N=538

B15	NORMAL	CLI	LD	BOTH
+	65	4	6	10
-	430	11	22	33
TOTAL	495	15	28	43
(%)	(13)	(27)	(21)	(23)

(2) The use of tumor antigens for the detection of developing angiosarcoma, although a provocative possibility, is far too non-specific in its present stage of development to provide any reasonably useful means of detecting early cancer development. Until there is better evidence that tumor antigens provide sufficient specificity to prevent cross reactivity with other tissue materials, this approach will not appear to be a clinically useful one.

(3) The present data suggest that the HLA-B15 antigen may reflect an increased susceptibility to liver injury, especially of chemically-induced origin. Confirmation of these findings and the determination of the effectiveness of this marker in screening those with increased susceptibility to chemical liver injury must yet be determined by prospective study.

(4) The leukocyte adherence inhibition test, unfortunately, was not adequate in its technical development nor sufficiently reproducible to be useful as a screening test for either colon cancer or liver angiosarcoma.

All of the above immunological studies, with the possible exception of HLA marker, have provided little supportive evidence that chemical injury interferes with human immunocompetence as determined by our present methods of study. Therefore, studies of the immunological system for screening purposes appear to be of little, if any, value in the early identification of chemical injury or disease.

(5) The identification of Factor VIII production by human and animal angiosarcoma tumors provides further evidence for the need to develp screening methods to detect endothelial rather than hepatocytic cell injury or dysfunction since these are the cells of origin that malignantly transform when exposed to vinyl chloride and other agents such as arsenic.

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PROGRAM B

STUDY OF HEPATIC BIOCHEMICAL AND ENZYMATIC SYSTEMS FOR THE IDENTIFICATION OF VINYL CHLORIDE AND OTHER CHEMICAL INJURY IN CANCER DEVELOPMENT: ANIMAL AND HUMAN STUDIES; Investigators - J.T. Du, C.H. Tamburro and M.T. Tseng

ANIMAL STUDIES:

B1. Characterization Of Hepatic Enzyme Changes In Rats With Prolonged Vinyl Chloride Exposure: Decreased Glucose-6-Phosphatase Activity

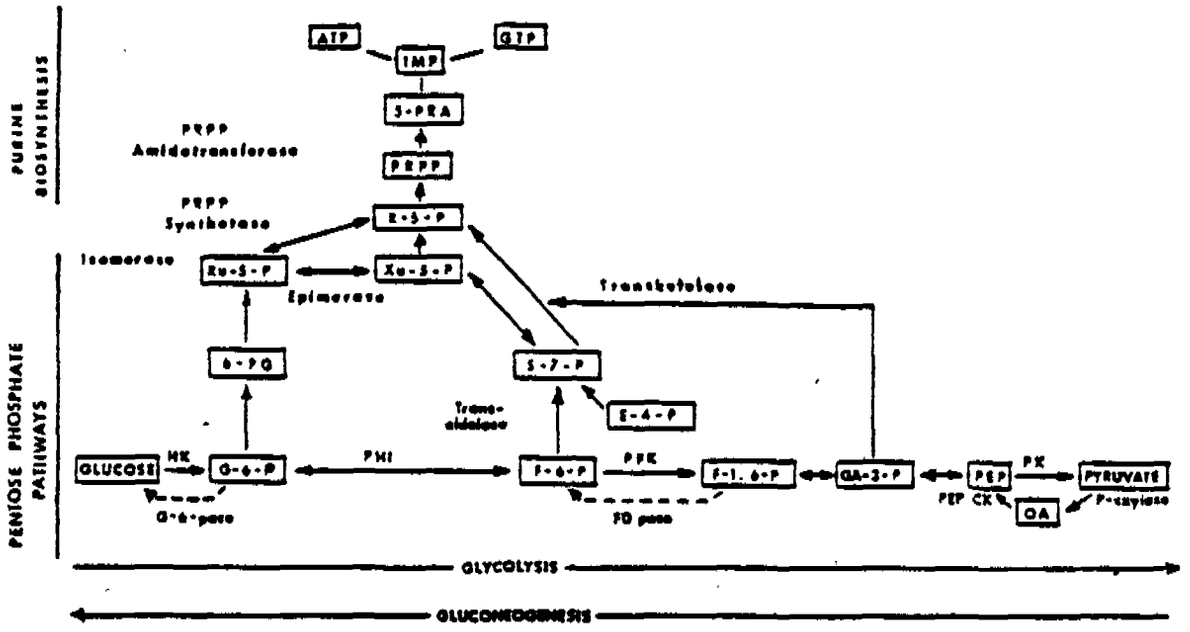
B2. Morphological Alterations In Livers Of Rats Exposed To Vinyl Chloride

Background

Vinyl chloride has been shown to induce an identical malignancy in both animals and man. Vinyl chloride-induced liver cancer in animals, therefore, provided an excellent model for the study of chemically-induced carcinogenesis. The rat model provides the opportunity to further elucidate the mechanism and the pathogenesis of this particular chemical agent, whose major oxidation occurs in the hepatocytes (primary liver cells) while inducing a malignant transformation in an adjacent, different cell type (endothelial lining cell of the sinusoids). Vinyl chloride had been shown to cause angiosarcoma and primary hepatocellular carcinoma in laboratory animals (Maltoni, 1975, 1976), and angiosarcoma in man (Creech and Johnson, 1974). However, little was known regarding the biochemical changes that vinyl chloride mediated to induce hepatocellular injury and subsequent malignant transformation of the mesenchymal cells.

Enzymatic alterations in primary liver parenchymal cell cancers (hepatocellular carcinoma, hepatoma) have been studied extensively. Weber and Convery (1966) and Weber and Lea (1967) proposed that there was a biochemical pattern which could be related to the biological behavior of neoplastic cells. Using a spectrum of hepatomas with different growth rates, a basis for underlying neoplastic cell transformation was proposed as being related to the cell's progressive alterations in the molecular pattern which led to progressive increases in the growth rate of the tumors. Figure 1 outlines the pathways.

FIGURE 1 WEBER'S MOLECULAR CORRELATION CONCEPT OF NEOPLASIA



Key enzymes of glycolysis were shown to increase while key enzymes in gluconeogenesis decreased with tumor growth. In addition, the pentose phosphate biosynthetic pathway, glucose-6-phosphate dehydrogenase (Weber and Morris, 1963) and transaldolase (Heinrich et al., 1974) were shown to be increased in all hepatoma studies. Furthermore, the activity of phosphoribosylpyrophosphate (PRPP) synthetase and glutamine PRPP aminotransferase, the first two enzymes channeling ribose-5-phosphate into purine, DNA and RNA synthesis were also increased. The PRPP sythetase was increased in rapidly growing hepatomas and the transferase in all the hepatomas irrespective of growth rates (Weber et al., 1975). Decreased enzyme activity was observed at an early stage of carcinogenesis before the morphological signs of a tumor appeared in the liver, and the activity of enzymes dependent upon the rate of hepatoma proliferation correlated with progressive malignant transformation.

These findings and the lack of any long-term sequential studies of animals exposed to vinyl chloride directed us to our first three objectives.

Objectives

1. To determine the sequential hepatic enzymatic changes in animals chronically exposed to vinyl chloride and their correlation to conventional clinical tests of liver function.

2. To determine if these sequential enzymatic patterns, especially with regard to carbohydrate metabolism and nucleic acid synthesis, would correlate with vinyl chloride-induced liver injury and ultimate malignant transformation.

3. To correlate the sequential, morphological changes with these enzymatic findings.

Research Results

1. Characterization of Hepatic Enzyme Changes

Sprague-Dawley adult male rats were chronically exposed to vinyl chloride. The initial studies characterized the metabolic alteration in the liver by studying various organelle enzyme markers which included glucose-6-phosphatase (microsomal), cytochrome oxidase (mitochondrial) and glucose-6-phosphatase dehydrogenase (cytosol). In addition, standard serum biochemical clinical liver studies of the liver (liver "function" tests) were performed including aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), alkaline phosphatase, lactic acid dehydrogenase, total protein, and bilirubin. Additional studies of subcellular enzymes related to nucleic acid synthesis--glutamine PRPP aminotransferase, and glycolysis--phosphofructokinase were done. In vivo protein synthesis was assessed by ^{3}H-leucine incorporation.

The results of these studies illustrated that rats exposed to high chronic doses of vinyl chloride had a 25% lower activity of glucose-6-phosphatase, a key enzyme for gluconeogenesis after about 71 hours of exposure and a 50-100% increase in glucose-6-phosphate dehydrogenase, a rate limiting enzyme in the pentose pathway, after 84 hours of exposure.

These studies demonstrated that (a) metabolic alterations in the liver cell are induced by vinyl chloride exposure, (b) gluconeogenesis is altered as reflected by a lower glucose-6-phosphatase and a higher glucose-6-phosphatase dehydrogenase activity, and (c) that these changes occur in a fashion similar to the lower gluconeogenic and higher pentose shunt activity of hepatomas. Although this could result in an enhanced production of PRPP and ultimately more nucleic acid synthesis, our studies of PRPP did not document this as a subsequent finding. These enzymatic changes, although similar to the biochemical changes seen in rapidly growing primary hepatocellular carcinomas, most likely reflect liver cell adaptation to vinyl chloride exposure but do not appear to significantly increase DNA synthesis. In addition, these changes were sufficiently limited so as not to be reflected by circulating enzyme levels traditionally used for the detection of clinical hepatocellular injury (Figure 2).

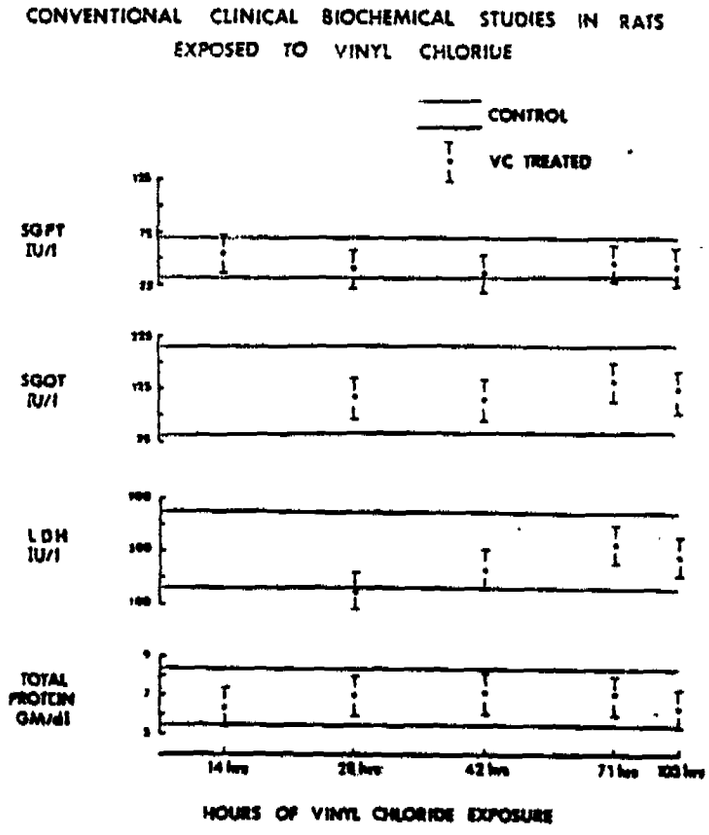


FIGURE 2

2. Morphological Alterations: Light and Electron Microscopic Assessment

Sequential morphological assessment was performed in both of the chronic experiments utilizing light and electron microscopy.

The enzymatic findings discussed in B1 and subsequently in B3 were associated with light microscopic findings which showed a marked increase in liver cell polyploidy, double nucleated cells and areas of focal hepatocellular hyperplasia. There was no evidence of hepatocellular injury, necrosis, or increased fibrosis. The morphological changes seen, however, reflect an increased liver cell regeneration which was not measurable by the techniques used to measure DNA synthesis in our experiments.

The electron microscopic changes demonstrated a proliferation of the smooth endoplasmic reticulum without evidences of changes in the hepatocytic cell, cell membrane, nucleic acid or rough endoplasmic reticulum structures.

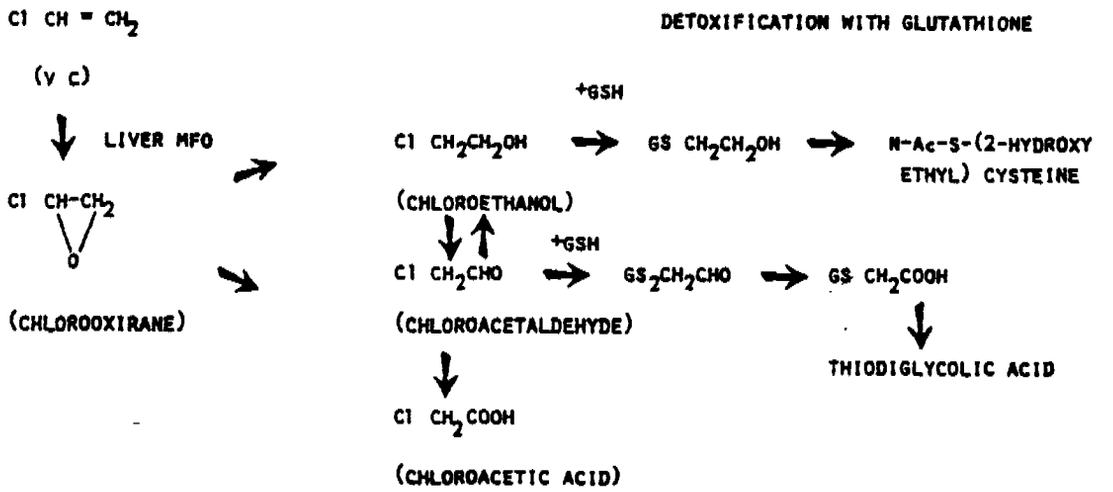
There was no evidence of increased collagen deposition in the space of Disse which has been reported by others during terminal or final stages of angiosarcoma development in animals.

B3. Alterations in the Oxidizing and Detoxifying System of the Rat Liver After Prolonged Exposure to Vinyl Chloride.

Background

Vinyl chloride metabolism had been shown to be blocked at low dosage levels (50 ppm) by inhibiting the alcohol dehydrogenase system and at higher levels (200 ppm) by metabolism via the microsomal mixed function oxidase (MFO) system (Hefner et al., 1975, Bolt et al., 1975). Vinyl chloride's major metabolites, chlorooxirane and chloroacetaldehyde, have been identified as probable ultimate carcinogens (Barbin et al, 1975; Van Duuren, 1975; and Jaeger et al., 1974). Further studies (Elmore et al., 1976; Malaveille et al., 1975; McCann et al., 1975) demonstrated the mutagenic activity of chlorooxirane and chloroacetaldehyde on microorganisms and the nonmutagenicity of chloroethanol and chloroacetic acid, other metabolites of vinyl chloride.

Detoxification of these metabolites was reflected by a reduction in non-protein sulfhydryl content of the liver (Hefner, 1975) and tracer studies showing major urinary metabolites of vinyl chloride to be N-acetyl-S-hydroxyethylcysteine and thiodiglycolic acid (Watanabe et al., 1976). The proposed metabolic fate of vinyl chloride is illustrated:



In these experiments, the enzymes studied related to vinyl chloride oxidation, included P-450, NADPH-cytochrome-C-reductase and mixed function oxidase. Those enzymes related to detoxification include non-protein sulfhydryl content (NPSC), glutathione content (GSH), glutathione reductase (GR), glutathione-epoxide-S-transferase (GEST, glutathione-E-transferase), and glutathione-aralkyl-S-transferase (GAST, glutathione A&B transferases).

Objective

1. To determine the sequential changes of key hepatic oxidizing and detoxifying enzymes with prolonged vinyl chloride exposure.

Research Results

The oxidizing and detoxifying capability of rat liver cells during prolonged exposure to chronic high doses of vinyl chloride demonstrated a 25-50% higher activity of glutathione reductase, the enzyme which regenerates reduced glutathione to detoxify the metabolites of vinyl chloride. Although our acute and single vinyl chloride exposure studies demonstrated a decrease in the nonprotein sulfhydryl content (48%) similar to that reported by Hefner (1975), repeated or chronic exposure to higher levels showed a progressive increase which was statistically significant after 70 hours of exposure (Figure 3).

Glutathione-epoxide-S-transferase (GEST) and glutathione-aralkyl-S-transferase (GAST), the enzymes capable of conjugating the toxic vinyl chloride metabolites, were significantly elevated following vinyl chloride exposure; glutathione reductase (GR), the enzyme to regenerate glutathione, was also elevated significantly following vinyl chloride exposure (Figure 4).

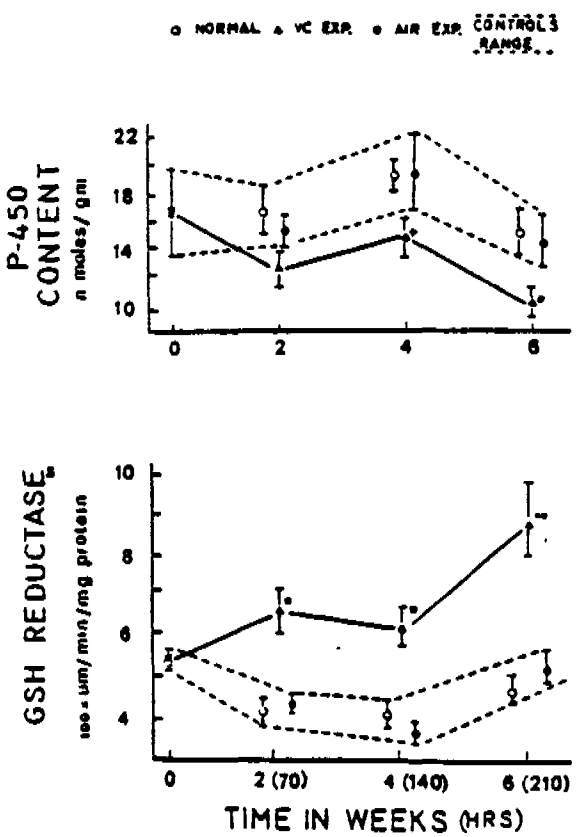


FIGURE 3

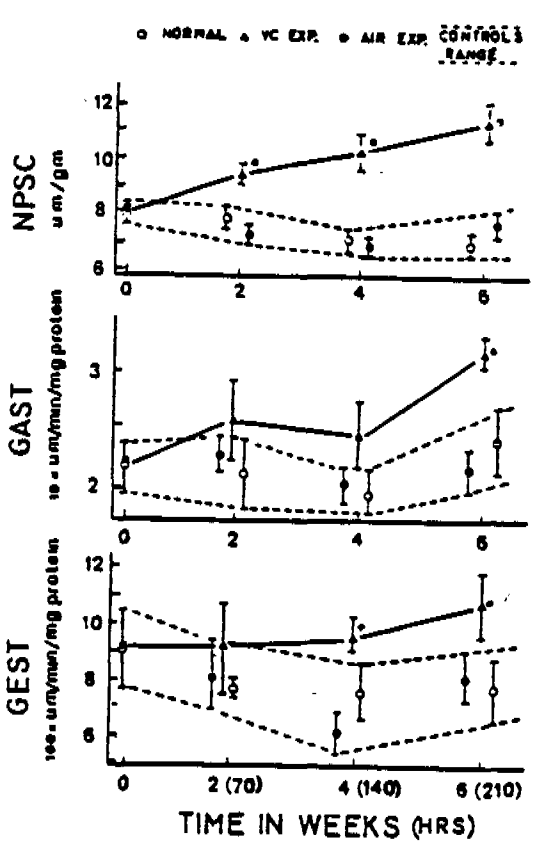


FIGURE 4

The oxidizing capability as reflected by cytochrome P-450, the major protein involved in vinyl chloride metabolism, however, was reduced significantly only after prolonged vinyl chloride exposure (Figure 3), supporting reports in vivo (Reynolds et al., 1975) and in vitro (Guengerich and Strickland, 1977; Ivanetich et al., 1977) that vinyl chloride metabolites destroyed P-450. No differences were found in the conventional clinical serum biochemical tests or in other cellular enzyme studies (cytochrome-C oxidase, NADPH-cytochrome-C-reductase and mixed function oxidase).

These changes seem to reflect early hepatic cell adaptation to chronic vinyl chloride exposure by increasing their capacity for detoxification and decreasing their capability to further metabolize vinyl chloride into its toxic intermediates--both provide a means of natural defense to a toxic substance. This entire detoxifying sequence is shown in Figure 5 as a composite of the detoxifying enzyme changes over a prolonged period of exposure.

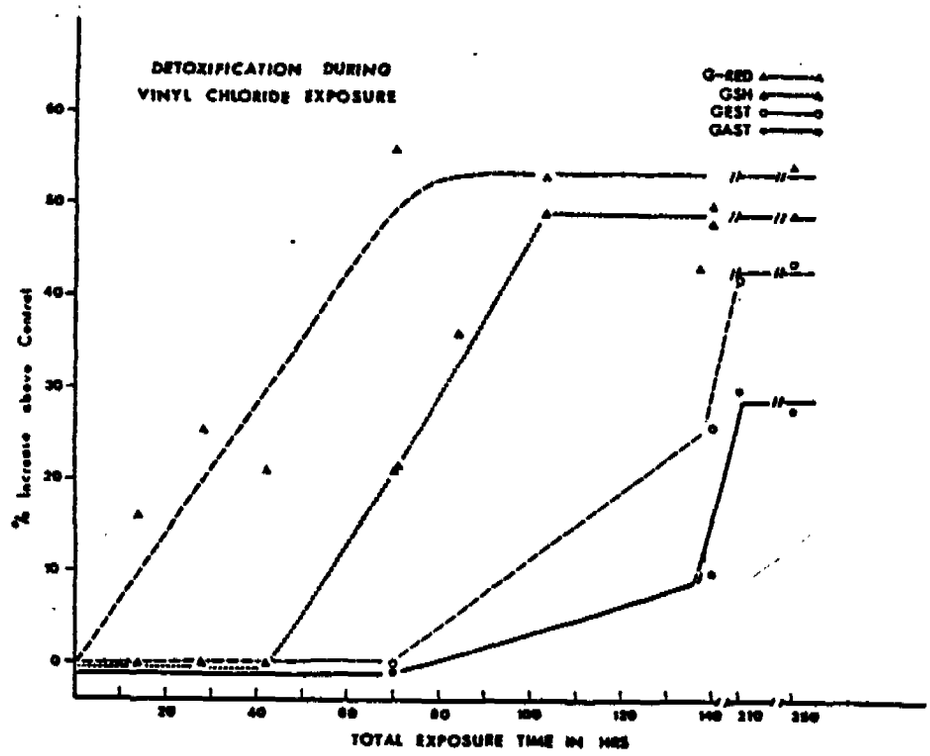


FIGURE 5

It is interesting to note that there is an earlier increase in the GEST transferase than the GAST transferases with prolonged exposure. This may reflect an earlier (in time) need for adaptation to excessive production of the epoxide and subsequently the accumulation of the aldehyde metabolite. Whether this reflects the maximum adaptability as indicated by a leveling off of enzyme after 200 hours, or whether these changes reflect an adaptation to

prevent cellular injury as well as prevent primary hepatocellular malignant transformation would require even longer sequential studies for confirmation; these were beyond our time constraints.

B4. Oxidizing and Detoxifying Ability of Liver Mesenchymal or Parenchymal Cells in the Metabolism of Xenobiotics.

Background

Even though the malignant transformation induced by vinyl chloride occurred in the mesenchymal cells of the liver (endothelial cells), all metabolic studies up to this time had been directed at the liver as a whole or to the parenchymal cell--the hepatocyte. There had been no differentiation between the oxidizing capability of the hepatocytes that demonstrated injury and the endothelial cells which were malignantly transformed. Little was also known regarding the variable ability of these two cell types in the removal of the ultimate carcinogens or active metabolites. Therefore, our next set of studies was directed toward the ability of two different cell types to metabolize vinyl chloride.

Objective

1. To study the comparative ability of the hepatocytes versus the mesenchymal cells to metabolize vinyl chloride and determine the detoxifying capability of the various liver cell types.

Research Results

Enzymatic studies of the detoxifying activities of the subcellular fraction of liver cells, both hepatocytic and mesenchymal included the study of GEST, GAST, GR, as well as P-450 and mixed function oxidase capability of the various cell fractions. The effectiveness of the isolation of the hepatocytes and mesenchymal cells was confirmed by studying pyruvate kinase activity.

Mesenchymal cells were shown to have about half the GEST activity, 7% of the GAST activity, less than half of the microsomal function oxidase activity, and 73% of the GR capability compared to the hepatocytes. The activity per million cells indicated that the nonhepatocytes had about 1/70 MFO activity, 1/65 GEST activity, 1/500 GAST activity, and 1/45 GR activity. These results demonstrate that the nonhepatocyte has significantly less capability in both the activation and detoxification of vinyl chloride and other xenobiotics. The data supports the hypothesis that the endothelial cells have a more limited capability to detoxify vinyl chloride metabolites and that this limited capability provides a greater possibility for DNA injury and subsequent malignant transformation. Again, unfortunately much longer

experimental studies would be needed to eventually validate this hypothesis (Du et al., preprint attached in Appendix).

Relevance to Industry

(1) These chronic sequential studies are highly relevant to the chemical industry's environmental health problems. The sequential, biochemical changes that occur in the carbohydrate metabolism of the liver hepatocytes reflect adaptation of the liver cell to chemical exposure. This is further reflected by the histological changes which show polyploidy, double nucleated cells and areas of focal hyperplasia. As the clinical studies in this report will subsequently show, these changes are characteristic of chemical injury but do not necessarily reflect premalignancy. This would be consistent with the enzymatic studies failing to demonstrate any evidence of increased nucleic acid synthesis which is seen in the premalignant hepatoma studies of Weber and others. The important fact is that these changes cannot be detected by the standard federally-required biochemical studies, because these are directed solely at the hepatocyte. The hepatocyte may become injured but it does not usually malignantly transform.

(2) The oxidative and detoxifying results of this study demonstrate that the hepatocyte progressively adapts its ability to remove and detoxify the active metabolites. This occurs without evidences of hepatocellular injury, at least up to what would be equivalent to 6-20% of the working years of a chemical worker. The studies also emphasize the fact that at very high exposures, the detoxifying system is adaptive and the oxidizing system's capacity is reduced, thereby providing a relative defense against continued formation of toxic metabolites.

(3) The massive ability of the hepatocyte to oxidize as well as detoxify the active metabolites of vinyl chloride can well explain why there is no hepatocellular carcinoma development in humans with vinyl chloride exposure. A markedly reduced ability of the nonhepatocyte to detoxify the metabolites while still having the capacity to oxidize vinyl chloride into its active metabolites would support the higher probability of this cell becoming malignantly transformed. Since all clinical screening studies are directed toward the hepatocyte, these animal studies emphasize the insensitiveness of clinical biochemical studies to correctly identify early injury in the more susceptible cell, i.e., endothelial cell. More sensitive indicators of nonhepatocytic cell injury or dysfunction are needed for the detection of early chemical injury. These indicators must take into account the function of both types of liver cells.

(4) The morphological findings described in the animal experiments confirm earlier observations regarding the earliest histological manifestations of vinyl chloride injury. They support our clinical observations that these lesions can be used to identify chemical injury. Program D, on the morphormetric and histological analysis details the clinical results found in humans.

HUMAN STUDIES:

B5. Effectiveness of Indocyanine Green Clearance in the Detection of Liver Injury.

Background

The increasing concern over the potential health hazard of synthetic chemicals in the occupational environment has led the government to require, and many industries to establish, medical laboratory screening programs for their employees. The primary objectives of these screening programs is to identify potential work-related disease, so that intervention may reduce disability, morbidity, and mortality. Most screening studies are directed toward the detection of abnormalities in certain body systems. The specific tests used are most often selected on the basis of medical experience in symptomatic or hospitalized populations. Prior experiences utilizing non-specific, multi-phasic health screening have not proven to be effective in this area. The effectiveness of standard medical testing used predominately in asymptomatic individuals with subclinical low-grade disease--both work and non-work related--had not been determined. This study assessed the effectiveness of using clearance studies of anionic dyes in the detection of liver injury in such individuals.

Objective

1. To determine the effectiveness of ICG clearances in detecting hepatic injury.

2. To compare ICG sensitivity and specificity to standard clinical biochemical screening in the detection of liver injury.

Research Results

The study group (cohort) consisted of 969 male employees who were tested between June 1, 1976 and May 31, 1977 with standard biochemical studies of the liver which included alanine aminotransferase (ALT/SGPT), aspartic aminotransferase (AST/SGOT), gamma glutamyl transpeptidase (GGTP), alkaline phosphatase (AP), and total bilirubin (TB). In addition, these employees were also screened by ICG clearances at the 0.5, 2.5, and 5.0 mg/kg doses, and by two more specific liver enzyme tests (isocitric dehydrogenase [ICD] and sorbitol dehydrogenase [SDH]). This main cohort was divided into a "standard" and a "non-standard" population for purposes of analysis. The division was based upon the best medical opinion. The "standard" group demonstrated no clinical evidence of significant medical disease--occupational or non-occupational in origin, and "non-standard" included all the others.

A subcohort of 120 individuals who had undergone liver biopsies for medical reasons, whether related to work or not, were also studied. This subcohort population was divided into those individuals with and without

histological evidence of liver injury, and the former further subdivided into those with and without histological characteristics of chemical injury (see Program D2).

All employees had individual work histories and rank-ordered exposure indices for 22 different chemicals used within the work place.

The GGTP provided the highest positive predictive value as a screening test for non-standard individuals (population with medical disorder). It also provided the highest sensitivity and specificity sum among the 5 most frequently abnormal tests (AST/SGOT, ALT/SGPT, AP, ICG); 2 other tests also had high predictive values--the indirect bilirubin, due to the high number of congenital indirect hyperbilirubinemic individuals, and the triglyceride levels, most likely reflecting differences in age, weight, and diabetic status in the non-standard population.

The ICG clearance (0.5 mg/kg) espressed as mean t 1/2 in minutes (min.) demonstrated differences not only between standard and non-standard population but also differences between those with high and low mean exposure to vinyl chloride (Table 1).

TABLE 1

INDOCYANINE CLEARANCE IN A COHORT INDUSTRIAL POPULATION

SUBGROUP	NO.	MEAN MIN.	90%	100%
STANDARD	662	2.9	2.0-3.8	1.8-4.8
LOW VC EXPOSURE	453	2.9	1.8-4.0	1.7-4.3
NON-STANDARD	257	3.4	2.1-5.6	2.0-6.6
HIGH VC EXPOSURE	466	3.1	2.1-4.4	1.9-4.8

The biochemical tests which best correlated with the presence of hepatic disease in the chemical workers with clinical evidence of liver disease were ALT (SGPT), GGTP, alkaline phosphatase, and ICG clearance (i.e. these were the tests with the highest sensitivity in detecting latent liver disease). Sensitivity alone, however, is not an adequate indicator of a test's screening value, especially when used in asymptomatic individuals. More appropriate evaluation of the screening ability of the tests requires that both sensitivity and specificity be determined, i.e., identify the diseased individuals and correctly exclude the non-diseased individuals. The sensitivity and

specificity of the 4 most sensitive biochemical screening tests and the ICG clearances (all doses) demonstrated that GGTP although the most sensitive is the least specific; AP has the highest specificity but the lowest sensitivity. ICG clearances, even at the low dose, clearly remain the best test, i.e., in combined sensitivity and specificity for screening and detecting individuals with subclinical liver disease (Table 2 a,b,c).

TABLE 2

A

SENSITIVITY AND SPECIFICITY
OF INDOCYANINE GREEN CLEARANCE

NO. PATIENTS 1500

ICG 0.5 mg/kg

BEST MEDICAL ASSESSMENT	(+)	(-)	TOTAL
(+)	293	140	516
(-)	38	1923	2011
			2939

SENSITIVITY: 55.3%
SPECIFICITY: 95.6%

B

SENSITIVITY AND SPECIFICITY
OF INDOCYANINE GREEN CLEARANCE

ICG 2.5 mg/kg

BEST MEDICAL ASSESSMENT	(+)	(-)	TOTAL
(+)	45	28	73
(-)	19	396	410
			483

SENSITIVITY: 45/73 = 61.6%
SPECIFICITY: 396/410 = 96.5%

C

SENSITIVITY AND SPECIFICITY
OF INDOCYANINE GREEN CLEARANCE

ICG 5.0 mg/kg

BEST MEDICAL ASSESSMENT	(+)	(-)	TOTAL
(+)	78	6	84
(-)	9	38	47
			125

SENSITIVITY: 78/84 = 92.9%
SPECIFICITY: 38/42 = 90.5%

In addition ICG clearance demonstrates excellent correlation with the histological presence of liver disease and liver cancer (Figure 6).

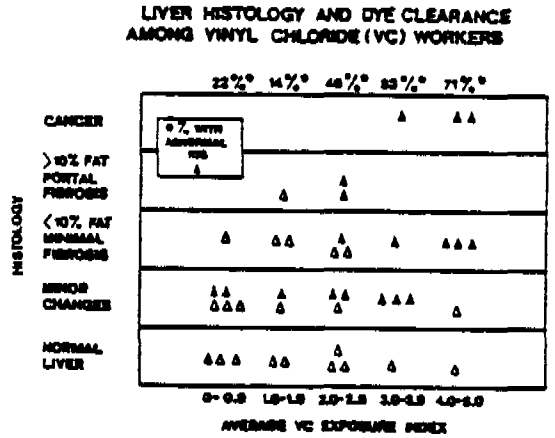


FIGURE 6

The subcohort biopsied population was additionally studied with regard to the sensitivity and specificity of the screening tests, the specific histological interpretation of the liver biopsy, and the relationship to vinyl chloride exposure. All biopsied individuals were subclassified into three groups: those with histological evidence consistent with chemical liver

injury, those with histological evidence of liver disease without evidence of chemical injury, and those with normal liver biopsies. Each group was then evaluated regarding the sensitivity and specificity of the biochemical tests and their total exposure to vinyl chloride based on a rank ordered scale.

Analysis of the subcohort biopsied group's work histories demonstrated that those individuals with chemical liver injury had the highest average rating for vinyl chloride exposure and were best identified by screening with ICG clearance tests.

B6. The Assessment of Bile Acids Vs. Indocyanine Green (ICG) Clearances in the Detection of Liver Injury in a Chemically-Exposed Worker Population

Background

Since highly sensitive and specific screening tests for the detection of latent hepatic injury were needed, studies were conducted to determine whether bile acid clearances could be utilized to detect occupationally-related hepatic injury. Bile acids are naturally occurring steroids which are synthesized and removed solely by the liver. These substances, in contrast to synthetic anionic dyes such as indocyanine green, have been reported to provide equal if not better, sensitivity in the detection of liver disease. The development of radioimmunoassays for bile acids provided an economical means of determining whether these natural substances could be used as effective screening measures in subclinical liver disease. Studies were conducted to determine the relative effectiveness of bile acid levels vs. ICG clearances in the detection of subclinical liver disease.

Objective

1. To determine the effectiveness of bile acid clearance in the detection of subclinical liver injury

2. To compare bile acids to ICG clearance in identifying chemical and non-chemical liver injury.

Research Results

A subcohort (64) of the worker population who had liver biopsies done for medical reasons and had both ICG clearances and serum bile acids--cholylglycine (CG) and conjugates of cholic acid (CCA)--performed, were studied. These individuals were subdivided on the basis of the histological features of their biopsies into individuals with chemical liver injury (CLI), individuals with non-chemical liver disease (NCLD), and individuals with no histological abnormalities (NBX). The mean plus or minus S.E.M. for ICG, CG and CCA in the CLI, NCLD, NBX, and a non-biopsied normal group are shown in Table 2.

TABLE 2

CORRELATION OF LIVER BIOPSY WITH
BILE ACID LEVELS AND ICG CLEARANCE

	CLI	NCLD	NBX	NON BIOPSIED NORMAL
ICG(ck) (0.5 mg/kg)	4.2 ± 0.6	3.2 ± 0.1	3.3 ± 0.2	3.1 ± 0.01
CG (ug/dl)	95.2 ± 28.3	27.3 ± 4.4	34.60± 7.1	14.9 ± 0.9
CCA (ug/dl)	89.7 ± 29.3	25.3 ± 4.5	52.6 ± 26.6	18.7 ± 1.2

Analysis of variance (on log transform data) showed significant differences for ICG clearance for the three biopsied groups and for all four groups. Values for the CLD were significantly different from the ICGs for the normal, but showed no significant difference between the NCLD and the NBX group. Analysis of variance for ICG clearance was not as discriminating for the 3 biopsied groups. ICG clearance gave a better separation (sensitivity) of the normal vs. abnormal at the 95 percentile level for the normal population in the serum bile acids.

This preliminary analysis, therefore, shows that fasting serum bile acids hold promise as a potential detector of early liver dysfunction, particularly chemically-induced, in those individuals who are asymptomatic. This test should be further investigated to determine how this natural substance might be more effectively utilized as a screening technique. Bile acids can be taken orally for clearance study and are not limited to intravenous route as is the case for synthetic anionic dyes such as ICG. Studies in this area continued to be pursued in similar populations.

Relevance to Industry

These clinical study results provide the best scientific basis upon which to recommend effective medical screening tests for the detection of occupationally-related injury to the liver. The results also provide sound scientific reasons for recommending modification of presently required federal testing in environments utilizing potentially hepatotoxic agents.

Although synthetic anionic dye presently provides the best means for detecting early latent liver injury, it is limited by the need to be injected (an invasive procedure). Although reactions to the injection of this dye have been minimal, it does present barriers to voluntary worker compliance. Therefore, development of a screening test which can effectively determine

overall hepatic function, can be given orally, and is a nonsynthetic, normal biological constituent approaches the most ideal test for screening. Bile acid clearance could be the least invasive and therefore the most acceptable method to test asymptomatic individuals undergoing screening. The bile acid studies described above are the basis for recommending its use in future screening. The standardization of these bile acid test techniques, however, requires further careful validation so that specific recommendations regarding their use will provide the most accurate delineation between those with disease and without disease and those with occupationally-related disorders versus naturally-occurring ones.

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PROGRAM C

STUDY OF GLYCOSAMINOGLYCAN CHANGES IN THE DETECTION OF HEPATIC FIBROTIC INJURY IN CHEMICAL EXPOSURE AND HEPATIC CANCER DEVELOPMENT; Investigators - C.E. Kupchella and R. Warick

ANIMAL STUDIES:

C1. The Study of Glycosaminoglycan Changes in Livers of Animals Bearing Metastasizing Hepatomas

C2. Significance of Tissue Glycosaminoglycan Elevations in Hepatic Fibrosis, Necrosis, and Regeneration

HUMAN STUDIES:

C3. Characterization of Glycosaminoglycan Patterns in Humans with Angiosarcoma

C5. Characterization of Glycosaminoglycan Patterns in the Identification of Chemical and Nonchemical Injury of the Liver

C4. Evaluation of Glycosaminoglycan Analysis of Urines of Chemically- Exposed Workers

Background

It is well established that glycosaminoglycans (GAGs) are involved in wound healing and scar formation. Although it is less certain what role they play, it is known that certain GAGs are elevated in malignant tumors including hepatic tumors, and it has been postulated that GAGs may be impor- tant determinants of tumor-cell properties. A number of laboratories, including ours, have established that urinary GAG excretions may serve as markers in the pathogenesis of chemical injury, fibrosis, and cancer. Despite the fact that urinary GAG analyses have long been used clinically to detect and diagnose genetically-determined metabolic disorders of GAG metabolism, a

systematic evaluation of the usefulness of urinary GAG patterns in the detection and diagnosis of acute and/or chronic, necrotic and/or fibrotic liver injury--or cancer--has never been made. Studies were begun to determine the practical utility of tissue and urinary GAG analysis in the detection and diagnosis of chemically-induced liver injury and cancers.

Objective

In Animals:

1. To determine the relationship between GAG patterns in tumor tissue (and urine) in animals with fast versus slow growing and in metastasizing versus non-metastasizing, chemically-induced, transplantable, hepatocellular tumors. (As an initial means of exploring the functional role of GAGs known to be elevated in hepatic cancer).

2. To determine the sequence of GAG changes (in tissue, urine and blood) associated with the onset of experimentally-induced fibrotic injury with special attention to any change coinciding with the transition from reversible to irreversible fibrosis.

3. To determine the degree to which chemically-induced necrosis of the liver results in altered urinary GAG excretion and confirm changes seen in tissue in earlier studies.

In Humans:

4. To compare the relative accuracy of the common methods of evaluating urinary GAGs and find the least expensive and least time-consuming method of urinary GAG analysis able to give good specificity and sensitivity.

5. To repeat in a double-blind clinical trial the evaluation of the ability of urinary GAG analysis to correctly identify active liver disease.

6. To evaluate urinary GAG patterns in groups of alcoholics with active liver injury over a time period.

7. To compare liver tissue and urinary GAG patterns in patients (a) with alcohol-injured livers, (b) hepatic angiosarcoma, (c) primary hepatocellular tumors, (d) livers with metastatic cancer, and (e) chemically-induced hepatitis and cirrhosis.

Research Results

In Animals:

Livers of animals bearing metastasizing hepatoma (5123tc) have 10-fold greater concentrations of a non-sulfated, neutral, uronic acid-positive

material than is found in the livers of animals bearing two others, non-metastasizing hepatomas (4).

Heparan sulfate and hyaluronic acid levels--but not heparin--are 3-4 times higher in experimentally transplanted hepatomas than in normal liver and urinary excretion reflects both the tumor GAG composition and the size of tumors (4, 8).

Hepatic necrosis is accompanied by significant tissue GAG elevations but hepatic regeneration is not (5).

Heparan sulfate (a type of GAG) is elevated in hepatic tissue undergoing experimentally induced fibrosis and heparan sulfate is elevated in the urine of experimental animals (3).

In Humans:

Human hepatic angiosarcoma and fibrotic liver disease are accompanied by elevated tissue GAGs (1).

The GAGs in the angiosarcomatous tumor tissue are different from those in fibrotic tissue adjacent to the tumor (2).

Angiosarcoma and hepatoma patients have characteristic urinary GAG patterns--patterns not found in normal controls (2).

Gross (non-fractionated) urinary GAG determinations give a better indication of liver disease than ultrasound analysis. Exacting urinary GAG analysis (fractionated) is able to differentiated active from inactive liver disease (7). Table 1 illustrates this for chrondrotin sulfate fraction.

TABLE 1

PRELIMINARY EVALUATION OF THE USE OF AN EXCLUSIVELY CHONDROITIN SULFATE EXCRETION PATTERN AS A SCREENING TEST FOR VINYL - CHLORIDE - EXPOSURE - ASSOCIATED LIVER INJURY *

TEST* RESULT	VINYL CHLORIDE EXPOSURE PLUS NON-ANGIOSARCOMA LIVER INJURY	
	+	-
+	7	3
-	2	29
SUM	9	32

SENSITIVITY = 77.7% (95% LIMITS 40.0% - 97.2%)

SPECIFICITY = 90.6% (95% LIMITS 75% - 98%)

*URINE FRACTIONATION IN WHICH THE CHONDROITIN SULFATE FRACTION IS POSITIVE BUT THE HYALURONIC ACID AND HEPARIN FRACTIONS ARE BOTH NEGATIVE.

Relevance to Industry

These studies address the need for useful screening tests for liver injury--urine tests of the type evaluated obviously fit the ideal of being non-invasive, have no associated morbidity/mortality, and do not require "time off" from work to perform.

They could provide useful screening tests for early injury and for differentiating active versus inactive disease.

GAG analysis may help assess methods of therapeutic intervention, i.e., the elucidation of the role of the GAGs in the pathogenesis of fibrotic liver disease and the identification of strategies by which fibrogenesis can be blocked and/or reversed.

Finally, they could provide useful means of identifying individuals at risk of chemical injury--by further characterization of GAG changes, one may be able to identify specifc injury due to chronic alcohol, chemical or other exposure agents in individuals with liver impairment.

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PROGRAM D

HISTOLOGIC AND MORPHOMETRIC ANALYSIS: A MEANS OF ACCESSING HISTOLOGICAL HEPATIC INJURY IN CHEMICAL WORKERS; Investigators - G.H. Barrows, R. Schrodt, and C.H. Tamburro

D1. Morphometric Assessment of Histological Lesions Characteristic of Vinyl Chloride Injury

D2. Computer-Assisted Morphometric Analysis as a Rapid Means of Determining Collagen Content

D3. Development and Assessment of Morphometric Method of Analysis of Collagen Content from Human Liver Biopsies - Relationship to Age

D4. Light Microscopic Assessment of Various Histological Lesions Found in Liver Biopsy Tissue Obtained from Vinyl Chloride Workers

Background

Observations made on liver biopsy specimens obtained from vinyl monomer polymerization workers have identified the association of periportal, subcapsular, and mid-zonal fibrosis, and activated sinusoidal lining cells with vinyl chloride exposure. Many have believed these findings to be early indicators of potentially dangerous chemical exposure since these histological findings are characteristic patterns in the terminal or late stages of disease.

The early stages of increased collagen deposition are often difficult to identify due to a lack of data concerning the normal collagen content and normal variation of collagen in the sinusoidal spaces. Other types of chemical workers' injuries, such as copper smelters' arsenic-induced injury, are also associated with increased collagen formation especially in the perisinusoidal areas (1-3, 6-7). In addition, there had been no systematic analysis of the collagen content of the liver's parenchyma based on age. Only subjective data was available regarding "normal" increases in the quantity of fibrous tissue within the aging liver. Therefore, objective criteria were needed to determine (a) whether the increased collagen deposition in industrial workers could be used as an indicator of developing cancer, and (b) whether this increased collagen could be identified solely on tissue obtained by liver biopsy.

Industrial vinyl monomer exposure, especially vinyl chloride, has been associated with various hepatic histological abnormalities. These have included hyperplasia of both the hepatocytes and sinusoidal cells. Early histological studies, mainly on autopsy material, have shown focal mixed hyperplasia (hyperplasia of the hepatocytes or sinusoidal cells) to be an early histological alteration associated with vinyl chloride exposure (4,7,8). A double-blind histological assessment was conducted on the liver biopsies of some 120 exposed chemical workers to substantiate this observation and determine its potential use in medical surveillance. Assessment of the degree and duration of the vinyl chloride exposure was determined utilizing rank ordered exposure indices as reported by Greenburg and Tamburro (9).

Objectives

1. Develop a clinically usable method for morphometric analysis of collagen content of liver biopsies.

2. Determine whether computer-assisted morphometric analysis could be used as the standard means of determining collagen content from human liver biopsies.

3. Morphometic assessment of normal collagen content and changes associated with increasing age.

4. Review and analyze light microscopic findings of liver tissue obtained from vinyl chloride workers to determine whether specific histological lesions were sufficiently characteristic to be identified and associated with vinyl chloride exposure.

Research Results

(1) Initially, a manual calculator-assisted approach was used for quanitation of trichrome stainable collagen in liver biopsies. Randomly selected areas of the liver biopsy were photographed. These were projected in an 16 x 16 cm area on a Hewlett-Packard 9864A digitizing plate and area calculated using a Hewlett-Packard 9815 calculator. Measurements of collagen content and distribution of the perisinusoidal spaces were made from a randomly selected number of complete grid sections in the various sublobular zones; they were prenumbered from left to right as shown in Figure 1A. The areas were measured in micrographs by superimposing an array of sampling points (Figure 1B) and measuring the number of those points which overlaid specific areas. Two randomly selected grid areas from 3 histological, (periportal, mid-zonal, central) and 4 anatomical locations by 2 methods were studied from 2 to 3 individuals in each bracket.

Portal to central distance was quantitated and divided into 3 sections: pericentral, midzonal, and periportal. A trained observer then manually

outlined all areas staining as collagen and the total sinusoidal area (Figure 2). This was repeated for a total of 3 areas in each biopsy sample. These randomly selected areas provided sufficient sampling to obtain a reproducible estimate of collagen content of various areas of liver lobule and were highly reproducible (Table 1).

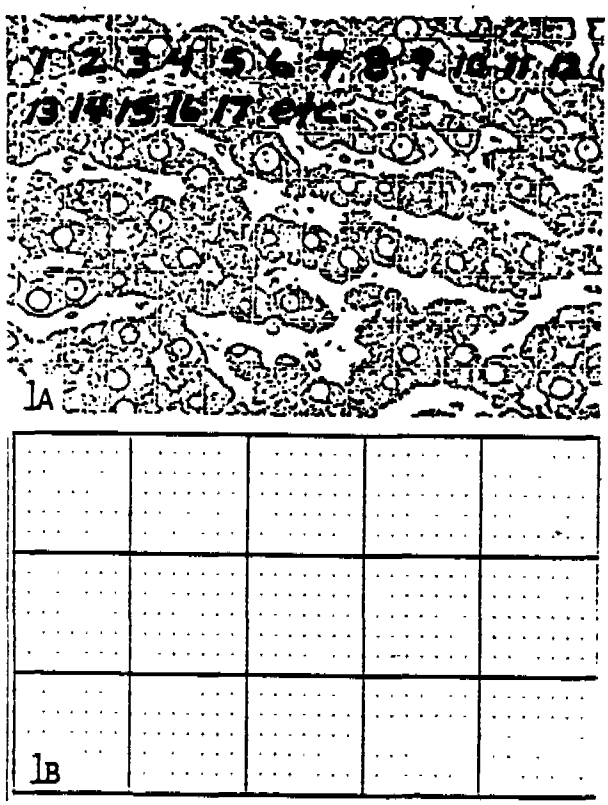


FIGURE 1

Manual collagen quantitation outline

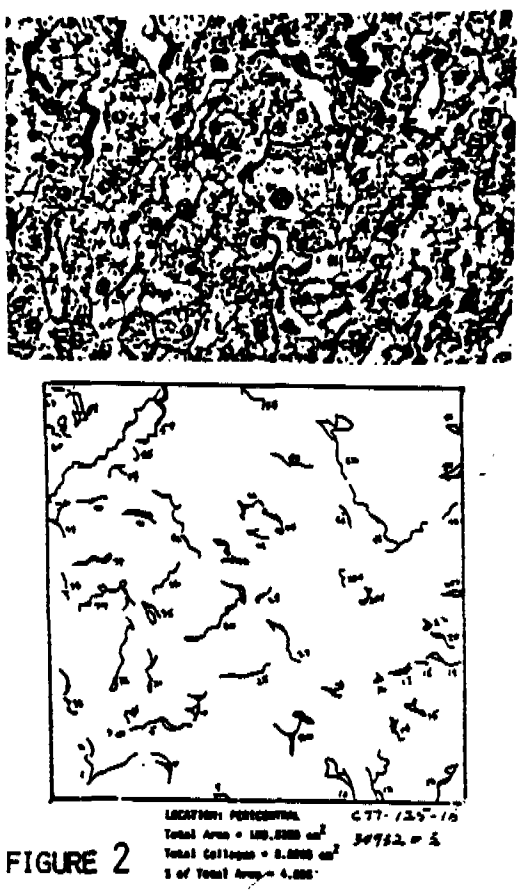


FIGURE 2

TABLE 1

REPRODUCIBILITY

Area Percent	Digitizer		Square Counting
	Inter assay	Intra Assay	
2.03	0.40	0.018	0.27
4.30	0.19	0.052	0.35
6.10	0.55	0.054	1.10
14.70	1.27	0.6075	2.95

(2) Later developments for computer quantitation promise to facilitate the execution of morphometric collagen determination. In this approach a video microscopic image is digitized to 4 bit precision, and held in digital video storage (Morphometrix-156 Image Analyzer). This digital image (Figure 3A, 3B) contains all density information on a 256 x 512 matrix. The amount of staining of a particular density can be rapidly quantitated by counting the percentage of a particular density which occupies an image. This approach allows rapid quantitation of the total biopsy and enables study of a large number of samples. A second advantage is the adaptability of this technique for 3-dimensional reconstruction and quantitation of liver fibrosis volume. Figure 3 illustrates a computer printout of a histological area and a drawing of the area morphometrically analyzed.

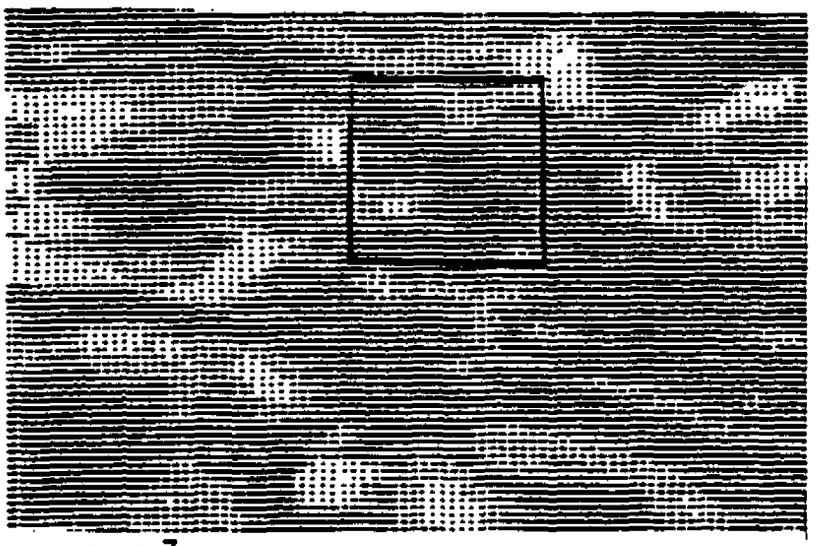


FIGURE 3A: Density printout of Figure 2, with 10:1 reduction (area in square corresponds to digital printout 3B).

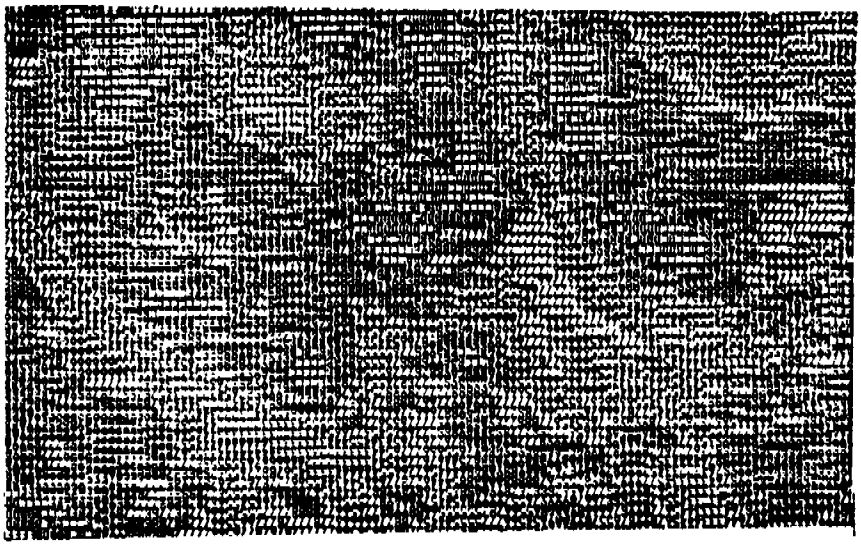


FIGURE 3B Digital densities of reticulin stained liver biopsy.

(3) Histological tissue was obtained for the study of liver collagen content at various ages from individuals dying of sudden death without historical, clinical or autopsy evidence of hepatobiliary disease, chronic congestive heart failure, excessive alcohol consumption, exposure to hepatoxins or infectious agents known to produce long term hepatic injury, drug abuse, or prolonged medical use of drugs known to be hepatoxic or potentially hepatoxic. The groups were divided into four age brackets: 16-30, 31-45, 46-60, 60 and older. Autopsy tissue was obtained from areas of the liver most frequently obtained in living patients by (A) percutaneous needle, (R, L) open wedge, and (D) transjugular biopsy. Needle and wedge samples were obtained from 4 areas of the liver as illustrated in Figure 4.

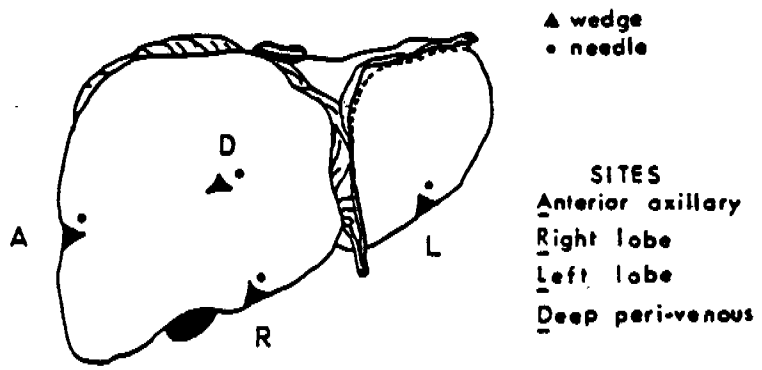


FIGURE 4

Two hundred and fifty three light micrographs derived from 30 tissue blocks from 8 normal individuals were studied. A Hewlett Packard 9864-A digitizer microcomputer was used to quantitate areas of trichrome stainable collagen. Collagen estimates varied from .2 to 6.1% (mean = 1.25) with considerable variation even in different biopsies from the same patient. Subcapsular biopsies have more collagen than deep biopsies; however, deep biopsies have more variation in collagen content. Collagen content appears to increase with age and more in midzonal regions than in portal areas (Table 2).

TABLE 2

PERCENT COLLAGEN RATIO IN THE VARIOUS LOBULE ZONES

AGE	PERICENTRAL / MIDZONAL	PERIPORTAL / MIDZONAL	PERICENTRAL / PERIPORTAL
15	11.8: 1	8.6: 1	1.4: 1
23	2.8: 1	1: 1	2.4: 1
40	2.8: 1	1: 1	2.3: 1
50	1.8: 1	1: 1	1.9: 1
51	1: 1	1: 1	1: 1

This increase appears to become detectable in the 4th and 5th decades (Figure 5).

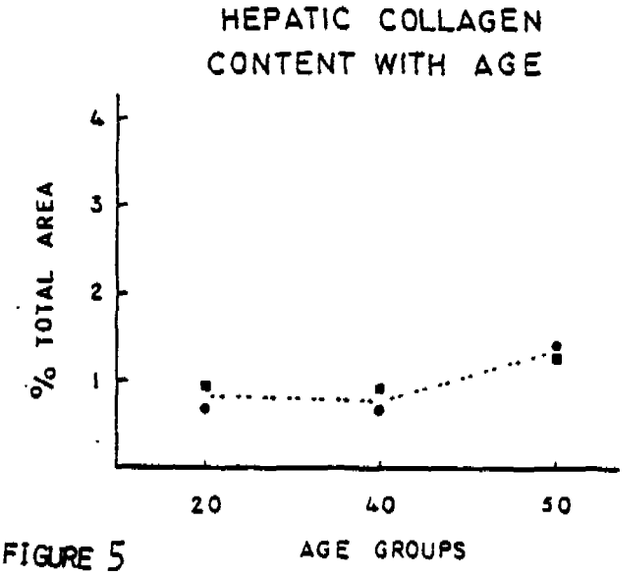


FIGURE 5

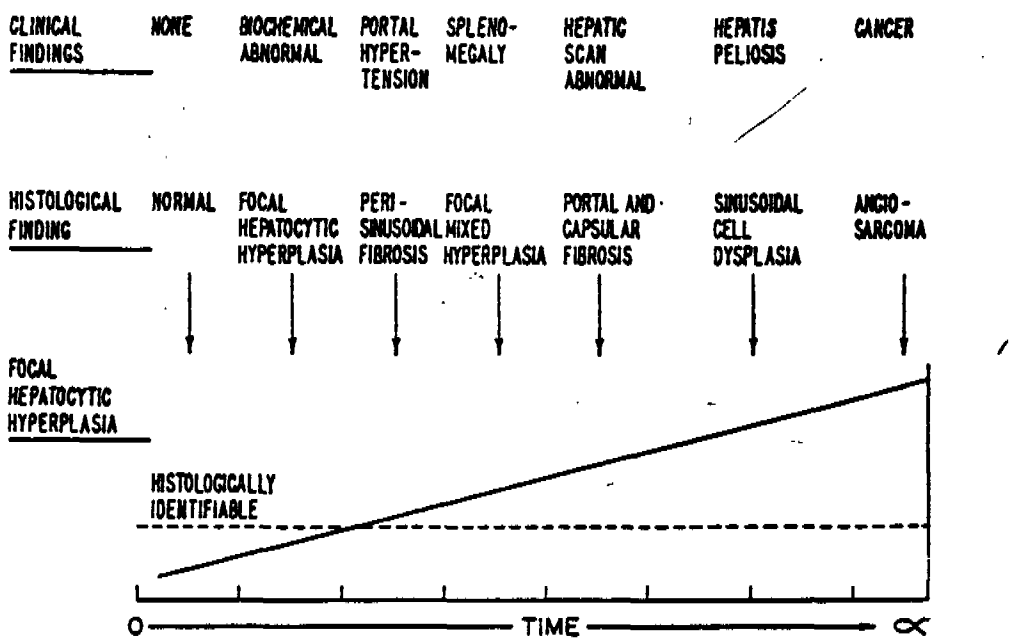
Differences in central, mid-zonal and portal collagen vary from 0.49 - 0.63 percent in the youngest age group and from 4 - 5.35 percent in the older age group. This histological data is consistent with the ICG clearances in normal adults which show a progressive decrease with age. The decreased functional capacity of normal aging liver may be due to an increased collagen deposition.

(4) Light microscopic assessment of liver tissue obtained from vinyl chloride workers with varying histological lesions was done. In order to substantiate the ability to identify early histological alterations indicative of chemical exposure, 93 liver biopsies from 78 individuals were investigated duplicatively in a double blind manner. These histological lesions included focal hepatocellular hyperplasia (Figure 6), focal mixed hyperplasia, sinusoidal dilitation (Figure 7), focal areas of increased reticulum (Figure 8), and focal subcapsular fibrosis (Figure 9). The progressive development of vinyl chloride liver lesions are illustrated in Table 3.

Thirty-five of these individuals were exposed chemical workers with hepatic screening test abnormalities, and 13 were exposed workers without hepatic screening test abnormalities who had had liver biopsies for non-liver related medical reasons. A group of 30 individuals who were not chemical workers, who had liver biopsies for nonhepatic-related illness during the same period of time at the same hospital, were compared. Twenty-three (48%) of the exposed workers had hepatic lesions consistent with exposure, 17 (35%) had only focal hepatocellular hyperplasia, and 6 (13%) had focal mixed hyperplasia or more advanced lesions.

TABLE 3

PROBABLE PHASE DEVELOPMENT OF VINYL CHLORIDE PRE-CANCEROUS LESIONS



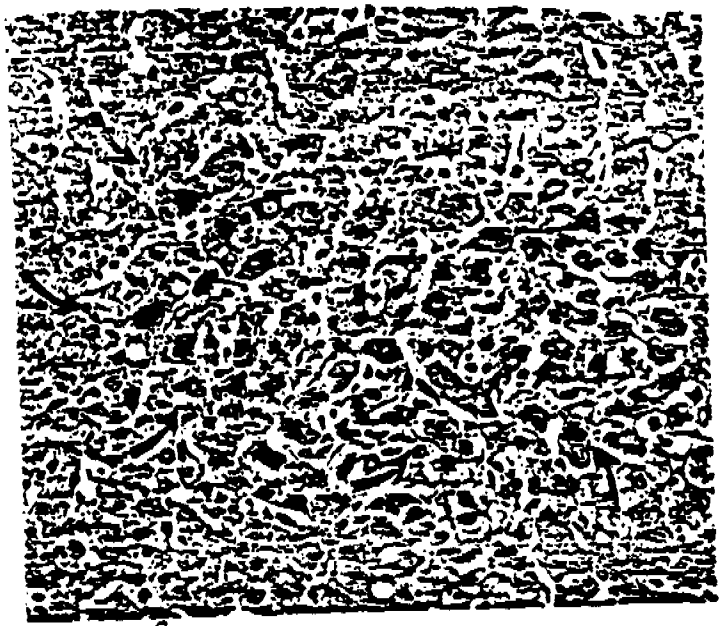


FIGURE 6

FOCAL HYPERPLASIA OF HEPATOCYTES WITH GREAT VARIATIONS IN THEIR SIZE AS WELL AS THEIR NUCLEI. THE LATTER ARE FREQUENTLY DOUBLE, POLYCHROMATOPHILIC OR VACUOLATED. H&E, 150x.

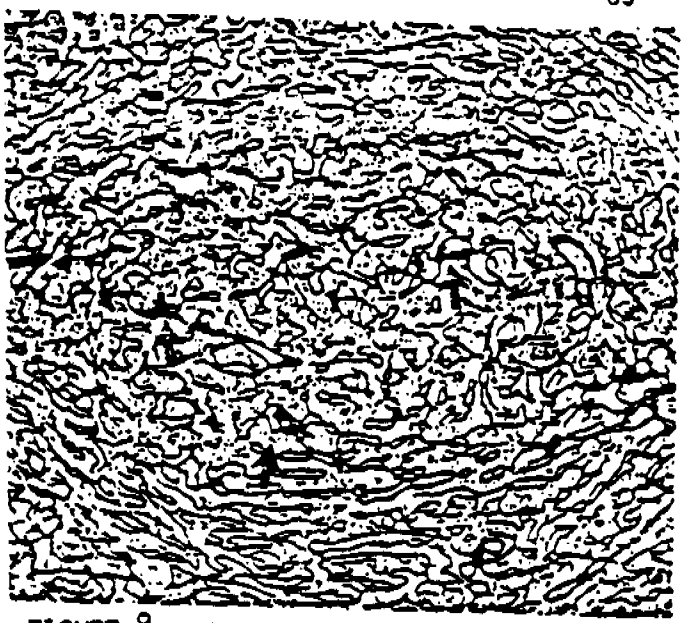


FIGURE 8

FOCAL INCREASE OF RETICULIN FRAMEWORK. SILVER IMPREGNATION, 100x.

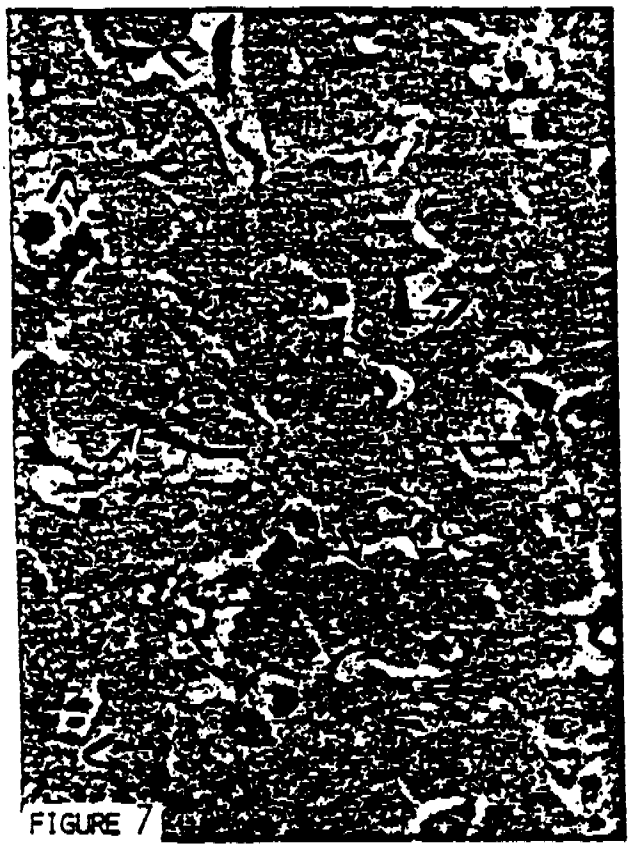


FIGURE 7

HYPERPLASIA OF SINUSOIDAL CELL (CURVED ARROWS) AND HEPATOCYTES (STRAIGHT ARROWS). (H&E, 100x)

MIXED HYPERPLASIA

FIGURE 9

FOCAL CAPSULAR AND SUBCAPSULAR FIBROSIS. ANILINE BLUE, 60x.

In contrast only 5 of the comparison group had similar findings. Four (13%) had focal hepatocellular hyperplasia, and 1 (3%) had focal mixed hyperplasia and sinusoidal dilitation. On subsequent biopsy this individual was found to have angiosarcoma and a history of using hair spray containing vinyl chloride as a propellent.

Ten individuals had 28 biopsies reviewed double blindly, and 10 individuals had 23 readings of the same biopsy; 21 of 23 (91%) duplicate readings and 27 of 28 (96%) multiple biopsy readings in the same individuals were identical. Only 18% of the individuals (3 of 17) had either duplicate and/or multiple biopsy readings which disagreed with their prior biopsy assessment. Focal hepatocytic hyperplasia, in addition to the previously described mixed hyperplasia, appeared to be the earliest identifiable change consistent with chemical exposure. These lesions can be consistently identified and are useful in screening chemical workers for evidence of chemical exposure.

Chemical exposure histories to 20 different occupational chemicals were analyzed with regard to the histological findings in the liver biopsy. Portal tract fibrosis, disruption of the limiting plate, and changes in the limiting plate of the portal area had a significant correlation in individuals identified as having histological features of chemical liver injury in contrast to those who had nonchemical liver disease. A larger percentage of individuals with histological evidence of chemical injury had higher levels of exposure to vinyl chloride (Figure 10). Changes in the sinusoidal areas also demonstrated the highest correlation with vinyl chloride's cumulative exposure rank months, but not with the cumulative exposure rank months of acrylonitrile, a comparison chemical.

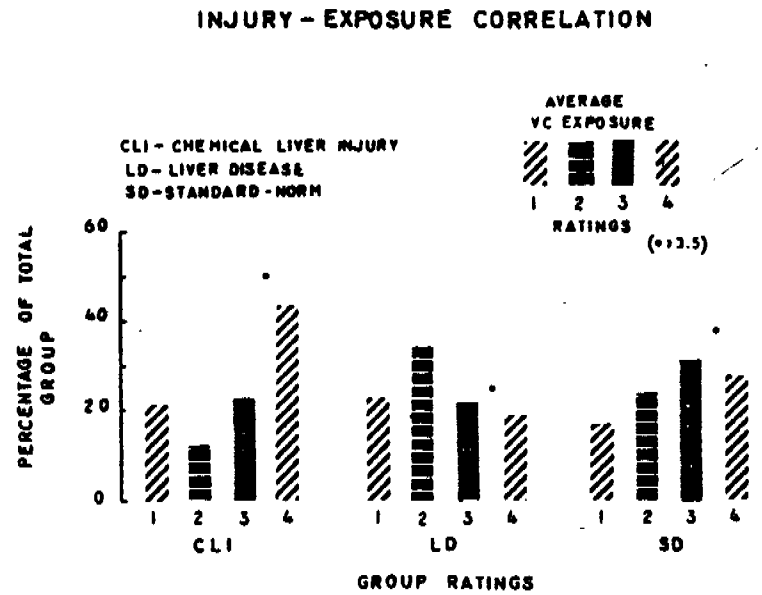


FIGURE 10

Relevance to Industry

These studies demonstrate some highly important and critical information regarding documentation of chemical exposure-induced liver injury:

(1) Rapid methods of quantitatively assessing total collagen content in human liver tissue have been applied. These can provide both retrospective as well as prospective analysis.

(2) Morphometric analysis of collagen can be performed for clinical purposes from human liver biopsies. This can be automated by using a computer for large volume work.

(3) The identification of increased liver collagen content with age demonstrates the need for better standards of "normality" before one can properly determine whether increased liver collagen is a reflection of chronic injury that may be work-related.

(4) There are histologically identifiable lesions that are consistent with chemical injury; these lesions can be separated from those histological findings of nonchemical liver injury. These characteristic chemical liver injury lesions are correlated with vinyl monomers, most significantly vinyl chloride. Therefore, at the present state of knowledge, one can reasonably determine the presence or absence of significant chemical exposure from the examination of human liver tissue. The present studies have not demonstrated any spontaneous progression in the earliest lesions, i.e., focal hepatocellular hyperplasia when found alone. . The present opinion is that lesion may simply reflect cellular adaptation to chronic exposure while mixed hyperplasia may indicate a higher risk of future cancer development.

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PROGRAM E

STUDIES OF VINYL MONOMER CHEMICALS AND THEIR METABOLITES USING CHEMICAL STRUCTURE AND SYNTHESIS IN THE DETERMINATION OF TOXICITY OF THESE AGENTS; Investigator - J. L. Wong

E1. Synthesis, Purification, and Utilization of Vinyl Chloride Metabolites in Mutagenicity and Carcinogenicity Studies

E2. Detoxification Studies of Vinyl Chloride and Its Metabolites

E3. Vinyl Chloride Metabolite Detection--Chloroacetic Acid

Background

The mutagenicity of vinyl chloride and its potential metabolites was studied in detail by a combination of chemical synthetic and microbiological assay techniques. We prepared and characterized the potential putative metabolites (chlorooxirane and four forms of chloroacetaldehyde), and Dr. Uldis Streips of Microbiology conducted the testing of these compounds in the Bacillus and Salmonella systems.

Since all literature methods for the preparation of chlorooxirane result in mixtures with contaminants such as chlorine, hydrogen chloride, ethylene oxide, polymers, etc., it was necessary to develop a method to prepare pure chlorooxirane.

In order to elucidate the intermediary metabolism of vinyl chloride, i.e., the metabolic activation and inactivation steps, to determine the significant end products of vinyl chloride in body tissues and fluids, the fate of chlorooxirane, the immediate P450 product of vinyl chloride, was studied using synthetic chlorooxirane to react with non-protein sulfhydryls.

Another prominent reactive property of chlorooxirane is its facile rearrangement to chloroacetaldehyde (CAA). In dimethylformamide at 25°C, it has a half-life of ~0.5 min in rearranging to CAA. Therefore, the mechanism of deactivation of CAA by cysteine was studied. The plausible cyclic product--3L-carboxy-2,3-dihydro-1,4-thiazine--may be the precursor of the urinary metabolites S-hydroxyethylcysteine and S-carboxymethylcysteine.

As a putative metabolite, CAA is well-known to react with adenine and cytosine nucleotides. We will demonstrate that CAA can also react with guanine derivatives at body temperature and physiological pH to yield covalent products. These products will alter the N-H hydrogen-bonding sites in the nucleic acid bonds, hence their formation in a biological host may be consequential in inducing the onset of neoplasia.

Since previous animal studies have not demonstrated chloroacetic acid in the urines of animals at low exposure levels but have been reported in humans at higher exposure levels, we intend to develop analytical methodologies which will help to verify this observation. Such studies may lead to a method of warning of environmental exposure to humans. The first target metabolite for analysis was chloroacetic acid.

Objective

1. To study the chemistry of vinyl chloride toxicity by identifying the intermediates of vinyl chloride metabolism and their reactions with cytoplasmic chemicals.

2. To determine the putative actions of the primary metabolites with regard to:

   a) the chemical reactions of metabolites, chlorooxirane (COR) and chloroacetaldehyde (CAA) with sulfhydryls (detoxification).

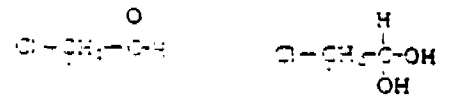
   b) reactions on nucleic acid constituents (mutagenesis and carcinogenesis).

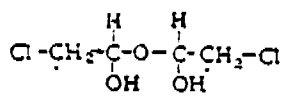
3. To develop analytical methods to detect a target metabolite--chloroacetic acid.

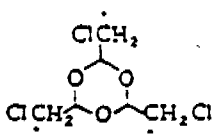
E1. Synthesis, Purification, and Utilization of Vinyl Chloride Metabolites in Mutagenicity and Carcinogenicity Studies

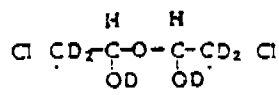
The detection study of the putative action of vinyl chloride is based on the hypothesis that such action comes from the modification of the nucleic acid materials by the primary metabolites COR and CAA. Although CAA is long known to react with nucleic acid bases such as cytosine and adenine to form the etheno derivatives, little is known about the reaction of CAA on the most reactive base, guanine. Our study of CAA with guanine base using analytical tools such as HPLC, GC-MS and FT-NMR have shown that the guanosine reaction is enormously complicated.

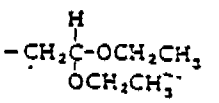
The hetero-bifunctional alkylating structures of 2-chloroacetaldehyde are shown below.











Studies of their reactivities have necessitated:

a. Synthesis of etheno-modified components of RNA and DNA— angular-etheno-guanine, linear-etheno-guanine, linear-etheno-guanosine, linear-etheno-deoxy-guanosine, etheno-adenine, etheno-adenosine, etheno-deoxy-adenosine, etheno-cytosine, etheno-cytidine, and etheno-deoxy-cytidine.

b. Synthesis of acidic hydrolytic products of etheno-modified bases— 2-aminobiimidazole, 2-formamidobiimdazole, E1-D-angular-etheno-guanine, and E1-D-linear-etheno-guanine.

c. Synthesis of alkaline decomposition products of etheno-modified nucleosides—2-amino-biimidazole nucleoside and 2-formamido-biimidazole nucleoside.

d. Synthesis of the deuterated analogues for detailed structural study—8-D-etheno-adenine, E1-D-etheno-cytosine, E1-D-angular-etheno-guanine, E1-D- etheno-adenine, and E1-D-linear-etheno-guanine.

The kinetics of guanosine with CAA at wide range pH value have been studied with careful pH control. The pK vs. pH plot showed that there are two plateaus, pH1 and pH8, and there is minimum at pH 4.5. From the product profiles, two different mechanisms can be suggested (Figure 1).

The putative action of vinyl chloride in respect to the physicochemical properties of the etheno derivatives of cytidine, adenosine, and guanosine is further studied. The ^{13}C-FTNMR spectroscopic properties are particularly revealing. By means of deuteration and proton undercoupling technique in acquiring the carbon-13 spectra, the various carbon chemical shifts are assigned and the ^{13}C-^{1}H coupling constants determined. From the latter data, the conformational preferences of the sugar moiety in the modified nucleosides are deduced as follows: (1) anti conformation for ethenocytidine

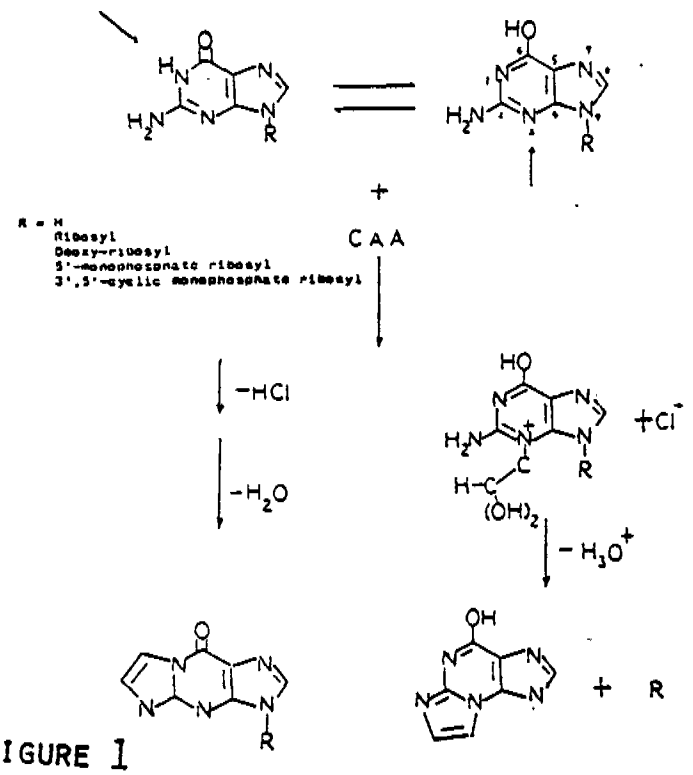


FIGURE 1

and (2) syn for etheno-adenosine. Since anti conformation of nucleosides is necessary for the formation and stability of the helix, the influence of the etheno bridge on the adenine nucleus in altering the ribosyl group to syn will impose considerable stress on the DNA chain which might result in biological damages.

E2. Detoxification Studies of Vinyl Chloride and Its Metabolites

A previous attempt by Gothe et al. to trap the putative metabolites COR and CAA in vitro from vinyl chloride with 3,4-dichlorobenzenethiol has led to the identification of the product as 3,4-dichlorophenylthioacetaldehyde. It was interpreted to be indicative of the formation of either COR or CAA. We have defined this experiment further. Thus, chlorooxirane in organic medium reacts slowly with 3,4-dichlorobenzenethiol to form 2-(3,4-dichlorophenylthio) acetaldehyde as 80 percent of the products. This reaction at room temperature takes about 7 days. At 60°C the reaction is completed in 21 hours giving the same products. (In both cases some disulfide $Cl_2Ph-S-S-Ph-Cl_2$ and other unidentified polymeric materials are observed.) The identity of the product was established by comparison of gas chromatography retention time with an authentic example, by PMR and by mass spectrum. In aqueous acetone (2:1) or aqueous acetonitrile (3:1), the reaction is much faster. As pH = 4, 55 percent conversion is observed in 15 min. which is the lifetime of COR under

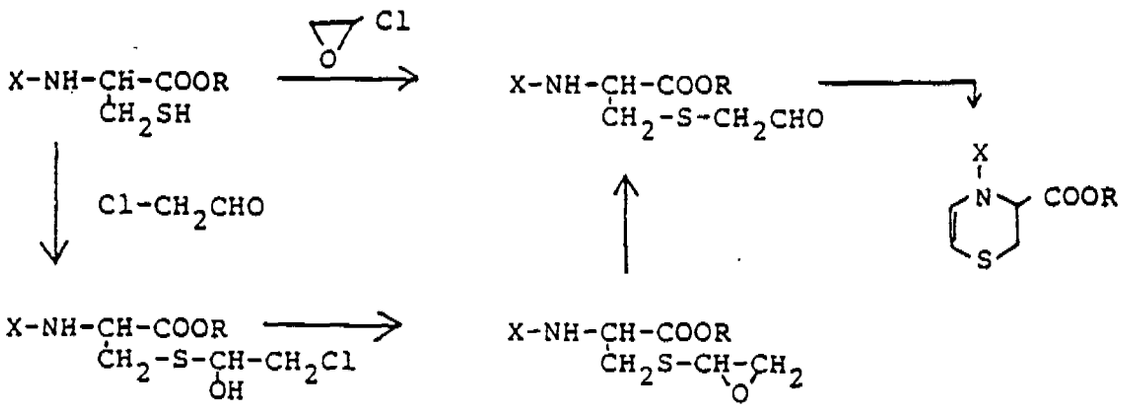
these conditions, and 80 percent conversion at pH = 7. The sole product identified from these reactions by high pressure liquid chromatography is the aldehyde indicated. On the other hand, chloroacetaldehyde in $CHCl_3$ gives another addition product, which was identified by PMR and IR and its facile reversion to the starting materials as $3,4\text{-}Cl_2Ph\text{-}S\text{-}CH(OH)CH_2Cl$. In aqueous acetone or aqueous acetonitrile, CAA did not react with the thiol within 1/2 hour, indicating its lower reactivity to the aromatic SH group compared to COR. These results, combined with the in vitro experiment by Gothe, have confirmed that chlorooxirane is an obligatory intermediate in the metabolism of vinyl chloride.

The detoxification of chlorooxirane was also studied in aqueous media at different pH's. N-Acetylcysteine was used as a typical cellular sulfydryl compound involved in detoxification. In aqueous solutions at pH4 and 7, their reaction was extremely fast at room temperature, yielding N-acetylcysteine-S-acetaldehyde. It was identified by ^1H and ^{13}C NMR and characterized as the 2,4-dinitrophenylhydrazone derivative. This aldehyde is probably the precursor of the urinary metabolites, S-2-hydroxyethylcysteine and thiodiglycolic acid.

The reaction of chloroacetaldehyde with N-acetylcysteine under controlled pH conditions in aqueous medium at 0°C produced an intermediate compound. Upon warming up the reaction mixture to room temperature, our previously identified thiazene was isolated as the final product. In order to elucidate the stepwise formation of the thiazene, N-acetylcysteine methyl ester was allowed to react with chloroacetaldehyde in chloroform at 0°C. The initial product, plausibly the hemithioacetal $H_3CO\text{-}CO\text{-}CH\text{-}(NHCOCH_3)CH_2S\text{-}CHOH\text{-}CH_2Cl$, eliminated HCl upon neutralization with aqueous sodium hydroxide to produce the corresponding epoxide $H_3COCOCH(NHCOCH_3)CH_2S\text{-}CHO\text{-}CH_2$. This structural assignment is supported by its PMR spectrum. Furthermore, when this epoxide was extracted into chloroform, it rearranged to the S-acetaldehyde $H_3COCOCH\text{-}(NHCOCH_3)CH_2\text{-}S\text{-}CH_2CHO$, identified by its pmr spectrum and by comparison with that formed from the reaction of N-acetylcysteine with chlorooxirane.

Even though chlorooxirane and chloroacetaldehyde eventually give the same final product with N-acetylcysteine, the rate of reaction and the intermediates in the two reactions are different. The chlorooxirane conjugates instantaneously with the sulfhydryl compound, while chloroacetaldehyde takes about 2 1/2 hours for a comparable reaction.

The comparative study of the reaction of chlorooxirane and chloroacetaldehyde with sulfhydryl compounds can now be summarized in the following reaction pathways with cysteines (unmodified, N-acetyl, and N-acetyl-methyl ester):



X=H, Ac
R=H, Me

Although both routes are converged to yield the cysteine S-acetaldehyde conjugate, their reaction rates are vastly different. In general, chlorooxirane completes its reaction within an hour while chloroacetaldehyde requires overnight. These chemical observations can be applied in (1) specific assays for the two putative metabolites in cellular studies of vinyl chloride, and (2) detoxification mechanism of cells exposed to the vinyl carcinogen.

E3. Vinyl Chloride Metabolite Detection - Chloroacetic Acid

The methodology includes gas chromatography and mass spectrometry. Gas chromatography results are as follows. Solid supports containing Carbowax and FFAP liquid phases have been used for direct analysis of carboxylic acids. Accordingly, 20 percent FFAP on chromosorb W passes chloroacetic acid at 200° with modest tailing of the peak. However, with this simple column, sensitivity is limited to concentration ranges of 0.2 - 2 mg/ml H_2O (200 - 2,000 ppm). Application of the improvements reported by Ackman (use of porous polymer solid support and use of formic acid in helium carrier gas) is expected to improve sensitivity by 100 fold.

Identification of chloroacetic acid by mass spectrometry was carried out. Chloroacetic acid can be identified by mass spectroscopy of water solutions as dilute as 0.02M (2mg/ml). The 2 doublets of peaks at m/e 49, 51 ($^{35}ClCH_2^+$ and $^{37}ClCH_2^+$) and 50,52 ($^{35}ClCH_3^{\cdot+}$ and $^{37}ClCH_3^{\cdot+}$) are distinctive because relative intensities within each doublet reflect the 76:24 isotopic ratio of $^{35}Cl:^{37}Cl$. Thus, the GC-MS combination technique should be most useful in the detection of the above.

Relevance of Industry

Broadly speaking, this is a study for early detection and prevention of industrial cancers. Our chemical methodologies (synthesis, structure, and analysis), aplied as an integral part of the multidisciplinary approach, contribute to elucidate specific molecular events in the effects of vinyl monomers on industrial workers. This information will form a rational basis for safer use of chemicals and design of preventive measures. Our molecular studies also provide the opportunity to develop useful marker(s) in the form of metabolites in the pathogenesis of chemical injury.

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PROGRAM F

ASSESSMENT OF ASSAYS FOR THE CARCINOGENIC POTENTIAL OF INDUSTRIAL CHEMICALS USING PROKARYTOIC AND EUKARYOTIC SYSTEMS. Investigators; U.N. Streips and G. Sonnenfeld

F1. Bacterial Assays for Testing Carcinogenicity and Mutagenicity of Industrial Chemicals.

Background

Our laboratory had instituted Salmonella reversion, Bacillus subtilis repair, and Bacillus subtilis forward mutations assays for screening the mutagenic potential of chemicals (1-3). For that reason we became an integral part, first, for B. F. Goodrich, then for the Manufacturing Chemists Association sponsored projects on industrial chemical risk assessment.

Objective

1. To screen a series of industrial and environmental - health-related chemicals for mutagenic potential.

2. To initiate with Dr. G. Sonnenfeld a rapid mammalian assay to better predict human-related bacterial chemical activity and carcinogenic potential.

Research Results

In our previous progress reports we have documented the work which delineated the involvement of chloroacetaldehyde and chlorooxirane as the probable active components of vinyl chloride-mediated carcinogenesis. In addition, we screened a multitude of other chemicals generated by Dr. John Wong (our collaborator in this study) or by other investigators in the university or industry (1-3). This is documented in Table 1. Recently, in collaboration with Dr. Gerald Sonnenfeld, a new, exciting, potential screen for carcinogens was developed (4,5). This involved the inhibition of interferon induction and was specific for several carcinogens (including chloroacetaldehyde) but not for non-carcinogenic analogues (including some mutagens). It should be illustrative of some of the molecular mechanisms of carcinogenesis attack on susceptible cells. We continue to develop this system even though our support from CMA has ended (6-8).

A screening laboratory (using microbial assays) for institutional service purposes is being maintained. We have proposed to the state of Kentucky that a regional chemical testing laboratory be funded to serve the industry and university needs in this part of the Commonwealth. That proposal is still pending.

TABLE 1

SUMMARY OF PERTINENT SUBSTANCES TESTED FOR MUTAGENICITY

		Salmonella	Subtilis
1.	Meta-chlorobenzoyl-cyclobutanecarbonyl peroxide	±	NR
2.	Benzoyl peroxide	NR	NR
3.	N-acetoxy-N-phenylacetamide	NR	NR
4.	N-cyclobutanecarboxyloxy-N-phenylacetamide	NR	NR
5.	bis-cyclobutane carboxyl peroxide	NR	NR
6.	Aflatoxin	+++	++
7.	Chloroethanol	NR	NR
8.	Benzo(a)pyrene	++	+
9.	4-nitro quinoline-1-oxide	+++	++
10.	Chlorocrotaldehyde	+++	++
11.	Epichlorohydrin	++	+
12.	Chloromitrane	+++	++
13.	Butane dioxoxide	++	+
14.	Styrene oxide	++	+
15.	1,4 epoxide butane	±	NR
16.	bis(Beta-chloroethyl)phenyl phosphate	NR	NR
17.	Beta chloroethyl phosphate (bis cyclohexylamine salt)	NR	NR
18.	Lung aspirates from smokers with cancer	±	NR
19.	Lung aspirates from smokers without cancer	±	NR

NR = no reaction + = mutagenic +++ = extremely mutagenic

± = borderline mutagenicity ++ = strongly mutagenic

To further elucidate molecular mechanisms of carcinogenesis, in collaboration with John Wong, we inititated a study of DNA-interaction with carcinogens, and with Ron Doyle, control of cell division processes in bacteria. Progress in this area has allowed us to secure a grant from the National Science Foundation to carry on with the work. Soon, we will be able to describe the cellular controls which maintain proper cell division in bacteria, then relate these findings to mammalian cells. At that time, we will be able to examine carcinogen attack on these processes and determine which events lead to loss of cell division control and neoplastic growth.

Support from the CMA has been instrumental in generating and maintaining research activity in our laboratory relating to carcinogenesis and cell cycle events. We deeply appreciate this support.

Relevance to Industry

With the tremendous number of chemical formulations generated yearly, a battery of rapid screens are crucial for examining the environmental and health-related impact of these chemicals. A regional laboratory can do such screening effectively and economically as shown by the results previously described. In addition we have demonstrated that such research also leads to innovation in terms of new assays (the interferon assay), and related research (the studies on cell division). Industrial support also has led to other granting. Valuable mutagenesis information has been provided to industry regarding crucial chemicals. In turn, industry has provided us with the opportunity to expand and solidify our research efforts. The interaction has been optimal.

F2. Preliminary Studies on the Use of Inhibition of Interferon Induction as an Indicator of Mutagenicity and Carcinogenicity of Chemicals

Background

Interferon, which was originally described as an antiviral agent, has since been shown to have several other activities, including an antitumor activity (9). Clinical trials are now in progress to determine the efficacy of interferon as an anti-cancer agent (10).

We became interested in the interactions of carcinogens, or cancer-causing substances, and interferon. Early studies by DeMaeyer and others suggested that there was indeed an interaction between carcinogens and interferon induction (11-13). Pretreatment of rat embryo fibroblasts with the carcinogens triethyleneamine, 4-nitroquinoline-N-oxide, benzo-(a)-pyrene, and 3-methylcholanthrene all inhibited the induction of alpha-beta interferon by viruses, without affecting the gross viability of the cultures or the replication of the viruses (11-13). Several poorly or noncarcinogenic analogs, such as benzo-(e)-pyrene, had no effect on the production of interferon (11-13). The effect was observed after only one day of incubation of very small amounts of carcinogen with the tissue cultures. In addition, Hahon and co- workers have shown that incubation of cell cultures with various forms of aflatoxin also inhibited the induction of interferon in the cultures (14). In this case, the degree of inhibition correlated with the in vivo carcinogenic potential of the aflatoxin form (14). Asbestos fibers and coal dust have also been reported to inhibit interferon induction in tissue culture (15,16). In vivo, injection of methylcholanthrene into mice inhibited the induction of alpha-beta interferon (17). These results created an interest in our laboratory to further study this phenomenon.

Objective

1. To repeat the early studies to determine if carcinogen pretreatment inhibited interferon induction.

2. To begin studies to determine if effects on interferon induction could be used as a reliable indicator of carcinogenic potential of chemicals.

3. To study the mechanisms of the observed effects.

Research Results

Our first step was to establish the system in a mouse culture model, since this was more appropriate to current interferon technology. Mouse embryo tissue cultures were prepared, treated with carcinogen or analogue for 24 hr., washed with fetal bovine serum-containing medium and with serum-free medium to help remove carcinogen, challenged with an interferon inducer (either Newcastle disease virus or poly I:C), and then assayed for antiviral activity. Earlier data was able to be duplicated and those findings extended. Several known carcinogens including 7,12-dimethylbenz-(a)-anthracene, benzo-(a)-pyrene, 2-aminofluroene, aflatoxin-B1 and the #4 fraction of tobacco smoke condensate all inhibited interferon induction (5). Styrene oxide, an important industrial chemical which was positive in the Ames Salmonella assay but negative in all Bacillus assays done to date (11), also inhibited interferon induction. Ethyl methanesulfonate (EMS) and methyl methanesulfonate, differ with respect to their carcinogenic potential as determined by tumor induction (18,19). MMS is a mutagen and highly carcinogenic, while EMS is a potent mutagen but rarely carcinogenic. In our assay, MMS strongly inhibited alpha/beta interferon induction, while EMS had no effect. Additional studies in our laboratory extended these findings to the potent carcinogen chloroacetaldehyde and its noncarcinogenic analogues chloroethanol and chloroacetic acid (4). Only chloroacetaldehyde inhibited interferon induction.

An additional finding of interest was the apparent requirement for activation of carcinogens before they could inhibit interferon induction. In a preliminary study, we were able to show that addition of reduced glutathione to our cultures blocked the inhibition of interferon induction by benzo-(a)-pyrene (4). Reduced glutathione can block the active metabolites of carcinogens (20), which suggested that these active metabolites were required for the effects of benzo-(a)-pyrene on interferon induction.

Relevance to Industry

We believe these studies to be highly relevant to the chemical industry because: 1) the studies yielded information on the effects of toxic chemicals on the interferon system, an important body defense against cancer, and 2) the studies may eventually lead to a new assay system for discriminating between carcinogenic and noncarcinogenic chemicals.

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PROGRAM G

THE STUDY OF TISSUE ANTIGENS AND ANTIBODIES IN THE DETECTION OF VINYL CHLORIDE INJURY; Enrique Espinosa, M.D.

G1. The Study of Tissue Antigens from Liver Tumors, Angiosarcoma, and Hepatomas.

G2. Circulating Antigens and Autoantibodies in Vinyl Chloride-Associated Liver Disease

Background

In hepatic fibrosis and angiosarcoma associated with vinyl chloride exposure of industrial workers, manifestations of the disease could not be detected in most cases until the process was far advanced (1). Normal values of liver function tests were reported in a case with significant vinyl chloride hepatic fibrosis (2), and only a small percentage of workers of a plant unit where 7 cases of liver angiosarcoma were diagnosed had abnormal blood screening tests (3). Thus, conventional liver function tests do not appear to be sensitive indicators of vinyl chloride liver disease. Development of more sensitive and specific methods for detecting the disease in early stages would be of great importance. An approach to this may be provided by antigenic studies in view of the tissue antigenic modifications which are known to occur in neoplasia. For example, in human carcinoma, loss of antigens have been reported in squamous cell carcinoma (4,5) and in ovarian carcinoma (6,7), loss of the ABH blood group isoantigens in some solid tumors (8,9), and of HL-A isoantigen in lymphoma (10). Also, tumor-specific transplantation antigens have been demonstrated in a number of experimentally induced tumors (11-14) as well as tumor-associated antigen in spontaneous tumors in man (e.g. 15-18). In such an antigenic study of liver angiosarcoma the question arises whether the fibrotic and angiosarcomatous livers contain antigens that are quantitatively or qualitatively different from those present in normal tissue and whether such changes could stimulate an immunologic response. If such hypothetical changes are demonstrable and proved to be specific, they may be of use in diagnosis of vinyl chloride related liver disease.

Objective

1. To test for liver antigenic changes in a) angiosarcomatous liver tissue, b) chemically-induced hepatomas, and c) cultured human liver carcinoma cells.

2. To test for abnormal serum antigens and autoantibodies in patients with histories of vinyl chloride exposure including individuals with liver angiosarcoma, liver dysfunction with fibrosis, and with normal liver function tests. Antigens studied included liver specific antigen (LSA) (19), and F-antigen (20,21), tissue antigens of wide organ distribution (22,23), and bile antigens (24). Autoantibodies were to nuclei, mitochondria and smooth muscle. Patients' sera were also tested by immunofluorescence for possible reactivity with rats exposed to vinyl chloride (25).

Research Results

1. Liver antigenic changes

a) angiosarcomatous liver tissue

Liver angiosarcomatous tissue was analyzed in this work for presence of neoantigens, normal tissue antigens and tumor bound immunoglobulins. Several normal tissue antigens were found by immunodiffusion to be present in the tumor. These included the previously described liver-specific antigen, (LSA) (19) and antigens which are shared with several tissues (22,23). In addition, a tumor associated protein antigen and a glycoprotein liver antigen found to be absent in angiosarcoma were identified and characterized (25).

Tissue distribution of liver angiosarcoma related antigen is shown in Table 1. Preparations containing tumor associated antigen were found to inhibit lymphocyte transformation tests in vinyl chloride workers suggesting that these workers have a decreased lymphocyte response to these preparations (26). Further, antigen coagulation Factor VIII was detected in the angiosarcoma cells, pointing to an endothelial cell origin of the tumor (25). Finally, immunoglobulin G bound to the angiosarcomatous tissue was demonstrated by immunofluorescence and elution experiments suggesting antibody stimulation by the tumor (25).

TABLE 1

TISSUE DISTRIBUTION OF LIVER ANGIOSARCOMA-RELATED ANTIGEN

TISSUE EXTRACT USED AS ABSORBENT	ANGIOSARCOMA-RELATED ANTIGEN*
Liver angiosarcoma	+
Liver	-
Kidney	-
Spleen	+
Lung	+

Antiangiosarcoma serum was absorbed with lyophilized tissue extracts, 100 mg/ml, and tested against angiosarcoma extract.

*Presence of antigen indicated by +.

b. chemically-induced hepatomas

Our finding of an antigen missing in VC-related liver angiosarcoma stimulated further studies of antigenic deletion in chemically-induced hepatomas and in cultured human liver carcinoma cells.

In studies performed with the fast growing and undifferentiated chemically-induced Morris hepatoma 7777, 2 liver antigens were found to be absent. These antigens were characterized and partially isolated. In studies of their occurrence in other tissues, one of these antigens (Antigen I) was shown to be present in kidney and spleen in addition to liver. The second antigen (Antigen II) was detected only in liver. Antigen II was found unrelated to liver-specific F-antigen, differing in a number of properties and in immunologic reactivity.

In studies of their subcellular distribution in normal liver, Antigen I appeared localized in cytosol (54%) and mitochondrial (38%) fractions. Antigen II was about equally distributed in cytosol, mitochondria and nuclei fractions with little amounts in microsomes. Antigen I has a electrophoretic mobility in immunoelectrophoresis close to that of serum gamma-globulins and Antigen II to that of serum alpha-globulins. The two antigens were completely inactivated with Pronase indicating that both antigens are proteins or protein associated. Both antigens were relatively thermolabile; they were partially inactivated following incubation at 56°C and completely inactivated at higher temperatures. Both antigens were completely inactivated when incubated in pH buffer lower than 3.5. In Sephadex-G200 gel filtration, Antigen I behaved like a protein of approximately 51,000 Daltons, using as standards serum albumin, ovalbumin, chymotrypsinogen and ribonuclease. The molecular size of Antigen II (determined on a Bio-gel A5m column) was approximately 240,000 Daltons, using aldolase, catalase and ferritin as markers.

The two antigens were found in the more differentiated and slower growing hepatomas 5123tc and 9618A at about the same concentration as normal liver. The fact that hepatoma 7777 is the fastest growing and least differentiated of the tumors studied suggests a possible functional relationship between the absent antigens and these properties (28). These antigenic deletions may be used as indicators in the early detection of liver tumors and in the evaluation of the rate of growth, histologic differentiation and metastatic properties of such hepatomas.

Another liver constituent which may serve as a sensitive indicator of chemically-induced liver tumors, liver-specific F-antigen, was studied. In studies on the behavior of this antigen in Morris hepatomas, it was found that different types of these chemically-induced tumors have quite different levels of F-antigen.

F-antigen appeared to be absent in the fast growing hepatoma 7777. In the slow growing hepatoma 9618A, the concentration was very low ranging from less than 2% to 10% of the normal liver concentration. The medium growing hepatoma, 5123tc, highly metastatic, had about twice the concentration as

normal liver. F-antigen of hepatoma 5123tc and of normal liver were found localized in the cytosol subcellular fraction and were determined to be immunologically identical and to have equivalent electrophoretic mobility and molecular weight (29).

Since the antigen was undetectable in the fast growing hepatoma and undetectable or very low in the slow hepatoma, the level of F-antigen does not appear to correlate with the rate of growth of these tumors. A possible relationship between metastatic properties and F-antigen is now being considered because the hepatoma with the increased concentration of F-antigen was by far the most highly metastatic. This may prove useful in treatment of tumors.

c) cultured human hepatoma cells

In studies on cultured human liver carcinoma cells (after establishing optimal conditions required for the maintenance in serum free media of PLC/PRF/5 human liver carcinoma cells) it was determined that these hepatoma cells, similar to the experimental Morris hepatoma 7777, are deficient in liver-specific F-antigen. Nevertheless, these cells, like normal liver cells, produce serum albumin, fibrinogen, transferrin, alpha-1 antitrypsin and alpha-2 macroglobulin as shown by immunodiffusion (30,31) and immunofluorescence (32). These data add further support to the clinical observation that tissue antigens are more useful for treatment and follow-up care than screening and early detection, and that antigenic deletions may prove useful in early screening.

2. Abnormal Serum Antigens and Autoantibodies

Serum autoantibodies to nuclei, mitochondria and smooth muscle were negative in all patients examined. In addition, serum from these patients did not show reactivity with liver from rats exposed to vinyl chloride. Liver-specific antigen LSA (19), bile antigens (24) and other tissue antigens (22, 23) associated with liver damage were not detected in these patients (25).

Relevance to Industry

These studies are relevant to chemical industry in view of the evidence provided showing important changes in the levels of angiosarcomatous liver-antigenic components in association with vinyl chloride exposure. Deletion of liver antigens were also demonstrated in chemically-induced hepatoma. In addition, these observations may eventually prove useful in the development of new approaches for screening of chemical-associated tumors.

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PROGRAM H

THE USE OF ISOLATED MAMMALIAN LIVER CELLS FOR THE STUDY OF CHEMICAL MONOMER METABOLISM; Investigator, R. C. Feldhoff

H1. Isolation of Mammalian Liver Cells for the Study of the Metabolism of Chemical Monomers

H2. The In Vitro Study of Albumin Synthesis by Liver from Human Biopsies

Background

Presently there is no simple or even complex method to assess the functional capability of the human liver to handle exposure to various chemicals and/or their metabolites. In general, the rat has proven to be the most useful experimental animal for studying hepatic metabolism. Perfused liver slices and isolated hepatocyte systems have been developed to better study the interrelationship and to control the myriad processes of liver metabolism. In the perfused liver the architecture of the organ is preserved but separated from other body influences. Newer in situ techniques allow the liver tissue to never be without a supply of oxygenated red cells. The same technique can also be used to prepare isolated liver cells enzymatically.

The intact liver consists of at least four cell types, whereas isolated liver cells are nearly homogeneous in population. These cell suspensions provide technical advantages in that a number of conditions can be tested at one time and many uniform serial samples can be collected. Preliminary investigations were therefore done to determine whether the human liver biopsy material can function in a manner equivalent to liver slices and whether these tissues would demonstrate similar findings to those of isolated cell suspensions. If this technique were made functionally feasible it could then be used to study the metabolism of xenobiotics using viable human liver tissue in an in vitro manner.

Objective

1. To determine if an in vitro adaptation could be developed which would utilize animal/human biopsy material for assessing the functional capability of human liver cells. Protein metabolism has been used as the standard indicator and criteria for comparison

2. To determine the usefulness of this technique in human biopsy material obtained for routine medical purposes via percutaneous and transvenous approaches.

Research Results

Our first step was to verify the clinical and biochemical conditions needed for handling human liver biopsy material obtained during routine medical procedures. Appropriate assay conditions are being determined to assess the functional capability of the liver at the time of the biopsy to synthesize retained and secreted protein. At the same time, the biopsy's histopathology will be assessed and correlated with the biochemical data. The technique used to study both protein synthesis and secretion is shown in Figure 1. The histopathology is illustrated in Figure 2.

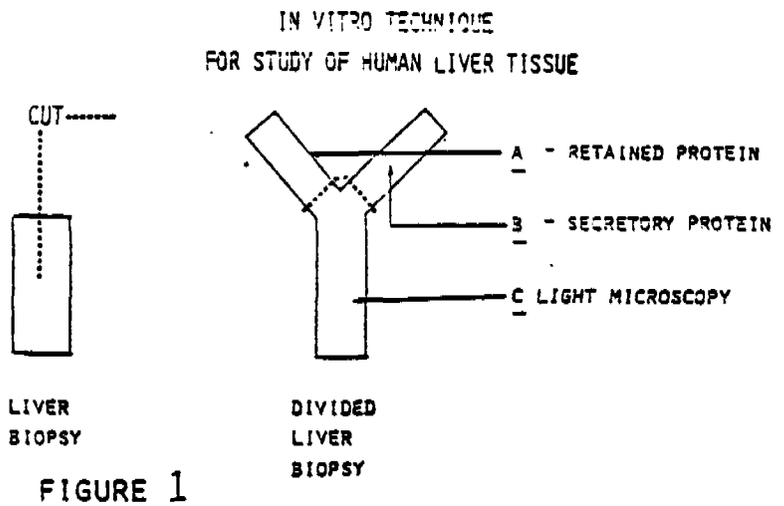

FIGURE 1

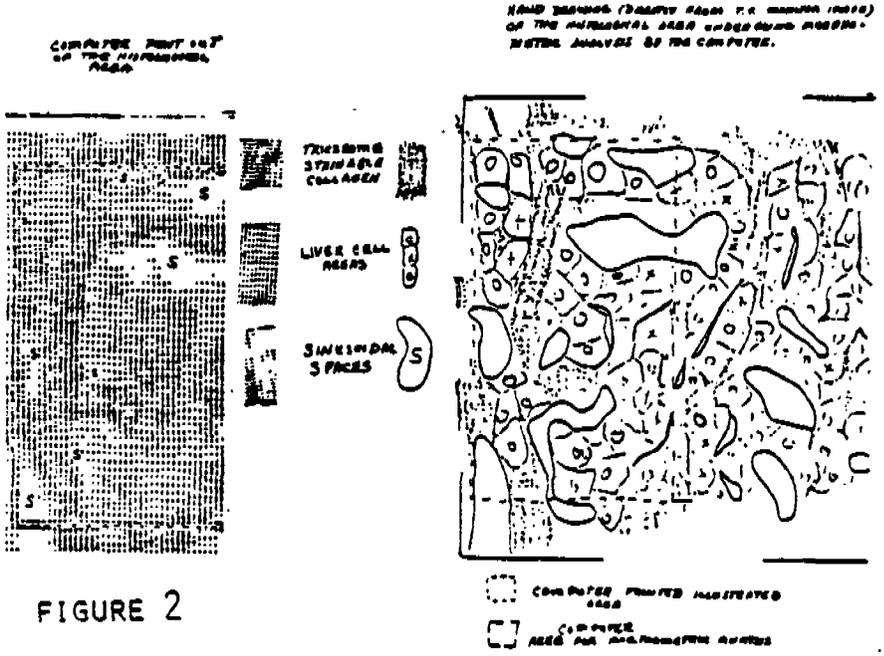

FIGURE 2

The initial experimental studies utilizing liver perfusion and the isolation of hepatocytes techniques provided the hepatocytic and nonparenchymal cells for Du and Tamburro's study of the oxidizing and detoxifying capabilities of these cells. Subsequent experiments have been used to quantitate the intracellular albumin synthesis, the rate of albumin secretion by the use of specific antibody fractions available for human and rat albumin. Initial results are illustrated in the rocket immunoelectrophoresis patterns from rat liver biopsy study (Figure 3) and the amino acid analysis is seen in Figure 4. The technique for liver cell identification after cell separation by the perfusion technique is illustrated in Figure 5.

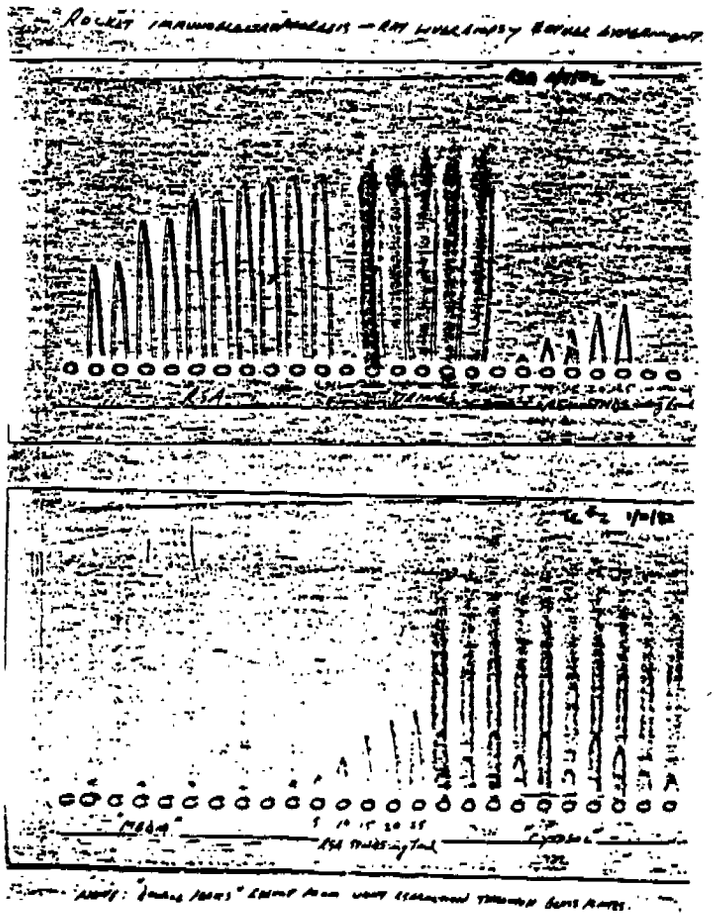

FIGURE 3

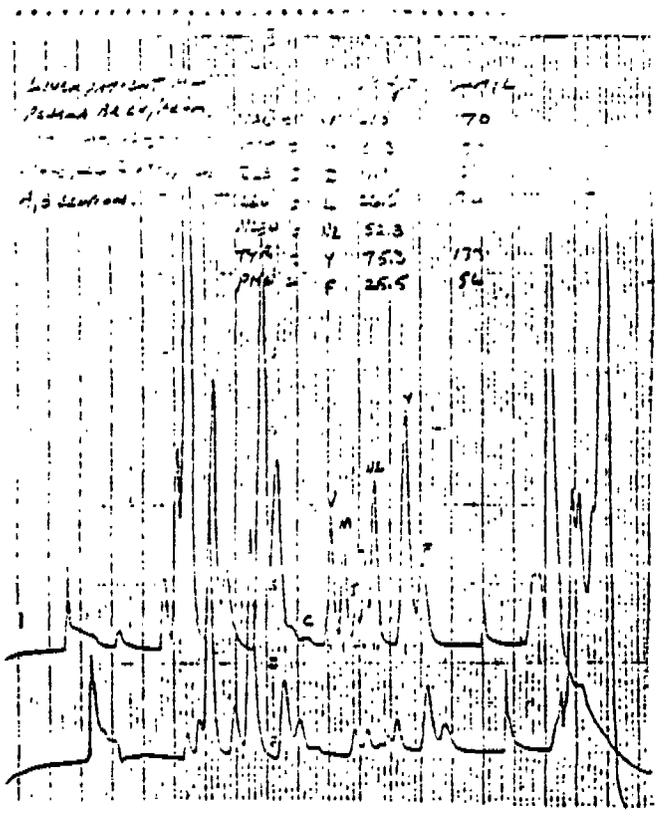

FIGURE 4

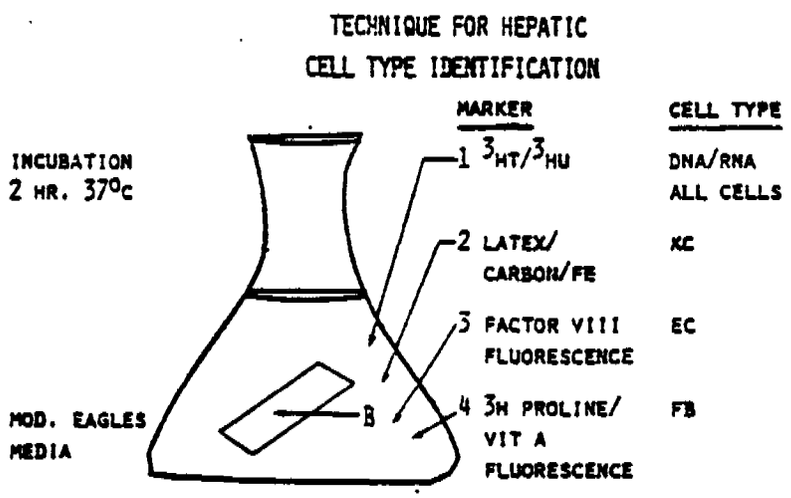

FIGURE 5

Relevance to Industry

Although this study was a last year addition to our initial proposal, it has provided the preliminary exploration into the adaptation of highly sophisticated laboratory techniques to human tissue in an in vitro system. While the work of these studies is still developmental, it provides promise for future clinical use. Such use could include determination of an individual liver's ability to oxidize and detoxify xenobiotics. The degree and nature of liver cellular injury or impairment could be determined in a quantitative biochemical fashion, and could be directly correlated with

morphological findings. These techniques could be also used to determine the degree of biochemical adaptations of the human tissue in individuals who have been exposed to xenobiotics. Finally, these methods could, on sequential biopsy, provide clinical information regarding whether these alterations persist or revert back to a normal state.

Although this is a very futuristic attempt to adapt presently developed methodology, it does accurately reflect the direction in which clinical research should be directed in order to better assess the human capability of sustaining environmental changes.

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THE STUDY OF TISSUE DISPOSITION OF INDUSTRIAL CHEMICALS: THE VINYL CHLORIDE EXAMPLE; Investigators - W.J. Waddell and C. Marlowe

II. The Use of Whole Body Autoradiography in Specific Tissues. Localization of Accumulated and Retained Industrial Chemicals and Their Metabolites

Background

Vinyl chloride has been classified by regulatory agencies as a carcinogen. Chronic exposure of workers to this carcinogen has resulted in angiosarcoma of the liver, as well as other tumors (Creech and Johnson, 1974). Several types of tumor also are observed after vinyl chloride exposure to animals (Maltoni and Lefemine, 1974). How or why these various body sites developed tumors was unclear. The need for methods which more accurately identify the sites of localization of chemical agents and their metabolites has obvious importance. The use of whole body autoradiography to identify the ultimate location of radioactively tagged chemicals was studied utilizing ^{14}C-vinyl chloride.

Objective

1. To determine the sites of localization and retention of the metabolites of vinyl chloride in mice.

2. Identification of the tissues of localization should help elucidate the mechanism of carcinogenic action of vinyl chloride.

Research Results

The sites of localization of radioactivity in tissues of the mouse following exposure to ^{14}C-vinyl chloride were studied by the technique of whole-body autoradiography (Waddell and Marlowe, 1977). Male CD-1 mice purchased from Charles River, weighing 25-31g., were exposed to ^{14}C-vinyl chloride in room air in a sealed chamber for 3 hours. The radioactive vinyl chloride was synthesized from ^{14}C-ethylene dichloride (New England Nuclear, Lot α1194-143, specific activity 3.2 mCi/mmole) immediately prior to exposure by the method of Wagner et al. (1975). The initial concentration of the vinyl chloride was 50 ppm with a specific activity of 1.84 mCi/mmole. At the end of the 3 hour exposure of the mice to ^{14}C-vinyl chloride the mice were removed to room air. Twenty minutes, 1, 3, 9 or 24 hours after removal of the mice to

[illegible] a mouse was briefly anesthesized with ether and sacrificed by freezing [illegible] in dry ice/hexane bath at -75°C.

The preparation of the radioactive vinyl chloride, exposure of the mice to this isotopic gas, and the sacrifice of the mice were done in collaboration with Dr. William T. Stott at Dow Chemical in Midland, Michigan. All remaining procedures were performed at the University of Louisville.

Whole-body sagittal sections of the mice, 20μ- and 40μ- thick were taken onto Scotch tape at -20°C. After freeze-drying, these sections were placed against Kodak AA X-ray film and allowed to expose in light-tight containers at -14°C for 6 to 39 weeks. The sites of localization of all nonvolatile metabolites of vinyl chloride were visualized in the developed X-ray film (autoradiograph) and reveals the in vivo disposition of these metabolites at the time of sacrifice of the mouse. These procedures for whole-body autoradiography, first described by Ullberg in 1954 do not allow thawing or contact with any solvents. Thus, there is no translocation nor loss of radioactivity. A complete description of these procedures have been published previously (Waddell and Marlowe, 1977).

Representative autoradiographs from each mouse were used as negatives to produce the prints included with this report. Therefore, white areas in the prints represent the sites of radioactive accumulation of the nonvolatile metabolites of ^{14}C-vinyl chloride.

The highest levels of radioactivity in the mice sacrificed 20 minutes and 1 hour after removal from the ^{14}C-vinyl chloride environment were observed in liver, pancreas, kidney, intestinal contents, urine and bile (Figures 1 and 2). Additional sites of localization of radioactivity in the 1-hour animal were thymus, thyroid and seromucous glands (Figure 2).

The concentration of metabolites in the organs of excretion decreased continually over the 24 hour period (Figures 3-5). By 9 hours after removal from the vinyl chloride, thymus, Harder's gland, epithelium of the esophagus and intestine, urine and sublingual gland retain the highest levels of radioactivity; moderate concentrations of radioactivity were observed in liver, kidney and intestinal contents (Figure 4). After 24 hours the primary organs of retentions of radioactivity were thymus and Harder's gland; some radioactivity could be visualized in liver and in esophageal and intestinal epithelium (Figure 5). Figure 6 shows the relative concentration of nonvolatile metabolites in the cortex and medulla of the thymus at the earliest and latest time intervals studied. Twenty minutes after removal from the vinyl chloride, the concentration of radioactivity in the thymus was only slightly increased above that of blood (Figure 1 and 6); however, by 1 hour the thymus showed the highest uptake of radioactivity in the body (Figure 2) and remained the highest throughout the 24 hour period (Figures 2 - 6).

^{14}C-VINYL CHLORIDE; 20 MIN AFTER REMOVAL

Figure 1: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 20 minutes after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE; 1 HR AFTER REMOVAL

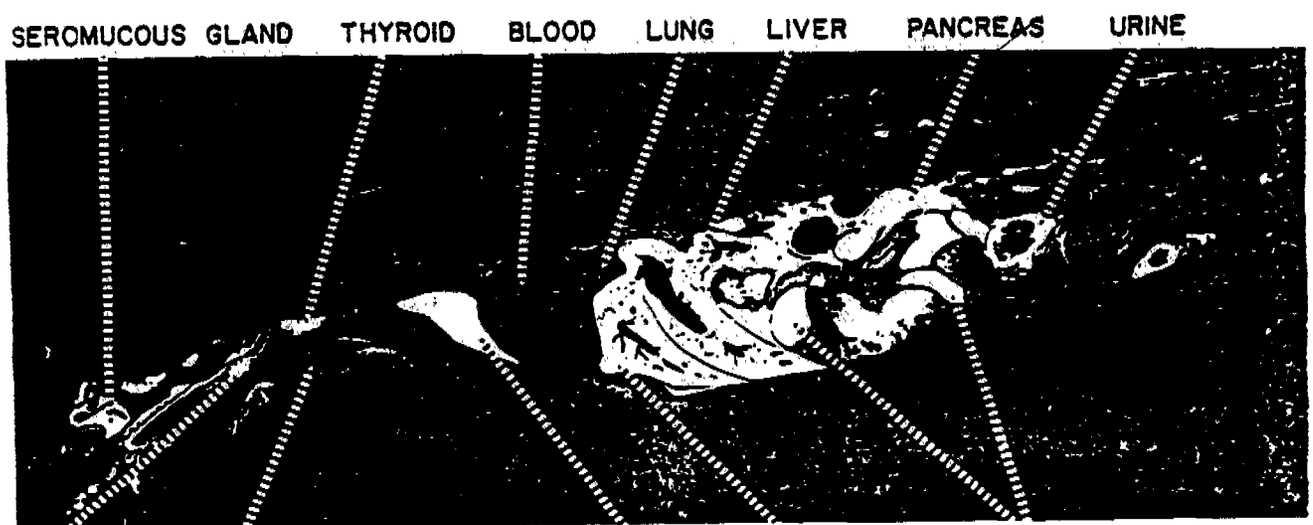

Figure 2: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 1 hour after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORICE; 3 HR AFTER REMOVAL

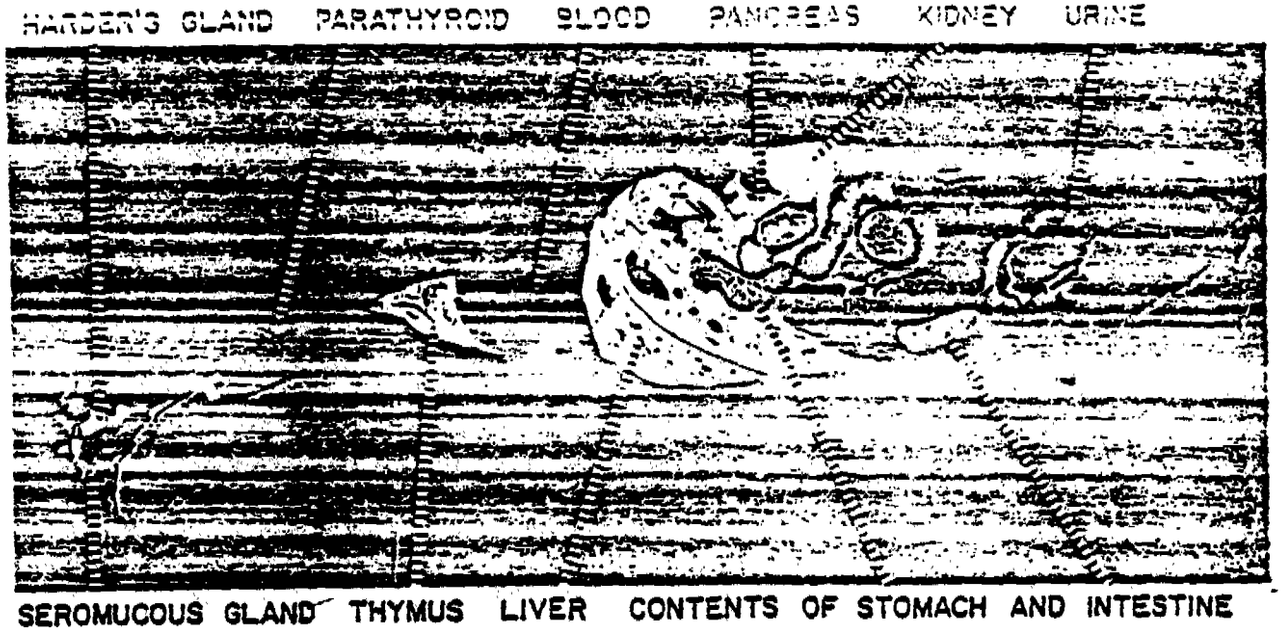

Figure 3: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 3 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE; 9 HR AFTER REMOVAL

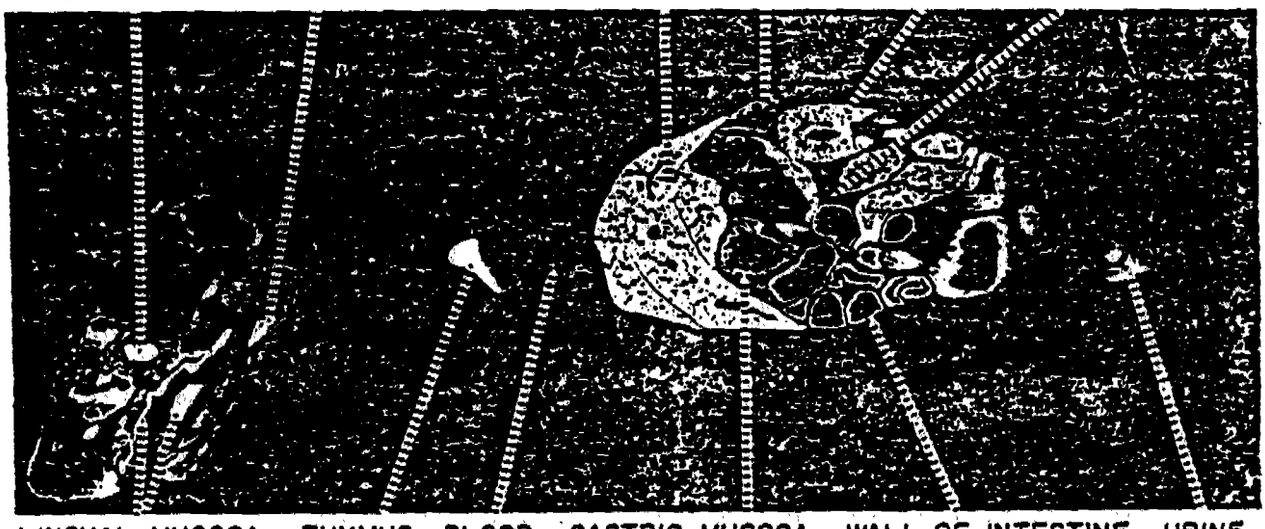

Figure 4: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 9 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE; 24 HR AFTER REMOVAL

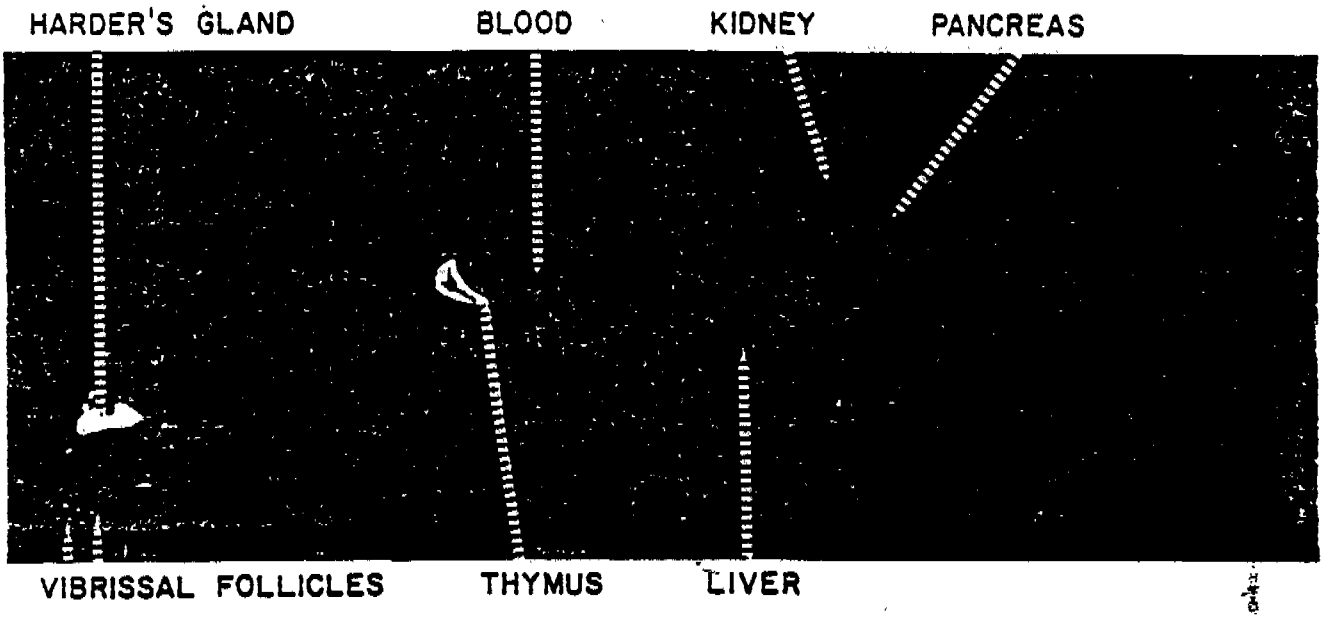

Figure 5: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 24 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE

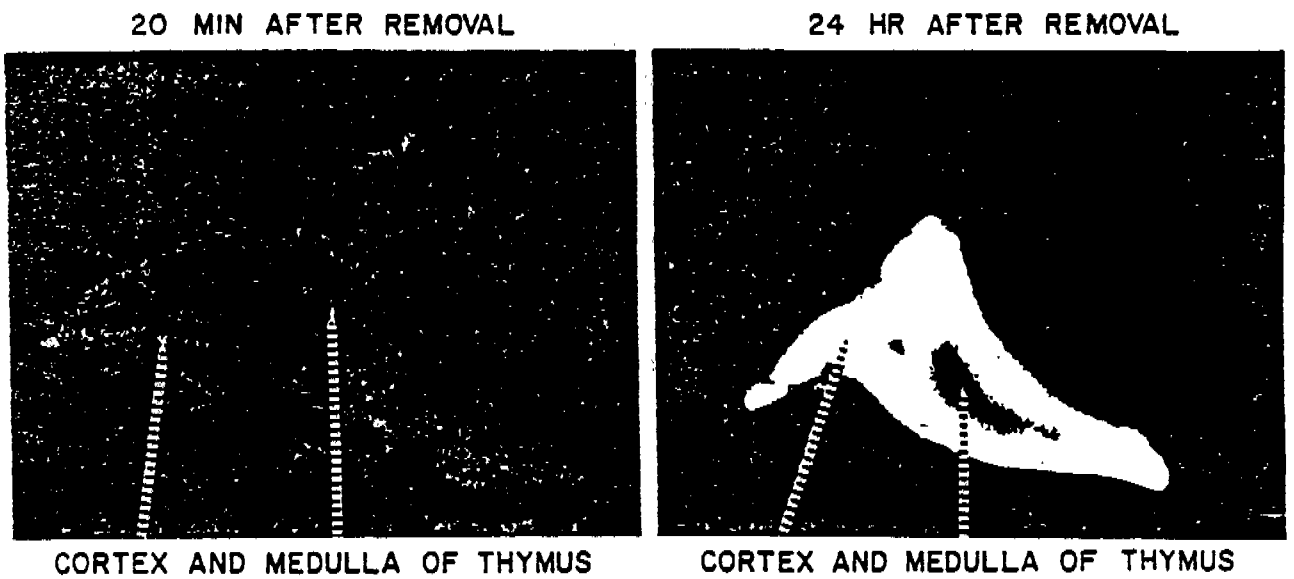

Figure 6: Prints of the thymus areas of whole-body autoradiographs from male CD-1 mice which were exposed to ^{14}C-vinyl chloride for 3 hours and then frozen 20 minutes or 24 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity. Note the high retention of the nonvolatile metabolites in the cortex of the thymus after 24 hours.

The sites of localization of the nonvolatile metabolites of vinyl chloride in the mouse are similar to those reported for the rat (Duprat et al. 1977). The high concentration of nonvolatile metabolites of vinyl chloride retained in the thymus after 24 hours suggests that there is covalent binding of these metabolites to molecules in the thymus. It is possible that these molecular interactions in the thymus could have an effect on the immune system. The high concentration in the thymus may be involved in the stimulation of the immune system in mice seen by Sharma and Gehring (1979) and in the reduction in peripheral T-lymphocytes seen in man by Ward et al. (1976).

Vinyl chloride may exert its carcinogenic action by a dual mechanism. It may suppress the immune surveillance system mediated by the thymus and concurrently damage several tissues including the liver.

Relevance to Industry

Whole-body autoradiographic studies on the biological disposition of industrial chemicals offers the most thorough approach to the study of the interaction of these chemicals with tissues in the body. Knowledge of these tissue interactions allows predictions of potential toxicity or, conversely, to a lack of toxic effect if the chemical is rapidly and completely eliminated (Waddell et al., 1977). When chemicals are retained in specific tissues as in the case with vinyl chloride, the tissue of retention gives an indication of possible mechanisms of toxic action. Vinyl chloride appears to be having a toxic effect on the immune system; the direct evidence for an interaction of the chemical with this system was not available until these studies were done.

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LISTS OF PUBLICATIONS, ABSTRACTS, PREPRINTS AND PUBLICATIONS IN PREPARATION

PUBLICATIONS

1. Fortwengler, Jr., H.P., Jones, D., Espinsoa, E. and Tamburro, C.H. (1981) Evidence of Endothelial Cell Origin of Vinyl Chloride-Induced Hepatic Angiosarcoma. Gastroenterology, 80:1415-1419.

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PREPRINTS

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2. Du, J.T., Eades, D.S., and Tamburro, C.H. Oxidative and Glutathione-Related Detoxifying Enzyme Capabilities in Hepatocytes and Nonhepatocytes of Rat Liver.

3. Kupchella, C.E., Greenberg, R.A. , Warick, R.A. and Tamburro, C.H. Preliminary Assessment of the Usefulness of Urinary Total Glycosaminoglycan.

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1. Tamburro, C.H., Miller, B. and Greenberg, R.A. Sensitivity and Specificity of ICG Clearance TEst of Hepatotoxicity.

2. Tamburro, C.H., Miller, B. and Chan, C. Indocyanine Green (ICG) Clearance Test Safety and Toxicity.

3. Fortwengler, P. and Tamburro, C.H. Use of HLA Tissue Typing in the Identification of Chemical Injury in Vinyl Monomer-Exposed Workers.

4. Fortwengler, P. and Tamburro, C.H. Immunocompetence of Humans Chemically Exposed to Finyl Monomer Chemical.

5. Barrow, G., Schroot, G.R. and Tamburro, C.H. Collagen Changes in Normal Aging Liver.

APPENDIX

PUBLICATIONS

GASTROENTEROLOGY 1981;80:1415–19

Evidence for Endothelial Cell Origin of Vinyl Chloride-Induced Hepatic Angiosarcoma

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Previous reports of hepatic angiosarcoma have not clearly defined the cellular type from which this tumor arises, as evidenced by the terminology of endothelioma, Kupffer cell sarcoma, endothelial cell sarcoma, and hemangioendothelial sarcoma, etc., which have been used interchangeably. In addition, there has been no consensus on the separate entity of Kupffer and sinusoidal endothelial cells. In the work presented here, evidence for the endothelial cell origin of this tumor is provided by the demonstration of factor VIII, a known endothelial cell marker, in the tumor cells. Fluorescence due to the presence of factor VIII appeared intense in the tumor sinusoidal cells of all four vinyl chloride-associated angiosarcomas studied, whereas normal liver sinusoidal lining cells showed negligible fluorescence.

Hepatic angiosarcoma has been associated with exposure to thorotrast (1), arsenic (2), androgenic-anabolic steroids (3), and vinyl chloride (4). However, most cases are of undetermined origin. Regardless of etiology, earlier studies (5–8) made no clear distinction between endothelial and Kupffer cells, and gave various histologic designations to this type of tumor, e.g., hemangioblastoma, Kupffer cell sarcoma, angiosarcoma, and endothelioma (9), thus raising the question of its true cellular origin. This question is of particular current interest in view of recent findings by electron microscopy which clearly support a distinct origin for Kupffer and endothelial cells (10), as well as indicating the absence of endothelial cell to Kupffer cell transition (11).

Factor VIII, a coagulation factor, was shown by Hoyer et al. (12), to be present in endothelial cells, megakaryocytes, and platelets. In the work presented here, using immunochemical evidence, the presence of abundant factor VIII in proliferating sinusoidal lining cells of angiosarcomatous liver tissue is demonstrated; it supports the endothelial cell origin of vinyl chloride-associated angiosarcoma.

Materials and Methods

Hepatic tissue studies were conducted in eight individuals—three with hepatic angiosarcoma related to heavy vinyl chloride exposure as production workers, one hepatic angiosarcoma associated with vinyl chloride used as hair spray propellant, and four normal subjects (traumatic death with no liver injury) who were used as controls.

Reagents. Purified human factor VIII was provided by the Louisville Red Cross Blood Center. Corresponding antiserum prepared in rabbits was from Behring Diagnostics (American Hoechst Corp., Somerville, N.J.). A single precipitin line in immunoelectrophoresis was seen with this material when tested against human plasma and purified factor VIII. Fluorescein-conjugated goat antiserum to rabbit IgG (GARI) was obtained from Meloy Laboratories, Inc. (Springfield, Va.).

Tissue specimens. Both normal and hepatic angiosarcoma tissue were obtained at postmortem examination within 30 min to 6 h of death. Portions of normal and tumor tissues were processed for standard histologic and immunofluorescent staining.

Immunofluorescence technique. Frozen sections (8

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This investigation was supported by a Research Grant from the Manufacturing Chemists Association.

This work was presented in part at the Federation of American Societies for Experimental Biology meeting in Dallas, Texas in April, 1979.

The technical assistance of Mr. Larry Wilder is greatly acknowledged. We would like to thank Dr. Hans Popper for his review of the histology and his suggestions.

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μm) were cut on a cryostat, air dried, fixed for 5 min in cold acetone and rehydrated in phosphate-buffered saline (PBS). The sections were then treated for 30 min with antiserum to factor VIII, washed with PBS, and stained with fluorescein-conjugated GARI for 45 min. After three 5-min washes in PBS, the sections were examined by immunofluorescent microscopy utilizing a Vanox microscope (Olympus Corporation of America), and an HB0200 UV illuminator with a 3-mm BF 12 excitor filter and a G530 barrier filter. Control sections were incubated with nonimmunized rabbit serum. Specificity of factor VIII staining was demonstrated by inhibition of the staining reaction after absorption of the anti-factor VIII rabbit serum with the purified factor VIII and by the negative staining reaction found using nonimmune rabbit serum.

Results

Factor VIII immunofluorescence of endothelial cells in human umbilical cord shows a homogeneous continuous staining pattern (Figure 1). This preparation served as a positive control. Sections from normal liver did not display any specific factor VIII immunofluorescence in the sinusoidal areas except for occasional spotty granular areas of minimal intensity along the sinusoidal borders (Figure 2). Only the linings of the hepatic arteries and veins consistently gave intense staining reactions. In contrast, vinyl chloride hepatic angiosarcoma tumor tissue demonstrated intense fluorescence occurring in the proliferating cells lining the enlarged sinusoids (Figure 3). These areas of specific immunofluorescence were limited to the sinusoidal spaces, were multifocal and occasionally occupied up to 20% of the tissue specimen. There was no demonstrable difference among the four angiosarcoma specimens as to the frequency of stained cells or the pattern of specific fluorescence. Adjacent tissue stained with H & E documents angiosarcomatous cells within the enlarged sinusoids lining the hepatocytic cords (Figure 4).

Figure 1. Umbilical cord, endothelial lining cells (arrows) showing factor VIII related immunofluorescence in white (× 100).

Figure 2. Fluorescent staining of normal liver showing minimal granular punctate factor VIII fluorescence in hepatic sinusoids (white arrows) (× 100).

Fluorescence was present in the cytoplasm and cell surface but not in the nuclei of the sinusoidal cells as illustrated in Figure 5. This distinguishable pattern of factor VIII immunofluorescent staining was seen in all the angiosarcomas examined and in none of the normal hepatic tissue.

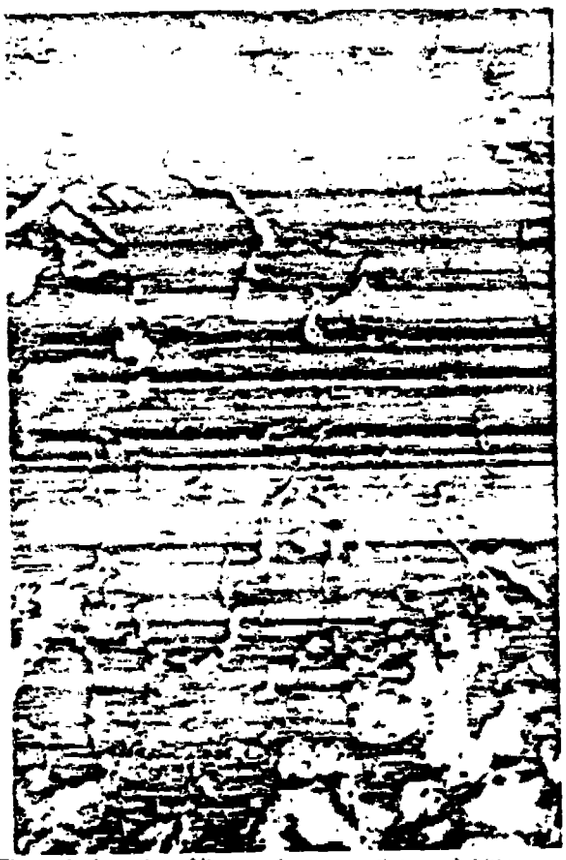

Figure 3. A section of liver angiosarcoma tissue exhibiting one of the multifocal areas displaying marked fluorescence in the proliferating sinusoidal cells (*white arrows*), with some nonspecific background staining of hepatocytes (× 200).

Discussion

Descriptions and definitions of hepatic angiosarcoma have varied in the past and have relied predominantly on morphologic criteria for identifying the various sinusoidal cells.

Some investigators have proposed that both Kupffer and sinusoidal endothelial cells are transitory in respect to each other and that Kupffer cells merely represent activated endothelial cells (5,13). In contrast, others support the view that the Kupffer cell is independent in nature and represents a hepatic macrophage (14,15).

As early as 1939, Miller considered the Kupffer cell as a class of endothelial cells and distinguished them as "endothelial cells of Kupffer (13)." He suggested the term endothelioma or endothelioblastoma for the tumors arising from Kupffer cells and hemangioendotheliomas or hemangioendothelioblastoma for those derived from vascular endothelium. In the first communication of a thorotrast-associated malignant hepatic vascular tumor, McMahon et al. (1), described it as composed of cells closely resembling Kupffer cells and indicated that some tumor cells were phagocytic. Nevertheless, the tumor was designated [illegible] in reporting a tumor, Baker et al. (12), made a distinction between the apparent maturation stages of sinusoidal lining cells, i.e. "Kupffer cells" and "normal resting sinusoidal endothelia." He noted that the tumor cells were phagocytic and therefore, described the tumor as originating from Kupffer cells, as did Burston (17). Edmonson's classical description of this tumor (9) makes no distinction between endothelial and Kupffer cells, but describes it as composed of malignant endothelial cells.

In the initial report of arsenic-associated "hemangioendothelial sarcoma" of the liver due to ingestion of Fowler's solution, Regelson et al. (18), indicated the presence of neoplastic endothelial cells but did not differentiate Kupffer from endothelial cells. On the other hand, Blackwell et al. (19), made no dis-

Figure 4. A frozen section of hepatic angiosarcoma tissue demonstrating individual and small groups of proliferating endothelial cells in dilated sinusoids (*black arrows*) (H & E × 400).

Figure 5. High-powered frozen section of angiosarcomatous tissue showing factor VIII immunofluorescence of individual proliferating cells lining hepatic cords (*white arrows*). Fluorescent staining of cytoplasm but not cell nucleus is seen in one of the cells (× 1000).

tinction between these cells but called the tumor Kupffer cell sarcoma.

In more recent reports involving vinyl chloride-associated angiosarcoma, the question of cellular origin of the tumor persists. This malignancy has been variously described as pleomorphic malignant endothelial cells (9), malignant spherical and spindle cells (20), "tumor cells" (21), sinusoidal cells (22), and as a tumor believed to be malignant Kupffer cells (23). Makk et al. (24) and Popper et al. (25) depicted this tumor as arising from sinusoidal endothelial cells.

In comparing the pathology of liver angiosarcoma due to vinyl chloride, thorotrast, and arsenic, Popper et al. (25) concluded from light and electron microscopy that the tumors were probably endothelial and suggested confirmation by use of histologic markers. An appropriate marker which concentrates in the endothelial cells is provided by coagulation factor VIII which differentially appears in the endothelial cells, megakaryocytes and platelets (12) and not in Kupffer cells which are peroxidase positive and phagocytic.

In the present work we have shown by an immunofluorescent procedure that Factor VIII is present in the proliferating cells of vinyl chloride-associated angiosarcoma, thus confirming their endothelial cell origin. Our observation that normal hepatic tissue displays intense factor VIII fluorescence in cross sections of vessels, but limited fluorescence of the sinusoidal endothelial cells in comparison to angiosarcomatous tissue, suggests that the aberrant cells may have an increased production or storage capacity for factor VIII or a decreased transport from the cell. In addition, staining for factor VIII may provide a means for identification of vascular cell involvement in hepatic tumors and could be used to identify specific endothelial cell changes and their relationship to chemical biotransformation in both animals and humans.

In view of these findings, we suggest that the term "endothelial cell angiosarcoma" be used as an appropriate term for this tumor.

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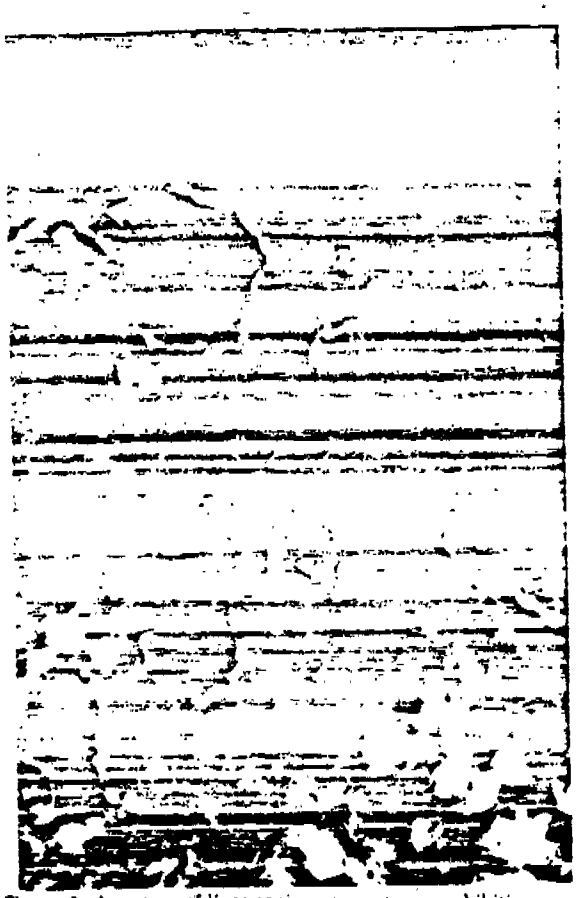

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BIOCHEMICAL ALTERATIONS IN LIVERS OF RATS EXPOSED TO VINYL CHLORIDE

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Sprague-Dawley rats were exposed to vinyl chloride to determine the earliest sequential biochemical changes occurring with liver injury before angiosarcoma development. Activity of glucose-6-phosphatase, a key gluconeogenic enzyme in the liver microsomal fraction, decreased 25% with respect to controls after 70 h of exposure. Glucose-6-phosphate dehydrogenase activity increased twofold after more than 100 h of exposure. Nonprotein sulfhydryl levels (glutathione and/or cysteine) showed a slight but progressive elevation, whereas glutathione reductase activity increased 50–60% during exposure to vinyl chloride. NADPH-cytochrome c reductase and mixed function oxidase were unchanged in the same microsomal fraction. There were no changes in seven conventional clinical biochemical liver tests or in four other markers of liver mitochondrial, cytosol, and microsomal function. No significant histological changes were found on light microscopic examination during this exposure period. However, with electron microscopy, dilation of rough endoplasmic reticulum was seen in the animals exposed for more than 137 h. These enzymatic changes are considered to reflect early hepatocellular adaptation to vinyl chloride exposure with very mild or limited hepatocellular injury in its earliest stage.

INTRODUCTION

Vinyl chloride has been shown to induce tumors, including angiosarcoma, in laboratory animals (Maltoni and Lefemine, 1975; Viola et al., 1971) and angiosarcoma in humans (Creech and Johnson, 1974). At high concentrations, vinyl chloride is believed to be metabolized by the microsomal mixed function oxidase (MFO) system of the liver to toxic metabolites (Bolt et al., 1975; Hefner et al., 1975; Johnson, 1967; Watanabe et al., 1976b, 1976c). Animals pretreated with phenobarbital, an inducer of

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MFO, and exposed to 5% vinyl chloride (50,000 ppm) had increased serum alanine aminotransferase, a conventional biochemical indicator of liver injury, and increased serum sorbitol dehydrogenase, a liver specific enzyme (Jaeger et al., 1974). However, there have been no sequential enzymatic studies to determine what progressive metabolic alterations of the hepatocyte occur during vinyl chloride exposure preceding the development of angiosarcoma. Although the hepatocyte is the major cell for oxidation and probably detoxification of vinyl chloride and its metabolites, angiosaccoma develops not in the hepatocyte but in adjacent mesenchymal cells. During this time, the hepatocytes undergo changes (focal nodular hyperplasia) suggesting focal regeneration, probably from limited hepatocellular injury (Tamburro et al., 1979).

The purpose of our study was to characterize the earliest sequential enzymatic changes that occurred in vinyl chloride exposure to elucidate the hepatocytes' role in vinyl chloride–induced injury and angiosarcoma development. Portions of this study have been presented elsewhere (Du and Tamburro, 1976).

METHODS

Animals and Experimental Design

Sprague-Dawley male rats (300–500 g) were randomly assigned before each experiment to either a group to be exposed to vinyl chloride gas (15,000 ppm) or a nonexposed control group kept outside the chamber in the animal room. Animals were fed standard laboratory chow pellets *ad libitum*.

Four sequential experiments were done. In experiments A and B, rats were exposed 2–4 h/d, 4 d/wk, for 1–2 wk with total accumulated exposure times of 14, 28, and 42 h. In experiment C, rats were exposed 4–8 h/d, 5 d/wk, for 2–3 wk with total accumulated exposure periods of 71 and 103 h. In experiment D, rats were exposed 6–8 h/d, 5 d/wk, for 3–4 wk with total accumulated exposure periods of 84 and 137 h.

The animals were exposed in a modified chamber consisting of an airtight vat that had been used for manufacturing polyvinyl chloride. An aliquot of vinyl chloride (185 g) was added to the vat per 4-d period to make an average concentration of 15,000 ± 4000 ppm. The air was constantly circulated by a stirrer. The chamber volume was 4400 l (1100 gal), so the respiration of the rats had a negligible effect on the composition of the chamber's atmosphere. Experiments with an added control group exposed only to air in an identical modified vat showed no differences from the controls kept in the animal room (Table 1).

All animals were anesthetized with ether, had blood drawn by cardiac puncture, and were sacrificed at the same time of day (within 3 h after final exposure) to avoid diurnal effects.

TABLE 1. Comparison of Hepatic Enzyme Activities in Animal Control Groups Based in Animal Quarters and in the Exposure Chamber.[a]

Enzyme	Air exposed	
	Animal room	Chamber[b]
Glucose-6-phosphate dehydrogenase	12.82 ± 1.32[c]	11.06 ± 1.78[c]
Glutathione reductase	4.77 ± 0.27[d]	5.28 ± 0.40[d]

[a] Values are means ± SEM ($n = 6$).
[b] Kept in chamber 7 h/d, 5 d/wk, for 6 wk; total, 210 h.
[c] Not significant.
[d] Not significant.

Sample Preparation

Liver was excised rapidly and was immediately rinsed in ice-cold 0.15 *M* KCl with 0.02 *M* Tris buffer, pH 7.4. A portion of the tissue was homogenized with 9 volumes of the cold KCl-Tris buffer in a Potter-Elvehjem homogenizer. Each sample was prepared from a single organ and kept at 4°C during preparation. Remaining liver was frozen rapidly and stored at −20°C. For assays with frozen tissue, livers from control and experimental rats were frozen in an identical manner for the same length of time.

Subcellular Fractionation and Biochemical Determination

Homogenate was centrifuged at 600 and 8000 X *g* for 10 min and at 100,000 X *g* for 60 min to obtain the nuclear, mitochondrial, microsomal, and cytosol fractions, respectively, by the differential technique of Schneider and Hogeboom (1950). Centrifugation was at 4°C in a Sorvall refrigerated RC5 supercentrifuge with a fixed angle rotor and a Beckman model L-5 ultracentrifuge with a swinging bucket rotor. Enzyme activities and cytochrome P-450 were determined in the isolated subcellular fractions: cytochrome oxidase in the mitochondrial fraction; NADPH-cytochome *c* reductase, mixed function oxidase, cytochrome P-450, and glucose-6-phosphatase in the microsomal fraction; and glutathione reductase and glucose-6-phosphate dehydrogenase in the 100,000 X *g* supernatant fraction.

Cytochrome P-450, NADPH-cytochrome *c* reductase, and cytochrome oxidase were determined in fresh samples; all other determinations were in freshly isolated fractions from frozen tissue. Glucose-6-phosphatase was estimated by measuring release of inorganic phosphate (Harper, 1965). Cytochrome P-450 was estimated by the maximum absorption difference between the dithionite-reduced cytochrome and its CO complex (Omura and Sato, 1964). The P-450 concentration was calculated by using 91,000

M^{-1} cm^{-1} as the extinction coefficient for the increase in peak height between 490 and 450 nm. Cytochrome oxidase was measured as described by Wharton and Tzagoloff (1967), and NADPH-cytochrome *c* reductase according to Degroot and Dunn (1964). Mixed function oxidase was determined according to Holtzman et al. (1968) by measuring the hydroxylation of analine. Nonprotein sulfhydryl was estimated by the method of Sedlak and Lindsay (1968). Rate of oxidation of NADPH by oxidized glutathione was used as a measure of enzymatic activity of glutathione reductase (Carlberg and Mannervik, 1975). Glucose-6-phosphate dehydrogenase was measured spectrophotometrically, as the rate of NADPH formation (Lohr and Waller, 1965). Assays were conducted under conditions of linearity with respect to both time and protein. Protein content was determined by the method of Lowry et al. (1951). Conventional clinical analyses of serum included aspartate aminotransferase (AST, SGOT), alanine aminotransferase (ALT, SGPT), alkaline phosphatase, bilirubin, albumin, cholesterol, and triglyceride, determined by the technicon sequential multiple analyzer computer (SMAC) system.

Materials

The NADPH, cytochrome *c*, and oxidized glutathione were obtained from Sigma Chemical Co., St. Louis, Mo. Analine and other chemicals were reagent grade. Double-distilled water was used throughout.

Light and Electron Microscopy

Small strips of liver were removed under ether anesthesia and immersed immediately in ice-cold 3% glutaraldehyde (pH 7.4). Tissues were sliced into small cubes and fixed for 2 h at 4°C. Subsequently, samples were washed overnight in phosphate buffer and postfixed in 1% osmium tetroxide for 1 h before being dehydrated in ascending alcohol and embedded in Epon. Tissue blocks were polymerized at 60°C for 2 d. Thin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate before examination on a Philips 300 electron microscope. For ultrastructural analysis, control rats and rats exposed for 42 and 137 h to vinyl chloride were reviewed.

For light microscopy, a block of tissue was fixed in buffered formalin and processed routinely for paraffin embedding. Sections 6 μm thick were stained with hematoxylin and eosin before examination.

RESULTS

The protein contents (milligrams per gram of liver) of the subcellular fractions in control and vinyl chloride-exposed groups were the same throughout the exposure; therefore, the enzymatic results were expressed as micromoles converted per minute per milligram of subcellular protein.

The statistical analysis (see below) showed certain significant

enzymatic differences between the exposed and control groups in glucose-6-phosphatase, glutathione reductase, and glucose-6-phosphate dehydrogenase.

Glucose-6-phosphatase. No significant differences in glucose-6-phosphatase activity were detected between the two groups up to 42 h; however, after 71 h, the glucose-6-phosphatase activity was significantly less in the exposed than in the control animals. Figure 1*a* shows the 25% decrease in mean activity of glucose-6-phosphatase after 71 h; this activity remained significantly lower up to 137 h. Figure 1*b* shows the 95% confidence intervals for *D*, the mean difference between the exposed group and the control groups, in each experiment.

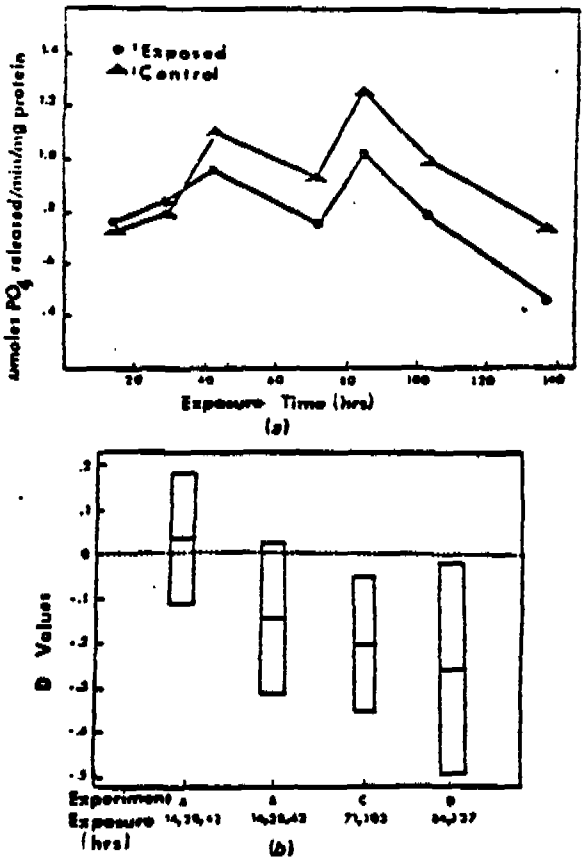

FIGURE 1. (*a*) Composite curve of group means of specific activity of glucose-6-phosphatase with respect to exposure time; (*b*) 95% confidence intervals for *D*, the mean difference between the exposed and control groups. There is no significant difference between the exposed and control groups until after 42 h of exposure. After 71 h the mean level of the exposed group is significantly less than that of the control group.

Glutathione Reductase. Figure 2, *a* and *b*, illustrates the significant differences ($p < 0.05$) between the exposed and control groups in all 4 experiments except at 71 h of exposure ($p < 0.06$). The glutathione reductase level was 25% greater in exposed than in nonexposed animals up to 71 h and approximately 50% greater after 84 and 137 h.

Glucose-6-phosphate Dehydrogenase. There was no significant difference between exposed and control groups from 14 to 42 h and at 71 and 84 h. However, after 103 and 137 h of exposure there was a statistically significant difference as shown in Fig. 3, *a* and *b*. After about 100 h f exposure, the mean glucose-6-phosphate dehydrogenase level in exposed animals was about twice that in nonexposed animals.

While these differences were occurring, none of the conventional biochemical liver function tests (e.g., serum aminotransferases, alkaline phosphatase) showed any significant change, nor were there any changes in cytochrome oxidase, mixed function oxidase, P-450, or NADPH-cytochrome *c* reductase.

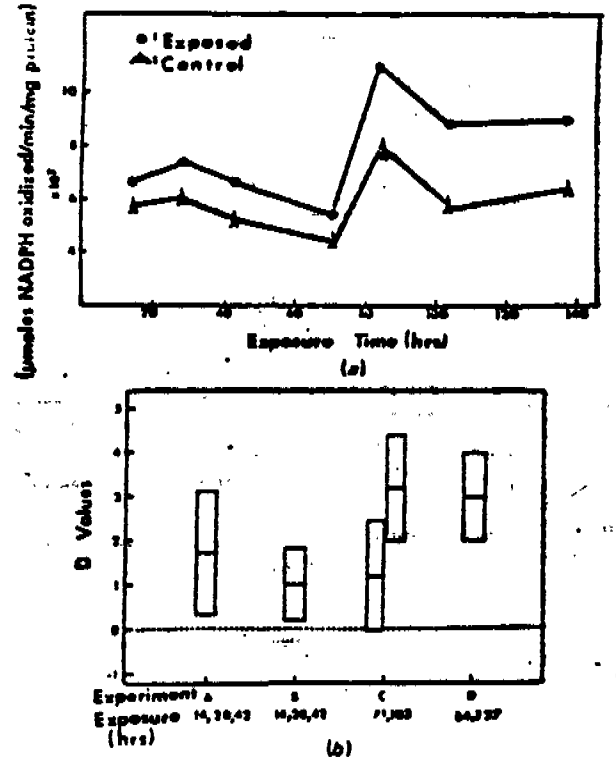

FIGURE 2. (*a*) Composite curve of group means of specific activity of glutathione reductase with respect to exposure time; (*b*) 95% confidence intervals for *D*, the mean difference between the exposed and control group. The mean value for the exposed group is significantly greater than that for the controls throughout the entire experiment ($p < 0.05$) except at 71 h of exposure.

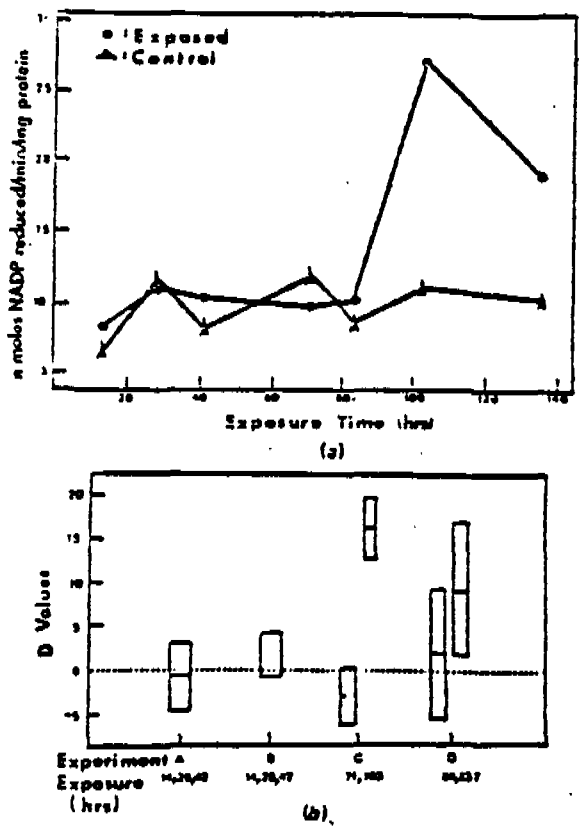

FIGURE 3. (*a*) Composite curve of group means of specific activity of glucose-6-phosphate dehydrogenase with respect to exposure time; (*b*) 95% confidence intervals for *D*, the mean difference between the exposed and control groups. The mean value for the exposed group is not significantly different from that for the control group until after 84 h of exposure.

The concentration of reduced glutathione in the livers of rats decreased to 52% of the control value after a single 2-h exposure to 15,000 ppm vinyl chloride (Table 2). However, the reduced glutathione concentration appeared to be the same or slightly elevated when the rats underwent multiple exposures. This elevation was not significant at the 5% level. The vinyl chloride-exposed rats suffered a weight loss of 4–13% and the control group gained 2–10% (Table 3). All rats survived to the termination of the experiment without noticeable ill effects.

Statistical Analysis of Results

The 95% confidence interval for group mean differences in each enzyme study was based on the error mean square from a two-factor analysis of variance with interaction, the two main effects being exposure time in hours and exposure-nonexposure. This analysis was performed for

TABLE 2. Effect of Vinyl Chloride Exposure on Concentration of Liver Nonprotein Sulfhydryl Compound in Rats

Exposure time (h)	Concentration ratio (exposed/control)
2[a]	0.52 (3)[b]
14[c]	1.02 (6)
28	0.96 (6)
42	0.82 (6)
71	1.21 (5)
84	1.07 (3)
103	1.49 (6)
137	1.29 (3)

[a] Single exposure.
[b] Number of animals in control or experimental group is shown in parentheses.
[c] Multiple exposures (4–8 h/d, 4–5 d/wk) for 14–137 h.

TABLE 3. Body Weights of Rats before and after Vinyl Chloride Exposure[a]

Accumulated exposure (h)	Time	Control	Change (%)	Vinyl chloride-exposed	Change (%)
14	Before	442 ± 6.2[b] (3)[c]	+3	453 ± 1.5 (3)	−7
	After	454 ± 9.5 (3)		421 ± 4.1 (3)	
28	Before	436 ± 9.2 (3)	+2	448 ± 6.0 (3)	−9
	After	445 ± 12.1 (3)		408 ± 3.0 (3)	
42	Before	433 ± 5.2 (3)	+5	450 ± 4.3 (3)	−9
	After	455 ± 6.4 (3)		410 ± 2.8 (3)	
71	Before	437.6 ± 12.2 (5)	+2	422.7 ± 9.2 (5)	−13
	After	447 ± 19.4 (5)		385.0 ± 4.2 (5)	
84	Before	412.7 ± 7.9 (3)	+10	425 ± 17.4 (3)	−6
	After	455.0 ± 6.8 (3)		399 ± 72 (3)	
103	Before	453 ± 12.9 (6)	+3	441.5 ± 6.8 (6)	−10
	After	465 ± 16.9 (6)		397.0 ± 5.7 (6)	
137	Before	404.7 ± 1.2 (3)	+5	415.0 ± 15.7 (3)	−4
	After	425.0 ± 5.2 (3)		398 ± 28 (3)	

[a] Multiple exposures to 1.5% vinyl chloride for 14–137 h, 4–8 h/d, 4–5 d/wk.
[b] Results are expressed as mean ± SEM.
[c] Number of animals is given in parentheses.

each of the four experiments. Overall significant ($p < 0.05$) differences between the exposed and control groups are found when the 95% confidence intervals (D values) *do not* contain zero.

In Fig. 1*a*, there is no significant difference ($p > 0.05$) between the 2 groups in experiments A and B because a D value of zero is within these two confidence intervals. However, for experiments C and D, the confidence intervals do not contain a D value of zero, indicating a significant difference ($p < 0.05$) between the exposed and control groups. When the D value is less than zero (as in Fig. 1*b*), the experimental group is significantly lower than the control throughout the experiment; when the D value is greater than zero (as in Figs. 2 and 3), the experimental group is significantly higher than the control. Further, in experiments C and D of Fig. 1*b*, a single confidence interval is shown even though there are two time periods of exposure each. This results from the nonsignificance of the interaction term in the analysis of variance, meaning that the observed difference at 71 h of exposure is not statistically different from the difference at 103 h. Similar conclusions hold for experiment D in Fig. 1*b*. Figure 2*b* shows 2 confidence intervals for the set of experiments C at 71 and 103 h because the interaction of the time and exposure factors was significant; that is, the difference between the exposed and control groups after 103 h of exposure was significantly greater than the difference after 71 h. This is also reflected by the differences of the mean values at 71 and 103 h as shown in Fig. 2*a*. For the same reasons, there are also two confidence intervals for the C (71 and 103 h) and D (84 and 137 h) sets of experiments in Fig. 3*b*.

Morphological Studies

Morphological light microscopic studies were performed in a blind (coded) and randomized fashion and did not show any evidence of hepatocellular injury or changes usually seen in the latter stages of vinyl chloride exposure.

Besides some variations in glycogen content, no difference in the fine structure of the hepatocytes was observed between controls (Fig. 4) and those exposed to vinyl chloride for 42 h. Dilation of rough endoplasmic reticulum was observed in a small number of hepatocytes after 137 h of vinyl chloride exposure (Fig. 5). Although no noticeable change in the amount of smooth endoplasmic reticulum was associated with this change, a concomitant increase in cytoplasmic density was evident in these cells. Another type of lesion found in *other* hepatocytes was characterized by the presence of small patches of clear spaces, which tended to aggregate near the cell periphery (Fig. 6). Such lesions usually affect the adjacent cell equally. No other cell type appeared to be affected by vinyl chloride exposure in this study.

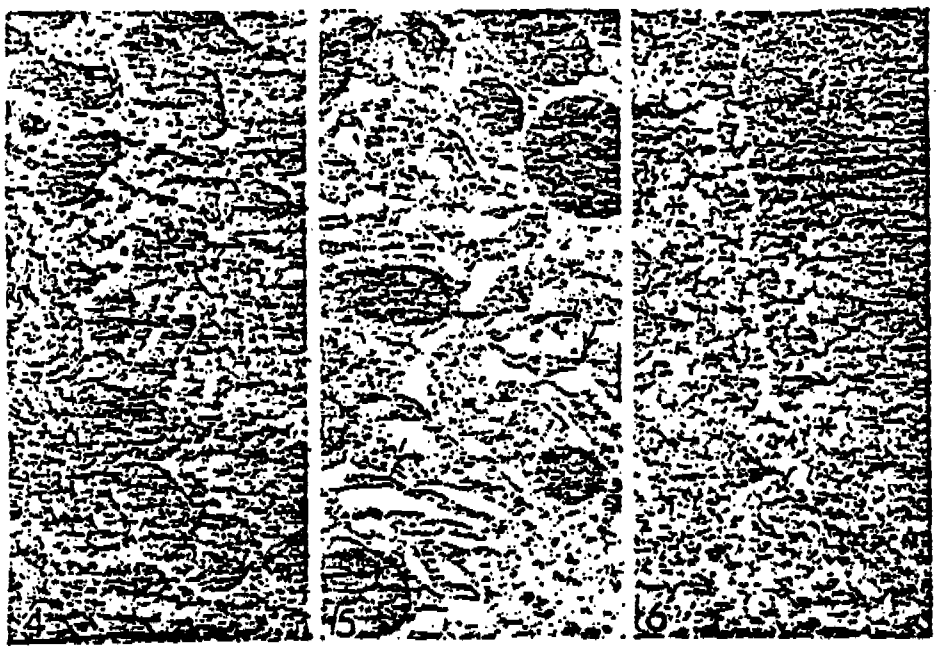

FIGURE 4. Portion of a hepatocyte from a control rat. A good complement of mitochondria and rough endoplasmic reticulum is shown (X12,000).
FIGURE 5. Dilation of RER (arrows) shown in hepatocyte 137 h after exposure to 15,000 ppm vinyl chloride during 2–3 wk (X25,000).
FIGURE 6. Subplasmalemmal lesions (*) in hepatocytes 137 h after exposure to 15,000 ppm vinyl chloride during 2–3 wk. The lesions are present in two adjacent cells and the clear spaces seem to coalesce (X12,000).

DISCUSSION

Decreased glucose-6-phosphatase activity and increased glucose-6-phosphate dehydrogenase and glutathione reductase activity in rat liver after exposure to vinyl chloride (as in our studies) may be the biochemical alterations indicative of early liver injury, adaptation to increased detoxification activity, or preparation for increased nucleic acid synthesis. These differences were observed before any abnormalities were detectable from conventional liver function tests, such as serum aminotransferases, or from changes in other subcellular organelle markers, such as mitochondrial cytochrome oxidase activity.

In primary hepatocellular cancer (hepatomas), the activity of key enzymes for gluconeogenesis (glucose-6-phosphatase, etc.) decreased with increased rate of tumor growth (Weber, 1974; Weber and Convery, 1966; Weber and Lea, 1967). In contrast, two enzymes for the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (Selmeci and

Weber, 1976; Weber and Morris, 1963) and transaldolase (Heinrich et al., 1974), increased in all hepatomas. Further, the activity of glucose-6-phosphatase decreased before and during the development of hepatomas when carcinogens such as nitrosamine and dimethylaminoazobenzene were fed to rats (Isok and Teras, 1973; Weber and Cantero, 1955). Whether our similar findings in this study are indicators of eventual cancer development (angiosarcoma) is not yet known. We used a shorter exposure period in order to identify the biochemical changes that would best reflect the morphological and cellular changes anticipated on the basis of previous human and animal studies. No attempt was made to determine what effects these shorter exposure periods would induce with long-term observation. This is now in progress. However, Maltoni and Lefemine (1975) showed that of 69 Sprague-Dawley rats exposed to 10,000 ppm vinyl chloride, 16 (26%) developed Zymbal gland carcinomas after 50 wk, 5 (8%) developed nephroblastomas after 59 wk, and 9 (15%) developed angiosarcomas after 64 wk.

The vinyl chloride-exposed groups lost weight (4–13%) whereas the control group gained weight (2–10%) during the entire experimental period. However, the differences in the three enzymes cannot be accounted for by lack of dietary food intake in the experimental group, because fasting increases glucose-6-phosphatase (Ashmore et al., 1954) and decreases glucose-6-phosphate dehydrogenase (Winberry and Holten, 1977), and we found decreased glutathione reductase and reduced glutathione content in fasted animals (unpublished data).

Rats exposed to a high dose (5%) of vinyl chloride (Reynolds et al., 1975) had decreased mixed function oxidase activity. An *in vitro* study (Ivanetich et al., 1977) showed that the metabolites of vinyl chloride from the microsomal enzyme system decreased the levels of cytochrome P-450. In our studies; the cytochrome P-450 content and the activity of mixed function oxidase and NADPH-cytochrome *c* reductase in rats repeatedly exposed to 1–2% vinyl chloride did not show any such changes. Subsequent experiments in which rats were exposed to 28,000 ppm vinyl chloride for 70–210 h in 2–6 wk showed a significant decrease of cytochrome P-450 concentration (in preparation). Differences in dose level or experimental design may account for these variations. Drew et al. (1975) found that the activity of mixed function oxidase depended on the time interval between cessation of exposure to vinyl chloride and sacrifice of animals.

The main detoxification route for vinyl chloride metabolism is thought to be conjugation with glutathione (Johnson, 1967); sulfur-containing urinary metabolites of radioactively labeled vinyl chloride have been found in rats' urine (Green and Hathway, 1975, 1977; Watanabe et al., 1976b, 1976c).

We found that under the present experimental conditions the non-protein sulfhydryl content of liver (glutathione and/or cysteine) tended to

increase in rats repeatedly exposed to vinyl chloride (Table 2). Although the difference was not significant in this experiment, subsequent exposure of rats to 28,000 ppm for 70, 140, and 210 h in 2, 4, and 6 wk led to a significant elevation of the nonprotein sulfhydryl content in liver (Du and Tamburro, 1978). Watanabe et al. (1976a) and Johnson (1967) found a depletion of nonprotein sulfhydryl content in rats after only a single exposure to vinyl chloride or chloroethanol. Hefner et al. (1975) found that a single vinyl chloride exposure reduced the glutathione content by one-half, but the decrease became smaller and even unnoticeable after repeated exposure to vinyl chloride. Fiala et al. (1976) showed that the reduced glutathione content increased after prolonged exposure of rats to various chemical carcinogens. These results are thought to reflect the exposed animals' attempt to make more glutathione to meet the unusually strong demand for detoxification.

Glutathione reductase was elevated in our system after rats were exposed to vinyl chloride for 42 h. This enzyme generates reduced glutathione from its oxidized form as a compensatory mechanism to maintain the level of glutathione, and the simultaneous elevation of glucose-6-phosphate dehydrogenase regenerates NADPH, which can be used as a cofactor for various synthetic pathways including nucleic acid synthesis. Elevated glutathione reductase activity was found in rats with primary hepatocellular cancer induced by diethylnitrosamine (Pinto and Bartley, 1973). This consistent increase in glutathione reductase activity after exposure to vinyl chloride suggests that it may be one of the earliest biochemical manifestations of exposure and injury.

These metabolic changes could also play a role in the early changes observed by light and electron microscopy. Hepatic lesions such as dilation of smooth endoplasmic reticulum and loss of microvilli were reported in mice as early as 1 mo after vinyl chloride exposure (Schaffner et al., 1976). In contrast, we observed dilation of rough endoplasmic reticulum and patchy lesions near the plasmalemma. The dilated rough endoplasmic reticulum could be related to the increased enzyme synthesis (i.e., glucose-6-phosphate dehydrogenase and glutathione reductase) induced by vinyl chloride. The absence of smooth endoplasmic reticulum proliferation in our animals suggests a comparatively milder effect in our short-term study. In a subsequent study where long-term effects of vinyl chloride exposure was assessed, dilation of smooth endoplasmic reticulum in hepatocytes was observed (in preparation). The nature of the patchy lesions found near the plasmalemma remains to be established. The location of the lesion, however, suggests that some toxic agents may be entering or exiting the hepatocytes in these sites.

We believe these early enzymatic changes reflect adaptation of the liver cell to early mild injury. The alterations in gluconeogenesis and in the pentose phosphate shunt appear to indicate the liver's adaptation while undergoing repetitive and prolonged exposure to the mildly toxic chemical

vinyl chloride, which, when inadequately metabolized, produces intermediates that lead to the formation of cancer.

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The Hepatic Role in Carcinogenesis and Its Early Detection—The Vinyl Chloride Model[1,2]

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The liver's role in vinyl chloride toxicity and carcinogenicity is providing a better understanding of the chemical carcinogenesis mechanism. A variety of both malignant and benign hepatic tumors has been demonstrated with prolonged exposure to vinyl chloride. The multi-system involvement of this carcinogen and toxin has provided a model for the study of chemical carcinogenesis common to both man and animal. Clinical studies have shown the usefulness of biochemical, radioisotopic, and radiological studies in the detection of toxic and carcinogenic lesions. Animal studies have demonstrated the biochemical metabolism by the liver of vinyl chloride-produced intermediates which are mutagenic in bacterial systems and may be the ultimate carcinogens. Hepatic subcellular enzyme studies prove preliminary evidence of cellular adaptation and increased detoxification. Disruption of this oxidization and detoxification balance may be the key to the malignant transformation of cells. A working hypothesis is presented which may explain the metabolism of vinyl chloride into mutagenic intermediates by the liver cell and the development of malignant transformation by extra hepatic sinusoidal lining cells, lung cells, and brain tissue.

INTRODUCTION

Currently there is a growing concern that chemical compounds may be responsible for most human cancer through environmental contact. Although 100,000 to 200,000 new chemicals are introduced into industry each year, little is known about their effects. These compounds are primarily synthetics and thus not natural to the environment. The view that industrial chemicals may be latent carcinogenic hazards has again been brought into sharp focus by the discovery of vinyl chloride-induced angiosarcoma. Vinyl chloride ($CH_2 = CH-Cl$ monochloroethyene, a gas) is the basic molecule or monomer of polyvinyl chloride and its co-polymers and one of the most important organic intermediates in the plastics industry. The resulting plastic resin, polyvinyl chloride, is used in innumerable consumer and industrial products, such as containers, wrapping film, electrical insulation, pipelines, credit cards, etc. Until recently (early 1970's) vinyl chloride was regarded as being relatively non-toxic [1,2]. Initially this opinion seemed to be supported by the facts that it was used transiently as an anaesthetic and had been used commercially in industry for many years [3].

The direct information on the toxicity of vinyl chloride to man was obtained from experiments by research workers on themselves, from the evaluation of its suit-

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[2]This article is the thirteenth in a series entitled, "Seminars on Liver Disease," that have been presented as part of the Training Program in Liver Disease at the Veterans Administration Hospital, West Haven, Connecticut. Dr. Harold O. Conn, Professor of Medicine, Yale University School of Medicine, and Director of the Training Program in Liver Disease, is guest editor.

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ability as an anaesthetic [4, 5], and from study of the cases of vinyl chloride poisoning contracted during industrial use [6, 7, 8, 9].

Sporadic studies with vinyl chloride polymerization workers demonstrated varying degrees of hepatic biochemical derangements, hematological abnormalities, and skin changes [9]. Acute short term exposures led to disturbances of the central nervous system, cardiac arrythmias, severe irritation of the mucosal membrane of the eyes and the respiratory tract, and in some cases—severe pulmonary edema with obstruction of the liver and kidneys. Chronic inhalation trials in animals clearly showed that vinyl chloride was toxic to the liver and kidneys as well as irritating to the mucosal membranes and the lungs. Microscopic examination of liver sections showed degeneration of the central lobules while the damage to the kidneys chiefly involved the tubule and the interstitial lining somewhat like that of carbon tetrachloride damage [10, 11, 12].

BACKGROUND

The literature, however, contained virtually no information on damage to man after chronic exposure until Filatova et al. reported disturbances of the blood vessels and nerves in individuals exposed with 20–300 ppm of vinyl chloride on a continuous basis [13]. Cordier et al. [14] and Wilson et al. [15] were the first to report the hitherto unrecognized disorder termed occupational acroosteolysis (AOL) which included the symptoms of tenderness of the fingertips, gradual destruction of bony integrity of the fingers and a Raynaud's-like phenomena.

Studies were initiated in animals to reproduce the acroosteolysis. In 1971, Violi et al. [16] while exposing animals to 30,000 ppm to induce acroosteolysis, accidentally discovered cancer. Maltoni et al. [17] while studying various levels of exposure demonstrated that angiosarcoma occured at 250 ppm; the Manufacturing Chemists Association's studies [18] demonstrated these liver cancers even at 50 ppm. At the same time, Dr. John Creech, at the B.F. Goodrich Chemical Company Plant in Louisville, Kentucky, discovered an hepatic angiosarcoma in one employee. Creech, recalling an earlier hepatic angiosarcoma at the plant, reviewed the medical histories of previous employees. Four additional angiosarcomas were found, further supporting the connection between vinyl chloride exposure and tumor development. Measures to control the levels of vinyl chloride exposure were then instituted following federal regulation.

THE CHEMICAL

Vinyl chloride's chemical structure is a double-bonded, 2-carbon halogenated hydrocarbon which has structural similarities to tri-chloroethylene, an inhalation anaesthetic. As previously noted, vinyl chloride was once considered as an anaesthetic but was discarded because it caused myocardial irritability. Some of its important physical properties include a low boiling point, a high specific gravity, a low solubility in water, and a half-life in air which ranges from 3–20 hours. Knowledge of these physical properties may be important in determining how this agent produces a cumulative effect in the environment which ultimately leads to cancer formation.

Vinyl chloride is both toxic and carcinogenic, as recognized by the wide variety of associated disorders which have been found among vinyl chloride polymerization workers and vinyl chloride-exposed animals. A yet incomplete list of these

TABLE 1
Occupational Vinyl Chloride
Exposure Associated Disorders

1. Thrombocytopenia
2. Reticulocytosis
3. Splenomegaly
4. Hepatic fibrosis
5. Scleroderma-like skin changes
6. Acro-osteolysis
7. Raynaud's phenomenon
8. Leukopenia
9. Hepatomegaly
10. Pulmonary functional impairment
11. Angiosarcoma
12. Cardiac arrhythmia
13. Nephroblastomas*
14. Zymbal gland carcinomas*
15. Large cell lung cancer
16. Brain cancer

*In rats only

associated disorders is shown in Table 1. Animal and epidemiological studies indicate the probability that cancer induction at other sites is also directly attributed to prolonged and excessive vinyl chloride exposure [19].

In order to develop effective methods of prevention, accurate knowledge of the pathogenesis of this environmental chemical in ultimately producing its most destructive effect—cancer—is needed.

EXPERIMENTAL ANIMAL STUDIES—TUMOR FORMATION

The carcinogenicity of vinyl chloride has been demonstrated in a variety of animals as well as in humans at exposure levels that vary from 50–30,000 ppm. As illustrated in Table 2, a variety of tumor types have been found in rats, mice, and hamsters. An extensive list of benign tumors have also been reported in mice, rats, and hamsters exposed to vinyl chloride. Maltoni's group has now demonstrated primary liver cell cancers in exposed newborn rats.

Direct hepatocellular injury as well as pulmonary, mucosal and skin injuries have been shown in directly exposed animals. Pretreatment with many agents increases the toxicity of vinyl chloride; they include phenobarbital, ethanol, polychlorinated biphenyls and pesticides such as hexachlorobenzene [20]. This relationship to vinyl chloride's ability to induce cancer is under study, particularly in view of the industrial environment which allows exposure to many chemicals to occur concurrently.

HUMAN STUDIES

Epidemiological studies in the human strongly suggest that exposure beyond 10 years is associated with increased cancer mortality, mainly digestive system cancers, primarily hepatic [21, 22]. There also appears to be a higher incidence of large cell carcinomas of the lung, brain glioblastoma multiforms, and lymphomas [23, 24], although there is some disagreement as to the interpretation of this aspect of the epidemiological data.

TABLE 2
Carcinogenicity of Vinyl Chloride
(Exposure: 50–10,000 ppm)

Tumor Type	Species
1. Liver—angiosarcoma	Adult humans, rats, mice and hamsters
2. Liver—hepatocellular carcinoma	Newborn rats
3. Lung—adenocarcinoma	Rats and mice
4. Lung—large cell carcinoma	Humans*
5. Mammary adenocarcinoma	Mice
6. Zymbal gland tumors	Rats
7. Nephroblastoma	Rats
8. Osteochondromas	Rats
9. Skin epitheliomas	Hamsters
10. Melanomas	Hamsters
11. Glioblastoma multiforme	Humans*
12. Lymphoma	Humans*

*Strongly suggested epidemiologically

The multisystem involvement of this carcinogenic and toxic chemical is further illustrated in man. Early physical findings of vinyl chloride-injury include hepatomegaly, portal hypertension, possible mild systemic pulmonary hypertension, bilateral midzonal pleural thickening of the lung, and splenomegaly with and without increased portal pressure [25]. These anatomical and physiological findings most frequently occur in the absence of the traditional clinical biochemical derangement of the liver. Screening studies of vinyl chloride workers during the past two and a half years have clearly illustrated the irregular and often delayed appearance of abnormalities in aspartate and alanine aminotransferases (SGOT and SGPT), alkaline phosphatase as well as other hepatocellular enzymes. Gamma glutamic transpeptidase (GGTP), believed to be a more sensitive indicator of hepatocellular injury, has proven to have too high a false-positive rate to warrant its use in the screens for hepatocellular injury. Sorbitol dehydrogenase (SDH) studies indicate that this enzyme, which is liver tissue specific, is too insensitive for primary screening but is useful for confirmatory testing.

Anionic dye clearance studies (Indocyanine Green, ICG) have demonstrated the highest specificity and sensitivity of all the primary screening procedures tested when performed at the 5 mg/kg dose level. At the traditional 0.5 mg/kg level, it has the same effectiveness as the aminotransferases and alkaline phosphatase combined. The frequency of abnormal ICG dye clearances increases with prolonged exposure to vinyl chloride as illustrated in Fig. 1 and correlates well with the cumulative exposure to vinyl chloride as well as the histological evidence of hepatocellular injury. These functional studies for detecting hepatocellular injury do not, however, identify the cause.

Clinical studies have illustrated the usefulness of radioisotopic liver-spleen scans as a primary screening procedure for *anatomical* lesions of the liver and spleen. This procedure has proven to be the single most reliable method of tumor detection. Sixteen of the 19 individuals with anatomical lesions were detected by liver-spleen scan. In contrast, only 32 of 950 normal individuals had scan abnormalities which further diagnostic studies proved incorrect. This method provides an 84% sensitivity and 97% specificity, with only a 3% false-positive rate.

Diagnostic angiographic studies of these radioisotopic abnormalities in 30 individuals have demonstrated 3 major lesions. The first is peliosis hepatis, illustrated in Fig. 2. These lesions are usually numerous involving the entire liver, and have a diffuse stain throughout the nodules which persists into the late venous phase without central hypovascularity [26].

A second, similar lesion has been discovered in individuals with splenomegaly, and named lienal peliosis (Fig. 3). These splenic lesions demonstrate a shortened celiac artery to portal vein circulation time, normal portal vein diameters, and increased spleen size. This has been found only in individuals with long-term vinyl chloride exposure.

The final lesion is that of angiosarcoma (Fig. 4). This tumor has characteristic angiographic features of central hypovascularity, midarterial puddling, and a prolonged peripheral tumor stain which continues up to 30–36 seconds after injection. These characteristic findings have allowed differentiation from other primary hepatocellular cancers, benign tumors and benign vascular lesions [26]. These angiographic lesions have been pathologically confirmed with the additional histological finding including peliosis hepatis, sinusoidal dilatation, and activated sinusoidal cells with increased deposits of collagen in the sinusoidal space of Disse. Exploratory wedge biopsies have in addition demonstrated increased subcapsular fibrosis with subcapsular bile duct proliferation plus the often described portal fibrosis [27].

VINYL CHLORIDE METABOLISM AND CARCINOGENESIS

Present biochemical knowledge indicates that vinyl chloride is most likely metabolized by the liver in a three step process [28]. At concentrations less than 50 ppm, vinyl chloride is metabolized by the alcoholic dehydrogenase system into chloroacetaldehyde and monochloroacetic acid,

$$Cl-CH=CH_2 \longrightarrow Cl-CH_2-CH_2-OH \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} Cl-CH_2-CHO \longrightarrow ClCH_2-COOH$$

An alternative pathway which appears to become operative at 220 ppm is oxidation by the peroxidase-catalase system.

$$Cl-CH_2-CH_2-OH \xrightarrow[\text{catalase}]{H_2O_2} ClCH_2-CH_2OOH \longrightarrow ClCH_2-CHO$$

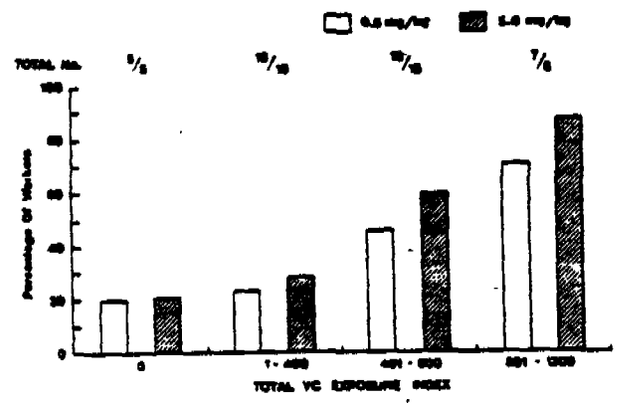


FIG. 1. Frequency of abnormal indocyanine green dye clearance among vinyl chloride workers utilizing 0.5 mg/kg and 5.0 mg/kg doses.

FIG. 2. Hepatic arteriogram: Venous phase. Changes of peliosis hepatis are present throughout the left lobe. The multiple nodular stains represent peliosis hepatis lesions (*arrows*) ranging from 2–3 mm to 2 cm in size.

FIG. 3. Splenic arteriogram: Venous phase (15 seconds). There are 3–4 circular and oval stains (*arrows*) in the superior and inferior-lateral portions of the spleen. Normal pancreatic stain occurs just above the splenic vein.

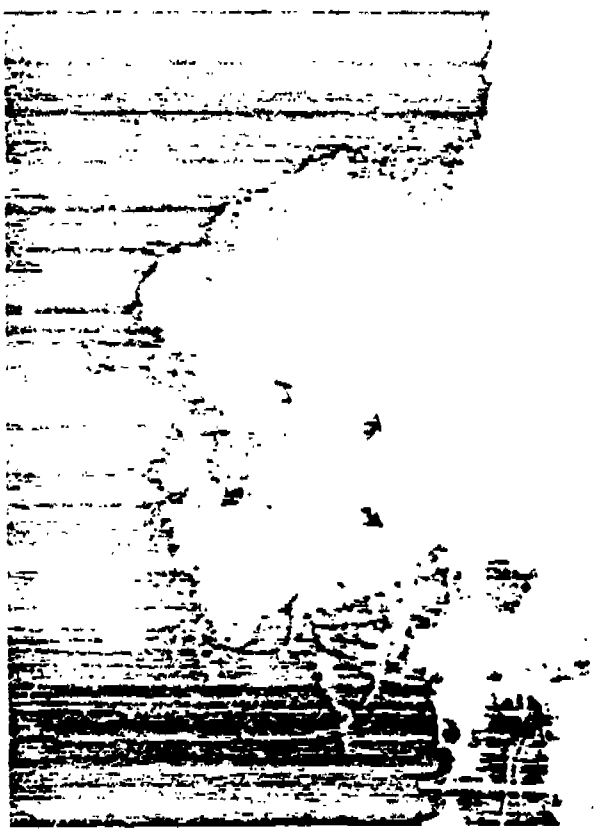


FIG. 4. Hepatic arteriogram: Venous phase. At approximately 14–15 seconds the peripheral stain is identified (*arrows*). lasting through the entire phase. Scattered areas of pooling are also present in and around the area of central hypovascularity.

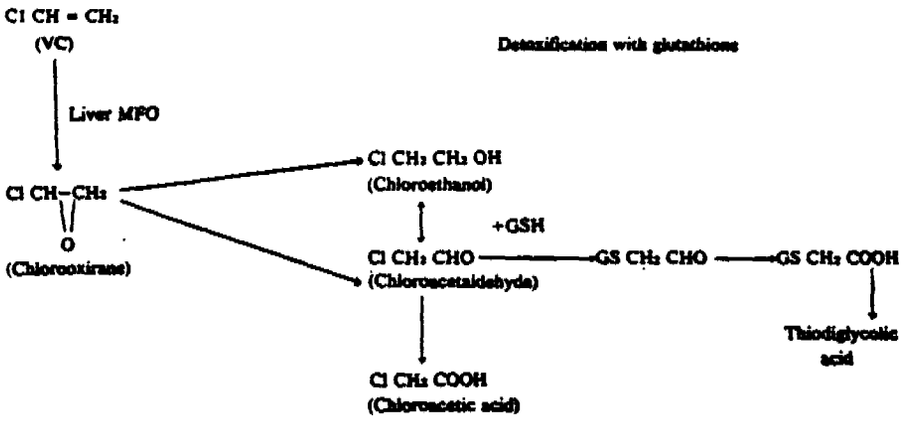
In this case chloroacetaldehyde is again formed. At higher levels oxidation appears to be by the mixed function oxidase system, forming chloroethylene oxide which spontaneously rearranges to form chloroacetaldehyde which then can be further oxidized to form monochloroacetic acid.

$$Cl-CH=CH_2 \xrightarrow{\text{oxidase}} CL-CH\!\!\!\diagup\!\!\!\diagdown CH_2 \longrightarrow Cl-CH_2-CHO \longrightarrow ClCH_2-COOH$$
(O)

As illustrated in Table 3, vinyl chloride oxidation intermediates chloroethanol and chloroacetaldehyde, at low doses, are most likely detoxified via the glutathione-cysteine conjugation system. This system, however, is saturable and at higher levels vinyl chloride is excreted via the lungs [28]. It appears that at higher vinyl chloride levels increased amounts of chloroethanol and chloroacetaldehyde are further oxidized to chloroacetic acid which is excreted in the urine. This is further supported by the absence of chloroacetic acid in urines of rats exposed to low, short term levels of vinyl chloride but found in urine of rats exposed to 5,000 ppm for an extended time and reported in workers exposed to levels greater than 250 ppm for a prolonged time [29, 30].

Elmore et al., utilizing a modified Ames system and pure synthesized vinyl chloride intermediates, has demonstrated that vinyl chloride, chloroethanol, and

TABLE 3
Proposed Metabolic Fate of Vinyl Chloride



chloroacetic acid are not mutagenic in bacteriological systems [31]. This may indicate that the vinyl chloride monomer is neither hepatotoxic nor carcinogenic until it has been metabolized to its intermediate forms by the liver and/or other tissues.

Alternatively, chloroethanol—the most transportable of the vinyl chloride metabolites—may be transferred or diffused to adjacent cells, such as the sinusoidal lining cells, where it could be converted to chloroacetaldehyde but less likely to be detoxified or further oxidized. Since vinyl chloride appears to bind the serum albumin, it may, itself be transported to and oxidized by extra-hepatic cells which are unable to fully oxidize or completely detoxify its metabolites, and thereby lead to molecular DNA injury and cancer formation at distant tissue sites.

These quandaries led to further study of the hepatochemical changes in rats undergoing progressively increased exposure to vinyl chloride. Subcellular enzymes and metabolites were studied in animals exposed to from 10-20,000 ppm vinyl chloride, ranging from 14–137 hours. Microsomal enzymes, including P-450, NADPH cytochrome *c* reductase, and mixed function oxidases were studied. Determinations of cytochrome *c* oxidase as the mitochondrial, tritiated-leucine incorporation as the protein synthesis and glucose-6-phosphatase as the carbohydrate metabolism markers were also done. Glutathione and glutathione reductase as oxidative and detoxification markers were determined in addition to the conventional clinical biochemical studies which included the aspartate (SGOT) and alanine aminotransferase (SGPT), alkaline phosphatase, bilirubin, lactic acid dehydrogenase (LDH), total protein, albumin, cholesterol, and triglycerides.

During the entire 137 hours of exposure there were no significant changes in the mitochondrial and the microsomal enzymes, the tritiated-leucine incorporation, or in the glutathione content. There was however, after 71 hours, a rise in the glutathione reductase and a concomitant fall in glucose-6-phosphate. This occurred without any histologically discernible changes in the hepatocytes by light microscopy nor any significant changes in the conventional clinical biochemical studies.

The discovery of a decreased glucose-6-phosphatase after "normalized" chronic exposure led to the study of enzymes in the pentose phosphate shunt pathway. Weber and Lea [32] had found similar changes for primary hepatocellular neoplasms, demonstrating that in a rapidly developing primary hepatocellular tumor, there is decreased gluconeogenesis with a reduction in the glucose-6-phosphatase, followed by an increase in glucose-6-phosphate dehydrogenase and transaldolase. These biochemical changes were also followed by an increase in purine biosynthesis (increased phosphoribosylpyrophosphate aminotransferase (PRPP) and increases in the production of ATP and GTP leading to increased nucleic acid synthesis.

Vinyl chloride-exposed animals showed no significant changes in the glucose-6-phosphatase dehydrogenase activity during the initial 84 hours of exposure. However, after 103 hours, there was significant increase in glucose-6-phosphatase dehydrogenase. Studies of PRPP, at least up to 137 hours, have as yet shown no significant changes. Studies are now underway using animals exposed to 130 to 250 hours to determine if the biochemistry in vinyl chloride injury is similar to that in primary hepatocellular tumors.

This, however, does not explain why the hepatocyte, which is the primary cell for oxidizing and detoxifying vinyl chloride, is not the primary target for cancer transformation. How do the hepatocytic biochemical changes, seen in the early phase of high vinyl chloride exposure, relate to the later morphological changes that occur in the adjacent sinusoidal cells?

MORPHOLOGICAL FINDINGS

Electron microscopic examination of liver sections of mice exposed from 1 to 6 months to 2,500–6,000 ppm vinyl chloride, for 5 hours/day, 5 days/week—a level known to induce angiosarcoma [33]—have demonstrated hepatocellular changes as early as one month. These changes included hypertrophy of the smooth endoplasmic reticulum (believed to reflect vinyl chloride metabolism) and, plasma membrane loss of microvilli with invaginations—possibly reflecting the movement of injurious metabolites across the membrane and out of the cell, allowing the metabolites to be picked up by the sinusoidal cells [34].

The sinusoidal cell reactions were multicellular. Increasing numbers and sizes of lipocytes were seen with little fibrosis. Macrophages were seen filled with phagosomes, sometimes containing long needle-like crystals. Although there were many mononuclear cells present, the main abnormalities were seen in the endothelial lining cells. In the early stages they are larger and thicker—possibly swollen. Later, they became bulky and in places, multi-layered containing increased organelles, especially mitochondria and endoplasmic reticulum. Later disruptions in the sinusoidal walls seen were consistent with beginning peliosis hepatis. The lining cells, probably the precursors of angiosarcoma, often resembled fibroblasts. However, their endoplasmic reticulum did not contain any collagen components. These observations by Schaffner et al. [34] give support to the suggestion that metabolites of vinyl chloride produced in the hepatocytes may be transported through the plasma membrane and enter sinusoidal lining cells, eventually leading to angiosarcoma. Attempts at screening for vinyl chloride hepatic injury might be better aimed at the endothelial cells and the hepatic sinusoidal circulation rather than the hepatocytes.

Our work in humans has identified similar findings. One major difference at

present is an increased collagen deposition, characteristic of human vinyl chloride injury and likely species specific. Light microscopic studies utilizing special stains on hepatic tissue from individuals with extensive exposure to vinyl chloride but without clinical biochemical hepatic abnormalities have shown distinctive midzonal increased deposition of collagen in the space of Disse [35]. Routine light microscopic studies using hematoxylin and eosin failed to easily demonstrate this midzonal increased collagen. The increased deposition along the hepatic cell surface is associated with larger sinusoidal space and activation of the sinusoidal lining cells illustrated by increased nuclear size and cytoplasmic content. The increased collagen deposition, when studied electron microscopically, demonstrates compression of the hepatocytes by the collagen bundles which initially give the appearance of *intra*-hepatocellular collagen bundles as illustrated in Fig. 5. The strands of collagen appear to compress the hepatocytes causing cords of hepatocytes to be broken and to coalesce with adjoining sinusoids eventually leading to peliosis hepatis-like lesions.

These observations led us to the study of the proteroglycan role in collagen formation in vinyl chloride-exposed workers. It had been suggested in the literature that glycosaminoglycans in blood and/or urine might be useful as means of early cancer detection since a number of studies had demonstrated the production of sulfated glycosaminoglycans with malignant states. Pathologists have often used this feature as a diagnostic aid in characterizing malignant vascular tumors of the skin

FIG. 5. Electron microscopy showing collagen (CB) bundles (*arrows*) invaginating into the hepatocyte, giving the appearance of inter-hepatocytic collagen. N = nucleus; S = sinusoidal space; IM = invagination into the cell membrane (*small arrows*).

[36]. Others have noted a strong positive Alcian blue glycosaminoglycan staining reaction in human angiosarcoma tissue [37]. This suggested that quantitative and qualitative determinations of glycosaminoglycan production in individuals with neoplasm, either by serum or urine, might be used to identify those at high risk or as an early indicator of neoplastic formation. The feasibility of glycosaminoglycan "spot test" for vinyl chloride production workers made this an attractive possibility for mass screening.

Urinary glycosaminoglycans, measured as uronic acid, were studied in individuals with alcoholic cirrhosis, viral hepatitis, secondary liver metastasis, hepatic angiosarcoma and normal controls.

The percentage of total glycosaminoglycans that was dialyzable and the percentage of unfractionated total that appeared in the hyaluronic acid, chondroitin sulfate, or in the heparin fractions was similar for all groups. However the distribution of positive fractions varied with the different groups studied.

Seven of the nine vinyl chloride-exposed individuals, other than those with angiosarcoma, had glycosaminoglycan positive chondroitin sulfate fractions with negative hyaluronic acid and heparin fractions whereas this occurred in only 3 of the 32 urines from other hepatic diseases [38].

In addition, study of the total tissue glycosaminoglycan levels in angiosarcoma tumors and fibrotic tissue adjacent to the tumor demonstrated that tumor tissue itself had higher levels of hyaluronic acid and heparin fractions as compared to the non-tumor adjacent tissue which had higher levels of chondroitin sulfate fractions. A similar relationship was found in cirrhotic liver tissue and normal controls. This data conforms to reports by others that both hepatic connective tissue disorder [39, 40, 41, 42, 43] and hepatic cancer [44] result in increased hepatic glycosaminoglycan levels. It may be significant that the angiosarcoma patient has half the urinary glycosaminoglycan excretion of patients with liver metastasis and that analysis of angiosarcoma tumor tissue exhibits half the glycosaminoglycan content reported by Kojima et al. [44] for hepatocellular carcinoma. The increases in liver and urinary glycosaminoglycans may well reflect the importance of these substances in the process of fibrogenesis and tumor growth. Although no significant differences were found in total glycosaminoglycans of vinyl chloride-exposed individuals with associated liver injury, there was a significant difference in the excretion patterns of these individuals. Seventy-eight percent of them had positive chondroitin sulfate fractions in contrast to only nine percent of the non-exposed liver injury cases. Thus the change in the glycosaminoglycan excretion pattern in individuals with pre-cancerous injuries may be of significant prognostic and diagnostic importance [45].

The urinary glycosaminoglycan excretion patterns in an angiosarcoma patient 3 months to 2 weeks prior to death demonstrated an increase in the urinary chondroitin sulfate fraction with a change in its composition as the disease progressed. During this time, the chondroitin sulfate composition showed a continuous increase in the ratio of the 1.25 M NaCl to the 1.5 M NaCl fractions. This was due to an increase in the 1.25 M eluate and a decrease in the 1.5 M eluate fraction and was 2.3 times greater than the controls. In the most advanced stage of the angiosarcoma the ratio increased to 13.7 times greater [46].

These very preliminary studies would suggest that alterations in the ratios of these fractions' compositions may be useful in evaluating the severity and subsequent progression of disease. Early lesions may produce small changes in the ratio which would become more pronounced as the disease worsened. The

determination of this ratio might also be useful in identifying those individuals with significant injury or continuing progression of disease despite changes in the environment. Finally therapeutic measures for intervention might be better evaluated for their effectiveness in arresting cancer development as reflected by the glycosaminoglycan changes which in turn reflect changes in collagen formation.

SUMMARY AND HYPOTHESIS

Vinyl chloride appears to enter the body through the respiratory tract, the skin, or by swallowing; it is absorbed and transported to the liver by the systemic and portal circulatory systems. In the liver, the primary site of metabolism, it is oxidized by various enzyme systems: alcohol dehydrogenase at lower exposure levels; the peroxidase-catalase system at intermediate levels; and mixed function oxidases at higher levels. At the higher levels, the mixed function oxidase system transforms vinyl chloride to chlorooxiranes which are then spontaneously transformed into choroethanol and chloroacetaldehyde.

These two intermediate metabolites, chloroethanol and chloroacetaldehyde, are detoxified by conjugation with glutathione and cysteine-SH groups and are excreted in the urine. At even higher doses increasing amounts of the chloroacetaldehyde are further oxidized to chloroacetic acid and excreted as an end product in the urine. However, when chloroacetaldehyde and/or the chlorooxiranes exceed the detoxification threshold of the hepatocyte, this leads to hepatocellular toxicity and/or stimulation of the sinusoidal cells. This acute event in turn acts as a stimulating mechanism for increased collagen deposition in the space of Disse and sinusoidal areas as shown by electron and light microscopy studies. The increase in the collagen depostion in sinusoidal spaces then leads to disruption of hepatic cell surface function, disruption of the hepatic cords, coalition of the sinusoidal spaces and eventual peliosis hepatis. These lesions alternately lead to sufficient vascular dysfunction to add further to the biochemical manifestation of hepatic cellular injury.

Since it is highly unlikely that the unstable chlorooxiranes are able to be transported to adjacent cells and that the chloroacetaldehyde would most likely be conjugated or detoxified within the hepatocyte, an intermediate form, such as chloroethanol, which is transportable from the hepatocyte, may then move on to the adjacent sinusoidal lining cells, or possibly even further to other extrahepatic tissue. At these extrahepatic sites, an intermediate, such as chloroethanol, may then be converted to chloroacetaldehyde. The extrahepatic tissue sites are most likely unable to further convert the chloroacetaldehyde to chloroacetic acid nor to detoxify it sufficiently, if at all, by their own detoxification systems. This would allow a longer contact period with the cell's DNA. In addition many of the extrahepatic cells normally are regenerating at faster rates than hepatocytes, thus increasing the possibility of DNA derangement and ultimate carcinogenesis.

Alternatively, vinyl chloride itself may be taken up by extrahepatic tissue, oxidized but incompletely detoxified, allowing the cell itself to become susceptible to direct DNA injury. In this or similar manner, chemical metabolites may induce injury to the DNA in rapidly replicating cells at sites beyond the liver, thus accounting for other cancers developing with vinyl chloride.

The recent work by Maltoni's group, showing that exposure of newborn rats to the same dose of vinyl chloride as adult rats, results in primary hepatocellular carcinoma (40–45%) rather than in angiosarcoma (8–12%), lends further support to

the concept that the hepatocyte's ability to resist cancer transformation is dependent upon its ability to detoxify the mutagenic/carcinogenic metabolite of vinyl chloride.

This review of our present knowledge of vinyl chloride injury and cancer formation in man is, at best, a very rough hypothetical outline. With continued investigation and study it will allow us to more accurately and completely fill in the missing pieces of this fascinating puzzle, thus leading us to a better understanding of the pathogenesis of chemically induced cancer in the biologically complex human system.

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The Effect of Repeated Vinyl Chloride Exposure on Rat Hepatic Metabolizing Enzymes[1]

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The Effect of Repeated Vinyl Chloride Exposure on Rat Hepatic Metabolizing Enzymes. DU, J. T., TSENG, M. T., AND TAMBURRO, C. H. (1982). *Toxicol. Appl. Pharmacol.* **62**, 1-10. Sprague-Dawley rats were exposed to 2.8% vinyl chloride for 2 (70 hr), 4 (140 hr), and 6 (210 hr) weeks to determine the sequential biochemical changes related to the oxidation and detoxification ability of hepatic tissue. Glutathione-*S*-transferase(s) activity using 1,2-epoxy-(*p*-nitrophenoxy)propane and *p*-nitrobenzyl chloride as substrates was elevated 17 to 24, 28, and 35 to 42% after 2, 4, and 6 weeks of exposure, respectively, suggesting enzyme(s) induction. Reduced glutathione, the major substrate required to conjugate the toxic metabolites of vinyl chloride, was also consistently elevated. Similarly, the activity of glutathione reductase, the enzyme necessary for the regeneration of reduced glutathione from its oxidized form, was also increased following vinyl chloride exposure. Cytochromes *P*-450, the major protein involved with vinyl chloride metabolism, was reduced after vinyl chloride exposure, confirming reports of others that vinyl chloride metabolites destroy *P*-450. No abnormalities of standard clinical biochemical blood tests of liver function were found during 6 weeks of vinyl chloride exposure. The only consistent ultrastructural modification was the dilation of endoplasmic reticulum. The biochemical and ultrastructural alterations could reflect early hepatocellular adaptation to vinyl chloride exposure.

Vinyl chloride, at high concentrations, has been shown to be carcinogenic in both laboratory animals (Maltoni and Lefemine, 1975; Viola *et al.*, 1971) and man (Creech and Johnson, 1974). Present data support the metabolism of vinyl chloride by hepatic microsomal mixed-function oxidase system into toxic intermediates, chloroethylene oxide (Bolt *et al.*, 1975; Hefner *et al.*, 1975; Kappus *et al.*, 1976) and chloroacetaldehyde (Gross and Freiberg, 1969). These two intermediates are considered to be the ultimate carcinogens (Barbin *et al.*, 1975; Jaeger *et al.*, 1974b; Van Duuren, 1975), to be mutagenic in bacterial systems (Elmore *et al.*, 1976; Greim *et al.*, 1975; Malaveille *et al.*, 1975; McCann *et al.*, 1975), to act as an alkylating agent by reacting with adenosine (Barbin *et al.*, 1975) and cytidine (Laib and Bolt, 1978), and to bind with protein (Bolt *et al.*, 1976; Kappus *et al.*, 1976; Watanabe *et al.*, 1978). Detoxification of these metabolites occurs mainly by conjugation with glutathione and is catalyzed by hepatic glutathione transferases; the conjugates are excreted in the urine as substituted cysteine derivatives (Watanabe *et al.*, 1976b,c; Green and Hathway, 1975, 1977). Chloroacetaldehyde can be further oxidized to chloro-

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acetic acid (Hefner *et al.*, 1975). These data are compiled in a metabolic scheme in Fig. 1 as an updated hypothesized metabolic fate of vinyl chloride in the adult rat. The metabolism of chloroethylene oxide via epoxide hydratase is not listed in the scheme because its product has not been identified.

There have been a few *in vivo* studies concerning the effects of vinyl chloride exposure on hepatic concentrations of glutathione (Hefner *et al.*, 1975; Watanabe *et al.*, 1976c; Du and Tamburro, 1978), cytochromes *P*-450 (Reynolds *et al.*, 1975) and on mixed-function oxidase activity (Drew *et al.*, 1975; Reynolds *et al.*, 1975). However, information about the sequential alterations in hepatic oxidation and detoxification of vinyl chloride, during prolonged exposure, simulating the occurrence in workers, is still lacking. It was reported previously that the enzymatic changes in rat liver following prolonged exposure to vinyl chloride were similar to those found in rat hepatoma (Du and Tamburro, 1976; Du *et al.*, 1979). The sequential biochemical changes related to the hepatic oxidation and detoxification of vinyl chloride following prolonged exposure are reported here.

METHODS

Animals and experimental design. Eight- to ten-week-old Sprague-Dawley male rats (~300 g), supplied by Laboratory Supply of Indianapolis, Indiana, were randomized prior to the experiment into three groups: a vinyl chloride-exposed group and the air-exposed group housed in identical chambers and a second control group housed in the University's Central Animal Care Center. The exposure level was 28,000 ppm vinyl chloride, 7 hr/day, 5 days/week for 2, 4, and 6 weeks. The exposure chambers were 4400-liter airtight vats. Vinyl chloride (~300 to 340 g) was added to the vat to give a time-weighted average concentration of 28,000 ± 1000 ppm. The chamber air was changed daily and the vinyl chloride concentration was determined by gas chromatography. The air was constantly circulated by a stirrer. The rats' respirations had negligible effect on the composition of the chamber's atmosphere because of the chamber's large volume. Animals were fed on standard laboratory chow pellets *ad libitum*.

All animals were anesthetized with ether, blood was drawn from the inferior vena cava, and the animals were

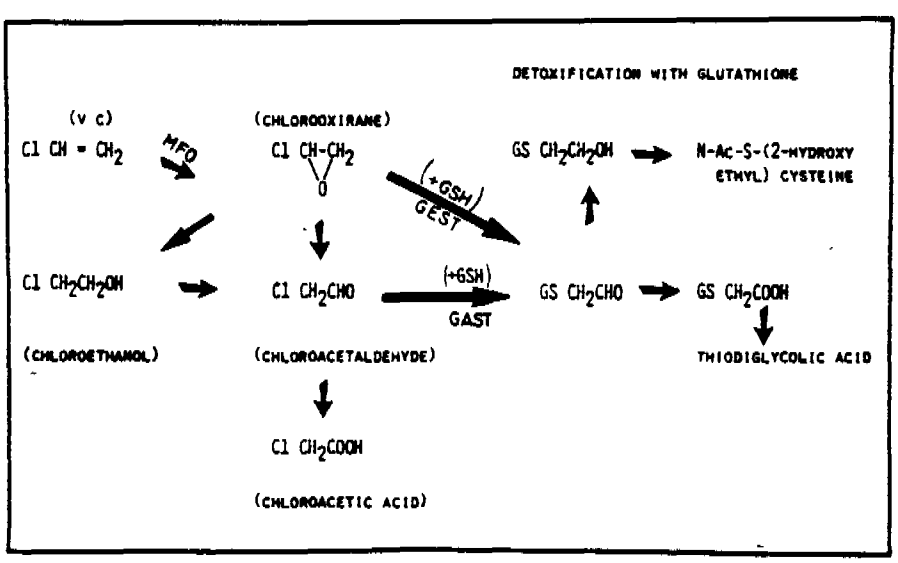


FIG. 1. The proposed metabolic fate of vinyl chloride. (GSH, glutathione; MFO, mixed-function oxidase; VC, vinyl chloride; GEST, glutathione *S*-epoxide transferase; and GAST, glutathione *S*-aldehyde transferase).

killed approximately 20 hr after exposure at 1:00 pm each day.

NADPH, glutathione, and glutathione disulfide were obtained from Sigma Chemical Company, St. Louis, Missouri; 1,2-epoxy-3-(*p*-nitrophenoxy)propane was purchased from Eastman Kodak Company, Rochester, New York; *p*-nitrobenzyl chloride was obtained from Matheson, Coleman and Bell, East Rutherford, New Jersey; benzphetamine was donated by the UpJohn Company, Kalamazoo, Michigan. Double-distilled water was used throughout.

Sample preparation and biochemical determination. Homogenates and subcellular fractions were prepared as described previously (Du *et al.*, 1979). Each sample was prepared from a single organ and kept at 4°C during preparation. The remaining liver was frozen rapidly in liquid nitrogen and stored at −70°C. Cytochromes *P*-450 concentrations were determined in the frozen microsomal fractions the day following sacrifice. The glutathione (GSH) concentration, as well as glutathione-*S*-transferase, glutathione reductase, and mixed-function oxidase activities were determined in the freshly fractionated frozen liver. For the assays using frozen tissue, the livers from control and experimental rats were frozen in an identical manner for the same length of time. Cytochromes *P*-450 concentration (Omura and Sato, 1964), nonprotein sulfhydryl content (Sedlak and Lindsay, 1968), and glutathione reductase activity (Carlberg and Mannervik, 1975) were determined in the microsomal or cytosol fractions by methods described previously (Du *et al.*, 1979).

Glutathione-*S*-transferase activity was determined using the 100,000 × *g* supernatant fraction. 1,2-Epoxy-3-(*p*-nitrophenoxy)propane and *p*-nitrobenzyl chloride were the substrates for glutathione-*S*-epoxide transferase and glutathione-*S*-aralkyl transferase (GAST), respectively. Enzyme activity was determined as described by others (Habig *et al.*, 1974; Kaplowitz *et al.*, 1975). All assays were linear functions of protein concentration and timed for at least 2 min. Solutions of 1,2-epoxy-3-(*p*-nitrophenoxy)propane and *p*-nitrobenzyl chloride were prepared in absolute ethanol; the final ethanol concentration in the incubation mixture was 0.5%. Mixed-function oxidase activity was estimated in the microsomal fraction by measuring NADPH disappearance in the NADPH-dependent demethylation reaction of benzphetamine (Lu *et al.*, 1972). The protein content was determined by the method of Lowry *et al.* (1951). The serum clinical liver tests including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, bilirubin, cholesterol, and triglyceride were determined by Technicon sequential multiple analyzer computer (SMAC) system.

Light and electron microscopy. Small strips of liver were removed under ether anesthesia, sliced into small cubes, placed immediately in ice-cold 1% osmium tetroxide (pH 7.4), and fixed for 2 hr at 4°C. Subsequently, samples were washed overnight in phosphate buffer, dehydrated in ascending alcohol, and embedded in Epon. Tissue blocks were polymerized at 60°C for 2 days. Thin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate before examination on a Philips 300 electron microscope. For ultrastructural analysis, three rats randomly selected from controls and groups exposed for 2, 4, and 6 weeks to vinyl chloride were studied.

For light microscopy, a block of tissue was fixed in buffered formalin and processed routinely for paraffin embedding. Sections 6 μm thick were stained with hematoxylin and eosin.

Statistical analysis. Analysis of variance was performed for the various groups at the different time periods and multiple comparisons were performed based on the results of the analysis of variance.

RESULTS

The protein content (mg protein/g liver) in the subcellular fractions in both control and vinyl chloride-exposed groups was the same throughout the exposure (data not shown); the enzymatic results, therefore, are expressed as micromoles of substrate converted per minute per milligram of protein.

There were no statistical differences between the normal and air-exposed groups in the glutathione and cytochromes *P*-450 contents or in any of the enzyme activities. The nonprotein sulfhydryl content (Table 1) was significantly elevated from 26 to 54% at 2, 4, and 6 weeks in the vinyl chloride-exposed group compared to both control groups. Although the nonprotein sulfhydryl content increased with exposure, the increases were not statistically significant. Glutathione reductase activity (Table 1) in the exposed group was increased by 53 to 77% at all three time periods. The increase in glutathione reductase was the same at 2 and 4 weeks of exposure but showed a further significant increase after 6 weeks of exposure. Glutathione-*S*-epoxide transferase (GEST, Table 1) and glutathione-*S*-aralkyl transferase (GAST, Table 1) activities were significantly higher than controls after 6 weeks of exposure, 37 and 45%, respectively. The cytochromes *P*-450 content, on the other hand,

TABLE 1

SEQUENTIAL CHANGES IN HEPATIC NONPROTEIN SULFHYDRYL, CYTOCHROMES P-450 CONTENT AND ACTIVITIES OF GLUTATHIONE REDUCTASE AND GLUTATHIONE-S-TRANSFERASES (EPOXIDE AND ARALKYL) IN RATS EXPOSED TO VINYL CHLORIDE[a]

	Treatment	Time (weeks) 0	2	4	6
Nonprotein sulfhydryl (μmol/g liver)	Normal control	7.9 ± 0.3	7.8 ± 0.4[b]	7.1 ± 0.4[b]	6.9 ± 0.4[b]
	Vinyl chloride-exposed	—	9.4 ± 0.2[b,c]	10.2 ± 0.6[b,c]	11.4 ± 0.6[b,c]
	Air control	—	7.1 ± 0.3[c]	6.9 ± 0.4[c]	7.9 ± 0.3[c]
Glutathione reductase (100 × μmol/min/mg protein)	Normal control	5.0 ± 0.2	4.3 ± 0.4[b]	4.2 ± 0.4[b]	4.8 ± 0.3[b]
	Vinyl chloride-exposed	—	6.7 ± 0.6[b,c]	6.3 ± 0.5[b,c]	8.9 ± 0.7[b,c,d]
	Air control	—	4.5 ± 0.2[c]	3.7 ± 0.3[c]	5.3 ± 0.4[c]
GEST (100 × μmol/min/mg protein)	Normal control	9.1 ± 1.6	7.8 ± 0.4	7.5 ± 1.0	7.7 ± 1.0[b]
	Vinyl chloride-exposed	—	9.1 ± 1.7	9.7 ± 0.7[c]	11.0 ± 1.3[b,c]
	Air control	—	8.1 ± 1.2	6.3 ± 0.7[c]	8.4 ± 0.9[c]
GAST (10 × μmol/min/mg protein)	Normal control	2.4 ± 0.4	2.1 ± 0.3	1.9 ± 0.2	2.4 ± 0.3[b]
	Vinyl chloride-exposed	—	2.6 ± 0.4	2.4 ± 0.3	3.2 ± 0.1[b,c]
	Air control	—	2.2 ± 0.2	1.9 ± 0.2	2.1 ± 0.2[c]
Cytochrome P-450 (nmol/g liver)	Normal control	17.0 ± 3.7	17.1 ± 1.7	19.7 ± 1.3[b]	15.5 ± 1.4[b]
	Vinyl chloride-exposed	—	13.2 ± 1.1	15.3 ± 1.3[b]	10.6 ± 1.0[b,c]
	Air control	—	15.5 ± 1.3	19.6 ± 2.7	14.3 ± 1.8[c]

[a] Rats were exposed to 28,000 ppm of vinyl chloride; normal controls and the air controls were exposed to air only. Each number represents the mean and the SEM from a group of six rats.

[b] Normal vs vinyl chloride-exposed, $p < 0.05$.

[c] Air control vs vinyl chloride-exposed, $p < 0.05$.

[d] Vinyl chloride (6 weeks) exposed vs vinyl chloride (2 and 4 weeks) exposed, $p < 0.05$.

was significantly lower than controls after 6 weeks of exposure to vinyl chloride (Table 1). No differences were found in the hepatic mixed-function oxidase activity or in the serum clinical liver tests. After 2 weeks of exposure, the two control groups had gained weight but the vinyl chloride-exposed group did not (Table 2). After 4 weeks of exposure, the normal control group housed at the animal care center had gained significantly more weight than either the air-control or vinyl chloride-exposed group. After 6 weeks of exposure, however, the vinyl chloride-exposed group failed to gain weight; the normal control group gained more than the air-control group (Table 2).

Morphological examination revealed polyhedral hepatocytes arranged in irregular plates interposed by vascular sinusoids in the livers of the control rats. This general cytoarchitecture was maintained after vinyl chloride exposure. Hepatocytes in control rats contained a prominent spherical nucleus, numerous ovoid mitochondria, stacks of rough endoplasmic reticulum (RER), some aggregates of smooth endoplasmic reticulum (SER), and varying amounts of lysosomes and glycogen particles (Fig. 2a). Few interstitial cells were scattered among the hepatocytes. These cells contained few cytoplasmic organelles and could be readily discerned at the light microscopic level by their hyperchromatic nuclei. The sinusoids were linked by fenestrated endothelium and some of the lining cells displayed phagocytic activity. After 2 to 6 weeks of vinyl chloride exposure, the principal organelle affected appeared to be the endoplasmic reticulum. Cisternae of the RER became dilated in a relatively small population of the hepatocytes in the 2-week treatment group. Four weeks after exposure to vinyl chloride, patches of dilated endoplasmic reticulum were prominently displayed in some hepatocytes (Fig. 2b). At this stage the SER was relatively unaffected. In the 6-week treatment group, vesiculation of SER and distention of RER were easily discernible in a large number of hepatocytes (Fig. 2c). However, other cell organelles showed no demonstrable change. These changes, though, are still beyond the resolving limit of the light microscope. The nonhepatocyte components showed minimum changes which

TABLE 2

BODY WEIGHTS OF RATS BEFORE AND AFTER VINYL CHLORIDE EXPOSURE[a]

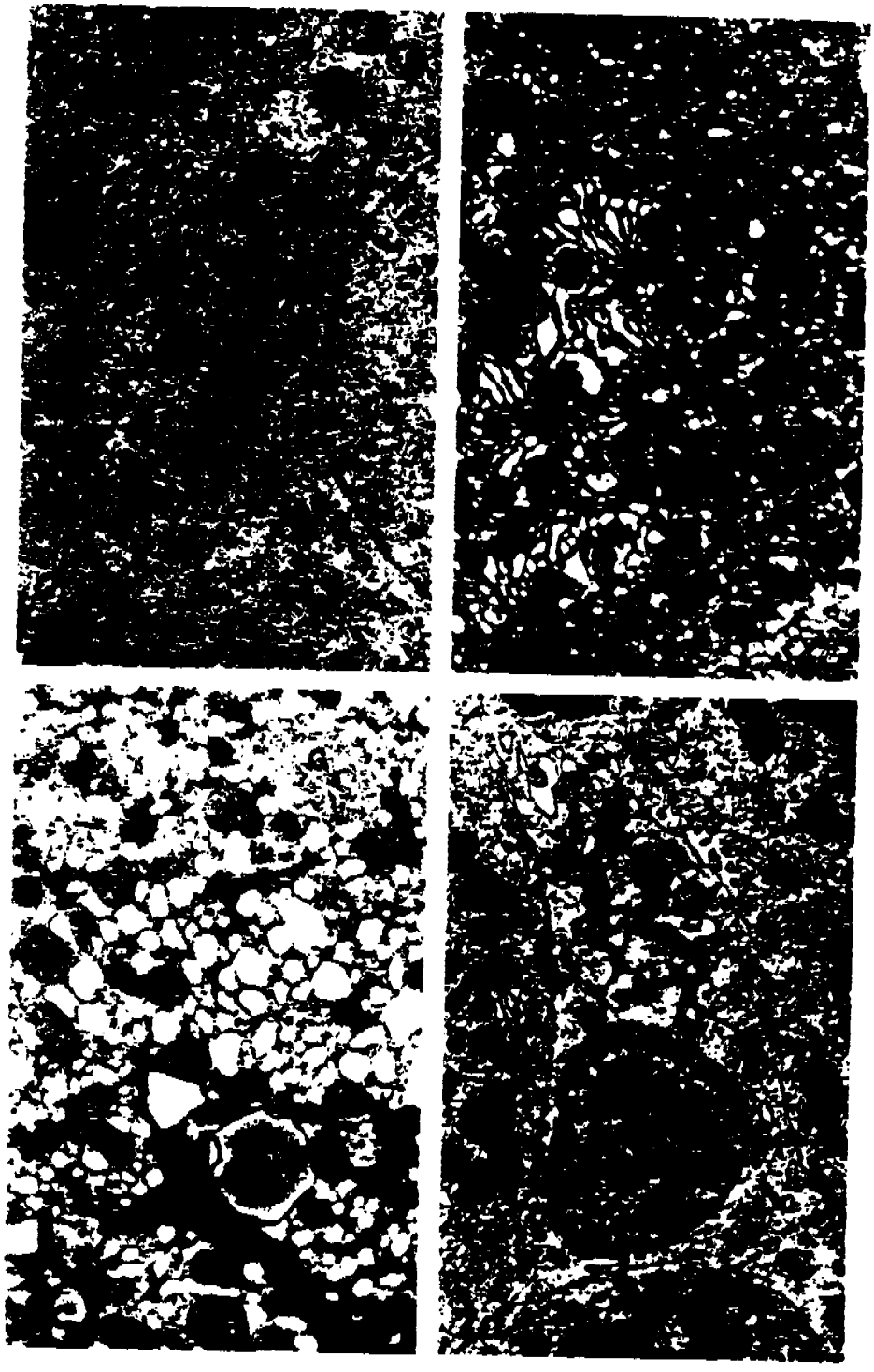
Duration (week)	Treatment	Initial weight (g)	Final weight (g)	Percentage gain
2	Normal control	400 ± 15	433 ± 16	8[b]
	VC-exposed	405 ± 12	396 ± 14	−2[b,c]
	Air control	396 ± 17	414 ± 18	5[c]
4	Normal control	398 ± 8	450 ± 15	13[b,d]
	VC-exposed	410 ± 16	421 ± 15	3[b]
	Air control	395 ± 10	419 ± 5	6[d]
6	Normal control	402 ± 14	486 ± 14	21[b,d]
	VC-exposed	396 ± 9	398 ± 10	<1[b,c]
	Air control	398 ± 14	449 ± 4	13[c,d]

[a] Analysis of body weight was by regression analysis followed by an analysis of variance on the residuals from the regression equation. (Residual = observed final weight − predicted final weight from regression equation.)

[b] Normal control vs vinyl chloride exposed, $p < 0.05$.

[c] Air control vs vinyl chloride exposed, $p < 0.05$.

[d] Air control vs normal control, $p < 0.05$.



were characterized by an increased accumulation of lysosomal-like substances in some of the sinusoidal lining cells as well as a greater tendency to accumulate lipids in the interstitial cells (Fig. 2d).

DISCUSSION

Glutathione conjugation is an important pathway for the metabolism of potentially harmful electrophilic metabolites of xenobiotics. Studies by Watanabe *et al.* (1976b,c) indicate this to be the major route for inactivation of the vinyl chloride metabolites.

Glutathione-*S*-transferases are a group of cytosol enzymes catalyzing the reaction of glutathione and electrophilic compounds to form less toxic and more water-soluble conjugates. Their activity during chronic exposure to xenobiotics, like vinyl chloride, could be a key determinate in the ultimate outcome of such exposures as illustrated by the longer arrow in Fig. 1. The increase in hepatic GEST activity at 4 weeks and the later increase in GAST activity at 6 weeks suggested that in the earlier stages of exposures most of the chlorooxirane intermediate is being adequately detoxified. During the later stages of chronic exposure, more chlorooxirane may become rearranged to yield more chloroacetaldehyde and, in turn, react with other available glutathione transferases or become further metabolized to chloroacetic acid. Alternatively, the excessive chlorooxirane could rearrange spontaneously to form chloroethanol and be further oxidized to chloroacetaldehyde, which in turn may react with glutathione, or be oxidized to monochloroacetic acid (Johnson, 1967). This would be consistent with the later increases in the aralkyl-transferases and the finding by Hefner *et al.* (1975) that monochloroacetic acid is found only in the urine of rats exposed for an extended time to higher levels (5000 ppm) of vinyl chloride. The increased use of alternate pathways, for chlorooxirane and chloroacetaldehyde detoxification, may reflect increased concentration of these active metabolites allowing greater opportunity for DNA injury.

A single exposure to vinyl chloride decreased hepatic nonprotein sulfhydryl compounds in rats (Watanabe, 1976a); similar decreases of glutathione concentrations were produced in rats by other xenobiotics such as 1,1-dichloroethylene (Jaeger *et al.*, 1974a; Reichert *et al.*, 1978) and acetaminophen (Mitchell *et al.*, 1973). In the present study, repeated exposure to vinyl chloride caused a significant increase of nonprotein sulfhydryl concentrations (Table 1) analogous to the elevation of glutathione concentrations seen after the administration of carcinogens to rats (Fiala *et al.*, 1976). In addition, the results showed that repeated exposure to vinyl chloride also caused an increase in hepatic glutathione-*S*-transferase activity (Table 1) similar to that seen after the administration of phenobarbital and 3-methylcholanthrene to rats (Mukhtar and Bresnick, 1976). These data suggest a mechanism for compensatory synthesis of hepatic glutathione and glutathione-*S*-transferases after repeated exposure to vinyl chloride.

The decreased concentration of cytochromes *P*-450 found in rats after repeated exposure to vinyl chloride (Table 1) is con-

FIG. 2. (a) Portion of a hepatocyte from control. Stacks of rough endoplasmic reticulum (RER) are separated by many ovoid mitochondria (M). Chromatin is finely dispersed in the nucleus (N). 9300×. (b) Hepatocyte after 2 weeks of vinyl chloride exposure. Dilation of RER appeared widespread in these two cells. Bile (B) canaliculus appeared unaltered in these rats. 5300×. (c) Four weeks after vinyl chloride exposure. Golgi complex (G) appeared unaffected while cisternal dilation continued. Distinction between SER and RER is complicated by the detachment of ribosomes. Lipid droplets (L) and glycogen (GL) often accumulated. 9500×. (d) A fat-storing interstitial cell is surrounded by several hepatocytes in a vinyl chloride-treated animal. Unlike lipid stored in hepatocytes, the shape of lipids (L) appeared irregular in these cells. 8000×.

sistent with work by Reynolds *et al.* (1975). This decrease in cytochromes *P*-450 content was also shown *in vitro* (Guengerich and Strickland, 1977; Ivanetich *et al.*, 1977) suggesting that a metabolite of vinyl chloride destroys the cytochrome. Mixed-function oxidase activity, with benzphetamine as substrate was unaltered.

With regard to the structural alterations produced by vinyl chloride, the present findings confirmed previous observations on the selective effect of this carcinogen in the endoplasmic reticulum (Du *et al.*, 1979). A gradual increase in the number of hepatocytes affected and the involvement of both smooth and rough ER were shown in this study. The endoplasmic reticulum is the primary site of protein synthesis and its dilation suggested the presence of a vinyl chloride-related effect. The lack of a concomitant Golgi hypertropy is noteworthy since this organelle serves as the site of glycosylation and packaging of many exportable proteins. It may be inferred that the vinyl chloride effect is mainly an intracellular phenomenon. The relatively late involvement of SER could reflect a further attempt at detoxification by the hepatocyte. The tendency for the interstitial cells to accumulate lipid and the heightened phagocytic activity correlate with increases in collagen formation and may be evidence of low-grade cellular injury. This may prove to be the initial histological response to vinyl chloride exposure.

Ultrastructural responses to chronic inhalation of vinyl chloride were reported by Feron *et al.* (1979). Unlike our findings, the principal effects observed were swollen mitochondria and some proliferation of SER. The mitochondrial swelling presumably reflects the extensive vacuolization observed by light microscopy. The discrepancy probably resulted from differences in the dose and duration in vinyl chloride exposure since Feron *et al.* (1979) exposed rats to 5000 ppm vinyl chloride for 52 weeks. Such an extensive mitochondrial lesion could result in biochemical modifications such as a change in ATPase or cytochrome oxidase levels. The only enzyme activity measured, glucose-6-phosphatase, was reduced.

The authors believed that the altered glutathione metabolism, as reflected by the increased nonprotein sulfhydryl content, and the increased activities of glutathione-*S*-transferases and glutathione reductase, in rat liver after repeated exposure to high doses of vinyl chloride represent an early hepatocellular adaptation to vinyl chloride exposure.

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Effectiveness of Federally Required Medical Laboratory Screening in the Detection of Chemical Liver Injury

by Carlo H. Tamburro* and Richard Greenberg*

The increasing concern of industrialized societies over the potential health hazard of synthetic chemicals in the occupational environment has led to goverment requirements for medical laboratory screening of workers. The specific tests for such screening programs are most often selected on the basis of medical experience which utilized them in symptomatic or hospitalized populations. Required screening tests for hepatic injury including cancer in vinyl chloride workers has been systematically and prospectively studied in an industrial population working with synthetic rubber and plastics. Approximately 1300 employees were studied over a five-year period. A cohort of 969 male employees, for the purposes of analysis, were divided into a "standard" and "nonstandard" population based upon the absence or presence of significant medical disease (including liver disease). A subcohort of 120 individuals was further identified based on availability of liver biopsy. Evaluation of federally required studies included alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGTP), alanine aminotransferase (ALT, SGPT), aspartic aminotransferase (AST, SGOT) and biliruhin (BR). Also studied were indocyanine green clearance (ICG) and radioisotopic liver spleen scans (L-S scans). The GGTP provided the highest positive predicted value as a screening test for identifying "nonstandard" individuals (individuals with all types of medical disease) followed by ICG, AST, ALT, L-S scan, AP, and BR.

In the identification of asymptomatic liver disease the GGTP had the least specificity due to a high false positive rate, while the AP provided the highest specificity. The ICG clearance however, provided the best combination of positive predictive value and sum of specificity and sensitivity. The AP provided additional increase in specificity as a follow-up study. There was no evidence that any of the other federally required tests added any additional benefit and did add significant increase in the false positive rate. These studies support the need for evaluating screening tests as to their sensitivity, specificity and positive predictive value, in asymptomatic individuals, before they are made established requirements.

Introduction

Industrialized societies throughout the world have become increasingly concerned over the potential health hazard of synthetic chemicals in the occupational environment. Governmental regulations have increased the number and types of medical laboratory screening required for a large variety of halogenated hydrocarbons as well as other potential environmental hazards. The primary objective of these screening programs is to reduce disability, morbidity and mortality in workers, especially as related to serious low-grade health hazards. In general, screening programs are instituted because of the presence in the work environment of a suspected or proven environmental toxin or carcinogen, which has the potential of producing low-grade injury over long periods of exposure.

Most screening studies are directed toward the detection of abnormalities in certain body systems. The specific tests are frequently selected on the basis of medical experience which utilized them in symptomatic or hospitalized populations. Prior experiences utilizing nonspecific multiphasic health surveillance screening and maintenance have not proven to be cost effective except under certain limited

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conditions (*1*). The cost effectiveness of such tests, however, in the determination of medical screening requirements, has played a limited role due to the potential seriousness of these occupational agents. Little attention has been paid as to whether the effectiveness of federally required screening provides the best or, more importantly, a necessary benefit when applied to asymptomatic and otherwise healthy worker populations.

The discovery in 1974 of hepatic toxicity and cancer formation in vinyl chloride workers provided the opportunity to systematically and prospectively study the effectiveness of federally required and federally recommended medical screening procedures for the detection of chemical liver injury, including cancer development (*2*). Table 1 lists the federally required medical screening procedures since 1974 for environments utilizing vinyl chloride or polyvinyl chloride. Table 2 lists the federally recommended studies for these same environments. This paper will present a preliminary assessment of the effectiveness of these federally required studies in the accurate detection and identification of chemically induced liver injury due to halogenated hydrocarbons, especially vinyl chloride.

Materials and Methods

The industrial population studied consisted of approximately 1200-1400 employees of a chemical plant whose two major products were synthetic rubber and plastics. The industrial plant had been in operation for over 35 years and had a predominance of male employees (96%), approximately 80-87% of the work force being white, 11-12% black, less than 1% of other racial origins. Turnover of the plant was approximately 10 to 15% per year with 65-70% of the work force having worked five years or more at the plant. Employee ages ranged from 18-65, with a mean of 52 years.

A cohort consisting of 969 male employees who worked continually from June 1, 1976 to May 31, 1977 was, for purposes of this analysis, divided into a "standard" and a "nonstandard" population. These designations were given on the basis of a review of all present standard medical data on each employee, including the federally required studies. Other screening studies of the medical surveillance programs were not utilized in the classification of overall medical status because, at that time, their clinical usefulness was unknown or controversial. All studies were performed on an annual basis; those individuals with ten years or more of employment were examined and screened semiannually. Compliance with medical screening studies during the five-year study period showed a continuous participation in the history and physical examinations by over 75% of the work force, laboratory tests and chest x-rays by 86%, and liver-spleen scans by 85%. Seventeen percent failed to undergo at least one history and physical examination, 9% did not have any of the radiological studies, and only 4% failed to have laboratory studies during this period. Approximately 40-50% of these individuals who did not undergo an examination claimed to have been examined by their private physician.

A subcohort of 120 individuals was further identified based on the availability of a liver biopsy performed for medical reasons, both related and not related to their work.

The term "standard" is used for those individuals who, based upon the best medical opinion, demonstrated no evidence of any significant medical disease, occupational or nonoccupational in origin. The "nonstandard" population included all others not included in the standard population.

The subcohort population was divided into those individuals with and without histological evidence of liver injury and further subdivided into those with and without histological features characteristic of chemical injury.

All employees had individual work histories. These consisted of a standardized job classification for all jobs within the plant since its opening and a

Table 1. Federally required studies for vinyl chloride workers.

History and physical
< 10 years as vinyl chloride worker—(annual)
> 10 years as vinyl chloride worker—(semiannual)
Biochemical studies
SGOT (AST)
SGPT (ALT)
GGTP
AP
TB

Table 2. Federally recommended (not required) studies.

Hepatic studies
LDH isoenzyme
Total protein
Protein electrophoresis
Hb_SA_0
Radioisotopic scan
Kidney dysfunction (urine examination)
Albumin
RBC
Exfoliative abnormal cells
Pulmonary system
FVC
FEV_1
Chest x-ray (PA and lateral)

rank ordering of exposure for 22 different suspected or potentially hazardous heptatoxic chemicals used within the work place (3-5). The agents were rank ordered on the basis of the intensity of exposure for each of the job classifications for each of the years that the plant was in operation. From this detailed work history, a cumulative exposure ran month ration (CERM) was determined for each employee for each of the 22 chemicals. All histological material was classified as to the presence or absence of liver disease, and to whether the abnormalities were consistent with chemical or non-chemical injury. This classification was conducted double blindly by three experienced physicians, two pathologists, and a hepatologist (6), without knowledge of any medical data, exposure or work history.

Results

Although 50 or more biochemical screening tests were performed during this study period, this paper will limit itself to the evaluation of the federally required studies, the indocyanine green clearance (ICG) study at the 0.5 mg/kg dose (7, 8) and radioisotopic liver and spleen scan (9). The

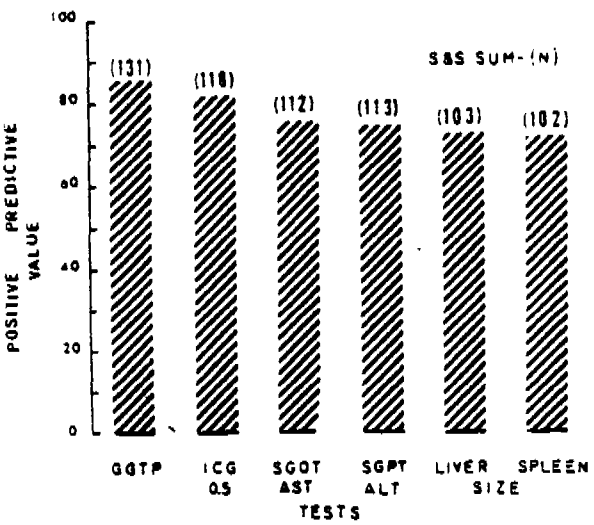

FIGURE 1. Positive predictive values of screening tests in identification of medical disease in an asymptomatic working population (N = 969). All those screening tests with positive predictive values of greater than 70 are shown except for indirect bilirubin (due to high number of congenital indirect hyperbilirubinemia) and triglyceride determination. Above each bar in the graph are shown the sum values for sensitivity and specificity of each test. They generally follow the same ranking.

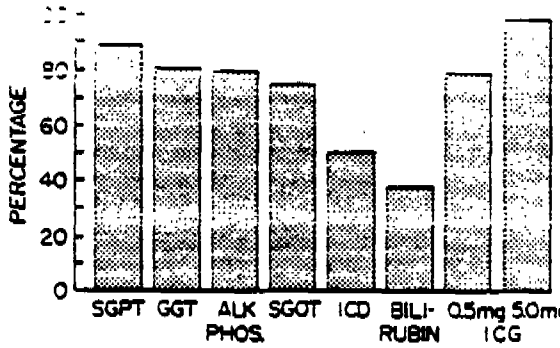

FIGURE 2. Frequency with which clinical biochemical tests correctly reflex the presence of hepatic damage in chemical workers suspected of having liver disease: (SGPT) alanine aminotransferase (ALT) (GGT) γ-glutamyl transpeptidase, (SGOT) aspartic aminotransferase (AST), (Alk. Phos.) alkaline phosphatase, (ICD) isocitric dehydrogenase (ICG) Indocyanine Green clearances at 0.5 and 5.0 mg/kg dose.

federally required biochemical studies include alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGPT), alanine aminotransferase (ALT/SGPT), bilirubin (ALT/SGPT), and the aspartic aminotransferase (AST/SGOT).

The positive predictive values of these screening tests in identifying medical disease (including liver disease) in this asymptomatic working population are shown in Figure 1. The GGTP provided the highest positive predictive value as a screening test for "nonstandard" individuals. It also provided the highest sensitivity and specificity sum shown in brackets. The predictive value of the other tests, in decreasing positivity were ICG, AST, ALT, liver and spleen scan, AP, and bilirubin.

Further evaluations were conducted on the subcohort population in whom we had both histological and biochemical data concerning hepatocellular damage. If one looks at only those individuals who received liver biopsies for suspected liver disease then one would find the percent of positive tests as illustrated in Figure 2. The ALT (SGPT), GGTP, AP and AST (SGOT) demonstrate a very high degree of sensitivity in identifying individuals with hepatic disease. As shown on the right, ICG clearances at the 0.5 mg/kg level provide a similar degree of sensitivity to SGOT and AP. The higher dose ICG clearance (5 mg/kg) appears to provide the most sensitivity for latent hepatic disease. These findings are consistent with the general medical experience with hospitalized patients.

Sensitivity alone however is not an adequat indicator of a test's screening value, especially when used in asymptomatic individuals. More appropriate evaluation of these tests' value as screening instruments are shown by their sensitivity, specificity

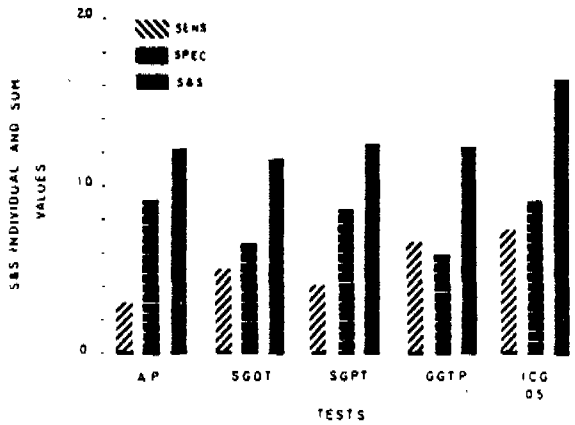

FIGURE 3. Sensitivity and specificity of various biochemical screening tests and their sensitivity and specificity sum values (S & S) based on 78 with biopsy documentation of their hepatic status and all of the biochemical screening studies listed. All screening tests with S & S sums less than 110 (e.g. bilirubin and isocitric dehydrogenase, are not illustrated.

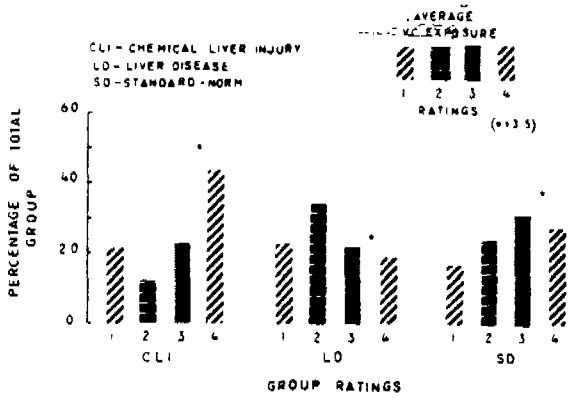

FIGURE 4. Correlation between the histologic findings on liver biopsy and each individual's average total vinyl chloride exposure based on their average CERM (Cumulative Exposure Rank Months) ratings. Rankings: 1 = lowest possible exposure; 2 = minimal exposure, low levels; 3 = moderate exposure; 4 = worked in areas subject to occasional high excursions, or frequently high and/or had intimate contact.

and sum values shown in Figure 3 in the biopsied subpopulation. Here again, γ-glutamyl transpeptidase and ICG clearance (0.5 mg dose) show the greatest sensitivity for identifying individuals with liver disease. However, GGPT had the least specificity, reflecting its high incidence of false positives. Specificity increased with the use of AST, ALT, and ICG clearance. The alkaline phosphatase provided the highest specificity, suggesting that mild or low grade chronic hepatic injury due to environmental agents may be activating hepatic AP synthesis in the absence of biliary tract obstruction or cholestasis. The ICG clearances, even at the low dose (0.5 mg/kg), clearly remains the test with the best combined sensitive and specific screening study for detection of individuals with subclinical hepatic disease.

This subcohort biopsied group was further examined on the basis of the histological interpretation of their liver biopsies and their work exposure to vinyl chloride. All biopsied individuals were subdivided into three groups: 19 with histological evidence consistent with chemical liver injury; 30 with histological evidence of liver disease, nonchemical liver injury; and 29 with normal liver biopsies. Each of the histological subgroups were further subdivided based on their vinyl chloride exposure, on a scale of 1 to 4 (Fig. 4).

The chemical liver injury group contained the highest percentage of individuals with the highest average rating (CERM) for vinyl chloride exposure. In contrast, with those with liver disease, nonchemical, and those with normal livers have a more even distribution of individuals relative to their degrees of vinyl chloride exposure.

In our previous studies we noted that almost all individuals with histologically specific lesion of vinyl chloride injury or angiosarcoma had a total average CERM rating of 3.5 or greater. The asterisk in Figure 3 indicates the percentage of individuals in each of the three histological groups with exposure ratings of 3.5 or greater. Again, the chemical liver injury group have the highest percentage of individuals with the high exposure ratings. This further supports previous work (*4*, *10*) identifying focal hepatocellular hyperplasia as the earliest histological characteristics of chemical injury in liver disease.

A study of the frequency with which these tests are positive among those individuals with liver disease, based on their histological findings (chemical versus nonchemical), provides additional data supporting the clinical observation that an increased AP has a greater specificity for chronic liver injury.

Figure 5 shows the ratio of the proportion of positive screening tests in those with histological chemical liver disease divided by the proportion of positive tests in those whose disease is not of chemical origin. All tests, independent of their sensitivity and specificity for liver injury, were more frequently abnormal in the presence of nonchemical, subclinical liver injury, except for AP. In contrast, AP was far more frequently abnormal in those individuals with chemical liver injury, which tended to be more chronic than acute and generally less severe.

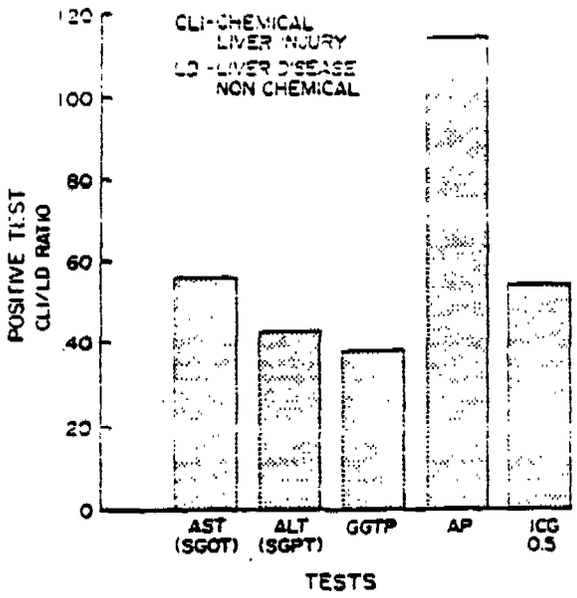

FIGURE 5. Frequency with which biochemical tests were abnormal in those with different type of hepatic injury expressed as a ratio: (CLI) chemical liver injury, (LD) liver disease, nonchemical.

Discussion

This preliminary systematic review of the positive predictive values and the sensitivity and specificity of federally and some non-federally required tests for chemical workers provides the first scientific and biological basis for the selection of medical screening tests for liver injury in occupational environments. Although these commonly used medical tests have been found by clinical experience to be effective as diagnostic tools in the symptomatically ill or hospitalized population, little clinical work has been done to determine their ability to accurately separate biological variations from early latent or underlying disease in asymptomatic individuals. Tests which provide very high false-positive rates (decreased specificity) such as GGTP, interfere with the screening process identification of the high risk worker by the extra time and cost required for repeat testing, the decreased productivity for the employer, the employees' increased anxiety, and by the loss of confidence in the effectiveness of the testing program by both employees and employer. Determination of the sensitivity and specificity of screening studies for asymptomatic individuals is essential if effective recommendations are to be made a federal requirement. This evaluation process also provided the best means of developing effective triage protocols for the screening program. For example, in this particular population of industrial workers, we have shown that the assessment of hepatic function is best accomplished by low dose ICG clearance (0.5 mg/kg). The ICG clearance is somewhat a more complicated technique (i.e., injection of substance and repeated blood sampling) but requires only 10 min to perform, and needs only one needle stick. In exchange it provides the best singular screening test for latent hepatic injury. If adequate medical facilities are not easily accessible, then ALT should be substituted. If either ICG clearance and/or ALT studies are found to be abnormal, an AP should be done and a diagnostic work-up instituted to determine the etiology (11).

The rationale for these recommendations is based on the actual study of chronic subacute chemical injury in an asymptomatic population, not preselected because of signs or symptoms. Therefore the test's ability to correctly differentiate disease from nondisease or one type of injury from another is more accurately determined. Chemical and environmental agents of low toxicity tend to produce repeated or persistent injury which accumulates over time. Tests which measure overall functional capacity quantitatively or semiquantitatively, rather than measuring acute low-grade injury over time are more likely to detect changes. For this reason, clearance or tolerance studies provide the best means for identifying latent hepatic disease, while enzyme studies like ALT, GGPT, and SGOT usually reflect acute cellular injury of higher grade or degree and cannot accurately reflex accumulative damage until very late in the disease process. Tests which provide information concerning the progression or nonprogression of injury will be far more helpful to the practicing occupational physician. They provide him/her with a better capability to discern between nonoccupational and occupational disease, and the best available reassurance for the worker of his or her safety while allowing the greatest possibility for continued productivity and employment.

Finally, these data provide a sound scientific basis upon which to modify federal requirements. The removal of specific testing requirements which, with field experience, prove not to have any significant positive predictive value, or effective sensitivity and specificity will aid in reducing overall cost and help maintain continued compliance by industry and workers. There may be theoretical reasons to maintain or continue some of the present federally required screening studies, but these reasons should be separately identified and not be confused with the purposes of the more effective test in the

detection of occupationally related liver injury.

The capabilities and limitations of any new tests or old tests for new screening purposes in detection of other potential occupational hazards, should be validated before making them a required screening procedure. This would lay the foundation for systematic determination of effectiveness of screening procedure against a proven standard. Unvalidated federal requirements provide the worker and employer with a false sense of security and safety by what is believed to be effective monitoring. More importantly, such a situation can lead to delay in effective correction of cause and disease prevention.

Conclusions

Biochemical screening for hepatic injury in asymptomatic chemical workers can be done most effectively by the use of liver specific clearance studies. ICG clearance provides the best combination of positive predictive value and sensitivity and specificity for functional hepatic testing at the present time.

None of the present federally required studies provide any significant degree of sensitivity without marked reduction in specificity in asymptomatic individuals.

The ALT (SGOT) is the most useful among those Federal tests presently required and the alkaline phosphatase may provide additional specificity as a follow-up study in those individuals with positive ICG or ALT screening studies.

There is no evidence that any of the other federal studies add any benefit and strong evidence that they significantly increase the false-positive results in well individuals.

All screening studies should undergo evaluation as to their positive predictive value and sensitivity and specificity in asymptomatic individuals before becoming permanent or established requirements.

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URINARY GLYCOSAMINOGLYCAN PATTERNS IN ANGIOSARCOMA OF THE LIVER

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Glycosaminoglycans extracted from 24-hour urine specimens from patients with hepatic angiosarcoma and from normal/controls were separated as cetylpyridinium complexes into "hyaluronic acid," "chondroitin sulfate," and "heparin" fractions, then further separated and characterized by anion-exchange chromatography and hyaluronidase susceptibility. The chromatographic pattern of the urinary chondroitin sulfate fraction in patients with angiosarcoma of the liver differed from those of controls in that there was a relative increase in the total amount of uronic acid in a hyaluronidase-resistant fraction and a decrease in a fraction susceptible to hyaluronidase digestion. These changes appeared to become more pronounced with advancing disease. Chromatographic patterns and determinations of hyaluronidase susceptibility indicated that the resistant fraction was heparan sulfate and that the susceptible fraction was chondroitin-4-sulfate and/or chondroitin-6-sulfate.

Cancer 40:3050–3053, 1977.

THE EMERGENCE OF ANGIOSARCOMA OF THE liver and its relationship to vinyl chloride exposure[4,7] has prompted a search for methods to detect this lesion. Although systematic screening programs are currently in operation,[10,18] there is still no single chemical indicator which is specific for angiosarcoma or for changes which may precede this disease.

The association of elevated tissue glycosaminoglycans (GAG) with tumors, including angiosarcoma, has been established.[1,9,16,18,22] Glycosaminoglycans are also known to be involved in normal connective tissue synthesis and collagen deposition and are elevated in connective tissue disorders.[13] Since angiosarcoma of the liver has both neoplasia and fibrogenesis in its etiology[17] GAG changes could be expected to serve to signal the appearance of early lesions and may be useful in evaluating advanced lesions.

Galambos[8] suggested that since the liver contributes very little to the overall connective tissue of the body, hepatic fibrogenesis should not be expected to result in significant increases in urinary GAG or collagen degradative or synthetic products. Preliminary studies in our laboratory, however, demonstrated an increase in both liver and urinary GAG in patients with angiosarcoma, chronic active hepatitis, and cirrhosis.[16] Most of the increase in urinary GAG occurred in the chondroitin sulfate fraction and, in contrast to what was found for normal and other diseases, the urinary chondroitin sulfate fraction was the *only* uronic acid positive fraction found in the urine of seven of nine cases of vinyl-chloride-exposure-associated liver injury other than angiosarcoma. This study was undertaken to characterize more completely the urinary "chondroitin sulfate" fraction in hepatic angiosarcoma.

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CLINICAL SUMMARIES

Case 1—(Hepatic Angiosarcoma—advanced)

A 46-year-old white male worked as a chemical helper in a vinyl chloride polymerization plant for thirteen years prior to the diagnosis of angiosarcoma. Twelve years after initial employment, the patient exhibited a persistent elevation of lactic dehydrogenase and underwent angiographic studies which demonstrated multiple areas of scattered tumor stain throughout both lobes of the liver with areas of central translucency consistent with the diagnosis of angiosarcoma of the liver.

Exploratory laparotomy and liver biopsy confirmed this diagnosis, and the patient was treated with adriamycin, cyclophosphamide, and methotrexate; an

initial response was associated with a decrease in the alkaline phosphatase activity, improvement in indocyanine green clearance and an increase in radioisotopic uptake in areas of previously defective uptake. After completion of the chemotherapy course, hepatic function deteriorated and the patient underwent partial hepatic lobe radiation (total dose of 5,000 rads) over a two-month period. Despite radiation therapy, the clinical course continued to deteriorate with the development of ascites, peripheral edema, increasing jaundice, hypoalbuminemia, and marked elevations of transaminases and alkaline phosphatase activities. This was followed by progressive hepatic failure, hepatorenal syndrome and hepatic coma. Autopsy findings showed extensive involvement of the liver with angiosarcomatous tissue extending into the diaphragm and metastasis to retroperitoneal and mediastinal lymph nodes, lungs, right adrenal gland and cerebellum. The right lobe of the liver demonstrated near elimination of the angiosarcoma, presumably due to the radiation treatment. Urinary GAG assays reported here were made on 24-hour urine specimens collected over the two-week period before death (Fig. 1).

Case 2 (Hepatic Angiosarcoma—moderately advanced)

A 54-year-old vinyl chloride polymerization worker was first employed as a polymerization vat cleaner 28 years prior to the diagnosis of angiosarcoma. Two years prior to diagnosis, the patient had persistent biochemical liver, abnormalities although he was otherwise asymptomatic with a normal liver-spleen scan. Angiographic studies showed peliosis hepatis. A liver biopsy revealed focal sinusoidal dilatation, mild chronic inflammatory reaction with portal fibrosis, Kupffer cell hyperplasia and dysplasia. Subsequent biopsies demonstrated continued sinusoidal dilitation, atypical and dysplastic Kupffer cells with premalignant changes. The peliosis hepatis pattern became more pronounced and multiple radioisotopic defects were evident on liver scan. A repeat biopsy one year after initial biochemical abnormality demonstrated malignant sinusoidal cells. The patient was treated with a combination of adriamycin, cytoxan, and methotrexate with limited clinical and biochemical response. Death was preceded by peripheral edema, ascites, progressive hepatic failure, and coma. The GAG analyses reported here were made 10 and 6 months before death (Fig. 1, [middle]).

MATERIALS AND METHODS

Twenty-four hour urine specimens were collected from two patients with angiosarcoma of the liver, and from two normal controls.

Urine specimens were stored at −70°C until analysis. Cetylpyridinium chloride (Sigma Chemical Company, St. Louis) was added to the entire 24-hour volume to precipitate the GAGs according to the method of DiFerrante.[8] The hyaluronic acid, chondroitin sulfate, and heparin fractions were eluted individually according to the method of Schiller *et al.*[19] Cetylpyridinium chloride was removed[14] and the GAGs were subjected to anion-exchange chromatography as described by Schiller *et al.*[19] Glycosaminoglycan fractions were applied to 1.0 × 44 cm AG1-X2 (200–400 mesh, chloride form) columns Bio Rad Laboratories, Richmond, California) and eluted stepwise with 0.0, 0.5, 1.0, 1.25, 1.50, 2.0, and 3.0 M NaCl. At a flow rate of 1.0 ml/min, approximately sixteen 10.3 ml fractions of each molar strength of NaCl were collected and a sample of each fraction was analyzed for uronic acid by the method of Bitter and Muir.[3] Standards of heparin (Nutritional Biochemical Company), chondroitin sulfate (Sigma Chemical Company), and hyaluronic acid (Nutritional Biochemical Company) were also evaluated by ion exchange chromatography.

The uronic-acid-positive fractions within each individual salt fraction were pooled, dialyzed to remove salt, and concentrated. The fractions eluted by 1.25 or 1.50 M NaCl were tested for

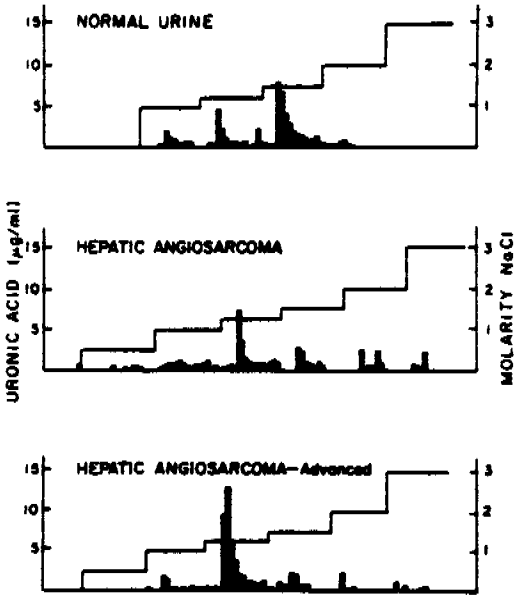

FIG. 1. Elution Patterns of the Urinary Chondroitin Sulfate Fraction. The glycosaminoglycans (GAG) in a 24-hour urine specimen were precipitated with cetylpyridinium chloride (CPC) and separated as 0.4 M NaCl soluble ("hyaluronic acid"), 1.2 M NaCl soluble ("chondroitin sulfate"), and 2.1 M NaCl soluble ("heparin") fractions. Each fraction was then subjected to anion-exchange chromatography. Shown here are typical 1.2 M (chondroitin sulfate) fraction elution patterns (Advanced = case 1).

Table 1

Source	Ratio of Total Uronic Acid Eluted in 1.25 M/1.5 M NaCl
Normal	0.364
Normal	0.316
Angiosarcoma, case 2, pre-chemotherapy[1]	0.843
Angiosarcoma, case 2, post-chemotherapy[2]	0.971
Angiosarcoma, case 1, advanced	5.000

NOTE: The glycosaminoglycans (GAG) in a 24-hour urine specimen were precipitated with cetylpyridinium chloride (CPC) and separated as 0.4 M NaCl soluble ("hyaluronic acid"), 1.2 M NaCl soluble ("chondroitin sulfate"), and 2.1 M NaCl soluble ("heparin") fractions. The CPC was removed from the 1.2 M NaCl-CPC-solubilized fraction and the GAGs further purified by anion-exchange chromatography. The total amount of GAG in the resulting 1.25 M and 1.50 M NaCl column-eluted fractions was determined and the ratio of the two fractions was calculated. ([1]One day prior to beginning of chemotherapy; [2]Two days following chemotherapy initiation.)

susceptibility to testicular hyaluronidase (Nutritional Biochemical Company) using a modification of the cetyltrimethylammonium-bromide, turbidimetric assay described by DiFerrante.[8]

Results

The major GAG fraction observed in all urines—both from normal controls or from patients with angiosarcoma—was the fraction solubilized by 1.2 M NaCl/1% cetylpyridinium chloride (the "chondroitin sulfate" fraction). Anion exchange chromatography of hyaluronic acid and heparin fractions revealed no qualitative differences between controls and angiosarcoma patients. The anion exchange column patterns of the 1.2 M NaCl solubilized GAGs are shown in Fig. 1. Chromatography of the urinary "chondroitin sulfate" fractions of patients with angiosarcoma yielded a comparatively large, uronic-acid positive peak in 1.25 M NaCl. The ratios of the total amount of uronic acid-positive material eluted with 1.25 M NaCl to the total amount eluted with 1.50 M NaCl are given in Table 1.

Table 2. Hyaluronidase Susceptibility

Source	Depolymerization %[1]
Heparin, standard	5.0
Hyaluronic acid, standard	93.1
Chondroitin sulfate, standard	97.6
1.25 M NaCl column-eluate, pooled fractions from angiosarcomatous patients	43.5
1.50 M NaCl column-eluate, pooled fractions from angiosarcomatous patients	100.0
1.50 M NaCl column-eluate, normal	100.0

[1] Glycosaminoglycans isolated from urine were tested for hyaluronidase susceptibility by measuring changes in turbidity developed with the addition of cetyltrimethylammonium bromide following incubation with hyaluronidase. Normal controls exhibited only minor amounts of 1.25 M NaCl column-eluted GAG and consequently do not appear in this table.

The susceptibility of the GAGs eluted with 1.25 or 1.50 M NaCl to hyaluronidase degradation is given in Table 2. The GAG eluted with 1.25 M NaCl was resistant to hyaluronidase, the enzyme producing only a 43% reduction in turbidity. The 1.50 M NaCl-eluted GAG fraction was 100% susceptible to hyaluronidase degradation.

Discussion and Conclusion

The chromatographic pattern found here for controls conforms to urinary glycosaminoglycan distributions reported by others.[12,21] These patterns suggest that there was a relative increase in urinary heparan sulfate and a decrease in chondroitin-4- and/or -6-sulfate in patients with hepatic angiosarcoma. This interpretation agrees with the Dowex 1-X2 chromatographic patterns reported by Kao and Leslie[12] and by others.[2,19]

Heparan sulfate is reported to be partially susceptible to hyaluronidase digestion,[20] and this correlates well with the observed 43% digestion of the GAG in our 1.25 M NaCl fraction. Since heparan sulfate has been shown to be associated with blood vessels,[20] an increase in the urinary excretion of this GAG is not surprising in this vascular lesion. Also, chondroitin-4- and -6 sulfates are reportedly eluted from Dowex 1-X2 columns with 1.50 M NaCl[2,19] and are susceptible to hyaluronidase[20] suggesting that our 1.5 M fraction is chordroitin-4- and/or chondroitin-6-sulfate.

Assuming that urinary GAG patterns described here are reflections of hepatic changes, it will be important to determine what processes these changes reflect, *i.e.*, those of neoplastic growth, fibrogenesis, or cell death. In this regard, it should be noted that 1) the ratio of heparan sulfate to chondroitin sulfate reported here for angiosarcomatous urine is similar to that reported for cirrhotic human liver *tissue* by Becker,[2] and 2) the shift from a hyaluronidase-

susceptible to a hyaluronidase-resistant GAG is consistent with the suggestion by Hutterer and Rubin[11] that the stabilization of collagen depends on a shift to a hyaluronidase-resistant GAG envelope surrounding the collagen bundle. Although Hutterer and Rubin attribute this to an augmentation of dermatan sulfate, Becker[2] reported that the GAG pattern in human cirrhosis was characterized by the augmentation of dermatan sulfate *and* heparan sulfate. If the observed changes in urinary GAG are reflective of vinyl chloride-exposure-associated fibrosis, the fact that fibrosis is a precursor of angiosarcoma[17] indicates that the observations reported here constitute a promising lead in early detection of vinyl-chloride-induced liver disease.

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Prevention and Detection of Cancer

PART I. PREVENTION

Volume 1. Etiology

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URINARY AND TISSUE GLYCOSAMINOGLYCAN PATTERNS IN HEPATIC ANGIOSARCOMA

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I. INTRODUCTION

The recent discovery of a relationship between vinyl chloride and angiosarcoma of the liver has received much attention (1-3). Although there are now systematic detection programs for vinyl chloride workers (3,4), there is as yet no specific chemical abnormality that serves as a good indicator of early, vinyl-chloride-induced liver injury and angiosarcoma. Alpha feto-protein has been a relatively valuable serological marker for hepatocellular carcinoma (5), but is has not as yet proven useful in the detection of angiosarcoma (6). New leads are needed if more specific tests are to be developed for angiosarcoma.

The literature suggests that the glycosaminoglycans in the urine and/or blood should be evaluated as a possible aid in early detection. The production of sulfated glycosaminoglycans is characteristic of malignant vascular tumors of the skin and some pathologists use this feature as a diagnostic aid (7). Barr and Bonin (8) observed a strong positive alcian-blue, glycosaminoglycan staining reaction in human angiosarcoma tissue and suggested than an attempt be made to qualitate and quantitate the production of glycosaminoglycans in the neoplasms, serum, and urine of those at risk. They pointed out that the urinary glycosaminoglycans may have diagnostic significance in angiosarcoma and, if so, a glycosaminoglycan spot test might easily be employed as a gross screening test of vinyl chloride production workers.

A number of other observations place the glycosaminoglycans in a relevant position with regard to angiosarcoma. Angiosarcoma is accompanied by connective tissue abnormalities (2,9) and changes in tissue, urinary, and blood glycosaminoglycans have been found to occur in many connective-tissue disorders -- including connective tissue disorders of the liver (10-14) -- as well as in hepatic cancer (15-17).

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The purpose of this study was to make a preliminary determination of the glycosaminoglycan patterns in tissue and urine associated with angiosarcoma of the liver and with vinyl-chloride-induced liver injury other than angiosarcoma and to compare these patterns with those in normal controls and those associated with other liver disease. Our goal was to evaluate the use of glycosaminoglycan patterns in the early detection of vinyl-chloride-induced liver injury and angiosarcoma and to explore the role of the glycosaminoglycans in the etiology of vinyl chloride injury.

II. PROCEDURES AND MATERIALS USED

Urine specimens were collected as occasional samples from: 9 normal controls; 9 individuals with histories of occupational exposure to vinyl chloride and having abnormal liver biochemical studies; 6 with "other" cancers prior to surgery; 3 with angiosarcoma; 8 with active viral hepatitis; 6 with cirrhosis; 2 with lung-liver metastases; and 4 with metabolic disorders of the liver (congenital and indirect hyperbilirubinemia).

In one case of angiosarcoma, 24-hr urines were collected on alternate days beginning 2 weeks prior to death.

Urine samples were collected without preservative and frozen at -76° until analysis. Specimens were divided into two 25 ml samples and one 5 ml sample. Urinary creatinine was measured on the 5 ml sample using a Technicon Autoanalyzer. The degree of urinary glycosaminoglycan polymerization was estimated by dialyzing one 25 ml sample for 24 hours in tap water; the sample was then treated identically to an undialyzed sample by the method of DiFerrante (18) using cetylpyridinium chloride as a precipitant. After resolubilization of the glycosaminoglycans in water, duplicate samples were assayed for total uronic acid by the modified carbozole reaction of Bitter and Muir (19). The remaining glycosaminoglycans were reprecipitated with cetylpyridinium chloride and separated into the wash, hyaluronic acid, chondroitin sulfate, and heparin fractions as described by Schiller et. al.(20). Each of the fractions was assayed for uronic acid (ug per mg of creatinine).

Autopsy tissue was obtained in 2 cases of hepatic angiosarcoma (tumor tissue and non-tumor tissue adjacent to tumor), 2 cases of cirrhosis, and in 3 control cases (gun-shot wound victims without liver pathology) and analyzed for glycosaminoglycans by a previously reported modification (21) of the method of Schiller et. al. (20). Uronic acid was determined in each of the hyaluronic acid, chondroitin sulfate, and heparin fractions.

Pieces of tissue were subjected to alcian-blue-periodic-acid-Schiff staining with and without hyaluronidase and diastase pretreatment. These procedures were carried out according to the methods described by Mowry (22).

Ascitic fluid was also obtained at autopsy in one case of angiosarcoma and analyzed for glycosaminoglycans. The fluid was centrifuged

and the sediment analyzed as tissue above. The supernatant was treated by the method for urine described above.

III. RESULTS

A summary of the urinary glycosaminoglycan measurement is given in Table I. Normal controls had the least urinary glycosaminoglycans (measured as uronic acid) of all groups. All other groups showed some elevation. The levels in angiosarcoma, hepatitis, cirrhosis, and liver metastases were significantly elevated ($P < .05$) over normal controls. The cirrhotic group exhibited the greatest variance in urinary glycosaminoglycans. No significant differences were found in urinary creatinine levels between groups.

There were no significant differences between groups in either the percentage of the total glycosaminoglycans that was dialyzable (Table I) or in the percentage of the unfractionated total that appeared in the hyaluronic acid, chondroitin sulfate, and heparin fractions.

Seven of 9 vinyl-chloride-exposed individuals other than those with angiosarcoma had positive chondroitin sulfate fractions with negative hyaluronic acid and heparin fractions. This was true in only 3 of 32 other urines evaluated in this same manner.

The pattern of daily glycosaminoglycan excretion prior to death due to angiosarcoma in one individual is given in Figure 1.

Total tissue glycosaminoglycan levels for angiosarcoma tumors, fibrotic tissue adjacent to tumors, cirrhotic liver tissue and normal liver tissue are shown in Figure 2. Fractional hyaluronic acid, chondroitin sulfate, and heparin levels are given in Figure 3.

Histochemically, angiosarcomatous tissue exhibited a strong alcian-blue positive staining reaction. Alcian-blue staining was only slightly less in "non-tumor" tissue adjacent to tumor masses. The staining reaction in tissue from normal liver was very weak and only slightly stronger in cirrhotic liver tissue. The strong alcian-blue reaction in angiosarcomatous tissue did not occur if sections were pretreated with hyaluronidase.

Ascitic fluid sediment was uronic-acid-positive in only the hyaluronic acid fraction -- 112 µg uronic acid per gram of dry, defatted sediment; ascitic fluid supernatant contained 1.7, 1.2, and 0.2 µg uronic acid per ml in the hyaluronic acid, chondroitin sulfate, and heparin fractions, respectively.

IV. DISCUSSION

The literature indicates that normal male creatinine excretion is 1.5 g per 24 hrs (23). Thus, our normal mean (Table I) of 3.2 ± .4 µg cetylpyridinium chloride-precipitable uronic acid per mg creatinine falls in the middle of the normal ranges reported by Varma et. al. (24).

2.6 - 4.7 μg/mg; DiFerrante and Rich (25), 2.9 - 4.3 μg/mg; and Rao and Leslie (26), 1.8 - 4.9 μg/mg.

Although our study was not controlled for age, Goldberg and Cotlier (27) have shown that urinary glycosaminoglycan excretion is constant from ages 20-70. Manley et. al. (28) have shown that the proportion of urinary glycosaminoglycans in the chondroitin sulfate fraction is constant from ages 20-70. Manley et. al. also reported that the chondroitin sulfate fraction is highest at birth and that it gradually drops until age 20, suggesting that urinary chondroitin sulfate reflects tissue growth.

The fact that we found no differences between groups in the creatinine concentration is significant in that it indicates that occasional samples do reflect 24-hour excretion when normalized to creatinine. Precedent for expressing glycosaminoglycan measurements as a function of creatinine content in occasional urine samples has been established by DiFerrante and Rich (25) and Pennock (29). Manley et. al. (28) have shown that the creatinine/uronic acid ratio is steady from ages 20-70.

Our results indicate that the liver diseases evaluated are accompanied by elevated urinary glycosaminoglycan excretion. Our tissue data suggests that this reflects liver-tissue glycosaminoglycan elevation and conforms to the reports by others that both hepatic connective tissue disorders (10-14) and hepatic cancer (15) result in increased hepatic glycosaminoglycan levels. It may be significant that the angiosarcoma patients had half the urinary glycosaminoglycan excretion of patients with liver metastases and that our analysis of angiosarcomatous tumor tissue exhibited half the glycosaminoglycan content reported by Kojima et. al. (15) for hepatocellular carcinoma.

While our data suggest that liver disease results in a decrease in the proportion of highly polymerized glycosaminoglycans, variance was large within each group and none of the differences between groups were statistically significant.

Although we have not completed the characterization of isolated glycosaminoglycan fractions, our data indicate: 1) that the chondroitin sulfates are the primary urinary glycosaminoglycans in both normal controls and in disease states; 2) that the chondroitin sulfates and heparin are the dominant glycosaminoglycans in normal and cirrhotic livers (Figure 3). Chondroitin sulfate is elevated in the fibrotic, non-tumor, portions of angiosarcomatous livers while heparin is the predominant glycosaminoglycan in tumor tissue. Hyaluronic acid is also apparently elevated relative to chondroitin sulfate in angiosarcomatous tumors (Figure 3); and 3) that hyaluronic acid is the sole glycosaminoglycan in ascites fluid sediment.

These qualitative data are in general agreement with those reported by others. Goldberg and Cotlier (27), Douglas et. al. (30), and Varma et. al. (24) have reported that the chondroitin sulfates are the predominant urinary glycosaminoglycans. Varma et. al. reported that 2/3 of urinary glycosaminoglycans are chondroitin-4-and chondroitin-6-sulfate and this agrees with our data on normal controls and on those with liver disease.

Kojima et. al. (15) reported that in hepatocellular carcinoma

tissue, chondroitin sulfates and hyaluronic acid were increased 33 and 10 times, respectively, over amounts found in healthy livers; the heparin and heparan sulfate proportions dropped. This contrasts with our data on angiosarcoma tissue, i.e. heparin and hyaluronic acid increased 5 and 10 times, respectively, over normal tissue; chondroitin sulfate levels rose but fell in proportion to other glycosaminoglycans. Galambos and Shapira (10) reported that the chondroitin sulfates are dominant in normal livers and in hepatic fibrosis, but Kojima et. al. (15) report that chondroitinase-resistant and hyaluronidase-resistant glycosaminoglycans are dominant. Kuroda et. al. (31) also reported that heparan sulfate is the dominant glycosaminoglycan in the normal liver. Our histochemical observation that nearly all of the increased alcian-blue positive material in angiosarcomatous livers was susceptible to hyaluronidase digestion suggests that the observed chondroitin sulfate elevation is due to chondroitin-4- and/or chondroitin-6-sulfate.

The increases in liver and urinary glycosaminoglycans may well reflect an important role of these substances in the process of fibrogenesis and in tumor growth. Galambos and Shapira (10) reported that hyaluronic acid was elevated during hepatic fibrogenesis. If a similar fibrotic process is operative in angiosarcoma, it may be that the observed tumor-tissue heparin increase is reflective of tumor growth. We did observe a four- to six-fold greater heparin level in tumor tissue than in adjacent, non-tumor tissue.

The observation that the chondroitin sulfates tend to be the exclusive uronic-acid-positive constituents in the urine of individuals is paradoxical in that those glycosaminoglycan fractions that are most elevated in angiosarcomatous tissue are those that are absent from the urine of individuals who may well have early, vinyl-chloride-induced liver injury. This pattern may be due to the selective action of lysosomal, glycolytic enzymes in the liver and/or may reflect the role of the chondroitin sulfates in early fibrotic changes in the liver. Certainly the potential usefulness of this pattern in early detection warrants the more complete evaluation now ongoing in our laboratory.

V. SUMMARY

Glycosaminoglycans were measured in urine and tissue of patients with hepatic fibrosis and hepatic cancer including vinyl-chloride-exposure-associated liver injury and angiosarcoma. Angiosarcoma, hepatitis, cirrhosis, and liver metastatic patients exhibited significantly elevated glycosaminoglycan excretion. Angiosarcoma tissue exhibited elevated glycosaminoglycan levels with the greatest increases in the heparin fraction. Histochemically, angiosarcomatous tissue gave a strong alcian-blue staining reaction which could be prevented by pretreatment with hyaluronidase. Although vinyl-chloride-exposure-associated liver injury other than angiosarcoma was not accompanied by a significantly elevated glycosaminoglycan excretion, this condition tended to be associated with a urinary glycosaminoglycan excretion pattern in which the chondroitin sulfate fraction was the only uronic-acid-positive fraction.

TABLE I. Urinary Glycosaminoglycan Levels in ug Uronic Acid per mg Creatinine by Liver Diseases Category

Patient group	Cases	ug uronic acid per mg creatinine (± 1 S.E.)	% uronic acid not dialyzable (± 1 S.E.)
normal control	9	3.2 ± .4	65 ± 5
vinyl chloride exposed	9	4.1 ± .4	39 ± 6
other cancer	6	4.5 ± 1.5	39 ± 6
other liver disease	4	5.1 ± 0.8	37 ± 13
angiosarcoma	3	7.6 ± 1.6	41 ± 22
hepatitis	8	8.5 ± 1.8	52 ± 14
cirrhosis	6	12.7 ± 3	53 ± 9
liver metastasis	2	13.8 ± .9	42

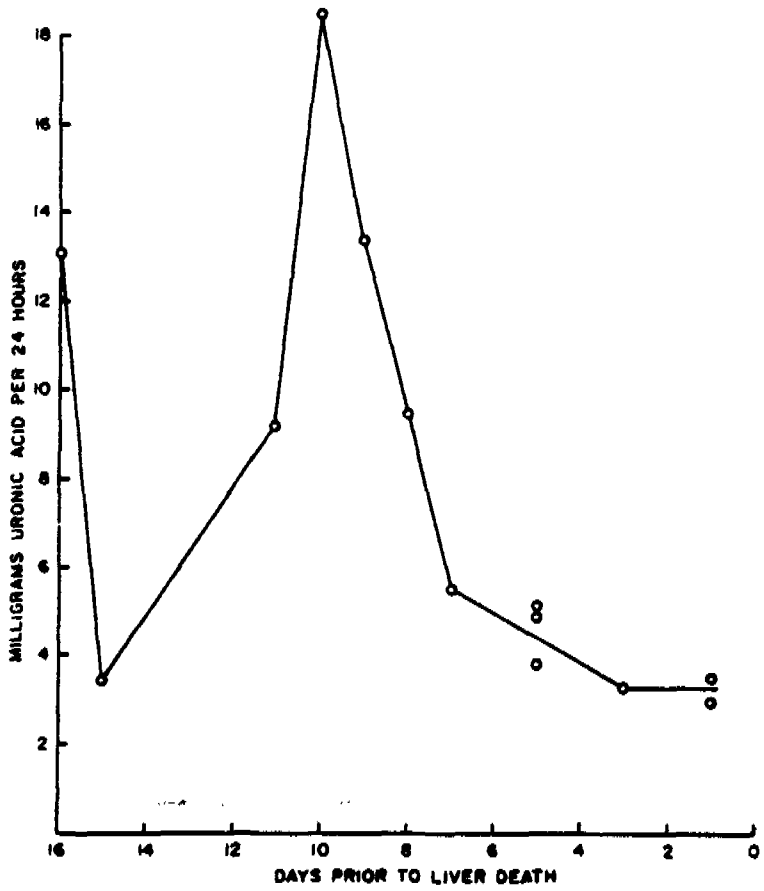

FIG. 1. Urinary glycosaminglycan output in one angiosarcoma patient during the 16-day period prior to death.

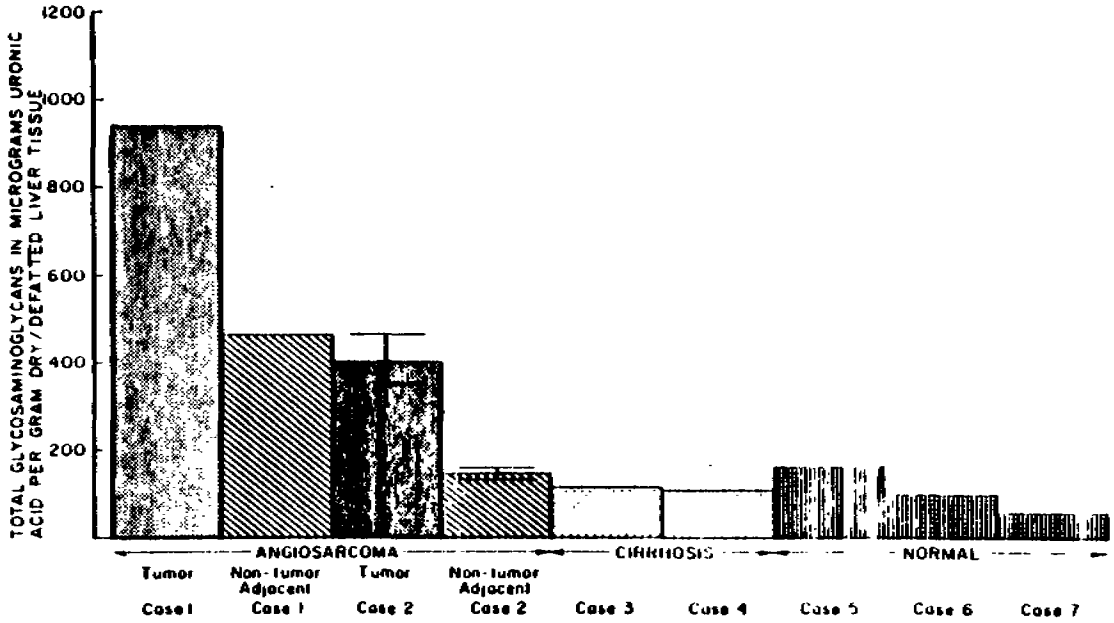

FIG. 2. Glycosaminoglycan concentration in angiosarcomatous, cirrhotic and normal human liver tissue.

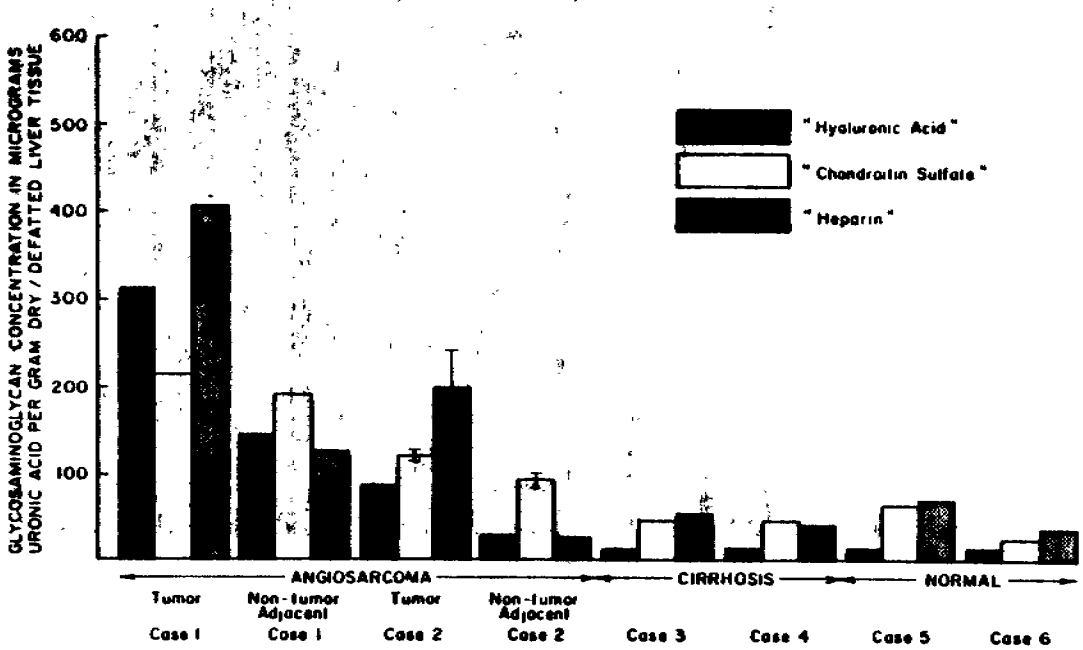

FIG. 3. Fractional concentrations of glycosaminoglycans in angiosarcomatous, cirrhotic and normal human liver tissue.

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Tissue and Urinary Glycosaminoglycan Patterns Associated with a Fast-, an Intermediate, and a Slow-growing Morris Hepatoma[1]

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ABSTRACT

The purpose of this investigation was to evaluate the glycosaminoglycans (GAG's) in different behavioral-histological types of i.m.-transplanted hepatomas and in the liver and urine of animals bearing these tumors. Groups of 10 Buffalo rats carrying fast-growing (7777), intermediate (5123tc), and slow-growing (9618A) Morris hepatomas were studied as the tumors reached 3 cm. Urinary and tissue GAG's were isolated by proteolysis, separated as cetylpyridinium complexes, and measured as uronic acid. The GAG's were further purified using anion-exchange chromatography and characterized with mucopolysaccharidases. Tissue GAG's were also evaluated histochemically using Alcian blue staining and mucopolysaccharidases. Tissue from fast-growing, intermediate, and slow-growing tumors exhibited greater GAG levels than did normal liver in the hyaluronic acid (0.4 M NaCl-soluble) fraction and in the chondroitin sulfate-heparan sulfate (1.2 M NaCl-soluble) fraction. The livers of tumor-bearing animals exhibited GAG levels similar to those of normal liver. Increased urinary GAG excretion was appreciated in animals bearing Tumors 5123tc and 9618A but not in those bearing Tumor 7777.

INTRODUCTION

An increasing number of reports cite the presence of comparatively high levels of GAG's[3] in both animal tumors (4, 7, 18, 22) and human tumors (5, 12, 16). There also have been reports citing qualitatively and quantitatively abnormal urinary GAG excretion in association with malignant tumors (6, 10, 11, 23–25). Although there have been attempts to establish any functional relationship between tumor GAG's and tumor cell properties (23, 25–27, 30, 31), the significance of elevated GAG's in malignant tumors remains obscure.

In view of the possibility that GAG's play an important role in the expression of one or more malignant cell properties, our purpose here was to evaluate the GAG patterns associated with transplantable hepatomas exhibiting different growth rates and metastatic properties. Because it has been suggested that tumor GAG may be contributed by normal host tissue in response to the presence of hepatic tumor (7), a secondary purpose was to evaluate the influence, if any, of "remote" hepatomas on the GAG's of the host liver; a third purpose was to evaluate the urinary GAG patterns in hepatoma-bearing animals.

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[3] The abbreviation used is: GAG, glycosaminoglycan.

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MATERIALS AND METHODS

Materials. Hyaluronic acid (umbilical cord) was purchased from Nutritional Biochemical Corp. (Cleveland, Ohio); chondroitin sulfate (whale and shark cartilage) and sodium heparin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Authentic samples of heparin, chondroitin 4-sulfate, heparan sulfate, and hyaluronate were also kindly supplied by Dr. M. B. Matthews, University of Chicago. Bovine testicular hyaluronidase was purchased from ICN Pharmaceuticals (Cleveland, Ohio), *Streptomyces* hyaluronidase was obtained from Calbiochem (La Jolla, Calif.), and *Proteus vulgaris* chondroitinase ABC was purchased from Sigma.

Experimental Design. Forty male Buffalo rats were shipped from Lab Supply Company (Indianapolis, Ind.) to Washington, D. C., where 10 were inoculated bilaterally (thigh) with Tumor 7777, 10 were inoculated with Tumor 5123tc, and 10 were inoculated with Tumor 9618A. These were shipped to Louisville with 10 controls. Throughout the study, animals were provided free access to food and water even when placed in metabolic cages for urine collections.

Urine collections were made twice each week from animals bearing Tumor 7777 and once each week from animals bearing Tumors 5123tc and 9618A. Collections were made alternatively on one-half of the animals in each group, with 5 control animal collections made each time a collection was made from tumor-bearing animals.

Tumors. Line 5123tc is a tissue culture variant of a moderately differentiated trabecular hepatocellular carcinoma induced with dietary administration of *N*-2-fluorenylphthalamic acid. Tumors were received and studied here in the 165th generation.

Tumor line 7777 is a poorly differentiated hepatocellular carcinoma induced by dietary administration of *N*-2-fluorenylphthalamic acid. This line was studied in the 159th transplant generation.

Tumor line 9618A is a well-differentiated hepatocellular carcinoma induced by 2-(4′-methyl)benzoylaminofluorene. This tumor was studied in the 13th generation.

Characteristics of these 3 tumors observed in our laboratory and selected characteristics reported by Hruban *et al.* (13, 14) are summarized in Table 1 (see also Fig. 1).

Extraction and Purification of GAG's from Liver and Tumor Tissue. Dry defatted tissue was subjected to proteolysis, trichloroacetic acid precipitation, and dialysis, and the GAG's were separated as cetylpyridinium chloride complexes into 0.03 M NaCl-soluble, 0.4 M NaCl-soluble, 1.2 M NaCl-soluble, and 2.1 M NaCl-soluble fractions as described by Schiller *et al.* (28) and measured as uronic acid (see Schiller *et al.*) by the carbazole method of Bitter and Muir (1).

Table 1

Characteristics of the transplantable hepatomas studied

Tumor line	Growth rate designation	Time (days) until tumor reached 3-cm-long axis	Histology	Metastatic potential[a]	General metastatic characteristic observed here	Collagen[a]
7777	Fast	18	Poorly differentiated	+++	Scattered lung micrometastases but no gross metastases evident at sacrifice	+
5123tc	Intermediate	35	Moderately differentiated	++	Multiple large metastases to lungs grossly evident in lungs of all animals at sacrifice	0
9618A	Slow	89–99	Well differentiated	0	No lung metastases evident by sampling at sacrifice	0

[a] According to the data of Hruban *et al.* (14).

Anion-Exchange Chromatography. Following uronic acid measurement, the uronic acid-positive material was pooled by tumor line with tumor and liver tissue material pooled separately. The cetylpyridinium chloride was removed as described by Korn (17), and then each fraction was dialyzed and subjected to Dowex 1-X2 (200 to 400 mesh) anion-exchange chromatography as described by Schiller *et al.* (28). The 0.0, 0.5, 1.0, 1.25, 1.5, and 2.0 M NaCl eluate fractions were eluted stepwise in 15 to 30 fractions (10 ml) each. One-ml samples were assayed for uronic acid to produce an elution profile. Chromatographic fractions were pooled across all tissue groups, dialyzed, and concentrated, yielding 6 pooled fractions which were then subjected to enzymatic characterization.

Enzymatic Characterization. Each of the fractions were subjected to digestion by *Streptomyces* hyaluronidase (digests only hyaluronic acid), bovine testicular hyaluronidase (digests hyaluronic acid, chondroitin, and chondroitin sulfate but not heparin, dermatan sulfate, or heparan sulfate), and chondroitinase ABC (digests hyaluronic acid, chondroitin, chondroitin sulfate, and derma sulfate but not heparin or heparan sulfate) as described by Kojima *et al.* (16).

Histochemistry. Small pieces of liver and tumor as well as lung and intestine in selected animals were fixed in Zenker's fluid, embedded in paraffin, cut at 6 μm, and subjected to hematoxylin and eosin, Alcian blue-periodic acid-Schiff (21), and Masson's trichrome (21) staining. Alcian blue-periodic acid-Schiff staining was also carried out with and without prior digestion in chondroitinase ABC and bovine testicular hyaluronidase (9). For the condroitinase ABC study, hydrated tissue sections were incubated with enzyme (16) for 2 hr at 37°; control sections were incubated in buffer only.

Statistical Analysis. Statistical analysis of tissue GAG data was carried out in a sequential format starting with an analysis of variance. Significant differences among the means were further evaluated using the Newman-Keuls procedure described by Snedecor and Cochran (29).

RESULTS

The amounts of GAG in Tumors 7777, 5123tc, and 9618A and in normal liver are presented by fractions in Chart 1. "Uronic acid" levels in the 0.03 M NaCl fraction were similar in Tumors 7777 and 5123tc but were significantly ($p < 0.05$) lower than the levels found in tumor 9618A and in normal liver. Uronic acid levels in the 0.04 M NaCl fraction isolated from the 3 tumors were similar and significantly higher ($p < 0.05$) than the levels found in normal liver. In the 1.2 M NaCl fraction, tumors exhibited 5- to 6-fold greater ($p < 0.05$) uronic acid levels than normal liver. In the 2.1 M NaCl fraction, uronic acid levels were similar for all tissues except that Tumor 9618A had significantly higher ($p < 0.05$) levels than did Tumor 5123tc.

There was a statistically significantly greater ($p < 0.05$) level of uronic acid in the 0.03 M NaCl fraction for the livers of animals bearing Tumor 5123tc compared to normal liver and the livers of animals bearing the other 2 lines. Except for this, differences among livers were unremarkable.

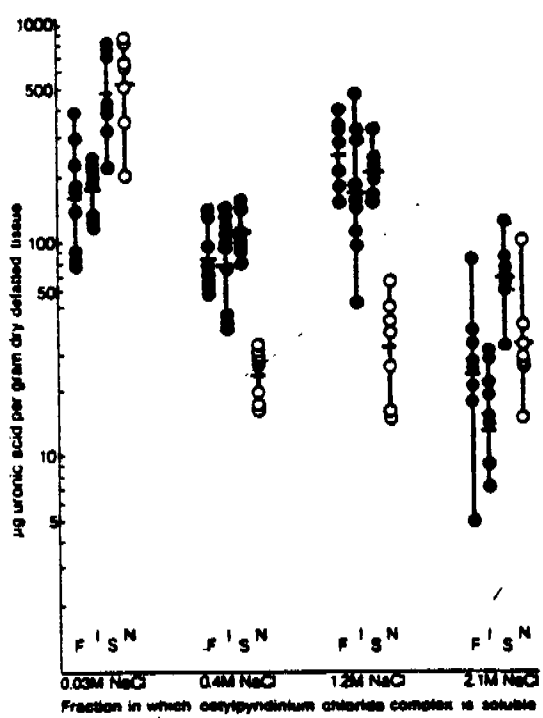

Chart 1. GAG levels measured as uronic acid in fast-growing (*F*) Tumor 7777, intermediate-rate (*I*) Tumor 5123tc, and slow-growing (*S*) Tumor 9618A and in the livers of non-tumor-bearing (*N*) animals for each of 4 sequentially collected salt fractions. *Bars*, geometric means. Differences between *F/I* and *S/N* in the 0.03 M NaCl fraction, between tumor tissue and normal liver in both the 0.4 M and 1.2 M NaCl fraction, and between *I* and *S* in the 2.1 M NaCl fraction are statistically significant ($p < 0.05$).

Chromatographic data for the 0.4 M NaCl and 1.2 M NaCl GAG fractions, those fractions which were appreciably larger in tumor tissue *versus* normal liver, are presented in Chart 2 together with the patterns obtained for these same fractions isolated from normal liver and the liver of tumor-bearing animals. These data indicate that, even though the uronic levels in the initial fractions were similar from tumor line to tumor line, there were some qualitative GAG differences in the fractions isolated from different tumor lines. This is even more apparent in composite Chart 3, which was derived by multiplying the mean of the individual uronic acid levels shown in Chart 1 by the percentage of distribution shown in Chart 2 and then summing within each chromatographic fraction.

Enzymatic Characterization of Tumor GAG's

The results of the enzymatic characterization of GAG's isolated from tumor tissue are summarized in Table 2. On the basis of the elution patterns reported by Kao and Leslie (15) and corroborated in our own study of authentic GAG's and on the enzyme susceptibilities for each fraction given in Table 2, we arrived at the identities of the predominant GAG in each chromatographic fraction given in Table 2, Column 7.

Histochemical Observations

Tumors. Each of the 3 tumor types exhibited significantly more intense Alcian blue staining than did normal liver or host liver; GAG's were generally distributed throughout the tumor tissue. Tumor sections subjected to hyaluronidase or to chondroitinase ABC exhibited reduced levels of Alcian blue staining consistent with biochemical measurements and the enzymatic characterization of chemically isolated fractions.

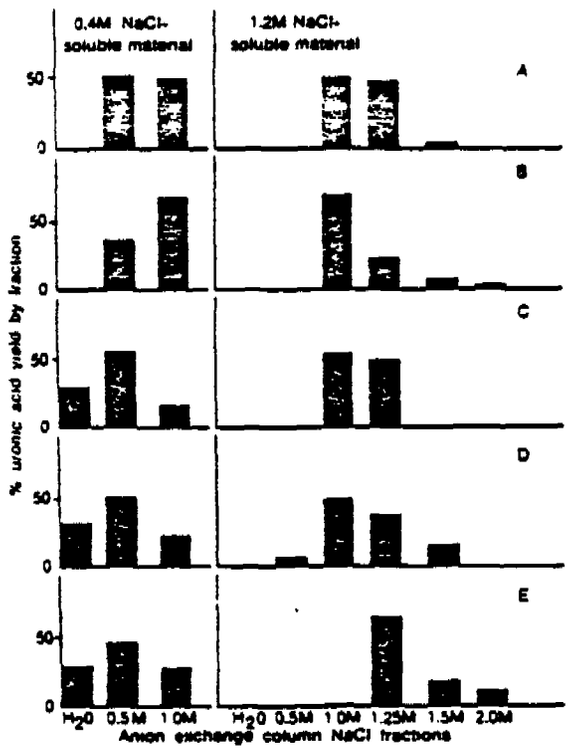

Chart 2. Anion-exchange chromatographic profiles for the GAG-cetylpyridinium chloride complexes solubilized initially in 0.4 M and 1.2 M NaCl. *A*, Tumor 7777; *B*, Tumor 5123tc; *C*, Tumor 9618A; *D*, pooled liver tissue from tumor-bearing animals; *E*, normal liver. Recoveries ranged from 88 to 107%.

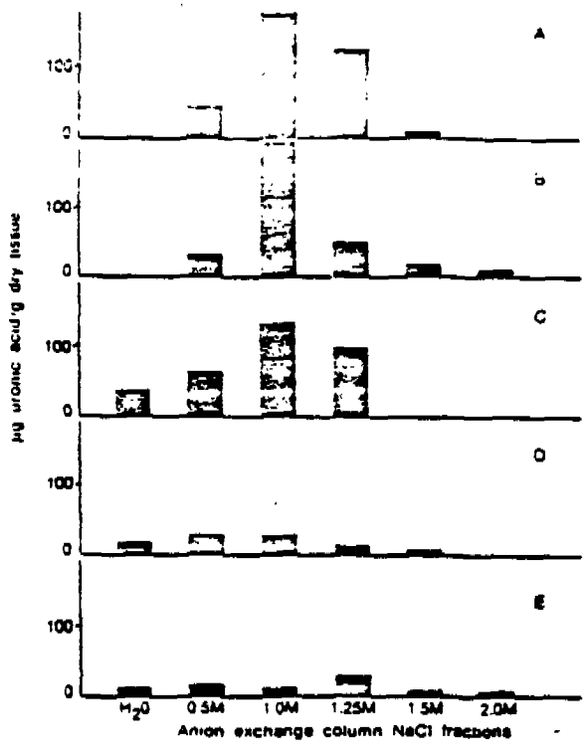

Chart 3. Anion-exchange chromatographic patterns obtained for the GAG's isolated initially as 0.4 M NaCl-soluble and 1.2 M NaCl-soluble cetylpyridinium chloride complexes. *A*, Tumor 7777; *B*, Tumor 5123tc; *C*, Tumor 9618A; *D*, livers of tumor-bearing animals pooled across tumor types; *E*, normal liver. This chart is a composite of Charts 1 and 2 and is based on the percentages (given in Chart 2) of the arithmetic mean of the individual uronic acid values (given in Chart 1) for each tissue type by fraction.

Host Livers. The livers of animals bearing Tumors 7777 and 9618A were histologically indistinguishable from normal liver. Liver tissue from animals bearing Tumor 5123tc consistently exhibited a slightly greater vacuolar appearance than did normal liver (Fig. 1B).

Urinary GAG Excretion

Over the entire study, mean total uronic acid excreted per pair of animals per 24 hr for control animals and animals bearing Tumors 7777, 5123tc, and 9618A were 118 ± 7 (S.E.), 116 ± 3, 203 ± 12, and 201 ± 7 μg, respectively. GAG excretion by animals bearing Tumors 5123tc and 9618A were statistically significantly ($p < 0.05$) elevated over controls and animals bearing Tumor 7777. There were 22, 8, 8, and 27 twenty-four-hr collections assayed, respectively. The small number of 7777 and 5123tc samples was due to the rapid growth of these tumors and time in transit after inoculation.

Urinary excretion profiles for animals bearing Tumor 5123tc over time and in relation to tumor size are illustrated in Chart 4. A similar pattern over a longer time span was observed in animals bearing Tumor 9618A. In both 5123tc and 9618A-bearing animals, uronic acid excretion appeared to be greater *after the tumors reached larger sizes*, but no regression with tumor size was apparent in either case.

Table 2

Identification of the predominant GAG in each of our anion-exchange chromatographic fractions

The identification (Column 7) of the predominant GAG or GAG's present in each of our anion-exchange chromatographic fractions (Column 1) was deduced from (*a*) solubilities of cetylpyridinium chloride complexes, (*b*) anion-exchange chromatographic patterns compared to those that we obtained for authentic GAG's and those reported by Kao and Leslie (15) (Columns 2 and 3), and (*c*) the susceptibility of each fraction to mucopolysaccharidases (Columns 4, 5, and 6).

Dowex 1-X2 fraction (M NaCl)	GAG's reported by Kao and Leslie (15) to be primarily eluted in this fraction	Other GAG s reported to be partially eluted in this fraction	% digested by *Streptomyces* hyaluronidase	% digested by bovine testicular hyaluronidase	% digested by chondroitinase ABC	Predominant GAG in our fraction
0.0			70–100	80–100	100	Hyaluronic acid
0.5	Hyaluronic acid (84)[a]	None	35–61	61	?	Hyaluronic acid
1.0			0	0	0	Heparan sulfate
1.25	Heparan sulfate (78)	Hyaluronic acid (12)	0	<2	0	Heparan sulfate
1.5	Chondroitin 4-sulfate (66)	Heparan sulfate (14) Heparin (18)				
	Chondroitin 6-sulfate (63)	Dermatan sulfate (19)	30	25	22	Heparan sulfate and/or heparin
2.0	Heparin (72)	Chondroitin 6-sulfate (19) Dermatan sulfate (67)				Heparin
		Chondroitan 4-sulfate (13)	0	?	0	

[a] Numbers in parentheses, percentage eluted per fraction.

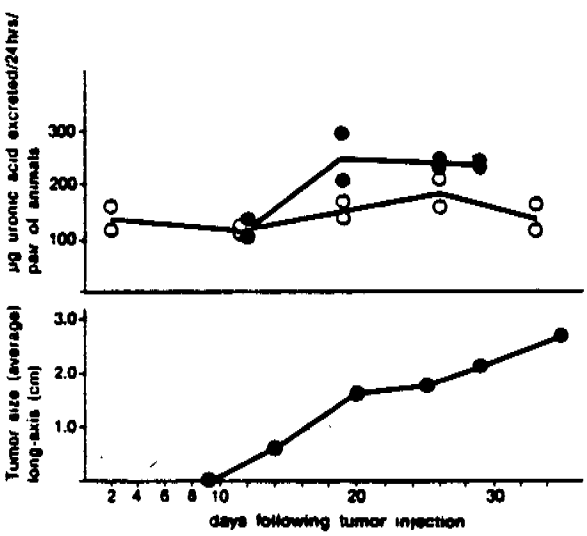

Chart 4. Urinary GAG excretion in relation to tumor growth for animals bearing Tumor 5123tc and in control animals. ●, tumor-bearing animals; ○, control animals.

We had sufficient urinary GAG's for anion-exchange chromatography only in the case of control animals and animals bearing Tumor 9618A. A chromatographic comparison of these 2 profiles indicated that the elevation in urinary GAG's occurred across all column fractions to about the same degree.

DISCUSSION

The results depicted in Charts 1 to 3 conform to the generalization (7, 16, 18) that tumors, including hepatic tumors, exhibit high levels of GAG's relative to the tissue of origin. Charts 2 and 3 suggest that differences between tumors may be related to the behavioral properties of the tumors. Chart 3 reveals a gradation from normal liver, through the liver of tumor-bearing animals, well-differentiated, slowly growing tumor tissue to faster-growing, metastatic tumor lines. The gradation shown in Chart 3 is even more striking if the patterns for tumor lines 7777 and 5123tc are inverted. Since tumor line 5123tc was more highly metastic than was line 7777 (Table 1), such an inversion would arrange the tissues according to metastatic potential and raises the possibility that the patterns, particularly Fraction 1.0 M, are related in some way to metastatic potential.

It is postulated that the 1.0 M NaCl chromatographic fraction contains an undersulfated form of heparan sulfate such as that described by Kuroda *et al.* (18) in AH109 hepatomas. Both Kuroda *et al.* (18) and Saito (25) reported that heparan sulfate is the major GAG constitutent in AH109A hepatic tumors. Kuroda *et al.* also reported that most of this heparan sulfate is eluted in 1.0 M NaCl in anion-exchange chromatography and that heparan sulfate is also the predominant GAG in normal liver and suggested that this may mean that the tumor heparan sulfate comes from tumor cells and not from connective tissue elements within the tumors.

The predominance of heparan sulfate in Morris hepatomas and in AH109A hepatomas do not conform with the fact that hyaluronic acid and chondroitin sulfate have generally been identified as the predominant GAG's in animal tumors (4, 7). These findings likewise do not conform with the report by Kojima *et al.* (16) that chondroitin sulfate and hyaluronic acid are the predominant GAG's in human hepatocellular cancer.

The possibility that tumor heparan sulfate is related in some direct way to tumor behavior has been raised by others. A role for sulfated GAG's in cell recognition and adhesion has been proposed by Dietrich *et al.* (8), and Chiarugi and Vannucchi (3) have proposed that cell surface heparan sulfate regulates both cell division and transport.

The histological appearance of the tumors studied here and the histological normalcy of the livers of tumor-bearing animals agree with the findings reported by Hruban *et al.* (14). Although the vacuolar appearance of the livers of animals bearing Tumor 5123tc is not abnormal in rodents, this characteristic was uniformly present in all Tumor 5123tc-bearing animals and was found in no others. The possibility exists that this structural feature is related to the high concentrations of the uncharac-

terized. 0.03 M NaCl-soluble, uronic acid-positive-material found in these livers.

The fact that urinary GAG excretion was elevated in animals bearing Tumors 5123tc and 9618A but not in animals bearing Tumor 7777 suggests that greater GAG excretion may reflect properties of certain tumors and is not simply an indirect result of tumor presence or tumor growth. Our levels of excretion of GAG in our control and experimental animals agree with levels reported for rats by Lehtonen *et al.* (19).

It remains to be determined what the source(s) of the elevated urinary GAG is (are). There have been reports of striking increases in GAG synthesis in tumor cells (see Ref. 20), but Kojima *et al.* (16) have suggested that decreased degradation may account for increased GAG in some tumors. Our investigations provide no evidence that the source of the abnormal GAG increases in tumor tissue and urine is anything other than the tumor cells themselves; however, a possibility that these results stem from an impaired ability of the liver to degrade circulating GAG's cannot be ruled out.

CONCLUSION

Our data clearly show that hepatomas 7777, 5123tc, and 9618A have GAG compositions that are appreciably different than that of normal liver. These data show that there are also qualitative differences in GAG between tumor lines and that heparan sulfate patterns parallel growth rate and possible degree of malignancy. Heparan sulfate is the predominant GAG in the hepatomas studied. The increased urinary excretion of GAG's for 2 of the 3 tumor lines examined suggests that urinary GAG analysis may prove useful in the detection and diagnosis of some hepatic tumors.

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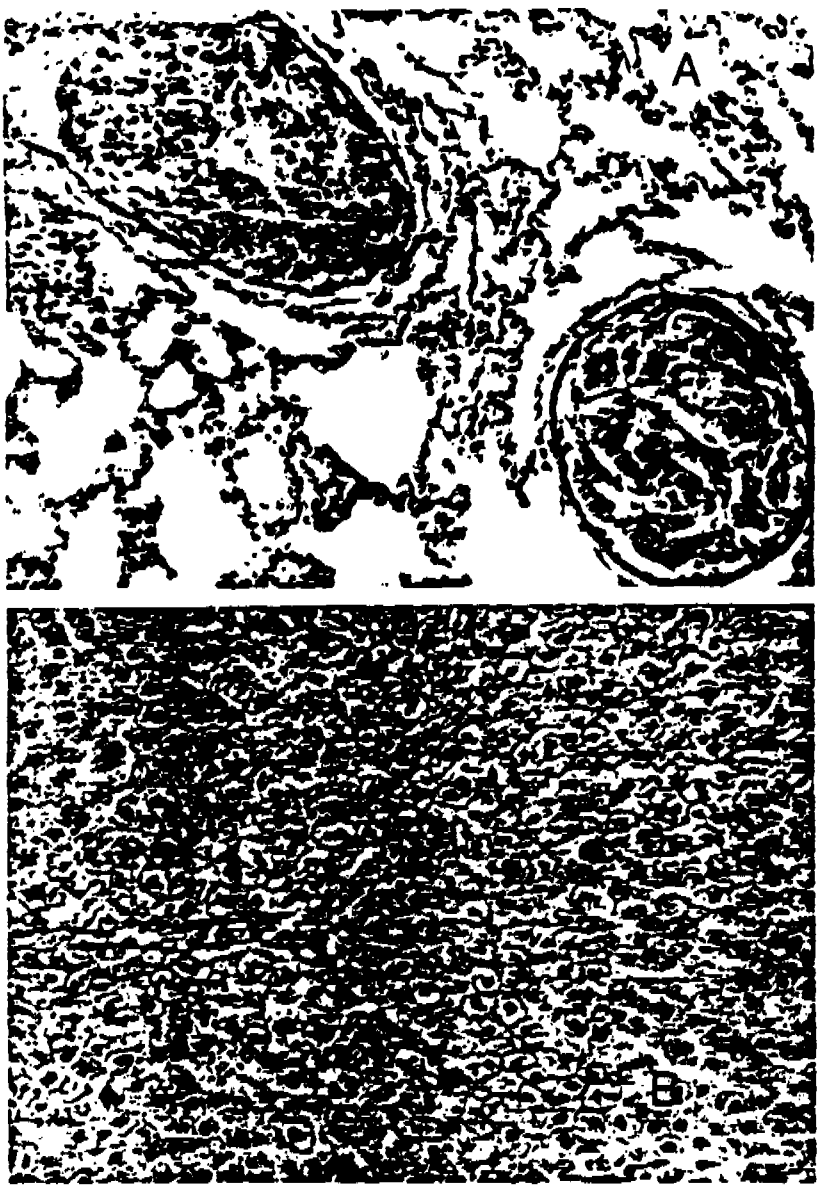

Fig. 1. *A*, typical lung metastases in animals bearing Tumor 7777. These were found more frequently and earlier and were larger in animals bearing Tumor 5123tc. No lung metastases were found in any of the animals bearing Tumor 9618A ×50. *B*, liver of animals bearing Tumor 5123tc showing clear cytoplasm-vacuolar appearance characteristic of such livers. ×50.

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EPIDEMIOLOGY STUDIES
SCREENING FOR THE EARLY DETECTION OF DISEASE IN INDIVIDUALS EXPOSED TO VINYL CHLORIDE

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SCREENING FOR THE EARLY DETECTION OF DISEASE IN INDIVIDUALS EXPOSED TO VINYL CHLORIDE

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ABSTRACT

A prospective collaborative study was conducted to compare the effectiveness of four clinical techniques in the detection of liver damage due to vinyl chloride monomer exposure. A chemically exposed and medically monitored worker population was identified by histopathological and biochemical documentation. Three techniques were non-invasive: a) grey scale ultrasonography of the liver, b) microvascular skin capillary assessment, and c) urinary analysis of glycosaminoglycan excretion. The fourth technique was the standard ^{99}mTc sulfur colloid radionucleotide liver spleen scan. The screening studies were performed on a randomly selected single cohort of chemical workers; some of whom were known to have disease. All four techniques were analyzed for their sensitivity and specificity as compared to results of the liver biopsy and biochemical blood test classification. Although all four screening techniques had a sensitivity and specificity sum greater than one, none were significantly better than could be explained by chance or the use of a biased coin. Reclassification of the population into those with more severe biochemical abnormalities improved the sensitivity of all screening tests, but only the sensitivity and specificity sum for the GAG test were statistically significant at the 0.05 level. There was no significant correlation between any pair of screening tests. None of the four screening tests agreed with the biopsy results better than might be obtained by biased coin or chance. These screening studies as presently constituted, do not provide sufficient sensitivity and specificity to warrant their use in community screening for subclinical asymptomatic hepatic injury due to chemical exposure.

INTRODUCTION

The initial reports of primary liver cancers (angiosarcoma) in rats exposed to vinyl chloride by Maltoni, et al (1) and the discovery of similar liver tumors in vinyl chloride polymerization workers by Johnson and Creech (2) has lead to considerable environmental concern regarding communities surrounding chemical industries which utilize potentially carcinogenic agents such as vinyl chloride. Several investigators have reported on various screening techniques as effective indicators of vinyl chloride chemical injury. Four such techniques - ultrasonography (3), radionucleotide scanning (4), nailbed capillary visualization (5), and glycosaminoglycan (GAG) (6) excretion - were reported to have some possible usefulness in detecting early chemical injury to the liver. In order to determine the useability of these techniques for community screening, the American Public Health Association (APHA) and Environmental Protection Agency (EPA) funded a multi-center collaborative study designed to determine the comparative sensitivity and specificity of these various techniques in detecting and identifying chemical related hepatic injury in asymptomatic individuals.

Materials and Methods

Population Selection

The chemical worker population consisted of 1,178 active (Group B, Figure 1) employees as of September 1, 1977, and 70 employees who had had liver biopsies regardless of current employment status (Group A, Figure 1); they were undergoing annual medical screening for the idertification of work-related disorders. Th medical screening consisted of an annual or semi-annual (for those employees with 10 or more years of employment) comprehensive history and physical examinations, laboratory screening studies consisting of 35 biochemical tests, chest and abdominal X-rays, and radionucleotide liver-spleen scan.

This population was selected for the collaborative study to determine the comparative effectiveness of four screening techniques in detecting liver damage as indicated by 1) past histopathological documentation of liver injury of 2) current hepatic dysfunction identified biochemically.

Three of the four techniques were included as potential non-invasive procedures suitable for determining the effects of vinyl chloride in a community population. These included 1) grey scale ultrasonography of the liver as developed by Taylor and colleagues (7, 13, 14), 2) a nailbed skin capillary evaluation of the middle and distal phalanges of the fingers as developed by Maricq and associates (8), and 3) urinary analysis of glycosaminoglycan excretions (GAG) as published by Kupchella and associates (9).

The fourth method included for comparison purposes was the standard radionucleotide liver-spleen scan utilizying ^{99}mTc colloid and interpreted by Whelan and associates (10).

The non-invasive screening studies were performed during a single week on a group randomly selected from all chemical company employees. The workers were selected on the basis of complete medical and work data for 1976-1977, and all those employees who had investigative liver biopsies performed during the screening program (1974-1977). The selection process for these workers is illustrated in Figure 1. The biochemical data and radioisotopic scans were part of the routine medical surveillance system for the employees. The pathological data was based on the last or most recent liver biopsy (s) which were performed for medical reasons, both related and not related to their work. Positive and negative results were determined as defined in Table 1 which list the technique, the evaluation or evaluators, and the criteria used. The employees targeted for examination were selected by simple random sampling from all available employees.

One hundred and twenty one of the targeted 170 employees (71 percent) participated. Twenty six declined to participate or could not be scheduled; 13 did not keep their scheduled appointment. After participation, nine employees were discovered to have been misclassified as biochemically abnormal. They had some biochemical abnormalities but not all (classified intermediate) and have been eliminated from the final analysis. Four biochemically abnormal individuals had abnormally low test values and they were included in the analysis.

Liver Biopsies

Liver biopsies were performed by the transjugular technique (11) and provided two to five biopsies from various areas of the liver. In addition, some individuals had second biopsies performed by the percutaneous or wedge biopsy via mini-laparotomy procedures. Pathological data was recorded in a computerized format identifying all histological abnormalities in a semi-quantitative fashion. Biopsies were read without knowledge of the individual's medical history or chemical exposure by two pathologists and a hepatologist with extensive experience in hepatic chemical injury. All biopsies were classified as 1) normal, 2) abnormal, a) chemical injury, and b) non-chemical injury.

The non-biopsy groups were drawn from those currently employed, and based on biochemical liver "function tests" individuals were sorted into positive, negative and indeterminate for hepatic disease. Only the positive and negative are included in this study.

The ultrasonic evaluation and its relative ability to identify hepatic damage due to vinyl chloride has been published elsewhere (3).

Microvascular techniques and the method of evaluation by Dr. Maricq and co-workers are also published in part (8).

The experimental work on GAG excretion in vinyl chloride workers and the techniques for differentiating the electrophoretic patterns in patients with

angiosarcoma and connective tissue damage of the liver has been published elsewhere (9). The effectiveness of radioisotopic (radionucleotide) scanning as a technique for identifying anatomical lesions in vinyl chloride workers is in preparation (12).

Method of Analysis

The biopsied group and the non-biopsied (biochemical) group were analyzed independently. For the biopsied group sensitivity and specificity were estimated for each screening test by assuming that the biopsy was correct. For the biochemical group the biochemical classification was assumed to be correct. For each analysis the data consisted of a simple cross classification. In a perfect screening test, the sum of sensitivity and specificity would be two. In a screening test which provided results no better than could be obtained by using a biased coin, the sum of sensitivity and specificity would be equal to one. We, therefore, estimated 95% confidence limits for the sum of sensitivity and specificity and observed whether or not one is included within these limits. As a test of statistical significance this is equivalent to the usual X^2 test for independent proportions.

Finally, in Table 4 we looked at the association (as measured by the phi coefficient) between each pair of screening tests. For these comparisons we used all employees who received both screening tests regardless of their biopsy status. In this case, we assumed both tests were subject to error and estimated the phi coefficient (r_ϕ) between them. Finally, for completeness, we give the biochemical classification for the 51 employees included in the biopsy group.

Results

Table 2 compares the histological and biochemical results for the 51 biopsied employees included in the study. Twenty-two of these employees had biochemical abnormalities as defined in Table 1. There was no significant

correlation between the biochemical and biopsy classification for the 23 employees with positive or negative biochemical classifications (χ^2_1 = 0.21; χ^2_1 = 0.20). The biochemical studies used in this analysis were those determined at the time of this and not at the time the biopsy was performed. In all instances of disagreement, the biopsy was positive and the biochemical results negative ($P < 0.001$).

Figure 2 provides the sensitivity and specificity for each of the screening tests when compared to biopsy results. In no case is the sum significantly greater than one, indicating that the results are not statistically significantly better than could be obtained using the biased coin. With the exception of the GAG studies, similar results are obtained when comparing the sum of sensitivity and specificity in the biochemical group (Figure 3). The sum of sensitivity and specificity for the GAG studies are just statistically significant with 95 percent confidence limits of 1.06 to 1.52. Table 3 gives the frequency distribution of the results of the GAG test for employees with normal and abnormal biochemical results. The distributions differ in their spread (variance) and not in their location (means).

Finally, the correlation matrix for the four tests are given in Table 4. There is no significant correlation, as measured by r_ϕ, between any pair of the screening tests. This is also true when they are sorted by biopsy status.

After reviewing the results, a reclassification of the 51 employees who had biopsies was assessed in regard to whether there was chemically induced liver damage. Ultrasonographic evaluation was reclassified by Dr. Taylor and the liver biopsies by Drs. Tamburro and Popper. Table 5 gives the results of this additional analysis which demonstrates that there was no agreement that could not be explained by chance ($P = 0.60$).

Discussion

The increasing industrialization in highly developed Western countries, such as the United States, continues to provide concern, not only for the health and safety of the industrial workers, but also for the surrounding communities in the areas of these industries. It is highly desirable to identify and validate the reliability of screening and diagnostic techniques which will identify the early development of injury due to the exposure of a variety of chemicals such as vinyl chloride. The assessment of newly developing techniques on a high-risk, exposed worker population, who have been carefully screened and prospectively followed, provide the most reliable method for determing both the sensitivity and specificity of these technical procedures in the asymptomatic subclinical high-risk exposed community population. The failure of such techniques to provide sufficient sensitivity in the presence of a required specificity is of critical clinical importance. This is especially so where the incidence of disease is relatively low and the population exposed large. Tests which provide a high sensitivity but of low specificity can and do medically stigmatize the population under surveillance leading to unnecessary anxiety and socioeconomic disturbances which can far outweigh the benefit of early detection of even serious disease in a smaller population.

Far too often screening techniques which have been developed in a highly diseased, clinically overt, hospitalized population are applied to an asymptomatic, clinically well-working populations without adequate determination of the sensitivity and specificity at this earlier stage of disease development.

In this study, all four techniques had, in the highly diseased hospitalized population, demonstrated either a sensitivity or specificity suggestive for the identification of underlying chemically related liver disease. Some techniques (Maricq and Kupchella) appeared ideal for community studies since they were non-invasive, relatively inexpensive, and provided a means of screening which would b highly accepted by a community.

This prospectively designed study has allowed us to estimate the ability of ultrasonography, nailbed capillary assessments, radioisotopic scanning, and glycosaminoglycan excretions to correctly predict the presence and absence of hepatic disease as documented by an exposed population. These studies clearly show that none of the four screening techniques sufficiently agree with either the biopsy, the biochemical results, or each other in a well defined population; they do not provide sufficient sensitivity or specificity to be useful as early indicators of chemical exposure injury.

The inclusion of nine individuals with intermediate biochemical results, who were originally misclassified as abnormal, would decrease the sum of sensitivity and specificity in all three screening tests. With their exclusion only the GAG tests provided results better than might be expected by chance ($P < 0.05$). Even the GAGs, from a pragmatic point of view, provide too high a false positive rate to be useful in its present stage. Possibly, with increased refinement and further study, this might provide a simple non-invasive technique for the identification of increased collagen changes related to chemical injury. More immediately, it should be duplicated to rule out chance.

Had we eliminated the four employees with an abnormally low biochemical test values, the sum of sensitivity and specificity for the GAGs and nailbed skin capillary screening tests would have been slightly reduced and that for the scan slightly increased. The GAG would still be of bordering significant ($X^2_1 = 3.73$), and the scan not significant ($X^2_1 = 1.46$).

Finally, the tests not only disagree with each other, but within the biopsy group there was no agreement between the biopsies and biochemical results. It should be noted, however, that the biochemical studies used in analysis were those chronologically closest to September, 1977, and not to the date of biopsy.

The test determinations and the biopsies may have been as long as three years apart. An analysis of the biochemical tests value done at the time of the biopsy would have more accurrately reflected the liver status, as shown by histology (15). It has been shown, in previously published studies, that biochemical and histological findings each correlate with chemical injury and chemical exposure (16, 17).

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Table 1

CLASSIFICATION	DETERMINED BY	CRITERIA
Biopsy	1) Dr. Popper, Pathologist 2) Dr. Makk, Pathologist 3) Dr. Tamburro, Hepatologist	a) Positive by consensus agreement if medically significant pathology is present. Negative otherwise. b) Pathology is of chemical or non-chemical origin. (18)
Biochemical	Liver "Function" Tests Group 1: SGPT, ICG, SGOT, ALK. PHOSPHATASE Group 2: GGTP, BILIRUBIN	Positive if two or more Group tests were abnormal or if one Group 1 and both Group 2 tests were abnormal. Negative if all six tests were normal. Intermediate otherwise.
Ultrasound	Dr. Taylor	Defined as negative if the overall impression was normal. Positive otherwise.
Microvascular	Dr. Maricq	Defined as negative if there were no microvascular abnormalities. Positive otherwise.
Glycosaminoglycans	Uronic acid = UG Uronic Acid / MG Creatinine	Defined as positive for values less than 2.0 or greater than 4.8. Negative otherwise.
Liver Scan	Dr. Whelan	Defined as positive if any pathological defect was detected. Negative Otherwise.

TABLE 2

COMPARISON OF BIOPSY AND BIOCHEMICAL DETERMINATIONS OF THE PRESENCE OF LIVER DISEASE

BIOPSY	BIOCHEMICAL		SUM	BIOCHEMICALLY INDETERMINATE	TOTAL
	POSITIVE	NEGATIVE			
POSITIVE	3	18	21	15	36
NEGATIVE	0	8	8	7	15
SUM	3	26	29	22	51

$r = 0.20966$ $\quad X_1^2 = 0.200$ N.S.

Matched $X_1^2 = (18 - 1)^2/18 = 16.1$ $\quad P < 0.001$

TABLE 3

FREQUENCY (F) AND RELATIVE FREQUENCY (R.F.)
OF GAGS FOR NORMAL AND ABNORMAL
BIOCHEMICAL RESULTS

GAG	Normal		Abnormal	
	f	R.F.	f	R.F.
< 2	4	0.111	6	0.250
2 < 3	16	0.444	7	0.292
3 < 4	13	0.361	6	0.250
4 < 5	1	0.028	1	0.042
5+	2	0.056	4	0.167
Sum	36	1.000	24	1.000
Mean	2.886		3.160	
Variance	0.746		1.776	

$t_{58} = 0.968$ N.S. T Test Independent Means

$F_{23,35} = 2.381$ $P < 0.05$ F Test Independent Variances (Two Tailed)

TABLE 4

CORRELATION MATRIX (Ro) BETWEEN FOUR SCREENING TESTS USED TO PREDICT THE PRESENCE OR ABSENCE OF LIVER DISEASE

	GAG ANALYSIS	CAPILLARY ASSESSMENT	ULTRASOUND STUDY	RADIOISOTOPIC SCAN
GAG ANALYSIS	.	0.08	-0.03	0.005
CAPILLARY ASSESSMENT	(113)	.	0.03	0.07
ULTRASOUND STUDY	(87)	(84)	.	0.06
RADIOISOTOPIC SCAN	(120)	(114)	(88)	.

None of the correlations are statistically significant (a = 0.05).
The correlations are given above the diagonal.
The sample size is given in parenthesis below the diagonal.

TABLE 5

RECLASSIFICATION OF BIOPSIED EMPLOYEES

FOR CHEMICALLY INDUCED ABNORMALITIES

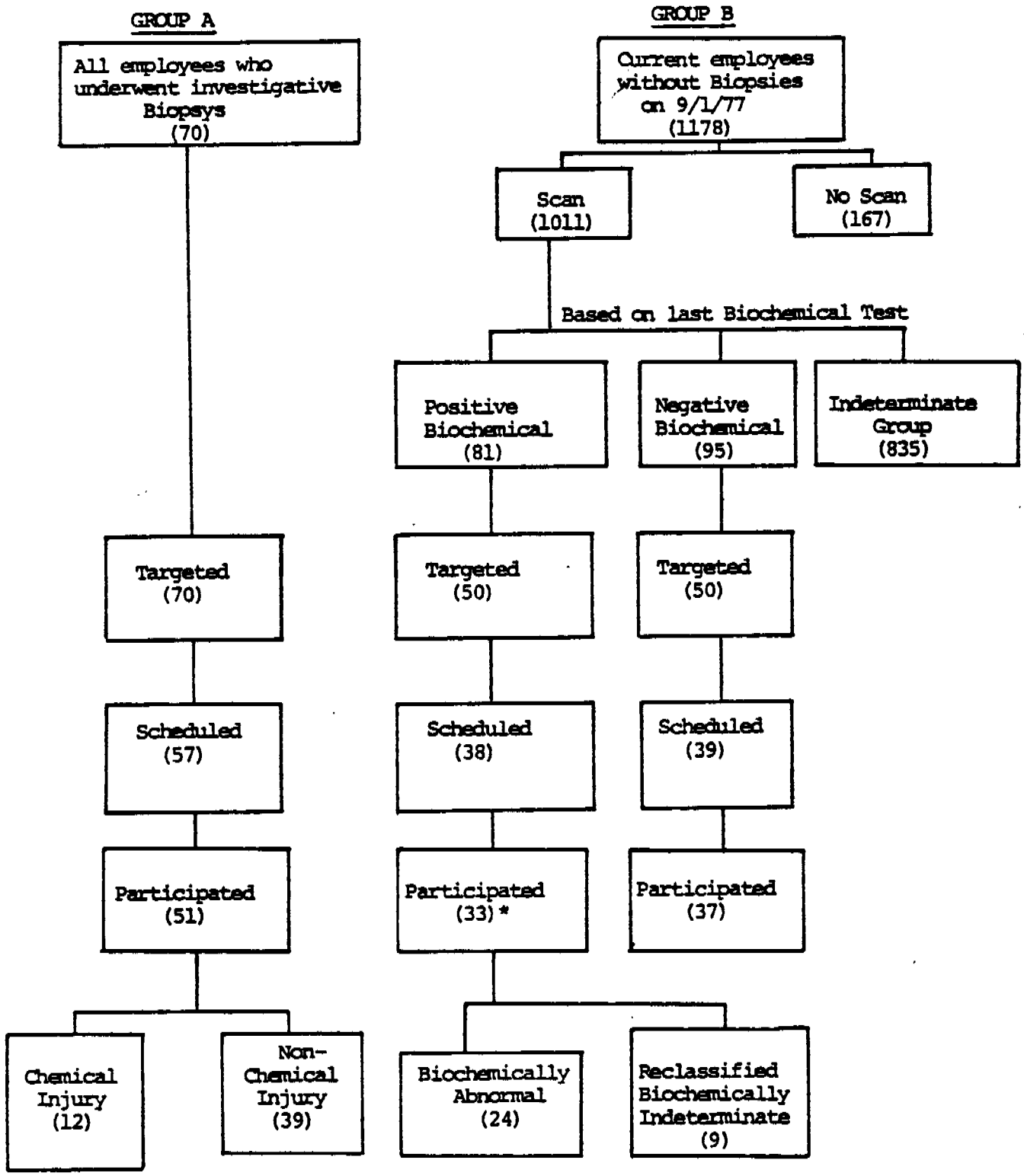
Biopsy	Ultra Sound		SUM
	Chemical Abnormality	Other	
Chemical Abnormality	7	5	12
Other	26	13	39
SUM	33	18	51

Specificity = 13/39 = 0.333

Sensitivity = 7/12 = 0.583

Sum = 0.916

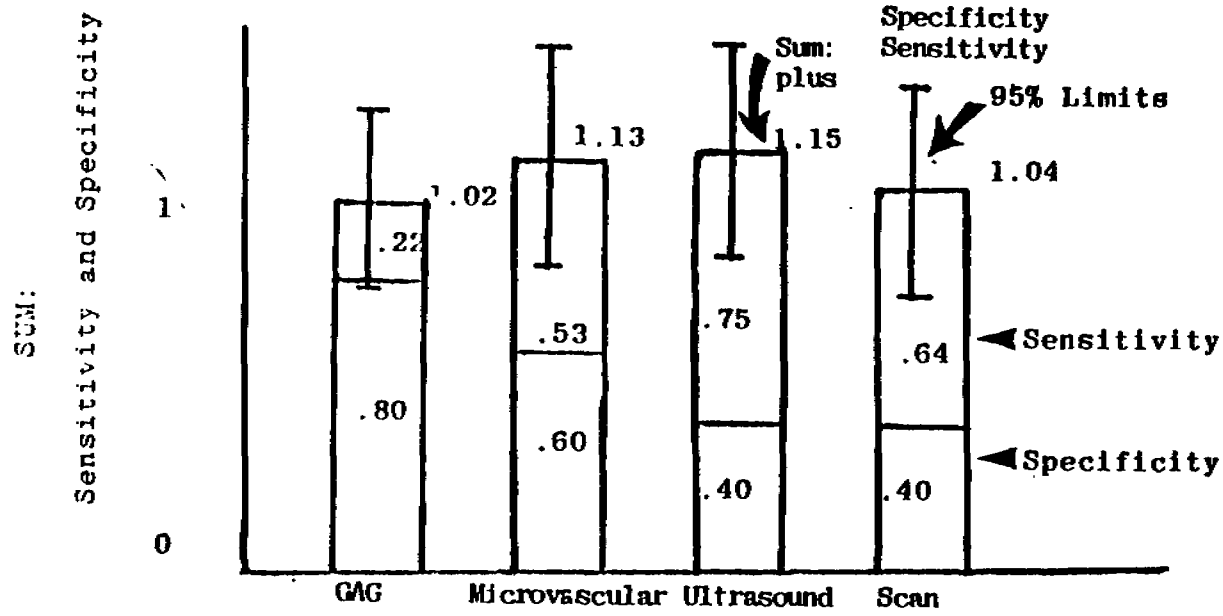
FIGURE 1



*Not seen by Doctor Taylor

FIGURE 2

SENSITIVITY AND SPECIFICITY FOR FOUR SCREENING TESTS FOR THE PREDICTION OF LIVER ABNORMALITIES (AS DETERMINED BY BIOPSY)

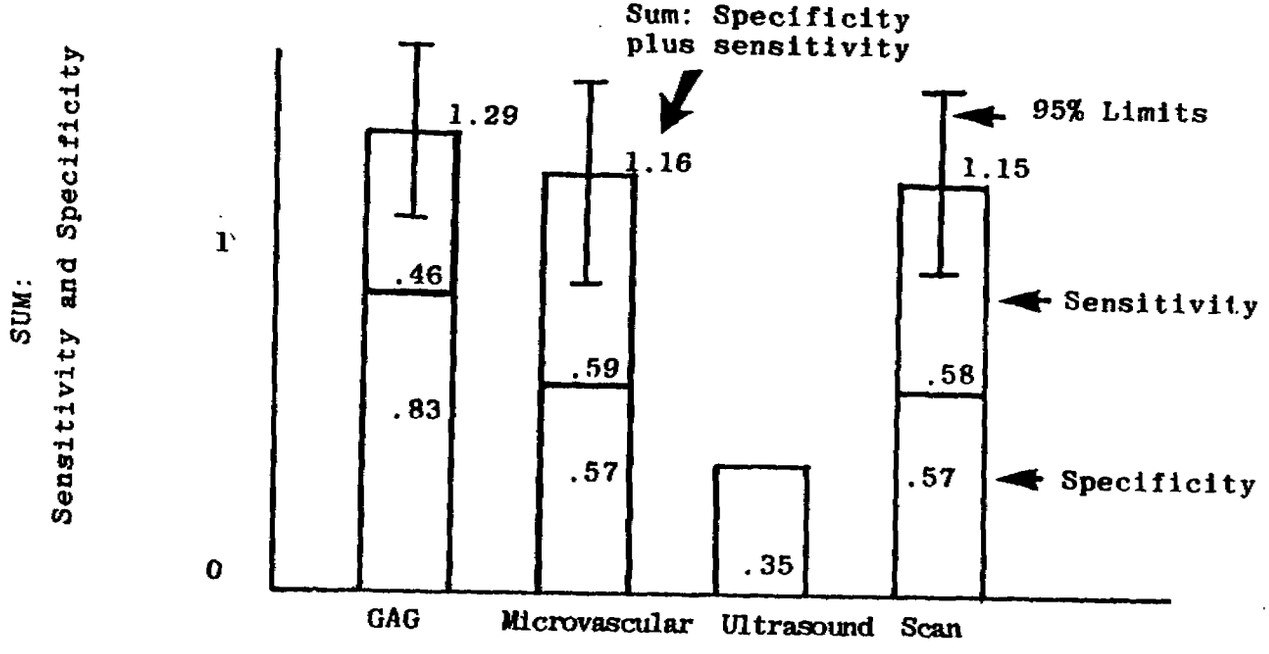


Number Employees	(51)	(49)	(51)	(51)
Positive Biopsies	36	34	36	36
Negative Biopsies	15	15	15	15

CMA 003199

Figure 3

SENSITIVITY AND SPECIFICITY FOR FOUR SCREENING TESTS FOR THE PREDICTION OF LIVER ABNORMALITIES (AS DETERMINED BY REVIEWED BIOCHEMICAL TESTS)



Number Employees	(60)	(57)	(37)	(61)
Positive Biochemical	24	22		24
Negative Biochemical	36	35		37

STUDIES ON THE MUTAGENICITY OF VINYL CHLORIDE METABOLITES AND RELATED CHEMICALS

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I. INTRODUCTION

The studies by Viola et al (26) and Maltoni et al (15) established the carcinogenic potential of vinyl chloride monomer. The detection of angiosarcoma in industrial workers exposed to polyvinyl chloride suggested a causal relationship between this chemical and the development of hepatic abnormalities (4,12). Hefner et al (7) have delineated the metabolic fate of inhaled vinyl chloride in rats and proposed that the epoxide, chlorooxirane, and chloroacetaldehyde were the carcinogenic intermediates. Their hypothesis has been supported by the work of several laboratories (3,14,16,19, Elmore, Wong, Laumbach and Streips, submitted for publication) using bacterial strains as mutagenic indicators.

In this communication we present additional data concerning the mutagenicity and the potential mechanisms of action of several vinyl chloride metabolites, including the previously unreported chloroacetadehyde monomer hydrate, chloroacetaldehyde dimer hydrate, and chloroacetaldehyde trimer. Epichlorohydrin, a mutagenic/carcinogenic (21,25) methylene homolog of chlorooxirane was also examined.

II. PROCEDURES AND MATERIALS USED

A. Bacterial Strains

The bacterial strains utilized in these studies are presented in Table I. The Bacillus subtilis strains were all maintained on AK agar (BBL). Salmonella typhimurium cultures were obtained from B. N. Ames (1) and were stored on Nutrient Agar (Difco) plus 5g NaCl per liter.

B. Mutagenicity Assays

The indirect assay utilized repair deficient strains of B. subtilis. The procedure was a modification of the "rec-assay" described by Kada et al (9). Cells were grown overnight in Nutrient Broth (Difco) at 37C in a rotary incubator shaker, then diluted tenfold in phosphate buffer (pH 7.0). The suspended cultures were streaked onto Nutrient Agar plates (Difco). Filter paper discs (6 mm) were saturated with the chemical solutions to be examined, then were placed onto the agar plates next to the streaked bacterial cultures. Following incubation at 37C overnight the plates were examined and the lethality and mutagenic potential of the test chemicals were assessed by comparing inhibition zones between the B. subtilis 168 wild type, a repair-capable strain and the various DNA repair-deficient strains. In all these studies 4-nitroquinoline-1-oxide (4NQO) was used as the positive mutagenic control.

Direct mutagenicity assays utilized the S. typhimurium tester strains described by Ames (1). The chemicals were examined by the methods of McCann et al (16). The cultures were grown in Nutrient Broth plus 0.5% NaCl overnight in a rotary incubator shaker at 37C. A mixture of the test chemical (0.1 ml) in dimethyl sulfoxide (DMSO) and 2 ml of soft agar (0.6% agar, 0.6% NaCl, 0.5 mM biotin, and 0.5 mM histidine) was added to 0.1 ml of the bacterial culture. The solutions were mixed thoroughly and overlaid onto minimal plates [Vogel-Bonner E medium (27), 1.5% agar, and 2% glucose]. Control samples were prepared by omitting the test chemicals. For the positive mutagenesis control, 4NQO was added to the mixtures in place of the test chemicals. All plates were incubated for 48 hr at 37C prior to the enumeration of revertant colonies.

C. Chemical Compounds

The chemical compounds utilized in these studies were prepared, purified, and analyzed by previously reported techniques (Elmore, Wong, Laumbach, and Streips, submitted for publication).

D. Preparation of DNA

Transforming DNA was isolated from B. subtilis cultures by the method described by Young and Wilson (29). In some of the experiments the cultures were pretreated for 15 min either with chloroacetaldehyde (16 mM) or epichlorohydrin (16 mM) prior to the extraction procedure. In alternate experiments S-9 liver homogenate mix was added to the compounds prior to addition to bacteria. The S-9 liver homogenate contains per ml, 0.3 ml of the S-9 fraction, 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-P, 4 mM NADP, and 100 mM sodium phosphate (pH 7.4). The DNA concentration in all lysates were assayed by the method of Richards (20).

E. Treatment of DNA In vitro with Chemicals

A sample of B. subtilis transforming DNA (0.9 ml) in standard saline citrate (SSC) (0.15 M NaCl-0.015 M trisodium citrate, pH 7.0) was combined with 0.1 ml chloroacetaldehyde (1.0 M in DMSO) or 0.1 ml

epichlorohydrin (1.0 M in DMSO). The mixture was allowed to react for 1 hr with occasional shaking. Following this treatment the treated DNA was dialyzed at 0C against three 500 ml changes of SSC for 24 hrs. In alternate experiments the DNA-chemical mixtures were placed in a dialysis bag and immersed in the S-9 liver homogenate mix. These samples were dialyzed in SSC as above.

F. Competent Cultures for Transformation Assays

The procedures for the development of competence were similar to those described (23). B. subtilis cells were grown in a modified Spizizen's minimal medium (GMI) (29) for 90 min at 37C after cessation of logarithmic growth in a rotary incubator shaker. The cells were then diluted tenfold into GMII medium (29) and incubated for an additional 60 min at 37C in the shaker. At this time the culture has attained maximum competence.

G. Transformation Procedures

A sample (0.1 ml) of extracted, treated or untreated DNA was added to 0.8 ml of the competent cultures and incubated at 37C for 30 min in the shaker. The reaction was terminated by the addition of 0.1 ml of deoxyribonuclease (500 µg/ml, Worthington Biochem. Corp.) for 15 min at 37C. The cells were plated on appropriate selective minimal media and incubated at 37C for 48 hrs.

III. RESULTS

A summary of preliminary mutagenesis screening experiments with potential vinyl chloride monomer metabolites and related compounds is presented in Table II. It is evident that chlorooxirane and chloroacetaldehyde are the ultimate mutagens in this system. These results agree with the published data (3,16). In addition, this table describes the mutagenicity of the other chemical forms of chloroacetaldehyde, notably a monomer hydrate, a dimer hydrate, and a trimer. The hydrate and dimer hydrate forms have been shown to form an equilibrium mixture by the spontaneous rearrangement of chloroacetaldehyde under physiological conditions (Elmore, Wong, Laumbach, and Streips, submitted for publication), and these hydrate forms must be regarded as potential metabolites of consequence. Purified dimer hydrate and trimer were synthesized under laboratory conditions. Neither acetaldehyde, chloroacetic acid, nor chloroethanol showed a significant level of mutagenicity in these assays. Other investigators have reported the mutagenicity of chloroethanol, however, either high concentrations or activation with microsomal enzymes was required for activity (3,16). Our results agree with those of McCann et al (16). These experiments suggest a molecular relationship involving the proximity of the chloride group to the aldehyde moiety for mutagenic activity. In this regard we are currently examining structurally analogous ketones, substituted with various halogens. Epichlorohydrin (1-chloro-2,3 epoxypropane) was also mutagenic in screens using the Salmonella tester strain TA100.

Further experiments examined the effect of proposed metabolites on several different DNA repair deficient strains of B. subtilis. Chlorooxirane and the different forms of chloroacetaldehyde were all found to specifically inhibit the growth of strain MC-1, which lacks recombination repair (17) (Table III). Epichlorohydrin was capable of moderate reactivity only in the presence of the S-9 fraction.

Quantitative mutagenesis assays with Salmonella strain TA100, an indicator for base-pair substitution mutations, revealed that chloroacetaldehyde monomer had the highest mutagenic capacity of all the reactive metabolites (Table IV). The monomer-dimer hydrates, dimer hydrate, and trimer show progressively decreasing mutagenic efficiency as evidenced by the higher chemical concentration required for eliciting maximum reversion. All forms of chloroacetaldehyde were very toxic, thus the mutagenic response of each compound was limited to a narrow range of concentrations. However, epichlorohydrin, a weak mutagen by comparison, has a broad mutagenic spectrum and a corresponding low toxicity.

Since the mutagenic activity of the compounds constituted strong evidence that DNA was a primary target of attack, we examined the interaction of chloroacetaldehyde and epichlorohydrin with transforming DNA. It is known that the biological activity of transforming DNA can be altered by exposure to physical and chemical agents (8,22). Previous studies have shown that chloroacetaldehyde can bind to DNA in vitro (11). Accordingly, transforming DNA isolated from B. subtilis 168WT was treated with either chloroacetaldehyde or epichlorohydrin as described in Materials and Methods. The treated DNA was examined in transformation assays utilizing several different auxotrophic strains of B. subtilis as the recipients. Data presented in Table V reveals that in vitro treatment of DNA with either compound has little or no apparent effect on the biological activity of this DNA in transformation.

Since both chloroacetaldehyde and epichlorohydrin demonstrated mutagenic activity in the Salmonella TA100 strain, we examined the effect of these two compounds on B. subtilis DNA in vivo. Transforming DNA was isolated from B. subtilis following a 15 min exposure to the mutagenic chemicals. The DNA concentration was calculated from these samples, and levels equivalent to those used in the in vitro assays were added to competent cultures. The results of these transformation assays are shown in Table VI. Two major effects are evident with chloroacetaldehyde in vivo treated DNA. First, there was a major depression of the biological activity in the transforming DNA. Secondly, the depression showed genetic marker specificity. Moreover, the DNA segments containing genetic markers which have previously been shown to be associated to macromolecular structures such as the cell membrane (6,24,28) or the cell wall (Streips, Doyle, Sueoka, Brown, and Fan, submitted for publication) were selectively protected from attack by chloroacetaldehyde and epichlorohydrin. The activity of epichlorohydrin was less in these experiments, however, the patterns of specific marker inactivation are quite similar. The addition of the S-9 mix to the chemicals prior to addition to the cells, did not cause significant alteration in transformation efficiency (results not shown). In some samples there was an effect on the transforming DNA by DMSO, therefore all transformation values were corrected to account for this parameter.

IV. Discussion

The major findings reported in this manuscript can be summarized: 1) We have confirmed the mutagenicity of chloroacetaldehyde and chlorooxirane, and extended it to include the additional potential metabolites, chloroacetaldehyde monomer hydrate, dimer hydrate and trimer, as well as the previously unreported chlorooxirane homolog, epichlorohydrin. 2) We have shown that recombination repair appears to be the mechanism for the correction of vinyl chloride metabolite elicited damage. 3) Chloroacetaldehyde causes a decrease in the biological activity of transforming DNA only if the cells are treated with the mutagen prior to the extraction of the DNA. In vitro studies showed no effect. 4) Epichlorohydrin apparently differs markedly from the vinyl chloride metabolites in mutagenic activity.

To understand the mutagenic potential of an environmental carcinogen, such as vinyl chloride and related chemicals,it is necessary to determine both its metabolic fate and probable mechanism of action for alteration of cellular processes. This report, as well as others, (3,14,16) has identified the potential active metabolites in vinyl chloride monomer mediated carcinogenesis. Furthermore, on the basis of a series of studies in microbial systems, we can postulate probable mechanisms of action of the vinyl chloride monomer metabolites and related chemicals. Understanding of these mechanisms is necessary for the development of possible blocking agents to the carcinogenic activity.

Recombination repair appears to be induced to correct DNA lesions caused by vinyl chloride monomer metabolites and epichlorohydrin. Salmonella strain TA100 which lacks excision repair (uvr$^-$), yet retains the capacity for recombination repair is capable of recovery and can express mutation following exposure. Furthermore, experiments with several repair-deficient B. subtilis mutants demonstrate that only the recombination repair mutant is specifically sensitive to the active metabolites, whereas the excision repair mutants and the wild type strain are relatively unaffected. The nature of the lesions may specifically evoke the recombination repair mechanism (10), or, alternatively, the chemical reactivity of the metabolites may directly suppress other repair. It is known that recombination repair is inducible, while other types of repair are mostly constitutive (5). Since chloroacetaldehyde has been shown to specifically interact with proteins containing -SH groups (J. Hoffman, personal communication), it is possible that the chemical could inactivate the constitutive repair enzymes leaving the repair to an inducible system.

The requirement for recombination repair of damage induced by these chemicals suggests the potential route of mutagenesis in bacteria. Recombination repair has been shown to be error prone (16). In this sense it resembles postreplication repair in mammalian cells (13). Thus, we can postulate that the analogous error prone repair pathway, postreplication repair, may function in mammalian cells in response to vinyl chloride metabolite elicited damage. A relationship between postreplication repair caused errors and somatic mutation and carcinogenesis has b en suggested in patients with the skin disease, xeroderma pigmentosum (13).

The increased inhibitory activity of the chloroacetaldehyde dimer and trimer forms for the other repair-deficient B. subtilis strains (Table III) may have been nonspecific killing of the cells, since all the strains other than MC-1 showed identical levels of inhibition. The necessity for metabolic activation of epichlorohydrin could reflect either a lack of permeability of the nonactivated compound or the requirement of a metabolite of this compound as the true mutagenic species.

Neither chloroacetaldehyde nor epichlorohydrin seemed to affect transforming DNA in vitro (Table V). Although several investigators have reported that CAA specifically modifies bases and causes mismatched base pairs (2,11), this reaction in vitro does not seem to affect the biological activity of the DNA. In contrast, DNA which was isolated from cells treated with either chloroacetaldehyde or epichlorohydrin (in vivo, Table VI) was severely affected. The overall biological activity of the transforming DNA is depressed, and it appears that the regions of the genome which are not protected by either the cell membrane or cell wall are most susceptible to attack and inactivation. It has also been postulated both in Escherichia coli and B. subtilis that the replication origin, terminus, and replication fork are all outer surface bound (18,24). Thus, these would be protected regions from chloroacetaldehyde attack and the nonreplicating DNA in the cytoplasm would be most susceptible. In this connection, recent experiments in our laboratory (Laumbach, Lee, Wong, and Streips, manuscript in preparation) have shown that chloroacetaldehyde causes enhanced mutation levels in cultures with nonreplicating genomes. This may imply that chloroacetaldehyde could be active in mammalian cells during growth stages where little DNA synthesis occurs.

The mode of action of epichlorohydrin, a known carcinogen (25), differs from that of the vinyl chloride monomer metabolites. Although epichlorohydrin causes similar base substitution mutations in Salmonella tester strain TA 100, it is a comparatively weaker alkylating agent based on quantitative assay. Epichlorohydrin also exhibits a lower toxicity level than vinyl chloride monomer metabolites, thus epichlorohydrin can demonstrate mutagenic activity through a wider range of concentrations. In addition, our laboratory has preliminary evidence that epichlorohydrin produces higher levels of mutation in Salmonella cultures which are actively replicating DNA than in cultures which have been arrested in DNA replication (Laumbach, Lee, Wong, and Streips, manuscript in preparation). The different activity spectra between chlorooxirane and its homolog epichlorohydrin points out the necessity for a multifaceted study of carcinogens.

V. SUMMARY

Our laboratories have utilized strains of B. subtilis and Salmonella typhimurium to investigate the mutagenicity of vinyl chloride metabolites and related compounds. The major findings reported in this manuscript are: 1) Confirmation of mutagenicity of chloroacetaldehyde and chlorooxirane. 2) Description of mutagenicity of additional potential metabolites of vinyl chloride, chloroacetaldehyde monomer hydrate, dimer hydrate, and trimer, as well as the mutagenic

carcinogenic chlorooxirane homolog, epichlorohydrin. 3) Recombination repair is postulated to be the mechanism for correcting vinyl chloride metabolite elicited damage. 4) Chloroacetaldehyde affects the transformation activity of DNA only if cells are treated with the mutagen prior to the extraction of the DNA. In vitro the chemical had no effect. 5) Epichlorohydrin differs from vinyl chloride metabolites in mode of action.

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Table I

Bacterial Strains

Bacillus subtilis	Genotype	Origin and Comments
RUB 783	purB6, leu-8, hisA1, metB10	U. Streips
BR 151	trpC2, lys-3, metB10	B. Reilly
BUL 709	ura-1, hisA1, leu-8, metB10	This laboratory
BUL 714	cysA, hisA1, leu-8, metB10	This laboratory
Hcr-9 (JB01-200)	trpC2	S. Okubo and W. Romig, hcr$^-$
MC-1	trpC2, recB2	S. Okubo and W. Romig, rec$^-$
FB-13	trpC2	C. Hadden, uvr$^-$
168WT	prototroph	A. Laumbach and I. Felkner

	Mutations in Strains				
Salmonella typhimurium	His$^-$	LPS	DNA Repair	R Factor	Mutation Detected
TA1535	hisB46	rfa	uvrB	-	base-pair substitution
TA100	hisB46	rfa	uvrB	pKM101	base-pair substitution
TA1537	hisC3076	rfa	uvrB	-	frameshift
TA1538	hisD3052	rfa	uvrB	-	frameshift
TA98	hisD3052	rfa	uvrB	pKM101	frameshift

STUDIES ON THE MUTAGENICITY OF VINYL CHLORIDE METABOLITES

Table II

Mutagenic Activity Assayed by Bacterial Test Systems

Compounds	Indirect Screen *B. subtilis* "Repair-Assay"	Direct Test[a] *S. typhimurium* Strain TA100
Acetaldehyde	NR[b]	NR
Chloroacetic Acid	NR	NR
Chloroethanol	NR	NR
Vinylidene Chloride	NR	NR
Vinyl Chloride	NR	NR
Chlorooxirane	+[c]	++[d]
Chloroacetaldehyde (monomer)	+++[e]	+++
Chloroacetaldehyde (monomer-dimer hydrates)	++	++
Chloroacetaldehyde (dimer hydrate)	+	+
Chloroacetaldehyde (trimer)	+	+
Epichlorohydrin	NR	+

[a] Experiments performed in absence of liver homogenate-mediated activation.

[b] NR no reaction detected

[c] + Reactive

[d] ++ Moderately reactive

[e] +++ Very reactive

Table III

"Repair-Assay" with Bacillus subtilis Strains

Compounds	Molar Concentration	Growth Inhibition in Millimeters[a]			
		168WT (hcr+, rec+)	MC-1 (hcr+, rec-)	Hcr-9 (hcr-, rec+)	FB-13 (uvr+, rec+)
Chloroacetaldehyde (monomer)	0.10	2.0	28.0	4.0	3.0
Chloroacetaldehyde (monomer-dimer hydrate)	0.115	NI[b]	23.0	NI	NI
Chloroacetaldehyde (dimer hydrate)	0.097	2.0	10.0	2.0	2.0
Chloroacetaldehyde (trimer)	0.096	7.0	15.0	6.0	7.0
Chlorooxirane	0.26	NI	10.0	NI	NI
Epichlorohydrin	0.997	NI	NI	NI	NI
Epichlorohydrin (plus liver homogenate)[c]	0.997	NI	3.0	NI	NI

[a]Average inhibition calculated from multiple experiments.

[b]No inhibition detected.

[c]9,000 x g supernatant (S-9) + NADPH generating system.

Table IV

Quantitative Mutagenicity Assay by Salmonella TA100 Reversion

Compound	Concentration in Soft Agar Layer mM/Plate[a]	Average Number Revertants/Plate[b]
Chloroacetaldehyde (monomer)	0.0004	265
Chloroacetaldehyde (monomer-dimer hydrate)	0.054	977
Chloroacetaldehyde (dimer hydrate)	0.490	311
Chloroacetaldehyde (trimer)	0.240	159
Epichlorohydrin	4.746	2856

[a]Highest effective non-toxic concentration for reversion.

[b]Spontaneous background revertants subtracted.

Table V

Effect of Chloroacetaldehyde and Epichlorohydrin of Transforming DNA *In vitro*

Recipient Strains [c]	Relative Transformation Efficiency[a] metB10	leu-8	cysA	hisA1	ura-1	trpC2	lys-8	purB6
Epichlorohydrin treated DNA								
BUL 714	.92	.97	.97	1.16				
RUB 783	.91	.60		.98				.77
BUL 709	.99	.95		1.02	.85			
BR 151	1.48					1.07	.62	
Chloroacetaldehyde treated DNA								
BUL 714	1.43	.92	.55	.91				
RUB 783	.93	1.45		.75				.89
BUL 709	.86	1.33		.90	.75			
BR 151						ND[b]	.77	

[a] Relative transformation efficiency calculated: $\frac{\text{number of transformants with treated DNA}}{\text{number of transformants with untreated DNA}}$

[b] Not determined.

[c] Conditions for competence and transformation as described in Materials and Methods.

Table VI

EFFECT OF CHLOROACETALDEHYDE AND EPICHLOROHYDRIN ON TRANSFORMING DNA IN VIVO

Recipient Strains[b]	Relative Transformation Efficiency[a] metB10	leu-8	cysA	hisA1	ura-1	trpC2	lys-3	purB16
Chloroacetaldehyde in vivo treated DNA								
BUL 714	.54	.09	.08	.36				
RUB 783	.33	.10		.35				.10
BUL 709	.53	.07		.32	.34			
BR 151	.37					.13	.11	
Epichlorohydrin in vivo treated DNA								
BUL 714	.55	.17	.25	.46				
RUB 783	.52	.15		.38				ND[c]
BUL 709	.59	.15		.50	.36			
BR 151	.37					.11	.19	

[a]Relative transformation efficiency calculated: $\frac{\text{number transformants with treated DNA}}{\text{number transformants with untreated DNA}}$

[b]Conditions for competence and transformation as described in Materials and Methods.

[c]Not determined.

VINYL CHLORIDE MUTAGENICITY AND CARCINOGENICITY VIA THE METABOLITES CHLOROOXIRANE AND CHLOROACETALDEHYDE MONOMER HYDRATE.

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SUMMARY

Mutagenicity tester strains of Bacillus and Salmonella were used to assay vinyl chloride in nutrient broth at a practical concentration level. Also screened without exogenous activation were seven potential metabolites of vinyl chloride in their pure forms as well as the related epichlorohydrin. Chlorooxirane, chloroacetaldehyde, chloroacetaldehyde monomer hydrate, chloroacetaldehyde dimer hydrate, chloroacetaldehyde trimer, and epichlorohydrin produced significant mutagenic activity in Salmonella typhimurium strains sensitive to base-pair mutation. A recombination repair deficient strain of Bacillus subtilis was inhibited in growth by these compounds, whereas excision repair deficient and wild type strains of Bacillus subtilis were relatively unaffected. On the basis of these assays a working hypothesis for the vinyl chloride carcinogenesis mechanism is proposed which involves chlorooxirane and chloroacetaldehyde monomer hydrate as the ultimate carcinogenic metabolites of vinyl chloride.

INTRODUCTION

The carcinogenic potential of vinyl chloride monomer 1 was initially established by Viola et al. [1] and Maltoni et al. [2] with inhalation experiments using laboratory animals. Detection of angiosarcoma in polyvinyl chloride workers suggested a causal relationship between industrial exposure to vinyl chloride and the development of pathological conditions in humans. This contention was supported by epidemiological data which revealed an association between exposure to 1 and the onset of hepatic abnormalities including angiosarcoma [3,4]. A report by Hefner et al. [5] on the metabolic fate of 1 in rats indicated that 67% of the vinyl chloride inhaled by the rats was metabolized and excreted in the urine. The metabolic products identified were N-acetyl-S-(2-hydroxyethyl)cysteine and thiodiglycolic acid [5,6] which are sulfhydryl conjugates of chloroethanol 2 and chloroacetic acid 3 respectively. Chlorooxirane 4 and chloroacetaldehyde 5 were speculated to be the carcinogenic forms. We report herein the mutagenicity and carcinogenic potential of these compounds in their pure forms.

Several reports [7,8,9] have appeared recently using the Salmonella tester strains to test vinyl chloride and several of its supposed metabolic derivatives. Vinyl chloride and chloroethanol were found to be mutagenic after activation by liver homogenates [9]. Direct mutagenicity of vinyl chloride was also reported by McCann et al. [7] and Bartsch et al. [10] at 20% v/v in air (200,000 ppm). Since the solubility of vinyl chloride in water at 25°C and 1 atm has been determined to be 7.79×10^{-4} mole fraction [11] or 2,900 ppm, we have conducted further testing at this concentration level to secure a practical

dose-response comparison with its proximate metabolites. Regarding the latter, the exact chemical forms of the proximate metabolites previously tested are often questionable. Chloroacetaldehyde, like formaldehyde [12], dichloroacetaldehyde [13], and chloral [14], can exist in combinations of four forms depending on the history of sample preparations: the monomer 5, the monomer hydrate 6, the dimer hydrate 7, and the trimer 8. McCann et al. [7] used vacuum distilled chloroacetaldehyde without a follow-up analysis of its content. This distillate may have consisted of chloroacetaldehyde monomer 5 and its cyclic trimer 8 if water was totally absent, or it may have been a mixture of chloroacetaldehyde hydrates 6 and 7 in an aqueous medium. Bartsch et al. [10] tested a commercial aqueous chloroacetaldehyde solution which, according to our analysis reported herein, had an acidic pH and approximately equal concentrations of the two hydrates 6 and 7. This solution was also contaminated by ethanol to the extent of 10%. We have therefore conducted individual assays of pure compounds, or assays of a known mixture of the specific forms of chloroacetaldehyde. Furthermore, the mutagenicity observed for chlorooxirane 4 [9] may be attributed to a chloroacetaldehyde hydrate rather than the chlorooxirane integrity. Under the 37°C aqueous testing conditions reported, chlorooxirane decomposed with a half life of 1.6 min [10] to chloroacetaldehyde. For this reason we have also screened epichlorohydrin 9 which is a stable chloro-epoxide homolog of 4 as a comparative assay to interpret the observations of the activity of chlorooxirane.

This investigation used the above-mentioned compounds 1 - 9 in mutagen assays without exogenous enzyme activation. A preliminary screen was performed using DNA repair-deficient mutants of Bacillus subtilis. This was followed by quantitative testing of the compounds for mutagenicity

with Salmonella typhimurium LT-2 strains [15]. Thus, a combination of these two screening procedures have led to information on the mutagenicity and carcinogenic potential of these compounds as well as their chemical mode of action.

MATERIALS AND METHODS

The bacterial strains used in the bioassays are shown in Table I. The Salmonella tester strains were designed to detect chemical carcinogens as mutagens [15]. The recombination and DNA repair-deficient Bacillus subtilis strains were used in the repair assays as an indirect test for mutagenicity [16,17]. Nutrient broth (Difco) and nutrient broth plus 0.5% NaCl was used for growth of stock cultures of Bacillus and Salmonella strains respectively. Nutrient agar (Difco) served as a solid medium for the growth of Bacillus strains in "repair-assays". The pour plates used with Salmonella strains consisted of molten (45°C) soft agar which contained 0.6% agar, 0.6% NaCl, 0.5mM biotin, and 0.5 mM histidine. The minimal agar plate was composed of Vogel-Bonner E medium [18], 1.5% agar, and 2% glucose.

Vinyl chloride gas was obtained from Matheson Scientific; aqueous chloroacetaldehyde (45% by wt.) from ICN Pharmaceuticals; epichlorohydrin from Matheson Coleman and Bell; and other chemicals from Aldrich Chemical Co.

Mutagenicity Assays

Salmonella - Vinyl chloride 1 was tested by the method of Ames [7]. A mixture of 0.1 ml of 1 in broth and 2 ml of top agar was added to 0.1 ml of cell culture. The solutions were then mixed and poured immediately onto the surface of a minimal medium plate. After incubation for 48 hrs at 25°C, colonies were counted and recorded. For compounds 2 - 9, sample solutions of known concentrations

were prepared in dimethyl sulfoxide (DMSO). A 0.1 ml aliquot of the sample solution was admixed with 0.9 ml of the tester strain culture. Then, a 0.1 ml sample of this mixture was added to 2 ml of molten soft agar and applied to the surface of a minimal agar plate. Control plates for detection of the spontaneous reversion rates were prepared for each tester strain by omitting only the compounds tested. Pour plates were incubated for 48 hr at 37°C before revertant colonies to prototrophy were counted. For positive mutagenesis control, plates containing the mutagen 4-nitroquinoline-N-oxide were used.

Bacillus - The "repair-assay" procedure was a modification of the "rec-assay" procedure of Kada et al. [19]. They were grown overnight in nutrient broth then diluted 10 fold in phosphate buffer (pH 7.0). Strains were streaked with pipettes onto nutrient agar plates. Filter paper discs (6 mm) saturated with test solution were placed upon the bacteria streaks. Following incubation for 24 hr at 37°C, growing bacteria were visible except in the inhibition zone. The lethality and mutagenic potential of compounds were assessed by comparison of inhibition zones between the 168 wild type strain and the DNA repair-deficient strain and the DNA repair-deficient strains. The control used was 4-nitroquinoline-N-oxide. Survival assays for 6 and 7 (cf. Fig. 3.) were made in MY-1 broth [17] solutions. Cultures were grown in tryptose blood agar base for 16 hrs, inoculated into MY-1 broth, and viable cell counts were done on TBAB agar plates.

Compound Synthesis and Purification

Chlorooxirane 4 prepared by the method of Walling and Frederick [20] was in higher purity (95% pure) than that by molecular chlorination [21] (50% pure). Thus, t-butyl hypochlorite and ethylene oxide at -10°C with 200 watt tungsten lamp irradiation yielded chlorooxirane 4; glpc (gas liquid phase chromatography)

t_R = 1.2 min at 50°C, infrared absorption (ν cm^{-1}) 900, 1250, 1320, and 1710 as described previously [21], and PMR as shown in Table II. Derivatization of 4 with an excess of acidic 2,4-dinitrophenylhydrazine solution gave glyoxal-bis-dinitrophenylhydrazone, mp 317-318°C (317°C reported [21]).

Chloroacetaldehyde monomer 5 was obtained in the purest form by cracking the chloroactaldehyde trimer 8 at 95°C and distilling it into dry DMSO; glpc t_R = 2.25 min at 100°C and PMR as shown in Table II.

Chloroacetaldehyde dimer hydrate 7 - A solution containing 49.5 ml of 38% hydrochloric acid, 30 ml of the 45% aqueous chloroacetaldehyde solution, and 55.5 ml of water was distilled over a 9 inch Vigreaux fractionating column. The fraction (1/5 of the initial volume) collected from 87-100°C was redistilled. This second distillate at 83-92°C crystallized after 3 days at -15°C. Upon sublimation of 60°C and 1 atm, white crystals of 7 were obtained; mp 55°C and PMR as shown in Table II.

Chloroacetaldehyde trimer 8 - Concentrated sulphuric acid (7.5 ml) was added to the 45% aqueous chloroacetaldehyde solution (5 ml) with vigorous stirring and external cooling (-5°C). The crystalline precipitate was filtered after standing overnight at -15°C, washed with 5 ml of cold 20% aqueous methanol, and recrystallized 5 times from cold methanol; mp 87-88°C, corresponding to that reported by Natterer [13], and PMR as shown in Table II.

Quantitation of vinyl chloride in nutrient broth - The concentration of vinyl chloride 1 in the broth solution was determined by an extraction method in conjunction with glpc. This method involved (1) establishing the

linearity of the response of 1 on glpc, (2) constructing a standard curve of vinyl chloride weight vs. peak area, and (3) extracting the broth with methylene chloride followed by glpc determination. (1) Linearity of response - A standard solution of 1 was prepared by condensing it (bp -13.4°C) at -78°C onto a known weight of methylene chloride in a 1 ml volumetric flask fitted with a serum cap. The condensed vinyl chloride was determined by weighing. A typical solution thus prepared was 0.2377 M and was subjected to glpc analysis by varying injection sizes from 1-9 µl. The correlation of vinyl chloride weight and peak area was made by a least square computer routine: slope = 0.877, with an index of correlation of 0.972 (ideal 1.00) up to 6 µl (0.1078 mg) of injection. (2) A standard linear curve using the above technique was established for 0.01-0.10 mg of vinyl chloride. (3) Extraction of broth - Vinyl chloride was allowed to bubble through the nutrient broth for 30 min at 25°C. A 1.0 ml broth sample was then extracted with 4 x 2 ml of methylene chloride, the extracts were combined and then made up to 10 ml in a volumetric flask with methylene chloride. Glpc analysis of this solution and application of the standard curve showed that there was 7.0 mg of 1 in the 10 ml solution, or the concentration of 1 in the broth was 0.0107 M. Repeated determination showed it to be 0.0105 M.

High Pressure Liquid Chromatographic (HPLC) Analysis of the Commercial 45% Aqueous Chloroacetaldehyde - Reverse phase HPLC on the commercial 45% chloroacetaldehyde solution (7 M, pH 2.6) resolved it into two components: t_R1 = 6.3 min and t_R2 = 8.8 min in a ratio of 40:60. The ratios of the two peaks on the chromatogram changed as the solution pH was varied by addition of 1 N NaOH at room temperature: 43:57 (pH 3.7), 44:56 (pH 5.0), 48:53 (pH 7.6),

and 56:44 (pH 8.2). The first eluted component at t_R1 = 6.3 min increased while the second at t_R2 = 8.8 min decreased at raised pH's. This indicated a retrograde aldol condensation type hydrolysis. The first peak was assigned to the monomer hydrate 6 and the second to the dimer hydrate 7. When the 45% solution was diluted to 0.44 M at pH 3.6, refluxed for 1 hr, cooled, and chromatographed, the ratio of t_R1:t_R2 became 33:67. This suggests an acid-catalyzed condensation of 6 to form the dimer hydrate 7. When these two components were analyzed by glpc, both emerged at the same retention time (t_R = 2.8 min at 60°C) as that of the monomer 5. Dehydration of 6 and 7 must have occurred under the glpc conditions thereby reverting them to the monomer form. As shown in Table II, the PMR spectra of 6 and 7 are resolved from one another. Thus, the PMR spectrum of the 45% commercial solution also revealed the presence of both hydrates 6 and 7 with approximately the same integrals.

Instrumentation

High Pressure Liquid Chromatography (HPLC) - A Waters Associates model 600 pump combination (dual) with a model 440 differential 254 nm ultraviolet detector were used for the analysis of aqueous chloroacetaldehyde solutions. A Porasil Bondapak $_\mu C_{18}$ 4 mm x 30 cm column was used with a 80:20 v/v 0.1 N $NH_4H_2PO_4$-MeOH isocratic eluant (pH 4.9) at 1 ml/min. These two components were also resolved on a Reeve Angel Partisil 10 ODS 4.6 mm x 25 cm column using the same eluant.

Gas Liquid Phase Chromatography (GLPC) - Analysis of vinyl chloride 1, chlorooxirane 4, and chloroacetaldehyde 5 were performed on a Carle model 9500 flame ionization gas chromatograph. A 10% SE-30 on Anakron 60/70 packed column was used at 30°C for vinyl chloride determination. A 20% Carbowax on chromosorb W column at 50°C and 40 ml/min of Helium was used for analysis of 4 and 5 unless specified otherwise. Both were 0.125 inch x 5 feet stainless steel columns.

Proton Magnetic Resonance (PMR) - Spectra were obtained using a Varian A-60 A and a Perkin Elmer R-12 spectrometer. Solutions of D_2O and DMSO-d_6 were used with 3-(trimethylsilyl)propanesulfonic acid sodium salt as internal reference. Tetramethylsilane was used as a reference in $CDCl_3$ and CCl_4. Probe temperature was 38 °C.

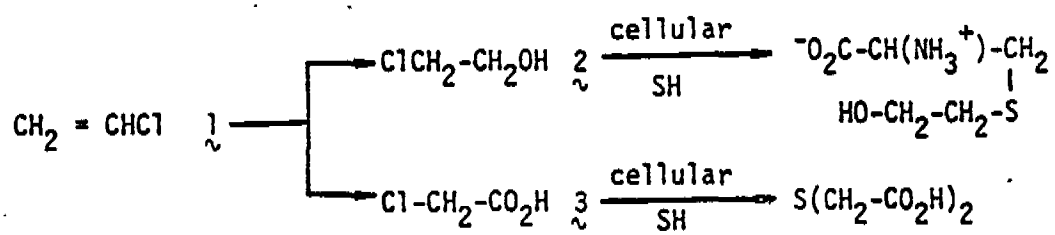
RESULTS AND DISCUSSION

Mutagenicity assays with Bacillus and Salmonella for compounds 1-9 are summarized in Table III. They are grouped into three categories in subsequent discussion. Further testing data are included in Tables IV-VI and Figures 2 - 4.

Nonmutagenicity of vinyl chloride, chloroethanol, and chloroacetic acid - Only high concentrations of vinyl chloride (20% v/v in air) have produced mutagenic action in previous assays with the Salmonella tester strains [9,10]. We have found that tests with both the Salmonella and the Bacillus cultures were negative within the practical solubility range of vinyl chloride in the nutrient broth under ambient conditions. Figure 1 shows the stability of a

presaturated vinyl chloride broth solution at 25°C and 1 atm. The initial concentration of 0.022 M of vinyl chloride 1 rapidly decayed by 50% in 15 hr. Thereafter the escape of 1 from the broth slowed considerably. In the next 45 hr there was a further decline of only 18%. Thus the bacteria strains (B. subtilis MC-1 and Salmonella JA 100) were exposed to the stabilized solution of 0.0106 M (723 ppm) of vinyl chloride in the nutrient broth. The negative activity observed was not unexpected since vinyl chloride lacks the electrophilic character common to many mutagens [23]. Although the direct mutagenicity of vinyl chloride at 200,000 ppm (20% v/v in air) observed previously [10] may be real, the chronic human exposure problem most likely requires metabolic activation of vinyl chloride to an electrophilic reactive form [8]. Also tested were chloroethanol 2 and chloroacetic acid 3 which probably are metabolic intermediates as shown in Scheme I. Both S-(2-hydroxyethyl)cysteine and

SCHEME I: Vinyl Chloride Metabolites



thiodiglycolic acid in Scheme I have been identified [6] as the urinary metabolites of vinyl chloride. Neither chloroethanol 2 nor chloroacetic acid 3 exhibited any mutagenic effects at 1 mM concentration in our mutagenicity assays (cf. Fig. 2). Although Bartsch et al.[10] found considerable mutagenic

activity with chloroethanol 2 for TA 1530 strain in the absence of microsomal activation, our observations corroborate with those of McCann et al.[7] who showed that 2 was weakly mutagenic directly even at high concentrations for the sensitive TA 100 and that it showed only trace activity for TA 1535. The enhanced activity of 2 after microsomal activation observed by these groups suggests that chloroacetaldehyde derivatives were being formed from chloroethanol. We have found potent mutagenicity and lethality with the various forms of chloroacetaldehyde as shown in Figures 2 - 4 and Tables IV-V.

Mutagenesis of chloroacetaldehyde, the monomer hydrate, dimer hydrate, and the trimer - When chloroacetaldehyde 5 was distilled into distilled water, a mixture of the monomer hydrate 6 and the dimer hydrate 7 was formed instantly as determined by HPLC and PMR spectroscopy. Analysis of the commercial 45% aqueous chloroacetaldehyde solution with the same techniques showed a 50:50 mixture of the two hydrates. Upon standing under dry conditions, the monomer 5 cyclized to form trimer 8. The trimer was sparingly soluble in water, but was disproportionated upon heating in water to form the hydrates 6 and 7.

Purified samples of chloroacetaldehyde 5, the commercial 45% chloroacetaldehyde solution containing a 50:50 mixture of the hydrates 6 and 7, the dimer hydrate 7, and the trimer 8, in DMSO solutions were tested for mutagenic potential. The data in Table IV summarizes the results of the repair assays with B. subtilis. Chloroacetaldehyde 5 and the monomer hydrate 6 specifically inhibited the growth of B. subtilis MC-1, a mutant lacking recombination repair of DNA. These unrepaired DNA lesions then led to cell death. However, compounds 5 and 6 did not inhibit the wild type B. subtilis or those mutant

strains (Hcr-9, FB-13) having the capacity for recombination repair. In contrast, purified samples of the dimer hydrate 7 and trimer 8 inhibited all of the mutants as well as the wild type, although MC-1 again demonstrated the most sensitivity. It is unlikely that 7 and 8 caused DNA lesions that are different from those by 5 and 6 because the excision repair mutants and the wild type were inhibited to an equivalent extent. Inhibition of these strains by 7 and 8 may result from a metabolic poisoning or cellular damage similar to that observed in Escherichia coli after exposure to vinyl chloride waste [24].

Viability assays on B. subtilis strains during short term exposure to the chloroacetaldehyde 5 revealed that recombination repair was essential for recovery (Figure 2). The B. subtilis strains with recombination repair capabilities displayed repair kinetics as evidenced by the shoulders in curves in Figure 3. The sensitive MC-1 strain lacking recombination repair yet having excision repair was rapidly killed following the exposure. We surmise that these DNA lesions caused by the chloroacetaldehydes were repaired solely by the recombination repair mechanism.

The direct mutagenic potential of the samples 4-9 was determined by the mutations expressed in Salmonella strain TA 100. The dose response relationships of these compounds (cf. Table V), and the dose-response curves (cf. Figure 4) were determined with strain TA 100. At high concentrations the dose response curves for all compounds became nonlinear because the toxicity of the chemicals reduced the number of potential revertants on the plates. The reduction of the cell population by high concentrations was confirmed by viable counts and observations of decreased background lawn on the test plates.

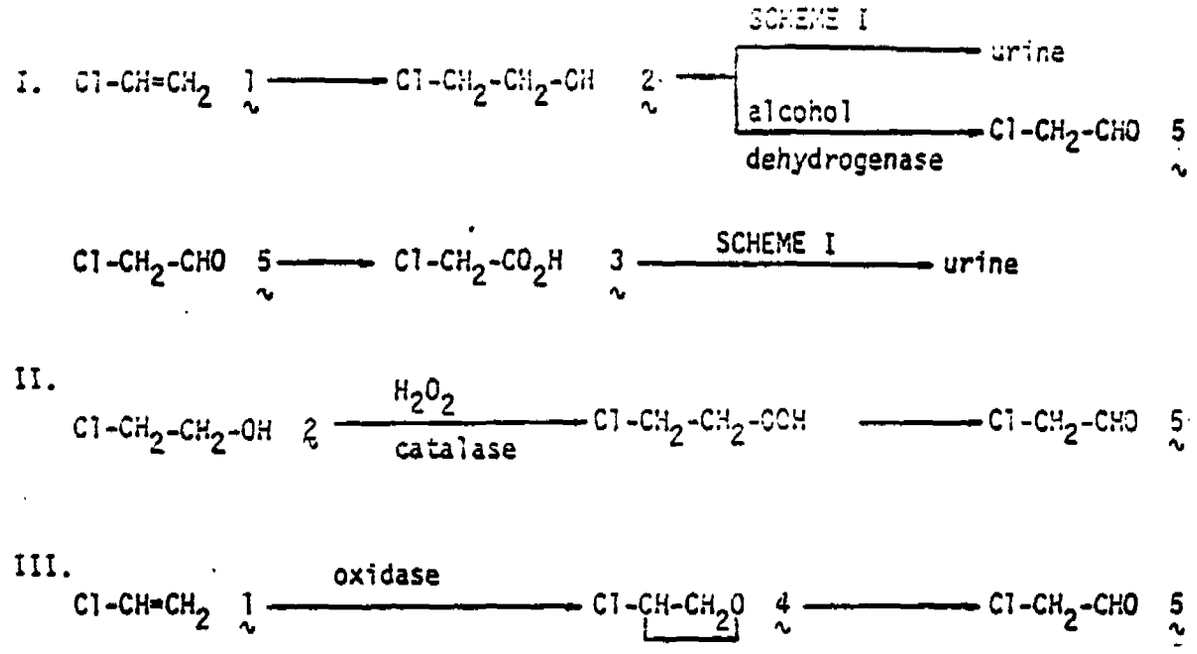
Data from the dose response curves (Figure 4) showed that chloroacetaldehyde 5 and the monomer hydrate 6 were more active than 7 and 8. The monomer 5 was the most mutagenic as predicted because its electrophilic carbonyl group remains intact. The restoration of the carbonyl character to the monomer hydrate 6 can be projected in hydrophobic environment of the cell membrane by loss of water, hence its mutagenicity is also explicable. The substantial activity of the dimer hydrate 7 can be attributed to its ready equilibrium with the monomer hydrate 6 in aqueous medium. The relationship of the activity of the cyclic trimer 8 to the chloroacetaldehyde action cannot be deduced on the basis of the curves in Figure 4. Nevertheless, these mutations were the base-substitution type and were expressed in Salmonella strains TA 100 and TA 1535. The mutagenic response in TA 1535 was very weak as compared to TA 100. It should be noted that the latter contains an "R" factor that enhances mutation by an error-prone recombination repair mechanism following DNA damage [7].

Comparison of mutagenic response of chlorooxirane with its methylene homolog epichlorohydrin - The prevailing opinion [5] is that chlorooxirane 4 is the primary metabolite of vinyl chloride and is derived from the action of the microsomal mixed function oxidase. This compound, prepared independently from chlorination of ethylene oxide, was found to rearrange readily to chloroacetaldehyde in aqueous or DMSO solution at ambient temperatures. A kinetic study of a 0.15 M solution of 4 in a D_2O-DMSO-d_6 (80:20) mixture at pD 7.1 and 4°C by PMR technique showed that the rearrangement followed first order kinetics, k (sec^{-1}) = 2.5×10^{-4}. This translates to a half life of 46.2 min

at 4°C as compared to 1.6 min at 37° C [9]. Such instability allows only limited testing in the cold as well as interpretation of the results. On the other hand, epichlorohydrin 9, which can be considered the epoxidation metabolite of allyl chloride, is a methylene homolog of 4. Both 4 and 9 are structurally bis-alkylating agents, hence comparing their mutagenic activities will lend insight into the mode of action of chlorooxirane [25,26]. Table IV tabulates the mutagenic response of these two compounds in the Bacillus repair assay and Table VI the Salmonella TA 100 reversion. Due to the instability of chlorooxirane at 37°C, the assays shown in Table VI were preincubated at 3°C in solution with the Salmonella TA 100 strain before plating to insure that chlorooxirane could reach the cells intact. Mutant strains of B. subtilis were not inhibited when exposed to high concentrations of epichlorohydrin 9. In contrast, chlorooxirane 4 selectively inhibited the rec⁻ strain MC-1 in a manner similar to chloroacetaldehyde hydrate 6. Tests with Salmonella showed that strain TA 100 was very susceptible to the mutagenic action of both epoxides 4 and 9. However 9 was less toxic to the tester strains than 4. The low toxic effects of 9 indicate a different type of DNA lesion compared to that caused by chlorooxirane 4. It is possible that 9 may react with DNA by a mechanism which does not cause potential lethal strand-scissions. On the other hand, chlorooxirane 4 may act on the bacteria via a NIH shift [27,28] to form chloroacetaldehyde 5 or 6. Conceivably, chlorooxirane can also behave as a diradical intermediate rather than a conventional S_N1 or S_N2 type alkylating agent in its reaction with DNA.

Vinyl chloride carcinogenesis mechanism hypothesis - Among the compounds tested in the metabolic Scheme-II, chloroacetaldehyde 5 and chlorooxirane 4

SCHEME II: The metabolic pathways of vinyl chloride [5].



were the most mutagenic with the lowest toxic side effects. Hence, they may qualify to be the active carcinogenic derivatives of vinyl chloride. Considering the aqueous milieu of the metabolic environment, however, the chloroacetaldehyde monomer hydrate 6 is a more realistic choice as an ultimate carcinogen than the monomer 5 which reacts immediately with water. Viability assays following exposure of the B. subtilis mutants to 6 indicated that the compound induced recombination repair. The strain with the rec$^-$ phenotype (MC-1) was immediately inactivated. All the rec$^+$ strains showed survival ability and repair kinetics of similar nature. It has been shown [27] that mammalian cells have postreplication repair of DNA which has many similar features to the recombination repair in bacteria. In addition, a relationship between this mammalian postreplication repair process and mutation as well as carcinogenesis

has been suggested [24]. Cells from patients with the skin disease xeroderma pigmentosum lack the ability to excise pyrimidine dimers and must rely on postreplication repair to remove these lesions [24]. It is believed that this error prone process of postreplication repair is responsible for the production of somatic mutations and cancer in patients with this disease. Since chloroacetaldehyde monomer hydrate 6 induces recombination repair in bacteria responding to lesions, it may also be capable of activating the error prone postreplication repair in exposed mammalian cells. At the molecular level, chloroacetaldehyde is known to react with N^1 and N^6 nitrogens of adenosine and N^3 and N^4 nitrogens of cytidine in single-stranded DNA [28,29]. It therefore appears that chloroacetaldehyde monomer hydrate 6 should merit our consideration as an ultimate carcinogenic metabolite of vinyl chloride.

The lower mutagenic activity of chloroxirane 4 compared to 6 may reflect the unstable nature of chlorooxirane as an α-chloroether. While the carcinogenic chloromethyl methyl ether is a bifunctional alkylating agent [30], the mutagenic activity of chlorooxirane cannot be so categorized, especially when it is compared with epichlorohydrin 9. One mode of action of chlorooxirane is a rearrangement to chloroacetaldehyde via the NIH shift [27,28]. Another is a homolytic ring cleavage to yield a stabilized diradical intermediate $ClCH\text{-}CH_2O$. Both are capable of reacting with DNA, thereby accounting for the mutagenicity of 4. In mammalian cells, chlorooxirane, being a reactive epoxide, could be trapped by a glutathione epoxide transferase [31] at a faster rate than the detoxification of chloroacetaldehyde via the less active aldehyde dehydrogenase [32] . Such detoxification of chlorooxirane could

explain the reported decrease in sulfhydryl level in liver cells during metabolism of short chain halo hydrocarbons including vinyl chloride [31]. Perhaps the lower mutagenicity of chlorooxirane 4 in these bacterial assays, compared to the chloroacetaldehydes, is also attributable to its being detoxified faster. We therefore consider both chlorooxirane 4 and the chloroacetaldehyde monomer hydrate 6 to be the ultimate carcinogenic metabolites of vinyl chloride. Their reactions with DNA causing mutations in Bacillus and Salmonella suggest a causal relationship with vinyl chloride carcinogenesis in human and laboratory animals.

Acknowledgements

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LEGENDS TO FIGURES

Fig. 1. Stability of vinyl chloride in nutrient broth at 25°C, 1 atm.

Fig. 2. Survival of Bacillus subtilis MC-1 after incubation with: 1.0 mM chloroethanol 2 (+—+); 1.0 mM chloroacetic acid 3 (□—□); 5.76 mM (concentration based on ClH_2CCHO) chloroacetaldehyde (45% aqueous solution) 6 and 7 (▷—▷); 0.1 mM 4-nitroquinoline-N-oxide (◇—◇); and untreated control cells (○—○). Mid-logarithmic cultures grown in MY-1 broth were incubated with compounds at 37°C. Samples were plated at the times indicated from which viable cell counts were recorded. The mutagen 4-nitroquinoline-N-oxide served as a positive mutagenicity control.

Fig. 3. Survival of Bacillus subtilis strains in the presence of 5.0 mM (concentration based on ClH_2CCHO) chloroacetaldehyde (45% aqueous solution) 6 and 7. Cultures were grown to mid-logarithmic phase in MY-1 broth and then incubated with the chloroacetaldehyde solution at 37°C. Samples were plated at the times indicated from which the percent survival was determined. FB-13 uvr^-, rec^+ (+—+); Hcr-9 hcr^-, rec^+ (◇—◇); 168M wild type (○—○); and MC-1 uvr^+, rec^- (▷—▷).

Fig. 4. Dose response curves with Salmonella typhimurium TA100. Sample solutions of known concentrations prepared in DMSO were mixed with tester strain culture and soft agar. Plates were poured, incubated at 37°C for 48 hrs, and then scored for revertant colonies to prototrophy. Chloroacetaldehyde monomer 5 (○—○); chloroacetaldehyde (45% aqueous solution-concentration based on ClH_2CCHO) 6 and 7 (◇—◇); chloroacetaldehyde dimer hydrate 7 (▷—▷); chloroacetaldehyde trimer 8 (×—×); and epichlorohydrin 9 (□--□).

Figure 1

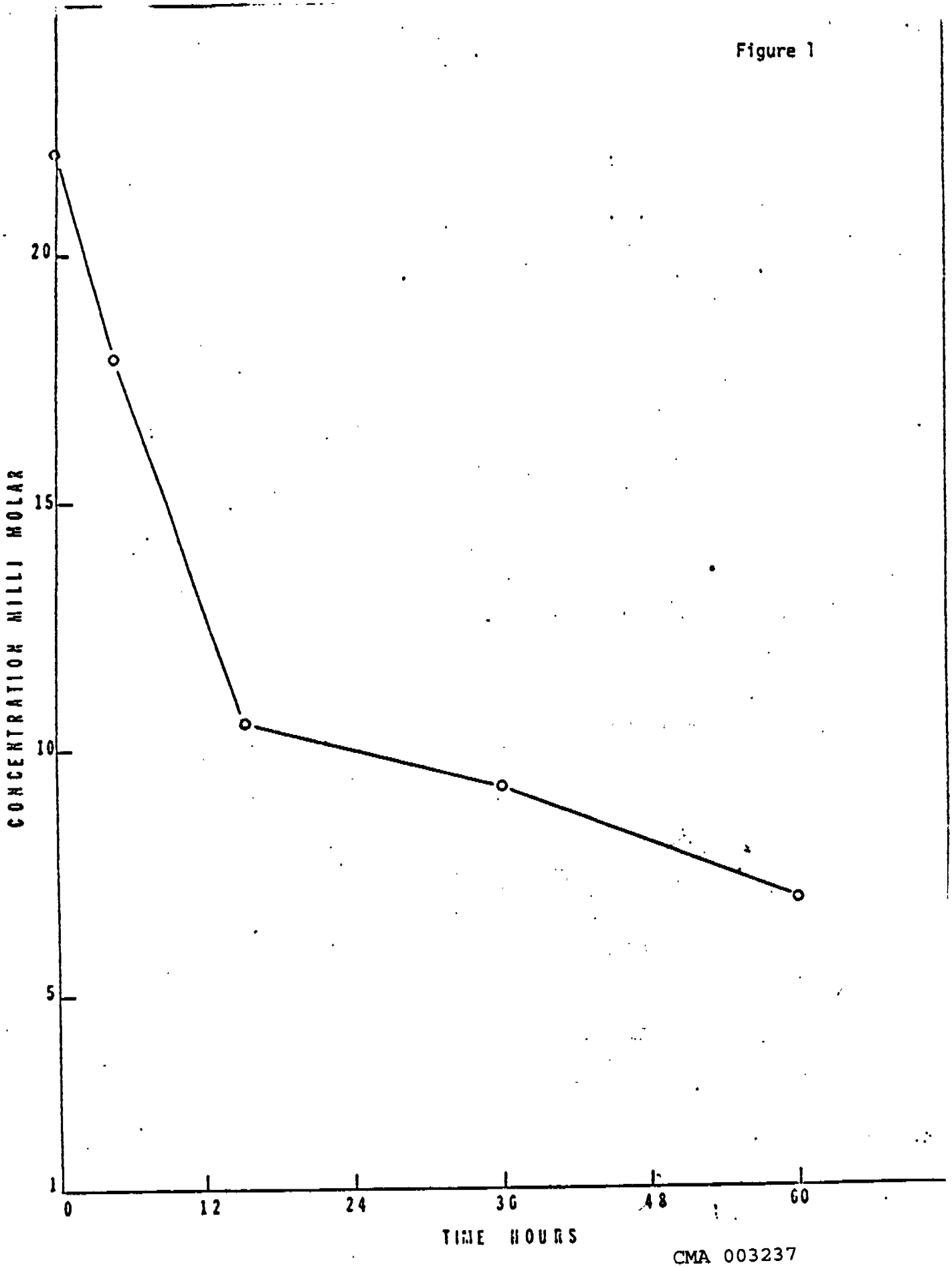

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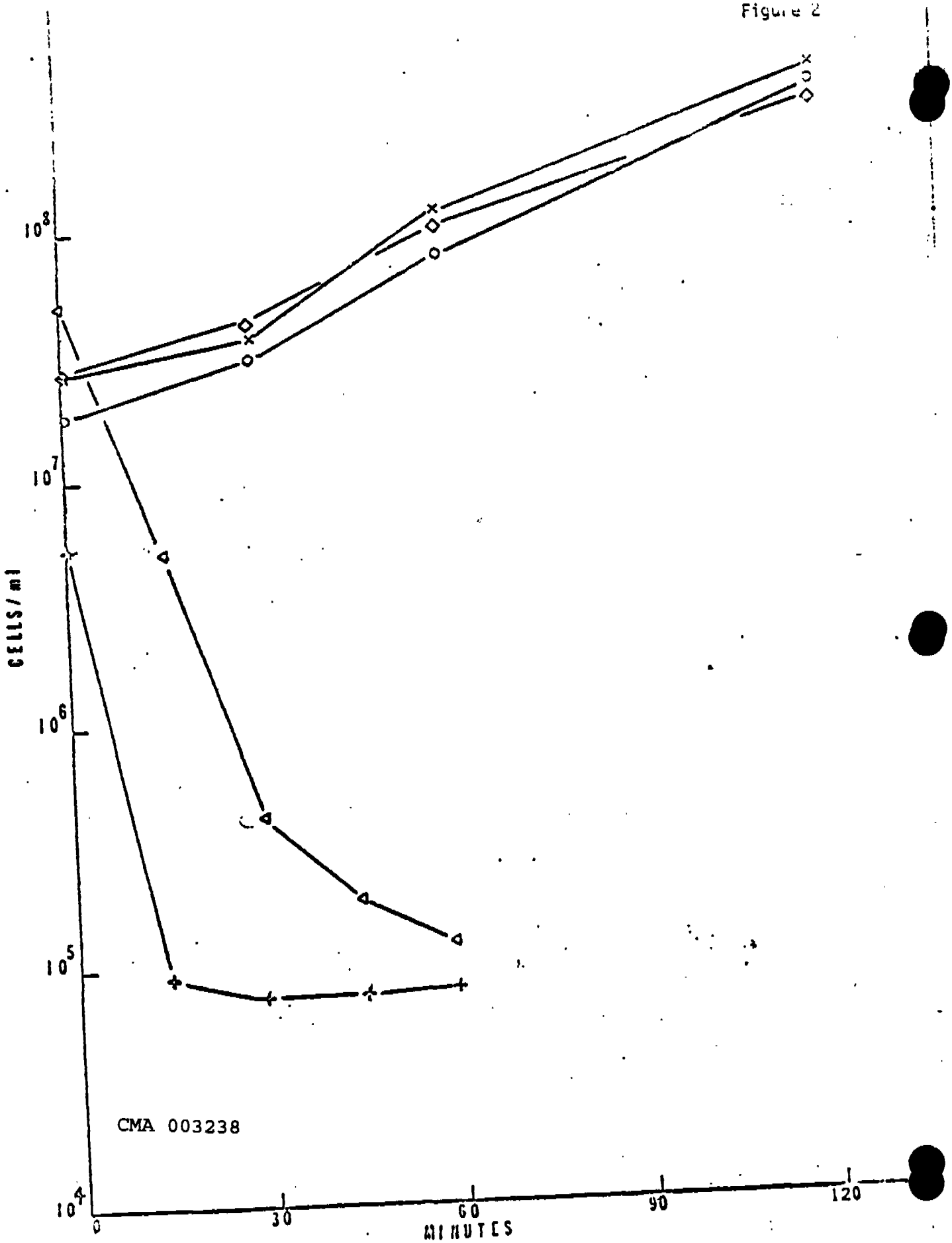
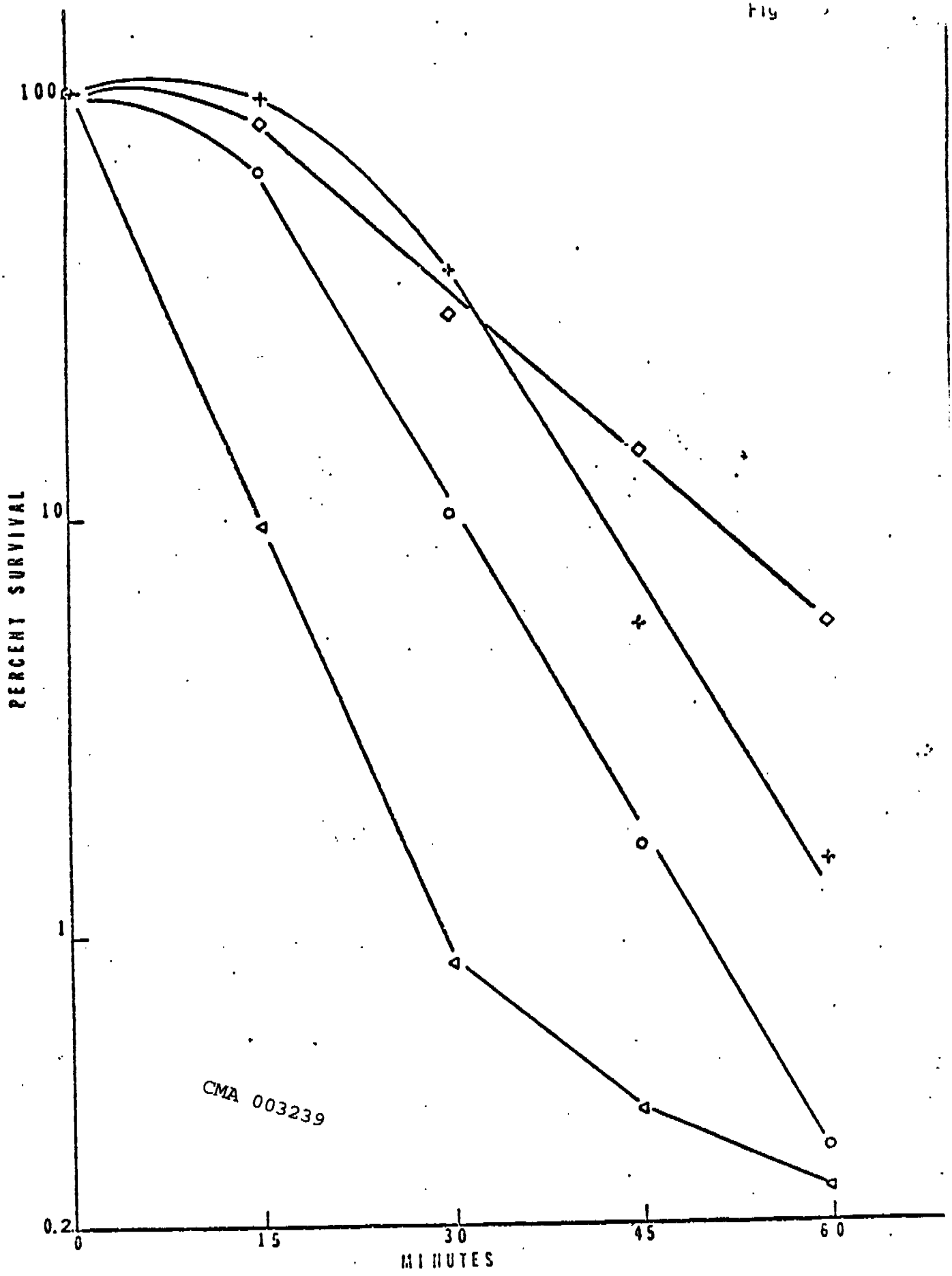
Figure 2





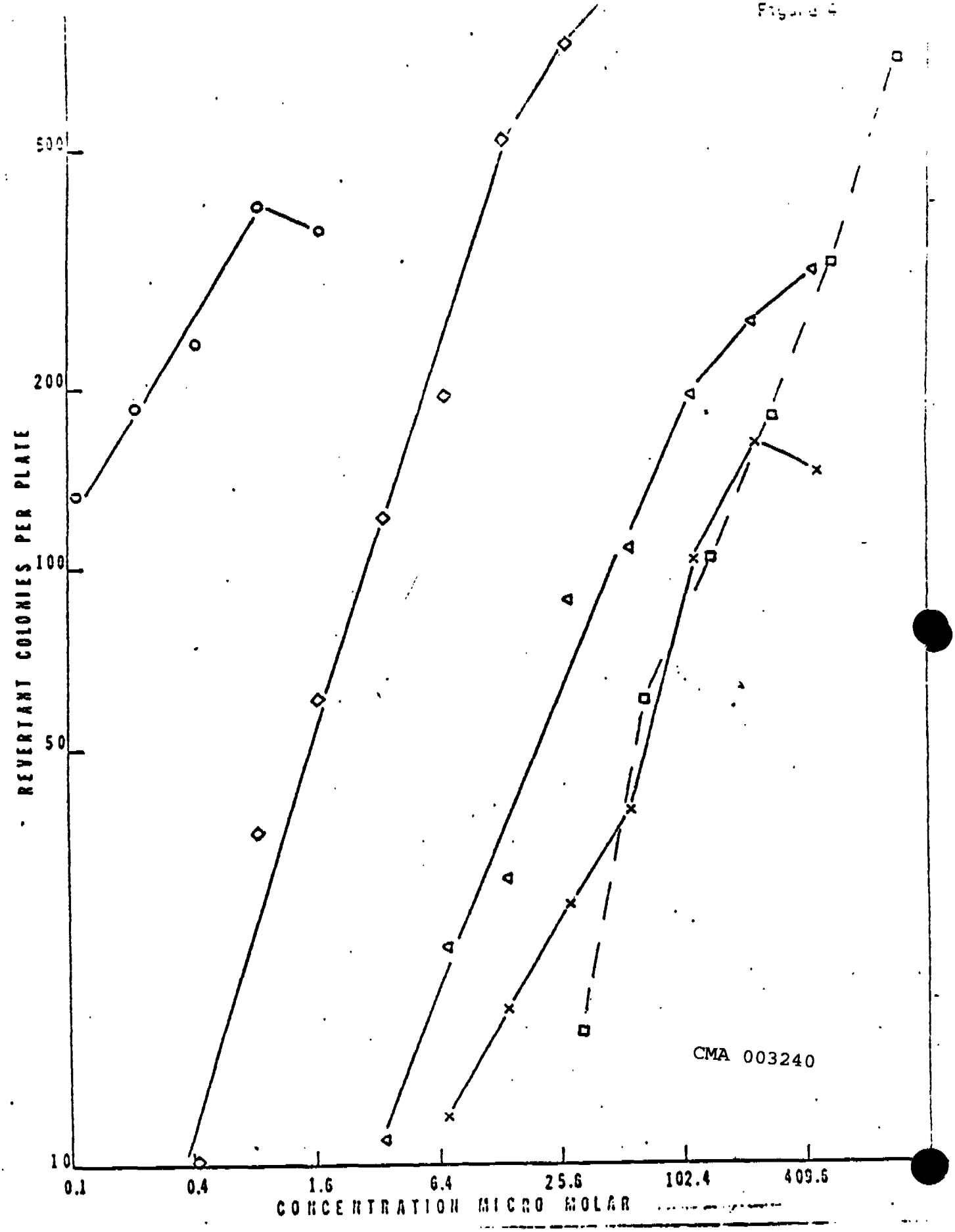

500
200
100
50
10
REVERTANT COLONIES PER PLATE
0.1
0.4
1.6
6.4
25.6
102.4
409.6
CONCENTRATION MICRO MOLAR
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TABLE I: Bacteria Tester Strains

A. Salmonella typhimurium LT-2 Tester Strains[a]

[a]All tester strains contain uvrB repair mutations which eliminate the excision repair system; mutations in the histidine operon; and rfa mutations which alter the cell wall by increasing permeability and eliminating pathogenicity.

[b]The resistance transfer factor, "R" factor, enhances the error-prone recombination repair system thus making the strains more susceptible to mutation [15].

[c]The strains susceptible to base-pair substitution contain mutations in the histidine G46 operon and those susceptible to frameshift mutation contain mutations in the histidine operon C 3076 (TA 1537) or D3052 (TA 1538, TA 98).

Strains	"R" factor[b]	Mutation detected[c]
TA 1535	-	base-pair substitution
TA 100	+	base-pair substitution
TA 1537	-	frameshift
TA 1538	-	frameshift
TA 98	+	frameshift

B. Bacillus subtilis Tester Strains

[a]Trp^- denotes a requirement for tryptophan; Mit-S denotes sensitivity to mitomycin C.

[b]hcr^+ denotes a host-cell reactivation DNA repair capacity; hcr^- lacks a host-cell reactivation DNA repair capacity; rec^+ denotes a recombination DNA repair capacity; rec^- lacks a recombination DNA repair capacity; uvr^- is sensitive to ultraviolet-induced DNA damage.

Strains	Phenotype[a]	DNA Repair[b]
168 M	Prototroph (wild type)	hcr^+, rec^+
Hcr-9	Trp^-	hcr^-, rec^+
FB-13	Trp^-	uvr^-, rec^+
MC-1	Trp^-, Mit-S	hcr^+, rec^-

TABLE II. PMR Spectra of Vinyl Chloride Derivatives

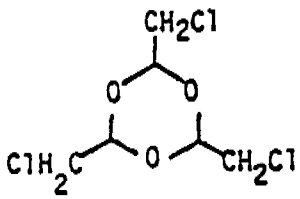
Compounds		Solvent	Spectra, δTMS=0 (J=Hz)
$ClHC(-CH_2O-)_2$	4	CCl_4	2.75 (q, CH, J=1.5) 2.85 (q, CH, J=2.4) 4.90 (q, CH, J=2.4,1.5)
ClH_2C-CHO	5	CCl_4	4.00 (d, CH, J=2.2) 9.57 (t, CH_2, J=2.2)
		DMSO-d_6	3.50 (d, CH) 9.60 (t, CH_2)
$ClH_2C-CH(OH)_2$	6	CD_3OD-D_2O 1:8 (pD 0.1)	3.60 (d, CH_2, J=5) 4.60 (t, CH, J=5)
$ClH_2C-CH(OH)-O-CH(OH)-CH_2Cl$	7	DMSO-D_6	3.55 (d, CH_2, J=4.9) 5.05 (t, CH, J=4.9)
		CD_3OD-D_2O 1:8 (pD 0.1)	3.60 (d, CH_2, J=4.5) 4.83 (t, CH, J=4.5)
	8	CCl_4	3.52 (d, CH_2, J=4.7) 5.08 (t, CH, J=4.7)
		DMSO-d_6	3.75 (d, CH_2, J=4.1) 5.45 (t, CH, J=4.1)

TABLE III: Summary of Mutagen Activity in Microbial Systems

NI = No inhibition of growth detected in Bacillus subtilis MC-1; NR = No increase of revertants in Salmonella typhimurium TA 100 compared to control; + =active; ++ = very active. Acetaldehyde, a potential metabolite of 1, and allyl chloride, the parent olefin of 9, were negative in these two systems.

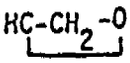
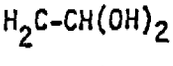
Compounds Tested	Bacillus subtilis Repair Assay	Salmonella typhimurium Reversion Assay
Control: 4-nitroquinoline-N-oxide	++	++
1 $H_2C=CHCl$	NI	NR
2 ClH_2C-CH_2OH	NI	NR
3 $ClH_2C-COOH$	NI	NR
4 	+	++
5 ClH_2C-CHO	++	++
6 $ClH_2C-CH(OH)_2$	++	++
7 $ClH_2C-CHOH-O-CHOH-CH_2Cl$	++	++
8 $(ClH_2C-\overset{|}{C}HO-)_3$	++	+
9 	NI	++

TABLE IV: Growth Inhibition of *Bacillus subtilis* Strains.

Inhibition was measured in mm after 24 hr at 37°C as described in text; NI denotes no inhibition

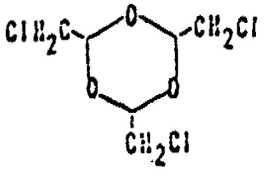
Mutagen		Molarity	168M	MC-1	Hcr-9	FB-13
ClH_2C-CHO	5	0.100	2.0	27.7	3.7	2.7
Chloroacetaldehyde (45% aqueous solution)	6 & 7	0.115	NI	22.5	NI	NI
ClH_2C-CH(O)-CH$_2$Cl	7	0.097	1.5	9.5	1.5	1.5
	8	0.096	7.0	14.5	6.0	7.0
ClHC-CH_2O	4	0.260	NI	10.0	NI	NI
ClH_2C-CH-CH_2O	9	0.113	NI	NI	NI	NI
4-Nitroquinoline-N-oxide (control)		0.001	10.0	18.0	15.0	15.0

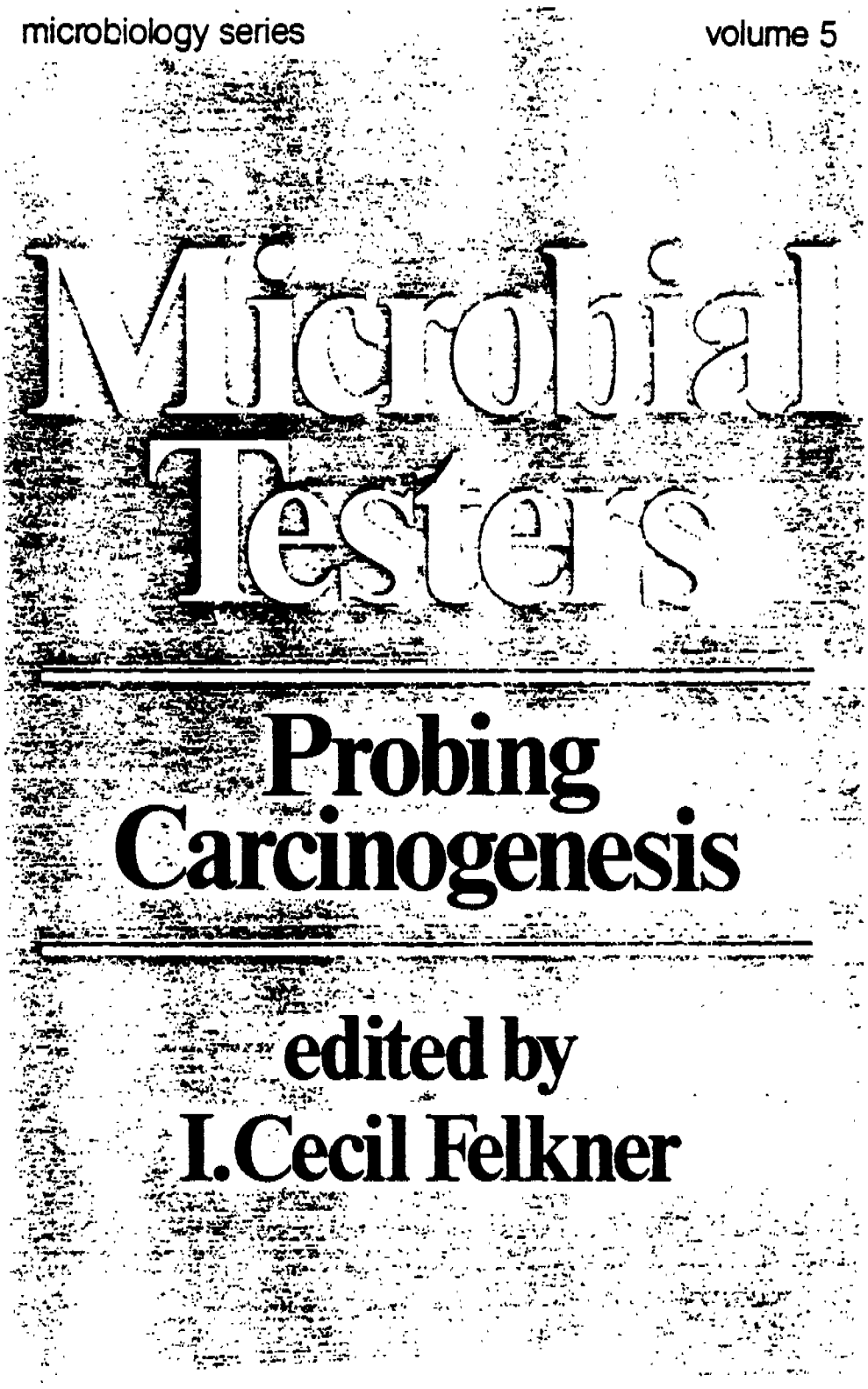
TABLE V. Relative Mutagenicity of the Four Forms of Chloroacetaldehyde with S. typhimurium TA 100

45% Aqueous Soln 6 : 7 50:50		Monomer 5		Dimer Hydrate 7		Trimer 8	
Molarity	Revertants	Molarity	Revertants	Molarity	Revertants	Molarity	Revertants
5.3×10^{-5}	977	1.3×10^{-5}	18	4.8×10^{-4}	311	4.8×10^{-4}	144
2.7×10^{-5}	723	6.7×10^{-6}	68	2.4×10^{-4}	259	2.4×10^{-4}	159
1.4×10^{-5}	512	3.3×10^{-6}	88	1.2×10^{-4}	193	1.2×10^{-4}	101
6.9×10^{-6}	194	1.7×10^{-6}	361	6.0×10^{-5}	107	6.0×10^{-5}	39
3.4×10^{-6}	120	8.4×10^{-7}	404	3.0×10^{-5}	88	3.0×10^{-5}	27
1.7×10^{-6}	61	4.2×10^{-7}	238	1.5×10^{-5}	30	1.5×10^{-5}	18
8.6×10^{-7}	36	2.1×10^{-7}	185	7.5×10^{-6}	23	7.4×10^{-6}	12
4.3×10^{-7}	10	1.1×10^{-7}	131	3.8×10^{-6}	11	3.7×10^{-6}	-0

TABLE VI: Reversion of S.typhimurium TA100 by Chlorooxirane 4 and Epichlorohydrin 9

Broth solutions of 4 or 9 with TA100 were preincubated at 3°C before plating. Duplicate plates were evaluated after 48 hrs at 37°C. The average number of revertant colonies per plate minus the number of spontaneous reversions were recorded.

Preincubation Time	Epichlorohydrin 9 (1.0 mM)	Chlorooxirane 4 (0.26 mM)
0 hr	186	31
1 hr	204	4
2 hr	297	6
4 hr	202	114
6 hr	154	44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6

BACTERIAL MUTATION MONITORS FOR ACTIVE METABOLITES OF CHEMICAL CARCINOGENS: *B. SUBTILIS* ASSAYS FOR MUTATION AND DNA REPAIR

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I. INTRODUCTION

Chemical monomers, such as vinyl chloride, styrene, and acrylylnitrile, are of great importance in many industrial synthesis reactions. They are the fundamental components of polymers used extensively in the economy of the industrial nations. The detection of tumors (e.g., angiosarcoma) in workers exposed to vinyl chloride in the industries of several nations suggested that there was a relationship between this industrial monomer and the development of hepatic disorders (13, 40). As a result, both industry and government have become increasingly active in research

concerning the potential carcinogenicity of extensively used chemical compounds. Unfortunately, the assays for tumor production are time consuming (70) and the number of existing and newly produced chemical formulations huge. Therefore, it is imperative that rapid assays for mutagenicity and potential carcinogenicity be performed to narrow the spectrum of chemicals which should be tested for tumor production in animal systems.

Epoxides are generally thought to be intermediates in microsomal, enzyme-mediated oxidation of olefinic compounds (29). As such, chlorooxirane and its rearrangement product chloroacetaldehyde (CAA) (20) were suspected to be the carcinogenic metabolites of vinyl chloride. Several laboratories have confirmed the mutagenicity of these compounds (4, 20, 39, 42, 45, 59). Our laboratory also tested the various multimer forms of chloroacetaldehyde, as well as chlorooxirane, and found them to be mutagenic (20). It is reasonable to assume that other industrial monomers may also form reactive metabolites upon oxidation.

In this chapter we describe several rapid assays, primarily utilizing DNA repair functions as the screening methodology, for the detection of biological activity in known mutagens [ethylmethanesulfonate (EMS) and methylmethanesulfonate (MMS)] as well as in the oxidation products of the chemical monomers vinyl chloride and styrene. Repair of mutagen-elicited lesions in DNA is important for the survival of affected cells. However, DNA repair has also become increasingly implicated in pathways leading to additional mutagenesis and possibly carcinogenesis (49, 50, 73).

The discovery that certain UV-sensitive mutants of Escherichia coli are not mutated following UV radiation led to the hypothesis of error-prone and error-free types of DNA repair (73, 75). There are three distinct pathways for the repair of damaged DNA following radiation or exposure to chemical agents (photoreactivation, excision repair, postreplication repair).

The process of photoreactivation involves the binding of the pyrimidine dimer by the photoreactivating enzyme and the "splitting" of the dimer following the exposure of the enzyme-dimer complex to photoreactivating light (37, 61, 66). Significantly, this mechanism of DNA repair does not result in the production of mutations and is therefore error-free (5, 74, 75).

Damaged DNA can also be repaired in the dark via the excision mechanism (10, 69). Excision repair (uvr genes) involves the enzymatic recognition and removal of the damaged DNA followed by repair replication (56). In this process, the single-strand gap produced following the removal of the damaged base or bases is filled in by a DNA polymerase which uses the undamaged strand as a template. In E. coli, the most efficient and accurate excision repair system requires, among other gene products, a functional DNA polymerase I (12, 24, 36). However, alternate pathways of excision repair have been demonstrated in cells lacking DNA polymerase I (44, 82, 84, 85). Excision repair is also virtually error-free, although there is a low frequency of mutation which has been ascribed to errors in the alternate pathways of this type of DNA repair (8, 53, 74). One of our rapid tests, the DNA repair assay, examines the ability of cells lacking this type of repair t survive chemical damage (Table 3).

Phot reactivation and excision repair are processes which rest re the integrity of the damaged chromosome prior to the replication of the DNA. Following the replication of the DNA, integrity can still be restored by the process(es) of postreplication repair. Postreplication repair is believed to involve the filling of gaps left in daughter strand DNA following the replication of damaged DNA (32, 62). The closing of these single-strand gaps is accomplished by at least two types of mechanisms in E. coli (83). First, a constitutive type of postreplication repair has been shown to involve recombination (22, 23, 63). Second, one or more types of postreplication repair are inducible and their mechanisms of action are unknown (64, 65). Postreplication repair has an error-prone element (6, 35, 65, 73, 74), which is an inducible, independent minor pathway. Specifically, mutants of E. coli lacking the products of the $recA^+$ and/or $lexA^+$ genes are unable to induce this error-prone repair system (48, 75). All postreplication repair in E. coli is deperdent on a functional $recA^+$ gene product (68) while inducible postreplication repair is also dependent on a functional $lexA^+$ ($exrA^+$) gene product (64). In addition to error-prone, inducible postreplication repair, recA and/or lexA mutations prevent the induction of prophage (9, 18), Weigle (72) or UV reactivation, and W mutagenesis (15, 48, 54), the induction of $recA^+$ gene product (21, 26, 27, 47, 64), the inhibition of exonuclease V (43), as well as other physiological changes following the inhibition of DNA replication (69, 74). The pleiotropic effects of these mutations led Radman (58) to propose the SOS hypothesis. This theory contends that damage to DNA and/or the inhibition of DNA replication results in the release of a signal which simultaneously activates various functions which aid the cells and/or prophage in survival (74). Therefore, error-prone repair is the result of an efficient but inaccurate repair mechanism which is induced in an effort t prevent cell death.

Based on the observations of certain inducible phenomena, the presence of an SOS system can be assumed in nonenteric bacteria (28, 30, 67, 76, 86), in lower eucaryotes (51, 57), as well as in mammalian cells in culture (7, 14, 34, 41). It is important to note that the components of SOS systems appear to differ from species to species. Specifically, an induced DNA modification system has been found in Bacillus subtilis (76), whereas in Haemophilus influenzae, W reactivation has been demonstrated although error-prone repair is not present (28, 38, 67). Thus, there appears to be significant divergence in the composition of SOS systems.

In mammalian systems the relationship between the mechanisms of DNA repair and mutagenesis is not as readily obtainable as in the prokaryotes. However, there do appear to be analogous mechanisms of repair in the two systems. Specifically, chemical damage (60) as well as photochemical damage (11) of DNA can be repaired by mammalian cells.

Recently, interest has begun to focus on the possible effect of inducible SOS-like systems on carcinogenesis (49, 74). In the tester systems developed by Ames and his colleagues (1, 2, 46), most of the carcinogenic agents tested have been shown to require a functional error-prone repair system

in order to generate mutations in the bacteria (46). A phage induction function has been proposed for their system (71). In addition, aflatoxin B_1 and 4-nitroquinoline-1-oxide (known carcinogenic agents) both induce prophage λ and therefore activate the bacterial SOS system (25, 31). Moreau et al. (49, 50) developed a tester system for carcinogenic agents which utilizes the induction of prophage λ. Based on their results, they have suggested that an SOS-like system, including induction of proteases, exists in eucaryotes, and that the activation of this system can lead to cancer. If this theory is correct, a model system in which precocious activation of SOS functions occurs should be a sensitive tester for potential carcinogens. The bacterium B. subtilis possesses many of the desired prerequisites for this type of sensitive tester system.

It has been demonstrated that when B. subtilis differentiates into its competent state, SOS functions are precociously activated (76, 77). In addition, competent B. subtilis cells have enhanced sensitivity to UV irradiation (55, 77, 80). This enhanced sensitivity is the result of the induction of defective prophages in the competent bacteria (Refs. 77 and 77a). Removal of the defective prophages and/or the inhibition of SOS induction eliminated the enhanced sensitivity of the competent cells to UV energy.

We have included this review to substantiate our rationale in utilizing several types of repair assays, each measuring a different step in the repair process, as a part of our battery of rapid assay systems. It is evident that bacteria have several methods for repairing both UV-induced and chemical damage to DNA. Therefore, assays which measure for the induction of error-prone repair (SOS) or for the necessity of repair (our series of repair assays) represent valuable information when considering the biological activity of potential mutagens and/or carcinogens.

II. MICROBIAL ASSAYS

A. Salmonella Reversion Test

The mutagenic capability of the postulated active derivatives of the chemical monomers vinyl chloride and styrene, as well as ethylmethanesulfonate (EMS) and methylmethanesulfonate (MMS), was examined using the Salmonella typhimurium tester strains described by Ames (1) using methodology already published by McCann et al. (45) and by our laboratory (20, 39) (Table 1).

Chloroacetaldehyde (CAA) was prepared, purified, and analyzed by previously reported techniques (20). Styrene oxide was prepared by treating styrene in methylene chloride with 1.2 equivalents of M-chloroperbenzoic acid at room temperature for several days. The solution was washed successively with aqueous $NaSO_3H$ and aqueous $NaHCO_3$, then dried and evaporated. Both EMS and MMS were obtained from the Aldrich Chemical Company (Milwaukee, Wis.).

Table 1. Bacterial Strains Used in These Studies

Strain	Relevant repair genotype	Source
S. typhimurium TA100	uvrB (R factor)	B. Ames
B. subtilis GSY1025	recA1	C. Hadden
B. subtilis MC-1	recB2	C. Hadden
B. subtilis GSY1627	recD27	C. Hadden
B. subtilis VUB214	recD27	C. Hadden
B. subtilis BD224	recE4	C. Hadden
B. subtilis HA106	recF7	C. Hadden
B. subtilis VUB133	recH342	C. Hadden
B. subtilis RUB827	polA5	R. Yasbin
B. subtilis FB13	uvr	C. Hadden
B. subtilis HC-9	hcr	C. Hadden
B. subtilis GSY1641	mtc-41	C. Hadden
B. subtilis 168W	wild type	A. Laumbach
B. subtilis RUB818	wild type	R. Yasbin

The assays with the S. typhimurium strain TA100, an indicator for base pair substitution mutations (45), revealed that all four chemicals had considerable mutagenic activity (Table 2). Our laboratory has shown previously that the chloroacetaldehyde consists of several different forms, all mutagenic in this assay (20). None of these chemicals caused significant reversion with strain TA98, a frameshift indicator.

B. Bacillus Repair Assay

The repair assay, using various repair-deficient strains (Table 1) of B. subtilis, has been described by our laboratory (20, 39). This is an indirect mutation assay, modified from the procedures described by Kada et al. (33). As discussed in the introduction to this chapter, when a mutagenic agent

Table 2. Mutagenicity of Chemical Monomers Assayed by Salmonella TA100 Reversion

Compound	Concentration in soft agar layer, mM/plate	Average number revertants/plate[a]
Chloroacetaldehyde	0.10	456
Styrene oxide	0.10	620
Methylmethanesulfonate	0.05	956
Ethylmethanesulfonate	0.10	295

[a]Spontaneous revertants have been subtracted.

affects DNA, the cell is required to repair the damage. If the cell is unable to express the repair capability, the majority of cells will not survive. Thus, a measure of the biological reactivity of a chemical is to test it against several strains, one being wild type for repair, the others being deficient in one of the repair systems. If the wild type strain survives but any of the repair mutants do not, the chemical is assumed to be reactive (see also Chapter 4).

In Table 3 we show the reactivity of CAA, MMS, EMS, and styrene oxide against a series of repair mutants of B. subtilis. First of all, styrene oxide does not appear to be reactive in any of these assays. This suggests that over a wide spectrum of concentrations tested, styrene oxide does not cause damage to DNA, which must be repaired by the available repair systems. Even when these tests were performed with cold incubation (see Chap. 4; Refs. 33 and 62), a procedure which increased sensitivity to CAA threefold, there was no discernible reaction with styrene oxide. On the other hand, all forms of CAA (20), MMS, and EMS were quite reactive in this system. However, these compounds affected only strains which

Table 3. Effect of Chemical Monomers on Repair-Assay with B. subtilis

Strain	Relevant repair genotype	Growth inhibition, mm[a]			
		Chloroacetaldehyde, 0.1 M	Styrene oxide, 0.1 M	MMS, 0.1 M	EMS, 0.1 M
GSY1025	recA1	13.5	NI	14.5	8.1
MC-1	recB2	11.3	NI	13.0	6.4
GSY1627	recD27	11.5	NI	11.2	4.8
VUB133	recD27	NI	NI	NI	NI
BD224	recE4	8.8	NI	16.4	6.1
HA106	recF7	6.1	NI	11.5	7.2
VUB133	recH342	NI	NI	3.0	1.5
Hcr-9	hcr	NI	NI	NI	NI
FB-13	uvr	NI	NI	NI	NI
GSY1641	mtc-41	NI	NI	NI	NI
168W	wt	NI	NI	NI	NI

[a]Growth inhibition was measured in millimeters from the edge of the disc to the first evidence of growth (radius). Values are an average of seven experiments. Inhibition due to toxicity, as measured on wt cells (168W), has been subtracted from these values.

Note: NI = no growth inhibition.

were deficient in the type of postreplication repair known as recombination repair. Moreover, only specific recombination genotypes recA, recB, recE, recF, and one strain of recD were affected by CAA, MMS, and EMS. One strain of recD, recH, as well as the excision repair-deficient mutants (her, uvr, polA) were inhibited slightly (EMS and MMS) or not at all (CAA). All strains were confirmed to possess the recombination deficiencies by transformation and transduction assays (19). These results suggest that CAA, MMS, and EMS may cause similar lesions, all of which require for repair certain components of the recombination repair pathway. In this regard, 4-nitroquinoline-1-oxide (4NQO) reacts identically in this system (Laumbach and Streips, unpublished data).

C. Bacillus Forward Mutation Analysis

An additional procedure for determining the mutagenic capability of a chemical compound is to examine its ability to cause forward mutations. This test serves the same purpose as the reversion assay described in Section II.A. Both are direct tests for mutation capability. However, the response is less limited for the forward mutation systems.

Cultures of B. subtilis 168 wild type (Table 1) were subjected to a 15-min exposure to CAA, styrene oxide, MMS, and EMS at various concentrations. The cultures were washed and allowed to incubate for 16 hr to express mutation to streptomycin resistance. The cells (0.1 ml) were plated on tryptose blood agar base (TBAB) plates for total viable count. Similar plates were overlaid with soft agar (0.8% agar) containing 1 mg/ml dihydrostreptomycin sulfate to determine the number of mutants. The results from this experiment are shown in Table 4. It is evident that CAA, MMS, and EMS

Table 4. Induction of Mutants Following Exposure to Chemical Monomers

Chemical	Treatment	Total number bacteria[a]	Total mutant colonies	Mutations per 10^8 cells	Relative mutation frequency[b]
Chloro-acetaldehyde	0	6.0×10^8	2.4	0.4	
	5 mM	5.5×10^8	70.8	12.9	32.3
Styrene oxide	0	7.0×10^8	3.1	0.4	
	5 mM	6.6×10^8	10.2	1.5	3.8
Methylmethane-sulfonate	0	6.0×10^8	2.4	0.4	
	5 mM	5.4×10^8	85.8	15.9	39.8
Ethylmethane-sulfonate	0	7.0×10^8	3.1	0.4	
	5 mM	5.2×10^8	68.3	13.1	32.8

[a] B. subtilis 168 wild type cells were used in all these experiments.

[b] Relative mutation frequency = $\frac{\text{\# mutations per } 10^8 \text{ cells no treatment}}{\text{\# mutations per } 10^8 \text{ cells with treatment}}$

are strong mutagens in this assay. Epichlorohydrin, a methylene homologue of chloroundrane, was also reactive. Styrene oxide showed very weak reactivity in initial tests and is being examined further in this system.

D. Bacillus Comptest

B. subtilis strain RUB827 (Table 1) was grown to competence using the procedure previously described (79), then exposed to DNA which had been previously isolated from strain RUB818 as described by Yasbin (78). After 25 min of incubation, followed by 5 min additional incubation with 100 ug/ml of deoxyribonuclease I, the competent culture was centrifuged (15,000g for 1 min) and resuspended in minimal salts for UV irradiation (76,77) or in GM2 (79) for treatment with chemical agents. The cells were exposed for 30 min to the chemical agents before they were plated on appropriate media. When needed, microsomal activation mixture was added to the competent cells and chemical agents according to the procedures of Ames, McCann, and Yamasaki (2). The total number of viable cells was determined by colony growth on minimal media with glucose, supplemented with all the amino acids (20 μg/ml) for which the bacteria are auxotrophic (79). The number of transformants were determined by colony growth on the same minimal media which was lacking either tryptophan or methionine.

Significantly, only 20% of a B. subtilis culture achieves the competent state (81). Therefore, in a "competent" culture of B. subtilis, 80% of the bacteria are noncompetent. The competent and noncompetent members of the culture can be separated by their growth on selective media following transformation. Thus, the number of noncompetent bacteria of strain RUB-827 can be determined on minimal media supplemented with both tryptophan and methionine. The competent cells can be calculated from the number of transformants (the number of colonies formed on minimal medium lacking either tryptophan or methionine) (52).

The precocious activation of the SOS system in competent B. subtilis makes these bacteria more sensitive to SOS-inducing factors than non-competent bacteria (Refs. 77 and 77a). This enhanced sensitivity is due to the fact that the resident prophages are induced at lower doses in the competent cells. In Figure 1 the data are presented for an experiment involving the UV irradiation of a competent culture of RUB827. The enhanced sensitivity of the competent cells was readily visible. This enhanced sensitivity can be expressed as the percent of transformation obtained after each fluence of radiation. If the sensitivity of the competent cells and the noncompetent cells to UV were identical, then the percentage of cells transformed should remain constant, independent of UV irradiation. However, if the competent cells are more UV-sensitive, then as the fluence increases,

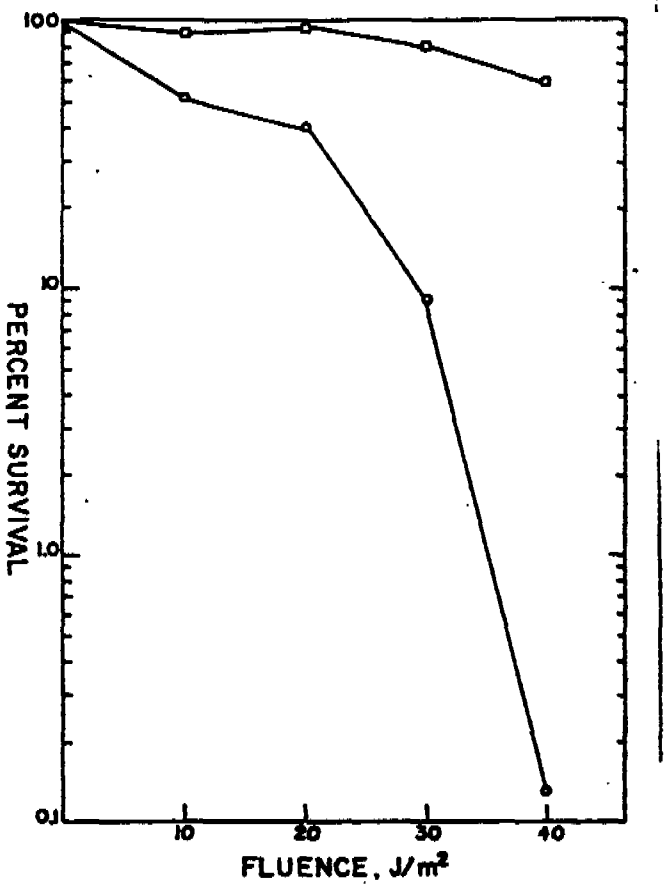

Figure 1. Survival of the total population of strain RUB827 (□) and of the competent portion of the culture (o) following UV irradiation.

the competent cells (as represented by the transformants) should be killed at a greater rate than the noncompetent cells. In Figure 2 the relative transformation efficiency is shown at the various UV fluences. As the fluence increased, the relative transformation efficiency decreased. The same types of results were obtained when the carcinogenic compound MMS was added to the competent cells (Figure 2). However, the mutagenic, but not carcinogenic, compound, EMS, did not produce a decreasing transformation efficiency with increasing dose (Figure 2). Thus, this assay appears to be highly specific for only those components which activate the SOS system. Chloroacetaldehyde, styrene oxide, and several other potential carcinogenic chemicals are being examined in this system at the present time.

Bacterial strain RUB827 was chosen for this assay because previous results (77) had shown that the polA5 mutation enhanced the sensitivity of this bacterial strain to SOS-inducing substances. Compounds are considered potentially carcinogenic when the relative transformation efficiency (the

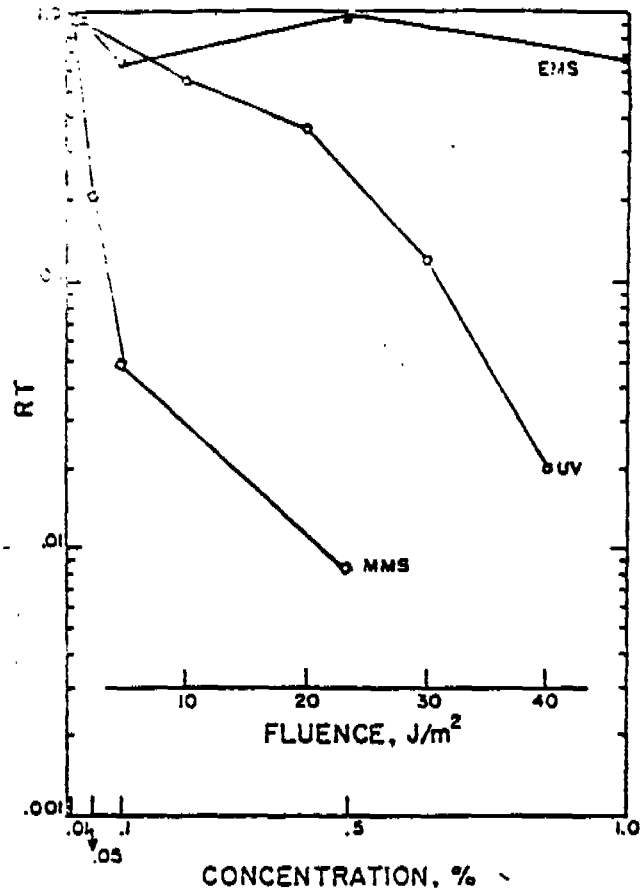

Figure 2. Relative transformation efficiency (RT) of strain RUB827 following exposure to various concentrations of MMS (□) and EMS (■) as well as following exposure to various fluences of UV irradiation (o). The RT is calculated by dividing the percent transformation at a given fluence or concentration by the percent transformation at 0 fluence or concentration.

percent transformation of the treated sample divided by the percent transformation of the untreated sample) is 0.05 or less.

This novel B. subtilis tester system measures the toxicity of agents as well as the ability of these same agents to induce the SOS system. Also, the Comptest identifies the concentrations at which the compounds have toxic and SOS-inducing capabilities. The main advantage of this assay is that it apparently distinguishes between substances which are mutagenic and those which activate SOS functions (potential carcinogenic compounds). This property of the Comptest can be noted readily when the relative activities of EMS and MMS are compared.

III. DISCUSSION

In this chapter we have described a battery of microbial (particularly B. subtilis) rapid tests for the assay of mutagenicity. The known mutagens EMS and MMS were used as standards in all these tests. In addition, we have also initiated a progressive study of industrial chemicals and their oxidation products using the described test systems. The initial results for chloroacetaldehyde and styrene oxide, the oxidative products of vinyl chloride and styrene, respectively, have been presented.

In all of the assays, except the Comptest, both EMS and MMS were highly reactive. It is interesting to note that EMS was not active in the Comptest, although MMS showed a high level of activity. This finding becomes highly significant when the carcinogenic properties of EMS and MMS are examined. EMS is not carcinogenic, whereas MMS is a potent carcinogen (17, 52). Thus, on the basis of these preliminary results, it seems promising that the Comptest may distinguish between carcinogens and noncarcinogenic mutagens. We are presently expanding this test to examine a whole battery of chemicals, including chloroacetaldehyde and styrene oxide, to verify this prediction. Since the Comptest specifically examines events associated with SOS induction, it may discriminate for chemicals which induce the error-prone repair systems. Such chemicals could include all the carcinogens. In this connection, our laboratories have also initiated the interferon induction inhibition (III) test (Ref. 3a). Interferon is an antiviral agent produced by mammalian cells and whole animals in response to viral infections. Preliminary studies in other laboratories have suggested that Newcastle disease virus (NDV) induced interferon production could be inhibited by pretreatment of cell cultures with known carcinogens such as benzo[α]pyrene (16). We have confirmed and extended these observations to include EMS, MMS, styrene oxide, and other known carcinogens. Our study (double-blind) has demonstrated that styrene oxide and MMS are active in this assay, but EMS shows no activity. This mammalian (including human cell) rapid assay may measure for SOS-like events in eucaryotic cells. Among these events could be the induction of protease, which we postulate could inhibit interferon induction in virus and polynucleotide-stimulated cells. Furthermore, several known carcinogens are active in this system, whereas noncarcinogenic analogs are inactive (Refs. 3, 3a, and 16).

An overall comparison of the various microbial tests performed in our laboratories is presented in Table 5. It is evident that a composite evaluation of many assays must be made when considering the biological activity of a chemical. This is shown by the variability in reactions of styrene oxide. It is interesting to note that this chemical is essentially nonreactive in the Bacillus tests performed so far, yet it is mutagenic in the Salmonella test and very active in the III assay. If this chemical is also inactive in the

Table 5. Composite Mutagenicity Spectrum of Chemical Monomers[a]

Chemicals	Salmonella	Comptest	Repair assay	Forward mutation
Chloroacetaldehyde	+	ND	+	+
Styrene oxide	+	ND	-	±
Methylmethanesulfonate	+	+	+	+
Ethylmethanesulfonate	+	-	+	+

[a]Results are expressed as (+) positive in the assay performed, (-) negative in the assay performed, and (ND) not determined. Styrene oxide was weakly reactive (Table 4) and must be considered to have borderline activity in the forward mutation assay (±).

Comptest assays, then it must be considered that styrene oxide may not enter Bacillus cells. Alternatively, styrene oxide may not elicit the types of repair we are examining in the B. subtilis system. An additional point must be made in regard to the repair assay. Two mutants with recD27 genotype gave dissimilar results when tested with reactive chemicals. Since they both were found to be Rec$^-$, we must assume that one or the other of these mutants does not have the recD27 genotype.

Chloroacetaldehyde has been highly reactive in all assays done to date and must be considered as the probable metabolite of vinyl chloride, responsible for the biological effects elicited by this industrial chemical. This conclusion has also been made by other laboratories testing vinyl chloride metabolites (4, 20).

In conclusion, we have described one rapid assay (Comptest) which may have the capability of detecting carcinogens. In addition, we are refining a rapid mammalian test (III), which also may identify carcinogenic chemicals. These two assays, along with the other tests described in this chapter, provide a battery of rapid procedures for screening a large number of chemicals for biological activity. As such, they are potentially valuable in our overall research programs dealing with environmental pollutants and industrial monomers.

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Selective Association of the Chromosome with Membrane in a Stable L-Form of *Bacillus subtilis*

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A stable L-form (Sal-1) of *Bacillus subtilis* was found to have retained a markedly modified chromosome-membrane association when compared to intact cells. The membrane-deoxyribonucleic acid complex of the L-form was similar to that of its parental strain in quantity and stability. Genetic analysis of the L-form membrane-deoxyribonucleic acid complex revealed enrichment for markers close to the replication origin, but not for internal markers, indicating preferential attachment of the origin of chromosomal replication to the membrane. These results are in close agreement with those found for the parental bacterial form. In contrast, the replication terminus region was not preferentially attached to the membrane of the L-form, even though it is enriched in the bacterial form. The association of the chromosome with the membrane at the replication terminus does not appear to be necessary for cell growth and separation, but because the L-form divides aberrantly, it may be one of the factors required for normal deoxyribonucleic acid segregation and septation.

Interaction between the chromosome and the cell surface has been well documented in procaryotes. A stable attachment of the chromosome to the cytoplasmic membrane has been demonstrated in gram-negative as well as gram-positive bacteria (15, 22). The DNA in the membrane-DNA complex was found to be specifically enriched for the replication point and for genetic markers close to the origin and terminus of chromosomal replication (10, 12, 19, 24, 27, 28, 34, 35). This attachment was proposed to aid in the segregation of the newly replicated chromosomes and in cell division (14). Further work with *Escherichia coli* (9), and in our laboratories with *Bacillus subtilis* (3, 26), has demonstrated a stable attachment of the chromosome to the (rigid) cell wall as well as to the cytoplasmic membrane. The DNA attached to the cell wall in *B. subtilis* was found to be enriched for genetic loci near the origin and terminus of chromosomal replication (26). The chromosome-membrane-cell wall complex has been proposed to be held together by specific binding proteins (5, 9, 11, 29, 30). This complex may create an apparatus functional in cell division processes.

Research with procaryotes lacking cell wall has demonstrated that the genome of *Mycoplasma gallisepticum* is also associated with the membrane, showing specific enrichment for the replication point and possibly the origin of chromosomal replication (20, 21). Electron micrographs have revealed the presence of chromosome-membrane association in protoplasts and unstable L-forms of *B. subtilis* (23). However, to date, genetic characterization of the chromosome-membrane complex in procaryotes without cell walls has been lacking.

In this report, we describe studies on the DNA-membrane association in a stable L-form of *B. subtilis*, Sal-1 (36). This organism requires 1.2 M NaCl for stabilization, does not have cell wall polymers, and does not revert to the bacterial form (6). Moreover, this L-form appears to have lost cell division control and undergoes aberrant cell division (R. W. Gilpin, personal communication), as do other stable L-forms (7, 33). Therefore, the L-form becomes a good model to test our hypothesis that loss of control in cell division may be due to the loss of one or more of the components in the cell division complex, such as the cell wall and proteins associated with the peptidoglycan. The present study offers the first evidence that a stable L-form retains attachment of the chromosome to the membrane. Furthermore, this attachment is specific only for the origin of chromosomal replication. The region for termination of genome replication appears to have no specific affinity for the surface of this L-form.

(This work is a part of the dissertation to be submitted by S. Horowitz to the Graduate Faculty of the University of Louisville in partial

fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used in this study are listed in Table 1. We thank H. Yoshikawa for strain TL8132, N. Sueoka for strain Mu8u5u16, J. Copeland for strain BC122, N. Harford for strains BD170, BD202, VUB45, and VUB41, S. Phillips for strain QB922, and J. Kane for strain BD82. All strains were maintained on tryptose blood agar base (TBAB) (Difco Laboratories, Detroit, Mich.). The stable L-form, Sal-1, kindly provided by R. W. Gilpin, is a derivative of *B. subtilis* BR151 (36) and retains the *metB10* marker (1). The L-form was maintained by passage in stabilizing liquid medium (below). The parental strain, *B. subtilis* BR151 *lys-3 trpC2 metB10*, was transformed to prototrophy for *lys-3* and *trpC2* with *B. subtilis* wild-type 168 DNA to maintain the same auxotrophic background as the L-form, and was designated as strain BUL404.

Media and growth conditions. The L-form, Sal-1, was grown in 15 ml of Sal-1 medium, a modified glucose minimal medium described by Spizizen (25) with 0.016% (wt/vol) $MgCl_2 \cdot 6H_2O$ (instead of 0.02% $MgSO_4 \cdot 7H_2O$–1.2 M NaCl) for stabilization, 0.05% casein hydrolysate, and 50 μg of L-methionine per ml. The culture was shaken in a rotary water bath (New Brunswick Scientific Co., New Brunswick, N.J.) at 37°C and 150 rpm. *B. subtilis* BUL404 was propagated in Sal-1 medium without NaCl, under the same growth conditions. Both cultures were grown for two or more generations in the exponential phase in the presence of deoxyadenosine (200 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and [2-^{14}C]thymidine (0.1 μCi/ml, 53 mCi/mmol; Schwarz/Mann, Orangeburg, N.Y.). Cell growth was monitored on a Klett-Summerson colorimeter with filter no. 54.

Lysis. The L-forms were harvested by centrifugation at 5,000 rpm for 5 min and then washed twice in cold NCP buffer (0.01 M sodium citrate, 0.08 M $K_2HPO_4 \cdot 3H_2O$, and 0.04 M KH_2PO_4, adjusted to pH 7.4 [12]), which contained 1.2 M NaCl. The cells were then suspended in a small volume of NCP plus 1.2 M NaCl buffer and lysed by osmotic shock due to the addition of NCP buffer without NaCl. The resulting lysate was a four- to eightfold concentration of the original culture, and contained a final concentration of 0.1 M NaCl. The *B. subtilis* BUL404 cells were harvested and washed as Sal-1, but in NCP buffer without NaCl, then concentrated four- to eightfold in NCP, and lysed by the addition of lysozyme (0.5 mg/ml; Worthington Biochemical Corp., Freehold, N.J.) for 5 min at 37°C. The lysate was then adjusted to 0.1 M NaCl. Both lysates were either left untreated, or were sheared for 30 s on a Vortex mixer (Fisher Scientific Co., Springfield, Mass.) set at position no. 6.

Renografin gradients. Linear (0 to 38%) Renografin density gradients (Renografin-76, from Squibb and Sons, Inc., Princeton, N.J., in NCP plus 0.1 M NaCl buffer) were prepared by the method of Ivarie and Pène (12). Samples (1.0 to 1.5 ml each) of the L-form or the parental strain lysates were layered onto 30-ml cold Renografin gradients, and the gradients were centrifuged in a Beckman L265B ultracentrifuge at 25,000 rpm, 2°C for 5.5 h, using an SW25.1 roter. After centrifugation, 1.2-ml fractions were collected into sterile tubes. Samples of these fractions (0.25 to 0.50 ml each) were counted in Multisol scintillation fluid (Isolab Inc., Akron, Ohio) in a liquid scintillation counter. The remaining volume of each of the peak fractions was maintained at 4°C for transformation experiments.

Transformation experiments. The procedures for the development of competence and transformation were those of Boylan et al. (2). Auxotrophic strains of *B. subtilis* (Table 1) were grown in GMI (2) at 37°C in a New Brunswick gyratory incubator shaker at 250 rpm. Cell growth was monitored on a Klett-Summerson colorimeter (filter no. 54) until the cessation of logarithmic growth. After 90 min of further

TABLE 1. *List of strains used*

Designation	Genotype	Derivation or source
Sal-1 (stable L-form)	*metB10*	R. W. Gilpin
BR7	*leuA8 trpC2*	B. E. Reilly
BR151	*trpC2 lys-3 metB10*	B. E. Reilly
RUB783	*leuA8 metB10 hisA1 purB6*	This laboratory
BUL201	*leuA8 purA16 hisA1*	This laboratory
BUL320	*leuA8 metB10 thr-5 hisA1*	BUL714 × BD170 *thr-5 trpC2*[a]
BUL401	*leuA8 gltA292*	QB922-*gltA292 trpC2* × TL8132-*leuA8 thyA thyB ts8132*[a]
BUL404	*metB10*	BR151 × W168[a]
BUL406	*metB10*	BR151 × Sal-1[a]
BUL502	*leuA8 thyA thyB*	168TT-*trpC2 thyA thyB* × Mu8u5u16-*purA16 leuA8 metB5*[a]
BUL705	*leuA8 metB10 lys-3*	BR151 × RUB783[a]
BUL709	*leuA8 metB10 hisA1 pyrA1*	RUB783 × VUB45-*hisA1 argC4 pyrA1*[a]
BUL714	*leuA8 metB10 cysA14 hisA1*	RUB783 × BD82-*cysA14*[a]
BUL717	*leuA8 purA16 metC3*	BUL201 × VUB41-*trpC2 metC3*[a]
BUL720	*leuA8 metB10 trpC2 purB6*	RUB783 × BD202-*trpC2*[a]
BUL726	*leuA8 citK5*	BC122-*trpC2 citK5* × Mu8u5u16[a]
BUL728	*leuA8 trpC2 lys-3*	BR151 × TL8132[a]

[a] Obtained by congression (17); general notation: recipient cell × donor DNA.

incubation, the cells were diluted tenfold into GMII (2) and incubated an additional 60 min, at which time the cultures were maximally competent. Peak fractions from the Renografin gradients served as donor DNA. These fractions were diluted in Spizizen's minimal salts solution to avoid saturation and to reduce the concentration of the Renografin (8). The competent cells were incubated with the DNA for 30 min at 37°C in the incubator shaker. Samples (0.1 ml each) were plated in triplicate on selective media and incubated at 37°C. Transformants were enumerated after 48 h of incubation. Each genetic marker was analyzed for enrichment from 3 to 16 times to attain statistical validity. In general, selection for transformants was done on glucose-minimal agar (25) supplemented with the appropriate amino acids and bases. Selection for the *citK5* transformants was on minimal agar plates which contained 0.1% sodium lactate (J. T. Baker Chemical Co., Phillipsburg, N.J.) instead of glucose. Selection for *thyA* transformants was by incubation at 46°C on plates without thymine (18). The *thyB* transformants were sensitive to growth at 46°C without thymine and were evaluated by subtracting the number of transformants (*thy*$^+$) at 46°C from those at 37°C (18). Selection for *gltA292* transformants was on glucose-minimal agar in the absence of 400 μg of glutamic acid per ml (4).

RESULTS

Distribution of labeled DNA on Renografin gradients. To determine the presence of a membrane-DNA (m-DNA) complex in the L-form, a culture of Sal-1 was grown in Sal-1 medium, and its DNA was uniformly labeled with [2-^{14}C]thymidine. The doubling time of the L-forms, as determined by Klett absorbance units, was approximately 2 h under these growth conditions. The L-forms were washed and then lysed by osmotic shock. For comparison, the parental strain BUL404 was also grown and labeled under similar conditions, having a doubling time of 40 min. The parental strain was also washed and then lysed by exposure to lysozyme (0.5 mg/ml) in hypotonic solution. A sample (1 ml) of each lysate was immediately layered onto a 0 to 38% linear Renografin density gradient. The remainder of each lysate was sheared by blending in a Vortex mixer for 30 s, and then 1.0- to 1.5-ml samples were applied onto Renografin gradients. The gradients were centrifuged and collected, and samples were assayed for radioactivity (Fig. 1). In the absence of shearing, the L-form DNA formed a distinct peak at the lower part of the gradient (Fig. 1A). The peak corresponds to the membrane-attached DNA (m-DNA) peak that was found in the unsheared lysate of the parental strain BUL404 (Fig. 1a). When sheared, part of the L-form DNA formed a slower-sedimenting peak (Fig. 1B) which corresponds to the free DNA (f-

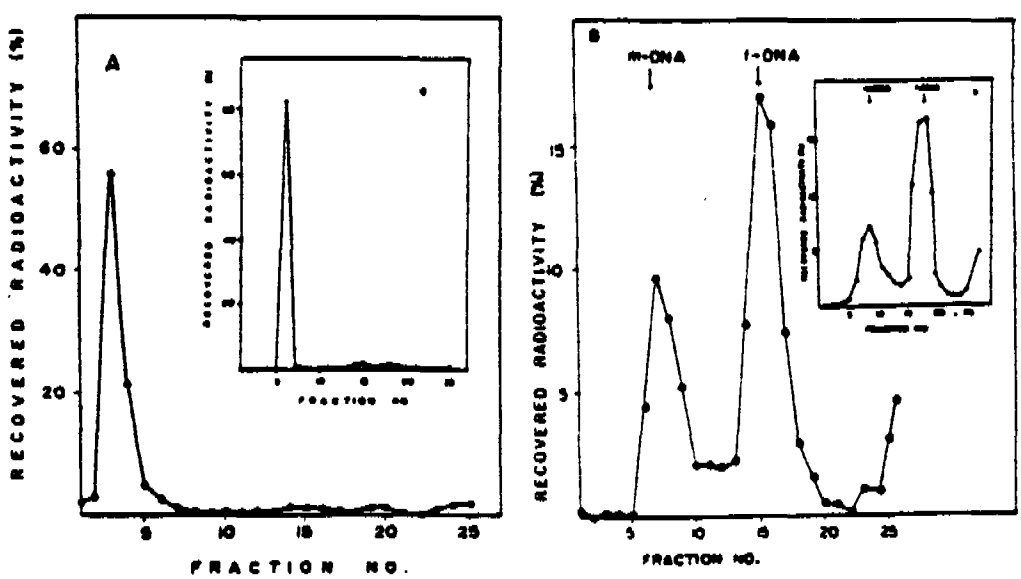

FIG. 1. *Distribution of the labeled DNA from the L-form, Sal-1, on Renografin gradients. The L-forms were grown, labeled, washed, and lysed as described. Unsheared (A) or sheared (B) lysates were sedimented on 0 to 38% Renografin gradients. Fractions were collected and counted for recovered radioactivity. In all samples, 90 to 100% of the input radioactivity was recovered. For comparison, the parental strain, B. subtilis BUL404, was grown, labeled, and washed under similar conditions, lysed by exposure to lysozyme (0.5 mg/ml) in hypotonic solution, and the unsheared (a) or sheared (b) lysates were sedimented on Renografin gradients as above. Sheared lysates demonstrate membrane-attached (m-DNA) and free (f-DNA) nucleic acid.*

DNA) peak of the parental strain (Fig. 1b). However, a significant proportion of the labeled DNA of the L-form was retained in the m-DNA peak as firmly attached DNA (Fig. 1B), as in the parental bacterial strain (Fig. 1b). The pattern of DNA distribution for the L-form, as well as the parental bacterial form, in unsheared and sheared lysates is in close agreement with that described by Ivarie and Pène for *B. subtilis* 168T⁻, using Renografin gradients (12). To compare the distribution of the L-form labeled DNA in the Renografin gradients to that of its parental strain, the results of several experiments were pooled, and the average percentage of DNA in each peak (± the standard error of the mean) was calculated. Approximately 80% of the labeled DNA in the unsheared L-form lysate is attached to the membrane (m-DNA) (Table 2). Upon shearing for 30 s, approximately 30% of the DNA remained firmly attached to the membrane (m-DNA), whereas about 55% of the DNA appears as free DNA (f-DNA). These results are statistically not different from those found in the unsheared and sheared lysates of the parental strain BUL404 as determined by an independent *t* test (Table 2). This observation suggests that the L-form has retained an m-DNA complex physically similar to that of the bacterial form.

To compare the stability of the m-DNA complex of the L-form to that of its parental strain, both lysates were subjected to different shearing times, and the resulting DNA distribution on Renografin gradients was observed. There was no significant decrease in the percentage of m-DNA for both the L-form and the parental strain when sheared for times ranging from 15 s up to 60 s (S. Horowitz, Ph.D. thesis, University of Louisville, Louisville, Ky., 1979). We, therefore, routinely used a shearing time of 30 s for the experiments described below.

Genetic analysis of membrane-attached DNA isolated from the L-form and its parental strain. The DNA bound to the membrane was used in transformation assays to determine the genetic composition of the m-DNA complex from the L-form and compare it to the profile found in membrane-associated DNA from the parental strain BUL404. Different chromosomal markers, chosen to provide an analysis of the entire chromosome, were examined, and the membrane enrichment indexes (MEI) were calculated by the method of Sueoka and Quinn (27). The marker *leuA8* was used as a standard in these calculations. The average MEI values for each marker tested (± the standard error of the mean) are presented in Table 3. For statistical analysis, a two-factor analysis of variance with repeated measures on one factor was run. The between factor was the 13 genes we had examined. The within factor was the parental (BUL404) *versus* L-form (Sal-1) condition. The Duncan range test was used for post hoc comparisons to find markers among which a significant difference occurred (31). The genetic and statistical analyses revealed that the DNA attached to the membrane in the L-form was enriched for the markers *purA16* and *cysA14*, which are close to the origin of chromosomal replication (16, 37), but not for the internal genes *hisA1*, *purB6*, *thr-5*, *leuA8* (standard), *metC3*, *pyrA1*, *lys-3*, and *thyA* (Table 3). These results showed preferential attachment of the origin of chromosomal replication to the membrane in the L-form. In this regard, the L-form m-DNA complex was not statistically dif-

TABLE 2. *Effect of shearing on m-DNA complexes from the L-form, Sal-1, and its parental strain, B. subtilis BUL404*[a]

Strain	Treatment of lysate	m-DNA (%)	f-DNA (%)
Sal-1	No shearing	81.33 ± 2.76	12.58 ± 2.77
	Sheared[b]	31.92 ± 1.01	56.09 ± 1.06
BUL404	No shearing	82.00 ± 1.28	12.41 ± 0.83
	Sheared[b]	34.08 ± 2.56	54.31 ± 2.08

[a] The procedures for the separation of m-DNA and f-DNA and the assays for radioactivity are as described in the legend to Fig. 1.

[b] The lysates were sheared for 30 s on a Vortex mixer set at position 6.

TABLE 3. *Genetic analysis of m-DNA isolated from the L-form, Sal 1, and its parental strain, B. subtilis BUL404*

Genetic marker examined[a]	Sal-1 MEI[b]	BUL404 MEI[b]
purA16	1.74 ± 0.11	1.55 ± 0.19
cysA14	1.78 ± 0.17	1.57 ± 0.23
hisA1	0.98 ± 0.13	0.90 ± 0.12
purB6	1.10 ± 0.12	1.06 ± 0.14
thr-5	1.17 ± 0.11	1.11 ± 0.13
leuA8	1.00	1.00
metC3	0.81 ± 0.10	0.92 ± 0.11
pyrA1	1.05 ± 0.10	1.06 ± 0.14
lys-3	1.17 ± 0.19	1.10 ± 0.21
thyA	1.07 ± 0.02	1.40 ± 0.17
trpC2	0.98 ± 0.06	1.57 ± 0.11
gltA292	1.16 ± 0.09	1.80 ± 0.18
thyB	0.82 ± 0.09	1.90 ± 0.48
citK5	0.96 ± 0.07	2.20 ± 0.24

[a] Recipient strains used in these experiments were BUL201, 320, 401, 502, 705, 709, 714, 717, 720, 726, 728, RUB783, and BR7.

[b] Calculated by the method of Sueoka and Quinn (27). MEI = [(*x*/*leuA8*) in m-DNA]/[(*x*/*leuA8*) in f-DNA], where *x* is the number of transformants for any marker tested, and *leuA8* is the number of transformants for the standard marker *leuA8*.

[c] $P < 0.01$ when compared to BUL404, by analysis of variance.

ferent from the m-DNA complex of the parental strain BUL404 (Table 3). In contrast, the replication terminus region of the L-form was not found to be preferentially attached to the membrane. The terminus markers *trpC2*, *gltA292*, *thyB*, and *citK5* were enriched in the m-DNA complex of the bacterial form, BUL404, but were not enriched in the L-form (Table 3). The MEI of the genetic markers tested in the m-DNA complex of the L-form and its parental strain are presented in relation to their relative positions in the bidirectional replication map of *B. subtilis* in Fig. 2. As a control, incubation of the parental lysate in the presence of 1.2 M NaCl did not bring about loss of the specific membrane attachment of genetic markers close to either the replication origin or the terminus.

Genetic analysis of m-DNA isolated from the hybrid BUL405. Since the L-form appeared to have lost the specific attachment of the terminus to the membrane, it was important to examine whether a chromosomal fragment from the terminus region of the L-form would be able to regain the specific attachment when placed in the parental, bacterial form. Therefore, we constructed a hybrid, BUL405, which is a derivative of the parental strain BR151. The hybrid contains the internal marker *lys-3* and the terminus marker *trpC2* from the L-form (Table 1). Strain BUL405 was grown, labeled, washed, lysed, and sheared as described above for the parental strain BUL404. Both m-DNA and f-DNA were isolated on Renografin gradients, and genetic analysis was performed. The internal marker, *lys-3*, of the hybrid, BUL405, was not enriched in the membrane and had an MEI value not statistically different from that of the parental strain, BUL404, and the L-form, Sal-1 (Table 4). The terminus marker, *trpC2*, was enriched in the membrane of the hybrid, BUL405, as it was in the parental strain. This enrichment, indicated by the MEI values, was significantly different from that found for the L-form *trpC2* gene which was not enriched in the membrane.

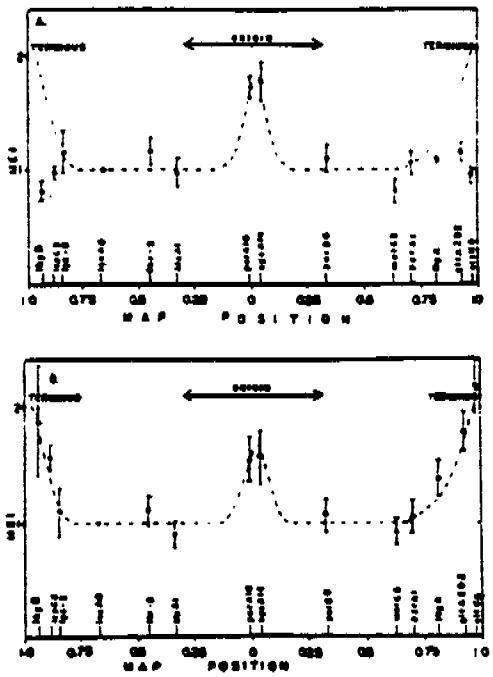

FIG. 2. *MEI for the genetic markers examined from m-DNA of the L-form, Sal-1, and its parental strain, B. subtilis BUL404, correlated to the map position of these markers in B. subtilis 168. The map positions for the different genes were calculated from the bidirectional replication map of B. subtilis 168 of Lepesant-Kejzlarova et al. (16) and Young and Wilson (37). The MEI values (± standard error of the mean) are taken from Table 3. (A) m-DNA from the L-form, Sal-1; (B) m-DNA from the parental strain, BUL404. The dotted line represents the areas of expected membrane enrichment of genetic markers, based on our results and those in the literature (10, 35).*

DISCUSSION

This study demonstrates that Sal-1, a stable L-form of *Bacillus subtilis* BR151, has retained an attachment of the chromosome to the membrane. In Renografin gradients, this attachment was similar in sedimentation, quantity, and stability to that of the parental bacterial form in both sheared and unsheared lysates. Furthermore, these results were in close agreement with the attachment found in another strain of *B. subtilis* (12). Our findings represent the first description of an m-DNA complex in a stable L-form, and it appears that the permanent loss of cell wall polymers did not prevent chromosome-membrane association. Such an attachment may be a universal feature of all procaryotes. Several laboratories have described both specific and

TABLE 4. *Genetic analysis of m-DNA isolated from B. subtilis BUL405*

Genetic marker examined[a]	BUL405 MEI	BUL404 MEI[b]	Sal-1 MEI[b]
lys-3	1.11 ± 0.08	1.10 ± 0.21	1.17 ± 0.19
trpC2	1.44 ± 0.15[c]	1.57 ± 0.11[c]	0.98 ± 0.05

[a] Recipient strains used in these experiments were BUL706, BUL728, and BR7.

[b] The MEI values were taken from Table 3 for comparison.

[c] $P < 0.01$ when compared to the MEI of the *trpC2* marker in Sal-1, using an independent *t* test.

nonspecific attachment of the chromosome to the membrane (10, 12, 13, 24, 27, 28, 34, 35). To determine whether the m-DNA complex from the L-form contained specific regions of the chromosome, we used samples of peak fractions from the Renografin gradients in transformation assays and calculated membrane enrichment indexes (MEI) (Table 3). Such genetic analysis revealed that the origin of chromosomal replication was preferentially attached to the membrane in the L-form, as in the parental bacterial form. In contrast, the specificity for the attachment of the terminus region had been lost in the L-form. This finding indicates that the two sites of attachment may be chemically and physically independent and may involve different binding proteins, as has been suggested previously (29, 34). The persistent binding of the origin region also suggests that this specific attachment to the membrane is of critical importance. The proportion of m-DNA (32%) found in the L-form, despite the loss of terminus attachment, could be explained by more extensive association of the origin, when compared to the parental form. Alternatively, radioactive counts due to nonspecific attachments (13) may mask the loss of radioactivity from terminus detachment.

Since the L-form, Sal-1, is known to divide aberrantly (R. W. Gilpin, personal communication), the loss of specific attachment of the terminus region to the membrane may be a contributing factor to the loss of division control in this organism. The integrity of the DNA-membrane-protein-cell wall complex was proposed to be crucial in maintaining normal cell division (26). Moreover, dynamic terminus attachment to the membrane in correlation with the cell cycle has been proposed to play an important role in cell division control in *B. subtilis* (32). All these factors lead to the speculation that loss of terminus attachment may be critical in the loss of normal DNA segregation and cell division processes.

Several possible factors could have been responsible for the loss of specific terminus attachment to the membrane in the L-form. The most probable are: a change in the affinity of the DNA in the replication terminus region of the chromosome for the membrane or for specific binding proteins; the permanent loss of cell-wall along with a disappearance of an outer surface site for terminus attachment (26); a loss or alteration of binding proteins which may aid in the specific attachment of the terminus to the membrane or cell wall (5, 9, 11, 29, 30); and, finally, the presence of a high concentration of salt (1.2 M NaCl) over many generations in the growth medium of the L-form, which might affect the terminus-membrane attachment, but not the origin-membrane association.

Results from genetic analysis of the hybrid BUL405 (Table 4) appeared to negate the first possibility and indicated that no change in the affinity of the L-form terminus region DNA had occurred, because the *trpC2* locus from the L-form was membrane-enriched when integrated into the parental strain. Nevertheless, this argument cannot be fully ruled out, since it is possible that only a very small fragment of the L-form *trpC2* region entered the BR151 strain—enough to make it tryptophan-independent, but not enough to make a significant change in the affinity of the terminus for the surface. It is also possible that the specificity of terminus attachment is determined by discrete sequences within the terminus region of the chromosome rather than by the whole region itself (35). The *trpC2* fragment, which originated from the L-form, although found in membrane preparations, may not be one of such specific binding sequences.

Partial loss of the specificity of DNA-membrane attachment in the L-form could also be due to the inactivation of binding proteins from the membrane and/or the cell wall in the chromosome-surface complex. Such proteins could have been lost or altered as a consequence of the removal of the cell wall, or during continued growth in the presence of high salt. Alternatively, it is possible that a mutation occurred in one or more of these proteins, because the L-form has undergone silent and apparent mutations since it was isolated (1). Furthermore, the profile of the membrane-protein composition (6) and the membrane-lipid composition in the L-form (S. Horowitz, Ph.D. thesis) was significantly different from that of the parental bacterial form when both were cultured under the same growth conditions.

In addition to the foregoing studies on the DNA-membrane complex from the L-form, we also have generated information which better defines the extent of the specific membrane attachment of the chromosome in *B. subtilis*. The specific attachment of the origin, as examined in transformation experiments by use of the markers *purA16* and *cysA14*, statistically confirmed that the *cysA14* gene was part of the origin-membrane complex (28), as is the *purA16* marker. In the terminus region, the *thyA* locus had an MEI value of 1.40 which appeared to indicate that it was membrane enriched, as noted by Yamaguchi and Yoshikawa (35). However, it did not pass statistical analysis, and therefore must be considered as an internal marker. Thus, it appears that the *thyA* locus may be the most proximal marker to the termi-

this region on the "right" side of the chromosome [illegible] and 0.7 min of the left side of the *B. subtilis* chromosome). The *trpC2* gene, previously considered to be an internal marker (12, 28), was significantly enriched in the membrane, and may be the beginning of the terminus region on the other side of the bidirectionally replicated chromosome. The *lys-3* marker, which is near to *trpC2*, was not membrane-enriched in any experiments. The observation of Neuhard et al. (18), describing the temperature sensitivity of thymidylate synthetase B in *B. subtilis*, enabled us to screen separately for *thyA* and *thyB* markers, thus adding the *thyB* gene to the list of terminus loci enriched in the membrane. The MEI of the different terminus markers were increased as the distance towards the terminus of replication was decreased, suggesting stronger chromosomal attachment near the terminus point. The *hisA1* and *purB6* loci which have appeared to be somewhat enriched in some of the previous reports (10, 24, 27, 28) were not found to be membrane enriched in our study, and belong to the internal region of chromosomal replication.

The attachment of the chromosome to the membrane, as demonstrated in the present work with strain BUL404, seems to be less extensive and less specific (lower enrichment values) than the attachment of the genome to the bacterial cell wall as reported by Streips et al. (26). These findings could indicate basic differences between the in vivo chromosome-surface complex (including cell wall) in bacteria and the complex isolated after cell wall removal.

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Genetic Analysis of DNA-Surface Interactions in *Bacillus subtilis*

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In a paper which has provided the foundation for many productive experiments in molecular biology and bacterial cell division, Jacob, Brenner, and Cuzin (11) proposed the "replicon" model to explain some of the mechanisms of DNA initiation and replication. One major concept of their proposal was that portions of the cellular genome were attached to the cytoplasmic membrane. That assumption made it possible to account for proper segregation of the genome into daughter cells concomitant with cell growth.

Subsequently, research in several laboratories demonstrated that the chromosome of various bacteria was indeed attached to the surface. Moreover, the attachment was at the origin and terminus of replication and at the replication point, as well as at several nonspecific points along the chromosome (4, 5, 9, 13, 17, 18). The specific association of the chromosome at sites involved in DNA replication reinforces the concept of a causal relationship among DNA-surface attachment, cell division, and chromosome segregation. To study this relationship further, we have investigated DNA-surface attachments in *Bacillus subtilis* under conditions in which normal cell division and DNA segregation are disturbed.

MEMBRANE-DNA COMPLEX IN A STABLE L-FORM

A stable L-form from *B. subtilis*, *sal-1*, has been propagated in liquid media since 1969 (19). The L-form grows in the absence of any cell wall and is stabilized by 1.2 M NaCl. *sal-1* and its salt-independent derivative, *sig-1*, have been shown to divide aberrantly, producing progeny with altered cytoplasm and DNA contents (6, 7). In this respect, these two L-forms are similar to other L-forms isolated from *B. subtilis* (14). We have used *sal-1* as a model for an organism which has lost proper cell division and DNA segregation control.

To determine whether the DNA-membrane attachment remained intact in these aberrantly dividing cells, we isolated DNA-membrane complexes by use of Renografin gradients (9). The complexes were then assayed for DNA content by transformation, and membrane enrichment indices were calculated (9). As shown in Fig. 1, when compared to spheroplasts (panel A), the L-form (panel B) retained enrichment for genetic markers close to the origin of replication, *purA16* and *cysA14*. Several internal markers were not enriched in either sample. However, selective enrichment for genes close to the terminus of replication, such as *trpC2*, *gltA292*, *citK5*, and *thyB*, was found to be lost in the L-form but not in spheroplasts. It is attractive to speculate that this loss of replication terminus attachment contributes to the loss of division control in the L-form.

There are several factors which could have contributed to the loss of replication terminus binding (9). Among the most probable alternatives are the absence of a cell wall or the presence of high salt (1.2 M NaCl) in the growth medium. If the loss of cell wall contributed to the dissociation of the terminus of replication from the surface, then it follows that the cell wall contributes to the makeup of the normal DNA-surface complex.

CELL WALL-DNA COMPLEXES

To examine the possibility that the cell wall is involved in attaching the chromosome to the surface of *B. subtilis*, we isolated cell walls at various stages of growth and examined the preparations for transforming activity (1, 15; R. J. Doyle et al., submitted for publication). Not only did isolated cell walls possess transforming activity, but the transformation was specifically enhanced for genetic markers around the replication origin and terminus (Fig. 1C). The extent of enrichment in the cell wall differs from that observed with membrane-DNA complexes. Thus, *hisA1*, *pyrA1*, and *sacA* were enriched in the cell wall samples, whereas the same markers were not enriched in membrane-DNA complexes (9, 15, 18). In contrast, *cysA14*, a marker which was shown to be membrane-enriched, was not found to be specifically attached to the cell wall (9, 15, 16). The significance of these differences is not obvious at the present time. There is an overall symmetry ap-

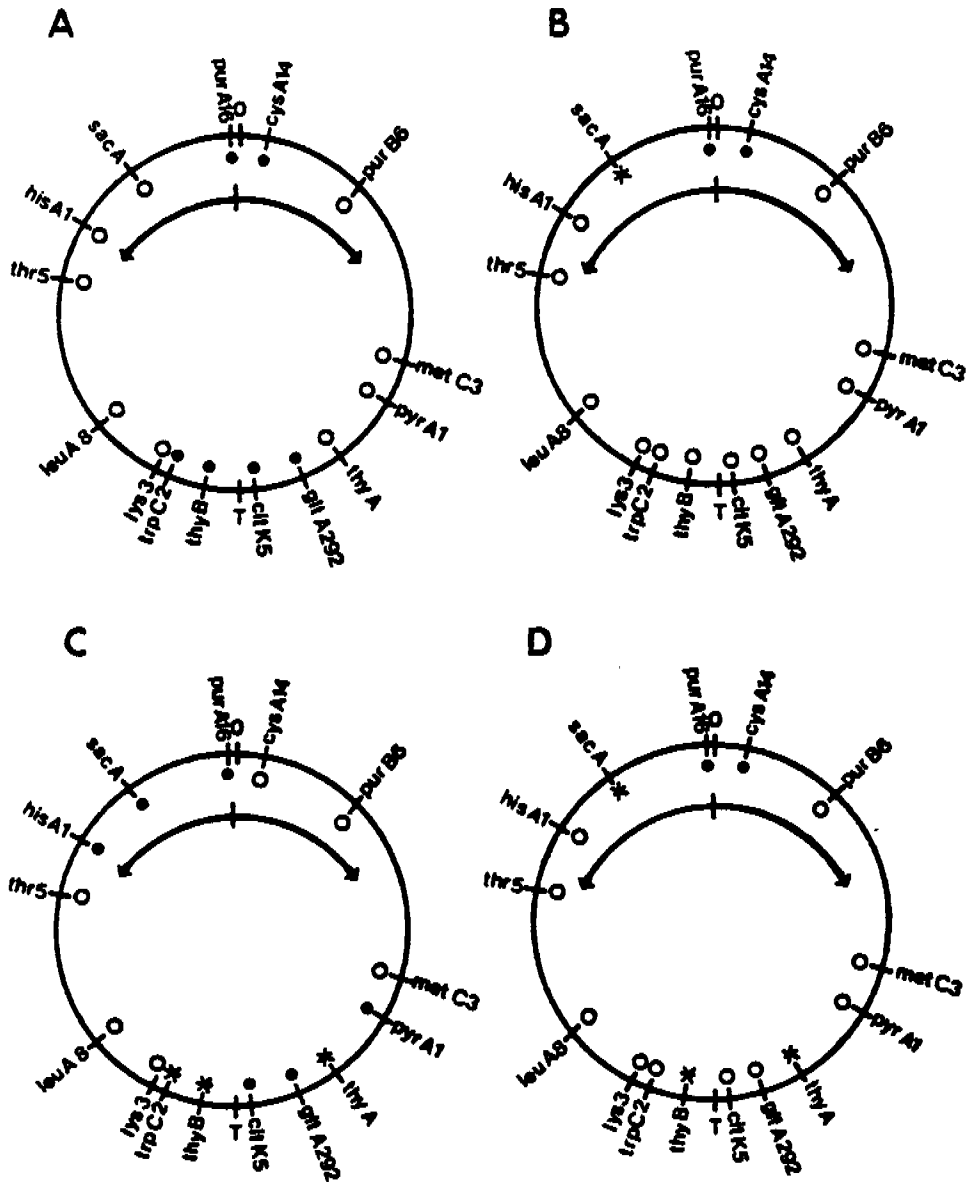

FIG. 1. *Genetic analysis of surface-DNA complexes in* Bacillus subtilis. *Genetic markers on the chromosome of* B. subtilis *and its L-form,* sal-1, *were examined for enrichment in membrane and wall preparations (9, 15). Represented are the enrichment maps for spheroplast membrane-DNA (A), L-form (sal-1) membrane-DNA (B), cell wall-associated DNA (C), and membrane-DNA from 1.2 M NaCl-treated cells (D). The genetic map of* B. subtilis *is that presented by Young and Wilson (20). Each marker is designated as enriched (●), nonenriched (○), or not examined (*). Replication origin (O), replication terminus (T).*

parent in the cell wall-DNA profile (Fig. 1C), which is not present in the membrane-DNA preparations (Fig. 1A and B). This loss of symmetry could be due to changes in the topography of the in vivo DNA-surface complex after removal of the cell wall. Along with these alterations it is possible that prolonged growth in the absence of a cell wall could also result in

the dissociation of the terminus from the membrane

EFFECT OF SALT ON MEMBRANE-DNA COMPLEXES

An alternate possibility for the loss of attachment of the replication terminus by the L-form is the presence of 1.2 M NaCl in the growth medium of this organism. A high concentration of salt may destroy the integrity of the surface-DNA complex. The effect of salt on morphology has been documented (3, 12).

We grew *B. subtilis* BUL 404 (*metB10*) in 1.2 M NaCl and isolated membrane-DNA complexes at various intervals. In Fig. 1D, we show the membrane-DNA profile from cells grown in 1.2 M NaCl for 120 min. It is obvious that growth in salt dissociated the replication terminus from the membrane in the bacterial strain. Recent experiments suggest that replication terminus dissociation occurs within the first 30 min after addition of 1.2 M NaCl to the culture growth medium. Moreover, after 120 min of growth in 1.2 M NaCl, the entire population of cells has assumed abnormal morphological characteristics, suggestive of changes in cell division control. Thus, we have been able to emulate the L-form state of replication terminus detachment and induce abnormal cell division by growing *B. subtilis* cells in high salt. Presumably, plasmolysis has occurred in these cells. Therefore, it is attractive to postulate that the replication terminus site may be labile to plasmolytic detachment of the membrane from the wall. Such removal could destroy the integrity of this surface-DNA site. In contrast, the replication origin-surface complex appears to be inviolate to the effects of high salt concentrations. Origin of replication binding has also been maintained in the L-form. This suggests that the replication origin-surface complex may be of primary and vital importance for cell survival.

DISCUSSION

On the basis of the foregoing series of experiments, several conclusions can be drawn concerning the surface-DNA complex in *B. subtilis*. First, the in vivo complex contains not only the chromosome and membrane, but also peptidoglycan. Second, of all the binding sites, the attachment of the origin of replication is of primary importance. This attachment is maintained in all samples assayed to date. Third, the replication terminus attachment appears to be labile to high salt and perhaps prolonged growth in the absence of the cell wall. Thus, the L-form and cells grown in 1.2 M NaCl have lost preferential attachment at the terminus of replication. Terminus attachment and normal morphology can be restored after removal of the 1.2 M NaCl (S. Horowitz et al., submitted for publication). Finally, once the attachment in the terminus region is lost, cells are observed to have altered morphology and division patterns.

These data suggest that the DNA-surface complex is a vital structure for the procaryotic cell and has a dynamic role in the maintenance of the cell cycle. The complex may regulate not only DNA replication but also DNA segregation and cell division events. If the complex is disturbed, then aberrancies in cell division processes occur. It is possible that information for the proper maintenance of the complex and its functions resides in the proteins found in both the cell wall and cell membrane (2, 8, 10). Such interactions, however, remain uncharacterized at the present time. The ability to manipulate the constitution of the DNA-surface complex and the morphology of cells leads to the possibility that the individual functional components may be isolated and studied.

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RESTRICTED CHROMOSOME-MEMBRANE ASSOCIATION IN A STABLE L-FORM OF BACILLUS SUBTILIS.

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ABSTRACT

A stable L-form (sal-1) of Bacillus subtilis BR151 was found to retain a chromosome-membrane association. The attachment of the chromosome to the membrane in the L-form is specific for the replication origin but not for the replication terminus. These results are in contrast to those obtained with the parental, cell-walled strain in which both the origin and terminus of chromosomal replication are preferentially attached to the membrane.

INTRODUCTION

Attachment of the chromosome to the cytoplasmic membrane and to the cell wall has been well demonstrated in cell-walled prokaryotes and is postulated to be instrumental in DNA segregation and division control [1,2,3,4]. This attachment is specific for the origin and terminus of chromosomal replication and for the replication point [1,4,5]. In Mycoplasma the chromosome was also found to be attached to the membrane [1,6]. Studies of unstable L-forms, utilizing electron microscopy, have also revealed chromosome-membrane association [7]. Yet, the genetic specificity of the chromosomal attachment in cell wall-deficient prokaryotes has not been studied. In this report, we demonstrate that a stable L-form (sal-1) of B. subtilis BR151, which has no cell wall polymers and divides aberrantly [8,9, R.W. Gilpin, personal communication], retains a chromosome-membrane association. The attachment is preferential for the region at the origin of chromosomal replication, but the specific attachment of the region close to the replication terminus has been lost.

MATERIALS AND METHODS

Bacterial strains. The stable L-form (sal-1) of B. subtilis BR151 [8,9] was kindly provided by R. W. Gilpin and retains the metB10 marker. The parental strain, B. subtilis BR151 lys-3, trpC2, metB10, was transformed to prototrophy for

lys-3 and trpC2 with B. subtilis W168 DNA to maintain the same auxotrophic background as the L-form. All strains used as recipients in the transformation experiments are listed elsewhere [10].

Media and growth conditions. The L-form (sal-1) was grown in sal-1 medium [10] which contains 1.2M NaCl for stabilization, according to a modification of the technique described by Gilpin et al [9], in the presence of deoxyadenosine (200 µg/ml) and ^{14}C-thymidine (0.1 µCi/ml, 53 mCi/mmole) for about two generations. The culture was washed in NCP buffer [11] plus 1.2M NaCl and lysed in NCP buffer by osmotic shock. The parental strain, B. subtilis BR151 metB10, was grown and labeled under the same conditions as the L-form but without the NaCl, washed in NCP buffer, and lysed by addition of lysozyme (0.5 mg/ml).

Renografin gradients. Lysates of the L-form and its parental strain were applied onto 0-38% linear Renografin density gradients [11] and were centrifuged, fractionated and counted according to a modification of the technique described by Ivarie and Péne [11].

Transformation procedures. The procedures for the development of competence and transformation were according to those described by Boylan et al [12]. Dilutions of the peak fractions from the Renografin gradients served as donor DNA.

RESULTS

Attachment of the chromosome to the membrane. Uniformly labeled DNA from an unsheared lysate of the L-form sal-1 (Materials and Methods) forms one distinct band at the lower part of the Renografin gradient. This band corresponds to the membrane-DNA (mDNA) peak which is found in the untreated lysate of the parental strain B. subtilis BR151 metB10 (results not shown). The DNA attached to the membrane of both the L-form lysate and the lysate from the parental strain contains about 80% of the label incorporated. Shearing the L-form lysate by vortexing for 30 seconds at setting no. 6 causes a decrease in the amount of labeled DNA which appears in the m-DNA peak and generates a slower sedimenting peak which bands at the upper part of the Renografin gradient. This new peak corresponds to the free DNA (f-DNA) peak of the parental strain (results not shown). The distribution of the uniformly labeled DNA from the L-form sheared lysate consists of about 55% label which appears as free DNA and about 30% which appears as firmly attached membrane associated DNA. This distribution is similar to that of the uniformly labeled DNA from the sheared

lysate of the parental strain [10].

Genetic analysis of the specificity of chromosome attachment. Since the DNA isolated in the Renografin gradients can be used in transformation assays, genetic analysis for the specificity of DNA attached to the membrane was performed, using peak fractions from the sheared lysate of the L-form and the parental strain as donor DNA. Genetic markers were tested throughout the chromosome and included markers at the origin and terminus regions of chromosomal replication as well as internal loci. The specificity of the attachment was determined by the membrane enrichment index (MEI), calculated according to Sueoka and Quinn [2] using leuA8 as a standard. Several transformation experiments were performed for each marker with both the L-form and the parental strain DNA peak samples, and the average MEI was calculated. An MEI value which equals or exceeds 1.40 was taken to indicate that the marker is enriched in the DNA attached to the membrane, hence preferentially bound. An MEI value close to 1.00 was accepted to show no specificity for the attachment to the membrane. The results from the transformation experiments are shown in Figure 1. None of the internal markers that we have examined has any specificity for association with the membrane in both the L-form and the parental strain. The origin region of chromosomal replication (purA16 and cysA14) in the L-form is preferentially attached to the membrane as is the case in the parental strain. However, the region close to the replication terminus (trpC2, thyB, citK5, gltA292, and thyA) does not appear to be attached to the membrane of the L-form although it is preferentially attached to the membrane in the parental strain.

DISCUSSION

This study demonstrates that the L-form, sal-1, retains a membrane-chromosome association in both sheared and unsheared lysates. This attachment is quantitatively similar to that of the parental strain and may indicate the importance of maintaining such an association for normal cell growth.

Genetic analysis for the specificity of the membrane-chromosome association in the L-form shows that the preferential attachment of the origin of chromosomal replication is retained. However, specificity for the attachment of the terminus region has been lost, when compared to the parental strain. This finding suggests that the two sites of attachment are different, and that the attachment of the origin is of primary importance. The loss of association of the terminus to the membrane in the L-form could be the

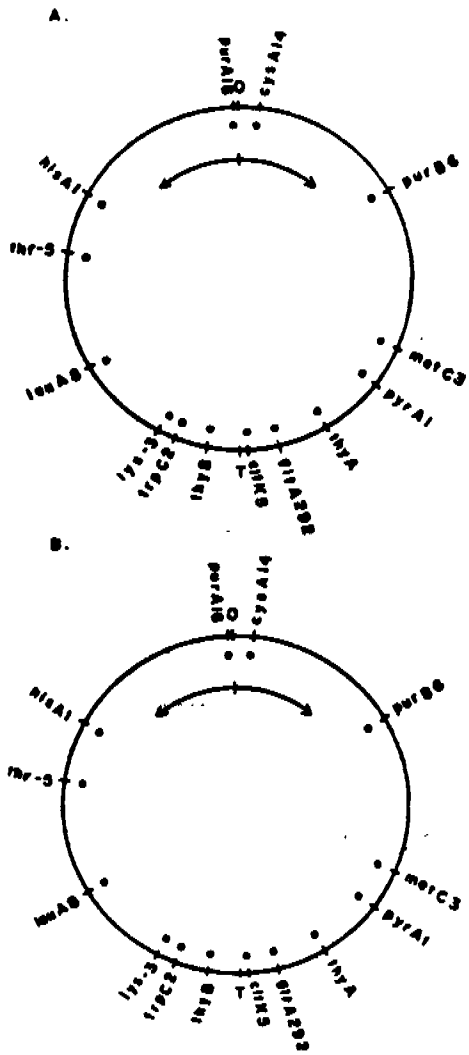

Figure 1. Genetic analysis of m-DNA from the L-form and its parental strain. Genetic markers along the entire chromosome of both the L-form and its parental strain were examined for specific enrichment in membrane samples (MEI) by transformation (Materials and Methods). Represented is the genetic map of the L-form sal-1 (A) and of the parental strain BR151 metB10 (B). The genetic map of Bacillus subtilis was constructed from those of Lepesant-Kejzlarova et al [13] and Young and Wilson [14]. Each marker is designated as enriched (●) or non-enriched (○) in m-DNA samples. Replication origin (O); Replication terminus (T).

result of one or more of the following physiological and/or genetic alterations. First of all, the loss of the cell wall may have removed an outer surface site for terminus attachment [15,16]. Secondly, a loss or alteration of a binding protein(s) which aids in the specific attachment of the chromosome to the membrane (or cell wall) could be reflected in the loss of association of the terminus region. Also, a temperate bacteriophage which inserts in this area of the chromosome [17] could participate in the binding of the genome. The L-form could have lost such a bacteriophage. Finally, the presence of a high concentration of salt (1.2M NaCl) throughout the growth cycle of the L-form could be responsible for the dissociation of specific, bound regions of the chromosome [3,9]. We are presently investigating all of these possibilities.

The chromosome of B. subtilis has been found to bind to the cell wall polymer [4,15]. It is attractive to speculate that any alteration of the integrity of the cell wall-protein-membrane-DNA complex would result in aberrant division and DNA segregation patterns. Our observation that the L-form, sal-1, of B. subtilis is altered in a part of the replication complex, the attachment of the terminus of chromosome replication, provides initial support for such a speculation. It also provides opportunity for further studies on the role of the bacterial outer surface in DNA replication and segregation, and cell division.

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INTERFERON: PROPERTIES AND CLINICAL USES
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INHIBITION OF INTERFERON INDUCTION AS A SCREEN FOR THE CARCINOGENIC POTENTIAL OF CHEMICALS*

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ABSTRACT

The induction of murine interferon by Newcastle disease virus has been shown to be inhibited by pre-treatment of fibroblasts with various carcinogens. The present study has extended the range of carcinogens to include chloroacetaldehyde. Chloroethanol and chloroacetic acid, rarely carcinogenic analogs of chloroacetaldehyde, had no significant effect on interferon induction by Newcastle disease virus. When polyriboinosinic-polyribocytidylic acid was used as an interferon inducer, induction of interferon was also inhibited by pretreatment of the cells with chloroacetaldehyde. Addition of reduced glutathione, which can trap active metabolites of carcinogens, abrogated the effect of a carcinogen on interferon induction, suggesting that fibroblasts can activate carcinogens in the inhibition of interferon induction system. Following further verification of the ability of the inhibition of interferon induction system to discriminate among chemicals on the basis of carcinogenic potential, this test could become part of a comprehensive battery of tests for chemical carcinogenicity.

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INTRODUCTION

Many different tests have been developed over the past several years to determine the carcinogenic potential of chemicals. Several of the early tests, such as the Ames Salmonella test (1), were able to determine the mutagenic potential of chemicals, but could not consistently discriminate between mutagens and carcinogens. Several recently developed assays, which involve determination of the activation of "SOS" repair in bacteria, have apparently been able to differentiate between carcinogens and mutagens (2,3). However, these tests suffer from the drawback that they are carried out in prokaryotic cell systems which may not be analagous to the systems operative following exposure of a mammalian cell to a chemical.

Mammalian tests to determine the carcinogenic potential of chemicals have been developed, but also suffer from several drawbacks. Transformation of cell cultures or induction of tumors in animal models require extended periods of time for the carcinogenic potential of a chemical to be expressed. In addition, if a chemical is carcinogenic only at a very low rate, massive quantities of animals would be required to observe the carcinogenic event (4).

DeMaeyer and DeMaeyer-Guignard have shown in early studies that pre-treatment of rat fibroblasts with several carcinogens including benzo-(α)-pyrene and 3-methylcholanthrene resulted in reduced interferon production when the cultures were challenged with vaccinia virus (5,6). Benzo-(ε)-pyrene, a rarely carcinogenic mutagen and an analog of benzo-(α)-pyrene, had no effect on interferon induction. We have recently extended these studies to a murine system and have shown that several other suspected carcinogens, including Aflatoxin-B_1, 2-aminofluorene, styrene oxide, and methyl methanesulfonate, all inhibited interferon induction by Newcastle disease virus (NDV) in mouse embryo fibroblasts (7). Ethyl methanesulfonate, a rarely carcinogenic analog of methyl methanesulfonate, also had no effect on interferon induction.

The present study represents an extension of the examination of the effects of carcinogens on the induction of interferon. Pre-treatment of mouse embryo fibroblasts with the postulated carcinogen chloroacetaldehyde resulted in

inhibition of interferon induction when the cultures were challanged with NDV. Treatment of the cultures with chloroacetic acid and chloroethanol, rarely carcinogenic analogs of chloracetaldehyde, did not significantly affect interferon induction. These data suggest that the inhibition of interferon induction system may be useful as an assay for the carcinogenic potential of chemicals, following more extensive studies and confirmation of results. In addition, chloroacetaldehyde treatment inhibited interferon induction by polyriboinosinic-polyribocytidylic acid (poly I:C), suggesting that carcinogen treatment does not disrupt the interaction of virus with the cell membrane resulting in inhibited interferon production. Finally, application of reduced glutathione to the cell cultures in conjunction with the carcinogen benzo-(α)-pyrene resulted in abrogation of the effects of the carcinogen on interferon induction. These data suggest that a system exists in fibroblasts which can activate carcinogens in the interferon inhibition system.

MATERIALS AND METHODS

Mouse Embryo Fibroblast Cultures: C57Bl/6 derived mice were bred and maintained in our laboratory. Fifteen to eighteen day old embryos were surgically removed from pregnant mice, minced, trypsinized in 0.25% trypsin 1-300 (ICN Pharaceuticals, Cleveland, OH) and then suspended in minimum essential medium (Grand Island Biological, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin and streptomycin and glutamine. Second or third passage culutres were used in all experiments and were plated in 25cm^2 tissue culture flasks (Falcon Plastics, Oxnard CA).

Chemicals: Chloroacetaldehyde, chloroethanol and chloroacetic acid were generous gifts of Dr. John Wong, Department of Chemistry, University of Louisville. Polyriboinosinic and polyribocytidylic acids were obtained from P-L Biochemicals, Milwaukee, WI. Benzo-(α)-pyrene was obtained from Aldrich Chemical Company, Milwaukee, WI. Reduced Glutathione was obtained from Calbiochem, La Jolla, CA.

Interferon Induction: Mouse Type I interferon was produced in fibroblasts with the Herts strain of Newcastle disease virus, as described elsewhere (8). After inactivation of residual inducing virus by pH 2 treatment at 4°C for four days, the tissue culture supernatants were assayed for antiviral (interferon) activity. Polyriboinosinic and polyribo-

cytidylic acid were complexed to poly I:C by heating at 45°C for one hour. Type I interferon was induced with poly I:C by adding 50 µg of poly I:C to tissue cultures for 90 min., and adding additional fresh medium to the mouse embryo fibroblast cultures. DEAE-Dextran was included with the poly I:C to insure maximum of induction of interferon (9). After twenty-four hours of incubation, the culture supernatants were harvested and assayed for antiviral activity.

Interferon Assay: Interferon titers were determined by plaque reduction on mouse L-929 cells using the Indiana strain of bovine vesicular stomatitis virus (10). The interferon titer corresponded to the reciprocal of the highest dilution of test sample that reduced virus plaques by 50%. One interferon unit in this assay equals 0.88 NIH-G-002-904-511 reference units.

RESULTS

Chloroacetaldehyde, chloroethanol, and chloroacetic acid were solublized in dimethyl sulfoxide (DMSO) and diluted to appropriate concentrations in tissue culture medium. The chemicals were then added to different confluent monolayers of mouse embryo fibroblasts. Following twenty-four hours of incubation at 37°C, the culture supernatants were removed and interferon was induced with NDV. The results shown in Table 1 indicate that only treatment with chloroacetaldehyde resulted in decreased interferon production of 50% or greater.

TABLE 1

EFFECT OF PRETREATMENT OF CELL CULTURES WITH CHLORO-ACETALDEHYDE AND ITS ANALOGS ON INTERFERON INDUCTION BY NDV

Treatment*	Interferon Titer	% Decrease
NDV Only	300	—
DMSO + NDV	500	—
Chloroacetaldehyde + NDV	69	77%
Chloroacetic acid + NDV	225	25%
Chloroethanol + NDV	209	30%

*All chemicals were applied at a concentration of 0.005 µM

Since the interferon assay has an innate two-fold variability due to the biological nature of the assay (11), only differences of 50% or greater were considered to demonstrate an effect of a carcinogen on interferon induction.

When chloroacetaldehyde, chloracetic acid and chloroethanol were applied to confluent monolayers and interferon was induced with poly I:C, inhibition of interferon induction of 50% or greater was again only observed in chloroacetaldehyde treated cultures (Table 2). Chloroacetic acid and chloroethanol had much less of an effect on the induction of interferon.

TABLE 2

EFFECT OF PRETREATMENT OF CELL CULTURES WITH CHLOROACETALDEHYDE AND ITS ANALOGS ON INDUCTION OF INTERFERON BY POLY I:C

Treatment*	Interferon Titer	% Decrease
Poly I:C only	430	—
DMSO + poly I:C	385	10%
Chloroacetaldehyde + poly I:C	187	57%
Chloroacetic acid + poly I:C	268	37%
Chloroethanol + poly I:C	304	29%

*All chemicals were applied at a concentration of 0.005 μm.

Benzo-(α)-pyrene was solublized in DMSO and applied to confluent monolayers of mouse embryo fibroblasts at a concentration of 0.5 μm. As has been previously reported for induction of interferon by NDV (7), this treatment resulted in a significant drop in the titer of interferon induced by poly I:C (Table 3). Addition of reduced glutathione with the benzo-(α)-pyrene resulted in abrogation, at least in part, of the inhibitory effects of benzo-(α)-pyrene on interferon induction by poly I:C (Table 3). Glutathione itself had minimal if any effect on the induction of interferon (Table 3).

TABLE 3

EFFECT OF CONCOMITANT ADDITION OF GLUTATHIONE AND BENZO-(α)-PYRENE ON INTERFERON INDUCTION BY POLY I:C

Treatment	Interferon Titer	% Decrease
Poly I:C only	266	—
Benzo-(α)-pyrene + poly I:C	76	71%
Benzo-(α)-pyrene + 0.1 μm glutathione + poly I:C	181	31%
Benzo-(α)-pyrene + 0.01 μm glutathione + poly I:C	150	43%
0.1 μm glutathione + poly I:C	347	—
0.01 μm glutathione + poly I:C	196	26%

Addition of glutathione to cultures of mouse embryo fibroblasts did not result in the induction of detectable levels of interferon.

DISCUSSION

The induction of Type I interferon has been shown to be inhibited by several carcinogenic chemicals (5-7). Of particular interest is the observation that when several pairs of highly carcinogenic chemicals and their rarely or non-carcinogenic analogs, e.g. benxo-(α)-pyrene and benzo-(ε)-pyrene, ethyl methanesulfonate and methyl methanesulfonat were tested, only application of the proven carcinogen resulted in the inhibition of interferon induction (5-7). We have now extended this observation to include the presumed carcinogen chloroacetaldehyde, and its rarely carcinogenic analogs chloro acetic acid and chloroethanol. After many additional pairs of carcinogens and analogs are tested in the future, the inhibition of interferon induction by chemicals may prove useful in the screening of chemicals for carcinogenic potential.

Dimethyl sulfoxide has previously been shown to have a non-statistically significant minimal effect on the induction of interferon by NDV (7). In the present study, no effect of DMSO on interferon induction was observed.

The mechanism of the inhibition of interferon induction was also examined in the present study. Previous work has shown that pre-treatment of rat fibroblasts with carcinogens did not inhibit the plaquing efficiency of vaccinia virus (5). Since poly I:C induction of interferon is also inhibited by pre-treatment of fibroblasts with carcinogens, it is not likely that the carcinogen treatment is affecting the binding of viruses to the cell membrane or the budding of viruses.

Many carcinogens must be activated by microsomal oxidases in order to form final active products before an effect in bacterial or mammalian systems can be observed (12). Benzo-(α)-pyrene is included among these chemicals. Reduced glutathione can trap these active products and prevent the occurance of a carcinogenic event (12). Application of reduced glutathione to the inhibition of interferon induction system resulted in at least partial abrogation of the effects of benzo-(α)-pyrene on interferon induction. These data suggest that fibroblasts must contain the type of activation system for carcinogens that renders them potent for the inhibition of interferon induction.

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DISCUSSION

UNKNOWN: Do you think that this effect is restricted only to these groups of carcinogens and works also with aflatoxins?

SONNENFELD: It does work with aflatoxin. We have shown it with aflatoxin previously as well and there has just been a report published using aflatoxins and the B1 aflatoxin.

UNKNOWN: And what about tumor promotors?

SONNENFELD: There have been reports using coal dust and also asbestos fibers as well, that there was reduced induction of interferon. The alfatoxin story is very interesting because these investigators at Western Virginia University used four different types of aflatoxin. One type being more potent carcinogen than the other and they found that the degree of inhibition of induction was related to the potency of the type of aflatoxin that they used.

DEGRE: Do you have any idea whether other cell types could be used for the same tests?

SONNENFELD: We have used fibroblast. DeMeyer has used fibroblasts. There has been one study in vitro, I cannot remember the chemical right off hand, but if mice were treated with this chemical they could not produce in vivo serum interferon. I do not know about other things. Of course, we would like to get into studies with Type II interferon as well in lymphoid cells but to this point this type of work has only been done using fibroblast.

GROB: You might have mentioned it but could you please repeat what the pretreatment schedule was before interferon induction.

SONNENFELD: What we would do was treat those cells with the carcinogens for twenty-four hours and then wash and make sure that there was no toxicity of the carcinogen.

UNKNOWN: Jay, just to support you, the emulsifiers that we used severely reduced the ability of cells to produce interferon. If you use them in very small concentrations in leukocyte culture, they cause chromosome breaks to a substantial degree higher than the normal controls.

SONNENFELD: Which might suggest some carcinogenic potential of the material.

UNKNOWN: Yes, I couldn't agree more on the need of shifting from a prokaryotic system to an eukaryotic system and I think that your approach is particularly exciting and promising, especially considering that you can use a human cell.

SONNENFELD: Yes, we have begun studies along those lines.

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Effect of Carcinogens and Analogs on Interferon Induction

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Key Words. Interferon · Carcinogen inhibition

Abstract. Pretreatment of mouse embryo fibroblasts with several chemicals, including 7,12-dimethylbenz-(α)-anthracene, 2-aminofluorene, aflatoxin B_1, benzo-(α)-pyrene, styrene oxide, and the No. 4 fraction of tobacco smoke condensate, resulted in severely reduced production of interferon when the cells were challenged with Newcastle disease virus. All of the above chemicals are proven or strongly suggested carcinogens. When the analogs methyl methanesulfonate, a potent carcinogen, and ethyl methanesulfonate, a weak carcinogen, were applied to cells, interferon induction was only inhibited by the methyl methanesulfonate. Therefore, carcinogens may directly inhibit the induction of interferon by Newcastle disease virus.

Introduction

Interferon is now being used in several clinical trials for the treatment of various types of tumors, including non-Hodgkin's-type lymphomas [1]. Therefore, it is of interest to determine the type of interactions that exist between interferon and tumor-inducing substances, such as carcinogens.

Early experiments by *DeMaeyer and DeMaeyer-Guignard* [2, 3] suggested that application of known chemical carcinogens, such as benzo-(α)-pyrene, to rat embryo fibroblast cultures prior to challenge with vaccinia virus resulted in inhibition of the induction of interferon by the virus. An additional finding of significance was that benzo-(ε)-pyrene, a weak carcinogen, had no effect on the induction of interferon. These data suggested that carcinogens might have a specific inhibitory effect on the induction of interferon.

The results of the present study confirm and extend these observations. Several known and suspected potent carcinogens, including methyl methanesulfonate (MMS), markedly inhibited the induction of interferon by Newcastle disease virus (NDV) in mouse embryo fibroblasts. The rarely or weakly carcinogenic analog of MMS, ethyl methanesulfonate (EMS) [4, 5] had little or no effect on the induction of interferon by NDV. Therefore, the new data suggest that interferon induction is inhibited by pretreatment of cell cultures with an extended group of potent carcinogens.

Materials and Methods

Mouse Embryo Fibroblast Cultures. B6 H-2^k lyt2.1 mice, a gift of *Thomas Huff* and *Samuel Wellhausen*, Department of Microbiology and Immunology, University of Louisville, Ky., were bred and maintained in our laboratory. 15- to 18-day-old embryos were surgically removed from mothers, minced, trypsinized in 0.25% trypsin 1-300 (Pharmaceuticals, Cleveland, Ohio) and then suspended in Gibco minimal essential medium (MEM) with 10% fetal calf serum. Second or third passage cultures were used in all experiments and were plated in Falcon 25 cm^2 tissue culture flasks, and were used immediately upon reaching confluency.

Chemicals. Aflatoxin B_1, 2-aminofluorene, benzo-(α)-pyrene, EMS and MMS were obtained from Aldrich Chemical Co., Milwaukee, Wisc. 7,12-Dimethylbenz-(α)-anthracene and No. 4 fraction of tobacco smoke condensate were received from the Kentucky Tobacco Health and Research Institute, Lexington, Ky. Dimethylsulfoxide (DMSO) was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Styrene oxide was kindly provided by Dr. *John Wong*, Department of Chemistry, University of Louisville, Ky.

Interferon Production. Mouse type I interferon was produced in fibroblasts with the Herts strain of NDV, as described elsewhere [6].

After inactivation of residual inducing virus by pH 2 treatment at 4 °C for 4 days, the tissue culture supernatant fluid was assayed for interferon activity.

Interferon Assay. Interferon titers were determined by plaque reduction on mouse L-929 cells using Indiana strain bovine vesicular stomatitis virus. The interferon titer corresponded to the reciprocal of the highest dilution of test sample that reduced virus plaques by 50%. One interferon unit equals 0.88 NIH HG-002-904-511 reference units [6].

Results

Viability of Mouse Embryo Fibroblast Cultures

Mouse embryo fibroblasts were allowed to grow into a confluent monolayer. After reaching confluency, different cultures were treated for 24 h with several concentrations of the chemicals used in this study. These chemicals included 7,12-dimethylbenz-(α)-anthracene, 2-aminofluorene, aflatoxin B_1, No. 4 fraction of tobacco smoke condensate, benzo-(α)-pyrene, MMS, EMS, DMSO and ethanol. Immediately after removal of the chemicals, the fibroblast cultures were stained with 2× neutral red, a vital stain. All of the cultures that were treated with the dosages of chemicals that were used in the study were >95% viable by staining criteria. Additional cultures that had chemical-containing medium replaced with fresh MEM remained viable for at least another 24 h.

Effect of Known Carcinogens on Interferon Induction

The known carcinogens 2-aminofluorene and 7,12-dimethylbenz-(α)-anthracene were diluted in MEM and added to confluent fibroblast cultures. After 24 h of incubation, the culture supernatants were removed. The cultures were then challenged with NDV for 24 h and the supernatants were harvested, pH 2 treated, and then assayed for interferon activity. Reductions in interferon titers 90% were obtained when compared to non-carcinogen treated controls (table I). Several different dosages of chemicals were initially used to determine the dosage for optimum effect without affecting viability of the cultures, and the data in table I represent the optimal dosages.

Effects of Several Chemicals on Interferon Induction

Mouse embryo fibroblasts were treated with several different dosages of various chemicals before attempted induction of interferon with NDV. Aflatoxin B_1, No. 4 fraction of tobacco smoke condensate, benzo-(α)-pyrene, and styrene oxide treatments all inhibited the induction of interferon by 90% or greater (table II).

Table I. Effect of carcinogens on interferon production

Treatment of fibroblasts	Antiviral titer	% decrease from control
Newcastle disease virus	1,000	–
7,12-Dimethylbenz-(α)-anthracene + NDV		
39.0 μM	100	90
3.9 μM	86	91
2-Aminofluorene + NDV		
0.050 μM	21	98
0.005 μM	247	81

Table II. Blind study of the effect of various chemicals on interferon induction

Chemical treatment[1]	Interferon titer ± SE	% decrease from control	p_{NDV}[2]
None (NDV control)	1,456 ± 455	–	–
Aflatoxin B_1 0.05 μM	105 ± 82	93	<0.05
No. 4 fraction, tobacco smoke[3] condensate, 10×	89 ± 20	95	<0.05
Benzo-α-pyrene, 0.05 μM	122 ± 74	92	<0.05
Styrene oxide, 0.05 μM	149 ± 60	90	<0.05

[1] Mouse embryo fibroblasts were pretreated with the appropriate chemical for 24 h. Supernatants were removed, cells washed and NDV applied for 24 h. Cell supernatants were then harvested and assayed for antiviral activity.

[2] Statistical analysis was performed by means of a Student's T analysis.

[3] Arbitrary dilutions of the No. 4 fraction of tobacco smoke condensate were used.

Table III. Blind study of the effect of a weak and a potent carcinogen on interferon production

Chemical treatment[1]	Interferon titer ± SE	% decrease[2] from control	p
None (NDV control)	1,456 ± 455	–	–
Ethyl methanesulfonate 0.05 μM	1,680 ± 294	–	>0.5
Methyl methanesulfonate 0.05 μM	137 ± 82	91	<0.05

[1] Experimental protocol as in table II.

[2] EMS and MMS were dissolved in ethanol only.

Table II represents the effects of optimum dosages of the chemicals used. DMSO was used as a solvent for all of the above chemicals. Pretreatment of fibroblast cultures with DMSO resulted in a small (0–40%), not

statistically significant decrease in the titers of interferon induced by NDV.

EMS and MMS were solubilized in ethanol. Pretreatment of mouse fibroblasts with ethanol had no effect on the titers of interferon induced by NDV. Pretreatment of the cell cultures with EMS had a minimal effect on the induction of interferon by NDV, while MMS pretreatment severely decreased the titer of interferon induced by NDV (table III).

Discussion

The results of the present study indicate that several well-known carcinogens, including 7,12-dimethylbenz-(α)-anthracene, benzo-(α)-pyrene, 2-aminotluorene, aflatoxin B_1, and the No. 4 fraction of tobacco smoke condensate, all inhibited the induction of interferon by NDV. Styrene oxide, an important industrial chemical, which was positive in the Ames assay, but negative in all *Bacillus* assays done to date, also inhibited the induction of interferon by NDV. Further studies of the carcinogenic potential of styrene oxide may be required.

DMSO has been used in clinical trials as a drug for the treatment of arthritis. However, these trials have been suspended due to the teratogenic potential of the chemical [7, 8]. It is of interest to note that pretreatment of cells with DMSO did not result in the significant inhibition of interferon induction by NDV.

EMS and MMS are closely related analogs which differ with respect to carcinogenic potential as determined by tumor induction [4, 5]. MMS is a highly carcinogenic mutagen, while EMS is a rare or weakly carcinogenic mutagen. In the present study, MMS strongly inhibited the induction of interferon by NDV, while EMS had no significant effect. In a recent study, fibroblasts were pretreated with four different forms of aflatoxin [9]. The degree of inhibition of interferon induction correlated with the proven carcinogenic potential of the aflatoxin forms, i.e. the more carcinogenic the form of aflatoxin, the greater the inhibition of interferon induction. In view of these findings and the previously reported discrimination between benzo-(α)-pyrene and benzo-(ε)-pyrene [3] and the presently observed discrimination between EMS and MMS, carcinogens may directly inhibit the induction of interferon.

The mechanism by which carcinogens inhibit the induction of interferon is not known. A toxic effect of the carcinogens to the cell cultures does not seem likely, since the results of this study indicate that the target cells are alive when they fail to produce interferon. However, a general effect of the carcinogens on DNA, RNA, or protein synthesis and differential dosage effects of potent and weak carcinogens cannot be ruled out and are the subject of future studies.

Other possible mechanisms include alteration of the cell membrane by the carcinogen resulting in reduced interaction of virus with the cells. Recent studies showing that pretreatment of cells with carcinogens inhibited the induction of interferon by poly-inosinic–poly-cytidylic acid, a nonviral inducer of interferon [10]. In view of those studies and the earlier finding that carcinogen pretreatment does not inhibit the replication of the viruses used to induce interferon [2], it is unlikely that virus-membrane interactions are severely disrupted by carcinogen treatment of cells.

Another possible mechanism could involve induction of a protease by the carcinogen in a manner analogous to bacterial 'SOS' repair systems [11]. This protease could degrade induced interferon, or interfere with the control mechanisms for interferon induction. The true nature of the proposed effect of carcinogens on interferon induction is the subject of extended studies.

The implications of these studies toward an understanding of the mechanisms of carcinogenesis are still obscure. It is possible to speculate that there may be a balance between interferon levels and oncogenesis and that balance may be upset by the action of a carcinogen. In addition, if further studies continue to indicate a differential effect on interferon induction by strong and weak carcinogens, the effect of a chemical on interferon induction may have some use in determination of the carcinogenic potential of that chemical.

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IMMUNOPATHOLOGIC OBSERVATIONS IN LIVER ANGIOSARCOMA

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I. INTRODUCTION

In hepatic fibrosis and angiosarcoma associated with vinyl chloride exposure of industrial workers, manifestations of the disease could not be detected in most cases until the process was far advanced (1). Normal values of liver function tests were reported in a case with significant vinyl chloride hepatic fibrosis (2), and only a small percentage of workers of a plant unit where seven cases of liver angiosarcoma were diagnosed had abnormal blood screening tests (3). Thus, conventional liver function tests do not appear to be sensitive indicators of vinyl chloride liver disease. Development of more sensitive methods for detecting the disease in early stages would be of great importance. An approach to this may be provided by immunologic studies. In such a study the question arises whether the fibrotic and angiosarcomatous livers contain antigens that are different from those present in normal tissue and whether such changes could stimulate an immunologic response. The purpose of this study was to search for antigenic changes in the angiosarcomatous tissue and to test for possible presence of an antibody response in the host.

II. PROCEDURES AND MATERIALS USED

A. Patients' Sera and Tissue Specimens

Serum samples from B.F. Goodrich Co. workers with histories of vinyl chloride exposure of several years included samples from two individuals with liver angiosarcoma, ten with liver dysfunction with fibrosis and ten with normal liver function tests. The patients with angiosarcoma died and the diagnosis was confirmed at autopsy and portions of tumor and neighboring liver tissues were obtained at autopsy. Patients with liver dysfunction with fibrosis included individuals with

abnormalities in liver function tests and fibrosis detected at biopsy. Tissues were also obtained from coroner's autopsies of healthy individuals a few hours after death by gunshot wounds.

B. Tissue Extracts and Antisera

Liver angiosarcoma and adjacent liver tissue and post-mortem tissues considered to be normal were frozen and stored at -70°C until used. Portions were cut, thawed and homogenized in 2-3 volumes of distilled water in a Potter-Elvehjem grinder in an ice bath until a smooth suspension was obtained. After centrifugation at 20,000 x G for 30 min, the supernatant fluid containing the aqueous extract was lyophilized. Albino rabbits were immunized with the tumor or normal liver extracts in Freund's complete adjuvant and sera collected and stored following procedures detailed elsewhere (4). Reaction of these immune sera with human serum or plasma was eliminated by absorption with 100 mg of lyophilized, pooled normal human serum /ml antiserum. Antisera were routinely absorbed in this manner prior to use. Additional absorption with tissue extracts was carried out with 100 mg lyophilized extract/ml antiserum. Absorptions followed a procedure described previously (5).

C. Treatment of Tissue Extracts

Enzymatic treatment of tissue extracts was carried out with Pronase and trypsin as previously described (6). Periodate oxidation was done according to Rajam et al. (7). Ammonium sulfate and cold ethanol fractionations were carried out as detailed (8).

D. Immunodiffusion and Immunofluorescence

Double immunodiffusion was carried out in 0.8% agarose in phosphate-buffered saline pH 7.2 (PBS) containing 0.1% sodium azide. Circular wells, 2 mm in diameter, 3 mm apart were used. Immunoelectrophoresis was performed according to Scheidegger (9) using 0.8% agarose in 0.025 M Veronal buffer at pH 8.2.

In immunofluorescent studies cryostat sections of rat kidney, stomach or intestine and liver (4 microns) were used as substrate for antimitochondrial, antismooth muscle and antinuclear antibodies. Liver sections from rats exposed 4, 8 and 14 days to 1-2% vinyl chloride for 4 hr/day were also used. The sections were covered with dilutions of patients' sera for 45 min at room temperature, washed twice in PBS for 10 min and then covered with fluorescein conjugated IgG fraction of rabbit anti-human immunoglobulins serum (Cappel Laboratories, Inc.) for 45 min and washed as before prior to examination. Cryostat sections of liver angiosarcoma and liver tissue considered to be normal (4 microns) were washed twice in PBS for 10 min to wash off nonfixed immunoglobulins. After drying,

sections were stained with fluorescein conjugated IgG fractions of rabbit anti-human IgG serum and goat anti-human IgM serum (Cappel Laboratories, Inc.). Sections were washed as above and examined under the fluorescent microscope. Representative frozen sections were stained with hematoxylin and eosin to allow correlation between immunofluorescence and the histological findings. The antigenic preservation of the tissues was indicated by the demonstration of their staining by antinuclear factor according to the indirect immunofluorescent procedure.

E. Elution of Tumor-bound IgG

The tumor and liver tissues were extracted five times with PBS to wash off nonfixed immunoglobulins and then extracted at pH 2.5 to release bound IgG according to a procedure applied in the elution of renal-bound antibody (10).

F. Circulating Tissue Antigens

Liver-specific antigen LSA (8), tissue antigens of wide organ distribution (4) and bile antigens (11) were tested in the patients' sera by immunodiffusion as described previously.

III. RESULTS

A. Angiosarcoma-related Antigen

To test for presence of new antigens appearing in liver angiosarcoma, antiangiosarcoma serum was absorbed with human serum and liver extract and tested by immunodiffusion with extracts of both normal liver and angiosarcoma tumor at varying concentrations. This absorption eliminated all reactivity with liver extracts prepared from five normal individuals but not with the angiosarcoma extracts where one line of precipitation remained (Fig. 1). This line of precipitation could still be seen after additional absorption of the antiserum with kidney extract. In contrast, absorption with the tumor extracts eliminated completely the angiosarcoma-related line of precipitation. Absorption with spleen and lung extracts also eliminated this line of precipitation. Thus, the antigen appeared to be of restricted tissue distribution and not angiosarcoma specific. The antigen was inactivated by trypsin and Pronase and thus appeared to be a protein or closely associated to protein. Incubation of the tumor extracts for 1 hr at 4°C in citrate buffer, pH 2.5, resulted in inactivation of the antigen whereas incubation in phosphate buffer, pH 5.0, neutral or alkaline pH up to pH 10.0, did not affect it. The antigen was shown to be relatively thermolabile. Incubation of the tumor extracts for 30 min in PBS at 25° and 56°C did not affect the antigen whereas incubation at 70°C and higher

completely inactivated it. The antigen precipitated mainly at 20-30% saturated ammonium sulfate and at ethanol concentrations of 30-70% (Table I).

B. Absence of a Normal Tissue Antigen in Angiosarcoma

Antiliver serum absorbed with human serum gave several arcs of precipitation in immunoelectrophoresis with extracts of normal liver and angiosarcoma tissue (Fig. 2a). These lines could not be seen following additional absorption of the antiserum with normal liver. In contrast, absorption with liver angiosarcoma extract failed to eliminate one of the arcs of precipitation (Fig. 2b). Thus, the tissue antigen related to this arc of precipitation appeared to be absent in the angiosarcomatous tissue whereas the antigens corresponding to the other arcs were present. The absent antigen in angiosarcoma was shown to be present in kidney and lung extracts in addition to liver by absorption and direct immunodiffusion tests. Physicochemical characterization studies indicated this antigen to be unaffected by Pronase and trypsin and inactivated by periodate treatment. The antigen was relatively thermostable withstanding incubation at 70°C for 30 min in PBS. The antigen was destroyed following incubation of the liver extract in citrate buffer at pH 2.5 or lower for 1 hr at 4°C; incubation at pH 5.0 or higher (up to pH 10.0) did not affect it. This antigen precipitated over a wide range of ammonium sulfate and ethanol concentrations (Table I).

C. Angiosarcoma-bound IgG

IgG fluorescent staining appeared in a linear pattern in the peripheral portion of the tumor cells suggesting in vivo binding by the tumor of the immunoglobulin. This staining is illustrated in Fig. 3. Fig. 4 shows the angiosarcomatous cells surrounding irregular vascular spaces. Relatively coarse, linear fluorescence was also present along some hepatic cords and strands of connective tissue. There was no evidence of IgM. Control post-mortem liver tissue did not show any significant fluorescence of bound IgG. Staining for IgG of the tumor sections did not change after several washings at pH 7.2 indicating that the IgG was firmly bound to the tumor. In contrast, sections showed marked diminution of staining after washing at pH 2.5. Elution of bound IgG from saline-extracted tumor homogenates was thus attempted at acid pH. With the five successive saline extractions the amount of saline soluble IgG gradually diminished to nondetectable levels; and at acid pH bound IgG was released from the homogenate. Similar treatment of liver homogenates did not demonstrate presence of bound IgG (Table II).

D. Circulating Autoantibodies and Tissue Antigens

Serum autoantibodies to nuclei, mitochondria and smooth muscle were negative in all patients examined. In addition,

serum from these patients did not show reactivity with liver from rats exposed to vinyl chloride. Liver-specific antigen LSA (8), bile antigens (11) and other tissue antigens (4) associated with liver damage were not detected in these patients.

IV. DISCUSSION

The immunologic characteristics of cancer have been under intense investigation during recent years, and antigenic differences between normal and malignant tissue are considered to be fundamental factors in the immunologic approach to cancer therapy and diagnosis. Liver angiosarcomatous tissue was thus analyzed in this work for presence of neoantigens, normal tissue antigens and tumor-bound immunoglobulins. Several normal tissue antigens were found by immunodiffusion to be present in the tumor, but one antigen of rather wide organ distribution was not detected. These findings are in agreement with observations in other tumors indicating that tumor cells contain many of the antigens of their original hosts and lack some normal tissue antigens. For example, immunohistochemical studies have shown the loss of kidney antigens in stilbestrol- and x-ray-induced kidney tumors (12), of skin antigen in 3-methylcholanthrene-induced mouse squamous cell carcinoma (13) and of certain muscle antigens in 20-methylcholanthrene-induced rat rabdomyosarcoma (14). By far the most extensively studied class of tumors are the chemically induced hepatomata where deletion of liver antigens have been shown in tumors induced with 4-dimethylaminoazobenzene (15, 16), diethylnitrosamine (15) and 2-acetomidofluorene in the rat (15, 17) and o-aminoazotoluene in the mouse (18). In human carcinoma, loss of antigens have been reported in squamous cell carcinoma (12, 19), loss of the ABH blood group isoantigens in some solid tumors (20, 21) and of HL-A isoantigen in lymphoma (22). In addition, it has been well documented that as cells transform from a normal state to malignancy they may gain new antigenic specificities. Tumor-specific transplantation antigens have been demonstrated in a number of experimentally induced tumors (23-26) as well as in spontaneous tumors in man (27-29). In the present report immunodiffusion analyses of liver angiosarcoma and other human tissues with rabbit antiangiosarcoma serum did not indicate the presence of a tumor-specific antigen but rather of an antigen found in lung and spleen but not in liver and kidney. This antigen is being further characterized in our laboratory.

Of particular interest is the demonstration of tumor-bound IgG by immunofluorescence and elution experiments. This finding must however be interpreted with caution and should be confirmed in biopsy specimens. The tumor-bound IgG may represent specific antitumor antibody, antibody fixed by the tumor tissue "nonspecifically" or part of both. Further speculation is premature until it has been shown that the staining pattern

is due to the deposition of a specific antibody, that the eluted antibody is specific or until the relevant antigen has been identified. Work is in progress to determine the precise significance of the finding of IgG in the tumor.

V. SUMMARY

Immunodiffusion analyses of human liver angiosarcoma associated with vinyl chloride exposure indicated presence in the tumor of an antigen not detected in normal liver and kidney but found to be present in lung and spleen. This antigen was shown to be a protein, inactivated by Pronase and trypsin, relatively susceptible to heating and to acid pH and precipitated mainly at 20-30% saturated ammonium sulfate and at 30-70% ethanol concentrations. The tumor was shown to contain several antigenic constituents of normal tissue but one normal tissue antigen was not detected. This antigen was characterized as a substance unaffected by Pronase and trypsin and inactivated by periodate. It was relatively thermostable, affected by acid pH and precipitated over a wide range of ammonium sulfate and ethanol concentrations. Tumor specimens obtained at autopsy contained bound IgG as shown by immunofluorescence and elution experiments suggesting possible in vivo binding of IgG to the tumor.

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TABLE I. Angiosarcoma-related Antigen and Antigen Absent from the Tumor in Ammonium Sulfate and Ethanol Fractions

Fraction tested	Presence of[a]	
	Angiosarcoma-related antigen[b]	Antigen absent from Angiosarcoma[c]
Ammonium sulfate:		
0-20% saturation	+	-
20-30% saturation	++	+
30-50% saturation	-	+++
50-70% saturation	-	++
Ethanol:		
0-20%	-	++
20-30%	-	+++
30-50%	++	+++
50-70%	++	++
SN[d]	-	+

[a] +++, ++, + indicate strength of double diffusion reaction in dilution assay.

[b] Detected in angiosarcoma fractions.

[c] Detected in liver fractions.

[d] SN = supernate of the 70% ethanol precipitation, dialyzed and lyophilized.

TABLE II. IgG in Saline and Acid Extracts of Angiosarcoma and Liver Tissues

Preparation tested	Weight solid extracted from 1 gm (wet weight) tissue (mg)	Presence of IgG[a]
Angiosarcoma:		
Saline extract 1	29.8	+++
Saline extract 2	8.6	++
Saline extract 3	6.2	+
Saline extract 4	5.6	-
Saline extract 5	6.1	-
Acid extract	6.1	++
Liver:		
Saline extract 1	47.4	+++
Saline extract 2	14.5	+++
Saline extract 3	8.6	+
Saline extract 4	7.4	-
Saline extract 5	6.4	-
Acid extract	9.0	-

[a] Tested by immunodiffusion at concentrations of the eluates ranging up to 2%. Present at concentrations 0.05-0.1% (+++); 0.2-0.5% (++); 1-2% (+); negative at 2% (-).

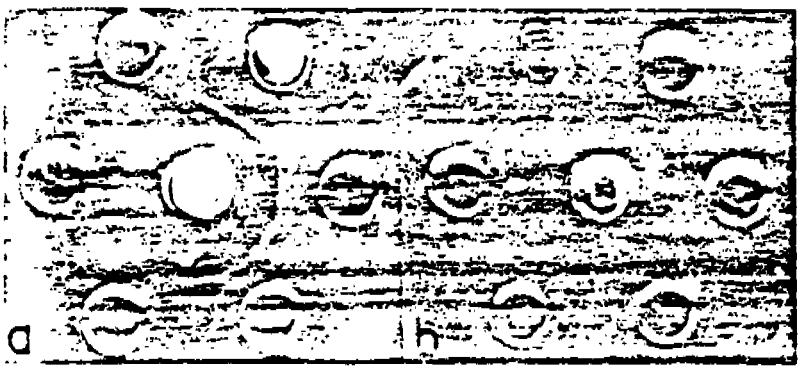

FIG. 1. Demonstration of angiosarcoma-related antigen. Peripheral wells have 2-fold serial dilutions of liver angiosarcoma extract (a) and normal liver extract (b). Dilutions are clockwise and start at 100 mg/ml in the upper right well. Central wells in each plate contain rabbit antiangiosarcoma serum absorbed with normal human serum and liver extract.

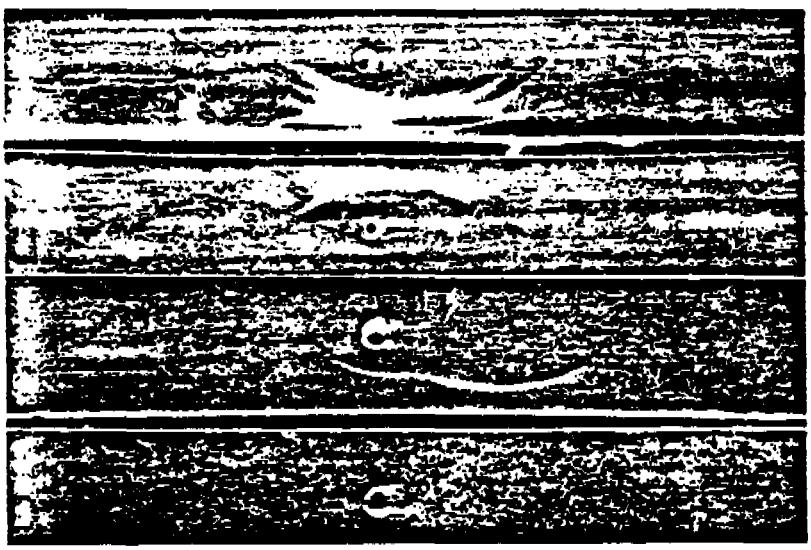

FIG. 2. Demonstration of tissue antigen absent in angiosarcoma. (a) Trough contains rabbit antihuman liver serum absorbed with normal human serum. (b) Trough contains the antiliver serum additionally absorbed with angiosarcoma extract. In both plates top wells have 10% solution of liver extract and lower wells angiosarcoma extract. Anode is to the right.

FIG. 3. Immunofluorescent staining of liver angiosarcoma by fluorescein conjugated IgG fraction of rabbit antihuman IgG serum (x 400).

Clin. exp. Immunol. (1976) 25, 410–417.

Circulating tissue antigens

III. IDENTIFICATION AND CHARACTERIZATION OF ANTIGENS OF LIMITED AND OF WIDE BODY DISTRIBUTION IN HUMAN GALL BLADDER BILE. PRESENCE IN SERUM OF PATIENTS WITH ACUTE HEPATITIS*

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(*Received* 19 *January* 1976)

SUMMARY

Three antigens shared by bile and tissues (BT-1, BT-2 and BT-3) and one shared by bile and saliva (BA) were identified in human gallbladder bile by immunodiffusion. The former were detected in all bile specimens examined, whereas the latter was detected only in half. BT-1 was limited in distribution to kidney, urine and bile; whereas BT-2 and BT-3 were widely distributed, mainly in liver, kidney, lung and bile. The antigens were not present in biles of other mammals tested, with the exception of BA which was also present in Rhesus monkey. All antigens were inactivated by Pronase, had relative electrophoretic mobilities of serum globulins and separated from each other in Sephadex G-200 gel filtration and ammonium sulphate fractionation. Ethanol inactivated BT-1 and precipitated the other antigens. BT-2 and BA were relatively resistant to boiling temperature and acid pH, whereas BT-1 and BT-3 were susceptible. Antigens BT-2 and BT-3 were detected in serum of patients with acute hepatitis but not of patients with other diseases or of normal controls.

INTRODUCTION

In earlier work both normal serum proteins and proteins apparently specific to the biliary tract have been identified in bile (Rawson, 1962; Hardwicke *et al.*, 1964; Clausen, Kruse & Dam, 1965; Yoon, Shim & Kil, 1966; Wales *et al.*, 1969; Englert, Wales & Straight, 1970). Yoon *et al.* (1966) and Englert *et al.* (1970) demonstrated by immunoelectrophoresis that rabbit antihuman bile serum absorbed with normal human serum was reactive with as many as three or four antigenic components of human bile. These antigens were considered to be bile-specific although a relationship to other body fluids and tissues was not investigated. We became interested in this relationship after finding that two of the tissue antigens detected in serum of patients with hepatitis (Espinosa, 1974) related to antigens in bile, as demonstrated in the present work. The primary objective of this investigation was the identification and characterization of both bile-specific antigens and antigens shared by bile and tissues.

MATERIALS AND METHODS

Body fluids, tissue extracts and patients' sera. Human gallbladder bile and tissues considered to be normal at post-mortem examination were obtained from coroner's autopsies of eight adult subjects within a few hours after death. Animal bile was obtained immediately after killing. Bile was centrifuged at 12,000 *g* for 30 min at 4°C. The supernate was lyophilized and kept in a desiccator until tested. Tissue saline extracts were prepared as previously described (Espinosa & Kaplan, 1970).

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Urine and saliva specimens were provided by five normal adults. Urine was dialysed against distilled water and lyophilized. Saliva was lyophilized. Serum samples were obtained from patients at Louisville's General and St Anthony Hospitals within the first week of hospitalization. Of the thirty-three patients tested, eighteen had acute hepatitis (five virus A, seven virus B, five alcoholic and one carbon tetrachloride intoxication), four liver cirrhosis, two obstructive jaundice, two liver angiosarcoma, five pneumonia and two pancreatitis. Serum samples from forty-eight normal blood donors were obtained from the local Red Cross blood centre. Samples were tested by double immunodiffusion as soon as obtained and kept frozen at −35°C when further testing was contemplated.

Preparation of antisera. Lyophilized bile and saline extracts of liver, kidney and cardiac and skeletal muscle were each dissolved in saline in a concentration of 20 mg/ml and emulsified in an equal volume of Freund's complete adjuvant. Albino rabbits were given weekly injections as follows: first injection, 0·4 ml of adjuvant mixture in the four footpads intradermally; second and third injection, 1 ml intramuscularly, divided between two sites; fourth injection, 1 ml of the extract solution (20 mg/ml) without adjuvant given intraperitoneally. Serum was prepared from blood withdrawn just prior to immunization and 10–15 days after the last injection; serum was stored frozen until used. Reaction of these antisera with normal human serum and plasma was eliminated by absorption with 100 mg lyophilized, pooled normal human serum/ml of antiserum. Absorptions were as previously described (Espinosa & Kaplan, 1968). Such absorbed antisera were used routinely in this work except as otherwise indicated. Both absorbed and unabsorbed preimmunization sera gave no reaction with either body fluids, tissue extracts or patients' sera in immunodiffusion tests.

Double immunodiffusion and immunoelectrophoresis. Double immunodiffusion was performed in 0·8% agarose in PBS, pH 7·2, containing 0·1% sodium azide. Circular wells 2 mm in diameter, 3 mm apart, were used. Immunoelectrophoresis was performed according to Scheidegger (1955) using 0·8% agarose in 0·025 M Veronal buffer at pH 8·2. The electrophoresis was carried out with ice-cooling at 5 mA/slide for 120 min. Both the double immunodiffusion and immunoelectrophoresis plates were incubated for 48–72 hr. Plates were then washed in the cold with daily changes of saline for 4–5 days and then photographed.

Fractionation of bile. Sephadex G-200 gel filtration of 1 ml of a 10% solution of lyophilized bile in PBS was performed at 4°C in an 85 × 1·5 cm column. PBS containing 0·02% sodium azide was used as eluant at a flow rate of 5–6 ml/hr; fractions of 2 ml were collected. In some cases, antigen-containing fractions were concentrated by lyophilization.

Ammonium sulphate and cold ethanol fractionations were carried out according to procedures detailed previously (Espinosa, 1973).

Treatment of bile. Treatment of the bile solution (100 mg/ml PBS) with Pronase was carried out with the enzyme, 5 mg/ml PBS, at 37°C for 24 hr. Controls included samples without enzyme, with enzyme but without incubation and with enzyme alone.

The effect of pH was tested by incubating solutions of lyophilized bile in citrate buffer at pH 2·5 and 3·5 and phosphate buffers at pH 5·0, 7·0, 9·0 and 10·0 for 1 hr at 4°C. Precipitation occurred at pH 2·5. For antigen testing, pH was adjusted to neutrality which brought precipitated material into solution.

RESULTS

Immunodiffusion analyses of bile

The eight bile samples were tested at varying concentrations with antisera to bile, kidney, liver, skeletal muscle and cardiac muscle by double immunodiffusion. With the antibile serum, all the bile samples gave a line of precipitation corresponding to the antigen designated BT-1; half of the samples gave an additional line related to the antigen referred to as BA. This is illustrated in Fig. 1. The BA line was thinner, continued to show at higher dilutions of bile and was closer to the antiserum well than the line of BT-1. Identity of each of these antigens detected in individual bile samples was demonstrated by the complete fusion of their corresponding lines of precipitation.

The antikidney serum gave one line with all samples. The antigen related to this line was shown to correspond to BT-1 as follows. This line gave a reaction of identity with that of BT-1 given by the antibile serum (Fig. 2); and both lines were eliminated following absorption of the antisera with either bile or kidney extract.

The antiliver serum gave two lines with all samples. These lines relate to antigens designated BT-2 and BT-3. The BT-2 line of precipitation is closer to the antiserum well than that of BT-3 (Fig. 3). These lines did not fuse with that of BT-1 or BA and, thus, appeared unrelated to these antigens. In addition, BT-2 and BT-3 separated from each other and from BT-1 and BA in immunoelectrophoresis and Sephadex G-200 gel filtration, as indicated below.

Antisera to skeletal and cardiac muscle were unreactive with bile.

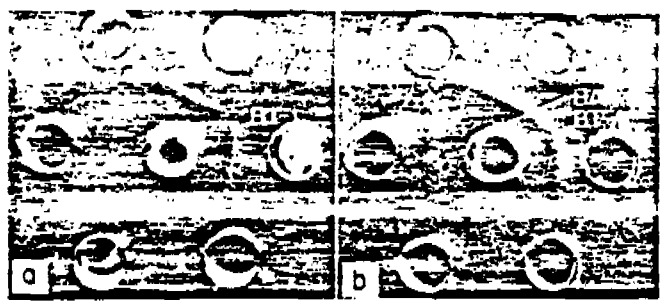

FIG. 1. Demonstration of bile samples containing antigen BT-1 (a) and antigens BT-1 and BA (b). Peripheral wells have two-fold serial dilutions of human gallbladder bile C74-180 (a) and C74-183 (b). Dilutions are clockwise and start at 100 mg/ml in the upper right well. Central wells in each plate contain antibile serum (AS-42). In this and succeeding tests, antisera were nonreactive with human serum and plasma constituents after specific absorption.

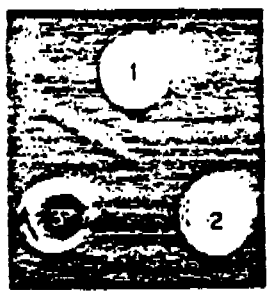

FIG. 2.

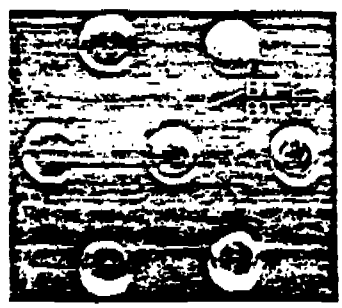

FIG. 3.

FIG. 2. Demonstration of identity of antigen BT-1 detected by antibile and antikidney sera. Well 1 contains antibile serum (AS-42); 2, antikidney serum (AS-38); and 3, 10% solution of bile C74-180.

FIG. 3. Double immunodiffusion test showing bile antigens BT-2 and BT-3. Peripheral wells contain, clockwise, two-fold serial dilutions of bile C74-180, starting at 100 mg/ml in the upper right well. Central well contains antiliver serum (AS-30).

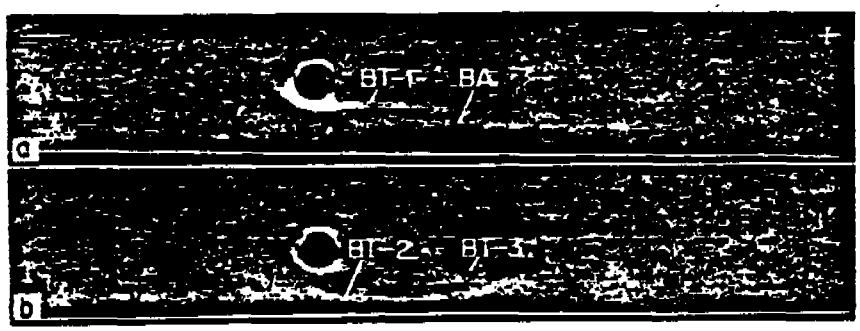

FIG. 4. Immunoelectrophoresis of bile C74-183. Wells in both plates contain 10% solution of bile C74-183. (a) Trough has antibile serum (AS-42). (b) trough has antiliver serum (AS-30).

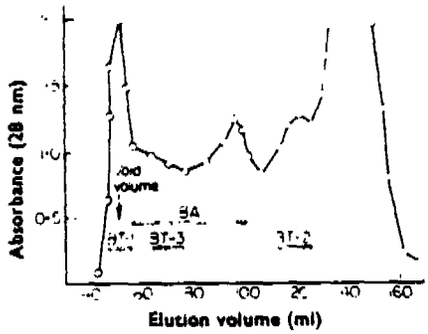

FIG. 5. Sephadex G-200 gel filtration of bile C74-183. See Materials and Methods section for details.

All these antigens separated from each other and moved towards the anode in immunoelectrophoresis (Fig. 4). Their electrophoretic mobilities ranged between that of alpha- and beta-serum globulins.

Separation of the antigens of bile was also accomplished in Sephadex G-200 gel filtration. BT-1 was detected close to the void volume of the effluent and BT-3, BA and BT-2 eluted later, in that order (Fig. 5). BA was detected over a wide zone, both in immunoelectrophoresis and gel filtration, indicating molecular heterogeneity.

Occurrence of antigens in tissues and body fluids

The relative amounts of lyophilized preparations of tissues and body fluids required in absorption tests to abolish the precipitin lines of antigens BT-1, BT-2, BT-3 and BA were compared—the more absorbent required, the less antigen it contains (Table 1). These results indicated that BT-1 is present in bile, kidney and urine; BT-2 and BT-3 are widely distributed mainly in liver, kidney, lung and bile. BA was detected in half of the biles examined and also in the saliva of three out of the five normal subjects

TABLE 1. Relative amounts of tissue extract and body fluid preparations required for absorption of precipitin lines of antigens BT-1, BT-2, BT-3 and BA

	Minimal weight absorbent (mg/ml antiserum) effective in abolishing precipitin line			
Absorbent	BT-1	BT-2	BT-3	BA
Liver	U	5	5	U
Kidney	20	5	20	U
Spleen	U	W	W	U
Lung	U	W	20	U
Skl. muscle	U	U	U	U
Heart	U	U	U	U
Uterus	U	50	U	U
Bile	50	50	20	5
Urine*	50	U	U	U
Saliva*	U	U	U	50

Antisera aliquots were absorbed with tissue extract or body fluid preparations (up to 50 mg/ml antiserum) and tested with bile.

U = unaffected line of precipitation; W = weakened line of precipitation.

* Obtained from live donors.

TABLE 2. Effect of temperature

Antigen	Temperature (30 min in PBS) 25°C	56°C	70°C	100°C
BT-1	+	±	−	−
BT-2	+	+	+	+
BT-3	+	−	−	−
BA	+	+	+	+

+, Unaffected; ±, partial inactivation; −, complete inactivation.

tested. This distribution of BT-1 and BA was confirmed by direct immunodiffusion tests of body fluids and tissue extracts with the antibile serum. Occurrence of BT-2 and BT-3 could not be studied by direct testing since the antiliver serum reacted with other tissue antigens not detected in bile.

Species specificity studies

In order to study the species distribution of the antigens, the antisera were absorbed with biles of various mammalian species and tested against two-fold dilutions of human bile. Absorption of the antisera with human bile abolished reaction with all antigens (with the exception of BA if biles not containing BA were used), thus confirming their presence in humans. In contrast, absorption with pig, dog, cat and guinea-pig biles did not significantly affect any of the lines of precipitation. Absorption with Rhesus monkey bile abolished only reaction with BA, thus indicating the presence of an immunologically related antigen in this species. These findings were confirmed by direct double immunodiffusion tests of the animal biles: none of the antigens could be shown, except BA which was demonstrated in monkey bile. However, immunodiffusion patterns comparing the reaction of human and monkey biles showed a line of fusion with a spur consistent with partial identity or a cross-reactive relationship of the BA from the two species.

Physicochemical properties

Treatment of bile with Pronase resulted in loss of the lines of precipitation given with the antisera. In the controls with no enzyme and with enzyme but without incubation, the antigens remained reactive. The control with enzyme alone was unreactive. This suggests that the bile antigens are proteins or closely associated to protein.

In order to study the effect of heating on the bile antigens, a 10% solution of bile in PBS was incubated at various temperatures for 30 min and tested with the antisera. As indicated in Table 2, BT-1 and BT-3 were relatively susceptible to heat treatment; in contrast, BT-2 and BA withstood boiling temperature.

All antigens were unaffected by treatment with neutral or alkaline buffers up to pH 10·0. Buffer at pH 5·0 partially inactivated BT-1 but did not affect the other antigens. Treatment at pH 2·5 and 3·5 resulted in complete inactivation of BT-1 and BT-3 and partial inactivation of BT-2. BA was unaffected even at pH 2·5.

As shown in Table 3, BT-1 was precipitated at ammonium sulphate concentrations between 30 and 70% of saturation, BT-2 at 70–100% and BT-3 at 30–50%. BA precipitated over a wider range of ammonium sulphate concentrations.

Treatment with ethanol (Table 4) resulted in inactivation of BT-1; the other antigens precipitated mainly at ethanol concentrations ranging from 30 to 70%.

BT-2 and BT-3 in patients' sera

Serum samples from thirty-three patients and from forty-eight normal blood donors were examined

TABLE 3. Antigens in $(NH_4)_2SO_4$ fractions

	$(NH_4)_2SO_4$ fractions (% saturated)				
Antigen	0–30	30–50	50–70	70–100	SN*
BT-1	–	+++	++	–	–
BT-2	–	–	–	++	–
BT-3	–	+	–	–	–
BA	–	++	++	+++	–

+++, ++, + Indicate strength of double diffusion reaction in dilution assay.
* SN = supernate of the 100% saturated solution.

TABLE 4. Antigens in ethanol fractions

	Ethanol fractions (v/v%)		
Antigen	0–30	30–70	SN*
BT-1†	–	–	–
BT-2	±	++	±
BT-3	–	++	–
BA	–	+++	+

+++, ++, +, ± indicate strength of double diffusion reaction in dilution assay.
* SN, supernate of the 70% ethanol precipitation, dialyzed and lyophilized.
† Ethanol susceptible.

TABLE 5. BT-2 and BT-3 in patients' sera

Group	Number of cases	Number of cases with circulating BT-2	Number of cases with circulating BT-3
Acute hepatitis:			
infectious	5	4	4
serum	7	6	6
alcoholic	5	4	4
carbon tetrachloride	1	1	1
Liver cirrhosis	4	0	0
Obstructive jaundice	2	0	0
Liver angiosarcoma	2	0	0
Pneumonia	5	0	0
Pancreatitis	2	0	0
Normal subjects	48	0	0

for the presence of the bile antigens by double immunodiffusion. BT-1 and BA could not be detected in any of the serum samples. As indicated in Table 5, BT-2 and BT-3 were detected in sera of most cases with acute hepatitis but not in other liver diseases, pneumonia, pancreatitis and normal subjects. Identity of antigens BT-2 and BT-3 detected in bile and patients' sera was shown by the fusion of their lines of precipitation and by their elimination following absorption of the antiliver serum with bile.

DISCUSSION

The present data have demonstrated presence in bile of antigens of limited and of wide body distribution. Of limited body distribution was antigen BA, detected in bile and saliva of certain individuals, and antigen BT-1 found to be present in bile, urine and kidney. A wide body distribution was shown by antigens BT-2 and BT-3 which were detected in bile, liver, kidney, spleen and lung.

Antigens previously demonstrated in bile include both normal serum proteins and apparently bile-specific proteins (Rawson, 1962; Hardwicke *et al.*, 1964; Clausen *et al.*, 1965; Yoon *et al.*, 1966; Wales *et al.*, 1969; Englert *et al.*, 1970). The reported number of bile proteins grew with increasing sensitivity of the techniques employed from four, by paper electrophoresis (Verschure, 1956; Wales *et al.*, 1969), to up to sixteen total proteins and four specific bile proteins, by disc electrophoresis combined with immunodiffusion and immunoelectrophoresis (Englert *et al.*, 1970). Three 'bile-specific protein components' were identified in human bile by Yoon *et al.* (1966) and up to four by Englert *et al.* (1970) by immunoelectrophoresis using rabbit antihuman bile serum absorbed with normal human serum. Since other body fluids and tissues were not tested, these studies did not rule out the possibility that such antigens are present in other body fluids and tissues as well as bile. Of the components identified by Yoon *et al.* (1966), one (alpha-1 biliprotein) moved slower and another (biliprealbumin) faster than serum albumin in immunoelectrophoresis; a third component (biliproalbumin) moved as serum albumin. One of the antigens described by Englert *et al.* (1970) moved as biliprealbumin and the three others in the post albumin zone which includes the serum globulins. The antigens described in the present work had immunoelectrophoretic mobilities ranging between that of alpha- and beta-serum globulins and thus may relate to the antigens described previously in the post-albumin zone. Antigens with albumin and prealbumin electrophoretic mobilities were not detected in this work. Neither was evidence obtained of antigens with strict specificity for bile. However, one bile antigen (BA) was found to be shared with saliva only. This antigen was not searched for in saliva in a preliminary study and was, thus, considered to be present in bile only (Espinosa, Shelton & Shaw, 1975).

The antigens described in this work separated from each other in Sephadex G-200 gel filtration and ammonium sulphate fractionation and, thus, appear to be distinct and different molecular entities. Both methods are being employed for their purification and isolation. All antigens were inactivated by Pronase, suggesting that they are proteins or closely associated to proteins. In addition, they were precipitated at 70% ethanol concentration, excepting BT-1 which was inactivated by ethanol and was generally rather labile. In contrast, BT-2 and BA were relatively resistant to boiling temperature and to acid pH.

Of special interest to liver disease is the finding of BT-2 and BT-3 in the blood of patients with acute hepatitis. These antigens are part of a group of circulating tissue antigens of wide organ distribution which have been found to be present in patients with liver injury (Espinosa, 1974). Two more of these antigens, referred to as CTA-2 and CTA-3, have been recently characterized (Espinosa, 1976). CTA-2 is a protein with a relative electrophoretic mobility of a beta-serum globulin and molecular weight in the range of 67,000–80,000; CTA-3 moves in immunoelectrophoresis like an alpha-1-serum globulin, is resistant to Pronase and behaves like a protein of molecular weight of about 300,000. Other circulating tissue antigens detected in liver disease include the following liver-specific antigens: (a) F-antigen of wide interspecies cross-reactive properties (Bodmer, 1969; Rosenmund, 1971; Smith & Iverson, 1973); (b) a protein located within the cytoplasm of hepatocytes and of molecular weight of about 190,000 (Meyer zum Büschenfelde & Miescher, 1972); and (c) LSA, a protein of electrophoretic mobility of serum gammaglobulins and molecular weight in the range of 82,000–93,000 (Espinosa, 1973). Significance of these findings in the pathogenesis and clinical assessment of hepatitis is worth investigating. These studies should include correlations of the levels of these antigens in diseases of the liver and of other organs and investigation of their autoimmunogenic properties. The possibility that bile antigens are related to autoimmune liver disease is suggested by the demonstration of cell-mediated immune response to a protein fraction of human bile in patients with primary biliary cirrhosis and active chronic hepatitis (Eddleston *et al.*, 1973).

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PATHOLOGY

LYMPHOCYTE TRANSFORMATION TESTS IN VINYL CHLORIDE (VC) WORKERS

H. Philip Fortwengler*, Michael E. Dever*, Carlo H. Tamburro*, and Enrique Espinosa, Univ. of Louisville School of Medicine, Louisville, KY 40201

Previous reports have shown circulating immune complexes in VC workers and a tumor-associated antigen in VC-related liver angiosarcoma. This suggests immune stimulation by a tissue or plasma antigen induced by or conjugated with VC or a metabolite. In this work, the in vitro lymphocyte reactivity for such antigens was tested in 79 VC workers including 25 with liver abnormalities, and 20 normal individuals having no exposure to VC. The liver angiosarcoma antigen preparation included the tumor-associated antigen and other tissue antigens as shown by immunodiffusion. Stimulation indices (SI) were calculated from cellular incorporation of tritiated thymidine. Mean SI in the normal individuals for antigens of angiosarcoma and normal liver tissues were 4.7 (SE±1.7) and 3.8 (±0.9) and in VC workers, 1.8 (±0.2) and 2.5 (±0.3) respectively. Mean SI for PHA and Con A in the normal individuals were 235 (±35) and 209 (±30) and in VC workers 201 (±23) and 180 (±19) respectively. Thus, these results suggest that VC workers have a decreased lymphocyte response to antigens of liver angiosarcoma and normal liver tissues rather than the hypothesized increased reactivity. This appears to be due to a lower overall lymphocyte responsiveness in these chemical workers.
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FACTOR VIII CONTENT AS EVIDENCE FOR ENDOTHELIAL ORIGIN OF VINYL CHLORIDE ASSOCIATED LIVER ANGIOSARCOMA (VCA). H. Philip Fortwengler*, Douglas Jones*, Carlo H. Tamburro* and Enrique Espinosa (SPON: G. Randolph Schrodt). Univ. of Louisville School of Medicine, Louisville, KY. 40232

To ascertain the endothelial cell origin of VCA we have looked for Factor VIII in the tumor since this factor appears to be specific for such cells (Hoyer et al., 1973). Frozen sections of three VCA and one idiopathic case were examined for presence of Factor VIII by indirect immunofluorescence. Sections of angiosarcoma demonstrated a strikingly increased specific fluorescence which lined enlarged sinusoids. This intense fluorescence was easily seen on low power(100x) as an irregular or splotchy pattern. Occasional striations of linear fluorescence which did not follow hepatic cords were also present. A similar pattern of staining was also given by the idiopathic angiosarcoma but was never seen in sinusoids of normal liver. These observations were further supported by the finding that absorption of Factor VIII antiserum with experimental angiosarcoma tissue resulted in complete inhibition of immunofluorescence. These findings demonstrate that VCA and idiopathic angiosarcoma include proliferating cells containing Factor VIII and therefore strongly support an endothelial cell origin of this tumor. (Supported in part by the Manufacturing Chemists Association.)

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USE OF DYE CLEARANCE IN DETECTION OF HEPATOCELLULAR INJURY AMONG VINYL CHLORIDE WORKERS. P. Fortwengler* and C. H. Tamburro. Department of Medicine, Digestive Diseases & Nutrition Section, University of Louisville, School of Medicine, Louisville, Kentucky.

Serious hepatic injury associated with exposure to potentially harmful industrial chemicals and the need for a sensitive method of early detection has been previously noted among vinyl chloride workers. (Creech, J. L., et al, Gastroent., 67:786, 1974). Forty-three of the 1,183 employees of this polyvinyl chloride production plant with biochemical, radioisotopic, and histological evidence of liver dysfunction were evaluated using indocyanine green (ICG) dye clearance, at both the 0.5 mg/Kg and 5.0 mg/Kg dose. Percentage disappearance rates (PDR) were determined by both blood and ear densitometry with automatic computer calculation of PDR. Liver histology was normal in 9, minor changes in 10, fat and minimal fibrosis in 14, portal fibrosis in 4, fibrosis and lobular distortion in 2, and angiosarcoma in 4. Individual biochemical tests correctly indicated hepatic status in 80-83% of the cases with 8% false positives and 10-15% false negatives. Radioisotopic studies alone correctly indicated liver status in only 55%, with 45% either false positive or false negative. The 0.5 mg/Kg dose of ICG correctly indicated liver status in 80% with no false positives, but failed to indicate liver dysfunction in 20%. However, at the 5.0 mg/Kg dose, all cases of liver dysfunction were detected. Ear densitometry values were essentially the same as blood valu s. Ear densitometry with electronic calculator allows a dye clearance d termination over 10 minutes without drawing blood. This data demonstrates an effective means for early detection of industrial chemical hepatotoxicity.

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DECREASED GLUCOSE-6-PHOSPHATASE ACTIVITY IN LIVER IN VINYL CHLORIDE EXPOSED RATS. J.T. Du* and C.H. Tamburro* (SPON: M. Fonda) Dig. Dis. & Nutr. Sect., Dept. Med., Cancer Center, Univ. of Louisville Med. Sch., Lou., Ky. 40201.

Increases in key glycolytic enzymes paralleling hepatoma tumor growth (Heinrich, et al., FEBS Letters, 42:145, 1974) and decreases in key gluconeogenic enzymes prior to and with the development of hepatomas (Isok, et al., Voprosy. Med. Khim 19:568, 1973) have been shown. We exposed adult Sprague-Dawley rats to 10,000-20,000 ppm of vinyl chloride (VC), 4-8 hrs./day, 5 days/wk. for 3-4 wks. (40-140 hrs. exposure) to induce liver injury and angiosarcoma formation. Glucose-6-phosphatase, a key gluconeogenic enzyme in the liver microsomal fraction, decreased 25% over control ($P<0.05$ from combined data). Other microsomal proteins and enzymes related to VC metabolism, i.e., P450, NADPH-cytochrome c reductase and mixed function oxidase were unchanged in the same microsomal fraction with no differences in either mitochondrial cytochrome oxidase or blood transaminases. The decrease in glucose-6-phosphatase is similar to the lower gluconeogenic enzyme findings in hepatomas. This may reflect an increase in glycolysis and ribose-5-phosphate production associated with de novo purine biosynthesis prior to tumor development, inhibition in enzyme synthesis, or increased breakdown due to VC exposure. Work is under way to determine the mechanism. The decrease in glucose-6-phosphatase may be an early biochemical lesion usable as an indicator of liver injury associated with the subsequent development of angiosarcoma. (B.F.Goodrich Grant) Supported by NCI Contract #NO1-CN-55212

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ELEVATED GLUTATHIONE CONTENT, GLUTATHIONE-S-TRANSFERASE AND GLUTATHIONE REDUCTASE IN LIVER OF RATS EXPOSED TO VINYL CHLORIDE Du, J.T.* and Tamburro, C.H.* (SPON: McGeachin, R. L.) Dig. Dis. & Nutr. Sect., Dept. Med., Cancer Center, Univ. Lou. Med. Sch., Lou., Ky., 40232

Vinyl chloride (VC) is believed to be metabolized to chloroethylene oxide (CEO) and chloroacetaldehyde, and detoxified by way of glutathione. Rats were exposed to 28,000 ppm VC, 7 hrs/day, 5 days/wk for 4 and 6 weeks, the activity of glutathione epoxide-S-transferase (GEST) was elevated 30 to 54% over normal control and air control (9.71± 0.68 vs 7.52± 0.97 and 6.30± 0.68) respectively. However, the activity of glutathione aralkyl-S-transferase (GAST) was not significantly elevated until 6 weeks of exposure to VC. The content of reduced glutathione was also elevated 45% in the VC treated group and the activity of the glutathione reductase, the enzyme to regenerate glutathione from the oxidized form was elevated 50%. These results demonstrate that VC exposed rats have the capacity to maintain glutathione reductase activity and glutathione concentration for detoxification. Further, it suggests that the primary route of VC metabolism is initial oxidation to CEO and then detoxification by GEST directly. With longer exposure and probable saturation of the direct route, there is greater rearrangement of CEO to chloroacetaldehyde, and detoxification with glutathione as supported by the delayed induction of GAST (Supported by a grant from Manufacturing Chemists Association).

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Serum Bile Acids (SBA) Screening For Chemical Hepatotoxicity. Gary Liss* and Carlo H. Tamburro* (SPON: William Waddell). NIOSH Robert Taft Laboratory, Cincin., OH. & Liver Research Center, Div. Occupational Health, Depts. of Medicine & Community Health, Univ. of Louisville, Lou., Ky. 40292.

Standard liver tests have limited ability to detect latent liver disease (Environ. Health Perspect. 1981; 41:117-112). Serum bile acids are more sensitive indicators of liver injury. During the medical surveillance of 1000 chemical workers 67 liver biopsies were investigated for histological evidence of chemical injury (Gastroenterology 1979; 77:A33); 15 had chemical liver disease (CLD), 27 nonchemical liver disease (NCLD) & 25 had normal biopsies (NBX). Fasting SBA-cholylglycine (CG) & conjugates of cholic acid (CCA) were studied by ^{125}I-radioimmunoassay in these groups and 416 "normal" workers. Mean ± S.E.M. for CG in the CLD, NCLD, NBX & normal groups were 95.17 ± 28.75, 27.25 ± 4.43, 34.60 ± 7.13 & 14.9 ± 0.88 ug/ml, respectively. Analysis of variance showed significant differences ($p<0.035$) in the 3 biopsy groups; $p<0.001$ for all 4 groups. The CLD values were significantly different in CG levels of the NBX ($p<0.02$). No significant difference in CG levels in NCLD and NBS groups; CCA showed similar results. Fasting SBA showed promise in detecting latent chemical liver injury.

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TISSUE AND URINARY GLYCOSAMINOGLYCANS (GAG) CHANGES IN HEPATIC FIBROSIS C. E. Kupchella, J. O. Jarvis, K. L. Curran, R. A. Greenberg, and C. H. Tamburro Cancer Center and Digestive Diseases and Nutrition Section, University of Louisville School of Medicine, Louisville, Kentucky.

GAG's are felt to play an important role in the collagen fibril formation and collagen bundle stabilization. It has been assumed that hepatic fibrogenesis contributes little to the total body connective tissue and would not be reflected in urinary excretion of GAG degradation or synthetic products. We have previously found, however, that increased urinary GAG's occur in humans with cirrhosis, hepatitis, and hepatic angiosarcoma (Kupchella, C.E., Tamburro, C. H., Clin. Res. 25:329, 1977; Curran, K. L., Kupchella, C. E., Tamburro, C. H., Cancer (in press), 1977). In the present study, changes in urinary and hepatic GAG's were measured in rats with CCL_4-induced hepatic damage. Highly significant increases ($P < .001$) in total GAG's and in the hyaluronic acid (HA), chrondrotin sulfate (CS), and the heparin (H) fractions were found in hepatic tissue after three weeks and persisted through nine weeks of exposure. Histological examination after three weeks showed hepatic necrosis with extensive fatty metamorphosis without histochemical (Trichrome-Alcian Blue-PAS) evidence of fibrosis. Control hepatic tissue GAG levels were 31 ± 5 µg (of uronic acid) per gram of dry defatted liver. CCL_4 treated livers showed a 2 to 4 fold increase in GAG levels; 81.4 µg at 3; 98 µg at 6; and 113 µg/g at nine weeks, respectively. Increases occurred mainly in the CS and H fractions. The urinary CS fraction was significantly ($P < .05$) elevated over the nine weeks of exposure; the HA fraction was elevated during the first two weeks only, and the H fraction excretion appeared to be directly related to CCL_4 injections. These data support our previous observations in humans and demonstrate that urinary GAG excretion pattern changes occur before histochemical evidence of collagen formation and suggest that urinary GAG patterns may be a useful indicator of early hepatic fibrogenesis.

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URINARY CHONDROITIN SULFATE FRACTION PATTERNS IN HEPATIC ANGIOSARCOMA

C. E. Kupchella, K. L. Curran, and C. H. Tamburro. Cancer Center, University of Louisville, Louisville, Kentucky.

This study was undertaken to evaluate the usefulness of urinary glycosaminoglycan patterns in the detection of hepatic angiosarcoma and in monitoring the course of this disease. Glycosaminoglycans extracted from 24 hr urines from 2 patients with angiosarcoma of the liver and from normal controls were separated as cetylpyridinium complexes into "hyaluronic acid," "chondroitin sulfate," and "heparin" fractions. These fractions were further purified by anion-exchange chromatography. The "chondroitin sulfate" fraction isolated from angiosarcoma patients consistently demonstrated an increase in the amount of glycosaminoglycan eluted in 1.25 M NaCl and a concomitant decrease in the 1.5 M NaCl elution peak. This "shift" was apparently related to the course of the disease and was unaffected by chemotherapy. As indicated by the following results, the ratio of 1.25 M to 1.5 M elution peaks may be of value in monitoring the course of angiosarcoma: 2 normal controls, .364 and .316; angiosarcoma (pre-chemotherapy), .843; angiosarcoma (post-chemotherapy), .971; angiosarcoma (advanced), 5.00. Characterization of the 1.5 M and 1.25 M NaCl elution peaks by hyaluronidase susceptibility and comparison with elution patterns reported in the literature suggest that the observed shift was from chondroitin 4 and/or chondroitin 6 sulfate to heparan sulfate. Preliminary data indicate that the excretion pattern in vinyl-chloride-associated liver injury other than angiosarcoma is characteristically different from patterns associated with hepatitis, cirrhosis, or metastases to the liver. These observations may be related to progressive connective tissue proliferation in angiosarcoma

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URINARY GLYCOSAMINOGLYCAN EXCRETION PATTERNS IN CHEMICALLY INDUCED LIVER INJURY AND CANCER C. E. Kupchella*and C. H. Tamburro**, Cancer Center, University of Louisville, Louisville, Kentucky.

Glycosaminoglycans (GAG) are essential compounds of connective tissue (CT) matrix and are increased with CT proliferation. Urinary GAG patterns were studied in 9 individuals with vinyl chloride (VC) induced chemical injury, 2 VC induced angiosarcomas, 8 viral hepatitis, 6 alcoholic cirrhosis, 7 non-hepatic cancers, and 9 controls. Hepatic histological and electron microscopic (EM) studies were obtained in all but normal controls which were studied biochemically, radioisotopically, and physically. Urines were analyzed for creatinine, total GAG, and uronic acid content. GAG levels (ug uronic acid/mg creatinine) in controls were 3.2 ± .4, in VC exposed 4.1 ± 4, and in alcoholic liver disease 5.1 ± 0.8. In contrast, in angiosarcoma they were 7.6 ± 1.6 and hepatic metastasis, 13.8 ± .9. Total GAG's were further separated into hyaluronic acid (HA), chondroitin sulfate (CS) and heparin (H) fractions. Although no significant differences were found in total GAG's 78% (7/9) of VC exposed had positive CS and negative HA and H fractions in contrast to 9% (3/32) of the other cases. Further characterization by anion-exchange chromatography showed a shift in the CS fraction composition. Total GAG's eluted at 1.25 M NaCl, were increased and at 1.50 M NaCl decreased. These changes became more pronounced as the disease developed. Enzyme digestion indicates that 1.25 M fraction is heparan sulfate (HS). EM studies showed increased sinusoidal collagen. Since HS is associated with blood vessels, its increased urinary excretion in early VC injury and angiosarcoma (vascular injury and sinusoidal cell cancer) may be used as an early indicator of chemical injury and liver cancer formation.

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TISSUE AND URINARY GLYCOSAMINGLYCANS IN TRANSPLANTABLE HEPATOMAS. C. E. Kupchella, K. L. Curran, E. Drake, J. Kennedy, and C. H. Tamburro. Cancer Center, and Division of Digestive Diseases and Nutrition, University of Louisville, School of Medicine, Louisville, Kentucky.

The purpose of this investigation was to evaluate: a) the glycosaminoglycans (GAGs) in different behavioral/histological types of intermuscularly transplanted hepatomas, b) GAG patterns in tumor tissue in relationship to degrees of fibrosis and necrosis, c) the GAG changes in the livers of tumor-bearing animals, and d) urinary GAG excretion as a function of tumor growth. Three types of Morris hepatomas, 7777, 5123tc, and 9618A, which differ in rates of growth, metastatic potential, fibrosis, and necrosis, were studied. Urinary and tissue GAGs were extracted as cetylpyridinium complexes and measured as uronic acid. Tissue GAGs were also evaluated histochemically using alcian blue staining with and without enzyme pretreatment. Tumor tissue exhibited four- to six-fold greater GAG levels in the hyaluronic acid (90 ± 10, 91 ± 12, and 111 ± 9 vs. 25 ± 3 µg uronic acid/g dry liver, respectively) and chondroitin sulfate (261 ± 29, 217 ± 51, and 208 ± 22 vs. 47 ± 7 µg uronic acid/g, respectively) fractions than normal liver; the heparin fractions did not differ significantly (31 ± 7, 17 ± 4 and 67 ± 11 vs. 39 ± 10). The livers of tumor-bearing animals exhibited slightly greater hyaluronic acid levels than normal liver. Moreover, increased urinary GAG excretion was evident after two weeks in animals bearing fast-growing tumors. The GAG tissue levels in fast vs. slow-growing tumors were not significantly different. This further supports our previously reported studies of urinary GAG excretion in human hepatic angiosarcoma (Curran, K. L., et al., Cancer 40 (6): 3050-3053) in suggesting that urinary GAG analyses may b useful in the detection, screening and diagnosis of hepatic cancer.

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Name Charles E. Kupchella, Ph.D., Associate Director, Cancer Center

Address 213 MDR Building, University of Louisville, P. O. Box 35260, Louisville, Ky. 40232

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URINARY GLYCOSAMINOGLYCAN PATTERNS IN HUMAN HEPATIC ANGIOSARCOMA, HEPATOMA, AND IN WORKERS AT RISK FOR ANGIOSARCOMA.

K. L. Curran, C. E. Kupchella, J. Sandoz, and C. H. Tamburro. Cancer Center and Division of Digestive Diseases and Nutrition, University of Louisville, School f Medicine, Louisville, Kentucky.

A previous study reported an abberation in glycosaminoglycans (GAGs) eluted from anion-exchange columns with 1.25 and 1.5 M NaCl in the urine of patients with hepatic angiosarcoma. A controlled pilot study examined urinary GAG patterns in workers at risk for angiosarcoma. Six individuals with a history of high vinyl-chloride exposure and documented liver disease were paired with individuals with a high exposure index to vinyl-chloride but n clinical liver disease. Similarly six persons with low exposure but abnormal liver function were paired with low exposure/normal liver function individuals. A 24-hour urine was collected from each individual and the GAGs analyzed by anion-exchange chromatography. Individuals with clinically active liver disease at the time of this study were found to have urinary GAG excretion patterns which were similar to those described in angiosarcoma. No significant differences were found between high vs. low vinyl-chloride exposure or between those with inactive liver disease and those with normal liver function. GAG excretion was also studied in an additional hepatic angiosarcoma and human hepatoma and confirm the reported urinary changes. These findings support the concept that urinary GAGs are increased only in active hepatic disease and may be useful in evaluating the degree of activity at the various stages of liver disease in humans.

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GLYCOSAMINOGLYCAN CHANGES ASSOCIATED WITH HEPATIC TUMORS: THE CONTRIBUTIONS OF REGENERATION AND NECROSIS. C. E. Kupchella, E. M. Secskas,* J. S. Kennedy,* and E. Espinosa*. Cancer Center and Department of Pathology, University of Louisville, School of Medicine, Louisville, Kentucky.

Although glycosaminoglycans (GAGs) have been shown to be elevated in many types of animal and human tumors including hepatic tumors, the cause and significance of these changes in neoplasia are still open questions. Regeneration and necrosis are operative in hepatic cancer and the purpose of this investigation was to evaluate the GAG changes associated with hepatic regeneration and hepatic necrosis. Regeneration was induced in male Sprague Dawley rats by partial hepatectomy and hepatic GAGs were evaluated at 4, 8 and 12 days post operatively. Necrosis was induced by: a) ligating the medium lobe, b) by resecting and placing median lobes in the peritoneal cavity and c) by resecting median lobes and incubating them *in vitro* in sterile saline. Analyses were carried out after 5 days of treatment. While regenerating livers exhibited GAG levels that were not statistically different from sham operated controls or non-operated controls, *in vivo* necrosis was accompanied by 3-4 fold increases in tissue GAGs. These data suggest that necrosis may make a substantial contribution to the elevated GAG levels found in some tumors.

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Early Detection of Disease in Individuals Exposed to Vinyl Chloride.

Richard Greenberg, MD. and Carlo Tamburro, MD. University of Louisville, Louisville, Kentucky

This is a collaborative comparison study of the effectiveness of ultrasound, nail bed capillary study and urinary glycosaminoglycan excretions in vinyl chloride workers with biochemical dysfunction or histopathologically documented injury.

It represents an effort to detect early morbidity in vinyl chloride workers. Included is Dr. Maricq's method of investigating capillaries of the middle and distal phalanges of the fingers, including the nailfold, as a screening test for early changes from vinyl chloride exposure. Another new method is Dr. Taylor's "grey scale" ultrasonographic method which has not yet been widely adopted for diagnosis of liver disease. The University of Louisville group reported that the first indicator of liver changes occurred in the vascular sinusoids and was reflected by the appearance of mucopolysaccharides in the urine.

The results of the massive clinical study in Louisville should soon be able to indicate whether more sensitive criteria of early disease may be forthcoming, especially from the liver function testing. The special value of the capillary and ultrasound method remain doubtful at this time for early detection of the effects of exposure to vinyl chloride.

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Computer Assisted Morphologic Quantitation of Collagen in Human Liver Biopsies.

Name(s) of authors, institutional affiliations and addresses:
G. H. Barrows, M.D., M.J. Joyce, G.R. Schrodt, M.D., R. Greenberg, Ph.D., C.H. Tamburro, M.D. Departments of Pathology, Epidemiology and Biostatistics, and Medicine, University of Louisville, Louisville, Kentucky 40202.

Underline name of author to notify and give Telephone (Area) 502-588-5341 Ext.
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Abstract — Double space.

Because unfixed liver is rarely available for analytical studies, appraisal of the extent of collagen must be made from appropriately stained sections of liver biopsy material leading to judgemental as well as sampling error. We have developed a computer assisted morphologic technique to evaluate the distribution of collagen in liver biopsy material. We examined multiple biopsy samples from patients dying suddenly with no history of liver disease to establish normal values. A Hewlett-Packard 9864-A digitizer and 9815-A micro computer were used to quantitate areas of trichrome stainable collagen within the biopsy samples. Random area selection and statistical analysis proved to be important considerations in determining the experimental model and these factors were readily incorporated into the calculator program. Stainable collagen estimates in normal liver varied from 0 to 6.1% (mean = 1.25%) in patients with no evidence of liver disease and showed considerable variation even in different biopsies from the same patient. As anticipated, subcapsular biopsies had more collagen than deep biopsy; however, the specimens from several deep biopsies often had significant variation in collagen content. Differences in central, mid-zonal and portal collagen could be obtained using this method. The computer assisted morphological technique for evaluating hepatic collagen appears to be readily adaptable to clinical studies. This study of normal liver [illegible] considerable care must be taken before the clinical diagnosis of significant fibrosis can be made.

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EARLY HEPATIC HISTOLOGICAL ALTERATIONS AMONG CHEMICAL (VINYL MONOMER) WORKERS. C.H. Tamburro, L. Makk, and H. Popper, Division of Digestive Diseases and Nutrition and St. Anthony Hospital, University of Louisville, Louisville, Ky., and Stratton Laboratory for Study of Liver Disease, Mt. Sinai School of Medicine, New York, N.Y.

Industrial vinyl chloride exposure is associated with hepatic subcapsular, portal, and perisinusoidal fibrosis and hyperplasia of both sinusoidal cells and hepatocytes. Earlier histological studies, mainly on autopsy material, indicated that focal mixed hyperplasia (of hepatocytes and sinusoidal cells) is the early histologic alteration indicative of exposure. To substantiate this observation and its potential use in screening workers, liver biopsies from 55 persons were investigated in double blind duplicative fashion; 34 were workers exposed to chemicals with hepatic biochemical abnormalities, and 21 were a comparison group composed of 8 non-exposed persons and 13 exposed workers without hepatic abnormalities who had biopsies for non-liver related reasons. Of the exposed workers with abnormalities, 12 (35%) had a hepatic lesion consistent with exposure; 6 (18%) had focal hepatocytic hyperplasia; 6 (18%) had focal mixed hyperplasia. In contrast, only 5 of the comparison group (2 exposed and 3 non-exposed persons) had similar findings; 1 exposed and 2 non-exposed had only focal hepatocytic hyperplasia. The other exposed worker had focal mixed hyperplasia as did the other non-exposed individual who, subsequent to the biopsy, was found to have angiosarcoma. Thus, only 19% of the comparison group demonstrated hepatic lesions with chemical exposure, half of whom had worked with chemicals. Of the 55 biopsies, 22 were re-read double blindly 2 to 4 times over a 2-year period. In addition, 9 of these individuals had second or third biopsies also read double blindly. Duplicate readings were identical in 86%, 9% had 1 positive and 1 questionable reading, and only 5% were read differently. Focal hepatocytic hyperplasia, in addition to the previously described mixed hyperplasia, appears to be the earliest identifiable changes consistent with chemical exposure and seem to be precursors of angiosarcoma. They are useful in the screening of chemical workers.

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Chloroacetaldehyde-induced Damage to Bacillus subtilis. A.D. Laumbach, U.N. Streips* and J.L. Wong. Bureau Foods, FDA and U. Louisville, Louisville, Ky.

Chloroacetaldehyde (CAA), a proposed metabolite of vinyl chloride metabolism, has been shown to be mutagenic in the Salmonella assay system, and to specifically inhibit the growth of a recombination-deficient mutant of Bacillus subtilis (Ellmore, et al BBA, 442: 405, 1976).

We have examined further the biological activity of this compound. First, CAA caused a significant increase in induced mutations to streptomycin resistance. Using B. subtilis 168, the relative mutation frequency (treated 5mM CAA/control) was 13.6, while using an her mutant of B. subtilis the frequency was 14.2. Secondly, a survey of the available repair-deficient mutants of B. subtilis revealed, that the only strains sensitive to CAA were of the rec-genotype. Furthermore, the several rec-mutants, show three types of response to CAA. Strong killing by CAA (inhibition of growth of 10 mm or more) was exhibited on the recA, recB, rec-4 (GSY1616), and recD27 (GSY1627) strains. Intermediate killing (3mm-8mm) was shown with strains bearing the recE, recF, and rec-13 (BD246) loci. All other strains examined (recC, recH, mtc-41, uvr, her, polA, as well as several repair-proficient cultures) grew in the presence of 1.1M CAA.

These studies are part of an ongoing program sponsored by the MCA to determine the molecular basis for vinyl-chloride induced carcinogenesis.

Chloroacetaldehyde-induced Damage to Bacillus subtilis. A.D. Laumbach, U.N. Streips*, and J.L. Wong. Bureau Foods, FDA, and University of Louisville, School of Medicine, Health Sciences Center, Louisville, Ky. 40232 (USA).

Chloroacetaldehyde (CAA), a proposed metabolite of vinyl chloride metabolism, has been shown to be mutagenic in the Salmonella assay system, and to specifically inhibit the growth of a recombination-deficient mutant of Bacillus subtilis (Ellmore, et al., BBA, 442:405, 1976).

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These studies are part of an ongoing program sponsored by the Manufacturing Chemists Association to determine the molecular basis for vinyl-chloride induced carcinogenesis.

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INHIBITION OF INTERFERON INDUCTION AS A SCREEN FOR THE CARCINOGENIC POTENTIAL OF CHEMICALS. Gerald Sonnenfeld, Mary Carol Barnes, and Uldis N. Streips, Department of Microbiology and Immunology, Univ. of Louisville School of Medicine, Louisville, Kentucky 40232.

Type I interferon was produced after challenge of mouse embryo fibroblasts with Newcastle disease virus. This production of interferon could be sharply reduced by pretreatment of the cells with the known carcinogens benz-a-pyrene, aflatoxin, 2-aminofluorine, and the #4 fraction of tobacco smoke condensate. This finding suggested that inhibition of interferon induction (I-I-I) may serve as an indicator for the carcinogenic potential of chemicals. We tested the screening potential of this assay by testing two chemical analogs, methylmethanesulfonate (MMS) a carcinogen and mutagen and ethylmethanesulfonate (EMS) a non-carcinogenic mutagen. Both of these chemicals are active in the Ames Salmonella assay but can be separated in "SOS" induction assays such as the Comptest. In the I-I-I assay, MMS was highly positive while EMS showed no inhbition of interferon induction. Thus, the I-I-I assay appears to have the potential to screen for carcinogens. Preliminary results using the carcinogen chloracetaldehyde and its non-carcinogenic analogs chloroethanol and chloroacetic acid support this idea. Following further verification of the discrimination potential of the I-I-I assay, this test could become an integral part of a comprehensive battery of tests for chemical carcinogenicity.

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Properties of 14 Week Maintenance Cultures of PLC/PRF/5 Cells. P.B. JOHNSTON*, E. Espinosa, S. Chia and S. Caple. Univ. of Louisville, Louisville, Ky 40232

This established cell line was originally described as being short lived within a given passage, with considerable cell loss by day 6 to 8. However, after 80 passages, we found that culturing in glass roller bottles at 0.25 RPM allowed maintenance for at least 14 weeks with cells exhibiting excellent morphology without sloughing. After treating monolayers with Hoechst 33258 DNA stain, intense cytoplasmic fluorescence was seen in a proportion of the cells, possibly a function of the subviral hepatitis B genome. HBsAg of culture supernates was monitored and solid phase RIA assay revealed 31 to 47 ratio-units/0.2 ml after 2 to 14 weeks in medium with 10% fetal calf serum. The 14 week old cultures were extremely well adapted to maintenance in serum free medium for 14 additional days when supplementing with 100 ng/ml of insulin, in contrast to 1 week cultures which survived well for only 5 days without serum. The HBsAg titers of supernates from these 1 week or 14 week cultures, were very similar to the previous harvests in 10% serum. In addition, both young and 14 week cultures were similar in their rate of acid production; and in that both elaborated serum albumin, fibrinogen, transferrin and alpha-2 macroglobulin, the proteins tested to date.

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LIVER-SPECIFIC F ANTIGEN IN TRANSPLANTABLE HEPATOMAS HAVING DIFFERENT GROWTH RATES. Enrique Espinosa, Sue Chia*, Sam Caple* and Charles Kupchella*. Dept. Pathology and Cancer Center, Univ. of Louisville School of Medicine, Louisville, KY 40232

In the course of studies of antigenic changes associated with liver neoplasia we measured the relative concentration of liver-specific F antigen in fast (7777), slow (9618A) and medium (5123tc) growing Morris hepatomas in comparison with its concentration in normal adult rat liver. F antigen was solubilized from each tissue by aqueous homogenization. The supernatant containing the antigen was then tested by a double diffusion dilution assay capable of detecting a minimum of 1-2% the concentration of antigen present in normal rat liver. The F antigen antiserum used was prepared according to Fravi and Lindenmann by immunizing CBA mice with BALB/c mouse liver extract. F antigen was undetectable in the fast growing hepatoma and ranged from less than 2% to 10% of the normal liver concentration in the slow growing one. The medium growing hepatoma showed similar or higher concentration than normal rat liver. Thus, the level of F antigen in these tumors does not appear to correlate with their rates of growth. (Supported in part by the Manufacturing Chemists Association)

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TWO LIVER ANTIGENS UNDETECTABLE IN A FAST GROWING LINE OF TRANSPLANTED HEPATOMA (MORRIS HEPATOMA 7777). Enrique Espinosa, Sam Caple*, Charles Kupchella* and Sue Chia*.
Dept. Pathology and Cancer Center, Univ. of Louisville School of Medicine, Louisville, KY 40232

During investigations of antigenic changes in liver tumors we examined fast (7777), medium (5123tc) and slow (9618A) growing Morris hepatomas for presence of liver antigens. Analyses of normal liver and hepatoma tissue saline extracts were performed by immunodiffusion methods using rabbit antiserum to rat liver extract absorbed with pooled normal rat plasma. Hepatoma 5123tc and 9618A gave immunoelectrophoretic patterns similar to normal liver. In contrast, hepatoma 7777 showed 2 arcs of precipitation missing. This was confirmed by the finding that after absorption of antiserum with hepatoma 7777 two lines of precipitation given by normal liver extract persisted whereas absorption with extract of normal liver, hepatomas 5123tc or 9618A abolished all lines of precipitation. The liver antigens found to be absent in hepatoma 7777 were characterized as proteins relatively unstable to heating and acid pH and unrelated to liver-specific F antigen. In Sephadex G-200 and Bio-Gel A-5m columns these antigens eluted as proteins of approximately 51,000 and 240,000 daltons respectively. The fact that 7777 is the fastest growing and least differentiated of the tumors studie suggests a possible functional relationship between the absent antigen and these properties. (Supported in part by the Manufacturing Chemists Association)

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OXIDATIVE AND DETOXIFYING ABILITY OF LIVER MESENCHYMAL PARENCHYMAL CELLS IN THE METABOLISM OF XENOBIOTICS

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The metabolism of xenobiotics, by the liver most often volves oxidation via mixed function oxidase (MFO) and s hydryl detoxification with glutathione. Hepatic toxici and carcinogenicity are modified by these two pathways. Vinyl monomer (e.g. vinyl chloride) exposure is associ with liver parenchymal cell (hepatocytic cell, HC) toxi and mesenchymal cell (sinusoidal cell, SC) carcinogenic even though the hepatocyte is the main cell for xenobic oxidation. The metabolic ability of these two cell gro was assessed by isolation of SC using pronase digestion and HC using collagenase profusion. Viability assessed 90% by trypan blue exclusion and cross contamination es mated (< 1%) by pryuvate kinase activity. MFO activity (demethylation benzphetamine), glutathione transferase (E) using (1,2, epoxy-(P-nitro nitrophenoxy) propane as substrate, glutathione transferase A,C and E (A) using nitrobenzyl chloride) as a substrate and glutathione tase (GR), were determined in subcellular isolated H SC fractions. Results based on protein content (N mole min/mg protein) were:

	E	A	GR	MFO
SC	42.9 ± 17	18.5 ± 6	47.4 ± 2	7.49 ± 2.
HC	82.5 ± 11	215.0 ± 61	65.0 ± 7	16.0 ± 5

and activity in mole/10^6 call were:

	E	A	GR	MFO
SC	1.16	0.5	1.28	0.026
HC	65.6	220.	52.	4.7

These results demonstrate both the greater average, as v as total, cellular ability of HC to oxidize xenobiotics. More importantly, it demonstrates an increased ability t detoxify the xenobiotic's toxic metabolites. This great ability may account for less severe HC injury and play a important role in preventing HC malignant transformatior In contrast, SC ability to oxidize but its lesser abilit to detoxity xenobiotic may account for SC's potential fc malignant transformation.

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Toxic, Carcinogenic & Safe Exposure Levels in Vinyl Chloride Induced Hepatic Angiosarcoma. Carlo H. Tamburro* and Richard A. Greenberg* (SPON: William Waddell). Depts. of Medicine & Community Health, Univ. of Louisville, Lou., Ky. 40292.

Vinyl chloride (VC) induced hepatic angiosarcoma (HA) has rapidly decreased since 1974. The relationship of latency & duration of exposure was studied in 21 North American (NA) cases and 62 worldwide (WW) reported cases; 92% of NA & WW cases had long exposures (LE) (10-33 yrs); 7 cases (8%) had short exposures (SE) (3.5-7 yrs). The mean duration in both LE & SE groups was 18.5 yrs. with 2 peaks at 15 & 25 yrs. Deaths from HA began in 1955, peaked in 1976 & rapidly fell thereafter. HA peaked in Canada (CA) in 1973 and USA 1974, France (FR) 1976, & Germany (GE) 1978. Operations began in CA in 1941 & USA 1939-44; FR 1941-57, & GE 1952-60. Exposure levels reported in 1940's of ∿2000 ppm; in 1950's ∿200-500 ppm, in early 1960's ∿50-500 ppm, late '60's & early '70's ∿50-250 ppm, & 1-10 ppm after '74. No relationship was found in total average exposures (TAE) range of 50 ppm; 200-1000 ppm range showed a direct relation of lower TAE to shorter latency, while higher TAE (1000-2000 ppm) had longer latencies. The data suggests VC has a biological threshold level, an optimum carcinogenic level and subcarcinogenic toxic level for occurrences of HA.

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PREPRINTS

EARLY HEPATIC HISTOLOGICAL ALTERATIONS AMONG CHEMICAL (VINYL MONOMER) WORKERS

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ABSTRACT

Earlier histological studies of industrial vinyl chloride exposure, obtained mainly on autopsy material, indicated that focal mixed (hepatocytes and sinusoidal cells) hyperplasia is the earliest histological alteration indicative of exposure. To substantiate this observation and its potential use in screening workers, 93 liver biopsies from 78 persons were investigated in double blind duplicative fashion; 35 were exposed chemical workers with hepatic screening tests abnormalities, and 13 were exposed workers without hepatic abnormalities who had liver biopsies for non-liver related reasons. A comparison group consisted of 30 individuals who were not chemical workers, but had liver biopsies for non-hepatic related reasons during the same time period. Of the exposed workers, 23 (48%) had a hepatic lesion consistent with exposure; 17 (35%) had only focal hepatocytic hyperplasia; six (13%) had focal mixed hyperplasia or more advanced lesions. In contrast, only five of the comparison group had similar findings; four (13%) had only focal hepatocytic hyperplasia and one (3%) had focal mixed hyperplasia and sinusoidal dilitation. On a subsequent biopsy, this individual was found to have angiosarcoma and a history of using hair spray containing vinyl chloride as propellant. Ten individuals had 28 multiple biopsies also read double blindly, and 10 individuals had 23 readings of the same biopsy; 21/23 (91%) duplicate readings and 27/28 (96%) multiple biopsy readings in the same individuals were identical. Only 18% of either duplicate and/or multiple biopsy readings had disagreements in their biopsy assessment. Focal hepatocytic hyperplasia, in addition to the previously described mixed hyperplasia, appears to be the earliest identifiable change consistent with chemical exposure and both lesions are present prior to the development of angiosarcoma. These lesions can be consistently identified and are useful in the screening of chemical workers for evidence of exposure.

INTRODUCTION

Industrial vinyl chloride and other vinyl monomer exposure is associated with various hepatic histological abnormalities. These include subcapsular, portal, and perisinusoidal fibrosis as well as hyperplasia of both hepatocytes and sinusoidal cells. Early histological studies, mainly on autopsy material, had shown focal mixed hyperplasia (hyperplasia of hepatocytes and sinusoidal cells) to be an early histological alteration associated with vinyl chloride exposure (1,2,3). To substantiate this observation and determine its potential use in medical surveillance screening of the exposed workers, liver biopsies from 78 individuals were studies in duplicate blind fashion to determine 1) if these early histological findings were associated with extensive vinyl chloride exposure, and 2) if these histological findings occur in non-exposed populations or were associated with other diseases.

MATERIALS AND METHODS

Liver biopsies from 48 vinyl monomer chemical workers with and without hepatic biochemical abnormalities were investigated. All 48 chemical workers had had hepatic biochemical studies performed on an annual or semi-annual basis. These included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), total bilirubin (TB), gamma glutamyl transpeptidase (GGT), prothrombin time (PT), and indocyanine green clearance (ICG). In addition, history and physical examinations, liver-spleen scans, and abdominal plain films had been performed annually. Thirteen of the chemical workers had hepatic biopsies for non-liver related reasons while 35 had biopsies performed because of screening biochemical and/or liver-spleen radioisotopic scan abnormalities.

A group of 30 individuals who were not chemical workers but had undergone abdominal surgery with liver biopsies, were used as a comparison population. All of the comparison population's liver biopsies were performed during the same three-year period, at the same hospital, by members of the same medical-surgical team, with the informed consent of the individuals. This group had similar hepatic biochemical studies with the exception of the GGT and ICG clearance. None had a history of extensive exposure of working with halogenated hydrocarbons, although one patient had a history of household contact with vinyl chloride via commercial home products.

Pathology

Earlier experiences with autopsy and animal material from vinyl chloride associated angiosarcoma have recognized certain hepatic lesions in the non-tumorous area of the liver parenchyma ( ). They include:

1. Hepatocytic changes consisting of foci of enlarged hepatocytes with increased amount of cytoplasm, and large hyperchromatic nuclei (Figure 1). These cells are intermixed with hepatocytes of normal and smaller sizes and create focal, indistinct nodular areas (Figure 2a). These areas are often associated with very slight increased sinusoidal dilitation and focal increase in the reticulin framework illustrated best by silver impregnation (Figure 2b).
2. Sinusoidal lining cell changes consisting of an increase in cell number associated with nuclear changes, particularly conspicuous in areas of sinusoidal widening. This proliferation of sinusoidal cells involves (a) Kupffer cells (macrophages) with PAS-positive granules; (b) fibroblasts with elongated, almost rectangular nuclei with loose vesicular chromatin patterns and inconspicuous

nucleoli with a moderate amount of cytoplasm which reacted on PAS staining and persisted after diastase reaction. These cells are interpreted as activated fibroblasts, are associated with an increase in fat storing sinusoidal cells and augmented perisinusoidal fibrosis tissue (Figure).

3. Fibrotic changes consisting of an excell of irregular connective tissue in the periportal zone, frequently arranged around proliferating bile ductules, and focal thickened subcapsular accummulation of corrective tissue into the parencyma (Figure 7).

These histological lesions are believed to be the three earliest hepatic findings associated with vinyl monomer chemical injury. The hepatocytic changes are referred to as focal hepatocytic hyperplasia or FHH. The sinusoidal cell changes (sinusoidal cell hyperplasia) associated with the hepatocytic changes are referred to as focal mixed hyperplasia or FMH.

For purposes of this study, early fibrotic changes were not classified since the majority of liver biopsies were of needle type, which could not be evaluated for subcapsular changes.

The liver biopsies were coded and read blindly to identify (1) histological evidence of hepatic disease; (2) any characteristics of non-chemical injury excluding steatosis; (3) steatosis with or without fibrosis and, (4) evidence of chemical injury identified by the following histological findings: a) focal

hepatocellular hyperplasia; b) focal increased reticulum associated with the hepatocytic hyperplasia; c) perisinusoidal fibrosis; d) focal hyperplasia; e) focal mixed hyperplasia associated with increased reticulum deposition; f) sinusoidal cell activation; g) sinusoidal dilitation; h) portal and capsular fibrosis; i) sinusoidal dysplasia; and, j) hepatic angiosarcoma.

The presence of focal hepatic cellular hyperplasia with focal increases in reticulum, in the absence of other evidences of hepatocellular disease or steatosis, were considered the minimum presumptive evidence of chemical exposure. The presence of focal mixed hyperplasia with increased reticulum and/or perisinusoidal fibrosis was considered evidence of chemical injury of a more advanced stage. Sinusoidal cell activation and dilitation of the sinusoids were considered evidences of chemical injury of an even further stage of advancement. The presence of subcapsular fibrosis, (on wedge biopsies only) and/or increases in both portal and sinusoidal fibrosis, with sinusoidal cell activation and sinusoidal dilitation was considered the most advanced stage of chemical injury characteristic of vinyl chloride or vinyl monomer exposure. Finally, sinusoidal cell dysplasia and/or evidence of malignant transformation, constituted definitive evidence of chronic vinyl chloride exposure and the terminal stages of injury.

Work and exposure histories were available for all the chemical workers. The work histories provide a total and average exposure data for 22 chemicals based on rank order exposure estimates. These exposure work histories consisted of an estimate of exposure to each of 22 different chemicals for all of the 350 job classifications used since the start of the chemical plant. Each of the chemicals exposure was ranked on a scale of zero to six for each of the 22 chemicals. These exposure ranking estimates were done separately

for each job for each year based on the best available information and industrial expertise. Each worker's cummulative exposure to each of the 22 chemicals was based on the various job classifications he/she held during their employment. A detailed explanation of this system and an actual in-the-field demonstration of the ability of this system to identify work-related liver injury (angiosarcoma) has been published elsewhere (4,5).

RESULTS

Thirty-seven percent (13/35) of the exposed workers with screening test abnormalities had hepatic lesions consistent with chemical exposure. Among the exposed workers without biochemical abnormalities 23% (3/13) had hepatic lesions consistent with chemical exposure. In the non-worker comparison group none of those with normal biochemical screening tests and 17/19 (89%) with abnormal biochemical screening tests had hepatic lesions consistent with chemical exposure. Of the remaining two, one had focal hepatocellular hyperplasia; the other had focal mixed hyperplasia and early peliosis hepatis. This latter individual was later found to have angiosarcoma possibly from vinyl chloride hair spray exposure. The former individual had no history of chemical exposure and/or alcohol consumption, but did have hepatobiliary tract disease in the form of biliary stones. Table 1 lists the hepatic lesions found in those with and without biochemical abnormalities for the entire group.

Reproducibility

An evaluation of the reproducibility of these biopsy readings was carried out in 17 individuals who had 32 biopsies. Seven individuals had only one biopsy (2 needle, 5 wedged) and 10 individuals had their biopsies read in duplicate. Four had needle biopsies with 9 readings and 6 had wedge biopsies with 14 readings (Table 2).

A comparison of the reproducibility of the biopsy readings is shown in Tables 3, 4 and 5. Table 3 illustrates the high degree of consistent readings between multiple biopsies in the same individual. In only one individual was one of the four biopsies read differently--the wedge was read differently from the three needle biopsies.

Table 4 makes a comparison between readings of multiple needle biopsies or multiple wedge biopsies from a single individual. In these cases there was consistent agreement in all readings.

Table 5 illustrates the consistent readings of the same biopsies over the three-year period. All the needle biopsy duplicate readings and 12 of the 14 wedge duplicate readings were the same.

At least 96% (43/45) of all readings, whether duplicate or from multiple biopsies, provided reproducible and consistent findings.

Correlation of Histological Lesions With Exposure

Biopsies from all the chemical workers were divided into three categories on the basis of final diagnosis:

1) individuals with no evidence of histological liver disease (N)
2) individuals who had liver disease but no evidence of chemical injury (LD)
3) individuals who had chemical liver injury identified by the histological criteria specified (CLI).

A comparison was made between the average vinyl chloride exposure ranking of each worker and his/her final histological diagnosis. The exposure rankings were not known to those making the final histological determination at any time during the three year period. As illustrated in Figure 6 the group of workers with evidences of chemical liver injury had the highest percentage of individuals with the highest ranking of exposure to vinyl chloride. Previous studies of

this worker cohort had shown that all the cases of angiosarcomas had an average vinyl chloride exposure ranking (AER) of 3.5 or greater. AER's of 3.5 or greater occurred in 45% of the CLI group. In contrast, those with liver disease (LD) alone, only 22% had a vinyl chloride exposure rating of 3.5 or greater and in the chemically exposed workers with no evidence of liver disease, 32% had a rating of 3.5 or greater.

Biochemical Studies

A study of the biochemical abnormalities among the two histological groups of chemical workers with evidence of liver disease is illustrated in Figure 7. The five screening tests with the best degree of sensitivity and specificity in identifying latent liver injury were reviewed. Indocyanine green clearances, at the 0.5 mg/kg dose, provides the most sensitive and the most specific of the laboratory screening study, followed by the alanine amiontransferase and aspartase aminotransferase, gamma glutamyl transpeptidase and alkaline phosphatase (6). However, specificity for chemical injury appeared to be associated more with abnormalities in the alkaline phosphatase, which was far more frequently abnormal in workers with chemical liver disease than in those with non-chemical liver injury. In contrast, the ALT, AST, GGT, and ICG were more frequently abnormal in those workers with liver disease of a non-chemical origin.

DISCUSSION

Figure 8 provides the most likely progression in the development of vinyl chloride and other vinyl monomers hepatic injury and cancer development.

These findings confirm those previously reported by Thomas and Popper (2) in their original pathological studies of vinyl chloride associated hepatic angiosarcoma and hepatic injury. Focal hepatocytic megalocytosis associated with focal increases in reticulum structure, in the absence of other evidence

of acute or chronic injury, appear to be the earliest change associated with chemical exposure. Subsequently, there occurs perisinusoidal fibrosis associated with a proliferation of fibroblasts and/or its precursor cell, the Ito cell. In later stages sinusoidal cell activation occurs involving both the macrophagic and endothelial lining cells. These changes are associated with focal sinusoidal enlargement. In the late stage mixed hyperplasia (hepatocyte and sinusoidal cells) becomes prominent, followed by sinusoidal cell dysplasia with palasading of cells, and finally malignant transformation.

Our data demonstrates that focal hepatocytic hyperplasia is the earliest consistently identifiable histological finding in industrial workers exposed to vinyl monomer chemicals. The very limited occurrence of these findings in non-chemical workers and the fact that focal mixed hyperplasia, a more advanced lesion, was only found in chemically exposed workers and in a non-chemical worker with vinyl chloride exposure (via hair spray), adds further confirmation that these lesions are related to prolonged or repeated periods of chemical exposure.

Whether these lesions should be viewed as precursors to the development of cancer or only as an early reflection of the degree and duration of chemical exposure cannot be determined at this point in time. The unchanging and stable status of screening studies over a seven year follow-up period and the histological similarities among the serial biopsies of these workers suggest that these early lesions are non-progressive when the affected individuals are removed from exposure (7).

The presence of sinusoidal cell dysplasia, however, has been associated with the later development of angiosarcoma as illustrated in one of our cases and may represent a true precancerous or a non-reversible lesion.

The reproducibility in identifying these histological lesions is quite high. Under double blind conditions there was consistent histological identification of chemical injury in both duplicate readings of single biopsies as well as single readings of multiple biopsies in the same individual. Inconsistency appears to occur more frequently among the duplicate readings of wedge biopsies and between wedge and needle biopsies than between duplicate readings of needle biopsies or readings of multiple needle or wedge biopsies in the same individual. This may be due to the increased time and attention needed for wedge section review in order to determine the absence or presence of these lesions anywhere in the specimen. There are, however, some pitfalls that require caution. The quality of the histological preparation is of vital importance. Technical artifact, excessive thickness, torn or fragmented specimens will greatly reduce the ability to correctly identify these lesions. The use of silver impregnation for assessment of the reticular structure is very useful in verifying focal areas of hepatocellular hyperplasia. The presence of fatty infiltration and/or chronic disease (hepatitis, granuloma, etc.) may prevent the identification of these lesions. We were, however, able to correctly identify the presence or absence of these early lesions in the six individuals who also had a concomitant granulomatous process (four individuals with granuloma were repeatedly read as negative and two individuals with granuloma were repeatedly read as positive for chemical liver injury).

The more frequent elevation of alkaline phosphatase in those individuals with histological evidence of chemical injury may provide some guidance in sequential uses of laboratory screening tests. During the first phase, tests with the greater sensitivity for identifying liver injury, such as the ICG and ALT can be used. Positive results can be followed up by using tests of greater specificity, such as the alkaline phosphatase, to further identify those at high risk of chemical injury.

CONCLUSIONS

Focal hepatocellular hyperplasia, in addition to the previously described focal mixed hyperplasia, appear to be the earliest identifiable changes consistent with chemical exposure. These histological lesions are useful in identifying high risk exposed chemical workers as part of a medical screening surveillance program. Biochemical studies may help identify which individuals warrant a liver biopsy for further identification of the cause of the liver abnormalities.

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OXIDATIVE AND GLUTATHIONE-RELATED DETOXIFYING ENZYME CAPABILITIES IN HEPATOCYTES AND NONHEPATOCYTES OF RAT LIVER

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ABSTRACT

Liver is the main organ in the body with the capacity to metabolize xenobiotics. Mixed function oxidase (MFO) and glutathione transferase(s) are the major enzymes involved. The activities of MFO (demethylation of benzphetamine), glutathione transferase(s) (glutathione S-epoxide transferase, GEST, 1,2-epoxy-[p-nitrophenoxy] propane as substrate; and glutathione S-aralkyl transferase, GAST, p-nitrobenzyl chloride as substrate), and glutathione reductase (GR) were determined in the subcellular fractions of isolated nonhepatocytes and hepatocytes. The nonhepatocytes were isolated by pronase digestion and the hepatocytes by collagenase perfusion. The viability of the cells were assessed by trypan blue exclusion and shown to be over 90% viable in the hepatocyte and nonhepatocyte fractions. Cross-contamination of isolated cells was estimated by the relative distribution and specific activities of the L- and M2-type pyruvate kinase isoenzymes and was found to be insignificant. The specific activity based on protein content showed that the nonhepatocytes had about 7% GAST activity, 50% GEST and MFO activities, and 73% GR activity compared to hepatocytes. Based on activities per million cells, the nonhepatocytes had approximately 1/450 GAST activity, 1/100 MFO activity, 1/60 GEST, and 1/40 GR activity as compared to hepatocytes. Based on distribution of enzyme activity in hepatic cells per gram liver, the nonhepatocytes had less than or equal to one hundredth the activities of MFO, GAST, GEST, and GR as compared to hepatocytes. This study demonstrates that the nonhepatocytes have the capacity to metabolize and detoxify xenobiotics but that they are significantly less capable than the hepatocytes in oxidation and glutathione-related detoxification.

INTRODUCTION

Liver is the main organ in the body in regard to the metabolism of xenobiotics including drugs and carcinogens. Hepatocytes are thought to be the cells that are mainly responsible for this metabolism, but little is known about the role of nonhepatocytes. These cells, mainly endothelial and Kupffer cells, because of their anatomical location may interact with xenobiotics before they even enter hepatocytes. Thus, it is important to compare nonhepatocytes to hepatocytes as to their enzymatic capability to oxidize and detoxify xenobiotics. In this study, we concentrated on the enzymes for oxidation (mixed function oxidase, MFO) and glutathione-related detoxifying enzymes (glutathione S-epoxide transferase, GEST; glutathione S-aralkyl transferase, GAST; and glutathione reductase, GR) in the microsomal and soluble fractions of isolated nonhepatocytes vs. hepatocytes as well as in whole liver tissue.

MATERIALS

Oxidized and Reduced Glutathione (GSSG and GSH respectively), Metrizamide, Bovine Serum Albumin (BSA Cohn Fraction V), NADPH, ADP, NADH, Phosphoenol pyruvate (PEP), DNAse, Fructose 1,6-diphosphate and Lactate dehydrogenase (pyruvate kinase free) were obtained from Sigma Chemical Co., St. Louis, MO. Gey's Balanced Salt solution was purchased from GIBCO, Grand Island, NY. 1,2-Epoxy-3-(p-nitrophenoxy) propane from Eastman Kodak Co., Rochester, NY., p-nitrobenzyl chloride from Matheson, Coleman and Bell, Norwood, OH. Benzaphetamine Hydrochloride was a gift from The UpJohn Co., Kalamazoo, MI. Collagenase Type II was purchased from Worthington Biochemical Crop., Freehold, NJ. Pronase E was purchased from E. Merck, Darmstadt, Germany. All other chemicals were reagent grade and were obtained from commercial sources. Double distilled water was used throughout the experiment.

Tests for Cell Purity and Integrity: The purity and integrity of cells isolated were tested by light microscopy (phase contrast), by trypan blue exclusion, and by the relative distribution and specific activities of the L- and M_2-type pyruvate kinase isoenzymes. The M_2-type pyruvate kinase (in the nonhepatocytes) is not activated by the addition of fructose-1,6-diphosphate but the L-type pyruvate kinase (solely in hepatocytes) is activated 11-fold (5).

Cell Homogenization and Fractionation: A Polytron homogenizer was used at setting 5 for 60 sec to obtain cell homogenates. The homogenate was then centrifuged at 8000 g for 10 min in a refrigerated Sorvall centrifuge. The supernatant was centrifuged at 100,000 g for 60 min at 4°C in a Beckman ultracentrifuge with a swinging bucket rotor. The rotors were equipped with microadaptors to enable the centrifugation of samples of 1 ml or less.

Enzyme Assays: The rate of GSH conjugate formation was determined for glutathione S-transferases. P-Nitro benzyl chloride was the substrate for GAST with $E=1900\ M^{-1}cm^{-1}$ at 310 nm; 1,2-epoxy-(p-nitrophenoxy) propane was the substrate for GEST with $E=510\ M^{-1}cm^{-1}$ at 360 nm. Glutathione reduc

tase was determined by the reduction of oxidized glutathione, GSSG, as measured by the disappearance of NADPH (6). Mixed function oxidase was estimated by measuring the disappearance of NADPH in the NADPH dependent demethylation of benzphetamine (7). All asays were done using the appropriate soluble or microsomal fractions of freshly isolated cells. The activities were linear functions of protein concentration and of time.

Protein Determination: Before homogenization, the cells were washed with Gey's balanced salt solution without BSA to completely remove the BSA contained in the perfusion medium. The protein content in the soluble, microsomal and homogenate fractions were then determined by Lowry's method (8).

METHODS

Animals: Sprague-Dawley male rats (300-400 g) were used.

Cell Isolations: Hepatocytes were isolated by a collagenase perfusion method (1). The liver was perfused in situ in a perfusion chamber, first with Krebs Hensleit bicarbonate buffer (KHBB) without calcium ion (pH 7.4) followed by KHBB with the addition of 0.02% (w/v) collagenase, 4 mM calcium and 2% bovine serum albumin for 40 min. The flow rate was 14 ml/min. at 37°C, and constant aeration was applied with 95% O_2-5% CO_2. After perfusion the liver was blanched completely, excised, rinsed in KHBB, blotted dry, and weighed. Glisson's capsule was removed, the cells were shaken loose by gentle agitation in KHBB with 2% BSA, and collected by centrifugation at 50 g for 5 min.

The nonhepatocytes were isolated essentially by the method of Mills and Zucker-Franklin (2) modified by Knook and Sleyster (3). The liver was first perfused with Gey's balanced salt solution (3 min), followed by the addition of pronase (0.2% w/v, 3 min) and then digested with pronase (0.2%, 60 min). In order to minimize DNA induced cell aggregation, 0.5 mg DNAse was added to the pronase digestion step. The digested tissue was filtered, and the cells collected by centrifugation at 300 g (3 min), washed and differentially centrifuged with concentrated Metrizamide (1400 g, 15 min) to remove red blood cells.(4) The cells were counted on a hemocytometer.

RESULTS AND DISCUSSION

Cell Viability and Purity: The cells used for the determination of enzyme activities in this study were shown to be greater than 90% viable, and there was essentially no hepatocyte contamination in the nonhepatocyte preparation as judged by the pyruvate kinase activation method (Table 1). In pure hepatocytes, pyruvate kinase is activated eleven-fold by the addition of fructose-1,6-diphosphate (1 mM) In nonhepatocytes, the activity is only slightly activated showing essentially no hepatocyte contamination (5).

Enzyme Activities: The GEST, GAST, GR and MFO activities from the soluble and microsomal fractions of isolated cells and from liver were determined and the specific activities are shown in Table 2. The specific activities in the enzyme-

isolated hepatocytes are higher than those obtained from the fractions of whole liver homogenate (Table 2) suggesting that the collagenase treatment does not appreciably affect the enzymes studied. The nonhepatocytes had about 7% GAST activity, 50% GEST and MFO activities and 73% GR activity as compared to hepatocytes; these results are based on protein content..

There have been no reports about the glutathione-related enzyme activities in nonhepatocytes. Cantrel and Bresnick (9) have studied benzpyrene hydroxylase in various hepatic cells by determining the fluorescence of the reaction product, 8-hydroxy benzpyrene, and found that the specific activity of benzpyrene hydroxylase in the nonparenchymal liver cells was only 7.5% of the activity of that in parenchymal cells. This is much lower than the ratio of MFO activities in nonparenchymal to parenchymal cells obtained here (50%). We measured the disappearance of NADPH in the NADPH-dependent demethylation of benzphetamine (7). The difference in these studies may be due to the use of different assay methods. Nevertheless both studies demonstrate that the nonparenchymal cells have less MFO activity than hepatocytes.

The subcellular fractions from nonhepatocytes were determined to contain 0.027 mg soluble protein per million cells, and 0.0063 mg microsomal protein per million cells; the subcellular fractions from hepatocytes contained 0.8 mg soluble protein per million cells and 0.29 mg microsomal protein per 10^6 cells. Thus, the enzyme activity expressed per million cells as shown in Table 3 was calculated from the specific activity previously shown in Table 2. The nonhepatocytes had approximately 1/450 GAST activity, 1/100 MFO based on cell number activity, 1/60 GEST activity, and 1/40 GR activity as compared to hepatocytes.

The total protein contents in nonhepatocytes and hepatocytes were determined to be 0.07 and 2 mg/per million cells respectively. The diameter of a nonhepatocyte is 8-11u (3) and that of a hepatocyte is 18-30u (10). Assuming the cells are spheres, the volume ratio of a hepatocyte to a nonhepatocyte is the ratio of the diameters cubed, or close to 27. Thus, the ratio of the protein content in the hepatocytes to nonhepatocytes is within the range of the volume ratio of the two types of cells.

The distribution of enzyme activity liver (Table 4) can be calculated from the activity expressed per million cells (Table 3) and the cell number per gram tissue. In the rat, there are approximately 100×10^6 hepatocytes and 54×10^6 nonhepatocytes per gram liver (3,11). Thus, the nonhepatocytes had less than or equal to one hundredth the activities of MFO, GAST, GEST and GR as compared to hepatocytes per gram liver. From this study, it is concluded that the nonhepatocytes have significantly less capability in the oxidation and glutathione-related detoxification of xenobiotics as compared to hepatocytes.

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Table 1. Determination of hepatocyte contamination in nonparenchymal cells of liver (pyruvate kinase activation method)

	Activation of pyruvate kinase by addition of F-1 6-P_2	
Tissue or Cell Type	This Lab	Van Berkel and Koster
Nonparenchymal Cell	1.41 0.18 (3)	1.008 (18)
Hepatocyte	9.4 11.8 (2)	11.77 (19)
Whole Rat Liver	6.79 0.70 (4)	6.52 (22)

Table 2. Enzyme activities of various types of hepatic cells and tissues

		Specific activity (nmol/min/mg protein)		
Enzyme	Fraction	Nonhepatocyte	Hepatocyte	While Liver Tissue
GEST	SOL.	42.9 17 (7)	82 11 (8)	74.1 (23)
GAST	SOL.	18.5 6 (6)	275 61 (8)	213 (18)
GR	SOL.	47.4 2 (4)	65 7 (10)	50.3 (34)
MFO	MC	7.49 2.3 (5)	16 5 (6)	12.6 (18)

Table 3. Enzyme activities of hepatocytes and nonhepatocytes

		Activity (nmol/min/10^6 cells)		
Enzyme	Fraction	Nonhepatocytes	Hepatocytes	Hepatocytes/nonhepatocytes
GEST	SOL.	1.16	65.6	56.6
GAST	SOL.	0.5	250	440
GR	SOL.	1.28	52	40.6
MFO	MC	0.048	4.4	96.7

Table 4. Distribution of enzymatic activity in various hepatic cells

		Activity (nmol/min/g liver)		
Enzyme	Fraction	Nonhepatocytes		Hepatocytes
GEST	SOL.	63	(1%)[a]	6,560 (99%)[a]
GAST	SOL.	27	(0.1%)	22,000 (99.9%)
GR	SOL.	69	(1.3%)	5,200 (98.7%)
MFO	MC	2.6	(0.6%)	464 (99.4)

a: % activity in each cell type per amount of liver

PREPRINT

PRELIMINARY ASSESSMENT OF THE USEFULNESS OF URINARY TOTAL GLYCOSAMINOGLYCAN EXCRETION IN THE DETECTION OF SUBCLINICAL LIVER DISEASE

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Summary

Urinary glycosaminoglycan excretion measured as uronic acid was studied in 60 asymptomatic vinyl monomer chemical workers. Thirty-six had normally and 24 had abnormally functioning livers as judged by a standard battery of biochemical liver tests. Urine samples were assayed for both creatinine and total cetylpyridinium chloride-precipitable uronic acid. Individual values were declared to be normal or abnormal based on the range 2.0 - 4.8 μg uronic acid/mg creatinine established as the normal range at the outset. The frequency distribution of glycosaminoglycans for the groups of normal and abnormal biochemistries were statistically significantly different. The distributions did not differ in their means ($t_{58}=0.968$), but did differ in their variability ($F_{23,35}=2.381$; $P<0.05$). The observation that liver disease increases the range of value for total urinary glycosaminoglycan excretion, but not the mean, suggests that this parameter is not a useful general screening test in asymptomatic individuals with hepatic dysfunction. The increased variance in urinary glycosaminoglycan excretion in individuals with liver disease may reflect or be related to a stage of the disease process. At certain stages of disease, glycosaminoglycan incorporation (into scar tissue for example) may predominate while in other stages there may be a predominance of glycosaminoglycan-releasing, tissue destruction and/or an impairment of the liver's ability to clear glycosaminoglycans from the blood. These possibilities warrant further study with greater differentiation of disease categories and stages and with resolution of specific glycosaminoglycans patterns.

INTRODUCTION

Changes in tissue, urinary and blood glycosaminoglycans (GAGs) have been found to occur in many connective tissue disorders including those of the liver (Galambos and Shapira, 1973; Koizumi, et al. 1967; Kojima, 1964; Rubin, 1966; and Patrick and Keenedy, 1964) and in hepatic cancer (Kojima, et al., 1975; Anghileri, 1974; Kupchella, et al., 1981; and Yamamoto and Teryama, 1973). In a previous report, we described elevated urinary excretion of uronic acid in a number of liver disease states (Kupchella and Tamburro, 1976) and showed that angiosarcoma patients exhibit a urinary GAG excretion pattern distinguishe from that of normal urine by relative increases in a hyaluronidase resistant fraction and a decrease in a hyaluronidase susceptible fraction (Curran et al., 1977). The purpose of this study was to assess the potential usefulness of total urinary GAG determinations in th identification of liver disease among chemical (iruylmonomer) workers other than hepatic angiosarcoma.

MATERIALS AND METHODS

One hundred non-biopsied workers (50 with and 50 without hepatic biochemical abnormalities) were selected at random from among more than 1200 vinyl chloride production workers at the Louisville plant of the B.F. Goodrich Company. Participation was voluntary and 36 workers without biochemical abnormalities and 24 workers with biochemical abnormalities elected to participate. Criteria used to determine the biochemical status of these workers' livers are summarized in Table 1.

Urine specimens were collected during routine screening and frozen until analysis. Twenty-five ml. amounts were subjected to urinary GAG analysis in triplicate as described by DiFerrante (1967) and 5 ml. samples assayed for creatinine using a Technicon Autoanalyzer. Uronic acid determinations of cetylpyridinium-chloride complexed GAGs were made according to the modified carbozole method of Bitter and Muir (1962) and expressed as micrograms of uronic acid per mg of creatinine. Based on our work with normal controls (Kupchella and Tamburro, 1976) and reports by others (Varma, et al., 1974; DiFerrante and Rich, 1956) we defined the normal range of urinary GAG excretion as 2.0 to 4.8 µg of uronic acid per mg of creatinine, assuming that the normal adult male excretes 1.5 grams of creatinine every 24 hours (Sunderman and Boerner, 1940). Our definition of normal range was established in advance of the study from previous data on a "normal" cohort (640 adult males examined annually for 3-5 yrs). The data includes physical examination, liver spleen scan, SMAC 20, chest x-ray, 0.5 mg/kg dose ICG clearance, negative HB_sAg serology, cbc and urine analysis. The GAG analysis was done blindly, i.e., clinical diagnosis of the patients from whom the urine was collected was not known.

RESULTS

Sixty GAG determinations were made, 6 of 36 (16.7%) workers with normal hepatic biochemistries had GAG excretions of less than 2 or equal to or more than 4.8 ug uronic acid/mg creatinine. This was true for ten of the groups of 24 (41.7%) workers whose biochemistries were abnormal. The frequency distributions are given in Table 2.

DISCUSSION

We attempted here to assess the diagnostic, and other aspects of the minimally expensive, minimally time-consuming method of determining urinary GAGs. Because we found that the effect of liv r disease on urinary GAG was to increase variance without appreciably altering the mean, we have clearly not identified a new diagnostic test. These results suggest however that liver disease may impact on urinary GAG excretion in multiple ways. Subclinical hepatis dysfunction may favor uptake or incorporation of GAGs (fibrotic tissu for example) while other diseases or disease stages are dominated by GAG-releasing tissue destruction and/or the impairment of the liver's ability to clear the blood of circulatory GAGs.

TABLE 1

Liver function tests used to determine "biochemical abnormality". Biochemical abnormality was established if two or more tests in group 1 were abnormal or if one from group 1 and two from group 2 were abnormal (negative otherwise).

GROUP 1	GROUP 2
- Alanine Amino Transferase (ALT)	Gammaglutamyl Transpeptidase (GTP)
- Indocyanine Green Clearance (ICA)	Bilirubin (total) (TB)
- Alkaline Phosphatase (AP)	
- Aspartate amino transferase (AST)	

TABLE 2

Frequency (f) and Relative Frequency (R.F.) of glycosaminoglycan excretion levels for workers with normal and abnormal biochemical results. A T-test of independent means (t_{58}=0.968) revealed no significant difference, however an F-test of the independence of variance (two-tailed) revealed significance ($F_{23,35}$=2.381) at the $P<0.05$ level.

	Normal		Abnormal	
GAG[a]	f	R.F.	f	R.F.
< 2	4	0.111	6	0.250
2 < 3	16	0.444	7	0.292
3 < 4	13	0.361	6	0.250
4 < 5	1	0.028	4	0.167
Sum	36	1.000	24	1.000
Mean	2.886		3.160	
Variance	0.746		1.776	

a µg uronic acid/mg creatinine

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