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
SCHOOL OF MEDICINE
GRADUATE SCHOOL
CANCER CENTER

REPORT FOR THE
CHEMICAL MANUFACTURERS ASSOCIATION
(FORMERLY MANUFACTURING CHEMISTS ASSOCIATION)

CONFIDENTIAL

Subject to Protective Order in
Ross v. Conoco, Inc., No. 90-1037
14th Judicial District Court
Calcasieu Parish, Louisiana

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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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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

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.

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SUMMARIES OF
RESEARCH PROGRAMS

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

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

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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

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.

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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

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

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HUMAN STUDIES:

C3. Characterization of Glycosaminoglycan Patterns in Humans with Angiosarcoma

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

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,

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

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

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.

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 sensiti[illegible] indicator of chemically-induced liver tumor. F antigen appeared to be abse[illegible] 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

PROGRAM G

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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

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

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.

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RESEARCH PROGRAMS AND RESULTS

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.

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.

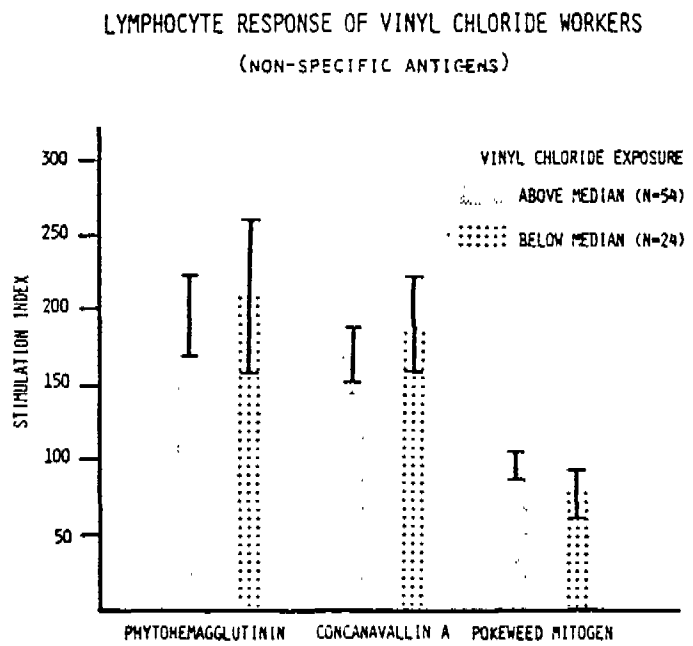

FIGURE 1

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).

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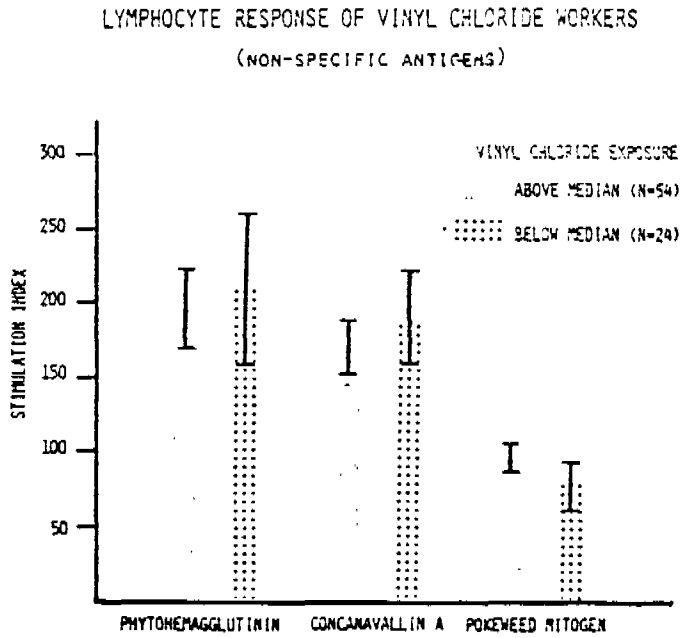

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Response of the other immune parameters in high and low vinyl chloride exposure are shown in Table 1. No statistically significant difference was found between workers with high and low vinyl chloride exposure, or between unexposed invididuals and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

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±171 (N=50)	1524±132 (N=24)	NONE
T-CELL ROSETTES AT 33°C (ABSOLUTE)	1227±80 (N=50)	1260±123 (N=24)	NONE

*STIMULATION INDEX
**SEM

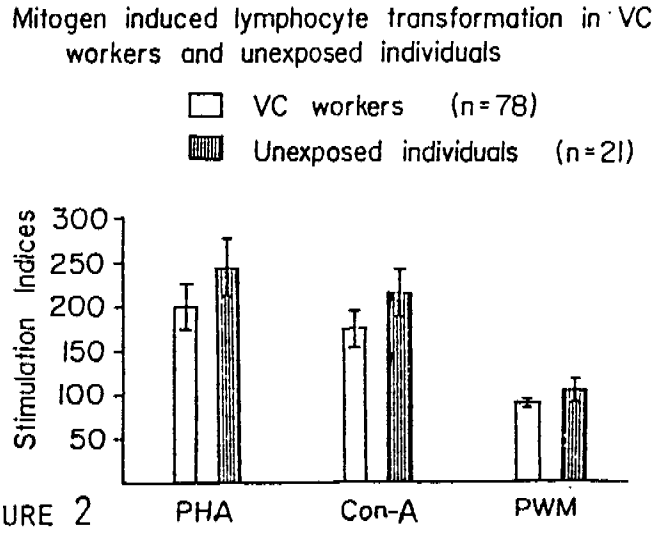

FIGURE 2

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)	174±35 (N=48)
CONCANAVALLIN A STIMULATION	195±36 (N=25)	162±29 (N=48)
POKEWEED MITOGEN STIMULATION	100±12 (N=25)	80±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)	62±2 (N=49)
T-CELL ROSETTES AT 4°C (ABSOLUTE)	1600±100 (N=25)	1500±100 (N=49)
T-CELL ROSETTES AT 33°C (PERCENT)	55±2 (N=25)	52±2 (N=47)
T-CELL ROSETTES AT 33°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.

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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)	128±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 P<.01 (N=40)
LYMPHOCYTE COUNT (PERCENT)	33±2 (N=36)	41±2 P<.001 (N=40)
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.

Research Results

Dr. Maluish came from Australia and spent a number of days helping us develop and further validate the methodology used. Despite multiple attempts we were not able to validate the reproducibility of the leukocyte adherence inhibition test, neither in colon cancer nor in liver angiosarcoma patients. Unfortunately, we must conclude that the leukocyte inhibition adherence test is not sufficiently reproducible to be used as a reliable indicator of the presence of tumors.

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

Background

New antigens arise on tumors formed as a response to carcinogens. Their presence on induced sarcomas was discovered by Foley (1953). This discovery in mice was verified and extended by Prehn and Maine (1957) who concluded that there were antigens particular to and specific for tumor tissue. Subsequently, evidence for specific tumor antigens was found in humans by Hellstroms, Vankey, Halliday and Maulish, Thompson, and others. The majority of the evidence suggests that tumor antigens found were distinctive for each histological type of tumor. This concept was applied to our cohort population for primary liver cancer.

Objective

1. To determine whether the human body mounts an immune reaction to developing cancer cells that can be identified by specific immune reactions with tumor-specific antigen.

Research Results

Lymphocytes (the cells responsible for immunity) were obtained from individual workers, isolated, and grown in the presence of liver reagents prepared from either normal individuals or individuals who had had angiosarcoma. A positive reaction was identified by incorporation of ^{3}H-thymidine into stimulated lymphocyte cultures as compared to unstimulated (control) cultures. Comparison of responses to a panel of tissue extracts (Table 6) by lymphocytes from vinyl chloride workers and normal non-chemical plant workers indicated that many unexposed individuals have reactivity to these tissue antigens. Lymphocyte reactivity against angiosarcoma tissue alone occurred more frequently among vinyl chloride workers (Table 7).

Research Results

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

Background

New antigens arise on tumors formed as a response to carcinogens. Their presence on induced sarcomas was discovered by Foley (1953). This discovery in mice was verified and extended by Prehn and Maine (1957) who concluded that there were antigens particular to and specific for tumor tissue. Subsequently, evidence for specific tumor antigens was found in humans by Hellstroms, Vankey, Halliday and Maulish, Thompson, and others. The majority of the evidence suggests that tumor antigens found were distinctive for each histological type of tumor. This concept was applied to our cohort population for primary liver cancer.

Objective

1. To determine whether the human body mounts an immune reaction to developing cancer cells that can be identified by specific immune reactions with tumor-specific antigen.

Research Results

Lymphocytes (the cells responsible for immunity) were obtained from individual workers, isolated, and grown in the presence of liver reagents prepared from either normal individuals or individuals who had had angiosarcoma. A positive reaction was identified by incorporation of ^{3}H-thymidine into stimulated lymphocyte cultures as compared to unstimulated (control) cultures. Comparison of responses to a panel of tissue extracts (Table 6) by lymphocytes from vinyl chloride workers and normal non-chemical plant workers indicated that many unexposed individuals have reactivity to these tissue antigens. Lymphocyte reactivity against angiosarcoma tissue alone occurred more frequently among vinyl chloride workers (Table 7).

TABLE 6

TISSUE EXTRACT PANEL

EXTRACT	NUMBER IN PANEL
ANGIO TUMOR	3
NORMAL LIVER	3
HEPATOMA LIVER	1
ANGIO "NORMAL" LIVER*	2
NORMAL KIDNEY**	2

*"NORMAL" AREAS OF LIVER TISSUE FROM ANGIOSARCOMA LIVERS.

**OBTAINED FROM THE SAME DONORS AS NORMAL LIVERS.

TABLE 7

LYMPHOCYTE REACTIVITY TO TUMOR AND NORMAL TISSUE PANEL

	VINYL CHLORIDE WORKERS		NON-VINYL CHLORIDE WORKERS		INTERPRETATION OF RESULTS
LYMPHOCYTES REACTIVE AGAINST	N	PERCENTAGE	N	PERCENTAGE	
ANGIO LIVER ALONE	6	16	0	0	INDIVIDUALS WITH POSSIBLE SPECIFIC ANTI-TUMOR REACTIVITIES
NORMAL AND ANGIO LIVER	7	18	8	57	INDIVIDUALS WITH NONSPECIFIC REACTIVITIES MASKING ANY POSSIBLE SPECIFIC REACTIVITIES
NORMAL LIVER ALONE	5	13	1	7	INDIVIDUALS WITH NONSPECIFIC REACTIVITIES
NONREACTIVE	20	53	5	36	INDIVIDUALS WITH NO TISSUE REACTIVITIES
TOTAL	38	100	14	100	

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)

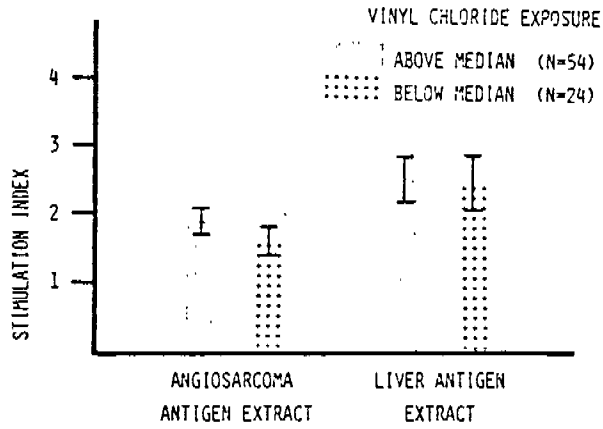

FIGURE 3

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

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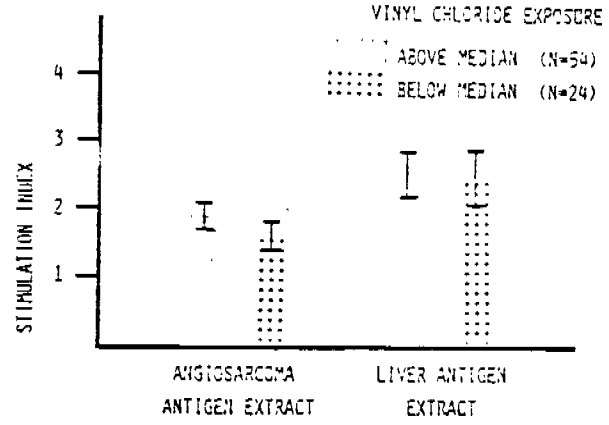

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On the chance that non-specific reactions might be obscuring the specific reactions, workers with vinyl chloride-suspected disease (working in a non-vinyl chloride environment--Pallet Plant) were compared to workers without known liver disease (working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

target antigens, immune response, tumor virus susceptibility, liver cyclic adenosine 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).

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

TABLE 9

HLA-A FREQUENCIES

HLA-A ANTIGENS	HEALTHY CONTROLS	WHO 1974 WORKSHOP	PALLET PLANT(BFG)	NON PALLET PLANT(BFG)
A1	34	32	46	30
A2	51	49	57	52
A3	23	22	14	21
A9	16	17	11	22
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%	177%	200%	200%

**NOT DONE

TABLE 10

HLA-B FREQUENCIES

HLA-B ANTIGENS	HEALTHY CONTROLS	WHO 1975 WORKSHOP	PALLET PLANT/BFG	NON PALLET PLANT/BFG
B5	10	11	11	9
B7	31	23	18	28
B8	27	20	21	20
B12	30	24	21	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	9	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	13
N	903	503	28	519
Total Frequency	155%	176%	201%	201%

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).

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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)

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.

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FIGURE 4

FIGURE 5

Relevance to Industry

These rather extensive, detailed studies of a well-defined human population provide very valuable data regarding the use of immunological screening tests for the detection and identification of chemically-related injury.

(1) There appears to be no significant evidence that the immunodepression or change in the antibody responding components or the cell-mediated responding components of the human immune system is affected by vinyl chloride exposure. Therefore, studying immunological parameters of type B or T cells will not provide any useful information in the early stages of disease even with excessive exposure.

(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.

References

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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

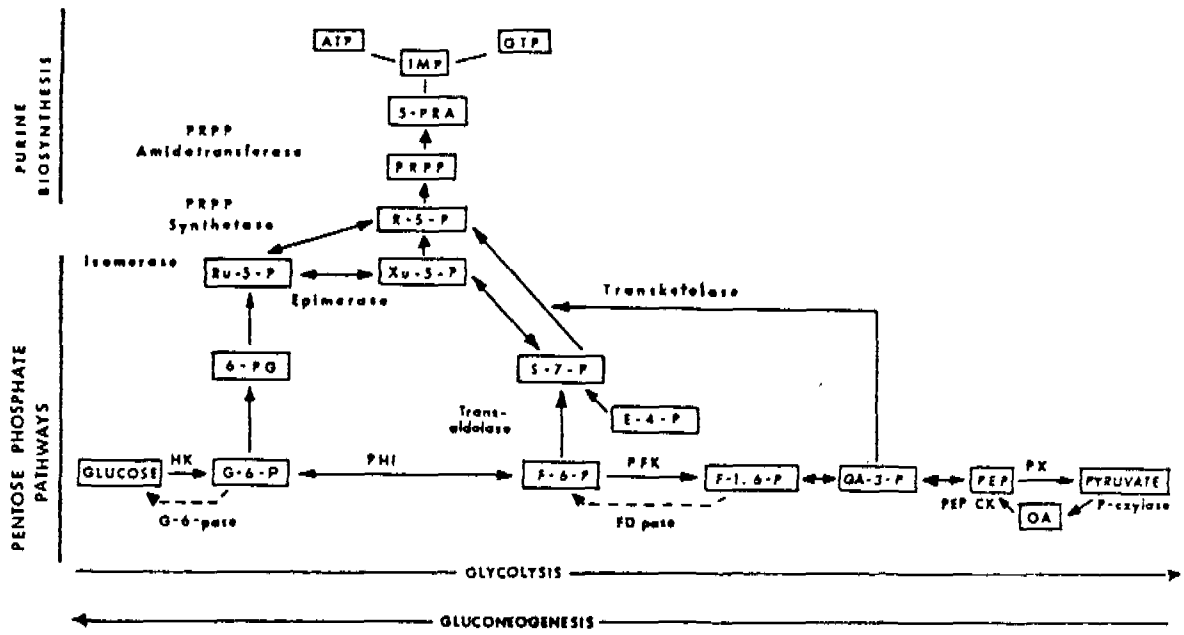
B2. Morphological Alterations In Livers Of Rats Exposed To Vinyl Chloride

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.

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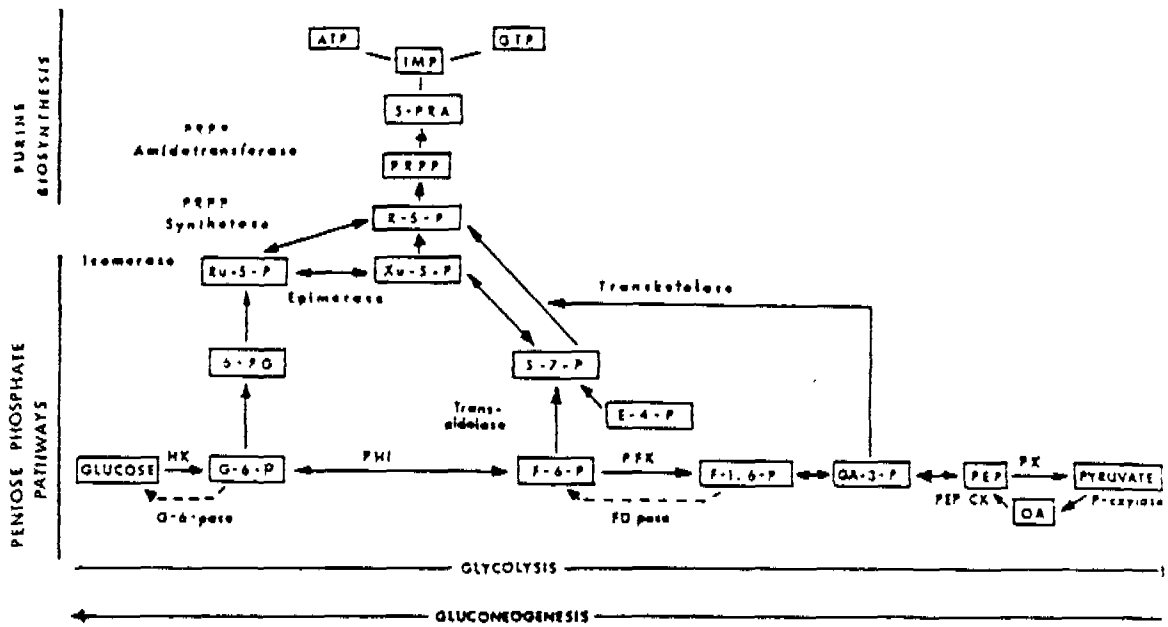

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1. To determine the sequential hepatic enzymatic changes in animals chronically exposed to vinyl chloride and their correlation to conventional clinical tests of liver function.

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).

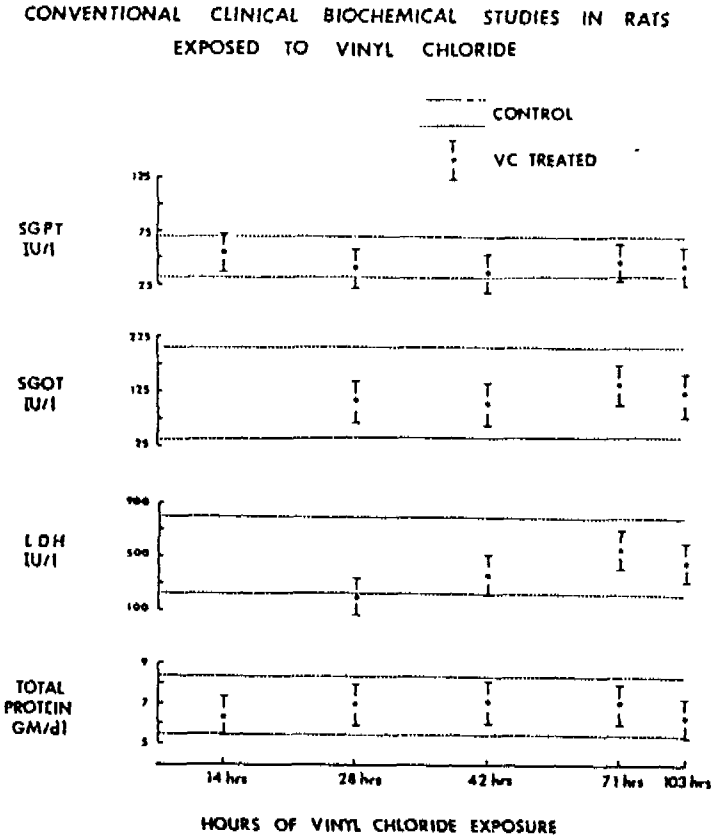


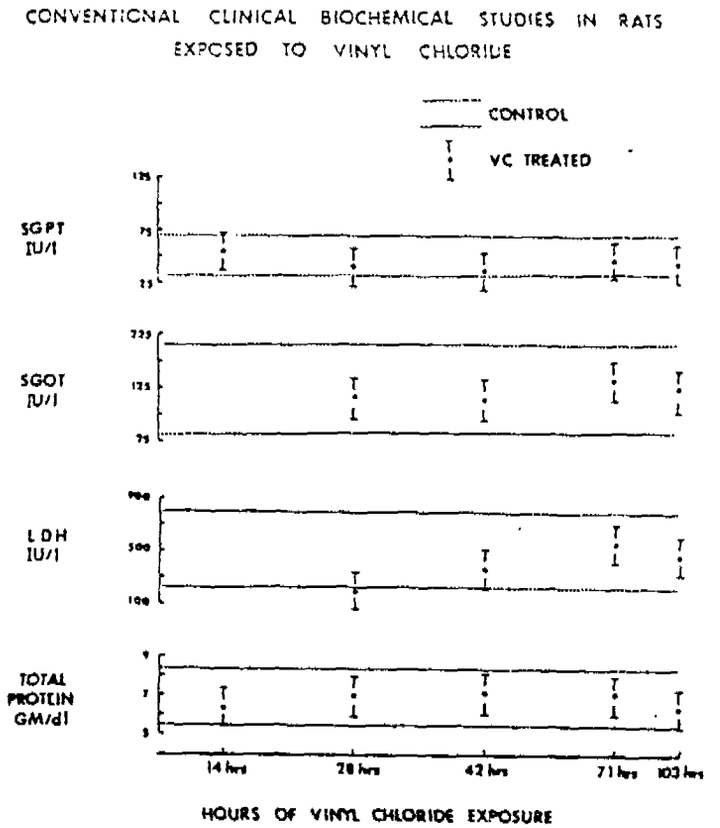
FIGURE 2

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.



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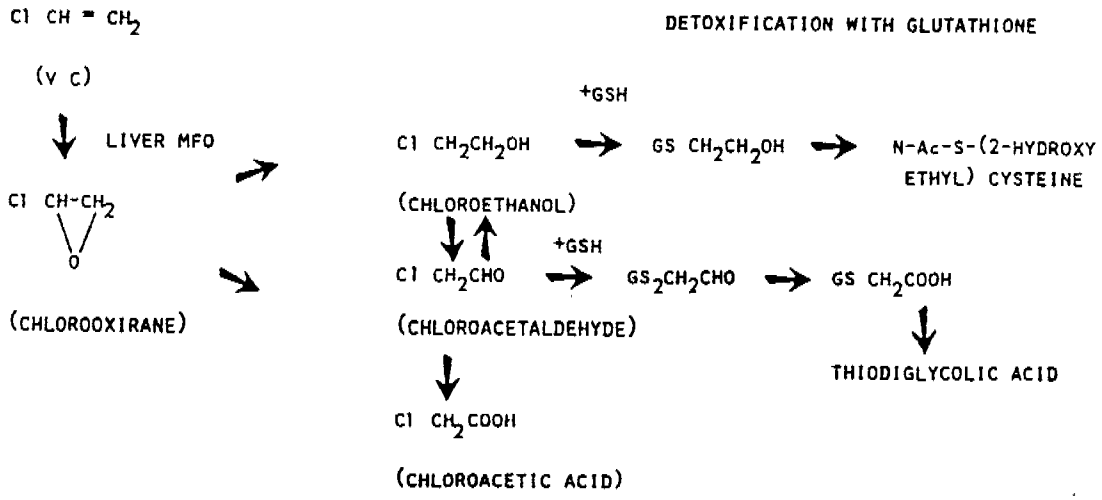
There was no evidence of increased collagen deposition in the space of Disse which has been reported by others during terminal or final stages of angiosarcoma development in animals.

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).

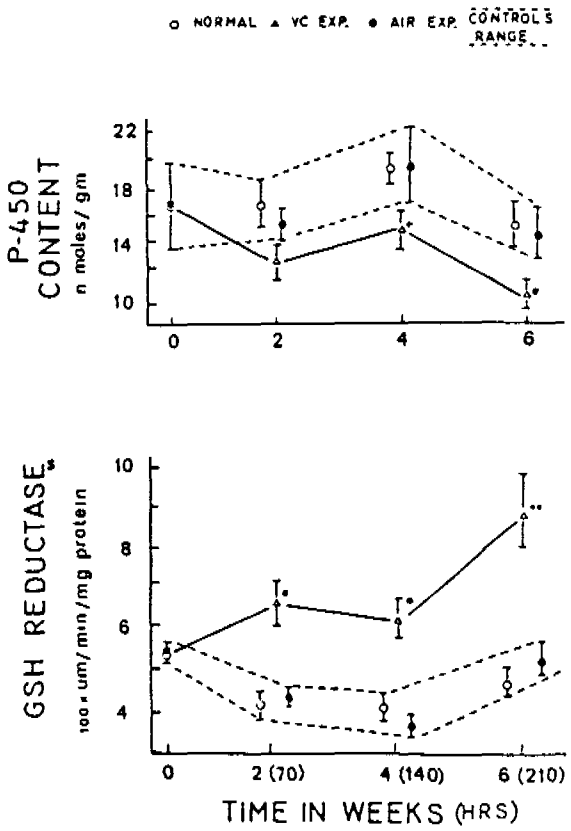
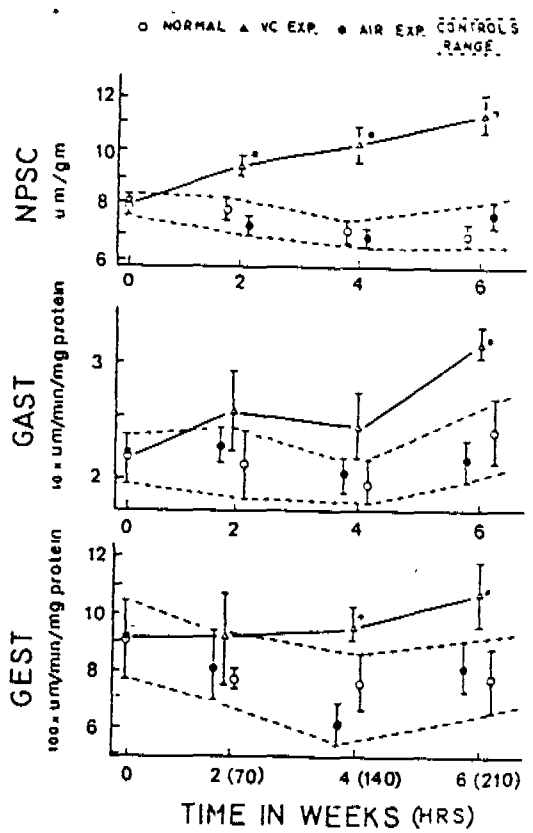


FIGURE 3



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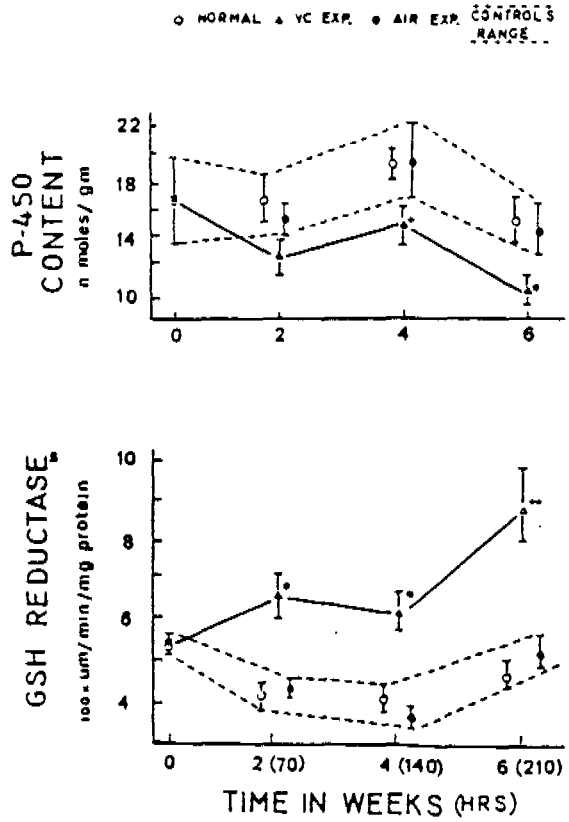

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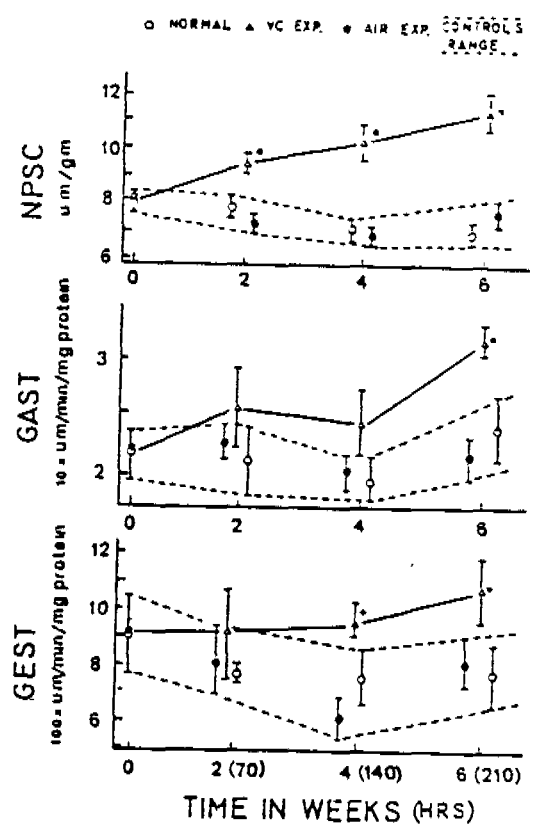

FIGURE 4

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.

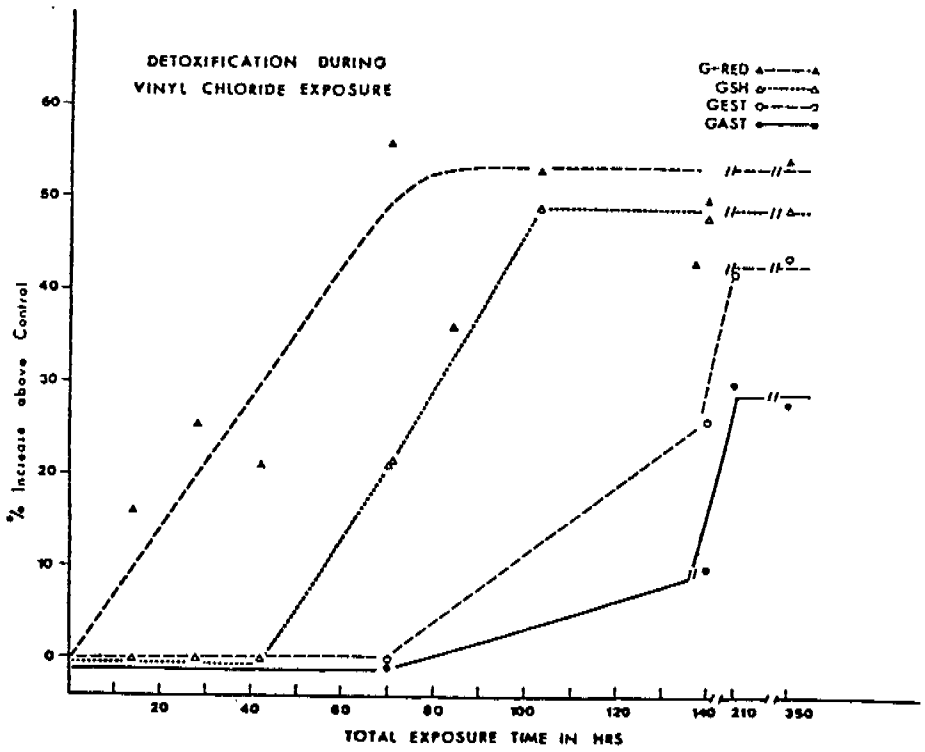

FIGURE 5

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 mes chymal 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

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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

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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
(+)	288	140	516
(-)	88	1923	2011
			2439

SENSITIVITY: 55.8%

SPECIFICITY: 95.6%

B

SENSITIVITY AND SPECIFICITY OF INDOCYANINE GREEN CLEARANCE

ICG 2.5 MG/KG

BEST MEDICAL ASSESSMENT	(+)	(-)	TOTAL
(+)	45	28	73
(-)	14	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
(-)	4	38	42
			126

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).

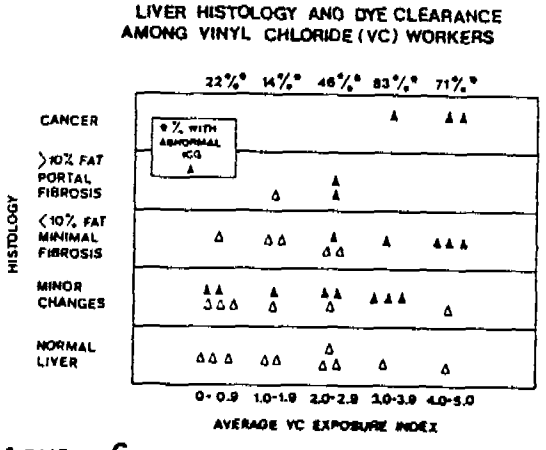

FIGURE 6

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

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).

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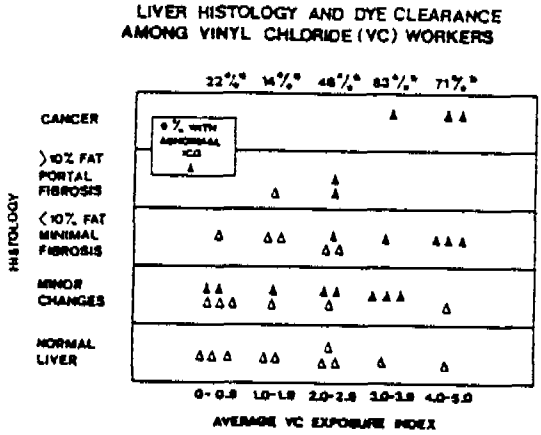

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The subcohort biopsied population was additionally studied with regard to the sensitivity and specificity of the screening tests, the specific histological interpretation of the liver biopsy, and the relationship to vinyl chloride exposure. All biopsied 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(ek) (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 (sensitivit[illegible] of the normal vs. abnormal at the 95 percentile level for the norm[illegible] 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

TABLE 2

CORRELATION OF LIVER BIOPSY WITH
BILE ACID LEVELS AND ICG CLEARANCE

				NON BIOPSIED
	CLI	NCLD	NBX	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.

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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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Subject to Protective Order in
Ross v. Conoco, Inc., No. 90-4837
14th Judicial District Court
Calcasieu Parish, Louisiana

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

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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

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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).

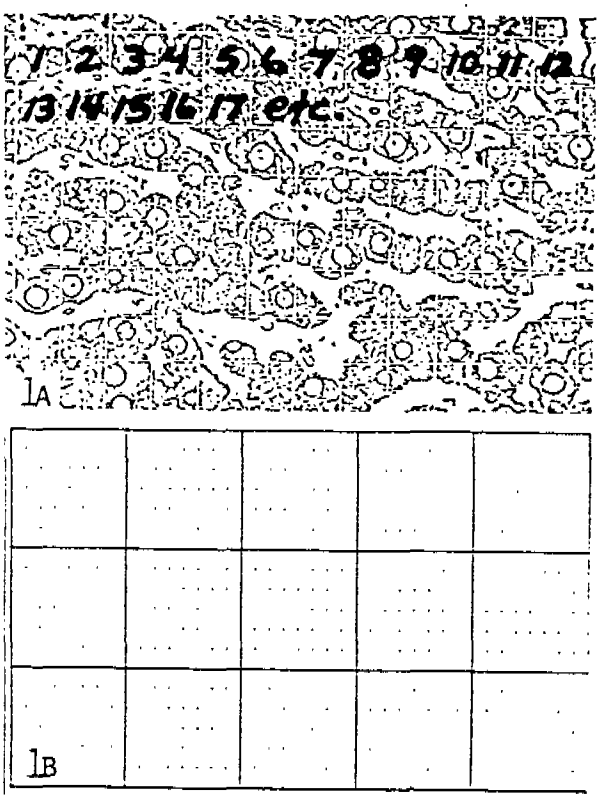

FIGURE 1

Manual collagen quantitation outline

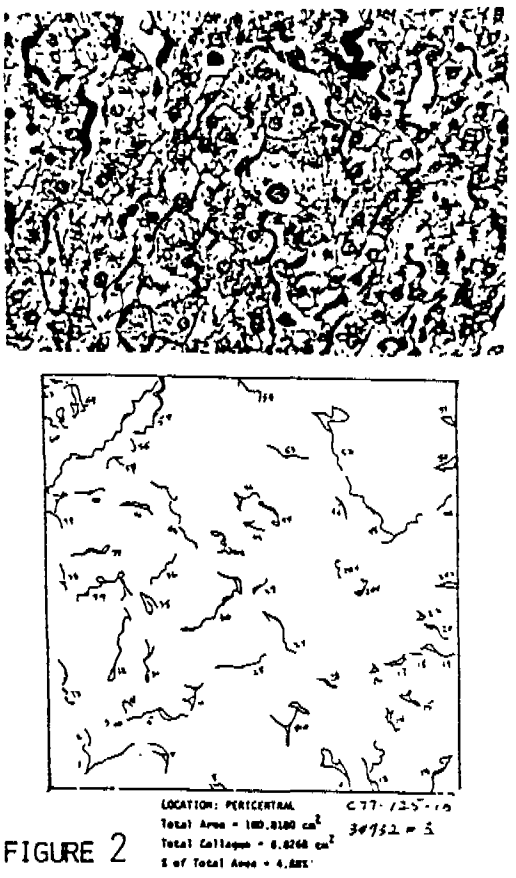

FIGURE 2

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.

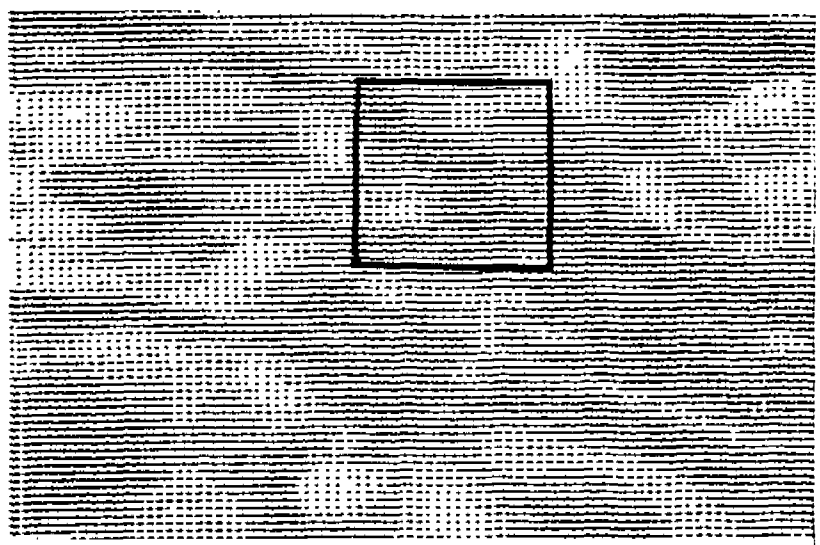

FIGURE 3A. Density printout of Figure 2, with 10:1 reduction (area in square corresponds to digital printout 3B).

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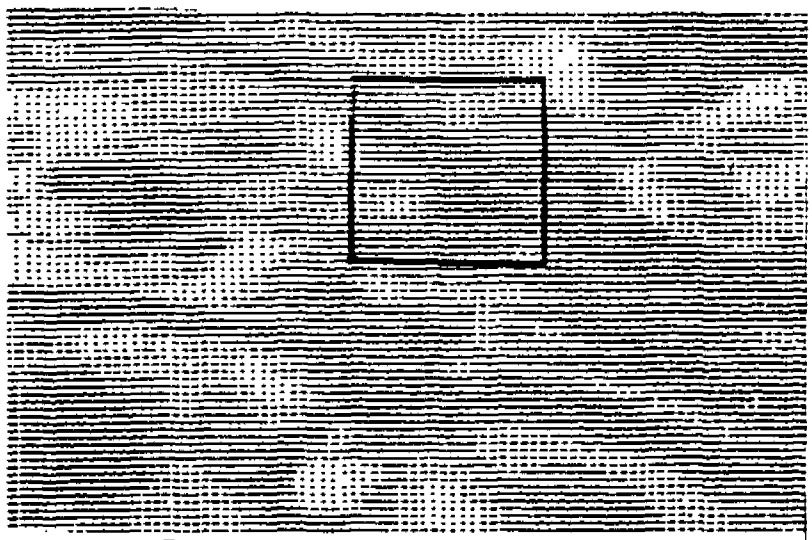


FIGURE 3A: Density printout of Figure 2, with 10:1 reduction (area in square corresponds to digital printout 3B).

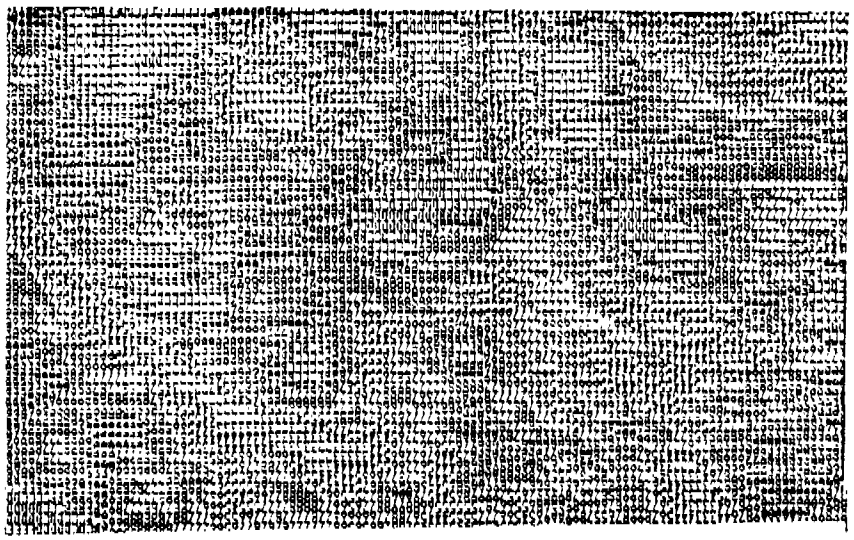


FIGURE 3B Digital densities of reticulin stained liver biopsy.

(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.

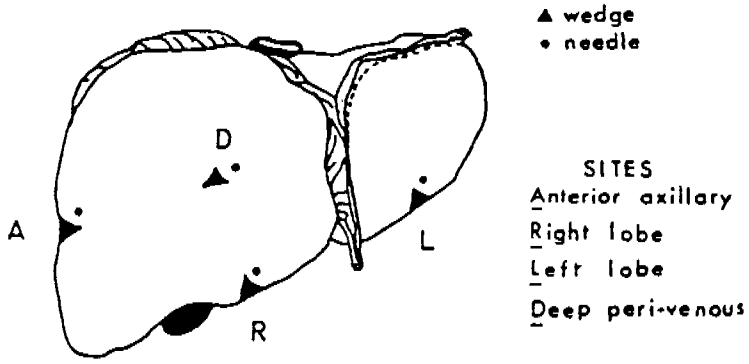


FIGURE 4

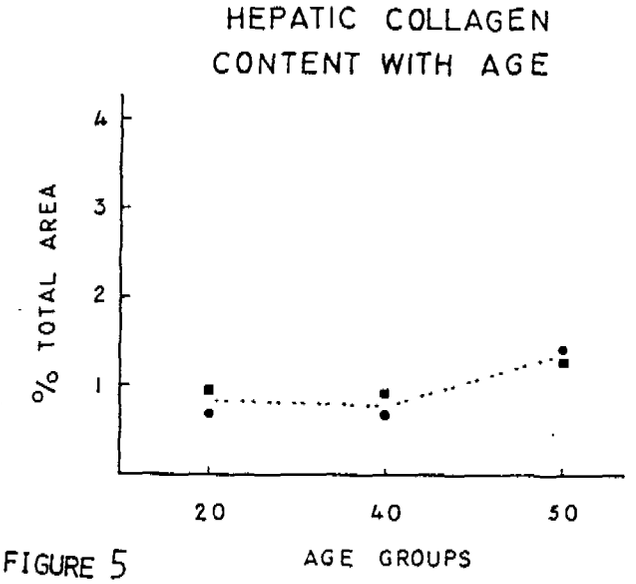
Two hundred and fifty three light micrographs derived from 30 tissue blocks from 8 normal individuals were studied. A Hewlett Packard 9864-A digitizer microcomputer was used to quantitate areas of trichrome stainable collagen. Collagen estimates varied from .2 to 6.1% (mean = 1.25) with considerable 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).



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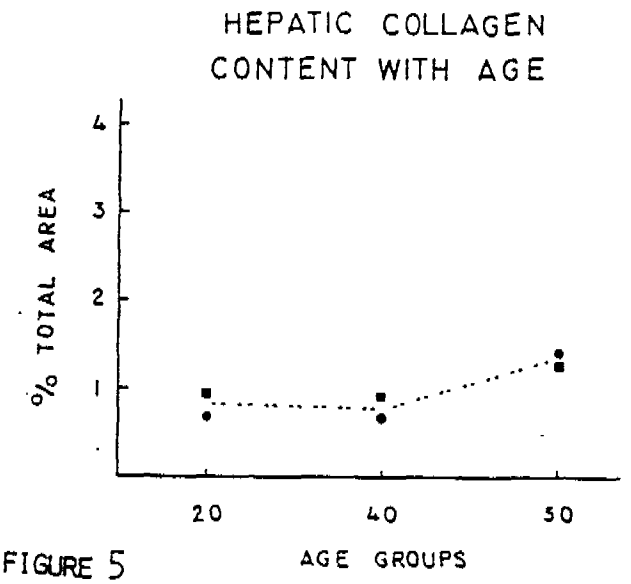


FIGURE 5

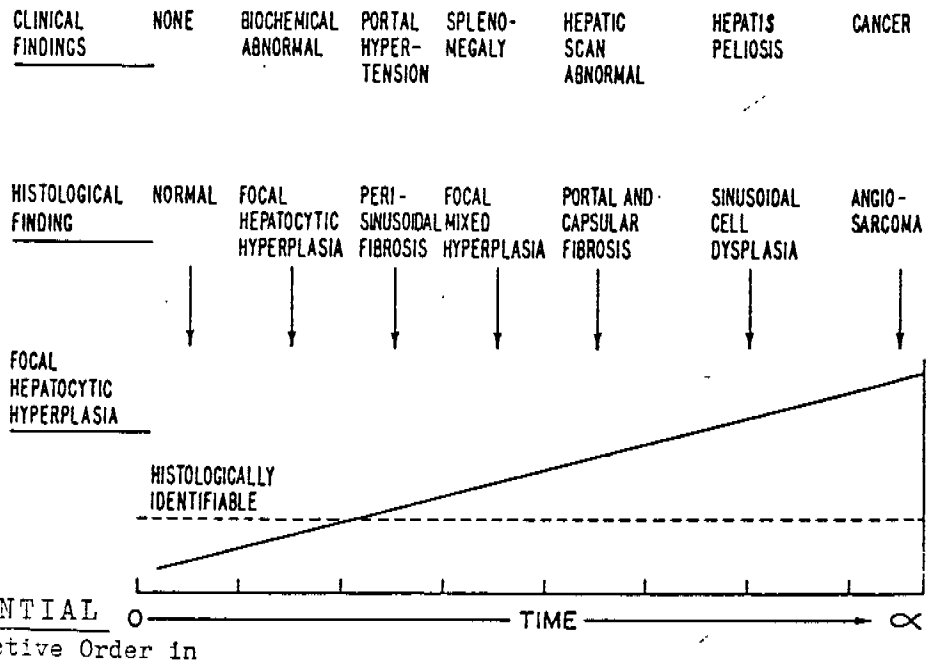
Differences in central, mid-zonal and portal collagen vary from 0.49 - 0.63 percent in the youngest age group and from 4 - 5.35 percent in the older age group. This histological data is consistent with the ICG clearances in normal adults which show a progressive decrease with age. The decreased functional 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



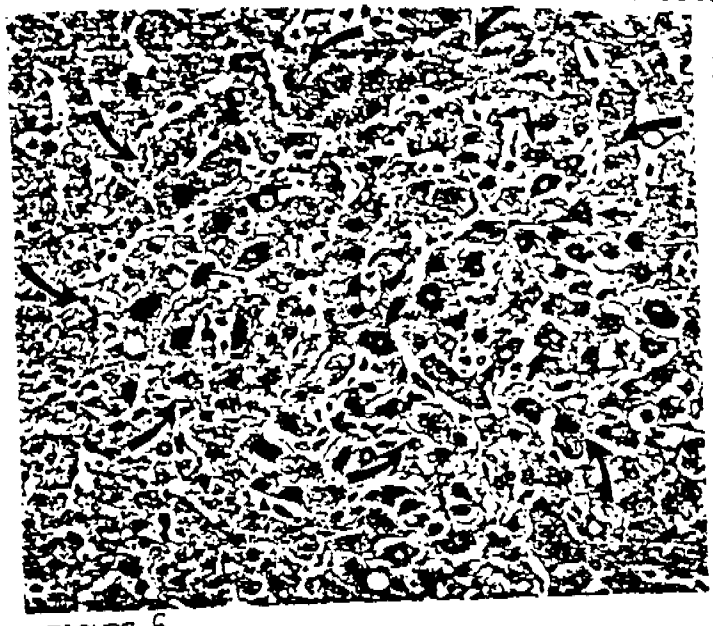


FIGURE 6

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.

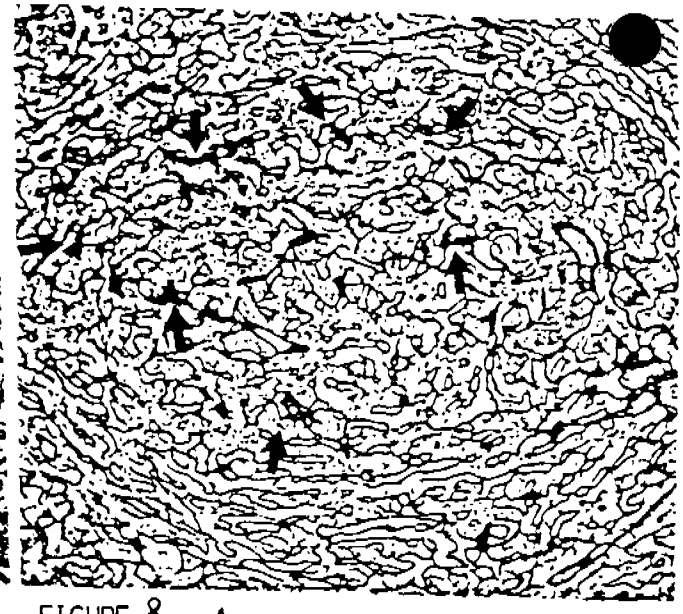


FIGURE 8

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

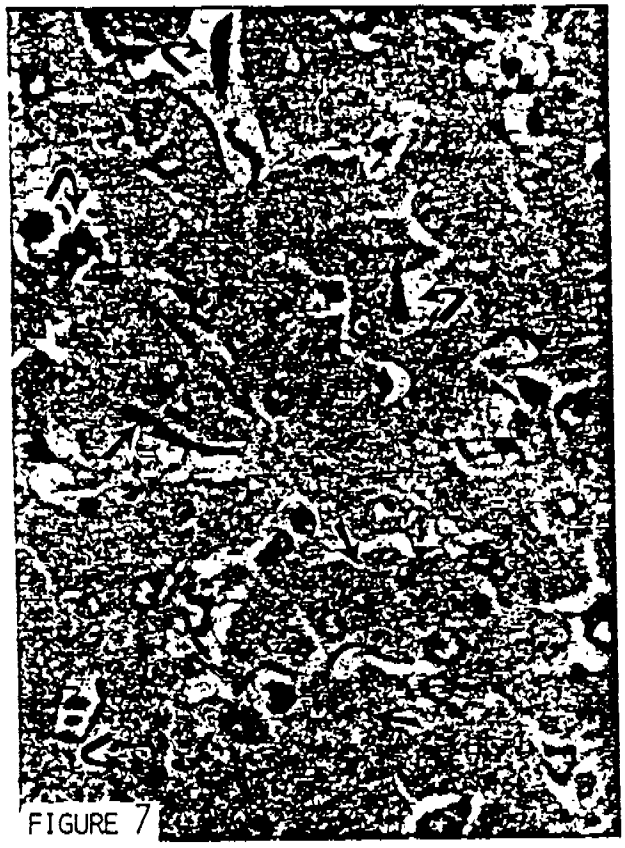


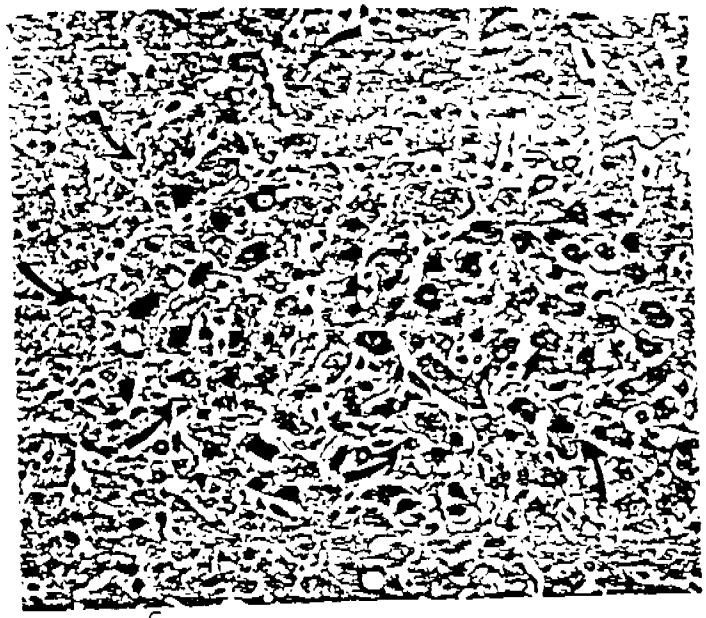
FIGURE 7

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.



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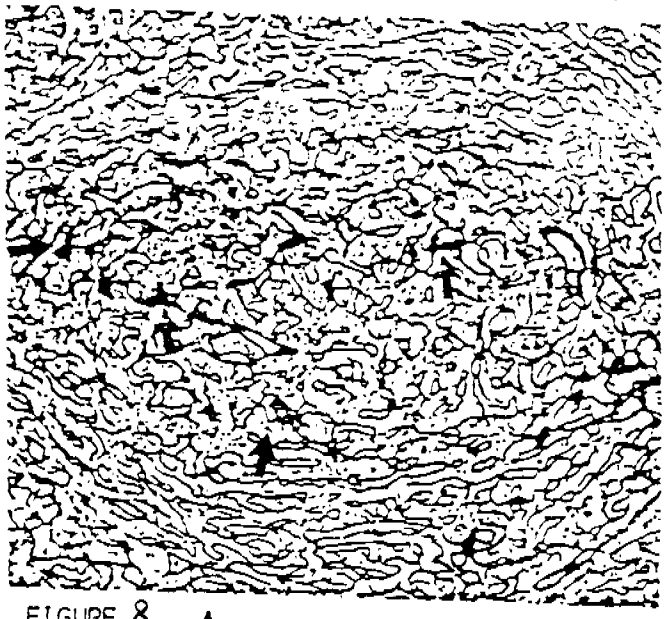

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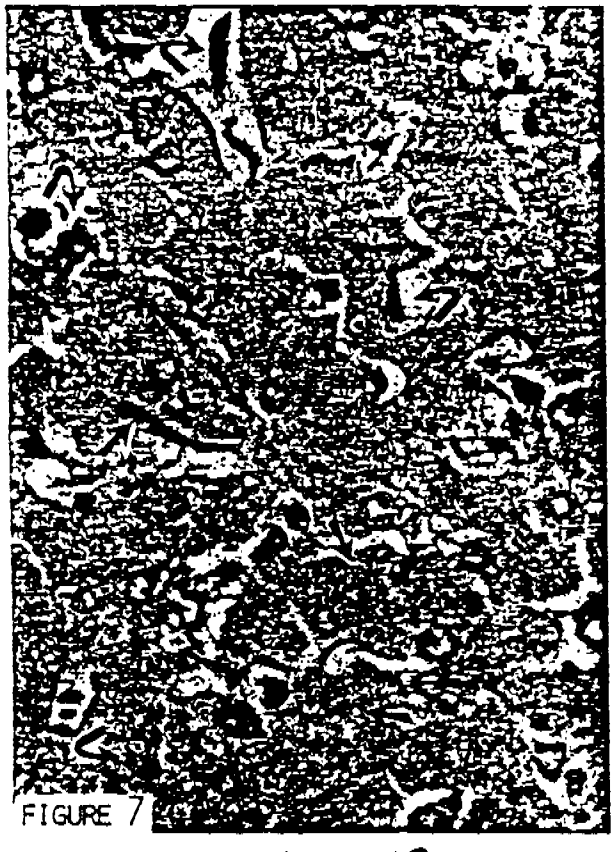

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In contrast only 5 of the comparison group had similar findings. Four (13%) had focal hepatocellular hyperplasia, and 1 (3%) had focal mixed hyperplasia and sinusoidal dilitation. On subsequent biopsy this individual was found to have angiosarcoma 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.

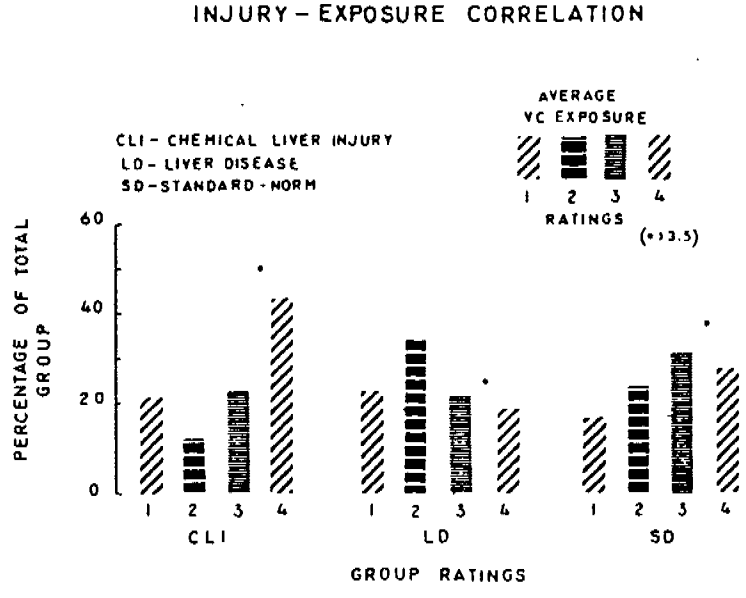

FIGURE 10

CONFIDENTIAL

Subject to Protective Order in
Ross v. Conoco, Inc., No. 90-4837
14th Judicial District Court
Calcasieu Parish, Louisiana

CMA 003527

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.

References

1. Creech, J.L., Jr. and Johnson, M.N. (1974) Angiosarcoma of Liver in the Manufacture of Polyvinyl Chloride. J. Occup. Med. 16:150.

2. Falk, J., Creech, J.L., Jr., Heath, C.W., Jr., Johnson, M.N. and Key, M.M. (1974) Hepatic Disease Among Workers at a Vinyl Chloride Polymerization Plant. JAMA 230:59.

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5. Makk, L., Delmore, F., Creech, J.L., Jr., et al. (1975) Clinical and Morphological Features of Hepatic Angiosarcoma in Vinyl Chloride Workers. Cancer 37:149-163.

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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).

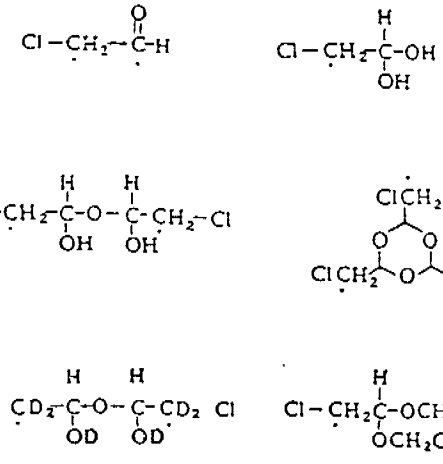
   b) reactions on nucleic acid constituents (mutagenesis and carcinogenesis).

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.

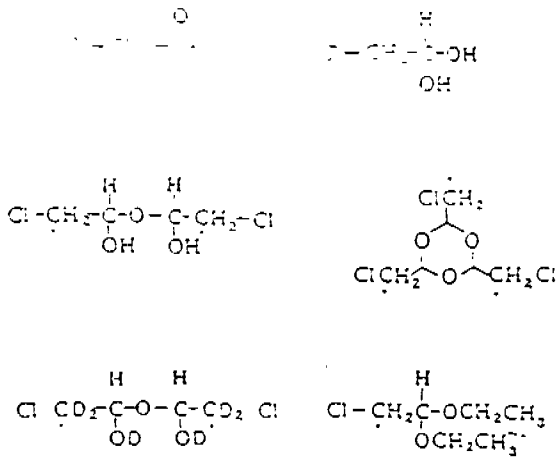
b. Synthesis of acidic hydrolytic products of etheno-modified bases--2-aminobiimidazole, 2-formamidobiimdazole, E1-D-angular-etheno-guanine, and E1-D-linear-etheno-guanine.

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



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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

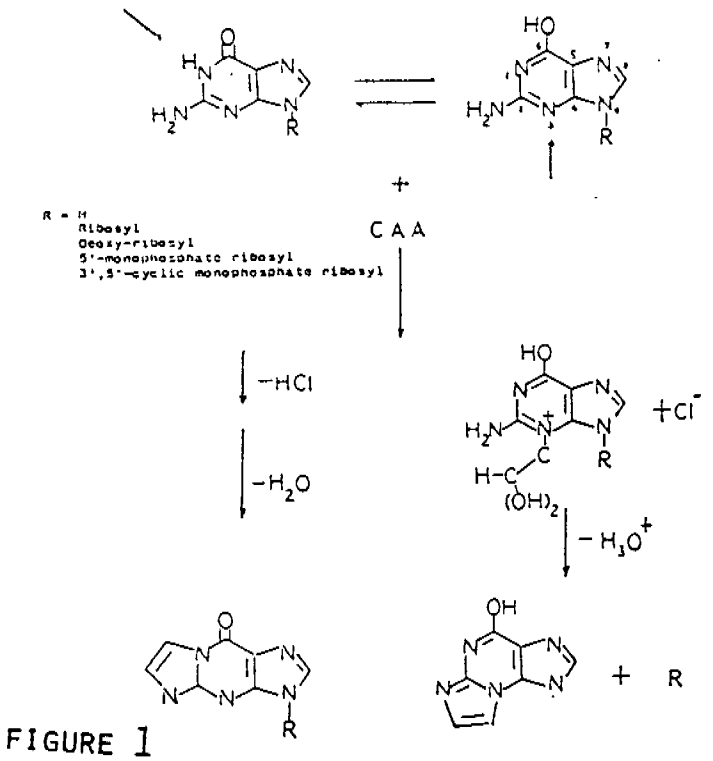

FIGURE 1

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):

these conditions, and 60 percent conversion in 24 hr. The diol product identified from these reactions by high pressure liquid chromatography is the dienyne indicated. On the other hand, chloroacetaldehyde gave another addition product, which was identified by NMR 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 CCR. These results, combined with the in vitro experiment by Gothe, have confirmed that chlorooxirane is an obligatory intermediate in the metabolism of vinyl chloride.

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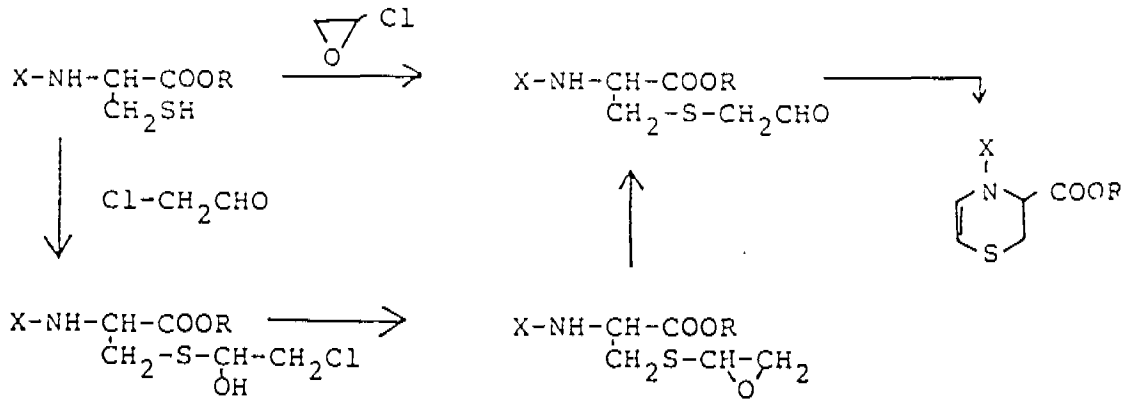

X=H, Ac
R=H, Me

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 ($^{35}ClCH_3^+$ and $^{37}ClCH_3^+$) 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-nicro quinoline-1-oxide	+++	++
10.	Chloroacetaldehyde	+++	++
11.	Epichlorohydrin	++	+
12.	Chlorooxirane	+++	++
13.	Butane diepoxide	++	+
14.	Styrene oxide	++	+
15.	3,4 epoxide butene	±	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.

A screening laboratory using microbial assays for carcinogenic genetic purposes is being maintained. We have proposed to the state of Kentucky that a regional chemical testing laboratory be formed 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.	[illegible]	NR	NR
4.	N-cyclobutanecarboxyloxy-N-methylacetamide	NR	NR
5.	Phenylcyclobutane carboxyl peroxide	NR	NR
6.	Aflatoxin	———	++
7.	Chloroethanol	NR	NR
8.	Benzo(a)pyrene	++	-
9.	4-nitro quinoline-1-oxide	+++	++
10.	Chloroacetaldehyde	+++	++
11.	Epichlorohydrin	++	+
12.	Chlorooxirane	+++	++
13.	Butane diepoxide	++	+
14.	Styrene oxide	++	+
15.	3,4 epoxide butene	±	NR
16.	Bis(Beta-chloroethyl)phenyl phosphate	NR	NR
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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-B_1 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.

Objective

1. To [illegible] 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.

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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

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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.

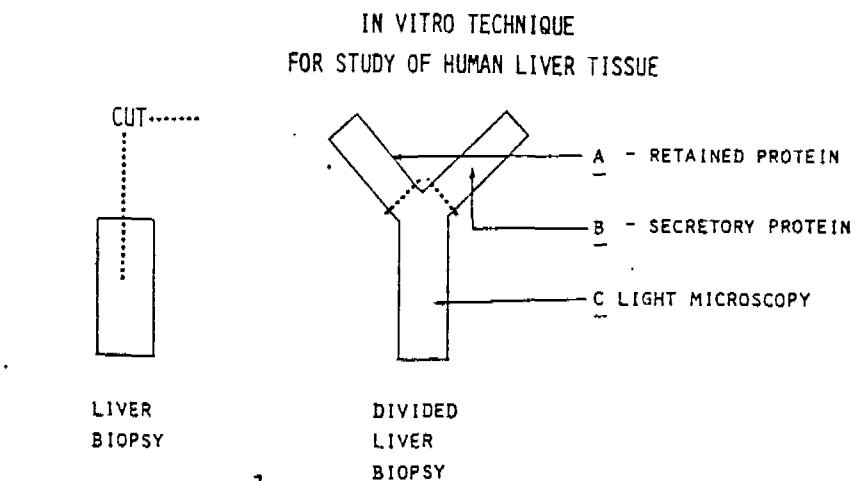

FIGURE 1

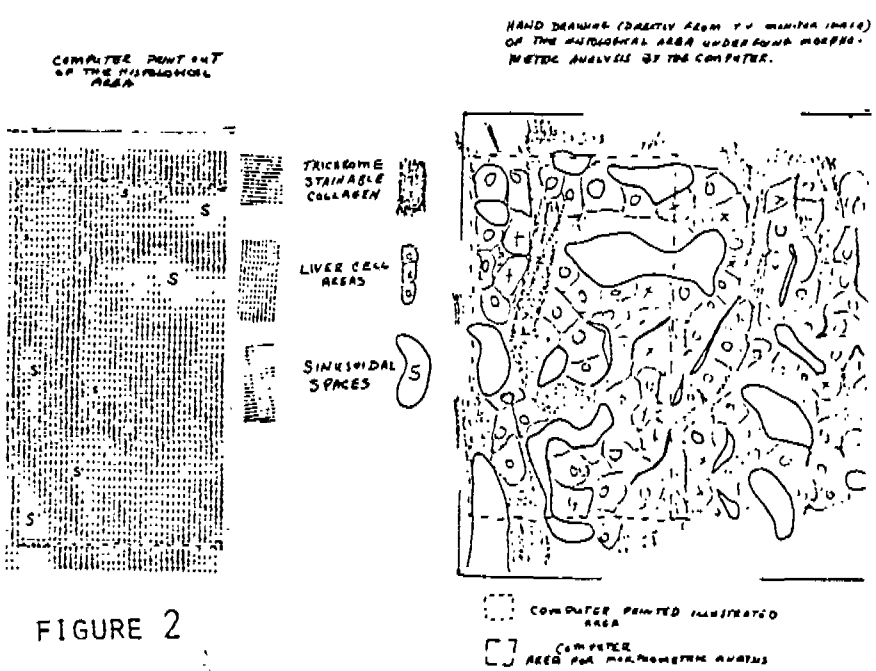

FIGURE 2

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

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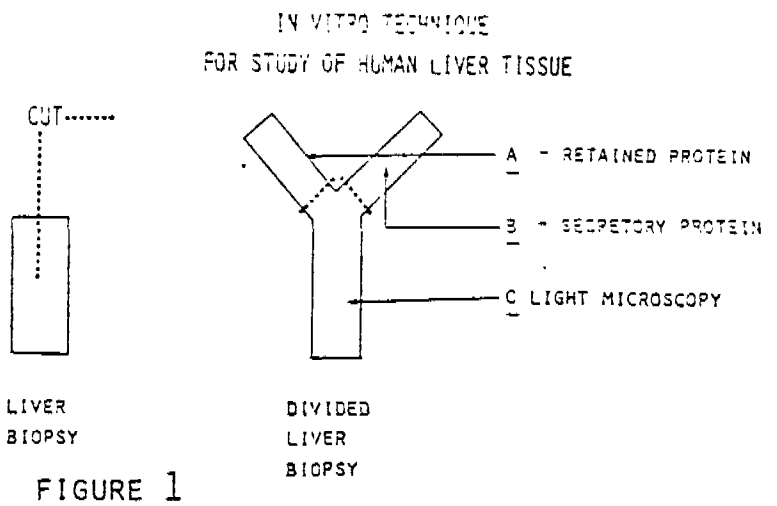

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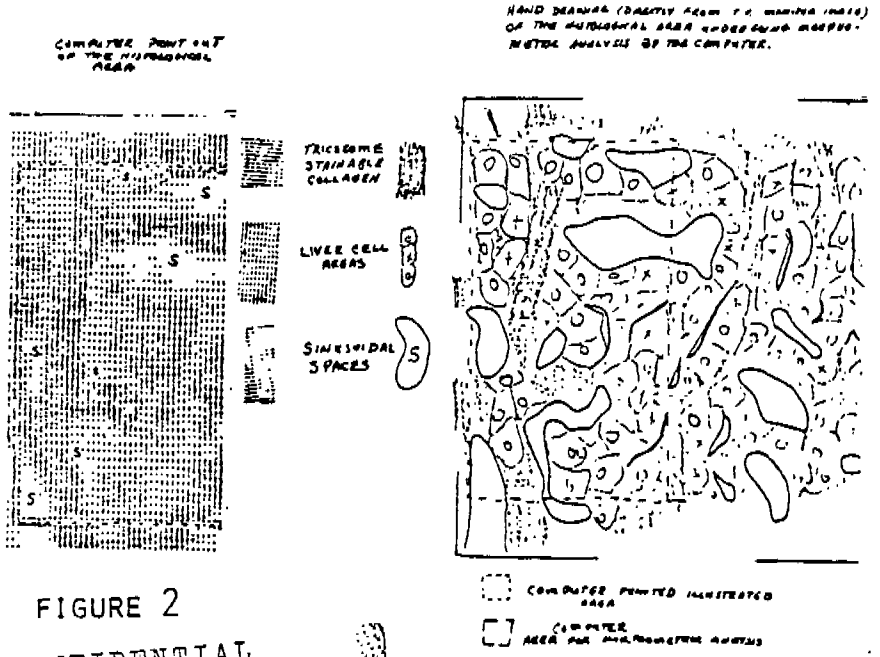


FIGURE 2

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.

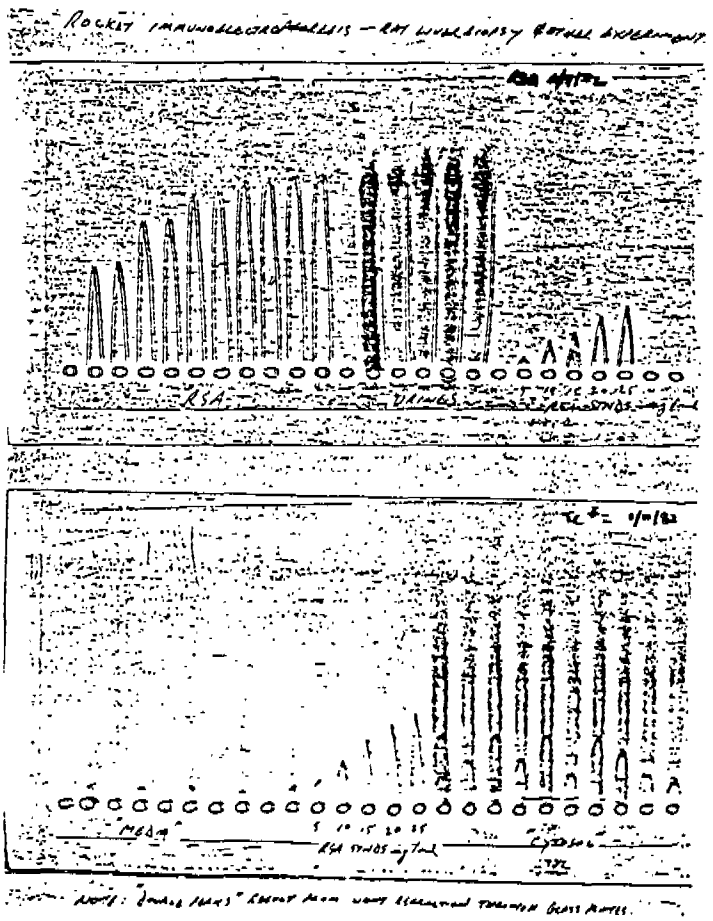


FIGURE 3

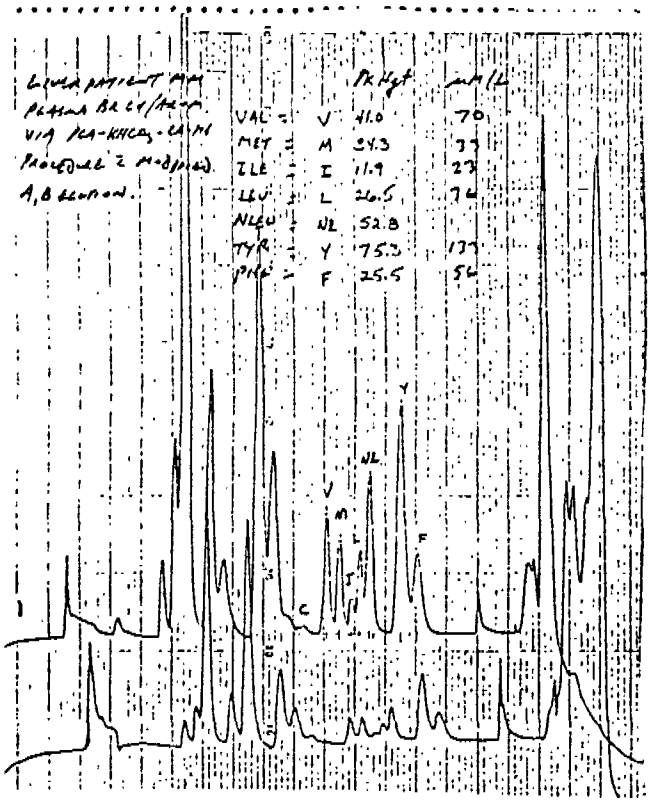


FIGURE 4

TECHNIQUE FOR HEPATIC
CELL TYPE IDENTIFICATION

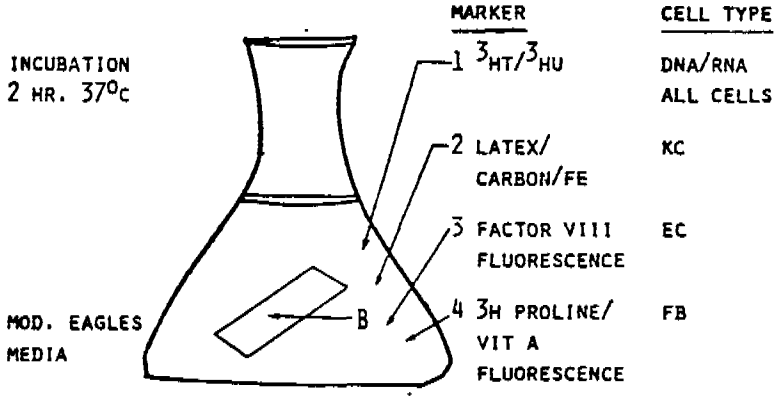


FIGURE 5

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

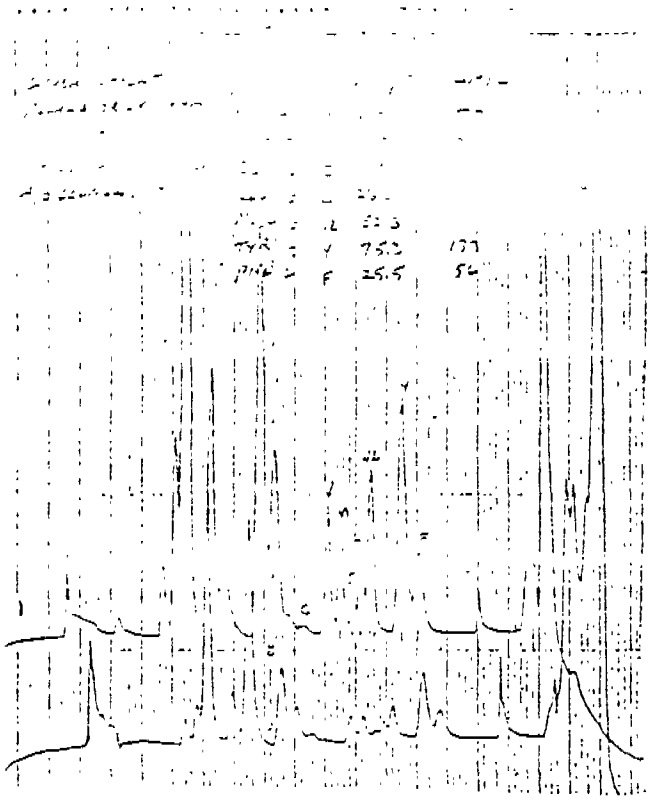
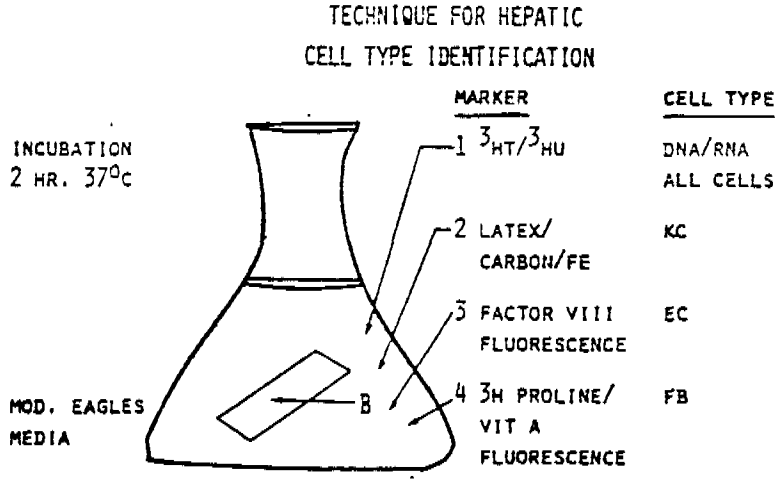


FIGURE 4



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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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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

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 a1194-143, 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

room air, a mouse was briefly anesthesized with ether and sacrificed by freezing in a 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 after 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).

[illegible] briefly anesthetized with ether and [illegible] a bath at -75°C.

The collection 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, 20u- and 40u- 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).

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

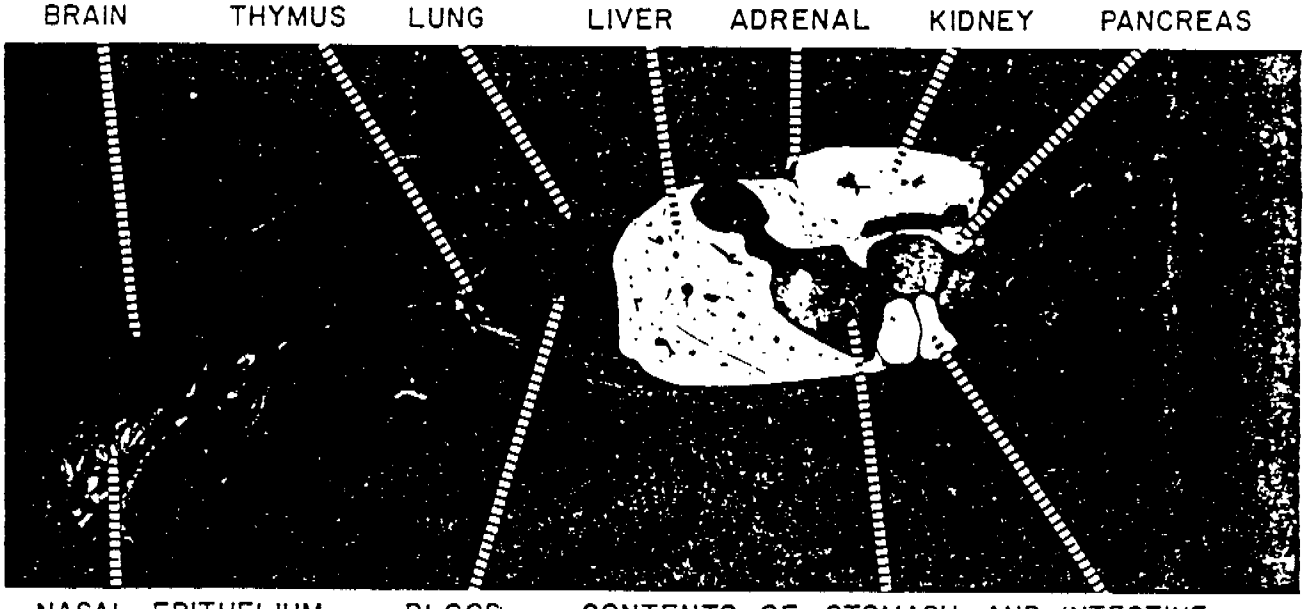

Figure 1: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 20 minutes after removal from the vinyl chloride environment. White areas correspond to radioactivity.

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

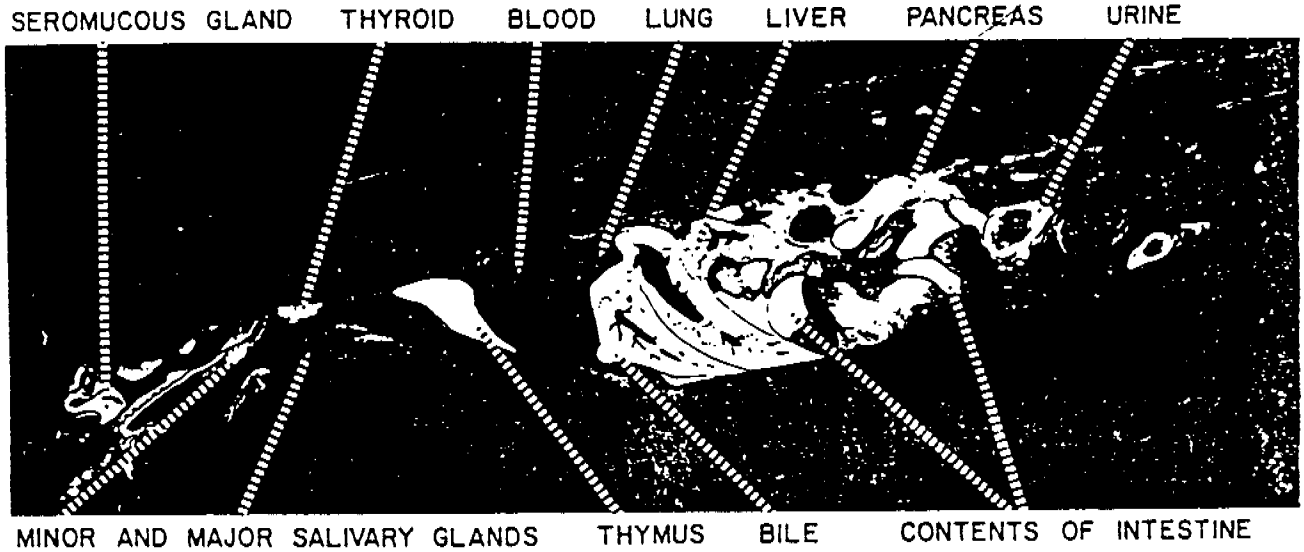

Figure 2: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 1 hour after removal from the vinyl chloride environment. White areas correspond to radioactivity.

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

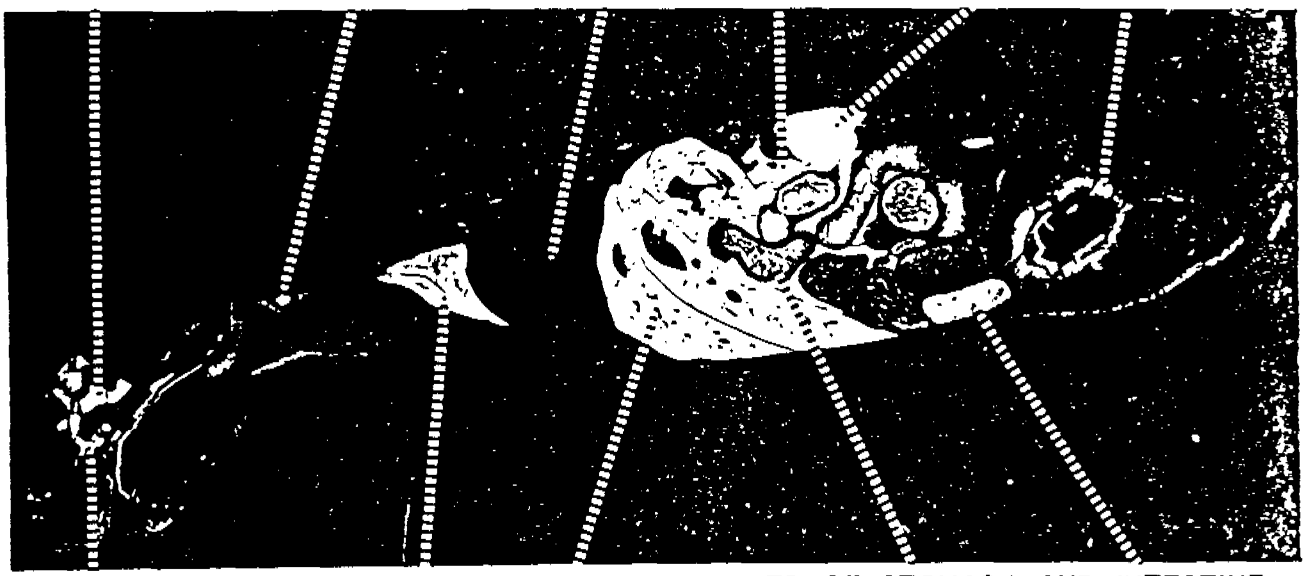

Figure 3: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 3 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

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

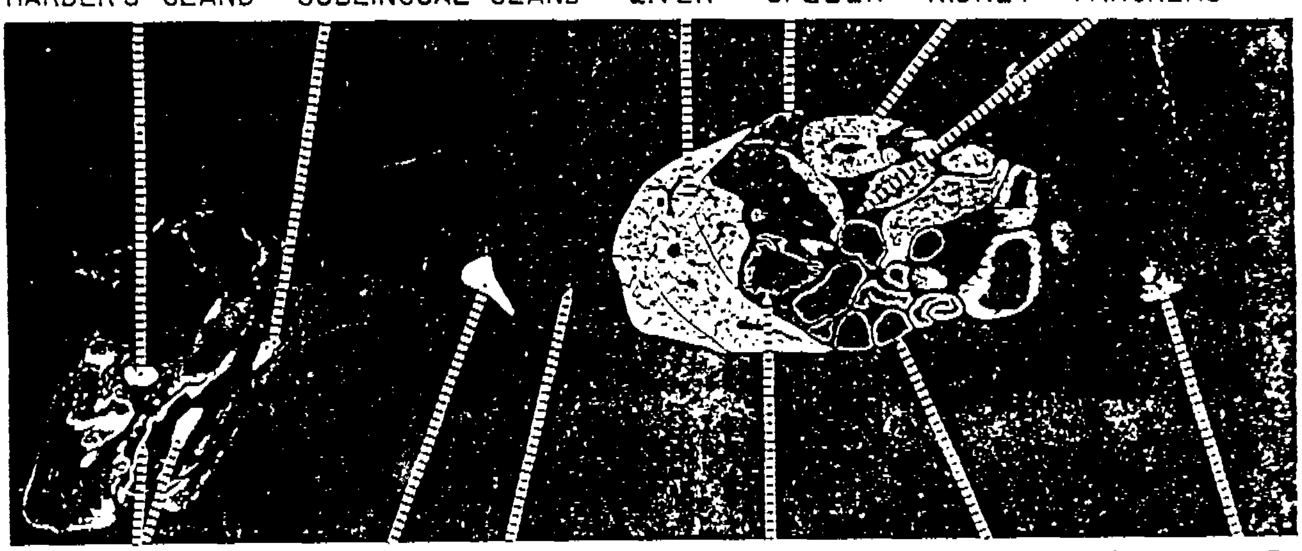

Figure 4: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 4 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE; 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

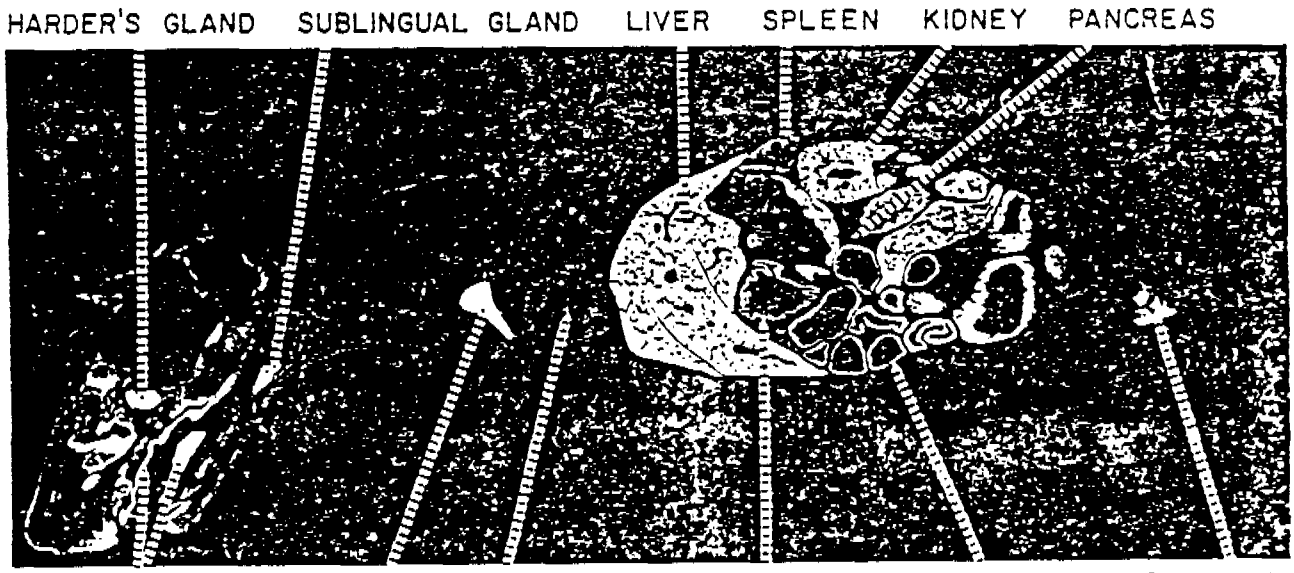

Figure 4: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 9 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

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

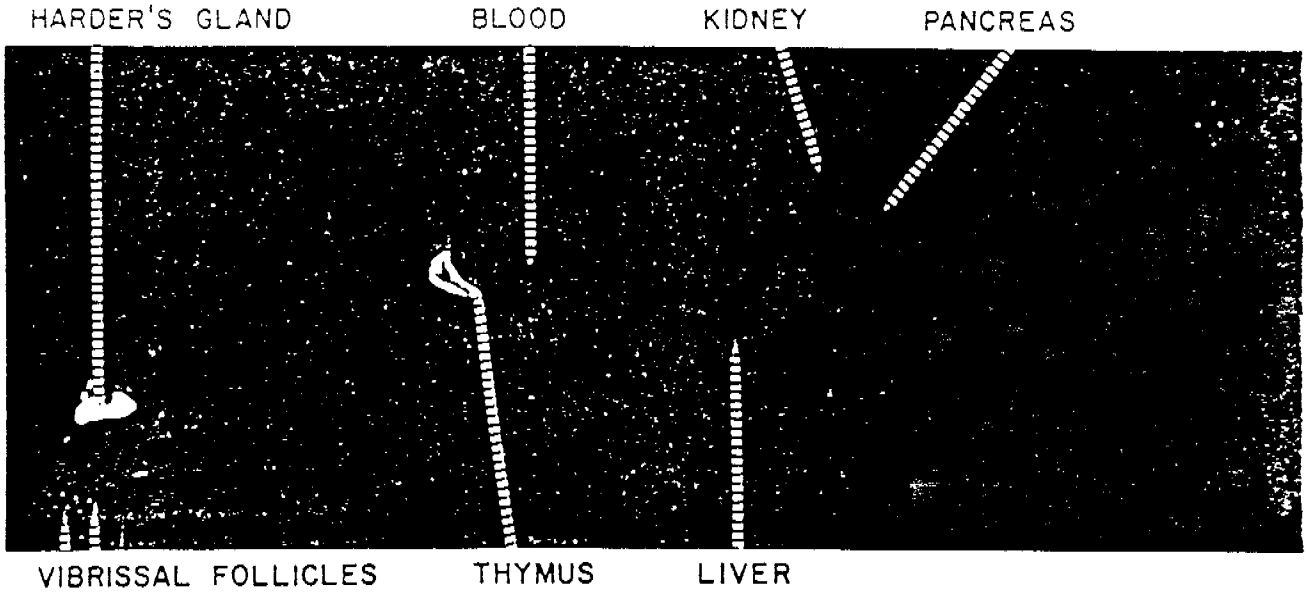

Figure 5: A print of a whole-body autoradiograph from a male CD-1 mouse which was exposed for 3 hours to ^{14}C-vinyl chloride and then frozen 24 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity.

^{14}C-VINYL CHLORIDE

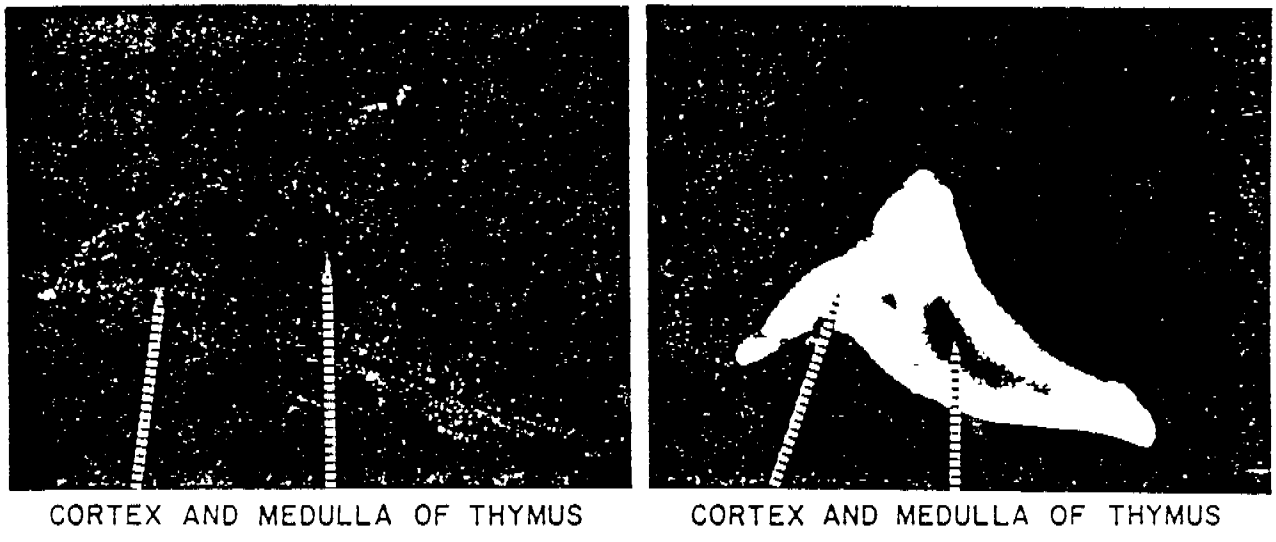

Figure 6: Prints of the thymus areas of whole-body autoradiographs from male CD-1 mice which were exposed to ^{14}C-vinyl chloride for 3 hours and then frozen 20 minutes or 24 hours after removal from the vinyl chloride environment. White areas correspond to radioactivity. Note the high retention of 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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The sites of localization of the non-volatile metabolites of vinyl chloride in the mouse are similar to those reported for the rat (Duprat et al. 1977). The high concentration of non-volatile metabolites of vinyl chloride remained 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).

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LISTS OF PUBLICATIONS, ABSTRACTS, PREPRINTS AND PUBLICATIONS IN PREPARATION

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PREPRINTS

1. Tamburro, C.H., Makk, L. and Popper, H. Early Hepatic Histological Alterations among Chemical (Vinyl Monomer) Workers.

2. Ou, 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.

PUBLICATIONS IN PREPARATION TITLES

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., Schrodt, G.R. and Tamburro, C.H. Collagen Changes in Normal Aging Liver.

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

APPENDIX

PUBLICATIONS

CONFIDENTIAL
Subject to Protective Order in
Ross v. Conoco, Inc., No. 90-4837
14th Judicial District Court
Calcasieu Parish, Louisiana

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 exciter 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 "endothelial cell sarcoma of the liver." Later, in reporting a tumor, Baker et al. (16), 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).

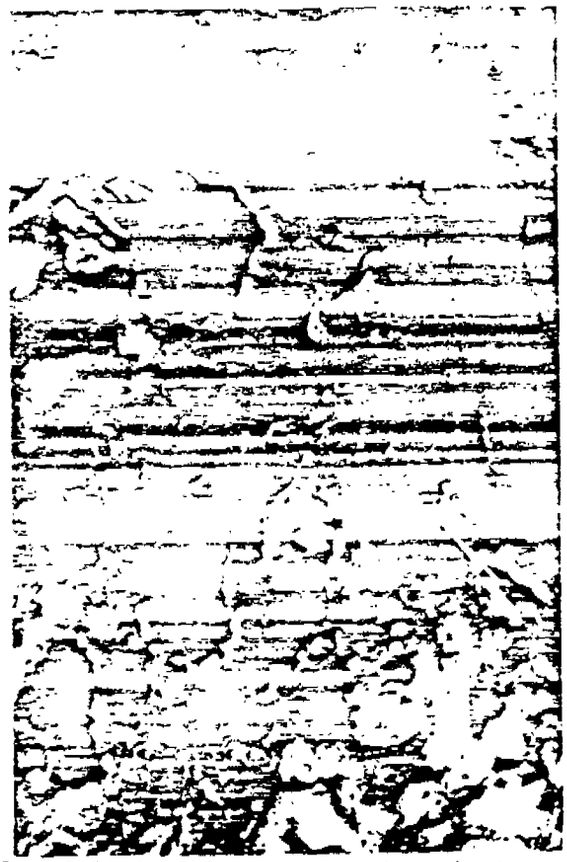

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Figure 5. High-powered frozen section of angiosarcomatous tissue showing factor VIII immunofluorescence of individual proliferating cells lining hepatic cords (white arrows). Fluorescent staining of cytoplasm but not cell nucleus is seen in one of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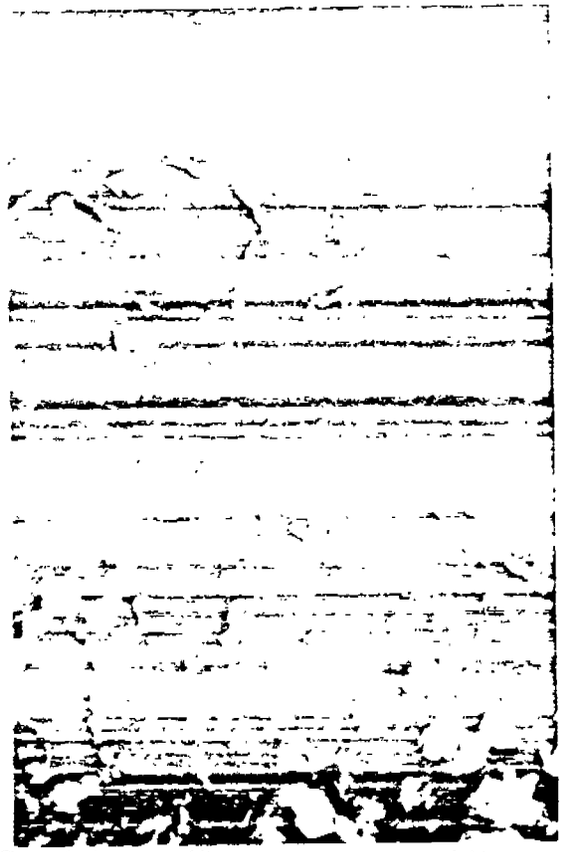

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BIOCHEMICAL ALTERATIONS IN LIVERS OF RATS EXPOSED TO VINYL CHLORIDE

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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

We wish to express our sincere thanks to the people in the B. F. Goodrich Plant in Louisville for their cooperation in the exposure studies, Dr. R. A. Greenberg for help with statistics, and Ms. Ruth Shelton for technical assistance.

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0098-4108/79/051119-14$2.25

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, angiosarcoma 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

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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.

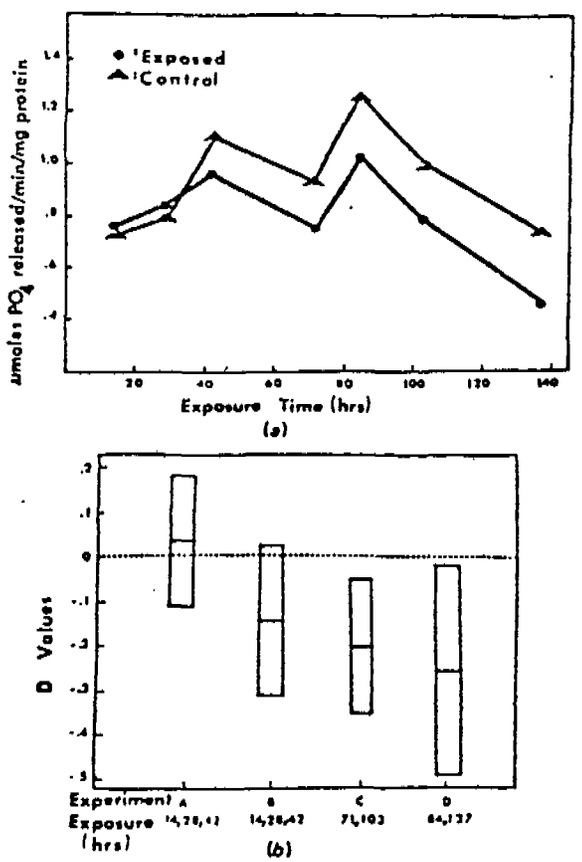

FIGURE 1. (*a*) Composite curve of group means of specific activity of glucose-6-phosphatase with respect to exposure time; (*b*) 95% confidence intervals for *D*, the mean difference between the exposed and control groups. There is no significant difference between the exposed and control groups until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

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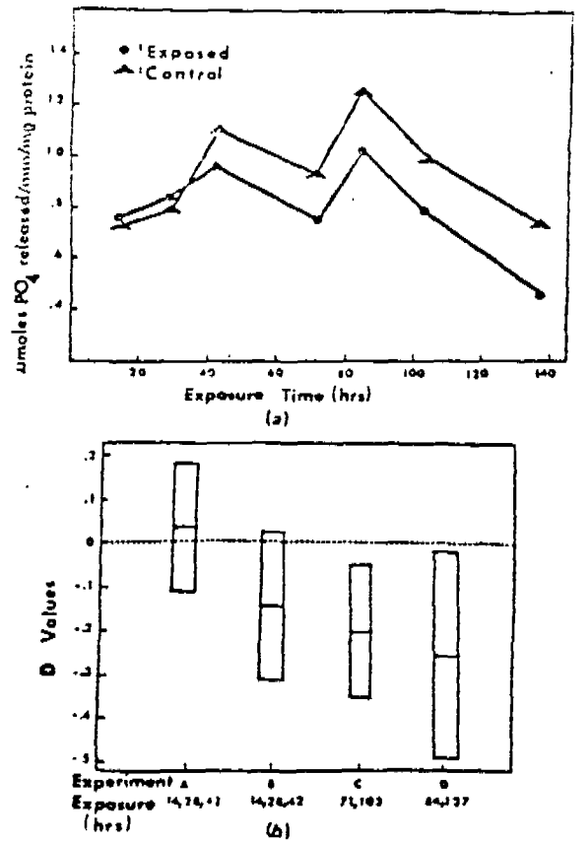

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Glutathione Reductase. Figure 2, *a* and *b*, illustrates the significant differences ($p < 0.05$) between the exposed and control groups in all 4 experiments except at 71 h of exposure ($p < 0.06$). The glutathione reductase level was 25% greater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

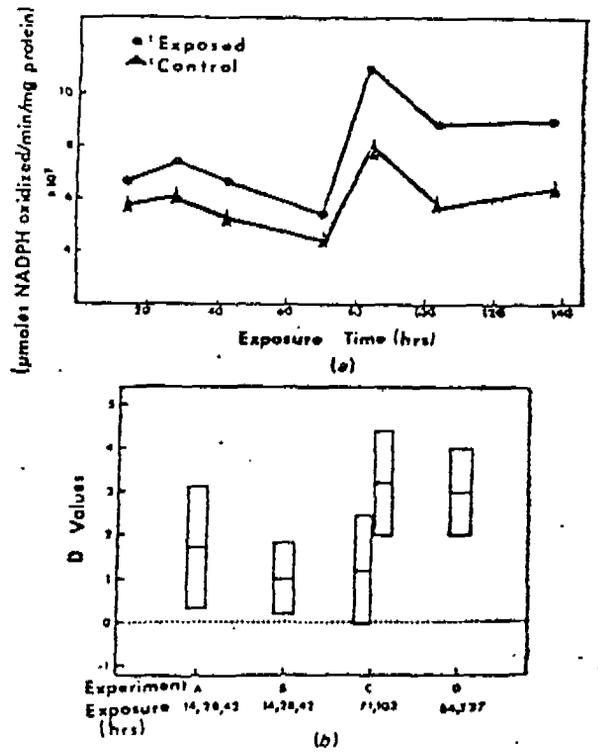

FIGURE 2. (*a*) Composite curve of group means of specific activity of glutathione reductase with respect to exposure time; (*b*) 95% confidence intervals for *D*, the mean difference between the exposed and control group. The mean value for the exposed group is significantly greater than that for the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

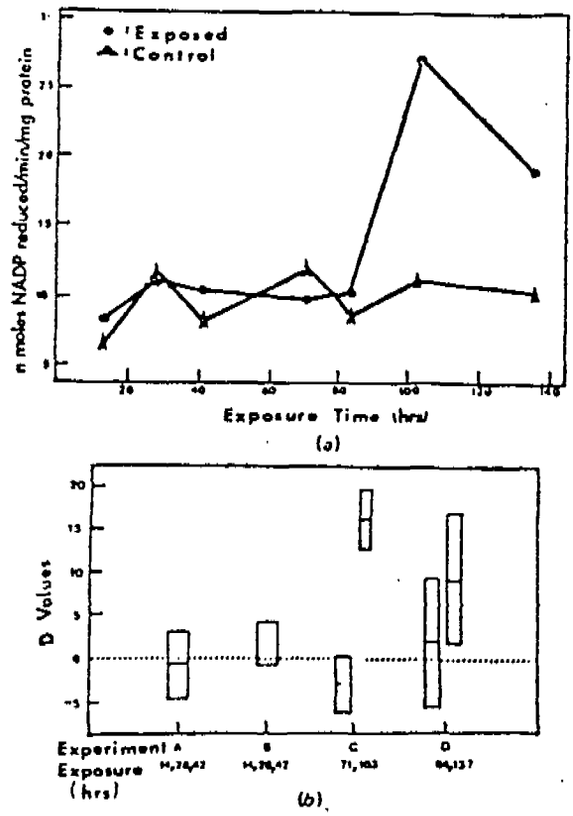

FIGURE 3. (*a*) Composite curve of group means of specific activity of glucose-6-phosphate dehydrogenase with respect to exposure time; (*b*) 95% confidence intervals for *D*, the mean difference between the exposed and control groups. The mean value for the exposed group is not significantly different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

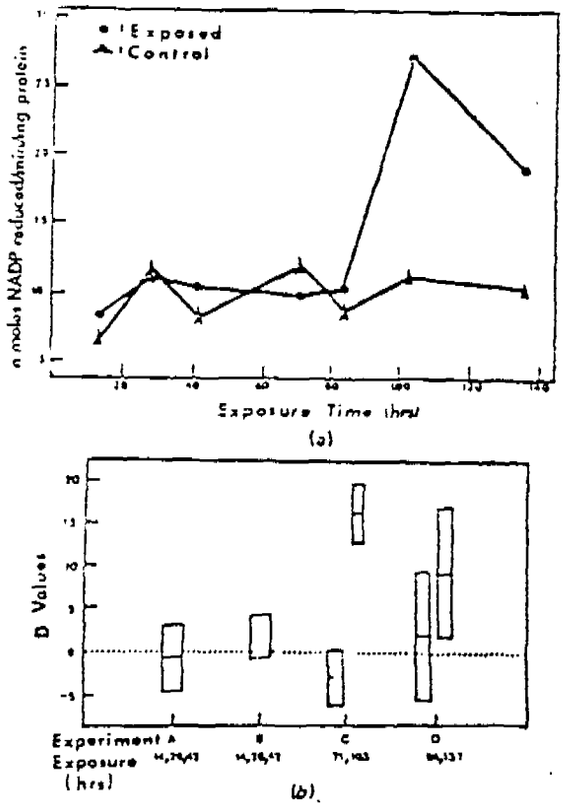

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TABLE 2. *Effect of Vinyl Chloride Exposure on Concentration of Liver Nonprotein Sulfhydryl Compound in Rats*

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 ± 22 (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*b*, 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.

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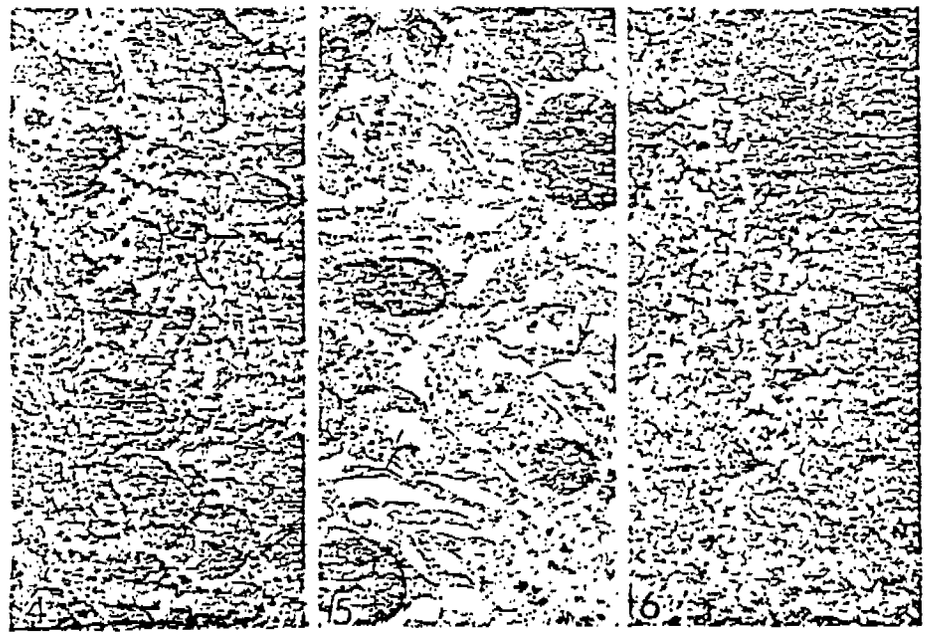

FIGURE 4. Portion of a hepatocyte from a control rat. A good complement of mitochondria and rough endoplasmic reticulum is shown (X12,000).

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 hr 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

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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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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 involvment 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.

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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 80 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.

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

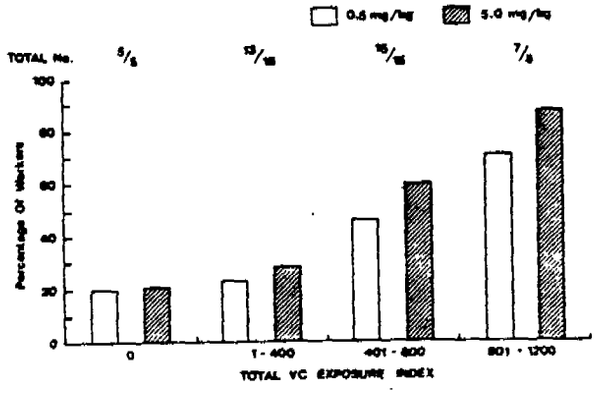

FIG. 1. Frequency of abnormal indocyanine green dye clearance among vinyl chloride workers utilizing 0.5 mg/kg and 5.0 mg/kg doses.

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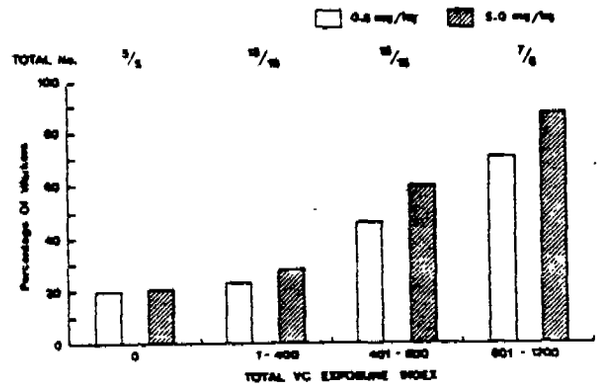

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FIG. 2. Hepatic arteriogram: Venous phase. Changes of peliosis hepatis are present throughout the left lobe. The multiple nodular stains represent peliosis hepatis lesions (*arrows*) ranging from 2–3 mm to 2 cm in size.

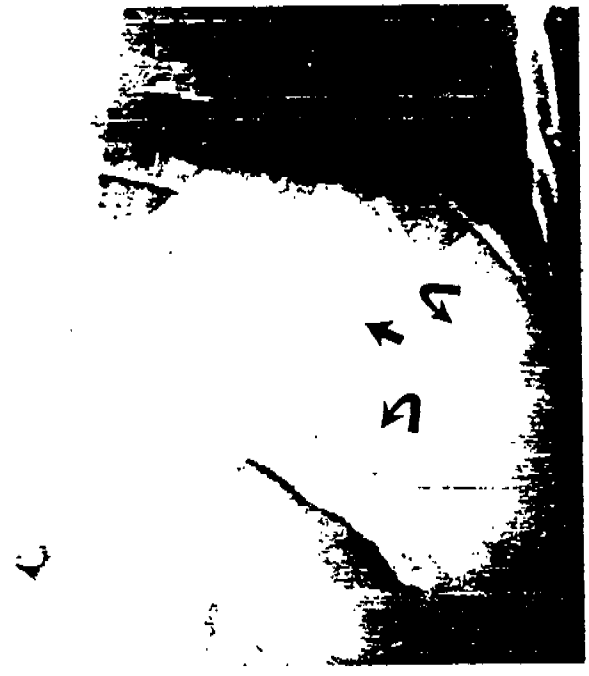

FIG. 3. Splenic arteriogram: Venous phase (15 seconds). There are 3–4 circular and oval stains (*arrows*) in the superior and inferior-lateral portions of the spleen. Normal pancreatic stain occurs just above the splenic vein.

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 puddling 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

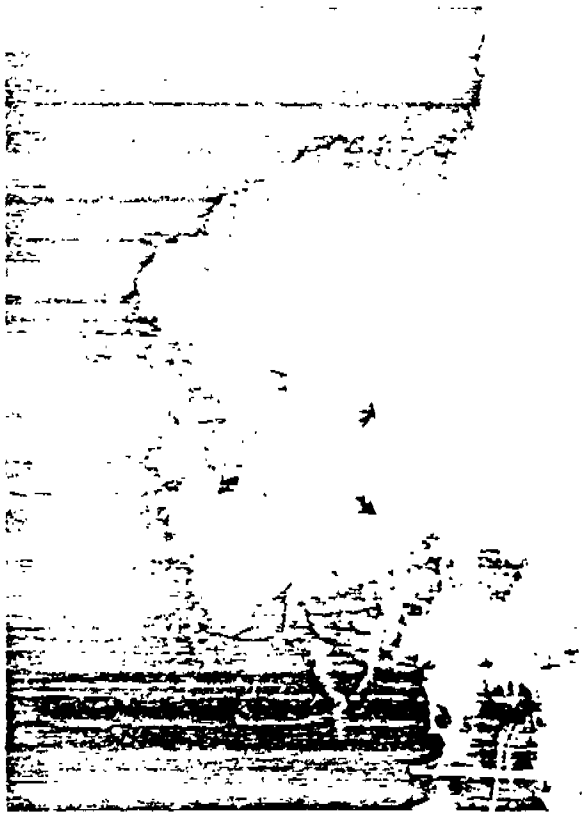

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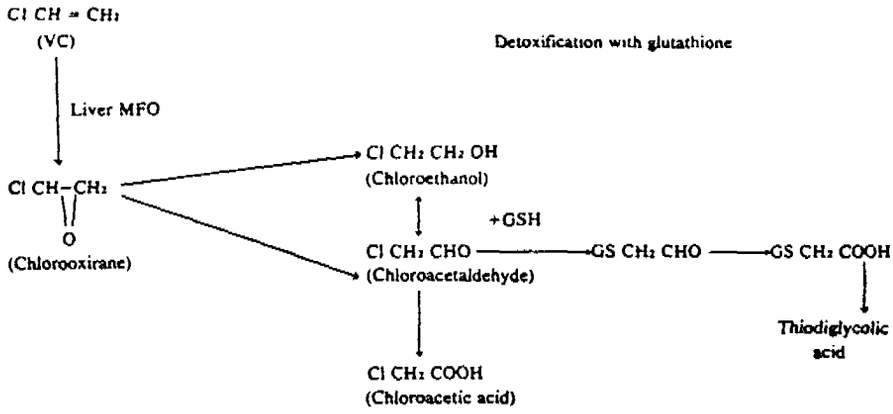
TABLE 3
Proposed Metabolic Fate of Vinyl Chloride



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 "simulated" 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

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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

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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

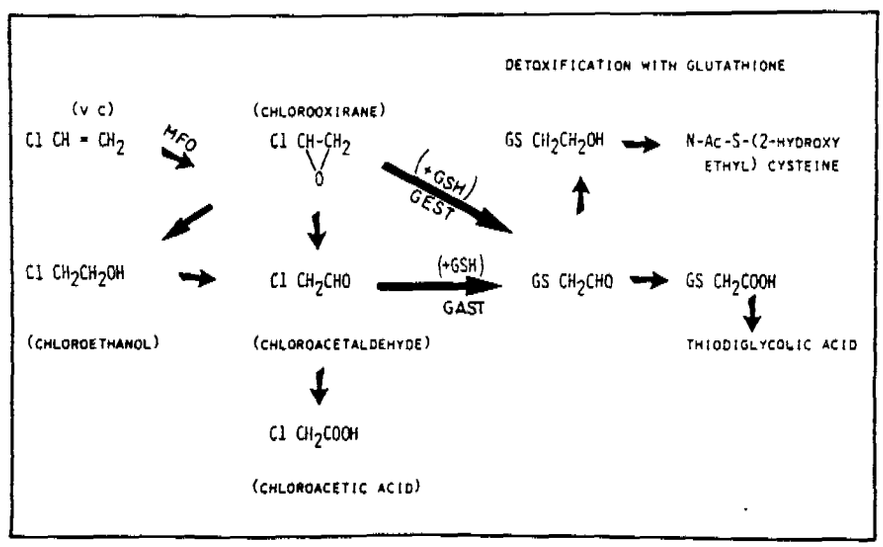

FIG. 1. The proposed metabolic fate of vinyl chloride. (GSH, glutathione; MFO, mixed-function oxidase; VC, vinyl chloride; GEST, glutathione *S*-epoxide transferase; and GAST, glutathione *S*-aldehyde transferase).

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,

was approximately 20 hr after exposure to 50 ppm vinyl chloride.

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TABLE I

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]

		Time (weeks)			
	Treatment	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,d]
	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[d]
	Vinyl chloride-exposed	—	9.1 ± 1.7	9.7 ± 0.7[c]	11.0 ± 1.3[c,d]
	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[d]
	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, $p < 0.05$.

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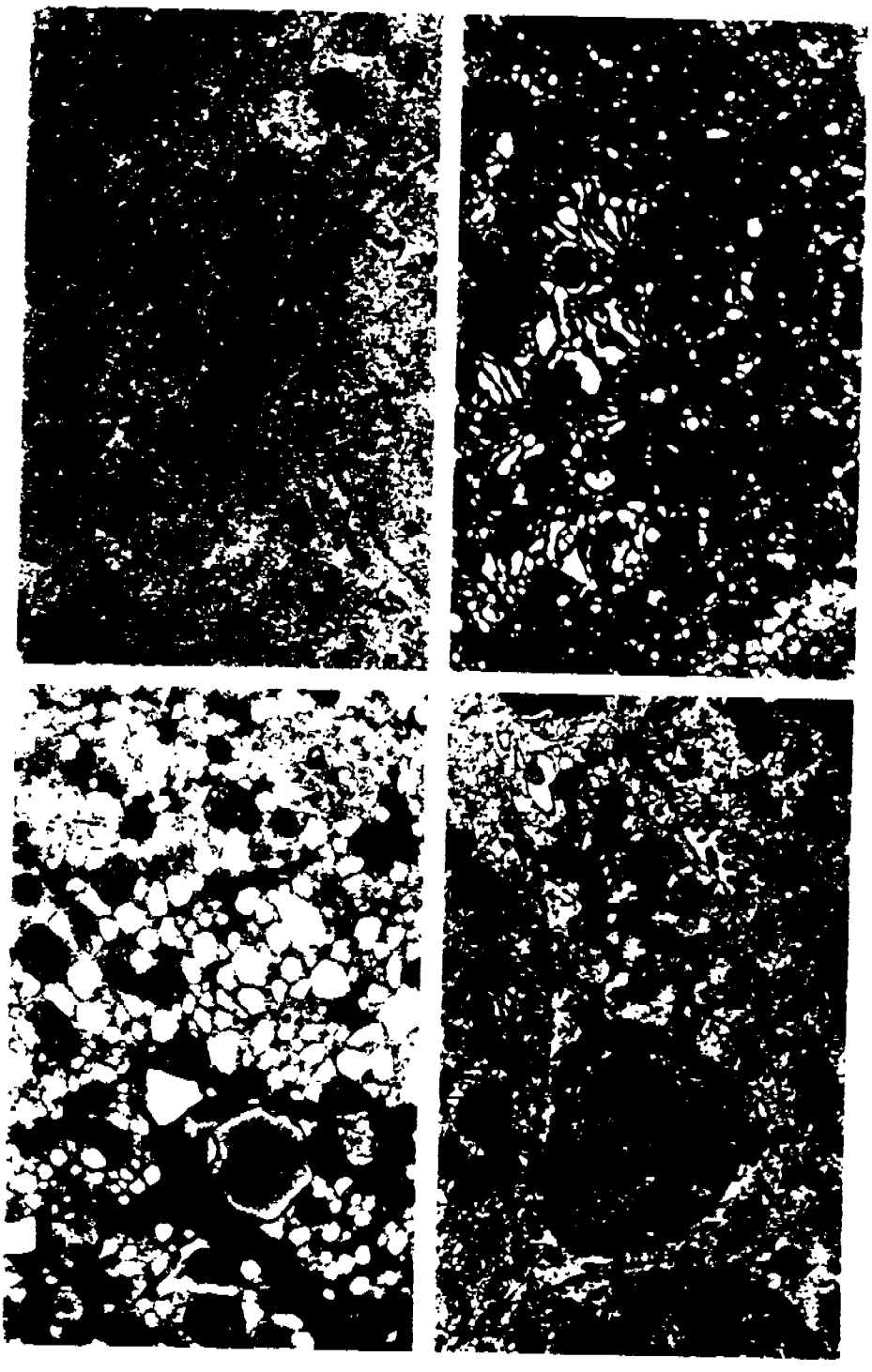

were characterized by an increased accumulation of lysosomal-like substances in some of the sinusoidal lining cells as well as a greater tendency to accumulate lipids in the interstitial cells (Fig. 2d).

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×.

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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 availabiliity of liver biopsy. Evaluation of federally required studies included alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGTP), alanine aminotranserase (ALT, SGPT), aspartic aminotransferase (AST, SGOT) and bilirubin (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_2
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 nonchemical 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

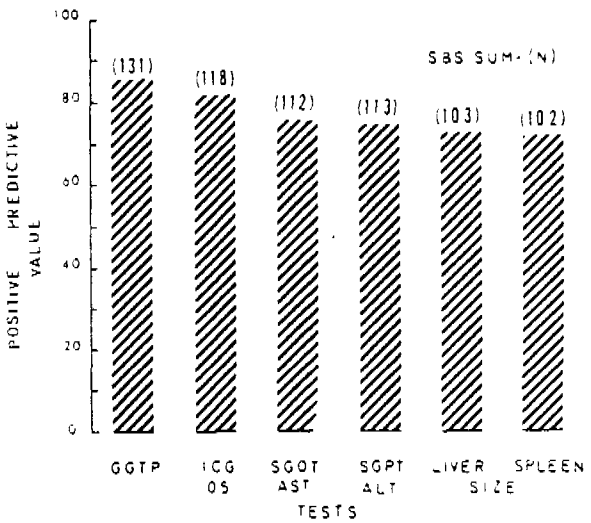

FIGURE 1. Positive predictive values of screening tests in identification of medical disease in an asymptomatic working population (N = 969). All those screening tests with positive predictive values of greater than 70 are shown except for indirect bilirubin (due to high number of congenital indirect 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.

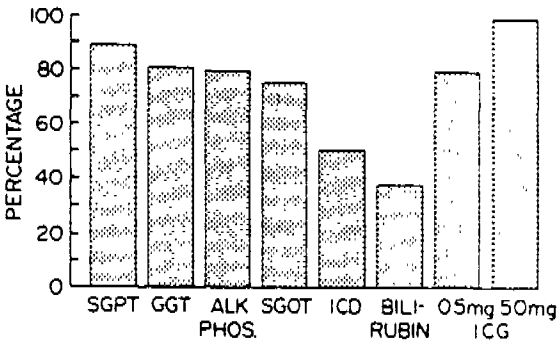

FIGURE 2. Frequency with which clinical biochemical tests correctly reflex the presence of hepatic damage in chemical workers suspected of having liver disease: (SGPT) alanine aminotransferase (ALT) (GGT) γ-glutamyl transpeptidase, (SGOT) aspartic aminotransferase (AST), (Alk. Phos.) alkaline phosphatase, (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 populati[illegible] are shown in Figure 1. The GGTP provided t[illegible] 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 adequate 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

pected or potentially hazardous neoplastic chemicals used within the work place (9,10). 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 nonchemical 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

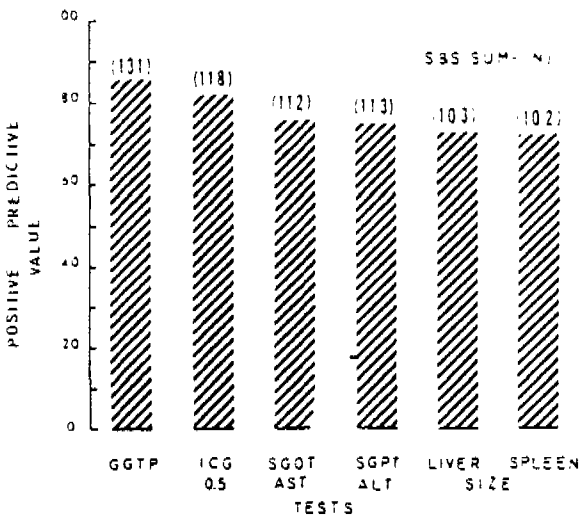

FIGURE 1. Positive predictive values of screening tests in identification of medical disease in an asymptomatic working population (N = 969). All those screening tests with positive predictive values of greater than 70 are shown except for indirect bilirubin (due to high number of congenital indirect 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.

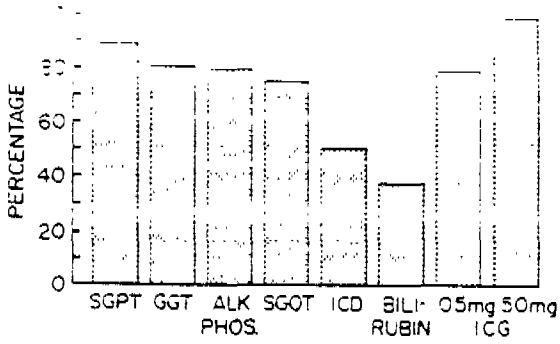

FIGURE 2. Frequency with which clinical biochemical tests correctly reflex the presence of hepatic damage in chemical workers suspected of having liver disease. (SGPT) alanine aminotransferase (ALT), (GGT) γ-glutamyl transpeptidase, (SGOT) aspartic aminotransferase (AST), (Alk. Phos.) alkaline phosphatase, (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 adequate 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

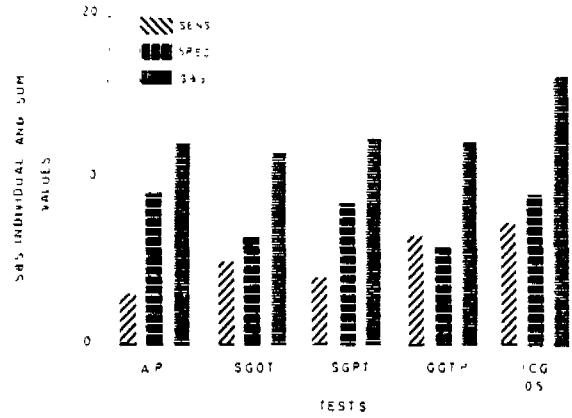

FIGURE 3. Sensitivity and specificity of various biochemical screening tests and their sensitivity and specificity sum values (S & S) based on 78 with biopsy documentation of their hepatic status and all of the biochemical screening studies listed. All screening tests with S & S sums less than 110 (e.g.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

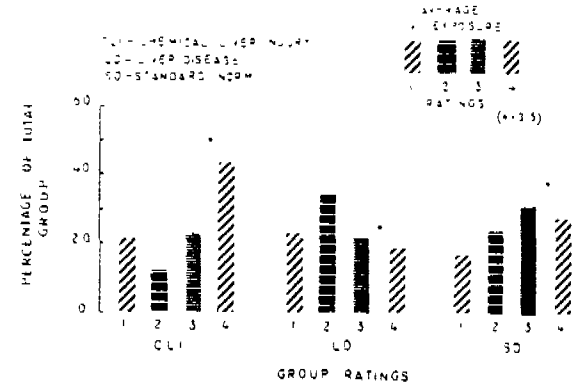

FIGURE 4. Correlation between the histologic findings on liver biopsy and each individual's average total vinyl chloride exposure based on their average CERM (Cumulative Exposure Rank Months) ratings. Rankings: 1 = lowest possible exposure; 2 = minimal exposure, low levels; 3 = moderate exposure; 4 = 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.

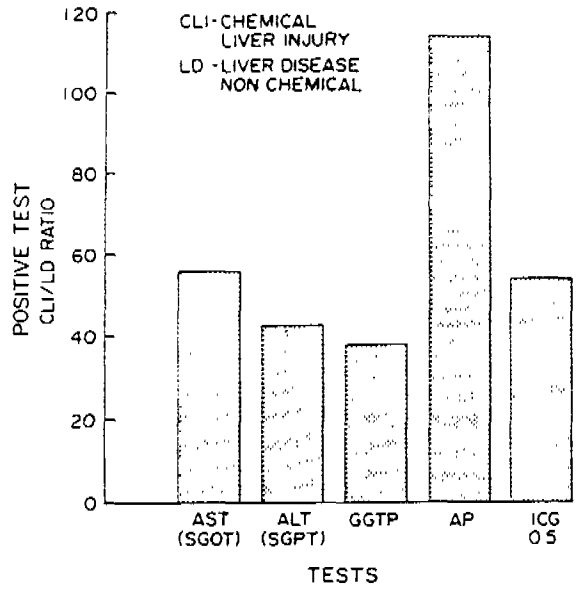

FIGURE 5. Frequency with which biochemical tests were abnormal in those with different type of hepatic injury expresses as a ratio: (CLI) chemical liver injury, (LD) liver disease, nonchemical

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

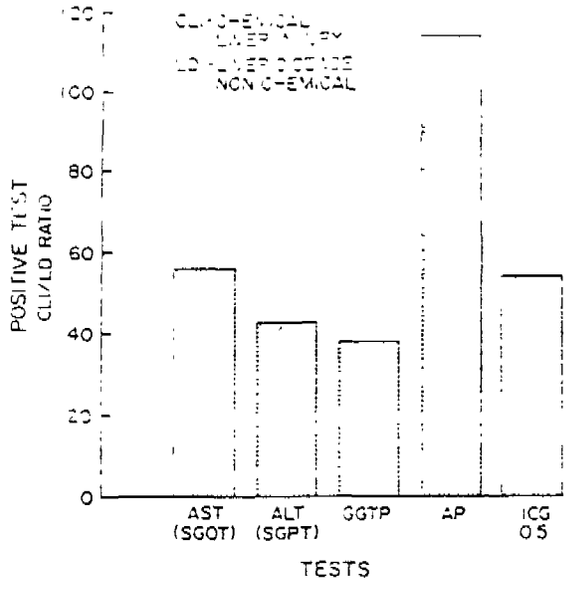

FIGURE 3. Frequency with which biochemical tests were abnormal in those with different type of hepatic injury expressed as a ratio (CLI) chemical liver injury, (LD) liver disease, nonchemical.

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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

KEVIN L. CURRAN, BA, MS, CHARLES E. KUPCHELLA, PHD, AND CARLO H. TAMBURRO, MD

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,[10,16] 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.[1,9,15,18,22] 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.[15] 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]).

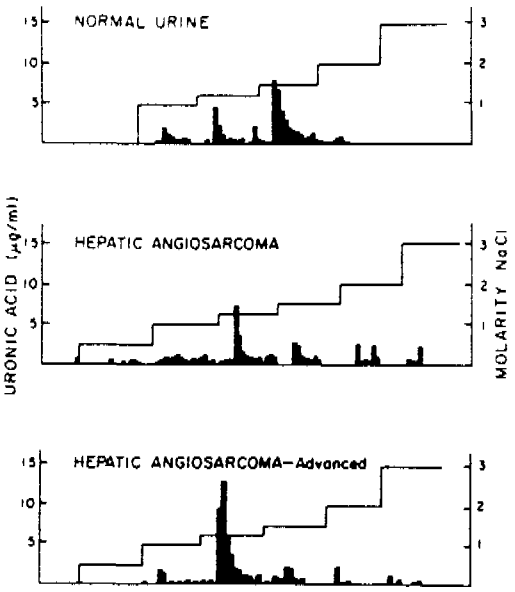

FIG. 1. Elution Patterns of the Urinary Chondroitin Sulfate Fraction. The glycosaminoglycans (GAG) in a 24-hour urine specimen were precipitated with cetylpyridinium chloride (CPC) and separated as 0.4 M NaCl soluble ("hyaluronic acid"), 1.2 M NaCl soluble ("chondroitin sulfate"), and 2.1 M NaCl soluble ("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).

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.[5] 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

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.[9]

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-

Source	[illegible] Uronic Acid [illegible] 1.25 M NaCl
Normal	0.754
Normal	0.516
Angiosarcoma, case 2, pre-chemotherapy[b]	0.843
Angiosarcoma, case 2, post-chemotherapy[c]	0.971
Angiosarcoma, case 1, advanced	5.600

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 [illegible] 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. [b]One day prior to beginning of chemotherapy. [c]Two days following chemotherapy initiation.

susceptibility to testicular hyaluronidase (Nutritional Biochemical Company) using a modification of the cetyltrimethylammonium-bromide, turbidimetric assay described by DiFerrante.[6]

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 [illegible] terns 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 %[a]
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

[a] 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 digestion, [illegible] 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^{0} 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 (µg 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

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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 - 4.8 μg/mg; and Kao 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.

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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 µg 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

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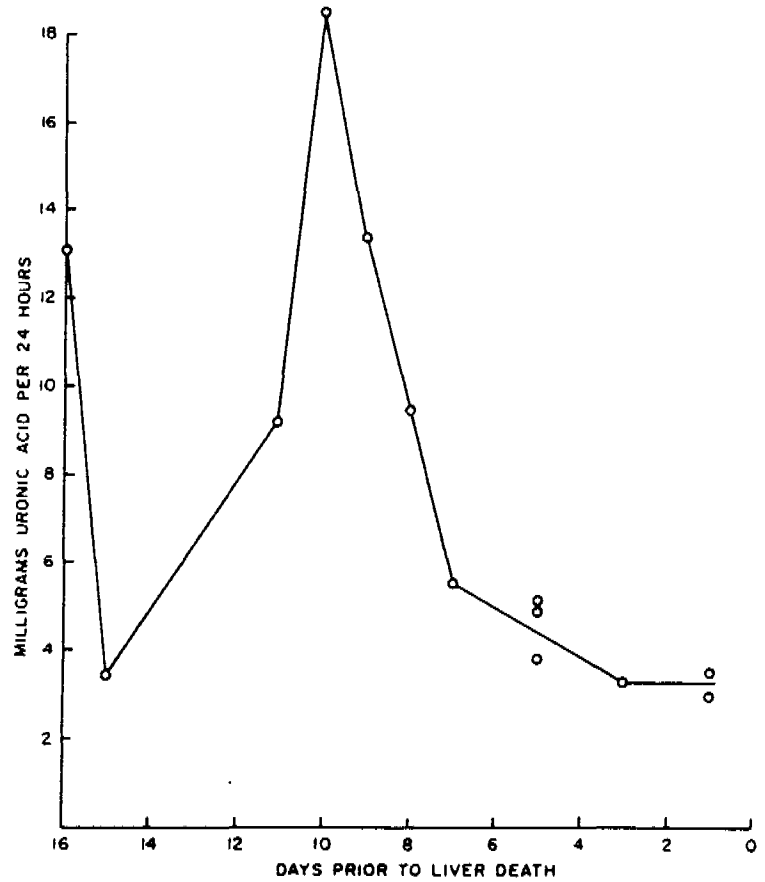


FIG. 1. Urinary glycosaminglycan output in one angiosarcoma patient during the 16-day period prior to death.

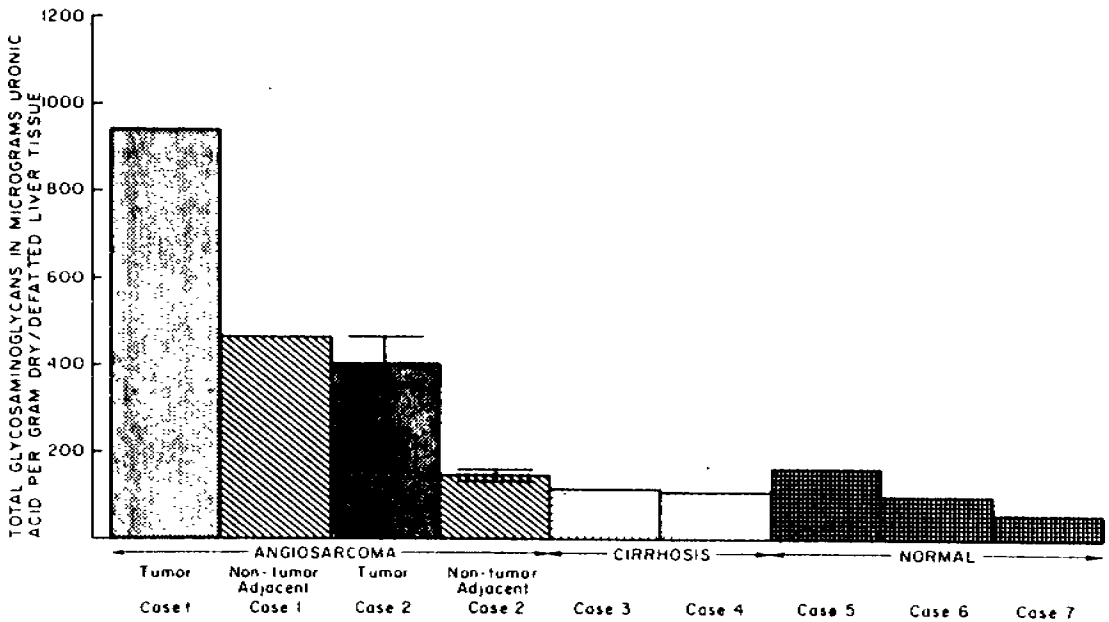
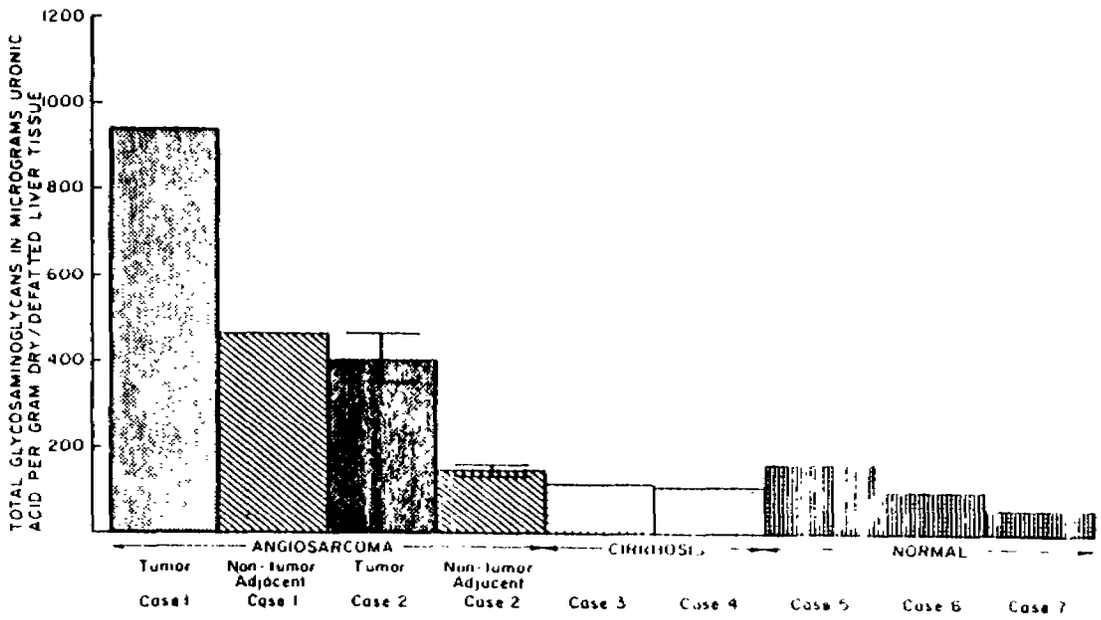


FIG. 2. Glycosaminoglycan concentration in angiosarcomatous, cirrhotic and normal human liver tissue.



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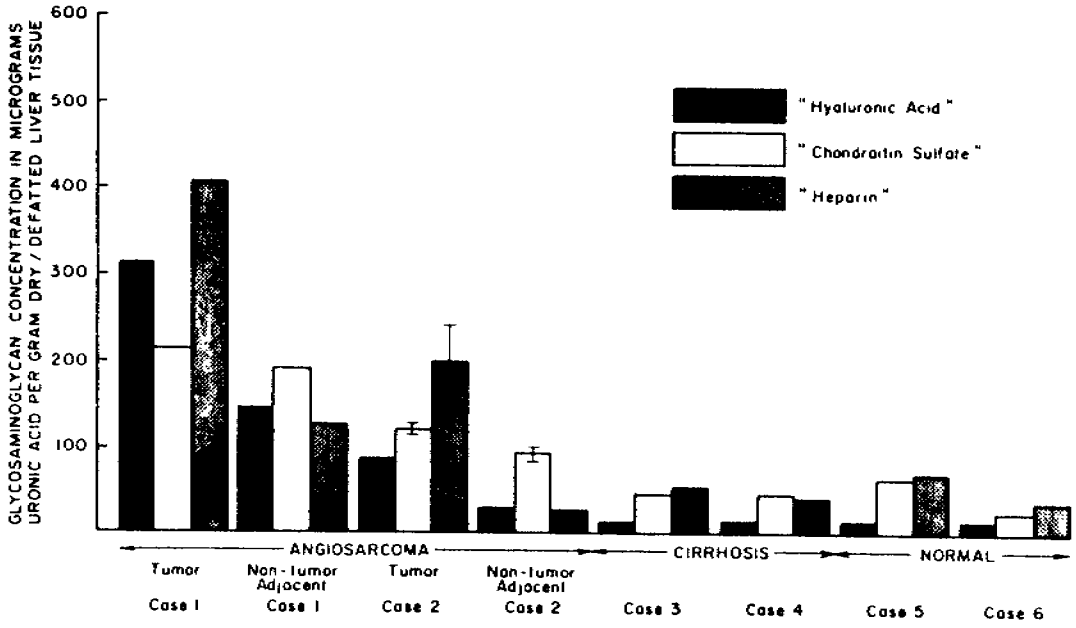

FIG. 3. Fractional concentrations of glycosaminoglycans in angiosarcomatous, cirrhotic and normal human liver tissue.

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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.

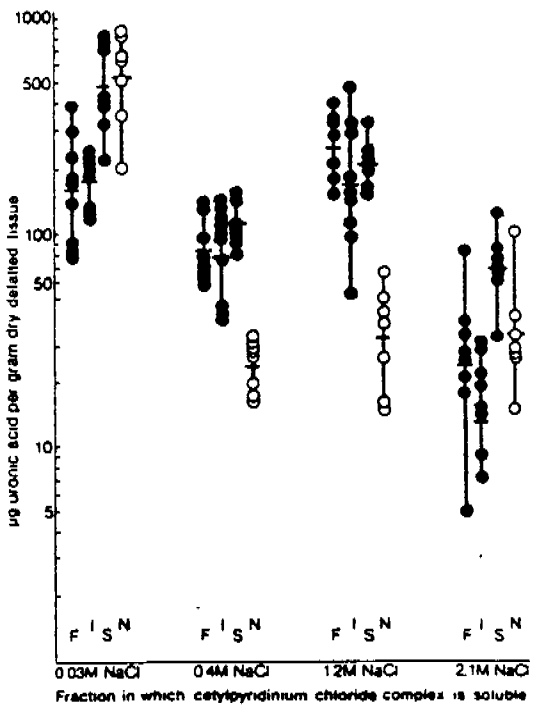

Chart 1. GAG levels measured as uronic acid in fast-growing (*F*) Tumor 7777, intermediate-rate (*I*) Tumor 5123tc, and slow-growing (*S*) Tumor 9618A and in the livers of non-tumor-bearing (*N*) animals for each of 4 sequentially collected salt fractions. *Bars*, geometric means. Differences between *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.

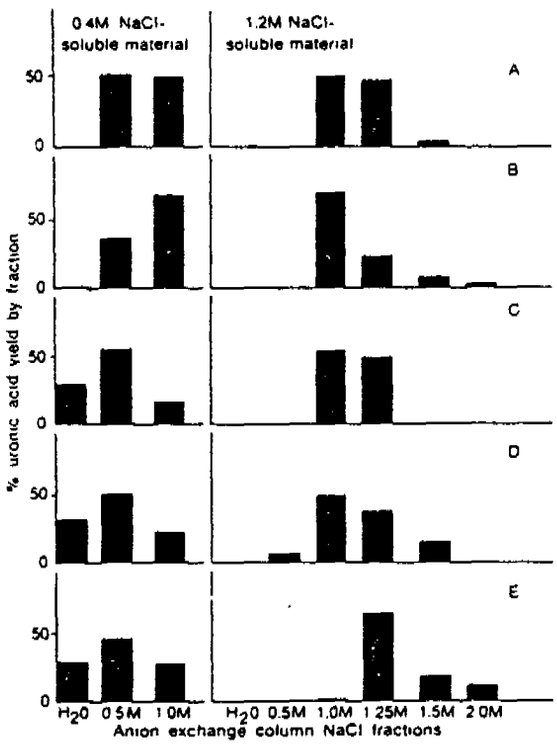

Chart 2. Anion-exchange chromatographic profiles for the GAG-cetylpyridinium chloride complexes solubilized initially in 0.4 M and 1.2 M NaCl. *A*, Tumor 7777; *B*, Tumor 5123tc; *C*, Tumor 9618A; *D*, pooled liver tissue from tumor-bearing animals; *E*, normal liver. Recoveries ranged from 85 to 107%.

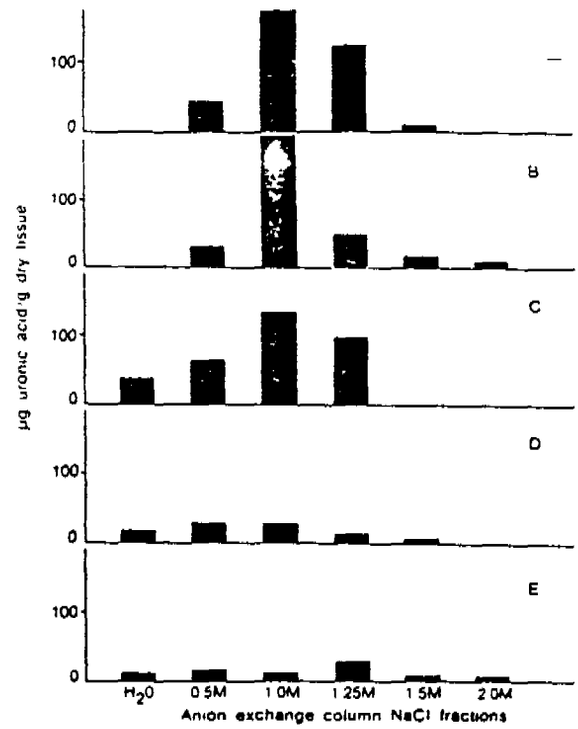

Chart 3. Anion-exchange chromatographic patterns obtained for the GAG's isolated initially as 0.4 M NaCl-soluble and 1.2 M NaCl-soluble cetylpyridinium chloride complexes. *A*, Tumor 7777; *B*, Tumor 5123tc; *C*, Tumor 9618A; *D*, livers of tumor-bearing animals pooled across tumor types; *E*, normal 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. 1*B*).

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.

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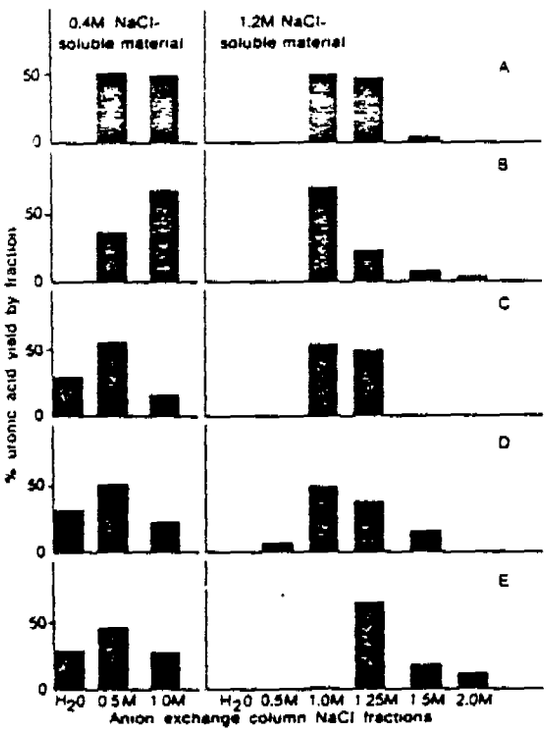

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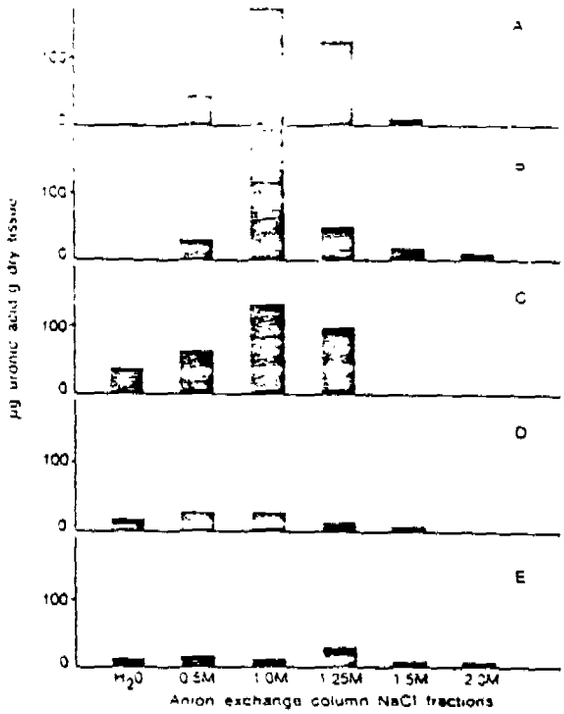

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Table 2

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.

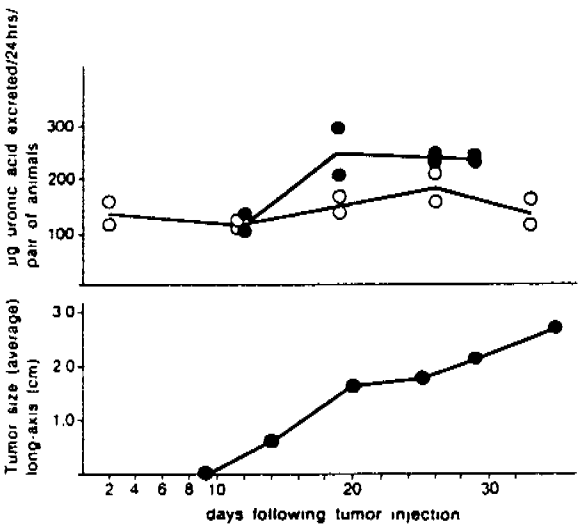

Chart 4. Urinary GAG excretion in relation to tumor growth for animals bearing Tumor 5123tc and in control animals. ●, tumor-bearing animals; ○, control animals.

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 urinary GAG excretion may reflect properties of certain tumors and is not simply an indirect result of the presence of tumor. Overall levels of excretion of GAG's 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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[illegible] 0.03 M NaCl-soluble, uronic acid-positive materials [illegible]

The fact that urinary GAG excretion was elevated in animals [illegible] 5123tc and 9618A [illegible]

[illegible] 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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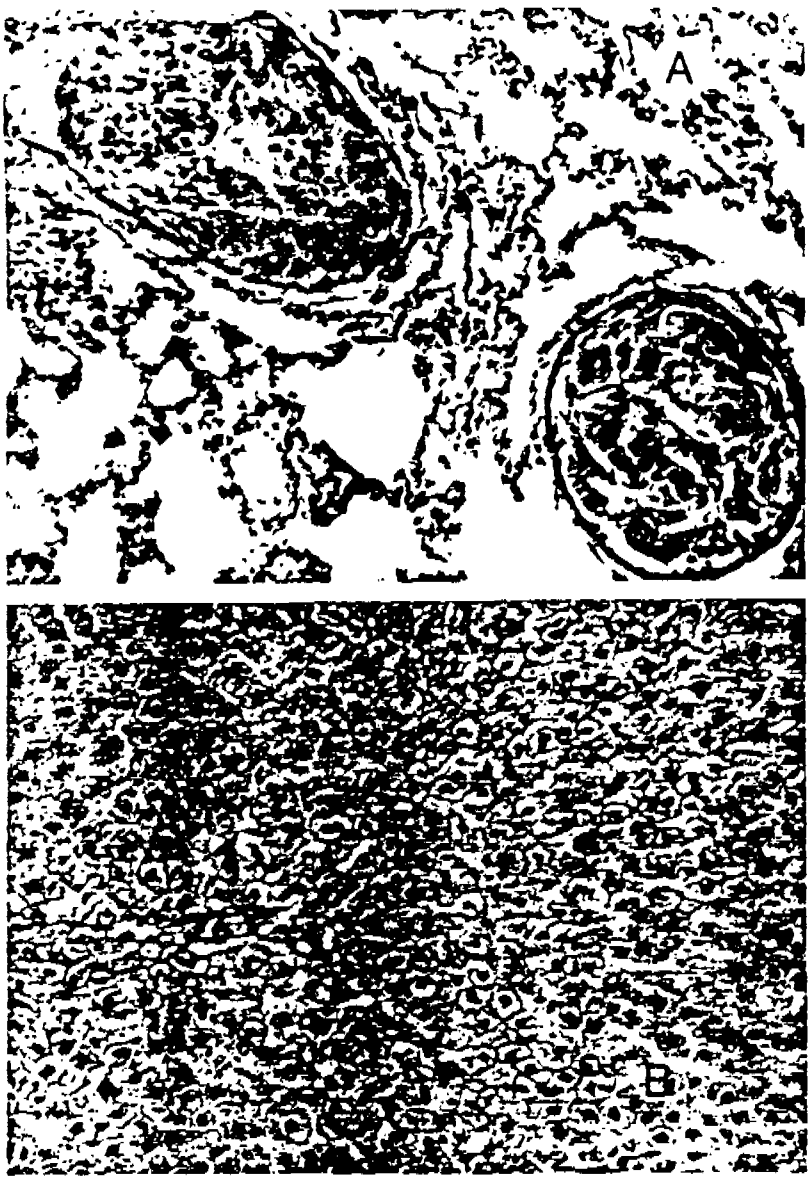

Fig. 1. *A*, typical lung metastases in animals bearing Tumor 7777. These were found more frequently and earlier and were larger in animals bearing Tumor 5123tc. No lung metastases were found in any of the animals bearing Tumor 9618A ×50. *B*, liver of animals bearing Tumor 5123tc showing clear cytoplasm-vacuolar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