CHEMICAL MANUFACTURERS ASSOCIATION

December 29, 1981

N. F. Elbert
Vice President
for Financial Affairs
University of Louisville
Louisville, KY 40208

RE: VC 7.0-DP/PREV-UL

Dear Mr. Elbert:

On March 1, 1979, the University of Louisville executed a contract for $287,784 with the Manufacturing Chemists Association (predecessor of the Chemical Manufacturers Association) in accordance with a University of Louisville proposal, "Research Techniques and Methods for Detection and Prevention of Carcinogenesis in Industrial Workers". (Exhibit I)

The Chemical Manufacturers Association has dutifully made interim payments on this contract totalling $267,784.00. Since the last invoice in April 1981, CMA staff and scientists have sought to obtain progress reports from the principal investigator on this project, Carlo H. Tamborro, M.D., with little success. While the Chemical Manufacturers Association has sought to foster this scientific research, terms of this contract have been flagrantly disregarded by the University of Louisville without even the professional courtesy of a letter of explanation.

The Chemical Manufacturers Association hereby notifies the University of Louisville of the following material breaches in this contract.

A. Failure to provide status reports.

B. Failure to complete research and submit final report pursuant to contractual terms.

Accordingly, the Chemical Manufacturers Association has the right to demand a complete reimbursement of all monies paid the University of Louisville as well as administrative expenses incurred in the supervision of this contract.

Your prompt attention to this matter is required.

Respectfully,

Patrick C. Joyce
Attorney

Enclosure: Exhibit I, Copy of Contract

cc: w/o Enclosure

Carol Stack, Ph.D., CMA

Charles F. Staggs
Assistant Controller
University of Louisville

Carlo H. Tamburro, M.D.
University of Louisville

VC 70-SP-PREJ-JL

UNIVERSITY OF LOUISVILLE
LOUISVILLE, KENTUCKY 40232

SCHOOL OF MEDICINE
DEPARTMENT OF MEDICINE
DIGESTIVE DISEASES AND NUTRITION SECTION

HEALTH SCIENCES CENTER
WALNUT & PRESTON STREETS

July 30, 1979

CONFIDENTIAL
Subject to Protective Order in
Ross v. Conoco, Inc., No. 80-4857
14th Judicial District Court
Calcasieu Parish, Louisiana

Mr. Joseph T. Seawell
Program Manager
Manufacturing Chemists Association
1825 Connecticut Avenue, N.W.
Washington, D.C. 20009

RE: Quarterly Report for the Manufacturing Chemists Association's Agreement with the University of Louisville for the 1979-80 Fiscal Year.

Dear Mr. Seawell:

The following described what has been completed during the past quarter of the Manufacturing Chemists Association's agreement with the University of Louisville entitled, "Research Techniques and Methods for Detection and Prevention of Carcinogenesis in Industrial Workers". Progress for each technical proposal will be reported separately.

Technical Proposal A -- Immunological Systems for the Detection of Vinyl Chloride and Other Chemical Injury. H. P. Fortwengler

Fortwengler's laboratory has continued evaluating three independent aspects of the immune system of industrial workers to determine whether cancer can be detected earlier or to identify those at high risk.

Part I. Evaluation of Immunocompetence of Humans Chronically Exposed to Vinyl Chloride.

The purpose of this study was to determine the immunocompetence of individuals that have undergone prolonged exposure to VC monomer and have developed liver lesions which, in some instances, are thought to presage the development of the cancer angiosarcoma. Immunological assays that have demonstrated usefulness in indicating immunodepression in cancer patients were used.

These tests evaluated the immunocompetence of individuals with possible pre-malignant lesions or other liver disease as demonstrated by biopsy. A comparison of the employees with demonstrated disease versus those employees with no clinical or laboratory evidence of disease showed no immunological difference between the two groups. A comparison of VC workers having high VC exposure (those with lifetime exposure above

the plant median VC exposure) with individuals having low exposure (below the plant median) demonstrated a slight pattern of immunodepression when lymphocytes were stimulated by PHA and Con-A. However, further evaluation of this data, even after additional immunological parameters were examined, indicated there is no statistical significance between the high and low exposure groups.

We are putting together data for a publication that indicates that there is no residual immunological depression as a result of chronic exposure to increased levels of VC as determined by the standard battery of immunological tests.

Part II. HLA Frequencies in V.C. Workers.

The microdroplet lymphocyte cytotoxicity test has been used in our laboratory to tissue type for 11 separate HLA-A antigens and 16 HLA-B antigens and their possible increased association with angiosarcoma or other chemically-related diseases. An increased number of tests have been completed: approximately 471 individuals from the Louisville vinyl chloride polymerization plant have had their lymphocytes isolated and typed.

Part III. A Search for Evidence of a VC-Induced Tumor Antigen.

A comparison of the responses between vinyl chloride plant workers and normal non-chemical plant workers indicated that there were many people in the general population having reactivity to hepatic tissue antigens irrespective of whether these antigens were from liver angiosarcoma or normal liver. Non-tumor specific reactions of this type may be due to sensitization by "natural" means, injections of human or animal substances, transfusions, etc.

Vinyl chloride workers were classified according to known VC exposure and tested for reactivity to reagents prepared from normal liver or angiosarcoma liver. Lymphocyte responses from all individuals tested having low VC exposure (below plant median exposure) were compared to responses from all individuals having high exposure. No quantitative statistical differences were noted between the reactivities of the two groups.

When viewed qualitatively, concomitant reactions by an individual's lymphocytes to both normal liver and angiosarcoma liver cannot be interpreted. Angiosarcoma reagent contains both normal and tumor antigen. Therefore, only those remaining individuals with reactions to either normal liver or angiosarcoma liver alone were compared further. The composition of that group of individuals reacting to the angiosarcoma liver reagent was striking--the only reactions obtained against this tumor preparation were from individuals with high VC exposure. That is, the individuals reacting to the tumor antigen reagent were all from the high risk group.

We have further determined that because of the small number of individuals reacting to angiosarcoma antigen, statistical validity of these results cannot be demonstrated. As a result, these data must be interpreted with caution.

In addition to testing for evidence of reactions to an angiosarcoma antigen, we have found that the tumors contain increased amounts of a normally occurring antigen, coagulation Factor VIII. Factor VIII is a large protein, often called antihemophilic factor, which was demonstrated by Hoyer et al. to be found differentially in endothelial cells, platelets and megakaryocytes. We have found FVIII in endothelial cells lining the lumens of arteries and veins in normal liver section when stained with rabbit anti-Factor VIII and FITC-conjugated anti-rabbit IgG. Normal sinusoidal linings displayed scanty fluorescense.

Sections of angiosarcoma, however, demonstrated a strikingly increased fluorescence which appeared lining enlarged sinusoids. The intensity and expanse of positive fluorescence seen in angiosarcoma tissue was never seen in sinusoids of normal liver. Control sections indicated that the fluorescence was due to a specific antibody to anti-Factor VIII. We, therefore, conclude that the cell which becomes neoplastic in hepatic angiosarcoma is the sinusoidal endothelial cell rather than the sinusoidal Kupffer cell.

Technical Proposal B -- Biochemical Enzymatic Systems for the Detection of Vinyl Chloride and Other Chemical Injury and Cancer Development in Industrial Workers. J. T. Du

Various liver cell types (hepatocytes, Kupffer and endothelial cells) have been isolated by enzyme digestion. The nonhepatocytes are being separated into Kupffer and endothelial cells by countercurrent elutriation. Enzymatic studies of the detoxifying activities of the subcellular fractions of liver cells, both hepatocytic and mesenchymal, are in progress. Enzyme studies include glutathione S-epoxide transferase (GEST), glutathione S-aralkyl transferase (GAST) and glutathione reductase (GR). In addition, mixed function oxidase (MFO), the microsomal enzyme responsible for oxidation of vinyl chloride, was also determined in isolated hepatocytes and mesenchymal cells. The extent of contamination of hepatocytes in mesenchymal cell preparation was investigated by Van Berkel and Kostel's method (Biochemical Characteristics of Nonparenchymal Liver Cells in Kupffer Cells and Other Liver Sinusoidal Cells, p. 299-306, ed. Wisse and Knook, Elsevier, North Holland Biochemical Press, 1977) and found not to be appreciable. The results showed that the mesenchymal cells had about half the GEST activity, 7% the GAST activity, less than half the MFO activity and 73% the glutathione reductase as compared to that of the hepatocytes. This preliminary result supports our hypothesis that the reason the endothelial cells become tumorous rather than hepatocytes is that the endothelial cells do not have the ability (or have an impaired ability) to detoxify vinyl chloride metabolites. Further experiments using isolated endothelial cells and labelled vinyl chloride metabolite can provide more definitive answers.

Technical Proposal C -- Glycosaminoglycan Changes in Earlier Detection of Fibrotic Injury and Hepatic Cancer. C. E. Kupchella

The objective of this proposal is to determine the usefulness of urinary and tissue glycosaminoglycan measurements in the detection of chemically-induced liver injury. A summary of the pertinent results follows:

1. Human hepatic angiosarcoma and fibrotic liver diseases are accompanied by elevated tissue GAGs. The GAGs in the angiosarcoma 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.

3. Heparin sulfate (a type of GAG) is elevated in hepatic tissue undergoing experimentally-induced fibrosis and heparin sulfate is elevated in the urine or experimental animals. Also, heparin 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. 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 other, non-metastisizing hepatomas.

5. Hepatic necrosis is accompanied by significant tissue GAG elevations, but hepatic regeneration is not. Gross (non-fractioned) urinary GAG determinations give a better indication of liver disease than ultrasound analysis. However, modifications in GAG analysis must be evaluated further as to specificity and sensitivity.

6. Exacting urinary GAG analysis (fractionated) is potentially able to differentiate active from inactive liver disease.

PRACTICAL SIGNIFICANCE:

These studies address the needs for:

a. Useful screening tests for liver injury and for active versus inactive disease -- urine tests of the type to be evaluated obviously fit the ideal of being non-invasive and having zero morbidity/mortality and not requiring "time off".

b. Methods of therapeutic intervention, i.e., the elucidation of the role of the GAGs in the pathogenesis of fibrotic liver disease may well lead to the identification of strategies by which fibrogenesis can be blocked and/or reversed.

Technical Proposal D -- Histological Systems of Detection. C. H. Tamburro, R. Schrodt

Drs. Schrodt and Tamburro continue to develop a means of analyzing light microscopic sections of liver tissue obtained from vinyl chloride workers to determine the feasibility of quantitating the amount of scar tissue in these individuals related to their exposure. Sinusoidal cell size and collagen deposits within the sinusoidal Space of Disse have been determined by utilizing a relatively newly developed Hewlett-Packard 9864-A digitizer and a 9815-A micro computer. With this equipment, Schrodt and Tamburro have been able to quantitate the areas of trichrome stainable collagen (fibrosis) within biopsy samples. These morphometric analyses have been done on randomly selected fields from biopsies obtained during medical evaluation in vinyl chloride workers. There are now some 110 biopsies approximately 50 of which have been reviewed. Morphometric analysis, however, has had to be delayed since there was no known standards of normal human collagen content known for human adults.

A study has been begun to determine the normal distribution and content of collagen at varying ages in normal individuals without history of chemical, viral or medical disease or injury of the liver. This study is one-third complete. Preliminary review of the data suggests that there may be an increase in the collagen deposition (fibrosis) in the normal human liver associated with age. If this holds true upon analysis after completion of the study, age corrected standards will have been established so that the data obtained from the vinyl chloride exposed human biopsies may be accurately interpreted.

Resumption of the morphometric analysis of the vinyl chloride exposed liver biopsies will be resumed upon completion of the normal control study.

Technical Proposal E -- Chemical Systems of Detection of Toxicity of Vinyl Chloride. J. L. Wong

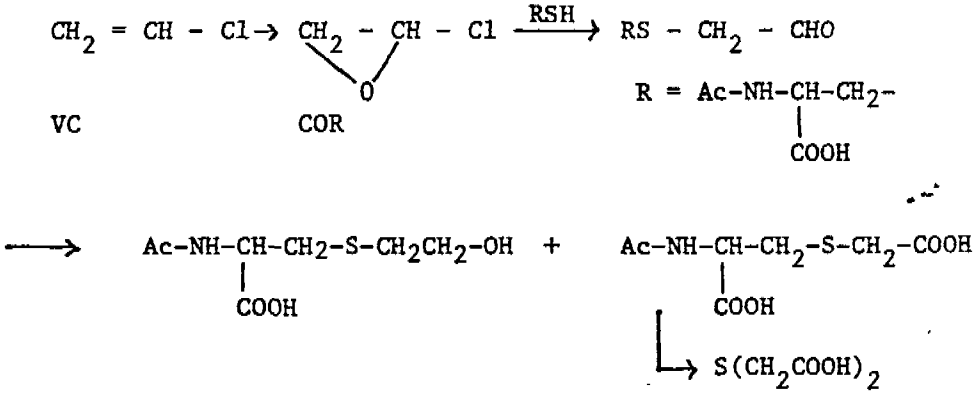
This study of vinyl chloride toxicity/carcinogenicity has been concerned with (1) the chemistry of vinyl chloride metabolism, i.e., the identity of the intermediates and their reactions with cytoplasmic chemicals, and (2) the putative actions of the primary metabolites on nuclear materials. The chemical reactions of two putative metabolites, chlorooxirane (COR) and chloroacetaldehyde (CAA) with sulfhydryls (detoxification) as well as with nucleic acid constituents (mutagenesis and carcinogenesis) have been studied in detail.

Part I. Detoxification Study. CMA 002646

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% 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% conversion is observed in 15 min. which is the lifetime of COR under these conditions, and 80% 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 $3,4-Cl_2Ph-S-CH(OH)CH_2Cl$. 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. Thus, the metabolic pathway of VC as shown below is strongly substantiated.




RSH - N-acetylcysteine, cysteine, glutathione

Part II. Mutagenesis and Carcinogenesis Study.

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. Alt[illegible]h

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. To continue our study on CAA with guanine base using analytical tools such as HPLC, GC-MS and FT-NMR, we have found that the guanosine reaction is enormously complicated. The reaction is very dependent on pH. At pH 6.5, the linear-ethenoguanosine is the immediate product which can further react with CAA. There were also at least 2 other minor products when the aqueous reaction was titrated accurately to pH 7.00 $\pm$ 0.01. There was one major product which is the same as one of the two minor products found at pH 6.3. This new major product is tentatively identified as the guanosine $N^1$-acetaldehyde, an intermediate to the etheno derivative.

The question of how does this etheno modification of the nucleic acid bases bring about physicochemical changes is also examined. By using $^{13}C$ FT-NMR with the special technique DIS (Deuterium Inductive Shifts) in the undecouple mode, the chemical shifts of etheno-bases, nucleosides, and deoxynucleosides were assigned and compared. Some significant electronic changes in the nuclei have been detected.

Part III. Significance

Broadly speaking, this study will lead to early detection and prevention of industrial cancers. Our chemical methodologies (synthesis, structure, and analysis), applied as an integral part of the multidisciplinary approach, will elucidate specific molecular events in the effects of vinyl monomers on industrial workers. This information will form a rational basis for safe 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.

Technical Proposal F -- Assays for the Carcinogenic Potential of Industrial Chemicals Utilizing Prokarystic and Eukaryotic Systems. U. N. Streips

In this quarter, we have primarily concentrated on the publication of our results, using data derived from our studies under the MCA contract. These publications include one paper by Barnes, Sonnenfeld, and Streips on our new Interferon assay. Also, there are two book chapters: 1) Streips, Laumbach, and Yasbin "Bacterial Mutation Monitors for Active Metabolites of Chemical Carcinogens: Bacillus Subtilis Assays for Mutation and DNA Repair" and (2) a chapter in a handbook for industrial physicians co-authored by Tamburro, Wong, and Streips, "Approaches to Occupational Cancer".

This activity is equally vital to our research programs since it establishes our laboratories as leaders in the field of chemical carcinogenesis. The MCA has been acknowledged in all these publications.

[redacted]
Subject to [redacted] Order in
Ross v. [redacted] No. 80-4637
14th Judicial District Court
Calcasieu Parish, Louisiana

Technical Proposal G -- Tissue Antigens and Antibodies in the Detection of Vinyl Chloride Injury. Enrique Espinosa

During this quarter part of the time was spent in the preparation and presentation of papers and manuscripts and in the continuation of part of our experimental work.

Part I. Papers.

Three of our papers which were referred to in the previous report were presented during the first week of April in the 1979 FASEB meetings, Dallas, Texas and one paper was presented on May 7 at the annual meeting of the American Federation for Clinical Research, Washington, D.C. During the second week of June, we presented the results obtained in our studies on the properties of cultured hepatoma cells at the 1979 Annual Meeting of the Tissue Culture Association, Seattle, Washington.

Part II. Experimental Work.

Further tests have been performed on the serum and liver antigens of the cell sheets and supernatant fluids of cultured hepatoma cells (PLC/PRF/5). The cell monolayers were assayed separately from culture supernates. Four day cultures in J. Alexander medium were shifted to serum-free medium containing insulin (100 ng/ml). Then daily harvests for five additional days were pooled; cell morphology remained excellent. These supernates were clarified by centrifugation and concentrated by ultrafiltration. The cells were scraped from the vessels into a small volume of water, homogenized and clarified. These concentrates were then tested by agarose double immunodiffusion against standard antiserums which were fully absorbed with fetal bovine serum. Both preparations were found to contain serum albumin, fibrinogen, transferrin, alpha-1 antitrypsin and alpha-2 macroglobulin but not immunoglobulins. This system may thus be used for the study of biosynthesis of serum proteins by hepatoma. The constituent of normal liver, liver-specific F antigen, had disappeared.

Technical Proposal I -- Vinyl Chloride Metabolism in Isolated Liver Cells. Richard C. Feldhoff

Sophisticated equipment for the preparation of isolated rat liver cells was ordered between March and May, 1979. Two of the scientific suppliers have yet to fill the orders for various production reasons. The majority of the equipment is present and we anticipate being operational in September. During the interim start-up period, we have proceeded to prepare and characterize specific antisera which will be used in the project. Rabbits were immunized over a 2-month period and are being bled at 2-3 week intervals. The antisera is being further purified by ammonium sulfate precipitation and by ion-exchange chromatography. A quantitative "rocket" immunoelectrophoresis assay has been set up to

measure the antigen in nanogram quantities. Radioactive vinyl chloride metabolites will be incubated with isolated liver cells and the uptake, intracellular transport and metabolism by various membrane and cytosol fractions will be investigated.

This completes the quarterly report from the University of Louisville Chemical Monomer Research Group. If there is need for further information or clarification, please do not hesitate to contact me.

Sincerely yours,

Carlo H. Tamburro, M.D.
Professor of Medicine
Chief, Division of Digestive
Diseases and Nutrition

CHT:nlh

VC 7.0-SP/PREV-UL

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Mr. Joseph T. Seawell
Program Manager
Manufacturing Chemists Association
1825 Connecticut Avenue, N.W.
Washington, D.C. 20009

RE: 2nd Quarterly Report for the Manufacturing Chemists Association's Agreement with the University of Louisville for the 1979-80 Fiscal Year.

Dear Mr. Seawell:

The following describes what has been completed during the 2nd quarter of the Manufacturing Chemists Association's agreement with the University of Louisville entitled, "Research Techniques and Methods for Detection and Prevention of Carcinogenesis in Industrial Workers". Progress for each technical proposal will be reported separately.

Technical Proposal A -- Immunological Systems for the Detection of Vinyl Chloride and Other Chemical Injury. H.P. Fortwengler

Fortwengler's laboratory has continued evaluating the immune system of industrial workers to determine whether cancer can be detected earlier or to identify those at high risk.

HLA Frequencies in V.C. Workers.

A compilation of HLA frequencies in V.C. workers is continuing to determine if a possible increased association of certain "tissue types" can be determined in individuals with angiosarcoma or other chemically related disease. To date, approximately 494 individuals from the Louisville vinyl chloride polymerization plant have had their lymphocytes isolated and typed for more than 27 different HLA-A and HLA-B antigens.

A Search for Evidence of a VC-Induced Tumor Antigen.

A search for evidence of a VC-Induced tumor antigen has lead to a finding that these tumors have an increased concentration of antigenic coagulation Factor VIII. Factor VIII is a known

marker for vascular lining cells of the endothelial type. Further experiments in animals gave us results indicating that normal endothelial cells found lining the liver sinusoids had little, if any, Factor VIII fluorescence (content). 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 cells, distinguished in histological cross section following engorgement with carbon particles, failed to demonstrate positive fluorescence. An increase in antigen Factor VIII in hepatic angiosarcomatous endothelial cells as compared to normal, leads us to believe that the aberrant cells have increased production or storage capacity for antigenic Factor VIII.

Technical Proposal B -- Biochemical Enzymatic Systems for the Detection of Vinyl Chloride and Other Chemical Injury and Cancer Development in Industrial Workers. J.T. Du

Staining techniques have been applied to various liver cell types to help identify the isolated liver cells. In the non-hepatocytes, the Kupffer cell is the peroxidase positive and the endothelial cell is the peroxidase negative cell. In a non-hepatocyte preparation, we found 18% peroxidase positive. After further separation of the non-hepatocytes by elutriation, only about 1% of the endothelial cell fraction was peroxidase positive.

We also did some experiments feeding chloroethanol, a vinyl chloride metabolite, to rats (30 mg/kg/day for 10, 20 and 40 days). The non-protein sulphydryl content, (mostly glutathione), was increased after 20 days, but the glutathione-related enzyme activities, glutathione transferase and glutathione reductase, were not altered, suggesting either the dose is not high enough or that chloroethanol and vinyl chloride metabolize via different routes. Further experiments with higher doses will be performed to elucidate the mechanism.

Technical Proposal C -- Glycosaminoglycan Changes in Earlier Detection of Fibrotic Injury and Hepatic Cancer. C.E. Kupchella

The original aim of this study was to determine the usefulness of urinary and tissue glycosaminoglycan (GAG) measurements in the detection of chemically-induced liver injury. We have made substantial progress toward this end and this has been described in previous reports. Within the period July 1979 - September 1979, specifically, we accomplished the following:

1) We have completed data reduction and have a manuscript now under review by all co-authors for January or February, 1980 submission to the Journal of Clinical Pathology relative to the following study. We developed complete urinary GAG excretion profiles from 24 hour urines from 24 vinyl chloride workers, half with and half free of liver disease. Our conclusion is that GAG profiles may reflect active liver disease. A copy of the final manuscript will be submitted with the final report.

2) We completed the laboratory work involved in a repeat of the study we reported in Gastroenterology 73:1229 in 1977. At the end of the quarter, we had not yet started data reduction on this project.

3) We completed an extension and a repeat of the Morris Hepatoma study. We reported in Gastroenterology 75:972, 1978.

In the earlier study, we evaluated three tumor lines; in 1979 we looked at these three plus three more. A paper will be submitted in January for presentation at next May's meeting of the American Federation for Clinical Research in Washington.

These studies address the role of GAGs in the behavior of hepatic malignancy as well as the usefulness.

Some recent presentations made as a result of our work are listed below.

Secskas, E., Kupchella, C.E., Kennedy, J. and Espinosa, E. Glycosaminoglycan Changes Associated with Hepatic Tumors: The Contributions of Regeneration and Necrosis. Presented at the Association for American Physicians/ American Society for Clinical Investigation/American Federation for Clinical Research National Meeting, Washington, D.C., May 7, 1979.

Kupchella, C.E., Drake, E. and Secskas, E. Glycosaminoglycan Patterns in Necrotic Liver, Regenerating Liver, and in Three Morris Hepatomas having Different Growth Rates. Presented at the Seventh Bi-Annual Hepatoma Conference, Washington, D.C., May 10, 1979.

Technical Proposal D -- Histological Systems of Detection. C.H. Tamburro, G. Barrows, R. Schrodt.

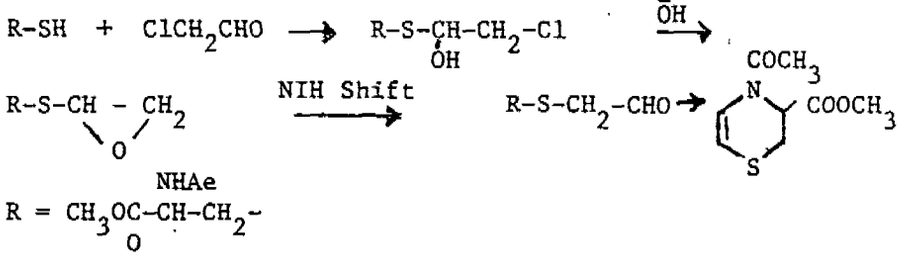
The means of analyzing light electron microscopic sections of liver tissue quantitatively for the amount of collagen (scar tissue) in individuals exposed to vinyl chloride has demonstrated a great variation in the amount of stainable collagen present. This can vary from non-detectable collagen without the use of special collagen stains, such as the trichrome, to 35-40% of the total biopsy being occupied by fiberous tissue in the terminal stages of the disease. At this point all 110 biopsies from the vinyl chloride workers have been reviewed as to the degree of collagen deposition present. Morphometric analysis previously had been delayed in order to develop standards for the normal human collagen content at various ages. The study of the normal distribution of collagen content at the various ages in individuals without history of chemical, viral or medical disease, or injury to the liver, has had 14 individuals accessed. Approximately 4 are in the under 25 age group, 6-7 in the 25-40 age group, and 3 in the 40-50 age group. Preliminary data demonstrates an increase in the collagen deposition in the normal human liver associated with age. In the younger age group less than 1% of the studied area is occupied by stainable collagen. In the older age group stainable collagen in the sinusoidal areas may range as high as 5-6%. There is considerable overlap between individuals but close agreement between biopsies in the same individual. At present, the lower age ranges has sufficient cases to develop standards. A few additional cases are needed in the upper ranges. The acquisition of these additional cases depends on the availability of accidental deaths occurring in normal individuals of the older age group without evidences of hepatic disease. However, sufficient data is available for us to resume the morphometric analysis of the vinyl chloride exposed worker's liver biopsies utilizing the Hewlett-Packard 9864-A digitizer and a 9815-A micro computer.

Technical Proposal E -- Chemical Systems of Detection of Toxicity of Vinyl Chloride. J.L. Wong

In the previous report we have delineated the reaction pathways of chlorooxirane with 3,4-dichlorobenzenethiol and N-acetylcysteine. In both cases, the major product is the sulfur conjugate S-acetaldehyde. Since chlorooxirane can spontaneously rearrange to chloroacetaldehyde, we have proceeded to investigate the reaction intermediates of the latter in the detoxification by cellular sulfhydryl compounds. 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 $H_3CO-\overset{O}{\overset{\|}{C}}-CH-(NHCOCH_3)CH_2S-\underset{OH}{\underset{|}{C}}H-CH_2Cl$, eliminated HCl upon neutralization with aqueous sodium hydroxide to produce the corresponding epoxide $H_3COCOCH(NHCOCH_3)CH_2S-CH\underset{O}{\diagdown\diagup}CH_2$. This structural assignment is supported by its pmr spectrum. Furthermore, when this epoxide was extracted into chloroform, it rearranged to the S-acetaldehyde $H_3COCOCH(NHCOCH_3)CH_2-S-CH_2CHO$, identified by its pmr spectrum and 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 reaction pathway for chloroacetaldehyde is proposed as follows.




These two metabolites are therefore both similar and different in their detoxification reaction with the RSH. Further study will continue to unravel the significance of either or both of chlorooxirane and chloroacetaldehyde in the mutagenesis/carcinogenesis problem of vinyl chloride.

Technical Proposal F -- Assays for the Carcinogenic Potential of Industrial Chemicals Utilizing Prokaryotic and Eukaryotic Systems. U.N. Streips in collaboration with G. Sonnenfeld

During the 2nd quarter we have concentrated on developing the interferon induction assay as a valid test for the carcinogenic potential of industrial chemicals. To this end the following results have been obtained:

1) Benzo-(a)-pyrene, #4 fraction of tobacco smoke condensate, 1,2-dimethylbenz (a) anthracene, 2-aminofluorine, aflatoxin-$B_1$, and styrene oxide all inhibited the induction of interferon by Newcastle disease virus.

2) MMS, a highly carcinogenic mutagen, inhibited the induction of interferon, while its analog, EMS, a rarely carcinogenic mutagen, had no effect on interferon induction.

3) Chloroacetaldehyde, the metabolite of vinyl chloride that is believed to be responsible for the actual carcinogenic event perpetrated by vinyl chloride, inhibited the induction of interferon by virus, but chloroethanol and chloroacetic acid, benign metabolites of vinyl chloride, had no effect on interferon induction.

These results suggest that the inhibition of interferon induction by chemicals may be a useful marker of the carcinogenic potential of a chemical, after extensive further study and collaboration of the results. The results were presented at the 1979 Annual Meeting of the American Society for Microbiology and at the International Symposium on Interferon of the Wadley Institutes of Molecular Medicine, and will appear in the form of two manuscripts. The Manufacturing Chemists Association has been recognized for its support to the completion of the data in the manuscripts.

Technical Proposal G -- Tissue Antigens and Antibodies in the Detection of Vinyl Chloride Injury. Enrique Espinosa

In order to further investigate antigen production by hepatoma application of the indirect immunofluorescent procedure to this question was investigated during this quarter. To accomplish this, several conditions for optimal growth of tumor cells sheets (PLC/PRF/5) in culture were studied. Satisfactory results were obtained by growing stationary cultures on coverslips in Leighton tubes at 37°C in a modified Eagle's medium containing 10% fetal calf serum. Cell morphology appeared excellent after 1/2 to 1/3 of confluency had developed in the coverslips. This was accomplished following seeding of 300,000 cells and culturing for 5-6 days. For the immunofluorescent staining the coverslips were then rinsed in Eagle's medium at room temperature and in cold ethanol in order to get rid of medium proteins. After fixation in ethanol (-75°C) for 10 minutes, The cells were tested for presence of several serum proteins (albumin, fibrinogen, transferrin, alpha 1-antitrypsin and alpha 2-macroglobulin). Specificity of the staining was

shown by absence of fluorescence with absorbed antiserum, non-immune serum or buffered saline. With each protein, the fluorescence was restricted to the cell cytoplasm and an intense degree of fluorescence indicated relatively high levels of these proteins in a large proportion of the cells. Other individual cells showed varied degrees of fluorescence suggesting heterogenicity of the cell population with respect to the synthesis of these plasma proteins. Demonstration of presence of these proteins in the tumor cells by immunofluorescence is in agreement with results obtained by immunodiffusion as previously reported and thus provide another approach for the study of serum proteins and possibly other protein antigens by hepatoma.

Technical Proposal I -- Vinyl Chloride Metabolism in Isolated Liver Cells. Richard C. Feldhoff

As reported for the preceding quarter we have experienced some difficulty in purchasing all of the special supplies needed for the in situ liver perfusion technique using our recently acquired liver perfusion apparatus.

In the meantime, we have continued to produce and purify rabbit anti-rat albumin antiserum. The antibodies which we are purifying will be used to quantitate the effects of ethanol and chemical monomers on the synthesis of serum albumin relative to total protein synthesis. Albumin is a constitutive product of the liver and perturbations in its rate of synthesis reflect alterations in normal cellular metabolic processes. Albumin synthesis has been reported to be particularly influenced by the levels of essential amino acids and ethanol. One of the primary intracellular effects is polysome disaggregation. Techniques are being developed to quantitatively recover undegraded polysomes from detergent-treated post-mitochondrial supernatants.

This completes the quarterly report from the University of Louisville Chemical Monomer Research Group. If there is need for further information or clarification, please do not hesitate to contact me.

Sincerely yours,

Carlo H. Tamburro

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