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Mr. Joseph T. Seawell
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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 angio-sarcoma 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 sulphhydryl 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 fibrous 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

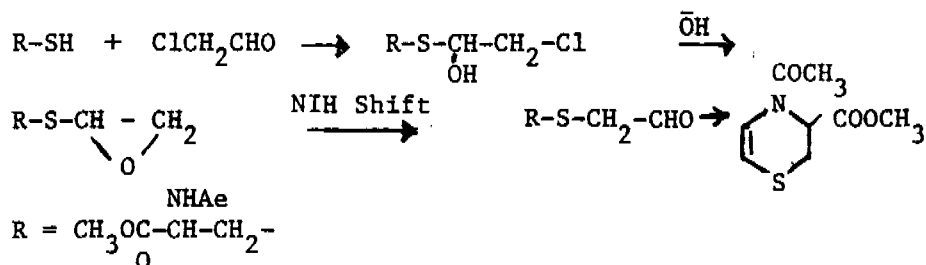
In the previous report we have delineated the reaction pathways of chlorooxirane with 3,4-dichlorobenzenethiol and N-acetylcysteine. In both cases, the major product is the sulfur conjugate S-acetaldehyde. Since chlorooxirane can spontaneously rearrange to chloroacetaldehyde, we have proceeded to investigate 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 $\text{H}_3\text{CO}-\overset{\text{O}}{\underset{\text{O}}{\text{C}}}-\text{CH}-(\text{NHCOCH}_3)\text{CH}_2\text{S}-\underset{\text{OH}}{\text{CH}}-\text{CH}_2\text{Cl}$,

eliminated HCl upon neutralization with aqueous sodium hydroxide to produce the corresponding epoxide $\text{H}_3\text{COCOCH}(\text{NHCOCH}_3)\text{CH}_2\text{S}-\underset{\text{O}}{\text{CH}}-\text{CH}_2$. This structural assignment is supported

by its pmr spectrum. Furthermore, when this epoxide was extracted into chloroform, it rearranged to the S-acetaldehyde $\text{H}_3\text{COCOCH}(\text{NHCOCH}_3)\text{CH}_2\text{S}-\text{CH}_2\text{CHO}$, 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-B₁, 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 heterogeneity 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.

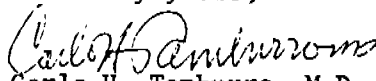
Technical Proposal I -- Vinyl Chloride Metabolism in Isolated Liver Cells.
Richard C. Feldhoff

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,



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