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April 6, 1979

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Ross v. Corl et al., No. 80-4937  
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: Annual Report for the Manufacturing Chemists Association's Agreement  
with the University of Louisville for the 1978-79 Fiscal Year.

Dear Mr. Seawell:

The following describes what has been completed during the second year 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

During the second year, 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.

It has been demonstrated that lymphoid cells (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 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.

The scientific literature is replete with demonstrations of immunodepression in cancer patients at various stages of disease. There is very little information, however, concerning

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immunoevaluation prior to diagnosis of frank malignancy. We used immunological assays that have demonstrated usefulness in indicating immunodepression in cancer patients. These tests include the enumeration of lymphocytes and lymphocyte function assays. (For details, see report for fiscal year 1976-77).

These tests have been used to evaluate 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 (1).

At this time, our preliminary interpretation of these results is 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.

An increased incidence of certain HLA types has been shown by several reports to be associated with susceptibility to various diseases. The first occupational disease-HLA correlation may have already been found: HLA-B27 antigen has been found more often in workers suspected of having the occupational disease asbestosis than among a control population.

The microdroplet lymphocyte cytotoxicity test has been used in our laboratory to HLA type approximately 429 individuals from the Louisville vinyl chloride polymerization plant. As individuals do not change their genetic complement of HLA antigens, these determinations need to be done only once and do not need to be repeated periodically as in various other biochemical assays.

Tissue typing for 11 separate HLA-A antigens and 16 HLA-B antigens and their possible increased association with angiosarcoma or other chemically-related diseases is about two-thirds completed.

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Our compiled data is being compared with the frequencies obtained by two other large HLA studies. Frequencies of the healthy individuals studied by Scott et al., 1977, and the World Health Organization compare favorably to the frequencies found in the study here at the University of Louisville.

A pattern of potential differences from normal frequencies has been seen in VC workers found to have liver disease. Although definitive statistical analysis will not be done until the study is completed, differences are being seen in the frequencies of antigens A9, A13, A15, and B17. Whether these particular antigens may be used to identify individuals susceptible to chemical injury will have to await completion of the study.

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

New antigens arise on tumors formed as a response to carcinogens. Their presence on methylcholanthrene-induced sarcomas was discovered by Foley in 1953. This discovery in mice was verified and extended by Prehn and Main in 1957 to conclude that there were antigens peculiar to and specific for tumor tissue. Subsequently, evidence for tumor antigens was found in humans by the Hellstroms, Vankey, Halliday and Maluish, Thompson and others. The majority of the evidence suggested that the tumor antigens found were distinctive for each histological type of tumor.

Since the body mounts an immune reaction to cancer, a cell-mediated test system (lymphocyte transformation) was used to search for evidence of specific immune reactions. Lymphocytes (the cells responsible for immunity) from the individual tested were isolated by centrifugation over Ficoll-Hypaque. These cells were then grown in the presence of a liver reagent prepared from either a normal individual or an individual who had angiosarcoma. A three-times increased incorporation of  $H^3$ -thymidine into stimulated cultures as compared to unstimulated cultures is a positive reaction.

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 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.

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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.

These results must be interpreted with caution because of the small number of individuals having reactions to only tumor antigen. We are now in the process of testing control subjects in replicate to determine if test and operator variations can be further minimized. Repeat determinations of selected exposed individuals will, as a consequence of reduced variation, take on greater statistical meaning.

In addition to testing for evidence of reactions to an "angiosarcoma antigen", we have found that the tumor cells contain antigenic coagulation Factor VIII. Factor VIII has been found in tumor tissue from three individuals having had angiosarcoma. Hoyer's finding in 1973 that only endothelial cells contain Factor VIII, when combined with our findings, indicate that the specific lining cell which becomes aberrant during the course of angiosarcoma is the endothelial cell (2).

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

#### Animal Studies

Animal studies are continuing to be conducted to determine which biochemical changes occur early in the course of vinyl chloride exposure and how the determination of these biochemical changes can be used to a) detect early injury, b) to demonstrate a level of exposure with no biological effect.

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The previous long-term experiment in Du's laboratory identified those metabolic pathways which best reflected vinyl chloride handling. The second phase of this study, extending exposure to over 250 hours, has verified the original findings and illustrated adaptation of the detoxifying mechanisms of rat liver with prolonged exposure at high levels (3).

Our second vinyl chloride exposure study exposed rats to 28,000 ppm VC for 70, 140 and 210 hours in durations of 2, 4 and 6 weeks. We examined the rat liver's ability to oxidize vinyl chloride by study of the microsomal P-450 enzyme system (mixed function oxidase) and detoxification mainly by conjugation via glutathione (GSH) and glutathione transferase. The results showed an elevation of glutathione reductase in the liver (the enzyme which regenerates reduced glutathione from its oxidized form), followed by an elevation of the concentration of reduced glutathione. This was later followed by an elevation of the detoxifying enzymes, glutathione epoxide-S-transferase (GEST) and glutathione aralkyl-S-transferase (GAST). These results suggest that the rats had the capacity to induce detoxifying enzymes as well as to maintain high glutathione concentrations to strengthen detoxification capability. These biochemical changes were evident while the conventional clinical liver tests showed no abnormalities (4).

Also, our finding a higher level of glutathione, and glutathione reductase after vinyl chloride exposure, may be an early adaptive biochemical mechanism which precedes the precancerous alteration. This is similar to Fiala's finding that administration of hepatocarcinogens to rats led to an increase in the concentration of glutathione in the liver and that the concentration remained high until the development of hyperplastic nodules (J. Natl. Cancer Institute, 57:591-598, 1976). Further, there was a decrease of P-450 concentration in the livers of rats exposed to vinyl chloride. The decreasing P-450 would help the animal to produce less toxic metabolites, and may reflect another means of biological adaptation and help understand why the liver cell does not become malignant.

The present working hypothesis to explain why the primary liver cell does not develop tumors in the adult animal is that it has the ability to adequately detoxify the carcinogenic metabolites of vinyl chloride. The adjacent sinusoidal lining cells most likely develop the tumor (angiosarcoma) because of their decreased ability to detoxify the metabolites. Further studies with isolated hepatocytes and endothelial lining cells and animal exposure studies will be performed to elucidate the mechanisms.

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#### Isolation Studies

Some preliminary studies of liver cell isolation have been started. Hepatocytes were isolated by collagenase perfusion and endothelial and Kupffer cells by Pronase digestion. We have successfully obtained cells of good viability. Newer techniques using ultracentrifugation have become available which will help further improve our yield and start the next phase of our studies.

Hopefully, the use of isolated various liver cells to determine their ability to oxidize and detoxify chemicals may be used to determine biological threshold limits in more realistic fashion.

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

Glycosaminoglycans (GAGs) are involved in wound healing and scar formation (fibrosis). Certain GAGs are elevated in malignant tumors including hepatic tumors, and it has been postulated that GAGs may be important determinants of tumor cell properties. A number of laboratories, including Kupchella's, have established that urinary GAGs may serve as markers in the pathogenesis of chemical injury, fibrosis, and cancer. Although urinary GAG analyses have long been used clinically to detect and diagnose genetically-determined metabolic disorders of GAG metabolism, a systematic evaluation 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.

The objective of this proposal is to determine the usefulness of urinary and tissue glycosaminoglycan measurements in the detection of chemically-induced liver injury.

Kupchella has reported a number of findings, (references 5-11), some of which are described in previously submitted MCA reports. A summary of the pertinent results follows. Abstracts previously not submitted are provided in the attached appendix.

1. Human hepatic angiosarcoma and fibrotic liver diseases are accompanied by elevated tissue GAGs. (5)
2. The GAGs in the angiosarcoma tumor tissue are different from those in fibrotic tissue adjacent to the tumor. (5)

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3. Angiosarcoma and hepatoma patients have characteristic urinary GAG patterns -- patterns not found in normal controls. (6)
4. Heparin sulfate (a type of GAG) is elevated in hepatic tissue undergoing experimentally-induced fibrosis and heparin sulfate is elevated in the urine of experimental animals. (7)
5. 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. (8)
6. 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-metastasizing hepatomas. (8)
7. Hepatic necrosis is accompanied by significant tissue GAG elevations, but hepatic regeneration is not. (9)
8. Gross (non-fractionated) 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. (10)
9. Exacting urinary GAG analysis (fractionated) is potentially able to differentiate active from inactive liver disease. (11)

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.

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- c. Tests to identify individuals at risk of chemical injury -- if we are able to find characteristic GAG changes reflective of chronic alcohol injury or other similar injury, we would have a way of identifying individuals with liver impairment in the screening of job applicants.

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

Scar tissue (fibrosis) is a common early finding associated with chemical injury to the liver as well as other organs. Vinyl chloride and other chemicals have been shown to produce mild injury undetectable by standard biochemical means but reflected by increase in fibrosis associated with prolonged exposure.

Drs. Schrodt and Tamburro have been developing 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 (12).

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

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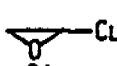
Technical Proposal E -- Chemical Systems of Detection of Toxicity of Vinyl Chloride. J. L. Wong

Wong's current study of vinyl chloride toxicity/carcinogenicity has been concerned with (1) the chemistry of vinyl chloride metabolism, i.e., the structure of the intermediates and their reaction with cytoplasmic chemicals, and (2) the putative actions of the primary metabolites on nuclear materials. Two putative metabolites, chlorooxirane (COR) and chloroacetaldehyde (CAA), have been shown to react in different ways with sulfhydryls (detoxification study) as well as with nucleic acid constituents (mutagenesis and carcinogenesis study). Radiolabeled derivatives are being prepared to observe disposition and conversions in animals and isolated liver cells.

#### Results and Discussion

The detoxification studies are summarized in Table I. The central question is how are the primary metabolites 1' and 2

TABLE I. DETOXIFICATION OF VINYL CHLORIDE

| PRIMARY METABOLITES      | COR <sup>1</sup><br>↓                                                                                                                                                                                                                                                               | CAA <sup>2</sup><br>↓                                                                                                                                                                                                                                                     |
|--------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| INTERMEDIARY METABOLITES | S-ACETALDEHYDE <sup>3</sup><br>THIAZENES <sup>5</sup> , ---                                                                                                                                                                                                                         | HEMITHIOACETAL <sup>4</sup><br>THIAZENES <sup>5</sup>                                                                                                                                                                                                                     |
| URINARY METABOLITES      | S-ACETIC ACID <sup>6</sup>                                                                                                                                                                                                                                                          | S-ETHYL ALCOHOL <sup>7</sup><br>CHLOROACETIC ACID <sup>8</sup>                                                                                                                                                                                                            |
|                          | <sup>1</sup>  <sup>2</sup> $\text{H}-\text{C}(=\text{O})-\text{CH}_2\text{Cl}$ <sup>3</sup> $\text{RS}-\text{CH}_2\text{CHO}$ <sup>4</sup> $\text{RS}-\text{CH}(\text{OH})-\text{CH}_2\text{Cl}$ | <sup>5</sup>  <sup>6</sup> $\text{RS}-\text{CH}_2\text{CO}_2\text{H}$ <sup>7</sup> $\text{RS}-\text{CH}_2\text{CH}_2\text{OH}$ <sup>8</sup> $\text{Cl}-\text{CH}_2\text{CO}_2\text{H}$ |

(RS FROM 3,4-DICHLOROBENZENETHIOL, N-ACETYL-CYSTEINE  
 R' = ACETYL, H)

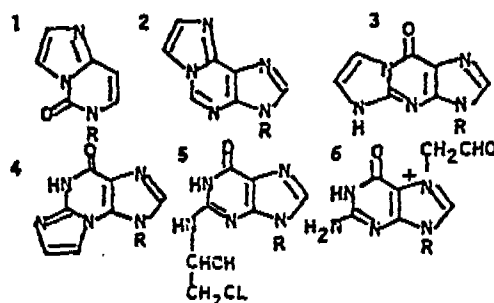
detoxified. Do they yield the same products or different ones? We have studied their reaction with sulfhydryl compounds 3,4-dichlorobenzenethiol and N-acetylcysteine. The benzenethiol was used by the Stockholm group in a preliminary study to detect the formation of CAA and COR from VC. The cysteine derivative is a cellular sulfhydryl component as well as a close analog of

glutathione. In the case of COR and benzenethiol, the sulfur-conjugation product S-acetaldehyde, compound 3, is formed. However, CAA and benzenethiol forms the hemithioacetal, compound 4. These two reactions are distinctly different. The formation of hemithioacetal is reversible but that of the S-acetaldehyde is not. With N-acetylcysteine, both COR and CAA yield the same cyclic condensation product, a dihydrothiazene-carboxylic acid, compound 5, in aqueous solution. This thiazene is a multi-step reaction product, formed much faster with COR than with CAA. It is plausible that 5 is the origin of the identified urinary metabolites: S-acetic acid, compound 6, and S-ethyl-alcohol, compound 7, and may itself be present in the urine. Furthermore, the COR reaction when titrated with hydroxide to maintain pH 7 yields a new product, the structure of which is yet undetermined. These results and continuation study will enable us to undertake a more comprehensive detection study of all the vinyl chloride detoxification products in biological specimens.

The detection study of the putative action of vinyl chloride is summarized in Table II. Our hypothesis is that such action comes from the modification of the nucleic acid materials by the

TABLE II. PUTATIVE ACTION OF VINYL CHLORIDE. REACTION  
 WITH NUCLEIC ACID BASES

|     |                                                                                                                                |
|-----|--------------------------------------------------------------------------------------------------------------------------------|
| CAA | ETHENO—C <sup>1</sup> , ETHENO—A <sup>2</sup><br>L—ETHENO—G <sup>3</sup> , A—ETHENO—G <sup>4</sup> , HEMIACETAL—G <sup>5</sup> |
| COR | G—7—ACETALDEHYDE <sup>6</sup> + ...                                                                                            |



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, compounds 1 and 2, very little is known about the reaction of CAA on the most reactive base guanine.

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By using a battery of modern analytical tools such as HPLC, GC-MS and FT-NMR, we have found that the guanine base in various forms, as the nucleoside, nucleotide and polyguanylic acid, gives rise to two tricyclic ethenoguanines, the linear etheno compound 3 and the angular etheno compound 4, and a third product which is possibly an intermediate, compound 5. Their ratios change with time and pH. It is worthy of note that some of them exhibit fluorescence properties which would allow direct detection in a cell nucleus. The reaction of COR with the guanine base is more tricky due to the instability of COR in aqueous medium. A multitude of products are formed which we have found to be different from those of the CAA reaction. One major product is tentatively identified as compound 6. It is important to note that this is the first indication that COR and CAA show different molecular events in their putative action.

#### 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 Prokaryotic and Eukaryotic Systems. U.N. Streips

In the second year of funding, Streip's laboratory has primarily developed and expanded testing capabilities relative to industrial chemicals. Thus, two new screening tests have been implemented: the Comptest and the III test which are indicators for SOS repair function. SOS repair is induced in bacteria following massive insult to DNA. This repair disregards normal DNA sequences and actually results in mutations. SOS repair is postulated to participate in the evolution of a neoplastic cell following chemical damage.

The Comptest examines SOS induction in the bacterium Bacillus subtilis and the III test determines the inhibition of interferon induction in mammalian cell lines (also a suggested SOS function). Since SOS repair is extremely error prone, we postulate that these tests will be specific for carcinogens, not just act as mutagen

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screens. These tests are described in an upcoming publication (14) and are summarized in Table III.

Table III  
COMPOSITE MUTAGENICITY SPECTRUM OF CHEMICAL MONOMERS<sup>a</sup>

| Chemicals               | Salmonella | Comptest | "Repair Assay" | Forward Mutation | III test |
|-------------------------|------------|----------|----------------|------------------|----------|
| Chloroacetaldehyde      | +          | ND       | +              | +                | ND       |
| Styrene oxide           | +          | ND       | -              | ±                | +        |
| Methyl methanesulfonate | +          | +        | +              | +                | +        |
| Ethyl methanesulfonate  | +          | -        | +              | +                | -        |

<sup>a</sup>Results are expressed as (+) positive in the assay performed; (-) negative in the assay performed; and (ND) not determined. Styrene oxide was weakly reactive in the forward mutation test and must be considered to have borderline activity in this assay (±).

As can be seen in Table III, the Comptest and the III test discriminate between ethylmethanesulfonate (EMS) and methylmethanesulfonate (MMS), both potent mutagens but only MMS is carcinogenic. We are currently testing chloroacetaldehyde and styrene oxide in both of these tests. We will, in the third year, be able to expand these new tests to be a part of our complete battery of rapid screens for the assay of a wide spectrum of industrial chemicals. In addition, our recent results should be applicable to industrial screening laboratories and result in better overall monitoring of environmental hazards.

At this time chloroacetaldehyde has been highly positive (15) in all assays tried and must be considered to be the active metabolite of vinyl chloride. Styrene oxide shows variable activity indicating it may have a different route of attack to cells than most other active chemicals. Since it is strongly positive in the III test, we will have to postulate that styrene oxide may be carcinogenic. This finding is being tested by the Comptest, and styrene oxide will be examined in whole animal systems for carcinogenesis.

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

Espinosa's finding of an antigen missing in VC-related liver angiosarcoma (detailed in the previous annual report) stimulated further studies of antigenic deletion in chemically-induced hepatomas and in cultured human liver carcinoma cells.

Some of the work in the past year has centered on the characterization of these liver tissue antigens in normal and chemically-induced diseased states. Two liver antigens found to be absent in the fast growing and undifferentiated chemically-induced Morris hepatoma 7777 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 (16). 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.

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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 behaviour of this antigen in Morris hepatomas, it was found that different types of these chemically-induced tumors have quite different levels of F-antigen.

F-antigen appeared to be absent in the fast growing hepatoma 7777. In the slow growing hepatoma 9618A, the concentration was very low ranging from less than 2% to 10% of the normal liver concentration. The medium growing hepatoma, 5123tc, highly metastatic, had about twice the concentration as normal liver. F-antigen of hepatoma 5123tc and of normal liver were found localized in the cytosol subcellular fraction and were determined to be immunologically identical and to have equivalent electrophoretic mobility and molecular weight.

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 (17).

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 (18). 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.

This completes the second annual report from the University of Louisville Chemical Monomer Research Group. If there is need for any further information or clarification, please contact me.

Sincerely yours,



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

CHT:vb

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HEALTH SCIENCES CENTER  
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Manufacturing Chemists Association  
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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

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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.

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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 fluorescence.

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-alkyl 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.

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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 of 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-fractionated) 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.

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

Drs. Schrodtt 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, Schrodtt 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.

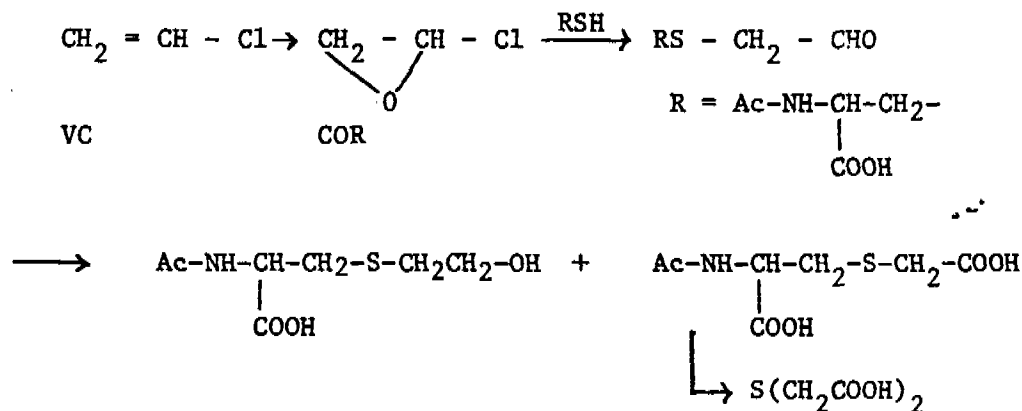
Part I. Detoxification Study.

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

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The detoxification of chlorooxirane was also studied in aqueous media at different pH's. N-Acetylcysteine was used as a typical cellular sulfhydryl 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  $^1\text{H}$  and  $^{13}\text{C}$  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

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

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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<sup>1</sup>-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 <sup>13</sup>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 Prokaryotic 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.

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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 antisera 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

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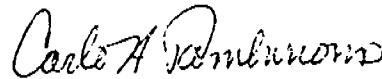
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measure the antigen in nanogram quantities. Radioactive vinyl chloride metabolites will be incubated with isolated liver cells and the uptake, intracellular transport and metabolism by various membrane and cytosol fractions will be investigated.

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  
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