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Mr. Joseph T. Seawell
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1825 Connecticut Avenue, N.W.
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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.

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.

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.

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.

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)

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.

- 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.

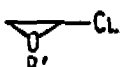
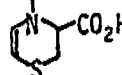
Technical Proposal E -- Chemical Systems of Detection of Toxicity of Vinyl Chloride. J. L. Wong

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

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

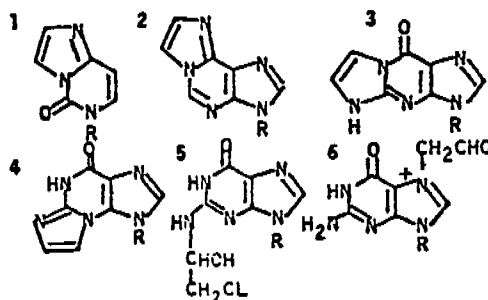
detoxified. Do they yield the same products or different ones? We have studied their reaction with sulfhydryl compounds 3,4-dichlorobenzenethiol and N-acetylcysteine. The benzenethiol was used by the Stockholm group in a preliminary study to detect the formation of CAA and COR from VC. The cysteine derivative 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 ¹ , ETHENO-A ² L-ETHENO-G ³ , A-ETHENO-G ⁴ , HEMIACETAL-G ⁵
COR	G-7-ACETALDEHYDE ⁶ + ...



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.

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

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

Table III
COMPOSITE MUTAGENICITY SPECTRUM OF CHEMICAL MONOMERS^a

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

^aResults 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.

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.
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APPENDIX

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A B S T R A C T

LYMPHOCYTE RESPONSE TO ANTIGENS OF LIVER ANGIOSARCOMA IN VINYL CHLORIDE WORKERS.

H. Philip Fortwengler, Michael E. Dever, Carlo H. Tamburro, and Enrique Espinosa, University of Louisville School of Medicine, Louisville, Kentucky 40232.

Angiosarcomatous liver tissue of vinyl chloride workers was shown by immunodiffusion and immunofluorescent procedures to include a tumor-associated antigen, antigens shared with normal liver and other tissues and tumor-bound immunoglobulin G. In addition, evidence has been presented for the existence of a tissue or plasma antigen induced by, or conjugated with vinyl chloride or a vinyl chloride metabolite. Possible lymphocyte sensitization to such antigens in 79 vinyl chloride workers including 26 with liver abnormalities was assayed by the lymphocyte transformation test. Antigen extracts used were prepared from the angiosarcomatous tumor and normal liver tissues. Twenty normal individuals having no exposure to vinyl chloride served as controls. Stimulation indices were calculated from cellular incorporation of tritiated thymidine. Mean stimulation indices in the normal individuals for antigens of angiosarcoma and normal liver tissues were 4.7 ($SE \pm 1.7$) and 3.8 (± 0.9) and in vinyl chloride workers, 1.8 (± 0.2) and 2.5 (± 0.3) respectively. Mean stimulation indices for PHA and concanavalin A in the normal individuals were 235 (± 35) and 209 (± 30) and in vinyl chloride workers 201 (± 23) and 180 (± 19) respectively. Thus, these results suggest that vinyl chloride workers have a decreased lymphocyte response to antigens of liver angiosarcoma and normal liver tissues rather than the hypothesized increased reactivity. This appears to be due to a lower overall lymphocyte responsiveness in these chemical workers in view of their decreased reactivity to PHA and concanavalin A. (Supported in part by the Manufacturing Chemists Association)

Federation Proceedings 27: 304, 1978

DECREASED GLUCOSE-6-PHOSPHATASE ACTIVITY IN LIVER IN VINYL CHLORIDE EXPOSED RATS. J.T. Du* and C.H. Tamburro* (SPON: M. Fonda) Dig. Dis. & Nutr. Sect., Dept. Med., Cancer Center, Univ. of Louisville Med. Sch., Lou., Ky. 40201.

Increases in key glycolytic enzymes paralleling hepatoma tumor growth (Heinrich, et al., FEBS Letters, 42:145, 1974) and decreases in key gluconeogenic enzymes prior to and with the development of hepatomas (Isok, et al., Voprosy. Med. Khim 19:568, 1973) have been shown. We exposed adult Sprague-Dawley rats to 10,000-20,000 ppm of vinyl chloride (VC), 4-8 hrs./day, 5 days/wk. for 3-4 wks. (40-140 hrs. exposure) to induce liver injury and angiosarcoma formation. Glucose-6-phosphatase, a key gluconeogenic enzyme in the liver microsomal fraction, decreased 25% over control ($P < 0.05$ from combined data). Other microsomal proteins and enzymes related to VC metabolism, i.e., P450, NADPH-cytochrome c reductase and mixed function oxidase were unchanged in the same microsomal fraction with no differences in either mitochondrial cytochrome oxidase or blood transaminases. The decrease in glucose-6-phosphatase is similar to the lower gluconeogenic enzyme findings in hepatomas. This may reflect an increase in glycolysis and ribose-5-phosphate production associated with de novo purine biosynthesis prior to tumor development, inhibition in enzyme synthesis, or increased breakdown due to VC exposure. Work is under way to determine the mechanism. The decrease in glucose-6-phosphatase may be an early biochemical lesion usable as an indicator of liver injury associated with the subsequent development of angiosarcoma.

Fed. Proc., 35, 1422 (1976).

Type Abstract in Space Below

TISSUE AND URINARY GLYCOSAMINGLYCANS IN TRANSPLANTABLE HEPATOMAS. C. E. Kupchella, K. L. Curran, E. Drake, J. Kennedy, and G. H. Tamburro. Cancer Center, and Division of Digestive Diseases and Nutrition, University of Louisville, School of Medicine, Louisville, Kentucky.

The purpose of this investigation was to evaluate: a) the glycosaminoglycans (GAGs) in different behavioral/histological types of intermuscularly transplanted hepatomas, b) GAG patterns in tumor tissue in relationship to degrees of fibrosis and necrosis, c) the GAG changes in the livers of tumor-bearing animals, and d) urinary GAG excretion as a function of tumor growth. Three types of Morris hepatomas, 7777, 5123tc, and 9613A, which differ in rates of growth, metastatic potential, fibrosis, and necrosis, were studied. Urinary and tissue GAGs were extracted as cetylpyridinium complexes and measured as uronic acid. Tissue GAGs were also evaluated histochemically using alcian blue staining with and without enzyme pretreatment. Tumor tissue exhibited four- to six-fold greater GAG levels in the hyaluronic acid (90 ± 10 , 91 ± 12 , and 111 ± 9 vs. 25 ± 3 μ g uronic acid/g dry liver, respectively) and chondroitin sulfate (261 ± 29 , 217 ± 51 , and 208 ± 22 vs. 47 ± 7 μ g uronic acid/g, respectively) fractions than normal liver; the heparin fractions did not differ significantly (31 ± 7 , 17 ± 4 and 67 ± 11 vs. 39 ± 10). The livers of tumor-bearing animals exhibited slightly greater hyaluronic acid levels than normal livers. Moreover, increased urinary GAG excretion was evident after two weeks in animals bearing fast-growing tumors. The GAG tissue levels in fast vs. slow-growing tumors were not significantly different. This further supports our previously reported studies of urinary GAG excretion in human hepatic angiosarcoma (Curran, K. L., et al., Cancer 40 (6): 3050-3053) in suggesting that urinary GAG analyses may be useful in the detection, screening and diagnosis of hepatic cancer.

Gastroenterology, 1979, in press

AMERICAN PUBLIC HEALTH ASSOCIATION
DECEMBER 1978 SAN DIEGO

3097

Early Detection of Disease in Individuals Exposed to Vinyl Chloride.

Richard Greenberg, MD, and Carlo Tamburro, MD, University of Louisville, Louisville, Kentucky

This is a collaborative comparison study of the effectiveness of ultrasound, nail bed capillary study and urinary glycosaminoglycan excretions in vinyl chloride workers with biochemical dysfunction or histopathologically documented injury.

It represents an effort to detect early morbidity in vinyl chloride workers. Included is Dr. Maricq's method of investigating capillaries of the middle and distal phalanges of the fingers, including the nailfold, as a screening test for early changes from vinyl chloride exposure. Another new method is Dr. Taylor's "grey scale" ultrasonographic method which has not yet been widely adopted for diagnosis of liver disease. The University of Louisville group reported that the first indicator of liver changes occurred in the vascular sinusoids and was reflected by the appearance of mucopolysaccharides in the urine.

The results of the massive clinical study in Louisville should soon be able to indicate whether more sensitive criteria of early disease may be forthcoming, especially from the liver function testing. The special value of the capillary and ultrasound method remain doubtful at this time for early detection of the effects of exposure to vinyl chloride.

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24 TISSUE AND URINARY GLYCOSAMINOGLYCANS IN
TRANSPLANTABLE HEPATOMAS OF DIFFERING
GROWTH RATES. Kupchella, C.E., Curran, K.L.,
Drake, E., Kennedy, J., and Tamburro, C.H.
Cancer Center and Department of Medicine,
University of Louisville, Louisville, KY. 40232.
The purpose of this investigation was to
evaluate the glycosaminoglycans (GAGs) in dif-
ferent behavioral/histological types of inter-
muscularly transplanted hepatomas and in the
livers and urine of animals bearing these tumors.
Fast (7777), medium (5123tc), and slow (9618A)
growing Morris Hepatomas were studied. Urinary
and tissue GAGs were extracted as cetylpyri-
dinium complexes and measured as uronic acid.
Tissue GAGs were also evaluated histochemically.
Tissue from fast, medium, and slow growing tumors
exhibited 4 to 6 fold greater GAG levels than
normal liver in the hyaluronic acid (90 ± 10 ,
 91 ± 12 , and 111 ± 9 vs. 25 ± 3 μ g uronic acid/g
dry liver, respectively) and chondroitin sulfate/
heparan sulfate (261 ± 29 , 217 ± 51 , and 203 ± 22
vs. 47 ± 7 μ g uronic acid/g, respectively) frac-
tions; the heparin fractions did not differ
significantly. The livers of tumor-bearing
animals exhibited slightly greater hyaluronic
acid levels than normal livers. Increased
urinary GAG excretion was evident after two
weeks in animals bearing fast-growing tumors.
These data together with those of our previously
reported studies of GAG excretion in human
hepatic angiosarcoma (Curran, K.L., et al.,
Cancer 40(6): 3050-3053) suggest that urinary
GAG analyses may be useful in the detection and
diagnosis of hepatic cancer.

Histologically these tumors consisted primarily of fusiform nevi cells with prominent eosinophilic cytoplasm embedded in a dense connective tissue stroma. Junctional activity, theque formation, and pigment were uncommon features. As a result desmoplastic nevi may be confused with reticulohistiocytoma, atypical fibroxanthoma, dermatofibroma, and even necrobiotic granulomas. Well defined intranuclear invaginations of cytoplasm occurred in 12 cases, and this was an important feature differentiating desmoplastic nevi from these fibrohistiocytic lesions. Desmoplastic malignant melanoma must also be considered in the microscopic differential diagnosis, but distinguishing features of desmoplastic melanoma include identification of preexisting malignant lentigo or acral lentiginous melanoma, atypical mitotic figures, and foci of necrosis. Clinically most desmoplastic nevi were dome-shaped nodules. The age range was from 7 to 53 years (mean 28); duration from 2 months to 15 years; male to female ratio, 6/8; and four patients gave a positive history of preceding trauma. Five lesions were located on the thigh or leg; and the rest, with the exception of sparing the palms and soles, were widely distributed. Desmoplastic nevi were compared to the ordinary variants of mixed spindle cell and epithelioid cell nevi in an attempt to define etiologic factors explaining a desmoplastic reaction. Initially, it was thought that patient age or preceding trauma might be important. No satisfactory explanation could be found since, with the exception of a predilection for occurrence on the lower extremities, the other clinical variables were not statistically different.

Computer-Assisted Morphologic Quantitation of Collagen in Human Liver Biopsies

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Because unfixed liver is rarely available for analytical studies, appraisal of the extent of collagen must be made from appropriately stained sections of liver biopsy material leading to judgemental as well as sampling error. We have developed a computer-assisted morphologic technique to evaluate the distribution of collagen in liver biopsy material. We examined multiple biopsy samples from patients dying suddenly with no history of liver disease to establish normal values. A Hewlett-Packard 9864-A digitizer and 9815-A microcomputer were used to quantitate areas of trichrome stainable collagen within the biopsy samples. Random area selection and statistical analysis proved to be important considerations in determining the experimental model and these factors were readily incorporated into the calculator program. Stainable collagen estimates in normal liver varied from 0 to 6.1 per cent (mean = 1.25 per cent) in patients with no evidence of liver disease and showed considerable variation even in different biopsies from the same patient. As anticipated, subcapsular biopsies had more collagen than deep biopsy; however, the specimens from several deep biopsies often had significant variation in collagen content. Differences in central, mid-zonal, and portal collagen could be obtained using this method. The computer-

assisted morphologic technique for evaluating hepatic collagen appears to be readily adaptable to clinical studies. This study of normal liver implies that considerable care must be taken before the clinical diagnosis of significant fibrosis can be made.

Uptake and Nuclear Transfer of 17-Fluorescein-Labeled Estradiol in Normal and Abnormal Human Endometrium

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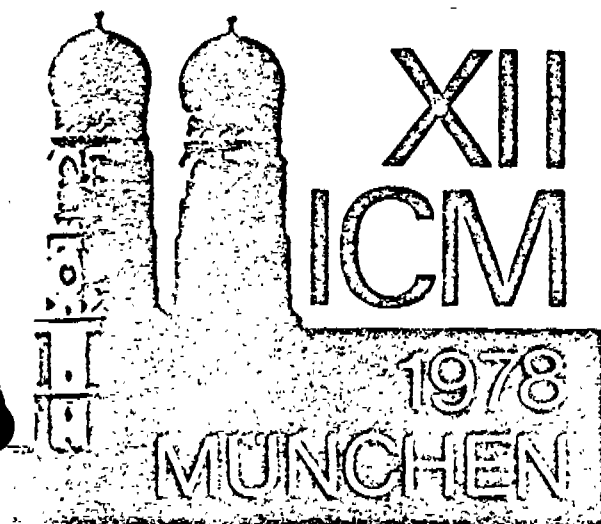
We have developed a 17-fluorescein-labeled estradiol which is specifically transferred to nuclei of target tissue (breast, endometrium) and inhibited by synthetic and natural estrogens. By *in vitro* incubation, patterns of temperature-dependent nuclear uptake may be observed. Marked cyclic variation is observed in normal human endometrium. With proliferative and early secretory endometrium, temperature-dependent nuclear labeling is present and appears to peak at late proliferative stages. Mid and late secretory endometrium, while having specific cytoplasmic labeling, do not exhibit temperature dependent nuclear labeling. This suggests that estradiol transport during mid and late secretory phases is inhibited or minimal. Abnormal endometrial samples exhibit a variety of patterns with the fluorescein-labeled estradiol. Endometrium with cystic hyperplasia reveals very little nuclear glandular staining, whereas in adenomatous hyperplasia strong nuclear uptake of the fluorescein-labeled compound is observed. Some cases of well differentiated endometrial carcinoma have strong nuclear uptake of the fluorescein-labeled estradiol whereas poorly differentiated endometrial carcinoma has little or no nuclear uptake. The method provides a good means of determining the endocrinologic behavior of glandular *versus* stromal elements of endometrium and suggests that in abnormal endometrium either variation in steroid uptake mechanisms or hormonal milieu may be important in the development of the lesion.

Aortic Infarction following Dissecting Aortic Aneurysm

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Aortic infarction was observed in 21 of 34 cases of dissecting aortic aneurysm. This lesion occurred as a central zone of necrosis, sparing media adjacent to the true and false lumina. This infarct followed rather than preceded dissection, took approximately 48 hours after dissection to develop, and did not organize with time. Lesions months to years in duration were similar histologically to those only several days old, and although there was some tendency to calcify in the older lesions, inflammation was consistently absent. The lesion occurred exclusively in the thoracic aorta. Histologically, aortic infarction was completely distinct from medial cystic necrosis (MCN). Aortic infarction was characterized by preserved elastic laminae with no increase in mucopolysaccharides, in contrast to MCN which was characterized by elastica disruption and increased mucopolysaccharides. When both MCN and aortic infarc-

**BACTERIOLOGY SECTION
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MYCOLOGY SECTION**



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In gram-negative bacteria the enzyme-independent type of penicillin resistance is assumed to be based on a permeability barrier to the outer membrane lipopolysaccharides, which prevents the function of the cell wall could not be established until now. From earlier studies we received some evidence for a possible role of the cytoplasmic membrane in this resistance mechanism. We therefore studied the binding of ^{14}C -benzylpenicillin (^{14}C -BP) to the membranes of resistant and sensitive *Staphylococci*.

First whole cells were incubated in buffer with increasing concentrations of ^{14}C -BP at 37°C for 30 min and then cytoplasmic membranes were prepared following the method of P. R. KAPLANACK (Voth. Enzymol. XXII, 99-120 (1971)). In membranes of sensitive staphylococci the binding increases sharply even at low penicillin-concentrations and levels out above 0.3 nmoles ^{14}C -BP/ml. In contrast, the binding to the resistant cells is reduced to a high degree. It reaches only 1-2% at 0.3 nmoles/ml. If the binding constants are calculated it can be seen that the number of binding sites is the same both in the resistant and in the sensitive membranes (20 nmoles BP/g membrane protein). However with the resistant membranes the affinity to penicillin is decreased by a factor of 250 to 700.

To exclude a permeability barrier in the cell wall the binding of penicillin to isolated membranes was examined. The incubation of the membranes with ^{14}C -BP was carried out under the same conditions as already mentioned. In this case, too, the binding to the resistant membranes was greatly reduced. This is also due to a high loss of affinity by approx. the same factor as above.

From these results a permeability barrier in the cell wall can be excluded. The cause for this type of resistance must thus be located in the membrane itself.

Therefore the membrane lipids were analysed. Differences were observed in the phospholipid fraction. The resistant membranes contain 40-50% more cardiolipin and 10-15% more phosphatidylglycerol than the sensitive ones. Additionally a cardiolipin derivative is found with the hydroxyl group of the central glycerol molecule acetylated. The other lipids are unchanged in quantity and in fatty acid composition.

From this it can be concluded that the intrinsic resistance of staphylococci is based on an altered membrane structure caused by increased lipophilicity.

K.9

Chloroacetaldehyde-induced Damage to *Bacillus subtilis*. A. D. LAUBACH*, U. N. SRIKISHA*, and J. L. WONG. Bureau Foods, FDA, and University of Louisville, School of Medicine, Health Sciences Center, Louisville, Kentucky 40232 (USA).

Chloroacetaldehyde (CAA), a proposed metabolite of vinyl chloride metabolism, has been shown to be mutagenic in the *Salmonella* assay system, and to specifically inhibit the growth of a recombination-deficient mutant of *Bacillus subtilis* (Fillmore, et al., 88A, 442: 405, 1976).

We have examined further the biological activity of this compound. First, CAA caused a significant increase in induced mutations to streptomycin resistance. Using *B. subtilis* 165, the relative mutation frequency (created μ /CA/Control) was 13.6, while using an *hcr* mutant of *B. subtilis* the frequency was 1.0. Secondly, a survey of the available repair-deficient mutants of *B. subtilis* revealed that the only strains sensitive to CAA were of the *rec*⁻ genotype. Furthermore, the several *rec*⁻ mutants, show three types of response to CAA. Strong killing by CAA (inhibition of growth of 10^{-6} or more) was exhibited on the *recA*⁻ *recB*⁻ *recC*⁻ (451616), and *recD27* (GSY1627) strains. Intermediate killing (10^{-5} - 10^{-6}) was shown with strains bearing the *recE*⁻ *recF*⁻ and *rec-13* (B0264) loci. All other strains examined (*recC*⁻ *recH*⁻ *recN*⁻ *rec-41*, *uvr*⁻ *hcr*, *polA*), as well as several repair-proficient cultures) grew in the presence of 0.1M CAA. The effects of CAA on the biological activity of DNA molecules in vitro are being studied.

These studies are part of an ongoing project sponsored by the Manufacturing Chemists Association to determine the molecular basis for vinyl-chloride induced carcinogenesis.

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30TH ANNUAL MEETING OF THE TISSUE CULTURE ASSOCIATION, INC.

Properties of 14 Week Maintenance Cultures of PLC/PRF/5 Cells. P.B.
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Louisville, Ky. 40232

This established cell line was originally described as being short lived with in a given passage, with considerable cell loss by day 6 to 8. However, after 80 passages, we found that culturing in glass roller bottles at 0.25 RPM allowed maintenance for at least 14 weeks with cells exhibiting excellent morphology without sloughing. After treating monolayers with Hoechst 33258 DNA stain, intense cytoplasmic fluorescence was seen in a proportion of the cells, possibly a function of the subviral hepatitis B genome. HBsAg of culture supernates was monitored and solid phase RIA assay revealed 31 to 47 ratio-units/0.2 ml after 2 to 14 weeks in medium with 10% fetal calf serum. The 14 week old cultures were extremely well adapted to maintenance in serum free medium for 14 additional days when supplementing with 100 ng/ml of insulin, in contrast to 1 week cultures which survived well for only 5 days without serum. The HBsAg titers of supernates from these 1 week or 14 week cultures, were very similar to the previous harvests in 10% serum. In addition, both young and 14 week cultures were similar in their rate of acid production; and in that both elaborated serum albumin, fibrinogen, transferrin and alpha-2 macroglobulin, the proteins tested to date.

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