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Boon v. Conoco, Inc., No. 80-4837

East Judicial District Court
Orleans Parish, Louisiana

AGREEMENT
between

Manufacturing Chemists' Association, Inc. and

University of Louisville

Reference Number: VC 7.0

1. Under this Agreement, the Research Contractor, University of Louisville is referred to as RC and Manufacturing Chemists' Association, Inc. is referred to as MCA.
 2. RC will conduct research in accord with Technical Proposal, "Research Techniques and Methods for Detection and Prevention of Carcinogenesis in Industrial Workers", Proposal Titles A. through I., Year 3, a copy of which is attached hereto and made a part hereof.
 3. RC will employ the staff, control the personnel, provide the facilities, and have exclusive control over the expenditure of funds provided by MCA under this Agreement. RC is and will be acting as an independent contractor in the performance of this Agreement, and shall be solely responsible for the defense against and payment of any and all claims against MCA for loss, personal injury or death, property damage, or otherwise, made by its employees or agents or by any other person or persons, arising out of or in connection with performance under this Agreement or any act or omission in connection therewith, unless caused by, or arising out of, MCA's negligence.
 4. While MCA commits to full public disclosure of scientific information developed through this project, RC will not publish nor otherwise release such information without prior MCA knowledge and review. This stipulation is not intended to inhibit privileged scientific interchange and consultation with other investigators in related fields or with the personnel of organizations participating in support of the project. If patentable discoveries should ensue, these shall be declared in the public domain without any retention of proprietary interest.
 5. ~~RC will furnish brief written progress statements to MCA, in accordance with the following schedule: what is projected for the near future as well as reporting on the essence of the research.~~
RC agrees to an oral presentation of the results of this research since the start of this research in 1977, such presentation to be made in Washington, D. C. or other similarly convenient location within the continental United States.
 6. Promptly upon completion of the project, RC will furnish to MCA one fine copy of its report on the project suitable for camera reproduction, and 35 copies. In addition, every four (4) months, RC shall furnish to MCA thirty-five (35) copies of a report giving the status of the ongoing studies.
 7. For the purpose of keeping informed*, personnel designated by MCA will have access to RC personnel and facilities engaged on the project as well as access to all raw data including wet tissues, slides and log books.
- * including a summary of the research conducted on this subject in the two years preceeding this Agreement,
** and for the purpose of monitoring the research or conducting an audit of the studies,

CMA 002129

8. MCA enters into this Agreement with the understanding that the project will be personally directed and supervised by Carlo H. Tamburro, M.D.

If any change in his status should arise that would affect his connection with the project, RC agrees to notify MCA in advance, in which event the Agreement becomes subject to renegotiation.

9. In support of this project MCA will provide funds in an amount not to exceed the lesser of actual project costs or \$297,734, payable as follows:
Payments will be made to RC bimonthly following receipt of an itemized statement detailing expenses incurred against the project during the preceding period, except that payment of the last twenty thousand dollars (\$20,000) will be withheld pending completion of the presentation described in paragraph 5 and receipt and acceptance by MCA of the final report and 35 copies referred to in paragraph 6 of this Agreement.

In no event will payments totaling more than \$267,764 be paid prior to the final payment. All invoices and progress reports will bear the Project Reference Number VC-7.0-DP/PREV-UL.

10. RC certifies that RC will comply with the Final Rule for Good Laboratory Practice Regulations -- Nonclinical Laboratory Studies -- as published in the Federal Register on December 22, 1978. RC also will notify MCA promptly of any audit affecting MCA contracted research at RC conducted by or on behalf of any government agency, and will provide MCA with a copy of audit results within thirty (30) days of completion of such audit. Also, RC will promptly notify MCA of the action of any governmental agency giving notification of disqualification, deficiencies or warning that there are discrepancies between the conditions and regulatory requirements of the performance of RC as a clinical or nonclinical laboratory.

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11.

~~10~~ This Agreement becomes effective **March 1, 1979**

and will remain in effect until completion of the research project which is expected approximately **twelve (12)** months thereafter. If unforeseen circumstances should make sooner termination desirable, it may be terminated by MCA on 30 days' written notice to RC. In such event, RC will present to MCA a detailed accounting of costs, including reasonable and necessary expenses incurred in terminating the project in an appropriate manner, with additional payment by MCA as limited by the provision of paragraph 9, or return of unused funds by RC, as the case may be, consistent with the results of such accounting and the provisions of this Agreement.

Accepted for:

University of Louisville

Signed *N. F. Elbert*

Name
Typed N. F. Elbert

Title Vice Pres. for Financial Affairs

Date 3/23/79

Accepted for:

Manufacturing Chemists' Association, Inc.

Signed *Gary C. Herrman*

Name
Typed Gary C. Herrman

Title Vice President-Treasurer

Date March 9, 1979

CMA 002131

TPFR2-1277



1275-1978-2-2

AUG 07 1978

UNIVERSITY OF LOUISVILLE
P.O. BOX 35260
LOUISVILLE, KENTUCKY 40232

SCHOOL OF MEDICINE
DEPARTMENT OF MEDICINE
DIGESTIVE DISEASES AND NUTRITION SECTION

HEALTH SCIENCES CENTER
WALNUT & PRESTON STREETS

August 2, 1978

Joseph T. Seawell
Vinyl Chloride Project Manager
Manufacturing Chemists Association
1825 Connecticut Avenue, N.W.
Washington, D.C. 20009

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Dear Mr. Seawell:

As discussed in our recent meeting here at the University of Louisville, I am submitting an updated third year (1979-80) proposal and its budget for the continuation of the grant entitled "Research Techniques and Methods For the Detection and Prevention of Carcinogenesis in Industrial Workers." I would appreciate your transmitting this to the members of the research committees for their consideration and continued support.

As we have done in the past, we are submitting this in two parts:

- Part I: A Summary of the Technical Proposals
- Part II: A Summary of the Budgets

In addition, there are appendices (reprints) which the investigators felt might be of interest to the group.

We have added two additional small proposals for the MCA members' consideration (Parts III and IV), as presented and discussed in our recent meeting here in Louisville.

As presented at our meeting, Dr. Waddell's work is related to total body autoradiographic study of the distribution of chemicals and their metabolites. Dr. Feldhoff's work is isolated to liver cell cultures for determining hepatotoxicity to chemicals and metabolites. These two proposals and their budgetary supports are included as special addenda. We would ask that the members might consider, for the third year, a small increase in the support, to allow these two very essential components to be added to our work in order for us to more fully understand and validate the studies already underway.

We appreciate this consideration on your part. Should there be any additional information needed, or any questions concerning this, please do not hesitate to let me know.

Sincerely yours,

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

CMA 002132

Research Proposal 1979-80

Univ. of Louisville

Work Copy

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P A R T A

TECHNICAL PROPOSAL

RESEARCH TECHNIQUES AND METHODS FOR
DETECTION AND PREVENTION OF
CARCINOGENESIS IN INDUSTRIAL WORKERS

UNIVERSITY OF LOUISVILLE

CANCER CENTER

LOUISVILLE, KENTUCKY 40201

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Technical Proposal A

Cellular Immunological Systems for the
Detection of Vinyl Chloride Injury

H. P. Fortwengler, M.S.

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H.P. Fortwengler, M.S. 2

Introduction.

14th Judicial District Court
Calcasieu Parish, Louisiana

The proposed cellular immunological research for year three funding consists of a continuation and advancement of work in progress. This ongoing research has been composed of three components which will be briefly introduced before giving more detailed information. The first component is directed toward the identification of those individuals who may be predisposed to chemical injury. Individuals with diagnosed chemically-related injuries as well as those at risk of injury are being tissue-typed to determine if certain genetic profiles can be correlated to increased incidence of disease.

The second component of the cellular immunological research is directed toward the identification of an immune defect in the lymphocytes, i.e. the protagonist of the immune system. Immune defects have frequently been reported in cancer patients and could possibly be an early signal that a cancerous lesion is imminent.

Our third research component is directed toward a search for evidence of a vinyl chloride (VC) induced tumor antigen. Using the responses of lymphocytes to hypothesized new products found in or on VC induced tumor antigen. Using the responses of lymphocytes to hypothesized new products found in or on VC induced tumors, we are seeking early specific clues that an individuals immune system has at least tried to challenge the tumor.

Cellular Immunological Studies. Part 1. HLA Frequencies in Vinyl Chloride Workers.

An increased incidence of certain HLA types has been shown by several reports to be associated with susceptibility to various diseases. Furthermore, Mulvihill in the Journal of the National Cancer Institute in 1976 stated that a challenge to science is to develop a means of screening potential employees for abnormal genotypes that predispose them to neoplasia after occupational exposure which is harmless to normal genotypes. 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.

HLA tissue typing procedures including the microdroplet lymphocyte cytotoxicity test have been initiated in our laboratory and we have typed approximately 300 individuals from the Louisville vinyl chloride polymerization plant. Individuals do not change their genetic complement of HLA antigens, so the determinations need not be repeated periodically as in various clinical assays. Tissue typing for 11 HLA-A antigens and 16 HLA-B antigens and their possible increased association with angiosarcoma or other chemically related diseases is about one-third completed.

A preliminary comparison has been made between the HLA frequencies of VC workers found to have liver disease and those without liver disease. Although the number of individuals tested is not large enough to allow statistical significance to emerge, potential difference in frequencies may be seen at antigens A9, B15, and B17.

As an additional procedural and population check, 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 our study here at the University of Louisville.

It has been clearly established that the larger the population studies the greater the chances of obtaining statistically significant data. It is anticipated that the accumulation of our HLA tissue typing data on the majority of the VC population plant workers will take an additional two years (three years total).

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Cellular Immunological Studies. Part 2: 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. Viola, et al, reported others reported the increased incidence of human angiosarcoma in VC production workers. It is the purpose of this study 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 angiosarcoma.

The scientific literature is replete with demonstrations of immunodepression as shown by one or more immune parameters in cancer patients at various stages of disease. There is very little information in the scientific literature concerning immunoevaluation prior to diagnosis of frand malignancy. We are using immunological assays that have demonstrated usefulness in indication immunodepression in cancer patients. These tests include enumeration of lymphocytes and certain lymphocyte subpopulations as well as tests for lymphocyte function. (For details, see annual report, year 1).

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 these diseased individuals with normal plant workers demonstrated nothing so far to suggest that the groups were immunologically different. A comparison of those VC workers having high VC exposure (defined as those with lifetime exposure above the plant median) with individuals having low exposure (below the plant median VC exposure) demonstrated a slight pattern of immunodepression when lymphocytes were stimulated by PHA and ConA. Preliminary evaluation of this data indicated there is no statistical significance between the high and low exposure groups. Even after additional immunological parameters were examined, no statistical differences could be found between the two groups.

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 our standard battery of immunological tests.

Further computer analysis of our data in addition to cocomitant correlations with data being received from clinical testing will give us more definitive statistical results. Definitive plans for the third year of this second component of the study must await the completion of computer programs and statistical analysis of data gathered to date. A more complete delineation of third year plans for this research component will appear in second year progress reports.

Cellular Immunological Studies. Part 3. 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.

In an effort to capitalize on the well established fact that the body mounts

an immune reaction to cancer, we are endeavoring to find evidence of these specific immune reactions and then utilize them to develop a relatively specific test for possible VC induced tumor development. Lymphocytes (the cells responsible for immunity) from the individual tested are being isolated by centrifugation over Ficoll-Hypaque. These cells are then grown in the presence of a liver reagent prepared from either a normal individual or an individual who had angiosarcoma. A positive reaction is considered a three times increased incorporation of H^3 -thymidine into stimulated cultures as compared to unstimulated cultures.

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 it was from angio 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.

The reactions of lymphocytes to normal and angio liver reagent were quantitated and a comparison of the responses of VC workers according to known VC exposure depending on whether they had exposure above or below the median plant exposure. No statistical differences were noted between the reactivities of the two groups.

On the chance that the non-specific reactions may be obscuring the specific reactions, all those VC workers having reactions to both angio liver and normal liver were grouped. Those with reactions to normal liver only were grouped as were those with no reactions. That left only those with reactions to angio liver, the group in which we are most interested. They have what appears to be a specific reaction to angio tumor extract, i.e., negative reactions to normal liver with concomitant positive reactions to angio tumor. The composition of this group was striking--the only reactions obtained with possible specific tumor reactivity were in the group of VC workers.

These results, although provocative, must be interpreted cautiously. In the third year of funding, the data will be extended by testing additional VC workers, but more importantly, by testing a comparison group of individuals having no known VC exposure. Our plans for the future, also include a slight reorganization of experimental design to ensure maximal statistical strength from the data obtained. Computer time and analysis will be used in the assessment of data obtained.

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Technical Proposal B

Biochemical Enzymatic Systems for the Detection
of Vinyl Chloride and Other Chemical Injury as a
Potential Means of Detection of Cancer in Humans

Julie T. Du, Ph.D.

CMA 002140

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Characterization of biochemical and enzymatic changes occurring in progressive exposure to vinyl chloride in animal experiments will be continued. These studies will enable us to further identify the sequential cellular enzymatic changes occurring with chemical monomer exposure.

As already has been reported, the changes in gluconeogenesis and in glucose 6-phosphate dehydrogenase already parallel the alterations seen in primary hepatocellular tumors. Our first experiments in animals exposed to one to two percent of vinyl chloride for 14 to 137 hours over a one- to four-week period, has shown this decrease in glucose 6-phosphatase and the increase in glucose 6-phosphate dehydrogenase accompanying the changes in glutathione reductase. These enzymatic changes occurred before there were any signs of physical abnormalities or any changes in the conventional liver biochemical tests performed during this period. These biochemical changes are similar to the alterations found in hepatomas, suggesting that the metabolic changes may predict impending physical changes.

In our human studies of vinyl chloride exposed individuals, Popper and Tamburro have identified focal hepatocellular hyperplasia and hyperplastic nodules as one of the earliest histological manifestations of prolonged vinyl chloride exposure.

Our present working hypothesis is that these early biochemical changes are indicative of a shift toward increased nuclei acid synthesis and hepatocellular replication, leading eventually to the hyperplastic areas seen in the human livers.

We are next planning to complete our study of the light microscopic slides of these animals and to determine whether similar morphological changes are occurring and are compatible with the enzymatic results already determined. These tissues have already been prepared for analysis and the first set is now being completed. These include selective groups from 28, 71, and 103 hours of exposure within paired controls. Similar studies will be completed with those animals from our second experiment which have a more prolonged exposure. We feel strongly, from our human studies, that these animal light microscopic evaluations will greatly assist us in understanding whether these changes seen in the human studies parallel those in the animals and are a correct reflection of the biochemical changes seen.

Our second major study is related to our working hypothesis that adult hepatocytes have adequate capability of detoxifying the carcinogenic metabolites of vinyl chloride, chloroxirane and chloroacetaldehyde, and thereby prevent the development of primary hepatocellular cancers. The adjacent cells, sinusoidal lining cells, have a decreased ability for detoxification and a greater susceptibility to DNA injury due to prolonged exposure of active metabolites, thereby leading to the development of angiosarcoma. This hypothesis is supported indirectly by the development of both primary hepatocellular carcinomas and angiosarcomas in newborn rats as reported by Maltoni, and by adult rats given significant, continuous alcohol ingestion with their vinyl chloride exposure, as reported by Radike and her group in Cincinnati.

CMA 002141

Toward this end our second experiment in animals exposed to two to three percent vinyl chloride for up to 6 weeks has shown an elevated glutathione-reductase in the liver, followed by an elevation of concentration of glutathione. This was later followed by an elevation of the detoxifying enzymes, glutathione epoxide-S-transferase (GEST) and later glutathione aralkyl-S-transferase (GAST). These biochemical changes were found before the conventional clinical tests showed abnormalities.

Vinyl chloride is believed to be metabolized by the microsomal P-450 enzyme system (mixed function oxidase) and detoxified mainly by way of glutathione via transferases. This suggests that the primary route of vinyl chloride metabolism is initial oxidation to chloroethylene oxide and then detoxification by GEST directly. The delayed induction of GAST suggests that the longer exposure and probable saturation of the direct route leads to greater rearrangement of the chloroethylene oxide to chloroacetaldehyde and detoxification with glutathione.

Fiala (Journal of the National Cancer Institute, 57:591-598, 1976) has found 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.

Our findings of a higher level of glutathione, and glutathione-related enzymes after vinyl chloride exposure may be an early biochemical precancerous alteration.

Our purpose was and still is to continue the exposure studies to identify the sequential cellular enzymatic changes occurring from early injury to tumor development.

The present working hypothesis for the nondevelopment of primary cell tumors in the adult animal is the ability of the adult hepatocyte to adequately detoxify the carcinogenic metabolites of vinyl chloride. The adjacent cells, sinusoidal lining cells, as well as newborn liver cells, most likely have a decreased ability of detoxification. This would explain why angiosarcomas occur in adult rats and primary hepatomas occur in newborn rats, as has been recently shown by Maltoni.

Our next set of experiments will be a prolonged exposure study of rats to vinyl chloride for 6 months or longer until the development of hyperplastic nodules and tumors. In addition to examining the enzymes and metabolites related to oxidation and detoxification of vinyl chloride and, the concentration of glutathione in the subcellular fractions of the total liver homogenate, we will also attempt to isolate the hepatocyte and endothelial cells from control and exposed animals. We intend to compare the cell's ability and capacity to handle the toxic agents, and to elucidate the mechanism of tumor formation in the endothelial lining cells.

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This requires a clear-cut isolation of liver tissue into hepatocytes and mesenchymal cells (Kupffer and endothelial cells). In order to obtain meaningful results, the mesenchymal fraction cannot have any contamination from hepatocyte debris. Kupffer and endothelial cells' densities are very close. A clear separation was not possible until the use of centrifugal elutriation introduced by Knook's group in Netherland recently (Knook, et al., Exp. Cell Res. 109:317-329, 1977). The method gives a high yield of endothelial cells and high viability. It involves perfusion with pronase which breaks hepatocytes specifically. For the isolation of hepatocytes, the original method of Berry and Friend (J. Cell Biology, 43:506-520, 1969), modified by Exton's group (Huston et al., J. Biol. Chem., 251:5200-5208, 1976) will be adapted. This method yields hepatocytes by perfusion of liver with collagenase.

Further, we are also working on developing methods to study the glutathione transferases and glutathione reductase in blood as possible means of detecting early changes. Liver tissue for light microscopy from these animals exposed to vinyl chloride will continue to be studied for correlations of these biochemical changes with morphological alterations.

In our next stage of animal exposure experiments, we plan to add weanling rats to the vinyl chloride animal group. In these animals, enzymatic assays, to document these hypotheses, will be performed in a manner similar to the adult rats. Both weanling and adult rats will be utilized for verification and further elucidation of the immunological and tissue antigen test systems by Dr. Espinosa's group. Urine from these rats will be used to further verify the urinary glycosomino glycan studies of Dr. Kupchella and the biological end-product analysis by Wong and Taylor.

The final portion of our studies will include the selective electron microscopic studies of these animals to further elucidate the morphological changes occurring at various stages of vinyl chloride exposure, and hopefully, to identify characteristic changes paralleling the enzymatic results already shown. These tissues have already been prepared for analysis and are awaiting our completion of our human electron microscopic studies so that we may have as complete a data base as possible for comparison.

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TECHNICAL PROPOSAL C

TISSUE AND URINARY GLYCOSAMINOGLYCAN CHANGES
RELATED TO VINYL CHLORIDE INJURY: USE AND EARLY
DETECTION AND DIAGNOSIS

CHARLES E. KUPCHELLA, PH.D.

CMA 002144

Project Rationale:

Work performed to date in our laboratory confirms the validity of the rationale described in original application, namely:

Since hepatic cancer has been shown to produce increases in liver glycosaminoglycans; since hepatic fibrosis has also been shown to produce such increases; since the types of GAG's associated with these two processes are not identical; and, if urinary GAG changes are found to reflect liver tissue GAG changes, urinary GAG analysis may serve as a means of early detection and perhaps a means of differential diagnosis. Further, if the GAG are found to play an important role in the pathogenesis of chemically-induced liver disease, perhaps GAG directed strategies can be developed for the control of these diseases.

Approach

It should be noted that our study continues to include both animal studies and studies of human clinical material. We continue to evaluate the urines and tissues of animals bearing transplantable liver tumors and the urines and tissues collected from other animal models, i.e., animals with necrotic livers, fibrotic livers, and livers undergoing regeneration. We are also evaluating human urine and liver tissue including:

- a) patients with hepatic angiosarcoma,
- b) patients with hepatocellular carcinoma,
- c) patients with fibrotic liver disease related to occupational exposure, i.e., the chemicals like vinyl chloride,
- d) patients with liver diseases (hepatitis, cirrhosis, etc., unrelated to occupational exposures,
- e) normal "controls".

It should also be noted that while our studies were stimulated by the vinyl chloride/hepatic angiosarcoma problem, our results will have relevance to hepatic cancer, hepatic fibrosis and other chemically-induced liver diseases.

Summary of Work Completed to Date

We have already found that:

- 1) Human hepatic angiosarcoma is accompanied by elevated hepatic GAG's. (1)
- 2) The GAG's in the angiosarcomatous tumors are different from those in fibrotic tissue adjacent to the tumors. (1)
- 3) Angiosarcoma and hepatoma patients have characteristic urinary GAG patterns -- patterns not found in workers with non-angiosarcomatous liver injury. (2)
- 4) Heparan sulfate (a type of GAG) is elevated in hepatic tissue undergoing experimentally induced fibrosis and heparan sulfate is evaluated in the urine of such animals. (3)

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- 5) yet uncharacterized, chondroitin sulfates and hyaluronic acid levels -- but not heparin -- are 3-4 fold higher in experimentally transplanted hepatomas than in normal liver and urinary excretion reflects both the tumor GAG composition and the size of tumors.
- 6) Livers of animals bearing metastasizing hepatoma (5123tc) have 50-fold greater concentrations of non-sulfated, neutral, uronic acid positive material than is found in the livers of animals bearing two other, non-metastasizing hepatomas.

Work to be Performed in Year-3

In our proposal "update" dated May 1977, we listed the following activities to be completed in year-3:

- 1) assess the contribution to urinary GAG patterns made by hepatic regeneration,
- 2) study the kinetics of GAG incorporation into connective tissue and GAG tumors,
- 3) study vinyl chloride injured animals, i.e., the urines and liver tissues of experimental animals with vinyl chloride induced fibrosis and angiosarcoma.
- 4) evaluate urinary GAG excretion in vinyl chloride workers with evidence of liver injury -- and "control" patients with non-occupationally related liver disease.

Some of these studies have already begun. Some studies originally planned to be conducted in year-2 will be conducted in the third year. We will also pursue a number of newly developed leads in the third year. Our current plan for the third year is to complete the following investigations:

Studies Already Initiated

1) THE VALUE OF URINARY GAG ANALYSES IN THE DIAGNOSIS OF LIVER DISEASE IN MAN: FIELD TESTING

As part of a double blind study intended to compare 1) urinary GAG analyses, 2) ultrasound diagnosis, and 3) capillary (nail bed) changes as rapid, non-invasive methods of detection; we evaluated 120 urine samples (occasional samples only -- not 24-hour urines) for urinary GAG's and declared each to be either normal or abnormal. The test population included 33 individuals with abnormal liver function tests and 36 abnormal biopsies.

While the GAG test provided the best screening for abnormal biochemical results, the statistical results were borderline, i.e., even with "occasional" random urine samples measuring only the total GAG content and expressing this as a function of creatinine content, the sum of sensitivity plus specificity was 1.20. When compared to an expectation of 1, this is of borderline significance ($z = 1.89$). We will repeat this study in year-3 focusing on specific GAG fractions now known to be affected most severely by liver disease.

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2) THE CONTRIBUTION OF NECROSIS TO THE INCREASED GAG LEVELS IN LIVER INJURY

We have tentative evidence that indicates that necrosis is responsible for the increased GAG levels in injured and tumor-bearing livers. Using both histochemical and biochemical methods, we plan studies in year-3 to definitively determine the effects on hepatic and urinary GAG's induced by chemical and ischemic necrosis.

3) URINARY GAG PATTERNS IN HUMAN ANGIOSARCOMA, HEPATIC FIBROSIS AND OTHER LIVER DISEASES

We will continue to accession patients with angiosarcoma, hepatoma, hepatitis, and cirrhosis to determine if the observation we reported in Cancer (40):2040 holds its specificity and sensitivity.

4) EXPERIMENTALLY INDUCED FIBROSIS AND REGENERATION

We will complete early in year-3 the ongoing studies of tissue and urinary GAG changes in animals undergoing hepatic fibrosis and hepatic regeneration -- as described in our previous update.

5) GAG PATTERNS IN EXPERIMENTAL HEPATIC TUMORS

We already have data showing that experimentally transplanted Morris Hepatomas have large amounts of GAG's and that this is reflected in urinary excretion. We are evaluating the relationship of these patterns to the behavior of these tumors. Results may lead to the development of GAG directed strategies useful in the control of hepatic cancer and perhaps cancer in general.

STUDIES TO BE INITIATED AND COMPLETED IN YEAR-3

1. Vinyl Chloride Exposure

We plan to study vinyl chloride exposed animals -- to compare observations with those obtained with CCl_4 treated and with hepatoma-bearing animals -- with respect to GAG patterns.

2. Metabolic Studies

Using radiolabelled precursors, we will study the synthesis of hepatic GAG's in response to fibrosis, necrosis, regeneration, and the pathogenesis of hepatic cancer.

Methods

We continue to use -- with only minor modifications -- the methods we described in our original grant application.

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Epilog:

Our results to date indicate that GAG changes may be among the earliest demonstrable changes to occur with liver injury. Our third year studies should confirm the degree of usefulness of urinary and tissue GAG measurements in early detection and differential diagnosis. These studies should help zero in on the role of GAG's in fibrogenesis and the pathogenesis of cancer and illuminate the way toward possible GAG directed strategies for the control of these diseases.

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TECHNICAL PROPOSAL D

ELECTRON MICROSCOPIC EVALUATION OF
LIVER INJURY FROM CHEMICAL WORKERS

G. RANDOLPH SCHRODT, M.D.

CARLO H. TAMBURRO, M.D.

CMA 002149

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TECHNICAL PROPOSAL D

ELECTRON MICROSCOPIC EVALUATION OF
LIVER INJURY FROM CHEMICAL WORKERS

Recent studies in this and other laboratories have suggested that in individuals exposed to vinyl chloride there is an increase in intralobular collagen (fibrous tissue) deposition. The more obvious increase in portal and subcapsular fibrosis is now well known. Our own preliminary data indicates that this collagen deposition occurs much earlier in the parenchyma of the liver rather than in the portal or subcapsular area.

The collagen deposits apparent in light microscopic studies only with special staining are more readily visualized with the electron microscope. Most of this collagen appears confined to the space of Disse along the surface of the hepatocyte. This occurs more frequently between the sinusoidal lining cell and the hepatocyte surface rather than between hepatocyte to hepatocyte junctions; not infrequently deep invaginations of the space of Disse creates the impression that collagen bundles are found within the hepatocyte. This gives the impression that the fibrous strands are actually compressing the hepatocyte.

As part of the total patient evaluation of individuals exposed to vinyl chloride, electron microscopic studies of the liver biopsies of a selected number of individuals have already been made. Following these initial observations, evaluation of the ultrastructural findings, with both the light microscope as well as the electron microscope, has been underway.

Observations in individuals who have developed angiosarcoma following extensive exposure to vinyl chloride have demonstrated a marked increase in the collagen deposition in the hepatic perisinusoidal spaces. This appears to eventually lead to the destruction of the hepatic cell cords and is associated with the activation of the sinusoidal lining cells with focal sinusoidal widening and sinusoidal cell atypia. This fibrosis and widening of the sinusoids with the sinusoidal atypia appear to be a premalignant indicator of hepatic injury due to vinyl chloride exposure. These studies are supportive of those findings by Kupchella et al. in which there are increased glucosaminoglycans in both tissue and urine samples of vinyl chloride-exposed individuals.

The importance of recognizing this early increase in collagen deposition in the perisinusoidal space and its lobular distribution may well characterize the vinyl chloride-exposed injury from other types of

CMA 002150

collagen deposits, such as those associated with alcohol, drugs and viral hepatitis. These other non-occupational diseases are associated with the hepatocellular injury and necrosis and subsequently have increases in collagen deposition. In contrast, it appears that the vinyl chloride injury is associated with earlier deposition of collagen in an unusual pattern (parenchymal distribution) and occurs before any significant hepatocellular injury.

Systematic comparison of the sinusoidal cell structures and the collagen content of the perisinusoidal spaces in the various zones of the hepatic lobule in individuals with vinyl chloride exposure, as well as exposure to other chemicals, will be continued during the second and third years of this grant proposal.

The use of a Hewlett-Packard 9815 desk-top calculator with surface digitizer with interface, has increased both the capability of studying larger numbers of individuals as well as increased the accuracy and rapid reproducibility of the manual methods. The calculator has been programmed to evaluate in a semi-quantitative manner the various ranges of collagen deposition seen in vinyl chloride and other chemically exposed workers. Three normal* individuals of various ages have had their hepatic collagen content studied. There appears to be a clear increased collagen deposition with increasing age. This "normal" data will play a significant role in the interpretation of our chemical worker data. Our chemical worker data will be analyzed using the work exposure histories of each individual for comparison of the collagen disposition to the other 19 chemicals to which they had potential exposure and in comparison to "normal" findings.

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*individual historical, clinical, or laboratory evidence of liver disease, and died from sudden traumatic causes.

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Technical Proposal E

Multivariate Analysis of Biological
End-Products and Vinyl Chloride Metabolite Synthesis

John L. Wong, Ph.D.

CMA 002152

Our study has been concerned with the chemical systems of detection of vinyl chloride (VC) toxicity. Since the vinyl chloride epoxide (chlorooxirane, COR) is an obligatory intermediate in the normal metabolism of the vinyl monomer and because of the high reactivity of the epoxide and its tendency to rearrange to chloroacetaldehyde (CAA) which is also reactive, we will continue to develop chemical methodologies pertaining to the study of: (1) detoxification of the primary metabolites (COR and CAA) in a cytoplasmic environment, i.e. identification of all plausible reaction products, so that the metabolic fate of the epoxide and the aldehyde can be determined as the host factors vary, (2) distribution of the metabolites COR, CAA, and their reduction product chloroethanol (CE) in whole animals and in cells, and (3) putative action of the primary metabolites COR and CAA on nuclear materials with the intention of relating the identity and quantity of the altered nucleic acid bases to pathological findings. Below illustrates our work plan for the coming year.

(1) Detoxification Study

The trapping of the epoxide and carbonyl compounds in the cytoplasmic environment by sulfhydryl compounds are generally the detoxification reactions. The identification of these sulfur-conjugates as urinary metabolites is still incomplete. There is 35 percent of the vinyl chloride carbon-14 activity in the urine unaccounted for. We have studied the reactions of COR and CAA 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 is formed. However, CAA and benzenethiol forms the hemithioacetal. 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 dihydrothiazinecarboxylic acid, at low pH. This thiazene is a multi-step reaction product. Its isolation may have solved the mystery of the unknown urinary metabolite as well as the origin of the identified urinary metabolites: the cysteine S-acetic acid and the S-ethyl alcohol. Basing on these model studies, two types of experiments will be conducted in collaboration with Dr. Tamburro's laboratory.

A. In Vitro Experiments: The cytosol and microsomal fractions will be isolated from hepatocytes and other nonhepatocyte cell lines. They will be used as such or fortified with glutathione to treat the primary metabolites COR and CAA, and the resulting mixtures will be analyzed for the known products described above as well as covalent binding to microsomes. Also, the thiazene compound which is a chemical equivalent to cysteine S-acetaldehyde, will be tested for its capability to generate the urinary metabolites S-acetic acid and S-ethyl alcohol in the presence of cytosol.

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B. In Vivo Experiments: Rats will be exposed to the metabolites COR, CAA, and CE (feeding or ip). Urine analysis for the metabolites and polyamine patterns will be made. It is noted that the latter has been associated with various cell injuries.

(2) Distribution Study

¹⁴C-labeled COR, CAA, and CE will be synthesized according to known methods. Exposure of whole animals to these tracer compounds followed by the freeze and slice method and autoradiography (with Dr. Waddell) will show the preferential accumulation of these metabolites in various organs. When bacteria and cell cultures are used (with Drs. Streips and Feldhoff), the distribution of the tracers in the cell compartments can be quantitated by scintillation counting. These distribution data will lend credence to the speculation concerning the pathogenesis of vinyl chloride.

(3) Putative Action Study

The detection study of the putative action of vinyl chloride has been started on the hypothesis that it is due to the modification of the nucleic acid materials by the primary metabolites COR and CAA. Although CAA is long known to react with nucleic acid bases such as cytosine and adenine to form the etheno derivatives, 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 gives rise to two tricyclic ethenoguanines, the linear etheno compound and the angular etheno compound, and a third product which is still unidentified. These products can disrupt nucleic acid structures hence their functions by means of steric and electronic perturbation. It is also worthy of note that their fluorescence properties may allow their direct detection in a cell nucleus. We will continue this line of structural and detection study involving CAA. 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. It is important to note that this is the first indication that COR and CAA show different molecular events in their putative action. Continuation work on the COR-reaction will unravel the structures of the major products. Furthermore, we will apply these metabolites to the bacteria systems (with Dr. Streips) in an attempt to correlate the mutation events with the extent and pattern of alkylations.

In summary, our strategy is to fully elucidate the molecular events in the metabolism of vinyl chloride, i.e. detoxification and putative action, and use it as a model to screen the mutagenic and carcinogenic potential of other industrial vinyl monomers. Preliminary results have shown promise in evolving a protocol for early detection and prevention of certain industrial carcinogenesis.

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TECHNICAL PROPOSAL F

ANALYSIS AND IDENTIFICATION OF
CARCINOGENIC POTENTIAL OF INDUSTRIAL CHEMICALS

ULDIS N. STREIPS, PH.D.

CMA 002155

ASSAYS FOR THE CARCINOGENIC POTENTIAL OF INDUSTRIAL CHEMICALS
UTILIZING PROKARYOTIC AND EUKARYOTIC SYSTEMS

INTRODUCTION

Our laboratory in collaboration with Dr. J. Wong, Department of Chemistry, has studied extensively the mutagenic properties of vinyl chloride and the potential metabolic derivatives of vinyl chloride. These studies have led to the identification of chloroacetaldehyde monomer hydrate as the probable mutagenic metabolite, and also have suggested that this chemical elicits replication repair in bacteria to rectify damage (1). Since replication repair is an error-prone process, this then may describe the potential carcinogenic mode of action of chloroacetaldehyde. In addition, Dr. Wong has discovered that formation of chloroacetaldehyde is inhibited in the presence of reducing agents such as cysteine, glutathione, and others: formation of chloroethanol, a nonmutagenic metabolite is favored. This finding suggests that the carcinogenic action of vinyl chloride may be blocked by appropriate chemicals. This hypothesis will be tested in vitro in the several experiments described in this proposal.

Although to date our work has centered on vinyl chloride and will continue to do so until this model system has been completely investigated, our laboratory has begun in collaboration with Dr. Wong, to examine other industrial monomers such as styrene and acrylyl nitrile and their metabolites by these same techniques.

OBJECTIVE

Our objective is to implement a fully comprehensive screening facility for potentially carcinogenic industrial chemicals and their metabolites. Once the carcinogenic compounds have been identified they will be examined in several types of assays by our research unit to define their mode of action and examine possible mechanisms for blocking their carcinogenic activity. The latter study will bear directly on potential routes of therapy to exposed populations.

SCIENTIFIC BASIS FOR STUDY

Although the mechanism for chemical carcinogenesis is still relatively obscure, it is fairly clear that many of the chemicals which elicit cancer (precarcinogens) do so as a result of metabolic conversion into more carcinogenic substances (proximate carcinogens) and finally to chemically reactive electrophilic compounds (ultimate carcinogens) which can react with nucleic acids, proteins, cellular components and are also mutagenic (2). Such seems to be the case of the potent carcinogens 4-nitroquinoline-1-oxide (4NQO) and its reduced form 4-hydroxylaminoquinoline-1-oxide (4HAQO) (3). Although it is important to determine all the parameters of carcinogenic action by any suspected compounds, mutagenesis can serve as an initial screen for potential carcinogens.

To screen for mutagenesis several systems using bacteria (4,5) and eukaryotic cells (6,7) as indicator strains have been developed. Of these systems probably the most comprehensive is the Salmonella reversion assay developed by Ames and coworkers (5,8). His tester strains can identify both base pair changes as well as frame shift mutations (5). Recently, these tests have come under attack as unreliable where human carcinogenesis is concerned (47). However, the accuracy of these tests in predicting carcinogens cannot be ignored.

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In addition, repair assays can be performed using recombination and repair deficient strains of B. subtilis and UV and X-ray sensitive strains of S. cerevisiae (9,10). These tests determine effects on DNA which may not result in mutation but instead result in a potentially lethal event due to structural damage to the DNA. Repair deficient strains cannot make the necessary corrections and as a result are preferentially killed (9). Our laboratory has utilized both the Ames and the repair screens to determine the mutagenic potential and probable mechanisms of action of chloroacetaldehyde, a metabolic derivative of vinyl chloride (1). In addition, tests for the induction of the SOS repair system (48) have recently been reported (49,50). These tests measure the induction of prophage in E. coli. Ron Yasbin has recently initiated a similar screen with B. subtilis utilizing the induction of PBSX in polA mutants. (Yasbin et. al, and Streips and Yasbin, in preparation). The investigators consider these screens to be more sensitive toward potential carcinogens than the reversion assays described by Ames. Since these experiments are directed at specifically monitoring the induction of error-prone repair pathways, they may in fact be more representative of carcinogenesis induction.

We must, of course, keep in mind the limitations of a single cell model system. There are several conceivable reasons why a compound may be mutagenic in one system but not another.

- 1) There may be permeability differences between the bacterial and mammalian cell. This probably is not important, since the usual mutagens are reasonably soluble in organic solvents, and the membrane probably acts as an organic solvent for the first step in mutagen attack on the cell. There should not be significant differences between the bacterial and mammalian cell membranes, for the solubilization of the mutagen, at least. In fact, solubility in both kinds of membranes may be very important step for mutational events, since both mammalian and bacterial DNA are connected to the membranes (1).
- 2) Mammalian repair systems may have a different efficiency than bacterial repair systems. This could be an important factor since very little is known about mammalian DNA repair machinery. Since bacterial assays are commonly done with repair-deficient strains to accentuate the mutational event, differences between bacterial and mammalian cell lines could be even more pronounced. This is one reason why the studies must be extended to mammalian cell lines for corroborative results.
- 3) Chromosomes with histones could have a different susceptibility than the bacterial chromosome. This probably is not very likely, since bacteria do have polyamines associated with their DNA, and the mammalian chromosome is not always covered by histones.
- 4) The mutagens could be differentially detoxified or metabolized. This is extremely likely. It is unlikely that bacteria could detoxify and/or metabolize whereas mammalian cells couldn't, since mammalian cells have a far wider variety of metabolic and detoxification systems than bacteria. However, the opposite is very likely true, and the host activation of potential mutagens has been demonstrated (12).

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To control for the possible differential effects between the two systems, especially in the activation of mutagens, the liver microsomal enzyme activated system was developed by Slater (12) and the host mediated assay by Legator and Malling (13). In the Slater system the potential mutagen is incubated with mouse liver homogenate either in the presence or prior to exposure to the indicator cells (12). Ames has modified this approach to include lung microsomal enzyme preparations as well (14). The Legator system can detect whether the animal can activate or detoxify compounds which might be mutagenic. The animal is administered the mutagen, then injected with the indicator microorganism. Mutagen and bacterium are administered by different routes. This allows the host to modify the mutagen prior to the time it reaches the bacteria. After a sufficient period of time of exposure, the bacteria are withdrawn, and the induction of mutants is determined. A comparison then can be made between the mutagenic action of the compound in vitro and in vivo (13).

Recently, Legator (7) has described an in vivo test utilizing Drosophila. He suggests there is a strong correlation between mammalian and Drosophila activation systems, and also that Drosophila is the only in vivo eukaryotic system which is both comprehensive, and where effects on chromosome alterations can be studied in short periods of time.

A combination of these several screens would probably allow in depth evaluation of the mutagenic potential of any chemical under investigation and also provide strong clues concerning that chemical mode of action in causing the genetic lesion.

Most of these tests utilize mutations which bring about the restoration of gene functions. These are called back mutation or reverse mutations and are restricted to only certain, highly specific sites within the gene. They must be near (as in the addition or deletion mutations) or at the precise site of the original mutations (as in the base substitution mutations). The frequency of reversions, as a consequence, is less than that of forward mutations. However, these can be screened rapidly and easily, simply by plating on a medium which normally would not support the growth of the original mutant organism. Reversion studies have been done in bacterial (5,12) and mammalian (15) cells. On the basis of these studies, the mutagenic properties of several compounds could be determined, and the type of original mutation identified (6). Most of the bacterial studies were done with repair deficient organisms to accentuate the occurrence of reverse mutations (5).

Nevertheless, following these initial screens, additional tests need to be made on suspected compounds to determine their carcinogenic activity. For instance, AF₂, a nitrofurant derivative is positive for DNA damage in repair tests and is mutagenic toward Escherichia coli, yet is negative in the Ames Salmonella screen (16). Furthermore, most mutagenesis studies are generally concerned with the mechanism of base sequence alteration due to a minority product or relatively rare event. In these studies products or structural changes occurring in high yield without regard for capacity to induce mutation are ignored (17). Thus, the action of carcinogens which result in structural changes of DNA, such as single-strand scissions, UV photoproducts, or alkylation of bases may involve many cell processes of repair and synthesis of which mutation may only represent a small fraction indicating successful completion of the task. The majority of events may result in non-mutagenic repair or cell death.

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Considerable research has been done to study physically the mechanism of action of several carcinogens. In most of these studies transforming DNA in the Bacillus subtilis system has been utilized. For instance, Strauss has described that the alkylating agent, methylmethanesulfonate (MMS) destroys transforming activity of DNA by causing single stranded breaks and depurination of the DNA molecule. If cells are treated with MMS and the DNA is extracted following the treatment, single stranded scissions predominate. When the experiment is performed on DNA in vitro, the DNA is inactivated at 50C, too low a temperature for thermal denaturation. This DNA is primarily inactivated by the formation of apurinic sites (18), although alkylation of bases and single strand scissions also occur. Furthermore, a uvr⁻ strain of B. subtilis which cannot repair UV damage, can partially repair MMS induced damage in transforming DNA. Presumably this strain is deficient in either excision, or repair replication but not in the last step, the ligation of the strands. Apparently, the MMS induced alterations require only this last step for restoration of activity.

Intensive research has also been done with the potent carcinogens 4NQO, and 4HAQO. Sugimura has described that 4NQO is converted to 4HAQO by rat liver enzymes (19). Both of these compounds are mutagenic (20), though 4HAQO is the more potent mutagen. The difference between these compounds can be resolved by investigating their effect on DNA; thus, 4HAQO effects greater damage on naked DNA than does 4NQO. It appears that a bacterial enzyme converts 4NQO to 4HAQO in vivo, because 4NQO is equally efficient in inactivating bacteria by damaging bacterial DNA (3). Here again the value of a transformation assay becomes apparent for the treatment of DNA in vitro or in vivo can give differential results (21). The action of 4HAQO has also been defined. It is active only in the presence of O₂, therefore the carcinogenic process must be the result of an interaction with a proximal compound formed by the oxidation process from 4HAQO (20). In animals, cancer can be induced by single injection. yet in vitro 4HAQO changes to an azoxy compound which becomes inactive within 10 minutes (20). Therefore, the time interval necessary for the determination of the fate of the cell is short.

The interaction of carcinogenic amines and amides as well as polycyclic hydrocarbons with transforming DNA in B. subtilis has been documented (3,22,23). In these studies it has been shown that all of these compounds interact specifically with the DNA causing it to lose biological activity. Moreover, this DNA, when introduced into recipient cells, brought in a significantly higher proportion of forward mutations than did control DNA samples. Furthermore, the biological activity was markedly decreased when the treated DNA was introduced into hcr⁻ strains which are deficient in enzymes required to repair UV light damage (23). UV damage of transforming DNA may have relation to the G+C content of the DNA. The greater amount of damage occurs in AT rich segments, primarily because of the significant proportion of thymine dimers (24). Chloroacetaldehyde, the probable carcinogen in vinyl chloride mediated neoplasia (1,25) has also been shown to interact with DNA specifically (26).

The action of carcinogens may also influence profoundly the physiology of test cells. This may be directly or indirectly attributable to the action of the carcinogen on the DNA. Mifuchi et al and Negai have described the induction of respiration deficient mutants of Saccharomyces cerevisiae by 4NQO (27,28). These cells also produced a taxohormone-like substance (27). In these properties they showed similarity to cancer cells.

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In conclusion, by integrating the several different screening procedures with basic research concerning the molecular basis for the carcinogenic attack, concrete conclusions may be drawn about the carcinogenesis potential of any chemical. Moreover, once the mode of attack upon the host cells is determined, methods for blocking carcinogenic action may be developed.

PLAN OF WORK

This section will be divided into two parts. First of all we will outline the mutagen screening facility, then describe the research component of our carcinogenic chemical analysis laboratory.

A. Mutagenicity screening

Established screening procedures:

1. Salmonella assay - We have obtained all the necessary Salmonella typhimurium assay strains from Bruce Ames (8) and this assay is fully implemented in vinyl chloride research performed by our laboratory (1).
2. Repair assay - We have four strains of B. subtilis which are deficient in various steps of DNA repair: polA, MC-1, hcr, and FB 13. These fall in the classical repair deficient classes described by Dubnau (29), and lack specific components of the repair-mechanism. Their use in vinyl chloride related studies by our laboratory is also documented (1). We have extended this study to include several recombination-deficient classes of B. subtilis, using CAA as the reactive chemical. Results from these studies are shown in Table 1.
3. SOS repair assay - Neither the Salmonella nor the Bacillus reversion tests directly address the carcinogenicity of the compounds tested. We have recently instituted a screen developed by Ronald Yasbin, which may approach, as closely as is possible in a bacterial system, the assay for carcinogenicity. This test will be described in detail in a forthcoming publication (U.N. Streips and R.E. Yasbin, Microbial Testers for Chemical Carcinogenesis, I.C. Felkner (ed). Marcel Dekker, N.Y.). Briefly, most investigators now envision that the carcinogenesis mechanism may be started by error-prone repair, the so-called SOS pathway. This happens when the cell cannot repair by normal means and now induces a repair system which fixes DNA lesions with no consideration for the correct template. As a result, not only has the chemical damaged the DNA of the cell, but the cell in attempting repair has created a mutational site. Therefore, any chemical which induces SOS repair is more than likely a carcinogen. The induction of SOS can be measured in several ways, but the induction of bacterial viruses is the easiest. Therefore, our test measures the extent of virus induction in a cell population as a measure of SOS inducing potential. When virus is induced, the cell dies. Therefore, in Table 2 100% survival is no SOS induction, less than 100% means SOS has been induced.

At the present time several laboratories are assaying various chemicals as well as other materials by the SOS test and correlating this data to known carcinogenicity. At this time the initial preliminary evaluation is that this test may be a very accurate predictor of the carcinogenic potential of a chemical.

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In Table 2 I present a comparison of the various systems. I have tested over 60 various substances in the last year by the Salmonella and Bacillus repair tests, but preliminarily only a few so far by SOS. I have chosen these four compounds for this presentation because they illustrate the variance of these microbial tests. All four compounds are mutagenic by the Salmonella test. All but styrene oxide are positive in the Bacillus repair assay, and seem to require recombination repair. Methyl methane sulfonate also is reactive toward the strain lacking polymerase I (polA). However, the preliminary indication from the SOS repair assay demonstrates significant differences between these compounds. Both CAA and MMS are quite reactive in this assay, but styrene oxide and EMS (ethyl methane sulfonate) are essentially non-reactive. This suggests that neither styrene oxide nor EMS can induce significant levels of SOS repair. It is interesting that recent reports show EMS not to be carcinogenic while MMS is a potent carcinogen (R. Yasbin, personal communication).

It is our intention in the next funding year to finish our studies with CAA, and elucidate the several mechanisms of action of this compound. In addition we will extend our now established research techniques to thoroughly test other vinyl monomers, notably styrene oxide and acrylonitrile. We will also extend our catalogue of compounds assayed by the SOS screen and draw more reliable conclusion on the applicability of this test for carcinogenesis screening.

All of these screens will be applied to any additional samples of industrial chemicals which will be provided for our laboratory either by the various investigators in the Department of Chemistry or any other source. Any screened chemicals which prove to be positive in one or more of these assays will be examined more fully, both by repeated screening and also by the research component of our laboratory utilizing the experiments described below. In this regard, due to its differential activity in our screens, styrene oxide is of great interest.

B. Examination of potential carcinogens

1. Assays using enucleated cells.

This is research performed in collaboration with Dr. Mansfield, a member of the Department of Microbiology and Immunology. Cytochalasin B, a metabolic product of the fungus Helminthosporium dematioides causes enucleation of cells (30). Following exposure to this antibiotic both enucleated cytoplasts and karyoplasts (nuclei) can be isolated. These techniques are currently being used routinely in our laboratory. These nuclei and cytoplasm can be treated with carcinogenic chemicals then fused, using Sendai virus (31), back to untreated cytoplasm and nuclei respectively. The fused products are then cultured in agarose and examined for one of several parameters of neoplastic transformation (32). These experiments will be implemented to examine potential carcinogenic chemicals isolated in the initial screen. This will add a mammalian system to our research program and also examine the effect of potential carcinogens on cytoplasmic components (i.e. membrane, mitochondria, etc) as well as nuclear material.

2. Transformation studies

Initially we will examine the direct effect of any mutagenic chemicals on transforming DNA. High molecular weight transforming DNA can be isolated by extraction of cells with phenol (33). This DNA will be placed in dialysis tubing and the tubing is immersed in a solution of the mutagen. The concentration of

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mutagen as well as pH can be regulated and varied. The mixture is incubated for various lengths of time. The dialysis tubing containing the DNA is removed and dialyzed against buffer to remove any unreacted mutagen. As a control, the dialyzing solution can be assayed for the presence of the mutagen on recombination deficient strains of B. subtilis. The resulting DNA samples will be analyzed spectrophotometrically to detect changes in concentration and absorbance spectrum. Radioactively labeled samples will be analyzed on neutral and alkaline sucrose density gradients to detect fragmentation and single stranded regions. Finally, the biological activity of these samples will be determined by transformation of both repair-positive and repair-negative strains of B. subtilis. (Initial experiments are shown in Tables V and VI of the publication attached to this proposal).

Secondly, repair-positive and repair-negative strains will be exposed to the mutagens. Following incubation with the mutagen the cells will be washed and the DNA will be isolated by phenol extraction. This DNA will be analyzed by the same techniques as were employed for the extracted DNA (above).

These experiments should identify the physical damage done to the DNA by the mutagenic fractions and whether the bacterial repair systems can repair the damage. By exposing whole cells to the mutagen, activation of the mutagen to a proximal form by the bacteria can be tested. In addition, all of these assays will be repeated in the presence of both liver and lung microsomal enzyme preparations according to Ames (14). Dr. Laumbach of our group has performed these types of experiments in testing the 4NQO mutagenesis system (23).

a.) Effect of DNA synthesis

Many mutagens act preferentially at the replication fork of DNA (34). Therefore, DNA synthesis is necessary for these mutagens to act. There are mutants of B. subtilis which are temperature sensitive for DNA synthesis (35). At the permissive temperature (37C) they grow normally, however, at the restrictive temperature (45C), even though proteins and RNA synthesis are normal. DNA synthesis is arrested. Mutagenic fractions will be incubated both with wild type cells and the temperature sensitive mutants. The effect on the viability of the two types of cells as well as on the DNA (assayed by the techniques described in the previous section) by the mutagenic chemicals will be determined. All experiments will be repeated in the presence of microsomal enzyme preparations.

Bacterial spores, as they germinate, initiate DNA synthesis and shortly thereafter excrete DNA in linear fashion starting with the replication origin (36). Therefore the appearance of specific markers in the supernatant fluid is a direct result of DNA replication. We will assay the effect of the chemicals both on the appearance and the physical structure of the DNA. This DNA can be used by transformation and can be examined for mutational events or alterations by the techniques described above.

b.) Specific bacterial sequences

If the mutagenic chemicals exhibit activity towards DNA, we can begin to determine if there are discrete regions which are preferentially attacked. This could be monitored by use of the restriction endonuclease degradation system. Restriction endonucleases hit specific sequences in DNA (37). Such sequences occur at random in DNA, but if you have a discrete segment of DNA such as bacteriophage genome, then degradation by the endonuclease will always result in the production of the same fragments. These can be resolved by gel electrophoresis (38). We are

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presently assaying the genome of the B. subtilis bacteriophage Ø29 in this manner. In addition, we have had success in isolating discrete segments of the B. subtilis chromosome both by isolating segments of DNA following excretion from germinating spores, and by isolating membrane bound DNA and removing the membrane (U.N. Streips and R.J. Doyle, manuscript in preparation). Both the bacteriophage and the bacterial systems can be used in assaying the effect of the mutagenic fractions on degradation patterns of those DNA segments by endonucleases. This work has been ongoing in our laboratory for the last year.

Untreated Ø29 DNA and bacterial DNA will serve as controls. In test samples both Ø29 and bacterial DNA will be exposed to the mutagen in dialysis tubing as described above. These tests will be done in the presence or absence of microsomal enzyme preparations. Following treatment, both control and test samples will be incubated with restriction endonucleases (we have in our laboratory EcoRI from E. coli, Hin from H. influenza, and Sma from Serratia) in appropriate buffers. The digested samples will be compared. With the help of Dr. Wong's group these experiments have already been initiated. We have several samples of phage DNA which has been methylated in vitro with CAA.

c.) Membrane interactions.

As mentioned in the previous section, we have routinely been isolating DNA-membrane complexes from B. subtilis. These complexes are enriched for markers near the terminus and near the origin of the chromosome (11,39). If the mutagenic chemicals alter membrane configuration, they may also alter the DNA-membrane association. To examine this parameter we will isolate DNA-membrane complexes from B. subtilis and an L-form of B. subtilis we have in the laboratory (40) which have been treated with the mutagenic chemicals. In these experiments, the cell wall is removed from the bacteria by lysozyme, then the resulting spheroplasts, as well as the L-form sample, are ruptured physically by dilution into buffer. The samples are washed exhaustively (15X) by centrifugation at 29,500 rpm, 20 minutes to remove all non-membrane bound DNA. The resulting samples also will retain enough nuclease to degrade all remaining DNA not covered by membrane, thus enriching the samples for the markers at the origin and the terminus (39). The membrane is removed from these samples by phenol extraction. The DNA can be assayed by transformation for biological activity. Treatment with the mutagenic chemicals may alter the regions protected by the membrane or could abolish the binding altogether. These possibilities could be evaluated in this system.

3. Membrane transport studies

These studies will be performed in collaboration with Dr. R.J. Doyle, a member of the department of Microbiology and Immunology.

In the membrane studies, the following cell types will be used: B. subtilis 168 (a gram positive bacterium), E. coli (gram negative) and S. cerevisiae (a eukaryotic organism). Cells will be grown in minimal media at 320 to logarithmic and stationary phases, centrifuged, then suspended in dilute buffer solutions. At this time, the mutagen will be introduced to the system. The mixture will be incubated for various periods of time, and the loss of protein or nucleic acid into the medium will be measured using previously developed procedures (41). This should establish whether gross membrane damage has occurred in these cells. Controls, without the mutagen will be run simultaneously.

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In addition, the transport of specific metabolites by mutagenic chemical treated cells will be examined. We will examine basically two types of membranes transport, energy-dependent or active transport; and energy-independent, or facilitated diffusion. For S. cerevisiae, transport of the non-metabolizable sugar, sorbose, can be determined (42). The sorbose transport system is particularly useful because the sugar enters S. cerevisiae via the facilitated diffusion systems and is not metabolically altered upon entering the cell (42). In B. subtilis, amino acid permease systems have been well characterized and will be utilized in our studies (43). This is done by use of isopr-pyl-thio- β -galactoside (IPTG) as described by Wilson et al (44). IPTG induced the lac operon system which controls both β -galactoside permease and β -galactosidase (44). Upon induction of E. coli by IPTG, lactose (or any β -galactoside) enters the cell by an active transport system. In studies done elsewhere (Argonne National Laboratory by R.J. Doyle) we have found that the best assay for galactoside transport in E. coli is based on the use of ortho-nitro-phenyl- β -D-galactoside (ONPG), a synthetic substrate which can be cleaved by β -galactosidase to yield a visible chromophore of high extinction. Other assays can be utilized where needed. We will use the established procedures in all these experiments. For example, cells are washed in buffered salt solutions, suspended to a pre-determined density, then subjected to the substance to be transported. The permeants (sorbose and amino acids) are ^{14}C labeled. At intervals, samples are withdrawn, quickly filtered, and washed. The amount of radioactivity in the residue is then determined. This is a facile and reliable procedure which can be used to determine whether membrane transport has been impaired in microbial cells. We will subject the cells to the chemicals and determine the effects of time, concentration, pH and temperature on both facilitated diffusion and active transport systems. Should these results appear promising we will then examine the effects of chemical mutagens on membrane transport of sugars by human red blood cells and by murine fibroblasts. We propose to perform these assays spectrally as described by Stein in his monograph (45). In the murine fibroblast system we will also assay for alterations of the specific surface histocompatibility antigens following exposure to the chemicals (46).

The described experiments are straightforward and can be performed quickly, thus, a significant study on the effects of mutagenic chemicals on biological membranes can be completed within a relatively short time. To our knowledge, no research has been published describing the effects of industrial monomer metabolites on membrane transport. We recognize that if a chemical interferes significantly with membrane transport, obvious health implications become apparent. For example, cells could not carry out normal metabolism if membrane transport has been impaired, thereby causing the cells to metabolize endogenous reserves or expire. If only a few components cause alterations of membrane transport it may then be possible to eliminate such injurious substances. That would be one of the long term goals of our research.

4. Blocking of the carcinogenesis process

This is potentially the most important section of the research component of this proposal, since this relates directly to possible therapeutic mechanism in vivo. Our collaborator, Dr. J. Wong, Department of Chemistry, has discovered that in the presence of reducing agents, such as cysteine, glutathione, etc., the potentially carcinogenic chlorooxirane is very quickly converted to non-mutagenic chloroethanol. In the absence of these agents, chlorooxirane forms the highly

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mutagenic chloroacetaldehyde. Utilizing this observation as a model system, we will examine the effect of chlorooxirane in all of the research studies outlined above in the presence and absence of reducing agents. In this way we will attempt to block the mutagenesis activity of this chemical. Pre-treatment of the various components in all of the assays will be approximately varied until optimal conditions are achieved. In these kinds of experiments we may discover not only mechanisms for blocking carcinogenesis, but also other blocking agents which may fit this role and be applicable to in vitro studies.

In this section we have described several series of experiments which would yield valuable information concerning the mutagenic potential of various chemicals which have been shown to be active in a preliminary screen. These experiments would examine both the effects of the chemicals on DNA and on the transport systems across the cell membrane. The ultimate aim of these studies is to identify the mutagenic chemicals, characterize their mode of action and seek methods for blocking their carcinogenic potential

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TABLE 1

REPAIR ASSAYS WITH B. SUBTILIS*

<u>Strains</u>	<u>Average Inhibition in mm/8 experiments</u>	<u>Strains</u>	<u>Average Inhibition in mm/8 experiments</u>
<u>REC A</u>		<u>REC H</u>	
recA1	13.5	recH342	NR
recA1	7.5		
<u>REC B</u>		<u>REC</u>	
recB6	13.5	rec-4	15.4
recB3	9.5	rec-13	5.4
recB19	16.5		
recB2	16.5	<u>MTC</u>	
recB2	11.3	mtc-41 (caf ^r)	NR
recB2	16.8		
<u>REC C</u>		<u>UVR</u>	
recC7	NR	uvr-35 (caf ^r)	NR
		hcr	NR
		uvr	NR
<u>REC D</u>		<u>WT</u>	
recD27	11.5		
recD27	NR	wt	NR
		wt	NR
<u>REC E</u>		wt	NR
recE61	6.7	<u>DNA POLYMERASE</u>	
recE4	8.8		
		pola	NR
<u>REC F</u>			
recF7	6.1		
recF18	7.4		
recF15	7.5		
recF15	8.42		
recF16	3.1		

* 100mM chloroacetaldehyde used in all these experiments

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TABLE 2

REACTIVITY OF CHEMICALS IN BACTERIAL ASSAYS

CHEMICAL	SALMONELLA REVERSION # REV/PLATE--CONTROL	SOS REPAIR % NONTREATED	W ⁺	SUBTILIS REPAIR ASSAY				
				REC ⁻	HCR ⁻	UVR ⁻	POL A ⁻	
				mm INHIBITION				
CAA (0.004M)	265	15%	0	18	2	2	3	
STYRENE OXIDE (0.01M)	342	100%	0	2	0	0	1	
EMS (0.001M)	312	80%	1	16	1	1	1	
MMS (0.001M)	355	1%	4	21	6	6	10	

U. N. Streips, Ph.D.

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Technical Proposal G

Tissue Antigenic Systems for Detection
of Chemical-Induced Carcinogenesis

E. Espinosa, M.D.

CMA 002171

Important alterations in liver antigens and antibodies in workers of the vinyl chloride industry have been described in previous work done in this laboratory. Of these changes, appearance in VC-associated liver angiosarcoma of an angiosarcoma associated antigen, an antigenic deletion and demonstration of tumor-bound immunoglobulin G appeared worth investigating in further detail for development of a test for the early detection of VC liver disease. In the course of these investigations 2 important questions arose which we propose to answer during the third year of support of this proposal.

The first question relates to the time required for the VC-associated antigenic changes to occur. This is of obvious great importance in order to determine the value of these alterations in the early detection of the disease. The second question pertains to the specificity of the antigenic changes as related to vinyl chloride.

In order to answer the first question, Sprague Dawley rats will be exposed to vinyl chloride monomer for up to 52 weeks. Some rats will be sacrificed on a weekly interval during the first six weeks of exposure and on a monthly interval thereafter. Non-exposed animals kept in similar conditions to the experimental one will be used as controls. Abnormalities found at sacrifice will be recorded and the livers will be examined histologically. Immunoglobulins bound to the angiosarcomas or the fibrotic livers identified at sacrificing will be examined by indirect immunofluorescence using frozen tissue sections. Serum prepared from the blood obtained from these rats will be examined for presence of antitumor antibody by the immunofluorescence procedure using frozen sections of the tumor as substrate. Liver extracts will be prepared in saline solutions and examined for antigenic changes. These will be tested by immunodiffusion using both anti-human and anti-rat angiosarcoma sera prepared in rabbits.

Our second question "Vinyl chloride specificity of the antigenic changes in the liver" will be answered in experiments using both animal and human tissues. In the animal experiments we propose comparing the antigenic changes induced by vinyl chloride with changes induced by other hepatocarcinogenic agents. An adequate experimental model for this study is Morris hepatoma; lines 7777, 5123tc and 9618A are being maintained in our laboratories for this purpose. Some of these experiments have already been initiated with the collaboration of Dr. C. Kupchella. In addition, possible relationship of these changes to early fibrosis and/or necrosis known to occur in VC-related liver disease will be studied in experiments using carbon tetrachloride liver fibrosis in rats. The antigenic changes will be studied also in human liver cirrhosis and liver tumors unrelated to vinyl chloride exposure.

Antigens from the tissues indicated above will be extracted by homogenization in saline and analyzed by immunodiffusion procedures. Antiserum to be used in the human tissue studies is already available and kept frozen at -35 C for further use. Antiserum to tissues and tumors of rat origin will be prepared in rabbits.

Human tissue extracts prepared from VC-associated liver angiosarcoma, hepatomas and liver cirrhosis will be compared for titer of angiosarcoma-associated antigen and for antigen deletions using immunodiffusion procedures. In the experiments using rat tissues, antigens appearing to be novel, absent, decreased or

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increased in concentration within the vinyl chloride, Morris hepatoma and liver fibrosis groups will be compared in order to establish their specificity as related to vinyl chloride. The chemical nature of these antigens will be determined by studying their susceptibility to proteases, nucleases, glycosidases and periodate. Other properties such as pH sensitivity, alcohol solubility, and heat stability will be determined. Molecular parameters to be estimated will include molecular weight, sedimentation and diffusion coefficients, molecular radius and frictional ratios. These will be measured by sucrose gradient ultracentrifugation and Sephadex filtration. The antigens in these procedures will be monitored by double immunodiffusion tests.

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P A R T B

BUDGET PROPOSAL

RESEARCH TECHNIQUES AND METHODS FOR DETECTION
AND PREVENTION OF CARCINOGENESIS IN
INDUSTRIAL WORKERS

UNIVERSITY OF LOUISVILLE
CANCER CENTER
LOUISVILLE, KENTUCKY 40201

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 4th Judicial District Court
 Orleans Parish, Louisiana

BUDGET SUMMARY

	Year 3
I. CLINICAL IMMUNOLOGY	
Personnel	31,523
Supplies and Expenses	21,371
II. CLINICAL BIOCHEMISTRY	
Personnel	23,922
Supplies and Equipment	14,373
III. CLINICAL BIOCHEMISTRY	
Personnel	9,476
Supplies and Equipment	8,690
IV. CLINICAL PATHOLOGY	
Personnel	--
Supplies and Expenses	8,508
V. MICROBIOLOGY	
Personnel	16,747
Supplies and Expenses	11,500
VI. CHEMISTRY	
Personnel	11,845
Supplies and Expenses	17,657
VII. PATHOLOGY	
Personnel	14,719
Supplies and Expenses	7,590

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	Year 3
VIII. FRINGE BENEFITS	(11,596)
IX. TOTAL (Personnel and Expenses include fringe benefits)	197,921
X. INDIRECT COSTS	62,813
TOTAL	260,734

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BUDGETM.C.A. PROPOSALCONFIDENTIAL

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I. CLINICAL IMMUNOLOGY	Year
H. Philip Fortwengler, Investigator	3
A. Toxicity/Tumorigenicity Study	
1. Personnel	
a. H. Philip Fortwengler, MS	15,965
b. Michael Dever, BS Research Technician	9,584
c. Linda Smith, BS Research Assistant	5,974
	31,523
2. Supplies and Expenses	
a. Animals, 300 per year	1,210
b. Animal Maintenance	1,315
c. Cell Culture Reagents	1,330
d. Sterile Culture Flasks	1,100
e. Chemical Reagents	550
f. Needles, Syringes	330
g. Sterile Cell Culture, Disposable Plasticware	990
h. Miscellaneous Expendables	330
SUBTOTALS	7,155
B. Immunocompetence Study	
1. Personnel	
(No additional cost)	
2. Supplies and Expenses	
a. Lymphocyte isolation reagents	1,650
b. Cell culture media	550
c. 3H isotopes	495
d. Scintillation reagents	1,210
e. Miscellaneous reagents, glassware	275
SUBTOTALS	4,180

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C. HL-A Tissue-Typing

1. Personnel	
(No additional cost)	
2. Supplies/Expenses	
a. Hamilton syringes	550
b. Tissue-typing antisera and plates	5,840
c. Miscellaneous reagents, glassware	<u>330</u>
SUBTOTALS	6,720

D. LAI Test for Angiosarcoma

1. Personnel	
(No cost)	
2. Supplies and Expenses	
a. Cell cultures	266
b. Cell maintenance	1,590
c. Miscellaneous reagents, glassware	<u>1,210</u>
SUBTOTALS	3,066

E. Xeroxing and publication costs 250

YEARLY TOTALS 52,894

II. CLINICAL BIOCHEMISTRY - ENZYME
 Julie T. Du, Ph.D., Investigator

A. Personnel

1. Julie T. Du, Ph.D.	13,622
2. Ruth Shelton, MS	<u>10,300</u>
SUBTOTALS	23,922

B. Supplies and Expenses

1. Laboratory Supplies	
a. Enzymes	1,200
b. Radioactive	<u>1,078</u>
SUBTOTALS	2,278

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2. Animal Expenses	
a. Animals	1,485
b. Animal care	
1. Short term (200)	2,112
2. Long term (300)	8,498
SUBTOTALS	12,095
YEARLY TOTALS	38,295

III. CLINICAL BIOCHEMISTRY

Charles E. Kupchella, Ph.D., Investigator

A. Personnel	
1. Charles E. Kupchella, Ph.D.	
(No Cost)	
2. Raya Warick, Technician	9,476
SUBTOTALS	9,476
B. Supplies and Expenses	
1. Lab/supplies/chemicals	3,590
2. Enzyme preparations	600
3. Glassware	1,200
4. Equipment maintenance	350
5. High pressure liquid	
chromatography columns	<u>1,050</u>
SUBTOTALS	6,790
C. Computer	1,000
D. Travel	<u>900</u>
YEARLY TOTALS	18,166

IV. CLINICAL PATHOLOGY

G. Randolph Schrodtt, M.D., Investigator

A. Personnel		
1. G. Randolph Schrodtt, M.D.		CMA 002179
(No cost)		
B. Supplies and Expenses		
1. Lab materials	2,700	
2. Chemicals	800	
3. EM preparation and		
tissue storage material	2,508	
4. Photography equipment		
and supplies	<u>2,500</u>	
YEARLY TOTALS	8,508	

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Year
3

V. MICROBIOLOGY

Uldis N. Streips, Ph.D., Investigator

A. Personnel

1. U. N. Streips, Ph.D.	2,000
2. Research Technician	8,087
3. Research Assistant	<u>6,660</u>

SUBTOTALS 16,747

B. Supplies and Expenses

1. Media	5,000
2. Glassware	1,000
3. Isotopes	2,100
4. Equipment maintenance	600
5. Publishing costs	<u>1,200</u>

SUBTOTALS 9,900

C. Travel to national meetings to
consult with experts in the field

1,600

YEARLY TOTALS 28,247

VI. CHEMISTRY

John L. Wong, Ph.D., Investigator

A. Personnel

1. John L. Wong, Ph.D. (No cost)	
2. Joseph P. Joseph, Ph.D. Post-doctoral	9,000
3. Research Assistant	<u>2,845</u>

SUBTOTALS 11,845

B. Supplies and Expenses

1. Chemicals, VPC, HPLC supplies	3,600
2. Glassware	3,700
3. Lab supplies	3,605
4. Publication costs	3,000
5. Equipment maintenance	<u>752</u>

SUBTOTALS 14,657

C. Travel to meetings to consult
(for 2)

3,000

YEARLY TOTALS 29,502

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Year
3

VII. PATHOLOGY

Enrique Espinosa, M.D., Investigator

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A. Personnel

1. Enrique Espinosa, M.D. (No cost)	
2. Virginia Ford, B.A. Research Assistant	9,980
3. Janice Gettelfinger Part-time Clerk-Typist	<u>4,739</u>

SUBTOTALS

14,719

B. Expenses and Supplies

1. Supplies/expendable	3,490
2. Rabbits, purchase and maintenance	2,300
3. Equipment maintenance	500
4. Publication costs	<u>650</u>

SUBTOTALS

6,940

C. Travel to other laboratories
for consultation (2 trips)

650

YEARLY TOTALS

22,309

CMA 002181

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14th Judicial District Court
Calcasieu Parish, Louisiana

ADDENDUM

Additional Technical Proposals for Consideration

CMA 002182

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Poss v. Conoco, Inc., No. 90-4837
14th Judicial District Court
Calcasieu Parish, Louisiana

Technical Proposal H

Whole-Body Auto Radiographic Study of Vinyl Chloride

W. J. Waddell, M.D.

CMA 002183

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Subject to Protective Order in W. J. Waddell, M.D. 47
Ross v. Conoco, Inc., No. 90-4877
14th Judicial District Court
Calcasieu Parish, Louisiana

Introductory Summary

Dr. Waddell proposes to obtain detailed data on the physical and chemical disposition of vinyl chloride in the mouse. The general procedure will be to administer the compound to the experimental animals by inhalation, orally and by parenteral routes and to sacrifice these animals after brief ether anesthesia at geometrically increasing time intervals by immersion in a mixture of dry ice and hexane. Whole sagittal sections of the frozen animals then will be taken on a microtome in a cryostat. No thawing or contact with any solvents will be allowed in order to prevent translocation or removal of the substance. After freeze-drying of the sections, they will be placed against X-ray film to allow the production of a whole-body autoradiograph.

The distribution of radioactivity in the specific tissues and fluids will be quantitated by a photometric densitometer which will compare the radioactivity in the autoradiograph with the specific area of the section. Using these quantitative data with the geometric time intervals will allow the calculation of elimination rates from each and every tissue and fluid of the animal. Kinetic data can be obtained by this technique which can be obtained by no other method. Procedures which dissect out organs and tissues are limited by the skill of the dissector; few if any dissectors can obtain clean preparations of islets of Langerhans, bronchial epithelium, pigmented epithelium of the eye, or corpora lutea. Each of these anatomic areas has, within recent years, been shown to accumulate and retain environmental compounds; the accumulation in each of these areas, as well as all areas of the body, can be quantitatively assessed by whole-body autoradiography. In some instances tissues will be removed either from the sections or from animals dosed in parallel experiments; these tissues will be solubilized and quantitated by liquid scintillation counting.

Selected areas of accumulation of radioactivity which are of interest will be studied further to identify chemically the nature of the radioactivity. These areas of the section will be removed by dissection, microscopically if necessary, and extracted with appropriate solvents. The extract will then be subjected to chromatography for identification of the chemical nature of the radioactivity. Studies in some animals may include removal of selected tissues or organs for autoradiography plus combustion or homogenization for extraction to quantitate the vinyl chloride and its metabolites.

Whole-body autoradiography offers the unique ability of studying the complete disposition of a substance in a single animal. Collection of feces, urine and expired air allows calculation of total loss from the body. Sectioning one-half of the animal provides quantitative information on the disposition of the total substance in every tissue of the body; the remaining half of the frozen animal can be used later for extraction and further quantitation of metabolites. Each animal can be used for total assessment.

Dr. Waddell and his colleagues have extensive experience in the study of drug and xenobiotic disposition by this approach and are in a position to offer this experience in correlating the biological disposition of vinyl chloride with its toxicities. Twenty-five consecutive years of research have been devoted by Dr. Waddell to research on the disposition and metabolism of drugs and xenobiotics. Most of the various techniques available for these various studies have been used.

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The facilities available in this laboratory for the proposed study include liquid scintillation counters, chromatography apparatus and equipment, and almost all of the supporting smaller items of equipment for such studies. Whole-body autoradiography for mice is currently in operation in the laboratory so that all of this equipment and apparatus is available. All of the supporting facilities, such as animal quarters, radiation and chemical safety rooms and protection, etc. are available.

Statement of Work; General Technical Approach

The initial studies will be done in pigmented mice. Since several compounds have been shown to have a remarkably intense and persistent affinity for melanin (in the pigmented epithelium of the eye, the brain, etc.) the initial experiments should be on animals containing melanin. The albino human constitutes such a small percentage of the population that the animal counterpart ranks low on priority for study. The mouse is usually preferred to the rat for initial study because of easier handling and lower cost for animals and isotopes without loss of any information on biological disposition. In fact the mouse has a gall bladder as does the human; however, the rat does not which makes examination of bile more difficult in this species. Metabolic differences in metabolism may be as wide among strains of rats or strains of mice as are such differences between mice and rats. Therefore, rats would be justified for the initial study only if other information had been obtained on a specific strain of rat which would enhance the informational yield of the study on the disposition of vinyl chloride.

The ^{14}C -labeled vinyl chloride would be given by inhalation, oral and intravenous routes of administration since these are most appropriate for obtaining valid pharmacokinetic data relating to its use in the environment. The intravenous route most often provides more precise data on the kinetics of distribution and elimination. One must be careful in the interpretation of data from intravenous administration of compounds with very limited water solubility even though this route frequently provides essential pharmacokinetic information. If the compound is solubilized in a system which does not maintain its solubility after dilution with blood, then the bulk of the material may appear in the lungs and spleen after intravenous injection (e.g., Kennedy and Waddell, Toxicol. Appl. Pharmacol. 22: 252, 1972; Waddell, et al., Toxicol. Appl. Pharmacol. 39: 339, 1977). This is due to a filtration by these tissues of the precipitate resulting when the material is injected. The papers cited above demonstrated this situation with the psychoactive compound Δ^9 -THC and the plasticizer DEHP.

Another important reason for including a parenteral route of administration is to clarify what may otherwise have been interpreted as another type of administration artifact. For example, when nicotine is given by inhalation or parenteral administration, the highest accumulation in the entire body is in the bronchial epithelium (Waddell and Marlowe, Drug Metabol. Disposition 4: 530, 1976). Had this compound only been given by its usual route of administration, i.e., inhalation, one might have concluded that the accumulation in the bronchial epithelium was simple an artifact from absorption through these cells. Another example is the intense accumulation of arsenic in intestinal epithelium following its intravenous administration (Deak, Csaky and Waddell, J. Toxicol. Environ. Health 1: 981, 1976). Had this element been given only by its usual route of exposure, i.e., oral, then one might not have suspected any specific interaction with enzymes in the intestinal epithelium. Instead, these studies lead to further investigation showing specific inhibition of glucose-6-phosphatase in these cells as a result of the distribution of arsenic.

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Following administration of ^{14}C -labeled vinyl chloride to the animal, time intervals for survival will be included which provide complete disposition data. In this laboratory, geometric time intervals on each side of 1 hour have been used routinely: i.e., 2 min., 6 min., 20 min., 1 hr., 3 hr., 9 hr., 24 hr., 3 days, 9 days and 27 days. Rarely, however, are all of these intervals used for the first series of animals; half or less may be sufficient, if the intervals are appropriately spaced and other information is already available on the kinetics of disposition of the substance. Conversely, further, longer intervals or even intervening intervals may be required as the dispositional data unfold.

The selection of the 3-fold geometric progression of time relative to one hour has proved to be a particularly useful framework. Plotting the concentration of a substance in a specific tissue or fluid against this type of time scale will allow a simple, direct estimate of the half-life of the substance in that biological compartment. (For further details see Waddell and Marlowe, Autoradiography. In: Drug Fate and Metabolism: Methods and Techniques, Marcel Dekker, Inc. New York, Vol. 1, Chapter 1, 1977).

The animals will be sacrificed by immersion in a bath of hexane cooled to the temperature of dry ice; the animals will have been lightly anesthetized with ether prior to freezing.

After freezing, the animals are never allowed to thaw and the tissues never are in contact with any solvents. These precautions are necessary to prevent any translocation or removal of the substance being studied. The frozen animal is sectioned on a microtome in a cryostat at -20°C . The whole, sagittal sections, 20 μ to 100 μ thick, are taken onto Scotch tape and allowed to dry in the freezer. The Slee model 250 cryostat, already in operation in this laboratory, will be used for studies in mice. All of the facilities and equipment for studies in mice are already in operation here.

After the sections have dried in the freezer, they can be removed to room temperature (if the substance being studied or its metabolites are not volatile). The sections are then placed against x-ray film in the darkroom, placed in light-tight boxes, and returned to the freezer for exposure of the film by the isotope in the tissue section. Currently the best film for ^{14}C and isotopes of similar energies is Kodak type AA. Until recently, it was not practical to use ^3H for whole-body autoradiography; however, a new film has been introduced in the last several months by Ceaverken AB in Sweden designed specifically for this use. It reduces the exposure time for tritium essentially to that required for ^{14}C and has proved to be free of artifacts for whole-body autoradiography. Conversations with representatives from Eastman Kodak indicate that a similar film will be introduced by this company in the near future.

After exposure of the autoradiograph, the section is removed in the darkroom and the x-ray film is developed. The sections can be stained with hematoxylin and eosin or any special stain if necessary to identify the sites of accumulation of the activity. Also, since the tissue has been subjected only to freeze-drying, the section can be used for histochemical studies to make further inferences correlating the disposition of the substance and its effect on the tissue (e.g., Deak, Csaky, and Waddell, J. Toxicol. Environ. Health 1:981, 1976).

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14th Judicial District Court
Calcasieu Parish, Louisiana

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Quantitation of the activity in specific organs and areas will be done with the C.H.G.1 densitometer manufactured by ADG instruments which is already in use in this laboratory. Photometric densities of the areas in the autoradiograph are compared on a scale relative to density of blood seen on the autoradiograph. The actual concentration in blood will then be determined by removal of a sample of blood from the section or frozen remains of the animal, solubilizing the blood, and counting it by liquid scintillation. Then the concentration in blood (or any other uniformly labeled reference tissue chosen) can be used with the relative photometric densities to give the concentration in any tissue or area. This technique provides information that cannot be obtained with any other method currently in use. For example, the concentration of radioactivity can be determined in the islets of Langerhans, bronchial epithelium, etc. Dissection of these tissues to obtain such areas without surrounding tissue is very difficult or impossible without allowing loss by diffusion or contamination. But the concentrations in these areas can be readily and simply obtained by the technique offered.

... The major error which results when organs are removed and sampled either by homogenization or excision of a small portion is that areas within the tissue are not identified. For example, if the whole lung is removed for analysis, the very high accumulation of nicotine (Waddell and Marlowe, Drug Metabol. Disposition 4: 530, 1976) or of certain PCB's in the bronchial epithelium is not observed (Brandt, Acta Pharmacol. Toxicol. 40 (Suppl. 11): 1977).

The precision of quantitation by this technique is adequate to allow calculations that can be done by no other method currently available. This approach was utilized recently to evaluate the distribution of water-soluble substances in specific cellular layers of the developing teeth of young mice. These quantitative data were sufficient to allow calculation of the pH gradients existing among these different layers of cells and structures (Lyman and Waddell, Am. J. Physiol. 232:F358, 1977; 232:F364, 1977).

It is necessary or desirable to identify chemically the nature of the radioactivity in the area seen in the autoradiograph. This will be done by excising the areas of interest from the 100 μ -thick sections which were contiguous to the 20 μ -thick sections used for autoradiography. These tissues will then be extracted with appropriate solvents and chromatographed by thin-layer chromatography. The thin-layer chromatographs will then be placed against x-ray film for localization for the different compounds. Quantitation can be further defined by removal of the material from the thin-layer plates and counting by liquid scintillation. For an example of the use of this method in the disposition of diphenylhydantoin see Waddell and Mirkin, Biochem. Pharmacol. 21:547, 1972.

Further details of the technique with whole-body autoradiography may be found in: Waddell and Marlowe, Autoradiography, In: Drug Fate and Metabolism: Methods and Techniques. Marcel Dekker, New York, Vol. 1, Chapter 1, 1977). Examples of its usefulness may be found in Waddell, Autoradiography in Drug Distribution Studies. In: Fundamentals of Drug Metabolism and Drug Disposition. Williams and Wilkins. Baltimore, Chapter 24, pages 505-514, 1971; Waddell, Ann. Rev. Pharmacol. 13:153, 1973 and in the chapter on Disposition of Drugs in the Fetus cited above. Reprints of any of these articles will be supplied on request.

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14th Judicial District Court
Calcasieu Parish, Louisiana CMA 002187

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Bess v. Gessco, Inc., No. 90-4837
14th Judicial District Court
St. Calcasieu Parish, Louisiana

Technical Proposal I

Vinyl Chloride Metabolism in Isolated Mammalian Liver Cells --

Richard C. Feldhoff, Ph.D.

CMA 002188

Vinyl Chloride Metabolism in Isolated Mammalian Liver Cells

Richard C. Feldhoff, Ph.D., Assistant Professor, Department of Biochemistry, University of Louisville School of Medicine, Louisville, KY 40232.

The Vinyl Chloride Study Group at the University of Louisville is investigating the metabolism of this important chemical monomer with a multifaceted yet interrelated attack. It is clear from the work of Tamburro, Wong, Streips, Du and others that the reactive vinyl chloride metabolites chlorooxirane and chloroacetaldehyde are produced in the liver and intimately related to vinyl chloride toxicity and carcinogenicity. The regulation of vinyl chloride metabolism at the cellular and molecular levels is however only poorly understood. An experimental approach which should facilitate vinyl chloride investigation, is the use of isolated mammalian liver cells.

The research program which is briefly described below would combine the knowledge and experience of the Vinyl Chloride Study Group at the University of Louisville with my own research training such that a model system can be developed where individual steps in the metabolism of vinyl chloride and other potential carcinogens can be routinely investigated following acute and chronic exposure. Initially, due to the lack of continuous human liver cell lines these studies would be performed with isolated rat liver cells and extended to human liver cells when they become more available.

The effects of ethanol consumption on the metabolism of vinyl chloride will be one aspect of the proposed studies. The mammalian liver is the principal organ responsible for the metabolism and/or detoxification of many drugs, hormones and other organic metabolites including potential carcinogens. The exposure of the liver to an hepatotoxin such as ethanol is likely to interfere with its ability to detoxify potential carcinogens. Many of the metabolic and hepatic effects of alcohol have been the subject of extensive investigations, however the concept of the potentiation of carcinogenesis by ethanol is relatively new and has not been adequately investigated. Indeed, recent animal experiments suggest that alcohol intake greatly increases the incidence of cancer when the animals are also exposed to vinyl chloride. In order to better protect industrial workers and the general public from chemically-induced cancers, it appears desirable to define pathways for metabolism and detoxification of vinyl chloride and other chemical monomers by the liver and to assess the role of alcohol consumption on these pathways.

Studies of vinyl chloride metabolism can be facilitated by reducing the complexities associated with whole animal experiments to the level of individual liver cells. Recently perfusion techniques have been developed which permit the bonds holding liver cells together to be cleaved when the enzyme collagenase is added to the perfusion medium. The perfusion procedure is depicted in Fig. 1. With the proper perfusion equipment, preparations of isolated liver cells can be readily obtained which exhibit very high viability and have been clearly shown by myself and others to retain normal liver specific functions. An example of some data which I obtained utilizing isolated liver cells is shown in Fig. 2. The synthesis and the secretion of albumin and many other plasma proteins is a specific function of the liver which the isolated cells retain. Additionally, as shown in Fig. 3, the intracellular morphology of the isolated cells when investigated by high resolution electron microscopy is identical to that of the intact tissue.

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There are also major technical advantages associated with the use of cell suspensions including 1) the ease of uniform sample collection (media or cells) as a function of time, 2) the ability to assay many different conditions or variables simultaneously and 3) the ability to employ radioactive metabolites at a much higher specific activity than is feasible in other *in vivo* systems.

With respect to vinyl chloride metabolism, isolated liver cells will be used to investigate:

- 1) the uptake, intracellular transport (binding proteins) and metabolism by different cell fractions of radioactive precursors (Feldhoff, Tamburro, Du, Wong, (Wittliff - cytosol receptors)).
- 2) identification and quantitation of metabolites released into the medium (Feldhoff, Wong).
- 3) effects of individual metabolites on normal parenchymal cell functions (Feldhoff, Tamburro); mutagenic capacities of newly identified compounds (Streips).
- 4) potentiation of carcinogenesis by concurrent exposure to hepatotoxins such as ethanol (Feldhoff, Tamburro).

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 14th Judicial District Court
 Calcasieu Parish, Louisiana

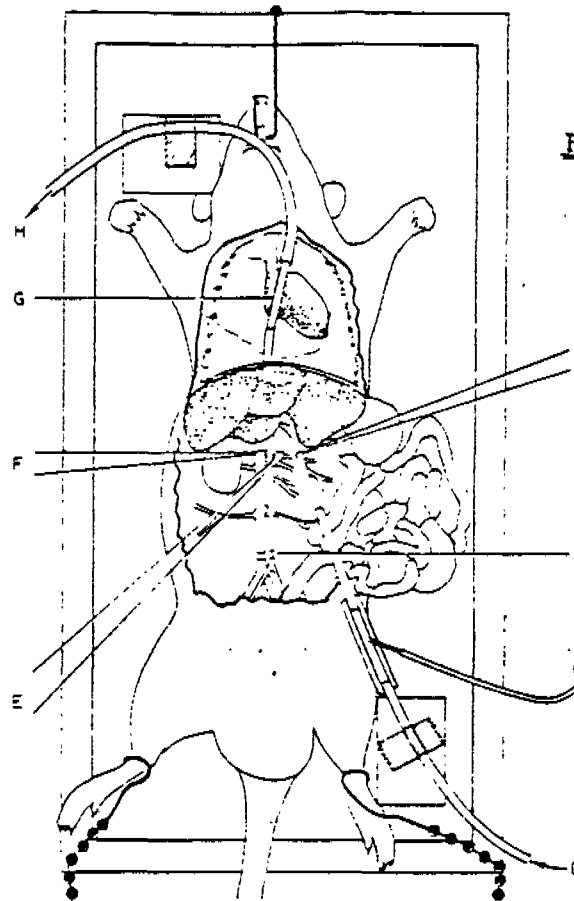


FIG. 1 Diagram depicting cannulation and perfusion procedures. A, ligature tying portal vein cannula and placed above entry of splenic vein; B, inferior vena cava and abdominal aorta sectioned after insertion of portal cannula; C, infusion tubing and cannula inserted into rubber tubing insert in inflow line; D, inflow line taped to aluminum block to hold portal cannula in place; E, ligature around celiac axis and superior mesenteric arteries, tied off after insertion of portal cannula; F, ligature around inferior vena cava above R renal vein, tied off after insertion of vena cava cannula; G, vena cava cannula inserted through right atrium into inferior vena cava, not tied; H, outflow line taped to aluminum block to hold vena cava cannula in place.

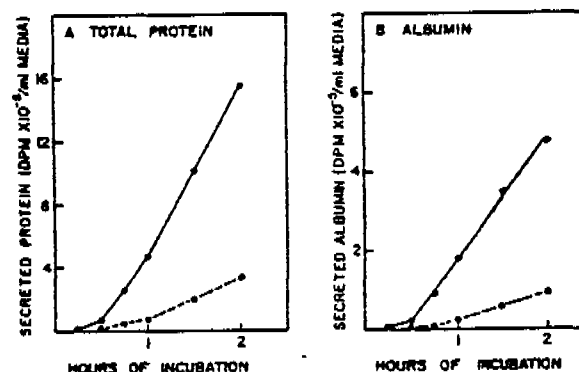
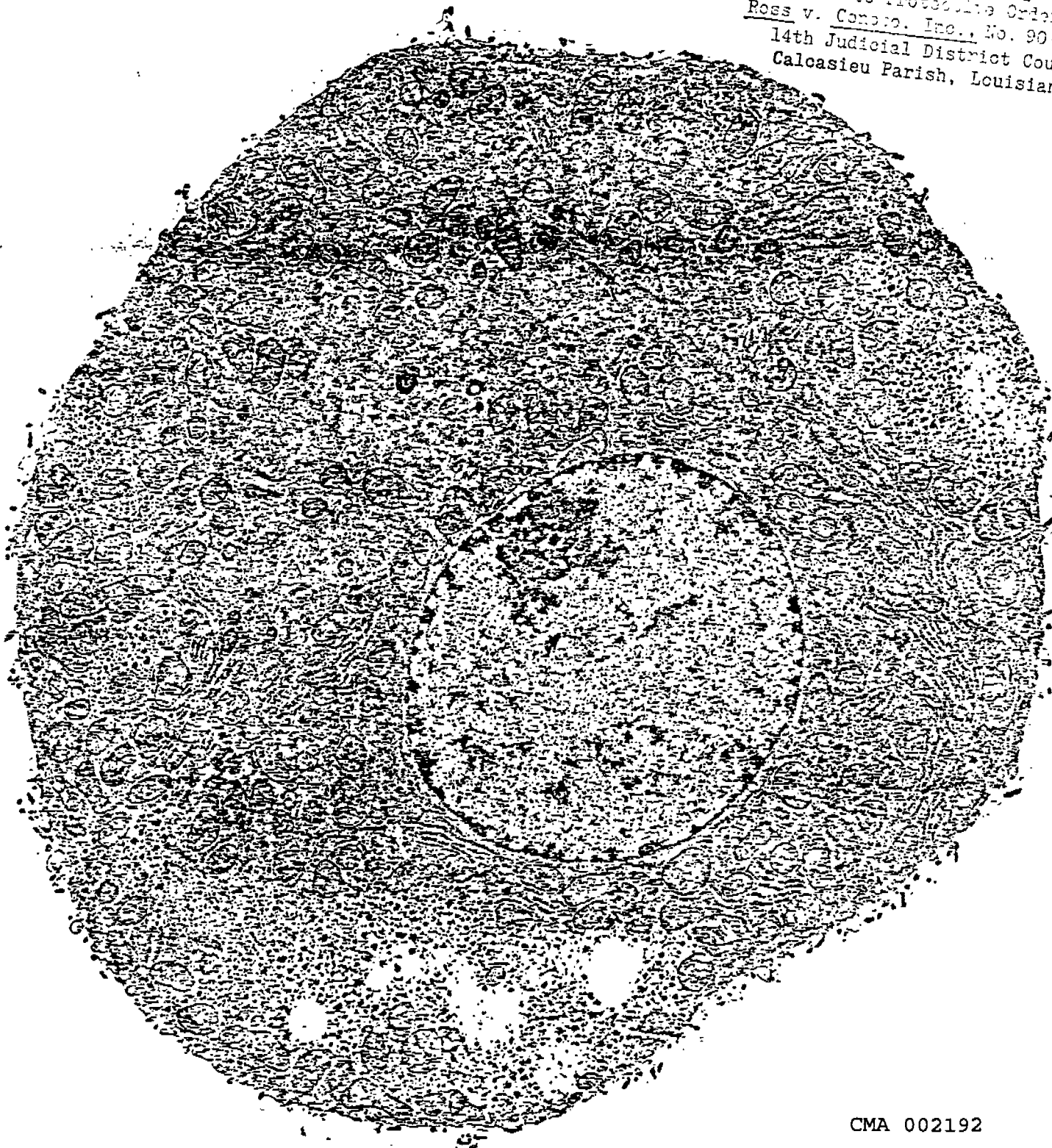


FIG. 2 Total protein and albumin secretion by isolated hepatocytes. Cells were prepared as described under "Experimental Procedures" and preincubated for 30 min in Krebs-Henseleit bicarbonate buffer containing 7.5 times the normal plasma levels of amino acids. Cells were transferred to 40 volumes of bicarbonate buffer containing normal plasma levels of amino acids and [3 H]leucine at 10 μ Ci/ml. At each time point, aliquots of the suspension were centrifuged and assayed for incorporation into total secreted protein and albumin as described under "Experimental Procedures." ●—●, hepatocytes from normal rats; ●- -●, hepatocytes from hypophysectomized rats.

CMA 002191

Fig. 3. Isolated rat hepatocyte. Cell suspensions were prepared by collagenase perfusion and then incubated for 2 hours at 37° C in Krebs-Heseleit bicarbonate buffer. Utilizing standard procedures the cells were fixed for 1 hour at 23° C in 0.1 M cacodylate buffer, pH 7.4 containing 2.7% glutaraldehyde. Cell preparations were dried in graded ethanol solutions, transferred to propylene oxide and embedded in Epon. The sections were stained with lead citrate and uranyl acetate. Magnification: 7700X. (Feldhoff, R.C. & Hillman, B., Unpublished observation).

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Addendum Proposed to Third Year Budget Only

Proposal H

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Ross v. Conero, Inc., No. 90-4837
14th Judicial District Court
Calcasieu Parish, Louisiana

Personnel

W. Waddell, Principal Investigator
Research Technician

8,500

TOTAL PERSONNEL 8,500

Supplies

Chemical, Biochemical
Glassware
Isotopes and Materials
Animals

500

1,000

2,500

1,000

TOTAL SUPPLIES 5,000

TOTAL \$13,500

CMA 002193

Proposal I

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Ross v. Conoco, Inc., No. 90-4337
 14th Judicial District Court
 Calcasieu Parish, Louisiana

Personnel

Richard C. Feldhoff, Ph.D., Principal Investigator	- 0 -
P. Patrick Hess, B.S., Research Assistant	5,500

Equipment

One perfusion box	3,700
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The apparatus consists of a Plexiglas box (120x40x40 cm) on a Formica-covered instrumentation box. The instrumentation includes an air circulating system, a motor for rotating the oxygenation chambers, a temperature control system and appropriate electronic controls.

The air in the Plexiglas box is continuously circulated and kept at 38° C by a thermostat whose probe is placed near the livers. A large number of precision components are included in the perfusion chamber such as cylindrical Plexiglas oxygenation chambers with a non-rotating module of 4 stainless steel tubes for influent and effluent media and gases, sliding stainless steel trays for each animal, bubble traps, etc.

Two perfusion pumps - Pharmacia (\$850 each)	1,700
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Supplies

Glassware	500
Chemicals, Biochemicals, Media	500
Animals and Animal Care	650
Radioisotopic Supplies	500
Disposable Sterile Tissue Culture Supplies	500

TOTAL	\$13,550
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CMA 002194

*Proceedings of the Third International Symposium on
Detection and Prevention of Cancer, New York. pp. 155-170*

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Ross v. Conoco, Inc., No. 99-4837
14th Judicial District Court
Calcasieu Parish, Louisiana

STUDIES ON THE MUTAGENICITY OF VINYL CHLORIDE METABOLITES
AND RELATED CHEMICALS

A. D. Laumbach, S. Lee, J. Wong, and U. N. Streips

Department of Microbiology and Immunology
University of Louisville School of Medicine
Department of Chemistry
Louisville, Kentucky 40201

I. INTRODUCTION

The studies by Viola et al (26) and Maltoni et al (15) established the carcinogenic potential of vinyl chloride monomer. The detection of angiosarcoma in industrial workers exposed to polyvinyl chloride suggested a causal relationship between this chemical and the development of hepatic abnormalities (4,12). Hefner et al (7) have delineated the metabolic fate of inhaled vinyl chloride in rats and proposed that the epoxide, chlorooxirane, and chloroacetaldehyde were the carcinogenic intermediates. Their hypothesis has been supported by the work of several laboratories (3,14,16,19, Elmore, Wong, Laumbach and Streips, submitted for publication) using bacterial strains as mutagenic indicators.

In this communication we present additional data concerning the mutagenicity and the potential mechanisms of action of several vinyl chloride metabolites, including the previously unreported chloroacetaldehyde monomer hydrate, chloroacetaldehyde dimer hydrate, and chloroacetaldehyde trimer. Epichlorohydrin, a mutagenic/carcinogenic (21,25) methylene homolog of chlorooxirane was also examined.

II. PROCEDURES AND MATERIALS USED

A. Bacterial Strains

The bacterial strains utilized in these studies are presented in Table I. The Bacillus subtilis strains were all maintained on AK agar (BBL). Salmonella typhimurium cultures were obtained from B. N. Ames (1) and were stored on Nutrient Agar (Difco) plus 5g NaCl per liter.

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B. Mutagenicity Assays

The indirect assay utilized repair deficient strains of *B. subtilis*. The procedure was a modification of the "rec-assay" described by Kada et al (9). Cells were grown overnight in Nutrient Broth (Difco) at 37C in a rotary incubator shaker, then diluted tenfold in phosphate buffer (pH 7.0). The suspended cultures were streaked onto Nutrient Agar plates (Difco). Filter paper discs (6 mm) were saturated with the chemical solutions to be examined, then were placed onto the agar plates next to the streaked bacterial cultures. Following incubation at 37C overnight the plates were examined and the lethality and mutagenic potential of the test chemicals were assessed by comparing inhibition zones between the *B. subtilis* 168 wild type, a repair-capable strain and the various DNA repair-deficient strains. In all these studies 4-nitroquinoline-1-oxide (4NQO) was used as the positive mutagenic control.

Direct mutagenicity assays utilized the *S. typhimurium* tester strains described by Ames (1). The chemicals were examined by the methods of McCann et al (16). The cultures were grown in Nutrient Broth plus 0.5% NaCl overnight in a rotary incubator shaker at 37C. A mixture of the test chemical (0.1 ml) in dimethyl sulfoxide (DMSO) and 2 ml of soft agar (0.6% agar, 0.6% NaCl, 0.5 mM biotin, and 0.5 mM histidine) was added to 0.1 ml of the bacterial culture. The solutions were mixed thoroughly and overlaid onto minimal plates [Vogel-Bonner E medium (27), 1.5% agar, and 2% glucose]. Control samples were prepared by omitting the test chemicals. For the positive mutagenesis control, 4NQO was added to the mixtures in place of the test chemicals. All plates were incubated for 48 hr at 37C prior to the enumeration of revertant colonies.

C. Chemical Compounds

The chemical compounds utilized in these studies were prepared, purified, and analyzed by previously reported techniques (Elmore, Wong, Laumbach, and Streips, submitted for publication).

D. Preparation of DNA

Transforming DNA was isolated from *B. subtilis* cultures by the method described by Young and Wilson (29). In some of the experiments the cultures were pretreated for 15 min either with chloroacetaldehyde (16 mM) or epichlorohydrin (16 mM) prior to the extraction procedure. In alternate experiments S-9 liver homogenate mix was added to the compounds prior to addition to bacteria. The S-9 liver homogenate contains per ml, 0.3 ml of the S-9 fraction, 8 mM MgCl₂, 33 mM KCl, 5 mM glucose-6-P, 4 mM NADP, and 100 mM sodium phosphate (pH 7.4). The DNA concentration in all lysates were assayed by the method of Richards (20).

E. Treatment of DNA In vitro with Chemicals

A sample of *B. subtilis* transforming DNA (0.9 ml) in standard saline citrate (SSC) (0.15 M NaCl-0.015 M trisodium citrate, pH 7.0) was combined with 0.1 ml chloroacetaldehyde (1.0 M in DMSO) or 0.1 ml

epichlorohydrin (1.0 M in DMSO). The mixture was allowed to react for 1 hr with occasional shaking. Following this treatment the treated DNA was dialyzed at OC against three 500 ml changes of SSC for 24 hrs. In alternate experiments the DNA-chemical mixtures were placed in a dialysis bag and immersed in the S-9 liver homogenate mix. These samples were dialyzed in SSC as above.

F. Competent Cultures for Transformation Assays

The procedures for the development of competence were similar to those described (23). *B. subtilis* cells were grown in a modified Spizizen's minimal medium (GM) (29) for 90 min at 37C after cessation of logarithmic growth in a rotary incubator shaker. The cells were then diluted tenfold into GMII medium (29) and incubated for an additional 60 min at 37C in the shaker. At this time the culture has attained maximum competence.

G. Transformation Procedures

A sample (0.1 ml) of extracted, treated or untreated DNA was added to 0.8 ml of the competent cultures and incubated at 37C for 30 min in the shaker. The reaction was terminated by the addition of 0.1 ml of deoxyribonuclease (500 µg/ml, Worthington Biochem. Corp.) for 15 min at 37C. The cells were plated on appropriate selective minimal media and incubated at 37C for 48 hrs.

III. RESULTS

A summary of preliminary mutagenesis screening experiments with potential vinyl chloride monomer metabolites and related compounds is presented in Table II. It is evident that chlorooxirane and chloroacetaldehyde are the ultimate mutagens in this system. These results agree with the published data (3,16). In addition, this table describes the mutagenicity of the other chemical forms of chloroacetaldehyde, notably a monomer hydrate, a dimer hydrate, and a trimer. The hydrate and dimer hydrate forms have been shown to form an equilibrium mixture by the spontaneous rearrangement of chloroacetaldehyde under physiological conditions (Elmore, Wong, Laumbach, and Streips, submitted for publication), and these hydrate forms must be regarded as potential metabolites of consequence. Purified dimer hydrate and trimer were synthesized under laboratory conditions. Neither acetaldehyde, chloroacetic acid, nor chloroethanol showed a significant level of mutagenicity in these assays. Other investigators have reported the mutagenicity of chloroethanol, however, either high concentrations or activation with microsomal enzymes was required for activity (3,16). Our results agree with those of McCann et al (16). These experiments suggest a molecular relationship involving the proximity of the chloride group to the aldehyde moiety for mutagenic activity. In this regard we are currently examining structurally analogous ketones, substituted with various halogens. Epichlorohydrin (1-chloro-2,3 epoxypropane) was also mutagenic in screens using the *Salmonella* tester strain TA100.

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Further experiments examined the effect of proposed metabolites on several different DNA repair deficient strains of *B. subtilis*. Chlorooxirane and the different forms of chloroacetaldehyde were all found to specifically inhibit the growth of strain MC-1, which lacks recombination repair (17) (Table III). Epichlorohydrin was capable of moderate reactivity only in the presence of the S-9 fraction.

Quantitative mutagenesis assays with *Salmonella* strain TA100, an indicator for base-pair substitution mutations, revealed that chloroacetaldehyde monomer had the highest mutagenic capacity of all the reactive metabolites (Table IV). The monomer-dimer hydrates, dimer hydrate, and trimer show progressively decreasing mutagenic efficiency as evidenced by the higher chemical concentration required for eliciting maximum reversion. All forms of chloroacetaldehyde were very toxic, thus the mutagenic response of each compound was limited to a narrow range of concentrations. However, epichlorohydrin, a weak mutagen by comparison, has a broad mutagenic spectrum and a corresponding low toxicity.

Since the mutagenic activity of the compounds constituted strong evidence that DNA was a primary target of attack, we examined the interaction of chloroacetaldehyde and epichlorohydrin with transforming DNA. It is known that the biological activity of transforming DNA can be altered by exposure to physical and chemical agents (8,22). Previous studies have shown that chloroacetaldehyde can bind to DNA *in vitro* (11). Accordingly, transforming DNA isolated from *B. subtilis* 168WT was treated with either chloroacetaldehyde or epichlorohydrin as described in Materials and Methods. The treated DNA was examined in transformation assays utilizing several different auxotrophic strains of *B. subtilis* as the recipients. Data presented in Table V reveals that *in vitro* treatment of DNA with either compound has little or no apparent effect on the biological activity of this DNA in transformation.

Since both chloroacetaldehyde and epichlorohydrin demonstrated mutagenic activity in the *Salmonella* TA100 strain, we examined the effect of these two compounds on *B. subtilis* DNA *in vivo*. Transforming DNA was isolated from *B. subtilis* following a 15 min exposure to the mutagenic chemicals. The DNA concentration was calculated from these samples, and levels equivalent to those used in the *in vitro* assays were added to competent cultures. The results of these transformation assays are shown in Table VI. Two major effects are evident with chloroacetaldehyde *in vivo* treated DNA. First, there was a major depression of the biological activity in the transforming DNA. Secondly, the depression showed genetic marker specificity. Moreover, the DNA segments containing genetic markers which have previously been shown to be associated to macromolecular structures such as the cell membrane (6,24,28) or the cell wall (Streips, Doyle, Sueoka, Brown, and Fan, submitted for publication) were selectively protected from attack by chloroacetaldehyde and epichlorohydrin. The activity of epichlorohydrin was less in these experiments, however, the patterns of specific marker inactivation are quite similar. The addition of the S-9 mix to the chemicals prior to addition to the cells, did not cause significant alteration in transformation efficiency (results not shown). In some samples there was an effect on the transforming DNA by DMSO, therefore all transformation values were corrected to account for this parameter.

IV. DISCUSSION

The major findings reported in this manuscript can be summarized: 1) We have confirmed the mutagenicity of chloroacetaldehyde and chlorooxirane, and extended it to include the additional potential metabolites, chloroacetaldehyde monomer hydrate, dimer hydrate and trimer, as well as the previously unreported chlorooxirane homolog, epichlorohydrin. 2) We have shown that recombination repair appears to be the mechanism for the correction of vinyl chloride metabolite elicited damage. 3) Chloroacetaldehyde causes a decrease in the biological activity of transforming DNA only if the cells are treated with the mutagen prior to the extraction of the DNA. *In vitro* studies showed no effect. 4) Epichlorohydrin apparently differs markedly from the vinyl chloride metabolites in mutagenic activity.

To understand the mutagenic potential of an environmental carcinogen, such as vinyl chloride and related chemicals, it is necessary to determine both its metabolic fate and probable mechanism of action for alteration of cellular processes. This report, as well as others, (3,14,16) has identified the potential active metabolites in vinyl chloride monomer mediated carcinogenesis. Furthermore, on the basis of a series of studies in microbial systems, we can postulate probable mechanisms of action of the vinyl chloride monomer metabolites and related chemicals. Understanding of these mechanisms is necessary for the development of possible blocking agents to the carcinogenic activity.

Recombination repair appears to be induced to correct DNA lesions caused by vinyl chloride monomer metabolites and epichlorohydrin. *Salmonella* strain TALOO which lacks excision repair (uvr⁻), yet retains the capacity for recombination repair is capable of recovery and can express mutation following exposure. Furthermore, experiments with several repair-deficient *E. subtilis* mutants demonstrate that only the recombination repair mutant is specifically sensitive to the active metabolites, whereas the excision repair mutants and the wild type strain are relatively unaffected. The nature of the lesions may specifically evoke the recombination repair mechanism (10), or, alternatively, the chemical reactivity of the metabolites may directly suppress other repair. It is known that recombination repair is inducible, while other types of repair are mostly constitutive (5). Since chloroacetaldehyde has been shown to specifically interact with proteins containing -SH groups (J. Hoffman, personal communication), it is possible that the chemical could inactivate the constitutive repair enzymes leaving the repair to an inducible system.

The requirement for recombination repair of damage induced by these chemicals suggests the potential route of mutagenesis in bacteria. Recombination repair has been shown to be error prone (16). In this sense it resembles postreplication repair in mammalian cells (13). Thus, we can postulate that the analogous error prone repair pathway, postreplication repair, may function in mammalian cells in response to vinyl chloride metabolite elicited damage. A relationship between postreplication repair caused errors and somatic mutation and carcinogenesis has been suggested in patients with the skin disease, xeroderma pigmentosum (13).

The increased inhibitory activity of the chloroacetaldehyde dimer and trimer forms for the other repair-deficient *B. subtilis* strains (Table III) may have been nonspecific killing of the cells, since all the strains other than MC-1 showed identical levels of inhibition. The necessity for metabolic activation of epichlorohydrin could reflect either a lack of permeability of the nonactivated compound or the requirement of a metabolite of this compound as the true mutagenic species.

Neither chloroacetaldehyde nor epichlorohydrin seemed to affect transforming DNA in vitro (Table V). Although several investigators have reported that CAA specifically modifies bases and causes mismatched base pairs (2,11), this reaction in vitro does not seem to affect the biological activity of the DNA. In contrast, DNA which was isolated from cells treated with either chloroacetaldehyde or epichlorohydrin (in vivo, Table VI) was severely affected. The overall biological activity of the transforming DNA is depressed, and it appears that the regions of the genome which are not protected by either the cell membrane or cell wall are most susceptible to attack and inactivation. It has also been postulated both in *Escherichia coli* and *B. subtilis* that the replication origin, terminus, and replication fork are all outer surface bound (18,24). Thus, these would be protected regions from chloroacetaldehyde attack and the nonreplicating DNA in the cytoplasm would be most susceptible. In this connection, recent experiments in our laboratory (Laumbach, Lee, Wong, and Streips, manuscript in preparation) have shown that chloroacetaldehyde causes enhanced mutation levels in cultures with nonreplicating genomes. This may imply that chloroacetaldehyde could be active in mammalian cells during growth stages where little DNA synthesis occurs.

The mode of action of epichlorohydrin, a known carcinogen (25), differs from that of the vinyl chloride monomer metabolites. Although epichlorohydrin causes similar base substitution mutations in *Salmonella* tester strain TA 100, it is a comparatively weaker alkylating agent based on quantitative assay. Epichlorohydrin also exhibits a lower toxicity level than vinyl chloride monomer metabolites, thus epichlorohydrin can demonstrate mutagenic activity through a wider range of concentrations. In addition, our laboratory has preliminary evidence that epichlorohydrin produces higher levels of mutation in *Salmonella* cultures which are actively replicating DNA than in cultures which have been arrested in DNA replication (Laumbach, Lee, Wong, and Streips, manuscript in preparation). The different activity spectra between chlorooxirane and its homolog epichlorohydrin points out the necessity for a multifaceted study of carcinogens.

V. SUMMARY

Our laboratories have utilized strains of *B. subtilis* and *Salmonella typhimurium* to investigate the mutagenicity of vinyl chloride metabolites and related compounds. The major findings reported in this manuscript are: 1) Confirmation of mutagenicity of chloroacetaldehyde and chlorooxirane. 2) Description of mutagenicity of additional potential metabolites of vinyl chloride, chloroacetaldehyde monomer hydrate, dimer hydrate, and trimer, as well as the mutagenic

carcinogenic chlorooxirane homolog, epichlorohydrin. 3) Recombination repair is postulated to be the mechanism for correcting vinyl chloride metabolite elicited damage. 4) Chloroacetaldehyde affects the transformation activity of DNA only if cells are treated with the mutagen prior to the extraction of the DNA. In vitro the chemical had no effect. 5) Epichlorohydrin differs from vinyl chloride metabolites in mode of action.

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Table I
 Bacterial Strains

<u>Bacillus subtilis</u>	Genotype	Origin and Comments
RUB 783	purB ⁶ , leu-8, hisA1, metB10	U. Streips
BR 151	trpC2, lys-3, metB10	B. Reilly
BUL 709	ura-I, hisA1, leu-8, metB10	This laboratory
BUL 714	cysA, hisA1, leu-8, metB10	This laboratory
Hcr-9 (JB01-200)	trpC2	S. Okubo and W. Romig, hcr ⁻
MC-1	trpC2, recB2	S. Okubo and W. Romig, rec ⁻
FB-13	trpC2	C. Hadden, uvr ⁻
168WT	prototroph	A. Laumbach and I. Felkner

<u>Salmonella typhimurium</u>	Mutations in Strains				
	His ⁻	LPS	DNA Repair	R Factor	Mutation Detected
TAL535	hisB46	rfa	uvrB	-	base-pair substitution
TAL00	hisB46	rfa	uvrB	pKM101	base-pair substitution
TAL537	hisC3076	rfa	uvrB	-	frameshift
TAL538	hisD3052	rfa	uvrB	-	frameshift
TA98	hisD3052	rfa	uvrB	pKM101	frameshift

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Table II

Mutagenic Activity Assayed by Bacterial Test Systems

Compounds	Indirect Screen	Direct Test ^a
	<u>B. subtilis</u> "Repair-Assay"	<u>S. typhimurium</u> Strain TA100
Acetaldehyde	NR ^b	NR
Chloroacetic Acid	NR	NR
Chloroethanol	NR	NR
Vinylidene Chloride	NR	NR
Vinyl Chloride	NR	NR
Chlorooxirane	+ ^c	++ ^d
Chloroacetaldehyde (monomer)	+++ ^e	+++
Chloroacetaldehyde (monomer-dimer hydrates)	++	++
Chloroacetaldehyde (dimer hydrate)	+	+
Chloroacetaldehyde (trimer)	+	+
Epichlorohydrin	NR	+

^aExperiments performed in absence of liver homogenate-mediated activation.

^bNR no reaction detected

^c + Reactive

^d ++ Moderately reactive

^e +++ Very reactive

Table III

"Repair-Assay" with Bacillus subtilis Strains

Compounds	Molar Concentration	Growth Inhibition in Millimeters ^a			
		168WT (hcr ⁺ , rec ⁺)	MC-1 (hcr ⁺ , rec ⁻)	Hcr-9 (hcr ⁻ , rec ⁺)	FB-13 (uvr ⁺ , rec ⁺)
Chloroacetaldehyde (monomer)	0.10	2.0	28.0	4.0	3.0
Chloroacetaldehyde (monomer-dimer hydrate)	0.115	NI ^b	23.0	NI	NI
Chloroacetaldehyde (dimer hydrate)	0.097	2.0	10.0	2.0	2.0
Chloroacetaldehyde (trimer)	0.096	7.0	15.0	6.0	7.0
Chlorooxirane	0.26	NI	10.0	NI	NI
Epichlorohydrin	0.997	NI	NI	NI	NI
Epichlorohydrin (plus liver homogenate) ^c	0.997	NI	3.0	NI	NI

^aAverage inhibition calculated from multiple experiments.^bNo inhibition detected.^c9,000 x g supernatant (S-9) + NADPH generating system.

Table IV

Quantitative Mutagenicity Assay by Salmonella TA100 Reversion

Compound	Concentration in Soft Agar Layer mM/Plate ^a	Average Number Revertants/Plate ^b
Chloroacetaldehyde (monomer)	0.0004	265
Chloroacetaldehyde (monomer-dimer hydrate)	0.054	977
Chloroacetaldehyde (dimer hydrate)	0.490	311
Chloroacetaldehyde (trimer)	0.240	159
Epichlorohydrin	4.746	2856

^aHighest effective non-toxic concentration for reversion.^bSpontaneous background revertants subtracted.

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Table V

Effect of Chloroacetaldehyde and Epichlorohydrin of Transforming DNA In vitro

Recipient Strains ^c	Relative Transformation Efficiency ^a							
	metB10	leu-8	cysA	hisA1	ura-1	trpG2	lys-8	purB6
<u>Epichlorohydrin treated DNA</u>								
BUL 714	.92	.97	.97	1.16				
RUB 783	.91	.60		.98				.77
BUL 709	.99	.95		1.02	.85			
BR 151	1.48					1.07	.62	
<u>Chloroacetaldehyde treated DNA</u>								
BUL 714	1.43	.92	.55	.91				
RUB 783	.93	1.45		.75				.89
BUL 709	.86	1.33		.90	.75			
BR 151						ND ^b	.77	

^aRelative transformation efficiency calculated: $\frac{\text{number of transformants with treated DNA}}{\text{number of transformants with untreated DNA}}$ ^bNot determined.^cConditions for competence and transformation as described in Materials and Methods.

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Table VI

EFFECT OF CHLOROACETALDEHYDE AND EPICHLOROHYDRIN ON TRANSFORMING DNA IN VIVO

Table VI

EFFECT OF CHLOROACETALDEHYDE AND EPICHLOROHYDRIN ON TRANSFORMING DNA IN VIVO

Recipient Strains ^b	Relative Transformation Efficiency ^a							
	metB10	leu-8	cysA	hisA1	ura-1	trpC2	lys-3	purB16
<u>Chloroacetaldehyde</u> <u>in vivo</u> treated DNA								
BUL 714	.54	.09	.08	.36				
RUB 783	.33	.10		.35				.10
BUL 709	.53	.07		.32	.34			
BR 151	.37					.13	.11	
<u>Epichlorohydrin</u> <u>in vivo</u> treated DNA								
BUL 714	.55	.17	.25	.46				
RUB 783	.52	.15		.38				ND ^c
BUL 709	.59	.15		.50	.36			
BR 151	.37					.11	.19	

^aRelative transformation efficiency calculated: $\frac{\text{number transformants with treated DNA}}{\text{number transformants with untreated DNA}}$

^bConditions for competence and transformation as described in Materials and Methods.

^cNot determined.

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