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SCHOOL OF MEDICINE
DEPARTMENT OF MEDICINE
DIGESTIVE DISEASES AND NUTRITION SECTION

HEALTH SCIENCE CENTER
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September 8, 1977

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September 8, 1977

Mr. George E. Best
Vice President, Secretary-Treasurer
Manufacturing Chemists Association
1825 Connecticut Avenue, N.W.
Washington, D.C. 20009

RE: First Annual Report for the Manufacturing Chemists Association's
Agreement with the University of Louisville

Dear Mr. Best:

The following describes what has been completed during the first year of the Manufacturing Chemists Association's agreement with the University of Louisville entitled, "Research Techniques and Methods for Detection and Prevention of Carcinogenesis in Industrial Workers". The technical proposal is again composed of seven parts. Progress for each part will be reported separately.

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

During the first year, Fortwengler's laboratory has been evaluating three independent aspects of the immune system of industrial workers exposed to vinyl chloride (VC). The object is to determine whether these various immunological functions can detect cancer development or identify high-risk groups.

Part I

Methods have been initiated and perfected for the study of a sub-population of lymphocytes (T cells) that are capable of eliminating foreign growths and tumors from the body. T-lymphocytes from VC exposed and unexposed, diseased and undiseased individuals have been enumerated microscopically by their ability to form rosettes. The functional state of these immune cells has been quantitated by their capacity to proliferate when stimulated by mitogens or antigens. A total of 82 vinyl chloride workers and about 20 apparently normal individuals have thus far been evaluated and preliminary statistical comparisons are under way.

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Part II

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 exposures which are harmless to normal genotypes.

An increased incidence of certain HLA types has been shown to be associated with susceptibility to various diseases. HLA-A27 antigen has been found more often in workers suspected of having the occupational disease asbestosis than among a control population.

During the first year procedures have been initiated to determine an individual's genetic tissue type. 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 HLA frequencies and their possible increased association with angiosarcoma or other chemically related diseases has been performed on almost 130 chemical workers with or without disease. Frequencies are being compiled and statistical evaluations will be performed after approximately 250 individual typings have been completed.

Part III

It is beyond doubt that new antigens arise on many 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, Fortwengler's group is endeavoring to develop a relatively specific test for angiosarcoma by utilizing reactions of immune cells to tumor antigens. Lymphocytes (the cells responsible for immunity) from the individual tested are being isolated by density gradient centrifugation. These cells are then grown in the presence of a liver reagent prepared from either a normal individual or an individual who had angiosarcoma. The test under development could be performed on a blood specimen in the lab, and if angiosarcoma antigens are recognized by the immune cells, could theoretically indicate that a person either has angiosarcoma or has been sensitized (pre-disposed) to it. Over 90 individuals have been tested for

reactivity to tumor antigen. Approximately eight percent of "pallet plant" individuals tested are reactive, two percent of the VC manufacturing plant workers are reactive, and none of the "normal" controls are reactive. "Pallet Plant" individuals are men who have had abnormal blood or liver function test results. To determine whether the results found are due to angiosarcoma antigens, or common antigens found on most tissues we have prepared a panel of tissue extract reagents derived from several normal livers, kidneys, or cancerous livers. These experiments, to determine specificity or non-specificity, are in progress and will be statistically analyzed in collaboration with the Project's biostatistician.

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

Part I - Human Studies

During the past year, Du's group has assayed more than 1,760 human sera for sorbitol dehydrogenase, an enzyme specific for the detection of liver malfunction. Comparison with the standard enzymatic studies (SGOT, SGPT, LDH) indicates that it is the most specific of liver function tests. However, its sensitivity is not great enough to utilize it for standard testing to detect injury from chemical exposure. Therefore, it is not recommended that this enzymatic study be used for screening purposes.

Serum bile acid clearances have been completed in 30 employees from B. F. Goodrich and in 50 non-vinyl chloride controls. Bile acids are normal human biochemicals whose clearance from blood can be used as a sensitive indicator of liver function. It is felt to be as sensitive as ICG clearance, a synthetic anionic dye presently being used for screening.

Radioimmunoassay for the determination of bile acids has recently been perfected. We are now testing this method's reproducibility and specificity in comparison to ICG clearance.

Part II - Animal Studies

In the past year, Du's group has completed and analyzed one series of the vinyl chloride exposure studies on rats. At a dose level of 10,000-20,000 ppm, the exposed group lost weight, increased glutathione reductase activity after 42 hours of exposure, decreased glucose-6-phosphatase activity after 70 hours of exposure, and increased glucose-6-phosphate dehydrogenase after about 100 hours of exposure. These

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enzymatic changes occurred before there was any sign of physical abnormalities or before any conventional liver function tests proved abnormal. These changes are similar to the alterations found in animals with hepatomas (Weber and Lea, 1966 in Advances in Enzyme Regulation, Vol. 2, Pergamon Press), suggesting that metabolic changes may be predictive of impending physical changes. The second series of vinyl chloride experiments in rats will extend the exposure period to 280 hours. The purpose is to see whether a correlation exists between physical and biochemical changes prior to and during the development of cancer.

In addition to vinyl chloride, rats have been exposed to chloroethanol, a vinyl chloride metabolite, to determine whether similar alterations in metabolism occurred. Rats were administered 30 mg/kg chloroethanol in drinking water for a 10 day period and various enzyme levels determined. The activity of glucose-6-phosphate dehydrogenase, glutathione reductase and gamma-glutamyl transpeptidase remained the same while the activity of glutathione epoxide-S-transferase increased 43% ($P < 0.05$). Glutathione epoxide-S-transferase is an indicator of the capacity of the liver cytosol to detoxify metabolites of vinyl chloride related compounds. The 43% increase after chloroethanol exposure demonstrates that the detoxification mechanism in rats is operational. The next series of experiments will determine if this enzyme is also elevated in animals exposed to high levels of vinyl chloride. This will allow an examination of the activity profile for rats exposed to vinyl chloride for varying periods of time to see if there are changes at the onset of carcinogenesis.

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

In previously submitted quarterly reports, a number of investigations conducted during the initial year of support from the Manufacturing Chemists Association were described. Kupchella's laboratory has:

1. Conducted a histochemical evaluation of angiosarcoma of the liver demonstrating a pronounced increase in glycosaminoglycans in the tumor and hepatic tissue adjacent to focal angiosarcoma tumors.
2. Completed a biochemical analysis of the angiosarcomatous tissue showing a significant elevation in tissue glycosaminoglycans.
3. Completed an evaluation of 50 human urine specimens from cases of angiosarcoma, cirrhosis, chronic hepatitis, and metastatic cancer of the liver, showing elevated urinary levels of

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glycosaminoglycan (the results of this investigation have been published: Kupchella, C. E. and Tamburro, C. H., Glycosaminoglycan Patterns in Hepatic Angiosarcoma, Proceedings of the Third International Symposium on the Detection and Prevention of Cancer, Marcel Dekker, Inc., New York, in press).

4. Completed a chromatographic evaluation of urinary glycosaminoglycans in angiosarcoma patients in both early and advanced stages of the disease and have demonstrated that there is progressive shift from a hyaluronidase-susceptible glycosaminoglycan fraction to a hyaluronidase-resistant glycosaminoglycan fraction (Currin, K. L., Kupchella, C. E., and Tamburro, C. H., 1977, Urinary Glycosaminoglycan Patterns in Angiosarcoma of the Liver, CANCER, accepted for publication, 4/4/77).

5. Also completed studies using rat carbon tetrachloride-induced fibrosis model demonstrating increases in both tissue and urinary glycosaminoglycans accompanying fibrosis of the liver (Kupchella, C. E., Jarvis, J. O., Currin, K. L., Tamburro, C. H., and Greenberg, R. A., Changes in Tissue and Urinary Glycosaminoglycans Accompanying Chemically Induced Hepatic Fibrosis in the Rat. This paper has been submitted to Biochim-Biophys. Acta).*

6. Completed studies of improvements in the procedure used to measure urinary glycosaminoglycans in urine. A new method that gives greater yield and less varient in less time than a widely used method of Diferrante has been developed. This work is being prepared for publication at the present time.

During the summer of 1977, that is in the most recent quarter, we have completed some preliminary assessment of the use of urinary glycosaminoglycan patterns in the early detection of liver disease, using both human material and a carbon tetrachloride animal model. These studies are described as follows:

Early changes in tissue and urinary glycosaminoglycan patterns that result from the induction of hepatic fibrosis by carbon tetrachloride were studied. Thirty-six Sprague-Dawley rats were separated into control groups and experimental groups to be sacrificed in groups of six at the end of one, two and

* A report of this work has also been accepted for presentation at the American Association for the Study of Liver Disease in Chicago on November 1, 1977.

Studies to be undertaken during the next period were described in the proposal update submitted on May 16, 1977. This described studies of the effects of vinyl chloride exposure on glycosaminoglycan changes, studies of the effects of partial hepatectomy to assess the degree to which regeneration contributes to altered glycosaminoglycan patterns, studies of the relationship of glycosaminoglycans to the irreversibility of fibrosis of the liver, studies of glycosaminoglycans in transplantable hepatomas in order to assess the relationship of glycosaminoglycan patterns to the growth rate of transplantable hepatomas with varying rates of growth.

Schrodt and Tamburro have spent the past year analyzing light and electron microscopic sections of liver biopsy tissue obtained from B. F. Goodrich employees. Sinusoidal size and cell changes have been determined utilizing a calculator digitizer for the morphometric

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analysis of the ultrastructures. Random selected fields were photographed and are being analyzed by the computer.

Once the 80 biopsies studied by light microscopy and the 60 for electron microscopy have been evaluated, they will be analyzed statistically to determine if there is a correlation between liver disease and the chemical exposure work history.

First stage review has been completed on 50 light and 15 electron microscopies; morphometric analysis has been completed on two (second stage review). This analysis is ongoing.

Technical Proposal E -- Chemical Systems of Detection

1. The mutagenicity of vinyl chloride and its potential metabolites was investigated in detail by a combination of chemical synthetic and microbiological assay techniques. The potential putative metabolites (chlorooxirane and four forms of chloroacetaldehyde) were prepared and characterized in Dr. Wong's laboratory; Dr. Uldis Streips of the Microbiology Department tested the compounds in Bacillus and Salmonella systems. Dose-related mutagenic activities of the above-mentioned metabolites as well as that of epichlorohydrin, a homolog of chlorooxirane, were compared and reported. See A) J. D. Elmore, J. L. Wong, A. D. Laumbach, and U. N. Streips, Biochim. Biophys. Acta, 442, 405 (1976), B) A. D. Laumbach, S. Lee, J. L. Wong, and U. N. Streips, Proceedings of the Third International Symposium on Detection and Prevention of Cancer, Vol. 1, Part 1, Marcel Dekker, Inc., New York, 1977. These results have added significantly to the understanding of pathogenesis of vinyl chloride and its potential in inducing genetic defects in humans.

2. Since all literature methods for the preparation of chlorooxirane result in mixtures with contaminants such as chlorine, hydrogen chloride, ethylene oxide etc., it was desirable to have double assurance that study of the reactivity of chlorooxirane would proceed with pure chlorooxirane. By using a specially designed apparatus, 99% pure chlorooxirane was prepared in gram quantities from ethylene oxide. This procedure can be adapted to the preparation of ^{14}C and ^3H labeled chlorooxirane which will be needed in several already-planned biological experiments.

3. In order to elucidate the intermediary metabolism of vinyl chloride, i.e., the metabolic activation and inactivation steps, which is essential in being able to properly determine the significant end products of vinyl

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chloride in body tissues and fluids, the fate of chlorooxirane, the immediate P450 product of vinyl chloride was studied. Synthetic chlorooxirane was used to react with non-protein sulfhydryls; chloroethanol was obtained. This unusual finding has explained the unknown pathway leading to chloroethanol formation which is a key metabolite in the biological conversion of vinyl chloride. This conversion reveals the diradical property of chlorooxirane. That the C-O bond of chlorooxirane is susceptible to homolysis is indicated by a study of its half-wave reduction potential. By means of polarography (dropping mercury electrode and saturated Calomel electrode), we have determined that $E_{1/2}$ (chlorooxirane) = -1.4v and $E_{1/2}$ (ethylene oxide) = -1.73v. This comparison shows that the three-membered ring of chlorooxirane should be reduced more readily than that of ethylene oxide.

4. Another prominent reactive property of chlorooxirane is its facile rearrangement to chloroacetaldehyde (CAA). In dimethylformamide at 25° C, it has a half-life of ~ 0.5 min. in rearranging to CAA. This reaction can be suppressed, however, in a different solvent, e.g., in carbon tetrachloride, the half-life is 24 hours. This type of stability data will be used to interpret the origin and distribution of vinyl chloride metabolites in a biological host.

5. The mechanism of deactivation of chloroacetaldehyde (CAA) by cysteine has also been studied. The derived cyclic product -- 3L-carboxy-2, 3-dihydro-1, 4-thiazine -- may be the precursor of the urinary metabolites S-hydroxyethylcysteine and S-carboxymethylcysteine. Further, that chloroethanol and chloroacetic acids do not react with cysteine at physiological pH's has been confirmed. This is a new and significant end product to look for in the body fluids and tissues of animals exposed to vinyl chloride or CAA.

6. As a putative metabolite, chloroacetaldehyde (CAA) is well known to react with adenine and cytosine nucleotides. We have demonstrated for the first time that CAA readily reacts with guanine derivatives at body temperature and physiological pH to form 1, N²-ethenoguanine. The rate of reaction is faster than the cytosine-CAA reaction. The tricyclic product has both of the N-H hydrogen-bonding sites in guanosine blocked. Considering the biological roles of GMP and the guanine residue in the nucleic acids, the formation of the new guanine-CAA product in a biological host may be consequential. All in all, these metabolic activation and inactivation data will form part of the basic picture which will facilitate the interpretation of the cellular and clinical symptoms, as well as help in the early determination of damage induced by vinyl chloride. This information is also essential in identifying urinary metabolites

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and in developing the specificity of the methodology for detecting these end-products in biological fluids and tissues.

7. Since previous animal studies have not demonstrated chloroacetic acid in the urines of animals at low exposure levels but have been reported in humans at higher exposure levels, analytical methodologies which will help to verify this observation are being developed. Such study can lead to the determination of specific threshold exposure levels and could greatly aid in the determination of what constitutes a safe environmental exposure for individual employees. The first target metabolite for analysis is chloroacetic acid. The methodology includes gas chromatography and mass spectrometry. Gas chromatography results: Solid supports containing carbowax and FFAP liquid phases have been used for direct analysis of carboxylic acids. Accordingly, 20% FFAP on chromosorb W passes chloroacetic acid at 200° with modest tailing of the peak. However, with this simple column, sensitivity is limited to concentration ranges of 0.2-2 mg/ml H₂O (200-2,000 ppm). Application of the improvements reported by Ackman (use of porous polymer solid support and use of formic acid in helium carrier gas) is expected to improve sensitivity by 100 fold.

Identification of chloroacetic acid by mass spectrometry: Chloroacetic acid can be identified by mass spectroscopy of water solutions as dilute as 0.02M (2 mg/ml). The two doublets of peaks at m/e 49,51 (³⁵ClCH₂⁺ and ³⁷ClCH₂⁺) and 50,52 (³⁵ClCH₃⁺ and ³⁷ClCH₃⁺) are distinctive because relative intensities within each doublet reflect the 76:24 isotopic ratio of ³⁵Cl:³⁷Cl.

Technical Proposal F -- Assays for the Carcinogenic Potential of Industrial Chemicals Utilizing Prokaryotic and Eukaryotic Systems

In the past funding year, significant advances in three major areas of interest concerning industrial mutagens/carcinogens have been made.

First of all, screening procedures have been improved by incorporating the direct, forward mutation assay (Tables 1 and 2). In addition, a bacteriophage induction assay was initiated (reported in second progress report). This latter test may well be the most sensitive microbial assay for the detection and identification of carcinogenic chemicals. In addition, implementation of a eukaryotic assay stem (sister chromatid exchange) is planned within the next few months.

Secondly, the assay systems have been applied not only to the vinyl chloride derivatives, but also to a wide range of medically and industrially pertinent chemicals. The chemicals tested appear in Table 3. However, this list is constantly

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being expanded as our screening procedures become more routine. Further chemicals which are to be examined (there are initial results on some already) are presented in Table 4. It is anticipated that in the next two years of the funding cycle, triple the number of chemicals will be tested.

Finally, vinyl chloride has been intensively used as a model chemical carcinogen in molecular assays for carcinogenesis mechanisms of action. Initial screens with repair-deficient bacteria (Table 5) show that only those cells lacking recombination repair (rec) are severely affected by chloroacetaldehyde (CAA), the true mutagen following metabolism of vinyl chloride (1). Other repair deficient strains, caf^R, uvr, hcr, polA, as well as the wild type (repair-proficient) bacteria are not affected. Furthermore, closer examination of the rec⁻ strains demonstrates that only a particular lesion in this pathway is vital in CAA mediated killing; recC7, recD27 (one strain), and recH342 are not affected by CAA.

These systems of carcinogenesis assays may yield some of the steps necessary for CAA lethality to microbial cells, and potentially carcinogenesis in mammalian cells. Therefore, CAA was sent to Dr. James Regan at Oak Ridge National Laboratories to test in some of his repair-deficient mammalian cell lines. This research could yield the molecular mechanism of vinyl chloride-induced carcinogenesis; this would then allow examination of specific blocking agents for this process.

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

Immunodiffusion analyses of angiosarcomatous liver tissue carried out in Dr. Espinosa's laboratory have demonstrated the following antigens: A) tumor-associated antigen; B) antigens with liver specificity, LSA-I and LSA-II; and C) antigens referred to as CTA-2 and CTA-3 which are shared with several tissues.

The efforts during the past year have centered on the characterization and isolation of these antigens in order to determine their possible utilization in the early detection of vinyl chloride injury and tumor formation. These immunologic analyses also revealed that a normal liver antigen is absent in the angiosarcomatous livers. In addition, tumor-bound immunoglobulin G was demonstrated to be present in the angiosarcomatous tissue.

The angiosarcoma associated antigen was characterized as a protein, inactivated by pronase and trypsin, relatively susceptible to heating and acid pH, and precipitated mainly at 20 to 30 percent ethanol concentration and at ammonium sulfate saturation of 30 to

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70 percent. Purification of this antigen by both ion exchange chromatography and Sephadex G-200 gel filtration has been partially accomplished. This is being done in order to develop a radioimmunoassay for this antigen to be used in patients with vinyl chloride associated liver injury.

Liver-specific antigens LSA-I and LSA-II are of special interest; their organ specificity makes them recommendable for the development of specific tests for liver injury. In the present studies these antigens were differentiated from each other as follows:

Firstly, LSA-I elutes from DEAE-cellulose columns with 0.01 M Na₂HPO₄ buffer at pH 7.6 whereas LSA-II binds to DEAE cellulose equilibrated with 0.01 M phosphate buffer at pH 7.6 and it elutes with 0.4 M Na₂HPO₄ at pH 6.8.

Secondly, LSA-I's electrophoretic mobility appears to be similar to serum gamma globulins while LSA-II's is between that of serum beta globulins and alpha globulins.

Thirdly, LSA-I was calculated by Sephadex G-200 gel filtration to have a molecular weight between 82,000-93,000 daltons and LSA-II between 127,000-150,000 daltons.

A purification procedure has been developed in part; further work is being currently pursued in order to apply these antigens to the detection of early chemical injury. These results are being prepared for publication.

CTA-2 antigen was detected in non-hepatic tissues as well as both angiosarcomatous and normal liver tissues. Characterization studies showed that this antigen is a protein susceptible to digestion by pronase and trypsin, relatively thermolabile, inactivated at acid pH and precipitated at ammonium sulfate concentrations between 30 and 50 percent saturation and at cold ethanol concentrations up to 70 percent. In immunoelectrophoresis it had an electrophoretic mobility of serum beta globulins. The molecular weight ranged between 67,000 and 80,000 and the diffusion coefficient was 5.4 to 6.0×10^7 cm²/sec both as measured by Sephadex G-200 gel filtration.

This antigen may be of special interest as a marker of acute liver injury since it was detected in serum of 62 to 79 percent of patients with acute hepatitis by the rather insensitive double immunodiffusion procedure. CTA-2 was detected early in acute hepatitis and disappeared from serum as the enzymes and bilirubin levels fell; it was detected in less than 28 percent of patients with other hepatic or non-hepatic diseases. Three cases with liver angiosarcoma and ten with liver dysfunction and fibrosis associated with vinyl chloride exposure were negative. This

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Calvin H. Tamburro, M.D.
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particular test therefore does not appear to be of clinical value in the assessment of vinyl chloride liver injury. A manuscript on this antigen entitled "Circulating Tissue Antigens IV. Occurrence in liver diseases and properties of a circulating tissue antigen" was submitted for publication.

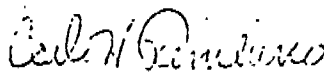
CTA-3 is another tissue antigen shared by angiosarcomatous livers and other tissues. Initial characterization studies have shown that in immunoelectrophoresis this antigen moves like an alpha-1 serum globulin, it is unaffected by treatment with pronase or trypsin, and it is relatively thermostable. Its relevance to vinyl chloride liver injury is under current investigation.

The liver antigen found to be absent in angiosarcoma was shown to have properties of a glycoprotein, since it is inactivated by periodate treatment and is unaffected by pronase and trypsin. In addition, it is relatively thermostable, affected by acid pH, and precipitates over a wide range of ammonium sulfate and ethanol concentrations. Further studies on the absence of normal liver antigens are continuing in order to determine how the absence or presence of certain antigens identified by antibody studies can be utilized in the early detection of vinyl chloride injury and tumor formation.

Finally, immunoglobulin G bound to the angiosarcomatous tissue was demonstrated by immunofluorescence and elution experiments suggesting antibody stimulation by the tumor. This may be another means of detecting the presence of angiosarcoma. Therefore, a procedure for the extraction of this immunoglobulin from the angiosarcomatous tissue was developed in order to further study the tumor specificity of this antibody and to identify the reactive antigen or antigens. These problems are still under investigation.

This completes the First Annual Report of the agreement between the Manufacturing Chemists Association and the University of Louisville. If there is need for any further information or clarification, please contact me.

Sincerely,



Carlo H. Tamburro, M.D.
Professor of Medicine
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