THE DOW CHEMICAL COMPANY

BENNETT BUILDING
2030 DOW CENTER
MIDLAND, MICHIGAN 48640

February 24, 1976

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MEDICAL SVCS.

Mr. Milton Freifeld
Manufacturing Chemists' Assoc.
1825 Connecticut Ave., N.W.
Washington, D.C. 20009

Dear Mr. Freifeld:

The attached protocol dated February 5, 1976 has been prepared as an extension of the metabolic studies coordinated by the Panel. You may recall that at the time the previous metabolic studies were reported, the Dow group thought it inappropriate to recommend further studies since there was no obvious next step to be taken. The Dow Toxicology Research Group now feels they have a reasonable approach to studying metabolism.

Therefore I request that a letter ballot be sent to The Technical Panel to determine their interest in re-establishing support for these proposed studies. The committee is aware, I'm sure, how valuable the metabolism data has been in discussing thresholds with the government agencies. The metabolic laboratory would like a rather prompt response so that they can start these studies or direct there interest to other problems.

The members should recall that the Panel is also considering research at the University of Louisville. It is also possible that we may wish to consider various types of human epidemilogical studies since there appear to be rather important.

Sincerely yours,

T. R. Torkelson
Chairman, MCA Tech. Panel on
Vinyl Chloride Research

ebg

cc:
MCA Technical Panel
on Vinyl Chloride Research

Attachment

ASI 000015252

PROTOCOL FOR CONTINUED STUDIES ON THE METABOLISM OF VINYL CHLORIDE

Prepared for the companies sponsoring
The Technical Task Group on Vinyl Chloride Research

February 5, 1976

Toxicology Research Laboratory
Health and Environmental Research
Dow Chemical U.S.A.
Midland, Michigan 48640

I. Introductory Summary

This document was prepared in response for the need to conduct additional studies on the metabolism of vinyl chloride (VC) in order to assess more definitely the hazard of low level exposure to VC. Numerous studies on the pharmacokinetics and metabolism of VC both in experimental animals and *in vitro* have indicated that the carcinogenesis of VC may be mediated by the metabolic formation of reactive metabolites. It appears that several metabolic pathways, dependent on the dose or exposure concentration, function in the biotransformation of VC in the body. Integration of the available information on the metabolism of VC has led to the theory that the carcinogenicity of VC is mediated by the reaction of metabolites of VC with intracellular macromolecules (DNA, RNA and protein). Although the fate of VC has been extensively studied, little information is available on the actual carcinogenic lesion and its relationship to metabolism.

This proposal describes a series of studies designed to characterize the potential *in vivo* macromolecular binding of ^{14}C-labeled VC in the liver of rats exposed by inhalation to

varying concentrations of VC. Parallel studies investigating in vitro metabolism and binding properties will also be conducted. If these initial studies are successful in elucidating 1) an effect consistent with induction of carcinogenesis by VC and 2) the primary metabolic pathways of VC biotransformation, then through additional studies it may be possible to relate the dose dependent metabolism of VC to carcinogenesis.

The proposed project on the metabolism of VC will draw on the broad spectrum of resources of The Dow Chemical Company. Primary responsibility for the project will lie with the personnel of the Toxicology Laboratory, located in Midland, Michigan. The research will be under the direction of Dr. Perry Gehring, Director of the Toxicology Research Laboratory; and Dr. Philip Watanabe, Senior Research Toxicologist. The Toxicology Research Laboratory has had extensive experience with VC (Hefner, et al., 1975, 1976; Wagner et al., 1975; Watanabe et al., 1976 a,b,c,d. The present studies are an extension of the previous work.

II. Technical Proposal

1. Introduction

Considerable effort has been devoted to research on VC since Creech and Johnson (1974) first associated the induction of liver disease and neoplasias in industrial workers exposed to VC. Studies in this laboratory have demonstrated that the fate of VC in the body following both ingestion and inhalation is dependent on the administered dose or exposure concentration (Watanabe et al., 1976a,b). These results are consistent with earlier studies (Hefner et al., 1975) which suggested that at least two metabolic pathways may be involved in transformation of VC in the body. Consideration of these results in toto led to the hypothesis that at low doses VC may be detoxified innocuously and as the dose increases the primary detoxicating metabolic pathway becomes saturated. When this occurs another pathway(s) become operant and ultimately form reactive metabolites which react with macromolecules leading to the development of cancer. This hypothesis is consistent with studies showing an enhanced mutagenic response in certain studies of Salmonella typhimurium and E. coli if fortified liver homogenates or microsomal enzymes are present (Bartsch et al., 1975; Malaveille et al., 1975; Rannug et al., 1975; Greim et al., 1975).

Many alkylating chemicals are detoxified in the body by conjugation with nonprotein sulfhydryl groups (primarily glutathione, GSH, Mitchell et al, 1973; Gillette, 1974). Urinary metabolites of VC appear to be primarily conjugates of cysteine. This indicates that covalent binding of reactive VC metabolites with hepatic GSH is a major detoxification mechanism. Furthermore, significant dose-related reduction of hepatic glutathione content occurred in rats exposed to 50-2000 ppm VC for 7 hours. In rats exposed for 7 hours to 10 ppm, no depression of hepatic GSH content was observed (Watanabe et al., 1976). It is reasonable to expect that as the GSH content of liver is decreased a larger fraction of the reactive metabolites are binding to intracellular macromolecules (protein, DNA and RNA) rather than to GSH. If it can be demonstrated that metabolites of VC interact in vivo with protein and nucleic acids which are responsible for cellular proliferation, and this interaction is a function of the dose dependent metabolism, then such results would be consistent with predicting a disproportionate increase in toxicity including cancer as the exposure level is increased.

It is necessary to emphasize at this juncture that pharmacokinetic and metabolism studies alone are insufficient

to assess toxicity. They are utilized to gain insight into the time related disposition of chemicals in the body which can explain in certain instances why toxic effects are produced at high doses and not at low doses. It is noteworthy that both of the studies conducted thus far, (indicating an altered fate of VC as the dose increases, and the dose related depression of hepatic GSH by VC) appear to correlate with the available data on the induction of carcinogenesis (Maltoni, et al., 1975a,b). Therefore this suggests that a threshold may exist for the production of cancer.

Recent reports have demonstrated that microsomal enzyme preparations form reactive metabolites of VC which bind to rat liver microsomes (Kappus et al., 1975) protein sulfhydryl groups, RNA (Bolt et al., 1975) and adenosine of DNA (Barbin et al., 1975). Reaction of VC metabolites with albumin has also been demonstrated to be mediated by a xanthine oxidase enzyme system (Bolt et al., 1975) in vitro. To determine whether the reaction of metabolites of VC with macromolecules may be pertinent to assessing the hazard of variuos magnitudes of exposure to VC, it is necessary to conduct studies in vivo.

Therefore the thrust of this research proposal is to characterize the macromolecular binding of VC to hepatic protein and nucleic acids following exposure to various concentrations of ^{14}C-labeled VC. Since the metabolism of VC appears to be intimately associated with its binding properties, additional in vitro studies will be conducted to elucidate the primary metabolic pathways involved in the biotransformation of VC.

2. Objectives

A. To determine the potential of reactive VC metabolites to bind with hepatic macromolecules (protein, DNA or RNA) in vivo following exposure in rats to various concentrations of ^{14}C-labeled VC.

B. To define the various metabolic pathways responsible for the biotransformation of VC. It is anticipated that in vitro techniques will be used to study both soluble (xanthine oxidase, glutathione transferases) as well as particulate (microsomal) enzyme systems.

If the studies outlined above are successful in elucidating 1) a VC induced effect which can be related to carcinogenesis

and/or 2) the primary metabolic pathways responsible for VC biotransformation. then additional studies to integrate the relationship between dose dependent metabolism and carcinogenesis may be warranted.

Due to the complex nature of the task at hand the proposed studies are outlined as a general approach. Individual tests will be screened and either pursued or terminated after an appropriate evaluation.

3. Test Material

Labeled 1,2-^{14}C-VC will be used in the majority of the proposed studies. The methodology for synthesis of ^{14}C-VC directly from 1,2-^{14}C dichloroethane (New England Nuclear) has been established previously (Wagner et al., 1975). The ^{14}C-VC will be synthesized directly prior to use and maintained in the gaseous state to prevent polymerization and degradation. The chemical purity of the labeled VC will be compared to authentic vinyl chloride (Matheson Gas Products, minimum purity 99.9%). The radiochemical purity will be established by liquid scintillation counting of trapped effluent fractions from the gas chromatograph.

4. Animals

Male Sprague-Dawley (Spartan substrain) rats will be used. Since alterations in hepatic glutathione levels may change the metabolic pattern of VC (Hefner, et al., 1974; Jaeger, et al., 1974) experiments will be initiated at a fixed time of day and food and water will be provided ad libitum.

5. Housing

All animals will be kept in rooms in which a constant temperature, humidity, and a 12 hour light-dark cycle are maintained. Noise and activity in the animal rooms are minimal and constant from day to day. The animal facility is approved by the American Association for Laboratory Animal Science and the American Association for Accreditation of Laboratory Animal Care.

6. Binding of ^{14}C-VC to Hepatic Macromolecules

a. Exposure Techniques

Rats will be exposed under dynamic conditions to ^{14}C-VC in a 30 l glass inhalation chamber. ^{14}C-labeled VC will be metered into the chamber air flow (~6 l/min) with a precision dual syringe pump. The nominal concentration of VC will be determined from the ratio of the rate at which the VC gas is dispensed and the total chamber air flow. The

analytical concentration of VC will be continuously monitored by recirculating a fraction of the chamber atmosphere through an infrared spectrophotometer (Wilks) set at 10.6 nm. Samples (1 ml) of the chamber atmosphere will also be subjected to gas chromatography periodically throughout the exposure. At corresponding intervals, the ^{14}C-activity will be determined by bubbling 1 ml aliquots of the chamber atmosphere into a scintillation solution and the radioactivity will be counted in a liquid scintillation spectrometer.

The inhalation chamber will be operated in a laboratory fume hood to prevent contamination of the working environment. After transit through the inhalation chamber the ^{14}C-VC will be absorbed on activated charcoal or trapped in an appropriate liquid solvent. These traps will be disposed of as radioactive waste according to standard regulations. Air samples from all experimental rooms will be sampled periodically by the Industrial Hygiene Department of The Dow Chemical Company.

b. Sample Preparation

Following a single 6 hour exposure to varying concentrations

of VC (10-1500 ppm) the rats will be killed immediately. An aliquot of liver will be sampled and used for determining hepatic nonprotein sulfhydryl content by a modification of the method of Sedlak and Lindsay (1968). The remaining liver will be frozen immediately on dry ice and stored at -20°C until analyzed. The remaining carcass will be analyzed for total radioactivity as described previously (Watanabe, et al., 1976a). Protein binding of ^{14}C-labeled VC to hepatic tissue will be determined by the method of Jollow et al. (1973). Binding of ^{14}C-VC to nucleic acids will be screened. Nucleic acids (DNA and RNA) will be isolated from rat liver following selected exposure levels (Kirby, 1957, 1962) and the ^{14}C-activity determined by liquid scintillation counting. If a significant interaction is observed additional exposures may be necessary to characterize the binding properties.

7. In Vitro Metabolism

Previous work in this laboratory (Hefner et al., 1975) indicated that the metabolic uptake of VC in rats exposed to less than 100 ppm was inhibited dramatically by pretreatment with ethanol. When exposed to greater than 220 ppm

VC,SKF 525-A, an inhibitor of the mixed function oxidase (MFO) enzymes, caused a slight inhibition of metabolic uptake. More recently Bolt et al. (1976), confirmed the effects of SKF 525-A, but also showed that 3-bromophenyl-4(5)-imidazole and 6-nitro-1,2,3-benzothiodiazole, potent inhibitors of cytochrome P-450 dependent MFO enzymes, completely blocked the metabolic uptake of VC in rats. Subsequent studies showed that VC binding to sulfhydryl containing proteins in vitro was facilitated by NADPH fortified MFO enzymes and the soluble enzyme xanthine oxidase. Therefore it appears that the metabolism of VC in vivo and binding properties in vitro are a function of both soluble and particulate enzymes. Considerable effort has been expended in studying the MFO mediated metabolism of VC while very little attention has been given to other metabolic enzyme systems. The present study proposes to investigate both soluble (xanthine oxidase, GSH transferases) as well as microsomal metabolism in vitro to gain further information on the potential metabolic systems involved in the biotransformation of VC. It is anticipated that various chemical trapping agents such as 3,4-dichlorobenzenethiol (Göthe et al., 1974), 4-(4-nitrobenzyl) pyridine (Barbin et al., 1975) or GSH will be used to derivatize reactive intermediates

which can then be examined for identification. Both radio-gas chromatography, liquid chromatography and gas chromatography-mass spectrometry are expected to be utilized extensively in metabolite identification. Following elucidation of *in vitro* VC metabolizing systems it may be appropriate to use selected inducers and inhibitors of the potential metabolic system to verify its function *in vivo*.

8. Conclusion

The proposed studies are designed to gain insight into the potential *in vivo* binding of VC to intracellular macromolecules (proteins and nucleic acids) which may be ultimately related to its mechanism of carcinogenicity. The secondary objective is to further characterize the biotransformation of VC by various enzyme systems *in vitro* in order to isolate reactive metabolites which may be related to the dose dependent metabolism and carcinogenicity of VC. The studies as outlined will be pursued only if preliminary results appear encouraging. This "experiment by experiment" approach is necessary to allow the flexibility required to optimize the return on investment to solve the complex problem of assessing the hazard of low level exposure to VC.

It is anticipated that an equivalent of 2 man years plus necessary support personnel will be required for the studies. This amounts to $115,000 over about a one year time period. Interim reports will be issued as the data warrants.

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