MANUFACTURING CHEMISTS ASSOCIATION

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JAN 31 1974

MEDICAL
DEPARTMENT

January 29, 1974

C O N F I D E N T I A L

To: TECHNICAL TASK GROUP ON VINYL CHLORIDE RESEARCH

Subject: Proposals for Additional Vinyl Chloride Monomer Research

Gentlemen:

The Research Coordinators, at their meeting of November 28, moved to ask the Technical Task Group on Vinyl Chloride Research to recommend to the Management Contacts that the sponsoring companies authorize the expenditure of an additional $80,000 for metabolism and teratology studies (to be conducted by Dow, at cost) and to alert them to the probable ultimate need for support of a continuing prospective epidemiology study at about $100,000 per year, and of three-generation reproduction studies and mutagenicity studies at a still-to-be-determined cost.

The proposed budget for the studies for which immediate support is solicited is

1.	Metabolic Studies (one year, best efforts study)	$50,000
2.	Teratology Study	20,000
3.	Contingency Funds	10,000
	TOTAL	$80,000

Enclosed herewith are the following documents, all of which are to be treated as sensitive and confidential, and restricted to those directly responsible for making the decisions on the subject proposals:

1. Report of the results of Dow's studies on the effect of exposure of rats to vinyl chloride on hepatic sulfhydryl levels,
2. A protocol for metabolism studies on vinyl chloride and

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3. A protocol for a teratology study on vinyl chloride.

A letter ballot on the Research Coordinator's request is enclosed. Please return promptly so that this item may be placed in the hands of the Management Contacts for an early decision on whether or not the sponsors will support one or both of the presently proposed programs.

Sincerely,

Kenneth D. Johnson, Ph.D.
Secretary, Technical Task
Group on Vinyl Chloride Research

KDJ:mb

Enclosures

cc: D. P. Duffield, M.D.
Mr. D. M. Elliott
Dr. Tiziano Garlanda

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Letter & Enclosures Copied by MCA
January 29, 1974.

DOW CHEMICAL U.S.A.

MIDLAND, MICHIGAN 48640

December 11, 1973

Dr. Kenneth Johnson
Manufacturing Chemists Association
1824 Connecticut Avenue N.W.
Washington, D.C. 20009

Dear Dr. Johnson:

Enclosed are the following: (1) Report of the results of our studies on the effect of exposure of rats to vinyl chloride on hepatic sulfhydryl levels, (2) A protocol for metabolism studies on vinyl chloride and (3) A protocol for a teratology study on vinyl chloride. Respectively, I request $50,000 and up to $24,000 to conduct the metabolism and teratology studies in the Toxicology Research Laboratory of The Dow Chemical Company. The cost for the teratology study will depend on the number of concentrations to which animals will have to be exposed. I predict a minimum of $16,000 and a maximum of $24,000 will be needed. These studies will commence as soon as a positive response to the requests are received. In order to obtain a decision, I rely on you to distribute these documents to the appropriate individuals. I request that the documents be distributed only to those individuals required for rendering a decision and that these individuals maintain the documents and their contents confidential.

Sincerely,

P. J. Gehring, D.V.M., Ph.D.
Chemical Biology Research
Toxicology Section
1803 Building
Phone (517) 636-1089

krr

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THE EFFECTS OF EXPOSURE OF RATS TO 50, 500 AND 5,000 PPM VINYL CHLORIDE ON THE SULFHYDRYL LEVELS OF LIVER

R. E. HEFNER, JR. AND P. J. GEHRING
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(THIS REPORT PREPARED FOR THE MANUFACTURING CHEMISTS ASSOCIATION TECHNICAL TASK GROUP ON VINYL CHLORIDE RESEARCH SUMMARIZES PRELIMINARY STUDIES AND IS TO BE CONSIDERED HIGHLY CONFIDENTIAL. RELEASE OF THIS INFORMATION IS TO OCCUR ONLY WITH THE CONSENT OF THE AUTHORS AND THE DOW CHEMICAL COMPANY).

THE EFFECTS OF EXPOSURE OF RATS TO 50, 500 AND 5,000 PPM VINYL CHLORIDE VAPOR ON THE SULFHYDRYL LEVELS OF LIVER

R. E. Hefner, Jr. and P. J. Gehring
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Midland, Michigan 48640

ABSTRACT

Exposure of rats to 50, 500 or 5,000 ppm vinyl chloride vapors for 7 hr/day for 5 consecutive days causes a decrease in the non-protein sulfhydryl content of the liver. The degree of this response was essentially equivalent at these exposure concentrations suggesting that this response is mediated via a "zero" order mechanism.

2-hydroxyethyl-L-cysteine and monochloroacetic acid were found in the urine of rats exposed to 5,000 ppm vinyl chloride. It is proposed that *in vivo* vinyl chloride is sequentially oxidized to 2-chloroethanol, chloroacetaldehyde and chloroacetic acid. The lack of dependency of the depression of the non-protein sulfhydryl content of the liver on exposure concentration of vinyl chloride is attributed to the "zero" order oxidation of 2-chloroethanol to chloroacetaldehyde via alcohol dehydrogenase. Chloroacetaldehyde spontaneously alkylates cysteine while other oxidation products of vinyl chloride do not.

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INTRODUCTION

In 1972, Viola *et al.* reported that tumors of the skin, lungs, and bones developed in rats exposed to an atmosphere containing 30 000 ppm vinyl chloride for 4 hours per day, 5 days per week for 12 months. In the study described herein the zero order kinetics of vinyl chloride metabolism would indicate that a dose response curve for tumor induction by various concentrations of vinyl chloride might be expected to have a very shallow slope. Thus, if this hypothesis is valid, tumor induction may be mediated via the metabolism of vinyl chloride.

Consideration of the possible fate of vinyl chloride in the body suggests that it may be oxidized sequentially to 2-chloroethanol, chloroacetaldehyde, and chloroacetic acid. *In vivo* oxidation of 2-chloroethanol to chloroaldehyde occurs via the same pathway as the oxidation of ethanol (Johnson, 1967). Both oxidations are mediated by alcohol dehydrogenase. Associated with the injection of 2-chloroethanol in rats is a reduction in the glutathione content of the liver, which is mediated via an alkylation of the free sulfhydryl group of the cysteine portion of this tripeptide (Johnson, 1967). Cysteine, per se, may also be alkylated. Neither 2-chloroethanol (Johnson, 1967) or chloroacetic acid (Gibson, 1973) alkylate glutathione *in vitro*, however, chloroacetaldehyde does, suggesting that the zero order oxidation of 2-chloroethanol to chloroacetaldehyde via alcohol dehydrogenase precedes the alkylation of glutathione and/or cysteine.

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Using the aforementioned rationale, we hypothesized that vinyl chloride will be oxidized to chloroacetaldehyde in vivo and cause a reduction in the non-protein sulfhydryl content, glutathione and cysteine, of various tissues. In a previous probe study, the non-protein sulfhydryl content of the liver of rats exposed to 15,000 ppm vinyl chloride was reduced (Hefner, and Gehring, NB T13.4-16-8).

How may such reactions be associated with tumor induction? It has been reported that the administration of cysteine or glutathione provides protection against the untoward effects of various aliphatic and aromatic mustards, triethylenemelamine, x-rays, and ionizing radiation (Calcutt, et al., 1963; Patt, 1964; Goldenthal, et al., 1959; Stacey, et al., 1958; Ball, 1966). Conceivably, cysteine and glutathione may provide a natural defense against tumor producing free radicals generated within the body, as well as synthetic or naturally occurring alkylating agents which are absorbed into the body. Thus, it is our hypothesis that the induction of tumors in rats exposed to vinyl chloride may be secondary to an induced reduction in the non-protein sulfhydryl content of various tissues.

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The primary objective of the study reported herein was to characterize the reduction of the non-protein sulfhydryl content of the liver of rats exposed to various concentrations of vinyl chloride. If our hypothesis is valid, the magnitude

Glutathione = $H_2NCH(CO_2H)CH_2CH_2CONHCH(CH_2SH)CONHCH_2CO_2H$

Cysteine $HSCH_2CH(NH_2)CO_2H$

2-amino-3-mercaptopropionic acid.

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of such reductions should not be markedly dependent on the exposure concentration. Secondary objectives were to establish some temporal relationships for the reduction of the non-protein sufhydryl content of the liver of rats exposed daily and to attempt preliminary identification of reaction products of cysteine and vinyl chloride in the urine of exposed rats.

METHODS

Groups of male Sprague-Dawley albino rats of Spartan strain weighing from 193 to 250 grams at initiation of the experiment were exposed to nominal concentrations of 5,000, 500, or 50 ppm vinyl chloride, for 1, 3, or 7 weeks and to 50 ppm for 1 week. The exposures were carried out in a glass-walled 160L chamber under dynamic conditions with the vinyl chloride being metered into the chamber airstream. Seven hour exposures were conducted on the first four of five consecutive days each week. On the fifth day, the duration of the exposure was reduced to 5 to 6 hours.

Body weights and food consumption were determined before each exposure and the rats were observed periodically for signs of toxicity. Between 1 and 2 PM, immediately following the fifth exposure of the designated week, the rats were killed and the livers removed and prepared for assay of sulfhydryl groups. Gross pathological examinations were conducted.

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Sulfhydryl Assay. The method used for free sulfhydryl assay was a modification of that described by Sedlak and Lindsay (1968). Exactly 500 mg of liver from each rat was homogenized for 1 minute in a Dounce tissue homogenizer containing 8 ml of 0.02M disodium EDTA. For the total sulfhydryl assay, a 0.5 ml aliquot of each homogenate was mixed with 1.5 ml of 0.2M tris pH 9.2 buffer, 0.1 ml of 0.01M DTNB [5,5'-dithiobis(2-nitrobenzoic acid)], and 7.9 ml of methanol. A reagent blank without liver homogenate, and a sample blank, without DTNB, were also prepared. The color generated via the release of nitro-mercaptobenzoic acid anion was allowed to develop for 15 minutes, and the samples were centrifuged for 15 minutes at 4,000 g. Absorbance of each sample was read against the respective sample blank at 412 nm with a Beckman DB spectrophotometer. Subsequently, the molar concentration of total sulfhydryl in the sample was calculated using an extinction coefficient determined from standards of known concentrations of glutathione or cysteine. A plot of absorbance versus concentration of cysteine or glutathione is shown in Figure 2. This plot coincides with that reported by Sedlak and Lindsay (1968).

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The non-protein sulfhydryl content of liver was determined after precipitating out the protein by addition of 1 ml of 50% trichloroacetic acid to a 5 ml sample of liver homogenate. Each sample was diluted with 4 ml of distilled water and after 15 minutes centrifuged at 4,000 g. A 2 ml aliquot of the

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supernatant was mixed with 4 ml of 0.4M tris pH 8.9 buffer. Immediately before reading the absorbance against a reagent blank, 0.1 ml of 0.01M DTNB was added. Subtraction of the non-protein sulfhydryl content from the total sulfhydryl content yielded a value for protein-bound sulfhydryl.

Metabolite Identification. Urine samples collected from rats exposed to 5,000 ppm vinyl chloride for 4, 5, and 7 weeks were analyzed for the presence of 2-chloroethyl-ℓ-cysteine, 2-hydroxyethyl-ℓ-cysteine, and 2-carboxymethyl-ℓ-cysteine (5 and 7 week samples only). Urine was collected for analysis by applying pressure to the posterior abdomen between the fifth and sixth hour of the fifth daily exposure on the designated week. Urine collected on the same day was pooled. For the three aforementioned compounds, 2 to 15 µl of urine were spotted directly on a 5 by 20 cm Baker-flex® silica gel plate. Also spotted were samples of urine collected from control rats and standard aqueous solutions as well as control urine to which approximately 1 µg/µl of each of the compounds had been added. The chromatograms were developed for 5 hours in a sealed glass tank containing n-butanol, acetic acid, water (80:10:10). After being air dried, the plates were sprayed with Ninspray® ninhydrin reagent and heated for 2 minutes at 80°C. The color of the spots and their Rf values were used to identify the compounds.

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A urine sample collected from rats exposed to 5,000 ppm vinyl chloride for 9 weeks was analyzed for the presence of chloroacetic acid. The 10 ml urine sample was acidified with 0.1 ml of 50% v/v H_2SO_4. Subsequently, the urine sample was extracted 3 times with 2 ml of diethyl ether. By evaporation, the diethyl ether extract was concentrated to 0.1 ml and 2 to 15 µl of the concentrated extract and an aqueous standard containing 5% w/v chloroacetic acid were spotted on an Eastman fluorescent silica gel plate. The chromatograms were develped in a sealed glass tank for 5 hours using the aforementioned solvent system. After drying the plates, they were examined under ultraviolet light to determine the location of spots. Subsequently, the plates were treated with ninhydrin as described previously.

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RESULTS

No antemortem or postmortem signs of toxicity were noted in rats exposed to any concentration of vinyl chloride used in these experiments. Analysis of food consumption data of rats exposed to nominal concentrations of 5,000 and 500 ppm vinyl chloride, and controls revealed no statistically significant differences. In addition, no significant difference was found in the water consumption of rats exposed to 5,000 ppm and controls. Throughout the durations of observation, the mean body weights of rats exposed to the various concentrations of vinyl chloride were essentially the same as those of the respective controls,

Figure 3. No gross pathological lesions were found in any of the rats exposed to vinyl chloride.

Sulfhydryl Content of Liver. In Tables 2, 3, and 4, respectively, are shown the total, protein, and non-protein sulfhydryl content of the liver of rats exposed to 50, 500, or 5,000 ppm vinyl chloride for the indicated durations. Values for concurrent controls are also given. The results in Table 4 show that exposure to either 500 or 5,000 ppm vinyl chloride was consistently associated with a statistically significant decrease in the non-protein sulfhydryl content of the liver. Even in the group of rats exposed to 50 ppm vinyl chloride for one week, there appears to be some reduction. However, because of a low value for the non-protein sulfhydryl content of one of the controls the reduction was not statistically significant. To resolve this, a second group of rats was exposed to 50 ppm vinyl chloride for one week and a statistically significant decrease in the non-protein sulfhydryl content of the liver was found, Table 5. The relative non-protein sulfhydryl value, for both control and exposed rats are higher than those found in previous assays. The samples of liver were homogenized more thoroughly in this study, 2 minutes instead of 1 minute, which may account for this apparent discrepancy.

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Figure 4 illustrates the percent depression of the non-protein sulfhydryl content of the liver as a function of exposure concentration and duration of exposure. The results indicate

that the reduction in the non-protein sulfhydryl content of the liver of rats exposed to 50, 500, 5,000 and 15,000 ppm vinyl chloride is independent of the exposure concentration. The result for rats exposed to 15,000 ppm was taken from a previous report (Hefner, Jr. and Gehring, NB T13.4-16-8).

Although not definitive, the depression of the non-protein sulfhydryl content of the liver of rats exposed to 500 and 5,000 ppm vinyl chloride appears to decrease as the duration of the exposure is lengthened. This suggests that repeated exposure to vinyl chloride may induce a metabolic adaptation which decreases the response.

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With regard to the total sulfhydryl and protein sulfhydryl content of the liver, the changes in rats exposed to vinyl chloride were sporadic and inconsistent, thus negating any definitive conclusions. This was not unexpected because the sulfhydryl groups of protein have been shown not to be readily alkylated unless denaturation of the protein renders them available for alkylation (Stacey et al., 1958).

Metabolite Identification. Figure 5 depicts a representative thin-layer chromatogram of urine collected from rats exposed to 5,000 ppm vinyl chloride. Chromatograms of urine from rats exposed for 4, 5, and 7 weeks were all the same. 2-hydroxyethyl-l-cysteine, Rf 0.26-0.28, appeared to be present all 3 times.

Rf values for 2-hydroxy-ℓ-cysteine in the chromatograms of standards were the same. In addition, the color of the respective spots was the same, reddish-purple after development with ninhydrin reagent. 2-chloroethyl-ℓ-cysteine and 2-carboxymethyl-ℓ-cysteine were not detected in the urine of rats exposed to vinyl chloride. Although the spots for 2-hydroxyethyl-ℓ-cysteine and 2-carboxymethyl-ℓ-cysteine were close, they were resolvable and the spot for 2-carboxymethyl-ℓ-cysteine was dark purple after treatment with ninhydrin reagent.

Figure 6 depicts a representative thin-layer chromatogram showing the presence of chloroacetic acid in urine collected from rats exposed to 5,000 ppm vinyl chloride for 9 weeks. Chloroacetic acid appeared to be present as visualized under ultraviolet light; the Rf value was 0.74. This value is the same as the Rf value obtained using a chloroacetic acid standard. As expected, the chloroacetic acid spots were not developed by ninhydrin reagent.

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DISCUSSION

This study shows that there is a depression of the non-protein sulfhydryl content in the liver of rats exposed to concentrations of vinyl chloride ranging from 50 to 15,000 ppm 7 hrs/day for 5 consecutive days. Within this range of concentrations, the degree of depression is not significantly influenced by the magnitude of the concentration to which rats are exposed.

Although not definitive, the degree of depression appears to decrease with additional weeks of exposure - 7 hrs/day, 5 consecutive days/week.

The depression of the non-protein sulfhydryl content of the liver of rats exposed to vinyl chloride occurred in the absence of concurrent signs of antemortem toxicity or gross pathological changes. Absence of definitive signs of toxicity is not surprising. In a previous study, no antemortem signs of toxicity or gross pathological changes were observed in rats exposed to 500 ppm vinyl chloride 7 hrs/day, 5 days/week for 4.5 months (Torkelson et al, 1961). A mild degree of central lobular degeneration in the liver and interstitial and tubular changes in the kidney were observed. In rats exposed to 100 or 200 ppm vinyl chloride 7 hrs/day, 138 to 144 times in 204 days, the only discernible effect was a slight increase in liver weight. Increased weights of the liver did not occur in rats exposed to 50 ppm.

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Whether a decrease in the non-protein sulfhydryl content of the liver constitutes a toxicologically significant parameter remains unknown. Hayes et al. report a correlation between the single dose lethality of monochloroacetic acid and depression of the non-protein sulfhydryl content of liver and kidney. Recently, Jaeger et al., 1974 reported a depression of the non-protein sulfhydryl content in rats exposed for 4 hrs to

1,1-dichloroethylene. Also demonstrated was an increased lethality in rats whose liver non-protein sulfhydryl content had been reduced by 16 hrs of starvation and subsequently exposed for 4 hrs to 1,1-dichloroethylene. Although these studies suggest that toxicity may be related to a reduction of the non-protein sulfhydryl content of liver and perhaps other tissues, a definitive association between the two remains to be established. It is not unreasonable to expect that the non-protein sulfhydryl content of tissues provides a pathway for the detoxification of various alkylating agents and free radicals generated normally within the body.

The results of the study reported herein provide initial support for our hypothesis that the tumorigenic activity of vinyl chloride in rats may be mediated via a reduction in the non-protein sulfhydryl content of liver. As rationalized in the introduction, such a reduction may depress the natural defense of the body against tumor development. The attractiveness of this hypothesis is augmented by the finding that the degree of the depression of the sulfhydryl group concentration of the liver is not dependent on the magnitude of the concentration of vinyl chloride to which rats were exposed. This finding also provides support for the hypothesis that a dose response curve for tumor induction by vinyl chloride might be expected to have a very shallow slope.

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The absence of a dose-response depression of the non-protein sulfhydryl content of the liver is also consistent with our hypothesis that this event is preceded by the oxidation of vinyl chloride to 2-chloroethanol and subsequently to chloroacetaldehyde. The oxidation of 2-chloroethanol to chloroacetaldehyde is mediated via alcohol dehydrogenase (Johnson, 1967) which is readily saturatable. Chloroacetaldehyde but not vinyl chloride (unpublished results) or 2-chloroethanol (Johnson, 1967) readily alkylates the sulfhydryl group of cysteine and glutathione. Therefore, the absence of a dose response for the depression of the non-protein sulfhydryl content of the liver may be expected if our hypothesis is valid. That vinyl chloride is oxidized via this pathway is supported by the finding of monochloroacetic acid in the urine.

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The finding of 2-hydroxyethyl-ℓ-cysteine but not carboxymethyl-ℓ-cysteine in the urine of rats exposed to vinyl chloride might at first appear to be inconsistent with our original hypothesis. If production of chloroacetaldehyde precedes alkylation of the sulfhydryl groups of glutathione and cysteine, the latter but not the former metabolite may be expected. Johnson (1967) reported carboxymethylglutathione but not 2-hydroxyethylglutathione in the urine of rats given 2-chloroethanol. In our studies, standards for the glutathione conjugates were not available; therefore, the urine of rats exposed to vinyl

chloride was not examined for their presence. Originally we had expected the cysteine conjugates to be like the glutathione conjugates reported by Johnson (1967).

It is conceivable that the alkylation of glutathione and cysteine with chloroacetaldehyde may not lead to the excretion of similar analogs. Perhaps, the conjugation of chloroacetaldehyde with cysteine will produce a thiazolidine ring which subsequently undergoes ring opening and hydrolysis to give 2-hydroxyethyl cysteine. The acetaldehyde glutathione conjugate cannot form such a structure because it is N,N'-disubstituted, (Figure 7).

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Another way to explain the finding of 2-hydroxyethyl-ℓ-cysteine but not carboxymethyl-ℓ-cysteine in the urine is that vinyl chloride may alkylate cysteine and glutathione directly to form 2-chloroethyl-ℓ-cysteine and 2-chloroethylglutathione. These products spontaneously form the hydroxyl analogs in an aqueous environment (Moppett and Martin, 1973). Direct alkylation of glutathione and cysteine by vinyl chloride occurs when vinyl chloride is bubbled through an aqueous-ethanol but not in an aqueous system (Martin and Moppett, 1973). Although unresolved, it is not expected that vinyl chloride will directly alkylate cysteine and glutathione in vivo. Furthermore, if the depression of non-protein sulfhydryl groups is mediated via this reaction, there should be a strong correlation

between the degree of depression and the magnitude of the exposure. Thus, it seems unlikely that vinyl chloride alkylates non-protein free sulfhydryl directly; however more data are needed to exclude this possibility.

Verification of our original hypothesis, as well as identification and verification of the metabolites of vinyl chloride is required, as the analytical procedures used in detecting 2-hydroxyethyl-L-cysteine and chloroacetic acid in the urine of rats exposed to vinyl chloride were only qualitative at best. Therefore, we propose a metabolism study using ^{14}C labeled vinyl chloride to further elucidate the validity of our hypothesis. Such a study would allow a definitive qualitative and quantitative identification of metabolites. Also additional studies are planned to determine what levels of exposure to vinyl chloride may be incurred without a reduction of the non-protein sulfhydryl concentration in the liver.

URL 00236

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URL 00237

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URL 00238

TABLE 1

TOTAL SULFHYDRYL CONTENT (TSH), 1 X 10^{-8} MOLES/MG, OF THE LIVERS OF RATS EXPOSED TO 5,000, 500, AND 50 PPM VINYL CHLORIDE FOR VARIOUS TIMES AND RESPECTIVE CONCURRENT CONTROLS

Duration of Exposure**	Concentration of Vinyl Chloride					
	5,000 ppm	Concurrent Controls	500 ppm	Concurrent Controls	50 ppm	Concurrent Controls
7 hours per day for 5 days	2.08 2.06 2.10 2.08 2.15	2.07 2.28 2.30	2.15 2.21 2.25 2.09 2.35	2.12 2.18 2.11 2.08	2.09 2.14 2.10 1.98	2.44 2.20 2.36
Mean±S.D.	2.09±0.04	2.22±0.13	2.21±0.10	2.12±0.04	2.08±0.07*	2.33±0.12
7 hours per day 5 days per week for 3 weeks	2.37 2.53 2.50 2.38 2.50	2.31 2.53 2.48	2.28 2.15 1.90 1.99	2.55 2.45 2.31		
Mean±S.D.	2.45±0.08	2.44±0.06	2.08±0.17*	2.44±0.12		
7 hours per day 5 days per week for 7 weeks	2.36 2.00 2.18 2.50 2.38	2.50 2.75 2.61	2.60 2.53 2.61 2.61	2.31 2.33 2.48		
Mean±S.D.	2.28±0.20*	2.62±0.13	2.59±0.04*	2.37±0.09		

*Significantly different using Students "t" test, $p<0.05$.

**Determinations made between 1 - 2 PM after completing 5 to 6 hours of the 5th and final weekly 7 hour exposure.

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TABLE 2 URL 00240

PROTEIN BOUND SULFHYDRYL CONTENT (PSH), 1 X 10^{-8} MOLE/MG, OF THE LIVERS OF RATS EXPOSED TO 5,000, 500, AND 50 PPM VINYL CHLORIDE FOR VARIOUS TIMES AND RESPECTIVE CONCURRENT CONTROLS

Duration of Exposure**	Concentration of Vinyl Chloride					
	5,000 ppm	Concurrent Controls	500 ppm	Concurrent Controls	50 ppm	Concurrent Controls
7 hours per day for 5 days	1.71 1.75 1.76 1.75 1.83	1.63 1.84 1.81	1.75 1.97 1.91 1.86 1.88	1.62 1.59 1.53 1.50	1.75 1.79 1.65	1.96 1.87 1.88
Mean±S.D.	1.76±0.05	1.76±0.12	1.87±0.08	1.55±0.05*	1.75±0.06	1.90±0.05
7 hours per day 5 days per week for 3 weeks	1.95 2.13 2.09 2.02 2.06	1.58 1.79 1.79	1.81 1.63 1.47 1.61	1.87 1.84 1.78		
Mean±S.D.	2.05±0.07*	1.72±0.12	1.63±0.14	1.83±0.05		
7 hours per day 5 days per week for 7 weeks	1.78 1.55 1.53 1.90 1.73	1.81 2.03 1.93	1.95 1.92 2.06 2.09	1.76 1.68 1.76		
Mean±S.D.	1.70±0.16	1.92±0.11	2.01±0.08*	1.74±0.05		

*Significantly different using Students "t" test, $p<0.05$

**Determinations made between 1 - 2 PM after completing 5 to 6 hours of the 5th and final weekly 7 hour exposure.

TABLE 3

NON-PROTEIN BOUND SULFHYDRYL CONTENT (NPSH), 1 X 10^{-8} MOLE/MG, OF THE LIVERS OF RATS EXPOSED TO 5,000, 500, AND 50 PPM VINYL CHLORIDE FOR VARIOUS TIMES AND RESPECTIVE CONCURRENT CONTROLS

Duration of Exposure**	Concentration of Vinyl Chloride					
	5,000 ppm	Concurrent Controls	500 ppm	Concurrent Controls	50 ppm	Concurrent Controls
7 hours per day for 5 days	0.37	0.44	0.40	0.50	0.34	0.48
	0.31	0.44	0.24	0.64	0.35	0.33
	0.34	0.49	0.34	0.58	0.32	0.48
	0.33		0.23	0.58	0.35	
	0.32		0.47			
Mean±S.D.	0.33±0.02*	0.46±0.03	0.35±0.09*	0.58±0.06	0.34±0.01	0.43±0.09
7 hours per day 5 days per week for 3 weeks	0.42	0.73	0.47	0.68		
	0.40	0.74	0.52	0.61		
	0.41	0.68	0.43	0.53		
	0.35		0.38			
	0.44					
Mean±S.D.	0.41±0.03*	0.72±0.03	0.45±0.06*	0.61±0.08		
7 hours per day 5 days per week for 7 weeks	0.58	0.69	0.65	0.55		
	0.45	0.72	0.60	0.64		
	0.65	0.68	0.55	0.71		
	0.60		0.52			
	0.65					
Mean±S.D.	0.59±0.08	0.70±0.02	0.58±0.06	0.64±0.08		

*Significantly different using Students "t" test, $p<0.05$.

**Determinations made between 1 - 2 PM after completing 5 to 6 hours of the 5th and final weekly 7 hour exposure.

TABLE 4

NON-PROTEIN BOUND SULFHYDRYL CONTENT (NPSH), 1 X 10^{-8} MOLE/MG, OF THE LIVERS OF RATS EXPOSED TO 50 PPM VINYL CHLORIDE FOR 1 WEEK AND CONCURRENT CONTROLS ***

	Concentration of Vinyl Chloride	
Duration of Exposure**	50 ppm	Concurrent Controls
7 hours per day for 5 days	0.759	1.296
	0.717	1.060
	0.770	1.300
	0.579	1.250
	0.484	.991
	0.732	
	0.747	
	0.671	
Mean ± S.D.	.682±.101*	1.179±0.144

URL 00242

*Significantly different using Students "t" test, $p<0.05$.

**Determinations made between 1-2pm after completing 5 hours of the 5th and final weekly 7 hour exposure.

***The relative NPSH values for both exposed and control rats are higher than those found in previous assays. This is most likely due to homogenizing each liver sample for 2 minutes, rather than 1 minute as in the previous assays.

Figure 1/
BEER'S LAW PLOT - CYSTEINE AND
GLUTATHIONE - DTNB COMPLEXES

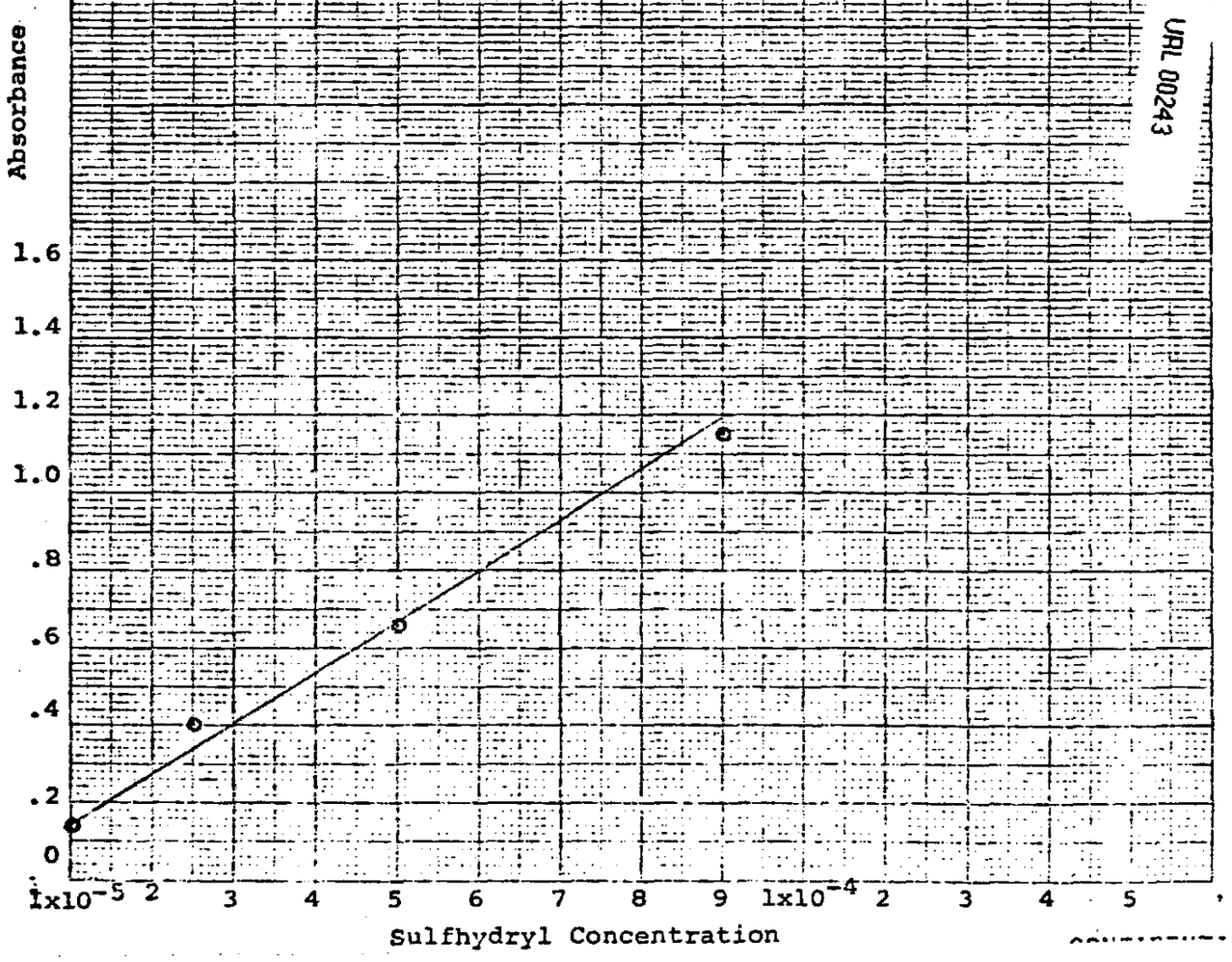

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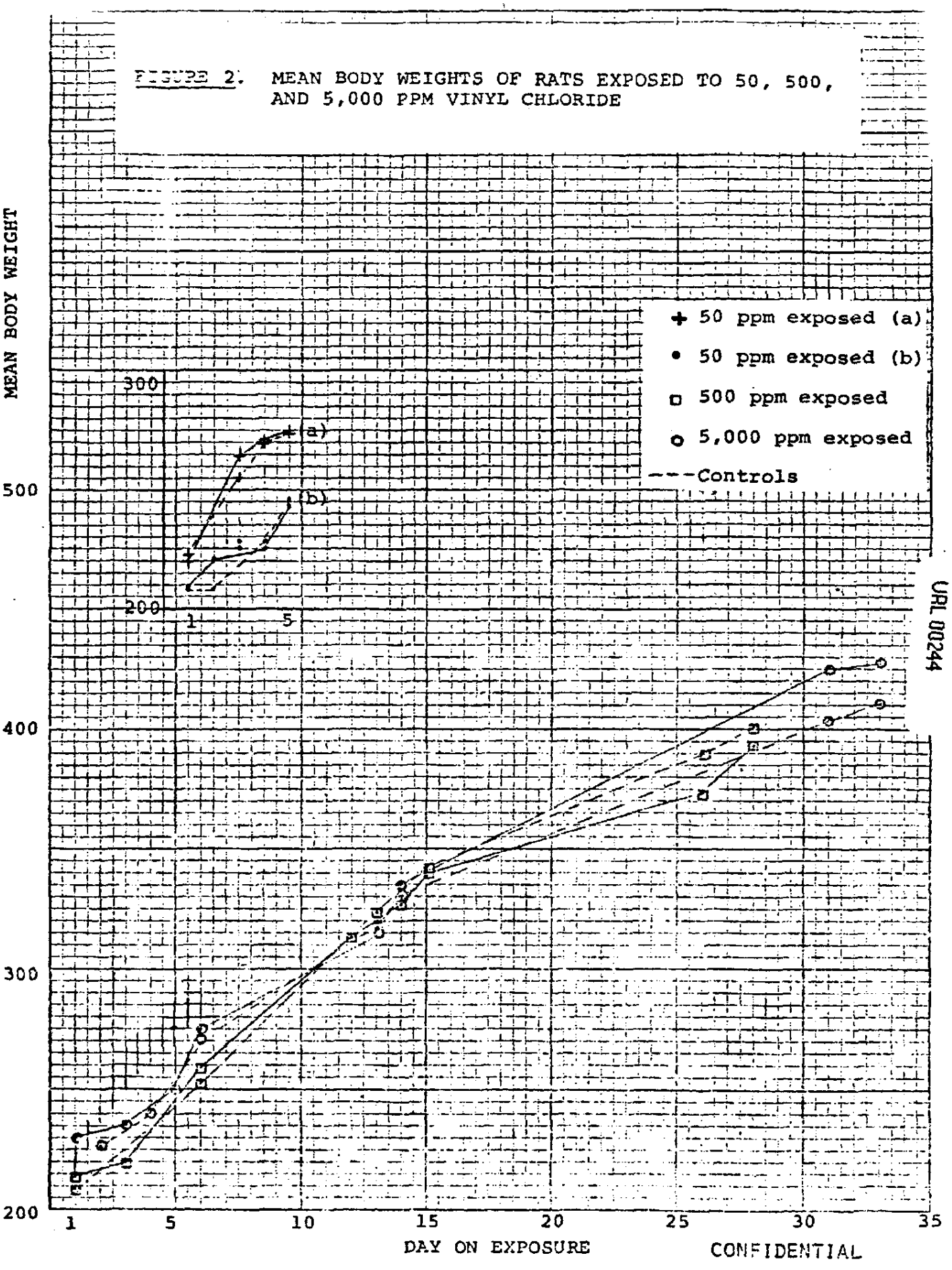
FIGURE 2. MEAN BODY WEIGHTS OF RATS EXPOSED TO 50, 500, AND 5,000 PPM VINYL CHLORIDE



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7 X 10 INCHES
KEUFFEL & ESSER CO.

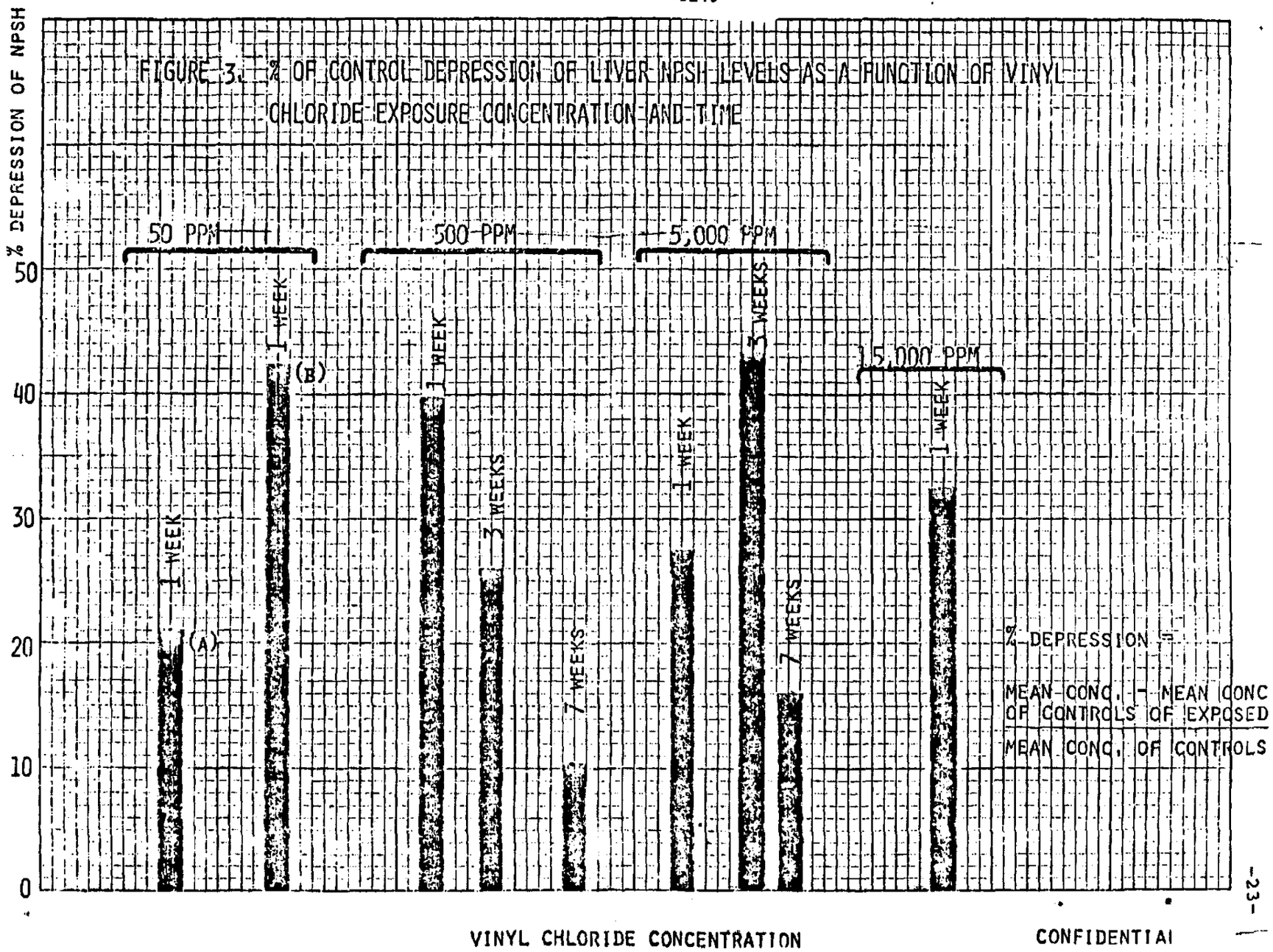

FIGURE 3. % OF CONTROL DEPRESSION OF LIVER NPSH LEVELS AS A FUNCTION OF VINYL CHLORIDE EXPOSURE CONCENTRATION AND TIME

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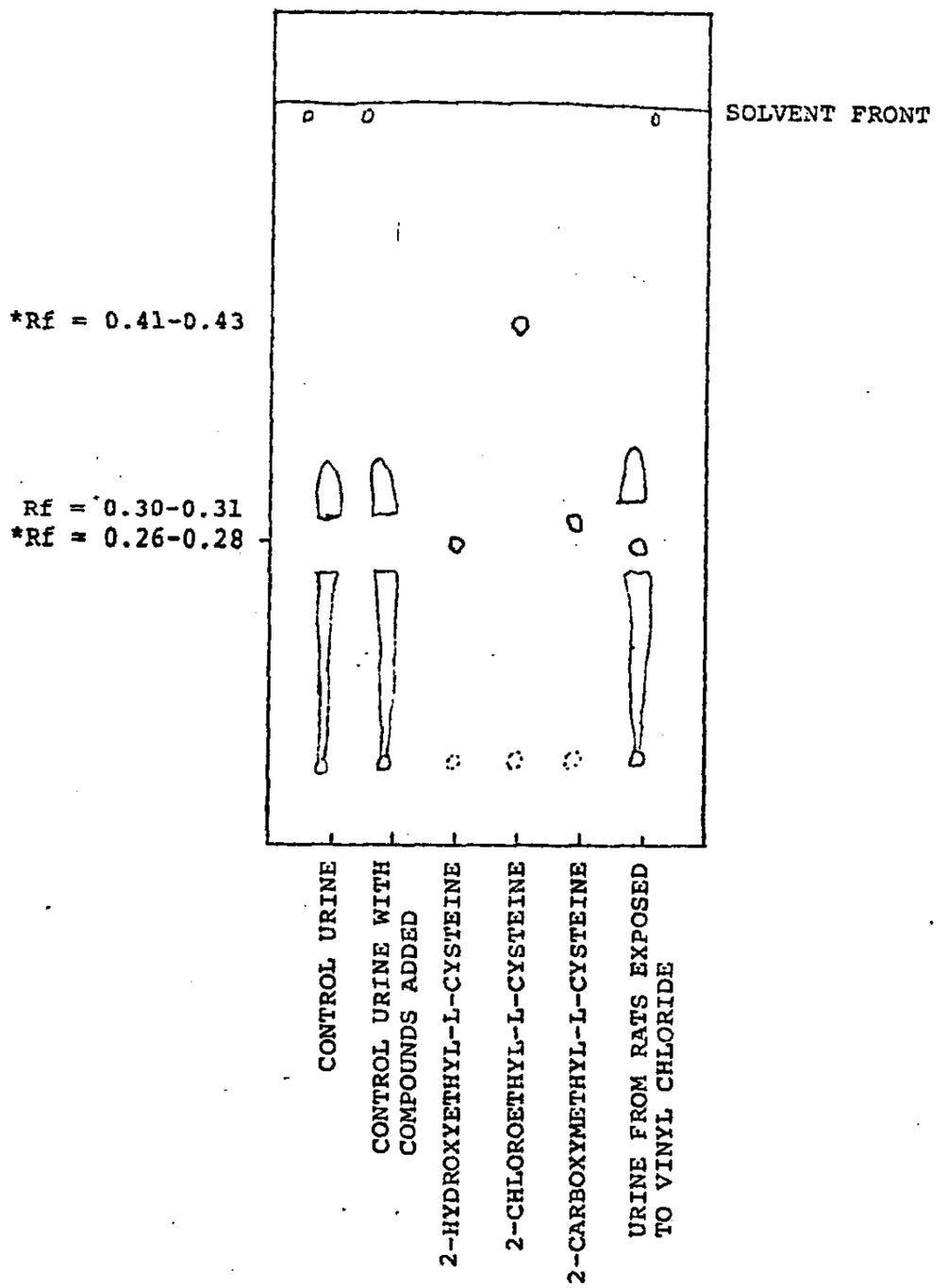
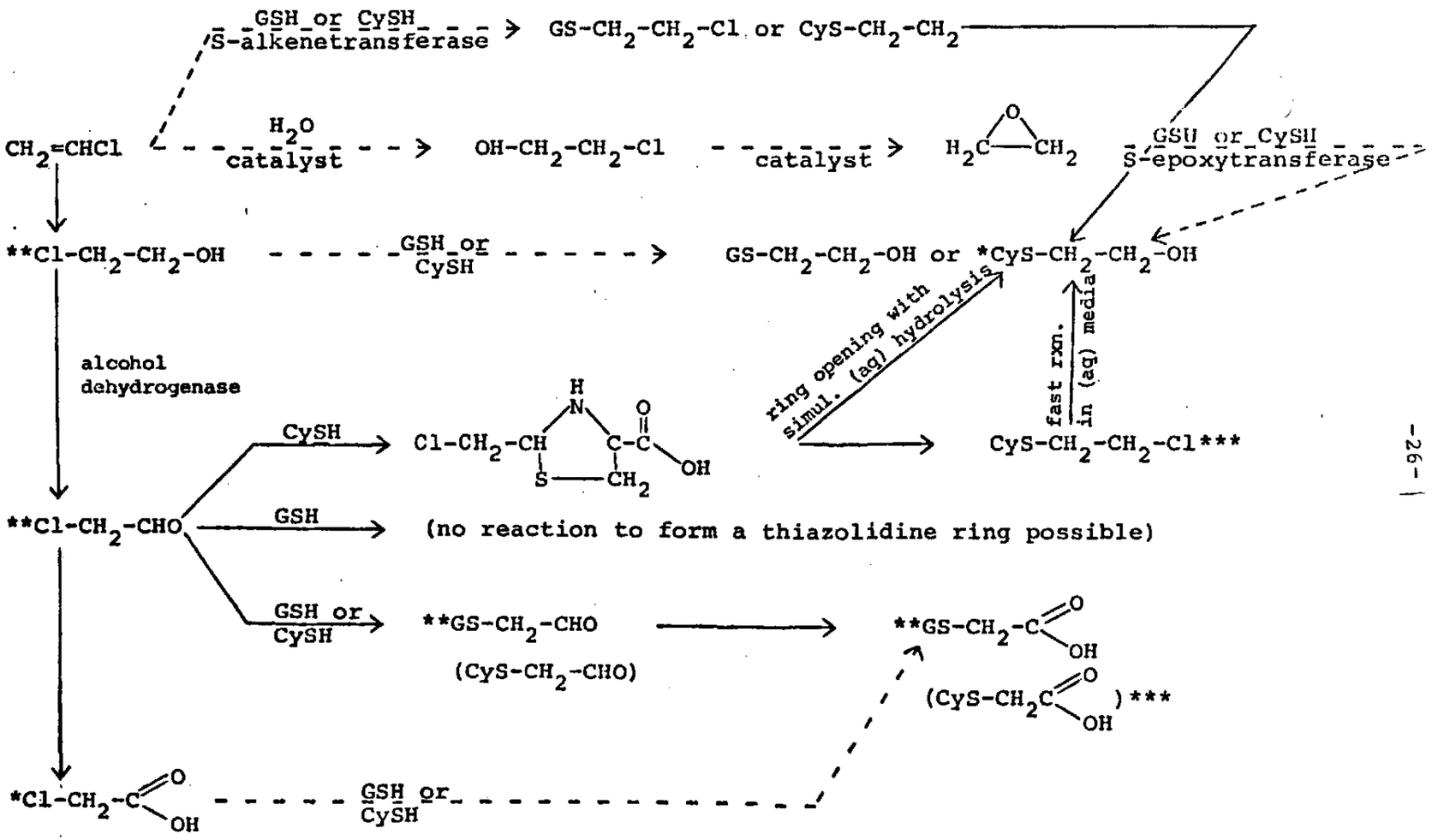
FIGURE 4. REPRESENTATIVE THIN-LYER CHROMATOGRAM OF URINE FROM RATS EXPOSED TO 5,000 PPM VINYL CHLORIDE FOR 4, 5, AND 7 WEEKS



*Value for 80, 10, 10 n-butanol, acetic acid and water. 2-hydroxyethyl-L-cysteine has a 0.13-0.14 Rf and 2-chloroethyl-L-cysteine has a .27-.28 Rf in 80, 20, 20.

URL 00246

FIGURE 6. POSSIBLE METABOLIC ROUTES FOR VINYL CHLORIDE



*Metabolites identified in this study
**GSH metabolites identified by Johnson (1967) using chloroethanol as a starting material
***Metabolites not observed in this study, but assayed for
- - - Energetically unfavorable reaction pathway

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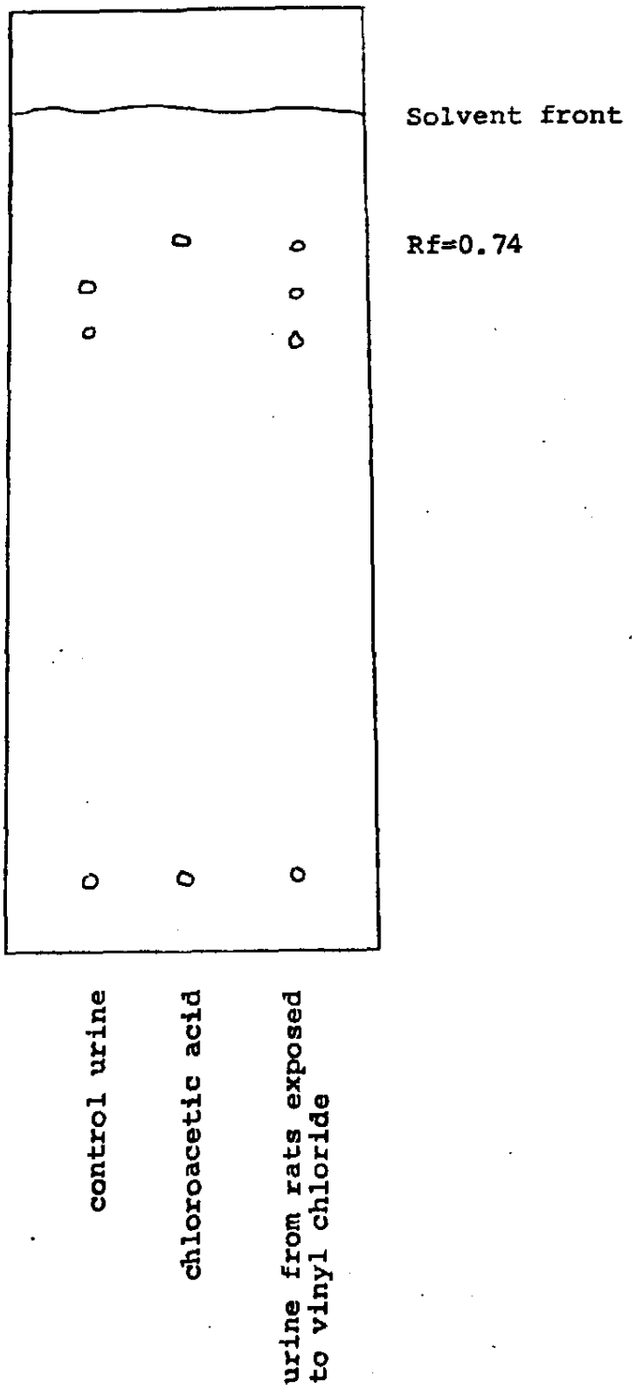
FIGURE 5

REPRESENTATIVE FLUORESCENT THIN-LAYER CHROMATOGRAM (AS VISUALIZED UNDER U.V. LIGHT) OF URINE FROM RATS EXPOSED TO 5,000 PPM VINYL CHLORIDE FOR 9 WEEKS



URL 00248

PROPOSED STUDIES ON THE METABOLISM OF VINYL CHLORIDE

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INTRODUCTION AND RATIONALE

The rationale for conducting studies on the metabolism of vinyl chloride is elucidated in the attached report. Briefly, vinyl chloride has been shown to be tumorigenic in rats exposed via inhalation. There is suggestive evidence that vinyl chloride is not the proximate carcinogen but rather the tumorigenic effect is mediated via its metabolism. Preliminary evidence indicates that vinyl chloride is oxidized in the body sequentially to 2-chloroethanol, chloroacetaldehyde and monochloroacetic acid. Except at low exposure concentrations the overall kinetics of this reaction is expected to be "zero" order. This explains the apparent lack of dependence of the incidence of tumors on the exposure concentration in rats exposed to 250 ppm and greater of vinyl chloride. If our hypothesis concerning the association between tumor production and metabolism of vinyl chloride is correct, it is expected that at some exposure concentration yet to be elucidated no tumors would be induced.

Aside from the rational described briefly above and more extensively in the attached report, it is highly desirable to characterize both the pharmacokinetics and metabolism of vinyl chloride in rats as well as other species including

URL 00249

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man. With acquisition of this information, the results of studies in rats and other laboratory animals can be used more reliably to assess the hazard incurred by humans exposed to vinyl chloride.

OBJECTIVES OF PLANNED WORK

1. Determine the rate of absorption, distribution and excretion of vinyl chloride in rats exposed to various concentrations via inhalation and perhaps via oral gavage.

2. Determine qualitatively and quantitatively the metabolism of vinyl chloride in rats exposed to various concentrations via inhalation. Particular attention will be given to characterizing the metabolites formed by oxidation of vinyl chloride (2-chloroethanol, chloroacetaldehyde and monochloroacetic acid) and the cysteine and glutathione conjugates of metabolites of vinyl chloride.

URL 00250

3. Determine whether significant differences in the metabolism of vinyl chloride exist in rats and dogs or monkeys. The intent is to use at least one other species to shed some light on the degree of differences in metabolism between species.

4. Determine how various agents and procedures affect the metabolism of vinyl chloride. For example, a few hours of starvation should decrease the availability of

cysteine and glutathione for conjugation with metabolites of vinyl chloride. Also, the administration of pyrazole which inhibits alcohol dehydrogenase should block the metabolism of vinyl chloride.

5. Obtain samples of urine from individuals exposed to vinyl chloride and determine whether similar metabolites are excreted by man and animals.

METHODS

It is anticipated that there will be only one significant deviation in the methodology used in these experiments and the methodology used in other metabolism experiments. In these experiments, it is intended that vinyl chloride will be administered via inhalation. Some work has been done to synthesize an apparatus that will allow exposures to be conducted in a recycled closed system. Since CO_2 must be removed from the system and O_2 added, our one remaining problem is to develop a method to remove CO_2 without affecting the concentration of vinyl chloride. Usually, an alkaline trapping solution is used; however, it is predicted that an alkaline trapping solution will degrade vinyl chloride. Currently, we are considering the use of carbonic anhydrase to convert CO_2 and H_2O to H_2CO_3.

URL 00251

In order to follow the metabolism of vinyl chloride, ^{14}C vinyl chloride will be used. Bids have been obtained

for its synthesis. If ^{14}C labelled cysteine and glutathione are needed, they will be purchased directly as they are commercially available.

Synthetic schemes have been formulated for preparation of glutathione and cysteine conjugates of potential metabolites of vinyl chloride. Some of the potential metabolites have been synthesized.

COST

It is requested that $50,000 be appropriated for this work. This amount will cover one year "best effort" work. Since it is impossible to predict the difficulties encountered in a study of this type, an absolute commitment cannot be made to completing all the work indicated above. The work will be done at cost and any money not spent will be returned. One year after being granted this request, a complete detailed report will be issued for review. Supplemental informal reports will be issued when sufficient data are gathered to justify issuance.

URL 00252

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PROTOCOL FOR A STUDY OF THE EFFECTS OF MATERNALLY INHALED VINYL CHLORIDE ON RAT AND RABBIT EMBRYONAL AND FETAL DEVELOPMENT

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INTRODUCTION

Vinyl chloride is widely used in the preparation of polyvinyl chloride resin, as a co-polymer in Saran and other plastics, as a solvent and as a chemical intermediate. A report of the effect of single exposures of mice, rats and guinea pigs to vinyl chloride by Mastromatteo et al, (1960) indicates that this compound has very low acute toxicity. Anesthesia is the primary significant effect of acute exposure to high concentrations (75,000-100,000 ppm). Use of vinyl chloride as a surgical anesthetic has been discouraged because of its undesirable effect on the circulatory system and its high flammability. The effect of repeated exposure of laboratory animals to vinyl chloride has been reported by Torkelson et al, (1961). Groups of animals were exposed 7 hours/day, 5 days/week for up to six months to either 500, 200, 100 or 50 ppm vinyl chloride in air. Detectable changes occurred at all but the lowest concentration. Repeated exposure for six months to 200 ppm resulted in histologic changes in the centrilobular area of the livers of rabbits but not in rats, guinea pigs or dogs. At 100 ppm, only slight

URL 00253

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liver enlargement was observed. In a study reported by Viola et al, (1971), rats were exposed to 30,000 ppm vinyl chloride vapor for 4 hours daily, 5 days/week for 12 months. Findings on these rats at or before the end of 12 months exposure included severe chronic hepatitis, interstitial pneumonia, as well as tumors of the skin, lungs and bones.

Reports of studies of the potential of vinyl chloride to have a deleterious effect on the developing embryo and fetus have not been found in the literature. The study described in this protocol has been designed to determine whether or not exposure of pregnant rats and rabbits has a deleterious effect on embryonal and fetal development.

URL 00254

EXPERIMENTAL PROCEDURES

A) Design

In an initial study, bred rats and rabbits will be exposed to twice the maximum excursion limit of vinyl chloride (TLV = 200 ppm). Twice the maximum excursion limit of vinyl chloride is 500 ppm (200 ppm x 1.25 x 2). Rabbits will be exposed on days 6 through 18 and rats on days 6 through 15 of gestation for 7 hours on each day. Groups of 30 rats and 15-20 rabbits will be exposed. A group of control rats and rabbits will be exposed in a chamber to filtered room air. If exposure to 500 ppm causes no evidence of maternal toxicity, embryotoxicity or teratogenicity, additional groups of rats and rabbits will be

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exposed to 5,000 ppm on the same days of gestation. If maternal toxicity is observed at 500 ppm, additional higher dose levels will not be studied, regardless of the effect on the embryo and fetus. If embryotoxicity or teratogenicity is observed at 500 ppm, additional lower concentrations will be studied at half-fold decrements until no embryotoxicity or fetal toxicity is evident. Thus, concentrations of 250, 125 or 62.5 ppm, etc, will be studied if a significant embryotoxic effect is observed at 500 ppm in the initial experiment.

B) Exposure Procedure

Exposure of pregnant animals will be carried out in stainless steel dynamic chambers of 3.7 cubic meter volume. The chamber atmosphere will be generated by metering gaseous vinyl chloride at a known rate into a metered stream of air into the chamber. The concentration of vinyl chloride in the chamber atmosphere will be calculated from the ratio of material delivery rate and the total chamber air-flow rate. The analytical concentration will be determined by infrared spectrometry (Beckman IR10). The wave lengths for analysis will be 10.6 and 11.2μ. The chamber concentration will be analyzed periodically during exposure. Combustion conductivity analysis will also be used to continuously monitor the exposure concentration.

URL 00255

C) Animals

Adult New Zealand white rabbits and Sprague-Dawley rats

will be used. The day of natural mating will be considered day zero of pregnancy. Animals will be housed individually in wire-bottom cages and maintained on commercially available laboratory animal chow ad libitum. Animals will not have access to water or food in the inhalation chamber during the exposure period. Food consumption will be measured at 3-day intervals during gestation.

D) Maternal Observations

Animals will be observed daily throughout the gestation period for indications of toxicity from the test material. The maternal body weight of rabbits will be recorded on days 6, 12 and 18 of gestation. The body weight of rats will be recorded on days 6, 10 and 16 of gestation. In addition, maternal body weights and the weight of the maternal liver will be recorded at the time of cesarean section, day 21 in rats and day 29 in rabbits.

URL 00256

E) Teratological Examination

On gestation days 21 and 29 in rats and rabbits, respectively, the pregnant females will be sacrificed by carbon dioxide inhalation and the fetuses will be removed by cesarean section. The following data will be recorded: 1) position and number of fetuses in utero; 2) number of live and dead fetuses; 3) number of resorptions; 4) number of corpora lutea; 5) individual pup weight and crown rump length and 6) gross external abnormalities.

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One-third of each litter will be examined immediately by dissection under a low-power microscope for evidence of soft-tissue abnormalities. Each pup in each litter will be eviscerated and sexed and placed in 95% ethanol, cleared and stained with Alizarin Red S for subsequent examination for skeletal anomalies.

F) Statistics

Statistical evaluation of the frequency of anomalies and resorptions among litters will be made by the Fisher Exact Probability test (Siegel, 1956). Analyses of maternal and fetal body weights and body measurements and liver weights will be made by an analysis of variance. Group means will be compared to controls using Dunnett's test (Steel and Torrie, 1960). The level of significance chosen for all cases is $P<0.05$. The litter is considered the experimental unit of treatment and observation.

URL 00257

G) Estimated Cost

The estimated cost of this study for both species is $8,000 $\pm$ 10% for each concentration to be studied.

H) References

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Viola, P. L. Bigotti, A. and Caputo, A. Oncogenic response of rat skin, lungs and bones to vinyl chloride. Cancer Res. 31, 516, 1971.

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ATTN DR W D HARRIS

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ONE OF THE COMPANIES SUPPORTING MCA-SPONSORED RESEARCH ON VINYL CHLORIDE HAS REASON TO SUSPECT MONOMER EXPOSURE AS POSSIBLE CAUSE OF DEATH OF DECEASED WORKERS. THIS COMPANY DESIRES TO ADVISE THE INDUSTRY PROMPTLY AS TO THE INFORMATION THAT IS AVAILABLE AND ACTIONS BEING TAKEN. WE ARE THEREFORE CALLING A MEETING OF INDUSTRY REPRESENTATIVES FOR THIS PURPOSE TO BE HELD IN MCA'S CONFERENCE ROOM ON FRIDAY, JANUARY 25 AT 9:00 AM.

WE STRONGLY URGE THAT EITHER YOUR MANAGEMENT REPRESENTATIVE OR TASK GROUP MEMBER ATTEND.

A C CLARK, VICE PRESIDENT
TECHNICAL DIRECTOR
MANUFACTURING CHEMISTS ASSN

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