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THREE SPECIFIC ENZYMATIC ALTERATIONS IN LIVER OF RATS EXPOSED TO VINYL CHLORIDE[1]

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[1]This work was supported by a grant from B. F. Goodrich Company, Akron, Ohio, and the Manufacturing Chemists Association, Washington, D.C.

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[3]Portions of this study have been presented in Fed. Proc., 35:329 (1976) Abstract.

Summary

Decreases in the activity of key gluconeogenic enzymes and increases in glucose-6-phosphate dehydrogenase and glutathione reductase have been shown in hepatoma. We exposed Sprague-Dawley rats to 10,000-20,000 ppm of vinyl chloride (VC), 4-8 hrs/day, 5 days/week for 1-4 weeks (14-137 hrs. of exposure) to induce potential liver injury and angiosarcoma formation.

Glucose-6-phosphatase, a key gluconeogenic enzyme in the liver microsomal fraction, decreased its activity 25% over control ($p < 0.05$). Glucose-6-phosphate dehydrogenase, an enzyme in the pentose pathway, increased its activity two-fold after more than 100 hrs. of exposure. Non-protein sulfhydryl levels (glutathione and/or cysteine) remained constant but the activity of the enzyme to regenerate the reduced glutathione, glutathione reductase increased 50-60% during exposure to vinyl chloride ($p < 0.05$) as a means of compensation thus holding the level of reduced glutathione constant. Other microsomal proteins and enzymes related to vinyl chloride metabolism, i.e. P-450, NADPH-cytochrome c reductase and mixed function oxidase were unchanged in the same microsomal fraction. Further, there were no changes in the liver function tests such as serum aminotransferases, nor in liver cytochrome oxidase, a mitochondrial marker for normal cell respiration. The decrease in glucose-6-phosphatase, and increase in glucose-6-phosphate dehydrogenase and glutathione reductase are similar to the enzymatic alterations in hepatomas. This may reflect an increase in ribose-5-phosphate production associated with de novo nucleic acid biosynthesis prior to the development of angiosarcoma.

Introduction

Vinyl chloride has been shown to induce tumors including angiosarcoma in laboratory animals (23,31) and angiosarcoma in man (4). At higher concentrations it is believed that vinyl chloride is metabolized by microsomal mixed function oxidase system (MFO) of the liver to toxic metabolites (2,13) and that these metabolites are detoxified by way of glutathione (13,18,33). When animals were pretreated with phenobarbital, an inducer of MFO, and exposed to 5% vinyl chloride, increased activities of serum alanine aminotransferase (SGPT), an enzyme used as a conventional liver function test, and serum sorbitol dehydrogenase, a liver specific enzyme, were observed (17). There have been, to date no serial enzymatic studies performed to determine the progressive metabolic alterations that occur with vinyl chloride injury before and during the development of angiosarcoma, a cancer of adjacent mesenchymal cells.

In this study, we examined the enzymatic profiles in relation to cellular function of the liver prior to tumor formation by exposure of rats to vinyl chloride. The approach includes: 1) conventional clinical liver function tests in serum, including aspartic aminotransferase (SGOT), alanine aminotransferase (SGPT), lactic dehydrogenase (LDH), alkaline phosphatase (AP), bilirubin, albumin, cholesterol and triglyceride; 2) microsomal proteins and enzymes related to the metabolism of vinyl chloride (P-450, NADPH cytochrome c reductase and mixed function oxidase) and glutathione content; the mitochondrial marker, cytochrome oxidase; the microsomal marker, glucose-6-phosphatase (G-6-P ase) and a pentose phosphate shunt enzyme, glucose-6-phosphate dehydrogenase (G-6-PD) in liver.

Materials and Methods

Materials

NADPH, cytochrome c and oxidized glutathione were purchased from Sigma Chemical Co., St. Louis, Missouri.

Aniline and other chemical reagents were ACS grade. Double distilled water was used throughout.

Animals and Experimental Design

A series of four experiments was conducted in which Sprague-Dawley male rats (300-500 g) were randomly placed into either a group exposed to vinyl chloride gas (10,000-20,000 ppm) or a non-exposed control group. The randomization was performed prior to each of the four experiments.

The animals were exposed in a closed chamber in which the atmosphere was constantly circulated. The chamber was a converted vinyl chloride polymerization vat, used for the manufacture of polyvinyl chloride in the B.F. Goodrich Plant, Louisville, Kentucky with a volume of 1,100 gallons; thus, the respiration of the rats had a negligible effect on the gaseous composition of the enclosed atmosphere. The exposure schedule was 4 or 8 hours per day, 5 days per week for 1-4 weeks. In the first experiment, three animals in each group were sacrificed after the animals had been exposed to vinyl chloride for total accumulated time periods of 14, 28 and 42 hours, a total of 18 animals including equal number of control. The second experiment was a repeat of the first. For the third experiment, there were 5 animals per group sacrificed after 71 hours of exposure and 6 animals per group sacrificed after 103 hours, a total of 22 animals. In the fourth experiment 3 animals per group were sacrificed after exposures of 84 and 137 hours, a total of 12 animals. The control animals were kept at the

University animal care facility in order to guarantee the total absence of vinyl chloride exposure. Two to three hours after exposure, the animals were anesthetized with ether, blood was drawn by cardiac puncture and the animals were sacrificed immediately. They were sacrificed at a fixed time of day to avoid diurnal effects.

Sample Preparation

The liver was excised rapidly and immediately rinsed in ice cold 0.15 N KCl with 0.02 M Tris buffer. A portion of the tissue was homogenized with 9 volumes of the cold KCl-Tris buffer in a Potter Elvehjem homogenizer. Each sample was prepared from a single organ and kept at 0° C during preparation. Remaining liver was frozen rapidly and kept at -20° C.

Subcellular Fractionation

The homogenate was centrifuged at 600 g, and 8,000 g, and 100,000 g to obtain nuclear, mitochondrial, microsomal and cytosol fraction, respectively, by the differential technique of Schneider and Hogeboom (27). Centrifugation procedures were carried out at $0-4^{\circ}$ C in a Sorvall supercentrifuge with a 9RA rotor and a Beckman Model L-5 ultracentrifuge with a swinging bucket rotor. Enzyme activities were determined in the isolated subcellular fractions. P-450, NADPH cytochrome c reductase and cytochrome oxidase were determined in fresh samples and glucose-6-phosphatase, mixed function oxidase, glucose-6-phosphate dehydrogenase and glutathione reductase were determined in freshly isolated fractions from frozen tissue.

Glucose-6-phosphatase was estimated (12) by measuring inorganic phosphate released. P-450 was estimated by the maximum absorption difference between the dithionite-reduced cytochrome and its carbon monoxide complex

(25). P-450 concentration is calculated by using 91,000 $M^{-1}cm^{-1}$ as the extinction coefficient for the increase in peak height between 490 and 450 nm. Cytochrome oxidase was measured as described by Wharton and Tzagoloff (39). NADPH cytochrome c reductase was determined by the method of Degroot and Dunn (5). Mixed function oxidase was determined by the method of Holtzman (15).

Non-protein sulfhydryl-containing compound was estimated by the method of Sedlak and Lindsay (28). The rate of oxidation of NADPH by oxidized glutathione was used as a standard measure of enzymatic activity of glutathione reductase (3). Glucose-6-phosphate dehydrogenase was measured spectrophotometrically as the rate of NADPH formation (20). All enzyme assays were validated. The reaction rate was proportional to time and sample size. The protein content of the fractions was determined by the method of Lowry et al. (21). Conventional clinical biochemical studies of serum including aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT), lactic dehydrogenase (LDH), alkaline phosphatase (AP), bilirubin, albumin, cholesterol and triglyceride were determined by Technicon Sequential Multiple Analyzer Computer (SMAC) system.

Results

Significant differences between the exposed and control groups were obtained in glucose-6-phosphatase, glutathione reductase and glucose-6-phosphate dehydrogenase.

A. Glucose-6-phosphatase. Chart 2A shows ninety-five percent confidence intervals for D, the mean difference between the exposed group and the control groups in each experiment. (The confidence intervals for groups

mean differences on the enzyme discussed are based on the error mean square from a two-factor analysis of variance with interaction, the two main effects being time in hours and exposure-nonexposure. Such an analysis was performed for each of the four experimental occasions).

There is no significant difference between the two groups over the first two experiments since D = 0 is included within both of the confidence intervals. For the third and fourth experiments, however, the level of glucose-6-phosphatase in the exposed animals was significantly less than that of the control animals. Note that for each of the experiments we show only a single confidence interval despite the fact that there are two or three time periods of exposure in each experiment. This results from the nonsignificance of the interaction term in the analysis of variance, meaning that the differences between the exposed and control groups are uniform over the time periods in each experiment. This uniformity is also evident from the parallel profiles in Chart 2B which is the composite curve of the group means of the specific activity with respect to time of exposure. From these experiments we observed that the mean activity of glucose-6-phosphatase decreased 25% after approximately 71 hours of exposure to vinyl chloride gas.

B. Glutathione reductase. Chart 3A shows the 95% confidence intervals for the difference between the exposed group and the control groups, and Chart 3B shows the composite curve of the group means of the specific activity with respect to exposure time. As shown by the 95% confidence intervals in Chart 3A, there is a significant difference between the exposed and control groups in all four experiments except at 71 hours of exposure, which has a p value of 0.06 in the third experiment. This experiment is shown with two confidence intervals because on this occasion the interaction

system to form chlorooxirane (2,13,19) and rearranges to chloroacetaldehyde (11). These two compounds are considered as the ultimate carcinogens (1, 17,30). They have been shown to be mutagenic in bacterial systems (8,10, 22,24), and to serve as an alkylating agent by reacting with adenosine (1) and to bind with protein (19).

Under normal circumstances, the main detoxification route for vinyl chloride metabolism is thought to be via glutathione. This view is supported by the early work of Johnson (18) and the recent discovery of sulfur-containing urinary metabolites of radioactive labeled vinyl chloride in rats (9, 33). The toxic metabolites can be further oxidized to chloroacetic acid (13). It is also possible that chlorooxirane can be detoxified by microsomal epoxide hydrase. This idea is supported by the work of Kappus' group who demonstrated an increase of irreversible vinyl chloride protein binding capacity after inhibiting epoxide hydrase by trichloropropene oxide (19). Based on these findings, we postulate the metabolic fate of vinyl chloride as shown in Chart 1.

Our study did not show a demonstrable change in the activity of mixed function oxidase, NADPH-cytochrome c reductase and the concentration of P-450 which are involved with the oxidation of vinyl chloride (2,13). Drew's group (5) has studied the effect of vinyl chloride exposure on the activity of benzphetamine demethylation and the concentration of cytochrome P-450 in rat liver. The result differed depending on the time interval between cessation of exposure and sacrifice of animals. When they sacrificed animals 18 hours after exposure, there was no difference in demethylatron nor in cytochrome P-450. However, when they sacrificed 4 days later, there was a decrease in both demethylation capacity and cytochrome P-450. From Drew's and our results, one can probably conclude that the enzyme system for vinyl chloride oxidation is

of the time and exposure factors was significant; i.e., the difference between the exposed and control groups after 103 hours was significantly greater than the difference after 71 hours. This can also be seen by the differences f the mean values at 71 and 103 hours as shown in Chart 3B. Hence, the level of glutathione reductase in exposed animals was about 25% greater than that in nonexposed animals up to 71 hours, and the observed difference was further increased to approximately 50% after 84 hours of exposure up to 137 hours.

C. Glucose-6-phosphate dehydrogenase. From the 95% confidence intervals of Chart 4A one can see that there is no significant difference between the groups over the first two experiments from 14 to 42 hours. In the third and fourth experiments, however, the difference at 71 and 84 hours was not significant whereas that at 103 and 137 was. The difference can also be seen in Chart 4B which is a composite plot of the group means of the specific activity with respect to exposure time. After about 84 hours of exposure, the mean level of glucose-6-phosphate dehydrogenase in the exposed animals was about twice that of the nonexposed animals.

The above differences occurred while other liver function tests, serum aminotransferases, LDH, alkaline phosphatase, etc., showed no significant changes. Nor could we detect any change in mitochondrial marker, cytochrome oxidase in liver. Liver microsomal enzymes related to the metabolism of vinyl chloride, mixed function oxidase, P-450 and NADPH cytochrome c reductase also remained unchanged.

Discussion

Our present understanding of vinyl chloride carcinogenesis implies that the compound at high levels is oxidized by liver mixed function oxidase

not inducible by its substrate, vinyl chloride. Jaeger, et al. (17) using serum, found elevated activities of both alanine aminotransferase and sorbitol dehydrogenase in rats pretreated with phenobarbital and exposed to vinyl chloride. However, they could not detect any elevation in these serum enzymes in rats pretreated with phenobarbital and exposed consecutively to vinyl chloride for 5 days and they suggested that the initial exposure to vinyl chloride seemed to protect against acute injury on re-exposure.

Under our experimental conditions, we found that the non-protein sulfhydryl content of liver (glutathione and/or cysteine) did not change in rats exposed to vinyl chloride. This finding is unexpected because Hefner and Watanabe (13,33) have found a depletion of non-protein sulfhydryl content in rats exposed to VC and Johnson (18) found a depletion of liver glutathione when chloroethanol was fed to rats. In a preliminary experiment, we found that the non-protein sulfhydryl content decreased sharply one hour after injection of chloroethanol, but the level went back to normal about 5 hours after injection and stayed normal afterwards (Du, J.T. and Tamburro, C.H.[5]; 40). Since we did not sacrifice our animals immediately after exposure; rather, we sacrificed 3 hours later, we might have missed the decrease seen by others. However, we did see an increase in the level f glutathione reductase, which converts the oxidized glutathione to reduced glutathione in all four experiments. This would serve two purposes: to maintain the level of reduced glutathione for detoxification as a compensation by enzyme and to supply $NADP^+$ as a co-factor for various synthetic pathways, including nucleic acid synthesis.

[5]Unpublished data.

In hepatoma the activity of key enzymes for gluconeogenesis have been shown to decrease in parallel with an increased rate of tumor growth (34,36,37). In contrast, two enzymes for the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (29,38) and transaldolase (14) were found to be increased in all hepatomas regardless of growth rate. In addition, the activity of glutathione reductase was increased in hepatomous liver induced by a chemical, diethylnitrosamine (26). Further, when carcinogens such as nitrosamine and dimethyl aminoazobenzene were fed to rats, the activity of glucose-6-phosphatase decreased prior to and with the development of hepatomas (16,35).

Our finding of a decreased activity in glucose-6-phosphatase and an increased activity in glucose-6-phosphate dehydrogenase and in glutathione reductase in rat liver after exposure to vinyl chloride may be an early biochemical alteration indicative of early liver injury associated with subsequent development of angiosarcoma. These differences were observed before any sign of abnormalities were detectable by conventional liver function tests, serum aminotransferases or by mitochondrial marker, cytochrome oxidase activity in liver.

In hepatocytes, chlorooxirane may rearrange to chloroacetaldehyde, and then be detoxified via glutathione, and subsequently metabolized and excreted in the urine (Chart 1). In situations when glutathione is insufficient for complete detoxification of these metabolites or the activity of the detoxification enzymes is too low to detoxify them, chloroacetaldehyde may be reduced to chloroethanol, and then be transferred to mesenchymal cells and there be reoxidized to the aldehyde form, but may not be oxidized further to the acid or detoxified by glutathione due to metabolic limits of these mesenchymal cells. Alternatively, the mesenchymal cell

may act metabolically similar to the hepatocyte in the oxidation of vinyl chloride, but may have a decreased or limited capability of detoxification leading to increased amounts of chloroacetaldehyde in contrast to the hepatocyte thereby allowing for increased possibilities of DNA injury.

There is an obvious difference in the cellular etiology of hepatoma (primary cell carcinoma) and angiosarcoma. The former is a cancer of hepatocytes and the latter, a cancer of mesenchymal cells. The concept that the hepatocyte's ability to detoxify the active metabolite of vinyl chloride, thereby protecting itself from DNA injury is given some further support by the observation of Maltoni et al. (personal communications, 1976) who have produced primary hepatocellular carcinoma in new born rats exposed to vinyl chloride and shown only the development of angiosarcoma in the adult rats. The newborn rat liver may have hepatocytes which are more vulnerable to the toxic metabolites. Studies have already begun to confirm Maltoni's observation and the enzymatic capabilities of hepatocytes from newborn rats. Whether these metabolic changes reflect a precancerous alteration in the hepatocytes regardless of the cell origin of the cancer, as seen in primary liver cell cancer, must yet be determined. Studies are underway to follow these enzymatic changes until induction of these two cell types of liver cancer.

Acknowledgement

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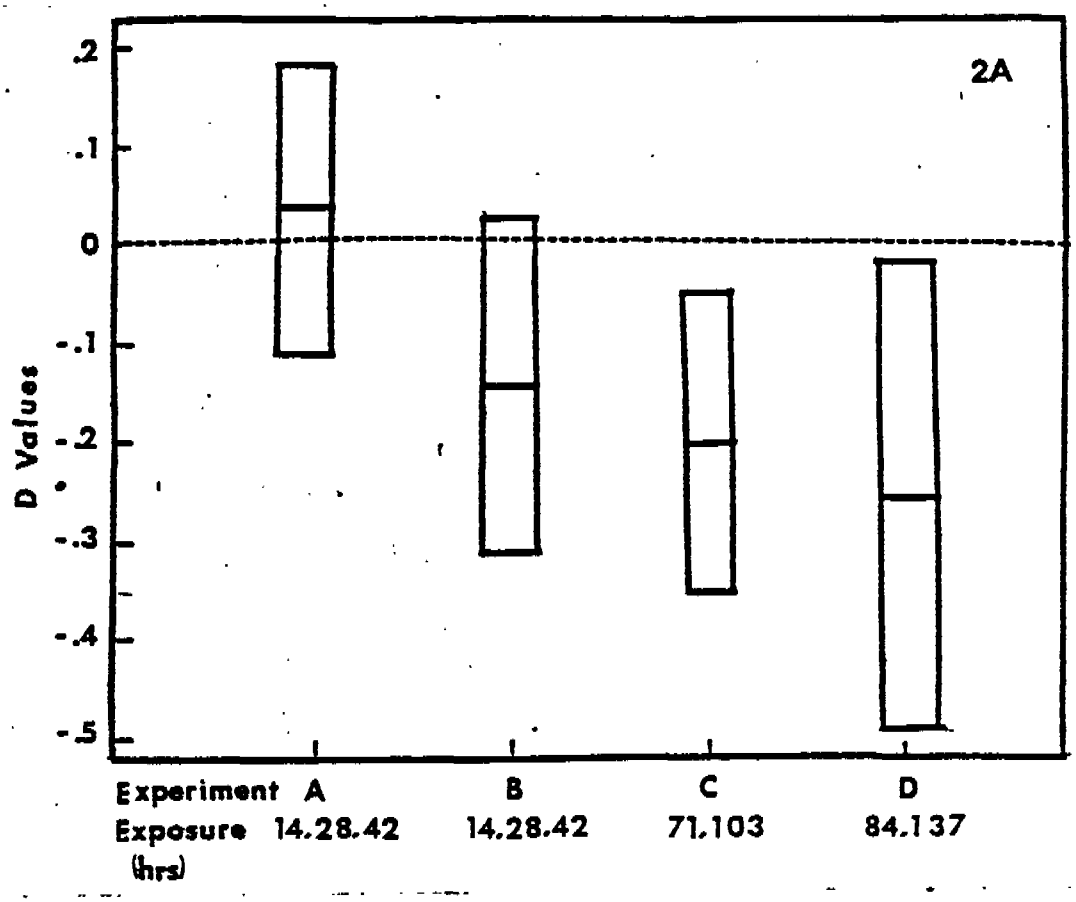
Chart 1. The proposed metabolic fate f vinyl chloride. GSH, glutathione; MFO, mixed function oxidase; VC, vinyl chloride. The detailed steps involved in the formation and detoxification of chloroethanol have not been demonstrated firmly in a biological system. Chlorooxirane can be reduced by sulfhydryl reagents such as cysteine and/or glutathione to chloroethanol in vitro without adding an enzyme.[4] It is likely that chloroethanol is first oxidized to chloroacetaldehyde, conjugated with glutathione to form $GSCH_2CHO$, and then reduced to $GSCH_2CH_2OH$ in vivo (18).

[4]Personal communication with Dr. John Wong, Professor, Department of Chemistry, University of Louisville, Louisville, Kentucky.

Cl CH = CH2
(V C)
LIVER MFO
Cl CH-CH2
O
(CHLOROOXIRANE)
Cl CH2CH2OH
(CHLOROETHANOL)
+GSH
Cl CH2CHO
(CHLOROACETALDEHYDE)
Cl CH2COOH
(CHLOROACETIC ACID)
GS CH2CH2OH
GS CH2CHO
GS CH2COOH
DETOXIFICATION WITH GLUTATHIONE
N-Ac-S-(2-HYDROXY ETHYL) CYSTEINE
THIODIGLYCOLIC ACID

Chart 2. Part 2A shows the 95% confidence intervals for difference between vinyl chloride exposed and control groups on glucose-6-phosphatase. The ordinate denotes D, the difference between the exposed and control group, and the abscissa denotes the exposure time in hours. Part 2B shows the composite curve of the group means of the specific activity of glucose-6-phosphatase with respect to time of exposure. Experimental rats, shown with 0, were exposed to 10,000-20,000 ppm of vinyl chloride for various periods of time; and control rats, shown with Δ, were unexposed. Detailed exposure schedule, methods and statistical analysis of results were described in the text. Each point represents the mean of 6 individual animals excepting periods of 71 hr. in which only 5 animals were used and 84 and 137 hr. in which only 3 animals were involved.


2A
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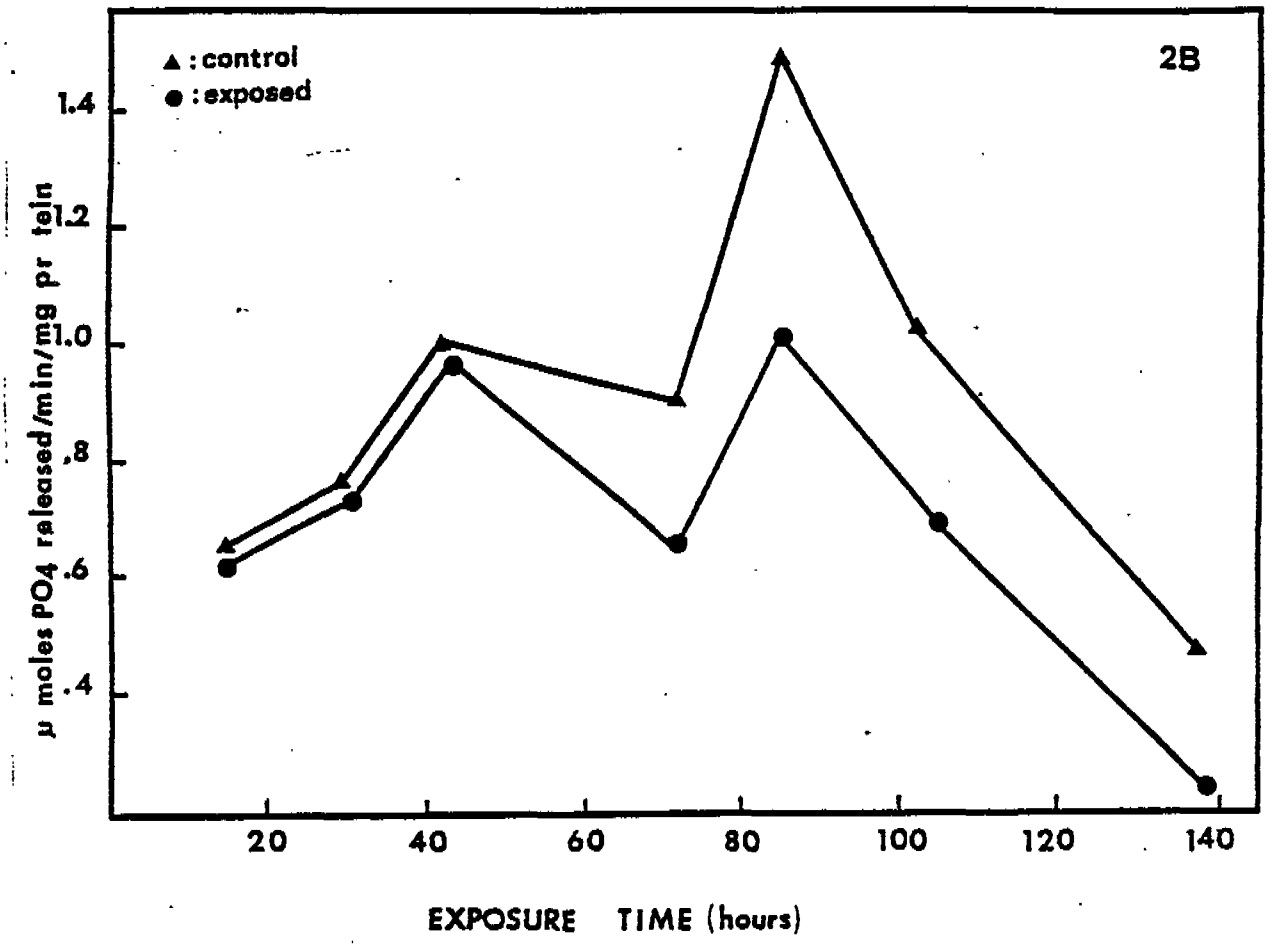
D Values
Experiment A B C D
Exposure 14.28.42 14.28.42 71.103 84.137
(hrs)


▲ : control
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2B
1.4
1.2
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.6
.4
µ moles PO4 released/min/mg pr tein
20
40
60
80
100
120
140
EXPOSURE TIME (hours)

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Chart 3. Part 3A shows the 95% confidence intervals for difference between vinyl chloride exposed and control groups on glutathione reductase. Part 3B shows the composite curve of the group means of the specific activity of glutathione reductase with respect to time of exposure. Experimental rats, shown with O, were exposed to 10,000-20,000 ppm of vinyl chloride for various periods of time and control rats, shown with Δ, were unexposed. Same animals were used as in Chart 2. See text and footnote to Chart 2 for other experimental and statistical details. The mean value of the exposed is significantly more than that of the control groups throughout the entire experiment ($p < 0.05$) except at 71 hours of exposure which has a p value of 0.06.

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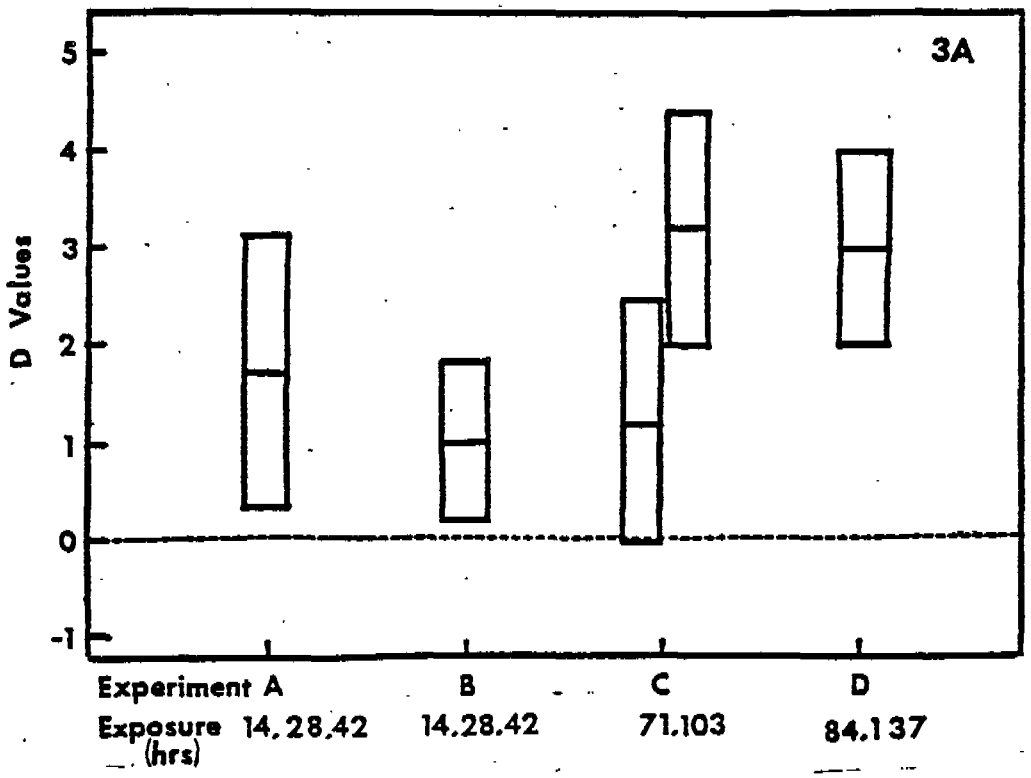
3A
D Values
5
4
3
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Exposure (hrs) 14.28.42
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71.103
84.137

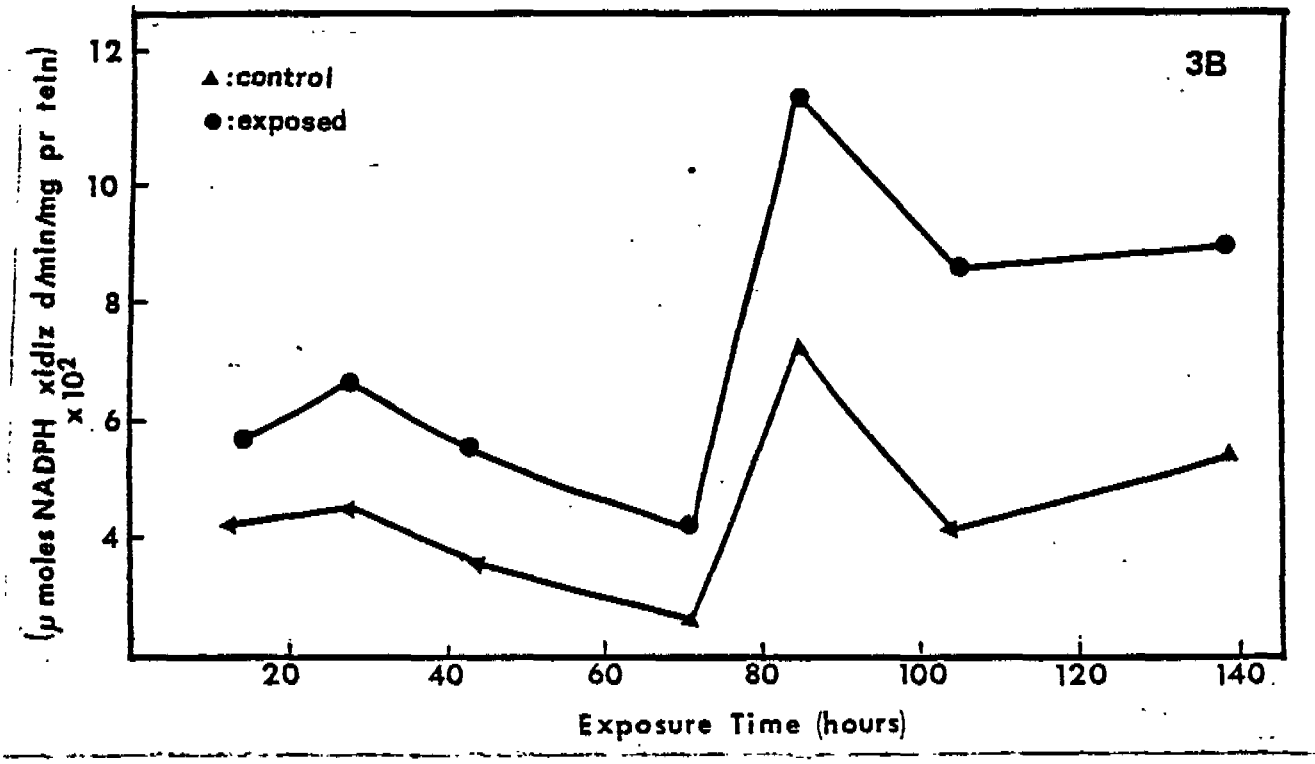
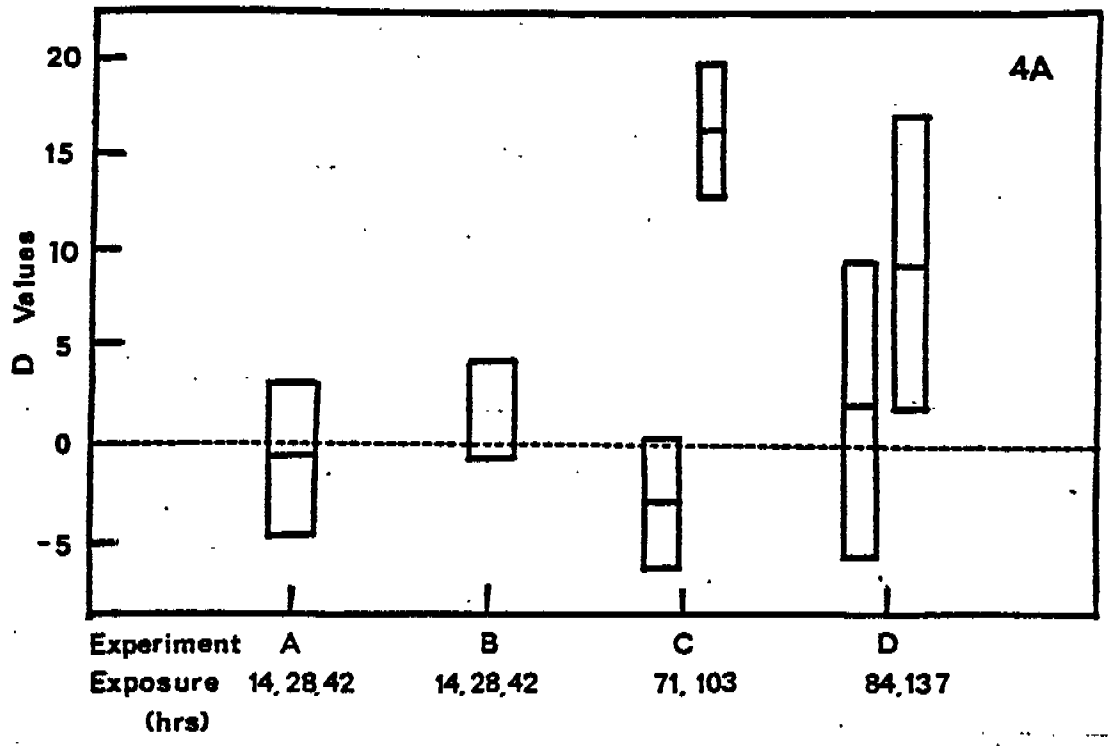
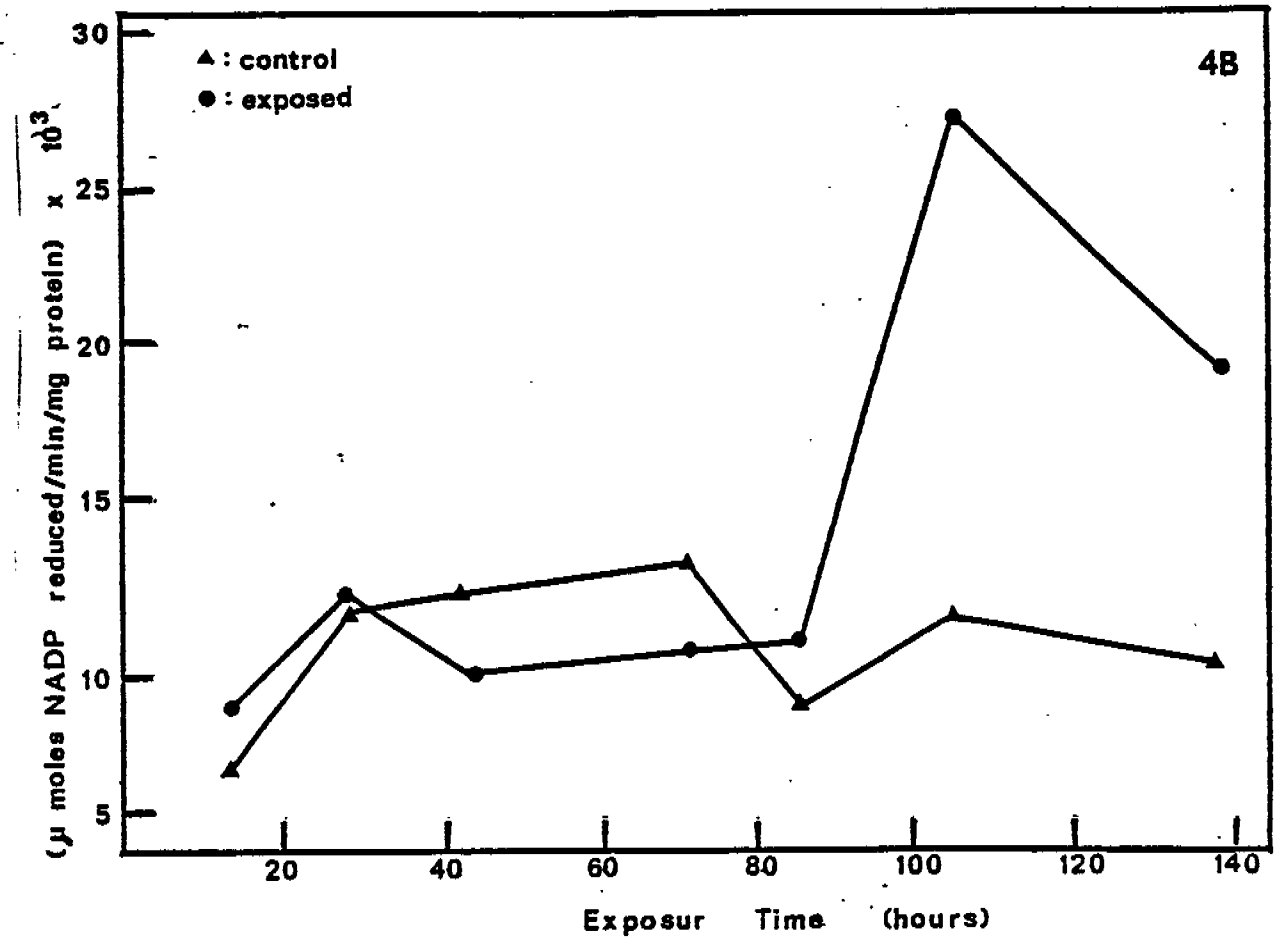

3B
▲:control
●:exposed
(μ moles NADPH xidiz d/min/mg pr tein) x 10²
12
10
8
6
4
20
40
60
80
100
120
140
Exposure Time (hours)

Chart 4. Part 4A shows the 95% confidence intervals for D, the mean difference between the exposed and control group. Part 4B shows the composite curve of the group means of the specific activity of glucose-6-phosphate dehydrogenase with respect to time of exposure. See footnote to Chart 2 about the experimental and statistical details. There is no significant difference between the experimental and control groups up to 84 hours, since D ≈ 0 is included in the confidence interval, but the mean level of exposed group is significantly more than that of the control after 103 hours of exposure.


4A
D Values
20
15
10
5
0
-5
Experiment A B C D
Exposure 14, 28, 42 14, 28, 42 71, 103 84, 137
(hrs)


4B
▲ : control
● : exposed
(μ moles NADP reduced/min/mg protein) x 10³
30
25
20
15
10
5
20
40
60
80
100
120
140
Exposur Time (hours)

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