

**ACUTE HEPATOTOXICITY AND
ENZYMATIC RESPONSE TO HYDRAZINE AND
1,1-DIMETHYLHYDRAZINE IN RATS**

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FOREWORD

This work was performed at the Ohio State University, Division of Occupational Medicine, Department of Preventive Medicine, under Contract AF33(657)1698, with the Toxic Hazards Branch, Physiology Division, Biomedical Laboratory, Aerospace Medical Research Laboratories, Wright-Patterson Air Force Base, Ohio. The work was conducted in support of Project 6302, "Toxic Hazards of Propellants and Materials." Charles F. Reinhardt, MD, was the principal investigator. The original data was presented in Dr. Reinhardt's thesis in partial fulfillment of the requirements for the degree of Master of Science. The thesis has been condensed and revised as a technical report by Mildred K. Pinkerton. The work began in September 1963 and was completed in May 1964.

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This technical report has been reviewed and is approved.

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ABSTRACT

The in vivo and in vitro effects of hydrazine and of 1,1-dimethylhydrazine (UDMH) on blood serum and liver tissue enzyme activities were studied. The specific enzymes investigated were lactic dehydrogenase, isocitric dehydrogenase, malic dehydrogenase and glutamic acid dehydrogenase. Activity levels were determined at 16, 24, 48 and 72 hours after intraperitoneal administration of the toxic agents to rats. Attempts were made to correlate enzyme levels with altered liver pathology. Results were compared with a concomitant study of the effects of carbon tetrachloride on the same enzymes and on liver pathology. Results indicated that the mechanisms of toxic action for these three chemical agents are probably dissimilar and that serum enzyme levels may not provide sensitive indications of altered liver pathology in the case of hydrazine or UDMH.

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SECTION I

INTRODUCTION

The current interest in the toxicity of hydrazine and 1,1-dimethylhydrazine (UDMH) has been generated by the fact that these two agents serve as high energy fuels to propel missiles. The toxicity of these compounds has been established by a number of investigators, and they constitute a health hazard for personnel using them at military establishments as well as for civilian workers at manufacturing sites.

Hydrazine has been established as a hepatocellular toxic agent, although there is doubt as to whether less than an LD-1 dose produces liver damage. This work attempts to determine whether hydrazine and UDMH produce liver damage after an acute exposure to sublethal concentrations of these agents. Even more important is development of an indicator of liver damage, since at this level of exposure, clinical evidence of illness might not be readily apparent. This might be done by measuring a biochemical response such as a change in serum enzyme activity. Dinman et al (ref 1) and others (ref 2) have shown experimentally that there is a definite measurable serum enzyme response following the liver damage which is produced by an acute exposure to carbon tetrachloride. Recently, Patrick and Back (ref 3) observed a significant elevation in serum glutamic oxaloacetic transaminase (SGOT) in monkeys following multiple injections of hydrazine. The dose ranged from 5 to 20 mg/kg body weight for a total of 4 to 20 injections.

Acute exposure to sublethal amounts of carbon tetrachloride in humans gives a significant elevation of serum glutamic oxaloacetic transaminase (ref 4). The elevation is probably due to liver damage with escape of the enzyme into the serum. Determination of serum enzyme activity levels plays an important role in diagnosis in modern medicine. These determinations offer measurable evidence of tissue damage or disease which may not be apparent at the clinical level.

The magnitude of the enzyme response might be correlated with the degree of exposure and the severity of liver damage. Cornish and Block (ref 5) reported that in the case of carbon tetrachloride, the magnitude of enzyme response bears a direct relationship to the degree of exposure. Wirtschafter and Tsujimura (ref 6) attempted to correlate SGOT activity with hepatocellular injury produced by carbon tetrachloride. They found that a high SGOT value was always accompanied by marked liver damage, while, conversely, a low value was not a reliable indication for the presence or absence of pathological liver changes.

In view of the present exposure potential to Hydrazine and UDMH, it is expedient to learn the answers to these questions.

The basic hypothesis of this study was that hydrazine and UDMH would produce liver damage under the conditions imposed by the experiments. The technical approach involved microscopic examination of liver tissue as well as measurement of serum and liver enzyme responses. The enzymes studied were lactic

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dehydrogenase (LDH), malic dehydrogenase (MDH), isocitric dehydrogenase (IDH), and glutamic dehydrogenase (GDH).

A concomitant study using carbon tetrachloride as an established known hepatotoxic agent was done in order to provide a standard reference point for correlation with hydrazine and UDMH relative to the production of liver damage and its magnitude, not only as judged by pathological change but also by enzymatic response.

SECTION II

METHODS AND MATERIALS

Experimental Animals

Approximately 200 healthy, male rats of the Wistar strain, 12 weeks old, and weighing 200-300 grams were used in all studies. The animals were housed at a temperature range of 70-75° F and were maintained on an ad libitum diet of nonmedicated Purina Laboratory Chow and water. They were kept in this stress-free environment for about 5 days prior to use. The animals were randomly assigned to groups of 6 animals each, except for the control group which contained 21 animals.

Exposure

The experimental groups were exposed to 3 doses of hydrazine and 2 of UDMH. The intraperitoneal (i.p.) route of administration was used in all cases. Witkin (ref 7) and Back (ref 15) have indicated that the route of administration has little influence on the toxicity of UDMH and hydrazine. The dose regimens were as follows:

Hydrazine:	Dose 1	A single injection of 45 mg/kg
	Dose 2	Two injections of 45 mg/kg, 24 hours apart.
	Dose 3	A single injection of 50 mg/kg
UDMH:	Dose 1	A single injection of 80 mg/kg
	Dose 2	Two injections of 70 mg/kg, 24 hours apart

Hereafter, all doses will be referred to as Hydrazine, dose 1, 2 or 3; and UDMH, dose 1 or 2.

The solutions for injection were freshly prepared each day from commercially obtained hydrazine and UDMH. The stock concentrations were hydrazine, 1000 mg/ml, and UDMH, 781.6 mg/ml. Both concentrations were stored in tightly stoppered brown bottles.

These injection doses were chosen because it was necessary to work at a dose level which would produce few, if any deaths. The single injection doses are close to the LD-1 level of the dose-response slopes for both hydrazine and UDMH.

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Four groups of 6 rats each were exposed to 2000 ppm carbon tetrachloride for 6 hours. These rats served as a positive response comparison group, since serum enzyme changes and liver damage have been shown to occur at concentrations less than those used in this study (ref 1).

One group of 6 rats was fasted for 16 hours, after which they were killed in order to determine the effects, if any, of fasting on the various experimental parameters. Another group of 6 rats was pretreated with Phenergan, 12.5 mg/kg, i.p. 1 hour prior to injection of hydrazine, dose 1. This was done in order to determine whether Phenergan helps to maintain cell permeability in hydrazine poisoning as it does in carbon tetrachloride intoxication (ref 8). The animals were killed 16 hours after hydrazine injection.

Blood and liver samples were obtained at 16, 24, 48 and 72 hours after exposure to the various agents, except for the fasting and the Phenergan-treated groups. The control and experimental animals were killed in a like manner.

Materials

Hydrazine (Practical Grade), 1,1-dimethylhydrazine (Practical Grade), and pyruvic acid were obtained from Eastman Organic Chemicals, Rochester, New York. Carbon tetrachloride (Technical Grade) was obtained from the Dow Chemical Company, Midland, Michigan. Phenergan was obtained from Wyeth Laboratories, Philadelphia, Pennsylvania, and all enzymes, substrates, and related reagents were obtained from the Sigma Chemical Company, St Louis, Missouri.

Preparation of Serum and Liver

The rats were anesthetized with ether and an incision was made in the inguinal area. The tissues were bluntly dissected until the femoral artery and vein were exposed. The vessels were cut and the blood was collected in a test tube. The abdominal contents were then exposed and the entire liver was removed. Random portions of the liver were taken for microscopic sections, and a 1-gram block of liver tissue was obtained after being blotted on filter paper. The tissue was then homogenized. The time required to obtain the specimens was approximately 5 minutes or less.

Samples were kept cool by placing them immediately in an ice water bath. The blood was centrifuged at 3000 rpm at 5° C for 15 minutes; the serum was removed and recentrifuged to remove all traces of red blood cells.

The 1-gram blocks of liver were homogenized in 9 ml of ice cold 0.25 M sucrose by hand, 20 excursions being used for each homogenate.

The liver slices for microscopic section were fixed in 10% formalin solution or Carnoy's solution. Those fixed in formalin were stained in hematoxylin-eosin (H & E) and oil red O, while those fixed in Carnoy's solution were stained in Best's carmine.

Enzyme Assay and Activity

In the spectrophotometric procedures, the change in absorbency at 340 mu was measured by a Beckman, Model DU spectrophotometer, using a Gilford optical

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density converter with a Bristol recorder. A temperature of 22° C was maintained in the cuvette chamber by a water cooling jacket. All reagents were prepared immediately before use and were preincubated at 22° C before assay.

In the LDH assay, standard phosphate buffer, M/15, at pH 7.4 and reduced nicotinamide adenine dinucleotide (NADH), 1 mg/ml, were used. After NADH oxidation by endogenous substrate, pyruvic acid was added to a concentration of 0.00033M, and recording was initiated immediately thereafter. Conditions for the assay of MDH were identical to those employed for LDH adding oxaloacetic acid to a concentration of 0.00025 M in place of pyruvic acid.

Isocitric dehydrogenase was determined essentially by the method of Wolfson et al (ref 9) and glutamic dehydrogenase by the method of Snoke (ref 10). The blank contained 1.5 ml of 0.33 M Tris buffer, pH 8.0; serum or homogenate, and distilled water to a final volume of 3.0 ml. The experimental cell contained the same quantity of serum or homogenate and buffer; 0.2 ml NAD, 2.5 mg/ml; 0.2 ml L-glutamic acid, 14.7 mg/ml, and distilled water to a final volume of 3.0 ml. The pH of the glutamic acid solution was adjusted to 8.0 with NaOH. The glutamic acid solution was added after there was no more observable NAD reduction by endogenous substrates.

Serum activity of LDH, MDH, ICD, and GDH is expressed as the change in absorbency per ml of serum per minute, times 1000 at 22° C. Liver activity of these enzymes is expressed as the change in absorbency per mg of protein per minute, times 1000 at 22° C. In order to present the liver enzyme activity in these units, it is necessary to determine the amount of protein present per ml of liver homogenate. This was done using the Biuret reaction according to the method of Gornall et al (ref 11).

Enzyme Inhibition

A series of in vitro determinations was performed in an attempt to show whether hydrazine and UDMH inhibit enzyme activity directly. Purified enzyme and substrate were allowed to react at concentrations that produced less than a maximum reaction velocity. The appropriate concentrations of enzyme and substrate were determined by combining different concentrations of each until a linear relationship was established by plotting the reciprocal of the substrate concentration against the reciprocal of the velocity. Once a suitable enzyme concentration was established, it was maintained constant while the substrate concentration was varied until the linear relationship was established. Four different substrate concentrations were used in each case, thus providing 4 points to establish the line. Each point on the various plots represents a single determination.

The line established when the enzyme and substrate were combined alone represents the control. Dilute solutions of hydrazine or UDMH were then added to the enzyme approximately 1 to 2 minutes before addition of the substrate. The change in absorbency was then determined in the same manner as described under enzyme assay. The same concentrations of enzyme and substrate were used in the experimental determinations as for the control. The results were graphed according to the plot of Lineweaver and Burk (ref 12). The reciprocal of the substrate concentration was plotted on the abscissa while the reciprocal of the velocity was plotted on the ordinate.

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The addition of albumin (bovine), 1 mg/ml, in the final 3.0 ml volume was performed in an attempt to show whether hydrazine and UDMH might be bound by albumin and rendered inactive.

A different approach to the question of inhibition was prompted by a study done by Cornish and Block (ref 5), to determine whether carbon tetrachloride produced serum enzyme inhibition. A sample of normal rat serum was divided into multiple equal portions. The activity of the 4 enzymes under study was determined in one of these portions to serve as the control. Hydrazine or UDMH was added to the other portions to give two of the same concentrations as were present in the cuvette in the in vitro study; enzyme activity was then redetermined after 1 to 2 minutes.

SECTION III

RESULTS

Enzyme Activity

Control serum and tissue enzyme activities are summarized in table I.

TABLE I

CONTROL ENZYME ACTIVITIES
(LL and UL represent lower and upper confidence limits)

Enzyme	Source	Mean	LL(.05)	UL(.05)
LDH	Serum	524	421	626
	Liver	1060	958	1163
MDH	Serum	716	561	870
	Liver	1353	1149	1558
ICD	Serum	9.0	7.1	10.9
	Liver	113	95	132
GDH	Serum	5.7	4.7	6.7
	Liver	6.7	5.0	8.4

Experimental serum and tissue enzyme changes from control values are presented in a gross fashion in table II. The mean enzymatic activities and the confidence limits (6 animals) are shown in figures 1-5.

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TABLE II
EXPERIMENTAL SERUM AND TISSUE ENZYME RESPONSE

AGENT and DOSE	HOURS	E N Z Y M E S							
		LDH		MDH		ICD		GDH	
		Serum	Liver	Serum	Liver	Serum	Liver	Serum	Liver
H Y D 1	16	(+)	(-)	+	(-)	+	(-)	(+)	(-)
	24	(+)	(-)	+	(-)	-	(-)	(-)	(-)
	48	(+)	+	(+)	(+)	-	+	+	+
	72	(+)	+	(+)	(+)	+	(-)	-	-
R Z I N 2	16	-	(-)	(-)	(-)	(-)	(-)	-	(-)
	24	(+)	(-)	(+)	+	-	(-)	+	-
	48	+	(-)	+	+	-	(-)	+	(-)
	72	(+)	-	+	+	-	(-)	+	+
E 3	16	(+)	(-)	-	(-)	(-)	(-)	-	(-)
	24	+	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	48	-	(-)	(-)	-	(-)	(-)	-	(-)
	72	(+)	(-)	(-)	+	(-)	-	-	(-)
U D 1	16	(+)	-	(+)	+	+	-	(-)	-
	24	+	(-)	-	-	(-)	(-)	(-)	-
	48	+	(+)	(-)	(+)	-	(+)	-	-
	72	-	-	(-)	+	(-)	(-)	-	(-)
M H 2	16	+	(-)	-	(+)	(-)	-	-	(-)
	24	(+)	(-)	+	(+)	(-)	-	(-)	-
	48	(-)	-	(-)	+	(-)	-	(-)	+
	72	+	-	(-)	(+)	(-)	-	+	+
C C L ₄	16	(+)	(-)	(+)	(-)	(+)	(-)	+	(-)
	24	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
	48	+	(-)	+	(-)	+	(-)	(+)	(-)
	72	-	(-)	-	-	+	(-)	-	(-)

Note: The experimental results as related to the control values are summarized in a gross fashion. The significant ($\alpha = .05$) changes are in parentheses. The others merely show the trend. Each symbol is based on the mean of six determinations. The dosages are as described in "Methods and Materials." "Hours" indicates time elapsed following exposure.

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The mean enzyme activities and the confidence limits ($\alpha = .05$) are shown in figures 1 - 5. The stippled area represents the confidence interval for the control, the control mean being shown by the solid vertical line within this area. The circled dots show the significant ($\alpha = .05$) variations from the control. Units of enzyme activity are as described in "Methods and Materials."

In figures 1 - 4, the doses of hydrazine and UDMH are denoted by the large numbers 1, 2, 3, and 1, 2, respectively. These doses correspond to those described in "Methods and Materials."

The upper limits of the liver LDH and MDH activity 48 hours after exposure to dose 1 of UDMH are shown numerically since these points lie off the graph.

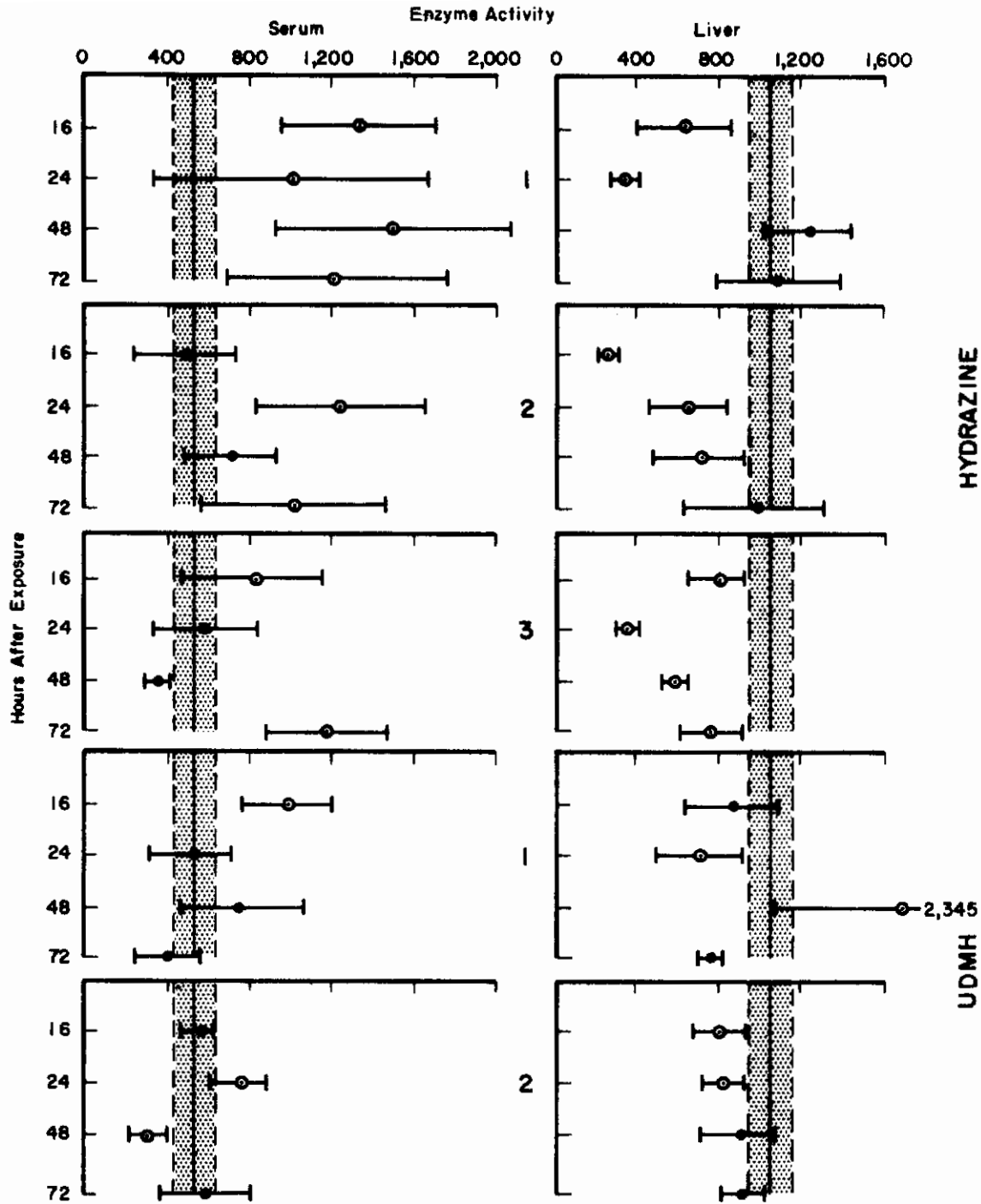


Figure 1. Lactic Dehydrogenase

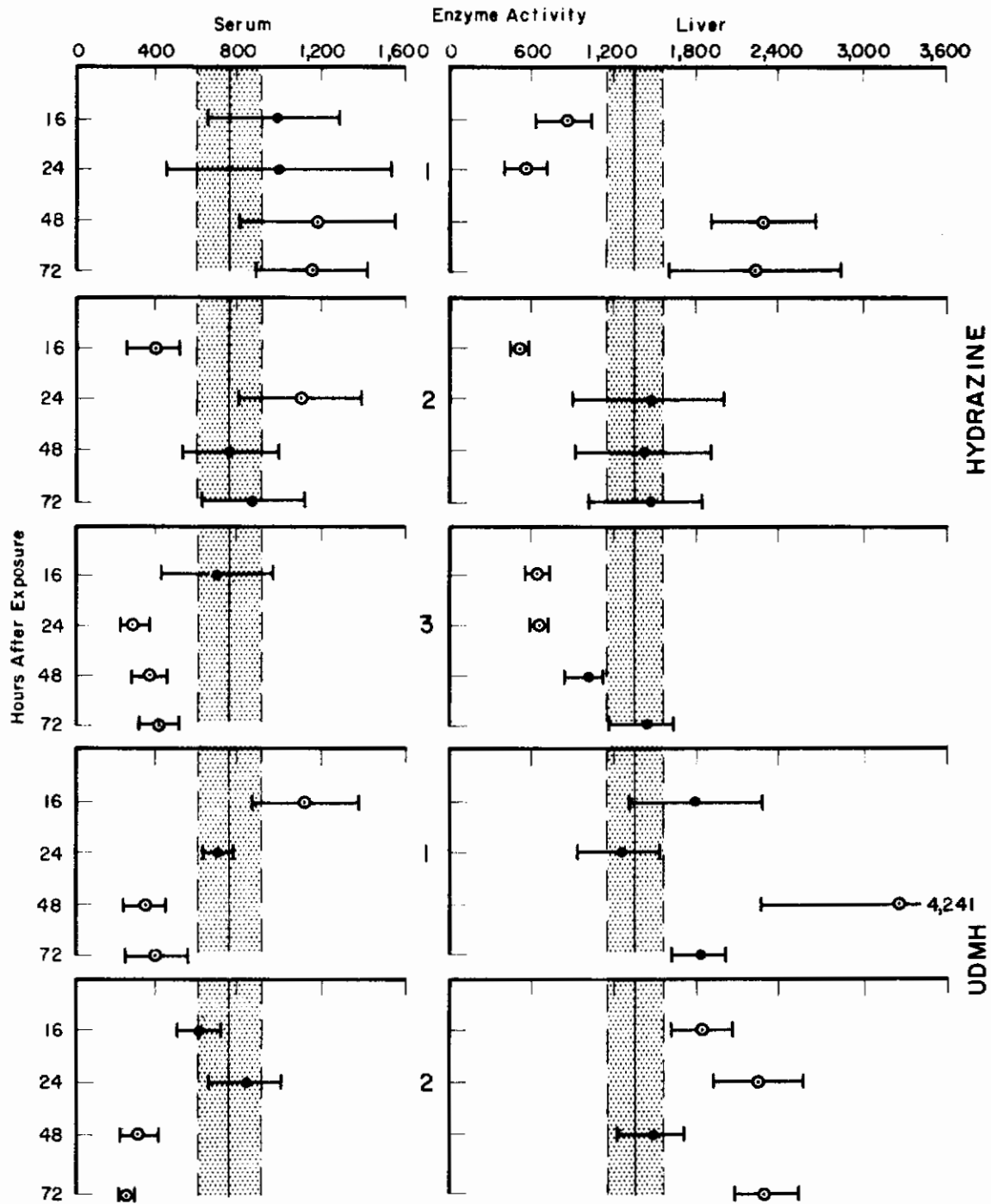


Figure 2. Malic Dehydrogenase

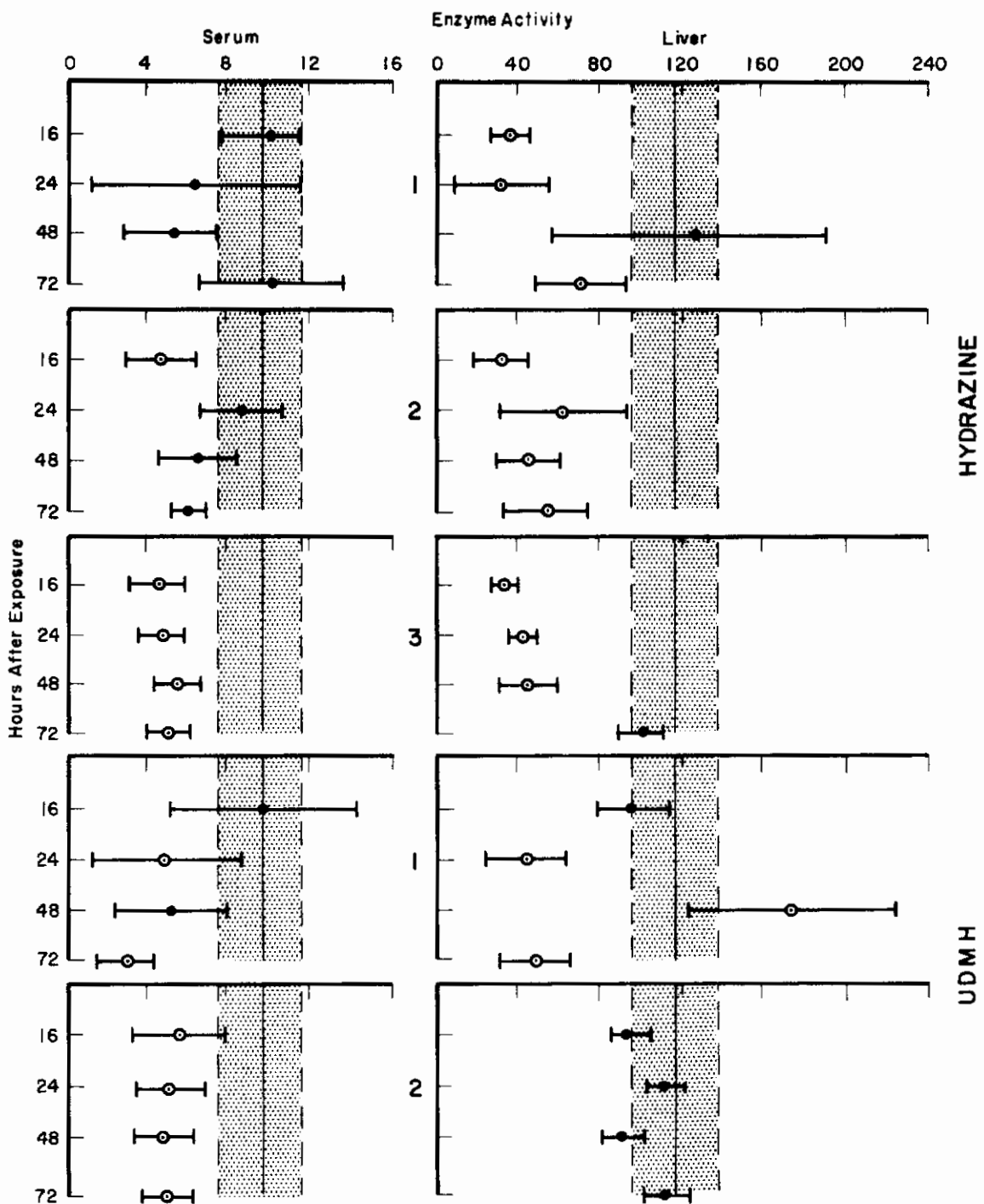


Figure 3. Isocitric Dehydrogenase

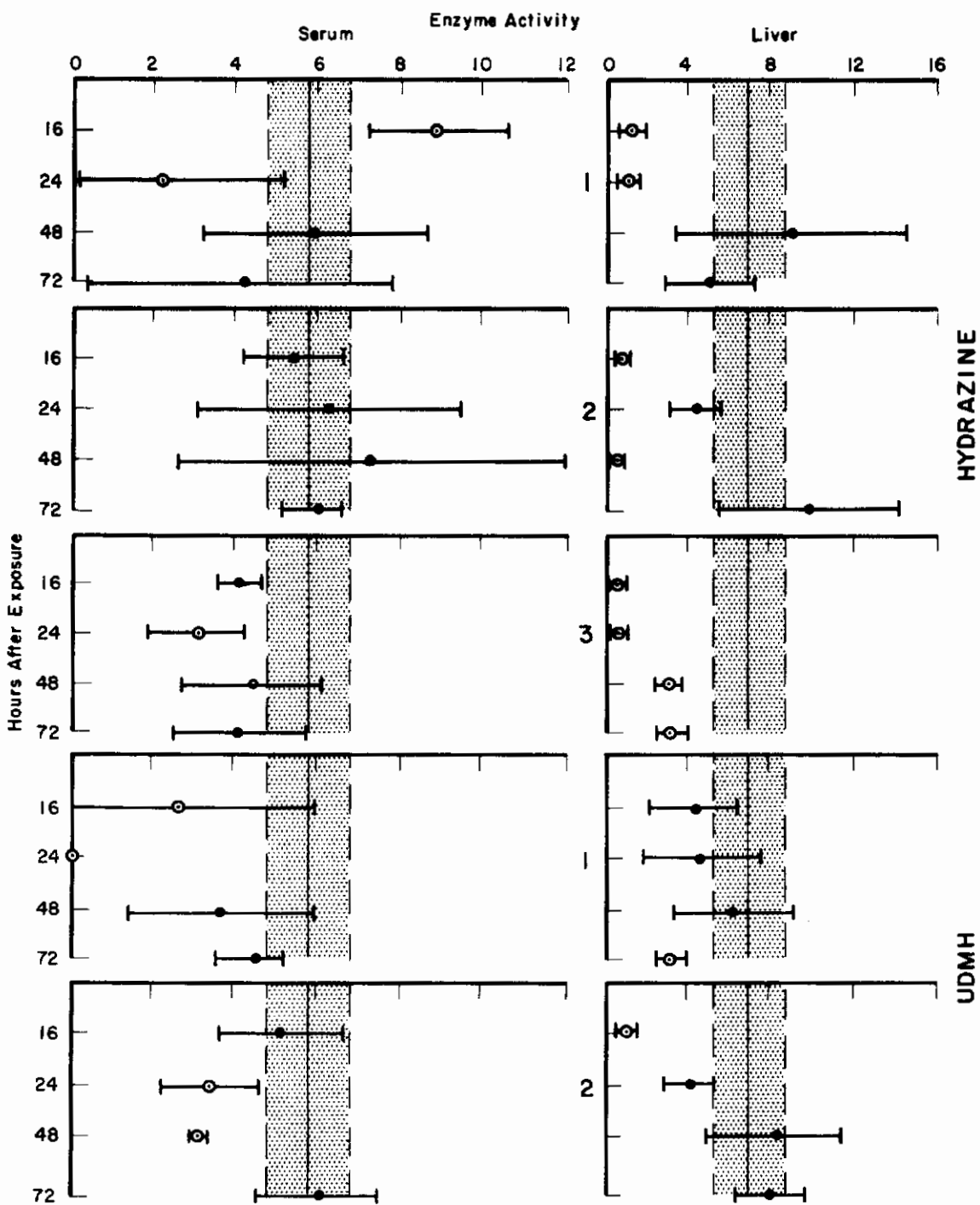


Figure 4. Glutamic Dehydrogenase

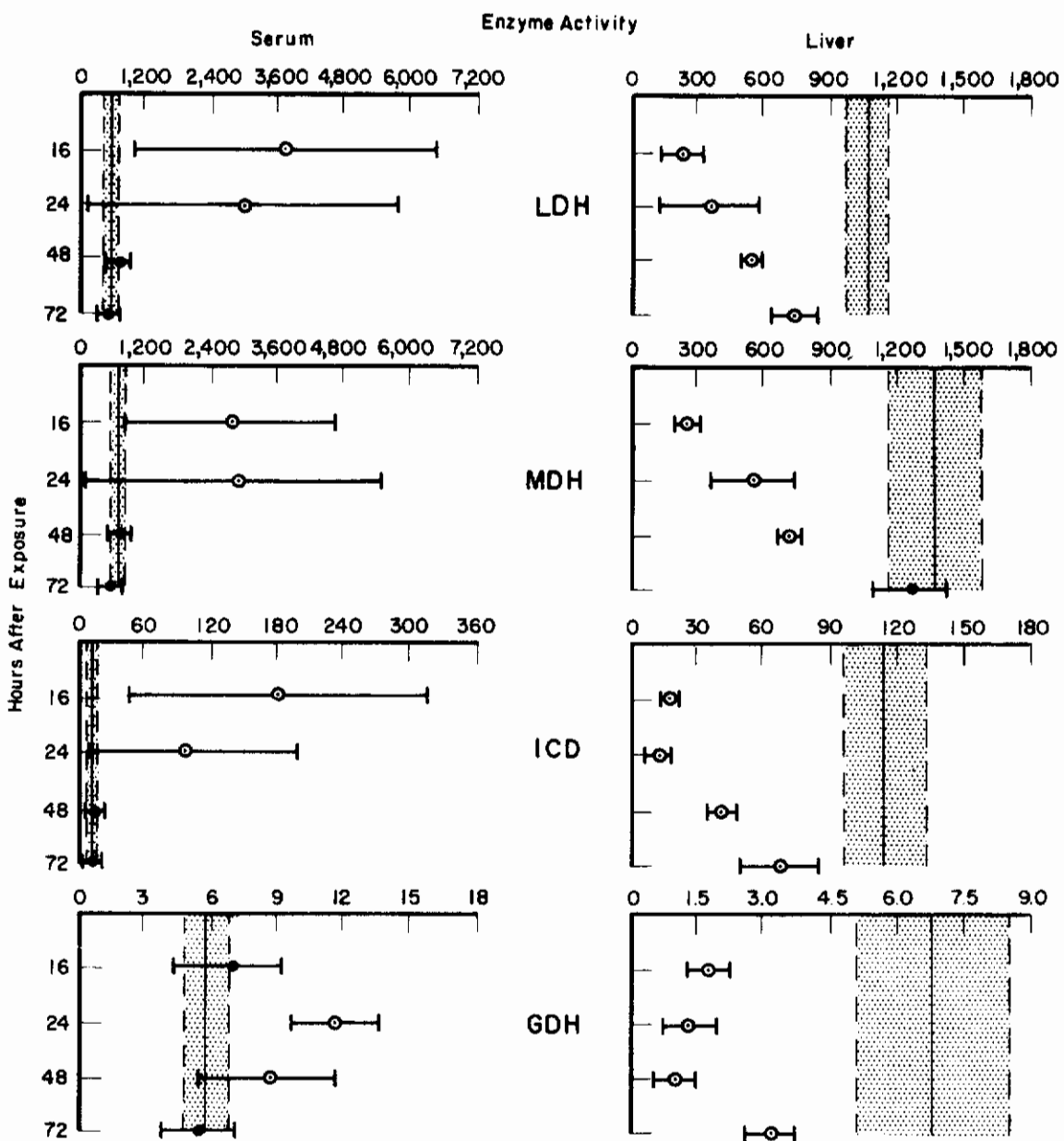


Figure 5. CCL₄ 2000ppm

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Inanition

Since the animals had a period of anorexia of about 16 hours duration following the administration of hydrazine, a study was done to determine whether fasting per se for this period of time had any effect on enzyme response and cellular structure. Samples of blood and liver were taken immediately after the fasting period. The rats were allowed water ad libitum. The results are shown in table III.

TABLE III
ENZYME RESPONSE TO FASTING FOR 16 HOURS

	LDH		MDH		ICD		GDH	
	Serum	Liver	Serum	Liver	Serum	Liver	Serum	Liver
Control	524	1060	716	1353	9.0	113	5.7	6.7
Fasting	483	2407	550	1559	2.5	102	5.3	2.9

Phenergan Pretreatment

One group of 6 rats was pretreated with Phenergan prior to the administration of hydrazine. The animals were killed 16 hours after the administration of hydrazine because this was felt to be a representative period of the greatest enzymatic response following a single injection exposure. The results are shown in table IV

TABLE IV
EFFECT OF PHENERGAN PRETREATMENT ON ENZYME RESPONSES

	LDH		MDH		ICD		GDH	
	Serum	Liver	Serum	Liver	Serum	Liver	Serum	Liver
Control	524	1060	716	1353	9.0	113	5.7	6.7
Hz + Phenergan	425	367	342	670	6.5	34	5.2	0.4
Hz (dose 1)	1350	623	950	844	9.3	36	8.8	1.2

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In Vitro Inhibition

The question arose concerning the possibility of direct enzyme inhibition by hydrazine and UDMH since they are highly reactive compounds and might be expected to interfere with protein activity. The results are shown graphically in figure 6.

Inspection of the graphs reveals that inhibition did occur. There was complete inhibition of GDH and ICD activity by a 6.66×10^{-4} M concentration of UDMH. ICD was also completely inhibited by the same concentration of hydrazine.

The addition of albumin (1 mg/cc) to hydrazine and UDMH prior to their addition to the enzymes did not prevent the inhibition.

Hydrazine and UDMH produced no apparent inhibition when added directly to normal rat serum to give concentrations equal to the two higher ones used in the in vitro study. The results are shown in table V.

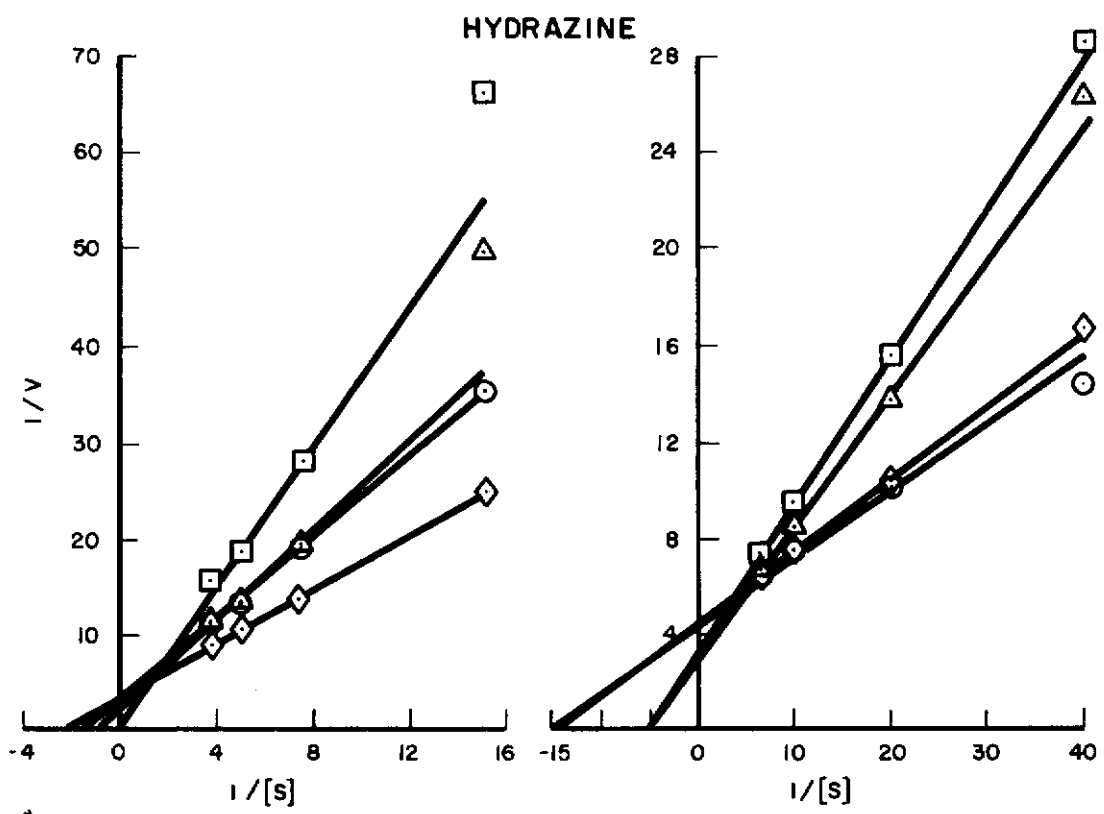
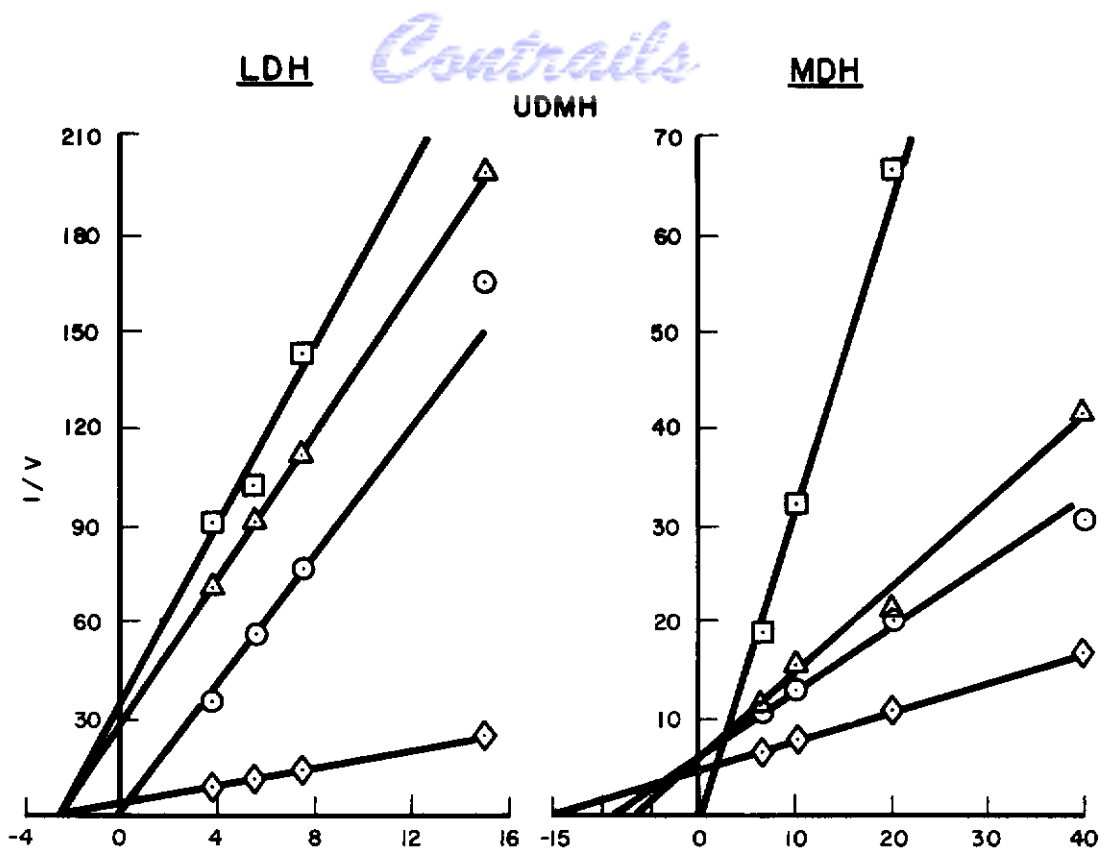
TABLE V

EFFECT OF HYDRAZINE AND UDMH ON SERUM ENZYME ACTIVITY

	SERUM ENZYME ACTIVITY			
	LDH	MDH	ICD	GDH
Control Serum	550	590	10	6
Serum with Hydrazine $3.33 \times 10^{-4}M$	575	590	10	7
Serum with Hydrazine $6.66 \times 10^{-4}M$	675	650	10	7
Serum with UDMH $3.33 \times 10^{-4}M$	525	650	8	8
Serum with UDMH $6.66 \times 10^{-4}M$	675	635	11	9

The in vitro direct inhibition results are shown graphically in figure 6. The reciprocal of the millimolar substrate concentrations is plotted on the abscissa while the reciprocal of the observed reaction velocity is plotted on the ordinate.

The method of plotting is that of Lineweaver and Burk (ref 12). The point where the extended line meets the abscissa equals $-\frac{1}{K_m}$.



- ◇ Indicates control curve.
- Indicates presence of Hydrazine or UDMH in $1.66 \times 10^{-4}M$ concentration.
- △ Indicates presence of Hydrazine or UDMH in $3.33 \times 10^{-4}M$ concentration
- Indicates presence of Hydrazine or UDMH in $6.66 \times 10^{-4}M$ concentration

Figure 6. In Vitro Direct Inhibition Results

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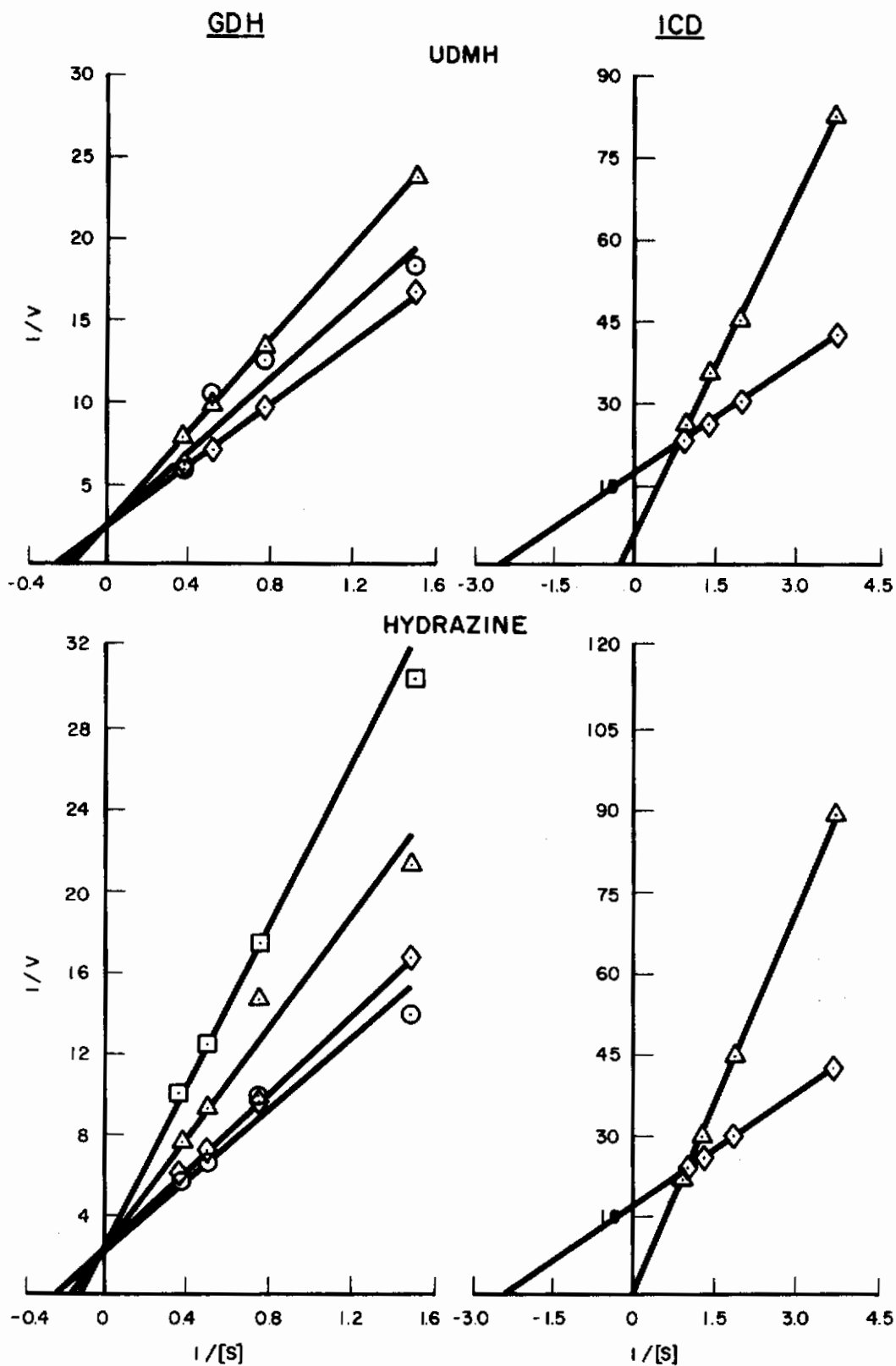


Figure 6 (continued)

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Pathological Examination

Controls. H & E stained liver sections showed no vacuoles. Fat stains revealed periportal fat in small amounts in several slides. There was a wide variation in Best's carmine-stained sections. The glycogen present was evenly distributed. The experimental animal tissues which manifested no change greater than controls were rated as essentially negative.

Hydrazine, dose 1. At 16 hours the H & E stained sections showed small vacuoles in the periportal and midzonal areas. There was no glycogen depletion. The picture was essentially unchanged at 24 hours. Oil red O stains at 48 hours confirmed periportal and midzonal fat. There was no glycogen depletion. By 72 hours the microscopic picture was essentially normal.

Hydrazine, dose 2. At 16 hours the H & E slides showed vacuoles in the periportal and midzonal areas. Fat stains confirmed that moderate-to-heavy amounts were present in the periportal and midzonal regions. There was complete absence of glycogen on Best's carmine-stained slides. The findings were about the same at 24 hours, but there was considerably more fat than in dose 1. After 48 hours there were only a few vacuoles; the fat and glycogen stains were nearly normal. The changes seen at 24 hours had cleared for the most part. At 72 hours there were still some vacuoles, and in some of the oil red O stains more fat was present in the midzonal areas than in the controls. Most of the glycogen stains were normal, but glycogen was absent in two of them. There was considerably more fat in this 72-hr group than was seen at 48 hours; however, it was not nearly as striking as at 16 and 24 hours. The discrepancy at 48 and 72 hours may have been a sampling phenomenon or perhaps these animals had not eaten well prior to being killed.

Hydrazine, dose 3. At 16 hours the H & E stained slides displayed vacuoles in the periportal and midzonal areas (figure 7). The oil red O stains confirmed that moderate-to-heavy amounts of fat were present in the periportal and midzonal areas (figure 8). Glycogen was decreased on the Best's carmine-stained sections (figure 9). Fat accumulation was somewhat less than in dose 2, but slightly greater than in dose 1. After 24 hours H & E and oil red O stains indicated periportal and/or midzonal fatty change in all animals. There was absence of glycogen in all animals. By 48 hours the liver was returning to normal. The changes were less pronounced than at 16 and 24 hours. The liver was essentially normal at 72 hours (figures 10, 11, 12).

The changes associated with hydrazine seemed most prominent at 24 hours with a return to normal at 72 hours. There was no necrosis associated with hydrazine. For the most part the fatty change was moderate. There was no distortion of hepatic cells by large vacuoles. The fasted animals manifested depletion of glycogen. Pretreatment with Phenergan appeared to have no effect on fatty change.

UDMH, doses 1 and 2. There were no pathological abnormalities related to the experiment.

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Carbon tetrachloride. The changes were fairly uniform at 16 hours. There was considerable fat around the central veins. A few cells at the periphery of the area revealing fatty change had undergone "balloon degeneration," ie, cellular swelling and pyknosis of nuclei. There was no actual necrosis; there was absence of glycogen. At 24 hours there was pericentral necrosis and/or severe cellular swelling (figure 13). The same cells which showed "balloon degeneration" were still present in the midzonal areas. The changes were more severe than at 16 hours. Fat was present around the central veins (figure 14) and glycogen was absent in these areas (figure 15). At 48 hours there was either a proliferation of Kupffer cells or infiltration of mononuclear cells around the central veins. Regeneration was in progress as evidenced by numerous mitotic figures around the damaged area. The "balloon cells" have dropped out, leaving empty spaces. There was much less fat around the central veins and glycogen was increasing. By 72 hours cellular debris had been cleared away. The mononuclear cell infiltrate was decreased and repair was almost complete (figure 16). Glycogen was nearly normal, except that it was slightly diminished around the central veins (figure 17).

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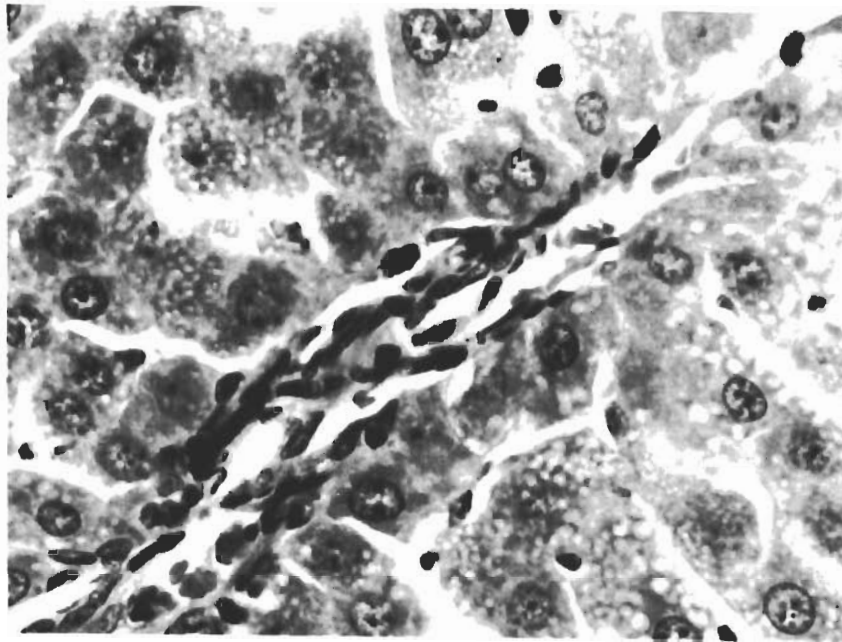


Figure 7. Vacuoles in the Periportal and Midzonal Areas. Hematoxylin and Eosin; X 680 (16 hours after exposure, hydrazine, dose 3).

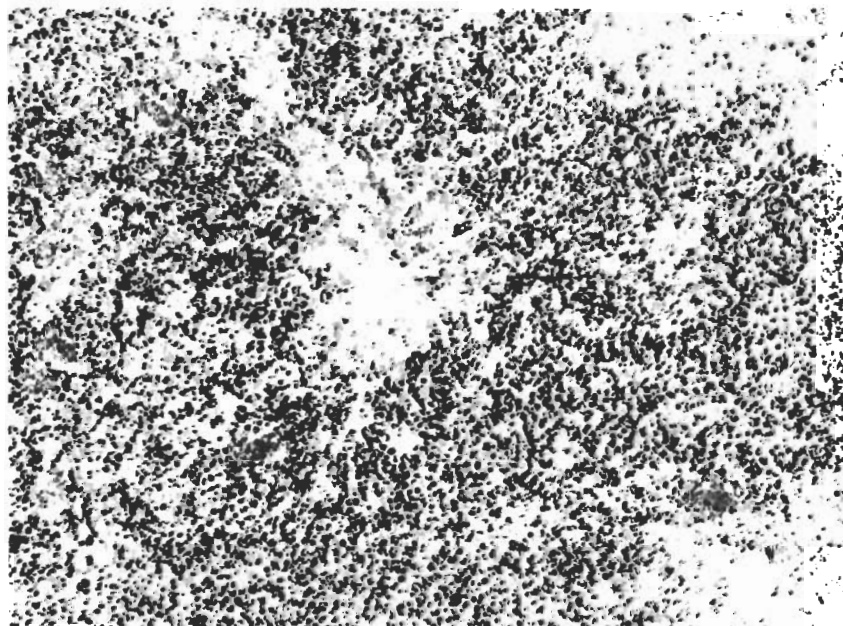


Figure 8. Fat Accumulation in the Periportal and Midzonal Areas. Oil red O; X 100 (16 hours after exposure, hydrazine, dose 3).

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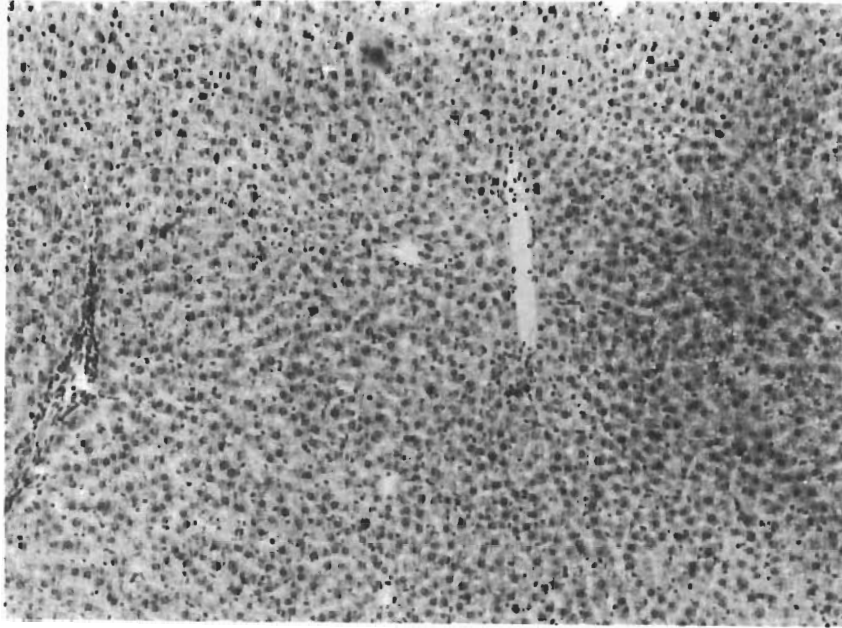


Figure 9. Glycogen Depletion Generalized.
Best's carmine; X 100 (24 hours after
exposure, hydrazine, dose 3).

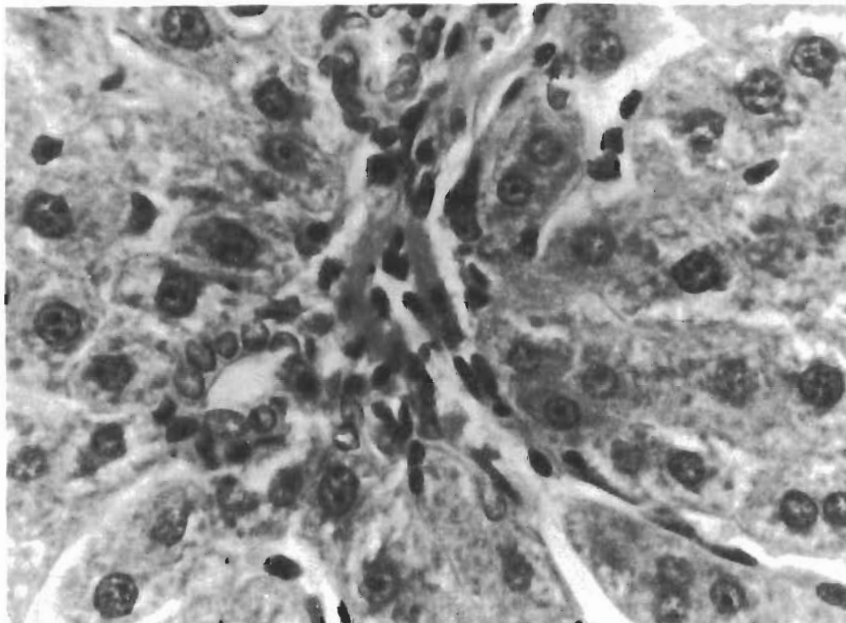


Figure 10. Absence of Vacuoles with Return to Normal.
Hematoxylin and eosin; X 680.
(72 hours after exposure, hydrazine, dose 3).

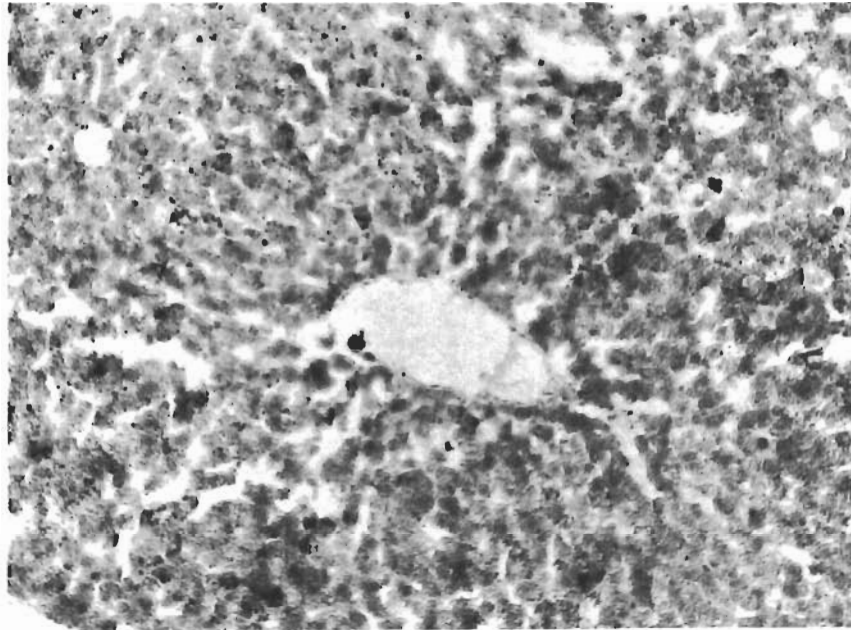


Figure 11. Fat Accumulation Almost Gone.
Oil red O; X 100
(72 hours after exposure, hydrazine,
dose 3).

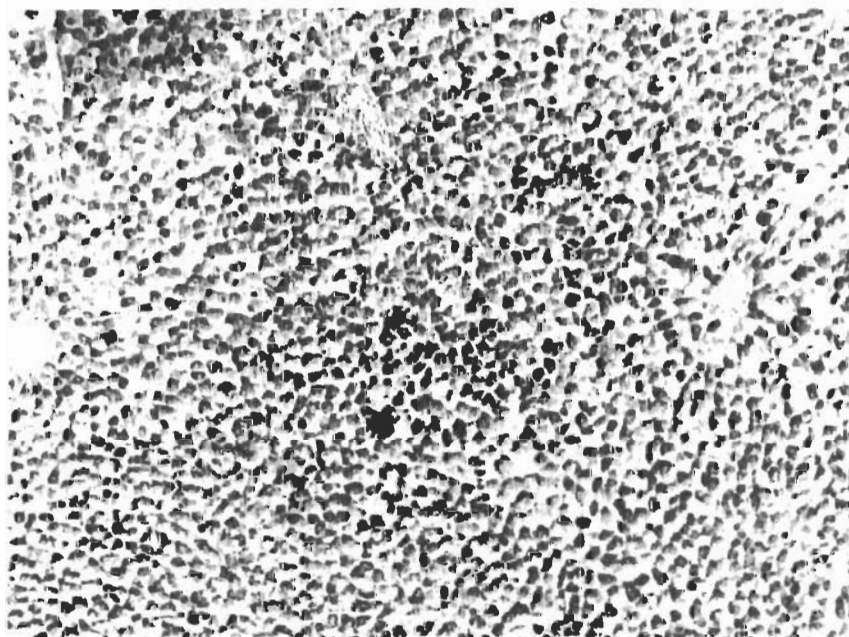


Figure 12. Glycogen Returning to Normal.
Best's carmine; X 100.
(72 hours after exposure, hydrazine,
dose 3).

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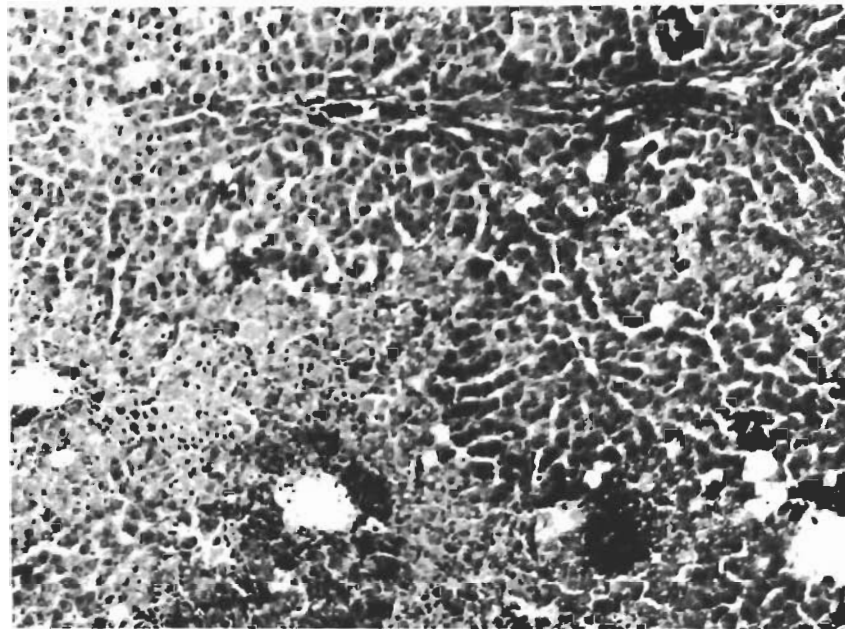


Figure 13. Necrosis in the Peri-Central Area.
Hematoxylin and eosin; X 100.
(24 hours after exposure, 2000 ppm
 CCl_4).

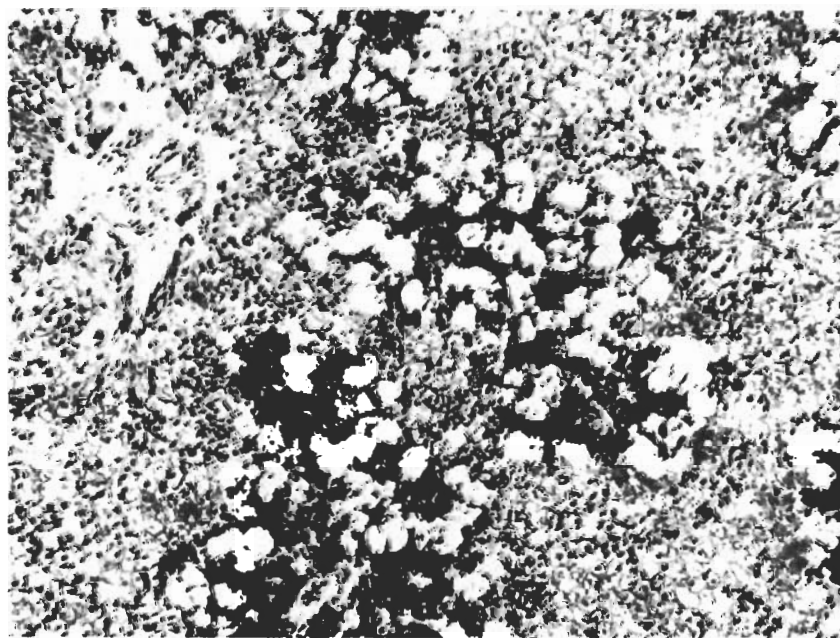


Figure 14. Fat Accumulation in the Peri-Central Region.
Oil red O; X 100
(24 hours after exposure, 2000 ppm CCl_4).

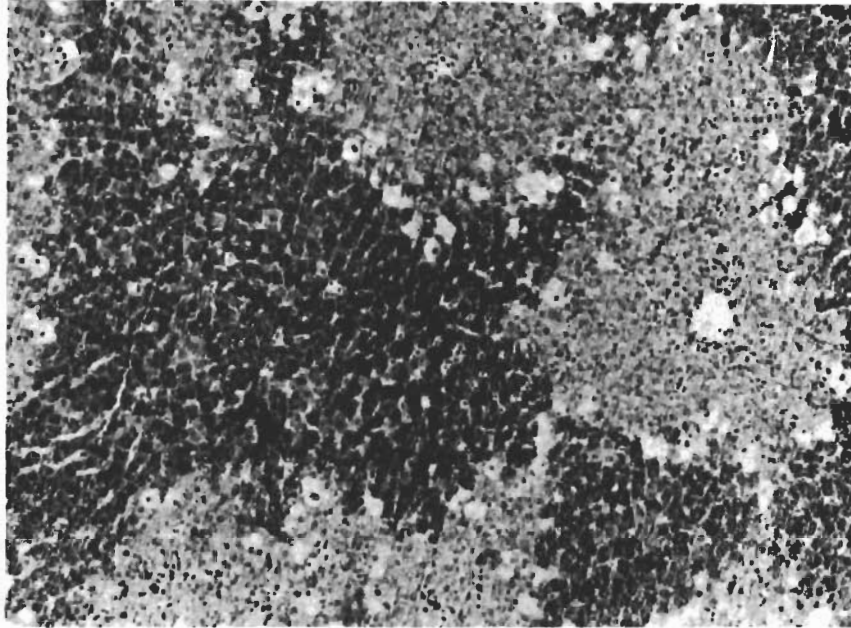


Figure 15. Glycogen Depletion in the Peri-Central Region. Best's carmine; X 100. (24 hours after exposure, 2000 ppm CCl_4).

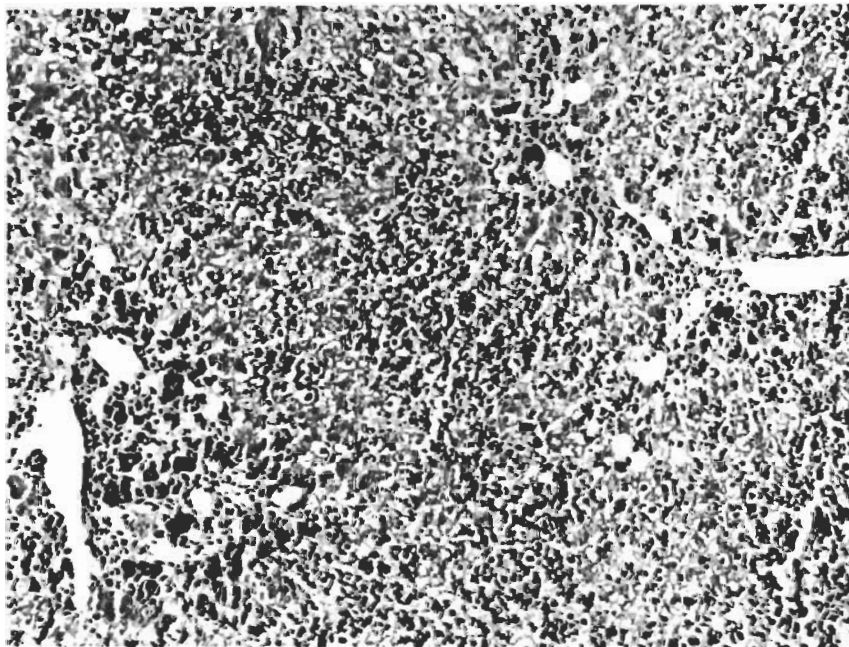


Figure 16. Return to Normal Almost Complete. Cellular Debris cleared and Mononuclear Cell Infiltrate is Decreased. Hematoxylin and eosin; X 100 (72 hours after exposure, 2000 ppm CCl_4).

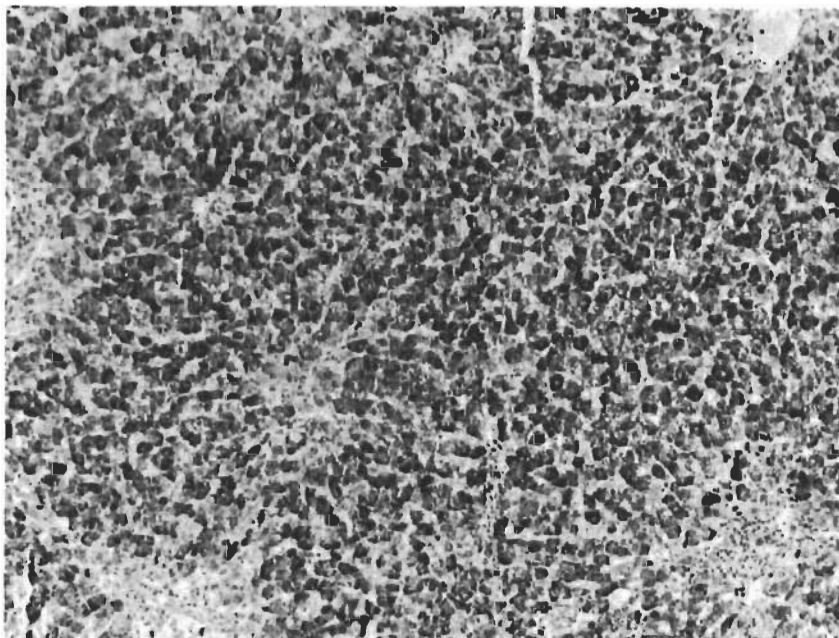


Figure 17. Glycogen Reaccumulation, Slightly Diminished Around Central Vein.
Best's carmine; X 100.
(72 hours after exposure, 2000 ppm CCl_4).

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SECTION IV

DISCUSSION

Enzyme Activity Response

The results indicate that increasing doses of hydrazine do not cause an increase in serum LDH, MDH, ICD, or GDH activity. In fact, except for LDH, the larger doses seem to cause a depression in serum enzyme activity, contrary to the effect of increasing doses of carbon tetrachloride which has been shown to cause increasing response with increasing dosages (ref 13). Liver tissue enzyme response to hydrazine did bear a direct relationship to magnitude of dose in that the higher doses of hydrazine caused general enzymatic depression for a longer duration than did the lowest dose of hydrazine.

There was essentially no correlation between magnitude of dose and degree of either liver or serum enzyme response after administration of UDMH.

The serum and liver tissue enzymes response to carbon tetrachloride exposure was definite and followed a pattern. Serum enzyme activities of LDH, MDH, and ICD were significantly increased at 16 and 24 hours, while GDH activity was significantly elevated at 24 and 48 hours after termination of the exposure. By 72 hours, serum activity had returned to essentially normal values for each of the enzymes. The liver activity was significantly decreased at each of the hourly intervals after exposure for all the enzymes studies, with the exception of MDH activity at 72 hours. The response progressively decreased at 48 and 72 hours, and all values approached control levels.

A comparison of the magnitude of enzyme response to each toxic agent might be useful for predicting severity of liver damage caused by each agent. Serum enzyme activities, when elevated, were at most only two and a half times the control value after exposure to hydrazine. The greatest elevation was that of LDH following exposure to hydrazine, dose 1. The maximum elevation of serum enzyme activity after exposure to UDMH was twice that of the control value and occurred only with LDH and MDH at 16 hours. Exposure to carbon tetrachloride produced much greater and more consistent increases in the serum enzyme activities. These elevations with carbon tetrachloride usually occurred 16 and 24 hours after exposure. In contrast, the serum enzyme activity following exposure to the higher doses of hydrazine was significantly lower than the control values.

It is interesting to compare the magnitude of depression of the liver enzyme activities following exposure to the various agents. When the level of enzyme activity is expressed as a percentage of the control value, the activity of LDH, MDH and ICD, after exposure to all three doses of hydrazine, ranged from 25 to 33 percent of control, while GDH activity varied from 3 to 20 percent. Exposure to UDMH depressed LDH activity 50 to 75 percent, while there was no depression of MDH activity; in fact, an elevation of MDH activity was noted after both doses of UDMH. Liver ICD activity ranged from 33 to 80 percent following exposure to UDMH, and GDH activity ranged from 12 to 50 percent. Exposure to carbon tetrachloride brought about a depression in LDH and MDH activity in liver tissue to 20 percent of the control value. The activity of

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ICD was depressed to 10 percent and GDH activity was 12 percent. These degrees of depression represent the maximum amount of liver enzyme depression observed following the exposure and usually occurred at 16 to 24 hours after exposure.

From these comparisons, it is apparent carbon tetrachloride produces a more pronounced enzyme response, especially in the serum, than does hydrazine or UDMH. The magnitude of the liver enzyme response is also greatest with carbon tetrachloride, with the exception of the depression in GDH activity following exposure to hydrazine, doses 2 and 3. UDMH provoked the least response, with hydrazine being intermediate but more comparable to UDMH. These comparisons are made with the full realization that the responses to each agent are comparable only to the extent that all doses used were of sublethal magnitude.

The diminished or near-normal serum enzyme activity occurring with the higher doses of hydrazine and UDMH probably indicates a different mechanism of toxicity from that of carbon tetrachloride, where the magnitude of serum enzyme activity increases with increasing doses. At least initially, the primary effect of carbon tetrachloride appears to be upon cell permeability (ref 2, 14). This allows efflux of tissue enzymes into the serum and thus gives an increase in serum activity with a concomitant decrease in tissue activity.

The decreases in serum enzyme activity which occurred following exposure to the higher doses of hydrazine may have resulted from a primary toxic effect upon enzyme synthesis, although some disturbance of cellular permeability cannot be discounted and may account for the concomitant decrease in both serum and liver enzyme activity. Since the lowest dose of hydrazine did produce a few apparently significant elevations in serum enzyme activity, an increase in cell permeability may have occurred along with either slight or no inhibition of enzyme synthesis.

UDMH may cause some inhibition of enzyme synthesis as reflected by below-control liver enzyme activity and, in a few instances, a concomitant decrease in serum enzyme activity. Such inhibition, however, is certainly not of the same degree caused by hydrazine, although the individual enzyme activity responses to a given dose of either hydrazine or UDMH were quite variable, and the numbers of animals used were small.

Inanition

The fact that hydrazine had an anorexic effect on the rats for approximately 16 hours after exposure prompted an investigation of the effect of fasting upon enzyme activities. As stated in RESULTS, there was elevation of liver LDH activity and a decrease in ICD serum activity. The elevated LDH activity in liver due to fasting conditions certainly did not interfere with the experimental results, since LDH liver activity was nearly always depressed at 16 hours after exposure. The decrease in ICD serum activity in fasting rats may be a factor to consider at 16 hours. The effect of fasting may have been prolonged in those animals receiving 2 injections of hydrazine. There is no readily apparent explanation for the changes observed. Cornish and Block (ref 5) observed an elevation of serum GOT activity in fasting rats although they did not postulate a causal relationship.

Phenergan Pretreatment

Pretreatment with Phenergan was done in order to compare the effect of pretreatment in hydrazine intoxication with that previously observed in carbon tetrachloride poisoning. It was also done to help define the liver lesion produced by hydrazine. Rees et al (ref 8) have shown that Phenergan pretreatment followed by exposure to carbon tetrachloride reduces Ca^{++} imbibition and the efflux of liver enzymes into the blood without reducing liver lipid.

The findings in this study are in agreement with those of Rees and his co-workers in that pretreatment with Phenergan reduced the efflux of liver LDH enzyme into the serum without reducing liver lipid, as shown microscopically. With pretreatment the serum enzyme activity was essentially the same as or below the control value for each enzyme, in contrast to being elevated in the absence of Phenergan pretreatment. This finding tends to support the hypothesis that hydrazine, dose 1, does alter cell permeability to some extent. This is supported by the observation that with Phenergan pretreatment, the percent of decrease in the efflux of the enzymes (except for GDH) was considerably greater than the percent of enzymatic depression in the liver. Phenergan may in some manner lead to decreased enzyme synthesis or cause an aggravation of the hydrazine-induced lesion since there is a greater decrease in intrahepatic enzyme activity with Phenergan pretreatment than without.

Inhibition Studies

The results of the inhibition study indicate that hydrazine and UDMH are capable of causing direct in vitro inhibition of purified enzyme activity at concentrations of approximately 10^{-4} M, although there is an insufficient amount of data to allow for exact characterization of the type(s) of inhibition observed. With the exception of the effect of UDMH on LDH activity, the type of inhibition appears to be competitive. This statement is based upon reference to the Lineweaver-Burk plot. However, competitive inhibition usually occurs when the inhibitor has a molecular configuration similar to that of the substrate, and this is not the case with hydrazine or UDMH.

The instances of complete inhibition noted may reflect inactivation of the enzyme by actual denaturation of the protein or an adverse effect of improper pH. The pH of hydrazine and UDMH as added to the enzymes was 9.4 and 10.0, respectively. The optimum pH for an enzyme-substrate reaction varies with each enzyme-substrate system.

Figure 6 demonstrates a decrease in the general level of velocity when hydrazine and UDMH are added to the enzyme. The greater the concentration of the inhibitor, the more the reaction velocity is slowed. All of the enzymes were inhibited significantly or completely by the higher concentrations of hydrazine and/or UDMH used. ICD was inhibited completely by the highest concentration of either hydrazine or UDMH, while GDH was inhibited completely only with the highest concentration of UDMH. The significant inhibition refers to a significant increase in the slope of the line when compared to the control.

There is proportionately greater inhibition with decreasing substrate concentration or, in other words, the greater the substrate concentration, the less

Conclusions

the inhibitory effect of a given concentration of inhibitor. As mentioned previously, this only holds true for competitive inhibition. As can be seen, the Michaelis Constant tends to increase in cases which appear to exhibit competitive inhibition.

The significance of the in vitro inhibition observed with hydrazine and UDMH at the approximate concentration of 10^{-4} M can better be interpreted in relation to this experiment if some estimation can be made of the tissue and serum concentrations of these agents at the time the animals were killed. If hydrazine and UDMH do produce direct inhibition of enzyme activity at concentrations similar to those found in tissue and serum, it might prove helpful in the interpretation of the experimental results.

Back et al (ref 15) have studied the tissue distribution and concentration of UDMH in rabbits at various intervals after the intraperitoneal administration of 50 mg/kg. At 18 and 24 hours after exposure, they found 115 μ g and 110 μ g of UDMH per gram of liver tissue, and 11.35 μ g and 6.04 μ g per ml of plasma. No data were obtained beyond 24 hours. An attempt was made to compare the concentrations of UDMH used in vitro with the concentrations presented by Back and co-workers. This is only a gross comparison, at best, but we estimated that the in vitro inhibition studies used concentrations greater than the actual tissue concentrations by a factor of 10 to 100 times. Further difficulties arise when the tissue levels reported by Back are used to estimate those concentrations which might have been present in these experiments; namely, a difference in species and dose of UDMH.

The fact that hydrazine and UDMH produced no inhibition when added directly to normal rat serum in concentrations equal to the two higher ones used in the in vitro study indicates that they are not an important factor in inhibiting enzyme activity directly at the concentrations used in this experiment.

Microscopic Examination of Liver Tissue

There are two important findings which should be noted. One is that while hydrazine did produce microscopic liver changes, there was no evidence of necrosis. The other important finding is the complete absence of pathological alteration attributable to UDMH. The alterations observed with hydrazine were intracellular fat deposition in the midzonal and periportal areas and glycogen depletion. Amenta and Johnston (ref 16) observed glycogen depletion on rats four hours after exposure to hydrazine while untreated, fasted animals showed no glycogen depletion after four hours of fasting. The untreated, fasted animals showed marked glycogen depletion by 24 hours. They demonstrated that after 24 hours, fasted rats exposed to hydrazine could accumulate glycogen in the centrilobular area following parenteral administration of glucose. Their observations seem to indicate that hydrazine can cause glycogen depletion by a direct effect. This may indicate a possible primary locus of action since some investigators feel that the smooth ergastoplasm in liver cells is associated with glycogen synthesis and release (refs 17, 18). This is not to say that the anorexia associated with hydrazine plays no part in the observed glycogen depletion.

Recknagel and Lombardi (ref 19) have suggested that the normal mechanism for the release of fat from the liver cell is associated with the microsomal

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fraction which contains the ergastoplasm. Interestingly enough phosphorylcholine-glyceride-transferase is a microsomal enzyme. They (ref 19) do not postulate the specific secretory mechanism, but such mechanism may be closely related to a disturbance in phospholipid synthesis. Such a disturbance might well be caused by the inhibition of phosphorylcholine-glyceride-transferase by carbon tetrachloride or the high intracellular calcium level which occurs in carbon tetrachloride poisoning. This disturbance in phospholipid synthesis may offer the answer to the "fatty liver" associated with carbon tetrachloride intoxication.

A similar disruption of normal biochemical function may be involved in explaining the accumulation of liver lipid which is a prominent feature of hydrazine intoxication. This would indicate that the primary locus of action of hydrazine might be on the ergastoplasm. This is in support of the site of action suggested in the discussion of glycogen depletion and the magnitude of pathological alteration increased with increased doses of hydrazine.

Relationship of Microscopic to Enzymatic Changes

Hydrazine occupies an intermediate position between carbon tetrachloride and UDMH with regard to the degree of enzymatic response and pathologic alteration which it produces.

UDMH produced a few elevations in serum enzyme activity and many depressions in liver enzyme activity in the absence of any pathologic alteration. This indicates that nonpredictable changes may occur on the biochemical level without concomitant anatomical changes. Of course, examination of structure at the subcellular level by electron microscopy might reveal structural changes. The number and magnitude of enzymatic changes which followed the administration of UDMH was the least when compared with hydrazine and carbon tetrachloride. This observation supports the microscopic findings.

The overall picture indicates that tissue recovery is fairly rapid, being almost complete in 72 hours for all three doses of hydrazine and for the single dose of carbon tetrachloride. The enzyme activity may or may not have returned to normal at this time, but the trend was in that direction.

The finding that mean elevations in some serum enzyme activity occurred in the absence of necrosis with hydrazine and UDMH is in agreement with results observed by other investigators (ref 1). This was also true with carbon tetrachloride, since a significant elevation in serum enzyme activity of LDH, MDH and ICD occurred at 16 hours. The microscopic picture at this time revealed no necrosis; however, necrosis was present at 24 hours after exposure to carbon tetrachloride. The magnitude of elevation of serum enzyme activity was no greater at 24 hours except for GDH which reached its peak activity then, but was not significantly elevated at 16 hours. This might be expected, since GDH is a 100 percent mitochondrial enzyme, although mitochondrial damage has been shown not to be the initial lesion in carbon tetrachloride poisoning (ref 20).

The increased magnitude of serum enzyme activities in carbon tetrachloride exposure when compared to the hydrazine and UDMH response cannot be attributed to necrosis alone, since the difference in magnitude was observed at 16 hours, while necrosis was not observed until 24 hours following exposure. The

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microscopic appearance of the liver indicated greater damage from carbon tetrachloride at 16 hours than from hydrazine at 16 hours. This may or may not account for the greater magnitude of serum enzyme activity in the case of carbon tetrachloride.

UDMH apparently has little effect on the liver at the dose levels used in this experiment. Measurement of any serum enzyme activity is of no practical value as a diagnostic procedure for an acute exposure to low doses of UDMH. Back et al (ref 15) point out that to date probably the best method of determining significant exposure to UDMH is a correlation of blood and urine levels of this compound.

The situation for hydrazine is a little different. Possibly an increase in serum LDH activity and/or a decrease in serum MDH activity might serve as supportive indices of liver damage. It is questionable whether below-normal levels of ICD and GDH serum activity would be helpful, because of the small changes obtained and the range of normal values. The chance for laboratory error to be a significant factor is real and should not be overlooked. Nevertheless, decreased tissue activity might be useful for experimental purposes in determining the hepatotoxicity of toxic compounds under investigation.

Any possible application of these experimental results to man and his environment must consider that these findings in rats cannot be applied directly to man. The results may, however, offer ideas as to how this current problem of exposure potential can be approached. Clinical application of these methods and analyses might help in determining when workers are being exposed to damaging or potentially dangerous concentrations of hydrazine.

SECTION V

CONCLUSIONS

The question of hepatotoxicity following an acute exposure to sublethal concentrations of hydrazine and UDMH was studied in rats. Histological examination of the liver by ordinary light microscopy and measurement of the serum and liver enzyme responses were used as indicators of hepatotoxicity. Correlations between enzyme response and histological change were made whenever possible. A concomitant study was performed in which rats were exposed to a sublethal concentration of carbon tetrachloride in order to show the effects of a known hepatotoxic agent.

The effects of fasting as they related to this experiment were studied, as well as the effects of Phenergan pretreatment prior to exposure to hydrazines. The question of direct in vitro enzyme inhibition by hydrazine and UDMH was considered and investigated.

Hydrazine produced acute reversible liver changes at the doses administered. This was shown by microscopic alterations (periportal and midzonal fat accumulation and glycogen depletion). There was no evidence of necrosis. Serum and liver enzyme responses were inconclusive.

Conclusions

Results indicate that an elevation of serum LDH, MDH, ICD, or GDH enzyme activity is not a reliable indicator of acute liver damage following exposure to sublethal concentrations of hydrazine. Indeed, a more reliable indicator might be a below-normal level of enzyme activity, if sufficiently predictable normal ranges have previously been established.

UDMH did not produce histological alteration of the liver under the conditions of this study. There were some apparently significant liver enzyme responses which suggest that UDMH may have a slight effect at the biochemical level at the doses given. The significant serum enzyme responses were limited in number and magnitude, and followed no pattern. Therefore, the measurement of these specific serum enzyme activities is unlikely to be a satisfactory method of determining an acute exposure to UDMH at or near the dose levels employed in these experiments.

Phenergan pretreatment did not prevent the basic lesion in hydrazine intoxication, but it did prevent the efflux of LDH enzyme from liver into the serum.

In conclusion, direct in vivo inhibition of these enzymes by hydrazine and UDMH was not a significant factor at the dose levels employed in this study; however, these agents were shown to be capable of producing in vitro inhibition at higher concentrations. This phenomenon may have been a function of pH alone.

The effects of carbon tetrachloride supported those reported by other investigators. There was definite histological alteration with necrosis. This was accompanied by highly significant serum and liver enzyme response.

Under the conditions of this experiment, UDMH has little if any significant effect upon the rat liver. Hydrazine and carbon tetrachloride were shown to produce reversible liver changes, with the effect of hydrazine being mild to moderate in contrast to the marked liver damage produced by carbon tetrachloride.

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