Biochemical Pharmacology, Vol. 19, pp. 1743–1752. Pergamon Press. 1970. Printed in Great Britain

CHANGES IN LIPIDS OF RAT LIVER AFTER HYDRAZINE INJECTION*

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(*Received* 6 *June* 1969; *accepted* 9 *October* 1969)

Abstract—Hydrazine (1·1 m-mole/kg, i.v.) in adult male rats fasted for 48 hr caused the previously reported prompt elevation of serum free fatty acids (FFA) and depletion of liver glycogen, followed in 12–24 hr by a 10-fold elevation of liver triglyceride. In the liver, total phospholipid and total cholesteryl ester increased slightly for 24 hr, but total free cholesterol remained unchanged. In the serum, phospholipid rose above control levels at 24 and 48 hr. Cholesteryl ester levels, however, fell below control levels at 8–24 hr, then rose at 48 hr, while free cholesterol levels appeared unchanged. In consequence, the esterified cholesterol of serum in hydrazinized rats fell to a mean of 57 per cent of total serum cholesterol at 12–24 hr. This evidence of liver malfunction occurred during the period when the liver was glutted with triglyceride. Since mean serum triglycerides were normal at 8–12 hr and only twice control levels at 24 hr, the data support the postulate that hydrazine embarrassed the secretion of triglyceride from the liver, possibly by decrease of lipid-binding capacity of lipoproteins.

THE ADMINISTRATION of adequate doses of hydrazine to experimental animals affects the central nervous system, as the resulting convulsions dramatically illustrate.[1] Less dramatic but equally characteristic metabolic consequences that have been observed include: lipemia;[2] elevation of blood free fatty acids (FFA),[3] lactate and pyruvate;[4] depletion of liver glycogen;[5] and massive infiltration of fat into the liver.[6, 7] This liver fat is primarily triglyceride.[3] Blood glucose levels may be initially elevated[8] or depressed[5] depending, in anesthetized dogs, on the initial level of liver glycogen,[4] but ultimately hypoglycemia supervenes in severely hydrazinized animals. Protein[9] and amino acid[8, 10] utilization is decreased. Several mechanisms may underlie these and other metabolic effects of hydrazine and its methyl derivatives.[11] A study of the effects of hydrazine on the levels of carbohydrate and various lipids in serum and liver of fasted rats was therefore undertaken in a search for further clues to the underlying metabolic mechanisms.

EXPERIMENTAL

Treatment of rats. Adult male Sprague–Dawley rats were studied in groups of ten

* The research reported in this paper was conducted by personnel of the USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, United States Air Force, Brooks AFB, Texas. Further reproduction is authorized to satisfy the needs of the U. S. Government.

The animals involved in this study were maintained in accordance with the *Guide for Laboratory Animal Facilities and Care* as published by the National Academy of Sciences—National Research Council.

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over a period of 6 months. Members of each group were selected to weigh within 10 g of the mean of the group, but the mean weights varied from group to group so that the range of body weights included in the entire study varied from 227 to 393 g. Each group was fasted 48 hr before being injected with hydrazine (1·1 m-mole/kg, i.v. via tail vein) or saline (as control). Between the times of injection and sacrifice, each member of ths group was housed separately in a metabolism cage. Drinking water, but not food, was provided. One hydrazine-injected and one saline-injected rat were decapitated at 1, 2, 4, 8, 12 or 48 hr after the injection. Not all time intervals could be included in each group, but over the 6-month period fourteen groups were studied to obtain data from ten hydrazine-treated and ten control rats for each of the seven selected time intervals. If an analysis of one of the pair was lost, all data from the other rat were discarded.

Hydrazine solutions. Fresh dilutions of liquid hydrazine (95%, from Matheson, Coleman & Bell) were prepared just before injection of each group. A suitable volume of liquid hydrazine was titrated to pH 7·4 with 2 N HCl, diluted with water to 0·22 M, and injected in a volume of 5 ml/kg body weight to provide a dose of 1·1 m-mole hydrazine/kg. Controls were injected with 5 ml saline/kg body weight.

Analytical methods. After decapitation, the first milliliter of blood was collected in a heparinized beaker and the remainder without anticoagulant. The liver was excised promptly. A piece was frozen in dry ice–acetone for glycogen determinations and the remainder was used for lipid analyses. Standard methods were used to measure blood glucose[12] and lactate,[13] plasma FFA[14] and liver glycogen,[15] which was expressed as glucose equivalents. A separate sample of nonfrozen liver and of nonheparinized blood was used for lipid analyses. Serum and liver lipids were extracted in $CHCl_3$–CH_3OH (2:1, v/v), separated into free and ester cholesterol, triglyceride, and phospholipid fractions on small columns of silicic acid, and measured quantitatively as previously described.[16]

Statistical analyses. An analysis of variance was performed for each variable in order to test whether the time response differed significantly between the control and hydrazinized groups and to provide standard errors of the differences between means to evaluate which differences were significant.[17] For any given mean value, there were two applicable standard errors of the difference between means—one for comparing means within the same group but at different times (e.g. controls at 4 hr vs. controls at 24 hr), and the second for comparing means from different groups at the same time (e.g. controls at 4 hr vs. hydrazinized group at 4 hr). Since these two standard errors were quite similar in value for any given variable, only one value, the larger one, was plotted in the figures (Figs. 1–4). Any means that differed by more than twice the applicable standard error were significantly different at the 0·05 probability level. No differences were considered significant unless probability values were 0·05 or less.

RESULTS

The data are summarized in Figs. 1–4, which present the mean values ± the standard error of the differences between means for both control and hydrazine-treated groups.

Effects of fasting. Since the rats were fasted for 48 hr before the start of the experiment, the data from control groups exhibit the effects of fasting for 49–96 hr. During the first 4–8 hr of this period, the serum glucose and lactate levels in controls were

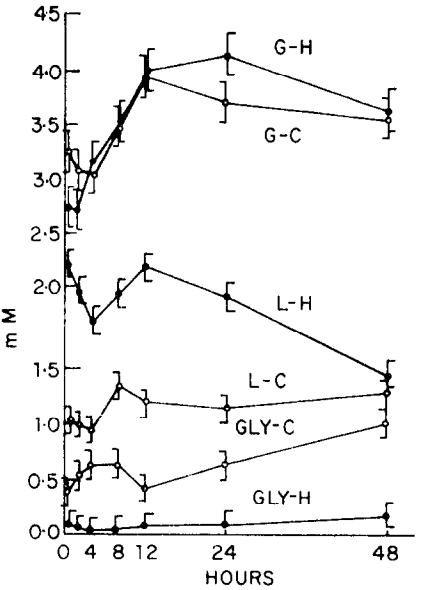

FIG. 1. Mean carbohydrate levels in groups of ten hydrazine-treated (H) and ten control (C) rats sacrificed at each time interval. Serum glucose (G) and lactate (L) are expressed in millimolar concentrations; liver glycogen (GLY) levels are glucose equivalents in units of millimoles/kg of body weight. Brackets enclose ± the standard error of the difference between means. See explanation in Experimental section (Statistical analyses).

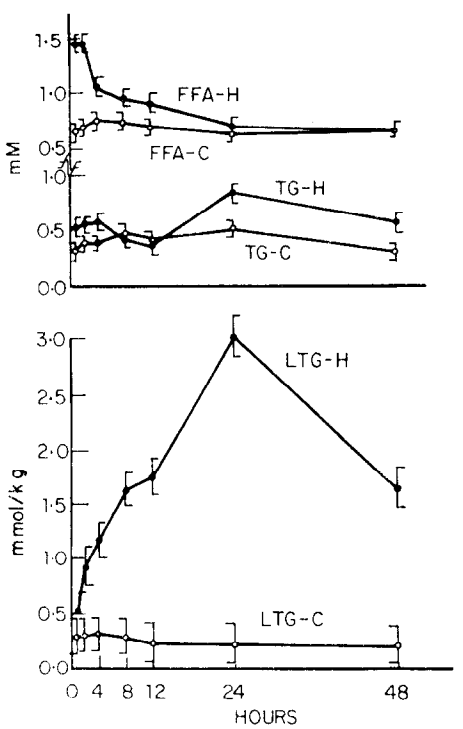

FIG. 2. Mean serum free fatty acid (FFA), serum triglyceride (TG) and liver triglyceride (LTG) levels in hydrazine-treated (H) and control (C) groups. Liver triglycerides are expressed as millimoles/kg of body weight. Brackets enclose ± the standard error of the difference between means. See explanation in Experimental section (Statistical analyses).

depressed (Fig. 1). Liver glycogen levels were low, but restoration of glycogen stores occurred during the last 24 hr of the experiment (Fig. 1). This rise is unexplained. The rats had no access to food during the entire 96-hr period (48-hr fast + 48-hr experiment). Analytical artifact was ruled out by repeat glycogen analyses of both early (1–4 hr) and late (24 and 48 hr) samples performed simultaneously. The late rise is therefore accepted as real.

Levels of FFA of serum and of triglycerides of both serum and liver were unchanged in controls during this period of fasting (Fig. 2). However, esterified cholesterol and phospholipid in serum were slightly higher in controls during the first 2–8 hr than during the 12–48 hr period of the study, while serum free cholesterol was less affected (Fig. 3). Liver cholesterol esters were unchanged in controls during the 48 hr of the study, while both phospholipid and free cholesterol decreased during the final 36 hr (Fig. 4).

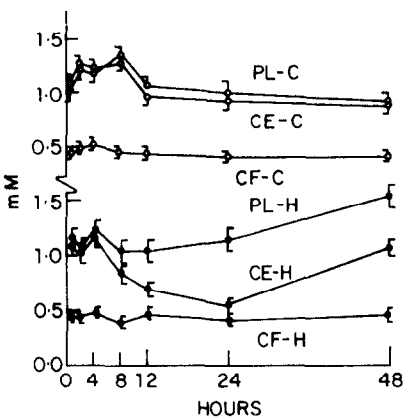

FIG. 3. Mean serum phospholipid (PL), cholesteryl esters (CE) and free cholesterol (CF) levels in hydrazine-treated (H) and control (C) groups. Brackets enclose ± the standard error of the difference between means. See explanation in Experimental section (Statistical analyses).

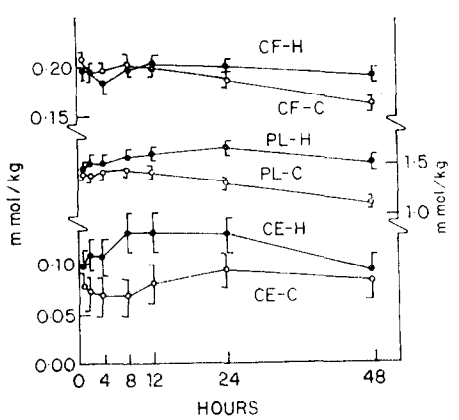

FIG. 4. Mean levels of liver lipids expressed as millimoles/kg of body weight. Abbreviations are given in the legend of Fig. 3. Brackets enclose ± the standard error of the difference between means. See explanation in Experimental section (Statistical analyses).

The data in Table 1 show that there was a trend toward decreasing liver size in controls during the last 36 hr of the study. Therefore, the amounts of liver lipids in each rat were calculated per gram of liver in that rat to determine whether the trends shown by the various lipid means were different when calculated on this basis. Mean free cholesterol and phospholipid, expressed per gram liver, steadily decreased with time, while the mean for cholesteryl esters decreased progressively to a minimum at 8 hr, then gradually returned to the starting level at 48 hr. These changes were small; the difference between maximum and minimum levels was significant only at the 5 per cent level. Mean triglyceride levels for controls, calculated per gram of liver, did not change significantly during the experiment. Evidently, the proportion of liver substance lost during prolonged fasting was approximately equal to the proportions of triglyceride and of cholesteryl esters lost, but exceeded by the proportions of phospholipid and free cholesterol lost.

TABLE 1. LIVER WEIGHTS OF CONTROL AND HYDRAZINE-TREATED RATS SACRIFICED AT DESIGNATED TIMES AFTER INITIAL INJECTION*

Time (hr)	Liver wt. (g/100g body wt.)	
	Hydrazine-treated	Control
1	2·98	2·70
2	3·02	2·60
4	3·05	2·74
8	3·25	2·79
12	3·39	2·83
24	3·68	2·60
48	3·08	2·31

* Each value is the mean of ten animals. S. E. of difference between means is 0·10 for comparison between hours and 0·09 for comparison of hydrazine and control groups.

Effects of hydrazine. The commonly recognized effects of hydrazine are summarized in Figs. 1 and 2. Blood glucose levels in hydrazinized rats were initially depressed, but reached control levels at 4 hr and were comparable to controls thereafter, except for a questionable elevation at 24 hr. Liver glycogen levels in this group were even lower than in controls at 1 hr after injections, and these levels were still severely depressed at 48 hr, in contrast to the significant replenishment evident in controls at that time.

Mean blood lactate levels in hydrazinized rats were almost double the control levels throughout the first 24 hr of the study.

The effects of hydrazine on lipid metabolism included early elevation of plasma FFA, which was still detectable at 12 hr, and a marked elevation of liver triglyceride, which was maximal at 24 hr (Fig. 2). This increase in liver triglyceride was accompanied by a slight elevation of serum triglyceride above controls at 1–4 hr and at 24–48 hr.

Levels of serum cholesteryl esters and phospholipids showed remarkable parallelism in changes in controls (Fig. 3). In the hydrazinized rats, this parallelism was not as close after 4 hr. Serum cholesteryl esters fell to significantly lower levels at 8–24 hr after injection of hydrazine than after saline. Although serum free cholesterol levels were significantly lower at 8 hr and higher at 48 hr in hydrazine-treated animals than

in controls, the overall trend of the means was similar to the trend of the controls, but with somewhat wider variation.

In the liver, changes in phospholipid and free cholesterol were closely parallel in both control (Fig. 4, PL-C vs. CF-C) and hydrazine-treated (Fig. 4, PL-H vs. CF-H) groups. To avoid the impression that liver phospholipid and cholesterol levels were approximately equal, the reader is warned that the scale for phospholipids in Fig. 4 has been reduced 10-fold to facilitate comparison of changes in levels of phospholipid and cholesterol.

Despite parallel changes in some liver lipids in both control and hydrazine-treated groups, the actual levels of liver lipids in the hydrazine-treated groups were frequently higher than in controls. Liver phospholipids were elevated above controls after the first 8 hr, but the difference is greatest later in the experiment because levels fell in controls but rose to a peak at 24 hr after hydrazine injection (Fig. 4). Free cholesterol was significantly higher in hydrazine-treated rats than in controls at 24 and 48 hr. In contrast to the trend in control levels, after hydrazine treatment, liver cholesteryl esters increased during the first 8 hr and remained elevated through the next 16 hr.

Hydrazine treatment increased the liver size progressively with time up to a maximum at 24 hr, after which liver size declined (Table 1), but still remained significantly greater than in controls at 48 hr. The maximum liver size at 24 hr coincided with the peak in liver triglyceride levels. When liver lipids were calculated per gram of liver weight instead of per kilogram of body weight, in the hydrazine-treated group the ester cholesterol was generally higher and the free cholesterol and phospholipid were lower than in the controls. These relationships represent the supranormal levels of cholesteryl esters and the dilution of less elevated free cholesterol and phospholipid levels by triglyceride in the hydrazine-poisoned livers.

The per cent of total serum and liver cholesterol comprised by the esterified cholesterol at various times in treated and control groups is summarized in Table 2. Significant depression occurred in serum of hydrazinized animals at 12 and 24 hr, but

TABLE 2. MEAN VALUES OF CHOLESTERYL ESTERS AS PER CENT OF TOTAL CHOLESTEROL IN LIVER AND SERUM*

Time (hr)	Liver			Serum		
	N	Hz (%)	S (%)	N	Hz (%)	S (%)
1	9	32·2	30·1	10	73·0	73·3
2	10	36·1	26·6	10	72·1	71·7
4	10	37·1	26·0	10	70·6	70·9
8	10	38·0	24·9	10	68·8	72·8
12	10	38·7	25·8	10	57·2	69·3
24	9	39·0	32·4	9	57·8	69·6
48	10	31·7	30·9	10	69·0	69·7
S.E.M. for differences between means in same rows (Hz vs. S)†		3·17			1·79	
S.E.M. for differences between means in same column (hr)†		3·94			2·06	

* Hz = hydrazine-treated group; S = saline-treated controls; S.E.M. = standard error of the mean; N = number of pairs of rats.

† S.E.M.'s assume ten animals per mean and are expressed as per cent.

values returned to control levels at 48 hr. Concomitantly, in the livers of the hydrazinized group, the mean per cent of esterified cholesterol was increased above controls in the 2 through 24 hr samples, with the greatest difference at 8–12 hr.

DISCUSSION

The data presented in Figs. 1–4 bring together several of the primary effects of hydrazine that have been previously documented in separate studies by other investigators, and show the concomitant changes observed in controls. The metabolic hallmarks of hydrazine poisoning—glycogen depletion[5] and fat infiltration[6] in the liver—are evident in those figures, along with elevation of plasma FFA[3] and lactate.[4]

It is striking that these effects of hydrazine in rats fasted for 48 hr are similar to those previously reported for rats fasted for 1 day. Evidently the hydrazine exerts these effects on metabolic processes, the component enzymes of which had been maintained in a functional state despite the prolonged fast. The recovery of liver glycogen levels in controls during the last 36 hr of the study is also evidence that the enzymes required for gluconeogenesis and glycogenesis were able to function after prolonged fasting in rats.

The early hypoglycemia caused by hydrazine was similar to that seen after a dose of 1·2 m-mole/kg,[18] but less pronounced than after a dose of 2·0 m-mole/kg.[19] This hypoglycemia may reflect the inhibition of glyconeogenesis of hydrazine reported by Lewis and Izume[20] and confirmed by Fortney *et al.*[19] In the present experiments, the failure of liver glycogen levels to recover as the controls did during the 48-hr observation period was further evidence in favor of inhibition of gluconeogenesis. The occurrence of normal or supranormal blood glucose levels at 12–48 hr was therefore surprising. These levels may reflect either the experimental result[19] that hydrazine did not completely block, but only partially inhibited, gluconeogenesis or the fact that the observations of Fortney *et al.*[19] were limited to one point in time, viz. 1 hr after injection of hydrazine.

The early elevation in serum FFA levels, followed by the accumulation of triglyceride in the liver in hydrazine-treated animals, undoubtedly reflects the uptake of serum FFA by the liver and incorporation into triglyceride, as previously shown by Trout.[18] This process would require either a greater quantity or a higher turnover rate of phosphatidic acid in the liver. The analytical procedures used would have measured the phosphatidic acid in the phospholipid fraction from the silicic acid column.[21] The higher quantities of liver phospholipid in hydrazinized than in control animals may therefore include higher levels of phosphatidic acids. Detailed studies of the phospholipid fraction are needed to evaluate this possibility.

The striking parallelism between phospholipid and cholesteryl esters in serum and between phospholipids and free cholesterol in liver in controls is evident in Fig. 3. It is noteworthy that the correlation with phospholipid is not as close for the serum free cholesterol and liver ester cholesterol, both of which are the quantitatively lesser forms of cholesterol in their respective tissues. These relationships were maintained in the livers but not in the serums of the hydrazinized group. The drop in serum cholesteryl esters in this group was maximal at 24 hr, when the mean per cent of serum cholesteryl esters fell to 58 per cent. This fall in per cent of serum cholesteryl esters was accompanied by a decrease in the level of serum cholesteryl esters. These

decreases may reflect, *inter alia*, decreased activity of lecithin:cholesterol acyltransferase[22] or liver damage, or both, caused by hydrazine.

The mean levels of serum total cholesterol were unaffected by hydrazine treatment except at 8 and 24 hr, when they were depressed. At 24 hr, the mean total cholesterol in hydrazinized rats was 75 per cent of the level in controls. This value agrees closely with the decrease observed by Amenta and Dominguez.[23] Because this decrease was temporary and because it coincided with the maximum glut of triglyceride in the liver, temporary inhibition of lipid secretion by the liver is a likely explanation.

Hydrazine affected both free and ester cholesterol levels in the liver. The total amount of liver free cholesterol, expressed as μmoles per gram of body weight, was not significantly different from that of controls. However, because hydrazine increased the liver size, the level of free cholesterol per gram of liver was significantly below control levels throughout the experimental period. In contrast, cholesteryl esters were increased in both concentration per gram of liver and in total amount of liver cholesteryl ester per gram of body weight in the hydrazinized group. These opposite changes resulted in only minor increases in liver total (free plus ester) cholesterol. Because of the dilution of the cholesterol with triglyceride in hydrazine-poisoned livers, the total cholesterol content per gram of liver (instead of per kilogram of body weight) was significantly ($P < 0{\cdot}05$) lower than in control livers at 24 and 48 hr. Roberts and Simonsen[24] reported that hydrazine decreased the total cholesterol content of liver in mice, but their cholesterol levels were expressed in milligrams per gram of liver. In our results, this apparent decrease was not a decrease in total cholesterol content of the liver, but was a dilution of cholesterol per gram of liver caused by the increase in liver weight resulting from the accumulation of triglyceride. There is, therefore, no evidence for decreased cholesterol synthesis in our hydrazinized rats. On the contrary, the increased cholesteryl ester levels that were observed are evidence against inhibition of synthesis. They imply increased esterification or decreased excretion from the liver, or both. Of these alternatives, decreased excretion is supported by the observation that mean serum cholesteryl ester levels fell while liver levels rose during the first 8 hr of the study.

Decreased secretion of lipoprotein is an attractive hypothesis to explain the accumulation of liver lipids, but this hypothesis has been both contradicted and supported by experimental findings. Amenta and Dominguez[23] found some decrease in the capacity of the liver to secrete triglyceride 4 hr after injections of hydrazine. Trout[25] also studied this capacity, but found no decrease during a period of 1·5–3 hr after the injection of hydrazine. Since he showed that the rate of triglyceride secretion from the liver was decreased by glucose feeding, the conflicting results of the investigators may reflect different nutritional aspects of the experiments. In the data presented above, despite the 10-fold increase in liver triglyceride 24 hr after injection of hydrazine, serum triglyceride was increased only 2-fold at 24 hr, and not significantly increased above controls at 8 and 12 hr. These relationships support the interpretation that secretion of triglyceride by the liver was inhibited after hydrazine administration.

The possibility that fat accumulation in the liver was secondary to a decrease in utilization of triglyceride seems unlikely, since Bitter *et al.*[9] showed that in rats, hydrazine increased the combustion of fat to meet the energy requirements of the animal.

Earlier, Amenta and Johnston[26] had shown that amino acid incorporation into

protein is supranormal in hydrazinized rat liver and had concluded that deficiency in liproprotein synthesis was unlikely. That conclusion was supported by Banks and Stein,[27] who found higher RNA and protein levels in livers of hydrazinized rats than in controls. Therefore, if it occurs, deficient secretion of triglyceride probably is not secondary to interference with protein synthesis.

If there is no deficiency in protein or lipoprotein synthesis in hydrazine-poisoned liver, why should secretion of triglyceride be inhibited? One possibility arises from the fact that hydrazine has been shown to inhibit catalase[28] and peroxidase.[29] Clark *et al.*[30] found that peroxidized lipoproteins lose lipid-solubilizing capacity. On this basis, one of us[11] postulated that hydrazine and monomethyl hydrazine, by inhibiting catalase and peroxidase, might elevate liver peroxide sufficiently to cause some loss of lipid-protein affinity, and thereby inhibit secretion of lipid from the liver, despite adequate protein synthesis.

Still another mechanism may play a role in the accumulation of liver lipid. Hydrazine inhibits several decarboxylases and transaminases that require Vitamin B_6 as a cofactor.[31] Since the enzyme that converts phosphatidyl serine into phosphatidyl ethanolamine is a pyridoxal phosphate-requiring decarboxylase,[32] it is a plausible hypothesis that synthesis of phospholipid or, at least, of phosphatidyl ethanolamine and of lecithin would be inhibited by hydrazine. The data presented in Fig. 4 can be interpreted either to support or to contradict that hypothesis. Since liver phospholipid levels were significantly elevated, it can be argued that hydrazine did not inhibit phospholipid synthesis. Furthermore, it is evident that phosphatidic acid metabolism must have been adequate to permit conversion of the increased load of incoming fatty acids into triglycerides. On the other hand, it can also be argued that phosphatidic acid levels may have been increased, as evidence by the elevated liver phospholipids, and that this increase concealed inhibited synthesis of selected phospholipids. Deficient supply of these phospholipids, resulting in incorrect proportions of lipids needed for lipoprotein structure, could limit the capacity of the liver to secrete lipids and account for the deficient lipid-secreting capacity in the liver in the face of supranormal protein synthesis.

There are, therefore, at least two possible mechanisms by which hydrazine could inhibit lipid secretion by the liver. Further experiments are required to establish the extent to which each contributes to the net effect of hydrazine.

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