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Effect of Thioacetamide-Induced Sublethal Hepatic Cell Injury On Protein Synthesis.* (30219)

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In acutely injured cells, increased contents of water (cloudy swelling) and lipid (fatty metamorphosis) are readily detected histologically and document the impairment of water and lipid metabolism and transport. The effect of cell injury upon cytoplasmic proteins is less readily examined in intact cells. Nevertheless, since the time of Virchow, morphologists have generally believed that in mildly injured cells, such as in cloudy swelling, alterations in quantity or state of cytoplasmic proteins do occur.

Recent studies indicate that one of the more significant functional alterations accompanying some forms of cell injury is impairment of protein synthesis(1,2,3,4). These studies, linked with current concepts of the role of ribonucleoprotein (RNA-protein) in the cytoplasmic synthesis of proteins(5) and with the feasibility of examination of ribosomes with electron microscopy, have introduced an additional parameter in the study of cell injury.

To correlate cytoplasmic ultrastructural and biochemical abnormalities related to protein synthesis in injured cells, an investigation of the effects of a sublethal dose of thioacetamide (TAA) upon the liver was carried out. The biochemical mechanism of the injurious effect of TAA upon the cell is not well known. Rather(6), Laird(7) and Kleinfeld and Von Haam(8) suggested that it caused an increase in nucleoprotein synthesis. Their studies were aimed at the tumorigenic effect of TAA and were carried out at a time when recovery from earlier injury might have been in effect. Thoennes and Bannasch(9) showed that in the earlier stages of the injury by TAA, hepatic cell ribosomes disappeared, while later the ribosomes became abundant. While these observations did not reveal the exact mechanism of cell injury by TAA, they suggested that it might be due to impaired protein synthesis.

Thioacetamide has been shown to cause hepatic cell injury and cell death if given in sufficient amount to rats(10). In our studies, a dosage of TAA which would produce cell injury with no or minimal necrosis was sought, and the early effects upon hepatic RNA-protein and serum albumin synthesis were evaluated by radioactive amino acid uptake. Simultaneously, the ultra-structural changes in hepatic cells were examined.

Methods. Seventy-two male Holtzman rats weighing about 150 g and fasted for 16 hours

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TABLE I. Radioactivity Incorporation Data in 5½-Hour Groups of Animals.

Time after leucine-C_{14} injection	Animal group and number of animals	Specific activity of serum albumin		P value	Specific activity hepatic total protein		P value	Specific activity hepatic-RNA proteins		P value	Percent recovery radioactivity in hepatic amino acids	
		Average	Range		Average	Range		Average	Range		Average	Range
30 min	TAA (7)	1.24	.84-1.84	.1	2.75	1.39-4.04	.01	5.34	4.53-6.25	.3	1.45	1.07-2.03
	Control (8)	1.66	1.33-2.48		2.97	2.52-4.30		5.09	4.40-5.63		1.34	1.02-1.79
90 min	TAA (8)	1.99	1.33-2.18	.05	1.98	1.42-2.73	.5	5.20	4.31-6.43	.01	1.17	.82-1.50
	Control (8)	2.37	1.64-3.33		2.80	1.90-3.49		4.17	3.51-4.94		1.23	.83-1.75
150 min	TAA (9)	2.16	1.81-2.44	.4	2.00	1.48-2.59	.2	5.18	3.74-6.45	.7	1.08	.77-1.47
	Control (8)	2.43	1.51-3.46		2.58	1.52-4.12		4.58	3.59-6.08		1.21	.86-1.43

were used. A trial of doses varying from 10 to 200 mg/kg indicated that 25 mg/kg was the amount which produced acute hepatic cell injury with minimal necrosis. This amount of TAA was administered by stomach tube to 36 rats lightly anesthetized with ether. Thirty-six animals were treated identically except that they were given water instead of the solution of TAA. At intervals of 5½ and 48 hours, using light ether anesthesia, control and TAA-treated animals were given 2 μc of C^{14}-L-leucine by tail vein. At 30, 90, and 150 minutes after leucine injections groups of 4 to 8 animals (Tables I and II) were sacrificed by withdrawing blood from the aorta. The livers were obtained for electron microscopic and chemical studies.

Tissue from the liver was fixed in neutral buffered formalin. Hematoxylin eosin (H and E) and azure B (AzB) stains on paraffin block sections were performed. One cu mm blocks of liver from each animal were fixed in Palade's solution(11) and prepared for Maraglas embedment(12). Thick sections (1 μ) were May-Grünwald stained and studied with light microscopy for orientation within the hepatic lobule. Thin sections were then obtained with diamond knives and lead-stained by Millonig's method(13). Sections were studied with an RCA EMU-3G microscope.

Hepatic tissue was weighed and then homogenized with a Branson sonifier. Liver homogenates were treated with perchloric acid for collection of free amino acid, total hepatic protein, and RNA-protein fractions(14). Total hepatic protein and RNA-protein were determined quantitatively using a biuret method(15). Radioactive counts were determined from these samples, and the specific activities of the total hepatic protein and RNA-protein were calculated. The percent recovery of radioactivity in the hepatic free amino acid fraction was also determined.

Serum proteins were determined quantitatively with a biuret method(15). Serum proteins were separated with a Spinco Model R paper electrophoresis apparatus, and the albumin content was determined using an Analytrol. The paper segments containing albumin were cut from the paper strips and

used directly for radioactivity determinations.

All radioactivity measurements were carried out using a Nuclear Chicago liquid scintillation system. The results of radioactivity measurements were subjected to Student's "t" test for statistical significance.

Results. Following administration of TAA the animals recovered promptly from the anesthesia, continued to be active, and appeared normal. There was no outward evidence of an hepatotoxic effect.

Histologic sections of liver tissue in the animals sacrificed at 6 to 8 hours after TAA showed cloudy swelling. There was slight swelling of the centrilobular cells which appeared more nearly rounded than normal. In H and E stains the cells in the central ⅓ of the lobule (in terms of radius) had paler cytoplasm than more peripheral cells, and very fine vacuoles about 2 μ in size were visible. AzB stains disclosed the loss of basophilic aggregates in these cells, although a fine, diffuse azurophilia was still present (Fig. 1 and 2). At 48 hours, minimal central necrosis was observed. It was estimated on a volume basis that less than one per cent of the cells in the lobule had undergone necrosis. At 48 hours a small zone 3 to 4 cells wide remained centrally in which azurophilic aggregates were still missing from the cytoplasm, while the more peripheral cells revealed prominent basophilic aggregates, nuclear hypertrophy, and increased size and number of nucleoli (Fig. 3 and 4).

Electron microscopy at 6 to 8 hours after TAA revealed a variety of evidences of acute hepatic cell injury; however, with the exception of ribosome changes, these cytoplasmic ultrastructural changes are not the object of this report. Ribosomes were markedly decreased in number in the cells near the central vein. Many of the ribosomes which remained were decreased and more variable in size than normal, and they tended to be detached from the endoplasmic reticulum. Whereas clusters of ribosomes or polyribosomes were numerous in the liver cells of normal control rats in areas where the ER was sectioned tangentially (Fig. 5), such polyribosomes were absent in the TAA treated animals (Fig. 6).

TABLE II. Radioactivity Incorporation Data in 48-Hour Groups of Animals.

Time after leucine-C_{14} injection	Animal group and number of animals	Specific activity of serum albumin		P value	Specific activity total hepatic protein		P value	Specific activity hepatic-RNA proteins		P value	Percent recovery radioactivity in hepatic amino acids	
		Average	Range		Average	Range		Average	Range		Average	Range
30 min	TAA (4)	1.80	1.49-2.04	.3	3.10	2.67-3.96	.7	6.42	5.51-7.01	.5	1.38	1.29-1.54
	Control (4)	1.50	.76-1.88		3.04	2.14-3.92		7.64	6.11-7.27		1.45	1.36-1.57
90 min	TAA (4)	3.09	2.28-3.60	.7	3.01	1.75-3.62	.8	6.30	6.00-6.61	.9	1.41	1.19-1.50
	Control (4)	2.65	2.30-2.95		2.89	2.52-3.45		6.28	5.64-6.98		1.26	.93-1.57
150 min	TAA (4)	2.91	2.07-2.56	.2	2.39	1.88-2.68	.05	5.97	4.77-6.86	.6	1.26	1.15-1.43
	Control (4)	2.27	2.07-2.56		3.04	2.84-3.39		5.53	5.13-6.02		1.24	1.09-1.36

At 48 hours the cells immediately adjacent to the central vein still revealed loss or decrease of ribosomes and other evidences of cell injury. More peripherally located cells contained greater concentrations of ribosomes and polyribosomes than in normal controls (Fig. 7). Complex, concentric and whorled arrays of endoplasmic reticulum were present in many of the central injured cells as well as in more peripherally located cells.

The incorporation of the radioactive label into the hepatic cell fractions and in serum albumin for the 5½- and 48-hour groups are shown in Tables I and II, respectively. In the 5½-hour group, the specific activity of serum albumin was significantly less at 30 and at 90 minutes in the TAA group. The smaller value for radioactivity at 150 min-

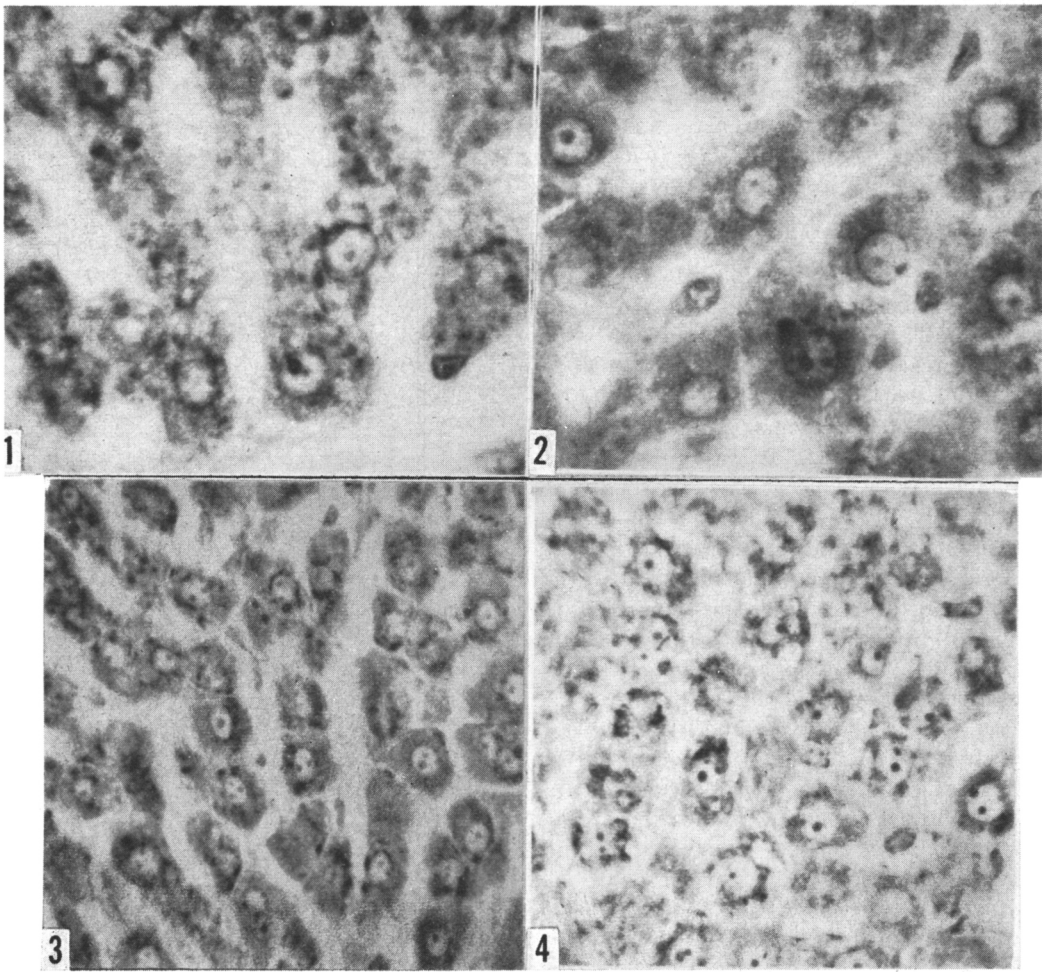

FIG. 1. Photomicrograph of azure B stain of liver of 6 hr control animal. Lower portion is near the central vein. Azurophilic aggregates are prominent in cytoplasm. Magnification, 900.

FIG. 2. Azure B stain of liver 6 hr after administration of thioacetamide, showing cells near the central vein. They are swollen and slightly more nearly rounded than normally. While they exhibit diffuse cytoplasmic azurophilia, there are no azurophilic aggregates. Magnification, 900.

FIG. 3. Photomicrograph of azure B stain of liver of 48-hr control animal. Basophilic aggregates are prominent. Magnification, 400.

FIG. 4. Azure B stain of liver 48 hr after administration of thioacetamide. Cells in lower part are near central vein. Basophilic aggregates are present in many cells, becoming more prominent toward periphery. Enlargement of nuclei and nucleoli is apparent. Magnification, 400.

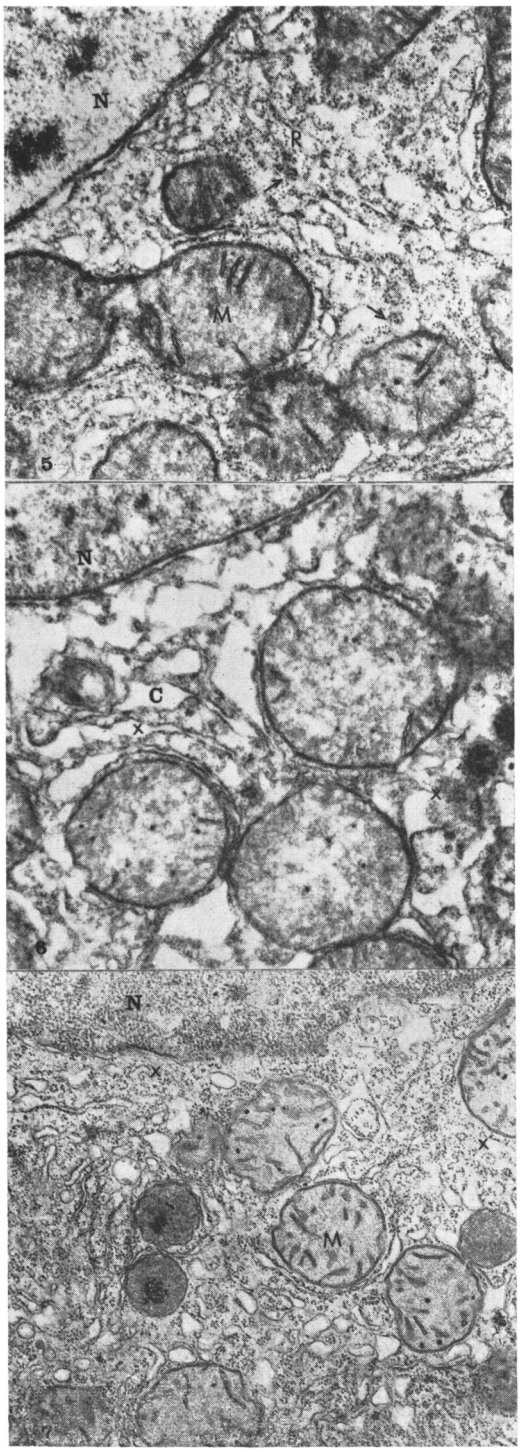

FIG. 5. Electron micrograph of liver cell from 6-hr control animal. A portion of nucleus (N) is present. Mitochondria (M) appear normal, and ribosomes (R) and polyribosomes (arrow) are abundant. Magnification, 19,000.

FIG. 6. Electron micrograph of portion of swollen liver cells from near central vein 6 hr after thioacetamide. A portion of nucleus (N) is shown. Mitochondria are swollen and their matrix is less dense and homogeneous than normal. Ribosomes and polyribosomes are absent in some areas, and sparse elsewhere. In some areas smaller than normal ribosomes of variable size are visible (X). There is slight distention of the cisternae (C) of the ER. Magnification, 19,000.

FIG. 7. Electron micrograph of portion of liver cell about mid portion of lobule 48 hr after thioacetamide. A portion of nucleus (N) is present. Nuclear granules are increased in number and more compactly arranged than in controls. Mitochondria (M) appear normal. Ribosomes and polyribosomes (X) are more numerous than in controls. Magnification 16,000.

utes in the TAA group is not significant. Hepatic RNA-protein was significantly higher in the TAA group at 90 minutes, but not significantly different from controls at the 30- and 150-minute intervals. On the other hand, in the total hepatic protein the specific activity was significantly lower in the TAA group at 90 minutes. The percentage recovery of radioactivity in the free amino acid fractions in the TAA group was not significantly different from controls.

The specific radioactivity in the total hepatic protein in the 48-hour TAA group was significantly lower than in controls at 150 minutes, but not at the other time intervals. While values for radioactivity tended to appear higher in serum albumin and hepatic RNA-protein at 48 hours, the differences were not significant, and there were no differences in the hepatic free amino acid fractions.

Discussion. Our observations indicate that mild sublethal hepatic cell injury, conforming with the state of cloudy swelling, is produced at 6 to 8 hours after small peroral doses of TAA. In addition to a variety of cytoplasmic alterations to be described, this cell injury is characterized by loss of cytoplasmic azurophilic clusters and loss and disposal of ribosomes from the endoplasmic reticulum. The radiochemical studies in these animals indicate that the rate of incorporation of labeled amino acid into serum albumin and into hepatic total protein is decreased, while it is increased in hepatic RNA-protein.

Guidotti and others(16) found an increased *in vitro* amino acid incorporation in hepatic

cell proteins in liver slices from rats in which cloudy swelling had been produced by endotoxin. They suggested that the increased amino acid incorporation indicated a state of hyperfunction. They did not, however, evaluate the rate of incorporation of amino acid into serum albumin. Our studies suggest that the increased incorporation in hepatic RNA-protein which occurred in conjunction with cloudy swelling produced by TAA at 5½ hours might be a consequence of the decreased rate of incorporation into serum albumin, rather than indicating a state of cellular hyperfunction.

In studies of cell injury produced by dimethylnitrosamine, Brouwers and Emmelot (17) and Mukherjee and others(18) found that decreased amino acid incorporation occurred in liver microsomal preparations. The latter authors observed concomitant alterations of arrangement of the ER, but did not describe loss or dispersal of ribosomes as we observed in TAA-treated animals. On the other hand, Porter and Bruni(19) found loss of ribosomes to be an early effect of dimethylnitrosamine.

Loss of ribosomes from hepatic cells has been described as the result of a variety of causes of cell injury. In addition to dimethylnitrosamine(18,19,20) it has been noted in the liver after prolonged fasting(21), and after hepatic injury due to hypoxia(22), carbon tetrachloride(23) and ethionine intoxication(24). This seemingly ubiquitous occurrence of ribosomal loss suggests that almost any form of sublethal cell injury may have as a net result the impairment of cytoplasmic synthesis of protein, at least temporarily. As a corollary, a biochemically specific inhibitory effect directly upon protein synthesis in injured cells probably cannot be predicated alone either upon ribosomal loss or demonstrated impairment of labeled amino acid uptake.

In correlating hepatocellular ultrastructural with concurrent biochemical observations, a complicating factor is introduced by the variability of cell injury in different portions of the liver lobule. The zone of injured cells in our experiments involved the inner third of the lobule which represents only a small fraction of all liver cells. Any demonstrated decrease in protein synthesis probably must be attributable to decreased activity in this zone of injured cells. It cannot be affirmed, however, that even the peripheral cells were uninjured to some degree, even though they appeared normal under the electron microscope.

On the other hand at 48 hours after injury those cells more peripherally located in the lobules contained an increased number of ribosomes and polyribosomes. Such changes could indicate an increased capacity to take up amino acids and synthesize protein. While these alterations were found peripherally in the lobules, the central zone contained cells in which the features of earlier injury were still present. Accordingly, the net protein synthesizing activity of the liver at this stage would be highly unpredictable. Thus, it is not surprising that at 48 hours the radiochemical studies did not reveal any statistically significant differences in net amino acid incorporation in serum albumin or in hepatic RNA-protein in the TAA or control groups.

Summary. Electron microscopic and radiochemical studies were performed in mild sublethal hepatic cell injury (cloudy swelling) produced by small peroral doses of thioacetamide. Azurophilia, ribosomes and polyribosomes decreased or disappeared in injured cells at 5½ hours. At 48 hours the cells in the outer portion of the lobule contained a marked increase in ribosomes and polyribosomes, while centrally located cells continued to show evidence of the earlier injury. At 5½ hours after thioacetamide there was a decreased uptake of the label from administered C^{14}-L-leucine in serum albumin and in total hepatic protein while it was increased in the hepatic RNA-protein. These findings were interpreted to indicate that incorporation of amino acid into hepatic RNA-protein is not measurably impaired by mild injury due to thioacetamide, but that synthesis or secretion of serum albumin is impaired. At 48 hours, amino acid incorporation in total hepatic protein was decreased in thioacetamide treated animals, but the incorporation in hepatic RNA-protein and in serum protein did not differ from controls. The interpreta-

tion of results at 48 hours was complicated by the joint presence of persistently injured centrilobular liver cells and peripherally located liver cells which contained an increased number of ribosomes.

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Strength of Suckling Stimulus and Maintenance of the Mammary Gland.* (30220)

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Selye(1) first demonstrated the importance of the nursing stimulus in the maintenance of mammary parenchyma during lactation. He showed that involution which normally occurs upon removal of young from the mother could be delayed despite the fact that milk removal from the gland was prevented by ligation of galactophores. More recently, Tucker and Reece(2) have determined deoxyribonucleic acid (DNA) content of teat-ligated and non-ligated rat mammary glands and found that DNA content of ligated glands was significantly less than that of corresponding glands of intact lactating rats at comparable stages of lactation. However, they neglected to indicate whether or not the degree of involution of teat-ligated glands was comparable to that which normally occurs upon removal of the litter. Mizuno(3) found that DNA content of ligated glands of mice was comparable to corresponding glands of intact lactating control mice while the DNA content of the non-ligated glands was significantly greater than that of corresponding control glands.

Since there appears to be a discrepancy between the results obtained in rats and mice and since the number of young per litter employed in previous experiments was either not reported or was the same for all groups,

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