Cellular aspects of ethanol-induced fatty liver: a correlated ultrastructural and chemical study

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SUMMARY Fatty liver induced by acute intoxication was studied chemically, histologically, and electron microscopically in rats. Six hours after administration of ethanol and corn oil, hepatic lipids (mainly triglycerides) had increased by 32%. Electron microscopy revealed concurrent marked accumulation in cytoplasmic vesicles of droplets measuring 500–2500 A. In control animals receiving an isocaloric amount of glucose plus corn oil, lipid droplets also appeared to enter liver cells in vesicles; they were visible mainly in peripheral portions of the cytoplasm. In alcohol-treated animals, however, the small lipid particles were more numerous and were present in vesicles throughout the cytoplasm. These smaller droplets appeared to fuse, forming larger droplets, and others were contiguous with the normally occurring larger storage lipid droplets. A possible explanation, that these changes represent an ethanol-induced impairment of the metabolism of lipid entering the liver cells, is discussed.

KEY WORDS liver · lipid · uptake · ethanol · acute intoxication · rat · electron microscopy · hepatic steatosis · particulate lipid · chylomicrons · β-lipoproteins

ETHANOL AS A POSSIBLE acute hepatotoxic agent has been the subject of a number of recent investigations (1–4). These studies have indicated that, aside from the conventional concept of nutritional deficiency resulting from caloric replacement in chronic alcoholism, an additional influence arises from the acute toxic effects of ethanol itself. Older experiments showed that alcohol administration, even when accompanied by an adequate supply of choline and other dietary requirements, might cause fatty liver in rats (5). The employment of better controlled approaches, especially the use of isocaloric glucose administration in control animals, has thoroughly established that acute ethanol intoxication produces fatty liver (1–4). It has recently been observed in man, as in experimental animals, that isocaloric replacement of carbohydrate by alcohol will produce a fatty liver despite adequate dietary intake, supporting the concept of the toxic effect of ethanol (6). The metabolic effects of alcohol on the liver have recently been reviewed (7, 8).

Most of the recent inquiries into the pathogenesis of the alcohol-induced fatty liver have been concerned with the biochemical derangement which occurs. What this defect or defects may be has not yet been established. Horning et al. (9) and Brodie et al. (10) suggested that acute alcoholic intoxication in rats caused increased mobilization of fatty acid from adipose tissue stores, which resulted in excessive accumulation of fat in the liver. Lieber and Schmid (11), however, presented evidence indicating that increased hepatic fat content was due to increased concentrations of $NADH_2$ within the hepatic cell, which caused increased triglyceride synthesis. Nikkila and Ojala (12) demonstrated that incorporation of palmitic acid-1-C^{14} into liver triglyceride increased 4 hr after ethanol administration, and postulated that even when the plasma free fatty acid level is not elevated an increased formation of hepatic triglyceride is an important factor in the pathogenesis of the ethanol-induced fatty liver.

Reboucas and Isselbacher (13) concluded that the acute ethanol fatty liver was the result of impaired lipid transport. Elko, Wooles, and Di Luzio (14) and Poggi and Di Luzio (15) studied lipid mobilization from epididymal fat pads during acute ethanol intoxication. They found no increased fatty acid release from adipose tissue during ethanol intoxication; and their studies indicated that the hepatic steatosis was possibly due to depression of triglyceride metabolism by the liver cells. Recently, Lieber et al. (6) have also reported that fatty liver developed in man and rats before there was any

indication of decreased hepatic triglycide release or excessive peripheral fat mobilization, further supporting the concept that the initial events leading to an alcoholic fatty liver result from a direct effect of alcohol on lipid metabolism in the liver.

During the accumulation of liver triglyceride following ethanol administration there is essentially no alteration in the plasma triglyceride level. However, when triglyceride is administered to ethanol-treated rats, hypertriglyceridemia develops (16).

Because the evidence implicated the liver cell as the site of impairment of some as yet unclarified biochemical mechanism in triglyceride metabolism, we decided to study the liver cell during acute ethanol intoxication with a superimposed absorptive lipid load, with the aid of electron microscopy. In addition, parallel studies on certain histochemical and chemical lipid parameters were performed in the same experiments.

METHODS

Holtzman female rats weighing about 150 g were fasted for 16 hr. Solutions were administered by stomach tube under light ether anesthesia. Six control animals received 2.25 ml/100 g body weight of a 1:1 mixture of corn oil and a solution of glucose. The mixture was isocaloric with the ethanol and corn oil mixture used in experimental groups. Six experimental animals received 2.25 ml/100 g body weight of a 1:1 mixture of ethanol (50% v/v) and corn oil (4.5 g ethanol per kg body weight). Two animals were fasted for 16 hr, lightly anesthetized, and then maintained for the total duration of the experiments (6 additional hr) as "fasting controls." Six additional rats receiving stock diet ad lib. were used as normal fed controls for morphologic comparisons only.

Animals receiving ethanol and corn oil became less active, moderately ataxic, and sluggish in reflexes. They nevertheless responded to stimuli, respiration was not depressed, and none of the animals became comatose. Six hours after the administration, all the animals were sacrificed by ether anesthesia and withdrawal of blood by aortic puncture. Liver weights were determined, and liver samples were subjected to histological examination, histochemical enzyme studies, electron microscopic study, and chemical determinations of total lipid, triglyceride, phospholipid, and total cholesterol. Serum total lipids, triglycerides, phospholipids, and total cholesterol were also determined chemically.

Histological examination was carried out on sections from paraffin-embedded blocks which were subjected to hematoxylin and eosin and periodic acid–Schiff stains. Cryostat sections were also stained for fat using Sudan black B. Histochemical enzyme studies were performed on cryostat sections of fresh tissue blocks which had been quenched in liquid nitrogen. The following enzymes were studied: succinic dehydrogenase (17), NAD-diaphorase (18), NADP-diaphorase (19), and ATPase (20). Tissue for electron microscopy was prepared by fixation in Veronal-buffered 2% osmium tetroxide, dehydration in graded alcohols, and embedment in Maraglas (21). Thin sections were prepared with diamond knives, and both lead-stained and unstained sections were viewed with an RCA-EMU-3 microscope.

Total lipids in serum and in liver homogenate were determined from methanol–chloroform extracts. Phospholipids were quantified by calculation from phosphorus analysis of the extracts (22), cholesterol by the method of Sperry and Webb (23), and triglycerides by the procedure of Van Handel and Zilversmit (24). The results of chemical determinations were statistically analyzed using the "*t*" test, placing the confidence limit at 95%.

RESULTS

Chemical Lipid Analyses

Acute ethanol intoxication accompanied by the administration of corn oil resulted in a definite increase in

TABLE 1 INFLUENCE OF ETHANOL ADMINISTRATION ON HEPATIC LIPIDS

Experimental Group	Total Lipids		Triglycerides		Phospholipids		Total Cholesterol	
	g/100 g Liver	Difference from Control and *P* Value	g/100 g Liver	Difference from Control and *P* Value	g/100 g Liver	Difference from Control and *P* Value	g/100 g Liver	Difference from Control and *P* Value
Controls receiving glucose and corn oil (6)	5.17 (4.61–5.62)		0.56 (0.48–0.63)		3.86 (3.60–4.21)		0.43 (0.32–0.59)	
Animals receiving ethanol and corn oil (6)	6.83 (6.25–7.51)	+1.66 $P = 0.001$	1.50 (1.32–1.83)	+0.94 $P = 0.001$	4.06 (3.83–4.22)	+0.20 $P = 0.20$	0.51 (0.40–0.62)	+0.08 $P = 0.20$
Fasting animals (2)	5.11 (5.01–5.21)	−0.06	0.48 (0.43–0.54)	−0.08	3.79 (3.60–4.00)	−0.07	0.46 (0.45–0.46)	+0.03

* Numbers in parentheses indicate number of animals used.

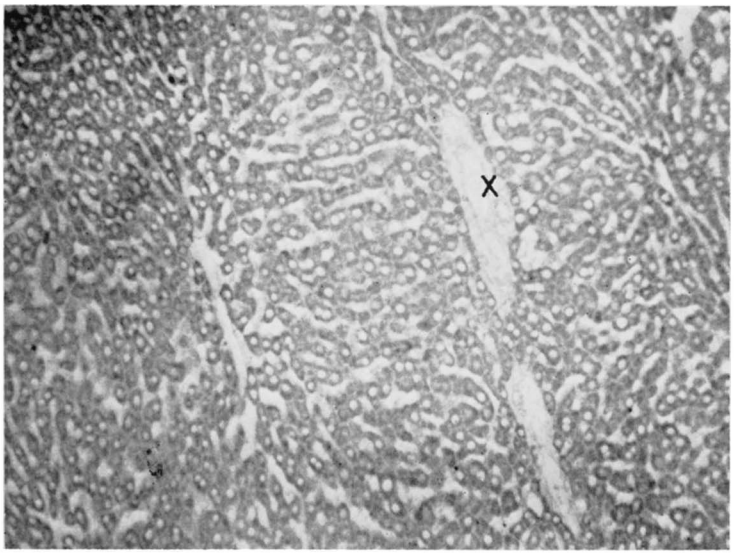

FIG. 1. Photomicrograph of liver of control rat given glucose and corn oil, stained for fat (Sudan black B). The lobule periphery is indicated by "X" in this and in Figs. 2 and 3. No visible fat droplets are present. Magnification 100.

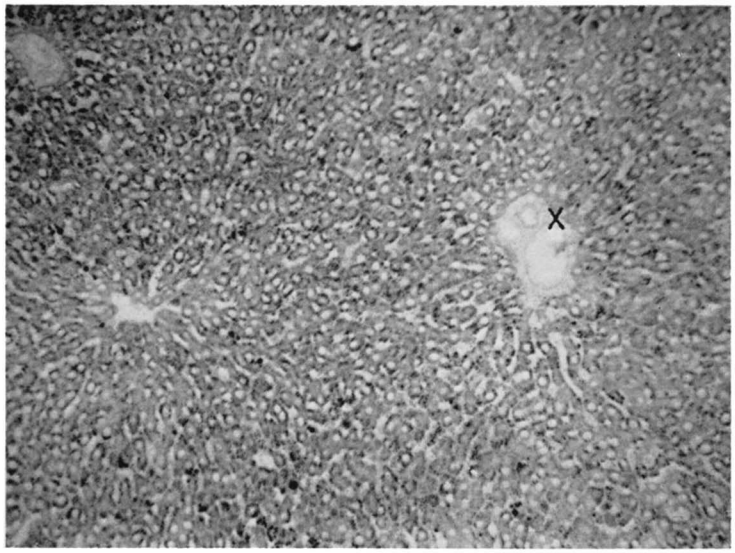

FIG. 2. Photomicrograph of liver of rat given ethanol and corn oil, stained for fat. Numerous fine fat droplets are seen in the liver cells. The droplets are concentrated along the sinusoidal walls, and they are of panlobular distribution. Magnification 100.

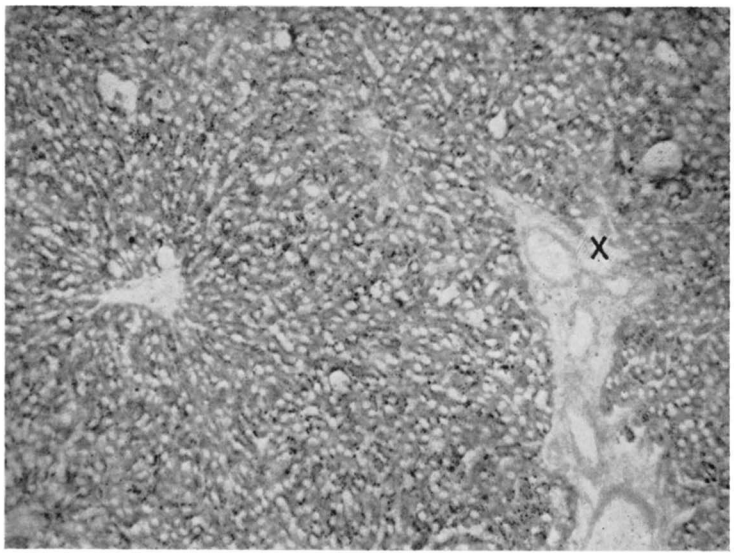

FIG. 3. Photomicrograph of liver of rat fasted for 22 hr, stained for fat. A few small fat droplets are present in liver cells near the lobule periphery. Magnification 100.

total hepatic lipids, the average concentration of which was 32% higher than that in animals receiving isocaloric glucose and corn oil (Table 1). The increase in total hepatic lipid was due almost entirely to an increase in hepatic triglyceride. Changes in hepatic phospholipid and cholesterol were not significant.

In fasted control animals hepatic triglyceride, phospholipid, and cholesterol values were essentially similar to those in rats fed glucose and corn oil.

Serum lipids in the different experimental groups varied from those in control groups only slightly (Table 2). Animals receiving only isocaloric glucose and corn oil did not have significantly higher serum triglyceride values than did fasted controls, although total lipids in the former group were slightly elevated and the confidence value was 94%. In rats receiving ethanol and corn oil, serum phospholipid values were no different from those in animals receiving isocaloric glucose and corn oil, but serum cholesterol was significantly decreased in the alcohol-treated rats.

Histological Changes in Liver

There were no detectable changes in hepatic cell size, granularity of cytoplasm, or cytoplasmic basophilia in the experimental group compared with animals receiving glucose and corn oil or fasted animals. In comparison with normal rats maintained on stock diet ad lib., PAS stains revealed reduction of hepatic glycogen in the group receiving glucose and corn oil, in ethanol-treated animals, and in fasted controls. However, the glycogen reduction was more marked in animals receiving ethanol. Sudan black B stains indicated a definite increase in number of hepatic cell lipid droplets in rats receiving ethanol. Minute fat droplets were also seen in the hepatic

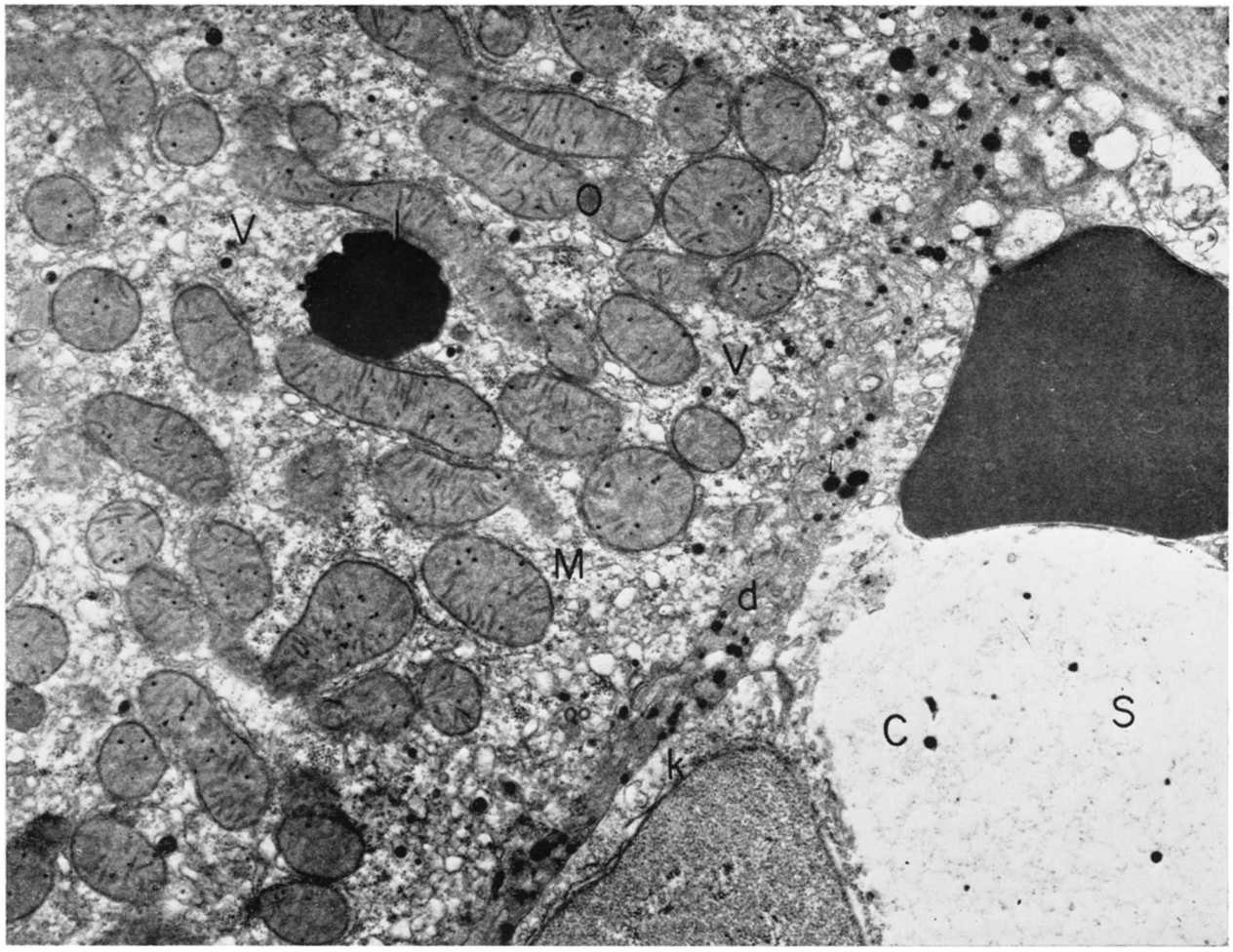

FIG. 4. Electron micrograph showing portions of sinusoid and liver cell from control rat which received corn oil and glucose. The sinusoid (*S*) contains chylomicrons (*C*), measuring up to 2000 A. A portion of a Kupffer cell (*K*) is present. Many small fat droplets are located in the space of Disse (*d*). Fat droplets up to 1000 A diameter are found in vesicles of the hepatic cell cytoplasm (*V*), especially in the zone near the sinusoid. A few are present in vesicles deeper in the cell (*V*), but most of the more deeply placed vesicles are empty or contain very small particles (*O*). The liver cell also contains a few larger droplets (*l*), believed to be stored triglyceride. Mitochondria (*M*) are normal in appearance. Dense intramitochondrial granules, which occur normally, are present in this and in the following illustrations. Magnification 14,000.

TABLE 2 INFLUENCE OF ETHANOL ADMINISTRATION ON SERUM LIPIDS

Experimental Group	Total Lipids		Triglycerides		Phospholipids		Total Cholesterol	
	mg/100 ml Serum	Difference from Control and *P* Value	mg/100 ml Serum	Difference from Control and *P* Value	mg/100 ml Serum	Difference from Control and *P* Value	mg/100 ml Serum	Difference from Control and *P* Value
Controls receiving glucose and corn oil (6)*	392 (312–486)		131 (119–168)		108 (81–133)		125 (101–156)	
Animals receiving ethanol and corn oil (6)	393 (332–460)	+1 $P = 0.6$	194 (147–241)	+63 $P = 0.01$	92 (83–108)	−16 $P = 0.10$	94 (82–110)	−31 $P = 0.05$
Fasting animals (2)	300 (284–316)	−92 $P = 0.05$	110 (126–155)	−21 $P = 0.2$	87 (84–90)	−21 $P = 0.2$	102 (101–102)	−23 $P = 0.1$

* Numbers in parentheses indicate number of animals used.

cells of fasted rats, but none were found in the control animals (Fig. 1). In the ethanol-treated group (Fig. 2), fine sudanophilic droplets measuring about 1 μ in size were noted. The fat droplets were located mainly in those portions of the hepatic cell cytoplasm adjacent to the sinusoidal walls. This alteration was panlobular in distribution, but slightly more marked in liver cells at the periphery of the lobules. In fasted animals, droplets were smaller and less numerous, and were located in the peripheral portions of the lobules (Fig. 3).

Histochemical stains for succinic dehydrogenase, NAD- and NADP-diaphorases, and ATPase showed no

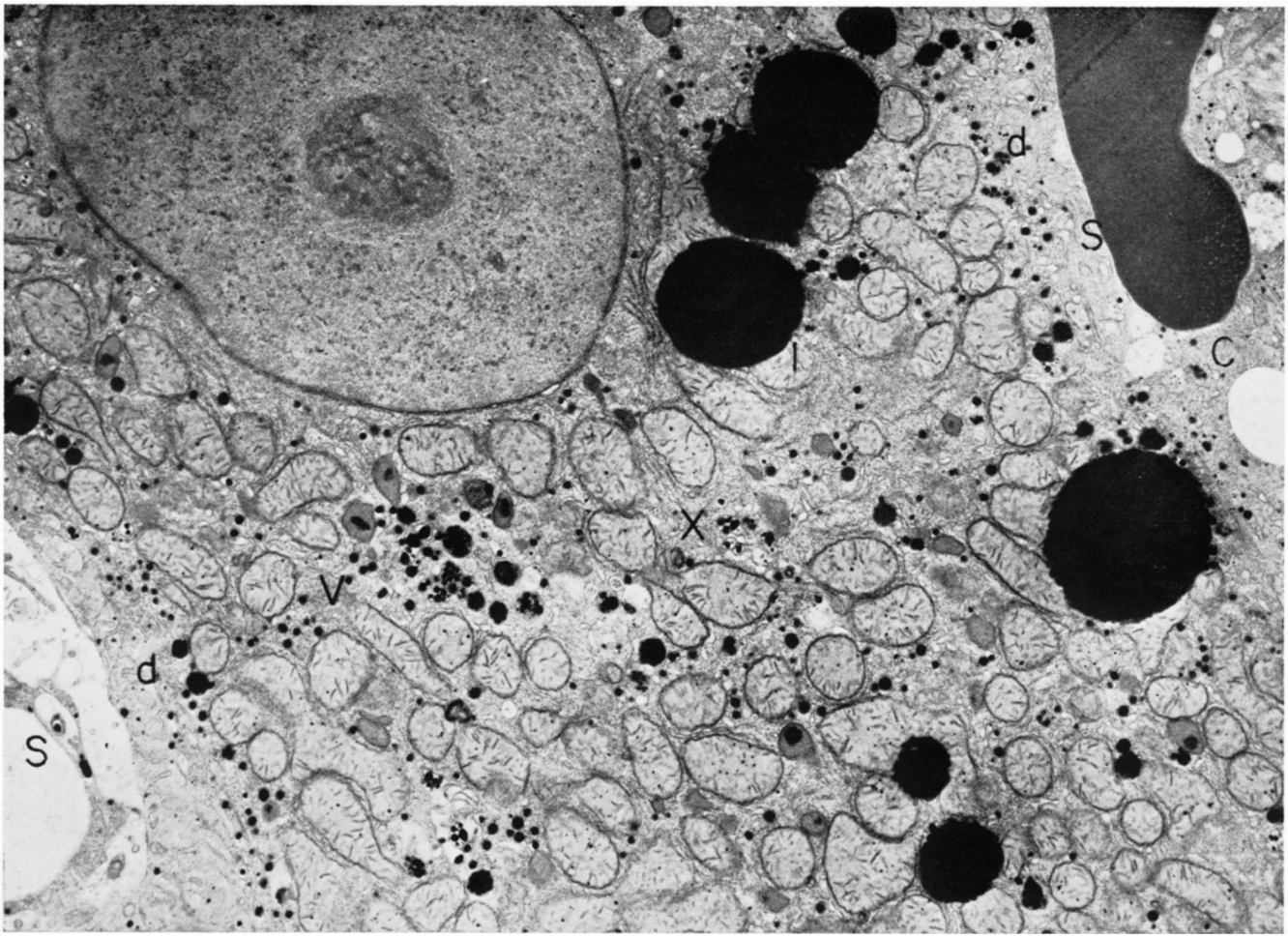

FIG. 5. Electron micrograph of portions of liver cell and of sinusoids in an animal given ethanol and corn oil. Chylomicrons (*C*) are present in the sinusoids (*S*), and small lipid droplets are present in the space of Disse (*d*). A greater number of lipid droplets is present in vesicles (*V*) of the liver cell than in control animals; many of the relatively large particles persist deep in the cytoplasm (*X*). Lipid storage droplets (*l*) are larger and more numerous than in the control animals. Magnification 10,000.

reduction in these enzymes in the alcohol-treated animals.

Electron Microscopic Changes in Hepatic Cells

In alcohol-treated animals, the main structural changes were related to cytoplasmic lipid particles and droplets. No definite mitochondrial alterations could be seen on comparison with control groups.

In *control rats* receiving glucose and corn oil, the hepatic sinusoidal blood usually contained a few chylomicrons ranging up to 4000 A in diameter (Fig. 4). The spaces of Disse contained somewhat smaller lipid droplets or chylomicrons. Membrane-lined vesicles at the hepatic cell membrane adjacent to the space of Disse were abundant, and many of these vesicles contained lipid particles measuring 500–2500 A. Similar vesicles were also present in the deeper cytoplasm but they decreased in number and size toward the nucleus. Occasional small osmiophilic particles were seen in the vesicles in the peripheral portion of the cytoplasm, and they were smaller (to the disappearing point) in more deeply situated vesicles. A few large droplets of osmiophilic lipid up to 2 μ in size were randomly located in the cytoplasm. Occasionally it was possible to see a membrane surrounding these larger droplets, or collections of membrane arrays located around segments of the periphery of the droplets, but in most instances their dense osmiophilia precluded any determination of an outer associated membrane. No abnormalities of the mitochondria, endoplasmic reticulum, ribosomes, or Golgi apparatus were seen. Glycogen granules were present in the cytoplasm, but were decreased in amount in comparison with normal fed animals.

In *animals fed ethanol and corn oil* (Figs. 5–7) mitochondria were similar in size and structure to those present in fasting rats and in animals fed glucose and corn oil. No abnormalities in the endoplasmic reticulum, ribosomes, or Golgi apparatus were seen. Glycogen was markedly decreased in the cytoplasm in ethanol-treated rats. Numerous complex arrays of fine tubules and non-

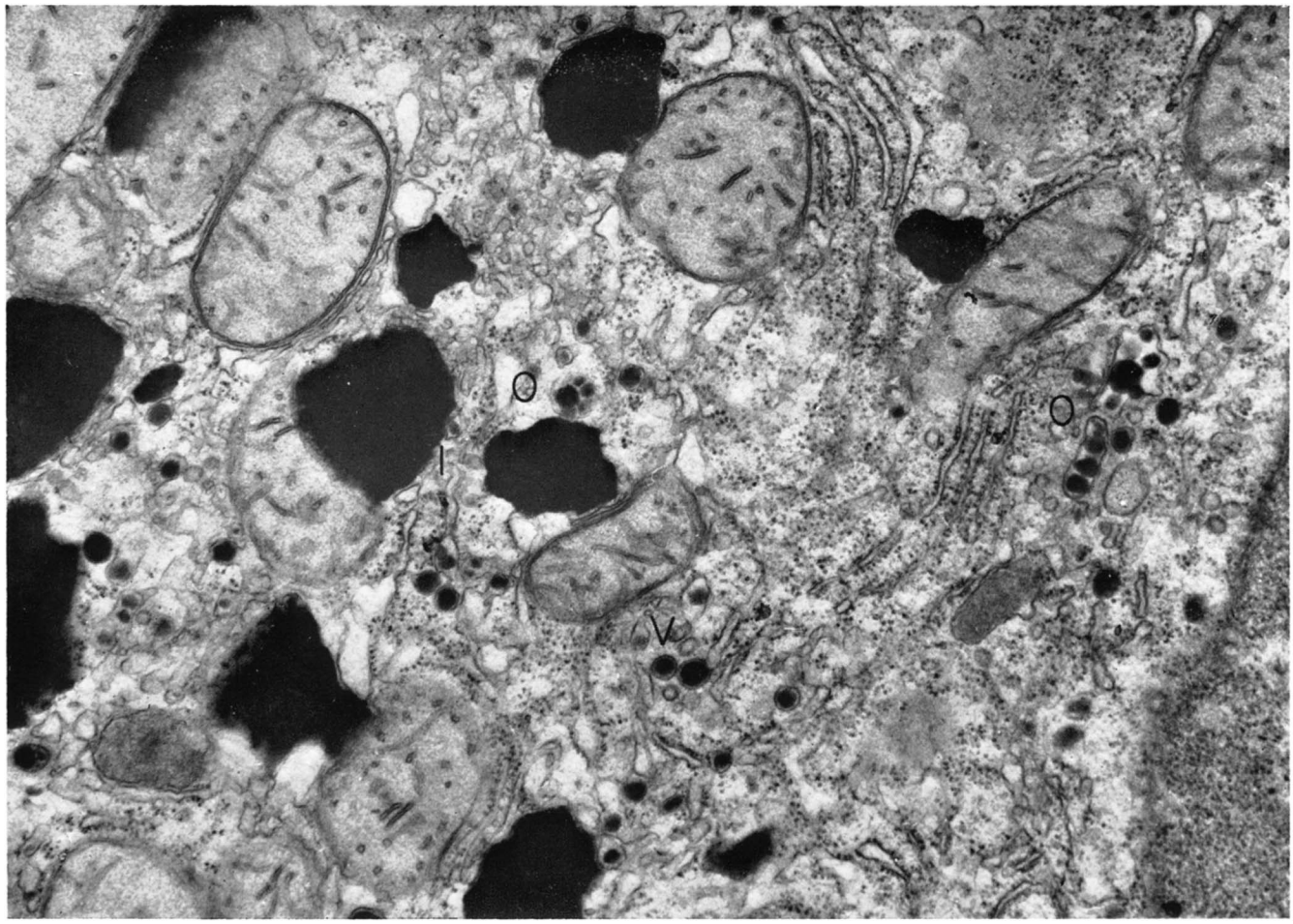

FIG. 6. Electron micrograph of portion of liver cell from rat given ethanol. The left portion of the photograph is near the sinusoid, which does not appear in the field. Lipid droplets are seen in cytoplasmic vesicles (*V*) and persist in deep portions of the cell. Some vesicles contain clusters of small lipid droplets (*O*), some of which appear to be fusing to form large droplets. Many of the larger storage lipid droplets (*I*) are present. Mitochondria and endoplasmic reticulum appear normal. Magnification 27,500.

granular membranes were noted in the cytoplasm. A few monoparticulate and rosette forms of glycogen granules were sometimes present in these areas.

Lipid particles and larger lipid droplets were more abundant in the hepatic cell cytoplasm, and the spaces of Disse contained a greater number of small chylomicrons than in the group fed glucose and corn oil. There was marked increase in the number of lipid particles in membrane-lined vesicles within the liver cells. In contrast to the control animals, these intravesicular lipid particles were present throughout the cytoplasm, showing little or no tendency to diminish in size and number deeper in the cytoplasm. The larger (1–2 μ) lipid droplets were still larger and more numerous than in controls. Small cytoplasmic vesicles containing lipid particles 500–800 A in diameter were frequently seen in close proximity to or in actual contact with these larger droplets. Lipid particles in the smaller vesicles sometimes appeared to be in contact with the outer part of the larger lipid droplets. The margins of the larger droplets were frequently irregular and tented in appearance.

The hepatic cells of rats which had been fasted for 22 hr contained numerous minute cytoplasmic osmiophilic particles (Fig. 8). These were present in approximately equal concentration in all portions of the cell and were quite uniform in size. They averaged 530 A in diameter, and were located in vesicles with nongranular membranes. Similar minute particles were present in these fasted animals in the space of Disse and in hepatic sinusoidal blood.

In all groups of animals studied, the Golgi vesicles of liver cells were found to contain oval or ellipsoid osmiophilic particles 400–600 A in size (Fig. 9). These resembled the small osmiophilic particles inside cytoplasmic vesicles in other portions of the liver cells, except for their ellipsoid shape.

DISCUSSION

Our findings on the chemically determined lipid content

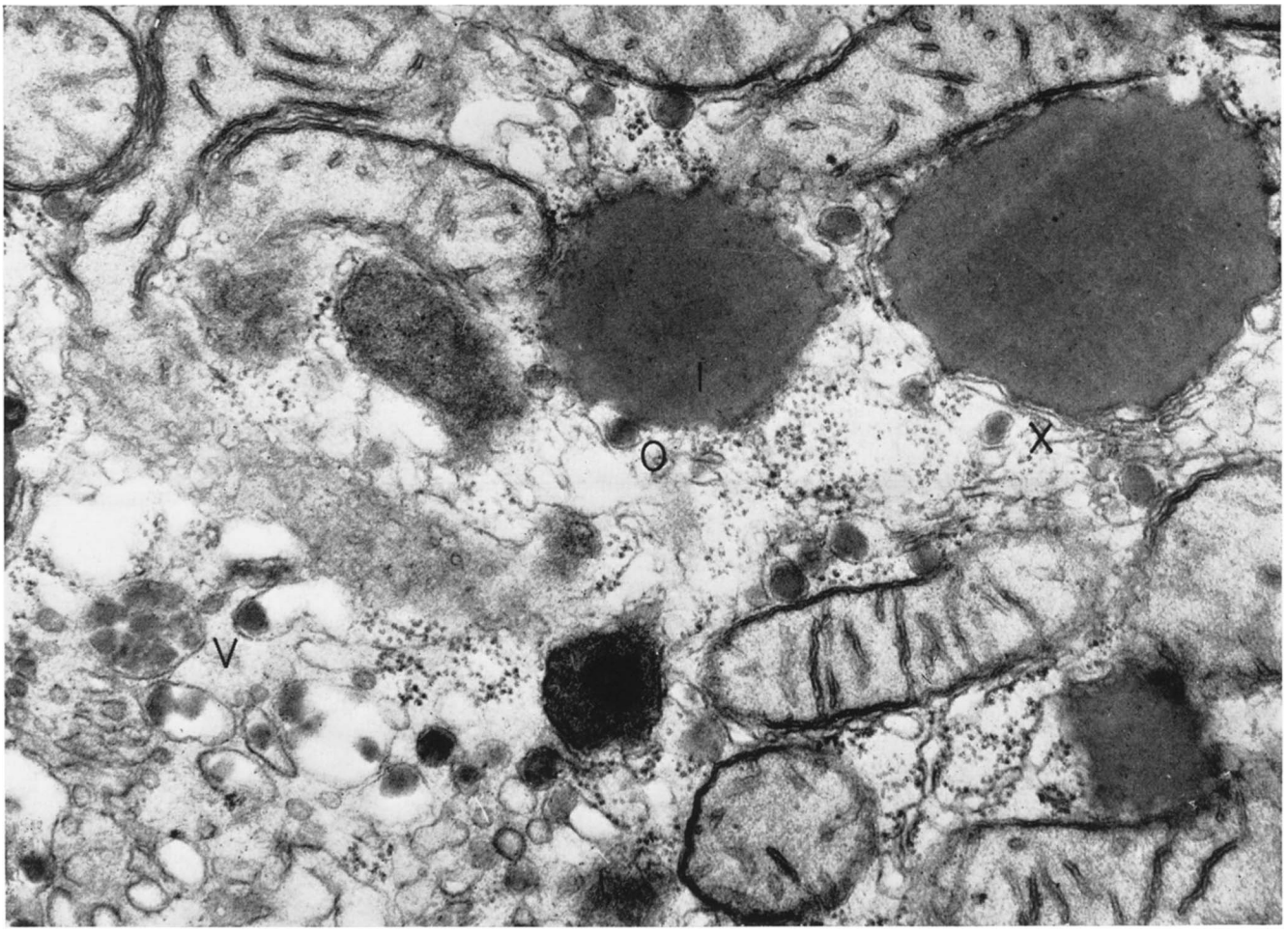

FIG. 7. Portion of liver cell from rat treated with ethanol and corn oil. The tissue has been stained with lead, resulting in relatively less contrast in the lipid droplets. Large storage lipid droplets (*I*) are present, and are partially surrounded by nongranular membranes (*X*). Numerous small intravesicular lipid droplets (*V*) are present also, and in several areas (*O*) they approach and appear to fuse with the large fat droplets. On the outer contour of the large droplets are numerous tented projections. Magnification 44,000.

of liver during acute ethanol intoxication in the presence of an alimentary load of triglycerides corroborate those of several recent studies (1–4). They indicate an impairment of the usually balanced and efficient mechanisms of supply to, and incorporation of, lipid by the liver cells, and the subsequent delivery of triglyceride from the liver cells as a component of lipoprotein. Whatever the nature of this impairment, it is manifested by an increased hepatic triglyceride content.

The present studies, conducted early in the development of the fatty liver, were not designed specifically to elucidate the nature of the chemical defect in lipid metabolism which occurs during acute ethanol effect. Rather, the intent was to study the hepatic cellular role in this process at the ultrastructural level.

Histologic studies showed no evidence of marked hepatocellular injury resulting from the effect of acute ethanol intoxication, and there was no gross loss of any of the cytoplasmic enzymes studied. These observations suggested that the resulting cellular disorder and lipid accumulation were related more to the biochemical processes of lipid transport and mobilization than to a structural cellular defect. The discussion, therefore, is directed toward particulate lipids in hepatic cells and the effects of acute ethanol intoxication upon these as revealed by electron microscopy.

During the intestinal absorption of administered fat in both control and alcohol-treated rats, numerous small lipid droplets were observed in the sinusoids and in the space of Disse. These droplets are believed to be formed in the intestines and brought to the liver (25). The similarity in size range of these particles to that seen in intestinal mucosa and in lacteals during fat absorption (26) lends support to this belief. The time of their appearance in the hepatic sinusoids during the process of intestinal absorption is also compatible with this interpretation of their origin.

The increased hepatic triglyceride content in acute ethanol intoxication is accompanied by a marked accumulation of lipid particles in membrane-limited

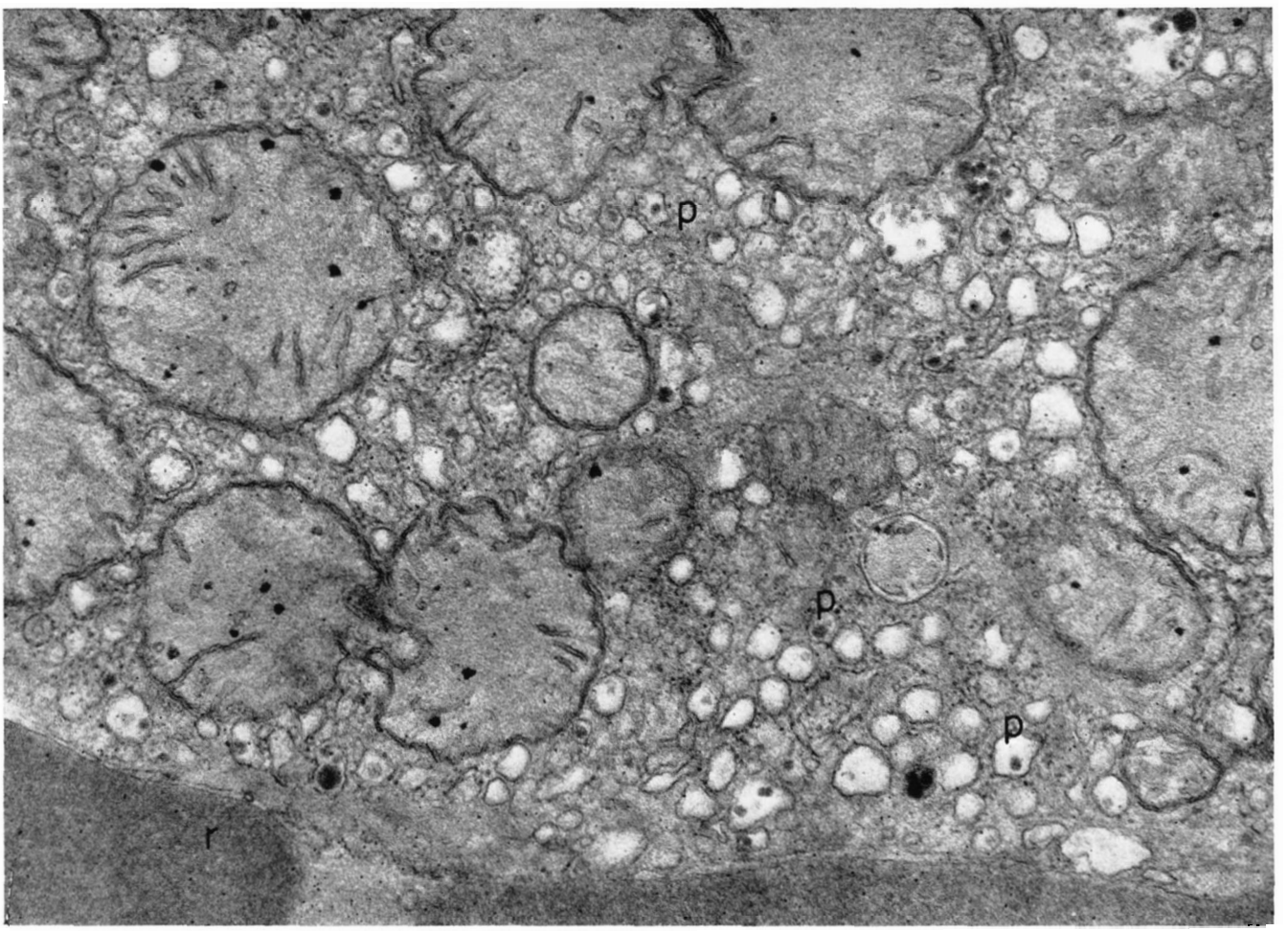

FIG. 8. Electron micrograph of liver from rat fasted for 22 hr. The portion of the sinusoid shown is occupied by a red blood cell (*r*). A few minute osmiophilic particles (*p*) about 500 A in size are present throughout the cytoplasm of the liver cell. These are located in membrane-limited vesicles. Magnification 44,000.

vesicles throughout the liver cell cytoplasm. In other forms of hepatic steatosis a similar deposition of accumulated cytoplasmic lipid occurs (27, 28). It could be argued that intravesicular particles represent lipid which has been taken into liver cells from the blood by pinocytosis; or that they are lipid-containing particles, such as β-lipoproteins, which are being synthesized within the liver cell preparatory to secretion into the blood. It is also possible that hepatic cell intake and output of particulate lipid both occur simultaneously, and that both processes may be represented by intravesicular cytoplasmic lipid droplets.

In control rats fed glucose and corn oil we observed lipid particles in vesicles located mainly in the peripheral portion of the liver cell cytoplasm, while in ethanol-treated animals intravesicular lipid particles were more numerous and more widely distributed throughout the liver cells. In alcohol-treated animals smaller particles frequently were partially fused to form larger droplets and were often contiguous with very large, storage-type lipid bodies. One explanation for these observations, although it cannot be substantiated by morphological evidence alone, is that in the normal animal lipid usually enters the liver cell within pinocytotic vesicles and is then metabolized, while during ethanol intoxication the intracytoplasmic metabolism and disappearance of lipid particles taken up by liver cells are impaired.

The evidence that chylomicron triglyceride is taken up as such by liver cells has recently been discussed by Dole and Hamlin (29) and is based mainly on the studies of Borgström and Jordan (30), Reiser et al. (31), and Stein and Shapiro (32). These studies indicate that triglyceride from tagged chylomicrons appeared in liver cells without molecular rearrangement, which would have occurred if lipolysis had taken place. Disappearance of intravesicular lipid particles in liver cells is consistent

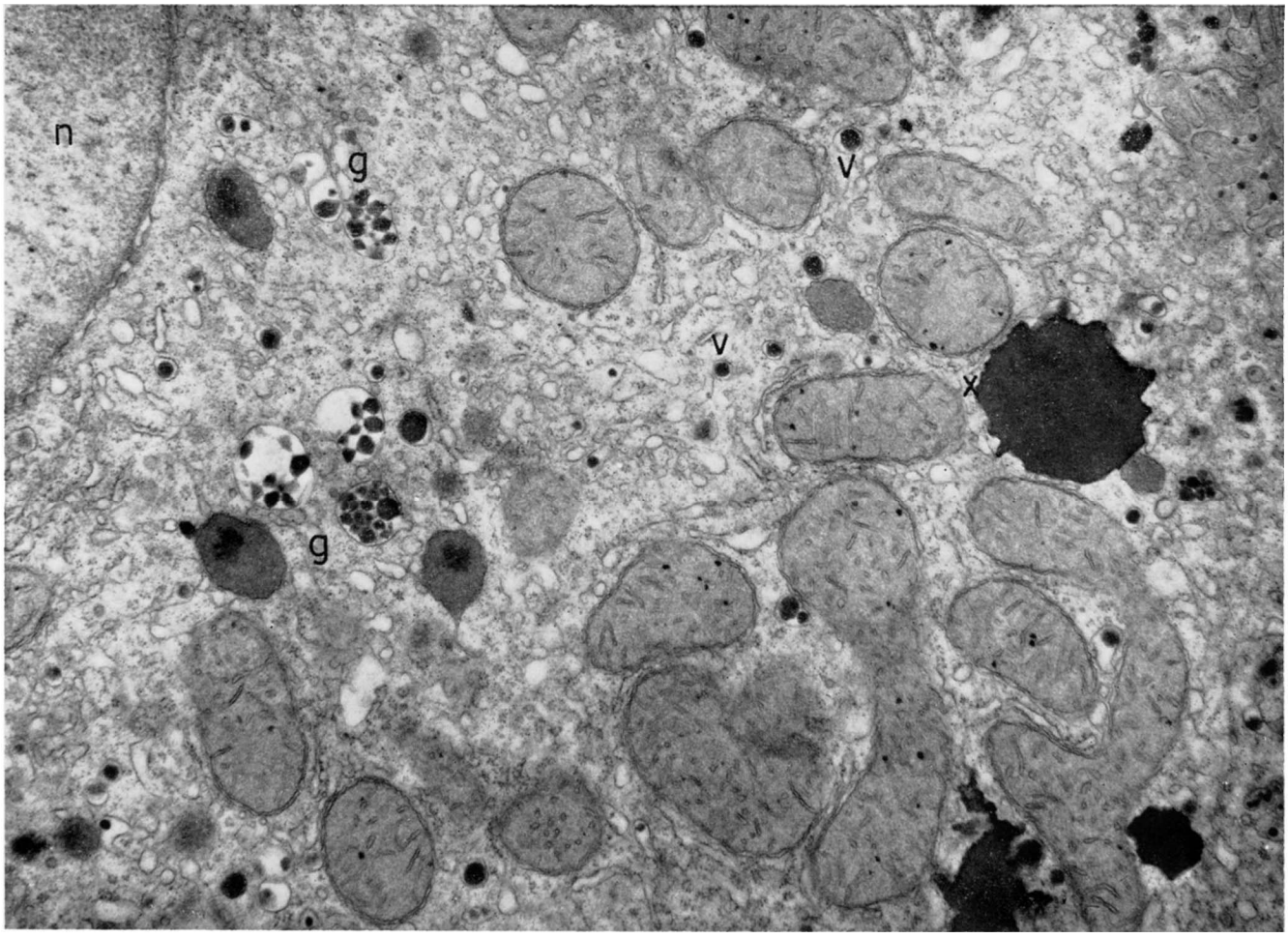

FIG. 9. Electron micrograph of portion of liver cell of rat which received ethanol and corn oil. A portion of nucleus is present (*n*). Intravesicular lipid droplets (*v*) measuring about 600–1000 A are noted in the cytoplasm, in addition to larger droplets where smaller ones have fused (*x*). Two areas of vesicles (*g*) which resemble the vesicular elements of Golgi apparatus are seen. The bile canaliculus nearby is not shown. The vesicles contain osmiophilic droplets averaging about 500 A in size. Some of these are slightly ellipsoid but most are spherical. The relationship of these droplets of lipid to lipoprotein synthesized or taken up by the liver cell is unknown. Magnification 20,000.

with the concept of subsequent degradation of triglyceride (30, 33), followed by diversion of resulting fatty acid into biological oxidation (34), its reassemby as stored triglyceride, or its incorporation into lipoprotein for secretion from the liver cell into the blood (33).

Further speculation upon the mechanism of impairment of those mechanisms of intracellular lipid metabolism resulting from acute ethanol intoxication is unwarranted on the basis of the circumstances and parameters of investigation employed in this study. Nevertheless, the origin of intravesicular lipid particles in liver cells encountered in these experiments may be further examined. Lack of knowledge of the morphologic appearance and site of formation of β-lipoprotein within the liver cell is a hindrance to the interpretation of the observation of small cytoplasmic lipid particles. Low density β-lipoprotein particles have been reported to have an average diameter of 350 A (35). In animals absorbing corn oil, the lipid particles which we observed in hepatic cell vesicles measured 500–2500 A. Therefore, at least the larger particles observed were probably not β-lipoprotein. On the other hand, in both control and alcohol-treated animals which were absorbing triglyceride from the intestine, the increased hepatic cell uptake of chylomicron lipid would lead one to expect an increased rate of hepatic cell lipoprotein synthesis and secretion. During the process, lipoprotein particles might be expected to become apparent in the liver cells. Thus some of the smaller intravesicular lipid particles seen in fat-absorbing controls and ethanol-treated animals could very well represent low density β-lipoprotein.

The possibility that lipoprotein particles being synthesized in the liver cells might be visible is supported by our observations in fasting animals. In this situation the problem of distinguishing between chylomicron-derived lipid particles and particles representing lipoprotein being synthesized does not exist. In 22 hr fasting animals we observed in the liver cells very small intravesicular lipid particles whose size (average 530 A) does not differ markedly from the size which has been reported for β-lipoprotein (350 A).

It is apparent in our study of the effect of ethanol on the liver that the concurrently increased intestinal absorption of triglyceride is an important factor. It has been shown, however, that fatty acid mobilized from adipose tissue can be the source of increased triglyceride in ethanol-induced fatty liver (10, 13, 14) in animals which are not absorbing fat. Recently Hartroft and Porta (36) described ultrastructural changes in liver cells in rats acutely intoxicated with ethanol which were apparently not absorbing fat from the intestine. They described mitochondrial swelling and the gradual accumulation of "hollow lipid spherules" which gradually became "solid lipid droplets" during the development of hepatic steatosis. Their studies might be presumed to reflect the origination of hepatic cell lipid as synthesized lipoprotein, in contrast to ours where absorbed fat is the major source of increased hepatic lipid.

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