Effects of Choline-Deficient Diets on the Rat Hepatocyte

Electron Microscopic Observations

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UNDERSTANDING THE PATHOGENESIS of the accumulation of unusually large amounts of triglycerides induced in hepatocytes by the administration of choline-deficient diets has been the object of active investigations at the morphologic [1,2] and biochemical levels.[3] In the electron microscopic image, the most obvious hepatic lesion induced by the choline-deficient diet is an unusually high number of large lipid droplets (LD), apparently free of membranes, in the ground substance of the cytoplasm. These LD are the counterpart of the pool of liver triglycerides known as the storage fat or floating fat. Another prominent deviation in the fine structure of choline-deficient hepatocytes is in the membranous systems of the cytoplasm, the rough endoplasmic reticulum (ER) and the Golgi system. It is interesting that electron microscopic studies,[4,5] in conjunction with biochemical technics, have provided evidence that granules 40–80 mμ in diameter, which are segregated normally within the lumen of these two systems, are the precursors of the very low density lipoprotein (VLDL) in plasma. These lipoproteins are known as the rapidly metabolizing pool of liver lipids and carry the triglycerides synthesized by the liver into the plasma. Equally interesting are biochemical studies [6–8] reporting that a prominent factor in the pathogenesis of the fatty liver of choline deficiency is a delay in the export of the triglycerides complexed to protein in the VLDL. These observations raise the problem of whether abnormalities in one lipid compartment are causally related to abnormalities in the other compartment, but, to our knowledge, this

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problem has not been explored in previous morphologic studies of choline deficiency. For instance, there is no clear fine structural information on the liver cell compartment where lipid material begins to accumulate and whether this initial accumulation is or is not associated with abnormalities of the membranous systems involved in the transport of VLDL. The present electron microscopic study is focused primarily on providing information on this point and on determining whether and in what manner deviation in these membranous systems contributes to the accumulation of lipid material in the ground substance of the cytoplasm. A brief report on the present observations has already been published.[9]

Materials and Methods

Seven groups of male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Massachusetts) weighing 222–250 g at the beginning of the experiments were used. Group I received Purina Laboratory Chow. The other diets used in these studies are shown in Table 1. These deficient diets have been designed to be critically low in "methyl donors." The peanut meal protein is low in methionine, which is a methyl source, and this has been supplemented with lysine and threonine to improve the quality of the protein and make it adequate, with the exception of methionine. The diets are also low in Vitamin B_{12}, which is required for the synthesis of methyl groups.

Table 1. Composition of Diets Used

Component	Percent in diet					
	II	III	IV	V	VI	VII
Extracted peanut meal*	32.00	32.00	32.00	32.00	19.00	19.00
Glucose	47.50	47.50	47.50	47.50	53.50	53.50
Jones Foster salt mix[10]	4.00	4.00	4.00	4.00	4.00	4.00
Coconut oil	15.00	15.00	4.00	4.00	15.00	15.00
Corn starch	—	—	24.75	24.75	—	—
Vitamin mix†	5.00	5.00	5.00	5.00	5.00	5.00
Threonine	0.26	0.26	0.26	0.26	0.26	0.26
Lysine HCl	0.73	0.73	0.73	0.73	0.73	0.73
Amino acid mixture‡	—	—	—	—	7.00	7.00
Choline chloride	—	0.50	—	0.50	—	0.50

* General Biochemicals, Chagrin Falls, Ohio

† Containing Vitamin A and D powder (325,000 IU/g and 32,500 IU/g), 5 g; thiamine·HCl, 200 mg; riboflavin, 400 mg; pyridoxine·HCl, 200 mg; Ca pantothenate, 1.25 g; niacinamide, 2.0 g; folic acid, 50 mg; biotin, 10 mg; menadione, 25 mg; α-tocopheryl succinate, 500 mg; glucose to make 250 g.

‡ Containing, in grams, DL-tryptophan, 3.0; DL-threonine, 5.0; DL-isoleucine, 14.0; L-leucine, 20; L-lysine·HCl, 9.0; L-cystine, 5.0; DL-phenylalanine, 16.0; L-tyrosine, 13.0; DL-valine, 13.0; L-arginine·HCl, 20; L-histidine·HCl, 6.0; DL-homocystine, 10.0; glycine, 108; L-glutamic acid, 108.

The three diets vary in their fat and methionine content, which influence the degree of fatty liver produced. Diet II and Diet IV are similar except that Diet II contains 15% fat (coconut oil) and should be more lipogenic than Diet IV which contains 4% of coconut oil. Diet VI also contains 15% fat but the methionine content of the diet was lowered by decreasing the content of peanut meal. The protein thus removed was replaced by a methionine-free amino acid mixture. Previous experience with these diets has shown that the fatty liver produced is completely prevented by supplements of methionine or choline and partially by supplements of Vitamin B_{12}.[11]

To reduce individual variations in hepatocyte fine structure due to variation in the feeding cycle, animals had free access to food from 6:00 PM to 9:00 PM, and their livers were removed for fixation 12 hours after food removal—*ie*, at 9:00 AM the next day. In Experiment 1, three choline-deficient rats (Group II) and 1 control rat (Group III) were sacrificed at the following times: 12 and 36 hours, and 3, 4, 5, 7 and 28 days. In Experiment 2, two rats from each choline-deficient group (Group IV and VI) and 2 rats from each control group (Group V and VII) were sacrificed for study at the following times: 12 and 36 hours, and 4, 7, 10 and 35 days.

Fragments of livers removed from rats kept under light ether anesthesia were fixed in buffered glutaraldehyde followed by osmium, or in osmium alone. After dehydration in alcohols, tissues were embedded in Epon 812. Sections were cut with diamond knives on a MT-1 Porter-Blum microtome, stained with uranyl acetate and lead citrate or with lead oxide alone and examined in a Philips 200 and a Philips 300 electron microscope.

To establish the presence of lipid in the granules within the membranous systems of the cytoplasm of the hepatocyte, blocks of tissues of the same size as those used for routine electron microscopic studies, after they had been fixed and dehydrated, were processed for lipid extraction according to these procedures: (1) chloroform-methanol (2:1) for 6 hours (3 changes, 2 hours each); and (2) acetone for 6 hours (3 changes, 2 hours each). Blocks of tissue kept in phosphate buffer for 6 hours served as controls. After lipid extraction, blocks were processed according to the standard technic for electron microscopy.

The present report is based on analysis of about 1600 micrographs.

Results

Hepatocytes of the Normal Rat

Since rat hepatocytes under normal conditions have been the object of many investigations,[12] we shall review only those details of fine structure most pertinent to the primary objective of the present study.

The normal hepatocyte of rats fed Purina chow, as seen in thin sections for electron microscopy (Fig 1), usually exhibits from 0 to 6 LD in the cytoplasm. The presence of higher quantities of LD is exceptional. The hepatocytes where LD are absent or present in low (1–2) number are more common than the hepatocytes where LD are present in higher quantities. Under these normal conditions, hepatocytes with a relatively high number of LD do not exhibit any difference in cell organelles and systems compared to hepatocytes that do not contain LD at all. There is a physiologic range of variations in lipid content

which is *not* associated with any demonstrable fine structural variation in cell system and organelles. The LD are mostly spherical and range in diameter from 0.3 to 2.5 μ, a range similar to that of the LD in the initial phase of choline deficiency, but much smaller than that found in the early and in the prolonged phases.

In the normal hepatocytes evidence that LD fuse one with another is observed far less frequently than in the liver of the choline-deficient animals. Variations in density in different areas of a single droplet and in different droplets are observed (Fig 2). These variations, which reflect the inhomogeneous chemical nature of the materials contained in the LD, are present in tissues fixed in glutaraldehyde followed by osmium, as well as in tissues fixed in osmium alone. In tissues fixed in osmium alone, the margins of the LD are clearly free of membranes (Fig 8 and 12). In tissues fixed in glutaraldehyde followed by osmium, the margin of the LD appears as a continuous dense line (Fig 2), which could be interpreted, when seen at low power, to be representative of a membranous structure and, thus, could suggest that the lipid material is contained within a membranous system of the cytoplasm such as the ER. However, when the margins of the LD come in close contact with the ER membranes, inspection at high resolving power shows that the classical trilaminar structure of these membranes is not repeated in the line limiting the LD (Fig 2). These exhibit close anatomic relationships with the other cytoplasmic structures and components and one of these relationships is by far more common and more significant than others—*ie* the contact with glycogen (Fig 1). In this respect, as well as in not exhibiting a limiting membrane, the LD in the normal hepatocytes are similar to those in choline-deficient hepatocytes (Fig 6).

Among the other normal cell systems most pertinent to the primary object of the present study are the ER and the Golgi system with their granular content. The rough ER is concentrated preferentially over three or four areas, where its long cisternae are oriented parallel to one another in stacks. These cisternae exhibit almost constant diameter along their middle portions, but are slightly dilated at their extremities—*ie* they are polarized toward the Golgi system. At the opposite ends, the cisternae reticulate into the smooth portion, which is usually in between the glycogen particles. The dilated ends of the cisternae polarized toward the Golgi are remarkable in that they contain round granules 40–80 mμ in diameter (Fig 3). These granules, which according to the most recent morphologic and biochemical evidence are the precursors of plasma VLDL, are found in the following intra-

cellular and extracellular sites: (1) the Golgi vacuoles (Fig 4), where they are more numerous than in cisternae of the rough ER, (2) vesicles presumably released from the Golgi system, which establish contact with the plasma membrane at the cell surface facing the sinusoids (Fig 5), and finally, (3) the space of Disse. The present observations are thus in agreement with those described by others[4,5,13] in providing evidence that ER and Golgi membranous components, which normally consist of proteins and choline-containing lecithins, are involved in the segregation and transport of VLDL from the intracellular site of synthesis to the space of Disse. Thus, one would reasonably postulate that the structural integrity of the membranes of these two systems is an important factor in the outward transport of VLDL.

Hepatocytes of Choline-Deficient Rats

Initial Phase: 12 Hours

The prominent feature of the hepatocytes during this phase is an unusually high number of LD that are not associated with deviations in cell systems and organelles. This feature is illustrated in the hepatocyte in Fig 6, where there are as many as 14 LD. These range in diameter from 0.3 to 1.5 μ. Most of them are separated from one another by areas which contain cell systems, organelles and components, such as the ER, both rough and smooth, mitochondria and glycogen. Evidence of fusion between the LD in Fig 6 is not seen, even though the observation that the two LD in the central portion of the cytoplasm below the nucleus are close to one another suggests that they would eventually coalesce and give rise to a LD of larger size. From the inspection of choline-deficient hepatocytes in this phase, as well as in the ensuing phases when fusion becomes more common and leads to the appearance of large LD, it appears that fusion occurs most commonly between two or more LD, one of which, at least, being about 1 μ in size. It is exceptional when coalescence takes place between two or more LD that are all of small size (0.3 μ or less). Thus, there is evidence that LD grow by fusion, but there is also an indication that before fusion occurs, the initially minute LD increase in size by gradual external addition of material. It is, thus, these minute LD that provide the most significant information on the site of formation and the site of deposition of the lipid material, which begins to accumulate in abnormally large amounts during the initial phase of choline deficiency. At the low magnification of Fig 6 the smallest LD (*arrows*) are seen to be embedded in the glycogen areas. When these small LD are observed at higher magnification, they appear either separated from the

surrounding glycogen by small, round vesicles of the ER (Fig 7) or in immediate contact with glycogen (Fig 8). In neither instance is there evidence that the small LD are enclosed within membranes of the ER. The first important conclusion to be drawn from these observations is that the initial site of deposition of lipid material, which accumulates in large amounts in choline deficiency, is in the ground substance of the cytoplasm, in preferential association with glycogen. The second important conclusion is that this initial accumulation of LD is not associated with abnormalities in cell systems and organelles including the ER and the Golgi systems, which are involved in the transport of VLDL, and the mitochondria, which are active in the oxidation of fatty acids.

Early Phase: 2–10 Days

The prominent feature of the hepatocytes during this phase is that the accumulation of LD becomes associated with abnormalities of cytoplasmic cell systems and organelles. In addition to the LD, the most common deviation in 2- and 3-day choline-deficient hepatocytes is the dilatation (Fig 9 and 14) of the cisternal elements of the rough ER. Variations in the degree of dilatation of the cisternae from cell to cell and also from one part to another of a single cell are readily recognized at the second and third day of treatment. At times, dilatation is exhibited only by two or three of the cisternae that are parallel to one another, forming a stack. In other cells, the dilated cisternae are located in two or three distinct stacks. Dilatation of the ER becomes more common with increasing time on the diet.

Another abnormality of the rough ER that appears at the second day of choline deficiency is the fragmentation of the normally long cisternae into shorter round vesicles. Figure 9 illustrates this transformation of the rough ER in a 2-day choline-deficient hepatocyte. Only a few of the cisternae shown are as long as under normal conditions. Most of them are shorter; they are also dilated and interrupted at several sites. There is an unusually high number of round vesicles, which, being associated with ribosomes, belong to the rough ER. It looks as though the dilated cisternal elements of the rough ER cannot preserve their structural integrity and fragment into smaller, round vesicles.

It is interesting to determine the morphologic features of the VLDL in this abnormal ER. Are these abnormalities of the ER associated with morphologic expression of interference with the transport of VLDL? The rough ER in the choline-deficient hepatocyte in Fig 9

contains several VLDL particles similar to those of the normal hepatocyte. It is not uncommon, however, to observe also choline-deficient hepatocytes in which the shorter cisternae or round vesicles (Fig 10) of the ER contain granules that are similar in density to the normal VLDL, but differ from the latter in being 3–4 times larger. Unusually large granules, similar to those in the ER and which also appeared dilated, have been found in the Golgi system (not shown). On a few occasions, the large granules in the Golgi appeared to derive from the fusion of VLDL particles of regular size.

Tissues were also treated with lipid solvents. No significant difference was found between lipid extraction by acetone and that by chloroform and methanol. Both procedures were equally effective in removing the LD in the ground substance of the cytoplasm as well as the granules, either of normal size or of abnormally large size, within the ER. Removal of the LD and the granules by these solvents was obtained in control liver as well as in choline-deficient liver. The removal of the free LD in cytoplasm, by these solvents, does not require specific comments in view of the long-established fact that these LD consist mostly of triglycerides. The removal of granules within the ER and the Golgi system is in agreement with observations by Ashworth *et al*[14] in similarly treated normal liver and indicates that these granules contain at least a lipid component.

Our observations have thus far established that the cytoplasm of choline-deficient hepatocytes contains, like the normal hepatocyte, two distinct types of lipid-rich bodies (Fig 11): (1) the LD described earlier, not surrounded by membranous structures and, therefore, free in the ground substance of the cytoplasm; and (2) the granules unequivocally located within the lumens of the ER and of the Golgi system. However, in choline-deficient hepatocytes, both types of lipid-rich bodies are commonly larger than in the normal.

These observations raise two questions about the interrelationships between the two pools of liver lipids: (1) does the continuous accumulation of lipid material within the ER and the Golgi system give rise to LD, as has been claimed to occur in the hepatocytes of starved rats,[4] and (2) do VLDL granules fuse with the LD and thus contribute lipid material to the latter? In answer to the first question, we have not observed that lipid-rich granules within the membranous systems grow in size and lose their surrounding membranes so as to acquire the characteristic features of floating fat.

The interpretation of the observations pertinent to the second question is more complicated. Figure 12 shows that a VLDL granule (*long*

arrow) within a round vesicle is in contact with a LD. Before this observation is taken as evidence that the common fate of the VLDL granules is to contribute material to the LD, the secretory cycle of VLDL in the rat hepatocyte should be taken into consideration. The VLDL, after synthesis, are transported first from the rough ER to the Golgi system and, second, from this system to the space of Disse, constantly segregated within vesicles. These may establish contact with other cell organelles and components, including the LD. The LD in choline deficiency are more numerous and larger than under normal conditions so that the chances of contact with VLDL are increased. Thus, the contact between VLDL granules and LD is a casual event rather than an indication that lipid material is commonly transferred from one lipid compartment to the other. This interpretation is supported by the presence in the same micrograph (Fig 12) of another VLDL granule (*short arrow*), which is on its way towards the space of Disse and has not established any significant anatomic relation with LD or with other cell systems and organelles.

Another abnormality in the choline-deficient hepatocytes—the increase in the number of ribosomes free in the ground substance of the cytoplasm (Fig 13 and 14)—appears to be closely related to the abnormalities in the ER membranes. This increase is observed more clearly in the areas included between the ER cisternae than elsewhere. As a result of the decrease in the volume of the ER, the ribosomes normally associated with this system are left behind and are transferred into the immediately adjacent ground substance.

Mitochondria are also affected in the early stage of choline deficiency. We have not applied the methods used by other investigators [2,15] for measuring the size and the number of these organelles in choline deficiency. Nevertheless, we have noticed a distinct, though slight, degree of mitochondrial dilatation (Fig 9). Additional fine structural deviations in the mitochondria have not been observed in our material.

The lesions described in this section have not been seen to change in character within the time limits (2–10 days) of the early stages of choline deficiency. However, changes in the degree of two lesions have been observed. One change concerns the LD, which increase primarily in size to occupy increasingly larger portions of the cytoplasm. As a result cytoplasmic systems and organelles are compressed and concentrated in the cytoplasmic areas, which are relatively smaller than under normal conditions. The other change in time concerns the membranous systems of the cytoplasm. We have reported that at the

second day the cisternal elements of the ER begin to appear dilated and fragmented into shorter round vesicles. The whole system is also slightly reduced in volume. With longer time on the diet, the decrease in volume of the ER predominates over the dilatation and the fragmentation.

Prolonged Phase: 28–35 Days

The prominent feature of the hepatocytes during this phase is the remarkable decrease in the volume of the membranous systems of the cytoplasm. In the 35-day choline-deficient hepatocyte illustrated in Fig 15, the rough ER consists only of a few and very short cisternae. The same is true of the Golgi system (*GO*), a portion of which is seen in the lower part of the right margin of the picture.

The description presented includes the complete series (from 12 hours to 35 days) of lesions common to the three groups of choline-deficient rats examined. We have not discovered fine structural differences in the character of the lesions in relation to the variations in the amounts of the components of the three choline-deficient diets. The degree of certain lesions has, however, been found to vary in relation to the amount of the peanut meal and of fat in the diet. The highest degree of deviations of the membranous systems has been observed in the group (VI) of rats which received the lowest amount of peanut meal. The accumulation of LD free of membranes in the hepatocytes was higher in Groups II and VI, which received 15% coconut oil, than in Group IV which received 4% coconut oil. This difference in the degree of accumulation of LD was clear in the early and late phases of choline deficiency, but was not demonstrated in the initial phase. The livers of control animals fed choline-supplemented diets did not exhibit pathologic deviations in the structure of systems and organelles compared to the rats fed Purina chow.

Discussion

The rat hepatocytes in the choline-deficient conditions of the present study exhibit the following sequence of abnormalities:

1. *Initial Phase* (12 hours). An unusually high number of membrane-free LD accumulates in the ground substance of the cytoplasm, preferentially in contact with glycogen particles. During this phase, the accumulation of LD is not associated with fine structural abnormalities of cell systems and organelles.

2. *Early Phase* (2–10 days). The size of the LD increases continuously in association with dilatation and fragmentation of the ER

cisternae into shorter and mostly round vesicles that contain lipid-rich bodies larger, in many instances, than those characteristic of the normal condition. The Golgi system is also dilated and contains unusually large lipid-rich granules. There is a tendency for the ribosomes, usually on the ER membranes, to become dissociated from the latter and, therefore, to become dislocated into the ground substance of the cytoplasm. The mitochondria are dilated.

3. *Prolonged Phase* (28–35 days). The volume of the ER and the Golgi system decreases prominently.

In accordance with this sequence, the discussion will be concerned first with the site of formation of LD unassociated with abnormalities of systems and organelles. The second part of the discussion will be concerned with the effects of abnormalities in the membranous systems on the transport of VLDL and on the continuous accumulation of the LD.

The first observation relevant to determining the cell compartment in which liver triglycerides begin to accumulate is that the small LD—*ie* the precursors of the large ones, are found unequivocally in the ground substance of the cytoplasm. As for their relationships with the ER, these minute LD are, at times, in close proximity to the membranes of this system. It is not difficult to reconcile these findings with functional findings indicating that in the normal liver the esterification of fatty acids to triglycerides, which are the main component of the LD, is catalyzed by enzymes in the microsomal fraction [4] and by cofactors in the supernatant.[16,17] It could be reasonably expected that a product resulting from the interaction of microsomal and supernatant enzymes would be found in the ground substance of the cytoplasm of the hepatocyte studied *in situ* with the electron microscope. This is supported by another morphologic observation—*ie* the small LD are preferentially closely associated with glycogen particles. Triglycerides are mainly synthesized from fatty acids and glycerophosphate and the most common source, though not the only one, of glycerophosphate is glycogen [18] located in the ground substance of the hepatic cytoplasm. Thus, it appears to agree with a number of firmly established functional and biochemical observations that interpret the present morphologic observations as indicating that the fraction of liver triglycerides that begins to increase in the initial phase of choline deficiency is formed in association with the ER, but is deposited in the ground substance of cytoplasm rather than in the lumen of this system. It is the portion of triglycerides ordinarily exported as VLDL that is segregated within the ER.

These conclusions on the site of formation of the LD do not explain, however, why they increase in number in choline deficiency. The content of fatty acids, including those in the LD, in the normal and abnormal hepatocyte results from the interaction of a number of activities: (1) synthesis, (2) uptake, (3) oxidation and (4) export. The relative significance of these activities in the accumulation of LD in this initial phase of choline deficiency is impossible to establish by electron microscopic studies alone. Nevertheless, certain observations made in the present study are of significance in the elucidation of this problem. Since the mitochondria, normally active in the oxidation of fatty acids, and the ER and the Golgi system, normally active in the outward transport of VLDL, are free of fine structural abnormalities during the initial phase of choline deficiency, the increase in the triglycerides in the LD is not due to impairment of these two functions. We are, thus, left with the alternative that the higher content of triglycerides is the result of an increase in the synthesis and/or in the uptake of fatty acids. Indeed, Yoshida and Harper [19] have reported enhancement of intrahepatic synthesis of fatty acids, and perfusion experiments of isolated livers by Mookerjea [20] have demonstrated enhanced rate of fatty acid uptake as early as 24 hours after the initial administration of a choline-deficient diet.

The fine structural abnormalities of the ER and of the Golgi system have been considered [1] as a direct effect of the lack of dietary choline on the lecithins of these systems. This interpretation is supported and clarified by two additional observations. One is that the microsomal membranes of the hepatocyte are not stable structures, but exhibit a relatively rapid turnover rate.[21] The other observation is that lecithins synthesized by the hepatocyte are not only incorporated into cell membranes but are also secreted into plasma and bile both under normal conditions and, though at a lower rate, in choline deficiency.[22] This latter observation makes it clear that those lecithins that are extruded extracellularly cannot take part in the two processes of degradation and reutilization that take place during membrane turnover. Preservation of the normal structure and the normal volume of the membranous systems requires, therefore, constant supply of either lecithins or the building blocks of lecithins from extrahepatic (dietary) sources. A number of biochemical studies[23–25] have, however, demonstrated that in choline deficiency the synthesis of hepatic lecithins, or at least of certain species of lecithins, is greatly impaired. From all these observations it is concluded that the abnormalities of the ER and of the Golgi system in choline deficiency reflect the inability of the

hepatocytes to synthesize membrane lecithins in amounts adequate to preserve the structural and, therefore, functional properties of these two systems.

In the present discussion, the conclusion that lack of choline impairs the functional properties of the membranous systems of the cytoplasm calls attention, first of all, to the part played by the ER and the Golgi system in the outward transport of VLDL. The observations of significance with regard to this function are that (1) lipid-containing granules in the ER and the Golgi of choline-deficient hepatocytes are 3–4 times larger than those found under normal conditions; and (2) these unusually large granules are contained within abnormally dilated and fragmented portions of these two systems. The association of the increased dimension of the granules with dilatation and fragmentation of the ER and the Golgi system indicates that the lipid-containing granules accumulate and, by fusion, enlarge within the lumens of these two systems; this results from their inability to be transported as readily as normally due to the structural deviations of the membranes. These observations thus provide the morphologic basis for the "delay" in the transport of liver triglycerides shown in choline deficiency by several biochemical studies.[6–8] When our study is considered in this respect, it confirms and enlarges the observations of Estes and Lombardi [26] in a recent electron microscopic study of the early stages of choline-deficient liver.

However, no clear indication or suggestion is provided by our study that lipid material segregated within the membranous systems is the source of the storage fat. This is not taken to indicate that the deviations of the membranous systems of the cytoplasm have no effect in the accumulation of increasingly large quantities of LD. The membranous systems of the cytoplasm are functional not only in the transport of VLDL and in the separation of the two lipid compartments of the liver from one another. They are also the site of the enzymes active in the esterification of fatty acids to triglycerides. It is after triglycerides are formed in association with the ER [4] that a part of them is released, as discussed earlier, into the ground substance of the cytoplasm while another part is segregated within the lumen of the vesicular elements in order to be transported, after being complexed to protein, into the circulation. Various factors must control the amounts of triglycerides diverted into the two pools of liver lipids and it seems logical that one of these factors is the structural condition of the cytoplasmic membranous systems. We thus propose that in choline deficiency the structural deviations of the ER and the Golgi system con-

tribute to the accumulation of triglycerides in the floating fat by the following mechanism. It is well established that normal hepatocytes respond to increased production of triglycerides by increasing the rate of synthesis and the rate of transport of VLDL.[27–29] This concept implies, first, that more triglycerides than under normal conditions are segregated within the ER and, second, that the membranous systems of the cytoplasm active in the outward transport of triglycerides are morphologically free of defects. In choline-deficient hepatocytes, where the membranous systems are structurally abnormal, the synthesis and the uptake of abnormally high quantities of fatty acids will rapidly overload the transport mechanism of the VLDL and unusually high quantities of triglycerides will, therefore, be diverted toward the storage fat.

It thus appears that the "fatty liver" of choline deficiency results from the participation of a number of factors. Under the experimental conditions of the present study, the unusually high number of hepatic LD associated with fine structural abnormalities suggests that chronologically the first factor is the uptake and the synthesis of the abnormally high quantities of fatty acids shown by biochemical studies. The second factor is the structural abnormalities (dilatation and fragmentation) of the ER and of the Golgi system, which result in delayed transport of VLDL into the rapidly metabolizing pool of lipid and in diversion of continuously increasing quantities of triglycerides toward the floating fat. The morphologic alterations of the ER membranes may receive further significance from the observation that a portion of ribosomes normally attached to these membranes are transferred to the ground substance of the cytoplasm. This abnormality could affect either the synthesis or the anatomic distribution, or both, of those protein species normally synthesized by the ribosomes, which in choline deficiency have lost their association with the ER.

Another factor contributing to the accumulation of triglycerides is the dilatation of the mitochondria, which appears to be the morphologic basis of a functional deviation—*ie*, the impaired oxidation of fatty acids, demonstrated by biochemical studies in these organelles in the early stages of choline deficiency.[30,31] There are, finally, the additional dietary factors, such as the quantities of methyl group donors and of fat, which have been discussed elsewhere.[11]

Summary

In this electron microscopic study of the hepatocytes of rats fed choline-deficient diets for 34 days, the initial abnormality, observed

12 hours after the beginning of the experimental treatment, is the increase in the number of membrane-free cytoplasmic lipid droplets, which are formed in the ground substance of the cytoplasm and correspond to the pool of liver triglycerides known as the "storage fat." This initial increase in lipid droplets is not associated with abnormalities in organelles and systems and is interpreted as the morphologic expression of an abnormally high production of triglycerides, which according to biochemical studies results from enhancement in the uptake and intrahepatic synthesis of fatty acids.

The accumulation of lipid droplets becomes more severe with time and, from the second day, is associated with dilatation and fragmentation of the normally long cisternae of the rough endoplasmic reticulum into smaller, round vesicular units. These vesicles and the Golgi vesicles contain in many instances lipid-rich granules 3–4 times larger than the normal granules representative of very low density lipoproteins. As a result of dietary choline deficiency, the lecithin-containing membranes of the endoplasmic reticulum and Golgi system lose their structural integrity and, therefore, their ability to transport lipoproteins into the plasma at the normal rate. The lipoproteins accumulate in the lumen of the membranous systems and, as a result of fusion, become larger than under normal conditions. These morphologic expressions of interference with the transport of lipoproteins is in agreement with the information, provided by several biochemical studies, that in choline deficiency there is a delay in the outward transport of triglycerides into the plasma. However, the present study has not provided evidence that lipoprotein granules, or any other lipid material segregated normally within the membranous systems of the cytoplasm, become part of the storage fat—*ie*, the pool of liver triglycerides that increases in the largest amount in choline deficiency. It is proposed that the structural abnormalities of the membranous systems contribute to the accumulation of triglycerides in the floating fat by the following mechanism. The membranes of the ER are active not only in the transport of lipoproteins and in separating them from the floating fat, but are also the site of enzymes active in the esterification of fatty acids to triglycerides. It is after formation at the endoplasmic reticulum that the triglycerides are, in part, diverted to the floating fat and, in part, segregated within the lumen of the endoplasmic reticulum. The well-established concept that normal hepatocytes respond to increased production of triglycerides by increasing the rate of synthesis and the rate of transport of lipoproteins requires, among other factors, the structural and functional integrity of the mem-

branous systems active in the transport of lipoproteins. In choline-deficient hepatocytes, the structural deviations of these membranous systems preclude the increase in the rate of transport of lipoproteins required by the increased production of fatty acids and, therefore, abnormally high quantities of triglycerides are diverted to the floating fat.

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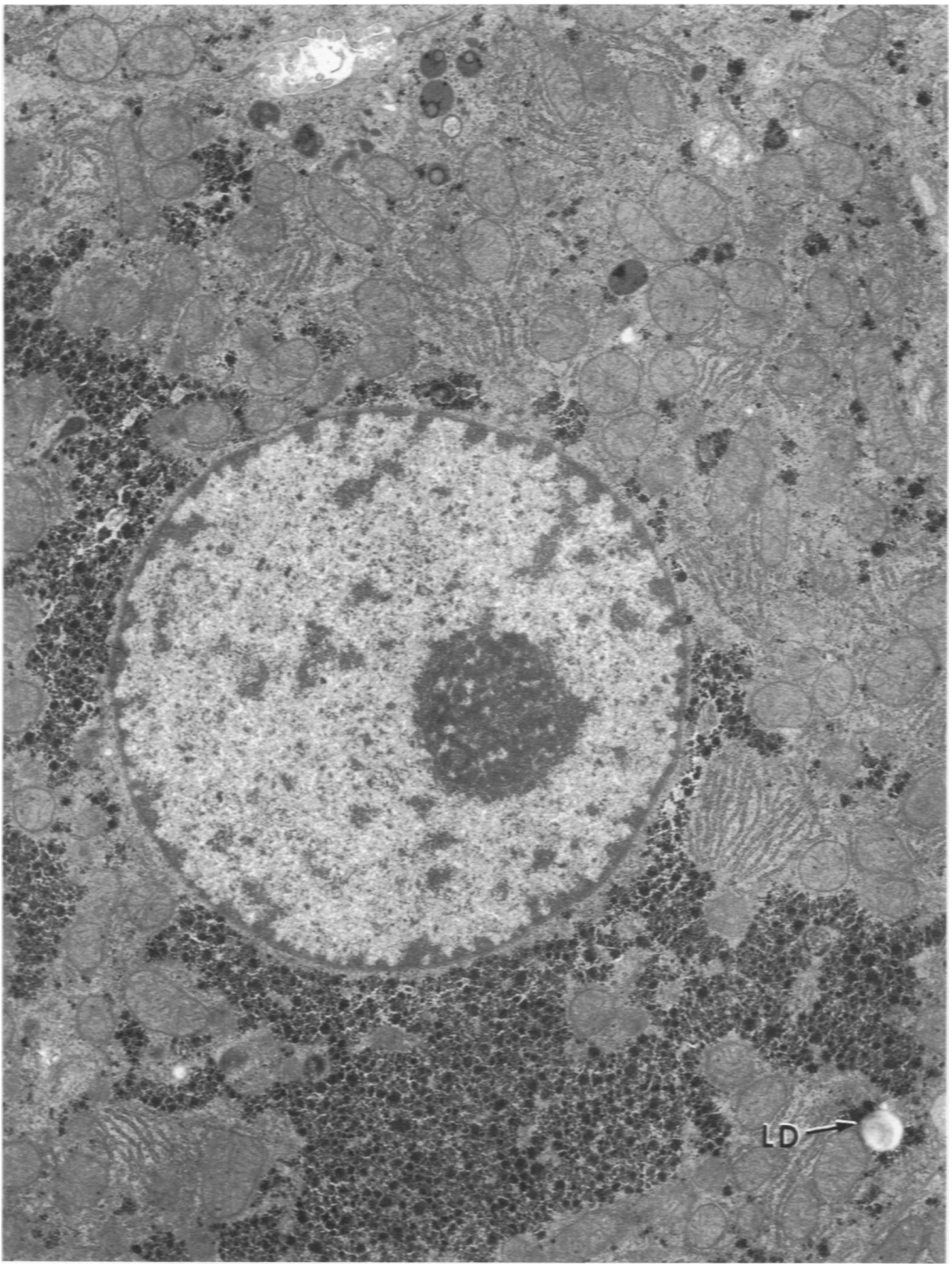
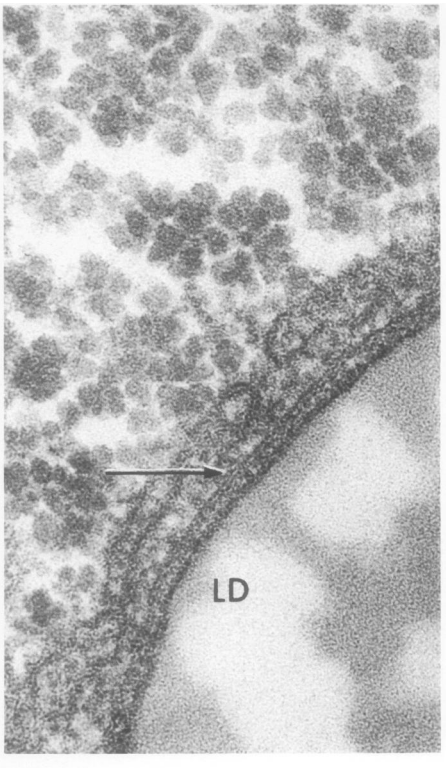


Fig 1.—Low-power view of hepatocyte of rat fed choline-supplemented diet. This micrograph is used as a base line for interpreting the nature and degree of some of the deviations induced by administration of choline-deficient diets. At this level of magnification, presence of lipid droplet *(LD)* in the cytoplasm in close anatomic relationship with glycogen and narrow lumen of cisternae of ER are readily noticeable. Glutaraldehyde fixation. Uranyl and lead stain. × 9900.



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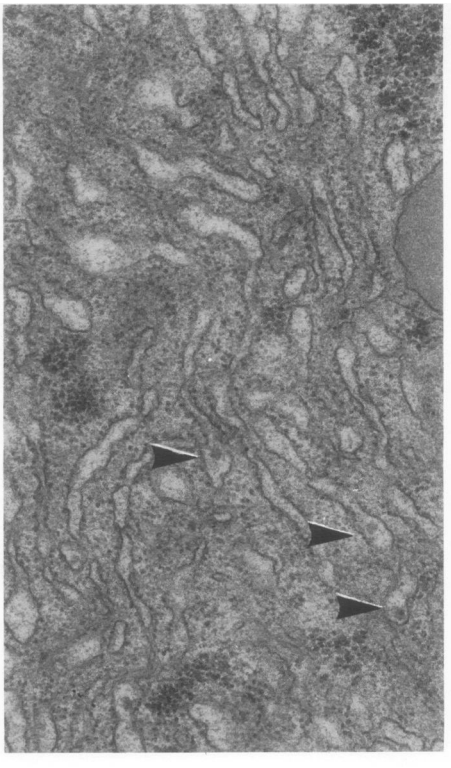

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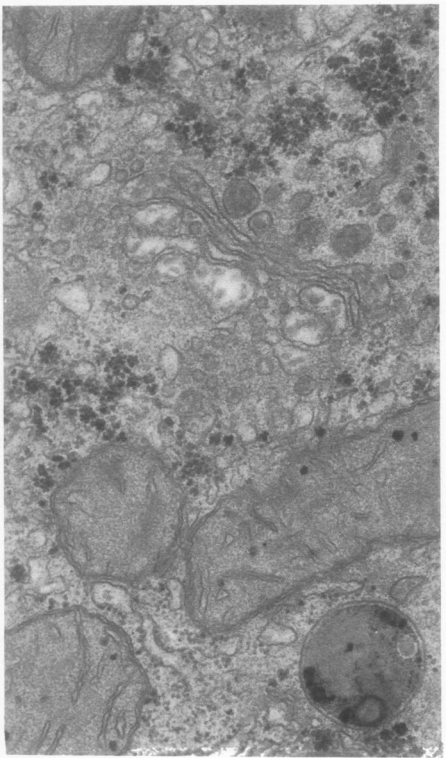

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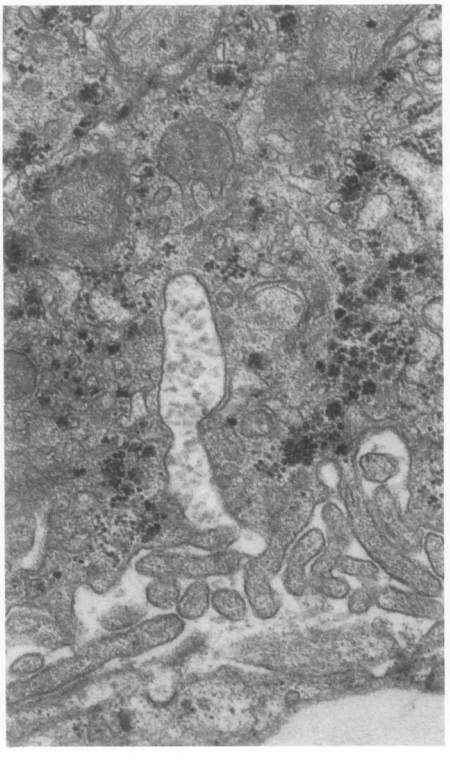

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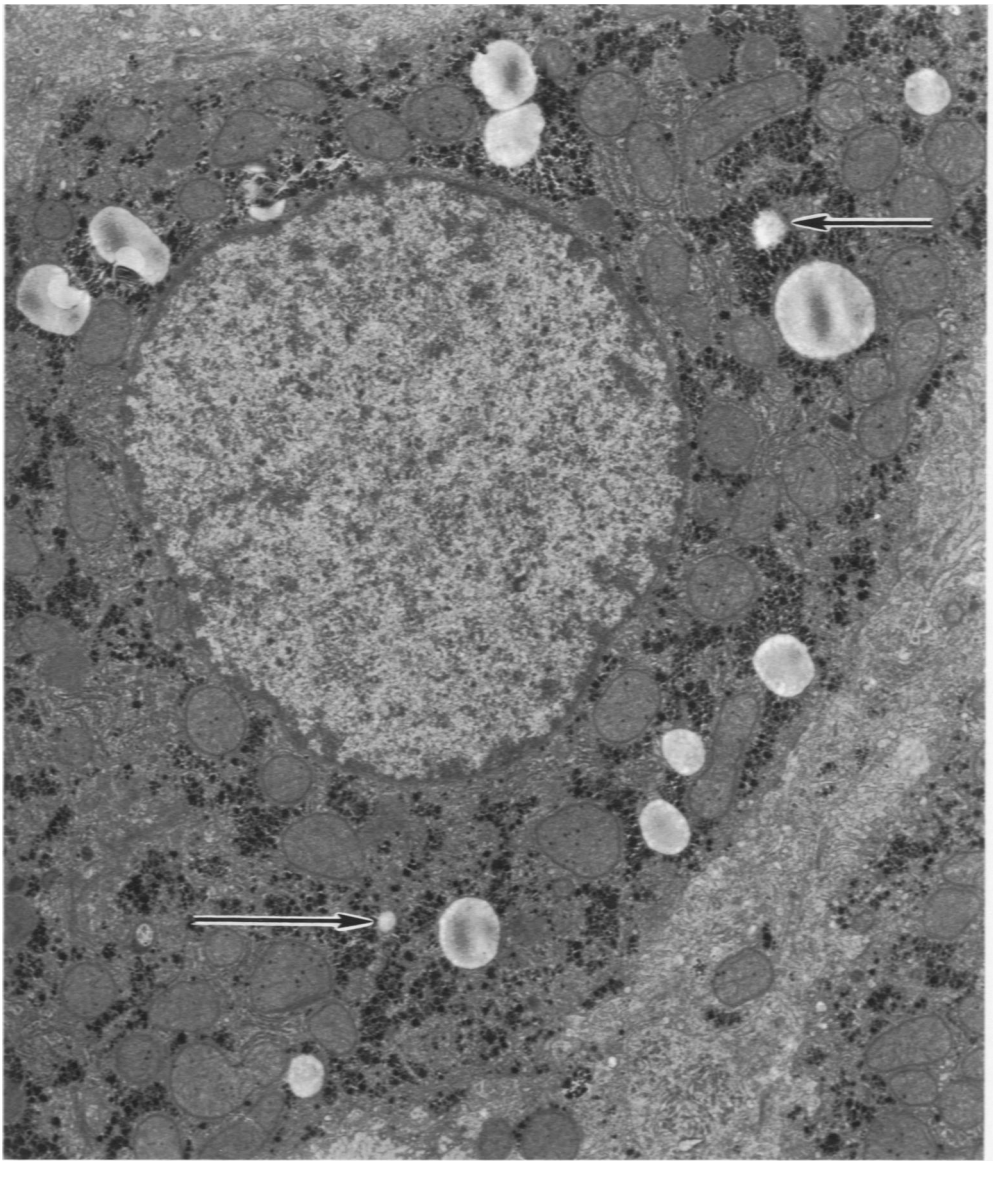


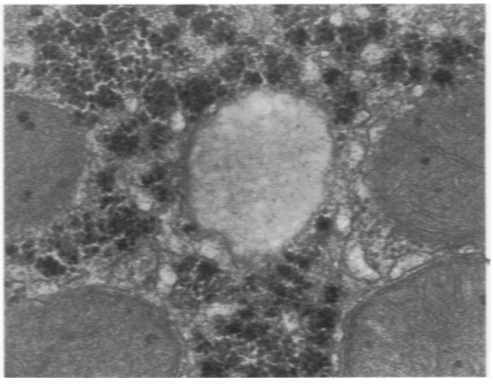
Fig 6.—Low-power view of hepatocyte of rat sacrificed 12 hours after administration of choline-deficient diet. Increase, with respect to normal hepatocyte **(Fig 1),** in number of lipid droplets representative of floating fat is the only abnormality of cell depicted here. Lipid droplets, which exhibit low density and are of different size, are in close anatomic association with glycogen *(very dense particles).* Smallest lipid deposits *(arrows)* are considered representatives of relatively early stages of formation of large droplets. Glutaraldehyde fixation. Uranyl and lead stain. × 9600.

Fig 2–5 depict morphologic representatives of the two pools of lipids in hepatocyte of choline-supplemented rats. **Fig 2.**—Portion of relatively large lipid droplet *(LD),* representative of floating fat, is located in very close proximity to membrane of ER *(arrow)* but is not segregated within this system. Dense line marking margin of LD does not duplicate trilaminar structure of ER membrane. Glutaraldehyde and *OsO*$_4$ fixation. Uranyl and lead stain. × 171,000. **Fig 3–5** illustrate second pool of lipids—*ie,* very low density lipoproteins that appear in form of dense granules 40–80 mμ in diameter, segregated within lumen of membranous systems of cytoplasm. Lipoprotein granules are observed first within rough ER **(Fig 3),** second in Golgi system **(Fig 4)** and finally at site of extrusion into space of Disse **(Fig 5).** *OsO*$_4$ fixation. Lead stain. × 31,000.

Fig 7 and 8 show in greater detail relatively early stages in formation of floating fat in choline-deficient hepatocytes.

Fig 7.—Liver fixed in glutaraldehyde. LD is adjacent to glycogen particles from which it is separated by vesicles of ER. **Fig 8.**—Liver fixed in OsO_4. LD, including smallest one *(arrow)*, are in immediate contact with glycogen. These observations indicate that floating fat is deposited in ground substance of cytoplasm and not within lumen of the ER membranous elements. Uranyl and lead stain. Fig 7, × 34,200. Fig 8, × 21,000.

Fig 9.—Hepatocyte of rat fed choline-deficient diet for 2 days. Cisternae of rough ER are mostly dilated and shorter than under normal conditions. Several vesicular elements of this system are round and contain very low density lipoprotein granules, which in both size and morphology, are similar to those seen under normal conditions. Mitochondria are slightly dilated. OsO_4 fixation. Uranyl and lead stain. × 20,200.



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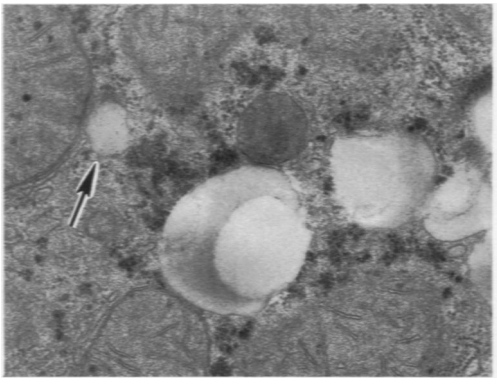

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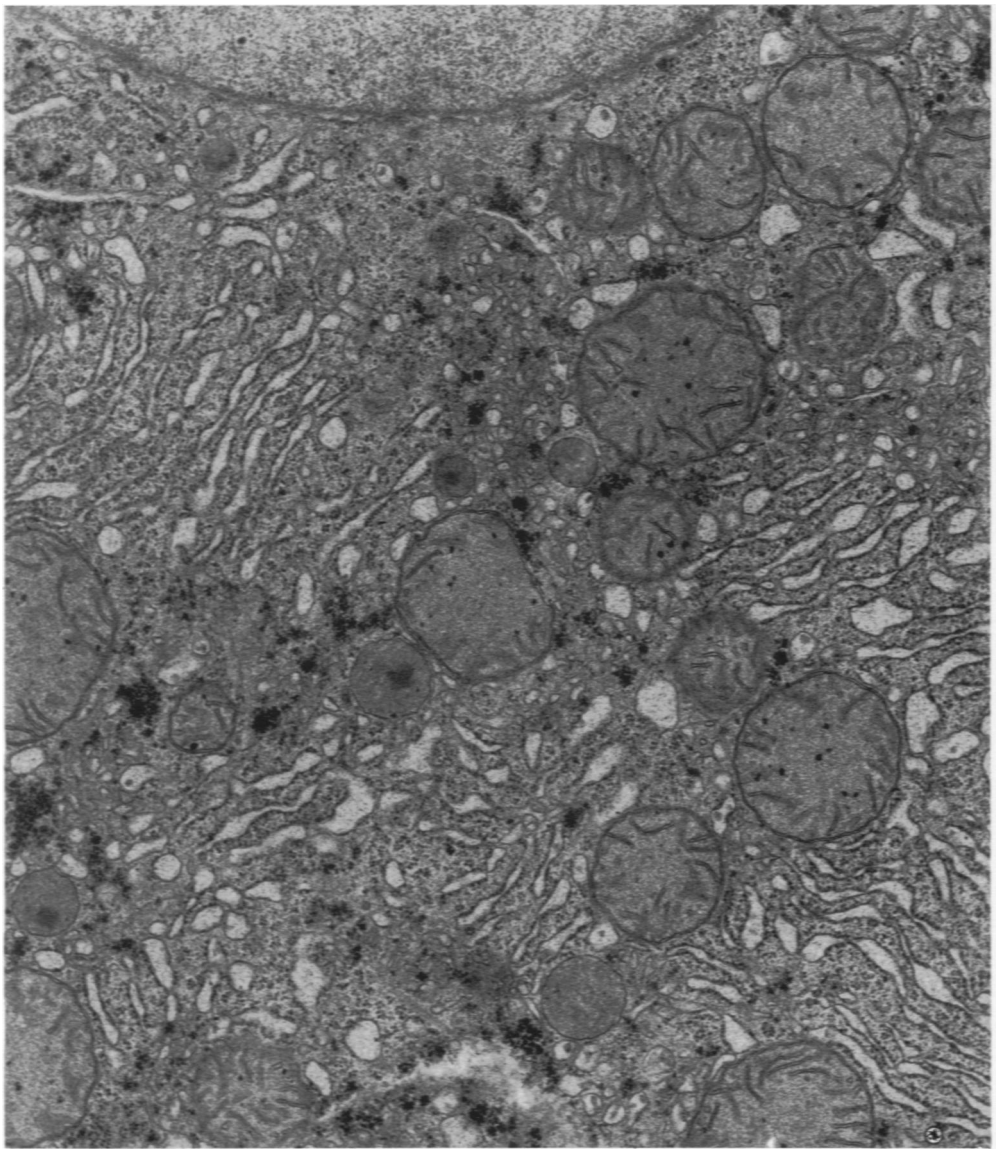

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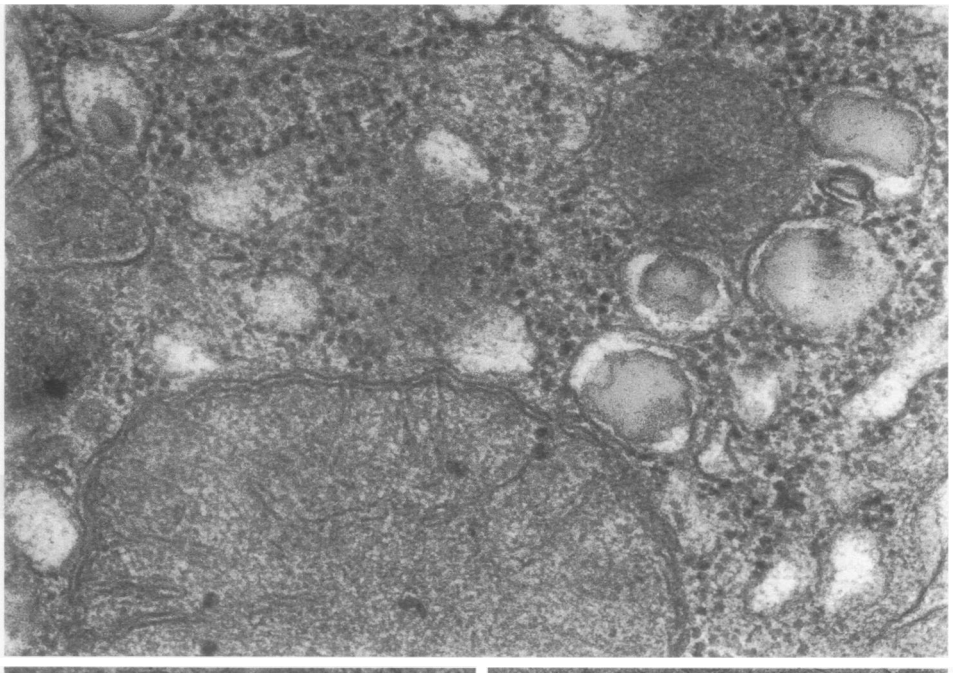

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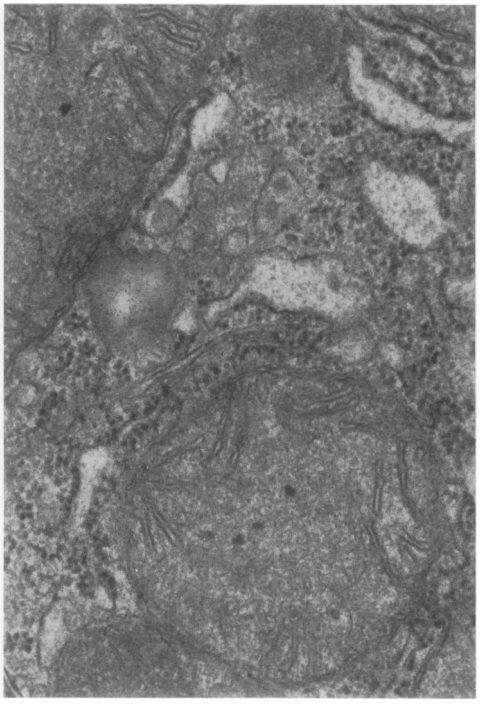

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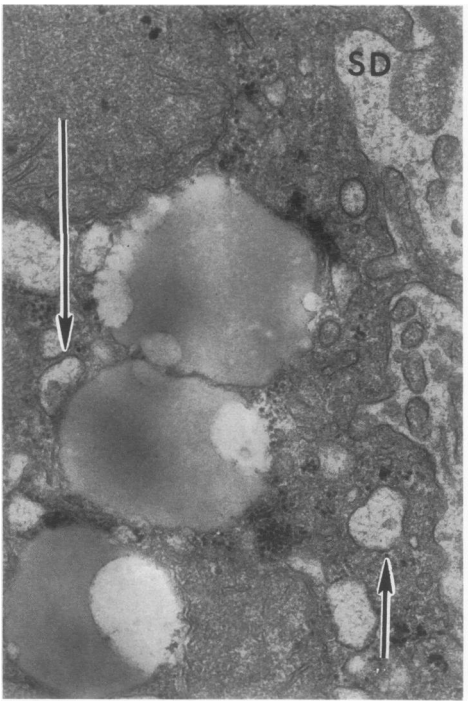

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Fig 10.—Two-day choline-deficient hepatocyte. Dilated vesicular elements of ER contain abnormally large granules. OsO_4 fixation. Uranyl and lead stain. × 68,000. **Fig 11.**—Representatives of the two pools of liver lipids appear side by side in choline-deficient hepatocyte: a membrane-free droplet and lipoprotein granules in cavities of ER. OsO_4 fixation. Uranyl and lead stain. × 40,000. **Fig 12.**—In this choline-deficient hepatocyte, lipoprotein granule *(long arrow)*, on its way toward the space of Disse *(SD)*, establishes contact with large lipid droplet free in cytoplasm. See text for other details. OsO_4 fixation. Uranyl and lead stain. × 40,000.

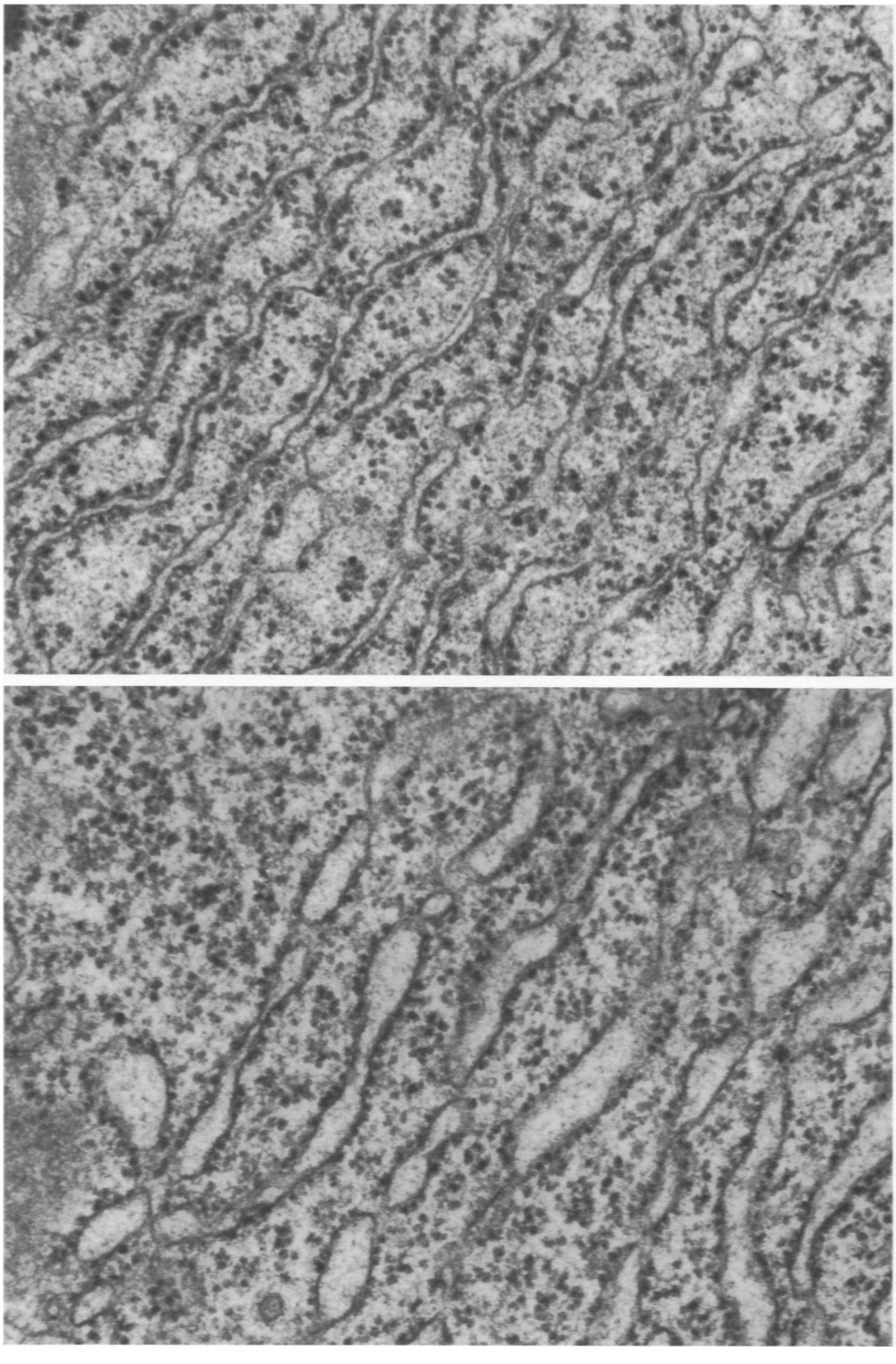

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Fig 13 and 14.—Comparative inspection of a normal hepatocyte **(Fig 13)** and a choline-deficient hepatocyte **(Fig 14)** clearly shows that latter has higher number of ribosomes free in ground substance of cytoplasm. Also clearly evident is dilated condition of cisternae of the ER in choline-deficient hepatocyte. *OsO*$_4$ fixation. Uranyl and lead stain. × 64,700.

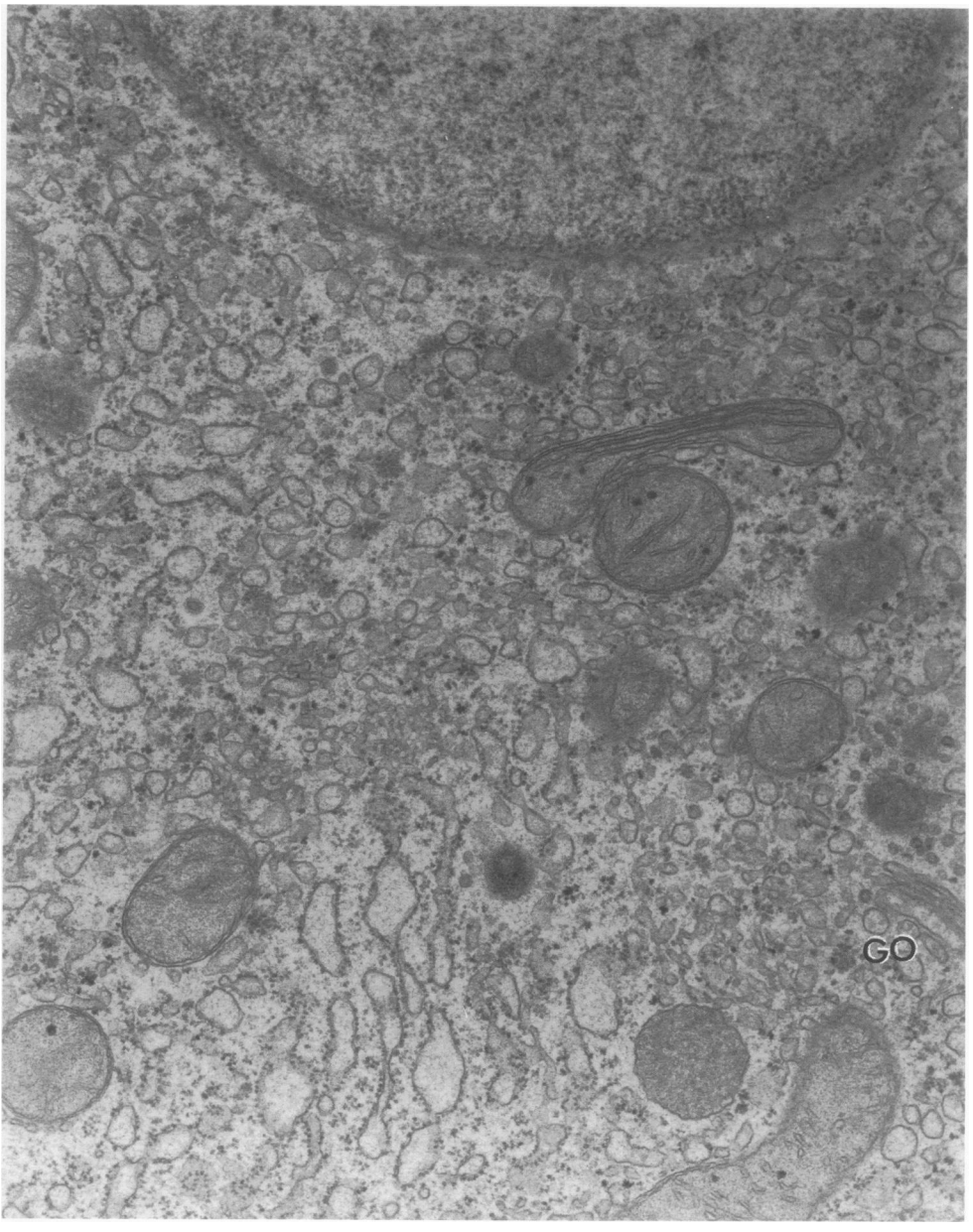

Fig 15.—Relatively large portion of rat hepatocyte in prolonged phase (35 days) of choline-deficient treatment. Prominent abnormality is decrease in volume of lecithin-containing cytoplasmic membranous systems, the ER and Golgi (*GO*). OsO_4 fixation. Uranyl and lead stain. × 26,000.