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HISTOLOGICAL AND ELECTRON MICROSCOPIC INVESTIGATION OF THE LIVER IN THE CHOLINE DEFICIENT GUINEA PIG

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ABSTRACT

Young guinea pigs fed a choline deficient diet were employed for light and electron microscopic studies. They showed severe growth retardation, muscular weakness, diarrhea and subcutaneous hemorrhage. About 73% of the deaths occurred within 5 weeks and survival time varied from 8 to 305 days. The symptoms of animals died within 5 weeks were more acute than the remainders. The gross appearance of the dead animals was characterized by marked emaciation, disappearance of the adipose tissue and congestion of the liver. Fat deposition in the liver was found microscopically in 17 of 48 animals. The light microscopic examination revealed definite degenerative changes of the liver cells including vacuolation, swelling and necrosis, and these findings became prominent with the duration of choline deficiency. In the electron microscopic examination the most conspicuous changes occurred in mitochondria of the liver cell. Mitochondria decreased in number gradually as choline deficiency progressed and manifested some characteristic features at each period of the experiment. Two types of mitochondrial swelling could be observed, the first type was recognized at the early stage and the second type developed at the terminal stage after the 10th week of choline deficiency. In the first type, mitochondria became balloon-like but there was no essential destruction of its fundamental membranous structures. In the second type, mitochondria became gigantic and membranous structures were more or less distracted. Occasionally there found protrusion of mitochondria, dissolution of mitochondrial membrane, disarrangemenet of cristae and appearance of small vesicles in mitochondrial matrix at the terminal stage of choline deficiency. On the other hand, the animals of the recovery experiment showed notable improvements on the above-mentioned symptoms and changes of the liver.

INTRODUCTION

Since McHeney[1] described the retardation of the growth of choline deficient young rats, there have been a pretty amount of studies on effects of choline deficiency. Fatty infiltration of the liver is one of the most remarkable findings of the deficiency, and the lipotropic action of choline has been discus-

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sed variously. Choline seems to be necessary for the synthesis of phospholipid[2], and the phospholipid derived from choline acts on stimulation of fat-utilization of the liver cells[3,4].

On the other hand, Robertson[5] proposed that all biological membrane systems were composed of so-called unit membranes, each of which contained a lipid bilayer coated on each side with an unfolded protein monolayer. Phospholipids are suspected to play an important role on the maintenance of the membrane structure and the function. Submicroscopic changes of the membrane systems must be also attended to studies of the liver in choline deficient animals.

The observation of electron microscopic changes of the liver in the choline deficient rat was first published by Hartroft and his coworkers[6] and they reported that choline deficiency caused the enlargement of mitochondria, which was quickly repaired when choline was replaced. This finding has been mostly supported by some further investigators[7,8], but there still remains much more problems especially on the electron microscopic techniques and interpretations, which will be discussed later in this paper.

Experimental choline deficiency has been carried out mostly in rats or some other species, but few in guinea pigs. The dietary choline requirement of the guinea pig has been determined by Reid[9]. Young and Lucas[10] have pointed out the inability of the guinea pig to utilize methionine, betaine or ethanolamine as a substitute for choline. The guinea pig is therefore less resistant to dietary choline deficiency and the findings on dietary choline deficiency in this animal is much more striking than other animals. Histological and histochemical investigations on the liver of the choline deficient guinea pig were presented by Casselman *et al.*[11], Young *et al.*[10], Mori[12] and Miyakawa[13]. We have no electron microscopic investigation of the choline deficient guinea pig.

In the present study the author aimed at determining the susceptibility of guinea pigs to dietary choline deficiency and describing light and electron microscopic findings of the hepatic lesions with attentions to the membrane system of the mitochondria of the liver cells.

MATERIALS AND METHODS

76 young guinea pigs of the GIFU uniform strain, male and female, weighing 150 to 250 g, were used in this experiment. They were placed individually in screen bottom cages in air-conditioned chambers. From 1 week before the experiment all the experimental animals have been fed the basal diet, the composition of which is shown in Table 1, then choline chloride was taken off from the basal diet. Control animals were maintained all the time on the basal diet. Food and water were supplied *ad libitum*.

TABLE 1. Composition of the Basal Diet

Vitamin-free Casein	300 g	Thiamin	100 mg
Corn oil	73 g	Riboflavin	35 mg
Sucrose	183 g	Pyridoxine HCL	35 mg
Cellophane spangles	150 g	Calcium pantothenate	125 mg
Corn starch	200 g	Niacin	200 mg
Potassium acetate	25 g	Biotin	1 mg
Magnesium oxide	5 g	Folic acid	25 mg
Brigg's salt*	60 g	Vitamin B_{12}	160 mg
Ascorbic acid	6 g	Vitamin A acet.	12 mg
Inositol	2 g	Vitamin D_2	0.08 mg
Choline Chloride	2 g	Vitamin E	20 mg
		Vitamin K	25 mg

* $CaCO_3$-15.0; K_2HPO_4-9.0; Na_2HPO_4-7.3; $Ca_3(PO_4)_2$-14.0; $MgSO_4$ 7 H_2O-5.0; $FeC_6H_5O_7$ 3 H_2O-0.40; $MnSO_4$ 4 H_2O-0.42; KI-0.04; $ZnCO_3$-0.02; $CuSO_4$ 5 H_2O-0.02; (g per kg).

The experimental animals were divided into two groups. In the 1st group (37 animals), the experiment was continued as long as the animals lived. After they died, they were employed for light microscopic study. Animals of the 2nd group (39 animals) were killed at each of the experimental periods for light and electron microscopic studies. Two of these animals had been fed choline deficient diet for 30 days and thereafter they were fed the basal diet for 25 days, to investigate recovery process.

For electron microscopic study the liver was taken out from living animals. Immediately small sections of the liver tissue were fixed for 2 hours in cold (0–4°C) 1% aqueous osmium tetroxide in acetate veronal buffer (pH 7.4) with sucrose added to make an isotonic solution. The sections were dehydrated in alcohol and embedded in Epon. The preparations were cut at 20–60 mμ with glass knives by JUM-5 microtome, stained with lead hydroxide for 15 minutes and examined with a Hitachi HU 11-A type electron microscope. To count the number of mitochondria in each liver cell, the specimens were cut 0.5 μ thick and stained with Altmann's method. For light microscopic study the sections of the liver tissue were stained with H.E., P.A.S. and fat stains.

RESULTS

Symptoms and growth rate

The symptoms of choline deficiency appeared at the beginning of the 2nd week. Roughness of fur, muscular weakness, abdominal distension, subcutaneous hemorrhages, diarrhea and inactivity were noticed as described Reid[14] and Mori[12]. Appetite, however, was kept fair until far later stage. Normocytic, normochromic anemia was found in many animals killed at each experimental period[15].

These symptoms developed as the deficiency progressed, but considerable variations were noticed among individual animals.

The body weight gain of the deficient animals was much less than that of control animals (Table 2). The average body weight gain by the end of the 10th week was 7.8 g in the deficient animals and 32.0 g in control animals. Animals of the recovery experiment showed retardation in growth (weekly body weight gain: 13.3 g) for the first 30 days of choline deficiency, but a remarkable improvement in their growth rate (weekly body weight gain: 39.6 g) was observed for the following 25 days.

Animals died at various periods from the 8th to the 191st experimental day. The mean survival time of 37 such dead animals was 37.7 days. About 73% of the deaths occurred within 5 weeks. The number of the deaths and the initial body weight in each experimental week were shown in Table 3. The initial body weight was not concerned with survival times.

The symptoms of animals died within 5 weeks were more acute, and the retardation in growth was more remarkable (Fig. 1). In the guinea pigs which survived this acute stage a slight improvement of the symptoms was seen and they could be kept alive more several months.

TABLE 2. Average of Body Weight Gain in Grams Weekly

Week	1	2	3	4	5	6	7	8	9	10
Experimental Animals	+10.9 (58)	+2.2 (52)	+10.4 (42)	+11.1 (31)	+10 2 (24)	+6.1 (21)	+2.8 (12)	+7.5 (8)	+16.1 (5)	+3.5 (4)
Control Animals	+44.7 (6)	+28.8 (6)	+30.7 (6)	+36.2 (6)	+27.8 (6)	+43.2 (6)	+21.0 (6)	+22.3 (3)	+31.0 (2)	+29.0 (2)

The figures in parentheses indicate the number of animals.

TABLE 3. Death Rate and Initial Body Weight

Weeks	Number of Dead Animals	Death Rate (%)	Initial Body Weights (g)
2	6	16.3	165.4
3	8	21.6	184.9
4	8	21.6	204.0
5	5	13.5	226.0
6	1	2.7	232.0
7	1	2.7	152.0
8	2	5.4	242.0
9	2	5.4	185.0
10	1	2.7	186.0
13	1	2.7	185.0
23	1	2.7	202.0
28	1	2.7	182.0

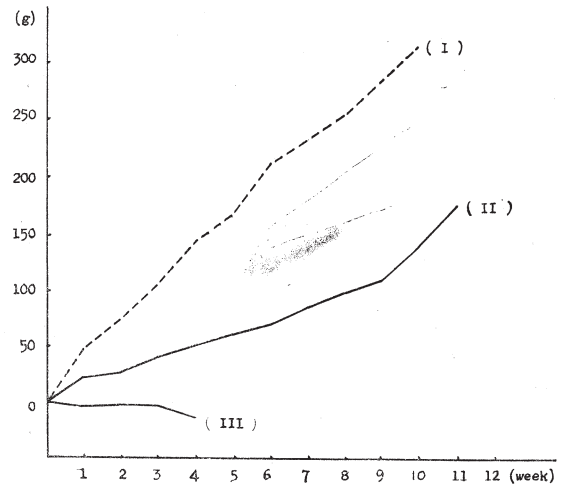

FIG. 1. Average of body weight gain.
(I) Control animals. (II) Choline deficient animals survived more than 5 weeks. (III) Choline deficient animals died within 5 weeks.

Gross observations

(*A*) *Liver:* The livers of choline deficient guinea pigs were rather poor in macroscopic changes, although severe congestion and hemorragic foci were frequently observed in those of dead animals.

Marked fatty infiltration of the liver was found in only 4 animals of the 31st, 69th, 86th and 191st experimental day. The livers of dead animals were usually dark brownish and those of killed animals were brownish in colour. In contrast, the liver of control animals was mostly dark reddish in colour. The outer surface of the liver was smooth and no local lesion was noticed except occasional small hemorrhagic foci.

The liver weights of choline deficient animals were smaller than those of control animals, however, the liver/body weight ratios were almost normal or slightly larger than control animals, especially in dead animals (Table 4). In the animals recovered from choline deficiency the liver weights and the liver/body weight ratios were almost similar to those of control animals.

(*B*) *Others:* There were seen marked emaciation, disappearance of the adipose tissue and atrophy of the skeletal muscles in dead animals. Hemorrhagic foci were frequently observed in the subcutaneous tissue, skeletal muscles and lungs, but rarely in the kidneys and adrenal glands. Congestion was found in the kidney, spleen, and lungs of dead animals. In some cases a small amount of fluid was found in the abdominal cavity. Most intestines were distended and their walls became very thin.

TABLE 4. Average of Liver Weight
Choline deficient animals

Week	Number of Animals	Final Body Weight (g)	Liver Weight (g)	Liver Weight per 100 g Body Weight
2	4 (5)	215.5 (164.8)	8.8 (6.7)	4.1 (4.1)
3	2 (4)	220.0 (143.0)	8.2 (6.0)	3.7 (4.2)
4	8 (8)	245.6 (184.1)	9.6 (8.4)	3.8 (4.6)
5	4 (4)	253.0 (207.5)	9.2 (9.8)	3.6 (4.7)
6	4 (1)	214.8 (278.0)	10.7 (15.0)	5.0 (5.4)
7	7 (0)	218.0 (—)	9.4 (—)	4.3 (—)
8	2 (2)	271.4 (270.0)	9.6 (16.8)	3.5 (6.2)
9	0 (2)	— (186.5)	— (7.4)	— (4.0)
10	0 (1)	— (320.0)	— (15.9)	— (5.0)
11	1 (0)	156.0 (—)	6.6 (—)	4.2 (—)
13	0 (1)	— (392.0)	— (31.2)	— (7.9)
23	0 (1)	— (512.0)	— (25.3)	— (4.9)
28	0 (1)	— (430.0)	— (24.4)	— (5.7)
47	2 (0)	373.5 (—)	15.2 (—)	4.1 (—)
Total	34 (30)	237.9 (280.7)	9.7 (15.2)	4.0 (5.4)
Animals of the recovery experiment				
Total	2	434.0	17.9	4.1
Control animals				
2	2	173.0	7.2	4.2
3	3	189.6	6.8	3.6
4	4	182.5	7.0	3.8
5	3	221.3	8.7	3.9
6	3	282.0	12.8	4.5
7	2	311.5	14.1	4.5
8	6	308.0	13.2	4.3
9	4	309.3	13.1	4.2
10	3	351.0	12.9	3.7
11	2	496.0	16.8	3.4
13	0	—	—	—
23	1	510.0	16.1	3.2
28	0	—	—	—
47	2	707.0	27.3	3.9
Total	35	336.8	13.0	3.9

(The figures in parentheses indicate the value of dead animals)

Light microscopic observation of the liver

In general the histological changes of the liver became more remarkable as choline deficiency progressed. The degeneration of the liver cells was moderate in the early stage, but after the 5th experimental week the degenerative changes of the liver cells took place more prominent and some of regenerative changes modified the histological findings.

In dead animals the changes due to congestion were intensive in general. The liver cells, however, showed essentially the same changes both in killed and dead animals.

(*A*) *Early stage* (*during the first 5 weeks*): In this stage the liver cells of choline deficient animals had fine granules or vacuoles in the cytoplasm and swelled slightly or moderately. A few of them showed necrosis in some of dead animals. Fatty infiltration of the liver occurred diffusively throughout the lobule in only 8 of 30 animals, but was mild except a severe one.

It was the most characteristic change in this stage that the central veins and the sinusoids were widely distended with blood in all dead animals (Photo. 1). Moderate dilatation of the spaces of Disse, swelling of Kupffer cells and disarrangement of the liver cell cords were seen usually in dead animals, but rarely in killed animals. Sometimes focal hemorrhages occurred in lobules and the hyalinosis of small arteries was noticed in the periportal spaces (Photo. 8).

In a few animals some erythroblasts and eosinophils appeared in the sinusoids and a small number of lymphocytes were scattered in the periportal spaces.

(*B*) *Late stage* (*after the first 5 weeks*): In this stage changes of the liver cells were more marked than in early stage, especially after 50 days of choline deficient experiment. The liver cells swelled usually and showed remarkable vacuolation (Photo. 2).

Fatty infiltration of the liver was demonstrated in 9 of 18 animals. In 3 of them it was very severe. Sudanophilic droplets tended to be distributed in the central regions of lobules (Photo. 5). Fat appeared initially beneath the sinusoidal margins in the cytoplasm of liver cells as tiny droplets (Photo. 6) and finally filled up the liver cells as large droplets. In several cases a large amount of glycogen was present within the cytoplasm of liver cells (Photo. 7).

Occasionally liver cells affected with necrosis and some of them showed small necrotic foci. Nuclear degenerations were observed in many liver cells. It was generally noticed that the liver cells were stained not uniformly and the disarrangement of the liver cell cords was seen, but few dissociation of liver cells was observed.

There was evidence of slight regeneration in several liver cells and in such cases multinuclear liver cells were noticed.

In the guinea pig of the 86th day in choline deficiency, the liver showed exceptionally severe changes. In this case an abundant large fatty droplets were scattered diffusively throughout the lobules. The liver cells were greatly vacuolated and some became atrophic or necrotic, their nuclei showed karyolysis, karyorrhexis and pycnosis. The liver showed severe central congestion and some hemorrhagic foci were found scattered. The peripheral zone of the hepatic lobules was occupied by intensive proliferation of bile ducts, which could be regarded as a sort of regenerative process following the liver cell

damage (Photo. 3, 4). Periportal fibrosis did not develop markedly.

In dead animals, marked dilatation of central veins and sinusoids was observed as well as in the early stage. The infiltration of small round cells was found in the periportal spaces and at the periportal zone of the lobules there were various degrees of regenerative proliferation of bile ducts or liver cells. Dilatation of interlobular ducts and hyalinosis of small arteries were seen in a few animals. Erythroblastic foci appeared in the sinusoids in half of all the cases.

In animals of the recovery experiment, no remarkable change was observed except slight swelling and vacuolation of the liver cells and multinuclear liver cells.

Electron microscopic observations of the liver

In the liver of choline deficient guinea pigs electron microscopic changes were found in the liver cells especially in their mitochondria. The changes of the mitochondria varied with progress of choline deficiency, but rather charateristic for each stage. Other organelles of the liver cells showed no definite changes.

(*A*) *Mitochondrial changes in the early stage:* It was strikingly evident in electron micrographs that mitochondria became swollen like a balloon and that was seen already in the guinea pig of the 8th day in choline deficiency. Mitochondria became more spherical in shape and increased in size on cut surface, especially in the liver cells of the deficient animals on the 4th or the 5th week (Table 5). The largest mitochondria in this stage was found in the liver cells of the animals at the 29th day in choline deficiency and its area

TABLE 5. Average Number and area of Mitochondria

Experimental Week	Number of Animals	Number of Mitochondria*	Mitochondria Area and its Standard Deviation
Choline deficient animals			
2–3	5	24.6	1.320±0.488
4–5	9	20.6	1.964±0.880
6–7	2	16.8	1.268±0.482
8–9	2	19.9	1.613±0.632
10–	3	16.2	3.533±1.670
Total	21	19.6	1.940±0.830
Animals of the recovery experiment			
Total	2	23.8	1.242±0.406
Control animals			
Total	5	28.1	0.958±0.353

* in 100 μ^2 of cytoplasm cut at 0.5 μ in thickness.

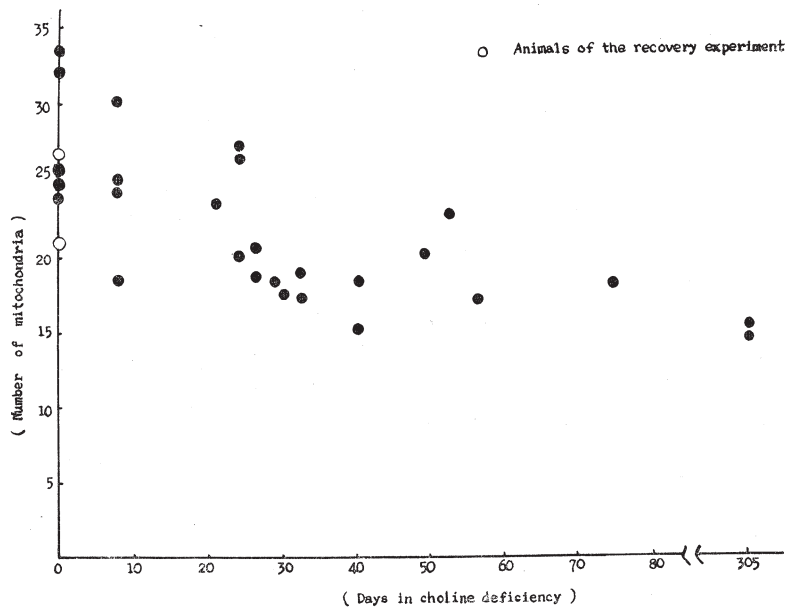

FIG. 2. Number of mitochondria in 100 μ^2 of cytoplasm cut at 0.5 μ in thickness.

was 5.70 μ^2 on cut surface.

The membranes of these swollen mitochondria were smooth and poor in folds. Cristae were almost entirely absent in the central region of mitochondria, however, cristae in each one mitochondria seemed not to decrease in number (Photo. 9). Most mitochondria had moderate density and appeared almost similarly dense to those of control animals (Photo. 14). In the mitochondrial matrix there were several small granules (150 to 400 Å in diameter).

In the animals on the 2nd or the 3rd week in choline deficiency the number of mitochondria in the cytoplasm of the liver cell was about the same as that of control animals, on the 4th and the 5th week, however, it was rather decreased (Table 5) (Fig. 2) (Photo. 10, 19).

(*B*) *Mitochondrial changes in the late stage:* The changes of mitochondria of the animals on the 6th to the 9th week in choline deficiency were definitely different from these animals of more than 10 weeks.

In the liver cells of the animals on the 6th to the 9th week, mitochondria were smaller both in size and number than those of the animals in the early stage of choline deficiency (Photo. 15). The density of the mitochondrial matrix increased markedly (Photo. 20). However, they were similar in appearance of mitochondrial membrane and cristae to the animals of the early stage, and in these matrices some granules were also found (Photo. 16, 20).

On the contrary, in the animals in more than 10 weeks of the deficiency mitochondria appeared to tend to conglomerate and reduced to half in number, but increased remarkably in size (Table 5) (Fig. 2) (Photo. 17, 18). Of all experimental animals the largest mitochondria was found in the liver cells of the animal on the 305th day in choline deficiency. It was almost ten times as large as normal ones and 14.50 μ^2 in area on cut surface. In general mitochondria was round but sometimes irregular in shape.

Mitochondrial membranes appeared wavy, and the limiting membrane or both of the limiting and inner membranes showed discontinuity in places (Photo. 22). In such places, small vacuoles were observed in the peripheral region of the mitochondria and the mitochondrial membranes surrounded the vacuoles. Therefore, the mitochondria were bizzare in shape, as if the vacuoles encroached upon the mitochondria (Photo. 26).

Sometimes mitochondria showed protrusions which projected into adjacent ones (Photo. 22). There were two modes of the mitochondrial joining: one was direct (Photo. 26) and the other was indirect joining (Photo. 25). The latter was a joining through a vesicle or a lamellar membranous small body.

In general cristae were destroyed and decreased in number, but in some places they were elongated and rather increased in number. Sometimes mitochondria was divided into 2 or 3 spaces by newly formed septal membrane, which showed the continuity with the inner mitochondrial membrane (Photo. 23). Cristae were absent in the central region of most of swollen mitochondria. In some of mitochondria the cristae were arranged in lamellae near the mitochondrial membrane (Photo. 24).

Mitochondrial matrix was usually pale and homogenous, had no granule, but sometimes reduced its density in a form of small vesicle, especially near the mitochondrial membrane (Photo. 26).

There were on rare occasions some mitochondria remained normal in size and shape on the same electron micrography. These seemed to be in marked contrast to other degenerative liver cells.

(*C*) *Changes in elements other than mitochondria:* As choline deficiency progressed, the enlarged lysosomes with a diameter more than 1.0 μ were increased in number. They were charaterized by the presence of internal, lamellar bodies or remnants of cellular organelles, such as mitochondria (Photo. 27). The acid phosphatase activity was observed being localized in the areas of the lysosomes (Photo. 28).

Microbodies were slightly increased in number, but in general they were poor in electron microscopic changes. Besides, many small round bodies were found throughout the cytoplasm of some liver cells in the animal at the 305th day in choline deficiency. They were surrounded by simple membranes and had large, dense, amorphous matrices (Photo. 29).

The rough surfaced endoplasmic reticulum somewhat decreased in amount in all choline deficient cases. The decrease was most remarkable in the liver cells which contained an abundant glycogen in the cytoplasm (Photo. 19). The endoplasmic reticulum seemed to have a tendency to localize in the neighborhood of mitochondria. Occasionally there were some dilated rough surfaced endoplasmic reticula (Photo. 21). Some parts of them had small vacuoles which measured up to 1.0 μ and contained light, homogenous substance. Sometimes they showed degranulation.

The smooth surfaced endoplasmic reticulum increased relatively in the glycogen areas, however, other changes were rarely observed.

Several large vacuoles measuring up to 3 μ were recognized only in the liver cells of the animals at an agonal stage. They were of variable shapes, surrounded by simple membranes and seemed usually to be empty.

Fatty droplets were seen frequently in the cytoplasm of the liver cells (Photo. 20). However, they were not large in amount in all animals examined with electron microscope and appeared regardless of the periods of choline deficiency.

A large amount of glycogen was observed in the cytoplasm in several cases and compressed all organelles except smooth surfaced endoplasmic reticulum to peripheral regions.

Dilatation of the bile capillary appeared not so frequently. No significant change was observed in perisinusoidal microvilli.

(*D*) *Changes of the liver cells in the animals recovering from choline deficiency:* Mitochondria seemed to have a definite tendency to be restored to normal in size and shape, but a slight degree of enlargement remained in some cases and also somewhat reduced in number comparing with those of control animals (Table 5) (Photo. 11, 12) (Fig. 2).

There was an increase of both smooth and rough surfaced endoplasmic reticula. Occasionally the latter increased remarkably in some parts. (Photo. 30)

Lysosomes were similar in shape and number to those of the controls.

DISCUSSION

Resistance of the guinea pig to dietary choline deficiency

The guinea pig is different from some other species in the response to dietary choline deficiency and also in choline metabolism. When dietary choline is absent or inadequate, in rats[16] methionine and betaine act as methyl donors in the conversion of ethanolamine to choline. However, in guinea pigs[10] none of methionine, betaine and ethanolamine is utilized individually as a substitute for choline. Young and Lucas[10] have suggested that this inability of the guinea pig is probably due to the inability to place the first

methyl group on the ethanolamine moiety of choline. It has been determined by Reid[9] that the young guinea pig has a definite dietary requirement for choline not only for growth but also for survival.

The survival time of the choline deficient guinea pig is reported by several authors and is thought to be from 21 to 67 days. Miyakawa[13] has examined the response of the "germ-free" guinea pig to dietary choline deficiency and emphasized that the "germ-free" guinea pig has longer survival time than the conventional animal in choline deficiency.

In the first group of our experimental animals about 73% of guinea pigs died within 5 weeks and the survival time was from 8 to 191 days. In the second group of our animals a few lived more than 300 days in choline deficiency, so the survival time of our guinea pigs was much longer than that described by previous authors. As compared with the conditions used by previous authors, the composition of our diet is almost similar but the initial body weight of our animals is somewhat larger. The latter, however, is not concerned with the survival time. Differences of the survival times in various experimental groups must be, therefore, caused by the different susceptibility of an individual animal to dietary choline deficiency. The factors which determine the different susceptibility of each animal must be resolved in further investigations, and the experiment using "germ-free" guinea pigs could be effective in these connections.

In any way, we could observe rather chronic effects of choline deficiency in our guinea pigs, of which none has yet experienced, and as described above, the findings in chronic deficiency are evidently different from the acute deficiency.

In choline deficiency the guinea pig usually dies suddenly. There are only a few reports concerning the cause of death, and Reid[9] has pointed out that retention of a large amount of blood in the heart was regularly found in the low choline animals. In the present study a severe congestion was observed in the livers of all dead animals. From the above facts it is suggested that the sudden death of the choline deficient guinea pig may be caused chiefly by acute circulatory disturbances.

Fatty liver resulting from dietary choline deficiency

Choline is necessary for the synthesis of lecithin. According to Kennedy[2], in the synthesis of lecithin the active forms of choline are phosphoryl choline and cytidine diphosphate choline (CyPPcholine), and diglyceride acts as the acceptor substance which is capable of reacting with CyPPcholine to accept the phosphoryl choline moiety. This information places diglyceride in a key metabolic postion as the major precursor of both neutral fat and phospholipids[17]. On the other hand, Artom[4] has pointed out the enhancement of fatty acid oxidation in the liver under the action of choline-containing phospholipids

formed from choline *in vivo*. And it has been shown that the enzyme system for fatty acid oxidation is located within mitochondria[18)19)]. From the above investigations it is probably safe to say that the fatty liver resulting from dietary choline deficiency is ascribed to increased synthesis of triglyceride, decreased synthesis of lecithin and decreased fatty acid oxidation. From a morphologic standpoint, some authors[20)] have divided the fatty livers of different etiologic types into two broad groups 1) those associated with mitochondrial abnormalities, and 2) those in which the mitochondria remain morphologically normal and in which some abnormalities in other cytoplasmic organelles occur. At this point of view, the fatty liver in choline deficiency belongs to the 1st group.

In many previous papers the fatty liver resulting from choline deficiency has been described not only in rats[21)22)23)] but in some other species[24)25)]. However, in choline deficient guinea pigs some authors[9)13)26)] have failed to produce the fatty liver and the other authors[10)11)12)] have succeeded in.

Handlar[26)] correlated this failure with the low choline oxidase content of the guinea pig liver. If so, choline deficiency could not easily be produced in the guinea pig by dietary means and so his suggestion disagrees with many previous studies. Young[10)] has proposed the sufficient time for the fat to accumulate in the liver by reason of presence of the fatty liver in the animal in a chronic state of choline deficiency. In the present study, although fatty infiltration of the liver is observed more frequently in the late stage than in the early stage (during the first 5 weeks), presence of the livers without fatty infiltration even in the late stage may disagree with Young's opinion. The fact that fatty liver can not easily be produced in the choline deficient guinea pig must be explained by some other unknown reasons.

In choline deficient guinea pigs of the present study, the light microscopic examination revealed definite degenerative changes of the liver cells including vacuolation, swelling and necrosis, and these findings became prominent with the duration of choline deficiency. The electron microscopic examination revealed some characteristic features of mitochondria at each period of the experiment. Moreover, the animals recovering from the deficiency showed notable improvements on the above-mentioned changes of the liver cells. These facts would indicate that the liver cells were surely injured by choline deficiency, although the fatty metamorphosis can be observed rather infrequently in guinea pigs.

In choline deficiency in guinea pigs disappearance of the adipose tissue has been observed in company with or without fatty infiltration of the liver[9)10)]. This is in sharp contrast to rats which show essentially normal deposition of body fat whether the diet has been choline-deficient or not. In many fatal cases of guinea pigs in choline deficiency severe diarrhea and dilatation of the intestine were observed, so that a poor absorption of dietary fat can not

be excluded as one of the factors causing marked disappearance of the body fat.

Mitochondrial changes of the liver

According to Green and his coworkers[27], the mitochondrial membrane is composed of structural—and contractile—protein networks and of lipid which is predominantly in the form of phospholipids micelles bounded hydrophobically to the protein. By some investigators[28] it has been shown that certain lipids in mitochondrial membrane have a quite specific function in electron transport, translocation mechanisms, and swelling and contraction.

It is possible that the disorder of phospholipids metabolism in mitochondrial membrane results from dietary choline deficiency. Kasuga[29] has found decrease of lecithin and sphyngomyelin and increase of saturated fatty acid in phospholipids obtained from the fatty liver of choline deficient rats. Tani[30] has pointed out decrease of the choline-containing phospholipids/cholesterol ratio in liver mitochondria in our choline deficient guinea pigs. In some investigations[31,32] uncoupling of oxidative phosphorylation, increased ATPase activity and decrease of ATP have been shown in liver mitochondria of choline deficient rats. Ozawa[33] has examined the respiratory response of isolated liver mitochondria to ADP in the animals of the present study and obtained the results as follows: the respiratory control index and ADP : O ratio do not change except at the terminal stage of choline deficiency, however, the oxygen consumption under aerobic conditions or in the presence of uncoupling agent increases on the 4th week in choline deficiency but decreases after this period.

On the other hand, some authors have described the morphological abnormalities of mitochondria in the liver of choline deficient animals, which are characterized by an apparent tendency to ensphere and to enlarge. Ashworth *et al.*[7] and Hartroft[34] have considered these morphological changes of mitochondria as results of imperfection of phospholipid synthesis. Grisham[20] has suggested that the disruption of mitochondrial structures in choline deficiency may be associated with impairment of actions of the enzymes therein localized. In our findings it must be noteworthy that the morphological changes in mitochondria are correlated to the changes of respiratory response of the isolated mitochondria to ADP throughout the experimental period.

Mitochondrial swelling is an unspecific phenomenon which occurs under various pathological conditions including starvation[35], acute hypoxia[36,37] and some intoxications[38,39]. It has also been described in deficiencies of some dietary elements such as vitamin B[40], vitamin E[41], essential fatty acids[42] and proteins[43]. In the present study we can find two types of mitochondrial swelling, the first type is recognized at the early stage of choline deficiency and the second type could be seen at the terminal stage after the 10th week of the deficiency. In the first type mitochondria undergoes swelling and

becomes balloon-like, but there is no essential destruction of its fundamental membranous structures. In the second type mitochondria becomes gigantic, the density of the mitochondrial matrix decreases and the membranous structures are more or less distracted. These two types of the mitochondrial swelling have been also observed in choline deficient rats by Hartroft[34], Ashworth[7] and others[20], but the findings in our guinea pigs are much more striking than the animals of them.

Occasionally protrusion of the mitochondrial membrane, which projects to adjacent mitochondria, were found in guinea pigs after the 10th week of choline deficiency. Sulkin and others[41] have described for the first time such a change in the liver of vitamin E deficient rats, and considered it rather specific for vitamin E deficiency. The same change has been observed, however, under many other conditions[44][45], so that the protrusion of the mitochondrial membrane must be regarded as rather a commen metamorphosis of mitochondria associated with unspecific degenerative processes of the injured cells.

As above mentioned, the mitochondrial swelling of the first type can be seen at the early stage, and the second type developes at the terminal stage of choline deficiency. Between these two phases, namely from the 6th to 9th week of choline deficiency we can see usually a middle phase, in which mitochondria decreases in number and also in size. Thereby the density of the mitochondrial matrix increases markedly. Such a transitional involution of mitochondria does not mean the restoration to a normal condition but it must be rather regarded as the expression of one of further advanced degenerative processes.

Enlargement of lysosomes and their increase in number seem to be associated with degeneration of mitochondria and it is not a rare finding that the enlarged lysosomes contain mitochondrial remnants.

As somewhat specific changes of cytoplasmic membranes other than mitochondria, Ashworth[7] has pointed out the appearance of incomplete endoplasmic reticulum in the liver cells of choline deficient rats. We could not, however, ascertain this finding in choline deficient guinea pigs of any stages.

SUMMARY

1) Dietary choline deficiency of the young guinea pig resulted in severe growth retardation, muscular weakness, abdominal distension, diarrhea, anemia and subcutaneous hemorrhage. These symptoms were improved in the animals of the recovery experiment.

2) About 73% of the animals died within 5 weeks and survival time varied from 8 to 305 days. The initial body weight did not concern with the survival time. The animals died within 5 weeks showed severer growth retardation than the remainders.

3) The gross appearance of the dead animals was characterized by marked emaciation, disappearance of the adipose tissue, dilatation of the intestine, congestion of the liver and atrophy of the skeletal muscles. The marked fatty liver was found in only 4 of all animals.

4) The light microscopic examination revealed vacuolation, swelling and necrosis of the liver cell. Fatty infiltration of the liver was found microscopically in 17 of 48 animals and sudanophilic droplets tended to be distributed in the central regions of lobules. Occasionally small hemorrhagic foci and small arterial hyalinosis were observed in the liver. Regenerative changes of the liver cells were noticed in the late stage of choline deficiency (after the first 5 weeks).

5) In the electron microscopic examination mitochondria manifested some characteristic features at each period of the experiment.

During the first 5 weeks mitochondrial changes appeared at the 8th day and the changes were enlargement in volume and loss of crista in the central region of mitochondria. Mitochondrial membrane was poor in folds and mitochondria became balloon-like.

During the 6th to 9th week mitochondria decreased in size and number, but its matrix density was increased.

In more than 10 weeks mitochondria reduced in number and usually gigantic mitochondria appeared, which had wavy mitochondrial membrane, pale matrix, a few cristae but no matrix granule. In some places mitochondria formed protuberances. In other places it was divided into several spaces by the septa. Occasionally there observed small vesicles in mitochondrial matrix, dissolved mitochondrial membrane and cristae in lamellar arrangement.

6) Significant changes in other organelles consisted with an appearance of enlarged lysosomes which contained internal lamellar bodies or remnants of cellular organelles, reduction of rough surfaced ER and relative increase of smooth surfaced ER. In one case many small round bodies were found, which had a simple limiting membrane and large, dense, amorphous matrix.

7) In the animals of the recovery experiment the changes of the liver cells were characterized by mitochondrial restoration to normal in size and shape, and increase of endoplasmic reticulum, especially of rough surfaced ER.

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EXPLANATION OF FIGURES

PHOTO. 1. The liver of the 31st day in choline deficiency. The central vein is widely distended with blood (H.E. stain; 120×).

PHOTO. 2. Same case as the above. The liver cells swell and show vacuolization (H.E. stain; 1 200×).

PHOTO. 3. The liver of the 86th day in choline deficiency. Disarrangement of the liver cell cords and remarkable vacuolization of the liver cells can be seen (H.E. stain; 120×).

PHOTO. 4. Same case as the above. Bile ducts appear intensitive proliferation in the peripheral region of the lobule (H.E. stain; 500×).

PHOTO. 5. Same case as the above. Sudanophilic droplets are distributed in the central region of the lobule (Sudan III stain; 120×).

PHOTO. 6. The liver of the 32nd day in choline deficiency. Tiny fat droplets appear beneath the sinusoidal margins in the cytoplasm of the liver cells (Sudan III stain; 1200×).

PHOTO. 7. The liver of the 45th day in the deficiency. A large amount of glycogen is present within the cytoplasm of the liver cells (P.A.S. stain; 500×)

PHOTO. 8. The liver of the 41st day in the deficiency. The small artery shows hyalinosis and the bile duct dilates moderatly in the peripotal space (H.E. stain; 500×).

PHOTO. 9. The liver cells of the control animal. Mitochondria are small in size but large in number (Altmann-Schridde's method; 1 200×).

PHOTO. 10. Liver mitochondria of the control animal. Mitochondria are moderately dense and contain well-formed cristae. Several dense granules are seen in mitochondrial matrices (Electron micrograph; 2 000×).

PHOTO. 11. The liver cells in the animal of recovery experiment. Mitochondria somewhat reduce in number (Altmann-Schridde's method; 1 200×).

PHOTO. 12. Same case as the above. Mitochondria are almost restored to normal but a slight degree of enlargement remains (Electron micrograph; 2 000×).

PHOTO. 13. The liver cells of the 8th day in choline deficiency (The early stage.). Mitochondria are larger than those of control animal but similar in number (Altmann-Schridde's method; 1 200×).

PHOTO. 14. The liver cells of the 24th day in the deficiency. Mitochondria becomes spherical and mitochondrial membrane is poor in folds. Cristae are almost entirely absent in the central region of mitochondria (Electron micrograph; 20 000×).

PHOTO. 15. The liver cells of the 40th day in the deficiency (The middle stage.). Mitochondria moderately decrease in number (Altmann-Schridde's method; 1200×).

PHOTO. 16. Same case as the above. Mitochondria has marked dense matrix and is smaller in size than those in the early stage. There are several dense granules in mitochondrial matrix (Electron micrograph; 20 000×).

PHOTO. 17. The liver cells of the 305th day in the deficiency (The terminal stage.). Mitochondria appear swollen and reduce to half in number (Altmann-Schridde's method; 1 200×).

PHOTO. 18. Same case as the above. Mitochondria increases remarkably in size and has a pale matrix. Mitochondrial membrane appears wavy. A large vesicle is observed in the mitochondrial matrix (Electron micrograph; 20 000×).

PHOTO. 19. The liver cell of the 24th day in the deficiency. Mitochondria become balloon-like and have moderately dense matrices. Several dense granules can be found in its matrices. The rough surfaced endoplasmic reticulum are localized in the neighborfood of mitochondria (Electron micrograph; 15 000×).

PHOTO. 20. The liver cell of the 40th day in the deficiency. Several fatty droplets are observed among mitochondria. Mitochondria are rod or round in shape (Electron micrograph; 8 000×).

PHOTO. 21. The liver cell of the 26th day in the deficiency. There are some dilated

rough surfaced endoplasmic reticulum among enlarged mitochondria (Electron micrograph; 15 000×).

PHOTO. 22. The liver cell of the 305th day in the deficiency. Most mitochondria appear swollen; their matrices are pale and their membranes frequently form protrusions which project to adjacent mitochondria. Dissolution of mitochondrial membranes is noticed at the point of juncture in the upper right portion of the micrograph. Two mitochondria are divided into two spaces by septal membrane (Electron micrograph; 10 000×).

PHOTO. 23. Same case as the above. Septal membrane which divides mitochondria shows the continuity with the inner mitochondria membrane (Electron micrograph: 21 000×).

PHOTO. 24. The liver cell of the 74th day in the deficiency. Cristae are arranged in lamellar figure near mitochondrial membrane (Electron micrograph; 20000×).

PHOTO. 25. The liver cell of the 305th day in the deficiency. There is seen a lamellar membranous body between two mitochondria (Electron micrograph; 25000×).

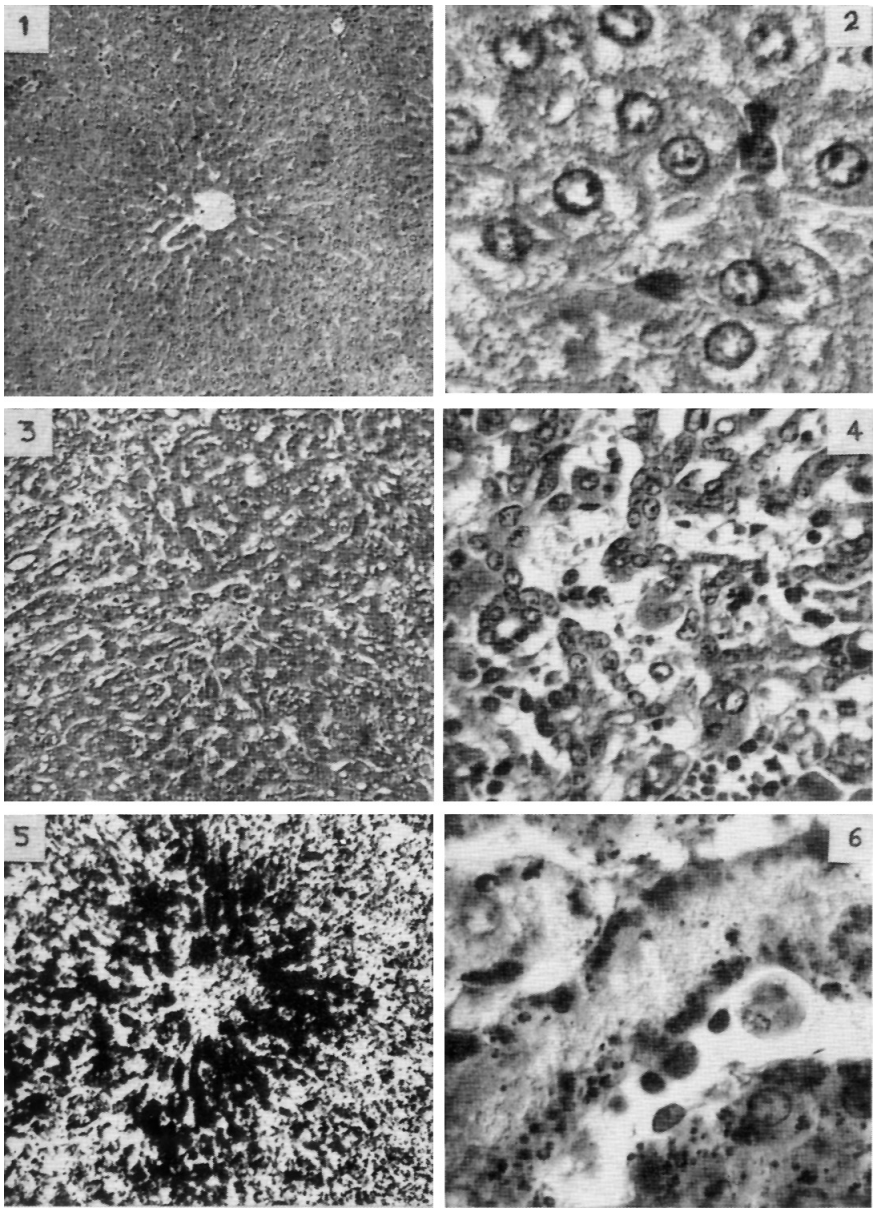
PHOTO. 26. Same case as the above. Mitochondria are bizzare in shape, as if the vacuoles encroached upon the mitochondria. Mitochondrial membrane forms small protrusion which extend into the left mitochondria. In some places mitochondrial matrices reduce their density in a form of small vesicle (Electron micrograph; 18 000×).

PHOTO. 27. Same case as the above. Lysosome contains a remnant of mitochondria (Electron micrograph; 25 000×).

PHOTO. 28. The liver cell of the 30th day in the deficiency. The acid phosphatase activity is observed in the area of lysosome (Gomori's modification: Electron micrograph; 12 000×).

PHOTO. 29. The liver cell of the 305th day in the deficiency. There are seen many small round bodies throughout the cytoplasm (Electron micrograph; 9 000×).

PHOTO. 30. The liver cell in the animal of recovery experiment. The rough surfaced endoplasmic reticulum are well developed (Electron micrograph; 9 000×).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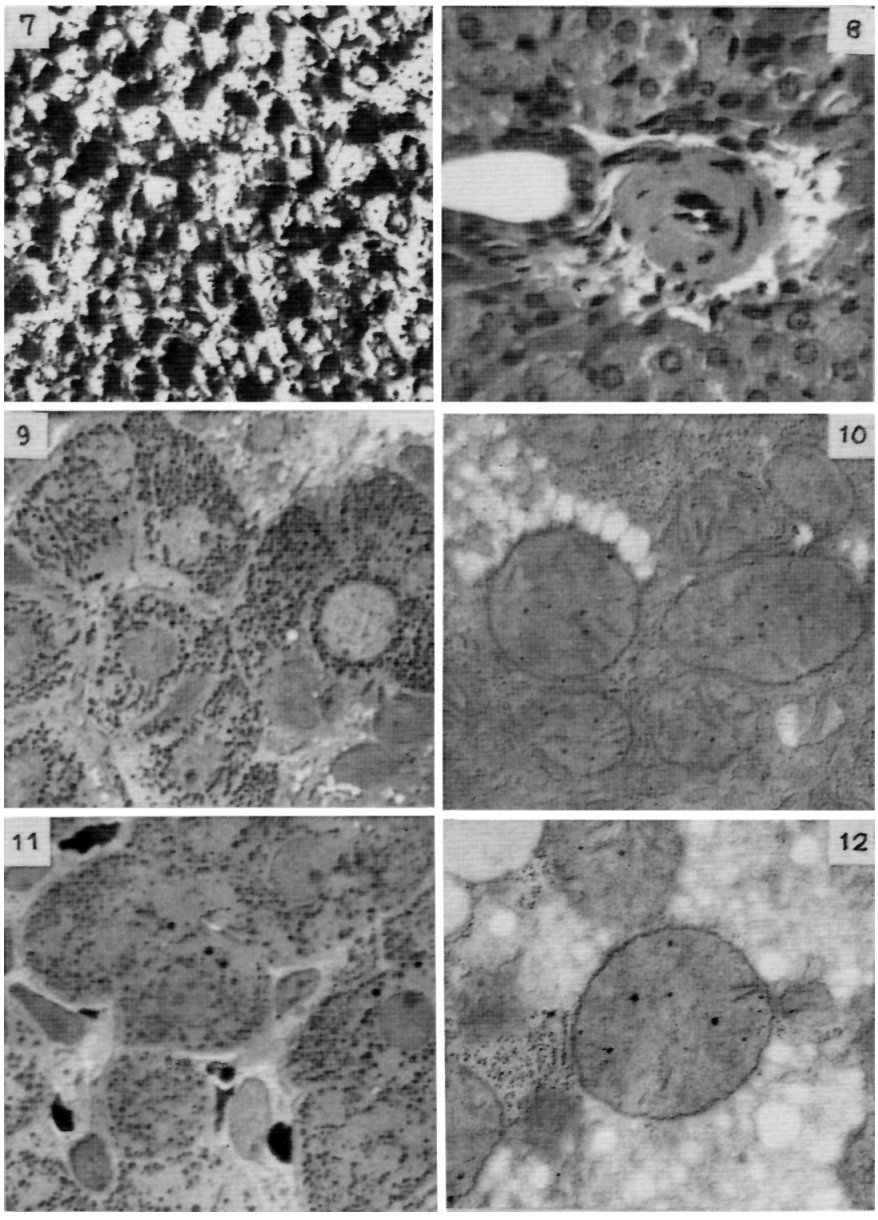
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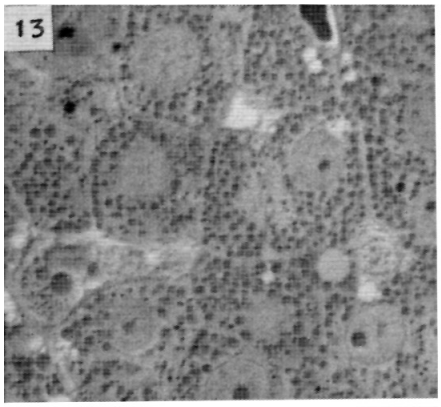
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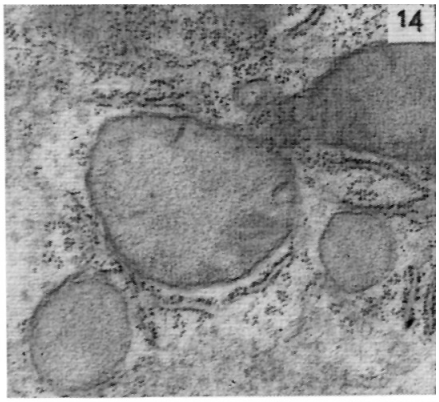
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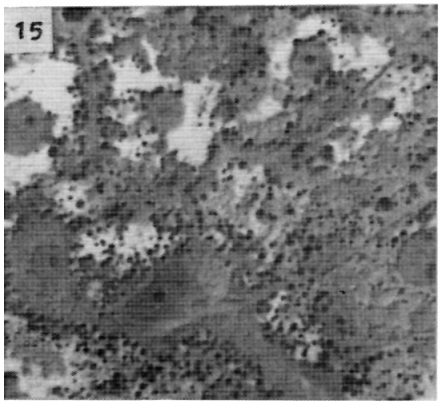
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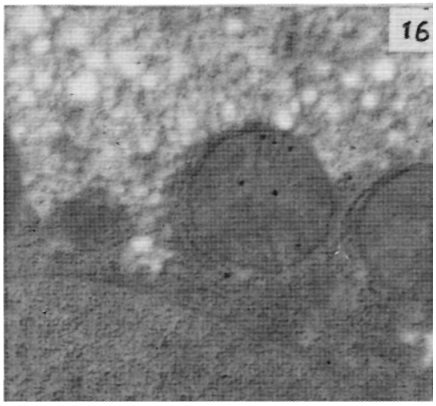
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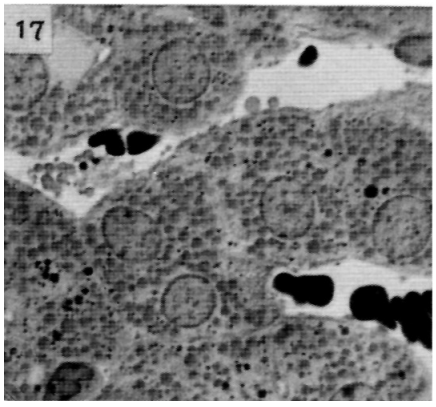
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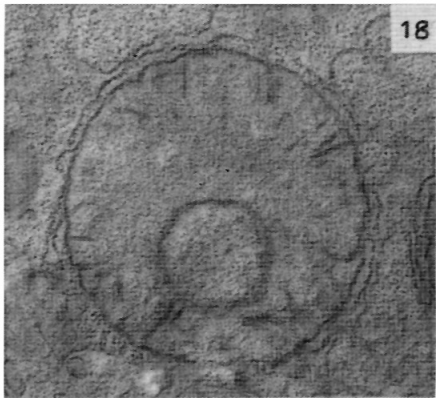
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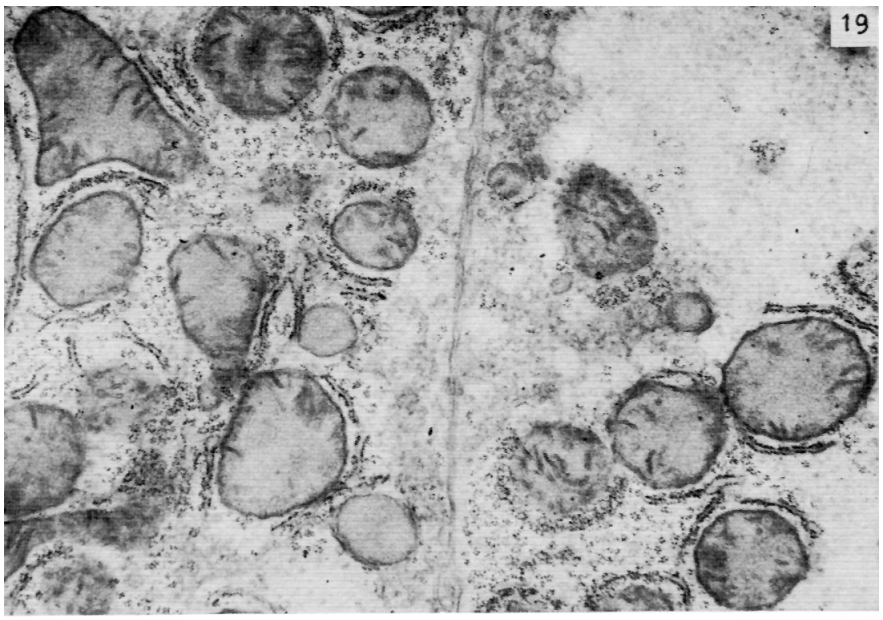
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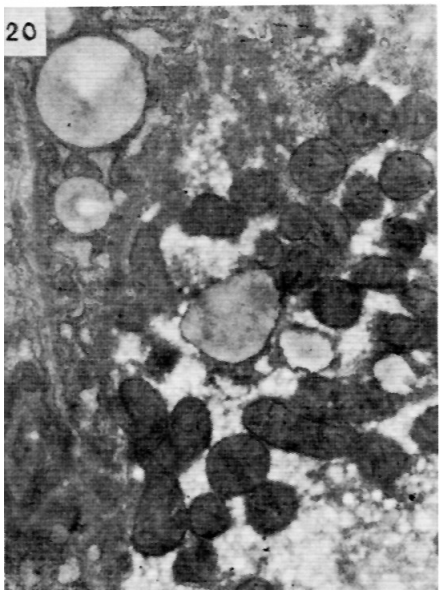
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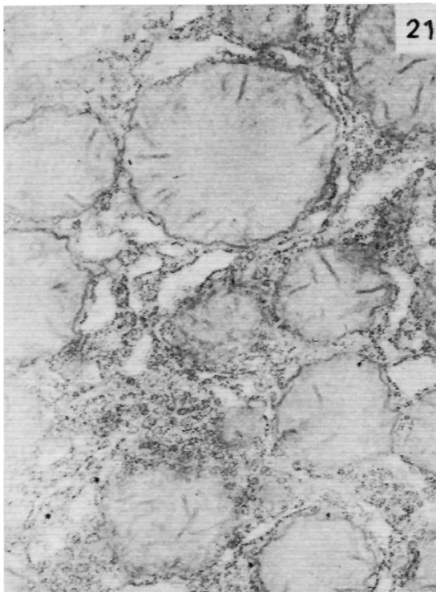
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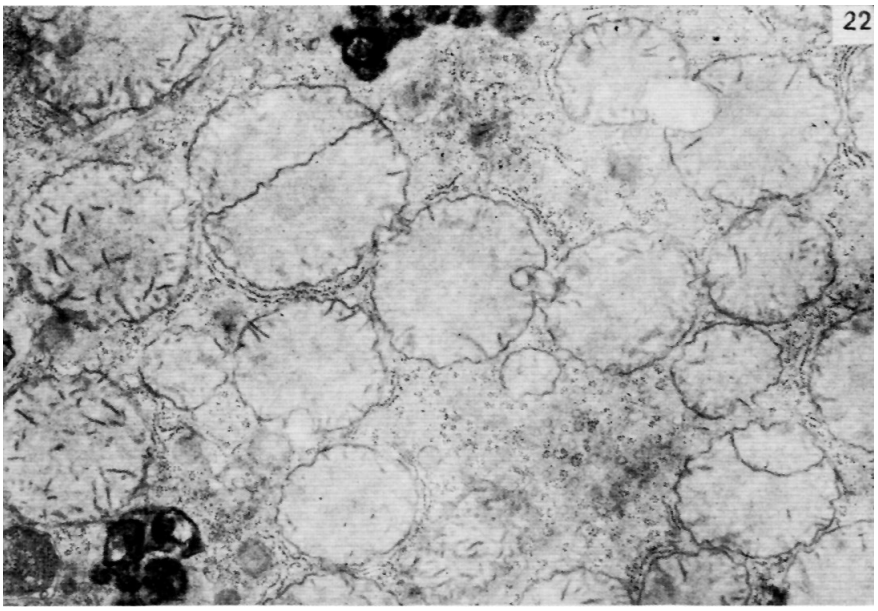
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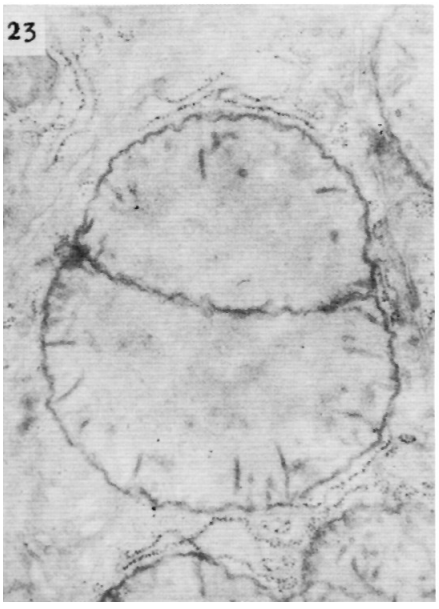
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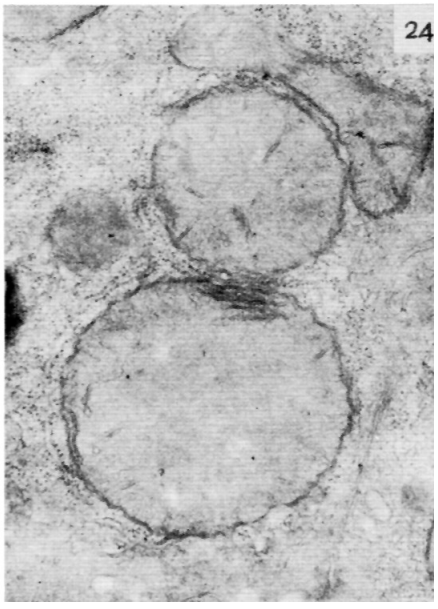
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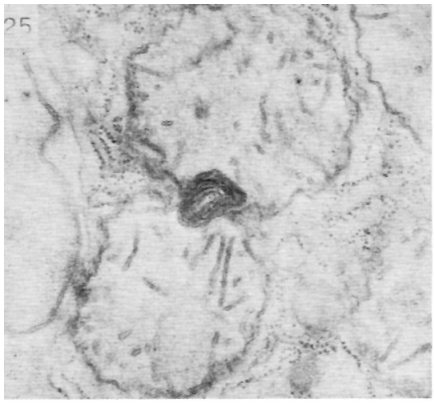
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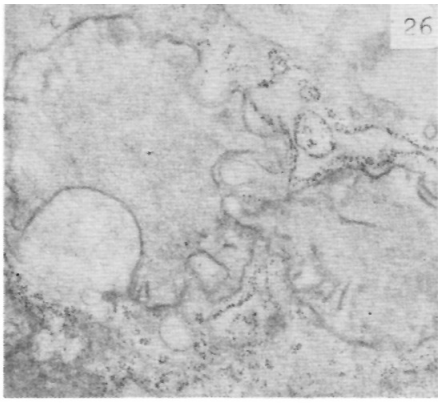
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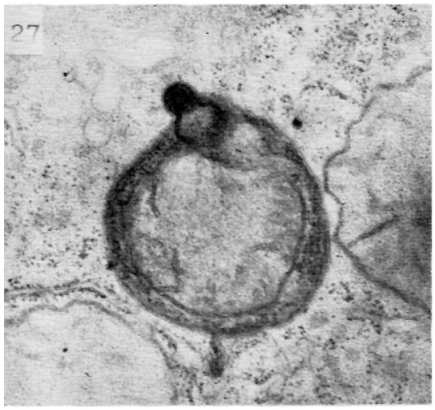
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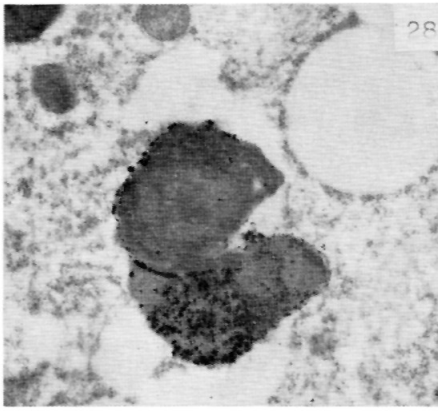
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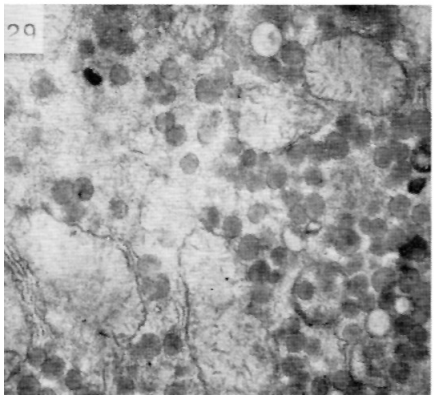
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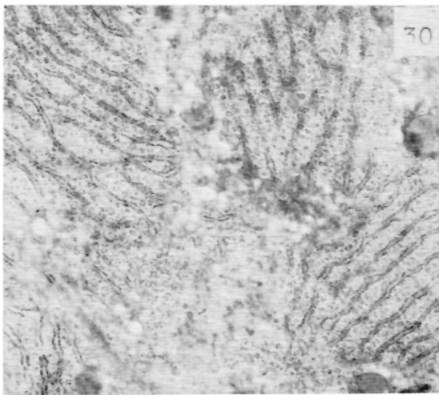
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