Diurnal changes in liver and plasma lipids of choline-deficient rats

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ABSTRACT Early effects of choline deficiency were studied in rats. Nonphospholipid ("neutral lipid") and phospholipid were measured in plasma and in three fractions of a liver homogenate: sediment, supernatant fraction, and "floating fat."

A single choline-deficient meal caused significant aberrations from the typical diurnal changes observed in the lipid fractions of the controls. These changes occurred in the following sequence: (*a*) failure of phospholipid to increase, after feeding, in the sediment fraction; (*b*) increase of neutral lipid, compared with controls, exclusively in the floating fraction; and (*c*) failure of neutral lipid to return to control levels. The rate of accumulation of neutral lipid increased during the first 4 days of deficiency.

The occurrence of NADH–cytochrome *c* dehydrogenase in the floating fat and the absence of succinate dehydrogenase activity point to microsomal origin of the floating fat.

Early effects of choline deficiency on plasma lipids were limited to phospholipid, and occurred later than changes in the liver. Plasma nonphospholipid levels were unchanged during the first 2 days; this does not support impaired secretion or transportation of glyceride as the cause of fatty liver in the early stages of choline deficiency.

KEY WORDS diurnal changes · lipids · liver · plasma · choline deficiency · early · floating fat · subcellular particles · NADH–cytochrome *c* reductase · fatty liver · rat

In a previous study (1) a decrease of serum phospholipid was observed in early choline deficiency of the rat. It was further established that this fall of serum phospholipid coincided with the increase of a hepatic lipid fraction characterized by its instant solubility in cold nonpolar solvents (2) or by its appearance as a creamy layer ("floating fat") when a liver homogenate is subjected to high centrifugal forces (5). Recent studies on liver lipid metabolism (3) have shown that labeled palmitate equilibrates more slowly with the floating fat than with the metabolically more active microsomes and mitochondria. Hence we decided to measure the effects of choline deficiency on hepatic lipid metabolism both in the floating fat and the lipid associated with the particulate matter and cytoplasm in rat liver homogenates.

Spiro and McKibbin (4) have explored the effects of choline deficiency on the distribution of lipid in the particulate matter of rat liver. These authors used fasted rats and pooled liver homogenates; they observed no changes in the phospholipid content or distribution in any cellular fraction. Nevertheless, there was a possibility that the effects of choline deficiency would be more conspicuous and characteristic at the early stages. Furthermore, fed rats might be more suitable as test animals than fasted ones, in view of our observation (1) that rats did not show a fall of serum phospholipid in choline deficiency after 16 hr of fasting.

In the present paper our interest is focused on the effect of a complete diet on neutral lipid and phospholipid in the compartments of the liver cell at known time intervals after food intake. The findings were compared with analogous data obtained after ingestion of a choline-deficient diet.

METHODS

Animals

Male albino rats obtained from Canadian Breeding Laboratories, St. Constant, Laprairie City, P.Q., and weighing 100 ± 10 g were housed in individual cages. Food (commercial ground chow, Maple Leaf Mills Ltd., Toronto) and water were offered ad libitum. The food was placed in a tray, which was attached to the beam of a balance through an opening in the top of the cage. The food tray was counterbalanced by weights corresponding to 80% of the usual daily food intake. With the aid of an electric contact, this arrangement

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permitted an automatic recording of the time at which the rats had consumed 80% of the expected food intake.

Dietary Treatment

All animals were fed the commercial chow diet until they had reached 140 ± 10 g and then a semisynthetic, complete diet (see below) was offered. The control group received this diet for 9 consecutive days and the "choline-deficient" group for 7 or 8 days, with two or one additional feeding(s) of the same diet without choline. The composition of the semisynthetic diet (diet No. IV) has been described in an earlier publication (1). It has the same total protein content as the commercial chow diet (19%); the methionine content, 0.3%, is adequate for optimal growth of rats weighing 140 g (24). The diet (25 g) was offered at 4 PM and the food trays were again removed on the following morning at 9 AM. Food consumption was calculated as the difference between the amount of diet offered, less the amount of scattered food and the amount of diet left in the food trays at 9 AM.

The lipid changes in the liver and plasma of the rat during a 24 hr period were measured at 7 PM, 12 PM, 5 AM, 9 AM, 2 PM, and 7 PM. At each of these time points six rats were killed and their tissues analyzed as shown in the following section. In order to study the effects of a second feeding of the deficient diet, four more groups of rats were used at 12 PM, 9 AM, 2 PM, and 7 PM.

PROCEDURE

Analysis

The rats were anesthetized with ether and bled from the abdominal aorta. The blood was collected in a heparinized syringe and the plasma separated by centrifugation for 30 min at 500 × *g* at room temperature. The livers were excised, blotted, and weighed. The chilled livers were homogenized at 2° in a Potter-type Teflon homogenizer with three times their weight of ice-cold isotonic KCl (0.154 M KCl containing 8 ml of 0.04 M $KHCO_3$ per liter).

The sediment, supernatant fraction, and floating fat were obtained by centrifugation of two 12-ml samples from the homogenate of each rat liver, in the No. 40 rotor of a Spinco Model L centrifuge at 80,000 × *g* (average) for 40 min at 1°. The recovery of the individual fractions from the tubes was carried out at 2–4°. With the help of a 2 ml syringe and a No. 24 needle the floating fat was removed together with a measured amount of the supernatant fraction. The "floating lipid" values were afterwards corrected for the small amount of lipid contained in the supernatant fraction. The supernatant solutions from two tubes were combined and recentrifuged at 80,000 × *g* for 30 min; 10 ml of the purified supernatant fraction was taken for lipid analysis. The sediment was quantitatively transferred with a small amount of water into a 250 ml conical flask.

The total lipid was extracted from all fractions with 150 ml of methylal (freshly distilled)–absolute ethanol 1:2 [a modification of the procedure recommended by Delsal (25) for the extraction of serum lipid]. The mixture was allowed to stand overnight at room temperature and the extract was decanted. The residue was mixed with 120 ml of absolute ethanol, heated to boiling, cooled, and filtered into the same flask in which the first extract had been placed. The extracts were evaporated to dryness in vacuo. The subsequent treatment of the crude lipid leading to the determination of total lipid, phospholipid and, by difference, nonphospholipid followed known procedures (26).

The separation of the liver lipid fractions was made in duplicate. The values for lipid in the supernatant fraction and in blood plasma were from single determinations in each rat. Nonphospholipid data were obtained by difference; since the nonphospholipid is composed of 90–95% triglyceride (including a small amount of free fatty acids) and 5–10% cholesterol and its esters, this difference is considered to be representative of triglycerides in liver and plasma. In place of the unwieldy term "nonphospholipid" we shall use the term "neutral lipid."

NADH–Cytochrome c Reductase and Succinate Dehydrogenase in Purified Floating Fat

The rat liver homogenates were prepared with 0.25 M sucrose, 0.001 M EDTA and 0.05 M Tris buffer [tris (hydroxymethyl) amino methane], pH 7.5. The supernatant fraction, obtained after preliminary centrifugation for 12 min at 12,000 × *g* (average) in a Servall centrifuge at 1°, was recentrifuged in a No. 30 rotor of a Spinco L preparative centrifuge for 40 min at 80,000 × *g* (average). The floating fat was recovered with a syringe and a No. 24 needle, resuspended in buffered 0.25 M sucrose, and again recovered by centrifugation as before. After four such purifications a suspension of the floating fat was prepared in a suitable volume of isotonic sucrose so as to obtain about 50 mg of lipid and 0.2–0.3 mg of protein per ml. After 10-fold dilution, 0.1–0.2 ml of this suspension was sufficiently transparent for the optical tests for NADH–cytochrome *c* dehydrogenase ($NADH_2$:-cytochrome *c* oxidoreductase, EC 1.6.2.1) activity according to Stotz (6). The same suspensions were used for the determination of succinate dehydrogenase (EC 1.3.99.1) activity according to Mahler (7).

In controls designed to measure the stability of the optical density of our lipid suspensions, no change was observed for periods three times as long as those required for the enzyme tests. For protein determination, 1 ml of the lipid suspension was diluted with 1 ml of 2

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N NaOH and left for 4 hr at room temperature. The solubilized protein was separated from insoluble sodium soap by filtration. In the filtrate the protein was determined colorimetrically (8). These colorimetric data compared well with the values obtained directly from suspensions of lipid in KCl by micro-Kjeldahl procedure, after correcting the crude nitrogen values for the presence of phospholipid-nitrogen.

RESULTS

In order to study the early effects of dietary intake on liver and plasma lipid, the time interval between food intake and sampling must be known. This can be achieved either by giving the diet by intubation at a given time and then killing the animals at known time intervals, or by measuring the rate of food consumption during the time before the animals are sacrificed. A practical adaptation of the second alternative was chosen, since it avoided the effects of stress during forced food intake and interference with the rats' nocturnal feeding habits.

It was found that the rats had consumed of their own accord 80% (i.e., 14.5 ± 1.5 g) of their daily food intake by 4 AM ± 1.2 hr (SD). The rate of food intake up to this time was 1.2 ± 0.2 g/hr. The remaining 3.5 ± 1.4 g of food was consumed at a lower rate: 0.71 ± 0.31 g/hr. These data are the averages of individual time and food intake measurements obtained from 35 rats, weighing 180–190 g and fed a complete diet. The amount and rate of food consumed up to 4 AM ± 1.2 hr were higher when a choline-deficient diet was fed: after a single feeding 15.5 ± 1.5 g (13 rats) and after two consecutive feedings 15.6 ± 1.5 g (14 rats) were consumed at a rate of 1.4 ± 0.4 g/hr in both groups. These values were significantly different from the controls (food consumption: $P = 0.05$ and 0.02, respectively, rate: $P = 0.02$ and 0.01, respectively). In comparison to the control, no significant difference in the amount and rate of food intake was observed in the choline-deficient groups between 4 and 9 AM: food uptake amounted to 3.9 ± 0.4 g in both deficient groups and was consumed at a rate of 0.76 ± 0.25 g and 0.78 ± 0.34 g/hr. (The P values in relation to the controls were: food intake $P > 0.5$, $P = 0.5$, and for food intake per hour $P = 0.3$, $P = 0.3$.)

The observation that the rats eat according to a physiologically determined time schedule is not new, but it has not always been taken into consideration in collecting samples of tissue for analysis. The finding warrants presentation of the following relationships between the amount and rate of food intake and diurnal alterations in liver and plasma lipids.

Liver Lipid Changes

The most striking early change in hepatic lipids caused by choline deficiency was a 2- to 6-fold increase in the neutral lipid of the floating fraction (Fig. 1). Study of the diurnal changes reveals several interesting relationships. The first characteristic alteration was a failure of the phospholipid in the sediment fraction of the choline-deficient group to show the small but significant postprandial increase observed when a complete diet was consumed (see data in Fig. 1 for midnight of the first day; for difference between control and choline-deficient, $P = 0.008$). This event precedes, by 5 hr, the first significant increase in neutral lipid in the floating fraction. These changes become more marked on the 2nd day of choline deficiency. It is of interest that choline deficiency did not alter the amount of neutral lipid in the sediment fraction. The neutral lipid in the floating fraction of the deficient group showed a considerable postprandial decrease on the 2nd day, but it failed to reach the level shown by the control.

After a single choline-deficient meal the amount of phospholipid in the floating fraction was not measurably affected (Fig. 2). By the 3rd day, however, the amount of phospholipid in this fraction had increased significantly. When the data are expressed as per cent of lipid, this increase is not apparent; indeed, because of dilution by glyceride, the phospholipid in the floating fraction appears to have decreased.

Supernatant Fraction

The amount of total lipid in this fraction is considerably smaller than that of any other hepatic lipid fraction; it varied between 5 and 11 mg in the liver of a rat of 100 g body weight, with a phospholipid content ranging from 25 to 40%. Significant diurnal variations were noted in the choline-supplemented group. However, neither neutral lipid nor phospholipid in this fraction revealed a clear-cut pattern of differences in response to choline deficiency.

Plasma

Plasma phospholipids (Fig. 3) show a distinct change even after a single choline-deficient meal, and the depression becomes more pronounced by the 2nd day of choline deficiency. Considerably greater diurnal variation was found in the plasma phospholipid of rats fed the complete diet. Thus the time of sampling with respect to the last meal can be of considerable importance in affecting one's decision as to the significance of the difference.

Neutral lipids in the plasma (Fig. 4) were not significantly altered by choline deficiency, except at 7 PM.

The values of both neutral lipid and phospholipid are characteristic for our rats after adjustment to the diet described here. They are higher than corresponding lipid levels of the same type of rats fed a commercial

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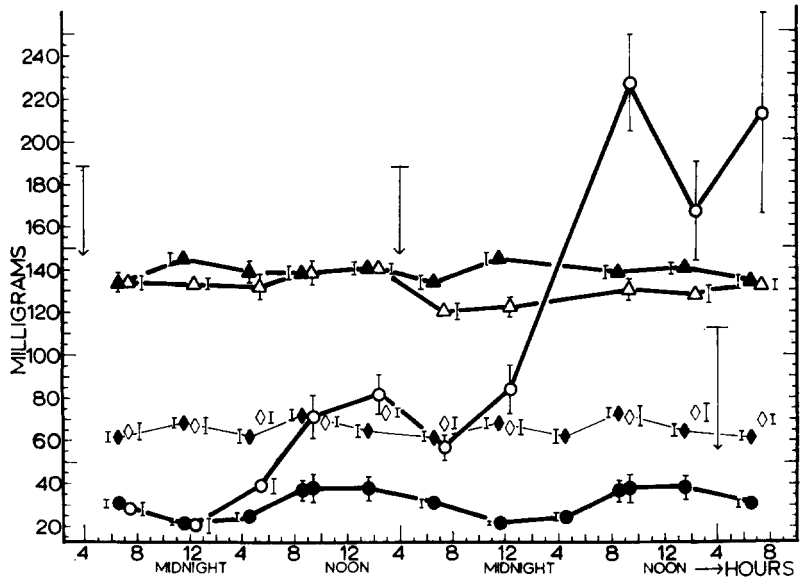

FIG. 1. Time course of lipid changes in the sediment and floating fractions of rat liver. Times at which diets were offered are shown by the arrows (all rats had received choline-supplemented diets up to the time indicated by first arrow). Values are means for homogenates of six rat livers and are normalized for the liver of a 100 g rat. The vertical bars denote 2 × SEM.

Sediment Fraction. Phospholipid: ▲—▲, choline-supplemented; △—△, choline-deficient diet. Neutral lipid (nonphospholipid): ◆—◆, choline-supplemented; ◇—◇, choline-deficient.

Floating Fraction. Neutral lipid: ●—●, choline-supplemented; ○—○, choline-deficient.

chow diet, namely 172 mg of neutral fat and 115 mg of phospholipid per 100 ml of plasma.

Association of Floating Fat with NADH–Cytochrome c Reductase

In purified suspensions of globular fat from the liver of CCl_4-treated rats, Christie and Juda (27) have observed succinoxidase activity. This finding was taken as evidence to support the assumption that the globules are derived from mitochondria. We were interested in investigating the possibility of a similar relationship between the particulate matter in the liver and the floating fat of normal and choline-deficient rats. Since the data shown by the specific activity–time curves for mitochondria, microsomes, and floating fat (3) point to

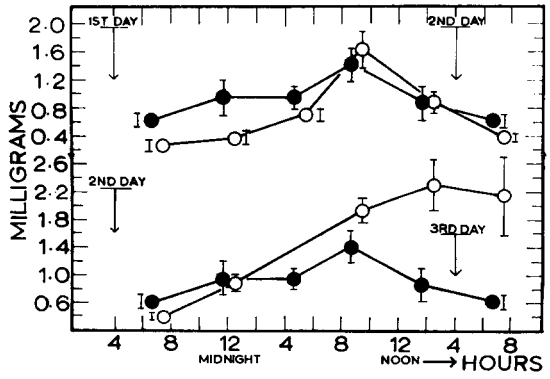

FIG. 2. Hepatic floating fat: changes of phospholipid during 1st and 2nd days. ●—●, Choline-supplemented; ○—○, choline-deficient diet.

Presentation of data as in Fig. 1.

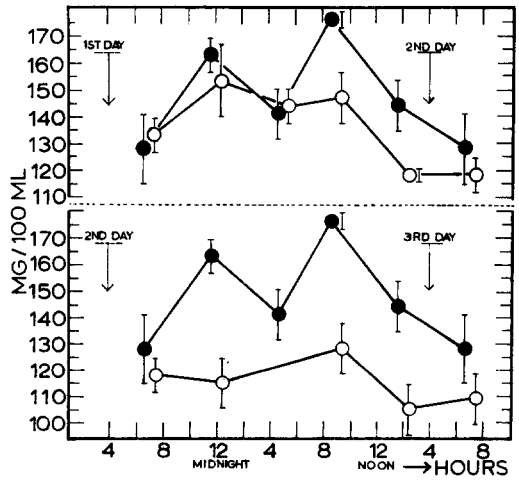

FIG. 3. Plasma phospholipid levels during the first 2 days of choline deficiency. The arrows at the 1st, 2nd, and 3rd day refer to the times at which the animals were offered their respective diets. For comparison the control values obtained with the supplemented group during the 1st day are plotted again in the lower curve for the second 24-hr period. Number of animals: 6. ●—●, Choline-supplemented; ○—○, choline-deficient diet.

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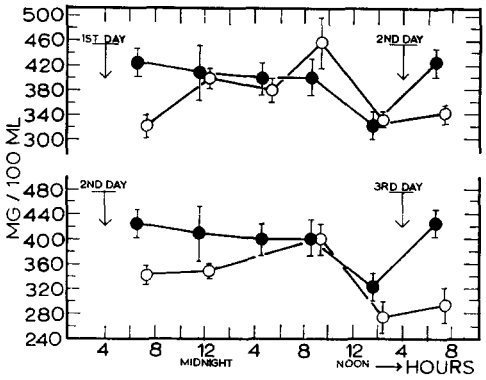

FIG. 4. Plasma neutral lipid (nonphospholipid) levels during the first 2 days of choline deficiency.

Presentation of data as in Fig. 3. Number of animals: 6. Symbols as in Fig. 3.

microsomal rather than mitochondrial origin of the floating fat, we tested the purified suspensions of floating fat for the presence of both (microsomal) NADH–cytochrome *c* reductase and (mitochondrial) succinate–cytochrome *c* dehydrogenase. The results are shown in Tables 1 and 2.

The specific activity (activity per milligram of protein) of NADH–cytochrome *c* reductase in the floating fat suspensions was similar to that shown by a suspension of rat liver microsomes. The yield of the microsomal enzyme increased in proportion to the increased formation of floating fat due to choline deficiency. In contrast, no succinate–cytochrome *c* dehydrogenase activity was found.

TABLE 1 NADH–CYTOCHROME *c* REDUCTASE ACTIVITY OF FLOATING FAT SUSPENSIONS

Source of Enzyme	μg Protein	Protein as % of Lipid	Enzyme Activity	Specific Activity
			units	*units/mg protein*
Microsomal suspension	10.3	—	0.036 ± 0.003 (4)*	3.5
Floating fat				
Choline-supplemented	12.3	1.8	0.031 ± 0.002 (4)*	2.5
Choline-deficient	11.6	1.1	0.038, 0.038	3.3

Suspension of floating fat prepared from the livers of 10 male rats weighing 180 ± 10 g. Assay mixture: NADH (Boehringer, Mannheim, W. Germany), 0.4 μmole in 0.4 ml of 0.1 M phosphate buffer pH 7.2; cytochrome *c* (Sigma, St. Louis, Mo.), 0.2 ml (40 μmole); KCN 0.02 M, 0.2 ml; 0.1–0.2 ml of test suspension (about 10 μg of protein); and 0.1 M phosphate buffer, pH 7.2, to give a total volume of 3.0 ml. The dehydrogenase activity is given as the initial rate of cytochrome *c* reduction, i.e., within 2 min after the addition of the enzyme; units, increase of optical density (at 556 mμ) per minute (±SEM). Temperature, 25°.

* The figures in parentheses refer to the number of measurements.

TABLE 2 SUCCINATE–CYTOCHROME *c* DEHYDROGENASE ACTIVITY OF FLOATING FAT SUSPENSIONS

Suspension Tested	Amount of Protein	Enzyme Activity
	μg	*units*
Rat liver mitochondria	(Corresponding to yield from 20 mg liver)	0.024
Floating fat		
Choline-supplemented	12.3	0.000
Choline-deficient	11.6	0.000

Mitochondrial suspension prepared from liver of a 200 g rat according to Schneider (28); same floating fat suspensions as for Table 1. Assay mixture: 0.05 ml of 0.3 M sodium succinate (150 μmoles); 0.2 ml of cytochrome *c* (40 μmoles); 0.2 ml of 0.02 M KCN; 0.1–0.2 ml of suspension of floating fat (ca. 10 μg of protein); and 0.1 M phosphate buffer, pH 7.2, to give a total volume of 3.0 ml. Enzyme units as in Table 1; temperature, 25°.

DISCUSSION

Rats eating a complete diet ad libitum showed a remarkably consistent feeding pattern: the time at which 80% of their normal daily intake was consumed was constant. Others have noted a diurnal cycle of change of rat liver glycogen (9, 10) and of enzyme activities in rat liver mitochondria (11). It is not surprising, therefore, that a diurnal cycle of changes should be found in the lipids of rat liver and plasma.

The diurnal hepatic lipid changes first appeared in the sediment fraction and were followed by lipid changes in the floating fraction. The same sequence of events was observed by Stein and Shapiro (3) in the relationship between triglycerides in mitochondria or microsomes and those of floating fat after intraportal injection of palmitate-^{14}C into the rat. These authors' data suggest a precursor–product relationship between triglycerides in microsomes and in floating fat. Our observation that suspensions of floating fat exhibit NADH–cytochrome *c* reductase activity support such a relationship. Succinate–cytochrome *c* dehydrogenase activity, which would point to mitochondrial origin, was not observed.

Effects of Choline Deficiency

The first aberration detected in hepatic lipid metabolism after the intake of a choline-deficient meal was absence of the postprandial increase in phospholipid shown by the choline-supplemented control (Fig. 1). This impairment of phospholipid synthesis is probably not limited to the liver; in fact, there is a considerable body of evidence (12–15) suggesting that impairment of synthesis of phospholipid or its precursors in the gut may precede analogous events in the liver. The data in Fig. 1 leave no doubt that the subsequent event, the accumulation of neutral lipid, occurs preferentially, if not exclusively, in the floating fraction.

The first stage of a more lasting accumulation of neutral lipid in the floating fraction is characterized by

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the failure of the postprandial decrease (2–7 PM) to bring the neutral lipid down to the same level as the control. Intake of a curative choline-supplemented meal does permit the neutral lipid to return to control levels (unpublished observations), so that it may be inferred that the failure of the neutral lipid to fall to the expected postprandial level is caused by the opposing effect of a second choline-deficient meal.

The amount of neutral lipid found in the floating fraction after the 2nd day of ingesting a choline-deficient diet is much greater than after 1 day (Fig. 1) and the depression of phospholipid (which always precedes the increase in glycerides) is also greater than on the 1st day. The decrease in phospholipid observed on the 1st day (12.7 ± 4 mg. at midnight) is very similar in magnitude to the increase in neutral lipid in the floating fraction (14.2 ± 4 mg, at 5 AM). This is in agreement with Kennedy's (16) suggestion of a switch from phospholipid to triglyceride synthesis from the common precursor 1,2-diglyceride.

The mechanism by which triglyceride accumulates during the first few days, with increasing rapidity (Fig. 5), is receiving further study.

Existence of Floating Fat In Vivo

The floating fraction, i.e. a suspension of globular fat obtained from the liver after homogenization, is no artifact. Evidence for the presence of free globules of fat in the hepatic cell of the living animal has been provided by microcinematography of livers of choline-deficient rats (17). The observation that palmitate-^{14}C injected into the rat (3) or the rabbit (18) gave rise to the formation of microsomal and mitochondrial triglyceride with a specific activity different from that of the triglyceride in floating fat appears to establish that prior to homogenization of the tissue, floating fat exists in the intact liver as an independent entity.

Plasma

A decrease of plasma phospholipid occurs as early as the 1st day when rats consume a choline-deficient diet. The absence of any significant change in neutral lipid was unexpected. Even after 2 days of choline deficiency, no decrease of neutral lipid was observed, although an even more pronounced fall of plasma phospholipid had taken place. Lombardi and Schotz (19) and Haines and Mookerjea (20) have also noted the lack of any change of plasma triglyceride during the earliest phases of choline deficiency. Consumption of a choline-deficient diet for at least 5 days (20) or longer (21) is required to produce a decrease in plasma neutral lipid, i.e., these changes occur long after the first increase of hepatic neutral lipid.

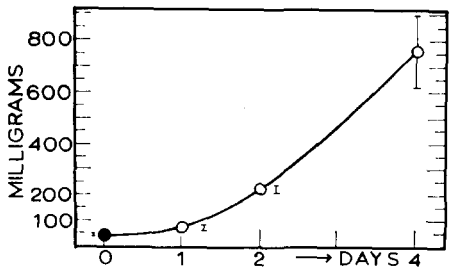

FIG. 5. Accumulation of neutral lipid in hepatic floating fat. The lipid levels were measured at 9 AM and are normalized to 100 g rat.
Number of animals: 6. Symbols as in Fig. 1.

Unless one invokes the possibility that during the early stages of deficiency the analytical technique is not capable of detecting impairment of lipid transport (22) it seems unlikely that the accumulation of neutral lipid in the liver is due to impaired release, at least in the early stages. Perfusion studies of normal and choline-deficient livers (22) have demonstrated that both uptake and oxidative catabolism of lipid within the liver are unchanged in choline deficiency. There remains the possibility of increased de novo synthesis of hepatic triglyceride from acetyl CoA. Some evidence in support of de novo synthesis of triglyceride after 3 days of choline deficiency has been offered by Yoshida and Harper (23), but evidence at still earlier stages of choline deficiency is required to substantiate the hypothesis.

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