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EFFECTS OF DIETARY PROTEIN, LIPOTROPIC FACTORS, AND RE-ALIMENTATION ON TOTAL HEPATIC LIPIDS AND THEIR DISTRIBUTION

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[WITH SPECIAL PLATE]

Interest in the distribution of fat within the liver lobule has been stimulated by the observation that in the widespread disease kwashiorkor the stainable fat is found predominantly in the periportal regions. This subject has recently been reviewed by Brock (1954). It is, of course, a long step from the distribution of fat in the liver of the human infant to that seen in the rat, even when an attempt is made to utilize diets similar to those that produce the characteristic lesions in the human liver. The initial distribution of fat under standardized experimental conditions may not be the same as that which is seen at a later stage. Our lack of knowledge of the anatomy and physiology of the liver lobule prevents us from explaining the mechanisms which determine the localization of fat even when this is sharply defined and consistently reproducible. However, experimental work is steadily revealing new facts that are contributing to the solution of these problems.

In choline deficiency in the rat the first appearance of stainable fat is in the area around the central veins (Lillie, Ashburn, Sebrell, Daft, and Lowry, 1942; Glynn, Himsworth, and Lindan, 1948; Hartroft, 1950). In fasted rats given anterior pituitary extract the initial distribution of the fat is definitely periportal (Best, Hartroft, and Sellers, 1952). Some years ago Dible reported that in rats and rabbits during starvation the distribution of fat is sometimes mainly periportal (Dible, 1932; Dible and Libman, 1934). Shils and Stewart (1954a) have drawn attention to the periportal distribution of fat in the livers of rats fed corn-meal diets and remarked upon the similarity of the lesion to that described by many clinicians in children suffering from kwashiorkor. A similar distribution has been noted in rats consuming other plant materials, such as rice, cassava, and wheat flour (Shils, Friedland, and Stewart, 1954).

Several reports of fatty livers not responding to the usual supplements of choline or its precursors have appeared recently (Wang, Hegsted, Lapi, Zamcheck, and Black, 1949; Litwack, Hankes, and Elvehjem, 1952; Sauberlich, 1953; Singal, Hazan, Sydenstricker, and Littlejohn, 1953; Harper, Monson, Benton, and Elvehjem, 1953; Harper, Monson, Benton, Winje, and Elvehjem, 1954; Winje, Harper, Benton, Boldt, and Elvehjem, 1954). The basal diets fed to the rats in which this condition has been encountered have usually been low in protein. Under these conditions choline has often failed to maintain the liver fat within the normal range, although it has generally prevented any great increase in total liver lipids.

We wish to report some effects of varying the nature and amount of protein in the diet on the total lipids and distribution of stainable fat in the livers of rats and on the lipotropic effect of choline in these diets. Histological studies suggest that the abnormal lipid whose accumulation is not prevented by choline has a different distribution from that seen when the deficiency involves choline or its precursors. It was also noted, during a study of the accumulation and removal of stainable fat in periportal regions, that re-alimentation after diminished food intake due to dietary (protein) deficiency resulted in a transitory fatty liver, the demonstrable fat being exclusively periportal in distribution.

Methods

Five separate experiments will be reported involving some 300 albino (Wistar) rats. The animals were kept in individual cages with a false bottom of coarse wire screen. Fresh food and water were supplied each day; food intake was recorded daily and rats were weighed weekly. Unless otherwise stated, the animals were fed the diets *ad libitum* for three weeks and were then killed by stunning and exsanguination. Livers were removed at once and weighed. Portions were taken for histological examination, and the remainder of the tissue was analysed for total lipids (extraction with hot alcohol, rectification of the lipid residue with a mixture of petroleum ether and chloroform 3:1 v/v). The percentage composition of the basal diets fed in each experiment is shown in Table I. It should be noted that in these studies vitamin B_{12} (300 μg. per 100 g.) was added to the vitamin mixture described by Ridout, Lucas, Patterson, and Best (1954), thus providing 3 μg. per 100 g. of ration. Basal diets are identified by the numerical suffix -0 (Tables I, II, III, and IV).

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Histological Examination

The distribution of demonstrable fat in the livers was studied in frozen sections (5 μ) of formalin-fixed tissues using Wilson's trichrome–oil red 0 technique. Paraffin sections (5 μ) of Bouin-fixed tissues were stained with haematoxylin and eosin.

The sections were examined under a code number by one of us (W. S. H.). The amount of demonstrable lipid was graded from 0 to ++++ (Special Plate, Figs. 1 to 8). Although in Tables II, III, and IV the distribution of the lipid is recorded merely as periportal or centrolobular, the degree of localization varied considerably. The term "periportal" was applied not only when the fat-containing cells were restricted to regions immediately surrounding the portal vein and hepatic artery alone, but also when this distribution extended much farther towards the central vein but did not envelop it. In almost all cases, however, the lobular distribution of the lipid was remarkably clear and well defined, as shown in the figures. Often there was evidence of increased glycogen deposition, especially in the periportal regions (cf. Ramalingaswami, Sriramachari, and Patwardhan, 1954).

Dietary Conditions

In *Experiment I* the lipotropic effect of choline was compared in diets containing different amounts of protein (casein). In these diets (Ia, Ib, Ic, and Id, Table I) the total methionine was kept constant at 0.54% by adding sufficient supplementary DL-methionine. This amount of methionine is equal to that present in the last diet, which contains 18% casein. Groups of six male rats (weighing 70 to 90 g.) were fed these basal diets supplemented with choline chloride as shown in Table II.

In *Experiment II* the rats were fed an animal protein (fibrin), a vegetable protein (soya bean), and mixtures of the two (Table I). Again, in this experiment enough DL-methionine was added to all diets (except IIe) to bring the total to 0.54%. Groups of eight male rats (65 to 80 g.) were fed these basal diets supplemented with choline chloride as shown in Table II.

Experiment III permits comparison of the effect on the distribution of stainable fat in the livers of feeding a low-protein diet (3% casein) without supplementary methionine (Diet IIIa) and with supplementary methionine (Diet Ia). A similar comparison was made in high-protein diets containing less than half as much methionine (250 mg.) as does 18% casein (Diets IIIb and Id respectively). Groups of ten female rats (65 to 90 g.) were fed the basal diets IIIa-0 and IIIb-0 (Table I), alone or supplemented with choline chloride (0.5%), as shown in Table III. Four groups were fed *ad libitum*; rats of the fifth group (IIIb-0 p.f.) were fed the same average amounts of food each day as were consumed by those in Group IIIa-0.

In *Experiment IV* the effect was studied of prolonged consumption of a diet very low in vegetable protein (soya protein 2% plus peanut protein 1%). This ration (IVa-0, Table I), which contains only 32 mg. methionine per 100 g. of mixture, was variously supplemented with choline chloride (0, 0.36%, and 1%, Table III) and fed *ad libitum* for 85 days to male rats (180 to 200 g.).

Experiment V was planned to supply data on the time relationships of both the accumulation of periportal liver lipids in rats on a low-protein diet and their removal when the animals were transferred to a "curative" diet high in protein (Table I, Diets Va and Vb respectively). Forty female rats (65 to 80 g.) were fed the low-protein diet (Va-0, 3% casein), which was supplemented with methionine to make it equivalent in that respect to the "curative" diet containing 18% casein; both diets contained 0.5% choline chloride. Pairs of rats were killed after being fed the low-protein diet for 2, 4, 8, and 16 days, respectively. The remaining 32 rats were transferred to the "curative" diet (Vb containing 18% casein). Groups of eight rats were killed after consuming the "curative" diet for 3, 7, 14, and 21 days, respectively. In addition 25 similar rats were fed a commercial stock ration ("master fox chow," Toronto Elevators, Ltd.). During the first 16 days these rats were pair-fed with those given the low-protein diet. Five rats were killed at the end of this time, and the remainder were then allowed to eat the chow *ad libitum*; groups of five rats were killed after 3, 7, 14, and 21 days, respectively.

Results

Livers from many normal rats of our colony have been examined during past years. The livers were subjected to chemical analysis and sections treated with oil red 0 were scrutinized carefully. Normal values for total liver lipids by our methods of extraction and rectification range from 5 to 8%, the mean and standard error being 6.62 ± 0.09. The values for 20 male rats were 5.19 to 7.48, mean 6.31 ± 0.14; and for 38 females, 5.9 to 8.03, mean 6.79 $\pm$ 0.10. No histologically demonstrable fat could be seen in most livers (Plate, Fig. 1a). Recently, since the above experiments were completed, we have been reinvestigating the "normal" rats of our colony and have found a more frequent appearance of fat in at least some hepatic parenchymal cells. This fat often occurs only as a few to many fine droplets per cell, but occasionally some cells (2 to 12 per high-power field) are seen to be filled with fat (Plate, Fig. 1b). These latter cells appear to be distributed at random within the hepatic lobules. This increased frequency of appearance of cells containing stainable fat is being investigated further.

Experiment I

Most of the stainable fat in the livers of rats consuming the basal diets low in casein (Ia-0, Ib-0, and Ic-0) was in periportal regions, with only a trace in centrolobular areas (see Table II and Plate, Fig. 2). It should be noted that these diets contained considerable methionine. We believe that adequacy of this choline precursor in these basal diets has been important in keeping the centrolobular areas clear. Even with 0.36% of choline chloride the amount of fat in periportal regions was not significantly reduced (cf. Plate, Fig. 2b). As the amount of protein in the diet was increased, less total lipid and periportal fat was observed; only traces of periportal fat could be detected when the diet contained 18% casein (Id-0). The lipotropic effect of a given amount of choline (as estimated by chemical analysis of liver tissue) improved as the protein content of the diet increased, but even large doses of choline were unable to prevent some deposition of abnormal fat so long as the protein in the diet was low.

Experiment II

The limited effectiveness of choline chloride in maintaining normal liver lipids is seen in other diets low in protein —for example, soya protein and a mixture of soya protein with fibrin. Diet IIa-0 (9% soya protein) gave total liver lipids of 9.0% (Plate, Fig. 3); even 0.36% choline chloride failed to reduce this value significantly. As in Experiment I, the deposition of centrolobular fat was prevented by choline, but the stainable fat in periportal regions was much less affected. Fibrin at 9% (Diet IIb-0) produced lower total lipids (by chemical analysis 6.7%) and considerably less stainable fat in periportal regions than did the soya protein, but the livers were not normal. Addition of choline chloride did not appreciably alter the findings (Plate, Fig. 4). A mixture of these two proteins at a total protein level of 9% (Diet IIc-0) also permitted the deposition of considerable periportal fat that was not affected by choline, although some prevention of lipid deposition could be shown analytically. Twice as much of the same protein mixture (that is, soya protein 12% plus fibrin 6%, Diet IId-0) resulted in a more nearly normal liver: only a trace of stainable fat could be detected in periportal regions and only a faint trace in the centrolobular area. Choline reduced the total lipid, but did not alter appreciably that seen in periportal regions.

The results when rats were fed Diet IIe-0 are of particular interest (Plate, Fig. 5). This ration was the same as Diet IId-0 except that the supplementary methionine was omitted. In the absence of this choline precursor the liver became very fatty (27% total lipids), the bulk of the stainable fat appearing in centrolobular position (+++ to ++++). Since the distribution of fat was diffuse throughout the lobule the amount in periportal regions was difficult to estimate, but it was recorded as +. A small supplement of choline chloride (0.12%) reduced the total lipids to 6.5% —that is, within the normal range—but a small amount of stainable fat could still be seen around the central vein as well as in the periportal region. Had more choline been given, this centrolobular fat would probably have been eliminated.

Experiment III

The well-established fact that choline deficiency leads to an accumulation of fat in the centrolobular region was confirmed, as was the newer observation that stainable fat in the periportal region is connected with protein inadequacy. The ration containing 3% of casein without supplementary methionine (Diet IIIa-0) was not eaten well; it caused a

TABLE I.—*Percentage Composition of Basal Diets*

(All diets contained a constant portion amounting to 6.02%*)

Component	Diet I				Diet II					Diet III		Diet IV	Diet V	
	a	b	c	d	a	b	c	d	e	a	b		a	b
Casein	3	6	9	18	—	—	—	—	—	3	3	—	3	18
Fibrin	—	—	—	—	—	9	3	6	6	—	—	—	—	—
Soya protein†	—	—	—	—	9	—	6	12	12	—	10	2	—	—
Peanut meal‡	—	—	—	—	—	—	—	—	—	—	10	2	—	—
Corn (maize) oil	5	5	5	5	5	5	5	5	5	2	2	—	2	2
Beef fat	—	—	—	—	—	—	—	—	—	10	10	—	10	10
Primex§	—	—	—	—	—	—	—	—	—	—	—	20	—	—
L-Cystine	—	—	—	—	—	—	—	—	—	—	—	0·27	—	—
DL-Methionine	0·45	0·36	0·27	0	0·45	0·33	0·41	0·28	0	0	0	0	0·45	0
Choline chloride	—	—	—	—	—	—	—	—	—	—	—	—	0·50	0·50
Sucrose	To make total 100 in each case													

* The constant portion consisted of "celluflour" (a non-nutritive powdered cellulose to supply bulk, obtainable from Chicago Dietetic Supply House, Chicago, Ill.) 2%, cod-liver-oil concentrate 0.010%, (Ayerst, McKenna, and Harrison, Ltd., Montreal; contains 200,000 i.u. vitamin A and 50,000 i.u. vitamin D per gramme), α-tocopheryl acetate 0.010%, salt mixture 3%, and sucrose-vitamin mixture 1%. The last two items are described fully by Ridout *et al.* (1954). Diet IV contained extra tocopheryl acetate to give a total of 0.035%.

† "Alpha protein" (Soya Products Division, Glidden Co., Chicago, Ill.) washed with water to remove traces of sulphite.

‡ Defatted peanut meal, washed with 50% ethanol to remove free and bound choline.

§ "Primex" (hydrogenated vegetable oil), obtainable from Proctor and Gamble, Ltd.

TABLE II.—*Effect of the Nature and Amount of Dietary Protein on the Lipotropic Potency of Choline*

(Sufficient DL-methionine added to keep total constant at 0.54% in all diets except IIe-0. Male rats (65 to 90 g.) fed rations for three weeks)

Experiment and Diet No.	Choline Chloride (%)	Average Daily Food Intake (g.)	Average Change in Weight (g.)	Total Lipids (% Liver Wt.)	Lobular Distribution of Stainable Fat			
					Portal		Central	
					Range	Mode	Range	Mode
Ia–0 (6)*	0	5·6	−8	12·5	+/+++	++±	0/+	f.tr.
Ia–1 (6)	0·12	4·6	−9	16·0	+/++++	++±	0/±	f.tr.
Ia–2 (6)	0·36	4·9	−10	9·9	±/++	+	0/tr.	0
Ib–0 (6)	0	5·9	+5	9·8	±/+++	+	0	0
Ib–1 (6)	0·12	7·1	+15	11·3	±/++	+	0/±	0
Ic–0 (6)	0	9·6	+54	10·7	+/++	±+	0/±	f.tr.
Ic–1 (6)	0·12	10·5	+61	9·1	+/+++	++	0/±	0
Ic–2 (6)	0·36	10·2	+61	6·8	+/++	+	0/f.tr.	0
Id–0 (6)	0	11·9	+93	8·9	f.tr./±	±	0/±	±
Id–1 (6)	0·12	12·1	+105	6·6	0/±	f.tr.	0/tr.	0
Id–2 (6)	0·36	11·8	+100	5·8	0/±	0	0/tr.	0
IIa–0 (6)	0	6·7	+10	9·0	++/++++	+++	0/+	tr.
IIa–1 (8)	0·12	6·7	+10	8·7	+/++	+±	0/+	tr.
IIa–2 (6)	0·36	7·0	+12	7·7	+/++++	++±	0/tr.	0
IIb–0 (8)	0	9·7	+55	6·7	±/++	+	0/±	tr.
IIb–1 (8)	0·12	10·4	+63	5·8	tr./+±	+	0/±	0
IIb–2 (6)	0·36	10·4	+58	5·8	f.tr./+±	+	0/±	0
IIc–0 (6)	0	11·0	+49	10·7	+/+++	++±	0/+	±
IIc–1 (8)	0·12	11·4	+52	9·3	±/++++	+++	0/++	tr.
IIc–2 (6)	0·36	10·1	+46	8·4	tr./+++	++±	0/v.f.tr.	0
IId–0 (8)	0	13·7	+102	7·9	v.f.tr./±	tr.	v.f.tr./tr.	v.f.tr.
IId–1 (8)	0·12	13·0	+109	6·0	f.tr./tr.	tr.	0/v.f.tr.	v.f.tr.
IId–2 (6)	0·36	14·0	+119	5·5	tr./±	tr.	v.f.tr./f.tr.	v.f.tr.
IIe–0 (8)†	0	9·9	+55	27·2	+/++	+ (?)	+++/++++	+++
IIe–1 (8)	0·12	12·0	+67	6·5	0	f.tr.	±/+	±

* The figures in parentheses are the number of rats used in the experiment. f.tr. and v.f.tr. = faint and very faint trace.

† Six out of the eight rats in this group, which were fed the basal diet *unsupplemented* with methionine, died with kidney lesions and fatty livers.

TABLE III.—*Effects of Choline in Diets Inadequate or More Adequate with Respect to Protein*

(Diets IIIa and IIIb contained 3% and 18% respectively, of casein. Diet IV contained 3% protein (2% soya protein plus 1% peanut protein supplied as defatted meal)

Experiment and Diet No.	Choline Chloride (%)	No. of Rats	Average Daily Food Intake (g.)	Average Change in Weight (g.)	Total Lipids (% Liver Wt.)	Lobular Distribution of Stainable Fat	
						Portal	Central
IIIa–0	0	10*	5·7	−12	19·9	+ to ++	++ to +++
IIIa–1	0·50	10*	5·1	−13	5·8	+	±
IIIb–0	0	10*	10·3	+45	23·1	+	++ to +++
IIIb–Opf†	0	10*	6·0	+8	14·3	+ to ++	++ to +++
IIIb–1	0·50	10*	11·1	+60	5·2	±	v. faint tr.
IVa–0	0	10‡	7·0	−75	26·1	+ (?)	+++ to ++++
IVa–1	0·36	15‡	6·3	−74	7·3	±	0
IVa–2	1·0	15‡	6·6	−76	5·8	±	0

* Female rats (65 to 90 g.). † Pair-fed with rats in IIIa–0. ‡ Male rats (180 to 200 g.).

TABLE IV.—*Rate of Appearance of Stainable Lipids in Periportal Areas and Effect on Liver Lipids of Realimentation with an Adequate Diet after a Period on a Similar Ration Low in Protein*

(Forty female rats, 65 to 80 g., fed Diet Va-0 containing 3% casein; pairs of rats killed after periods shown. After 16 days the remaining rats were fed Diet Vb-0, containing 18% casein, for periods shown. Both rations contained 0.54% total methionine and 0.5% choline chloride)

Experiment and Diet No.	No. of Rats	Period Fed (Days)	Average Daily Food Intake (g.)	Average Change in Weight (g.)	Total Lipids (% Liver Wt.)	Lobular Distribution of Stainable Fat	
						Portal	Central
Va–0	2	2	7·1	−2	5·4	+	0 to f.tr.
	2	4	5·6	−10	6·2	0 to +	0
	2	8	5·0	−9	7·1	+ to ++	0
	2	16	4·8	−16	9·1	++	0
Vb–0	8	3	9·6	+19	18·0	+++	0
	8	7	9·3	+38	11·0	++	0
	8	14	10·0	+64	6·3	0 to +	0
	8	21	10·8	+91	5·1	f.tr.	0

moderate loss in weight and resulted in the development of a fatty liver. The total lipids reached 19.9%; there was considerable fat in periportal regions, with even more in centrolobular areas (Plate, Fig. 6). A similar ration containing 0.5% choline chloride resulted in a liver almost free from centrolobular fat but with considerable fat remaining in periportal regions. The total lipids were, however, only 5.8%. Diet Ia-0, a similar ration but with added methionine, was eaten in almost identical amounts and resulted in much lower liver lipids (compare 12.5% with 19.9%), the difference being mainly due to absence of fat from the central areas, as might have been predicted. Diet IIIb-0, methionine-poor but containing 18% of protein, was eaten more freely, permitted a fair gain in weight, and caused a fatty liver, with the abnormal lipids mainly centrolobular, as was anticipated. Paired feeding reduced the total lipids somewhat, but did not change the distribution of stainable fat appreciably. Inclusion of choline chloride (Diet IIIb-1) improved slightly both food intake and gain in weight, and almost completely prevented any stainable fat from appearing in the centrolobular region; a little remained in portal areas.

Experiment IV

Prolonged consumption of a diet low in protein and free from choline produced a very fatty liver, some of the lipid appearing in periportal areas, but most of the abnormal fat being centrolobular (Table III). The centrolobular deposition of stainable lipids was prevented by choline. Thus the effects of a low-protein diet on stainable liver lipids were essentially the same whether the rats were fed for three weeks or three months.

Experiment V

Development of a fatty liver upon realimentation has been noted frequently (for example, MacFarland and McHenry, 1945; Best, Lucas, Patterson, and Ridout, 1951), but the distribution of stainable fat in the present experiment proved interesting, being exclusively periportal. The low-protein diet (3% casein) resulted in a decreasing food intake (Table IV), in spite of adequate vitamins (including choline) and methionine. Despite the diminishing caloric intake and consequent loss of weight, the livers became increasingly fatty, the deposition of fat occurring in periportal regions (Plate, Fig. 7). When the rats were transferred to a similar diet in which the protein was made more adequate (casein 18%) they ate twice as much and began to gain weight. Coincident with this sudden increase in food consumption during the first three days on the "curative" ration there was a dramatic increase in the total liver lipids (to 18%), all of it occurring in periportal regions (Plate, Fig. 8). With continued intake of about the same amount of food, however, the abnormal fat soon disappeared, the liver appearing normal both histologically and chemically at the end of three weeks. No such increase in liver lipids occurred following realimentation in rats pair-fed a commercial ration throughout both the period of decreasing food intake and of realimentation.

Discussion

The data presented above show that protein inadequacy leads to the prompt appearance of stainable fat in periportal regions of the liver. It is a well-established fact that choline deficiency results in a rapid accumulation of abnormal lipids, mainly glyceride in nature, in centrolobular areas; the present data confirm this. The essential difference in the lesions can best be recognized when the deficiency is of mild degree and of brief duration; more severe or more prolonged deficiencies lead to a more generalized distribution of stainable fat, often obscuring the original site of appearance of the lipid.

DESCRIPTION OF SPECIAL PLATE

Photomicrographs (×133) of frozen sections of rats' livers stained with oil red 0 to demonstrate fat.

FIG. 1.—Livers of young rats (Wistar) fed the stock ration ("master fox chow"). In our strain under these dietary conditions there is usually no stainable fat (Fig. 1*a*), but occasionally some cells contain fine droplets and a few are filled with fat. When present these fat-filled cells are distributed at random within the lobule (Fig. 1*b*).

FIG. 2.—Lipid distribution typical of rats fed rations low in protein (made adequate with respect to methionine): Fig. 2*a* without choline (Diet Ia-0), and Fig. 2*b* with supplementary choline (Diet Ia-1). The choline is without significant effect on the periportal fat (++ to +++).

FIG. 3.—In the livers of rats fed Diet IIa-0 (soya protein 9%) there is abundant periportal accumulation of fat (++ to ++++). This photomicrograph illustrates the mode (+++).

FIG. 4.—The periportal deposition of lipids was the least when fibrin (Diet IIb-0) was the protein fed. The figure illustrates the appearance of the liver when the ration is supplemented with choline (Diet IIb-2). The findings (+) are essentially the same in the absence of choline.

FIG. 5.—Stainable fat in relatively large amounts (+++ to ++++) present in centrolobular and non-portal regions of livers of rats fed rations low in choline (Diets IIe-0 and IVa-0).

FIG. 6.—Dual distribution encountered in rats fed rations low in both choline and protein (for example, Diet IIIa-0): stainable fat present in relatively large amounts (++ to +++) in centrolobular regions of the liver, and in smaller quantities (trace to +) in periportal areas.

FIG. 7.—Stainable fat (+) present in periportal regions only. Rat consumed a low-protein ration (Diet Va-0) supplemented with both choline and methionine for 16 days; food intake was low. A portal field is just to the right of and below centre.

FIG. 8.—Abundant stainable fat present in periportal fields, but none occupies cells around central veins (one at left). A portal triad, cut longitudinally, is shown on the right. Rat consumed for three days excessive amounts of a ration containing 18% casein (methionine and choline believed adequate—Diet Vb-0). For 16 days previous to the three-day period the rat had eaten only small amounts of the same ration (Diet Va-0) as that consumed by the rat whose liver is shown in Fig. 7.

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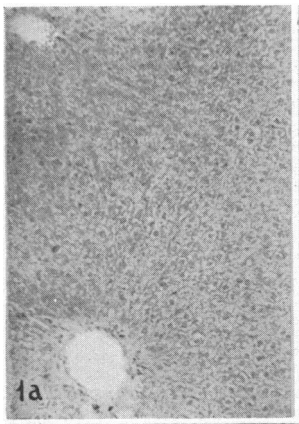

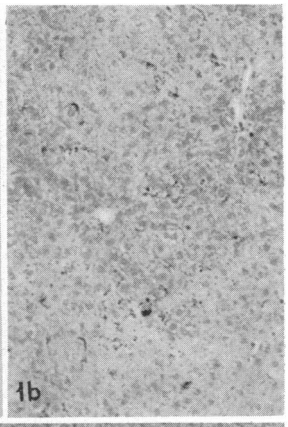

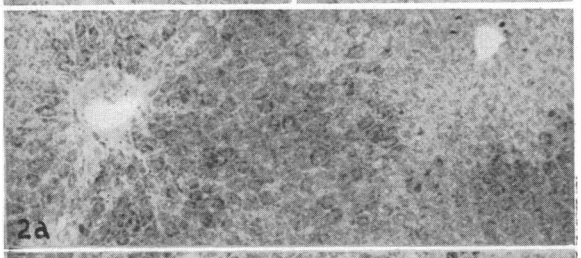

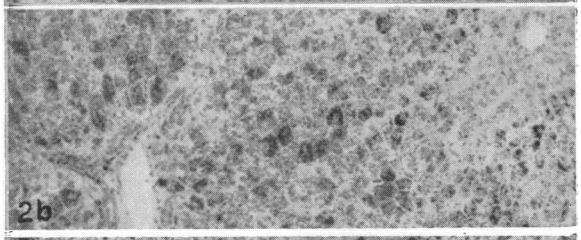

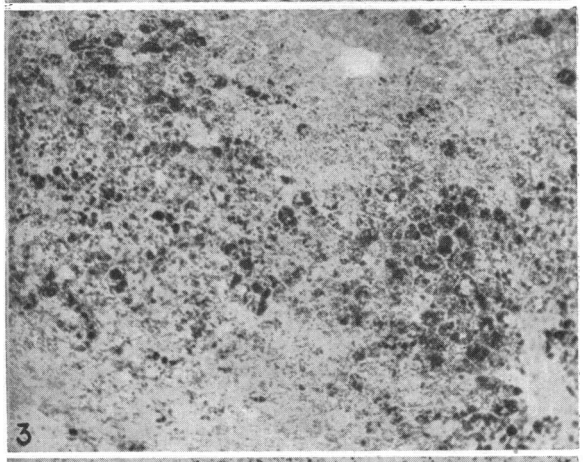

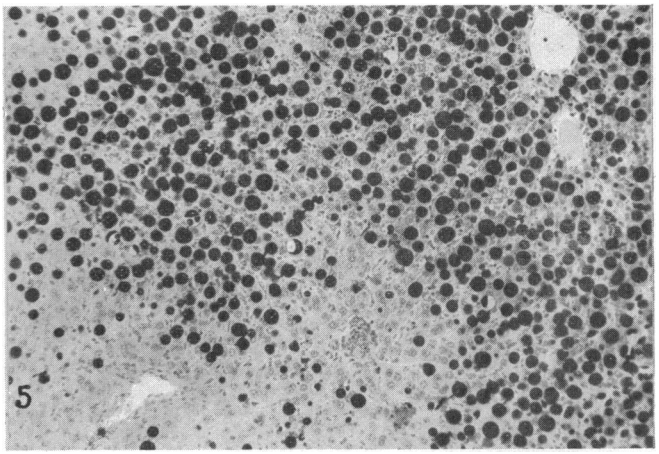

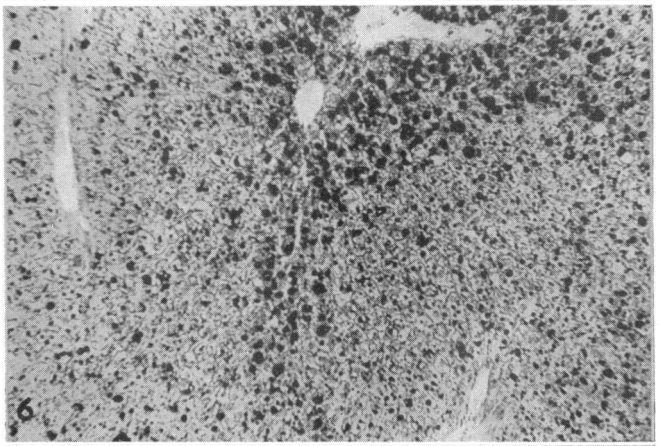

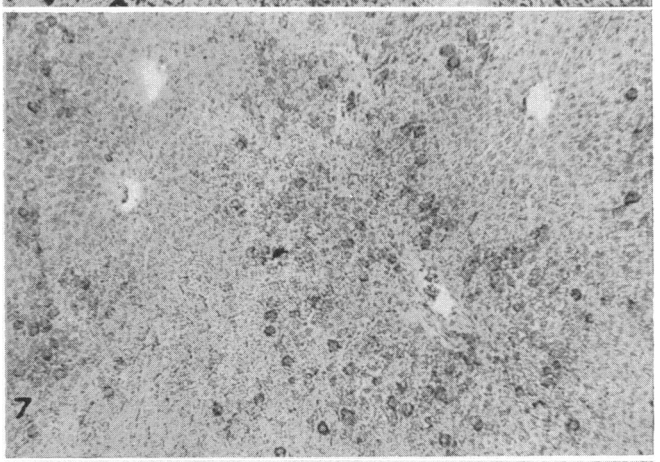

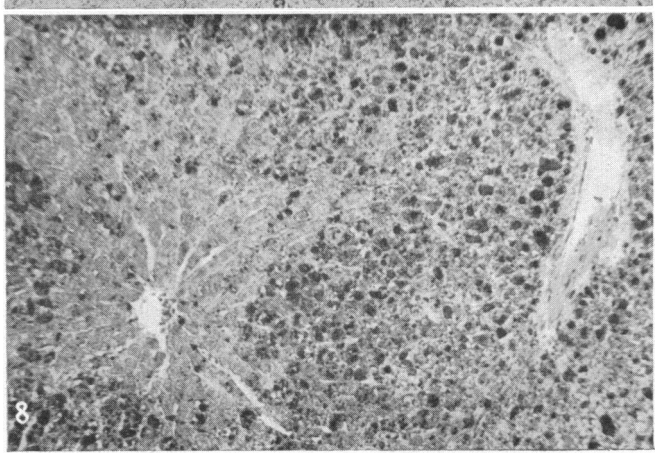

For complete protection of the hepatic cells in the portal region the amount of protein necessary depends upon its nature, fibrin being much more effective than casein, which is better than soya protein. The deleterious effect of diets low in protein on the cells in the portal region may be related to some inadequacy of essential amino-acids (cf. Lucas and Ridout, 1955). The effect of protein on hepatic cells around the central veins appears to be attributable largely, if not solely, to its content of the choline precursor methionine. Protein inadequacy in regard to protection of the liver appears to begin at about 12% in the diet when casein is fed, and below 6% in the case of fibrin (cf. data of Winje *et al.*, 1954); choline inadequacy begins at about 0.12% (expressed as chloride). It should be emphasized that these values may vary considerably, depending upon the strain, age, and sex of the rat, upon the adequacy of the diet with respect to other essential nutrients, upon environmental temperatures, and on other factors less well understood.

These observations may help to resolve two anomalies in the literature—namely, the occasional discrepancy between the chemical and histological estimations of liver fat and the lack of response of certain types of dietary fatty livers to the lipotropic agents. The term "fatty liver" has somewhat different connotations according to the viewpoint of the user. To the pathologist who is concerned with the appearance of the organ the liver is described as fatty if fat is evident microscopically in suitably stained sections. Not every cell need contain stainable fat. Cells in certain regions only may be affected, yet the organ would be referred to as a "fatty" liver. The biochemist, on the other hand, is concerned with the results of the chemical analysis—that is, with the quantity of lipids extractable from the tissue. Only when the analytical figure is increased appreciably above the normal range of values for the species would the chemist describe the liver as "fatty." The pathologist may see stainable fat, sometimes in considerable amount, before there is any significant increase in the extractable lipids. An erroneous impression of the magnitude of the increase above normal may be obtained unless attention is paid to the extraction procedure used, and especially to the base to which the analytical data are calculated, whether it be to fresh tissue, dry tissue, fat-free tissue, or dry, fat-free tissue. We have discussed elsewhere the merits and disadvantages of each of these (Ridout *et al.*, 1952). There are situations—for example, in protein depletion—where the values might better be expressed as percentages of body weight, or, better still, referred to the desoxypentosenucleic acid (D.N.A.) content of the liver. Campbell and Kosterlitz (1948, 1950, 1952) found that, although ribonucleic acid and phospholipid are lost from the liver in protein deficiency, the D.N.A. content of the liver of the adult rat varies linearly with body weight and is not affected by the protein content of the diet. Where effects of variations in dietary protein are under study, and particularly where a considerable change in the number of functional liver cells is apparent (as in necrosis or cirrhosis), the D.N.A. content of the liver should prove a useful basis for comparing changes in lipids and other hepatic components. However, at present in our laboratory the common practice is followed of referring the data to the fresh weight basis. All values are also calculated to the dry, fat-free basis, as this sometimes permits more useful comparisons, but these data are not always reported.

In choline deficiency hepatic lipid values of 15 to 40% of fresh liver weight are commonly observed. Figures of 25 to 45% reported in rats fed diets low in protein (for example, Harper, Benton, *et al.*, 1954) are expressed as percentages of *dry* liver weight. When referred to the fresh weight the total hepatic lipids are no more than about 8 to 18% (usually below 14%). These are certainly fatty livers, but quantitatively they are of mild degree as compared with those seen in choline deficiency. A considerable amount of stainable fat may appear in periportal areas with minimal elevation of total lipids as determined chemically. It has been suggested (Lucas and Ridout, 1955) that this unmasking of normal structural lipids (fat phanerosis)* may be caused by improper formation of lipoproteins due to protein deficiency. Protein deficiency may seriously interfere with the formation of many vital factors which the lack of choline might not affect to the same extent. (Cf. Campbell and Kosterlitz, 1948, 1950, 1952.) While fibrosis or cirrhosis has not yet been produced in our rats by protein deficiency in the presence of abundant choline, even after a year, it is possible that these lesions may appear in more prolonged experiments, as Ramalingaswami (1954) has suggested.

With regard to the second anomaly, our findings confirm and supplement those from other laboratories that fatty livers due to diets low in protein do not respond to lipotropic substances. However, it is not surprising that a fatty liver due to protein deficiency is not cured by giving supplements of choline or its precursors.

In our opinion it has been obvious for many years that the clinical use of choline and methionine to repair the liver damage produced by multiple deficiencies—that is, of protein, minerals, vitamins, etc.—is doomed to failure because most hospital diets supply an abundance of the lipotropic agents. This does not decrease in any way the potential clinical significance of experimental work on the lipotropic factors; it merely means that in the laboratory it is possible to make choline the limiting factor by the design of the experiment. The clinician rarely encounters so simple a situation. If he supplied the missing amino-acids other than methionine and made good the other deficiencies except that of choline, this substance would be the limiting factor in the clinical situation.

The importance of the balance of amino-acids in the diet in the study of fatty livers was noted in this laboratory 10 years ago by Beveridge, Lucas, and O'Grady (1944, 1945), who pointed out that the lipotropic activity of a protein is determined not only by its content of methionine but also by the nature and quantity of the other amino-acids present. While studying changes in liver composition during protein depletion Wang *et al.* (1949) fed protein-free rations to rats and observed a fatty liver not completely preventable by choline (at the dosage used, which was high—0.50% choline chloride in the diet). Dick, Hall, Sydenstricker, McCollum, and Bowles (1952) suggested that some fatty livers may result from failure of certain cell functions when protein anabolism is reduced—that is, they may represent a non-specific effect of protein deficiency rather than a specific lack of any one essential amino-acid. When Harper, Benton, *et al.* (1954) observed an anti-lipotropic effect of methionine in rats fed threonine-deficient diets containing choline, they too concluded that the extent of fat deposition in the liver depends on the balance as well as on the absolute amounts of amino-acids in the ration. The fatty livers caused by diets deficient in threonine and lysine (Dick *et al.*, 1952; Singal *et al.*, 1953; Harper *et al.*, 1953), even with moderate amounts of choline in the food, are examples of fatty livers of non-specific origin—that is to say, due to inadequate or imbalanced protein—and the lesions would doubtless prove to be periportal in distribution. Indeed, Niño-Herrera, Harper, and Elvehjem (1954), reporting upon the histological differentiation of fatty livers produced by threonine or choline deficiency, stated:

> "In the livers of choline-deficient animals the fatty infiltration is diffuse and is most severe in the vicinity of the central vein. In the livers of animals receiving low-protein diets containing choline the distribution of fatty cells results in a network appearance in which zones of normal cells are interspersed among the zones of fatty cells and only occasionally is the fatty infiltration not

*This is not the place to attempt a full discussion of the vigorously debated subject of lipophanerosis. Modern biochemical and physicochemical studies of the lipoproteins have revealed the possibility of dissociation of these compounds to give free lipids, but the conditions governing this process are not well understood.

severe around the central vein of the lobule. The magnitude of the fatty infiltration is much greater in rats fed a diet deficient in choline."*

Shils and Stewart (1954b) have observed a periportal distribution of fat in livers of rats fed diets consisting principally of corn meal, rice, or cassava, and found that lysine and tryptophan prevented this fatty liver. These observations are in agreement with our hypothesis that protein deficiency or amino-acid imbalance causes the periportal type of lesion.

Shils and Stewart (1954a) suspected that the periportal accumulation of lipids seen in rats fed corn meal may be due to some unique property of corn proteins. Since the lesion was later seen in rats fed corn, rice, wheat, or cassava, Shils, Friedland, and Stewart (1954) believed that it is typical of plant products. Our finding of this periportal type of distribution whenever the amount of dietary protein is low, regardless of the source of the protein fed, confirms and extends their results but alters the conclusions.

Shils and Stewart (1953) reported observing a sex difference in the distribution of stainable fat in the liver lobule: in male rats the total concentration of hepatic lipids was higher than in females and the lipid appeared first in periportal areas; in females the fat was found first in central areas. Female rats responded somewhat to threonine; males did not (Shils and Stewart, 1954b). In our experiments, admittedly with different proteins, we have not noticed any significant difference in the distribution of stainable fat in the livers of male and female rats.

In a personal communication Ramalingaswami, who with his colleagues has published several thoughtful and stimulating papers on liver injury in protein malnutrition (for example, Sriramachari and Ramalingaswami, 1953; Ramalingaswami *et al.*, 1954), has informed us that the fat content of diets low in protein and choline may influence the localization of the fatty deposits in the hepatic lobule. After rats were fed for about three months on a low-fat, low-protein, choline-deficient diet he observed that the microscopical appearance of the livers presented a striking similarity to the picture in kwashiorkor.

In our present study the observation that may be of greatest practical significance concerns the abrupt increase in total liver lipids associated with a sudden improvement in the quality and quantity of food intake, even when the "curative" ration is adequate in protein and rich in choline (Experiment V). Liver fat increased within three days from 9 to 18%, all stainable fat being present in periportal regions. This is the largest amount of extractable lipid ever observed by us when the stainable fat was present in periportal regions exclusively. A similar increase in liver fat was not observed in other rats pair-fed a commercial stock ration, suggesting that an unidentified factor prevents the sudden but transient increase in liver lipids confined to the periportal regions. These experiments, which may have their clinical counterparts in the treatment of alcoholic subjects with certain "good" diets, reveal the presence of factors in addition to protein and choline that may be concerned in the deposition and localization of fat in the liver.

Summary

In rats fed diets containing a moderate amount of fat and 9% or less of protein, supplementary choline failed to prevent completely the accumulation of fat in periportal regions of the liver.

Lack of adequate protein resulted in the appearance of fat in the periportal areas, while deficiency of choline (or precursors) caused an accumulation of fat in the cells bordering on the central vein. Protein inadequacy has not produced the massive accumulation of hepatic lipids seen in choline deficiency.

When rats were transferred on to an "adequate" diet (containing 18% casein and 0.5% choline chloride) after a period on a low-protein ration (3% casein) there was a dramatic appearance of a transient fatty liver (total lipids 18%), the fat appearing in periportal positions only. During three weeks on the same ration the hepatic lipids returned to normal. Other rats pair-fed the same amounts of a commercial ration did not develop fatty livers, so that the transient appearance of periportal fat is not entirely due to the increased food intake. Certain points of potential clinical interest have been discussed briefly.

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We wish to thank Dr. W. G. Bruce Casselman and Dr. Jean M. Patterson for the exceptional amount of help they have given us in this study.

(A preliminary account of some of these findings was read at the 37th Annual Conference of the Chemical Institute of Canada, Toronto, June 21 to 23, 1954.)

*The literature concerning the site of lipid accumulation in threonine deficiency is contradictory, however. Dick and his associates reported that it occurs in the cells around the central veins. Unfortunately the histological techniques used by Niño-Herrera *et al.* were unsuitable for lipid studies. Using histochemical methods, Kerbel and Casselman in this laboratory have repeated the experiments relating directly to threonine deficiency and have found that the distribution of fat is clearly periportal.

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In 1953 in England and Wales 40 people were killed in quarry accidents and in Scotland 5. In 1952 these totals were 49 and 4 respectively. The injured numbered 104 in 1953 in England and Wales and 11 in Scotland, as compared with 118 and 14 respectively in 1952. The average death rate per 1,000 persons employed in all quarries in England, Scotland, and Wales was 0.99 in 1951, 0.85 in 1952, and 0.73 in 1953.—*Report of H.M. Inspectors of Mines and Quarries for 1953* (H.M.S.O., 2s.).