Effect of Choline Deficiency on the Fatty Acids of Liver and Body Lipids in the Rat

By C. SIVA RAMAN

National Chemical Laboratory of India, Poona

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Changes in the nature and amounts of tissue lipids are of special significance in elucidating the mechanism by which choline prevents liver-fat deposition. Numerous investigations have been concerned mainly with changes in the relative proportions of the various lipid fractions. Liver-fat accumulation during choline deficiency in the rat is characterized almost exclusively by an increase in glycerides with a proportionate rise in cholesterol content, mostly in the form of cholesteryl esters (Best, Lucas, Patterson & Ridout, 1946). The total phospholipin content of the liver is generally not altered, although a decrease in the proportion of choline-containing phospholipins has been observed by several authors (Stetten & Grail, 1942; Artom & Fishman, 1943; Fishman & Artom, 1944, 1946; Campbell, Olley & Blewett, 1949).

animals. The saturated acids of carcass lipids, like the solid acids of liver glycerides, were influenced by the composition of the dietary fat. There was no apparent relationship between the total fatty acid content of the carcasses and the percentage of lipids in the livers.

The present paper deals with the effect of choline on the amount and nature of the fatty acids of the phospholipin and glyceride fractions of liver and carcass of rats maintained on diets free from unsaturated fatty acids. In the light of current views on the function of choline it appeared probable that the exclusion of unsaturated acids would give a clearer indication of the changes taking place in choline deficiency. With the object of studying the effect of choline under conditions wherein fat deposition is due on the one hand to assimilation from

Table 1. *Percentage composition of basal diets*

Diet	Casein*	Fully saturated glycerides	Glucose	Cellulose†	Salt‡ mixture
High-fat	5	30	61	0	4
Fat-free	5	0	89	2	4

* Light white (British Drug Houses Ltd.) extracted with ethanol.

† Prepared from filter paper (Whatman no. 1) pulp.

‡ Steenbock-Nelson salts 40 (Steenbock & Nelson, 1923).

Much less attention has been given to the study of variations in the nature of the fatty acids of liver lipids. Channon & Wilkinson (1936) investigated the effect of the chemical nature of food fat on the nature of the fatty acids deposited in the liver of rats when various fats were fed for 2 weeks at a 40 % level with a choline-deficient, 5 % casein diet. The total liver lipids were fractionated into phospholipins and glycerides and the fatty acids of each fraction separated into solid and liquid acids by the Twitchell (1921) procedure. The results indicated that in the glyceride fraction the proportion and mean molecular weights of the solid and liquid acids and the iodine numbers of the liquid acids were influenced by the nature of the food fats, while in the phospholipins only the molecular weights of the fatty acids varied with the type of dietary fat. Channon, Jenkins & Smith (1937) also investigated in detail the total carcass fatty acids in these

dietary fat and on the other to synthesis from carbohydrate, two different basal diets were used, one containing 30 % of fully saturated glycerides, while the other was fat-free.

EXPERIMENTAL

The choline-free basal diets were made up as shown in Table 1.

The fully saturated glycerides (iodine value, i.v., 0) used in the diets were prepared from buffalo depot fat. The fat (after removal of free fatty acids) was dissolved in lots of 500 g. in acetone (5 ml./g. fat), left overnight at room temperature (approximately 30°) and the separated fat obtained by filtration. The insoluble portion was redissolved in acetone (3 ml./g. fat) and left overnight at room temperature. The treatment resulted in the deposition of a crop of about 150 g. with i.v. of about 13. The final crystalline fraction was oxidized in batches of 100 g. for preparation of fully saturated glycerides according to the method of Hilditch & Lea (1927) and the neutral, completely saturated

glycerides separated from the acidic oxidation products. The preliminary crystallizations from acetone ensured complete oxidation in one operation.

Young albino rats weighing 110–150 g. were placed in individual cages over wide-mesh screening in groups of eight, animals of the same sex being distributed evenly between the groups. Each animal received in addition to the basal diets described above, the following vitamin supplements: thiamine hydrochloride, 25 μ g.; riboflavin, 20 μ g.; pyridoxine hydrochloride, 25 μ g.; nicotinic acid, 100 μ g.; vitamin A, approximately 150 i.u. and vitamin D, approximately 25 i.u. The water-soluble vitamins were fed by tube. Vitamins A and D were prepared as a fat-free concentrate by saponification and ether extraction of a

matter by precipitation with acetone and $MgCl_2$ (Sinclair, 1930). Portions of the various extracts corresponding to a total of about 200 mg. phospholipin fatty acids (approximately 12 g. liver and 35 g. of carcass) were mixed and taken to dryness at 40–45° under reduced pressure in an atmosphere of N_2 . The residue was extracted with ether (about 60 ml. with washings) and poured into 420 ml. acetone. To facilitate complete precipitation of the phospholipins, a saturated solution of $MgCl_2 \cdot 6H_2O$ in ethanol (2 ml.) was added with stirring. After centrifuging for 5 min. at low speed the supernatant acetone-ether was poured out, the precipitate stirred with 100 ml. of fresh acetone, centrifuged again for 1 min. and the extracts combined. The residue was stirred into 40 ml. ether and the phospholipin fraction re-

Table 2. Average food intakes and weight records

	High-fat diet		Fat-free diet	
	Basal	Basal + choline	Basal	Basal + choline
Average initial body weight (g.)	115	116	141	141
Average final body weight (g.)	99	106	137	140
Average food intake (g./rat/day)	5	5	7	7
Liver (average % body weight)	4.07	2.93	3.37	2.99

Table 3. Characteristics of liver lipids of rats

((a) Values expressed as percentage fresh liver weight; (b) values expressed as mg./100 g. body weight.)

	High-fat diet				Fat-free diet			
	Basal		Basal + choline		Basal		Basal + choline	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Phospholipin fatty acids	1.198	48.77	1.698	49.72	1.572	52.91	1.833	54.77
Glyceride fatty acids	22.13	901.8	0.982	28.72	10.78	363.1	2.709	80.95
Unsaponifiable matter	1.227	50.00	0.146	4.27	0.514	17.32	0.356	10.65

commercial preparation; the ether was sprayed on to the food and the solvent removed *in vacuo*. The animals on the choline-containing diets received in addition 23 mg. choline chloride, corresponding to 20 mg. of choline per rat per day. The diets were fed *ad lib*, and the daily food intakes recorded. After 30 days on the experimental diets the animals were weighed, killed by stunning and their liver and carcass lipids analysed.

Analysis of liver and carcass lipids

Extraction of liver and carcass lipids. The livers were removed immediately after killing, weighed and ground with sand under ethanol. The pooled livers from each group were extracted in the Soxhlet with ethanol and finally with $CHCl_3$. The ethanol and $CHCl_3$ extracts were separately made up to volume and samples combined for analysis.

Each carcass, after removal of the liver, was worked up immediately as follows: the head, tail and gastro-intestinal tract were removed and the remaining carcass weighed, cut into small pieces and stirred with about 3 vol. of hot acetone. The carcasses from each group were pooled, dehydrated further by boiling with two more lots of acetone and the residue extracted for 3 hr. in the Soxhlet with fresh acetone. The dehydrated material was then sucked dry at the pump, the residue ground to a powder and re-extracted in the Soxhlet with ethanol and the extraction completed with $CHCl_3$. The solvents were separately made up to volume and samples from the various extracts combined for analysis.

Separation of phospholipins. The phospholipins were separated from neutral fat ('glycerides') and unsaponifiable

precipitated and washed as before. To verify the complete absence of glycerides from the phospholipin fraction a sample of the phospholipins obtained as described was in certain cases precipitated a third time. The analysis of phospholipin fatty acids resulted in almost identical values for solid acid content whether two or three precipitations had been made.

Analysis of phospholipin and glyceride fatty acids. The fatty acids were obtained from the phospholipin and glyceride fractions according to methods described by Sinclair (1930, 1940), the separation and liberation of the acids being carried out in an atmosphere of N_2 . Approximately 200 mg. fatty acids from both the fractions were analysed by the Twitchell procedure as modified by Sinclair (1940), a Hirsch funnel equipped with an outer hot-water jacket being used, however, for filtering the ethanolic solutions of the lead salts as recommended by Horning & Eckstein (1946).

Iodine numbers of fatty acid fractions were determined according to Yasuda (1931), and the mean molecular weights by titration with 0.05 N-sodium ethoxide in ethanol (Fowweather, 1926).

RESULTS

The food intakes and weight records of the various groups are set out in Table 2.

Rats on the high-fat diets lost weight, the loss being more pronounced on the choline-free diet, whereas animals on the fat-free diets maintained

Table 4. *Carcass lipids*

(Values expressed as percentage of carcass weight.)

	High-fat diet		Fat-free diet	
	Basal	Basal + choline	Basal	Basal + choline
Phospholipin fatty acids	0.4490	0.4742	0.5152	0.5344
Glyceride fatty acids	1.517	2.793	11.87	12.77
Unsaponifiable matter	0.526	0.513	0.581	0.564

Table 5. *Effect of diet on phospholipin fatty acids of liver and carcass*

	High-fat diet		Fat-free diet		Stock
	Basal	Basal + choline	Basal	Basal + choline	
LIVER					
Solid acids:					
%	44.3	31.6	38.7	30.9	33.0
I.V.	3	4	4	2	6
Mol.wt.	273	270	282	282	—
Liquid acids:					
%	55.7	68.4	61.3	69.1	67.0
I.V.	266	228	223	202	218
Mol.wt.	338	330	326	328	—
CARCASS					
Solid acids:					
%	33.2	34.3	29.8	30.5	—
I.V.	3	6	3	5	—
Mol.wt.	266	270	265	265	—
Liquid acids:					
%	66.8	65.7	70.2	69.5	—
I.V.	178	179	147	144	—
Mol.wt.	341	334	320	340	—

Table 6. *Effect of diet on glyceride fatty acids of liver and carcass*

	High-fat diet		Fat-free diet		Stock
	Basal	Basal + choline	Basal	Basal + choline	
LIVER					
Solid acids:					
%	29.4	28.5	28.3	24.0	24.8
I.V.	5	8	8	6	9
Mol.wt.	269	271	272	272	—
Liquid acids:					
%	70.6	71.5	71.7	76.0	75.2
I.V.	108	124	99	95	107
Mol.wt.	298	295	289	291	—
CARCASS					
Solid acids:					
%	42.7	37.6	34.1	33.8	—
I.V.	8	6	6	7	—
Mol.wt.	289	290	264	262	—
Liquid acids:					
%	57.3	62.4	65.9	66.2	—
I.V.	98	99	94	90	—
Mol.wt.	295	290	275	278	—

their body weights during the period of the experiment. The average food intakes of animals in all the groups, however, were approximately the same when calculated for the 100 g. rat.

The composition of the liver lipids is shown in Table 3 and of the carcass lipids in Table 4.

In animals on the high-fat diets an inverse relationship is apparent between the glyceride fatty acid contents of liver and carcass, choline reducing the glyceride content from 22.13 to 0.982 % in the

liver and increasing it from 1.517 to 2.793 % in the body depots. The values indicate that under the experimental conditions choline is necessary for the normal distribution of fat between the liver and the body. On the fat-free diets, the glyceride acid content of carcass is unaffected by choline, the values being 11.87 % without and 12.77 % with the supplement. Choline has apparently no effect on deposition of fat in body depots when fat is derived from dietary carbohydrate. On both types of diet

choline is without influence on the amount of phospholipin fatty acids of liver and carcass and the unsaponifiable matter of carcass.

Tables 5 and 6 record the variations in the nature of the fatty acids of the phospholipin and glyceride fractions. Values for liver fatty acids from a group of stock animals of approximately 100 g. body weight are included for comparison.

DISCUSSION

The results obtained are best discussed under two separate heads.

Effect of choline on liver lipids. As was expected, choline deficiency caused excessive deposition of liver fat in animals on the high-fat as well as on the fat-free diets, the liver-fat content (total fatty acids + unsaponifiable matter) being 24.5 % in the former and 12.9 % in the latter. The addition of choline to the diet reduced the values to 2.8 and 4.9 % respectively, indicating that the moderate liver-fat accumulation which takes place on a fat-free diet is possibly less susceptible to the corrective action of choline.

Interesting changes were observed in the nature of phospholipin fatty acids of liver during choline deficiency. In the absence of choline the proportion of solid acids increased from 31.6 to 44.3 % on the high-fat diet and from 30.9 to 38.7 % on the fat-free diet. The degree of unsaturation of the liquid fatty acids of liver phospholipin was also influenced by dietary choline, the iodine number of the liquid acids being considerably higher in choline deficiency (Table 5).

Although the effect of choline is most marked on the glyceride content of the liver it is apparent from the results described in Table 6 that choline does not have any appreciable influence on the fatty acid composition of liver glycerides. The percentages of the solid fatty acid in liver glycerides are approximately the same in animals on high-fat diets, the values being 28.5 % with choline and 29.4 % without choline and only slightly different from each other in those on the fat-free ration, the corresponding values being 24.0 and 28.3 % respectively. As between the high-fat and fat-free diets, the unsaturation of the liquid acids of liver glycerides is greater on the former diet, the iodine numbers in the two cases being 108 and 99. Supplementation with choline raises the former value to 124, but appears to have no significant effect on the latter.

Effect of choline on body lipids. It is of interest to note the high lipid content of the carcass of animals on the fat-free diet as compared to those on the high-fat ration (Table 4). This is evidently due to synthesis of fat from dietary carbohydrate as is borne out by the low molecular weights of the glyceride fatty acids (Table 6). Spadola & Ellis (1936) and Longenecker (1939) observed a high proportion

(40–45 %), of low molecular weight fatty acids, of the C_{16} series in particular, in the body fat of animals maintained on a low-fat, high-carbohydrate ration and came to the conclusion that such high proportions of the lower fatty acids are characteristic of fat synthesized from carbohydrate. The results obtained in the present investigation would indicate that choline has no role in this synthetic process since animals on the basal fat-free diet have the same high carcass-glyceride content and the characteristic low mean molecular weight of the glyceride acids as the corresponding choline-supplemented group.

In contrast to the observations noted above on the effect of choline on the fat content and phospholipin composition of liver, choline administration produces little or no effect on either of the lipid fractions of carcass fat.

SUMMARY

1. The effect of choline on the fatty acids of liver and carcass lipids has been investigated on rats maintained on two different basal diets, one containing 30 % of fully saturated glycerides, the other being fat-free.

2. Animals on the high-fat diets lost in body weight, the loss being more pronounced in the absence of choline. Choline effectively prevented fatty livers and caused a slight increase in the low carcass-fat content of these animals.

3. Rats on the fat-free diets maintained their body weight and had a carcass-fat content markedly higher than animals on the high-fat diets. Fat accumulation in the liver in the absence of choline was moderate and at the same time not completely prevented by addition of choline.

4. Choline had a marked effect on the nature of liver phospholipin fatty acids: (i) the proportion of solid fatty acids in phospholipin fatty acids of liver was considerably higher in choline deficiency both on the high-fat diet and fat-free ration, (ii) iodine numbers of the liquid acids of liver phospholipin were higher in the choline-deficient groups.

5. No changes in the nature of the fatty acids of liver and carcass glycerides or of carcass phospholipins were noticed. In the case of the liver glycerides the effect of choline was apparent only on the content and not on the fatty acid composition.

6. The mean molecular weights of the liquid acids of carcass glycerides in animals on fat-free diets were characteristic of body fat derived from dietary carbohydrate. Choline, evidently, has no role in this synthetic process, since animals on such diets had the same high carcass-glyceride content both in the presence and absence of choline.

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The Effect of Light on Xanthine Oxidase Activity

BY S. M. SIEGEL* AND R. L. WEINTRAUB

Chemical Corps Biological Laboratories, Frederick, Maryland, U.S.A.

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It has been postulated, on the basis of studies of activation by light, that a flavin enzyme is involved in the indolylacetic acid oxidase system (Galston, 1950; Galston & Baker, 1951). The only report of light activation of a flavin enzyme appears to be that of Bernheim & Dixon (1928); working with a crude preparation of xanthine oxidase they found either activation or inactivation according to the conditions of the particular experiment. In a later study of the same enzyme only an inactivating effect of light was observed (Toyama, 1933). It has seemed important, therefore, to re-investigate the effect of light on xanthine oxidase.

EXPERIMENTAL

Enzyme. Milk from which casein had been removed after precipitation by rennin served as the source of enzyme in the majority of the experiments. In a few instances comparative tests were made with whole milk.

Substrate. Xanthine or hypoxanthine was employed as substrate. Preliminary studies indicated that maximal activity was obtained with 5×10^{-5} M-substrate.

Hydrogen acceptor. Methylene blue (MB), at 1×10^{-5} M, or nitrate, at 5×10^{-3} M, was used as hydrogen acceptor. The measurements were carried out in 0.05 M-phosphate buffer of pH 8.8 in the case of MB and of pH 5.5 with nitrate.

Measurement of enzyme activity. With MB, enzyme activity was measured by following spectrophotometrically with an Evelyn photoelectric colorimeter (620 m μ . filter) the time course of decolorization of the dye in evacuated Thunberg tubes. Activity was conveniently expressed as the reciprocal of the time required for 25 or 50 % reduction. To measure the rate of nitrate reduction, a series of identical

tubes was prepared and the nitrite determined successively in individual tubes, using a modification of the method of Parker (1949). The reaction rates were measured at 25° with MB and at 37° with nitrate.

Irradiation. The solution of enzyme in buffer, with or without hydrogen acceptor, was irradiated in open tubes immersed in a water bath at 25–27°, frosted tungsten filament lamps serving as the sources. The tubes were illuminated from two opposite sides and were shaken frequently. The non-irradiated control tubes, which were wrapped with aluminium foil, were manipulated concurrently in exactly the same way. At the highest intensities and longest exposures, the temperature of the illuminated solutions containing MB rose as much as 8° above that of the dark controls; tests showed that holding the enzyme solution in darkness at the elevated temperature did not affect its activity subsequently at 25°.

Procedure. Immediately after irradiation the tubes were closed and evacuated, the zero time transmission determined, the substrate tipped in, and readings commenced.

RESULTS

Light was found to influence the activity of xanthine oxidase only if an added photoreceptive pigment was present during the irradiation. Fig. 1 illustrates a typical experiment with MB.

Riboflavin also was found capable of serving as a photoreceptor in mediating light inactivation of xanthine oxidase. A comparison of the effectiveness of the two pigments was obtained as follows. Various amounts of riboflavin were added to portions of enzyme solution and MB to others. After irradiation at 1800 ft.c. for 5 min., MB was added to the riboflavin samples and the activities of all were measured in the usual manner. Riboflavin does not

* Present address: Department of Botany, University of Chicago, Chicago, Illinois.