The Effect of Varying Amounts of Dietary Cholesterol and of Choline upon Liver Lipids

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The early investigations of the effects of feeding cholesterol or foods rich in cholesterol have been reviewed by Duff (1935). The role of the lipotropic factors in protecting the liver against excessive deposits of lipids following the ingestion of cholesterol has been discussed by Best & Ridout (1939) and by McHenry & Patterson (1944).

In spite of the volume of work which has been carried out upon the effects on liver lipids of feeding cholesterol, no paper has been found which describes a systematic study of feeding graded doses of this substance. To obtain some information on the subject, twenty-one synthetic diets were prepared containing various amounts of cholesterol, up to 1.6%. Six of the diets contained no choline. The lipotropic effect of supplementary choline chloride was noted at two dietary levels (equivalent to 0.08 and 0.32% choline) and of inositol at 0.32%. Even at the highest level of cholesterol intake, choline had a marked effect in preventing a rise in the glyceride fraction and exerted a considerable but less pronounced effect on the cholesteryl esters of the liver. The lipotropic action of inositol under these conditions was negligible.

Several rations typical of those consumed by average families in this country were analysed for cholesterol and choline. The values found for cholesterol are possibly slightly high since it is known that certain other sterols are precipitated by digitonin, and some of these give a colour with the Liebermann-Burchard reagent. Although cholesterol is found only in products of animal origin, many phytosterols and sitosterols possess such similar properties that 'apparent cholesterol' values are often obtained when foods of vegetable origin are analysed by the Schoenheimer-Sperry method. To that extent, estimations made on pooled food mixtures may be too high. The choline content of these rations appeared adequate to protect the livers of rats consuming such diets against excessive accumulation of glycerides or cholesteryl esters. If one assumes that these ordinary diets are not harmful to the livers of persons consuming them, one might be tempted to conclude that the choline requirement (as percentage of dry dietary ingredients) of the rat and of man is similar. It is obvious, however, that further work may reveal

many discrepancies between the effects of cholesterol and choline upon the liver of the rat and of man.

EXPERIMENTAL

White rats (210) of the Wistar strain (80–120 g. in weight) were divided among twenty-one comparable groups. The animals, in individual cages fitted with a false bottom of coarse wire screen, were group pair-fed for 3 weeks. Weighed amounts of fresh food were supplied daily in feed trays designed to minimize spilling; left-over food and scatter were weighed the following morning. From these data the average daily individual food consumption was calculated. Fresh water was always available.

The basal diet (essentially free from choline) had the following percentage composition: casein (fat-free, vitamin-free) 8, gelatin 12, salt mixture no. 185 (McCullum & Simmonds, 1918) 5, cellu flour 2, 'vitamin powder' 1, sucrose 60, beef fat 10, corn oil 2 and cod-liver oil concentrate 0.015. The 'vitamin powder' consisted of thiamine hydrochloride 500 mg., riboflavin 250 mg., pyridoxine 200 mg., calcium pantothenate 1000 mg. and nicotinic acid 1000 mg. made to 1000 g. with very finely powdered (100 mesh) sucrose. Rats eating 10 g. of food per day receive 50 µg. thiamine hydrochloride and proportional amounts of the other vitamins daily. The cod-liver oil concentrate (Ayerst, McKenna and Harrison Ltd., Montreal), contained 200 000 i.u. vitamin A and 50 000 i.u. vitamin D/g.

Several groups of diets were prepared as shown in Table 1, containing five levels of crystalline cholesterol (British Drug Houses Ltd.) namely, 0.1, 0.2, 0.4, 0.8 and 1.6%. The cholesterol was incorporated in these diets (as in similar fat-free ones) by grinding it in a mortar with small portions of the dry ingredients; this mixture was spread by means of a fine sieve over the main bulk of the dry mixture. The 'vitamin powder' and lipotropic supplements were treated similarly. Cod-liver oil concentrate dissolved in light petroleum was sprayed over the combined dry ingredients; after evaporation of the solvent the warm fats were poured over this dry mixture and blended thoroughly. Diets 6 to 11 contained 8 mg. of choline (supplied as the chloride in amounts 1.15 times the weight of the base) in 10 g. of diet, which was the anticipated daily food intake. This amount of choline was chosen since other experiments (Best, Lucas, Ridout & Patterson, 1950) had suggested that in rats on such a basal diet, 8 mg. of choline/10 g. of food are sufficient to maintain practically normal liver lipids. Similar rations with four times this amount of choline (diets 12 to 17) were fed to see whether the greater amount was sufficient to protect against the development of the so-called 'cholesterol fatty liver'. Diets 18 to 20 contained 0.32% inositol.

After the animals had been fed the rations for 3 weeks they were stunned and their livers were removed immediately and weighed. The pooled livers from each group of ten rats were ground in a Waring Blender with some acetone, extracted several times with hot absolute ethanol and the lipids in the extracts were taken up in light petroleum. The analytical details have been described previously by Best, Lucas, Patterson & Ridout (1946).

It is interesting to compare the dietary levels of cholesterol and of choline in these experimental diets with those in

average wet weights and the average fat-free, dry weights of the livers of each group so that each lipid component may be calculated to either basis. It is necessary under certain circumstances, as will be pointed out later, to compare the results expressed as percentage of the fat-free dry weight, in order to interpret the data properly.

Table 1 shows that in the liver lipids of the rats on the hypolipotropic basal diet glycerides have been

Table 1. *Effects of dietary cholesterol on rats*

(Diets fed for 3 weeks to groups of 6 F., 4 M.; 80–120 g.)

Group no.	Supplements (%)	Mean food intake (g./day)	Mean wt. change (%)	Liver		Composition of liver lipids (mg./liver)				
				Mean wet wt. (g.)	Mean fat-free dry wt. (g.)	Total lipids	Cholesterol			Glyceride
							Free	Total	Ester*	
	Choline, 0									
0	Cholesterol 0	8.4	-5	6.0	0.991	1892	11.4	25.0	22.9	1700
1	0.1	8.9	-3	6.4	1.078	1829	12.0	34.4	37.9	1612
2	0.2	9.4	+5	7.5	1.176	2464	13.8	57.9	74.3	2219
3	0.4	9.7	+5	7.4	1.121	2716	15.0	100	143	2379
4	0.8	8.9	-4	8.6	1.138	3453	16.3	162	245	3008
5	1.6	8.8	-6	8.8	1.093	3929	18.6	208	320	3411
	Choline, 0.08									
6	Cholesterol 0	9.7	+10	4.5	1.024	390	9.6	12.8	5.4	224
7	0.1	9.7	+12	4.6	1.060	362	9.9	14.4	7.6	181
8	0.2	9.6	+16	5.0	1.064	549	11.6	32.0	34.3	338
9	0.4	9.8	+18	5.1	1.087	641	12.8	81.0	115	324
10	0.8	10.3	+22	5.8	1.222	795	14.8	126	187	414
11	1.6	9.8	+12	5.0	1.063	946	14.7	141	213	555
	Choline, 0.32									
12	Cholesterol 0	9.7	+22	4.6	1.088	351	10.1	12.5	4.0	174
13	0.1	9.5	+17	4.6	1.069	323	9.9	14.0	6.9	144
14	0.2	9.0	+12	4.7	1.067	321	10.4	18.0	12.8	125
15	0.4	9.9	+17	4.6	1.044	432	12.4	49.0	61.6	201
16	0.8	9.4	+14	4.7	1.050	477	13.8	64.6	85.6	210
17	1.6	10.1	+28	5.1	1.095	601	14.8	110	160	237
	Inositol, 0.32									
18	Cholesterol 0.1	9.5	+2	5.9	0.999	1754	11.1	35.6	41.2	1544
19	0.2	9.5	+4	5.7	0.972	1631	11.3	43.8	54.7	1413
20	0.4	9.9	+15	6.3	1.087	1815	13.8	88.5	126	1492

* Cholesteryl esters were calculated as oleate by multiplying bound cholesterol by 1.68.

natural rations consumed by man. To obtain data on this latter point representative servings of all foods and beverages offered to the nurses of a local hospital were collected on three separate days through the courtesy of the dietitian. The mixture for each day was thoroughly blended and analysed for solids, ash, total lipids, free and total cholesterol, phospholipins (light petroleum-soluble P × 25), choline (by microbiological assay with the cholineless mutant no. 34486 of the bread mould, *Neurospora crassa*) and inositol (with the Hansen no. 1 strain of *Saccharomyces cerevisiae* American Type Culture Collection, Washington D.C. (A.T.C.C.) 7921).

RESULTS

The effects of the different intakes of cholesterol are presented in Table 1 as absolute weights (mg. per liver) of the lipid components, together with the

doubled by the largest dose of cholesterol (1700–3411 mg.) and cholesteryl esters have increased 14-fold (23–320 mg.). Free cholesterol increased only slightly (11.4–18.6 mg.) and phospholipins, which are not shown in the table, remained essentially constant. When the results are expressed as percentage of wet weight, the rather large changes in the absolute values are less obvious, e.g. glyceride increased from 28.6 to 38.6 % of wet liver weight, cholesteryl esters from 0.39 to 3.6 %, free cholesterol from 0.19 to 0.21 %.

The effects of increasing amounts of dietary cholesterol upon total lipids and cholesteryl esters are plotted in Fig. 1 as percentage of wet weight. Both fractions were increased markedly by increasing dietary cholesterol when the rations were free from choline (groups 0–5; curves A and A'). The

presence of 0.08 % choline (groups 6-11; curves *B* and *B'*) as anticipated, greatly reduced the total lipids at each level of cholesterol intake but, unexpectedly, the cholesteryl esters appear to have been increased, e.g. cholesteryl oleate as percentage of wet weight is greater in group 11 (4.30 %) than in group 5 (3.62 %). A simple explanation for this apparently anomalous observation is presented later in the paper.

are expressed as percentage of fat-free, dry weight (see curves at right-hand side of Fig. 2) or as absolute weight, the lipotropic effect of choline upon the cholesteryl esters is clear.

For the benefit of those unfamiliar with normal values for liver lipids when expressed as percentage of dry fat-free tissue, the following data for ten rats from our stock colony may be reported: total lipid 20-32 % (av. 25 %), glyceride 9-18 % (av. 12 %),

Fig. 1. Influence of dietary cholesterol on liver lipids. The curves on the left-hand side show the total lipids, as percentage of fresh liver; the curves on the right give the cholesteryl esters as percentage of fresh liver. Curves labelled *A* depict data from rats on diets without choline, curves labelled *B* show data from rats given diets containing 0.08 % choline and curves labelled *C* are drawn from data for rats on diets containing 0.32 % choline (supplied as chloride in amounts 1.15 times the values mentioned).

The data are plotted in Fig. 2 to show the lipotropic effect of increasing amounts of dietary choline in rations containing different amounts of cholesterol. Curves *A*, *A'* and *A''* refer to rats ingesting cholesterol at 1.6 % dosage, curves *B*, *B'* and *B''* to 0.8 % cholesterol, and curves *C*, *D*, *E* and *F* to 0.4, 0.2, 0.1 and 0 % respectively. The curves on the left-hand side illustrate the influence of choline upon the glyceride and cholesteryl ester fractions, in the basal ration and at two dietary levels of cholesterol intake, expressed in the usual way as percentage of wet weight of liver. It is obvious from the figure (curves *A''*, *B''* and *F''*) that the percentage of glycerides has been greatly reduced by 0.08 % of choline, but, as was noted previously, with the lower dose of choline an unexpected increase seems to have occurred in the bound cholesterol (curves *A'* and *B'*). In Table 1 the actual reduction in weight of the glyceride fraction can be seen to be from 3411 to 555 mg. (groups 5 and 11). Largely due to this removal of glyceride there is a reduction in weight of the liver from 8.8 to 5.0 g. Although the smaller dose (0.08 %) of choline reduced the weight of cholesteryl esters from 320 to 213 mg. per liver, the values expressed as percentage of wet weight have increased (from 3.62 to 4.26 %). When the results

Fig. 2. Influence of dietary choline on liver lipids of rats ingesting different amounts of cholesterol. Cholesteryl esters are shown as solid lines, glycerides $\times 1/10$ as broken lines. Data shown on the left-hand side are expressed as percentage of fresh liver weight, on the right-hand side as percentage of fat-free, dry liver. Curves *A*, *A'* and *A''* refer to diets containing 1.6 % cholesterol, *B* to 0.8 %, *C* to 0.4 %, *D* to 0.2 %, *E* to 0.1 % and *F* to an essentially cholesterol-free diet.

total cholesterol 0.9-1.2 % (av. 1.0 %), free cholesterol 0.7-1.0 % (av. 0.87 %), cholesteryl esters (calculated as oleate) 0.18-0.52 % (av. 0.26 %) and phospholipins 10.2-12.5 % (av. 11.8 %).

Analysts are well aware of the different impressions that one may obtain from different methods of expressing analytical findings. Biochemists and physiologists are less conscious of the erroneous notions that can arise if data are expressed as percentage when some component (not considered or mentioned because it is not one of interest at the moment) undergoes a large alteration simultaneously with the one being followed. The two methods of presenting the data for cholesteryl esters shown in Fig. 2 illustrate how certain statements in the literature may be wrong, due to neglect to consider the effect upon cholesteryl esters of a simultaneous large decrease in the amount of glycerides in the liver.

Channon & Wilkinson (1935) were puzzled by the fact that increasing the casein content of a hypolipotropic diet (containing 20 % fat and 2 % cholesterol) from 5 to 30 % caused an increase in cholesteryl oleate. The data of Beeston, Channon & Wilkinson (1935) show a similar effect. The explanation given above probably applies; in their published data a large decrease in glycerides may be seen to

have occurred, but since no absolute values are given it is impossible to verify the suggestion. The increase which some workers have noted in phospholipin under the influence of choline has a similar explanation. Actually, the weight of phospholipin in mg. per liver (or better, percentage of fat-free, dry liver tissue if the rats are not of uniform weight) remains essentially constant, but as glycerides are lost and the liver weight decreases, the value expressed as percentage of wet weight increases.

Thus, it should be emphasized that whenever a lipotropic substance produces a great decrease in the amount of glycerides in the liver, an erroneous impression may be gained of the nature of the changes in other lipid components if the values are expressed as percentage of wet liver weight.

done several years ago, that there is a sex difference in the response of rats to hypolipotropic diets, and that consequently there is an apparent difference between males and females in the lipotropic effect of inositol (Best, Ridout, Patterson & Lucas, 1951). Further, the livers were pooled before analysis, and with no knowledge as to the variability of the results it is impossible to estimate the significance of the relatively small differences observed when inositol was added to the diets. A reliable interpretation of the data can be made under these circumstances only when large and consistent differences are found, as was the situation with choline.

The cholesterol and choline values found by analysis of the nurses' diets, with other pertinent analytical data, are shown in Table 2. It may be

Table 2. *Lipid components of some human rations*

(Amounts ingested, g./day.)							
Sample	Total lipid	Cholesterol			Phospholipin	Choline	Inositol
		Total	Free	Oleate			
1	87	0.625	0.513	0.189	5.78	0.305	1.04
2	96	1.125	1.013	0.189	7.51	0.534	1.16
3	83	0.400	0.313	0.147	2.89	0.267	0.49
Mean	89	0.717	0.613	0.175	5.39	0.369	0.90
Expressed as percentage of dry solids in diet							
1	19.1	0.137	0.112	0.041	1.27	0.067	0.23
2	19.1	0.223	0.202	0.038	1.49	0.106	0.23
3	17.5	0.085	0.066	0.031	0.61	0.057	0.10
Mean	18.6	0.148	0.127	0.037	1.12	0.077	0.19

The rats in these experiments were fed the diets for only 3 weeks, but in other studies previously reported similar diets containing 0.5 % cholesterol were fed for periods of 8 and 16 weeks (Ridout, Lucas, Patterson & Best, 1946). Glycerides in the liver were maintained at normal levels by choline throughout the experiment, and a marked lipotropic effect on the cholesteryl esters was still demonstrable even if they were distinctly above normal after the rats had been on the rations for 4 months (4.5 % without choline, 1.0 % with choline, 0.05 % being normal).

Groups 18 to 20 illustrate the effects of increasing amounts of cholesterol (0.1, 0.2 and 0.4 %) in diets liberally supplied with inositol (0.32 %). Total lipids remained essentially unchanged at 29–30 % of wet liver weight. No great decrease in lipids due to the presence of inositol, such as occurred with choline, is to be noted, e.g. 0.4 % of dietary cholesterol in the basal hypolipotropic diet produced total liver lipids of 36.5 %, while inositol reduced this to only 28.7 % (groups 3 and 20). No significant decrease in cholesteryl esters was observed, but the glyceride fraction did seem to be decreased (compare 2379 mg. with 1492 mg. per liver). Unfortunately, it was not recognized, when these experiments were

recorded also that the total wet weights (including beverages) were 2980, 3020 and 3035 g. (mean 3012 g.) of which the total solids amounted to 456, 504 and 472 g., respectively (mean 477 g.). The ash contents of these diets were found to be 11.1, 11.6 and 10.6 (mean 11.1 g.). The data in Table 2 are first presented as grams consumed daily and then in the more useful form (for comparative purposes) of percentage in the dry solids.

DISCUSSION

These results require but little discussion. It might be mentioned that the cholesterol-rich diets containing liver or egg yolk fed by Blatherwick, Medlar, Bradshaw, Post & Sawyer (1931, 1932, 1933*a, b*) and by Okey, Yokela & Knock (1933–34) and Okey & Yokela (1936), contained quantities of lecithin providing amounts of choline considerably outside the range examined in the present study. It is therefore impossible to make exact comparisons, but it is obvious from Fig. 2 that the slope of the curves relating liver cholesteryl esters to dietary choline is rapidly decreasing as choline increases. Thus considerably larger doses of choline, while exerting a marked lipotropic effect, would

probably leave the cholesteryl esters much above normal.

The original experiments of Best and his colleagues on the effects of lecithin and of choline were conducted with high-fat diets devoid of cholesterol: the excessive deposition of liver lipids was readily prevented under these conditions.

As shown in Fig. 2 (curves F' and F'') the large accumulation of glycerides (28.6 %) and the moderate increase in cholesteryl esters (0.39 %) that resulted from feeding the basal hypolipotropic diet free from cholesterol are both completely prevented by choline. The ready response of both fractions to the lipotropic influence of choline in this situation (i.e. the so-called 'fat fatty liver') may be contrasted with that in the case of the 'cholesterol fatty liver' in which choline, although it has almost as marked an effect on the glycerides (curves A'' and B''), inhibits only partially the deposition of cholesteryl esters at the higher intakes of cholesterol (cf. curves A' and B' , or better, curves A to F in Fig. 2).

The average choline content (0.077 %) of the nurses' diets was found by coincidence to correspond almost exactly with the lower level of choline (0.08 %) used in the experiments reported above. It must be remembered that natural diets contain choline precursors (methionine, betaine and possibly thetins) besides the choline which can be estimated microchemically or microbiologically. Mere knowledge of analytical data for these substances does not permit an estimate to be made of what proportion is actually used for lipotropic purposes, but undoubtedly some is so used and supplements the dietary choline. While it is dangerous to assume that data obtained from rats can be applied to man, it is interesting to note that the average percentage of choline found in the diets of the nurses is just about that required to maintain normal levels of glycerides and cholesteryl esters in the livers of rats consuming

diets containing proportions of cholesterol similar to those found in the nurses' rations.

SUMMARY

1. Crystalline cholesterol was incorporated at five concentrations between 0.1 and 1.6 % in hypolipotropic diets and in two similar sets of diets containing 0.092 and 0.368 %, respectively, of choline chloride. Three diets were prepared containing different levels of cholesterol with 0.32 % inositol. Liver lipids were determined after these rations had been fed to rats for 3 weeks.

2. A large deposition of glycerides and some increase in cholesteryl esters occurred in the livers of the rats consuming the basal hypolipotropic diet free from cholesterol.

3. Increasing amounts of dietary cholesterol caused a progressive increase in the accumulation of both glycerides and cholesteryl esters in the livers.

4. The larger amount of choline was able to maintain liver glycerides within or only slightly above the normal range at all these intakes of cholesterol.

5. Cholesteryl esters in the liver were slightly elevated even at the larger intake of choline when the diet contained 0.2 % of cholesterol; further increases in dietary cholesterol caused progressive increases in the deposition of cholesteryl esters.

6. Data are given for the lipid components (including cholesterol and choline) of several human diets.

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