The Effect of Feeding Cholesterol without Fat on the Plasma-lipids of the Rabbit. The Role of Cholesterol in Fat Metabolism

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The extensive studies reported on the biological effects of cholesterol administration are chiefly concerned with changes brought about in the lipid composition of tissues. In the blood only the cholesterol fractions have been systematically examined after cholesterol feeding (cf. Anitschkow & Cowdry, 1933); only a few workers have examined the other blood lipids. Page & Bernhardt (1935), who fed rabbits with cholesterol dissolved in olive oil, reported that besides free and esterified cholesterol the phospholipids were increased in the plasma. Weinhouse & Hirsch (1940) found that phospholipids and neutral fats, as well as the cholesterol fractions, were increased in the serum of rabbits after cholesterol feeding. Vermeulen, Dragstedt, Clark, Julian & Allen (1942) also found that there was a great rise in the 'non-cholesterol fraction' of the serum lipids of rabbits fed cholesterol dissolved in sunflower-seed oil. The studies quoted were concerned chiefly with the experimental production of atheroma; the plasma-lipid values were estimated only after a very long period of cholesterol administration and no attempts were made to elucidate the mode of action of cholesterol.

Most of the changes in tissue- and blood-lipids reported as having been brought about by exogenous cholesterol cannot strictly be attributed to cholesterol alone (except those found by Weinhouse & Hirsch), but to cholesterol plus a high fat diet. It was generally held until recently that cholesterol is not appreciably absorbed from the alimentary tract unless fats are administered with it (cf. Bloor, 1943, pp. 106–8). This is apparently true only for crystalline cholesterol, for Dömösi & Egyed (1939) have shown that cholesterol is readily absorbed from the alimentary tract of rabbits if it is administered in the form of an amorphous suspension in water. The total blood-cholesterol level of their rabbits, fed 1 g. of cholesterol daily, rose in 4–5 days from the normal of about 80 to 300–400 mg./100 ml., and in 3–4 weeks the rabbits developed atheroma in the aorta and pulmonary arteries and the total blood-cholesterol levels reached 1300–1800 mg./100 ml. Dömösi & Egyed made no observations on non-cholesterol lipid fractions in the blood and their earliest observations were made after 4 days of cholesterol administration.

The experiments to be reported here were originally designed to study the effect of cholesterol on the kidney lipids. It might be mentioned that while cholesterol readily induces fatty livers—'cholesterol-fatty liver' (Best & Ridout, 1933; Chanutin & Ludewig, 1933; Okey, 1933; Best, Channon & Ridout, 1934; Channon & Wilkinson, 1934)—in the kidney only negligibly small amounts of cholesterol or neutral fat are deposited in the tubular epithelium during cholesterol feeding. In these rabbits' kidneys cholesterol accumulated only in the interstitial cells and capillary endothelium. The plasma-lipids during the experiments were also investigated and results were obtained which are thought to cast some new light on the rather obscure role of cholesterol in fat metabolism.

The changes observed in plasma-lipids in the present experiments can be attributed entirely to the action of cholesterol as no fats, save the small amounts contained in the usual vegetable diet, were administered to the rabbits.

MATERIAL AND METHODS

Rabbits of about 3000 g. weight were used. Their diet consisted of bran, oats, cabbage and water *ad libitum*. They were fed daily through a stomach tube 0·5–1·0 g. of amorphous cholesterol suspended in water. Cholesterol purified by recrystallization was converted into its amorphous form by dissolving suitable quantities in hot glacial acetic acid and pouring the hot solution into a large volume of cold water (cf. Dömösi & Egyed, 1939). Amorphous cholesterol precipitated immediately and the acid was removed by washing several times on a Buchner funnel. Suspensions of the acid-free amorphous cholesterol were prepared in tap water so that 20 ml. of the suspension contained 1 g. of cholesterol.

Blood was taken from the lateral ear-vein at varying intervals. Neutral potassium oxalate (3 mg./ml.) or heparin were used as anticoagulants. The plasma was separated by centrifuging and was immediately extracted.

Analytical procedures. The plasma was extracted by boiling with 30–40 volumes of an ethanol-ether mixture (3 : 1, v/v) by the method of Bloor (1928). The filtered extracts were evaporated to dryness under reduced pressure (20–40 mm. of Hg) at an external temperature of 30–40°. The dry residue was re-extracted with light petroleum (b.p. 40–60°) and the various lipid fractions were isolated from the latter extract. Phospholipids were precipitated with acetone and $MgCl_2$ and were determined by

the dichromate oxidation method of Bloor (1929). The supernatant fluid obtained after phospholipid precipitation was divided into a smaller and a larger sample: the former was used for the determination of free cholesterol and the latter, after saponification, for the determination of total cholesterol and of non-phospholipid fatty acids (derived from neutral fats and cholesteryl esters). For the saponification, the larger sample of the acetone supernatant was first evaporated to dryness on a hot plate and then 5 ml. of ethanol and 0·3 ml. of aqueous KOH, containing 40 g. KOH/100 ml., were added. The mixture was kept in an incubator at 75° for 2 hr. After completion of hydrolysis most of the ethanol was evaporated off on a hot plate and then about 2 ml. of distilled water were added. The last traces of the ethanol were removed by further heating and current of air. The mixture was made acid with 5% (w/v) HCl and extracted with light petroleum. Suitable samples of this extract were used for the determination of total cholesterol and non-phospholipid fatty acids. For the latter, samples of the light petroleum extract were evaporated to dryness on a hot plate and the residue was dissolved in 10 ml. ethanol. The fatty acids were titrated at 75° with 0·02 N-aqueous NaOH, using phenolphthalein as indicator. The average molecular weight of the fatty acids was assumed to be 275.

Free and total cholesterol were determined by the digitonin method of Popják (1943).

Ester cholesterol was calculated as the difference between total and free cholesterol, and neutral fat by the formula:

$$\text{neutral fat} = (\text{non-phospholipid fatty acids} - 0{\cdot}72 \times \text{ester cholesterol}) \times 1{\cdot}05.$$

The iodine values were determined by Yasuda's (1931–2) micromodification of the pyridine sulphate dibromide method.

RESULTS

The results obtained were very uniform and for this reason detailed data of representative experiments only will be presented.

Early changes in plasma-lipids after administration of cholesterol

No changes were found in the lipid content of the plasma until 24 hr. after feeding 1 g. of cholesterol, but after 24 hr. the plasma usually appeared slightly opalescent and the analyses showed a considerable increase in all the lipid fractions, e.g. free and esterified cholesterol, phospholipids and neutral fat (Table 1). Even 48 hr. after the single dose of cholesterol the plasma-lipid values were still elevated, but after 1 month without further cholesterol the plasma-lipids of this rabbit returned to slightly below the original levels. There is no explanation available at present of the 24 hr. delay in the plasma-lipid changes after ingestion of cholesterol. Possibly (*a*) in the rabbit the contents of the stomach may be retained for long periods, (*b*) cholesterol, although absorbed earlier than 24 hr. after the administration, may not reach the blood because of storage in the tissues, possibly in the liver. The observation of Aylward, Channon & Wilkinson (1935) that cholesteryl esters in the liver of rats increase steadily from the 4th hr. onwards, after the ingestion of cholesterol-containing food, suggests that probably the second explanation is more valid. This might indicate that the effects of cholesterol on plasma-lipids are mediated through the liver.

Plasma-lipids during prolonged feeding of cholesterol

Two typical experiments are recorded in Tables 2 and 3. It is apparent that after a few days of cholesterol administration high degrees of lipaemia were obtained; the increase in the cholesterol fractions was paralleled by an increase in other lipids. With an increasing lipid content the plasmata became more and more opalescent until finally they resembled milk. Two points in these tables require special mention. When the animals received only 0·5 g. of cholesterol per day their plasma-lipid values were maintained at a high but fairly constant level: at about values recorded in Table 3 after administration of $8 \times 0{\cdot}5$ g. of cholesterol. This indicates that with the daily intake of 0·5 g. of cholesterol there was an equilibrium between absorption and elimination (deposition in tissues, excretion and destruction). The lipaemia, on the other hand, continually increased whenever the

Table 1. *Plasma-lipids of rabbit Ch* 1 *before and after feeding* 1 *g. of cholesterol*

(Values are mg./100 ml. plasma.)

	Non-phospholipid fatty acids	Neutral fat	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters*
				Free	Ester	
Before cholesterol	103	71	102	24	48	35
24 hr. after cholesterol	338	238	207	50	154	111
48 hr. after cholesterol	301	154	224	51	214	154
1 month after cholesterol	84	68	90	16	27	19

* Calculated as ester cholesterol × 0·72.

Table 2. *Plasma-lipids of rabbit Ch* 1 *during prolonged cholesterol feeding* (1 *g. of cholesterol/day*)

(Values are mg./100 ml. plasma.)

Total dose of cholesterol (g.)	Non-phospholipid fatty acids	Neutral fats	Phospho-lipids	Cholesterol		Fatty acids of cholesteryl esters*	Ratio
				Free	Ester		Ester cholesterol/Free cholesterol
None	84	64	90	16	27	19	1·69
2	363	163	227	138	289	208	2·10
6	783	89	592	262	968	698	3·70
8	900	118	600	309	1093	788	3·54

* Calculated as ester cholesterol × 0·72.

Table 3. *Plasma-lipids of rabbit Ch* 5 *during prolonged cholesterol feeding*

(The animal received 0·5 g. of cholesterol per day for the first 8 days; then the dose was increased to 1 g. per day. Values are mg./100 ml.)

Total dose of cholesterol (g.)	Non-phospholipid fatty acids	Neutral fats	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters*	Ratio ester/free
				Free	Ester		
None	181	149	134	22	54	39	2·46
8 × 0·5	395	269	296	132	193	139	1·46
8	975	713	460	214	411	296	1·92
11	955	455	690	366	724	522	1·98
60	1840	368	927	631	1955	1490	3·10

* Calculated as ester cholesterol × 0·72. The concentration of cholesteryl esters can be obtained from the data by the addition of ester cholesterol and fatty acids of cholesteryl esters.

animals were given 1 g. of cholesterol/day; this rise continued for as long as 5–8 weeks, after which a plateau followed by a gradual decline was usually observed. The experiments were not continued beyond 3 months. The plasma-lipid values recorded in Tables 2 and 3 were by no means the highest observed, but an occasional rabbit showed less pronounced increases in plasma-lipids, although no 'resistance' was observed such as that reported by some investigators (cf. Wright, Robinson & Trikojus, 1944).

The second point is that while the phospholipids and non-phospholipid fatty acids (the latter not being a separate lipid fraction, only a convenient laboratory measure) always increased parallel with the rise in cholesterol, the behaviour of neutral fats was variable. In rabbit Ch 1 (cf. Table 2) the neutral fats showed only a moderate to slight increase, while in rabbit Ch 5 (cf. Table 3) there was a far greater increase. It is evident from the last columns of Tables 2 and 3 that the two rabbits in question differed in the degree to which they were able to esterify cholesterol, this being higher in rabbit Ch 1 than in rabbit Ch 5. The same relationship was observed in other animals, and the general conclusion can be drawn that animals which markedly esterify cholesterol will respond with a slight to moderate increase, and those which esterify cholesterol to a lesser degree with a greater rise in plasma-neutral fats during cholesterol administration.

The ratio of ester to free cholesterol commonly increased with the rising of plasma-total cholesterol up to *c.* 1200 mg./100 ml.; when the total cholesterol reached values higher than this, the degree of esterification usually decreased. There were, however, some exceptions to this rule as the example recorded in Table 3 shows.

Mode of action of cholesterol

The fact having been established that the administration of cholesterol causes without exception a great increase in the fatty acid compounds of the plasma, the origin of these fatty acids remains to be explained. Dietary fat can be readily exeluded as the source, since the fat content of bran, oats and cabbage is insufficient to account for the increases in the plasma. Two alternatives remain: (*a*) that the fatty acids came from mobilized depot fat, and (*b*) that there was an increased synthesis of fatty acids from carbohydrates. The second alternative could not be investigated owing to lack of facilities. Data will be presented, however, which are thought to show that probably the major portion, if not all, of the fatty acids built into the various lipids of the plasma during cholesterol administration are derived from the fat of connective tissue.

The first indication that connective tissue fat might be the source of the fatty acids in the plasma was provided by direct observation of the fat-depots of the experimental animals. The rabbits used were kept in the department for several months before the experiments, during which time they were fed *ad libitum* and they built up large amounts of adipose tissue. The nutritional state of rabbits can

be well assessed from the total weight of the abdominal and scapular fat (cf. Dible & Popják, 1941). Ten control animals, from the same stock as that used for the cholesterol-feeding experiments, possessed 200–250 g. (fresh weight) of adipose tissue. Twenty-five cholesterol-fed animals, on the other hand, killed at varying intervals during the experiment (3–12 weeks on cholesterol) had only very little (0–50 g.) reserve fat. The depletion of fat depots, moreover, was related to the duration of the experiment: the longer the period of the cholesterol feeding the smaller the amount of the body adipose tissue. The appearance of the remaining fatty tissue was very characteristic: it was slightly oedematous ('gelatinous') and greyish-white or brown, appearances which in the experience of morphologists suggest a rapid depletion.

In spite of the apparent loss of 150–200 g. of adipose tissue the animals either maintained their body weight, or lost only 50–100 g. in the course of several weeks. This may indicate that at the time of increased fat utilization there was a deposition of other body constituents, but it is more probable that the maintenance of body weight (or only slight loss) while the animals lost up to 200 g. of adipose tissue was due to water retention. Determinations of water and electrolyte content of skeletal muscle and serum showed that there was a considerable increase in the volume of the extra-cellular phase of the muscle; the muscles were often visibly oedematous. The changes in water and electrolyte equilibria in these animals will be reported in a later publication.

Although there was no indication that the rabbits consumed less food during cholesterol administration, more exact experiments were made to ascertain that the diminution of the depot fat was not caused by a decreased food intake.

Four rabbits were placed in separate metabolism cages and were given a weighed amount of the stock diet (2 parts oats + 1·5 parts bran) in excess of their requirements; 100 g. (fresh weight) of cabbage were also given daily. The food left over was collected daily and weighed. After a 3-week control period the rabbits were given 1 g. of cholesterol/day and their food consumption and body weight measured.

The results of two typical experiments are shown in Fig. 1, in which the average food consumption and body weights are recorded for 3-day intervals. The recorded food consumption represents the dry weight of the oats and bran mixture only; the rabbits ate all the cabbage offered. The mean food consumption, calculated from 3-day averages, of rabbits 75 and 76 during the control period was 100 ± 6 g. and 102 ± 8 g. respectively, and during cholesterol administration 114 ± 9 g. and 109 ± 14 g. respectively. During the third week of the control period there was a slight fall in food consumption and body weight (Fig. 1) which might have been due to the fact that in the smaller space of the metabolism cages the movements of the rabbits were more limited, resulting in a loss of appetite. After cholesterol feeding was started the daily food intake rose to levels slightly above those of the control period and the body weights were maintained at about the initial levels. After

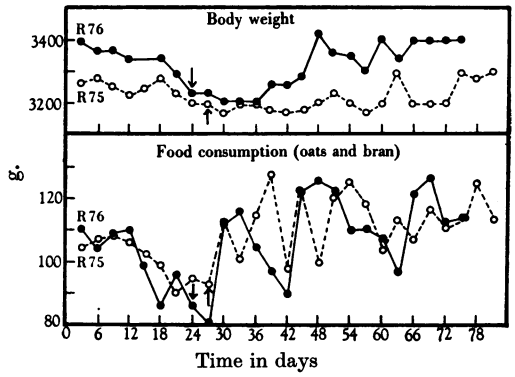


Fig. 1. Food consumption and body weights of rabbits 75 (o - - - o) and 76 (•—•) before and during cholesterol feeding. Cholesterol feeding was started at ↑.

12 weeks on cholesterol the rabbits were killed; the total amount of the dissected adipose tissue (omental, mesenteric, pelvic, perirenal and scapular fat) in rabbit 75 was <10 g. and in rabbit 76, 40 g. The other two experiments yielded essentially the same results. Five of the control rabbits from the same stock, with similar body weights and kept on the same diet, were killed at the same time and all of them had more than 200 g. of fatty tissue.

It is evident therefore that cholesterol-fed rabbits do utilize more fat than those without cholesterol. The amount utilized in excess of normal is however no more than about 1 g./day/kg. rabbit.

Although the evidence brought forward is more circumstantial than direct, the depletion of the fat depots was observed with such regularity that it leaves little doubt in the writer's mind as to its real significance.

Lipids of lymph during cholesterol administration

Observations on the lipid content of lymph during cholesterol administration give further support to the view that cholesterol causes an increased mobilization of depot fat.

The flow of lymph in the cysterna chyli of cholesterol-fed rabbits was so rapid that 1–2 ml. of lymph could be easily collected with a syringe in about 5 min. One representative observation out of three is shown in Table 4, in which the lipid content of lymph and blood plasma obtained simultaneously from rabbit 42 are compared. The striking feature of the comparison is that while the lymph contained less phospholipids and cholesterol, its neutral fat content was nearly five times greater than that of the plasma. Since the animal received no fat in its diet it is inferred that the neutral fat in the lymph

Table 4. *Lipid content of lymph and blood plasma of rabbit 42, after receiving 1 g. of cholesterol/day for 1 month*

(Values are mg./100 ml.)

	Non-phospholipid fatty acids	Neutral fats	Phospholipids	Cholesterol		Fatty acids of cholesteryl esters*
				Free	Ester	
Lymph	770	664	174	102	191	138
Blood plasma	747	140	510	198	852	614

* Calculated as ester cholesterol × 0·72.

was derived from the adipose tissues. This observation agrees with the data summarized by Bloor (1943, p. 363) that mobilized fat enters the lymphatics first and is then carried into the blood. That the lipids found in the lymph are not necessarily derived from intestinal absorption was clearly shown with the aid of radioactive phosphorus by Reinhardt, Fishler & Chaikoff (1944), who found that phospholipids are exchanged between blood plasma and lymph. Taking this fact into account, the relatively high phospholipid content of the lymph shown in Table 4 can be accounted for by such an exchange and does not require the postulation of synthesis by the intestinal mucous membrane. The high neutral fat content of the lymph, on the other hand, cannot be explained by the same mechanism, since the concentration in the plasma was less than in the lymph. Whether a circulation of the cholesterol fractions between blood and lymph exists cannot yet be decided.

Source of excess phospholipids during cholesterol administration

It has been shown by Fishler, Entenman, Montgomery & Chaikoff (1943) with the aid of ^{32}P, that plasma-phospholipids are synthesized exclusively by the liver. In the present experiments a ten-fold rise in plasma-phospholipids was not an unusual finding and in view of the work of Fishler *et al.* the cause of this increase must be an increased synthesis of plasma-phospholipids by the liver under the influence of cholesterol. The mean total phospholipid content of the liver of 15 rabbits fed cholesterol for 2–12 weeks was $3{\cdot}39 \pm 0{\cdot}52\%$ and that of 12 normal rabbits $3{\cdot}10 \pm 0{\cdot}25\%$ of the moist weight. The mean weight of the livers of the cholesterol-fed rabbits was 102 ± 24 g. and that of the controls 65 ± 16 g. It may be calculated then that the livers of normal rabbits contained an average of 2·02 g. and those of the cholesterol-fed rabbits 3·46 g. of phospholipid. The increase in plasma-phospholipids, therefore, cannot be regarded as caused by a mere displacement of liver phospholipids by the accumulation of cholesterol in this organ, but must be attributed to new and increased synthesis. These observations may seem contradictory to the findings of Okey (1933, 1944) and Best *et al.* (1934) that the concentration of phospholipids in 'cholesterol fatty livers' is decreased. This discrepancy is probably due to the fact that in these authors' experiments cholesterol was fed in a high fat diet which resulted in a deposition of large amounts of neutral fat as well as cholesterol in the liver. The changes in liver lipids in the present experiments will be reported separately, but it may be mentioned that the feeding of cholesterol alone, without fat, resulted in an accumulation of only small amounts of neutral fat in the liver.

A further discrepancy arises from the finding of Perlman & Chaikoff (1939) that feeding of cholesterol in a high fat diet for 30 hr. depresses the phospholipid 'activity' in the liver of rats, as measured by radioactive phosphorus. They did not measure, however, the radioactivity of plasma-phospholipids and it may well be that under the influence of cholesterol the newly synthesized phospholipids are poured rapidly into the blood.

Iodine values of plasma-fatty acids during cholesterol administration

In an endeavour to obtain further evidence on the source of the plasma-fatty acids, their iodine values were determined in 8 rabbits at weekly intervals during cholesterol administration. It was thought that if the mobilized depot fat fatty acids are built directly into phospholipids the iodine value of the phospholipid fatty acids should approximate that of depot fat fatty acids; if, on the other hand, the cholesteryl esters act as carriers of fatty acids from the depots, their fatty acids should have an iodine value close to that of the fatty acids of adipose tissue.

All the results were the same; two representative experiments are recorded in Tables 5 and 6. During the administration of cholesterol the iodine value of the phospholipid fatty acids decreased markedly, i.e. these phospholipids contained more saturated fatty acids than before the experiment. The changes in the iodine values of these fatty acids recorded in Table 5 were the least observed; in other experiments iodine values as low as 66 were found.

When the administration of cholesterol was discontinued for one week (Table 6), the plasma-lipids

Table 5. *Plasma-lipids and their iodine values in rabbit* 53 *before and during administration of* 1 *g. of cholesterol per day*

(The interval between successive determinations was 1 week.)

	Non-phospholipid fatty acids								
	Total		Fatty acids of cholesteryl esters*	Fatty acids of neutral fats†	Neutral fats	Phospho-lipids	Iodine value of phospho-lipid fatty acids‡	Cholesterol	
	(mg./100 ml.)	Iodine value	(mg./100 ml.)	(mg./100 ml.)	(mg./100 ml.)	(mg./100 ml.)		Free (mg./100 ml.)	Ester (mg./100 ml.)
Before cholesterol	98	88·0	25	63	66	134	110·6	20	35
During cholesterol feeding	450	85·0	353	97	102	361	89·5	134	495
	552	98·0	443	109	114	521	84·0	195	615
	1150	99·5	884	266	279	860	87·0	438	1228
	1146	100·6	864	282	296	835	81·9	427	1200
	1261	116·2	1054	207	207	1010	82·0	636	1464

* Ester cholesterol × 0·72.

† Non-phospholipid fatty acids − (ester cholesterol × 0·72).

‡ Calculated from the iodine values of whole phospholipids assuming that fatty acids make up 69% of the phospholipid molecule.

Table 6. *Plasma-lipids and their iodine values in rabbit* 75 *before and during administration of* 1 *g. of cholesterol per day*

(The interval between successive determinations was 1 week.)

	Non-phospholipid fatty acids								
	Total		Fatty acids of cholesteryl esters	Fatty acids of neutral fats	Neutral fats	Phospho-lipids	Iodine value of phospho-lipid fatty acids	Cholesterol	
	(mg./100 ml.)	Iodine value	(mg./100 ml.)	(mg./100 ml.)	(mg./100 ml.)	(mg./100 ml.)		Free (mg./100 ml.)	Ester (mg./100 ml.)
Before cholesterol	114	83	46	68	71	113	106	26	64
During cholesterol feeding	686	98	503	183	192	564	75	236	699
	799	100	649	150	157	730	78	312	902
	1048	118	788	260	273	796	72	417	1094
	Cholesterol discontinued for 1 week								
After 1 week without cholesterol	722	118	646	76	79	586	91	281	897
	Cholesterol feeding resumed								
After 1 week of resumed cholesterol feeding	1660	97	1013	647	679	988	72	524	1407

showed a decline and the iodine value of the phospholipid fatty acids increased; on the resumption of the feeding of cholesterol, plasma-lipids rose again and the iodine value of the phospholipid fatty acids decreased. The iodine value of the crude depot fat fatty acids of rabbits fed on bran, oats and beet pulp was found (Dible & Popják, 1941) to be 66 ± 7 (range 60–86). The iodine values of the phospholipid fatty acids during cholesterol administration, therefore, are close to those of the adipose tissue. It is inferred from these findings that the fatty acids of mobilized depot fat have been built into plasma-phospholipids. It is of some interest to note that, while the iodine values of the fatty acids of plasma-phospholipids during cholesterol administration varied between 66 and 80, the iodine value of the fatty acids of liver phospholipids was 95–100. There appears to be a selective 'secretion' into the blood by the liver of the phospholipids containing the more saturated fatty acids.

The iodine values of the non-phospholipid fatty acids showed the reverse of what was found for phospholipid fatty acids. The greater proportion of these fatty acids during the experimental period was derived from cholesteryl esters (cf. Tables 5 and 6) and their iodine values therefore represent more closely those of the fatty acids of cholesteryl esters; whereas before the administration of cholesterol they were more representative of the neutral fat fatty acids. Bloor, Blake & Bullen (1938) have shown for normal human plasma that the degree of unsaturation of the fatty acids increased in the

order: neutral fat < phospholipid < cholesteryl ester. It can be inferred from the data presented that this is also true in the rabbit, except that under the conditions of the present experiments the degree of unsaturation of the phospholipid fatty acids is nearly the same as that of neutral fats, presumably because during the rapid transfer of the fatty acids from neutral fats to phospholipids no significant degree of unsaturation occurred.

If the fatty acids of cholesteryl esters were ultimately also derived from adipose tissue fat, as the depletion of fat depots suggests, a considerable degree of unsaturation had taken place. Whether this unsaturation is a preliminary step in the breakdown of the fatty acids cannot be decided. The conflicting views on the subject have been recently reviewed by Bloor (1943, pp. 314–17).

relationship between blood-total cholesterol and phospholipids was found by Mayer & Schaeffer (1913) and Bloor (1914), and between total cholesterol and total fatty acids by Terroine (1914), Bloor (1914) and Bang (1918), and more recently by Peters & Man (1943). These relationships are relatively constant among members of the same species in the postabsorptive state.

The data of the experiments were analyzed in respect of these ratios (total cholesterol/phospholipid; total cholesterol/total fatty acids), but it was found that, while the ratios were fairly constant for normal animals, in cholesterol-induced lipaemia no such constancy existed. When plasma-total cholesterol values were plotted against phospholipids, or against non-phospholipid fatty acids (or total fatty acids), the points approximated to a smooth

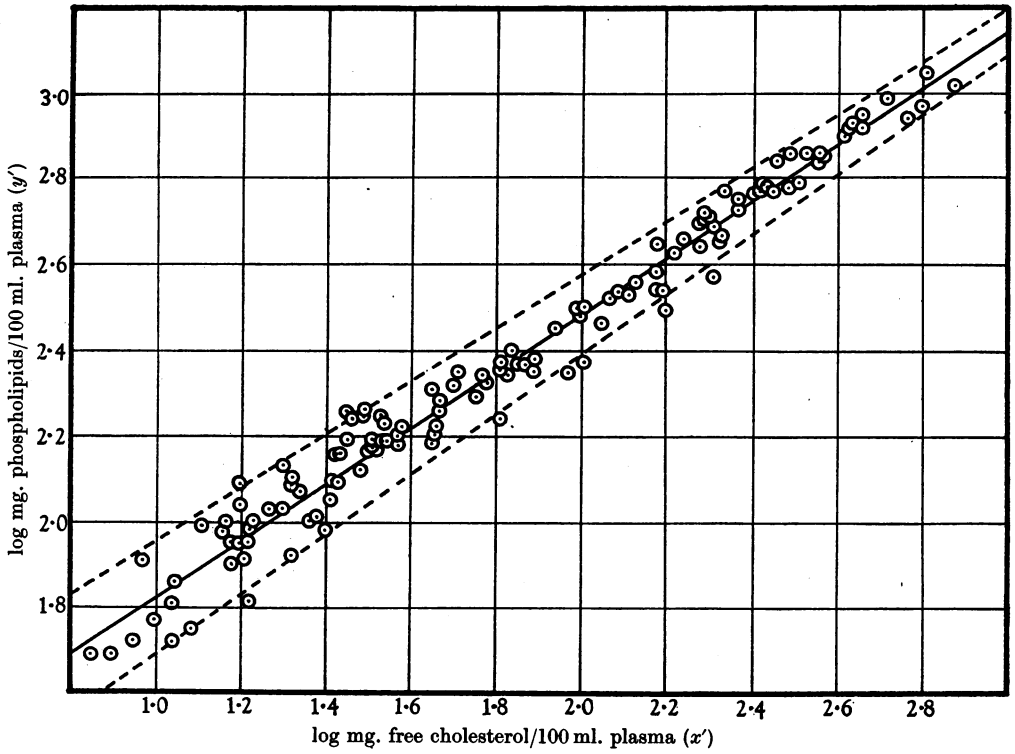


Fig. 2. Relationship between log plasma free cholesterol (x') and log phospholipids (y') in normal and cholesterol-fed rabbits. The continuous straight line represents the equation $y' = 0{\cdot}660x' + 1{\cdot}1536$ calculated by the method of least squares. The interrupted lines, showing the maximum limits of variation, represent the equation

$$y' = (0{\cdot}660 \mp 0{\cdot}03)\, x' + (1{\cdot}1536 \pm 0{\cdot}15).$$

Interrelation between cholesterol and plasma-lipids. Universality of the relationship

It was thought that if cholesterol is the cause of the described changes in plasma-lipids, a quantitative relationship should exist between plasma-cholesterol and the other lipids. A fairly constant

curve (not a straight line) up to total cholesterol values of 600–800 mg./100 ml. Above these values, however, no definite relationships could be detected. It is of some interest, therefore, that a quantitative relationship was found between plasma free cholesterol and phospholipids, and free cholesterol and non-phospholipid fatty acids, over the entire

range of the experimental values. Moreover, as will be shown, the equations expressing these relationships are applicable not only to the data of this investigation, but hold true for values of a number of species in the postabsorptive state and even in some pathological conditions.

A linear relationship was found between the common logarithm of plasma free cholesterol and the logarithm of phospholipids on the one hand, and the logarithm of non-phospholipid fatty acids on the other. The first relationship is illustrated in Fig. 2,

The similar equation relating the logarithms of plasma free cholesterol and of non-phospholipid fatty acids (Fig. 3), calculated also by the method of least squares, was found to be

$$z' = 0{\cdot}752x' + 1{\cdot}061, \qquad (3)$$

where z' = log non-phospholipid fatty acids (mg./100 ml.). The maximum variations from the mean are given by the equation

$$z' = (0{\cdot}752 \mp 0{\cdot}046)\, x' + (1{\cdot}061 \pm 0{\cdot}217). \qquad (4)$$

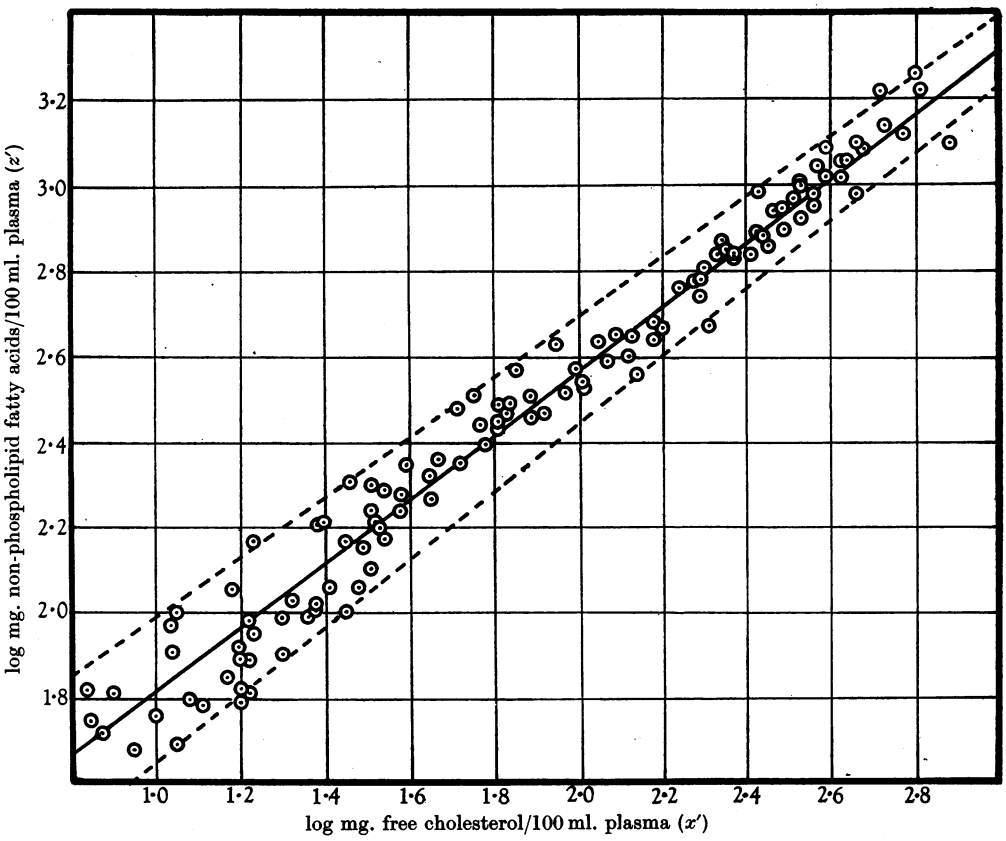


Fig. 3. Relationship between log plasma free cholesterol (x') and log non-phospholipid fatty acids (z') in normal and cholesterol-fed rabbits. The continuous straight line represents the equation $z' = 0{\cdot}752x' + 1{\cdot}061$ calculated by the method of least squares. The interrupted lines, showing the maximum limits of variation, represent the equation $z' = (0{\cdot}752 \mp 0{\cdot}046)\, x' + (1{\cdot}061 \pm 0{\cdot}217)$.

in which the continuous straight line represents the equation calculated by the method of least squares

$$y' = 0{\cdot}660x' + 1{\cdot}1536, \qquad (1)$$

where y' and x' are the common logarithms of plasma-phospholipid and plasma free cholesterol (y and x, both as mg./100 ml.) respectively. The two interrupted lines, giving the maximum observed variations from the mean, correspond to the equation

$$y' = (0{\cdot}660 \mp 0{\cdot}03)\, x' + (1{\cdot}1536 \pm 0{\cdot}15). \qquad (2)$$

By taking the antilog of equations (1) and (3) equations of the type $y = ax^b$ are obtained, which express the concentrations of plasma-phospholipids (y) and of non-phospholipid fatty acids (z) as functions of plasma free cholesterol (x):

$$y = 14{\cdot}240x^{0{\cdot}660}, \qquad (5)$$

$$z = 11{\cdot}508x^{0{\cdot}752}. \qquad (6)$$

These equations together with the observed points are illustrated in Figs. 4 and 5, where it can be seen that about 80 % of all the points fall within ± 10 %,

and 90% within ±15% of the values given by equations (5) and (6). In a preliminary communication (Popják, 1945) slightly different numerical values were given for the coefficient and exponent of x in equation (5). Since then more data have been obtained which allowed the more accurate calculation of these constants.

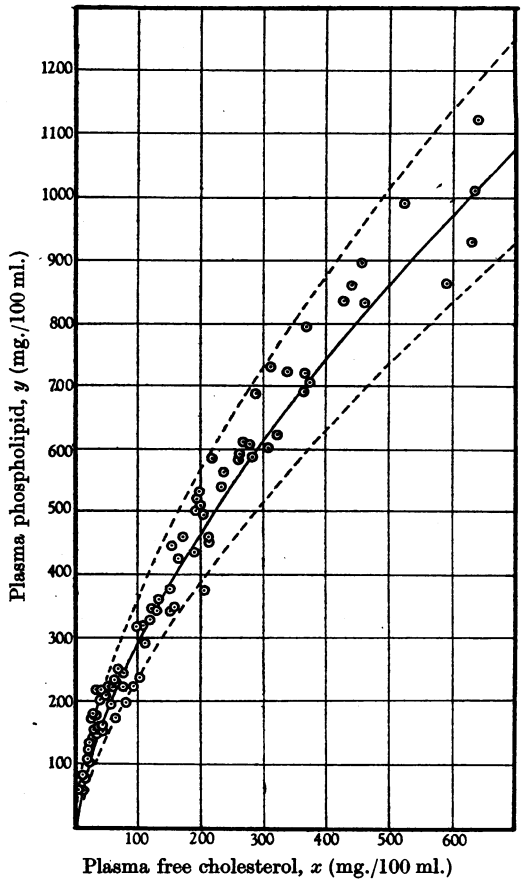


Fig. 4. Relationship between plasma free cholesterol and phospholipids in normal and cholesterol-fed rabbits. The continuous curve represents the equation $y = 14{\cdot}24x^{0{\cdot}666}$.

It follows from equations (5) and (6) that a similar quantitative relationship must exist between plasma free cholesterol and plasma-total fatty acids (Z) also, which should be represented by the equation

$$Z = 20{\cdot}797x^{0{\cdot}720},$$

as derived from (5) and (6).

These results would be of a very limited interest if they were applicable only to rabbits under the conditions of the present experiments. Plasma-lipid values for a variety of species reported by other workers were collected and compared with values defined by equations (5) and (6). These data are shown in Table 7 and illustrated in Figs. 6 and 7. It should be mentioned that the different methods employed for lipid determinations by various

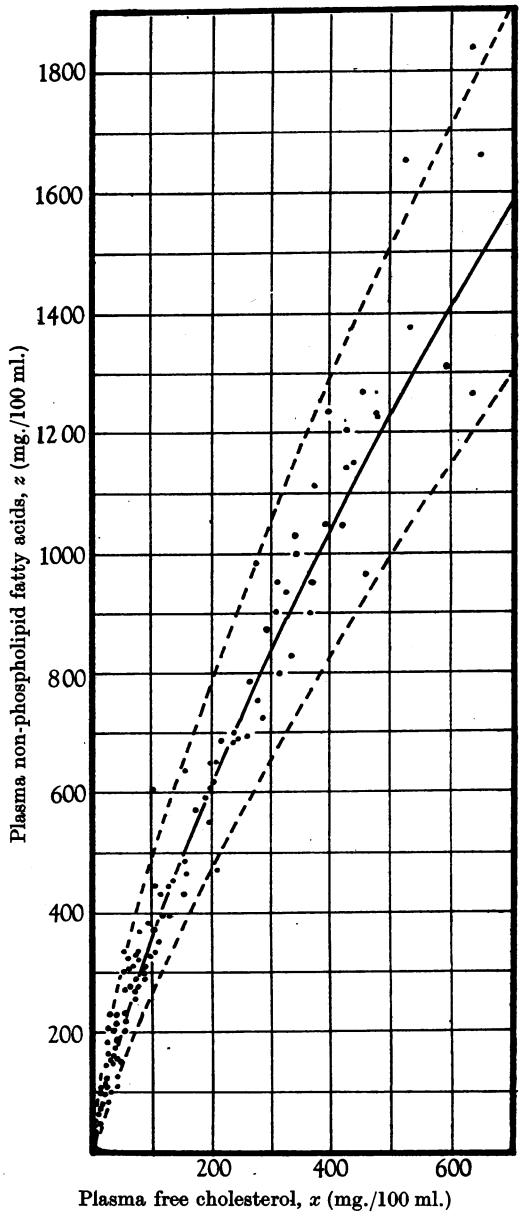


Fig. 5. Relationship between plasma free cholesterol and non-phospholipid fatty acids in normal and cholesterol-fed rabbits. The continuous curve represents the equation $z = 11{\cdot}508\ x^{0{\cdot}752}$.

investigators may give different results (cf. Bloor, 1943, pp. 121–22); for this reason, only those data were selected from the literature which were obtained on plasma by methods similar to those used

Table 7. *Mean plasma free cholesterol, phospholipid and non-phospholipid fatty acid values in different species as found by various investigators*

(Values are mg./100 ml.)

Species	Condition	Free cholesterol	Phospholipids	Non-phospholipid fatty acids	Author
Human adult	Normal	47±7	196±23	228*	Boyd (1936)
Human newborn	Normal	14±7	61±32	98*	
Human adult	Normal	53±10	195±37	227*	Boyd & Connell (1937)
Human adult	Hyperthyroidism	41±8	125±35	178*	
Human adult	Normal	50±12	190±34	—	Boyd (1937)†
Human	Nephritis	74	233	—	
Human	At term of pregnancy	65±15	248±43	—	Boyd (1934)
Human	Normal	70·2±13·9	235±40·1	—	Davis, Stern & Lesnick (1937)
Human	Arteriosclerosis and angina pectoris	86·4±14·1	280±38	—	
Human	Normal	46±8	165±28	202*	Boyd (1942)
Cockerel	Normal	34±9	155±34	254*	
Cat	Normal	30±10	132±53	137*	
Albino rat	Normal	21±8	83±24	95*	
Guinea-pig	Normal	11±2	51±12	81*	
Sheep	Normal and in pregnancy	16±9	90±33	67±20	Barcroft & Popják (1944–5)
Rabbit	Normal	22±9	118±46	117±66	From data of this investigation
Rabbit	On 28th day of pregnancy	5±1	20±5	43±5	Popják (1946)

* These values were calculated when possible from the author's data by the formulae: (neutral fat ×0·95 + ester cholesterol ×0·72), or (total fatty acids − phospholipids ×0·69).

† These data were presented in charts; these were magnified and values read off the scale.

in this investigation. The continuous curves in Figs. 6 and 7 are the initial portions of equations (5) and (6) drawn on a large scale. Within the range represented fall all the normal and some pathological values for the various species. It can be seen that there is very good agreement between the curves obtained from this investigation and the values reported by other workers for a variety of species (human, cat, cockerel, rat, guinea-pig, rabbit, and sheep). It remains to be seen how far these equations can be applied to pathological conditions, but, from a limited number of publications which give the results of differential lipid analyses by methods similar to those employed by the author, it appears that equation (5) expresses the relationship between plasma free cholesterol and phospholipids even in some pathological conditions. In Fig. 8 cases of diabetes mellitus, subacute nephritis and myxoedema are illustrated. The values reported by Rubin (1939) are of particular interest as they were obtained on one person, a 14-year-old diabetic girl, during her recovery from diabetic coma. The phospholipid values were calculated by Rubin from lipid phosphorus, a procedure which yields usually 10–15% higher values than Bloor's dichromate oxidation method. Taking this into account, there is an excellent agreement with the experimental data.

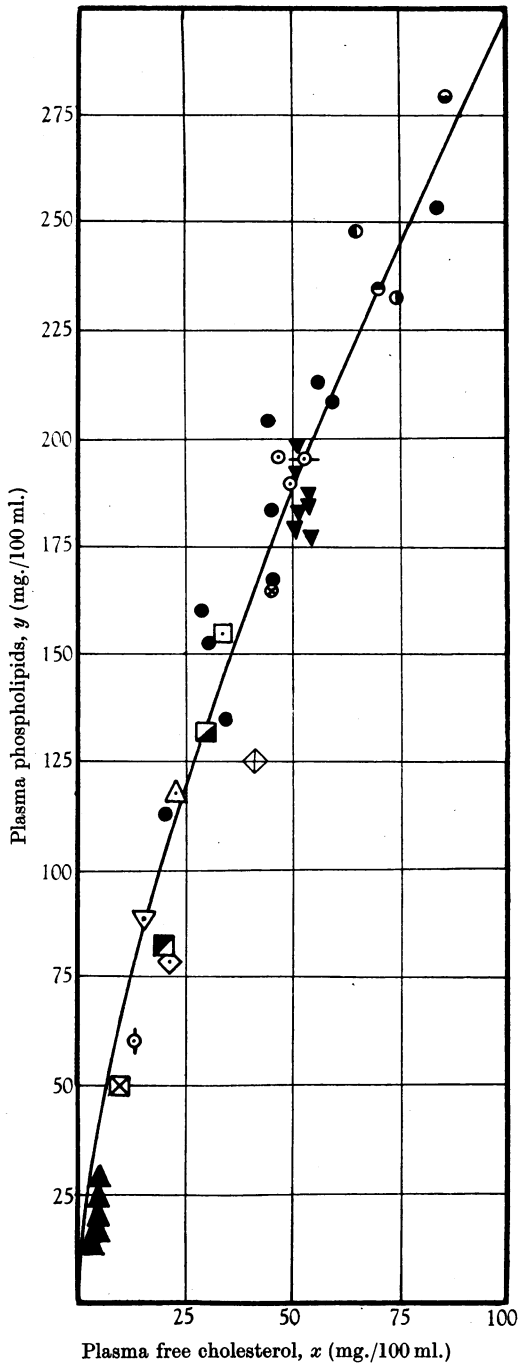
Equation (6) expresses the relationship between plasma free cholesterol and non-phospholipid fatty acids in the postabsorptive state and in such metabolic disturbances as hypo- and hyperthyroidism, but the equation cannot be applied to conditions in which a disturbance of the blood neutral fats occurs, e.g. diabetes mellitus.

It is realized that the numerical values of the exponent and coefficient of x in equations (5) and (6) depend on the methods employed for the determination of the lipids. This point may be illustrated with some further data from the literature. In fig. 9 the log of serum free cholesterol (x') is plotted against the log of serum lipoid phosphorus (p') as calculated from the values found by Man, Kartin, Durlacher & Peters (1945) in patients with various forms of liver disease. It can be seen that there is a good approximation to linearity although there is a greater scattering of the points than in Fig. 2, in which the results of the cholesterol-feeding experiments were illustrated. At least part of this scattering is probably due to a subjective error on the writer's part since the values were presented by Man *et al.* in small-scale charts from which it was

difficult to read off the figures accurately. The straight line in Fig. 9 was drawn visually as the best fit to the points and its equation

$$p' = 0{\cdot}819x' - 0{\cdot}528 \qquad (7)$$

was obtained graphically.



It follows that the antilog of equation (7) should express serum lipid phosphorus (p) as function of serum free cholesterol (x):

$$p = 0\ 297x^{0{\cdot}819}. \qquad (8)$$

DISCUSSION AND CONCLUSIONS

The results presented confirm the finding of Dömösi & Egyed (1939) that fat is not required for the absorption of cholesterol. The concentration of plasma cholesterol reaches much higher values and in a considerably shorter time after the administration of amorphous cholesterol than after feeding cholesterol dissolved in fat. Moreover by eliminating the necessity of a high fat diet the method permits the study of the effects of cholesterol alone.

It is concluded from the results obtained that cholesterol initiates a series of events which manifest themselves in an increased mobilization of depot fat, an increased synthesis of plasma-phospholipids by the liver and an increased utilization of fatty acids. The low iodine values of the phospholipid fatty acids during cholesterol administration, coinciding with the depletion of depot fat, are taken to indicate that these phospholipids were synthesized from the fatty acids of adipose tissue. The high iodine value of the fatty acids of cholesteryl esters suggests that it is unlikely that these esters act merely as carriers of fatty acids from the depots. If desaturation is the primary step in the breakdown of fatty acids, it appears more probable that cholesteryl esters are intimately linked with fatty acid oxidation (cf. Bloor, 1943).

The effects of exogenous cholesterol are considered to shed light on a general function of cholesterol in the normal organism, since it was found that plasma-phospholipid and non-phospholipid fatty acid levels can be expressed as functions of plasma free cholesterol, not only in cholesterol-fed animals, but also in a variety of species in the postabsorptive state.

Fig. 6. Relationship between plasma free cholesterol and phospholipids in different species in the postabsorptive state and in some pathological conditions. The continuous curve represents the equation $y = 14{\cdot}24x^{0{\cdot}660}$ obtained from the cholesterol-feeding experiments. The points are those reported by various investigators. Key to symbols: ⊙ human adult (Boyd, 1936, 1937); ⊙ human new-born (Boyd, 1936); ▼ human, diurnal variations (Boyd, 1935); -⊙- human adult, normal and ⊕ in hyperthyroidism (Boyd & Connell, 1937); ◓ human adult, normal and ◒ in arteriosclerosis and angina pectoris (Davis, Stern & Lesnick, 1937); ◐ human in pregnancy (Boyd, 1934); ◑ human in nephritis (Boyd, 1937); ⊗ human, ⊡ cockerel, ◪ cat, ◩ albino rat, ⊠ guinea-pig, ◇ rabbit (Boyd, 1942); △ rabbit (this investigation); ▲ rabbit on 28th day of pregnancy (Popják, 1946); ● human, individual values (own data); ▽ sheep (Barcroft & Popják, 1944–5).

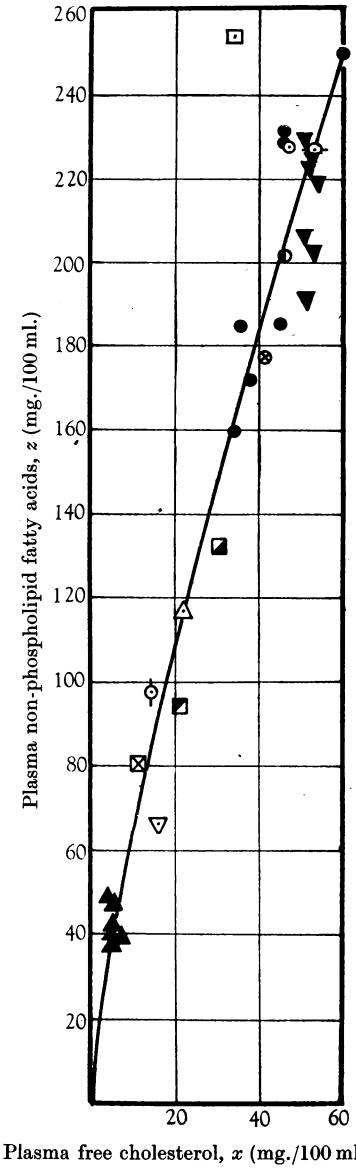


Fig. 7. Relationship between plasma free cholesterol and non-phospholipid fatty acids in various species. The continuous curve represents the equation $z = 11{\cdot}508x^{0{\cdot}752}$ obtained from the cholesterol-feeding experiments. The values shown are those reported by different investigators, or were calculated from their data. Key to symbols: ⊙ human adult and ⊙ new-born (Boyd, 1936); ⊙ human adult normal and ⊗ in hyperthyroidism (Boyd & Connell, 1937); ◐ human, ⊡ cockerel, ◪ cat, ◪ albino rat, ⊠ guinea-pig (Boyd, 1942); ▽ sheep (Barcroft & Popják, 1944–5); △ rabbit (this investigation); ▲ rabbit on 28th day of pregnancy (Popják, 1946); ● human, individual values (own data); ▼ human, diurnal variations (Boyd, 1935).

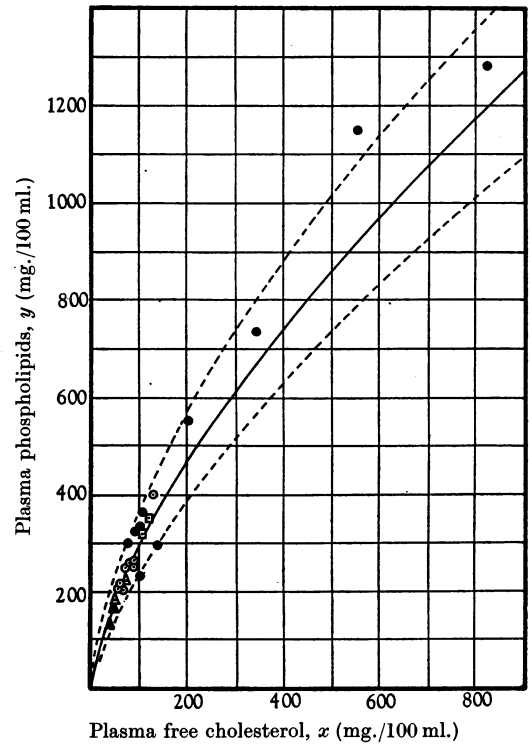


Fig. 8. Relationship between plasma free cholesterol and phospholipids in cases of diabetes mellitus, subacute nephritis, myxoedema, and thyrotoxicosis. The curves are the same as in Fig. 4. Key to symbols: ● diabetes mellitus during recovery from coma (Rubin, 1939); ⊡ subacute nephritis, △ treated diabetes mellitus, ⊙ myxoedema, ▲ thyrotoxicosis (own data).

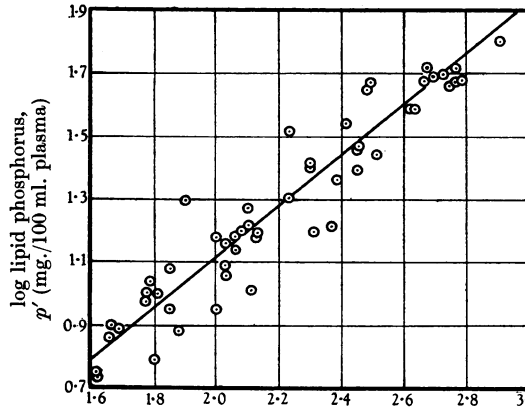
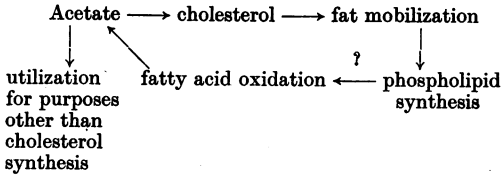


Fig. 9. Relationship between log mg./100 ml. serum free cholesterol (x') and log mg./100 ml. serum lipid phosphorus (p') in cases of liver diseases in human patients. Log values calculated from data of Man *et al.* (1945). The straight line was drawn visually as the best fit to the points and is given by equation $p' = 0{\cdot}819x' - 0{\cdot}528$.

Bloor (1914) was led to the belief, from the analysis of the lipids of a large number of human blood samples, that in the postabsorptive state there is a very efficient regulation of the lipid constituents of the blood. It is suggested here that the regulator of the plasma lipid levels is cholesterol, on the basis of its actions observed in the present experiments, and that this biological principle is common to many species. The fact that the quantitative relationship between plasma free cholesterol and phospholipids is the same even in some pathological conditions as in the cholesterol-feeding experiments and in the normal post-absorptive state, suggests that in these pathological conditions the primary disturbance is an increased synthesis of cholesterol.

If the present findings are correlated with the recent discovery by Bloch & Rittenberg (1942, 1944, 1945) that cholesterol is synthesized from acetate in the animal body, the different normal plasma-lipid levels of the various species (and in some pathological conditions) could be explained by a single factor, i.e. by the different amounts of acetate available for cholesterol synthesis. This hypothesis can be represented schematically:



Experiments which will be reported later, show that this hypothesis in essentials is correct, since hypercholesterolaemia has been produced in experimental animals by the feeding of sodium acetate. The way in which this controlling mechanism is linked with other factors influencing fat metabolism (lipotropic factors, etc.) cannot be conjectured.

SUMMARY

1. The effects of feeding cholesterol, in the absence of a high fat diet, on the plasma-lipids of rabbits were studied.

2. Amorphous cholesterol fed in a watery suspension is well absorbed by rabbits and causes a rise in the free and esterified cholesterol, phospholipids and neutral fats of the plasma 24 hr. after the ingestion. During prolonged cholesterol feeding all plasma-lipids show a progressive increase.

3. The advantages of feeding amorphous cholesterol without added fat are (*a*) that the effects of cholesterol alone can be studied, and (*b*) that induced lipaemia develops more rapidly and reaches higher levels than with the customary high fat cholesterol diets.

4. The fat reserves of the cholesterol-fed animals became depleted in 3–12 weeks in spite of an undiminished food intake.

5. The concentration of neutral fat was higher in the lymph than in the plasma of cholesterol-fed rabbits, indicating an increased mobilization of depot fat.

6. The iodine values of the phospholipid fatty acids of the plasma decreased during cholesterol feeding, nearly reaching those of the rabbits' adipose tissue; on the other hand, the iodine values of the non-phospholipid fatty acids increased. It is suggested that the fatty acids of plasma-lipids during cholesterol administration were derived from mobilized depot fat.

7. Quantitative relationships were found between plasma free cholesterol (x) and phospholipids (y) on the one hand, and non-phospholipid fatty acids (z) on the other. These relationships, in the rabbit, can be expressed by the equations $y = 14{\cdot}24x^{0{\cdot}660}$ and $z = 11{\cdot}508x^{0{\cdot}752}$ respectively. It is shown with the aid of data reported by others that these equations are applicable also to plasma-lipid values of species other than rabbit, including human, in the post-absorptive state and also in some pathological conditions.

8. It is inferred that plasma free cholesterol acts as a regulator of plasma-lipid levels by determining the rate of mobilization of fat from the depots, and the synthesis of plasma-phospholipids. Cholesteryl esters may play a part in fatty acid oxidation.

9. A hypothesis is advanced which seeks to explain the different plasma-lipid levels in the various species by a single factor, i.e. by the different amounts of acetate available for cholesterol synthesis.

I wish to thank Miss Muriel Kendall for her skilful technical assistance.

REFERENCES

Anitschkow, N. & Cowdry, E. V. (1933). *Arteriosclerosis*, 1st ed. New York: Macmillan Co.

Aylward, F. X., Channon, H. J. & Wilkinson, H. (1935). *Biochem. J.* **29**, 169.

Bang, I. (1918). *Biochem. Z.* **90**, 383.

Barcroft, J. & Popják, G. (1944–5). Unpublished.

Best, C. H., Channon, H. J. & Ridout, J. H. (1934). *J. Physiol.* **81**, 409.

Best, C. H. & Ridout, J. H. (1933). *Amer. J. Physiol.* **105**, 6.

Bloch, K. & Rittenberg, D. (1942). *J. biol. Chem.* **145**, 625.

Bloch, K. & Rittenberg, D. (1944). *J. biol. Chem.* **155**, 243.

Bloch, K. & Rittenberg, D. (1945). *J. biol. Chem.* **159**, 45.

Bloor, W. R. (1914). *J. biol. Chem.* **19**, 1.

Bloor, W. R. (1928). *J. biol. Chem.* **77**, 53.

Bloor, W. R. (1929). *J. biol. Chem.* **82**, 273.

Bloor, W. R. (1943). *Biochemistry of Fatty Acids*, pp. 106–8, 121–2, 314–17, 363. New York: Reinhold Publishing Corporation.
Bloor, W. R., Blake, A. G. & Bullen, S. S. (1938). *J. Allergy*, **9**, 227.
Boyd, E. M. (1934). *J. clin. Invest.* **13**, 347.
Boyd, E. M. (1935). *J. biol. Chem.* **110**, 61.
Boyd, E. M. (1936). *Amer. J. Dis. Child.* **52**, 1319.
Boyd, E. M. (1937). *Canad. med. Ass. J.* **36**, 18.
Boyd, E. M. (1942). *J. biol. Chem.* **143**, 131.
Boyd, E. M. & Connell, W. F. (1937). *Quart. J. Med.* **6**, 231.
Channon, H. J. & Wilkinson, H. (1934). *Biochem. J.* **28**, 2026.
Chanutin, A. & Ludewig, S. (1933). *J. biol. Chem.* **102**, 57.
Davis, D., Stern, B. & Lesnick, G. (1937). *Ann. int. Med.* **11**, 354.
Dible, J. H. & Popják, G. (1941). *J. Path. Bact.* **53**, 133.
Dömösi, P. & Egyed, M. (1939). *Magy. orv. Arch.* **40**, 242.
Fishler, M. C., Entenman, C., Montgomery, M. L. & Chaikoff, I. L. (1943). *J. biol. Chem.* **150**, 47.
Man, E. B., Kartin, B. L., Durlacher, S. H. & Peters, J. P. (1945). *J. clin. Invest.* **24**, 623.
Mayer, A. & Schaeffer, G. (1913). *J. Physiol. Path. gén.* **15**, 984.
Okey, R. (1933). *J. biol. Chem.* **100**, lxxv.
Okey, R. (1944). *J. biol. Chem.* **156**, 179.
Page, I. H. & Bernhardt, W. G. (1935). *Arch. Path.* **19**, 530.
Perlman, I. & Chaikoff, I. L. (1939). *J. biol. Chem.* **128**, 735.
Peters, J. P. & Man, E. B. (1943). *J. clin. Invest.* **22**, 707.
Popják, G. (1943). *Biochem. J.* **37**, 468.
Popják, G. (1945). *J. Path. Bact.* **57**, 280.
Popják, G. (1946). *J. Physiol.* In the Press.
Reinhardt, W. O., Fishler, M. C. & Chaikoff, I. L. (1944). *J. biol. Chem.* **152**, 79.
Rubin, S. H. (1939). *J. biol. Chem.* **131**, 691.
Terroine, E. F. (1914). *J. Physiol. Path. gén.* **16**, 212.
Vermeulen, C., Dragstedt, L. R., Clark, D. E., Julian, O. C. & Allen, J. G. (1942). *Arch. Surg., Chicago*, **44**, 260.
Weinhouse, S. & Hirsch, E. F. (1940). *Arch. Path.* **30**, 856.
Wright, L. E. A., Robinson, A. R. & Trikojus, V. M. (1944). *Aust. J. exp. Biol. med. Sci.* **12** [-13], 209.
Yasuda, M. (1931–2). *J. biol. Chem.* **94**, 401.

The Mechanism of Selective Fermentation of *d*-Fructose from Invert Sugar by Sauternes Yeast

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It has long been known that brewer's and baker's yeasts preferentially ferment glucose from a mixture of equal parts of *d*-glucose and *d*-fructose, whereas Sauternes yeast selects *d*-fructose (for literature cf. Sobotka & Reiner, 1930*a*). As was shown previously (Gottschalk, 1943*a*), of the various *d*-fructose modifications present in a fructose solution at equilibrium, only fructofuranose is a suitable substrate for fermentation. This reactive substance, constituting approximately 22% of the total fructose concentration at 25° (Gottschalk, 1943*b*), has about twice the affinity of *d*-glucose for hexokinase (Gottschalk, 1944). These findings satisfactorily explain the selective fermentation, at room temperature, of glucose from invert sugar by brewer's and baker's yeasts as the result of the unequal distribution of hexokinase between its substrates *d*-glucose and *d*-fructofuranose, the higher concentration of the aldehyde sugar outweighing the greater affinity of fructofuranose for hexokinase (Gottschalk, 1945*a*). In conformity with this interpretation it was found possible to equalize the relative rates of fermentation by baker's yeast of *d*-glucose and of *d*-fructose from invert sugar by raising the proportion of fructofuranose without changing the total concentration of *d*-fructose (Gottschalk, 1945*b*).

The object of this communication is to investigate the factor or factors responsible for the phenomenon that Sauternes yeast in contrast to the industrial yeasts preferentially ferments *d*-fructose from invert sugar.

Materials and Methods

A pure culture of Sauternes yeast was obtained from the collection of the Institut Pasteur, Paris, through the courtesy of Professor R. H. Hopkins, Birmingham. A stock culture of champagne yeast (*Saccharomyces ellipsoideus*) was kindly supplied by the Roseworthy Agricultural College, South Australia. Both yeast strains were kept in an active condition by subculturing on grape juice-agar slopes (1·5% agar; pH 5·8). For the experiments cultures were grown at 27° in grape juice cleared and sterilized by repeated filtration through Seitz pads. After three 48 hr. passages of the culture through 10 ml., 10 ml. and 100 ml. of the medium respectively, the yeast was sown into a series of 'medicine flats' (450 ml. volume) each containing 100 ml. of medium. After incubation for 4 days the contents of the bottles were pooled, the yeast centrifuged off at 5°, washed twice with water on the centrifuge, once on the Buchner funnel and then compressed. For experiments with living yeast the cake thus obtained was stored at 4° and used within the next three days. Brewer's (ale) top yeast (*Sacch. cerevisiae*) was collected at the Carlton Brewery, Melbourne, from the vats 64 hr. after pitching and was washed and compressed as above. The dry weight of the compressed yeast is recorded in Table 3. Both the wine and