Phospholipid was the only fraction which appeared to be relatively constant in its rate of deposition.

The effect of increasing the concentration of cholesterol in the diet of male rats may be seen in Fig. 2, where data for total liver lipids and for cholesteryl esters are presented as percentage of fresh liver weight. More complete data about the composition of these livers are available in Table 2. The increases which occurred in each component were calculated (as in Table 1) and the percentage composition of the material deposited in the livers under each of the dietary regimens was calculated. These data are shown in Table 3. Again it may be seen that regardless of the amount of cholesterol in the diet, the material deposited in the liver consists principally of glycerides and of water. As might be anticipated, the deposition of cholesteryl esters increases with increasing amounts of dietary cholesterol. In these male animals the cholesteryl esters continued to rise throughout the period of observation. At the start of the experiment, the absolute amount of free cholesterol was about 10 mg. and that of cholesteryl esters about 3 mg., making a total of 13 mg. (cf. Table 2). In the case of rats consuming the diet containing 0·8 % cholesterol, the absolute amounts of free cholesterol at 21 and 90 days were 20 and 66 mg., respectively, and the corresponding values for cholesteryl esters were 209 and 1278 mg. Since the liver gained 5·53 g. in weight in the first 21 days and cholesterol plus esters account for 216 mg. of this, they represent 3·9 % of the material deposited in this interval (cf. Table 3). When rats were maintained on the same diet for a total of 90 days the cholesterol plus ester portion increased to 10·1 % of the material deposited during the period of 21–90 days. The female rats used in the first experiment did not exhibit the same increase. Actually, in female rats, the rate at which cholesteryl esters were accumulating decreased with time (after 21 days).

SUMMARY

1. Analyses have been made of the lipids accumulating in the livers of rats fed a hypolipotropic diet containing different amounts of crystalline cholesterol (0, 0·2, 0·5, 0·8 and 1·6 %, respectively) and the rates at which the different components accumulate in the liver have been determined.

2. The material deposited during the first 3 weeks consisted mainly of glycerides and of water, these two accounting for between 80 and 90 % of the increase in weight of the liver. When the cholesterol-containing diet was fed for longer periods, the nature of the material being deposited changed: the amount of glyceride decreased and that of water increased, but the sum of these two continued to account for about 80 % of the gain in liver weight.

3. Although the percentage of total lipids in the livers reached a more or less limiting value after feeding the diets for about 3 weeks, the absolute amount of glycerides and of cholesteryl esters continued to increase throughout the period of observation.

4. In the livers of rats consuming diets containing cholesterol, cholesteryl esters increased about 200-fold in absolute amount, but since the normal concentration is low, the cholesteryl esters never accounted for more than a small percentage of the total lipid material deposited.

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Preventive and Curative Studies on the 'Cholesterol Fatty Liver' of Rats

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Some preventive and curative studies of the so-called 'cholesterol fatty liver' were made almost twenty years ago. This early work has been reviewed by Best & Ridout (1939) and by McHenry & Patterson (1944). Increased knowledge of dietary requirements indicated the desirability of re-investigating this type of fatty liver in rats fed more adequate diets. The preventive effect of choline chloride and of inositol in improved rations containing graded doses of cholesterol was reported recently from this laboratory (Ridout, Lucas, Patterson & Best, 1952). These experiments revealed two points upon which further data seemed desirable: (1) the effect of a change in the nature of the dietary protein, and (2) the effect of somewhat larger doses of choline chloride. Preventive and

curative experiments under these new conditions have now been completed and the curative effects of betaine, of vitamin B_{12} and of inositol have been determined.

The protein portion of the basal diet used in the recent study (Ridout *et al.* 1952) consisted of casein (8 %) and gelatin (12 %). Although gelatin is devoid of tryptophan and almost lacking in tyrosine and cystine, the amount of casein used supplied nearly enough tryptophan for young adult rats. Gelatin contains large amounts of glycine and of proline, which some authors have claimed to be deleterious when present in excess. Half of the gelatin was therefore replaced by soybean protein in the present study. This mixture has the same methionine content as the other diet but provides a better assortment of amino acids by increasing tryptophan, threonine, phenylalanine and histidine. Biotin, folic acid, *p*-aminobenzoic acid and 2-methyl-1:4-naphthoquinone were added to the vitamins supplied previously and a new salt mixture was also adopted. These changes did not affect appreciably the general conclusions. When considerable amounts of cholesterol were present in the diet, even high concentrations of choline chloride in the ration failed to prevent a considerable accumulation of cholesteryl esters in the liver.

The main purpose of the present communication is to describe some curative experiments with lipotropic substances in rats with 'cholesterol fatty livers'. These were produced by feeding (for a preliminary period of 3 weeks) the basal diet to which 0·5 % cholesterol had been added. Cholesterol was then withdrawn from all diets. Some rats were continued on the basal ration; other groups were given different lipotropic substances for various periods of time. In the livers of rats maintained on the cholesterol-free basal diet, there was a gradual fall in cholesteryl esters but glycerides remained high throughout the experimental period of 10 weeks. At the dosage level used, supplements of choline and of betaine were about equally effective in producing a decrease in glycerides. The removal of cholesteryl esters from fatty livers was accelerated by dietary choline or betaine but was still a slow process. Vitamin B_{12} exerted a considerable lipotropic effect upon the glyceride fraction, but the reduction of cholesteryl esters was less definite. Inositol had no significant effect on either fraction.

EXPERIMENTAL

The care of the animals and analytical procedures have been described in previous publications (Ridout *et al.* 1952; Ridout, Lucas, Patterson & Best, 1954); the composition of the basal diet is given in the latter. Inositol was omitted from the 'sucrose–vitamin powder' there described.

Preventive experiment. Male white rats (250) of the Wistar strain (100–120 g. in wt.) were divided among twenty-five comparable groups. The animals, in individual cages, were fed the test diets *ad lib.* for 3 weeks.

Several groups of diets were prepared in which different amounts of crystalline cholesterol (0, 0·2, 0·8 and 1·6 %) were added to the basal ration. Choline chloride was added to certain of these diets in amounts corresponding to 0·04, 0·08, 0·16, 0·32, 0·64 and 1·28 % of choline (factor, choline chloride: choline = 1·15). Supplementary betaine hydrochloride (0·211 and 0·422 %, amounts molecularly equivalent to 0·16 and 0·32 % choline, respectively) was added to two diets containing 0·2 % cholesterol. To another diet containing the same amount of cholesterol, 0·32 % of inositol was added. Four groups were fed the test diets for 3 months; two of these groups were given the ration containing 0·2 % cholesterol with and without 0·64 % choline, respectively; the other groups were fed the ration containing 0·8 % cholesterol with and without 1·28 % choline, respectively.

Curative experiment. Male white rats (140) of the Wistar strain (125–150 g. in wt.) were fed for a preliminary period of 3 weeks, the basal diet to which 0·5 % crystalline cholesterol had been added. At the end of this time the rats were distributed at random among fourteen groups. One group was killed and the liver lipids were analysed to determine the degree of fatty deposition and the amount of cholesteryl esters present. Cholesterol was then withdrawn from the diet of the remaining groups. Four groups were continued on the basal ration for periods of 14, 28, 42 and 70 days, respectively; another four groups were fed for corresponding periods the basal diet to which 0·368 % of choline chloride had been added. An equimolecular amount of betaine hydrochloride (0·422 %) was added to the diet offered to two other groups for 14 and 42 days, respectively. The three remaining groups were fed, for 42 days, diets containing 0·32 % inositol, choline (0·32 %) plus inositol (0·32 %), and 10 μg. of vitamin B_{12}/100 g., respectively.

RESULTS

A comparative study of the effectiveness of the different lipotropic agents was made in rats given the diet containing 0·2 % cholesterol (Fig. 1). Choline and betaine were almost equally effective in preventing the accumulation of total lipids (glycerides), while inositol had no effect. Choline at lower dosages was less effective in preventing the deposition of abnormal amounts of cholesteryl esters; e.g. with 0·08 % of dietary choline the esters accumulated to 5 times the normal concentration, although total lipids were maintained at essentially normal values. A larger dose of choline (0·64 %) did maintain the esters within the normal range at this intake of cholesterol (see Fig. 2). Under these dietary conditions supplementary betaine at moderate dosage (0·32 %) appeared to be as effective as choline, but at lower dosage (0·16 %) it seemed less effective in preventing the accumulation of cholesteryl esters. A proper comparison of their relative lipotropic activity can only be made by constructing more complete dose/response curves. Data are available which indicate that under some conditions betaine is much less effective than choline (Best, Lucas, Ridout & Patterson, 1950).

The main purpose of this preventive experiment was to study the effects of larger doses of choline on the glycerides and cholesteryl esters in the livers of rats ingesting cholesterol-rich diets. Choline is markedly effective in preventing the accumulation of glycerides at all intakes of cholesterol (Fig. 2*a*). In contrast, large doses of choline are unable to prevent a considerable accumulation of cholesteryl esters in the liver when the diet contains excessive amounts of cholesterol (0·8 or 1·6 %). This does not mean that choline has no lipotropic effect on the esters: rats consuming the diet containing 1·6 % cholesterol had liver cholesteryl esters of 17·4 % (on a dry, fat-free basis); these are reduced to 9·3 % by choline. Even prolonged consumption of choline-rich diets does not prevent the deposition of excessive amounts of cholesteryl esters in the liver. For example, when rats were fed a diet containing 0·8 % cholesterol for 3 months, the esters reached 49 % dry, fat-free liver (5·9 % of fresh liver); with 1·28 % choline present in the ration there was a great reduction, although the ester content was still high, viz. 11 % (2·6 % fresh liver). Normal values are about 0·5 % dry, fat-free liver (about 0·1 % fresh liver weight).

Curative experiment. The nature of the lipotropic supplements and their effectiveness in curing the 'cholesterol fatty liver' are presented in Table 1. The lipid components are given as absolute weights (mg. per liver), together with the average fresh liver weights and the average dry, fat-free weights of the liver for each group so that the percentage of each component may be calculated on either basis. The effect on total liver lipids of removing cholesterol from the diet is shown in Fig. 3*a* as percentage of fresh liver weight, and the effect on cholesteryl esters (expressed as percentage dry, fat-free tissue) is shown in Fig. 3*b*. The relative effectiveness of the several lipotropic agents on total lipids and cholesteryl esters may be seen in these figures.

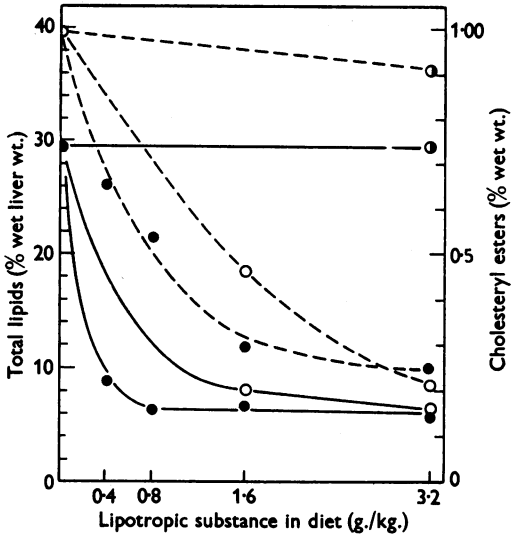

Fig. 1. Comparison of activity of lipotropic agents in diet containing 0·2 % cholesterol. Groups of ten rats fed rations for 3 weeks. Total lipids (—), cholesteryl esters (– – –), choline (●), betaine (○), inositol (◑).

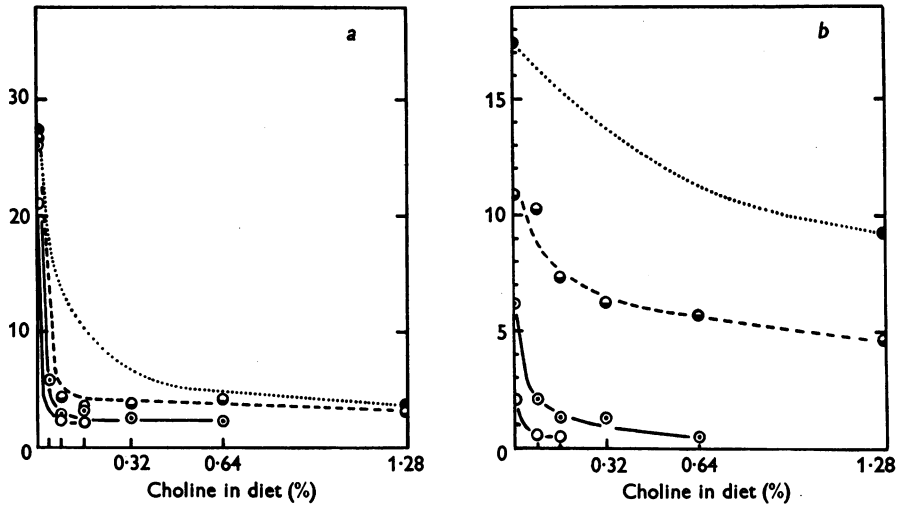

Fig. 2. Effect of choline on liver lipids of rats consuming diets containing different amounts of cholesterol. Curves in left-hand figure show glycerides as % fresh liver when diet contains no cholesterol (○), 0·2 % (◉), 0·8 % (◒) and 1·6 % (●) cholesterol. Curves in right-hand figure show cholesteryl esters as % dry, fat-free liver under corresponding conditions.

Table 1. *Curative effects of lipotropic supplements in rats with 'cholesterol fatty livers'*

Basal diet supplemented with 0·5% cholesterol fed for a preliminary period of 3 weeks to groups of ten male rats 125–150 g. Average daily intake of cholesterol-free diet during curative period, 10–11 g.

Group no.	Supplements	Period on curative diet (days)	Mean liver weights		Composition of liver lipids (mg./liver)			
			Wet (g.)	Fat-free dry (g.)	Total lipids	Cholesterol		
						Free	Total	Ester†	Glyceride
0	None (basal)	0	10·7±0·7	1·47±0·07	3872±362	22±1·2	203±25	305±41	3303±336
1	—	14	15·1±0·8	1·74±0·08	6261±429	31±2·0	239±23	351±35	5576±388
2	—	28	15·1±0·7	1·87±0·06	6026±436	30±1·2	203±11	292±19	5373±431
3	—	42	18·1±1·0	2·19±0·09	7449±547	35±2·1	182±19	248±29	6817±513
4	—	70	16·2±0·7	2·03±0·06	6384±531	33±1·3	153± 9	203±14	5814±520
5	Choline (0·32%)*	14	10·1±0·7	1·64±0·08	2695±295	23±1·4	192±20	284±32	2109±255
6	—	28	8·6±0·4	1·61±0·08	1403±149	19±1·0	110±12	154±19	960±129
7	—	42	7·8±0·4	1·56±0·09	832± 99	17±0·8	66± 9	83±16	470± 86
8	—	70	6·3±0·3	1·36±0·06	452± 41	14±0·7	21± 2	13± 3	192± 31
9	Inositol (0·32%)	42	15·5±0·7	1·97±0·05	5693±441	29±1·4	152±12	207±20	5116±421
10	Choline (0·32%)*+inositol (0·32%)	42	7·9±0·5	1·62±0·10	579± 85	16±1·2	64± 7	80±10	210± 55
11	Betaine (0·32%)*	14	11·1±0·5	1·72±0·08	3132±238	27±1·7	189±24	272±39	2528±195
12	—	42	10·1±0·7	1·89±0·11	1096±118	19±0·9	72±10	89±16	672± 99
13	Vitamin B_{12} (10 μg.)	42	11·3±0·3	2·03±0·04	2207±140	26±0·9	136±12	186±20	1619±134

* Choline was supplied as chloride (0·368%) and betaine as a molecularly equivalent amount of hydrochloride (0·422%).

† Cholesteryl esters were calculated as oleate by multiplying bound cholesterol by 1·68.

The curves in Fig. 3, however, do not give all the information which is necessary to evaluate properly the relative lipotropic effectiveness of choline, betaine, inositol and vitamin B_{12}. As is shown in Table 1, the livers of the rats fed the basal diet continue to increase in size for 6 weeks after cholesterol is removed from the ration; although the amount of cholesteryl esters decreases, the glycerides (and total lipids) continue to increase. The fact that total lipids and liver weight are increasing in the same proportion gives the erroneous impression (Fig. 3*a*) that in rats on the basal diet no change in total lipids occurs after about 2 weeks.

Although it may seem in Fig. 3 that inositol has failed to exert any significant curative action on either total lipids or cholesteryl esters, the livers are slightly smaller and the absolute weights of these components are reduced proportionately. Vitamin B_{12} had a considerable lipotropic effect: the size of the livers was greatly reduced as well as the percentage of total lipids. The action of vitamin B_{12} was mainly on the glycerides (reduced at 42 days from 6817 mg. per liver in the basal group to 1619 mg.). Some removal of cholesteryl esters from the liver was noted, but the effect of vitamin B_{12} on this fraction was less pronounced. Supplements of choline, of betaine, and of choline plus inositol were about equally effective in hastening the removal of total lipids. Over 6000 mg. of glycerides and about 160 mg. of cholesteryl esters had been removed by each of these supplements at 42 days. Restoration of normal composition is a very slow process, however, for even after a period of 6 weeks on these curative diets, the percentage of glycerides still present in the livers is about twice normal (5·9% fresh liver weight compared with the normal range of 2–4%) and cholesteryl esters are nearly 8 times normal (0·79% compared with the normal range of 0·07–0·14%). The only rats that were kept on a curative diet for as long as 70 days were those receiving the supplement of choline (group 8); in these the percentage of glycerides in the liver was back to normal. However, even after 10 weeks on this curative ration, cholesteryl esters were still slightly above normal (0·20% of fresh liver weight).

DISCUSSION

Lipotropic findings obtained in animals suffering from multiple dietary deficiencies are difficult to interpret. As we have pointed out elsewhere (Best, Lucas, Ridout & Patterson, 1950) the ideal ration for the study of lipotropic phenomena should be complete in every respect, except for the lipotropic agents, which should be absent. Methionine is an essential amino acid and cannot be omitted from the diet, but the lowest concentration of methionine compatible with the general well-being of the rats

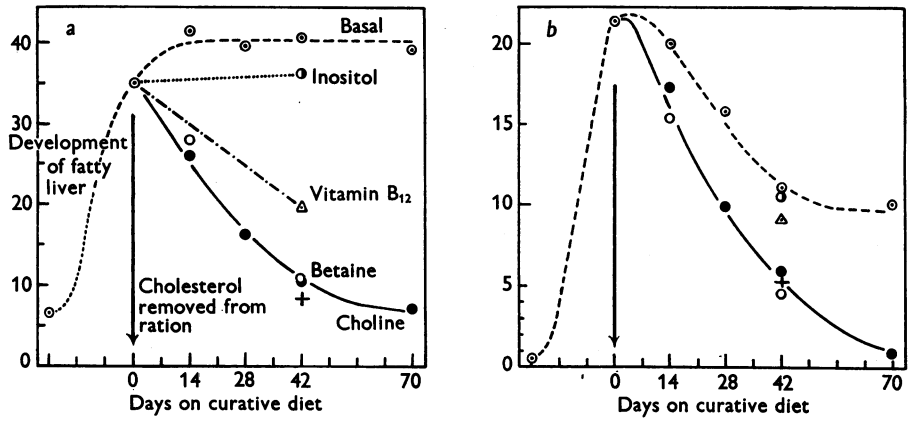

Fig. 3. Development and cure of 'cholesterol fatty liver'. Fatty liver produced by feeding diet containing 0·5% cholesterol for 3 weeks. Groups of ten rats fed curative diets (cholesterol-free) for periods shown. Curves in left-hand figure depict total lipids as % fresh liver in rats continued on hypolipotropic basal diet (⊙), and in rats on other diets containing supplementary inositol 0·32% (◑), vitamin B_{12} 10 μg./100 g. (△), betaine 0·32% (○), choline 0·32% (●) and choline 0·32% plus inositol 0·32% (+). Curves on the right-hand side give corresponding data for cholesteryl esters expressed as % dry, fat-free liver.

has been sought, and all the known essential dietary factors for rats have been included in the rations adopted for these studies. The improved supply of essential amino acids, minerals and vitamins in this more complete ration permitted moderate gains in weight (1–1·5 g./day) where the former diet barely maintained weight. No greater improvement in growth was expected as methionine, which totalled 360 mg./100 g. in both rations, appeared to be one of the limiting amino acids in both diets. The animals on the improved ration seemed healthier, appearing well groomed, bright-eyed and lively.

Even large intakes of choline are unable in preventive studies to maintain liver cholesteryl esters in the normal range when the cholesterol intake is excessive. While this may appear to support the contention that the presence of cholesterol in a diet is deleterious and to be avoided, it is a matter of clinical experience that at reasonable intakes of cholesterol the amounts of lipotropic agents (choline, betaine, methionine and vitamin B_{12}) present in usual mixed rations are adequate to maintain the liver lipids within the normal range (see also Ridout *et al.* 1952).

The impression obtained in these present experiments, that betaine is just as effective as choline in both preventive and curative studies, is probably an artifact due to the lack of data in a suitable dosage range. Under other conditions where data were available to construct a complete dose/response curve, betaine was distinctly less active than choline when the comparison was made, as it should be, on the steep part of the response curves (Best *et al.* 1950).

The first attempt to use choline in the cure of a cholesterol fatty liver was made by Channon & Wilkinson (1934). In the 12-day test period adopted, no beneficial effect of choline was noted. By prolonging the curative period to 41 days, Best & Ridout (1935, 1936) established that choline does accelerate the removal of cholesteryl esters from the liver. They showed that the primary effect of choline is on the glycerides and that the removal of cholesteryl esters is a much slower process. The present paper contains more complete data on the rate of removal of the lipid components under the influence of choline and supplies new information on the curative effects of betaine, of vitamin B_{12} and of inositol. The findings are clear-cut and require no further comment.

The role of vitamin B_{12} in lipotropic phenomena is still not clearly defined. The protective effect of vitamin B_{12} on the renal lesions in young rats described by Schaefer, Salmon & Strength (1949) (see also Strength, Shaefer & Salmon, 1951) has been seen in our laboratory, but in other rats on similar diets with a lower lipotropic background no beneficial effect whatever from vitamin B_{12} was observed (Best, Lucas, Patterson & Ridout, 1953; cf. Stekol & Weiss, 1950). Similarly, in older rats, others have demonstrated that by altering the dietary background one may either obtain or fail to elicit a lipotropic effect with vitamin B_{12} (Drill & McCormick, 1949; McCormick & Drill, 1950; György & Rose, 1950; György, 1951; Bennett, Joralemon & Halpern, 1951). Until more is known about the relationship of vitamin B_{12} to the biosynthesis of labile methyl groups and to the *in*

vivo formation of choline, it is unwise to advance reasons for the inconsistent findings. In the present curative study, the liptropic effect of vitamin B_{12} was very distinct, at least on the glyceride fraction; the effect on cholesteryl esters was less definite.

SUMMARY

1. Crystalline cholesterol was added at different concentrations (0·2, 0·4, 0·8 and 1·6 %) to a hypolipotropic diet. The livers of rats fed these rations and of other rats fed similar rations containing various lipotropic supplements were analysed for lipid content.

2. At moderate intakes of cholesterol (0·2 %) sufficient dietary choline or betaine were able to prevent the excessive deposition of both glycerides and cholesteryl esters.

3. In rats consuming diets rich in cholesterol, even considerable concentrations of choline (1·28 %) in the diet failed to prevent an excessive accumulation of cholesteryl esters in the livers.

4. In a curative experiment, betaine hydrochloride at a concentration of 0·42 % in the diet was about as active as an equimolecular amount of choline in hastening the removal of glycerides and cholesteryl esters from 'cholesterol fatty livers'. The limited data suggest that had the effect of lower dosages been compared, betaine would have been found to be less effective than choline.

5. Vitamin B_{12} exerted a considerable curative lipotropic action on the glycerides but much less on the cholesteryl ester fraction of liver lipids.

6. Inositol was without any appreciable lipotropic effect on either fraction in either type of study.

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Effects of Lipotropic Substances on the Cholesterol Content of the Serum of Rats

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Some investigators believe that there is a causal relationship between atherosclerosis and the level of serum cholesterol. The well-known effect of lipotropic agents upon lipid deposits in the liver has suggested to many workers that these substances might be useful in treating or preventing the lesions in the blood vessels. The literature concerning the effects of lipotropic substances on serum cholesterol is very inconsistent. This prompted us to determine free and total cholesterol in the sera of certain rats used in the lipotropic study reported in the preceding paper (Ridout, Lucas, Patterson & Best, 1954).

Some of the discrepancies in the literature may be related to our observation that under certain conditions, ingestion of diets rich in cholesterol leads to a marked postprandial elevation in the bound serum cholesterol when the food contains choline. Our findings indicate that the same substances that prevent or cure fatty livers are also concerned in the