XLII. PROTEIN AND THE DIETARY PRODUCTION OF FATTY LIVERS.

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THE observation by Best *et al.* [1932] that fatty livers could be produced in rats which received a diet consisting of mixed grain with 40 % of beef fat provided a means whereby the curious action of choline in preventing and curing such dietary fatty livers has been extensively studied [Best and Huntsman, 1935]. On a number of occasions however use of this diet has failed to produce in our rats fatty livers of an intensity similar to that recorded by the Toronto workers, while its deficient nature may cause weight losses in the experimental animals. Since the amount of liver fat is changed by starvation, it may also be influenced by this type of undernutrition and complicating factors in the interpretation of results may thereby be introduced.

A governing factor in fatty liver production on diets containing 40 % of fat has been shown to be the choline content [Best and Huntsman, 1935]. With this finding in mind an attempt was next made to produce dietary fatty livers in rats under conditions in which no loss of body weight occurred. For this purpose a diet of caseinogen 20, starch 30, beef fat 40, salt mixture 5, marmite 5 and vitamins A and D was given. Both the caseinogen and starch had been exhaustively extracted by alcohol and ether and save for the small amount of choline present in the marmite—1.75 mg. per rat per day—this diet was choline-free. Fatty livers did not result. This amount of choline is of no importance since the mixed grain diet of Best, Hershey and Huntsman [1932] which causes fatty livers in rats contains enough choline to give each animal about 8 mg. per day. This failure suggested that some factor other than the choline content was influencing the production of fatty livers on diets containing 40 % of fat, and consideration of various results in the literature suggested the level of protein intake as the most probable. In this connection the wide variations in the relative amounts of glyceride and cholesteryl ester appearing in the "cholesterol" fatty liver caused by feeding diets containing 1–2 % of cholesterol were considered. Calculation of the approximate compositions of the diets used by different workers [Okey, 1933, 1, 2; Chanutin and Ludewig, 1933; Best *et al.*, 1934; Channon and Wilkinson, 1934] indicated that the protein content of the diets might be a factor in the control of the amount of liver glyceride. Accordingly the effect of varying the protein content of the diet at the expense of the carbohydrate, with a constant fat content (40 %) and an adequate supply of vitamins and salts, has been studied. The object was to evolve a diet which would cause fatty livers without loss of body weight if possible and to study the part played by protein in fatty liver production. Further, in order to study any effect of protein in controlling the fatty liver produced by feeding rats on pure sucrose [Best and Huntsman, 1935], two further diets were included; these were fat-free and contained 5 and 30 % of protein respectively with 85 and 60 % of glucose hydrate. All the diets used have been substantially choline-free.

These experiments have been used at the same time to attempt to elucidate a further point in fatty liver production. Great variations in the percentage of liver fat among individual rats under the same fatty liver-producing dietary regime often occur and the extent of these variations can be readily seen by reference to the paper of Best and Huntsman [1935] where variations from 5.5 to 26.6 % among individuals from one experiment are recorded. This degree of variation must have some significance. The frequency of its occurrence is such as to make the use of large numbers of animals necessary, apart from rendering interpretation of results somewhat uncertain. The degree of fat infiltration occurring in the liver in starvation in rats and rabbits has been shown to be related to the amount of fat present in the depôts [Dible, 1932; Dible and Libman, 1934]. It was possible that the variations in the amount of fat in the fatty livers of rats on a high fat diet were related to the amount of depôt fat. This has been investigated by a study of the fat content of the liver and carcass of each individual animal in these experiments.

EXPERIMENTAL.

The general plan of the experiment was to feed groups of rats on various diets for 21 days. The animals were then killed by a blow on the head 12 hours after the last meal. The livers were removed and weighed for analysis, the gastro-intestinal tracts removed and the carcasses stored in a refrigerator until they could be analysed.

The diets used are described in Table I. The caseinogen was B.D.H. "fat-free casein" which had been further extracted three times with boiling industrial alcohol. The choline content of the constituents of the diets was determined by assay on the isolated rabbit intestine after hydrolysis and acetylation. Save for the marmite, the constituents were choline-free. The marmite contained 350 mg. of choline per 100 g. and since it constituted 5 % of all the diets, each animal in the different groups ingested about 1.5 mg. of choline per day. The average actual amounts of choline ingested by the animals in each group are recorded in Table II.

Table I. *Description of the diets.*

Group ... Diet	A	B	C	D	E	F	G	H
Caseinogen	0	5	10	20	30	50	5	30
Beef dripping	40	40	40	40	40	40	0	0
Glucose hydrate	50	45	40	30	20	0	85	60
Marmite	5	5	5	5	5	5	5	5
Salt mixture	5	5	5	5	5	5	5	5
Cod-liver oil								

One drop per rat every 3 days

The fat content of the individual livers was determined by ethereal extraction after acidification of the livers following saponification with aqueous NaOH. After evaporation to dryness of the washed ethereal extract the weight of the "fat" was obtained. Separation of this material into its constituent fatty acids and unsaponifiable matter was not carried out because experiments showed that the combined weights of unsaponifiable matter and fatty acids obtained by further alcoholic saponification were in all cases 98 % of that of the original material. The fat content of the carcasses was determined either by a similar method or by saponification of the alcoholic extract of the minced carcass. In the latter cases the minced carcasses were treated many times with successive volumes of boiling alcohol and the combined extracts saponified and treated as already described.

Results.

In Table II are recorded data concerning the number of rats used in each group, their changes in weight during the experiment, the average food intake per rat per day, the caloric value of this food and its choline content.

Table II. *Weight records of the animals and the food intake.*

Group	...	...	A	B	C	D	E	F	G	H
Protein % of diet	...	...	0	5	10	20	30	50	5	30
No. of animals	...	...	6	6	6	6	6	5	6	6
Av. weight at beginning of experiment (g.)			141	143	141	136	139	148	141	136
Av. weight at end of experiment (g.)			122	147	153	179	182	186	140	146
Gain or loss (g.)			-19	+4	+12	+43	+43	+38	-1	+10
Av. food intake per rat per day (g.)			8.3	9.2	8.0	9.0	9.0	8.5	11.7	13.5
Caloric intake per rat per day (Cals.)			48.0	53.4	46.4	52.5	52.5	50.6	44.5	51.1
Choline intake per rat per day (mg.)			1.46	1.70	1.42	1.59	1.59	1.50	2.07	2.38

Diets A to F contained 40 % fat; G and H were fat-free.

In Table III the percentages of "fat" (total fatty acids and unsaponifiable matter) present in the livers and the carcasses of the individual animals are set out.

Table III. *The percentage of "fat" in the livers and carcasses.*

		(g./100 g.)							
Group	...	A		B		C		D	
		liver	carcass	liver	carcass	liver	carcass	liver	carcass
		9.93	10.30	22.83	16.13	6.74	17.12	6.55	—
		7.35	8.92	8.53	19.20	10.35	18.94	4.48	12.04
		7.59	17.44	15.83	18.00	7.19	14.21	7.91	12.27
		8.55	11.25	7.68	20.16	6.68	13.54	4.94	—
		10.54	15.08	7.67	16.16	7.70	—	7.41	—
		9.71	11.84	12.40	15.94	5.78	—	7.75	16.78
Mean		8.95	12.40	12.49	17.60	7.35	—	6.50	—
Group	...	E		F		G		H	
		liver	carcass	liver	carcass	liver	carcass	liver	carcass
		4.54	—	—	—	3.07	7.81	4.79	17.06
		6.05	15.39	4.21	11.14	3.42	8.42	4.46	8.13
		6.73	19.15	5.56	15.61	3.57	7.16	4.20	16.57
		6.21	—	6.18	15.57	5.01	9.74	4.84	14.16
		7.01	14.34	6.67	17.60	6.88	11.64	4.67	—
		6.20	23.02	5.40	17.73	6.01	13.94	4.85	4.68
Mean		6.12	—	5.60	15.53	4.66	9.78	4.64	—

DISCUSSION.

1. *Diets (B-F) containing 40 % of fat with varying percentages of protein.* The wide variations in the percentages of fat in the individual livers are seen in Table III. For discussion purposes the mean values are recorded in that table, although the limitations caused by the relatively small numbers of animals are recognised. The average figures for the "fat" content of the livers of the animals receiving 5, 10, 20, 30 and 50 % of protein are 12.49, 7.35, 6.50, 6.12

and 5.60 % respectively. These diets were substantially choline-free, for the choline intake of each rat in the different groups varied only between 1.42 and 1.70 mg. per day. As mentioned in the introduction, this amount of choline is considered to be of no significance in the interpretation of the results and further, since it is virtually constant at 1.5 mg. per rat per day, any possible effect is the same for all the groups. It is clear therefore that the amount of protein in the diet is a factor controlling the amount of fat appearing in the liver irrespective of any effect of choline, the degree of fat infiltration increasing with decreasing protein content. The 12.49 % of fat in the livers of animals which received 5 % of protein is similar to the figure often recorded by the Toronto workers as occurring in rats receiving the mixed grain diet with 40 % of fat. Such a diet would on our calculations contain 6 % of protein. The decreasing amount of fat appearing in the liver with increasing protein intake explains also our previous failures to produce fatty livers in rats on diets of high fat content but choline-free, because in all these cases protein constituted at least 20 % of the diet. These investigations further provide a method for producing fatty livers in rats by dietary means on diets free from choline without the possibility of complications due to weight loss. Thus the diet containing 5 % of protein and 40 % of fat resulted in the livers of the animals containing 12.49 % of fat at the end of 3 weeks with an average weight increase of 4 g. per animal.

This control of the glyceride content of the liver by the protein content of the diet has been further confirmed in experiments on the "cholesterol" fatty liver. We administered to one group of rats a diet containing caseinogen 5, fat 20, glucose 65, marmite 5, salt mixture 5, vitamins A and D, with 2 % cholesterol. To a second group the same diet was given save that the protein content was raised to 30 % at the expense of the sugar, which was reduced to 40 %. After 3 weeks the animals were killed and their livers analysed. The results obtained are recorded in Table IV.

Table IV. *The liver lipoids of rats fed on a diet containing 20 % fat and 2 % cholesterol, but free from choline.*

	(g./100 g. liver.)	
	Group J	Group K
Percentage protein in diet	5	30
Phosphatide	1.79	2.54
Glyceride	25.42	5.54
Free cholesterol	0.294	0.452
Cholesteryl oleate	4.42	7.91

The figures in Table IV appear amply to confirm the finding of the control of glyceride deposition in the liver by the level of the protein intake, for whereas the glyceride content of the livers of animals receiving 5 % of protein (group J) is 25.42 %, that of group K, which received 30 % of protein, is only 5.54 %. It is of interest to note that the amount of cholesteryl esters is some 80 % higher in group K, compared with group J, but at this stage no interpretation of this finding can be provided.

These results show therefore that in the "cholesterol" fatty liver the relative amounts of glyceride and cholesteryl esters depend in the case of choline-free diets on the protein content of the diet, the glyceride fraction being far more extensively involved and in the reverse direction. They explain the varying degree to which these two constituents occur in the livers of rats fed on diets containing cholesterol as reported by different workers, a question which has

been discussed in a previous paper [Channon and Wilkinson, 1934]. A number of other points arise from consideration of Table IV and other data concerning this experiment: (a) The total fatty acid content of the livers of group J (protein 5 %, fat 20 % with cholesterol 2 %) is 27.5 %. On the other hand that of group B in Table III (protein 5 %, fat 40 % without added cholesterol) is 12.49 %, a figure half as great. This increase does not seem to be related in any way to the cholesteryl ester content of the livers, because the livers of group K in Table IV contained 80 % more cholesteryl esters than those of group J. (b) In group J the phosphatide percentage, 1.79 %, is the lowest which we have yet encountered in any group of fatty livers, just as the "fat" content is the highest. (c) The liver weights expressed as a percentage of the body weights were, in group J 5.55 % and in group K 3.54 %; the former figure again is the highest we have encountered and is to be compared with the normal figure of about 3 %. (d) The normal iodine value of rat liver fatty acids is about 115. Those of group J (total liver fatty acids 27.5 %) had an iodine value of 78.7, while in group K (total liver fatty acids 10.52 %) the iodine value was 80.7. These figures illustrate the fact that under the conditions of these experiments the iodine value does not fall in proportion to the degree of fat infiltration. Thus in group J where the livers contained about nine times the normal amount of fatty acids, the iodine value has fallen only from 115 to 78.7, while in group K (liver acids three times the normal amount) the iodine value was 80.7. Admixture of one part of the original liver acids (i. v. 115) with 8 parts of those of the food (i. v. 40) would yield a mixture of acids having iodine value 48. Clearly therefore the liver acids of group J have not been derived by direct absorption of the mixed food acids. Further they contained 29.5% of solid acids of iodine value 13 (Twitchell) a percentage differing little from the normal. Whether the unsaturated liver acids, which in group J amount to 1.58 g. per animal, of iodine value 106, have been derived by preferential absorption of the unsaturated acids of the food or *dépôt* fats, cannot be determined. More detailed evidence along these lines may however throw light on any possible desaturation process.

The general finding that the protein content of the diet controls the degree of glyceride infiltration of the liver may have a number of explanations, of which mention will be made of one only. In view of the known action of choline and of betaine in preventing fat infiltration [Best and Huntsman, 1932] it may imply that certain amino-acids may be converted into these substances in the tissues. Best and Huntsman [1935] draw attention to the fact that Engeland [1909] suggested that betaines might arise in the tissues by methylation of amino-acids. It is possible that a number of other compounds of types similar to betaine and choline, which might have physiological actions in controlling the liver fat level, may arise from different amino-acids, although at the present time choline and betaine themselves are alone known to be active. Thus choline itself might be derived from glycine, as was suggested by Rosenfeld [1930] as a result of his study of the synthesis of lecithin by chickens. Another possible precursor of choline might be serine. It is of interest however that the protein used in our experiments, caseinogen, contains very little glycine or serine. We are therefore continuing these studies by producing fatty livers in rats on a diet containing 5 % caseinogen and are adding to the diets supplements of these and other amino-acids. In a previous paper [Aylward *et al.*, 1935] studies were made of the changes in the lipoids of the livers of rats following a fat- and cholesterol-containing meal free from and with added choline. The diets used contained 20 % of caseinogen and the decrease in liver phosphatide at the seventh hour in

the livers of the animals receiving the choline-free diet was much greater than on the diet which contained choline. If this finding be confirmed, it would imply that the action of protein in preventing fat infiltration was much slower than that of choline itself and may suggest that the conversion of some precursor into a substance having the same action as choline is being relatively slowly effected.

A further point of interest is that although the percentage of liver fat decreases with increase of the protein percentage in the diet, even in group F (protein 50 %) it is still 5.66 %, a figure which is to be regarded as about 1 % higher than the normal.

It will be observed from Table III that in the animals of group A, which received no protein in the diet, the livers contained 8.95 % of fat, *i.e.* substantially less than did those of rats which received 5 % of protein. We do not propose to discuss this figure because the weight losses of the animals, an average of 19 g. in 21 days, are too great to permit of any deductions being made with certainty.

With the results recorded in Table III are to be compared those of Best *et al.* [1935]. These workers obtained fatty livers in rats receiving a diet of 15 % of protein and 20 % of fat for 21 days (average figure 10.5 %). According to our results such a diet should not produce fatty livers, for the figures in Table III suggest that, if 15 % of protein were present in the diet with as much as 40 % of fat, the fat content of the liver would be about 7 %, whereas the fat content of their diet was only 20 %. It is not unlikely that these discrepant findings are to be ascribed to the weight losses of the animals of Best *et al.*, which were 9 g. per animal in a period of 21 days, the diet used being deficient in vitamins. The experiments of Best and Ridout [1933] in which rats received a grain diet with 20 % of fat did not result in fatty livers. Since the protein content of this diet would be only 8 %, it might have been expected from our data that fatty livers should have resulted, even though the fat content of the diet was only 20 %. These particular results cannot be compared with our own, however, because the average weight loss of the animals was 23 g. in 31 days, while the diet was such that each animal received about 8 mg. of choline per day, facts which complicate any comparison.

2. *Diets (G-H) containing no fat with 5 % and 30 % of protein.* No difference in the fat contents of the livers of these groups occurred, nor did fatty livers result, the average value being 4.65 %. From the point of view of future experiments it should be pointed out that the individual variation among the livers of the animals which received 30 % of protein was very much less than in any of the groups in this experiment. This suggests such a diet to be very suitable as a preparatory one for ensuring the greatest possible constancy of the fat content of the liver before further dietary treatment.

Best and Huntsman [1935] found that normal animals receiving exclusively sucrose developed fatty livers. The present results show that a diet of 5 % protein and glucose does not cause fatty livers. We think the explanation of these differences is again to be found in complicating factors of weight loss, for whereas our animals had an average weight loss of 1 g. in 21 days, the rate of weight loss of those of Best and Huntsman was rapid.

3. *The relationship between the amount of depôt fat and the amount of liver fat.* The figures recorded in Table III show that no relationship exists between the amount of depôt fat and that of liver fat. If the ratios of the percentage of the carcass fat to that of the liver fat be calculated for the animals which received a 40 % fat diet with 0, 5, 10, 30 and 50 % of protein, the average figures re-

sulting are 1.38, 1.40, 2.17, 2.94 and 2.77; if the individuals of one particular group are considered (group B, 40 % fat, 5 % protein) the ratios of carcass fat to liver fat are 0.70, 2.25, 1.14, 2.62, 2.11, 1.28. It is clear therefore that these results provide no evidence for the view that fat infiltration in the liver under these particular dietary conditions is influenced by the amount of depôt fat, and in this they are to be contrasted with the starvation experiments referred to in the introduction. This question of individual variation must be of significance and needs further investigation.

SUMMARY.

1. Groups of rats have been fed on complete diets containing 40 % fat with varying amounts of protein (from 5 to 50 %) for a period of 3 weeks and their livers and carcasses analysed for fat content. The diets were substantially choline-free, but each rat actually received 1.5 mg. of choline per day, which was present in the yeast extract used to supply vitamin B complex.

2. The amount of fat appearing in the liver was conditioned by the amount of protein in the diet irrespective of any action of choline.

3. The diet containing 40 % of fat and 5 % of protein is adequate for weight maintenance and for the production of livers containing 12.49 % of fat in 3 weeks.

4. This control of the glyceride content of the "fat" fatty liver has been confirmed by experiments on the production of the "cholesterol" fatty liver, for livers of animals receiving a diet containing 5 % of protein, 20 % of fat and 2 % of cholesterol contained at the end of 3 weeks 25.42 % glyceride and 4.42 % cholesteryl esters. On the other hand the livers of another group receiving the same amounts of fat and cholesterol but with 30 % protein, contained 5.54 % glyceride and 7.91 % cholesteryl esters.

5. The possibility of the action of the protein in controlling liver fat being due to its providing amino-acid precursors which are converted either into choline or betaine or substances of similar physiological action is discussed.

6. No relationship exists between the amount of fat infiltration in the liver and the amount of the depôt fat.

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