105. THE ACTION OF SULPHUR-CONTAINING AMINO-ACIDS AND PROTEINS ON LIVER FAT DEPOSITION

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AN investigation of the effect of pure amino-acids on the degree of fatty infiltration into the livers of rats maintained on high-fat, choline-free diets was a natural extension of the observation of the lipotropic action of proteins [Channon & Wilkinson, 1935; Best *et al.* 1936; Channon, Loach *et al.* 1938]. The discovery of the effect of cystine in causing marked acceleration of liver fat deposition [Beeston & Channon, 1936; Tucker & Eckstein, 1937] and of the contrasting finding that methionine prevented fat deposition to a considerable extent [Tucker & Eckstein, 1937; Channon, Manifold & Platt, 1938, 1; Best & Ridout, 1940] were the firstfruits of this method of approach. At the present time nothing is known regarding the means whereby these two amino-acids exert their opposing effects on fat deposition; in the case of methionine, however, it may be assumed as a working hypothesis that this amino-acid is involved in choline synthesis. In this connexion, interesting observations have been made by du Vigneaud *et al.* [1939], who found that supplements of homocystine were without effect on the growth of young rats receiving a diet deficient in methionine, but that the further addition of choline resulted in good growth; in contrast, the addition of triethylcholine had no effect and, since Channon & Smith [1936] found this latter compound to possess lipotropic activity, it must be concluded that the action of choline in these growth experiments is not to be associated with its effect in preventing liver fat deposition. These results suggest that choline can supply methyl groups for the conversion of homocystine into methionine for growth-promoting purposes. Du Vigneaud *et al.* made the further tentative suggestion that the action of methionine on liver fat might be due to its provision of methyl groups for choline synthesis. Such a suggestion is not improbable, for on present evidence the demethylation of methionine to homocystine is a likely initial step in the catabolism of this amino-acid. In a previous paper, an endeavour was made to make a quantitative estimate of the action of methionine in terms of choline [Channon, Manifold & Platt, 1938, 1], and the present paper records the results of further studies designed to throw light on its mode of action; evidence on this question has also been sought by the use of *S*-methylcystine and homocystine. In the case of the contrasting action of cystine in increasing fat deposition, no simple theory suggests itself, and preliminary experiments on the level of blood fatty acids in animals receiving high fat diets with and without cystine were carried out to see if the accelerating action of cystine on liver fat deposition could be correlated with blood fat changes.

Since methionine exerts a preventive action on fat deposition in the liver, the further question arises as to whether the action of proteins in this respect is

solely a function of their methionine contents. This question is not easy to study because, as already pointed out, one at least of the amino-acids contained in proteins, namely cystine, markedly increases fat deposition. The behaviour of any protein in affecting fat deposition might therefore be due to the net result of the actions of these two amino-acids, a suggestion first made by Tucker & Eckstein [1937]. Study of the cystine and methionine contents of proteins in relation to their lipotropic actions will not confirm the accuracy of this suggestion unless quantitative information is available concerning both the relationship of the amount of each amino-acid ingested to the effect on liver fat and the effect of adding mixtures of the two amino-acids to fatty-liver diets. Experiments to be reported here, in which varying amounts of cystine and methionine were added to fatty-liver-producing diets, were designed to throw light on this point.

EXPERIMENTAL

In all the experiments groups of 10 rats of 150–200 g. weight were used; the groups had the same average body weight at the commencement and in the different experiments contained animals either of one sex or of the two sexes evenly divided. Experiments were carried out both on the “fat” and on the “cholesterol” fatty liver. The basal diets used in producing the “fat” fatty liver followed the general principles laid down by Channon & Wilkinson [1935] and were of the type: caseinogen 8, beef dripping 40, glucose 46, salt mixture 5, cod liver oil 1 part and aneurin 10 μ g. per rat per day. This diet varied from experiment to experiment only in the proportion and nature of the protein, the glucose being varied at the expense of the protein. In discussing the separate experiments we shall refer to the diets by their protein contents, e.g. 8% egg albumin diet. In Exp. 1 the “cholesterol” fatty liver was studied, the diet being caseinogen 8, beef dripping 20, glucose 64, salt mixture 5, cholesterol 2, cod liver oil 1 part with 10 μ g. aneurin per rat per day.

Except in Exp. 1 the individual livers were analysed by the usual saponification method, while in the “cholesterol” fatty liver study, pooled minced livers were extracted with alcohol and ether and the ether-soluble material analysed [Channon, Manifold & Platt, 1938, 1]. Total blood fatty acids were determined by the method of Stoddart & Drury [1929] as modified by Stewart & Hendry [1935]; blood samples were collected by severing the main neck vessels after stunning the animals.

dl-S-Methylcysteine was prepared, according to du Vigneaud *et al.* [1934]. (Found: C, 35.14; H, 6.62; N, 10.50; S, 2.38%. $C_3H_7O_2NS$ requires C, 35.50; H, 6.52; N, 10.44; S, 2.39%.) The formyl derivative melted at 119°.

dl-Homocystine was a sample kindly given to us by Dr du Vigneaud.

Determinations of the cystine and methionine contents of the proteins used were carried out by the method of Baernstein [1936].

RESULTS

Liver fat

In previous work [Channon, Manifold & Platt, 1938, 1], it was found that a daily intake of 40 mg. methionine per rat had no effect in preventing fat deposition on diets which resulted in liver fat of 15%; in contrast, amounts of this order were effective when control diets resulting in a liver fat content of 20–25% were used. The object of Exp. 1 was to study the effects of smaller intakes of methionine and, for the reason just given, the diet was designed to

cause very fatty livers. The use of a basal diet supplemented with cystine fulfilled this condition and also provided information as to the effect of adding mixtures of cystine and methionine to a low-protein diet. It was also important to confirm the observation of Beeston & Channon [1936] that there was no relation between the amount of cystine fed and the liver fat resulting (intakes of 7–80 mg. per rat per day were studied). Clearly a definite knowledge of the effect of small amounts of cystine is essential for complete understanding of the part played by dietary protein in the control of liver fat.

Accordingly, 13 groups of rats were maintained on the cholesterol diet. One group served as control, five received supplements of 0.05, 0.10, 0.15, 0.20, 0.25 % cystine, four received 0.25 % cystine and 0.20, 0.30, 0.40, 0.50 % methionine and three groups received 0.25 % cystine and 0.01, 0.02, 0.03 % choline. The last three groups were included with a view to assaying the effects of smaller amounts of methionine than previously used in terms of choline. All the experimental data are collected in Table 1.

Table 1. *The effects of cystine and methionine in fatty liver production*

		Exp. 1. Period of feeding 19 days				
Group		Liver wt. as % of final body wt.	Cystine content of the diet %	Methionine content of the diet %	Total fatty acids	
					% fresh liver wt.	g. in liver of 100 g. rat
1	Control	5.34	0.04	0.22	21.70	1.17
2	+ 0.05 % cystine	6.05	0.09	0.22	27.10	1.63
3	+ 0.10 % cystine	6.09	0.14	0.22	28.30	1.72
4	+ 0.15 % cystine	6.24	0.19	0.22	27.22	1.75
5	+ 0.20 % cystine	6.44	0.24	0.22	26.20	1.69
6	+ 0.25 % cystine	6.16	0.29	0.22	25.85	1.61
7	+ 0.25 % cystine + 0.20 % methionine	4.47	0.29	0.42	13.92	0.62
8	+ 0.25 % cystine + 0.30 % methionine	4.29	0.29	0.52	13.68	0.59
9	+ 0.25 % cystine + 0.40 % methionine	4.64	0.29	0.62	13.53	0.61
10	+ 0.25 % cystine + 0.50 % methionine	4.22	0.29	0.72	13.14	0.56
11	+ 0.25 % cystine + 0.01 % choline	5.57	0.29	0.22	25.43	1.42
12	+ 0.25 % cystine + 0.02 % choline	4.94	0.29	0.22	18.67	0.95
13	+ 0.25 % cystine + 0.03 % choline	5.32	0.29	0.22	20.53	1.09

The five concentrations of cystine administered have all produced the same increased degree of liver fat deposition, the figures varying only from 1.61 to 1.75 g. of fatty acids in the liver of the 100 g. rat. Thus 0.25 % added cystine has had no greater effect than 0.05 %, which corresponds to an intake of 4 mg. extra cystine per day. In confirmation of previous findings, these small supplements of cystine have increased liver fat by some 50 % over its already very high value. As with the cystine groups, the liver fat values of the animals receiving both cystine and increasing amounts of methionine show no differences among themselves. Since the average figure for liver fat of the methionine groups, 0.59 g., is not only considerably less than that for the group receiving cystine alone, 1.68 g., but also no more than one-half that of the control group, 1.17 g., it is clear that a daily extra intake of 16–40 mg. methionine not only inhibits the effect of cystine but even reduces the liver fat much below the control value. In group 11, a daily intake of about 0.8 mg. choline has resulted in the weight of liver fatty acids, 1.42 g., differing but little from the corresponding group 6. In groups 12 and 13 which probably ingested 1.6 and 2.4 mg. choline daily, the weights of liver fatty acids are 0.95 and 1.09 g.; these variations are insignificant and the failure to obtain different effects from doses differing so

little as 2.4 and 1.6 mg. is not unexpected. Making the reasonable assumption that 2 mg. choline have restored the liver fat to the control figure of 1.17 g., then 2 mg. choline have prevented the cystine action. From the results of this experiment, it is not possible however to make any certain estimate of the activity of methionine in terms of choline.

Table 2. *Analysis of liver lipins of Exp. 1*

Group		% of fresh liver			Wt. in liver of 100 g. rat		
		Free cholesterol	Cholesterol-oleate	Glyceride	Free cholesterol	Cholesterol-oleate	Glyceride
1	Control	0.49	4.86	20.44	0.03	0.26	1.09
2-6	+ cystine	0.32	4.25	26.22	0.02	0.26	1.63
7-10	+ cystine + methionine	0.31	3.28	12.71	0.01	0.14	0.56
11	+ 0.25 % cystine + 0.01 % choline	0.34	4.34	24.61	0.02	0.24	1.37
12	+ 0.25 % cystine + 0.02 % choline	0.37	4.26	18.40	0.02	0.21	0.92
13	+ 0.25 % cystine + 0.03 % choline	0.33	4.09	19.60	0.02	0.22	1.04

In order to obtain a more complete picture of these effects, detailed analyses were carried out on the control, choline, pooled cystine and pooled methionine groups. The phosphatide figures are omitted from the table since they showed no variations; the results for the glyceride content reflect the figures for total fatty acids which have already been discussed; the free and esterified cholesterol figures are, however, of interest. The control, cystine and cystine + methionine groups gave 0.03, 0.02 and 0.01 g. free cholesterol and 0.26, 0.26 and 0.14 g. cholesteryl oleate respectively in the 100 g. rat liver. These figures confirm our previous observation that the administration of methionine with cholesterol-containing diets markedly decreases the deposition of cholesterol, both free and esterified; cystine, on the other hand, exerts no effect on cholesterol [Channon, Manifold & Platt, 1938, 1].

In Exp. 2, the effects of still lower methionine dosages were studied; this experiment was similar in plan to Exp. 1 except that the groups receiving graded cystine supplements were omitted. Nine groups of rats were maintained on an 8 % caseinogen diet, one serving as control, and the other eight receiving 0.20 % cystine. In addition, three of these groups received 0.01, 0.02, 0.03 % choline respectively, and another four 0.05, 0.10, 0.15, 0.20 % methionine. All the results are recorded in Table 3.

Table 3. *Effects of choline and methionine on the "cystine" fatty liver*

Exp. 2. Period of feeding 17 days								
Group		Intake of supplements in mg. per rat per day			Liver wt. as % of final body wt.	“Fat” in fresh liver %	“Fat” in liver of 100 g. rat g.	
		Cystine	Methio- nine					Choline
1	Control	—	—	—	5.02	24.60	1.23	
2	+0.20 % cystine	16.6	—	—	6.44	29.80	1.91	
3	+0.20 % cystine +0.01 % choline	17.6	—	0.9	6.02	25.45	1.53	
4	+0.20 % cystine +0.02 % choline	18.0	—	1.8	5.20	25.20	1.31	
5	+0.20 % cystine +0.03 % choline	15.4	—	2.3	5.35	25.30	1.35	
6	+0.20 % cystine +0.05 % methionine	17.2	4.3	—	5.48	29.50	1.62	
7	+0.20 % cystine +0.10 % methionine	16.6	8.3	—	5.48	28.11	1.54	
8	+0.20 % cystine +0.15 % methionine	17.6	13.2	—	4.80	21.47	1.03	
9	+0.20 % cystine +0.20 % methionine	16.8	16.8	—	4.87	22.28	1.08	

The addition of 0.20 % cystine to the basal diet has resulted in an increase of some 5.6 % in liver fat as compared with the control value. A daily intake of only 0.9 mg. choline per rat (group 3) has brought about some decrease in the extra fat deposition caused by cystine, while 1.8 or 2.3 mg. choline (groups 4 and 5) has virtually overcome the effect of cystine, for the values do not differ significantly from the control figure of group 1. This confirms the finding of the previous experiment, that 0.02–0.03 % choline is sufficient to prevent the increased deposition of liver fat caused by cystine; as in Exp. 1 no difference is seen in the effects of 0.02 and 0.03 % choline. In the groups which received both cystine and methionine, daily extra intakes of 4.3 and 8.3 mg. methionine (groups 6 and 7) have both exerted some preventive action while extra intakes of 13.2 and 16.8 mg. (groups 8 and 9) not only prevent the increase due to cystine but also lower the liver fat as compared with the control group. One important point is that the livers of the groups receiving 0.15 and 0.20 % methionine contain the same amounts of fat; 21.47 % and 1.03 g., 22.28 % and 1.08 g. respectively; in the previous experiment groups receiving supplements of methionine ranging from 0.20 to 0.50 % also gave values not differing from each other. The conclusion to be drawn is that under these conditions the lipotropic action of methionine is independent of the dose, provided that the dose is greater than a certain minimum value.

The results of this experiment enable a reasonably accurate comparison of the relative effects of methionine and choline to be made. Taking as standard the amount of either substance required to prevent the action of 0.20 % cystine, it appears that about 10 mg. methionine are equal in action on the extra deposition of liver fat caused by cystine to about 2 mg. choline.

In Exps. 1 and 2, the inclusion of 0.02 and 0.03 % choline was found to overcome the effects of cystine. Since the results in neither case differentiated between the effects of these two dosages, it seemed of value to attempt to define the effective dose more clearly. The results of Exp. 3 in which 9 % caseinogen was used in the diet, are presented in Table 4.

Table 4. *Effect of choline on the "cystine" fatty liver*

Exp. 3. Period of feeding 16 days

Group		Choline intake mg./rat/ day	Liver wt. as % of final body wt.	"Fat" as % of wet liver wt.	"Fat" in liver of the 100 g. rat, g.
1	Control	—	4.47	17.34	0.80
2	Control	—	4.26	18.48	0.83
3	+0.25 % cystine	—	5.58	24.23	1.55
4	+0.25 % cystine + 0.01 % choline	0.90	4.87	20.11	1.01
5	+0.25 % cystine + 0.02 % choline	1.88	4.59	16.24	0.78
6	+0.25 % cystine + 0.03 % choline	2.74	4.05	10.05	0.43

In this experiment the addition of 0.25 % cystine has caused a 90 % increase in liver fat; this increase is completely prevented by 1.9 mg. choline per rat per day: a further increase in choline intake to 2.7 mg. has resulted in a profound decrease of liver fat to 10.05 % and 0.43 g. The finding that 1.9 mg. choline are the effective daily intake of choline necessary to overcome the effects of cystine thus confirms the figure of 2 mg. which was deduced from the results of Exps. 1 and 2. Thus, in the prevention of the effects of cystine, choline is five times more effective than methionine on a weight basis.

To correlate these observations of the effect of mixtures of cystine and methionine on liver fat deposition with those of the lipotropic action of proteins, the effect of adding cystine to diets of increasing caseinogen content was studied. Four groups of animals received a 40 % fat diet containing 8, 12, 15 and 25 % caseinogen respectively while four more groups received the same diets supplemented by 0.25 % cystine. The results are presented in Table 5.

Table 5. *The effect of caseinogen on the "cystine" fatty liver*

Exp. 4. Period of feeding 19 days						
Group	Caseinogen content of diet %	Dietary content of cystine and methionine, derived from caseinogen %		Added cystine %	"Fat" % fresh liver	"Fat" in liver of 100 g. rat g.
		Methionine	Cystine			
1a	8	0.22	0.04	—	21.1	1.00
1b	—	—	—	0.25	29.2	1.66
2a	12	0.33	0.06	—	12.0	0.53
2b	—	—	—	0.25	21.7	1.04
3a	15	0.41	0.075	—	12.7	0.52
3b	—	—	—	0.25	16.2	0.72
4a	25	0.68	0.125	—	7.6	0.29
4b	—	—	—	0.25	8.1	0.30

The results of groups 1a–4a illustrate the now well-established lipotropic action of caseinogen; greater importance however lies in the fact that the addition of cystine results in an increased fat deposition at all but the highest protein intakes. Thus in group 1b cystine has caused a 66 % increase in the already high fat content of the liver, in group 2b the increase approaches 100 %, in group 3b it is 40 % while in group 4b, in which the protein content of the diet is 25 %, the cystine action disappears.

All the experiments so far described have been carried out with caseinogen as the dietary protein. Channon, Loach *et al.* [1938] showed that when egg albumin was substituted for caseinogen in low protein diets, the livers became much more fatty, and they suggested that possibly the greater cystine content of albumin might be responsible for the behaviour of this protein. In order to obtain evidence on this question, two experiments were carried out to see whether the addition of cystine to diets containing egg albumin would increase the deposition of liver fat. The relevant data of these experiments are given in Table 6, and all that needs to be added is that in Exp. 6 an 8 % albumin diet was used while in Exp. 7 the diet contained 10 % albumin.

Table 6. *Effect of supplementing an albumin diet with cystine*

Exp.	Period of feeding days	Group	Dietary content of cystine and methionine %		Liver % body wt.	"Fat" % fresh liver wt.	"Fat" in liver of 100 g. rat
			Methionine	Cystine			
6	17	1. Control	0.31	0.20	5.5	25.23	1.43
		2. +0.25 % cystine	0.31	0.45	5.4	26.55	1.52
7	18	3. Control	0.39	0.25	5.8	23.90	1.47
		4. +0.5 % cystine	0.39	0.75	6.1	25.13	1.61

Clearly, cystine does not exert its action on liver fat deposition when egg albumin is the dietary protein, for in neither experiment has the addition of

cystine resulted in amounts of liver fat differing significantly from the control figures. This may indicate that the very fatty livers resulting from albumin diets are a result of their high cystine contents—in these two experiments 0.20 and 0.25 % respectively.

Finally, in Table 7 are presented the results of experiments with *S*-methylcysteine, taurine and homocystine, a preliminary report of which has already been published [Channon, Manifold & Platt, 1938, 2]. The diets used were as follows: Exp. 8, 5% caseinogen; Exp. 9, 8% egg albumin; Exp. 10, 8% caseinogen.

Table 7. *Effects of S-methylcysteine, homocystine and taurine on fatty liver production*

Exp.	Period of feeding days	Group	Liver % body wt.	"Fat" % fresh liver wt.	"Fat" in liver of 100 g. rat
8	11	1. Control	4.1	16.36	0.68
		2. +0.28 % <i>S</i> -methylcysteine	4.5	16.48	0.79
		3. +0.2 % taurine	4.3	17.26	0.79
9	18	4. Control	6.3	27.01	1.82
		5. +0.5 % <i>S</i> -methylcysteine	6.7	29.21	1.98
10	17	6. Control	3.9	17.03	0.70
		7. +0.25 % cystine	5.7	32.95	1.98
		8. +0.25 % homocystine	4.5	23.07	1.08

In Exp. 8 equimolecular proportions of *S*-methylcysteine and taurine were administered and these compounds have clearly exerted no influence on liver fat deposition. Neither the percentages of fat in the liver, 16.48 and 17.26 respectively, nor the weights per 100 g. rat, 0.79 g. in both cases, are different from the control values, 16.36 % and 0.68 g. This inactivity of taurine was confirmed in a further experiment which will not be presented here in full; suffice it to say that the percentages of glyceride in the control and taurine groups were respectively 13.24 and 13.04 while the weights per 100 g. rat were 0.57 and 0.60 g.

Bearing in mind that it was not possible to demonstrate a convincing preventive action of methionine when the control group contained about 15 % fat in the liver, the next experiment with *S*-methylcysteine was designed to provide optimum conditions for the exertion of a preventive action. Accordingly, in Exp. 9 the 8 % egg albumin diet which invariably produces very fatty livers was used, and the control liver fat values were 27.01 % and 1.82 g. Here again, *S*-methylcysteine has exerted no effect on the liver fat for the values, 29.21 % and 1.98 g., do not differ significantly from the control figures.

One experiment was carried out with homocystine and the results indicated that this amino-acid behaved in a manner qualitatively similar to that of cystine. Whereas 0.25 % cystine produced a spectacular rise from the control values of 17.03 % and 0.70 g. to 32.95 % and 1.98 g., 0.25 % homocystine gave values of 23.07 % and 1.08 g. Further study of the action of homocystine was not made, since Singal & Eckstein [1939] and du Vigneaud *et al.* [1939] later found that homocystine markedly increases liver fat deposition.

Blood fat

Table 8 shows that the feeding of rats fasted for 24 hr. with a 40 % fat diet causes no changes in the amount of fat in the blood at any time up to 31 hr. after the meal, and, surprisingly, there is no indication of a blood fat curve or transient lipaemia. While changes in blood fat of many species of animals have been recorded in the literature, we have been unable to find observations on the rat with which the present results might be compared. The lack of any increase

in blood fat may be connected with the very slow rate of absorption of fat from the intestine with diets such as this, for we have repeatedly observed large amounts of unabsorbed fat in the alimentary tract many hours after feeding. It was this observation which led us to adopt the 16-hr. period after feeding for killing in the second experiment and even at this time the alimentary tracts of the majority of the animals still contain much unabsorbed fat. The results of the next experiment show very clearly the progressive increases of fat deposition in the liver with time.

Table 8. *Variations in blood fat of rats after a fatty meal and during the production of fatty livers*

Exp. 11					
Group	No. of rats	No. of hours after feeding at which animals were killed	Total fatty acids in milli. equiv. per litre of whole blood		
1	6	—	12.3		
2	4	2.0	11.2		
3	6	5.0	10.0		
4	6	8.0	11.4		
5	5	10.5	11.4		
6	6	14.0	11.1		
7	6	17.0	11.3		
8	6	23.0	11.0		
9	6	31.0	10.9		

Exp. 12					
Group	Period on fatty liver diet days	Added cystine %	"Fat" % fresh liver wt.	"Fat" in liver of 100 g. rat g.	Total fatty acids in milli. equiv. per litre of whole blood
Control	—	—	3.22	0.13	9.6
1a	1	—	4.69	0.16	11.1
1b	—	0.25	4.96	0.18	10.4
2a	3	—	8.74	0.30	9.3
2b	—	0.25	10.91	0.40	9.2
3a	7	—	14.22	0.56	9.2
3b	—	0.25	17.91	0.73	9.2
4a	14	—	23.91	1.06	9.0
4b	—	0.25	28.75	1.50	8.8
5a	21	—	27.08	1.36	8.9
5b	—	0.25	32.77	2.13	8.9

Thus the amount of fat begins to increase in the control groups within 24 hr. and progressively rises in 21 days from the zero figure of 0.13–1.36 g. on the 21st day. Similarly the effect of cystine is demonstrated at every stage, the amount of fat increasing progressively to 2.13 g. The effect of cystine appears to increase with time, for after 24 hr. it has caused a 12 % increase over the control and this steadily rises to 57 % after 21 days. In striking contrast to these liver fat changes, the blood fat figures show no change and it is noteworthy also that at every stage the blood fat of the cystine groups is identical with that of the controls. It may of course be that different results would have been obtained if the animals had been killed at a different hour after their last meal, but in view of the fact that much fat remained unabsorbed at this stage this seems unlikely.

DISCUSSION

Table 1 shows clearly that the effect of cystine is demonstrated on 8% caseinogen diets containing as little as 0.05% added cystine. The daily intake of cystine from a diet containing 8% caseinogen would be about 3 mg., and the extra intake of 4 mg. added cystine profoundly increases the fat deposition. The results clearly show also that increasing the added cystine to five times this amount has no further effect on liver fat; this is in confirmation of the results previously reported [Beeston & Channon, 1936]. While the mode of action of cystine remains to be determined, the fact that its effect is unrelated to the intake is a factor which must be borne in mind.

The considerably increased fat deposition caused by cystine does not seem to be the result of a process differing from that which causes the deposition of fat in the livers of animals on the control high-fat diet but rather an acceleration of this process. This seems to be borne out by the fact that apparently no damage to the liver results, since the extra fat is removed readily by the same substances which normally prevent deposition, for 0.02% choline in the diet or 0.32 g. caseinogen per rat per day is sufficient to prevent the cystine action.

The results of Exp. 12 show that the acceleration of liver fat deposition caused by cystine cannot be associated with any changes in the amount of blood fat. This may mean that cystine exerts its effect in the liver itself, but at the present time no evidence exists which can serve as the basis of a theory to account for this action.

If it be assumed that methionine exerts a preventive action on liver fat deposition by supplying methyl groups for the synthesis of choline or of the active compound derived from choline, then three molecules of methionine would be required for the synthesis of one molecule of choline if complete conversion occurred; from this it follows that 3.3 mg. methionine should be equivalent in its action to 1 mg. choline. In Exp. 2 in which the amount of methionine used was such as to demonstrate the maximum action, 10 mg. methionine were equivalent to 2 mg. choline, i.e. a ratio by weight of 5:1. While this ratio derived from the results of Exp. 2 is the only certain finding on this question, support for it is given by the results of Exp. 1. In this experiment, 0.02% choline had an action equivalent to two-thirds of that of 0.20% methionine, and in the light of many previous experiments, it is reasonable to believe that 0.04% choline would have exerted an effect equal to this percentage of methionine. If this be correct, the ratio would again be 5:1. In view of the many difficulties of obtaining accurate assays in terms of choline, of substances having an action on liver fat, this ratio is to be regarded as being in quite reasonable agreement with the theoretical requirement, 3.3:1, and this finding provides, therefore, superficial evidence at least in support of the theory that methionine may act as a choline precursor.

On the other hand it must be emphasized that this ratio only obtains when the minimum amount of methionine required to exert maximum lipotropic action is considered. The fact that further increases above a certain minimum in the amount of methionine added to fatty-liver-producing diets exert no more action is one difficulty preventing the ready acceptance of this theory. In this respect methionine resembles cystine and differs from choline, the action of which is related to the amount administered. A second difficulty, probably associated with the first, lies in the fact that the action of methionine is limited in extent. In only one of our experiments has liver fat been reduced as low as 12% by methionine and when the control value is very high, methionine admini-

stration has not reduced it to a figure lower than 15 %, and a liver containing 15 % fat is still very fatty; in contrast, the use of a suitable amount of choline or lipotropically active protein will invariably reduce liver fat to well below 10 %. These points can be explained in one of two ways; either methionine exerts its effect quite independently of choline or some factor other than the provision of methyl groups becomes limiting in the rate of synthesis of choline.

On the other hand our finding that *S*-methylcystine is without action lends support to the hypothesis of choline synthesis, for du Vigneaud *et al.* [1934] and Brand *et al.* [1935; 1937] showed that demethylation is probably not a stage in the metabolism of this compound. If this be true, *S*-methylcysteine could not supply methyl groups for choline synthesis. It must be remembered however that in the one experiment with homocystine reported here, this amino-acid increased liver fat deposition, having an action similar to but less intense than that of cystine. Further Singal & Eckstein [1939], in experiments on mice, find homocystine and cystine to be equally active in this respect, while du Vigneaud *et al.* [1939] also find that homocystine stimulates the deposition of liver fat. The primary product of the demethylation of methionine is homocysteine and the results of du Vigneaud *et al.* [1939] suggest that this compound is readily converted into homocystine. Hence in the demethylation of methionine to produce choline, there would be produced at the same time homocystine; the former compound would prevent fat deposition and the latter increase it. If the action of homocystine is like that of cystine, rising to its maximum at a very low level beyond which increasing amounts have no further effect, its action would be overcome as increasing amounts of choline were synthesized. This might account for the activity of methionine relative to that of choline being less than that expected from theoretical consideration.

Interesting observations on a problem of biological methylation have been made by Borsook & Dubnoff [1940]. These authors found that the conversion of glycocyamine into creatine by liver slices was increased some 50 % in the presence of methionine but that neither any other amino-acid nor choline nor betaine had any effect. While methionine can provide methyl groups for the formation of creatine, choline is apparently unable to do so. The very different conditions of these experiments forbid their comparison with those of du Vigneaud *et al.* [1939] but the work provides definite evidence that methionine can act as a methylating agent in biological systems. Summarizing the findings presented in the present paper, it can be said that if methionine plays a part in the synthesis of choline, then some other dietary factor is also involved.

The results of these studies throw light on the question of whether the lipotropic action of proteins is solely dependent upon their cystine and methionine contents, as first suggested by Tucker & Eckstein [1937]. The fact that in Exp. 4 the extra weight of fat deposited in the liver as a result of the intake of the same amounts of added cystine progressively decreased as the content of dietary caseinogen was increased, strongly suggests, in view of the known lipotropic action of caseinogen, that the cystine present in proteins is intimately concerned in the production of fatty livers by dietary methods. In this connexion it is of interest to note also that in Exp. 4 group 2*b* which received 12 % caseinogen with 0.25 % added cystine gave the same results as group 1*a* receiving 8 % caseinogen alone. It is possible therefore to equate these effects; the extra daily intake of 0.32 g. caseinogen by group 2*b*, as compared with group 1*b*, has thus neutralized the effect of cystine. Since the results of Exp. 3 showed that the cystine effect was prevented by 2 mg. choline, this finding shows that 1 g. caseinogen has an activity of approximately 6 mg. choline, a value in agreement

with that previously obtained [Beeston *et al.* 1936]. Thus whether the lipotropic action of caseinogen in terms of choline be derived from experiments concerned with fatty livers produced by diets of varying protein content or from those in which yet further fat is deposited in the liver as a result of cystine feeding, the same result holds. Furthermore, the results of Exps. 5 and 6 showed that the addition of cystine to basal albumin diets produced no further increase in the already large amount of fat deposited in the livers; similar observations when gliadin was the basal protein were reported by Tucker & Eckstein [1938]. Clearly, the cystine contents of these two proteins are so high that further supplementing exerts no effect. In our opinion, these findings are of importance in showing that the cystine content of dietary proteins is one of the factors normally concerned in dietary fatty liver production; we are gradually accumulating evidence, however, that cystine is not the only protein constituent acting in this way.

The limitations of the action of methionine have been discussed in detail already, and it is clear from what has been said that methionine cannot be solely responsible for the action of proteins in preventing liver fat deposition. That this amino-acid is of significant importance in this connexion is suggested by two facts; first, the proteins classified by Channon, Loach *et al.* [1938] in order of descending lipotropic activities, fall in the approximate order of descending methionine contents as Tucker & Eckstein [1938] pointed out; secondly, the latter authors showed that arachin, which contains very little methionine, has no lipotropic properties whatever.

On the other hand, results of the present experiments reveal the more complex nature of the problem. The lipotropic action of high fat diets containing caseinogen increases as the percentage of caseinogen is increased to 30 % at which point the maximum effect of caseinogen is reached. The lipotropic action of the diets thus increases until caseinogen is providing to the diet 0.83 % methionine. In contrast, while the results in Table 3 show that added methionine exerts an increasing effect up to 0.15–0.20 %, those in Table 1 show that a further increase beyond 0.20 % exerts no further effect. The limit of action is thus reached when the *total* methionine content of the diet is 0.42 %. It does not seem possible therefore that methionine can be the sole active agent in the caseinogen molecule, a finding amply borne out by the results of Best & Ridout [1940]. These authors found that the administration of the amounts of cystine and methionine contained in 30 % caseinogen exerted virtually no effect on fat deposition in contrast to the profound effect of 30 % caseinogen itself. Since methionine is probably not the only lipotropic constituent of caseinogen, two possibilities arise; either that some other amino-acid also exerts a lipotropic action, or alternatively, added methionine is incapable of exerting its full action in the absence of some other protein constituent. In connexion with the former possibility previous work in our laboratory has shown that alanine, arginine, aspartic acid, histidine, hydroxyproline, leucine, lysine, proline and valine have no lipotropic action, although a slight action was observed with tyrosine [Beeston & Platt, 1939]. Thus of twelve amino-acids only methionine and to a far less extent tyrosine prevent fat deposition in the liver. We have no evidence bearing on the second possibility although we wish to mention that the production of aminoethanol, possibly from an amino-acid, might be the limiting factor. Among the number of compounds which have been reported to be active in the last few years, only one, betaine, seems likely to be of physiological importance, and it is interesting in this connexion that Carter & Melville [1940] found the betaines of serine, threonine and *allo*threonine to be inactive. It must not be

forgotten also that proteins may contain substances other than cystine which increase fat deposition.

It is clear that much further work is required before the lipotropic action of proteins can be explained. There are already two variable factors acting in opposite directions, namely cystine and methionine, and the logical deduction from the present evidence is that there are yet others. The isolation of the substances concerned is an essential preliminary and for this reason we are studying the action of fractions prepared from protein hydrolysates; results already obtained confirm the belief that lipotropic action of proteins is a far more complex story than the cystine-methionine hypothesis would suggest.

SUMMARY

1. The greatly increased fat deposition in the liver caused by the addition of cystine to diets low in caseinogen and high in fat is seen when as little as 4 mg. of cystine are administered to the rat daily. No further increase results from increasing the cystine intake. The cystine effect can be demonstrated in progressively decreasing intensity at all levels of dietary caseinogen up to 25 %. Dietary caseinogen exerts its normal lipotropic action on fat deposition caused by cystine; similarly 1.8 mg. choline per day will effectively prevent the cystine action. The increased fat deposition caused by cystine progressively increases with time up to 21 days. The cystine effect is not observed when the dietary caseinogen is replaced by albumin.

2. No changes were observed in the amount of fat in the blood of rats receiving diets high in fat and low in protein, either with or without added cystine.

3. The preventive action of methionine on liver fat deposition on an 8 % caseinogen diet increases until the amount of added methionine is 0.20 % of the diet; further additions of methionine to the diet do not have any increased effect. At its optimum, the intensity of action of methionine is one-fifth that of choline.

4. *S*-Methylcysteine and taurine are without effect on liver fat deposition; in contrast, homocystine has an action qualitatively similar to that of cystine.

5. The bearing of these findings on the respective modes of action of cystine and methionine in liver fat deposition and on the part played by these two amino-acids in accounting for the lipotropic action of proteins are discussed.

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