by H_2O was used to compensate for the colour of the original urine. The concentration of catechol was calculated by reference to a standard curve prepared with urinary solutions of stibophen of known concentrations.

Fig. 1. The excretion of stibophen by subject M.C. ● antimony, ○ catechol, both from a 5 ml. dose of stibophen (6.3%). ▲ catechol, from a dose of sodium catechol disulphonate equivalent to 5 ml. of stibophen (6.3%).

RESULTS

The results (Table 1, Fig. 1) show that in the normal subject catechol excretion was almost

complete in about 6 hr., whereas the Sb excretion was very much slower, and followed a different course. The rates of excretion were not affected by changes in urine volume. In Khalil's experiments, his colour test failed after 6 hr. because the catechol portion of the molecule had been completely eliminated. The function of the catechol appears to be to keep the circulating Sb in solution in a non-toxic form while it is being absorbed by the liver or excreted by the kidney. Meanwhile, excretion of the catechol takes place independently of that of the Sb and (as shown by the results) at the same rate as that of an equivalent dose of Na catechol disulphonate.

SUMMARY

1. Khalil's test for stibophen in urine has been modified for use as a quantitative method for the determination of the catechol fraction of the molecule.

2. The catechol fraction has been shown to be excreted very much more rapidly than the Sb of stibophen, elimination being complete in six hours in the normal subject.

Our thanks are due to the volunteers, especially Mr E. Rogers, who was also responsible for the catechol determination.

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The Action of Amino-acids and Proteins on Liver-fat Deposition

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Any explanation of the lipotropic action of proteins must take into account the now well-established facts that while cystine accelerates, methionine partially prevents the deposition of fat in the livers of rats maintained on diets high in fat and low in lipotropic factors. Agreement on the question as to whether the effects of these two amino-acids alone account for the lipotropic behaviour of proteins has, however, yet to be reached. Thus Best & Ridout [1940] and Channon, Manifold & Platt [1940] con-

clude that these two amino-acids are not the only factors involved; in contrast, Tucker, Treadwell & Eckstein [1940] claim that the cystine and methionine contents of the diets used by them suffice adequately to explain their results. More recently, Treadwell, Groothuis & Eckstein [1942] arrived at similar conclusions but also found that when methionine was present as the free amino-acid in the diet it appeared to be superior to that contained in protein (caseinogen) so far as lipotropic action is

concerned. Before final conclusions with regard to the action of proteins on liver-fat deposition can be drawn, it is essential to have information concerning both the qualitative nature and the intensity of the action of all the naturally occurring amino-acids. We have previously reported the results of studies of a number of these acids [Beeston & Platt, 1939] and the present paper records further experiments with other pure amino-acids. At the same time the question of the lipotropic activity of proteins has been attacked from a new angle by the use of fractionated protein hydrolysates. The results of these experiments, which are also presented, indicate the complex nature of the problem.

EXPERIMENTAL

General

The animal experiments and the analyses of liver fat were carried out with rats as experimental animals, according to methods developed in this laboratory [Channon, Manifold & Platt, 1938*b*, 1940]. The basal diets used to produce fat- or cholesterol-fatty livers varied in detail in the different experiments and are mentioned as each is described. Blood fibrin was prepared according to the method of Channon, Loach, Loizides, Manifold & Soliman [1938*a*].

Estimation of amino-acids

Determinations of the cystine and methionine contents of the proteins and the amino-acid fractions were carried out by the method of Baernstein [1936]; in the titration method we have found that the recovery of methionine was 91%, which agrees with the correction figure of 1.12 given by Kassel & Brand [1938]. The tyrosine contents of the proteins and fractions prepared from hydrolysates were determined by the method of Lugg [1937, 1938].

Preparation of amino-acids

The tyrosine used in these experiments was prepared from caseinogen, the cystine from hair; other amino-acids were obtained commercially.

Preparation of protein fractions

Caseinogen hydrolysate. Caseinogen was hydrolysed with H_2SO_4 and the hydrolysate neutralized with $Ba(OH)_2$ [Berg & Rose, 1929], filtered and concentrated *in vacuo*. The product was partly solid and partly an uncrystallizable syrup.

Butanol fractionation. A caseinogen hydrolysate prepared as above was concentrated until the volume was equivalent to 1 l. for every 500 g. original caseinogen. The solution was allowed to cool, and the residue obtained by filtration is referred to subsequently as Fraction I. The filtrate was then exhaustively extracted with equal volumes of butanol on a shaking machine at room temperature. The butanol extracts were concentrated *in vacuo* and the amino-acids filtered, washed with ether and dried. The yield of butanol-soluble amino-acids was roughly 20% of the weight of caseinogen used. Butanol-insoluble amino-acids remained in the form of a syrup on concentration and were used as such.

Copper salt fractionation. The butanol-soluble amino-acids were divided into three groups, according to their

solubilities in H_2O and dry CH_3OH , by the method of Town [1928, 1936]. These three groups are: (a) amino-acids with Cu salts insoluble in H_2O ; (b) amino-acids with Cu salts soluble in H_2O but insoluble in dry CH_3OH ; (c) amino-acids with Cu salts soluble in H_2O and in dry CH_3OH .

Assessment of results

Difficulties were encountered in deciding on the significance of differences which occurred in the amounts of liver fat of groups of rats receiving various dietary treatments. With carefully selected groups, each of not less than ten animals, there may be variation in the amount of fat deposited in the pooled livers even when the groups are under identical dietary treatment. These differences are greater when the livers become very fatty, and contain amounts of fat greater than, say, 1.5 g., as compared with the normal figure of 0.13 g. The differences, however, decrease rapidly as the amount of fat falls from these very high figures, so that when the liver fat is no more than three or four times the normal, they tend to disappear. Similarly with animals receiving supplements of substances under test; if the liver fat is very high, only substantial decreases can be regarded as significant. As the liver fat diminishes to 0.5–1.0 g., however, decreases of small magnitude become significant, for the ease of removal of fat from the liver diminishes as the amount of its contained fat decreases. Although methods of statistical analysis have been applied, we have found cases in which results which have subsequently been found to be significant have not been revealed so by mathematical methods. Considerable caution in interpreting apparent changes between control and experimental groups has, therefore, to be exercised, and even with great experience some degree of uncertainty in interpreting the results is inevitable.

RESULTS

Experiments with pure amino-acids

The first two experiments were designed to test the action on liver fat deposition of as many amino-acids as were available. In Exp. 1, five groups of rats were maintained on basal diet A (Table 1). Four groups received supplements of 0.2% aspartic acid, lysine, phenylalanine and serine respectively. In Exp. 2, 14 groups of rats received basal diet B (Table 1). The 12 experimental groups received supplements of 0.5% alanine, arginine, aspartic acid, histidine, hydroxyproline, leucine, lysine, phenylalanine, proline, serine, tryptophan and valine respectively.

Table 1. *Composition of basal diets (g./100 g.)*

(In addition to the following constituents all diets contained 1 g. cod-liver oil and 5 g. salt mixture/100 g. diet.)

Diet	Caseinogen	Albumin	Glucose	Beef dripping	Mar-mite	Cholesterol	Aneurin μg./rat/ day
A	5	0	46	40	3	0	0
B	0	10	44	40	0	0	10
C	5	0	64	20	5	0	0
D	0	8	54	30	0	2	10
E	0	8	46	40	0	0	10
F	10	0	45	39	0	0	12
G	0	5	50	39	0	0	12

The amino-acids tested in Exp. 1 have clearly had no action on the level of liver fat (Table 2). In Exp. 2 also, no amino-acid save tryptophan has caused the weight of liver fat to differ from the control figure by a significant amount (Table 2).

Table 2. *The effect of dietary supplements of amino-acids on liver fat of the rat*

Group	Dietary supplement	Change in body wt. ($\pm$ % original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
Exp. 1. Basal diet A. Period of feeding 14 days. Amino-acid supplement = 0.2 %					
1	None	- 5	4.8	14.54	0.70
2	Aspartic acid	-13	4.5	15.87	0.72
3	Lysine	-13	4.7	13.60	0.64
4	Phenylalanine	- 3	4.6	14.57	0.67
5	Serine	- 7	4.5	15.06	0.67
Exp. 2. Basal diet B. Period of feeding 18 days. Amino-acid supplement = 0.5 %					
6	None	0	5.6	23.93	1.43
7	None	- 3	6.0	23.87	1.51
8	Alanine	0	5.0	23.54	1.25
9	Arginine	+ 2	4.9	24.85	1.28
10	Aspartic acid	+ 2	5.0	24.63	1.24
11	Histidine	+ 1	5.7	27.62	1.58
12	Hydroxyproline	+ 3	5.6	26.95	1.51
13	Leucine	+ 3	5.0	23.39	1.22
14	Lysine	+ 1	5.8	25.95	1.58
15	Phenylalanine	- 3	5.6	24.25	1.43
16	Proline	+ 3	5.6	22.03	1.30
17	Serine	0	4.7	27.26	1.26
18	Tryptophan	0	4.8	20.69	1.05
19	Valine	- 3	5.8	25.97	1.53

The decrease caused by tryptophan from 1.50 to 1.05 g. is noteworthy, but unfortunately further supplies of tryptophan were not available, and the necessary confirmation of this result could not, therefore, be sought.

Glutamic acid. Exps. 3-10 were concerned with a more detailed study of the effect of glutamic acid on the cholesterol- and fat-fatty liver. It was found that glutamic acid exerted some effect in preventing glyceride deposition in the cholesterol-fatty liver, but to our surprise had little significant effect on the fat-fatty liver (Table 3).

The results of Exps. 3 and 4 show that although 0.24 % glutamic acid and its mono-sodium salt had little, if any, action on the levels of liver fat, 2.4 % of both of these substances definitely decreased the glyceride fraction. The decreases in Exp. 3 from 1.12 g. for the control group to 0.70 g. for the 2.4 % glutamic acid group, and in Exp. 4 from 0.57 to 0.38 g. in the corresponding groups, thus show that this amino-acid possesses some lipotropic action. The results of Exp. 5 agree with those of Exps. 3 and 4 and define the effect of glutamic acid more closely. Thus the effect shown by 3 % of the Na salt is shown equally well at the lower level of 0.5 %. A demonstrable effect is therefore shown by a 0.5 % supplement of glutamic acid, but no further effect

Table 3. *Effect of glutamic acid on the cholesterol-fatty liver*

Group ₃	Dietary supplement	Change in body wt. ($\pm$ % original wt.)	Liver (% of final body wt.)	Fatty acids	
				In 100 g. fresh liver g.	In liver of 100 g. rat g.
Exp. 3. Basal diet C; period of feeding 17 days					
20	None	- 7	5.3	21.03	1.12
21	0.24 % glutamic acid	- 6	4.6	20.08	0.91
22	0.4 % glutamic acid	-10	4.5	15.58	0.70
Exp. 4. Basal diet C; period of feeding 21 days					
23	None	- 5	4.3	13.24	0.57
24	0.24 % mono-sodium glutamate	- 8	4.1	13.53	0.56
25	2.4 % mono-sodium glutamate	-11	4.1	9.25	0.38
Exp. 5. Basal diet D; period of feeding 18 days					
26	None	- 5	5.6	25.34	1.42
27	0.5 % mono-sodium glutamate	- 4	5.1	21.44	1.09
28	3.0 % mono-sodium glutamate	-10	4.8	20.65	0.99
Exp. 6. Basal diet D; period of feeding 17 days					
29	None	- 8	6.7	31.88	2.15
30	0.5 % glutamic acid	- 4	5.8	26.19	1.53
31	2.5 % glutamic acid	- 5	5.7	27.95	1.59
32	10 % glutamic acid	- 6	5.4	26.44	1.44
33	0.58 % mono-sodium glutamate	- 7	5.9	31.06	1.84
34	2.88 % mono-sodium glutamate	- 7	5.6	30.40	1.69
35	11.5 % mono-sodium glutamate	- 6	5.7	28.91	1.65
36	1.44 % NaHCO ₃	- 3	6.4	31.47	2.00

is induced by increasing the amount of the supplement above this level.

The results of Exp. 6 show that glutamic acid has decreased the fat deposition in groups 30-32, although the fact that the decrease does not run parallel with the amount of the supplement is again noteworthy. The results for groups 33-35, in which the mono-sodium salt was used, also show some decrease in the amount of fat deposited in the 100 g. rat liver, while group 36 has served as a further control to group 35. While the effects of glutamic acid in preventing glyceride deposition in the cholesterol-fatty liver are not of great magnitude, the results, which are in agreement, are significant.

The results of Exps. 7-10 show conclusively that neither glutamic acid nor mono-sodium glutamate have any action on the fat-fatty liver. In Exp. 7 glutamic acid has raised the fat content of the liver of the 100 g. rat 0.70-0.98 g., but this result was not substantiated in Exps. 8-10, where in no case do the animals on supplemented diets show any difference from those on the control diet (Table 4).

Tyrosine. At first sight the results (Table 5) would seem to indicate that tyrosine possesses a small lipotropic action, the level of liver lipids (of the 100 g. rat) having been lowered by 0.34 g. in 1.01 g. by 0.5 % tyrosine in Exp. 12 (groups 52

Table 4. *Effect of glutamic acid on the fat-fatty liver*

Group	Dietary supplement	Change in body wt. ($\pm$ % original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
Exp. 7. Basal diet A; period of feeding 14 days					
37	None	-5	4.8	14.54	0.70
38	0.2% glutamic acid	+1	4.6	21.56	0.98
Exp. 8. Basal diet E; period of feeding 18 days					
39	None	-4	4.1	14.80	0.60
40	5% mono-sodium glutamate	-5	4.2	13.98	0.58
Exp. 9. Basal diet E; period of feeding 18 days					
41	None	-2	4.9	19.34	1.01
42	3% mono-sodium glutamate	-2	4.8	20.79	1.07
Exp. 10. Basal diet E; period of feeding 16 days					
43	None	+2	5.9	28.64	1.68
44	0.5% glutamic acid	-1	5.8	29.66	1.72
45	2.5% glutamic acid	-1	5.8	31.36	1.80
46	10% glutamic acid	+2	5.4	30.07	1.62
47	0.58% mono-sodium glutamate	+4	5.6	29.91	1.68
48	2.88% mono-sodium glutamate	0	6.1	28.58	1.75
49	11.5% mono-sodium glutamate	0	5.5	28.25	1.55

and 53). This result was substantiated in Exp. 13, where 0.3% tyrosine lowered the liver lipids (of the 100 g. rat) from 1.15 g. in group 54 to 0.71 g. in group 55. 3.0% tyrosine, however, lowered the

Table 5. *Effect of tyrosine on the fat-fatty liver*

Group	Dietary supplement	Change in body wt. ($\pm$ % original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
Exp. 11. Basal diet A; period of feeding 14 days					
50	None	-5	4.8	14.54	0.70
51	0.2% tyrosine	-6	4.7	12.23	0.57
Exp. 12. Basal diet E; period of feeding 18 days					
52	None	-2	4.9	19.34	1.01
53	0.5% tyrosine	0	4.2	15.85	0.67
Exp. 13. Basal diet F; period of feeding 18 days					
54	None	-5	4.8	24.02	1.15
55	0.3% tyrosine	-7	4.1	17.58	0.71
56	3.0% tyrosine	-2	4.4	21.10	0.93
Exp. 14. Basal diet G; period of feeding 18 days					
57	None	-5	6.5	30.11	1.96
58	0.1% methionine	-6	6.0	31.04	1.87
59	0.3% tyrosine	-8	6.4	32.39	2.06
60	0.1% methionine + 0.3% tyrosine	-4	5.3	28.44	1.50

liver lipids by only 0.22 g. When these results were statistically analysed, the figures for the tyrosine groups were not significantly different from those for the control group. Exps. 11 and 14 do not give any indication of a possible lipotropic action of tyrosine. It would appear, therefore, that tyrosine has little if any action on the liver lipids of animals with fat-fatty livers.

Table 6. *Effect of tyrosine on the cholesterol-fatty liver*

Group	Dietary supplement	Change in body wt. ($\pm$ original wt.)	Liver (% of final body wt.)	Fatty acids	
				In 100 g. fresh liver g.	In liver of 100 g. rat g.
Exp. 15. Basal diet C; period of feeding 17 days					
61	None	- 7	5.3	21.03	1.12
62	0.3% tyrosine	- 2	4.3	16.41	0.71
63	3.0% tyrosine	-10	4.3	13.62	0.58
Exp. 16. Basal diet C; period of feeding 21 days					
64	None	- 5	4.3	13.24	0.57
65	0.3% tyrosine	- 6	3.9	10.98	0.43
66	3.0% tyrosine	- 7	3.9	11.21	0.43
Exp. 17. Basal diet D; period of feeding 18 days					
67	None	- 5	5.6	25.34	1.42
68	0.5% tyrosine	- 8	5.6	26.31	1.47
Exp. 18. Basal diet D; period of feeding 16 days					
69	None	- 6	7.1	33.11	2.35
70	0.5% tyrosine	- 4	7.2	30.33	2.18
71	None	- 6	8.2	34.73	2.83
72	0.5% tyrosine	0	8.2	33.83	2.76

In only one of these four experiments with the cholesterol-fatty liver does tyrosine exert any significant effect on the liver lipids (Table 6), this being in Exp. 15, where 0.3% tyrosine lowers the liver lipids of the 100 g. rat from 1.12 g. in group 61, to 0.71 g. in group 62, and 3.0% tyrosine lowers the level to 0.58 g. (group 63). In the other three experiments tyrosine exerts no significant effect on the amount of liver fat. These findings appear to confirm the above conclusion that tyrosine lacks any lipotropic activity. In one experiment (Exp. 14) 0.3% tyrosine and 0.1% methionine together have exerted an action on the level of liver lipids which is greater than that exerted by 0.3% tyrosine or 0.1% methionine alone, and it was this result which led to a belief that in conjunction with other amino-acids tyrosine may influence the level of liver lipids. In later experiments, therefore, when amino-acid fractions of equivalent methionine and cystine content were compared for action on liver lipids, the tyrosine contents were also equalized, so that the true effect of amino-acids other than these three should become apparent.

Experiments with protein hydrolysates

It was now becoming obvious that the problem of the lipotropic action of proteins should be tackled from another angle, and the most obvious method was the examination of the action of various fractions of the protein molecule, formed by hydrolysis on the liver lipids of animals with fatty livers, since it is plain from the work of Channon *et al.* [1940] that cystine and methionine alone cannot explain the lipotropic action of proteins.

Casein hydrolysate. Up to the present there has been only one recorded observation concerning the lipotropic action of a protein hydrolysate. Carter & Melville [1940] found that hydrolysed caseinogen, with tryptophan added to replace that lost during hydrolysis, had the same activity as the original caseinogen. The amount of the amino-acid added appears to have been arbitrary, and no actual determinations were made. In this work, however, no account was taken of the possible loss of cystine and methionine during hydrolysis. We have found that as much as 50 % of these amino-acids may be lost during hydrolysis by the method employed. This result is, therefore, of doubtful value.

We, therefore, compared the lipotropic action of unhydrolysed caseinogen with that of hydrolysed caseinogen to which methionine and cystine and tyrosine were added in amounts to ensure an intake of these amino-acids equal to that provided by the original caseinogen. Possible effects of tryptophan were, for the present, ignored.

Three groups of rats (73-75) received basal diet G. Group 73 served as control, group 74 received a supplement of 15 % caseinogen, and group 75 the hydrolysis products equivalent to a 15 % supplement of caseinogen with the addition of sufficient cystine, methionine and tyrosine to bring the intakes of these amino-acids up to the level of the caseinogen group. The analytical figures concerning the caseinogen and its hydrolysate are recorded in Table 7.

Table 7. *Amino-acid content of caseinogen and hydrolysed caseinogen*

Material	Total content (g.) of				
	Solids	Nitrogen	Cystine	Methionine	Tyrosine
Caseinogen	315	40.05	1.55*	8.37*	18.60*
Total hydrolysis products of 315 g. caseinogen	269	36.50	0.60	6.86	12.26
Recovered after hydrolysis (% of total)	85	81	39	82	66

* Based on the following percentage contents: cystine 0.5; methionine 2.75; tyrosine 6.0.

Table 8. *Effect of hydrolysed caseinogen on the fat-fatty liver*

Exp. 19. Basal diet G; period of feeding 16 days

Group	Dietary supplement	Change in body wt. (± % original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
73	None	-9	5.95	33.94	2.02
74	15 % caseinogen	+7	3.98	13.49	0.54
75	Hydrolysate of 15 % caseinogen	-5	3.85	14.28	0.56

The results (Table 8) show that supplemented hydrolysed caseinogen has the same lipotropic action as the unhydrolysed caseinogen, for even though the animals receiving the hydrolysate lost 5 % of their weight, while those receiving caseinogen gained 7 %, previous experience shows that these differences are not of sufficient magnitude to influence the liver-fat deposition. Thus the conclusion to be drawn from this experiment is that if there is present in caseinogen one or more compounds which affect liver-fat deposition, other than cystine, methionine and tyrosine, these further compounds are stable to acid hydrolysis. Since tryptophan is in part destroyed during hydrolysis, this result implies that this amino-acid plays only a minor, if any, part in liver-fat deposition.

Butanol fractions of caseinogen hydrolysates. At this point we tested the action of fractionated caseinogen hydrolysates, and the groups of amino-acids soluble or insoluble in butanol were chosen as the most readily prepared fractions for the initial experiments.

When the action of caseinogen and the butanol-soluble and insoluble fractions of the amino-acids derived from it were compared, additions of cystine, methionine and tyrosine were again made so that the intake of these 'active' amino-acids was the same as that in the group receiving caseinogen. It also was considered desirable to equate the N intake with that of the caseinogen group; suitable amounts of gelatin were therefore added, since gelatin has been shown to be without lipotropic action except at high levels [Best, Grant & Ridout, 1936; Channon *et al.* 1938*a*]. An additional group received 10 % gelatin also supplemented to bring the intake of the 'active' amino-acids to the same level as that of the caseinogen group. A further group received blood fibrin, since it was desirable to obtain more information regarding the action of this protein than had previously been obtained [Channon *et al.* 1938*a*].

The analytical data for the various amino-acids, and for the protein fractions used in this experiment, are presented in Table 9. The basal diet G (Table 1) was used and was supplemented in the five experimental groups with 10 % caseinogen (77), 6 % butanol-soluble amino-acids and 4 % gelatin (78), the butanol-insoluble amino-acids equivalent to 10 % caseinogen with gelatin sufficient to equate the N intake to that of group 77 and 78 (79), 10 % gelatin (80) and 10 % blood fibrin in group 81. Cystine, methionine and tyrosine were added to groups 77-81 in amounts sufficient to ensure that the intake of these amino-acids in these groups was equivalent.

Table 10 shows that the addition of 10 % caseinogen has lowered the liver fat from the control figure of 1.96 g. (group 76) to 0.75 g. in group 77, while

Table 9. *Amino-acid content of proteins and of amino-acid mixtures used in Exp. 20*

Material	Content (%) of		
	Methionine	Cystine	Tyrosine
Gelatin	0.5	0.1	0.0
Blood fibrin	2.0	1.5	4.4
Caseinogen	2.75	0.5	6.0
Butanol-soluble amino-acids (yield: 20% of original caseinogen)	4.6	0.5	4.1
Butanol-insoluble amino-acids from 500 g. caseinogen (233 ml. of a non-crystallizable syrup containing 189 g. solids)	0.61	0.29	0.91

Table 10. *Effect of various proteins and supplemented fractions of hydrolysed caseinogen on the fat-fatty liver*

Exp. 20. Basal diet G; period of feeding 16 days

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
76	None	-8	6.51	30.16	1.96
77	10% caseinogen	+6	4.28	17.58	0.75
78	6% butanol-soluble amino-acids	-3	3.50	10.42	0.37
79	3.8% butanol-insoluble amino-acids	+3	4.72	24.44	1.15
80	10% gelatin	-1	4.05	19.72	0.80
81	10% blood fibrin	+8	4.87	21.35	1.06

in group 78, which received the butanol-soluble amino-acids, the amount of liver fat is even lower at 0.37 g.; while the difference between these two latter figures is not striking, it is statistically significant. Since groups 77 and 78 both received the same intake of the amino-acids known to be active, it is thus a reasonable deduction that the butanol-soluble fraction contains either more of a further amino-acid which decreases liver fat or less of one which accelerates liver-fat deposition than that present in caseinogen. This conclusion seems supported by the results for group 79 (Table 10), which received the butanol-insoluble amino-acid fraction, for while the amount of liver fat (1.15 g.) is again not strikingly different from that for the caseinogen group 77 (0.75 g.), the difference is in the reverse direction from that of the butanol-soluble group and is of similar magnitude. The fact that the amount of butanol-soluble amino-acids fed was three times greater than their yield from 10% caseinogen does not, of course, affect this conclusion, although it is impossible to deduce more from these findings than that the lipotropic action of caseinogen may not be accounted for by the actions of its contained cystine, methionine and tyrosine.

In apparent contrast with this conclusion are the results for group 80, which received gelatin which was also supplemented with cystine, methionine and tyrosine. Gelatin supplemented in this way, so

that the intakes of the above amino-acids were the same as in group 77, had an action indistinguishable from caseinogen, even though it is known to be without action when unsupplemented. While the results of this experiment seem in the light of previous experience to be convincing, their admittedly unexpected nature made it desirable to confirm them, and Exp. 21 was, therefore, carried out under the exact conditions of Exp. 20, save that the action of fibrin was not investigated further.

Table 11. *Effect of proteins and supplemented fractions of hydrolysed caseinogen on the fat-fatty liver*

Exp. 21. Basal diet G; period of feeding 17 days

Group	Dietary supplement	Change in body wt. (±% original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat g.
82	None	-10	5.40	26.78	1.45
83	10% caseinogen	+1	4.16	14.05	0.59
84	6% butanol-soluble amino-acids	-6	3.66	9.63	0.35
85	3.8% butanol-insoluble amino-acids	-1	4.38	18.88	0.83
86	10% gelatin	-2	3.91	17.01	0.67

The results of this experiment (Table 11) confirm in every particular those of Exp. 20. Particularly interesting from the immediate point of view is the confirmation of the finding that when gelatin, which alone is devoid of lipotropic action, is supplemented with cystine, methionine and tyrosine, its action is indistinguishable from that of caseinogen. It will be remembered that in groups 78 and 79 of Exp. 20, as well as in groups 84 and 85 of the present experiment, it was considered desirable to equate the N intake to that of the caseinogen groups, and for this purpose gelatin, a protein known to be lipotropically inactive, was used. As a result, the immediate conclusion from the previous and present experiments that it is possible to prepare from caseinogen hydrolysates fractions which, irrespective of their contents of cystine, methionine and tyrosine, exert actions of varying magnitude on liver-fat deposition, may not be valid because of the possible interfering action of the gelatin supplements. We therefore compared with that of caseinogen the action of a similar amount of gelatin alone, and supplemented with cystine, methionine and tyrosine in amounts which were such that the intake of these amino-acids was similar to those of the caseinogen group; at the same time study was made of the action of similar amounts of these amino-acids alone.

Basal diet G (Table 1) was supplemented as follows: group 95, 15% caseinogen; group 98, 15% gelatin; group 99, 15% gelatin plus sufficient cystine, methionine and tyrosine to make the level

of these amino-acids up to that present in the caseinogen supplement of group 95; group 100, cystine, methionine and tyrosine in amounts equivalent to those in the 15% caseinogen supplement.

Table 12. *Effect of gelatin and some caseinogen fractions on the fat-fatty livers*

Exp. 23. Basal diet G; period of feeding 16 days

Group	Dietary supplement	Change in body wt. ($\pm$ % original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat
94	None	-9	5.95	33.94	2.02
95	15% caseinogen	+7	3.98	13.49	0.54
96	2.6% fraction I	-3	3.74	13.44	0.50
97	9.8% fraction II	-4	4.15	14.38	0.59
98	15% gelatin	-7	5.38	33.09	1.78
99	15% gelatin + cystine, methionine and tyrosine	-4	3.53	13.15	0.46
100	Cystine, methionine and tyrosine equivalent to 15% caseinogen	-4	3.65	14.74	0.54

A comparison of the liver-fat deposition in group 95 (Table 12) with that in group 99 provides yet further confirmation that the action of gelatin, when supplemented with active amino-acids, is indistinguishable from that of an equivalent weight of caseinogen. The results for group 98 demonstrate again that unsupplemented gelatin is without effect. The result of most immediate interest is that given by group 100, where it is very clearly demonstrated that an intake of an amount of cystine, methionine and tyrosine equal to that present in a 15% caseinogen supplement has an action identical with that of a 15% caseinogen supplement itself. This result suggests that the lipotropic action of caseinogen depends solely on its content of these three amino-acids, and that it was purposeless to pursue the investigation further. On the other hand, the findings in Exps. 20 and 21 seem to be in conflict with this view. In both experiments the amounts of fat deposited in the livers of the groups receiving the butanol-soluble and insoluble fractions differ not only from each other, but also from that deposited in the caseinogen groups, by amounts which are statistically significant. This conclusion is supported by the findings of Exp. 23, which show that the additions of gelatin made in groups 78 and 79 of Exp. 20 and in groups 84 and 85 of Exp. 21, which were made in order to equate the N intakes to that of the caseinogen groups, has had no influence on the liver-fat deposition. If this be so, something other than cystine, methionine and tyrosine must account for the differences resulting from the administration of the fractions soluble and insoluble in butanol, and the investigation was therefore carried further.

Cu salt fractions of casein hydrolysate. In Exps. 20 and 21 the butanol-soluble amino-acids were fed

as a 6% supplement, an amount chosen because it equated, without further supplement, the methionine intake of the groups receiving it with that of the caseinogen groups. In order to attempt to obtain a more quantitative comparison with caseinogen this fraction was now fed in the amount which would be derived from the weight of caseinogen with which it was being compared; this procedure had been adopted in the case of the butanol-insoluble amino-acids in both Exps. 20 and 21 and was again repeated for this fraction. No attempt was made to equate the N intake for the reasons already discussed, and the use of gelatin was, therefore, discontinued.

At the same time it was felt desirable to compare further fractions from the butanol-soluble amino-acids with the unfractionated acids, since the results of Exps. 20 and 21 suggested that if there did exist one or more unknown lipotropically active constituents, the study of this fraction was likely to be most profitable. For this purpose the Cu salt separation described in the experimental section was used and three fractions of amino-acids prepared: (a) with Cu salts insoluble in H_2O , (b) with Cu salts soluble in H_2O and insoluble in dry CH_3OH , and (c) with Cu salts soluble in H_2O and soluble in dry CH_3OH .

Methionine, cystine and tyrosine estimations were carried out on each fraction and the analytical data for these three fractions and for the butanol-soluble and insoluble amino-acids are presented in Table 13.

Table 13. *Amino-acid contents of the fractions of caseinogen used in Exp. 22*

Material	Content (%) of		
	Cys-tine	Methio-nine	Tyro-sine
(a) Butanol fractionation			
Caseinogen	0.5	2.75	6.0
Butanol-soluble amino-acids (20% of caseinogen)	0.3	5.1	4.0
Butanol-insoluble amino-acids (183 g. from 500 g. caseinogen)	0.46	0.88	1.15
(b) Cu salt fractionation of butanol-soluble amino-acids (125 g.)			
Amino-acids with Cu salts insoluble in H_2O (42 g.)	0.43	5.34	4.40
Amino-acids with Cu salts soluble in H_2O and insoluble in CH_3OH (28 g.)	0.28	5.68	4.17
Amino-acids with Cu salts soluble in H_2O and soluble in CH_3OH (32 g.)	0.11	1.28	4.05
Total recovered after fractionation* (g./100 g. starting material)	76.0	66.5	86.0

* The total weight of the three fractions was 102 g., i.e. 82% of the weight of the starting material.

Although neither Town [1928, 1936] nor Caldwell & Rose [1934] claim that the Cu salt method gives a clean separation of amino-acids into three groups, the figures given in Table 13 for the cystine,

methionine and tyrosine contents of the three fractions show that in our hands the separation is not good. Town [1941] has recently stated that the presence of NH_3 and of the dicarboxylic amino-acids greatly interferes with the Cu salt separation, and that these substances must be removed before attempting the fractionation. Since this was not done in these investigations, it may account for the poor separation of the Cu salts of the above-mentioned amino-acids.

Basal diet G (Table 1) was supplemented as follows: group 88, 15% caseinogen; group 89, 3% butanol-soluble amino-acids, which represented the weight of these amino-acids derived from 15% caseinogen; group 90, 5.6% butanol-insoluble amino-acids, equivalent to 15% caseinogen; groups 91–93, the amino-acids obtained from the different Cu salt fractions of the butanol-soluble fraction in amounts derived from 3% of the latter, and thus equivalent to 15% caseinogen. In addition, groups 89–93 received supplements of methionine, cystine and tyrosine in amounts calculated on the basis of the analyses recorded in Table 13, so that these groups obtained the same intake of these acids as did group 88, which received 15% caseinogen.

Table 14. *Effect of caseinogen and fractions thereof and of fractions of the butanol-soluble amino-acids on the fat-fatty liver*

Exp. 22. Basal diet G; period of feeding 16 days

Group	Dietary supplement	Change in body wt. ($\pm$ % original wt.)	Liver (% of final body wt.)	Fat (% of fresh liver wt.)	Fat in liver of 100 g. rat
87	None	-9	4.79	26.52	1.27
88	15% caseinogen	+5	3.96	10.12	0.40
89	3% butanol-soluble amino-acids	-4	3.54	8.61	0.31
90	5.6% butanol-insoluble amino-acids	0	3.60	14.00	0.51
91	1% amino-acids with Cu salts insoluble in H_2O	-8	4.02	11.56	0.47
92	0.7% amino-acids with Cu salts soluble in H_2O and insoluble in CH_3OH	-6	4.39	21.61	1.00
93	0.8% amino-acids with Cu salts soluble in H_2O and soluble in CH_3OH	-6	3.93	16.75	0.66

The results (Table 14) confirm the presence in caseinogen of a factor or factors other than cystine, methionine and tyrosine, which plays some part in the lipotropic action of caseinogen. In spite of the fact that groups 88–93, inclusive, received equivalent intakes of cystine, methionine and tyrosine, the amounts of fat deposited in the livers of rats varied from 21.6% in group 92 to 8.6% in group 89.

These results introduce two possibilities: that there exists in caseinogen either a substance with a methionine-like action which is concentrated in the butanol-soluble amino-acids and from there into the

group of amino-acids having Cu salts insoluble in H_2O , since this group (91) has an action indistinguishable from caseinogen, or a substance with a cystine-like action, which cannot exert this action until it is separated—as for example by the copper-salt method—from some inhibiting factor. The result of group 92 lends some belief to the latter idea since the level of liver fat in this group is very much greater than that in the caseinogen group (89), although both groups received equivalent intake of the known active amino-acids.

From an examination of the analytical data for the butanol-soluble and insoluble groups of amino-acids, it will be seen that together these make up only a little over 57% of the original caseinogen, thus leaving approximately 43% unaccounted for. The action of this fraction on liver fat was investigated in Exp. 23. Two fractions of hydrolysed caseinogen were examined, fraction II, which is the sum of the butanol-soluble and insoluble amino-acids, and fraction I, which is the rest of the hydrolysed caseinogen. The hydrolysate from 315 g. caseinogen was concentrated *in vacuo* to 650 ml. and allowed to crystallize. The amino-acids which separated were filtered off and dried. This comprised fraction I. The filtrate was concentrated as far as possible by vacuum distillation, when about two-thirds of the material was obtained in the solid form and the rest in the form of a non-crystallizable syrup. The solids plus syrup constituted fraction II. Cystine, methionine and tyrosine determinations were carried out on these fractions and the analytical data recorded in Table 15.

Table 15. *Amino-acid contents of the fractions of hydrolysed caseinogen used in Exp. 23*

Material	Amounts		Total content (g.) of		
	g.	% of starting material	Cys-tine	Methio-nine	Tyro-sine
Caseinogen	315	100	1.55	8.37	18.60
Fraction I	52	17	0.10	3.41	8.84
Fraction II	206	65	0.39	3.11	2.09
Fractions I and II	258	82	0.49	6.55	10.39
Total recovered after hydrolysis (% of that originally present)	—	82	32	78	59

Groups 96 and 97 were supplemented by fractions I and II respectively in amounts equivalent to a 15% supplement of caseinogen. Groups 96 and 97 were supplemented with cystine, methionine and tyrosine in amounts such that the intakes of these amino-acids were equivalent to that of a 15% caseinogen supplement (group 95). The results for these groups have already been recorded in Table 12.

Clearly the amounts of fat in the livers of animals, in groups 95, 96 and 97 are indistinguishable, and the results support those for groups 95, 98, 99 and

100, in showing that cystine, methionine and tyrosine alone may account for the lipotropic action of caseinogen.

We have now arrived at the point where two series of experiments have indicated opposing solutions to the problem of accounting for the lipotropic activity of caseinogen.

DISCUSSION

The results of Exps. 20-22 make it clear that cystine, methionine and tyrosine alone cannot explain the lipotropic activity of caseinogen, since groups of rats receiving equivalent intakes of the above amino-acids have liver-fat levels which are significantly different. Furthermore, these differences are large enough for the conclusion to be drawn that there exists in the protein molecule some new factor which affects the level of liver lipids, and that this factor varies in amount in the different fractions examined. Although in Exps. 20 and 21 the results are complicated by the presence of gelatin, in Exp. 22 this is not the case, and here the differences are clearly demonstrated. The butanol-soluble amino-acids have a greater lipotropic action than an equivalent amount of caseinogen. Furthermore, the figures for the liver-fat levels of the groups of animals fed the amino-acids fractionated by the Cu salt method show very distinct differences, even though the cystine, methionine and tyrosine intakes were equivalent in the three groups.

The experiments with pure amino-acids have given no clue to a further factor which affects liver fat. Of the 14 amino-acids examined so far, none has shown any significant effect. Interesting in itself, although casting no light on the general problem, was the observation that glutamic acid exerts a lipotropic action on the cholesterol-fatty liver but not on the fat-fatty liver. There would appear to be no adequate explanation of this fact at present, but it may be bound up with the fact that both caseinogen and egg albumin, which were used as basal proteins, contain considerable amounts of glutamic acid, and at the levels at which they are fed contribute over 1% of that amino-acid to the diet. Glutamic acid is, therefore, the only substance which has so far been encountered in this laboratory which exerts an effect on the cholesterol-fatty liver and yet is without action on the fat-fatty liver. Tryptophan was found in one experiment to possess only a small lipotropic action, and this is not sufficient evidence for any concrete conclusion to be drawn about this amino-acid. Tyrosine also has a questionable action on liver fat.

Singal & Eckstein [1941] found that *dl*-valine, *dl*-leucine and *dl*-isoleucine had no effect on liver-fat deposition. In addition, it has been found in this

laboratory that glycine also has no action on the fatty liver [Manifold, 1939], so that the only amino-acids which have not been examined are threonine, nor-leucine, nor-valine and hydroxyglutamic acid, and the possibility exists that one of these may show significant action on the fatty liver.

The conclusions to be drawn from the results obtained with supplemented gelatin in Exps. 20, 21 and 23 would appear to be in opposition to that already drawn from Exps. 20-22, and indicate that cystine, methionine and tyrosine alone account for the lipotropic activity of caseinogen. Tucker *et al.* [1940] came to a similar conclusion when they found that the lipotropic action of a low-protein (5% caseinogen or edestin), high-fat diet, so supplemented with cystine and methionine that its content of these amino-acids was equal to that of a 20% caseinogen diet, was similar to that observed when the level of caseinogen in the diet was 20%. On the other hand, Best & Ridout [1940] found that cystine and methionine in amounts equivalent to a 30% caseinogen supplement had little lipotropic action compared with that of a 30% caseinogen supplement. Now Channon *et al.* [1940] showed that for both free cystine and methionine there is a limiting intake above which no further increase in the amounts of these amino-acids ingested had any influence on the level of liver fat, and these authors came to the conclusion that the action of cystine and methionine alone cannot explain the lipotropic activity of caseinogen.

A finding which further complicates this work is that of Treadwell *et al.* [1942], whose experimental data seem to indicate that diets supplemented with methionine, as the free amino-acid, were superior in lipotropic action to the diets containing equivalent amounts of methionine in the form of protein. Such a finding casts a certain amount of doubt on the conclusion drawn from the result of Exp. 23 (and of parts of Exps. 20 and 21) concerning the supplemented gelatin.

The balance of this work appears to be in favour of the existence of at least one unknown factor in caseinogen which affects the level of liver fat. The results with the butanol-soluble amino-acids would lead one to assume the presence of a lipotropic factor, whereas the results of Exp. 22 (groups 91, 92 and 93) lead to the conclusion that there exists another substance, with a cystine-like action, which is not manifest in the presence of certain other protein constituents.

It is abundantly clear that much further work is necessary before any definite conclusions may be drawn concerning this problem. A further factor which may influence the action of fractions of hydrolysed caseinogen is that we have no knowledge of the fate of the amino-acids which are partially destroyed during hydrolytic and other treat-

ments. The possibility exists that they may be converted into substances which have actions on the fatty liver different from that of the amino-acids in the original form. An example of this reversal of action is given by the work of Singal & Eckstein [1941], who found that whereas cystine accelerated liver-fat deposition, cystine betaine had the opposite action, and another substance having a lipotropic action was dithiodiglycollic acid.

It is to be regretted that conditions did not allow of our continuing this investigation, and consequently our results are presented in an incomplete form.

SUMMARY

1. Fourteen pure amino-acids were investigated with a view to ascertaining whether or not they had any effect on the deposition of fat in the livers of rats on diets high in fat and low in lipotropic factors.

2. Tryptophan was found to possess a small lipotropic action in one experiment, and tyrosine to

possess a small but questionable action on the fat- and cholesterol-fatty livers.

3. Glutamic acid was found to exert a lipotropic action on cholesterol- but not on fat-fatty livers.

4. The lipotropic actions of various protein fractions were studied. Evidence was obtained that some factor which affects liver-fat deposition, other than cystine, methionine and tyrosine, is present in the protein molecule. This factor can be concentrated in the butanol-soluble amino-acid fraction. Fractionation by the copper-salt method effected further concentration of the unknown factor and indicated the possible presence of a substance which accelerates liver-fat deposition. Conflicting results have been obtained in experiments with gelatin, and in one experiment with caseinogen fractions, and more work is necessary to elucidate the problem completely.

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A Biological Method for Estimation of Pantothenic Acid with Rats, in which Wheat Germ is included in the Basal Diet

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Investigations on the B₂ group of vitamins made with chicks fed on a heated diet of mixed grains, and with rats on synthetic diets supplemented by purified aneurin (vitamin B₁), riboflavin, and pyridoxine (vitamin B₆), have led to the discovery that the filtrates obtained when extracts of yeast, liver or rice bran are treated with Fuller's earth contain substances essential for growth of these animals

[Lepkovsky, Jukes & Krause, 1936; Elvehjem & Koehn, 1935]. These substances, generally known as filtrate factors, were called factor 2 by Lepkovsky and his colleagues. Chicks deprived of filtrate factors are checked in growth and develop a characteristic dermatitis; young rats also show subnormal increase in weight and a less specific skin affection with greying of the pigmented fur. In due course