THE EFFECT OF DIETARY PROTEINS AND AMINO ACIDS ON LIVER FAT

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The recognition of protein as a dietary factor affecting the outcome of investigations involving lipotropic substances (1–5) and the subsequent discovery that cystine (6) and methionine (7) exert marked effects on the level of liver fat in rats on low choline diets occasioned several attempts to determine whether these two amino acids alone were involved or whether others were also concerned in the phenomenon. Best and Ridout (8) and Channon, Manifold, and Platt (9) reported that dietary casein exerts a distinctly stronger lipotropic effect than does the methionine and cystine contained in it (suggesting the presence in protein of other active constituents), but Tucker, Treadwell, and Eckstein (10) found just the opposite. 2 years later Treadwell, Groothuis, and Eckstein (11) published further experimental data indicating that the free amino acids were more effective in reducing liver fat than were similar quantities fed as casein. The lipotropic effect of protein and amino acids is discussed briefly in a review (12) published shortly after the appearance of the paper of Treadwell et al., but at that time no explanation could be offered for these conflicting conclusions.

The data in Table I reveal at least five apparently small, but possibly highly significant differences between the conditions used by Best and Ridout and by Tucker et al. It was considered desirable to reinvestigate the matter (a) with a constant nutritional background, (b) at different levels of dietary casein, and (c) to equalize the total nitrogen intake of the rats on the two series of diets (i.e., those containing casein and those containing corresponding amounts of methionine and cystine as free amino acids) by adding to the diets of the latter group a protein known to produce little, if any, lipotropic effect. Gelatin appeared to be suitable for this purpose (3, 13, 14). Further, in order that the diets under comparison might be more nearly alike, it was proposed (d) to add essential amino acids to those of a second group of animals on the diets containing methionine plus gelatin to correct the known deficiencies of these rations. This was considered important, since a preliminary study, suggested by consideration of the data in Table I, had shown that methionine exerts a markedly greater lipotropic effect in the absence (or deficiency) of certain of the essential
amino acids than in their presence (14). Finally, the age of the rat has been shown to influence strongly the deposition of fat in the liver under certain conditions (14–16). This factor differed in the studies mentioned above but was kept constant in the present investigation.

EXPERIMENTAL

Fourteen test diets (Series 1 to 5, Table II) were prepared, in each of which the total protein was 35 per cent.¹ Four diets (Series 6), containing 40 per cent protein, and a control diet containing 40 per cent of gelatin, were subsequently added. Diet A in each series contained the amount of casein shown in Table II. Diet B in each series contained exactly the same total quantities of methionine and cystine as did the corresponding diets in Group A. The remainder of the nitrogenous moiety of the B diets was supplied by gelatin. Diet C in each series contained total methionine and cystine equivalent to that in Diets A and B, but included also supplements of those essential amino acids which are absent from or markedly deficient in gelatin, but are present in the casein-containing diets.

The scope of the experiment necessitated a compromise in the attempt to balance the sulfur-free essential amino acids in the various diets because

¹ Unpublished work of the authors had shown that in short term experiments the weight of young adult rats could be maintained on a high fat diet (fed ad libitum), the protein moiety of which was comprised of 10 per cent casein and 25 per cent gelatin.

### Table I

**Comparison of Experimental Conditions Used in Previous Attempts to Account for Lipotropic Action of Casein**

<table>
<thead>
<tr>
<th>Basal protein</th>
<th>Best et al.</th>
<th>Eckstein et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplements compared</td>
<td>Diet A</td>
<td>Casein, 30%</td>
</tr>
<tr>
<td></td>
<td>“ B</td>
<td>Methionine, 0.96%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cystine, 0.10%</td>
</tr>
<tr>
<td>Daily food consumption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>“ N intake</td>
<td></td>
<td>476 mg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>76</td>
</tr>
<tr>
<td>Essential amino acids</td>
<td></td>
<td>Abundant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grossly inadequate</td>
</tr>
<tr>
<td>Weight of rats, gm.</td>
<td></td>
<td>200</td>
</tr>
</tbody>
</table>

*This beef muscle powder, which contained 2.90 per cent methionine and 0.89 per cent cystine, contributed 145 mg. and 44.5 mg., respectively, of these amino acids per 100 gm. to both Diets A and B.*
of the excessive cost and current limited availability of the amino acids involved. Because 10 per cent of casein in such a diet prevented any weight loss when fed ad libitum, supplementary essential amino acids were added to the gelatin diets to make the total amount of each correspond with that in the diet (No. 1A) containing 10 per cent of casein. Additions of \((-\)-tryptophane, \(+(\)-isoisoleucine, \(-\)valine, and \(-\)-threonine were made, since these are either absent from gelatin or present in quantities very much smaller than in casein. Probably some histidine and phenylalanine should have been included in the supplements, but at the time (1943) they were not available. Because the analytical figures reported from different laboratories often vary considerably, rather arbitrary decisions had to be made as to the values adopted. Table III shows the values chosen and gives an estimate of the amino acid composition of five of the diets used. In Diet 6D not only were the methionine and cystine balanced exactly on Diet 6A, but the essential amino acid supplements were increased correspondingly, thus giving two casein levels (10 and 40 per cent) at which equivalence in this respect was approximated.

The percentage composition of the basal diet (referred to in Table IV as Diet 0) was gelatin\(^2\) \(^2\) 35, beef dripping 40, sucrose 18, salts 5 (McCollum

\(^2\)This gelatin contained 1.02 per cent methionine and 0.04 per cent cystine.

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**Table II**

*Supplements Used in Test Diets*

The supplements of casein and amino acids were added at the expense of an equal weight of gelatin. The amounts are given in per cent.

<table>
<thead>
<tr>
<th>Series No.</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Casein(^*)</td>
<td>Methionine</td>
<td>Cystine</td>
<td>Methionine</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>0.194</td>
<td>0.045</td>
<td>0.223</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>0.291</td>
<td>0.068</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>0.387</td>
<td>0.091</td>
<td>0.417</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>0.484</td>
<td>0.113</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>0.677</td>
<td>0.158</td>
<td>0.707</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>0.774</td>
<td>0.180</td>
<td>0.803</td>
</tr>
</tbody>
</table>

\(^*\)Labco casein (2.93 per cent methionine and 0.49 per cent cystine).

\(^†\)The essential amino acids added to the Group C diets were, in per cent of the diet, \((-\)-tryptophane 0.22, \(+(\)-isoisoleucine 0.30, \(-\)valine 1.58, \(-\)-threonine 0.74.

\(^†\)The supplements used in Diet 6D, which was fed at a later date, were based on more recent data and therefore differ slightly from the ratios used in the Group C diets: \((-\)-tryptophane 0.88, \(+(\)-isoisoleucine 1.20, \(-\)valine 5.14, and \(-\)-threonine 2.12 per cent, respectively.
Salt Mixture 185 (38)), agar 2, cod liver oil concentrate 0.015. When Series 6 was later added, a new basal diet was required to control it. This

**Table III**

*Amino Acids in Representative Diets*

Calculated in mg. per 100 gm. of the diet from data in the second and fourth columns and the supplements listed in Table II.*

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Casein Biblio-</th>
<th>Gelatin Biblio-</th>
<th>Diet No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
<td>1A</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.5 (17)</td>
<td>27.0 (30)</td>
<td>6000</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.5 (18)</td>
<td>9.2 (31)</td>
<td>2850</td>
</tr>
<tr>
<td>Serine</td>
<td>7.5 (19)</td>
<td>3.3 (32)</td>
<td>1580</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.9 (20)</td>
<td>1.4 (33)</td>
<td>740</td>
</tr>
<tr>
<td>Valine</td>
<td>6.3 (21)</td>
<td>2.5 (21)</td>
<td>1260</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.3 (21)</td>
<td>3.3 (21)</td>
<td>1760</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.1 (21)</td>
<td>1.7 (21)</td>
<td>1040</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6.7 (22)</td>
<td>3.4 (34)</td>
<td>1520</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>24.2 (23)</td>
<td>5.8 (34)</td>
<td>3870</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.1 (24)</td>
<td>8.9 (24)</td>
<td>2640</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.4 (25)</td>
<td>0.9 (25)</td>
<td>470</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.3 (25)</td>
<td>5.1 (25)</td>
<td>2000</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.49 †</td>
<td>0.04 †</td>
<td>57</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.93 †</td>
<td>1.0 †</td>
<td>543</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.2 (25)</td>
<td>2.6 (35)</td>
<td>1170</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.4 (26)</td>
<td>0 (34)</td>
<td>640</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>2.2 (27)</td>
<td>0 (34)</td>
<td>220</td>
</tr>
<tr>
<td>Proline</td>
<td>8.0 (28)</td>
<td>17.5 (36)</td>
<td>5180</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0 (28, 29)</td>
<td>14.7 (37)</td>
<td>3680</td>
</tr>
</tbody>
</table>

*For example, the methionine content of the diets is calculated as follows: in Diet 1A 293 mg. from 10 per cent casein plus 250 mg. from 25 per cent gelatin, totaling 543 mg.; in Diet 1B 348 mg. from 34.8 per cent gelatin plus 194 mg. in the supplement, totaling 542 mg.; in Diet 1C 319 mg. from 31.9 per cent gelatin plus 223 mg. in the supplement, totaling 542 mg.
† Unpublished analysis (J. M. R. B.).

The following B vitamins were injected subcutaneously daily in 0.5 cc. of physiological saline: 25 γ of thiamine chloride, 20 γ of pyridoxine, 20 γ of

* Obtained from Ayerst, McKenna and Harrison, Ltd., Montreal. It contains 50,000 I.U. per gm. of vitamin D and 500,000 I.U. per gm. of vitamin A.
riboflavin, 100 \( \gamma \) of calcium pantothenate, and 100 \( \gamma \) of nicotinic acid. Supplements of casein (Labco, fat-free, vitamin-free), methionine, cystine, and essential amino acids were given at the expense of the gelatin, as described in Table II. All diets within each series were balanced exactly with respect to total methionine and cystine content.

The major dry ingredients of the diets were mixed by hand and the minor ingredients were incorporated as follows: Each one was ground in a mortar

### Table IV

**Effect of Diets on Liver and Body Weights and on Liver Fat**

Fifteen rats were placed on each diet (except Diets 2A, 2B, 4A, and 4B, for which ten rats were used) for 21 days.

<table>
<thead>
<tr>
<th>Diet No.</th>
<th>Initial weight, average and range</th>
<th>Change in weight</th>
<th>Food consumed</th>
<th>Weight of moist liver (average)</th>
<th>Crude liver fatty acids (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>176 (145-209)</td>
<td>-26.5</td>
<td>7.0</td>
<td>6.33</td>
<td>1.28</td>
</tr>
<tr>
<td>1A</td>
<td>183 (116-213)</td>
<td>-5.2</td>
<td>6.9</td>
<td>8.32</td>
<td>2.13</td>
</tr>
<tr>
<td>1B</td>
<td>174 (117-215)</td>
<td>-27.2</td>
<td>6.9</td>
<td>5.81</td>
<td>0.73</td>
</tr>
<tr>
<td>1C</td>
<td>183 (126-226)</td>
<td>-11.1</td>
<td>7.1</td>
<td>9.73</td>
<td>2.98</td>
</tr>
<tr>
<td>2A</td>
<td>182 (117-198)</td>
<td>-6.2</td>
<td>7.1</td>
<td>7.71</td>
<td>1.55</td>
</tr>
<tr>
<td>2B</td>
<td>167 (139-230)</td>
<td>-30.5</td>
<td>6.7</td>
<td>5.41</td>
<td>0.50</td>
</tr>
<tr>
<td>3A</td>
<td>188 (123-202)</td>
<td>-4.6</td>
<td>7.1</td>
<td>6.63</td>
<td>0.73</td>
</tr>
<tr>
<td>3B</td>
<td>175 (138-236)</td>
<td>-27.3</td>
<td>6.8</td>
<td>5.67</td>
<td>0.50</td>
</tr>
<tr>
<td>3C</td>
<td>185 (154-213)</td>
<td>-14.1</td>
<td>7.0</td>
<td>6.74</td>
<td>1.11</td>
</tr>
<tr>
<td>4A</td>
<td>182 (127-198)</td>
<td>-3.0</td>
<td>7.2</td>
<td>6.63</td>
<td>0.57</td>
</tr>
<tr>
<td>4B</td>
<td>164 (149-215)</td>
<td>-24.2</td>
<td>7.0</td>
<td>5.77</td>
<td>0.72</td>
</tr>
<tr>
<td>5A</td>
<td>188 (127-207)</td>
<td>+1.3</td>
<td>7.7</td>
<td>7.13</td>
<td>0.51</td>
</tr>
<tr>
<td>5B</td>
<td>175 (133-220)</td>
<td>-26.7</td>
<td>7.4</td>
<td>5.72</td>
<td>0.57</td>
</tr>
<tr>
<td>5C</td>
<td>184 (134-226)</td>
<td>-7.8</td>
<td>7.2</td>
<td>6.83</td>
<td>0.80</td>
</tr>
<tr>
<td>6A</td>
<td>178 (119-247)</td>
<td>+9.3</td>
<td>6.9</td>
<td>7.24</td>
<td>0.48</td>
</tr>
<tr>
<td>6B</td>
<td>176 (117-244)</td>
<td>-29.2</td>
<td>6.6</td>
<td>5.38</td>
<td>0.40</td>
</tr>
<tr>
<td>6C</td>
<td>181 (112-254)</td>
<td>-7.6</td>
<td>6.4</td>
<td>6.94</td>
<td>0.78</td>
</tr>
<tr>
<td>6D</td>
<td>183 (119-254)</td>
<td>-15.6</td>
<td>5.7</td>
<td>6.10</td>
<td>0.59</td>
</tr>
<tr>
<td>7</td>
<td>179 (107-235)</td>
<td>-33.2</td>
<td>5.5</td>
<td>4.89</td>
<td>0.50</td>
</tr>
</tbody>
</table>

with a small portion of the dry mixture and the resulting powder was sifted through a 40 mesh screen over the main bulk of the diet, which was then blended thoroughly. The cod liver oil concentrate (dissolved in petroleum ether) was sprayed over the dry ingredients and mixing was continued until the solvent had evaporated. The complete dry mixture was incorporated, with vigorous agitation, into the melted beef dripping, and stirring was continued until the mass solidified. The diets were freshly prepared at about 7 to 10 day intervals and were stored in closed containers in a refrigerator.
Groups of rats (usually fifteen) of the Wistar strain, weighing from 107 to 254 gm., were placed on the test diets for 21 days. The rats in Groups A and C of each series were pair-fed with those of Group B in the same series. Group B was started 3 days ahead of the other groups and 3 day averages were used in determining the food to be given to Groups A and C. Usually an extra 0.5 gm. of the diet was offered daily to each of the pair-fed rats on Diets A and C to correct for the variable amount scattered. The actual average food consumptions are given in Table IV. If one of the control rats (Diet B) died, the corresponding rats on Diets A and C were pair-fed with the rat of closest weight on Diet B. The animals were killed by a blow on the head. Total crude liver fatty acids were determined in the usual way by saponification, acidification, and extraction with petroleum ether (b.p., 30–60°). For brevity the material so obtained is referred to in Table IV as liver fat.

DISCUSSION

Attempts to compare the previous studies (8, 11, 16) are complicated not only by the difference in the nature of the basal protein used and the different quantities of extra casein added, but also by the different vitamin supplements given (e.g., Best and Ridout supplied only thiamine chloride ("adequate amounts"), whereas Tucker et al. supplied the vitamin B complex as yeast tablets). The data here presented, covering the ranges of dietary casein used by the several groups of workers, offer a constant nutritional background with respect to total protein and vitamins, enabling comparisons to be made across the rows and down the columns of Table II.

Fig. 1 shows graphically all the values obtained by the analysis of the individual livers. The variation and the mode are easily seen by inspection, thus making possible a better assessment of the significance of any differences between the mean values reported in Table IV. Four values which were far removed from the others in their respective groups are shown. The failure to join them by a solid line to the other points for the same group indicates that they were not used in calculating the averages given in Table IV. Fig. 2 depicts the mean values obtained for liver fat (total crude fatty acids plus sterols) on the several diets used and shows the relative lipotropic effects over the whole range studied.

The liver fat values of the basal groups (Series 0 and 7) were unexpectedly low in view of the results of Best et al. (3) and of Beveridge et al. (14), who found that gelatin, when fed at a level of 20 per cent, exerted no demonstrable lipotropic effect. It should be recalled that Series 0 to 5 was run at a different time from Series 6 and 7 and hence any comparisons between them must be made with reservations; nevertheless, the marked decrease in liver fat produced by increasing the dietary gelatin from 35 to 40 per cent is noteworthy. Although gelatin alone (at a 20 per cent dietary level) per-
mitted a large accumulation of fat in the liver, Channon et al. (13) have shown that increasing amounts of gelatin added to a diet containing 8 per cent of egg albumin exert a progressive but limited action in decreasing liver fat.
The results on the Group A diets confirm previous reports that increasing the casein content of high fat, low choline diets occasions a progressive decrease in the liver lipids. The decrease is most marked between casein levels of 10 and 20 per cent, but an effect is noted up to the 40 per cent level, although even this quantity of casein does not bring the liver fat down to normal on these diets.

The average liver fat in the rats on the Group B diets is never very high, but the variation on any one diet is considerable, and there is no clear cut progressive decrease in liver fat with increasing dietary methionine, as is the case when comparable quantities of casein are fed (see Fig. 1).

Comparison of the results in Groups A and B reveals that at the 10, 15, and 20 per cent casein levels free methionine causes a greater lowering of liver fat than does a corresponding amount fed in casein (confirming Tucker et al.), but that at 25 and 35 per cent casein levels the casein-containing diets produce the greater lipotropic effect (confirming Best and Ridout). The curves in Fig. 2, which intersect at about 22 per cent casein, indicate that at this level and again at about 40 per cent the lipotropic effects of the two types of diet are about equal.

Although Diets A and B in each series were equalized as far as fat, total protein, and methionine and cystine were concerned, the diets of Group A contained reasonable quantities of the essential amino acids (supplied by the casein), while those of Group B lacked tryptophane entirely and were grossly deficient in valine, isoleucine, and threonine. When these defects were approximately corrected in Diets 1C and 6D and partially corrected in the other diets of Group C (in which the essential amino acids were kept constant throughout at levels approximately equivalent to those in the diet containing 10 per cent casein), the addition of increasing amounts of methionine lowered the liver fat progressively, based either on absolute weight of liver fat or on liver fat expressed as per cent of wet weight of the organ. The progressive decrease in liver fat with increasing amounts of the lipotropic factor, noted in the Group A diets but absent from those of Group B, is again clearly demonstrated in the Group C diets.

Comparison of the results of feeding Diet C with those from Diet B of each series reveals that, when a ration lacking or deficient in essential amino acids is supplemented with the quantities of these considered necessary to maintain weight in rats, the lipotropic effects of these diets are minimized and in some cases obliterated. These results, confirming the find-

4 Similar diets containing 10 per cent casein and 25 per cent gelatin, when fed ad libitum, sufficed to prevent loss in weight, as mentioned earlier (foot-note 1). Possibly due to the restricted food intake, the animals on the Group C diets did not maintain their weight, but the extensive losses noted on the Group B diets were considerably reduced.
ings recently published by Beveridge et al. (14), emphasize the importance of the nutritional adequacy of diets used in comparative studies of the lipotropic factors.

Increasing the quantity of the essential amino acids from those in a 10 per cent casein diet to those in a 40 per cent casein diet did not affect the liver fat appreciably (compare Diets 6C and 6D, Fig. 1).

Comparison of Curves A and C in Fig. 2 shows that all the groups that received the essential amino acids as supplements to the methionine plus gelatin diets had consistently higher liver fat levels than those on corresponding diets containing casein. These observations lead one to suspect that some lipotropic substance other than methionine occurs in casein, or that the synthetic amino acid preparations were antilipotropic, due either to the presence of some impurity or to the action of the unnatural forms of valine or threonine which were used.

In connection with the first suggestion, it should be noted that in the diets containing casein (Group A) one amino acid is present which is absent from those of Groups B and C. This amino acid is tyrosine. It is of considerable interest, therefore, to recall that Channon et al. and Beeston and Platt (9, 39) have suggested the possibility that tyrosine lowers liver lipids. More recently they have reported (40) that a diet containing methionine plus tyrosine gives a greater lipotropic effect than does either one alone. The evidence which they have presented is suggestive but not convincing. However, considering it in conjunction with the data here reported, there is obviously need for clarification of the point. It may be significant, in connection with the above diets, that casein contains a considerable amount of tyrosine, while the gelatin used in the basal ration has none. Experiments to examine the lipotropic action of tyrosine are being undertaken in this laboratory.

Some support for the second possibility appeared in a paper by Albanese and Irby (41), who reported that on a certain diet containing only essential amino acids (supposedly in the proportions found in casein) as the source of nitrogen the growth of young rats was subnormal; the mixture was inferior to a comparable amount of casein, or of casein hydrolyzed by acid or by pancreatic enzymes. They believed there was some evidence that the nutritive inadequacy of the essential amino acid diet may be due, at least in part, to the effects of the unnatural forms of certain amino acids, the non-utilizable enantiomorphs of which were postulated to be toxic. However, Kinsey and Grant (42) in a similar study obtained good growth at almost the same dietary level of total amino acid mixture. Several differences are apparent in the diets used and one or more of these may be of importance to the problem in hand. The fat component of the basal diet of Albanese and Irby consisted of cod liver oil and Crisco, at levels of about 4.5 and 17.5
EFFECT OF DIET ON LIVER FAT

per cent respectively. Kinsey and Grant fed cod liver oil and corn oil at levels of 2.0 and 10.0 per cent respectively. The former workers fed brew-

ers' yeast; the latter supplied the B vitamins as pure compounds. Further,
the ratios of the essential amino acids fed were quite different in the two
cases, and it is possible that isoleucine was deficient in the diet used by
Albanese and Irby. The more recent findings of Kinsey and Grant, con-

firming Rose (43), seem to be stronger evidence for the non-toxicity of
the unnatural enantiomorphs than does any evidence advanced in support of
the idea of a toxic effect. There may be some imbalance of the essential
amino acids in our diets, which like those of Albanese and Irby were
designed to simulate the ratios in casein. Their diets, like ours, are high
fat diets and, like ours, lack tyrosine, which may under these unusual
dietary conditions be an important factor. The problems raised by the
findings mentioned are of interest and will be investigated.

Beveridge et al. (14) and Horning and Eckstein (16) have compared the

lipotropic effects of free methionine and equivalent quantities fed as casein
in both young and adult rats. They found that free methionine was almost
equally effective in reducing liver fat in both age groups, but that casein (at
the level fed) was lipotropic in the adult rats only. The probable explana-
tion for these findings is that the amount of protein in the casein-supple-
mented ration supplied enough methionine for maintenance of adult rats
and left some over for lipotropic action, but in young rats the quantity of
methionine required for growth left little or none for other purposes.
However, the diets containing equivalent amounts of methionine (fed as
free amino acid), being deficient in essential amino acids, did not permit as
much growth in young rats and more methionine would thus be left for
lipotropic action. This explanation (which is similar to one suggested
originally by Griffith and Mulford (44), although theirs is stated in some-
what more general terms) is supported by the data in the present paper as
well as by those in several others (11, 14, 16) previously mentioned.

The animals in the experiments here reported were pair-fed and were
ingesting not only equal weights of food but equal quantities of protein (or
amino acid) nitrogen; yet the casein-fed rats more or less retained their
initial weight, or even gained, while those getting only the equivalent
amount of methionine (and cystine) lost from 24 to 30 per cent in weight.
The deficiencies of essential amino acids appear to have reduced the effi-
ciency (and probably changed the character) of the protein metabolism in
such a manner as to leave more dietary methionine free for lipotropic action.
In other words, the total nitrogen intake and the adequacy (or inadequacy)
of the sulfur-free essential amino acids both play a part in determining the
amount of methionine used for general metabolic purposes (i.e., for growth
or maintenance), and thus limit the amount available for lipotropic pur-

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poses. However, even when the casein-containing and methionine-containing diets were made as nearly identical as possible (cf. Diet 1A with Diet 1C and Diet 6A with Diet 6D), there is still a difference in the lipotropic actions of the two diets, indicating the probable existence in casein of an unrecognized factor influencing the deposition of fat in the liver.

The data in Table III may contain a clue as to the nature of this factor. The absence of tyrosine from all diets in Groups C and D has already been commented upon. It should be noted that in general the diets contain adequate amounts of the essential amino acids, as judged by Rose's (45) tentative estimates of the minimal percentages required in a normal diet for optimal growth, viz., threonine 0.6, valine 0.7, leucine 0.9, isoleucine 0.5, arginine 0.2, histidine 0.4, lysine 1.0, methionine 0.6, phenylalanine 0.7, and tryptophane 0.2. In spite of the apparently satisfactory supply of these factors, maintenance of weight was achieved on only two of the diets, Nos. 5A and 6A, containing 35 and 40 per cent respectively, of casein. The high fat content and absence of choline or the restricted food intake may be the cause of the weight losses on the majority of the diets, although some recent work suggests a deleterious effect of gelatin upon growth under somewhat similar conditions (46).

The elegant investigations of Griffith and his collaborators must be mentioned in connection with the explanation offered below for the findings reported in this communication. Griffith and Wade (15) demonstrated that a high fat diet is not necessary for the appearance of fatty livers and renal lesions in young rats and pointed out that the deposition of liver fat is "intensified on those diets permitting the better rates of growth." In 1940 Griffith and Wade (47) fed young rats on a series of low choline, high fat diets containing increasing quantities of casein and noted that the liver fat increased as the casein level was raised from 5 to 15 per cent; the incidence of renal lesions continued to increase as the casein level was raised to 25 per cent. Both lesions then decreased in severity as the percentage of casein was further increased, clearly indicating that factors other than the methionine-cystine ratio are involved. Mulford and Griffith (48) showed that the "toxicity" or antilipotropic effect of added cystine decreases as the percentage of casein in the diet increases and disappears at 30 per cent. They also found that the "toxicity" of 0.5 per cent of added cystine in an 18 per cent casein diet is proportional to the food intake, being absent at 2 gm. per day and very severe at 4.6 to 4.9 gm. per day. As early as 1941 Griffith (49) had drawn attention to the fact that the "toxic" effect of cystine is observed only on diets low in cystine and simultaneously low in choline or methionine (i.e., those with a dual deficiency, presumably of organic sulfur compounds and of labile methyl groups). He also pointed out, as had others (9), that the cystine effect is not proportional to the level
of supplementary cystine. Once the sulfur deficiency has been made good, further additions of cystine are without effect. The cystine supplement, by improving growth and stimulating metabolism, brings to light deficiencies previously unrecognized in the diet. With regard to the apparent antilipotropic effect of cystine, Griffith and Mulford (44) have commented that "the deposition of liver fat or the appearance of renal hemorrhage in experiments in which a dietary supplement increases the consumption of food or the rate of growth is not necessarily evidence of a direct antagonism between choline and the dietary supplement."

Thus Griffith has consistently maintained that factors other than the methionine-cystine ratio are important in establishing the lipotropic activity of a diet, and of these other factors he attributes particular importance to the adequacy of the ration.

Most of the facts presented in this report and in the papers referred to seem to be explained by a relatively simple hypothesis; viz., that the amount of dietary methionine available for lipotropic action is limited to that portion not utilized by metabolic processes of apparently higher priority, such as growth or maintenance. The amount required for these non-lipotropic activities is dependent upon the total protein intake and is further modified by the adequacy of the essential amino acids supplied in the diet. If the above hypothesis is accepted, it follows that the quantity of any protein fed and the nature and amount of the sulfur-free essential amino acids in the protein, as well as its methionine and cystine content, will influence its lipotropic activity because of their effects upon growth and maintenance.

The constant use of one protein (e.g., casein) in the basal diets may obscure the issue or delay the solution of the problem. It was therefore decided to conduct similar experiments with other proteins. Arachin, a globulin from peanuts, which is very low in methionine (50, 51) but otherwise adequate for growth (52), was selected.

Recently a report (53) has appeared which describes the use of arachin in a study of the lipotropic effect of methionine. The data published do not answer all the questions in which we are interested, but they do agree with many of the facts presented here and support the hypothesis presented in this paper concerning the rôle of the essential amino acids.

While the data here reported may not resolve all the anomalies to be noted in previous attempts to explain the varying lipotropic effects of different proteins, they do account for some of the difficulties and point the way for further research.

**SUMMARY**

1. Discrepancies in previous attempts to account for the lipotropic effect of casein, by feeding to one group of rats a certain amount of this protein
and to another group corresponding amounts of methionine and cystine as free amino acids, are accounted for by the finding that different results are obtained at different dietary levels of casein: below 22 per cent the free amino acids exert the stronger effect; above 22 per cent, the casein diet is more lipotropic.

2. The apparently superior lipotropic effect of free methionine over an equal quantity bound in casein (at casein levels below 22 per cent) is obliterated when the quantities of the essential amino acids in the two diets under comparison are made approximately equal. Thus the lipotropic effect of a diet is determined not only by its content of sulfur-containing amino acids but also by its adequacy in other respects.

3. The lipotropic activity of a protein is determined not only by its methionine and cystine contents, but also by the nature and quantity of the sulfur-free essential amino acids in the protein. These amino acids do not act directly, but through their well known influence on growth and maintenance they influence the formation of new tissue, thus modifying the amount of methionine left available for lipotropic action.

4. Some evidence is presented for the existence in casein of a lipotropic factor other than methionine. Indirect evidence suggests that tyrosine may be involved.

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