EFFECT OF AMINO ACIDS ON ANEMIA CAUSED BY DEAMINIZED CASEIN*

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Deaminized casein is grossly inadequate as the sole source of nitrogen, so in our studies of its nutritional properties it has been combined with other proteins. It has been shown in earlier papers (1, 2) that when deaminized casein is combined with gelatin or gliadin, or a laboratory preparation of lactalbumin, and fed to rats they become severely anemic, fail to grow, and die. Surprisingly enough, if deaminized casein is combined with normal casein in the diet, the rats do not become anemic and they grow normally. If normal casein is hydrolyzed and the hydrolysate combined with an anemia-producing ration, then the animals recover from the anemia and growth is normal. It is almost certain that the anti-anemic activity of hydrolyzed casein is explained by its amino acid content; the purpose of this paper is to report subsequent attempts to obtain the active agent in more concentrated form.

EXPERIMENTAL

In order to save time and expense the rats are first made anemic by the procedure of Elvehjem and Kemmerer (3) before the experimental period begins. They are then placed in individual cages and transferred to the experimental ration, No. 2149, made up as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deaminized casein</td>
<td>10.0</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>15.0</td>
</tr>
<tr>
<td>Corn-starch</td>
<td>52.5</td>
</tr>
<tr>
<td>Milk fat</td>
<td>12.5</td>
</tr>
<tr>
<td>Agar</td>
<td>2.0</td>
</tr>
<tr>
<td>Salts (4)</td>
<td>4.0</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>2.0</td>
</tr>
<tr>
<td>Water extract of yeast</td>
<td>2.0</td>
</tr>
</tbody>
</table>

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When the various amino acid mixtures to be tested were included in the ration, they displaced an equivalent weight of starch. In later descriptions of individual rations, therefore, only the kind and amount of the nitrogenous constituents will be specified, as the other constituents either remain constant or are readily calculated.

Fractionation of Hydrolyzed Casein—The methods are combinations of those applied previously by other investigators, and will be indicated by a brief description of the first instance in which the procedure now followed was used. 3 kilos of casein were hydrolyzed with 1490 gm. of concentrated sulfuric acid and 3 liters of water for each 500 gm. of casein. The sulfuric acid was then removed with barium hydroxide, and the amino acid mixture was concentrated to 20 liters. One-sixth of this was removed for another purpose but tests showed that it was effective in curing anemic rats. The remainder of the amino acid solution was concentrated to a volume of 4.5 liters. This was packed in ice for 2 days and then the soluble and insoluble amino acids were separated by filtration. The insoluble amino acids were washed with ice water and the washings added to the more soluble portion. When tested, it was found that the insoluble amino acids were only slightly effective in curing the anemic animals, and the soluble amino acids were highly effective.

Since the soluble amino acids contained most of the activity, they were subjected to further fractionation. Solid Ba(OH)₂ was added in 30 per cent excess (5) and the mixture was poured into 4 volumes of 95 per cent ethyl alcohol and packed in ice for 2 days. The dicarboxylic acid fraction which was precipitated was filtered off and washed with 70 per cent alcohol. The alcohol was removed from the filtrate by distillation under reduced pressure, and the excess barium was precipitated with sulfuric acid and filtered off. The dicarboxylic acid fraction was decomposed with sulfuric acid, and, after removal of the barium sulfate, both fractions were examined to see which contained the larger proportion of the antianemic agent. The dicarboxylic acid fraction was ineffective in curing anemia and it accelerated the rate of growth slightly or not at all. The amino acids not precipitated as barium salts by alcohol were very effective both in curing anemia and in supporting growth.

For the next stage in concentration two methods have been considered, the first of which is the butyl alcohol extraction method.
of Dakin (6). Half of the remaining amino acids was used, and it developed that the butyl alcohol-soluble portion had very slight activity and that the insoluble portion was highly active. All details will be omitted though, as this procedure was abandoned when it became apparent that the second method gave some promise of being more useful at this stage. In the second method the remainder of the amino acid mixture was subjected to copper salt fractionation as described by Caldwell and Rose (7). The three copper salt fractions obtained were freed of copper by hydrogen sulfide, and the solutions of the amino acids were evaporated to dryness. The amino acids whose copper salts are soluble in water were gummy, so they were partially converted into calcium salts in order to make them friable enough to be handled easily. 5 gm. of calcium hydroxide were added to the fraction that is soluble in methyl alcohol, and 20 gm. to the fraction which is insoluble in methyl alcohol. The weights of the three fractions are (1) copper salts insoluble in water, 27.0 gm.; (2) copper salts soluble in water and soluble in methyl alcohol, 50.0 gm.; (3) copper salts soluble in water but insoluble in methyl alcohol, 200.0 gm.

Inspection of the weights shows that the amount of amino acids whose copper salts are insoluble in water is relatively small, so if this fraction contains most of the active principle it must be very concentrated. At first therefore only 50 mg. daily of this fraction were supplied as a supplement to the basal diet. When this amount was found ineffective, it was increased to 100 mg. and later to 200 mg. daily, but even the latter amount was without retarding effect on the anemia.

The amino acids from the copper salts soluble in both water and methyl alcohol were supplied in amounts of 50 and 100 mg. daily, as a supplement to the basal diet, and also as 2 to 4 per cent of the ration. At none of these levels was this fraction effective in curing anemia.

There remains then Fraction 3, the amino acids whose copper salts are insoluble in methyl alcohol but soluble in water. The rations which contained this fraction were modifications of Ration 2149, obtained by substituting the amino acid mixture for an equal weight of starch. The following are the substituted amounts: Ration 3038, 5 per cent; Ration 3039, 10 per cent; Ration 3042, 2 per cent; Ration 3075, 3 per cent.

When the rats received the ration which contained 5 per cent of
Fraction 3, they recovered as quickly and grew as rapidly as when it contained 10 per cent, or as when it contained 10 per cent of casein itself. 2 per cent of this fraction was not enough, as only one of the three animals on this amount recovered. Another preparation of this copper salt fraction, which had been extracted still more thoroughly with methyl alcohol, cured anemia and supported growth at a level of 3 per cent. 100 mg. daily of this second preparation, as a supplement to Ration 2149, is not enough, as only one of the two animals observed recovered and it grew slowly. The other died at the end of 16 days. Additional data on the red cell counts and rate of growth are summarized in Fig. 1.

There is no doubt that Fraction 3 is the most active but it seemed possible that more than one factor might be concerned. If this supposition is correct, then a considerable part of one of the
active agents might be in either the fraction which is soluble in methyl alcohol or in the fraction which is insoluble in water. To test this hypothesis the methyl alcohol-insoluble portion was supplemented with each of the others. The methyl alcohol-soluble fraction seemed to increase the growth rate but the recovery from anemia was not markedly affected. The amino acids from the water-insoluble fraction were altogether ineffective. It is concluded then that whatever the number of amino acids concerned may be, Fraction 3 is the most concentrated source of all of them.

The total potency of Fraction 3 is much less than that of the casein from which it was obtained. A portion of the active agent may have been destroyed by the chemical procedures, but more probably it was lost in the various stages of the separation process. A clear cut separation of amino acids cannot be obtained by the procedures employed.

Amino Acid Supplements—The next problem is the identification of the active agent, and though this has not been achieved the preliminary experiments will be described briefly. It was hoped that the distribution of the amino acids in the various copper salt fractions would be a useful guide, and so previous reports on the composition of these fractions were taken as a starting point. The amino acids whose copper salts are soluble in water, but insoluble in methyl alcohol (7), are glycine, glutamic acid, arginine, alanine, hydroxyglutamic acid, histidine, serine, tyrosine, and lysine.

Of these nine it is probable that only six are present in Fraction 3 in any considerable amount. Most of the tyrosine was precipitated in the first concentration of the entire amino acid mixture. At a later stage the barium salts of glutamic acid and hydroxyglutamic acid were precipitated with alcohol. This precipitate probably contained in addition a considerable portion of glycine (8).

Our chief interest was at first confined to the six amino acids that should be present in the active copper salt fraction. Hogan and Ritchie (1) had failed to find antianemic activity in lysine, and Smith and Stohlman (9) had been equally unsuccessful with lysine and histidine. Arginine, alanine, and serine were regarded as the most promising, therefore, but histidine was included also
to confirm the report of Smith and Stohlman. Each of these was tested separately at a level of 50 mg. daily, as a supplement to the basal ration, but none effected any improvement in the anemic condition. It was then decided to try glycine, glutamic acid, and tyrosine, at levels of 100 mg. daily. Aspartic acid does not belong in Fraction 3 but since it was available it too was tried. All four were entirely inert.

Inasmuch as the results with the amino acids supposed to be in Fraction 3 had been disappointing, it was decided to try some of the others. It seemed probable that the substance sought is an essential amino acid, so threonine, valine, phenylalanine, leucine, and isoleucine were tried separately, at levels of from 50 to 100 mg. daily. None was effective. Three essential amino acids remain then which have not been investigated at this time, lysine, methionine, and tryptophane. The reason for omitting lysine has been mentioned; it had not shown curative properties in earlier trials. According to Hogan and Ritchie (1) methionine and tryptophane had offered no promise of usefulness, so these two were also omitted.

**Enlargement of Spleen**—In our experience the only abnormality of the internal organs that has been consistent is an enlargement of the spleen, which often becomes several times the normal size. This was first observed on postmortem examination of rats that had died while severely anemic, and it was supposed at the time that the spleen was in some way involved in the disappearance of red blood cells. However, further study showed that the spleen may enlarge during recovery. It seems more probable then that the increase in size is related to an accelerated rate of formation of erythrocytes, rather than to an increased rate of destruction. Hamre and Miller (10) observed that there is a relation between the size of the spleen and recovery from nutritional anemia.

Fig. 2 shows that the spleens of anemic rats are from 1 to 4 times as heavy as those from normal animals (11) of comparable weight. It should be pointed out though that there is no definite ratio, as the increase in size depends on both severity of the anemia and the length of the anemic period. Some of the rats for which results are shown in Fig. 2 had been given a curative treatment

1 Practically all of the amino acids used recently were obtained from commercial sources.
and there is no reason to suppose that the spleens had decreased in size during recovery. The data give no indication as to whether the increase in size is persistent.

Suitability of Proteins for Studies of Anemia Caused by Deaminized Casein—To digress from the main theme for a moment, it should be mentioned that different preparations of lactalbumin are not equally suitable for studies of this type of anemia. Most of the preparations permit a slow rate of growth with no increase in the number of red blood cells, but on a few preparations the animals will recover spontaneously. It is assumed that this difference is due to variability during the separation process. The skim milk is heated with live steam in 50 gallon barrels in order to precipitate the albumin, and for various reasons there may be considerable variation in the time during which the protein is exposed to high temperatures. Recent papers (12, 13) indicate that this variability may be a factor of some importance. A mixture of gliadin and gelatin may be used but as these proteins are expensive a search was made for a substitute. It
developed that either wheat or corn gluten is satisfactory, but wheat gluten is preferable, as it has a higher biological value. When this protein is used, Ration 3211, it displaces the lactalbumin of Ration 2149. As shown in Fig. 1 (Graph S), when rats receive deaminized casein and wheat gluten they not only become anemic, but they also fail to make any consistent gain in weight.

**DISCUSSION**

When it was first discovered that casein is effective, and lactalbumin ineffective, in preventing anemia due to deaminized casein, it was hoped that the percentages of amino acids they contain might explain this difference in behavior. According to the compilation published by Schmidt and Allen (14) six different amino acids are present in markedly smaller proportions in lactalbumin than in casein, as shown below.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Casein per cent</th>
<th>Lactalbumin per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>21.8</td>
<td>12.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Proline</td>
<td>7.6</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Hydroxyproline</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>7.9</td>
</tr>
</tbody>
</table>

As was mentioned previously, the possibility that glutamic acid, phenylalanine, tyrosine, and valine might be involved was tested by adding them directly to the anemia-producing ration, with negative results.

If any of the amino acids are involved in this syndrome, it seems highly probable that they belong to the group classified as essential, but all attempts made thus far to identify the active agents with this group have failed. On the other hand the anemia-producing substance of deaminized casein may be detoxicated by some non-essential amino acid, and thus the essential amino acids may not be concerned in the process. It may be also that there are unsuspected gaps in our knowledge of the constituent amino acids of casein and lactalbumin. Another possibility is that when deaminized casein is in the ration the animals require more of certain amino acids than they do when this protein is absent, and that the amino acids were offered in too small an amount to be effective. In future attempts to solve the problem chief reliance
will be placed on a more complete fractionation of the active copper salts, combined with the use of increased amounts of amino acids, individually and in combination.

Since it now seems certain that this type of anemia can be prevented by certain of the amino acids, a solution of the immediate problem can be anticipated with some confidence. One can also anticipate that the solution will raise other questions which at this time seem equally puzzling. Casein is highly effective as the antianemic agent. The laboratory preparation of lactalbumin, wheat gluten, or a mixture of gelatin and gliadin is not effective. Our present knowledge of the constituent amino acids of these proteins does not suggest a satisfactory explanation for this difference.

SUMMARY

1. Of the proteins examined casein is the most potent source of the antianemic agent. A considerable portion of the activity remains after hydrolysis. Lactalbumin is relatively ineffective, and both corn and wheat gluten are still more deficient.

2. The antianemic agent is not extracted from hydrolyzed casein by butyl alcohol.

3. The antianemic agent forms a copper salt soluble in water but insoluble in methyl alcohol.

4. The anemic animals were not cured by supplying them with amino acids supposed to be in this copper salt fraction. Supplements of the individual essential amino acids which were not expected to be in this fraction were equally ineffective.

5. The spleens of anemic rats were much enlarged.

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