AMINO-ACIDS IN NUTRITION.*

II. THE NUTRITIVE VALUE OF LACTALBUMIN: CYSTINE AND TYROSINE AS GROWTH-LIMITING FACTORS IN THAT PROTEIN.

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Since 1911, when Osborne and Mendel published their first work on "Feeding experiments with isolated food substances," there has been made accessible to investigation the problem of the biological evaluation of proteins as correlated with their chemical analyses. It is true that for a time the advance made in the solution of this problem was obscured by the lack of appreciation of the necessity of the presence of the water-soluble and fat-soluble vitamins, but this was soon rectified as information in connection with these vitamins was accumulated by Hopkins (1), McCollum and Davis (2), and by Osborne and Mendel themselves (3). However, with the incorporation of the water-soluble vitamin in the diet in the form of the extracts in which it is known new complications were introduced, inasmuch as this also meant the introduction of a small amount of nitrogen in unknown forms. Some of the earlier work in this field was, therefore, open to much criticism, as unquestionably in many instances the amino-acid deficiency of a protein was covered by the amino-acid nitrogen contained in the vitamin preparations used in excessive amounts. Even with the present technique of ration-compounding this difficulty has not been entirely circumvented, but it has at least been minimized by the use of vitamin preparations low in nitrogen and minimum in amount (4).

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In the early work of Osborne and Mendel, lactalbumin no more than edestin or other globulins was able to support growth, but here the fat-soluble vitamine was not incorporated in the diet and little else was to be expected. In 1916, in a series of experiments designed to test the comparative values of casein, lactalbumin, and edestin, these same workers found that lactalbumin was a protein of unusual value; in fact, 50 per cent more casein and 90 per cent more edestin than lactalbumin were required to produce the same increment in growth (5). Later, however, in another series of experiments they found lactalbumin, as well as cotton-seed globulin, cotton-seed protein, or squash seed globulin, invariably unable to support growth (6). These conflicting data were harmonized when it was appreciated by them that where failure had supervened yeast had been used as the source of the water-soluble vitamine, while where success had been obtained their so called protein-free milk had been used instead. Evidently, then, here occurred a concrete instance of supplementation of an "incomplete" protein by the nitrogen of a water-soluble vitamine preparation from milk. Possibly the statement of McCollum, Simmonds, and Parsons (7) that lactalbumin is a poor protein can also be harmonized on these general premises. Another suggestion has been made by Emmett and Luros (8), in which they state that lactalbumin is a complete protein and that their success was due to a vitamine, with a possible detoxifying effect, carried by the lactose and not introduced by other investigators. It scarcely appears necessary, however, in the light of present information to advance such hypotheses, in addition to those already advanced, which are based on the supplementary value of the nitrogen carried by the vitamine preparations. It becomes increasingly desirable, however, to establish definitely the specific amino-acids which limit the nutritive value of lactalbumin.

EXPERIMENTAL.

All the experiments were carried out on young rats according to the technique which has become a standard in this laboratory in the course of the last few years. As basal constituents there were used dextrinized corn-starch, agar-agar for roughage, Salt No. 32 for mineral constituents, filtered butter fat for fat-soluble
vitamine, and an alcoholic extract of ether-extracted wheat embryo for water-soluble vitamine. Before the experimental details were fully organized one ration containing yeast as the source of water-soluble vitamine was started, but this was later changed as the wheat embryo preparation was found to introduce only one-third as much nitrogen as the yeast. Although comparative supplementary values could not be forecast, it appeared to be desirable on general premises to introduce the minimum of nitrogen that might be of possible supplementary value.

As the individual proteins of milk may not be as definite entities as is ordinarily assumed, it appears desirable to describe the method of preparation of the lactalbumin used in some detail. The small amount of so-called albumin in milk as obtained by heat coagulation after the removal of the casein made it almost impossible to consider milk as a source for its preparation. Instead there was used the whey produced in the manufacture of cottage cheese by the lactic acid fermentation process. 1,000 to 2,000 pound lots of this were run through a centrifugal whey separator to remove small particles of casein held in suspension and then heated by steam to about 85°C. for 1 hour. Care was taken to avoid violent agitation, as otherwise the coagulated albumin particles do not coalesce and their collection is made difficult. After settling for 1 hour the supernatant liquors were run off through a large wire strainer covered with three layers of cheese-cloth. The albumin left in the vat was united with that caught by the strainer and after standing for 24 hours was decanted free from excess liquors and worked up repeatedly by hand with 40 liters of chloroformed tap water for a week. This was done to wash out soluble forms of nitrogen of possible supplementary nutritive value. The supernatant liquor was finally allowed to drain off through the strainer, and the albumin dried in a steam oven at a temperature not exceeding 75°C. Final purification with hot 95 per cent alcohol left a product faintly tinged with yellow. On analysis it was found to contain from 0.5 to 0.7 per cent nitrogen, 0.06 per cent phosphorus, and 14.3 to 14.7 per cent nitrogen.

Chart I, Lot 4.—Lactalbumin fed at an 18 per cent level in a low caloric ration and when supplemented with cystine to the extent of 1 per cent of the weight of the protein met the requirements for growth in the rat.

Chart II, Lot 5.—By comparison with Chart I, Lot 4, it is seen that when the cystine is omitted from the ration the growth of the animals is far from being satisfactory. Of the four animals fed on this ration one was able to grow at a rate which may be considered normal, but, on the other hand, three failed to continue their growth. This is to be taken as indicating a deficiency of the ration, for while the one animal was able to grow, that ability no doubt was resident in either a larger consumption of the ration, or else in a greater efficiency in conserving available sulfur complexes in the body.
Chart III, Lot 37.—A duplication of the experiment of Chart II, Lot 5, resulted in good growth of the four animals for a considerable period, but ultimately failure ensued in two animals out of the four in the time that the trial was continued. When at this point the ration was fortified with cystine there resulted prompt recovery. This emphasizes the point indi-

\[\text{In all charts, for "40 gm. ether-extracted wheat embryo" read "15 gm. ether-extracted wheat embryo."}\]
cated in the previous chart that cystine is a limiting factor in the biological efficiency of lactalbumin. The fact that the two animals did not respond to the addition of cystine does not detract from the value of the data, as this may well have been due to irreparable injury directly or indirectly caused by the amino-acid deficiency.
Chart IV, Lot 2.—Lactalbumin at a 12 per cent level supplemented with 1 per cent cystine supported a fair amount of growth.

Chart V, Lot 33.—When not supplemented with cystine at a 12 per cent lactalbumin level the growth performance was considerably inferior (Chart IV, Lot 2).
Chart VI, Lot 1.—When yeast was used as the source of the water-soluble vitamin, lactalbumin on a 12 per cent level supplemented with 1 per cent of cystine allowed the rats to make excellent growth. At “a” the yeast was replaced by an alcoholic extract of ether-extracted wheat embryo, such as was used in the other rations, and growth continued for the
duration of the trial. While in itself not conclusive, as the supplementary value of the yeast alone was not determined, yet Osborne and Mendel's findings that lactalbumin was a poor protein when yeast was used as a source of the water-soluble vitamine strongly suggest that the addition of cystine was here responsible for the excellent growth. The continuation of growth when the change in water-soluble vitamine from yeast to an alcoholic extract of wheat embryo was made bears out this suggestion.

Chart VII, Lot 25.—When supplemented with 1 per cent of cystine no such increment in growth is obtained with the lactalbumin fed at a 9 per cent level as when fed at a 18 or even 12 per cent level. At this low level of protein intake evidently other amino-acids are reduced in amount below the level where they can still allow growth to take place after the primary deficiency of cystine has been removed.

Chart VIII, Lot 31.—The amino-acid responsible for failure of growth in Chart VII, Lot 25, appears to be tyrosine. Growth was regular and long continued when the lactalbumin fed at a 9 per cent level was supplemented with tyrosine as well as cystine. From this it appears that when an alcoholic extract of wheat embryo is used as the source of the water-soluble vitamine tyrosine is the second growth-limiting factor in lactalbumin.

DISCUSSION.

It appears demonstrated that lactalbumin, if we accept as lactalbumin the proteins in milk removable by heat coagulation after the casein has been removed, is biologically an incomplete protein. This was brought out in feeding trials even when nitrogen in unknown forms had been introduced with the water-soluble vitamine in the ration, so that it constituted 2 per cent of the total protein nitrogen. Under the experimental conditions cystine proved to be an efficient supplement to the lactalbumin when the protein was fed at an 18 and at a 12 per cent level; yet lactalbumin is not unusually low in cystine, as it has been reported to have 1.30 per cent of its total nitrogen in the form of cystine or 1.74 per cent molecular cystine (9), which is about the average percentage of cystine in other proteins (10). Although arachin contains only about one-half as much cystine (0.88 per cent cystine nitrogen) as lactalbumin, no response to cystine additions was obtained in the case of arachin, even in the presence of tryptophane (11). This fact the author believes is further evidence to substantiate the theory set forth in a previous communication (11) that the efficiency of a protein may largely depend on its constitution as well as composition in amino-acids, and that
chemical analysis of amino-acid content may at times be inadequate to explain the nutritive failure or success obtained with certain proteins widely distributed in nature. It is quite possible that cystine is so oriented in the complex polypeptide chain of lactalbumin that when it is hydrolyzed by the enzymes in the digestive tract it is split into simpler peptides of such form that a great part of them escape further cleavage, and are, therefore, deaminized and converted to urea. Of course, it still leaves open the possibility that the animal organism requires more cystine than that actually contained in lactalbumin when that protein is fed at as high a plane of intake as 18 per cent of the total food.

Osborne and Mendel found lactalbumin to be a highly efficient protein when fed with protein-free milk, and the experimental data here presented show that cystine renders that protein of excellent nutritive value, when employing a synthetic salt mixture, and an alcoholic extract of wheat embryo for water-soluble vitamine, when without cystine it is of little value. The author is consequently of the opinion that protein-free milk must either carry cystine as part of its nitrogen of unknown source, or other forms of organically bound sulfur which the animal organism can readily transform into cystine. An analysis of protein-free milk showed a sulfur content of 0.2 per cent, of which the greater part was in organic combination.

While growth was induced by the addition of cystine to lactalbumin when fed at 12 and 18 per cent levels, such was not the case when the protein was reduced to a 9 per cent level. This brings out the fact that, while cystine is the primary growth-limiting factor in lactalbumin, other amino-acids may also limit its usefulness when the intake is sufficiently reduced. With the protein under consideration this proved to be tyrosine, as complete success was obtained when the lactalbumin was fortified with 5 per cent of its weight of tyrosine.

Prior to this work the experimental evidence does not give definite information with regard to the indispensability of tyrosine for growth. The first notable experiment on this subject was conducted by Abderhalden (12) on a dog. A preparation of the digestion products of casein, freed from tyrosine as completely as possible, was given to the animal. The dog lost 750 gm. in 9 days and there was a further loss of weight in
the 4 following days, though the loss of weight in the last few days may have been partly due to an insufficient intake of food. The loss of weight, however, was regained almost entirely when tyrosine was added to the previously consumed dietary. Abderhalden, therefore, came to the conclusion that tyrosine is an essential amino-acid for nutrition.

Recently Totani (13) continued the work on tyrosine with a view to the possible replacement of that amino-acid by its corresponding ketonic acid. Since Abderhalden fed his protein freed from tyrosine by crystallization only, Totani made a much more painstaking and laborious attempt towards removing all traces of tyrosine from a casein-hydrolysis mixture. He found that after repeated crystallizations the material still gave a positive Millon’s test. Mercuric sulfate and phosphotungstic acid were then used to precipitate out any residual amounts of tyrosine. He then found that the most efficient method of exhausting casein of its tyrosine content was to use a combined tryptic and acid hydrolysis. With all his most elaborate methods his final preparation of hydrolyzed protein was found to contain by colorimetric measurements 0.3 per cent tyrosine. Totani found that animals which received tyrosine did not produce any more growth than those that were on the practically tyrosine-free ration, and concluded from these experiments that tyrosine is not essential for nutrition, thereby taking a stand opposite to Abderhalden.

Totani also found that, while tryptophane improves somewhat the deficient character of gelatin, surprisingly, tyrosine, which is not found in that protein, does not; also that the addition of phenylalanine produces no effect just like tyrosine. This strengthens his evidence, he believes, that tyrosine is an amino-acid unessential for growth and that phenylalanine can be transformed into hydroxy-phenylalanine or tyrosine.

There are several objections to Totani’s work, the main being that his period of experimentation, 24 days, was altogether too short to get any idea of the possible outcome of such an investigation. Besides, as Totani admits himself: “It is, of course, an objection to my experiments that the actual amount of tyrosine left in the diet is uncertain.” He claims he could detect only 0.3 per cent tyrosine, but that figure may be much higher and we do not know yet about the minimum requirements of that amino-acid for growth. Moreover, in his control ration his rats did not grow normally, and from the experience in this laboratory on the requirements of the water-soluble vitamine, Totani’s alcoholic extracts of protein-free milk did not furnish enough of that factor in the diet. In his work on gelatin the records show loss of body weight in all cases. The addition of tyrosine brought no improvement, neither did the equivalent amount of phenylalanine, yet the investigator concludes that the latter may be transformed into the former.
The author believes that Totani's conclusions with regard to the possible synthesis of the benzene nucleus are unwarranted and are based on altogether inadequate data. On the contrary the experimental data here presented strongly suggest the indispensibility of tyrosine for growth. However, further experimental data are necessary to establish this point finally.

Reconsidering the supplementary value of protein-free milk, it is interesting to note that, although Osborne and Mendel claim they were unable to obtain a positive Millon's test on 2 or 3 gm. of preparations of protein-free milk (14) the author has repeatedly obtained positive tests for tyrosine in preparations of several tenths of a gm. of protein-free milk prepared exactly as described by Osborne and Mendel. The preparations contained only 0.60 to 0.65 per cent nitrogen, and, therefore, were certainly free from any notable amounts of casein or lactalbumin. When 28 per cent of the ration is made up of this protein-free milk the amount of tyrosine which may exist there either free or in peptide chain formation may be considerable.

SUMMARY.

1. Lactalbumin when fed as 12 and 18 per cent of a ration, carrying 2 per cent of the total protein in the form of nitrogen of unknown source in an alcoholic extract of wheat embryo to furnish the water-soluble vitamine, was found to be inadequate for growth.

2. When fortified with a weight of cystine equal to 1 per cent of that of the total protein, lactalbumin fed at an 18 and 12 per cent level was found to be of excellent nutritive value. Cystine, then, is the primary growth-limiting factor in that protein.

3. Lactalbumin, when fed at a 9 per cent plane of intake, even in the presence of 1 per cent of the total weight of the protein in the form of cystine, was found to be very deficient in nutritive value. However, when fortified in addition with tyrosine in amount equal to 5 per cent of the total protein, it was found to be of excellent nutritive value. Tyrosine, then, is the secondary growth-limiting factor in that protein.

4. Protein-free milk was found to have a total sulfur content of 0.2 per cent, the greater part of which is in organic form. The
results suggest that protein-free milk contains either cystine or organically bound sulfur which the animal organism can transform into cystine. Protein-free milk was found to give qualitative tests for tyrosine.

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