UTILIZATION OF NON-SPECIFIC NITROGEN SOURCES BY THE ADULT PROTEIN-DEPLETED RAT*

BY DOUGLAS V. FROST AND HARRY R. SANDY
(From the Abbott Laboratories, North Chicago, Illinois)

(Received for publication, October 27, 1950)

The rat repletion method of assay of protein value, developed by Cannon's group at the University of Chicago (2-4), has been applied to liquid protein hydrolysates and amino acid mixtures in this laboratory (5, 6). The method has the advantage of convenient volumetric portioning of the amino acid components of the diet. With properly conditioned depleted animals, there is quantitative acceptance of liquid protein hydrolysates of high biologic value over a wide range of nitrogen intake; i.e., up to 400 mg. of N per rat day. Whole, dry, powdered proteins fed in cups separate from the non-protein diet are also quantitatively consumed, and provide convenient standards of protein value for comparison with amino acid solutions (7).

In the present studies, solutions of mixtures of pure amino acids were generally fed at a level to supply 100 to 120 mg. of available N per rat day. This is equivalent to approximately 1 per cent of N in the diet, or only about 6 to 7 per cent of protein. This level is well below the minimum level at which maximum growth responses are obtained even with proteins of high biologic value such as lactalbumin, whole egg, and fibrin, and is, therefore, highly critical with regard to limiting deficiencies of individual amino acids.

Rose (8) suggested many years ago, and reemphasized recently, that it may be possible with the exact knowledge of individual essential amino acid requirements to devise mixtures of extraordinary value for parenteral use. Such mixtures must obviously contain those eight amino acids which Rose has shown essential for maintenance of N balance in adult men. In addition, one must consider appropriate sources of nitrogen to supply the body needs for the non-essential amino acids plus other nitrogen metabolites ordinarily supplied by food. The eight essential amino acids account for only 40 to 50 per cent of the composition of proteins of the highest biologic value. The proteins of lower biologic value have an even lesser ratio of the essential to non-essential amino acids. The question arises, then, as to the optimum nutritive ratio between essential and non-essential.

* Presented in part before the Division of Biological Chemistry of the American Chemical Society, Atlantic City, September, 1949 (1).
amino acids, or, more broadly, between essential amino acid N and other than essential amino acid N.

The principal objects of this investigation were to determine (1) whether the so called non-essential amino acids are necessary for optimum repletion in the adult depleted rat, (2) whether isonitrogenous replacement of the non-essential amino acids can be made with such non-specific nitrogen sources as ammonia or urea, and (3) approximately how much of the total N should be present as other than essential amino acid N for the best performance.

**EXPERIMENTAL**

Details of the rat repletion method as applied to the assay of liquid protein hydrolysates are given in previous papers (5, 6). The basal diet was redesigned in recent studies to correct any marginal deficiencies which might ensue through repeated use of the test animals. With the improved diet, it was found that rats could be carried through at least four cycles of depletion and repletion at the level of 240 mg. of N with no apparent injury. When rats were carried through successive assays at lower nitrogen levels, a 3 to 4 day period on a stock diet prior to redepletion was used.

The non-protein diet (Diet NP10) used in these studies is expressed in per cent as follows: sucrose 83, salts (Salt Mixture 1, U. S. P.) 4, CaHPO4·2H2O 1, agar 1.4, Primex 4.2, corn oil 4.6, cod liver oil 1.4, Wilson liver fraction 0.1, choline chloride 0.15, inositol 0.1, MnSO4·2H2O 0.01, CuSO4·5H2O 0.004, ZnSO4·7H2O 0.004, CoCl2·6H2O 0.0002, ascorbic acid 0.01, thiamine hydrochloride 0.0006, riboflavin 0.0009, nicotinamide 0.01, pyridoxine hydrochloride 0.0006, calcium pantothenate 0.004, p-aminobenzoic acid 0.006, folic acid 0.0006, biotin 0.00004, menadione 0.0004. The above diet differs from Diet NP previously used by an increased amount of phosphorus and calcium and the addition of copper, cobalt, manganese, p-aminobenzoic acid, folic acid, biotin, inositol, menadione, and a liver fraction intended to supply vitamin B12. The diet supplies 4.2 calories per gm. and contains 0.03 per cent nitrogen.

Five young adult male rats were used in each assay group and were prepared for assay by a drinking trial period, as previously described (5, 6). Comparisons were made only between groups which had a similar history of high intake and satisfactory weight response to a standard intravenous fibrin hydrolysate. Repeated use of the animals over a cycle of two assays lends itself to procedures for checking results both within and between groups of animals. Fig. 1 illustrates the repeated use of the same animals on the same supplements. Significant differences were estimated from the data.
by Fisher’s $t$ test (9). Reference is made only to those differences which appear to have high significance.

Precautions were taken as to the purity of the amino acids used, which were obtained from Merck, Dow, Van Camp, Winthrop, and Interchemical. Microbiological and chemical assays served as criteria of purity. Purified amino acid standards were kindly supplied by Dr. Max Dunn and by the Interchemical Corporation. The latter standards were furnished as part of a collaborative microbiological assay project sponsored by the Bureau of Biological Research of Rutgers University. As a further check,
Kjeldahl nitrogen determinations were made on representative mixtures. The nitrogen content found by analysis agreed closely with the theoretical values.

**Amino Acid Mixtures Patterned after Casein**—In preliminary experiments, amino acid mixtures were made to compare as closely as possible with the mixtures described by Frazier et al. (3), which were patterned after the composition of casein. Certain modifications had to be made to correct for the insolubility of tyrosine and cystine, as previously reported (5). 5 per cent amino acid solutions were fed to supply 0.24 gm. of N per rat day, in a volume of about 35 cc. No water was supplied in addition to the amino acid solutions.

The form of the amino acids used is indicated, except in the case of histidine, lysine, and arginine, which were added as the hydrochlorides. All solutions were adjusted with sodium hydroxide, as needed, to pH 5. The values for percentage composition in Tables I to IV are expressed as per cent of the physiologically active components in the dry mixtures. Thus the values show only one-half the amounts of DL-isoleucine, DL-threonine, DL-valine, and DL-alanine which were present. The composition of the first series of amino acid mixtures is shown in Table I, together with the 12 day average gain in weight, the standard error, and per cent intake of the allotted volume of solution.

As is seen in Fig. 1, there was a greater response to Mixture A₄ (fourteen amino acids), both in the original and repeat assays. At the time this experiment was done, it was not clear whether or not any of the non-essential amino acids were needed for best growth in rats. Our results were in good apparent agreement with those of Womack and Rose (10), which suggested a stimulatory value for glutamic acid.

When alanine and glycine were eliminated (Mixture A₅), the response was not different from that observed for Mixture A₄. However, when glutamic acid alone was added at a level comparable to that used in the solution of fourteen amino acids (Mixture A₄), the response was improved over that of either the solution with twelve amino acids (Mixture A₅) or that with ten amino acids (Mixture A₆). This finding appeared to support the idea that glutamic acid was indeed playing a supplementary rôle to the essential amino acids.

Contrary to the above finding, on the other hand, was the report of Frazier et al. (3) that a mixture of only the nine essential amino acids gave as good a repletion response as their sixteen amino acid mixture. According to these authors, neither arginine nor glutamic acid would be needed for a high rate of recovery in the adult protein-depleted rat. In view of our own findings, however, and the contemporary reports from Rose's laboratory with growing rats, we were particularly concerned about the rôle of arginine.
Mixture A₉ was made up of the nine essential amino acids except for arginine. The response to Mixture A₉, as shown in Table I, was poorest of all. The intake of allotted nitrogen was only 41 per cent, and the weight gain was less than half that of rats on the fourteen amino acid mixture. The response to the ten amino acid mixture (No. A₉), which included arginine, was significantly greater than the response when arginine was omitted. Thus, our data clearly indicated a nutritive role for nitrogen sources, other than the essential amino acids, for the adult rat.

**Table I**

<table>
<thead>
<tr>
<th>Repletion Response to Amino Acid Mixtures Fed in 6 Per Cent Solutions to Supply 0.24 Gm. of N per Day</th>
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<tr>
<td></td>
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<tr>
<td>Mixture A₉</td>
</tr>
<tr>
<td>L-Arginine</td>
</tr>
<tr>
<td>L-Histidine</td>
</tr>
<tr>
<td>DL-Isoleucine</td>
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<tr>
<td>L-Leucine</td>
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<tr>
<td>L-Lysine</td>
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<tr>
<td>DL-Methionine</td>
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<tr>
<td>L-Phenylalanine</td>
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<tr>
<td>DL-Threonine</td>
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<tr>
<td>L-Tryptophan</td>
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<tr>
<td>DL-Valine</td>
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<tr>
<td>L-Glutamic acid</td>
</tr>
<tr>
<td>DL-Aspartic</td>
</tr>
<tr>
<td>DL-Alanine</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Average 12 day gain, s.e., gm.</td>
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<tr>
<td>Intake of allotment, %</td>
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</table>

The specific nutritive significance of arginine or glutamic acid for the adult depleted rat was, however, still not clear. It appeared likely that other sources of non-essential amino acid nitrogen would play a supplementary role. To test this hypothesis, mixtures were made up in which glycine alone and a mixture of glycine plus alanine were used to replace arginine at an isonitrogenous level. Experiments were carried out with Cannon’s amino acid minima as detailed below rather than with mixtures patterned after casein.

**Tests with Cannon’s Amino Acid Minima**—When Cannon’s minimum requirement values became available (11), it was decided to continue work with this combination, as representing a maximally efficient mix-
the \(\text{d}\)-amino acid \(\text{N}\) of the unavailable isomers of isoleucine, threonine, and valine. All mixtures based on the minimum requirements were fed in 25 cc. of solution to supply 0.138 gm. of \(\text{N}\) per rat day.

The response to the minimum mixture alone at the twice minimum level was poor, as predicted from our previous results with mixtures of only the nine essential amino acids. Addition of arginine, a mixture of glycine and alanine, or glycine alone, all improved the weight response and intake markedly, as shown in Table II. In each case the addition was made to supply 19 per cent of the total \(\text{N}\) of the mixture. Glycine, or glycine plus alanine, appeared to be just as effective as arginine when
added to the minimum mixture. It was now clear that the requirement for other than essential amino acid nitrogen was relatively non-specific. It was also clear that the rat was unable to make efficient use of only the essential amino acids, and that the presence of non-essential amino acids greatly enhanced the feeding value of the mixture.

There was a large excess of essential amino acids over the requirement in all of these mixtures. To illustrate this point, one can compare the amino acid composition and response to a complete hydrolysate of fibrin fortified with tryptophan, as shown in Table II. This preparation, despite its lower content of the essentials, was more acceptable to the rats than any of the synthetic mixtures.

At this stage of the investigation, Rose, Oesterling, and Womack (12) reported that removal of glutamic acid from a nineteen amino acid mixture did not result in a significant drop in nutritive value. At the same time they clearly demonstrated the nutritive significance of the non-essential amino acids for the growing rat. This report, together with our contemporary findings, pointed to the possibility that the rôle of the non-essential amino acids is largely non-specific. It appeared desirable then to study various readily metabolized sources of amine, amide, or ammonia N, over and above the essential amino acids, for their capacity to supply the nitrogen necessary for synthesis in the body pool.
Urea and Ammonium Citrate As Non-Specific Nitrogen Sources—The ratio of non-essential to essential amino acid N in the above experiments was thought to be low, and in the next series of experiments a mixture was made in which arginine was added to supply 32.2 per cent of the total nitrogen. The results are shown in Table III, from which it will be seen that all replacements gave an improvement both in intake of allotted nitrogen and weight recovery over the minima alone. Glutamic acid, or the mixture of glutamic acid and arginine, appeared superior to arginine alone; however, the difference was not large and was not borne out by subsequent experiments to test this point. The N efficiency ratios calculated from the results, assuming the d-amino acid N of threonine, valine, and isoleucine to be excreted, is as high as that for lactalbumin; i.e., about 30 gm. in gain per gm. of N consumed. The response when urea was added was not as great as that with added arginine or glutamic acid.

Further experiments were set up to test the response to urea and diammonium citrate with glutamic acid as a reference. Again the mixtures were made so that they supplied 32.2 per cent of total N. The results are shown in Table IV. The intake of allotted nitrogen was 99 per cent for the mixture with urea; however, the weight response was significantly less for this mixture than for the preparations with diammonium citrate or glutamic acid. The probability that such differences in weight gain would be determined by chance alone is less than 1 in 100 by Fisher’s $t$ test.

### Table IV

<table>
<thead>
<tr>
<th></th>
<th>Minimum + urea</th>
<th>Minimum + ammonium citrate</th>
<th>Minimum + glutamic acid</th>
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<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>DL-Isoleucine</td>
<td>14.2</td>
<td>10.6</td>
<td>9.8</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>17.0</td>
<td>13.9</td>
<td>11.7</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>11.3</td>
<td>8.5</td>
<td>7.8</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>7.7</td>
<td>5.2</td>
<td>5.3</td>
</tr>
<tr>
<td>DL-Phenylalanine</td>
<td>8.5</td>
<td>6.2</td>
<td>5.9</td>
</tr>
<tr>
<td>DL-Threonine</td>
<td>9.9</td>
<td>7.4</td>
<td>6.8</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>2.8</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>DL-Valine</td>
<td>11.4</td>
<td>8.4</td>
<td>7.8</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>5.6</td>
<td>5.2</td>
<td>3.9</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>11.6</td>
<td></td>
<td>39.0</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
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<tr>
<td>Ammonium citrate</td>
<td></td>
<td>32.6</td>
<td></td>
</tr>
</tbody>
</table>

Average 12 day gain, s.e., gm... 32 ± 1.4 43 ± 2.4 40 ± 1.6
Intake of allotment, % 99 89 88
As a further check on nitrogen utilization in the above groups, nitrogen balance determinations were performed on two representative rats from each group of five. Urine collections were made in two equal 3½ day periods over the last week of assay. Strongly positive N balances were seen in each case, although least nitrogen retention was observed for the group on urea.

**Ratio of Essential Amino Acid N to Non-Specific N**—On the basis of preliminary experiments, it became clear that the ratio of essential to other than essential amino acid N is of importance when studying the efficiency of any given source of non-specific N. One-third substitution of the total N of the minimum mixture in the form of glutamic acid, arginine, or diammonium citrate, as already described, supports an excellent weight response, and an N efficiency ratio equal to that shown by proteins of the highest biologic value. One-fifth nitrogen substitution by arginine, glycine, or a mixture of glycine and alanine also gave a clear cut response above the minimum mixture alone. When ammonium acetate was used at 10, 20, and 30 per cent levels of nitrogen substitution, the increase in response over the minimum mixture alone was progressive toward the highest level of substitution. There was, however, not a clearly significant difference between the 20 and 30 per cent levels. Thus, the data suggest that at least 20 per cent of the nitrogen should be other than essential amino acid N to assure efficient utilization of the total nitrogen of an amino acid mixture.

**DISCUSSION**

Protein hydrolysates modified in various ways to meet the needs of parenteral nutrition have thus far provided the only practical preparations for intravenous amino acid therapy. Mixtures of pure amino acids have been too expensive to be commercially feasible. There have also been questions of the possible inhibitory effects of D,L-amino acids and of the requirements for nitrogen compounds other than the recognized essential amino acids. The present study has a bearing on these questions.

About 10 per cent of the nitrogen in commercial protein hydrolysates is ammonia N derived from the amide N of glutamine and asparagine. The metabolic fate of this nitrogen has been in question for some time. During the course of these studies, evidence for the utilization of ammonia N by the growing rat was reported by Lardy and Feldott (13) and by Rose et al. (14). All three studies confirm the findings of Foster, Schoenheimer, and Rittenberg (15) with regard to the metabolic availability of labeled ammonia N15. Obviously, the disposition of the ammonia by the body is primarily conditioned by the adequacy of essential amino acid intake together with the adequacy of other non-specific nitrogen sources available to the body pool.
Rose (8), in reviewing the classic studies at Illinois on amino acid requirements in man, refers to the preliminary work on rats in his laboratory with glycine and urea as the non-specific nitrogen sources. This work led to the use of these compounds to provide extra nitrogen in the specialized amino acid diets for humans.

The present studies with adult depleted rats differ from the Illinois and Wisconsin studies on growth in that a large excess of essential amino acids was offered under the conditions of our experiments. Substitution of part of the nitrogen of the essential amino acids by various other sources of nitrogen elicited a much greater response than that given by the essential amino acids alone. Thus, it became clear that the essential amino acids themselves are not readily available as sources of nitrogen for conversion to other generalized nitrogen components of the body. Schoenheimer (16) demonstrated that when urea containing N\textsuperscript{15} was fed to normal rats the urinary urea contained practically all of the N\textsuperscript{15}. Under the particular conditions imposed in our studies, however, urea appears as a more efficient source of extra nitrogen for general metabolic purposes than the mixture of essential amino acids.

A difficulty inherent in the use of racemic mixtures comes in the need for knowledge of the fate of the \textsuperscript{D} isomers. Ramasarma, Henderson, and Elvehjem (17) have assumed in their growth studies with amino acid mixtures that the \textsuperscript{D}-amino acids serve to supply non-essential amino acid N. On the other hand, Rose \textit{et al.} (14) have reported that the \textalpha{}-keto analogues of valine and isoleucine promote growth, presumably by undergoing asymmetric amination to the corresponding \textit{L}-amino acids. If the body were capable of deaminating the \textsuperscript{D} forms of these amino acids, the residues might then be expected to undergo inversion and asymmetric resynthesis by the body to the \textit{L} isomers. Such an inversion must happen in the case of those \textsuperscript{D}-amino acids which are utilized, as there is little evidence that \textsuperscript{D}-amino acids are present as components of animal tissue.

We have dealt in terms of total nitrogen in the present study, leaving open the question of the nitrogen of the \textsuperscript{D} isomers. Additions of \textit{L}-amino acids, urea, or ammonium salts, however, serve to dilute the \textsuperscript{D}-amino acid content. This creates variables both with regard to possible inhibitory effects and with regard to utilization of \textsuperscript{D}-amino acid N. It is impossible to assess the effects of these variables fully with the knowledge at hand.

The interesting question whether the presence of \textsuperscript{D}-amino acids exerts an inhibitory effect does not yet appear to be clearly resolved. The observation has been made in this laboratory (1) that protein hydrolysates are accepted with greater avidity by protein-depleted rats than any of the amino acid mixtures studied, all of which have contained the \textit{DL} forms of isoleucine, valine, methionine, and threonine. Support for the idea
that d-amino acids do exert inhibitory effects and are less well tolerated than l-amino acids is extensive (18–21). On the other hand, in recent studies designed specifically to throw light on the effects of d-amino acids, Van Pilsum and Berg (22) concluded that proportionately large amounts can actually be fed as components of d-amino acid mixtures without producing any growth-retarding or other deleterious effects.

These contradictory conclusions suggest that a second variable in addition to the optical forms of the amino acids studied may have been at work in the different laboratories. The requirement for an adequate source of other than essential amino acid N for optimum growth has not been generally recognized and the fulfilment of this requirement appears as a variable in certain of the studies cited.

**SUMMARY**

The nine amino acids, exclusive of arginine, required by the adult rat gave a poor response in protein-depleted rats when fed in 5 per cent solution as the sole source of amino acid nitrogen. Mixtures patterned after the composition of casein and in the proportions of Cannon’s minima for maximum rate of repletion were studied. When fed at a level to supply 0.138 gm. of N per rat day, the latter mixture supplied twice the minimum requirements of each of the nine essential amino acids. Isonitrogenous replacement of one-fifth to one-third of the nitrogen of this mixture by arginine, glycine, glutamic acid, ammonium acetate, urea, or diammonium citrate resulted in a marked improvement in avidity for the solutions and in weight response. Efficiency of such mixtures compares favorably with that of the best proteins; i.e., about 25 to 30 gm. gain in weight per gm. of N consumed. Urea proved somewhat less effective than the other compounds as a source of other than essential amino acid N.

The data suggest that best utilization occurs when at least 20 per cent of total N is present as nitrogen other than that of the essential amino acids. Such a source of nitrogen appears fairly non-specific. This is supplied equally well by ammonia itself or by sources of nitrogen capable of supplying ammonia metabolically.

The avidity for all amino acid solutions thus far studied is markedly less than for solutions of complete hydrolysates of proteins fortified with tryptophan. The reason for this difference is not yet clear. The presence of the unnatural optical forms of certain of the amino acids is one basis of difference. This question cannot be satisfactorily resolved, however, on the basis of present evidence.

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