16388

Method for Determining Amino Acid Adequacy of Liquid Protein Hydrolysates in Rats.

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Experiments designed to measure the nutritive value of hydrolysates given parenterally[1] and orally[2] for growth of young rats have not met with marked success. The method of depletion of adult rats described by Cannon, *et al.*[3,4,5] appeared to offer certain advantages. Adult protein-depleted rats will voluntarily consume large volumes of liquid food,[6] have a great avidity for nitrogenous foods, and typify the clinical condition wherein the use of intravenous protein hydrolysates is indicated.

Method. Young adult male rats, 140-220 g, were placed on the following depletion diet for 12 days: sucrose 83, salt mixture No. 1 (U.S.P.) 4, agar 1.4, primex 4.2, corn oil 4.2, cod liver oil 1.4, choline chloride 0.15, and inositol 0.14 g; thiamine hydrochloride 0.6, riboflavin 1.2, pyridoxine hydrochloride 0.6, calcium pantothenate 5.0, nicotinic acid 3.7, mixed tocopherols 2.5 and ascorbic acid 14 mg per 100 g diet. Average weight loss was 24% of body weight, range 21-28%. The non-protein diet was fed *ad libitum*. The 5% hydrolysates, or amino acid solutions, were fed in drinking tubes attached to the cages. Water was not generally supplied to groups fed the liquid hydrolysates.

To avoid the somewhat variable response during the first 3 days of the 12-day assay period the following practice was adopted. At the end of the 12-day depletion the rats are offered a standard partial acid hydrolysate of fibrin[7] *ad libitum* for 3 days. During this trial drinking period the rats rapidly increase their consumption to 40 cc or more per day. The rats are then returned to the non-protein diet for 3 days for redepletion. Rats which do not respond well can be discarded here. When the liquid hydrolysate is again offered to rats prepared in this way, they regularly consume 40 cc or more per day for 12 days.

The rats are weighed before the initial feeding at the beginning of the 12-day assay period, and again 24 hours after the last feeding. In this way the possibility of weighing just after the animal has consumed a large volume of solution is avoided. Because no water is offered more concentrated hydrolysates are diluted to about 0.65% nitrogen before feeding. This level of nitrogen represents about 5% hydrolysate solids.

In *ad libitum* feeding trials with acid hydrolysates of fibrin rats consumed an average of 50-60 cc daily, equal to 0.32-0.4 g N. Separate experiments indicated that the nitrogen efficiency ratio (ratio of weight gain to N intake) is at a peak at 0.24 g N per day. This level provides the equivalent of 1.5 g average protein per day. In the present study both *ad libitum* and restricted feeding methods are reported. Continuing studies have been carried out chiefly with restricted feeding—the depleted rats readily consume the allotment of about 35 cc of 5% hydrolysate equal to 0.24 g N in 10-20 hours.

Application. Ad libitum liquid hydrolysate feeding was compared with feeding the dried hydrolysate added to the diet at a level to supply 2% N. Comparison was also made with intact fibrin at a level which supplied

[1] Horwitz, A., Sachar, L. A., and Elman, R., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 118.

[2] Mueller, A. J., *Ind. Eng. Chem. Anal. Ed.*, 1945, **17**, 639.

[3] Wissler, R. W., Woolridge, R. L., Steffee, R. H., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

[4] Frazier, L. E., Wissler, R. W., Steffee, C. H., Woolridge, R. L., and Cannon, P. R., *J. Nutrition*, 1947, **33**, 65.

[5] Wissler, R. W., Steffee, C. H., Woolridge, R. L., Benditt, E. P., and Cannon, P. R., *J. Am. Diet. Assn.*, 1947, **23**, 841.

[6] Adolph, E. F., *Fed. Proc.*, 1947, **6**, 67.

[7] Frost, D. V., Heinsen, J., and Olsen, R. T., *Arch. Biochem.*, 1946, **10**, 215.

TABLE I.
Comparison of Different Methods of Feeding Protein Hydrolysate. Comparison with Whole Proteins.

Supplement	How supplied	No. of rats	Intake of allotted N, %	Weight gain Range, g	Weight gain Avg. g
Casein	Separate from diet: 0.24 g N/day	5	96	27-52	44
Fibrin	Separate from diet:				
	0.24 g N/day	4	100	58-60	59
	0.32 " "	5	99	53-74	68
	At 1.9% N in diet:	6	—	53-80	70
5% Fibrin† Hydrolysate	35 cc = 0.24 g N/day	6	100	44-55	49
	47 " 0.32 " "	5	100	47-65	56
	Ad libitum:	12	—	44-77	61
Hydrolysate dried	At 2% N in diet:	5	—	49-73	65

* Aminosol is the trade name for the intravenous partial acid hydrolysate of fibrin used in these studies.

1.9% N in the diet.

Further experiments were carried out wherein powdered, purified fibrin and casein were fed as separate supplements at levels of 0.24 and 0.32 g N per day for comparisor with a 5% partial acid hydrolysate of fibrir fed at similar levels of nitrogen. The dry proteins were fed in small cups attached tc

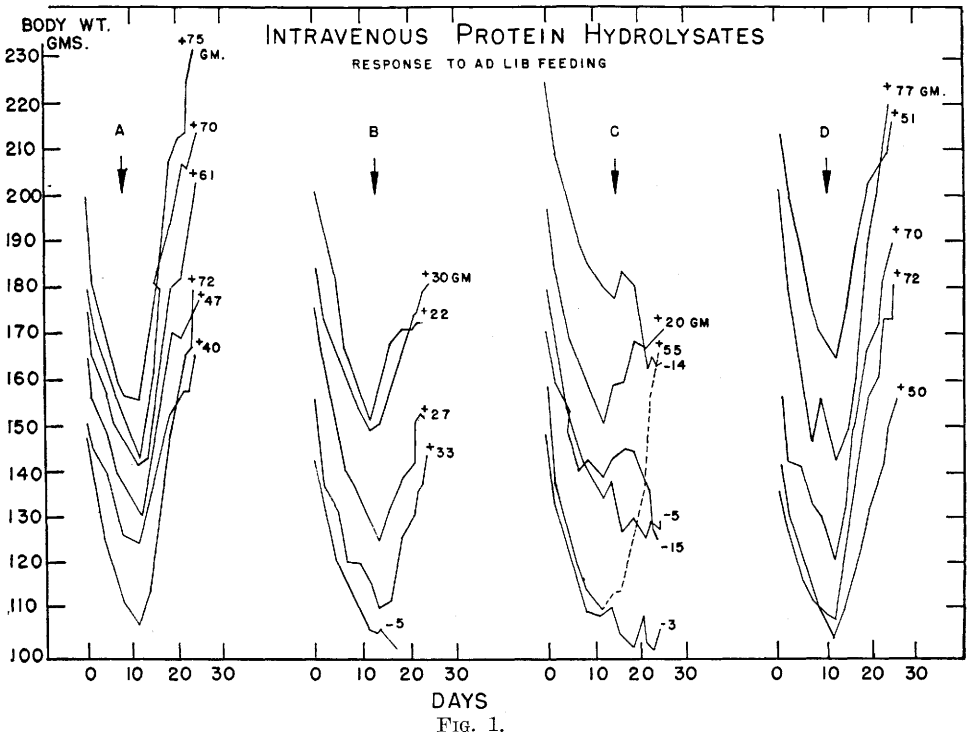

FIG. 1.

Weight changes of individual rats in 4 groups during depletion on non-protein diet and during repletion following *ad lib* feeding of 4 different protein hydrolysates. The marked weight gain of one of the rats on hydrolysate C is shown with a dotted line because it is quite different from the responses of other rats on this hydrolysate. The 12-day weight gains or losses of the individual rats are shown to the right of each curve.

TABLE II.
Essential Amino Acid Analysis of Four Intravenous Protein Hydrolysates and of Whole Egg (Calculated to 16% N).

	A %	B %	C %	D %	Defatted whole egg %
Isoleucine	4.6	4.9	3.6	6.3	5.7
Leucine	6.5	6.9	9.3	9.5	9.1
Valine	4.8	5.9	7.7	7.4	6.4
Threonine	6.7	3.3	7.1	4.4	4.9
Methionine	2.6	3.0	1.1	2.7	3.3
Cystine	2.0	1.2	1.7	1.4	2.4
Phenylalanine	3.4	2.2	3.5	4.7	5.5
Tryptophan	1.0	0.4	0.4	0.9	1.5
Lysine	8.4	6.8	7.8	7.6	6.0
Histidine	2.3	2.3	2.2	2.5	2.5
Arginine	5.7	3.2	3.5	2.9	6.4

Cystine was determined chemically by the method of Folin and Marenzi,[12] methionine by the method of White,[11] and tryptophan by the method of Graham *et al.*[10] All other amino acids were determined microbiologically by the method of Stokes *et al.*[9]

the cage walls by metal clips. Water was offered *ad libitum* to these groups. The rats accepted these particular dried proteins with avidity throughout; however, subsequent trials with other proteins have not been generally as successful. A summary of the above experiments is shown in Table I.

Protein hydrolysates designated A, B, C and D made from different proteins and known to differ in amino acid value were assayed. The hydrolysates were all made to contain .65% nitrogen before feeding. Both the protein-free diet and the hydrolysates were fed *ad libitum* to all groups. The depletion and growth responses are shown in Fig. 1.

The 4 hydrolysates were then subjected to analysis for the essential amino acids. As a reference protein of known high biological value defatted whole egg was analyzed simultaneously, as suggested by Block and Mitchell.[8] The analytical results and references to the methods used are shown in Table II. The limiting amino acids for hydrolysate B appear to be tryptophan and phenylalanine, and for hydrolysate C, tryptophan and methionine. No marked deficiencies appear in hydrolysates A and D. The magnitude of the repletion response appeared unrelated to the original weights of the rats over a rather wide range.

Amino Acid Mixtures in Solution. A mixture of 16 amino acids* patterned after casein (Ration A of Frazier, *et al.*)[4] was found to be somewhat less than completely soluble at a level of 5% solids in water. Adjustment of the pH to 5.5 or to 7.5 did not correct this problem. The solution used for feeding was pH 5.5. The solution was diluted to 4% solids and an equal amount of dextrose was added. On heating the amino acids went into solution; however, slight crystallization of tyrosine occurred again on cooling. Cysteine hydrochloride was added at a level of 0.6% of the amino acid solids to correct partially the known deficiency of total sulfur amino acids. This amino acid mixture was designated Amino Acid Solution A_1 and the composition is shown in Table III.

A preliminary comparison was made of the growth response of standard depleted rats to powdered casein and to the Amino Acid Solution A_1. The casein was fed in cups attached to the sides of the cages and the rats in this

[8] Mitchell, H. H., and Block, R. J., *J. Biol. Chem.*, 1946, **163**, 599.

[9] Stokes, J. L., Gunness, M., Dwyer, J. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

[10] Graham, C. E., Smith, E. P., Hier, S. W., and Klein, D., *J. Biol. Chem.*, 1947, **168**, 711.

[11] White, W. F., *J. Biol. Chem.*, 1945, **158**, 535.

[12] Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, 1929, **83**, 109; 1934, **106**, 311.

* Crystalline amino acids were obtained chiefly from Merck; also from Dow, Winthrop, Pfanstiehl and Monsanto. Purity was established by chemical or microbiological assay before use.

group were given water *ad libitum*. Both supplements were offered in amounts to supply 0.24 g N per rat day. Of 6 rats in this group, one failed to take the dry casein at all and was discarded, while the remaining 5 gained 27, 40, 48, 52 and 52 g.

Two rats, similarly prepared, received 48 cc per day of Solution A_1 and gained 52 and 55 g. The rats consumed 93% of their liquid allotments. This response is especially significant in view of the fact that the synthetic form of certain of the amino acids used, *i.e.* valine, threonine, isoleucine and phenylalanine have been shown to be incompletely used by the growing rat, the D-isomer being partly or completely rejected.[13] Further work with complete hydrolysates of fibrin fed similarly has indicated that responses of a very high order are regularly obtained with solutions containing only amino acids.

TABLE III.
Composition of Amino Acid Mixtures Fed in Solution.

	Sol. A-1 contained 4% of mixture, g/100 g	Sol. A-4 contained 5% of mixture, g/100 g
l-Arginine HCl	4.21	4.0
l-Histidine HCl	2.87	3.0
dl-Isoleucine	11.05	11.0
l-Leucine	10.29	10.0
l-Lysine HCl	8.05	8.0
dl-Methionine	2.98	5.0
dl-Phenylalanine	4.42	9.0
dl-Threonine	6.63	6.5
dl-Tryptophan	1.53	1.5
dl-Valine	11.9	12.0
dl-Alanine	4.76	6.0
dl-Aspartic acid	5.36	5.0
l-Cystine	0.31	—
l-Glutamic acid	20.16	14.0
Glycine	0.42	5.0
l-Tyrosine	5.44	—
	100.38	100.0

l-Cysteine hydrochloride monohydrate was added to Solution A-1 at a level equal to 0.6% of the amino acid solids.

An attempt was made to compensate for the insolubility of certain of the amino acids and to prepare a mixture of amino acids of equal nutritive value to amino acid solution A_1, but one which would be soluble to the extent of 5% in water. Solution A_4 (Table III) was developed along this line and was made up for feeding with 5% dextrose. The pH was 4.7. In order to demonstrate the effect of complete omission of one of the essential amino acids, mixture A_4 minus isoleucine was made up similarly.

The effect of feeding Solution A_4 and Solution A_4 minus isoleucine to standard depleted rats is shown in Fig. 2. Because they received no water other than that in the amino acid solution the rats were forced to take some of the incomplete mixture if only to satisfy thirst; however, they showed an actual aversion for the solution and drank an average of only about 2 cc per day. The response of the 6 rats to Solution A_4 was unusually erratic and only 70% average allotment was consumed. In repeat experiments responses of about 60 g have been obtained with much less variation than that shown by the present group. A gradual decrease in intake of this type of solution has been noted and suggests a possible inhibitory effect of the *D*-amino acids present.

Discussion. The data at hand suggest that adequacy of the 9 amino acids described by Rose[13] and established in the rat repletion assay by Frazier, *et al.*[4] is the most important single criterion which must be met if a hydrolysate is to be of high nutritive value. On failure of a hydrolysate by the rat repletion technic, one would look first for limitations in the essential amino acid composition, as has been done in the above experiments. Such questions as palatability to the rat and the role played by the so-called dispensable amino acids require individual study.

An advantage of the method is the ability to feed the liquid supplement accurately and rapidly. No wastage of the nitrogen component occurs and measurement of any liquid not consumed is convenient. The nutritive adequacy of the nitrogen component determines the appetite of the animal for the non-nitrogenous part of the diet. The practice of rationing only the nitrogen component at a level of 240 mg N per day appears to provide a fairly critical assay with a minimum of handling.

Summary. The 12-day weight response of

[13] Rose, W. C., *Physiol. Rev.*, 1938, **18**, 109.

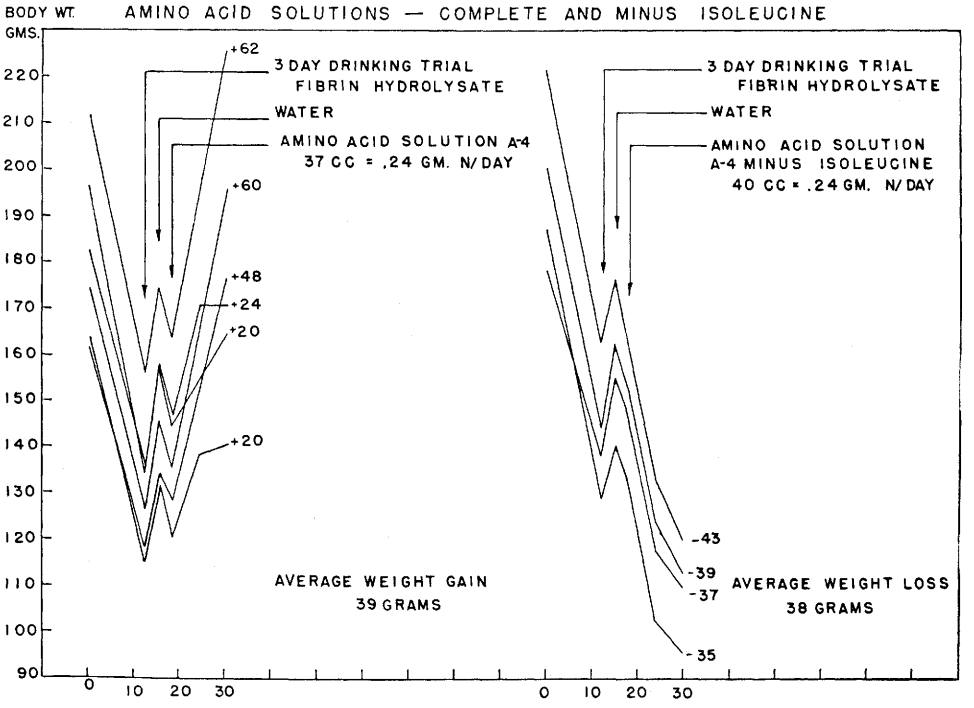

FIG. 2.

Weight changes of individual rats during 12-day depletion, 3-day orientation to liquid feeding, 3-day redepletion and final assay periods. Assay of a 5% amino acid solution patterned after the amino acid composition of casein (corrected for insolubility of tyrosine, cystine, and glutamic acid) versus the similar mixture minus isoleucine.

young adult protein-depleted rats to 5% protein hydrolysates fed as the sole source of amino acid nitrogen provides a basis for rapid assay. The response to liquid hydrolysate feeding approached that of dried hydrolysate or whole protein added to the diet. Pure amino acid solutions of good nutritive composition are readily accepted and support good weight recovery. The effect of the *D*-amino acids in such mixtures requires clarification.

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16389

Enzyme Studies on Human Blood. II. The Substrate in the Fibrinogen-Thrombin Reaction.*

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Divergent views exist in respect to the influence of fibrinogen concentration on the fibrinogen-thrombin reaction. An optimal range of substrate concentration for the velocity of the fibrinogen-thrombin reaction has been reported.[1,2,3,4] In this communica-

* Fibrinogen fractions were prepared from dried normal human plasma which had been processed from blood obtained from volunteer donors enrolled by the American Red Cross.