A microbiological method for assessing the nutritional value of proteins

2.* The measurement of 'available' methionine, leucine, isoleucine, arginine, histidine, tryptophan and valine

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The nutritive value of a food protein, measured in tests with laboratory animals, has no exact significance except when carefully qualified in terms of the species, strain and age of the test animals used, and of the particular physiological response selected for measurement. It must also be specified at what level the test protein is incorporated in the diet, and whether it provides all or only a part of the dietary protein. The values obtained are not uniquely descriptive, in the sense that proteins of widely different amino acid composition may be judged of equal nutritional merit. And it must be remembered that estimates of the nutritive quality of individual proteins cannot be extended to predictions of their value in mixed diets. Practical diets usually contain several proteins, whose combined nutritive value might well exceed a calculated mean value if amino acids deficient in one protein are balanced by a relative surplus in others. Supplementary relationships of this kind are of common occurrence. The problem of the nutritionist, when devising diets for man or beast, is to provide adequately and economically all the amino acids required. To do it he needs a means to evaluate proteins as sources of these amino acids, and especially of lysine and methionine which are commonly limiting. Much of the information available in the various tables of amino acid composition of foodstuffs is unreliable for this purpose. Protein foods that have been subjected to heating during processing, or to unfavourable conditions of storage, may be highly resistant to enzymic digestion in vivo, though yet susceptible to chemical hydrolysis, and for such materials the information obtained from the analysis of chemical hydrolysates may be wholly misleading. Within groups of similar proteins—as for example meat meals or fish meals of different manufacture—there may well be large differences in nutritive value but only small and irrelevant differences in amino acid composition. It has become evident that, when the main requirement of a 'grading' test is that it should indicate reliably any diversity of nutritive quality among samples of the same designation, then the use of tests which involve a preliminary chemical hydrolysis of the test samples is not appropriate.

Carpenter & Ellinger (1955) recognized that the value of heated proteins as sources of lysine may be very much less than would appear from measurements of lysine in the hydrolysed proteins. They developed a chemical method for measuring only the

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'available' lysine in the proteins, and in a modified form (Carpenter, 1960) this method shows great promise as an indicator of protein quality (cf. Boyne, Carpenter & Woodham, 1961). An object of the present paper is to describe a microbiological method for measuring 'available' methionine, leucine, isoleucine, arginine, histidine, tryptophan and valine.

EXPERIMENTAL

Test organism. The organism used for these tests was *Streptococcus zymogenes* NCDO 592, obtained from the National Collection of Dairy Organisms at the National Institute for Research in Dairying, Shinfield, Reading. It is vigorously proteolytic and has an absolute requirement for exogenous methionine, tryptophan, arginine, histidine, leucine, isoleucine, valine and glutamic acid. Of the 'essential' amino acids, lysine, threonine and phenylalanine were not indispensable for *Strep. zymogenes* though the omission of any one from the culture medium caused a marked fall in growth rate (cf. Ford, 1960).

Table 1. *Composition of the assay medium*

Basal medium		Amino acid supplement*	
Glucose (g)	12	L-glutamic acid (g)	1
K_2HPO_4 (g)	12	L-leucine (g)	0·5
Citric acid (g)	0·5	L-isoleucine† (g)	0·5
Sodium acetate (trihydrate) (g)	2·5	L-valine (g)	0·5
Tween 80‡ (ml)	1	L-lysine (g)	0·5
Solution of mineral salts§ (ml)	10	L-alanine (g)	0·5
Adenine (mg)	5	L-aspartic acid (g)	0·5
Guanine (mg)	5	L-arginine (g)	0·2
Uracil (mg)	5	L-methionine (g)	0·2
Xanthine (mg)	5	Glycine (g)	0·2
Thiamine (mg)	2	L-cystine‖ (g)	0·2
Pyridoxal ethylacetal hydrochloride (mg)	2	L-serine (g)	0·2
Riboflavin (mg)	2	L-tyrosine (g)	0·2
Nicotinic acid (mg)	2	L-proline (g)	0·2
Calcium pantothenate (mg)	2	L-histidine (g)	0·2
p-Aminobenzoic acid (mg)	2	L-phenylalanine (g)	0·2
Folic acid (mg)	0·2	L-threonine (g)	0·2
Biotin (μg)	10	L-tryptophan (g)	0·2
Ascorbic acid (g)	0·5	pH adjusted with N-KOH to 7·2	
Vitamin B_{12} (μg)	2	Water added to 250 ml	
pH adjusted to 7·2			
Water added to 200 ml			

* Added to the basal medium in the proportion of 2 vol. basal medium to 1 vol. supplement. The amino acid to be assayed was omitted.

† Allo-free.

‡ Polyoxyethylene sorbitan mono-oleate.

§ Contained $MgCl_2.6H_2O$, 20 g; $CaCl_2$, 5 g; $FeCl_3.6H_2O$, 0·5 g; $ZnSO_4.7H_2O$, 0·5 g; $MnSO_4.4H_2O$, 0·25 g; $CoCl_2.6H_2O$, 0·25 g; $CuSO_4.5H_2O$, 0·25 g; VSO_4, 0·25 g; Na_2MoO_4, 0·25 g, dissolved in 1 l. distilled water with addition of N-H_2SO_4 to clear.

‖ First dissolved separately in 10 ml boiling water by addition of N-KOH.

Assay medium. The assay medium was prepared from two stock solutions—the basal medium and an amino acid supplement—which were made up and stored separately at −20°. Their composition is shown in Table 1, which lists the 'complete' supplement of amino acids from which the particular amino acid to be assayed was

omitted. When required for use, the stock solutions were thawed and the solution of amino acids was warmed to dissolve the precipitate that had formed on cooling. The solutions were then combined in the proportions of two vol. basal medium to one vol. amino acid supplement.

Maintenance of stock cultures. The stock cultures were grown at 37° for 24 h, first in a broth comprised of basal medium supplemented with 200 mg Tryptone (Oxo Ltd)/100 ml, and then in stab culture in basal medium supplemented with 150 mg casein, 15 mg sodium glutamate and 1·5 g agar/100 ml. The stab cultures were stored at 2° and subcultured at intervals of 1 month.

Preparation of inocula. The assay tubes were each inoculated with one drop of 24 h culture (undiluted) grown at 37° in basal medium supplemented with 150 mg casein and 15 mg sodium glutamate/100 ml. The culture was maintained by daily transfer. The use of this heavy inoculum was necessary to ensure linearity of the dose-growth responses and the reproducibility of the results from test to test.

Materials. A variety of food proteins was examined, most of them of animal origin. Several (series WM, FM and MM) had been selected as representative of a wide range of sources and quality and distributed by the Agricultural Research Council in connexion with its collaborative investigation into the measurement of the quality of protein feeding-stuffs (cf. Anonymous, 1959; Boyne *et al.* 1961). The code numbers of samples are those uniformly used by the participants in this collaborative investigation.

Preparation of heat-damaged fish meal and dried skim milk. Samples of a white-fish meal and of a freeze-dried skim milk were spread in shallow (0·5 cm) layers on stainless steel trays and heated at 121° in steam for $\frac{1}{2}$, 1, $1\frac{1}{2}$, $4\frac{1}{2}$ or 18 h. On removal from the autoclave these samples, together with unheated 'control' samples, were dried at room temperature under reduced pressure and milled to pass a 60-mesh sieve.

Preparation of proteins for test. The proteins to be tested were ground as finely as was possible in a laboratory mill and rubbed through a 60-mesh screen. Two samples of each were then weighed, containing precisely 100 mg nitrogen, and prepared for test by the following procedures.

(*a*) For the measurement of 'total' methionine, leucine, isoleucine, arginine, histidine and valine: One of each pair of weighed samples was transferred to a Carius tube of about 25 mm internal diam. and 200 mm length, and 40 ml of 2 N-HCl were added. The tubes were sealed, placed horizontally in a steam autoclave and heated for 5 h at 115°. After cooling, the tubes were opened, and their contents brought to pH 7·0 with 10 N-NaOH and then diluted to 100 ml with water. The hydrolysates were now shaken vigorously to disperse uniformly any undissolved material. Finally, 10 ml portions were quickly taken and diluted to 100 ml with water.

It was considered undesirable to filter these test extracts.

This choice of conditions of hydrolysis was dictated by the need to avoid an inhibitory concentration of sodium chloride in the assays, and to minimize charring and darkening of the hydrolysates. The assay solutions contained about 0·5% sodium chloride; tests showed that the presence of $< 0{\cdot}75\%$ sodium chloride in the 'standard' solutions of amino acids caused no significant changes in the growth response curves.

In comparative tests, leucine and methionine were assayed in four whale-meat meals. Results obtained after hydrolysis for 18 h with boiling 6 N-HCl were broadly similar to those found after hydrolysis under the conditions described above. The values for leucine were about 15% higher, and those for methionine about 10% lower.

(*b*) For the measurement of 'available' methionine, leucine, isoleucine, arginine, histidine, tryptophan and valine: The other of each pair of samples was transferred to a glass-stoppered test tube of 50 ml capacity to which were added 20 ml of an aqueous solution containing, per l., 5 g sodium citrate, 30 mg sodium cyanide and enough N-H_3PO_4 to bring the pH to 7·0. The pH of the tube contents was adjusted to 7·0. The tubes were placed in a water-bath at 56° and to each was added 1 ml of a 1% (w/v) solution of crude papain (L. Light and Co., Colnbrook, Bucks.) in the citrate buffer. After incubation for 2 h with frequent shaking, the digests were diluted to 100 ml with water. Finally, after vigorous shaking, 10 ml portions were quickly taken and diluted to 100 ml with water. The amount of papain used contributed 1 mg nitrogen/100 mg nitrogen of the sample, which quantity has been disregarded in the calculations.

Preparation of the reference standard solutions of amino acids. Stock solutions of amino acids (L. Light and Co., Colnbrook, Bucks.) containing, per ml, 2 mg L-methionine, L-arginine, L-leucine, L-isoleucine (allo-free), L-histidine (free base), L-valine or L-tryptophan were made up in an aqueous solution containing 10% (v/v) ethanol and stored at 2°. From these stock solutions 'standard' solutions were prepared containing, per ml, 20 μg L-methionine, 20 μg L-arginine, 40 μg L-leucine, 20 μg L-isoleucine, 10 μg L-histidine, 40 μg L-valine or 5 μg L-tryptophan. For assaying amino acids in the acid hydrolysates, the 'standard' solutions were made up in a 0·46% (w/v) aqueous solution of sodium chloride; for the assays of 'available' amino acids the 'standard' solutions were made up in water. In point of fact, this duplication of the 'standard' solutions appears to have been unnecessary as the presence in them of $< 0{\cdot}75\%$ sodium chloride was later found to cause no significant change in the growth-response curves.

Procedure. The assays were set up in wire racks, each holding seventy-two 19 × 150 mm optically matched Pyrex test tubes. The 'standard' and the 'test' preparations were each added to four pairs of tubes in amounts of 1, 2, 4 and 8 ml. To each tube 3 ml of the assay medium (i.e. basal medium with the appropriate amino acid supplement, Table 1) were then added, and water to bring the fluid content to 11 ml. The racks of filled tubes were each covered with a folded towel held firmly in position by an aluminium lid. They were then heated in flowing steam for 20 min, cooled in a water-bath at 37°, inoculated, and returned to the water-bath to incubate for 48 h.

Measurement of growth response. After incubation for 48 h the racks of tubes were heated in flowing steam for 10 min and cooled to room temperature. The heated skim-milk powders gave highly coloured extracts, and for them the growth responses were assessed acidimetrically. For all the other foods examined, the responses were measured photometrically as follows. The assay tubes were stoppered and shaken vigorously and set aside for 2–3 min to allow air bubbles to rise and particulate food residues to settle. The optical densities of the cultures were then measured in the

tubes with a Lumetron Model 400 A colorimeter (Photovolt Corporation, New York) fitted with a 580 mμ glass filter.

Calculation of results. The results were obtained by the process of 'reading off' the standard response curves. The dose-response relationships were generally not rectilinear and it was not possible consistently to apply a simple linear transformation to the standard response curves. Each result represents six or eight observations, being a mean of values calculated from the growth responses in duplicate tubes at three or four dosage levels. These observations were not strictly independent, being made within a single test, upon a single preparation of the test protein. The between-assay standard deviation of an estimate of 'available' methionine in a whale-meat meal (WM13) and a fish meal (FM6) was about ±7%. For other amino acids, and for other types of test proteins, this figure might have been different. With cottonseed and linseed meals, for example, there are special difficulties of preparing and dispensing the test extracts and of estimating the growth responses that add considerably to the errors of measurement. No statistical assessment of the errors of the measurements was attempted, and probably none was warranted.

Definitions of terms. Relative nutritive value (RNV): the value of the protein for promoting the growth of *Strep. zymogenes*, relative to that of casein = 100 (cf. Ford, 1960).

Net protein utilization (NPU): rat-assay value, determined by the method of Miller & Bender (1955).

Gross protein value (GPV): chick-assay value, measured as described by Duckworth, Woodham & McDonald (1961).

RESULTS AND DISCUSSION

Fig. 1 shows the growth responses of *Strep. zymogenes* to graded limiting concentrations of arginine, valine, methionine, leucine, isoleucine, histidine, tryptophan and glutamic acid. The organism seems to offer a means for assaying any one of these amino acids, whether free or bound in protein. Detailed study was in part restricted, for lack of time and technical facilities, to only four of these amino acids, namely methionine, arginine, leucine and tryptophan.

Whale-meat meals. The twelve whale-meat meals (series WM, Table 2) were selected for this study because they showed wide differences in nutritive quality within one type of material (Bunyan & Price, 1960; Ford, 1960). They were very similar in their content of 'total' leucine and 'total' arginine, and probably also of 'total' lysine. The values for 'total' methionine showed greater differences, and correlated with the values representing the quality of the meals for the rat ($r = 0{\cdot}57$; $P = 0{\cdot}05$) and for *Strep. zymogenes* ($r = 0{\cdot}65$; $P = 0{\cdot}02$).

The values obtained for 'available' methionine, tryptophan, leucine and arginine were closely correlated with each other and with the rat-assay and the 'available' lysine values obtained for these same products by Bunyan & Price (1960) (Table 3).

None of these whale-meat meals was improved in value for *Strep. zymogenes* by supplementation with L-lysine (2%) and L-methionine (1%), separately or together. Further tests on two of the meals, WM5 and WM10, showed that no one of the

'essential' amino acids improved their nutritive value. From all this evidence it seemed probable that the differences in the nutritive quality of these meals reflected corresponding differences in the biological availability of several or all of their constituent amino acids.

Fish meals. The fifteen fish meals (series FM, Table 4) were more uniform in their nutritive quality than were the whale-meat meals. For ten herring meals in this

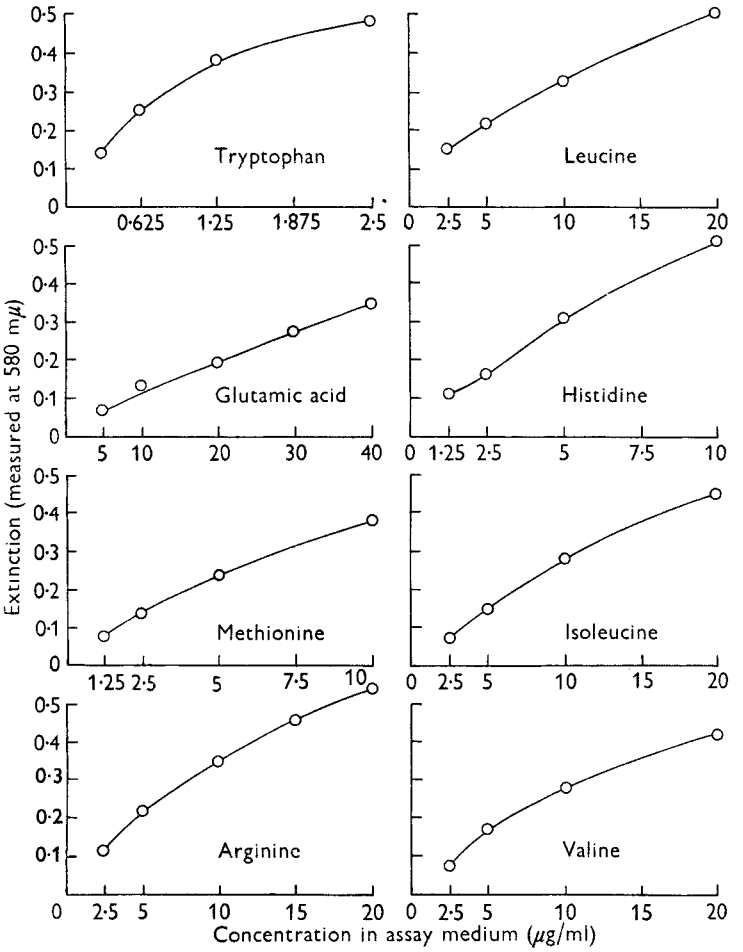

Fig. 1. Growth response of *Streptococcus zymogenes* to graded concentrations of methionine, arginine, tryptophan, valine, leucine, isoleucine, histidine and glutamic acid.

series, values for *Strep. zymogenes* ranged from 57 to 85, and GPV for the chick (Boyne *et al.* 1961) from 106 to 119. Boyne *et al.* also measured the 'available' lysine in the fish meals and obtained values which correlated closely with their chick-assay values. For the ten herring meals the coefficient of correlation (r) between GPV and 'available' lysine was 0·82. This finding was not unexpected, since the GPV is essentially a measure of biologically available lysine. It is of greater interest that the chick-assay values correlated also with 'available' methionine (r = 0·66) and 'available' tryptophan

Table 2. *Methionine, leucine, arginine, tryptophan and lysine contents* (*g*/16 *g N*) *of whale-meat meals*

Whale-meat meal, code no.*	Methionine		Leucine		Arginine		Lysine†		Tryptophan	Rat-assay value (NPU)‡	RNV§
	Total	'Available'	Total	'Available'	Total	'Available'	Total	'Available'	'Available'		
WM 1	2·5	1·0	9·0	3·4	5·6	2·5	8·8	4·5	0·30	25	55
WM 2	3·3	1·5	9·6	4·8	5·8	3·2	—	6·3	0·51	36	58
WM 3	3·2	1·7	9·9	5·3	5·5	3·3	—	7·0	0·59	44	62
WM 4	3·1	1·7	10	6·2	5·4	4·1	9·7	8·1	0·66	57	73
WM 5	2·5	1·6	9·9	5·6	5·7	4·1	—	7·1	0·53	37	55
WM 6	2·9	2·0	9·9	5·8	5·7	4·1	—	6·4	0·73	52	70
WM 7	2·2	0·81	8·5	1·9	5·5	2·0	8·1	3·3	0·14	17	26
WM 9	2·7	2·1	9·9	7·1	5·6	4·5	—	7·0	0·73	61	68
WM 10	2·5	1·5	9·9	5·2	5·6	3·2	—	5·4	0·51	35	52
WM 11	2·6	1·7	9·7	5·0	5·8	3·7	—	6·6	0·61	41	66
WM 12	2·9	1·6	10	4·3	6·1	3·3	8·9	5·1	0·59	31	62
WM 13	3·0	2·5	10	7·2	5·7	4·9	9·6	7·3	1·1	62	83

* For meanings of reference code numbers see p. 411.

† Values from Bunyan & Price (1960). Total lysine was measured microbiologically with *Leuconostoc mesenteroides* P 60; 'available' lysine was measured by the dinitrofluorobenzene method of Carpenter & Ellinger (1955).

‡ Net protein utilization (see p. 413); values from Bunyan & Price (1960).

§ Relative nutritive value (casein = 100) for *Streptococcus zymogenes* (see p. 413).

($r = 0{\cdot}63$). These values of r are not significantly different from that for lysine when the errors inherent in all the measurements and the small differences in quality between the samples are taken into account.

The chick-assay values and the 'available' lysine values correlated rather poorly ($r = 0{\cdot}44$) with the RNVs (Ford, 1960) for *Strep. zymogenes*, but here the point of interest is not that they correlated poorly but that they should have correlated at all, as *Strep. zymogenes* has no nutritional requirement for exogenous lysine. The RNVs correlated more closely with the values for 'available' methionine ($r = 0{\cdot}79$) and 'available' tryptophan ($r = 0{\cdot}89$), and also with 'available' arginine ($r = 0{\cdot}61$) and 'available' leucine ($r = 0{\cdot}54$). It is apparent that in this series of fish meals, as in the whale-meat meals, the differences in nutritive quality reflected corresponding differences in the availability of several or all of the constituent amino acids.

Table 3. *Correlations between the rat-assay values of twelve samples of whale-meat meal and their content of total methionine and 'available' methionine, leucine, arginine, tryptophan and lysine*

	'Available'					Total
	Methionine*	Leucine*	Arginine*	Tryptophan*	Lysine†	methionine*
Rat-assay value (NPU)	0·92	0·95	0·92	0·89	0·86	0·57 ($P < 0{\cdot}05$)
'Available' methionine	—	0·93	0·94	0·98	0·77 ($P < 0{\cdot}01$)	0·70 ($P < 0{\cdot}01$)
Leucine	—	—	0·96	0·90	0·89	—
Arginine	—	—	—	0·92	0·86	—
Tryptophan	—	—	—	—	0·75 ($P < 0{\cdot}01$)	—
Protein-quality rating for *Strep. zymogenes*	—	—	—	—	—	0·65 ($P < 0{\cdot}02$)

$P < 0{\cdot}001$ except where stated.
* Measured microbiologically with *Strep. zymogenes*.
† Measured chemically by the method of Carpenter, Ellinger, Munro & Rolfe (1957); values from Bunyan & Price (1960).

The availability of methionine, leucine and arginine in these fish meals was surprisingly low (Table 4). The values obtained, which ranged from about 50 to 70%, were not increased by prolonging the time of incubation in the assays and have been regarded as absolute, at least for *Strep. zymogenes*. Of course, higher animals, perhaps with 'stronger' digestive capabilities, might make more efficient use of these proteins. Different results might also be obtained according to the methods of biological evaluation adopted and the species of experimental animal used. Kraft & Morgan (1951), for example, showed that the nutritional availability of heat-damaged proteins was quite different for the rat and the dog. Because of all this, the microbiological-assay results are difficult to interpret in precise terms of biological availability for higher animals. They do perhaps warrant a suggestion that there may be considerable scope for improvement in the quality of commercial fish meals.

Miscellaneous protein foods. Table 5 shows values obtained for an assortment of

Table 4. *Methionine, leucine, arginine, tryptophan and lysine contents (g/16 g N) of fish meals*

Fish meal		Methionine		Leucine		Arginine		Tryptophan	Lysine†		
Code no.*	Description	Total	'Available'	Total	'Available'	Total	'Available'	'Available'	'Available'	RNV‡	GPV§
FM 1	Herring meal	2·9	1·8	8·0	5·0	7·0	4·5	0·55	6·4	64	112
FM 2		3·1	1·4	7·8	4·6	6·9	4·2	0·28	5·8	57	106
FM 5		3·2	1·9	8·4	5·2	7·0	5·3	0·56	6·5	70	119
FM 6		3·4	1·9	8·7	5·3	7·0	5·5	0·61	6·6	73	116
FM 8		3·2	1·5	8·0	4·4	5·5	4·1	0·49	7·1	68	117
FM 10		3·2	1·9	8·3	4·8	6·9	5·0	0·68	6·3	85	112
FM 11		3·1	1·5	8·6	4·9	7·2	5·0	0·56	6·2	67	113
FM 12		3·5	2·0	9·0	5·6	7·6	5·7	0·68	6·5	83	113
FM 13		3·0	1·3	8·3	4·7	6·9	5·1	0·32	5·9	61	108
FM 14		3·1	1·8	9·2	5·7	7·8	5·4	0·63	6·9	75	115
FM 19	White-fish meal	3·6	1·9	7·7	6·3	6·7	4·7	0·48	—	73	100
FM 21		3·6	1·8	7·8	5·6	7·1	5·3	0·51	—	71	98
FM 22		3·2	1·6	6·8	5·0	6·7	4·5	0·38	—	60	92
FM 15	Cod meal	3·2	1·9	7·4	5·1	6·9	4·7	0·48	6·1	71	110
FM 7	Pilchard meal	2·8	1·4	8·3	4·2	5·8	3·7	0·52	6·7	61	121

* For meaning of reference code numbers see p. 411.
† Measured chemically by the method of Carpenter *et al.* (1957); values from Boyne, Carpenter & Woodham, 1961.
‡ Relative nutritive value for *Strep. zymogenes* (see p. 413).
§ Gross protein value; values from Boyne *et al.* (1961) (see p. 413).

Table 5. *Methionine, leucine, arginine, tryptophan and lysine contents (g/16 g N) of miscellaneous protein foods*

Protein food*	Methionine		Leucine		Arginine		Tryptophan	Lysine†	
	Total	'Available'	Total	'Available'	Total	'Available'	'Available'	'Available'	RNV‡
Meat meal (MM 3)	1·0	1·0	5·4	3·0	6·7	4·0	0·18	3·6	32
Meat-and-bone meal (MM 10)	1·1	1·0	6·9	3·2	6·4	3·1	0·21	3·7	39
Meat meal (MM 16)	1·0	0·6	8·2	2·8	6·9	2·1	0·13	2·6	26
Meat meal (MM 18)	1·5	1·2	8·5	4·0	6·4	3·5	0·40	4·9	50
Indian fish meal (FM 3)	2·2	0·7	6·4	3·2	6·3	2·3	0·14	3·6	34
Crayfish meal (FM 4)	2·3	0·7	7·1	4·2	5·7	2·6	0·24	3·6	34
Cod-bone meal (FM 17)	2·7	1·6	4·7	3·7	7·1	4·9	0·30	4·9	49
Herring meal (GF 20)	3·5	2·3	7·5	5·9	5·4	4·7	0·69	—	67
Peruvian fish meal (GE 22)	3·5	1·8	7·7	4·7	5·8	3·3	0·44	—	58
Overheated tunny-fish meal (GF 627)	2·3	0·3	6·2	0·8	4·3	0·7	0·04	—	11
Rosefish meal (EBGH 2)	3·9	2·1	8·4	4·6	6·2	4·2	0·51	—	66
Menhaden meal (GG 3-3 A 85)	3·5	1·3	8·1	3·5	5·6	3·0	0·29	—	60
Pea flour	1·0	0·9	6·4	6·1	6·6	6·8	1·0	—	53
Cottonseed meal	1·7	1·3	5·9	5·1	9·3	9·1	1·2	—	71
Maize-gluten meal	3·3	2·1	15·0	9·7	4·3	3·5	0·4	—	71
Dried food yeast	1·6	1·2	6·7	4·6	3·8	3·0	0·7	—	82
Wheat gluten	1·6	1·6	7·0	6·5	3·6	3·5	1·1	—	69
Soya-bean meal	1·5	1·2	6·7	5·7	7·4	5·7	1·1	—	57
Groundnut meal	1·3	1·0	7·9	6·4	11·0	8·2	0·9	—	66
Wheat germ	1·5	1·5	4·7	4·5	6·3	4·8	1·0	—	71

* For meanings of reference code numbers see pp. 411, 419.
† Measured chemically by the method of Carpenter *et al.* (1957); values from Boyne *et al.* (1961).
‡ Relative nutritive value for *Strep. zymogenes* (see p. 413).

Table 6. *'Available' methionine, arginine and tryptophan, and total methionine and arginine, contents (g/16 g N) of fish meals*

Fish meal, code no.*	Value as sole protein source for chicks†	'Available'						Total			
		Methionine		Arginine		Tryptophan		Methionine		Arginine	
		MA	CA	MA	CA	MA	CA	MA‡	Ousterhout *et al.* (1959)‡	MA‡	Ousterhout *et al.* (1959)‡
GF20	6·7	2·3	2·7	4·7	3·0	0·69	1·3	3·5	2·6	5·4	5·7
GE22	3·8	1·8	—	3·3	1·6	0·44	1·4	3·5	—	5·8	—
EBGH2	6·6	2·1	2·6	4·2	4·7	0·51	0·9	3·9	2·9	6·2	6·7
GG3-3A85	4·2	1·3	2·3	3·0	4·0	0·29	1·1	3·5	2·6	5·6	8·0
GF627	0	0·3	0·7	0·7	0	0·04	0·3	2·3	1·7	4·3	3·8

MA, measured microbiologically with *Streptococcus zymogenes*; CA, chick assay (from Ousterhout, Grau & Lundholm, 1959).
* Code number used by Ousterhout *et al.* (1959).
† Percentage gain/day (from Ousterhout *et al.* 1959).
‡ Measured microbiologically after chemical hydrolysis.

protein foods. The meat meals (series MM) were notably different from the whale-meat meals in their amino acid composition. Guttridge (1961) measured with chicks the 'available' methionine in MM 3, MM 16 and MM 18. His values of 0·88, 0·36 and 0·90 g/16 g N are broadly similar to those found microbiologically with *Strep. zymogenes*. Ousterhout *et al.* (1959) also measured with chicks the biological availability of several amino acids in the fish meals GF 20, GE 22, GF 627, EBGH 2 and GG 3-3 A85. Their values for methionine, tryptophan and arginine are set out in Table 6, with estimates of the relative value of the meals when given to chicks as the sole protein source. If the microbiological-assay values are considered first, it seems that in these five samples, as in those listed in Table 4, the differences in 'biological value' reflected corresponding differences in their content of 'available' methionine, arginine and tryptophan. This direct relationship is less apparent when the chick-assay values for these amino acids are compared and an attempt is made to relate them to 'biological values'. Ousterhout *et al.* (1959) interpreted their findings as showing large differences in the availability of different amino acids. Thus, except in the severely heated sample GF 627 and in sample GE 22 for which information was incomplete, tryptophan and methionine were adjudged highly available to the chick. In contrast, the availability of arginine was said to be low and to vary markedly between samples.

It may be that the apparent high availability of the methionine resulted from erroneously low estimates of 'total' methionine, as the values obtained were markedly lower than those found by me (Table 6). If my values are taken as correct, then the ratios of 'available' to 'total' methionine were broadly similar to the corresponding ratios for arginine. There remains a puzzling discrepancy between the chick-assay and the microbiological-assay values for 'available' tryptophan. The microbiological-assay values gave an approximate measure of the nutritive quality of the meals when given to chicks as the sole source of protein, whereas the chick-assay values were relatively high and showed little correlation with nutritive quality.

Influence of heating on the availability of amino acids in dried skim milk and white-fish meal. Table 7 and Figs. 2 and 3 show some effects of heating in steam at 120° for up to 18 h on the nutritive quality of dried skim milk and a white-fish meal.

With the skim-milk powder the colour changed during heating, from white through yellow and brown to chocolate-brown. The solubility of the material diminished progressively, as did its nutritive value for *Strep. zymogenes*. The content of 'available' lysine fell sharply, and after 30 min heating only 14% remained. Of the 86% loss, about one-third was restored on subsequent hydrolysis with 2 N-HCl. The contents of 'available' arginine and histidine both fell to about 42% of the initial values during this 30 min heating. Further heating caused relatively greater damage to arginine than to histidine. As with lysine, only about one-third of the loss of arginine was restored on acid hydrolysis, whereas three-quarters of the lost histidine was recovered. The availability of methionine, valine, leucine, isoleucine and tryptophan also decreased during the heating, but at a slower rate than that of lysine, arginine and histidine. There was little or no fall in the 'total' methionine, valine, leucine and isoleucine even after heating for 18 h.

Table 7. *Influence of heating on the availability to* Streptococcus zymogenes *of some amino acids in dried skim milk and white-fish meal*

Material	Treatment*	RNV†	Arginine		Methionine		Leucine		Isoleucine		Histidine		Valine		Trypto-phan	Lysine	
			Total	'Available'	Total	'Available'	Total	'Available'	Total	'Available'	Total	'Available'	Total	'Available'	'Available'	Total‡	'Available'§
Freeze-dried skim-milk powder	Unheated	108	3·6	3·6	3·7	2·9	11·4	8·8	5·7	4·1	2·9	2·1	6·9	5·9	1·1	9·4	6·9
	Heated:																
	½ h 120°	64	2·2	1·6	3·8	2·0	12·3	7·4	5·6	2·7	2·5	0·83	7·0	5·1	1·0	4·5	1·0
	1 h 120°	44	1·4	1·1	4·0	1·8	12·0	7·1	5·7	2·5	2·5	0·80	6·8	4·8	0·87	3·5	0·90
	1½ h 120°	36	1·0	0·74	4·2	1·7	12·3	7·1	5·5	2·5	2·4	0·77	6·7	4·5	0·79	2·9	0·80
	4½ h 120°	14	0·50	0·25	3·9	1·3	11·0	6·2	5·2	1·7	2·1	0·62	6·6	4·0	0·54	1·4	0·43
	18 h 120°	0	0·17	0·08	3·2	0·60	10·1	1·9	4·5	0·64	1·9	0·38	6·4	0·96	0·06	0·6	0·46
White-fish meal	Unheated	73	6·5	3·1	4·0	1·8	7·1	4·5	4·3	2·2	2·0	1·0	4·8	2·9	0·41	—	—
	Heated:																
	½ h 120°	73	6·5	3·0	4·0	1·6	7·3	4·0	3·8	2·3	2·0	0·83	4·6	2·7	0·34	—	—
	1 h 120°	73	6·1	2·9	4·1	1·7	7·0	3·8	4·1	2·1	1·9	0·86	4·3	2·7	0·27	—	—
	1½ h 120°	73	6·3	2·9	4·0	1·7	7·3	3·6	4·1	2·0	2·0	0·85	4·4	2·7	0·27	—	—
	4½ h 120°	69	6·3	2·6	4·1	1·5	6·8	2·7	3·9	1·9	2·0	0·67	4·6	2·6	0·23	—	—
	18 h 120°	62	6·1	2·2	4·3	1·4	6·5	2·6	4·3	1·8	1·8	0·59	4·5	2·5	0·23	—	—

Values for amino acids are expressed as g/16 g N.

* For details see p. 411.

† Relative nutritive value for *Strep. zymogenes* (see p. 413).

‡ Measured microbiologically with *Pediococcus cerevisiae* P60 (formerly *Leuconostoc mesenteroides* P60) NCTC 521, as described by Barton-Wright (1952).

§ Measured chemically by the method of Carpenter *et al.* (1957).

The nutritive quality of milk powders deteriorates during relatively much milder conditions of heating than were imposed in the study described here, and certainly these extreme laboratory conditions would not normally be encountered in commercial processing. They were chosen to illustrate the different heat lability of the different amino acids. Using more 'practical' conditions, Mauron, Mottu, Bujard & Egli (1955) showed destruction of lysine, and in a lesser degree of methionine, during the manufacture of dried and condensed milks. Henry, Kon, Lea & White (1947–8) and Lea & Hannan (1950) had earlier shown that storage under warm and humid conditions

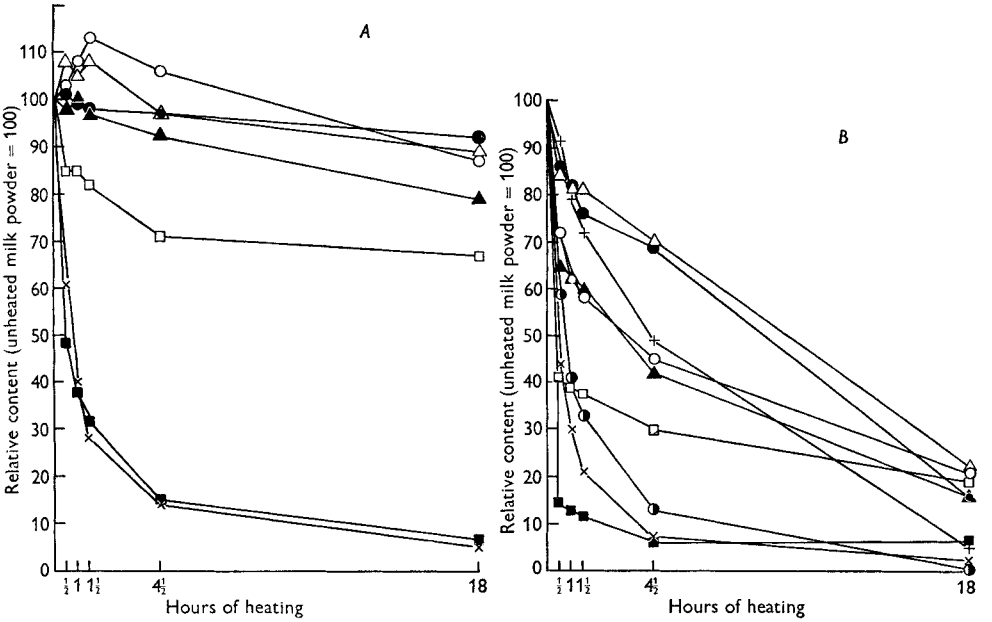

Fig. 2. Influence of heating in steam at 120° on the amounts of (*A*) total and (*B*) 'available' amino acids in freeze-dried skim milk. ○—○, methionine; △—△, leucine; ▲—▲, isoleucine; ×—×, arginine; □—□, histidine; ●—●, valine; ■—■, lysine; +—+, tryptophan; ◑—◑, relative nutritive value (see p. 413).

caused a rapid deterioration in the nutritive quality of the proteins of dried milk. They traced much, but not all, of this deterioration to lysine's becoming biologically unavailable through combination with lactose. Lea & Hannan (1950) studied the disappearance of amino acid groups in casein during reaction with glucose at 37° and 70% relative humidity. They found that after 5 days about two-thirds of the lysine had disappeared but other amino acids were only slightly affected. After 30 days the loss of lysine exceeded 90% and losses of other amino acids were then considerable. About 70% of the arginine and 50% of the methionine had reacted. As with the dried skim milk in the study now described, all the combined methionine was released during acid hydrolysis; two-thirds of the combined lysine was released, compared with only about one-third from the heated dried skim milk. None of the combined arginine was released on acid hydrolysis, whereas from the heated dried skim milk one-third of the combined arginine was recovered.

Patton, Hill & Foreman (1948) showed that heating casein for 24 h with boiling 5% glucose solution caused considerable loss of lysine, arginine and tryptophan. Lea & Hannan (1950) found no demonstrable loss of tryptophan under their conditions of test, but my study shows that in milk powder there was a significant and progressive loss of tryptophan with continued heating.

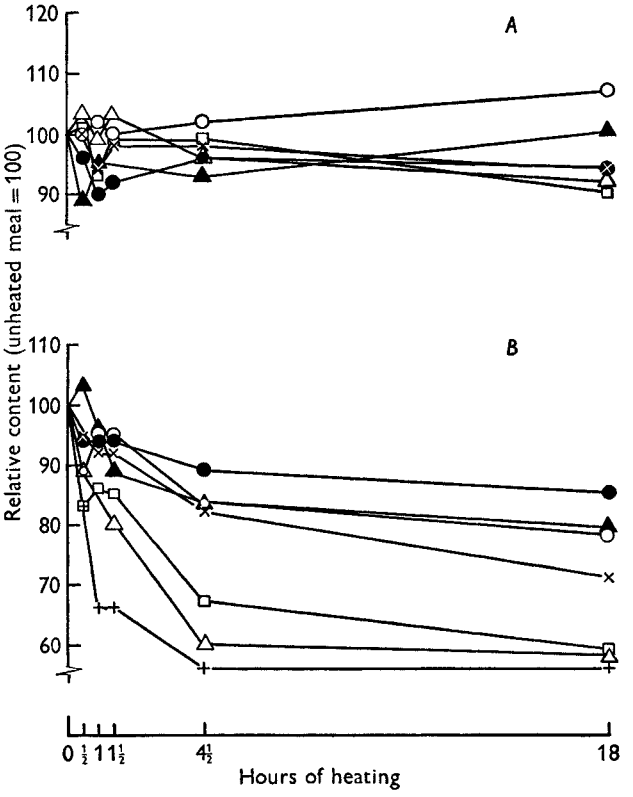

Fig. 3. Influence of heating in steam at 120° on the amounts of (*A*) total and (*B*) 'available' amino acids in white-fish meal. ○—○, methionine; △—△, leucine; □—□, isoleucine; ×—×, arginine; ■—■, histidine; ●—●, valine; +—+, tryptophan.

The pattern of the changes that took place during heating in the amino acid composition of the white-fish meal was different from that in the skim-milk powder. The content of 'available' lysine was not determined, but it has been shown (Carpenter, Morgan, Lea & Parr, 1962) that in fish meal, as compared with milk powder, lysine is relatively much more stable towards heating. The same is clearly true also of the other amino acids. Thus, after 18 h heating about 70% of the 'available' arginine remained, as against 2% in the skim-milk powder. Of the 'available' methionine, isoleucine and valine, about 80% remained, compared with 20% in the skim-milk powder.

There was little or no loss during heating of 'total' methionine, leucine, isoleucine, arginine, valine or histidine.

General considerations

Several methods have been proposed for computing the nutritive value of proteins from their amino acid composition. Block & Mitchell (1946–7) showed with an assortment of thirty-five proteins a high degree of correlation between 'chemical score' and nutritive value for the rat. The 'chemical score' was based on the value for the essential amino acid in the greatest deficit, relative to the content in whole-egg protein. For this calculation it was necessary to know the amounts of ten amino acids in the test protein, but McLaughlan, Rogers, Chapman & Campbell (1959) reported that for many common foods deficient in lysine or methionine, or in methionine and cystine, it was sufficient to determine only these three amino acids to obtain a fair measure of the nutritive value of the protein. FAO (1957) proposed that proteins be rated by comparison of their amino acid composition with that of a specified mixture of amino acids, calculated from the requirements of adult man. All these methods are useful for assigning proteins of different kinds into broad nutritional categories. Their weakness is that they fail to take into account all the considerations of digestibility and biological availability that are often of paramount importance.

It is evident in the findings reported in the present paper that within groups of similar proteins—as for example meat meals or fish meals of different manufacture—there may be large differences in nutritive quality but very small differences in amino acid composition. The differences in nutritive quality reflect subtler differences in the biological availability of the amino acids. In practice, the nutritionist's main requirement of a 'grading' test is that it should indicate reliably any diversity of nutritive quality among samples of similar designation. For this type of application, the advent of the more accurate methods of 'total' amino acid analysis offers only a more refined means of obtaining the wrong answer, though with greater precision than hitherto.

There is scope for improvement in the precision of the *Strep. zymogenes* assays, but a more important need is for comparative information from animal tests. From the evidence given in this paper one must suspect that, in the whale-meat and fish meals studied, the availability of amino acids to the rat must parallel closely the availability to *Strep. zymogenes*. But a great amount of biological testing needs to be done to establish or refute this supposition.

SUMMARY

1. *Streptococcus zymogenes* NCDO 592 is vigorously proteolytic and requires exogenous methionine, leucine, isoleucine, arginine, histidine, tryptophan and valine. It was used to measure the biological availability of these amino acids in a variety of food proteins.

2. In twelve whale-meat meals, differences in nutritive quality reflected corresponding differences in the biological availability of several of the constituent amino acids. Values obtained for 'available' methionine, tryptophan, leucine and arginine were closely correlated with each other and with the rat-assay and the 'available' lysine values obtained for these same materials by Bunyan & Price (1960).

3. It was apparent that within groups of similar proteins—as, for example, meat meals or fish meals of different manufacture—there may be large differences in nutritive quality but only small differences in 'total' amino acid composition.

4. A study was made of the effects of heating in steam at 120° for up to 18 h on the amino acid composition of dried skim milk and white-fish meal. The nutritive value of the skim-milk powder for *Strep. zymogenes* diminished progressively during heating. The content of 'available' lysine fell sharply and after 30 min heating only 14% remained. The contents of 'available' arginine and histidine both fell to about 42% of the initial values during this period. About one-third of the losses of lysine and arginine, and three-quarters of the loss of histidine, were recovered on subsequent hydrolysis with 2 N-HCl. The availability of methionine, valine, leucine, isoleucine and tryptophan also decreased during the heating, but at a slower rate than that of lysine, arginine and histidine. There was little or no fall in the 'total' methionine, valine, leucine and isoleucine contents even after 18 h heating.

In the white-fish meal the amino acids were relatively much more stable towards heat. Thus after 18 h about 70% of the 'available' arginine remained, as against 2% in the heated skim-milk powder. There was little or no loss of 'total' methionine, leucine, isoleucine, arginine, valine or histidine.

I thank my colleagues, Dr S. K. Kon and Dr J. W. G. Porter, for their helpful interest in this work, Mr J. H. Prentice for advice on statistics, and Dr K. J. Carpenter for the 'available' lysine values quoted in Table 7. I am greatly indebted to Mr J. P. Fordham for skilled technical assistance.

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