Brit. J. Nutr. (1964), 18, 449, *with* 1 *plate*

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

A microbiological method for assessing the nutritional value of proteins

3.* Further studies on the measurement of available amino acids

By J. E. FORD

National Institute for Research in Dairying, Shinfield, Reading, Berks

(*Received* 3 *March* 1964—*Accepted* 8 *April* 1964)

Within groups of similar proteins—for example whale-meat meals or fish meals of different manufacture—there may be large differences in nutritive quality but only relatively small differences in amino acid composition. From chemical and microbiological tests (Bunyan & Price, 1960; Ford, 1962) it appears that the differences in quality may not be specifically related to any one amino acid but reflect corresponding differences in the availability of several amino acids. Thus in twelve whale-meat meals of sensibly uniform amino acid composition but widely differing nutritive quality, values obtained for available lysine, methionine, tryptophan, leucine and arginine were closely correlated with each other and also with rat assay values representing overall protein quality (Ford, 1962).

Any one set of values would serve to grade these meals in order of nutritive quality—whether available lysine measured chemically by the dinitrofluorobenzene procedure of Carpenter (1960), or available methionine, tryptophan, leucine or arginine measured microbiologically as described by Ford (1962). We cannot infer from this that these chemical and microbiological values represent quantitatively the 'biological availability' of the amino acids. They must first be checked by comparison with the results of direct growth tests with animals. This will be a laborious undertaking but some beginnings have been made. Thus, Carpenter, March, Milner & Campbell (1963) have developed a chick growth assay for lysine, and found fair agreement between chemical and biological estimates of the available lysine in five fish meals. Miller, Carpenter & Morgan (1963) compared the results of growth assays with chicks for methionine in several meat meals and fish meals with values for available methionine obtained in microbiological tests. They found that the *Streptococcus zymogenes* procedure of Ford (1962) gave uniformly lower results than the chick assays. On increasing the concentration of papain used for the preliminary digestion the microbiological assay values increased, to agree closely with the chick results. The coefficient of correlation was then 0·97 ($P < 0.001$; ten samples). It is an open question whether these higher values on which the chick and microbiological tests agreed represented all the methionine that was potentially available. The microbiological results were maximum values in the sense that they were not further increased by treating the test samples with even greater amounts of papain. They might con-

* Paper no. 2: *Brit. J. Nutr.* (1962), 16, 409.

ceivably have been higher if different enzymes or combinations of enzymes had been used, or if the samples had been more finely ground, or if a different test micro-organism had been used. The present paper deals with the influence of these and other variables in the conditions of the microbiological tests upon the results obtained for available methionine and tryptophan in several protein concentrates, and for available methionine, tryptophan, leucine, isoleucine, valine and histidine in extracted leaf-protein concentrates.

EXPERIMENTAL

Assays with Streptococcus zymogenes

Strep. zymogenes NCDO 592 was obtained from the National Collection of Dairy Organisms at the National Institute for Research in Dairying, Reading. Assay procedures were as described by Ford (1962), with the following modifications.

Basal medium. The amount of K_2HPO_4 was increased from 12 to 18 g. L-cystine was dissolved separately in 10 ml boiling water by addition of HCl (not KOH as formerly recommended) before it was added to the amino acid supplement.

Preparation of inocula. Assay tubes were each inoculated with one drop of a 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 during the week; on Friday evenings it was transferred and put to incubate in a cupboard at room temperature (about 20°) until the following Monday.

Assays with Clostridium welchii (perfringens) *NCTC* 8426

Clost. welchii has been recommended by Boyd, Logan & Tytell (1948) for amino acid assay. It requires nine of the essential amino acids; it is fast-growing and, under the specified test conditions, it is virtually immune from bacterial contamination. Thus, drastic heat sterilization of the test extracts can be avoided. For general use these advantages are outweighed by the potential danger of the organism as a pathogen but, for this study, it offered a possible alternative means for assessing available methionine. The test conditions were similar to those used in the assays with *Strep. zymogenes* and the same assay medium was used except that Tween 80 was omitted. The culture was maintained and the inocula prepared as described by Boyd *et al.* (1948). Growth was assessed turbidimetrically.

Assays with Streptococcus faecalis *R (ATCC* 9790) *and* Pedicoccus cerevisiae *P* 60 (*NCDO* 521)

These assays were done as described by Barton-Wright (1963).

Preparation of protein for test

The test protein was ground in a laboratory mill and rubbed through a 40-mesh screen. A sample was taken, containing precisely 100 mg nitrogen, and transferred to a homogenizer tube (Pl. 1 A), to which were then added 10 ml of an aqueous solution containing, per l., 5 g trisodium citrate, 30 mg sodium cyanide and enough N-H_3PO_4 to bring the pH to 7·2. When homogenized the sample was transferred to a wide-

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

necked 1 oz McCartney bottle with an additional 5 ml citrate buffer used for rinsing the homogenizer tube and pestle, and adjusted to pH 7·2 in readiness for digestion with papain.

Alternatively, when pepsin was to be used, the sample was homogenized with 10 ml 0·05 N-HCl, transferred to a McCartney bottle by rinsing with an additional 8 ml acid, and adjusted to pH 1·8.

Papain. The papain was a partially purified grade (Schering, Berlin) reported by Rao, Sreenivas, Swaminathan, Carpenter & Morgan (1963) to have an activity of $5{\cdot}7 \times 10^{-6}$ Anson units/μg. It was dissolved (2%, w/v) in 0·5% citrate buffer which was then adjusted to pH 7·2.

Pepsin. Crystalline pepsin (L. Light and Co. Ltd, Colnbrook) was used, dissolved (2%, w/v) in warmed (37°) N/30-HCl.

Enzymic predigestion. After addition of papain or pepsin in the required amounts to the homogenized samples, metal stoppers with rubber inserts were screwed on to the bottles, which were then clipped into a rack in a water-bath at 56°, and mechanically turned end-over-end for 3 h, sixty times/min. The construction of this end-over-end shaker is illustrated in Pl. 1 B.

Further digestion of some pepsin digests with papain. After digestion with 1 ml pepsin solution some samples were further digested with papain. The pepsin digests were brought to pH 7·2. To each was added 1 ml of a solution containing, per ml, 50 mg trisodium citrate, 2 mg NaCN and enough N-H_3PO_4 to bring the pH to 7·2. Papain solution (1 ml) was then added, and the whole incubated at 56° for a further period of 3 h in the end-over-end shaker.

Further digestion with trypsin and erepsin. Some pepsin digests were further incubated with trypsin and erepsin. They were first transferred to glass-stoppered test tubes of 50 ml capacity and adjusted to pH 8·5 with N-NaOH. To each was added 2 ml of a 1% (w/v) solution of trypsin (1 × crystallized grade; L. Light and Co. Ltd, Colnbrook) in 1% (w/v) aqueous sodium bicarbonate solution and 0·5 ml toluene. The tubes were stoppered tightly and put to incubate in a water bath for 4 h at 50°, with manual shaking at frequent intervals. They were then cooled to room temperature and their contents adjusted to pH 7·8. To each were added 1 ml of a 1% (w/v) aqueous suspension of crude porcine erepsin (L. Light and Co. Ltd, Colnbrook) and a further 0·5 ml toluene. The tubes were returned to the water bath and again incubated for 4 h at 50°, and finally heated in flowing steam for 10 min to remove the toluene.

After enzymic digestion, all the preparations were adjusted to pH 7·2 and diluted with water to 100 ml. They were then shaken vigorously and 10 ml portions diluted to 100 ml with water.

Enzyme 'blanks' were included with all the tests. Because of their relatively low content of amino acids the final tenfold dilution with water was omitted.

Chemical hydrolysis. For the assay of the total amounts of methionine, histidine, leucine, isoleucine and valine in dried whole egg and in extracted leaf proteins, the samples were hydrolysed with 2 N-HCl (cf. Ford, 1962). For the assay of tryptophan, samples containing 100 mg nitrogen were heated in sealed tubes for 5 h at 121° with

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

40 ml 5 N-NaOH. The assay values were doubled to correct for the racemization that occurs under these conditions of alkaline hydrolysis, since DL-tryptophan has for *Strep. zymogenes* only half the activity of the L-isomer.

Dose levels. The test preparations were added to paired assay tubes in amounts of 2, 4, 6 and 8 ml, and the standard amino acid solutions at 0, 1, 2, 4, 6 and 8 ml.

Measurement of growth responses

In the main these responses were measured turbidimetrically. In some protein concentrates the availability of the amino acids, judged with *Strep. zymogenes*, has seemed to improve with fine grinding. To some extent this improvement might have been more apparent than real, and might have been caused by the residual turbidity of the test extracts. There is no obvious means of correcting for this error, except by assessing growth responses indirectly from measurements of titratable acidity. Results obtained by this means have been less precise than those calculated from turbidity measurements, which I have generally preferred as being directly related to the bacterial growth in the assay tubes.

Before measurement of turbidities, 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. Turbidities were then measured directly in the tubes, with a Lumetron Model 401 A colorimeter (Photovolt Corporation, New York) fitted with a 580 nm glass filter.

Acidimetric determinations were made with a Pye automatic titrator (W. G. Pye and Co. Ltd, Cambridge) set to titrate to pH 8·2. At this pH the rate of change of pH in the test cultures with added 0·1 N-NaOH was about 0·7 pH units/ml, compared with 0·17 unit/ml at pH 7·0.

Materials

The whale-meat meals and fish meals examined (series WM and FM) had been distributed by the Agricultural Research Council in connexion with its collaborative investigation into the measurement of the quality of protein feeding-stuffs (cf. Boyne, Carpenter & Woodham, 1961). The whale-meat meals were of special value for the purposes of my investigation, being uniform in origin and in amino acid composition but differing widely in nutritive quality and in the availability of their amino acids (Bunyan & Price, 1960; Ford, 1962). The fish meals were examined to check an earlier finding (Ford, 1962) that their methionine might be only 50–70% available. The leaf-protein concentrates were provided for test by Dr N. W. Pirie, F.R.S., and were included because, as with the fish meals, the test procedure originally described gave unexpectedly low values for the availability of several amino acids.

RESULTS AND DISCUSSION

In general, microbiological assay values must be qualified by reference to the particular assay organism and conditions of test employed. In *Strep. zymogenes* assays for available amino acids it is especially important to standardize the preparation of the samples.

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

Influence of particle size. Table 1 shows for comparison the values obtained with coarsely ground and with very finely ground samples for the available methionine and tryptophan in whale-meat meals and fish meals. Finer grinding of the test samples fairly consistently gave higher results as judged from extinction values, on average by 21% for methionine and by 33% for tryptophan. Some of this increase was illusory and could be attributed to the greater turbidity of the homogenized preparations; but it was partly real, as is evident from the results calculated from titratable acidities, which yielded on average 13% more for methionine and 25% more for tryptophan. The increases were relatively greater with the poorest of the meals, WM7 and FM26, which were also poorly digested by the rat (K. M. Henry, personal communication). The true nitrogen digestibility of WM7 was 58·7% and of FM26, 62%. After reduction of FM26 to a very fine powder in a ball mill its true digestibility appeared to increase to 68·8%, but with only six rats in each test group the difference was not statistically significant. It is clearly possible, however, that such fine grinding would also improve utilization in feeding tests with animals.

Table 1. *Available methionine and tryptophan (g/16g nitrogen) in whale-meat and fish meals. Influence of particle size on* Streptococcus zymogenes *assay results**

	Methionine				Tryptophan			
	40-mesh samples		Homogenized samples		40-mesh samples		Homogenized samples	
Meal	OD	TA	OD	TA	OD	TA	OD	TA
WM1	1·1	1·1	1·6	1·3	0·23	0·18	0·49	0·33
WM2	1·6	1·6	1·8	1·7	0·42	0·44	0·53	0·46
WM3	1·7	1·9	2·0	1·9	0·41	0·44	0·59	0·54
WM4	2·0	2·4	2·1	2·5	0·57	0·59	0·68	0·66
WM5	1·7	1·7	1·9	2·0	0·53	0·50	0·68	0·56
WM6	2·1	2·2	2·1	2·3	0·60	0·62	0·68	0·72
WM7	0·7	0·6	1·2	0·9	0·17	0·10	0·32	0·24
WM9	2·1	2·4	2·4	2·6	0·70	0·69	0·76	0·72
WM10	1·4	1·4	1·7	1·4	0·45	0·40	0·56	0·40
WM11	1·7	1·8	2·1	2·1	0·54	0·52	0·67	0·56
WM12	1·6	1·6	2·0	1·9	0·52	0·48	0·59	0·50
WM13	2·7	3·1	2·7	3·3	0·87	1·05	0·89	1·02
FM24	2·2	2·4	2·4	2·4	—	—	—	—
FM25	2·2	2·2	2·6	2·0	—	—	—	—
FM26	1·4	1·2	1·9	1·7	—	—	—	—

* The meals were ground to 40-mesh or homogenized (p. 450) and samples, containing 100 mg nitrogen, were predigested with 'purified' papain (1 ml 2%, w/v). Growth was assessed turbidimetrically (OD) and titrimetrically (TA).

Predigestion of the test protein. Strep. zymogenes is itself vigorously proteolytic, but pretreatment of the samples with papain or pepsin was a necessary preliminary in the tests. It speeded growth, particularly at the lower dose levels, and improved the linearity of the dose-response curves. Tests with casein (Ford, 1960) showed that after digestion with papain the proportion of the casein nitrogen incorporated into bacterial cells increased from 64 to 71%. As judged from the first two columns of figures in Table 2, the increase would probably be greater for a whale-meat meal than for casein.

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

This table shows the influence of enzymic predigestion on the available methionine measured in four whale-meat meals and two fish meals. At the 1 % level both papain and pepsin gave markedly higher values than were obtained for the undigested meals. At the 2 and 5 % enzyme levels the values were slightly higher still, and for WM 13 and FM 24 several of the values were about as high as the total methionine values measured after hydrolysis of the meals with acid. Digestion successively with pepsin and papain, or pepsin, trypsin and erepsin, gave no higher results than with 2 % papain alone.

Table 2. *Available methionine (g/16 g N) in whale-meat meals and fish meals. Effects of different pretreatments of the samples on* Streptococcus zymogenes *assay results*

		Predigested with								
	No enzymic pre-	'Purified' papain*			Pepsin*			Pepsin–	Pepsin– trypsin–	
Meal	digestion	1 %	2 %	5 %	1 %	2 %	5 %	papain†	erepsin†	2 N-HCl‡
WM 1	1·1	1·3	1·6	1·4	1·3	1·5	1·5	1·7	1·6	2·5
WM 3	1·2	1·6	2·0	2·1	1·8	2·1	2·0	2·1	1·9	3·2
WM 7	0·3	0·9	1·2	1·1	0·9	1·0	1·2	1·2	1·1	2·2
WM 13	1·6	2·5	2·7	3·0	2·4	2·5	2·6	2·8	2·5	3·0
FM 24	—	2·1	2·3	2·6	2·3	2·5	2·7	—	—	2·5
FM 26	—	1·4	1·6	1·7	1·5	1·6	1·8	—	—	2·7

* The meals were homogenized (p. 450) and samples, containing 100 mg nitrogen, were predigested with 0·5, 1 or 2·5 ml of 2 % (w/v) 'purified' papain or crystalline pepsin.
† See p. 451.
‡ See p. 451.

The use of large amounts of enzymes introduces technical difficulties with high 'blanks' and there seems to be no practical advantage in the use of combinations of enzymes, or in exceeding the 2 % level of papain or pepsin. Crystalline pepsin is cheaply available and is more effective than papain in bringing some food proteins into solution, especially leaf-protein preparations (p. 452) for which the papain procedure had given improbably low values. Purified papain has the advantage of being virtually free from methionine, but the crystalline enzyme is expensive, and the cheaper 'purified' grade used for these tests is no longer obtainable. Papain was more effective than pepsin for digesting milk proteins and gave a water-clear digest when used as described (Ford, in preparation). But, in general, with a variety of protein foods, the two enzymes gave broadly similar results when used at the 2 % level.

Table 3 shows revised values for available methionine in a series of fish meals, compared with values for the same meals given by Ford (1962). For these original tests the meals were ground to pass a 60-mesh screen, and samples containing 100 mg nitrogen were predigested with 1 ml of 1 % (w/v) crude papain. For the assays now presented the meals were homogenized and digested with 1 ml of 2 % 'purified' papain, which provided ten times greater proteolytic activity as measured by Anson's procedure. The new values are all higher than the original ones, the mean increase being 24 %.

Total and available amino acids in extracted leaf protein. Preliminary assays of extracted leaf-protein concentrates that had been subjected only to the mildest con-

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

ditions of processing during manufacture showed them to be surprisingly poor sources of available amino acids for *Strep. zymogenes*. Substitution of predigestion with pepsin for that with papain or increasing the quantity of papain caused a striking increase in the measured availability of methionine, histidine, tryptophan, leucine, isoleucine and valine. Table 4 shows the available amounts of these amino acids in dried whole egg and in preparations of the leaf proteins of wheat, tares and clover, measured after predigestion of the homogenized samples with 1 ml of 2% (w/v) pepsin. For comparison, it shows also total amounts measured in chemically hydrolysed samples.

Table 3. *Revised values for available methionine (g/16 g N) in fish meals*

FM1	FM2	FM6	FM8	FM10	FM12	FM14	FM15	FM19	FM21	FM22
Original values (Ford, 1962)										
1·8	1·4	1·9	1·5	1·9	2·0	1·8	1·9	1·9	1·8	1·6
Revised values										
2·2	1·8	2·3	2·0	2·3	2·4	2·2	2·3	2·3	2·4	2·1

For the original tests the meals were ground to 60-mesh and samples, containing 100 mg nitrogen, were predigested with 1 ml of 1% (w/v) crude papain. The revised values were obtained after digestion of the homogenized sample with 1 ml 2% (w/v) 'purified' papain.

Table 4. *Total and available* methionine, histidine, leucine, isoleucine, valine and tryptophan (g/16 g N) in dried whole egg and in extracted leaf proteins*

Source of protein	Methionine Total	Methionine Available	Histidine Total	Histidine Available	Leucine Total	Leucine Available
Dried whole egg	3·6	3·5	2·4	2·0	8·0	8·5
Wheat leaf protein	2·2	2·2	2·3	2·0	7·9	7·7
Tares leaf protein	1·8	1·6	2·6	2·0	8·6	8·3
Clover leaf protein	1·8	1·7	2·6	1·8	9·0	8·0

Source of protein	Isoleucine Total	Isoleucine Available	Valine Total	Valine Available	Tryptophan Total	Tryptophan Available
Dried whole egg	5·7	5·0	6·7	6·7	1·6	1·5
Wheat leaf protein	5·3	4·7	6·8	6·9	2·2	2·3
Tares leaf protein	5·3	4·5	6·6	6·6	2·0	2·0
Clover leaf protein	5·3	4·1	6·8	6·9	2·1	2·0

* The test samples, containing 100 mg nitrogen, were homogenized and predigested with 1 ml of 2% (w/v) crystalline pepsin.

It is evident from these results that the amino acids in the leaf proteins were intrinsically highly available, and that the low values obtained after less rigorous enzymic pretreatment reflected the relatively slow rate of digestion of the protein as a whole, at least in the microbiological test system. It may be, of course, that the values in Table 4 are now too high, in that they overestimate the availability of the amino acids to animals. In tests with rats Dr K. M. Henry (personal communication) found the true digestibility of nitrogen of these and similar leaf proteins to be only about 75–80%.

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

Results with different test micro-organisms. Strep. zymogenes, Strep. faecalis R, *Clost. welchii* and *Pediococcus cerevisiae* P60 (*Leuconostoc mesenteroides* P60) have all been recommended for the assay of methionine, and Table 5 shows values obtained with all four micro-organisms for the total and the available methionine in dried whole egg, freeze-dried fresh cod fillet, a commercial cod meal (FM 15), a whale-meat meal of excellent quality (WM 13) and another of very poor quality (WM 7). For this comparison somewhat drastic conditions of chemical hydrolysis were used, to ensure complete hydrolysis of the proteins. Samples containing 100 mg nitrogen were sealed in Carius tubes, each with 30 ml 6N-HCl, and heated for 18 h in a steam autoclave at 121°. This caused some destruction of methionine, the *Strep. zymogenes* values for WM 13 and FM 15 being 2·5 and 2·5, compared with 3·0 and 3·2 after milder acid hydrolysis (Ford, 1962).

Table 5. *Total and available* methionine (g/16 g nitrogen) in food proteins, measured with four different assay micro-organisms*

Source of protein	*Strep. zymogenes* Total	*Strep. zymogenes* Available	*Strep. faecalis* R Total	*Strep. faecalis* R Available	*Clost. welchii* Total	*Clost. welchii* Available	*Ped. cerevisiae* P60 Total	*Ped. cerevisiae* P60 Available
Dried whole egg	3·0	3·2	3·1	3·1	3·3	3·5	3·1	1·2
Freeze-dried cod fillet	2·7	2·8	3·3	2·4	3·2	3·1	2·8	1·2
Cod-meal (FM 15)	2·5	2·3	2·8	1·9	2·9	2·9	2·6	0·7
WM 7	2·0	1·1	2·4	0·6	2·2	1·5	2·0	0·3
WM 13	2·5	2·8	2·8	2·5	2·3	3·1	2·4	1·0

* The test samples, containing 100 mg nitrogen, were homogenized and predigested with 1 ml of 2% (w/v) crystalline pepsin.

The four test organisms gave broadly similar results for free methionine, as it occurred in these acid-hydrolysed extracts. In my experience, results obtained with *Strep. zymogenes* and *Ped. cerevisiae* P60 have been closely reproducible and in fair agreement. *Strep. faecalis* R and *Clost. welchii* have tended to give higher results, and with *Clost. welchii* the reproducibility has been inadequate for precise quantitative work.

Strep. zymogenes, Strep. faecalis R *and Clost. welchii* are all in varying degrees proteolytic and they all made efficient use of methionine in the pepsin digests of the four good-quality meals. *Strep. faecalis* R is relatively weakly proteolytic, and with these peptic digests the assay results showed a marked upward drift with increasing dose levels. From all the test levels, the mean available methionine values were somewhat lower than those obtained with *Strep. zymogenes*. In contrast, *Clost. welchii* gave higher results though these, like the values for total methionine, were unaccountably variable from assay to assay. All three micro-organisms graded the meals similarly as sources of available methionine. *Ped. cerevisiae* P60, however, could apparently make little use of the methionine as it occurred in the pepsin digests.

Conclusions. The apparently simple situation which with lysine allows the total amount of that amino acid present in a protein to be precisely assigned to 'available' and 'unavailable' portions is not at all typical for most of the other amino acids. For

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

some of these—the leucines for example—it is more difficult to conceive from their chemical constitution that heat processing could impair availability except indirectly, by reducing the accessibility of the protein, or of fragments of its peptide chains, to the action of proteolytic enzymes. Earlier work with whale-meat meals (Ford, 1962) showed that overall nutritive quality for the rat (net protein utilization or NPU) was closely correlated with the availability of several of the amino acids, measured microbiologically with *Strep. zymogenes*. The microbiological values, however, were correlated almost as highly with the true digestilibities of nitrogen of these meals, as given by Bunyan & Price (1960). For available methionine the coefficient of correlation with the digestibility values was 0·89 ($P < 0{\cdot}001$), and for available tryptophan 0·92 ($P < 0{\cdot}001$). A further close correlation, between DNFB lysine values (estimated with the aid of 2:4-dinitrofluorobenzene) and digestibility ($r = 0{\cdot}86$, $P < 0{\cdot}001$) calculated from data of Bunyan & Price (1960) seems especially interesting. It suggests that the molecular changes that accompany the fall in the content of DNFB lysine could directly determine the digestibility of these meals, and thus the biological availability of all their amino acids. One can envisage the formation during heating of a tangle of intramolecular linkages, not amenable to enzymic hydrolysis, involving the ϵ-amino groups of the lysine moieties distributed throughout the peptide chains. Other workers had earlier expressed much the same view. Thus Seegers & Mattill (1935) observed that beef liver, carefully dried at temperatures below 60°, suffered considerable loss in its nutritive value on being heated at 120° for 72 h. When samples of the heated and the unheated material were hydrolysed with acid the resulting amino acid mixtures were alike in their nutritive value. Digestion in vitro showed that the heated liver was resistant to tryptic digestion and the authors concluded that, during heating, new linkages were formed that were not susceptible to the action of proteases. Harris & Mattill (1940) identified the free amino groups, comprised in the main of the ϵ-amino groups of the lysine, as being the most likely agents in the formation of these enzyme-resistant bonds. They suggested that, under conditions favourable to dehydration, the free amino groups might form anhydride linkages with carboxyl groups to create indigestible compounds like the diketopiperazines. Mecham & Olcott (1947) investigated the effects of dry heat on several proteins. Like Harris & Mattill (1940) they found marked losses of amino nitrogen, decreased solubility and decreased rate of digestion by pancreatin. They explained the physical changes as resulting from the loss of polar groups, possibly by internal formation of amides or esters.

From closer examination of the results of Bunyan & Price (1960) it was apparent that nutritive quality in our whale-meat meals (expressed as NPU) was not simply a measure of their overall digestibility. The biological values of the meals varied just as widely as did the digestibilities, and a comparison of the two sets of figures showed them to be roughly proportional. The coefficient of correlation was 0·75 ($P < 0{\cdot}001$). Further calculation revealed that the DNFB lysine values (Bunyan & Price, 1960) and the available methionine and available tryptophan values (Table 1) also formed highly significant correlations with the biological values ($r = 0{\cdot}83, 0{\cdot}83$ and $0{\cdot}80$, $P < 0{\cdot}001$), as well as with the digestibilities (see above). Thus from the poorer meals, containing

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

relatively low proportions of available amino acids as judged by the chemical and microbiological tests, correspondingly low percentages of the absorbed nitrogen were retained by the rat. The whale-meat meals were broadly similar in their total amino acid composition. The differences in nutritive quality reflect the wide differences in the biological availability of several, and probably all, of their constituent amino acids (cf. Ford, 1962). Evidently much of the amino acid nitrogen absorbed from the poorer meals was not retained by the rat, and it is an interesting question whether this is explainable in terms of differences in the patterns of amino acids absorbed from the different qualities of meal, or whether, even after absorption from the gut, some proportion of the amino acids might still remain unavailable. Considering this latter hypothesis, it seems possible that some of the amino acids absorbed after digestion were not readily utilizable because they were locked up in indigestible peptide residues. It is still uncertain that peptides are in fact absorbed from the gut, but Fisher (1954) favoured the view that peptides form the 'currency' of protein metabolism, and constitute a large part of the substances passing into the body fluids during protein absorption. Whether or not this is so, it remains to be seen whether indigestible peptides may be released and absorbed during the digestion of heat-damaged proteins, and experiments with rats are planned to test the possibility, and to chart the patterns of free amino acids in the portal blood during the course of digestion of unheated and heat-damaged proteins.

Henry & Kon (1947–8) found with rats that in badly stored milk powder, though the lysine was largely unavailable, digestibility and the availability of other amino acids were reduced very little, if at all. In milk powder, unlike whale meat and other muscle proteins, the free amino groups are in a sense protected by the aldehyde groups of the lactose present in large excess, with which they will readily combine to form Maillard-type compounds, even under mildly adverse conditions of storage or heating.

Whatever may determine 'availability', it is clearly important that it should be carefully defined in terms of the particular system used for measuring it. Thus, in microbiological tests with the protozoon *Tetrahymena pyriformis*, J. M. Stott (personal communication) found the availability of lysine in a leaf protein concentrate to be low, whereas with the DNFB lysine procedure Dr K. J. Carpenter (personal communication) judged it to be much higher. Growth trials with pigs (Duckworth, Hepburn & Woodham, 1961) showed this same material to be equivalent in quality to a white-fish meal, and so supported the DNFB lysine results. Results with *Strep. zymogenes* were equivocal. By the standard test procedure (Ford, 1962) the availability of several amino acids was low but after more rigorous enzymic predigestion with pepsin or with higher concentrations of papain, higher values were obtained. The amino acids were intrinsically highly available but the protein as a whole was digested relatively slowly. It remains to be seen from further comparative testing whether rate of digestion may similarly influence the results of biological tests with animals.

The chemical assay for available lysine would take no account of rate of digestion, nor of the possibility that, after digestion of a heat-damaged protein, many of the lysine moieties reacting as 'available' would remain bound in indigestible peptide

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

residues, and inaccessible to the animal. It might therefore tend to overrate heat-damaged proteins of poor nutritive quality. The poor-quality fish meal, FM 26, was improved for *Strep. zymogenes* and perhaps for the rat by fine-grinding, whereas the DNFB lysine content was unaffected. In direct feeding experiments with rats, K. M. Henry (personal communication) obtained markedly lower values for the available lysine in the two poor-quality whale-meat meals, WM 1 and WM 7, than did Bunyan & Price (1960) with the chemical method. With chicks, however, Carpenter *et al.* (1963) obtained broad confirmation of their DNFB values for four fish meals.

After homogenization of the test samples and digestion with a higher concentration of papain, the *Strep. zymogenes* values for available methionine in the whale-meat meals (Table 1) averaged about 20% higher than those originally published (Ford, 1962). Both sets of values graded the samples in the same order and were correlated closely with the rat NPU values (Bunyan & Price, 1960). Results by either procedure were reproducible within 95% confidence limits of about ± 14%. Similarly, with the fish meals (Table 3), finer grinding and the use of a higher concentration of papain gave a uniform increase of about 24% in the available methionine values. It is not yet certain that these higher values represent the 'biological availability', which may itself vary with the species of test animal and the conditions of test. But, even so, it seems that results obtained with the original test procedure were too low, at least for certain classes of food proteins. Miller *et al.* (1963) found that their available methionine values obtained with *Strep. zymogenes* were initially lower than those obtained with chicks, but agreed closely with the chick results when a higher concentration of papain was used in the preparation of the samples. Until more comparative biological and microbiological testing of this kind has been completed on a wider variety of food proteins it will be premature to attempt to redefine an optimum procedure for preparing the samples for microbiological testing, except in terms of the conditions necessary to give the highest possible result.

SUMMARY

1. A study was made of the influence of enzymic predigestion and fineness of grinding of the test samples, and of other variables in the conditions of the microbiological tests, upon the values obtained for the content of available amino acids in several protein concentrates.

2. With whale-meat meals and fish meals, especially those of poorer quality, grinding very finely increased the values. So also did the use for predigestion of concentrations of papain higher than those originally recommended (Ford, 1962). For a variety of protein foods pepsin and papain, at 20 mg enzyme/100 mg sample nitrogen, gave broadly similar results. There was no practical advantage in the use of greater amounts of these enzymes, nor in the use of combinations of enzymes.

3. Comparison with the results of Bunyan & Price (1960) showed that, for the whale-meat meals, the microbiological assay values for available methionine and tryptophan, and the chemical assay values for available lysine, were all correlated closely with the digestibilities of the meals and also with their biological values.

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press

4. The findings are discussed in relation to the general question of what determines the biological availability of the amino acids in heat-damaged proteins.

I thank my colleagues, Dr K. M. Henry and Dr S. K. Kon, for their helpful interest in this work, and Mr K. J. Scott for skilled technical assistance.

REFERENCES

Barton-Wright, E. C. (1963). *Practical Methods for the Microbiological Assay of the Vitamin B Complex and Amino Acids.* London: United Trade Press Ltd.

Boyd, M. J., Logan, M. A. & Tytell, A. A. (1948). *J. biol. Chem.* **174**, 1027.

Boyne, A. W., Carpenter, K. J. & Woodham, A. A. (1961). *J. Sci. Fd Agric.* **12**, 832.

Bunyan, J. & Price, S. A. (1960). *J. Sci. Fd Agric.* **11**, 25.

Carpenter, K. J. (1960). *Biochem. J.* **77**, 604.

Carpenter, K. J., March, B. E., Milner, C. K. & Campbell, R. C. (1963). *Brit. J. Nutr.* **17**, 309.

Duckworth, J., Hepburn, W. R. & Woodham, A. A. (1961). *J. Sci. Fd Agric.* **12**, 16.

Fisher, R. B. (1954). *Protein Metabolism.* London: Methuen and Co.

Ford, J. E. (1960). *Brit. J. Nutr.* **14**, 485.

Ford, J. E. (1962). *Brit. J. Nutr.* **16**, 409.

Harris, R. L. & Mattill, H. A. (1940). *J. biol. Chem.* **132**, 477.

Henry, K. M. & Kon, S. K. (1947–8). *J. Dairy Res.* **15**, 341.

Mecham, D. K. & Olcott, H. S. (1947). *Industr. Engng Chem.* **39**, 1023.

Miller, E. L., Carpenter, K. J. & Morgan, C. B. (1963). *Int. Congr. Nutr.* VI. *Edinburgh. Programme*, p. 166.

Rao, M. N., Sreenivas, H., Swaminathan, M., Carpenter, K. J. & Morgan, C. B. (1963). *J. Sci. Fd Agric.* **14**, 544.

Seegers, W. H. & Mattill, H. A. (1935). *J. biol. Chem.* **110**, 531.

EXPLANATION OF PLATE

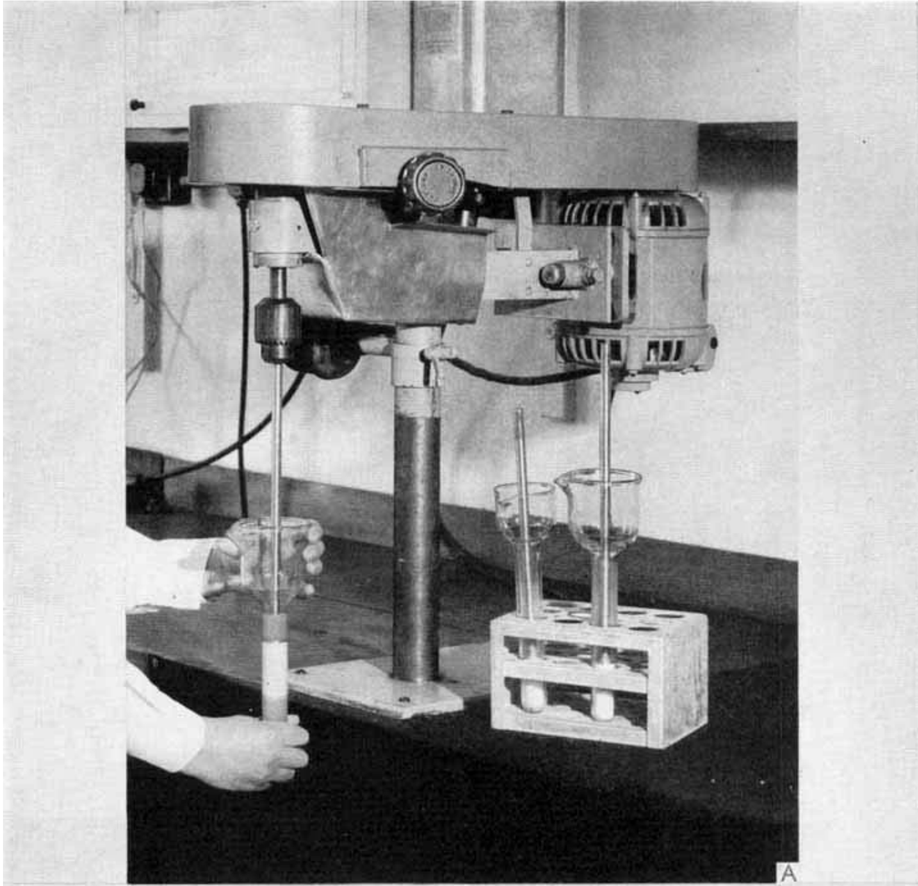
(*A*) homogenizer and (*B*) end-over-end shaker used in the preparation of samples for test.

A. The homogenizer tube is of heavy-wall Pyrex tubing, made uniformly ⅝ in. in bore by shrinking on to a stainless-steel former. The piston is of polytetrafluoroethylene (PTFE) and is 2½ in. long, half-rounded at one end and machined to move freely in the tube. It is drilled and tapped to screw on to a ⅜ in. Whitworth thread on the end of a ½ in. diameter stainless-steel rod, to abut against a 0·7 in. diameter thrust washer. In operation, the piston is rotated at 500–1000 rev/min through variable gearing on a ¼ hp electric drill. Meanwhile the homogenizer tube, containing the test sample in buffer solution, is pressed repeatedly up and down over the piston. PTFE is relatively soft, and its volume increases by about 1 % as its temperature rises through *c*. 20°. The pistons therefore wear quickly and tend to 'seize-up' in the tubes. A silica-filled PTFE is obtainable (Fluorosint; Polypenco Ltd, 68–70 Tewin Road, Welwyn Garden City, Herts.) which is much more satisfactory in these respects, but which generates greater heat during grinding. This is probably no disadvantage, but for the study described here the softer PTFE was used.

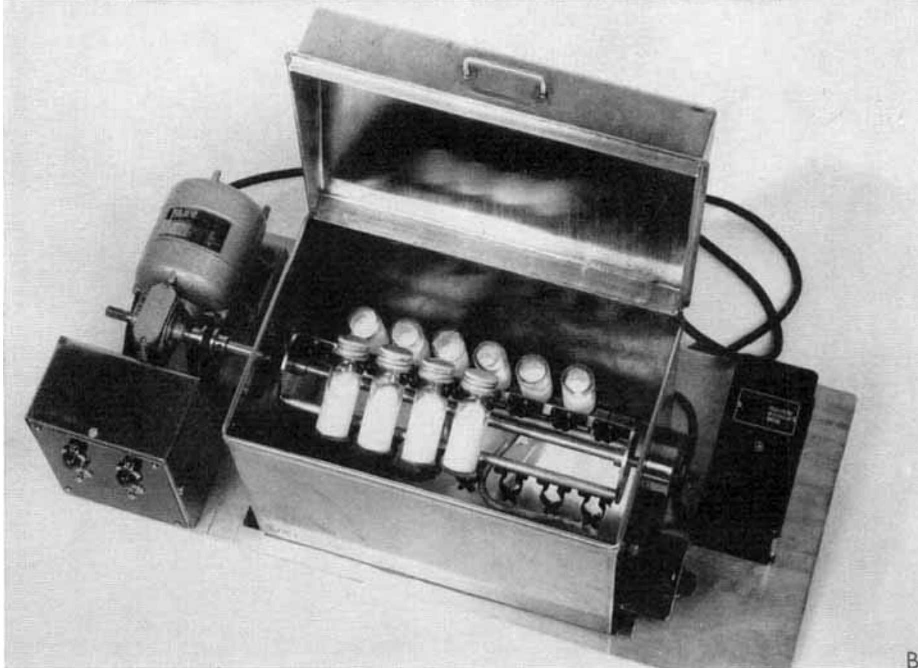
B. The water-bath is made of stainless steel and is 13 in. long, 8 in. high and 8 in. broad. A 500 W heater, controlled by a thermostat, maintains the water temperature at 56°. Wide-necked McCartney bottles of 1 oz capacity, containing the test preparations, fix into twenty-four plastic-covered spring clips mounted on a cylindrical cage which, with the attached bottles, is turned continuously during the incubation period by a geared fractional hp synchronous electric motor with an output shaft speed of 58 rev/min.

Printed in Great Britain

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press



A



B

J. E. FORD

(*Facing p.* 460)

https://doi.org/10.1079/BJN19640040 Published online by Cambridge University Press