https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

A microbiological method for assessing the nutritional value of proteins

4.* Analysis of enzymically digested food proteins by Sephadex-gel filtration

By J. E. FORD

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

(*Received* 8 *October* 1964—*Accepted* 27 *November* 1964)

Of the several characteristics that determine the nutritive quality of a manufactured protein food, its digestibility may be of prime importance. Bunyan & Price (1960) examined a series of twelve whale-meat meals which, though broadly similar in amino acid composition (Ford, 1962) showed wide differences in nutritive quality. They found in tests with rats that protein quality ratings (NPU) of the meals correlated closely with their true digestibilities of nitrogen, and also with their content of available lysine determined with fluorodinitrobenzene reagent (FDNB-lysine) as described by Carpenter (1960). Ford (1962) found that these same NPU and digestibility values also paralleled closely the contents of available leucine, methionine, arginine and tryptophan, measured microbiologically with *Streptococcus zymogenes*.

All these findings are consistent with the view, earlier expressed by Harris & Mattill (1940) and others, that in heat-damaged proteins digestibility, and thus the biological availability of all their amino acids, might be directly determined by the extent to which the free amino groups, comprised in the main of the ϵ-amino groups of the lysine, have combined with other reactive groups to form enzyme-resistant intramolecular linkages. But the differences in nutritive quality in the whale-meat meals cannot so simply be attributed entirely to the differences in their overall digestibility. The biological values of these meals varied widely and were roughly proportional to their digestibilities and to their content of FDNB-lysine, available methionine and available tryptophan. From the poorer meals, which contained relatively low proportions of available amino acids as judged in chemical and microbiological tests, correspondingly low percentages of the nitrogen absorbed from the gut were retained by the rat. It is a question of some fundamental interest whether this finding is explainable in terms of differences in the patterns of amino acids absorbed from the meals of different qualities or whether, even after absorption from the gut, some proportion of the amino acids might still remain locked up in indigestible peptide residues and be biologically unavailable.

The present paper describes the application of Sephadex-gel filtration, combined with microbiological assays, in a study of the effects of heating on the biological availability of some amino acids in fish protein. Portions of a preparation of freeze-dried cod fillets were heated under a variety of controlled conditions and were then

* Paper no. 3: *Brit. J. Nutr.* (1964), 18, 449.

digested in vitro, first with pepsin and then with papain. The digests were passed through a column of Sephadex gel, and in this manner were crudely resolved into fractions containing the different molecular species, ranging from free amino acids to undigested soluble protein. The distribution of some essential amino acids in the different fractions and their availability were determined by microbiological assay. Comparative tests were also made on two commercial anchovy meals, similar in their total amino acid composition but of widely different nutritive quality (Bunyan & Woodham, 1964), and on two whale-meat meals, also of widely different nutritive quality despite a broad similarity in amino acid composition.

In practical diets for livestock the high-protein constituents make an important contribution of several vitamins of the B-complex, and the effects of overheating during manufacture could well be as damaging to some of these as to the proteins. This study was therefore extended to include analysis of all the test proteins for riboflavine, pantothenic acid, nicotinic acid, thiamine, vitamin B_6 and vitamin B_{12}.

EXPERIMENTAL

Materials

A freeze-dried fish meal was prepared from fillets of cod (*Gadus callarias*). A 7 lb block of frozen fillets was thawed and minced with 4 l. water in a blender jar, and then dried over a period of 24 h in an evaporative spin-freeze drier (Record & Taylor, 1958). During the course of the drying the temperature of the material did not exceed 24°. After drying, the product was ground in a hammer mill, yielding a flocculent creamy-white powder.

Preparation of heat-damaged meals

Portions of the freeze-dried fish meal, each weighing 100 g, were spread in a shallow (about 0·5 cm) layer in each of two stainless steel trays. One tray was heated at 121° in steam for 24 h, the other at about 140° for 24 h in an air oven.

The steam-heated material had cohered to form a creamy-yellow coloured biscuit. This was broken into small pieces, dried at room temperature under reduced pressure, and ground to pass a 40-mesh sieve.

The oven-heated material had become yellow-orange in colour, but remained a flocculent powder.

All the preparations were stored at 2°, in screw-stoppered Polythene bottles.

Commercial fish meals and whale-meat meals

Besides the laboratory preparations of freeze-dried cod-fish meal, two Peruvian anchovy meals (FM 25 and FM 26) and two whale-meat meals (WM 7 and WM 13) were examined, each pair representative of good- and extremely poor-quality products of its kind. They were taken from a selection of samples 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).

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

Digestion in vitro with pepsin and papain

The pepsin was a twice-crystallized grade, prepared from the stomach mucosa of the pig (L. Light & Co. Ltd, Colnbrook, Bucks). It was dissolved (2%, w/v) in N/30-HCl. 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 citrate buffer of pH 7·2, containing a trace of cyanide (Ford, 1964).

The nitrogen content of the test proteins was determined by macro-Kjeldahl analysis. Samples of each, containing 1 g nitrogen, were weighed into glass-stoppered conical flasks of 500 ml capacity, to which were added 150 ml N/20-HCl. The contents of the flasks were allowed to stand for 30 min and their pH was adjusted to 1·7. To each flask were added 5 ml pepsin solution. The flasks were then placed for 24 h in a water bath at 37°, and their contents stirred every few minutes during the first 8 h of incubation.

After the 24 h incubation with pepsin, 0·5 g trisodium citrate was added to each flask, and enough N-NaOH to bring the pH value to 6·5. Next were added 25 mg NaCN, and the pH values were adjusted to 7·0; 5 ml of papain solution were added, and 5 ml of sulphur-free toluene. The flasks were stoppered tightly and incubated for a further 24 h at 37°, again with frequent swirling of the contents during the first 8 h of incubation. The contents of the flasks were transferred to 250 ml centrifuge bottles, with an additional 20 ml water used for rinsing the flasks, and centrifuged for 30 min at 1850 ***g***. The supernatant fluids were decanted. The residues were resuspended in 50 ml water and centrifuged again. The washings were decanted and combined with the digests, and the whole was evaporated under reduced pressure in a rotating film evaporator to about 90 ml, and the volume adjusted by the addition of water to 100 ml. The nitrogen content of the digests was now determined by macro-Kjeldahl analysis, and the volume of water required to bring its nitrogen content to 5 mg/ml was added to each digest.

The residues were dried at room temperature in a vacuum desiccator.

Preparation of gel column

Sephadex-gel-filtration medium was used, bead-form type G25, lot no. TP 4150, particle size 20–80 μm (Pharmacia, Uppsala, Sweden). Dry gel (100 g) was suspended in about 1200 ml 0·02 M-sodium phosphate solution of pH 7·6 and allowed to stand for 24 h, with occasional stirring. The column was prepared by the procedure described by Andrews (1964), in a vertical glass tube of 2·7 cm internal diam. The settled height of the bed of gel was 61 cm. The 0·02 M-phosphate buffer was employed for eluting the column. It was contained in a 5 l. aspirator bottle fitted with a Marriotte constant-pressure device. A flow rate through the column of about 150 ml/h was maintained by positioning the effective fluid level in the buffer reservoir 45 cm above the top of the gel bed. All the column runs were done at room temperature (about 21°).

Procedure for column runs

Portions (5 ml) of the various test solutions were applied in turn to the top of the column and allowed to enter the gel bed. Buffer (5 ml) was then applied and also allowed to enter the gel bed. A further 10 ml buffer were applied and the column was connected to the buffer reservoir. The buffer was now allowed to elute the gel and the effluent was collected in sixty 7·5 ml fractions, with a fraction collector.

Calibration of the column

By calibrating the column with 'markers' of known molecular weight, it was possible to judge approximately the order of size of the digestion products in the different fractions obtained from the protein digests (cf. Andrews, 1964). The following compounds were employed: cytochrome *c*, from horse heart, mol. wt 12400 (Sigma Chemical Co.); glucagon, mol. wt 3500 (Eli Lilly & Co.); insulin B chain, mol.wt 3483 and bacitracin A, mol. wt 1470 (both kindly given by Dr P. Andrews of this Institute) polymyxin B sulphate, mol. wt 1447 (Burroughs Wellcome & Co.); various amino acids (British Drug Houses Ltd). The compounds were dissolved in the buffer solution at a concentration of 1 mg/ml and applied to the column in 5 ml amount. Cytochrome *c* in the effluent fractions was estimated photometrically by measurement of extinction at 408 nm. Bacitracin A, polymyxin B sulphate and insulin B chain were estimated from extinction measurements at 215 nm. Amino acids were determined by addition of ninhydrin reagent (Moore & Stein, 1954) to the effluent fractions.

Measurement of available lysine

FDNB-lysine in the test proteins was determined by reaction with fluoro-2,4-dinitrobenzene as described by Carpenter (1960). The procedure does not measure free lysine or any N-terminal lysine residues in peptides, and so was not applicable to the enzymic digests. For these a microbiological assay method was employed, with *Streptococcus faecalis* R (*Streptococcus durans*, NCDO 1258) as the test organism. The method was similar to that employed with *Strep. zymogenes* (Ford, 1962) but with the following modifications. The basal medium was modified by substitution of sucrose for glucose, and by addition of hydroxylysine as recommended by Carpenter, Hartley & Ward (1964). The inoculum was grown in basal medium supplemented with 0·2% (w/v) tryptone (Oxo Ltd, London). Growth responses were assessed titrimetrically after 36 h incubation.

Measurement of methionine, leucine, isoleucine, valine, arginine and histidine

The amounts of these amino acids in the enzymic digests, and in the Sephadex effluent fractions, were determined by microbiological assay with *Strep. zymogenes* (cf. Ford, 1962).

The number of effluent fractions was halved by combining consecutive fractions: fraction 1 with fraction 2, fraction 3 with fraction 4, and so on. From each of these combined fractions two 7 ml portions were taken. One portion was suitably diluted with water and samples were taken and added directly to the microbiological assay

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

tubes, for the determination of available amino acids. The other 7 ml portion was added to a Carius tube of about 10 ml capacity, together with 1·5 ml 11·65N-HCl. The tube was sealed and heated in a steam autoclave for 20 h at 120°. It was then cooled and opened, and the pH of its contents was adjusted to 7·0. After suitable dilution with water, this acid hydrolysate was assayed for total amino acids.

Approximate estimation of total α-amino nitrogen

A portion of each of the above acid hydrolysates was taken for the estimation of the α-amino nitrogen in the fractions, by reaction with the modified ninhydrin reagent of Moore & Stein (1954). A standard response curve was prepared with known graded concentrations of leucine, and the amount of material present in each fraction was calculated in terms of 'leucine-equivalent' by reference to this standard curve.

Redigestion of Sephadex effluent fractions with papain

The question arose, whether 'unavailable' peptides isolated by gel filtration were susceptible to further enzymic hydrolysis. A second series of Sephadex fractions was prepared from each of the cod-fish meal digests, and consecutive fractions were combined as described above. Duplicate 7 ml samples were again taken from each of these combined fractions. One was suitably diluted with water, and portions were taken and added directly to the microbiological assay tubes for the determination of available amino acids. To the other 7 ml sample were added 1 ml of a solution containing, per ml, 20 mg trisodium citrate, 0·2 mg NaCN, and enough citric acid to bring the pH to 7·0, and finally 0·05 ml of papain solution. The mixture was incubated for 4 h at 56° and then diluted with water and added to the assay tubes. An enzyme 'blank' was included with each of the amino acid assays.

Microbiological assay of B-complex vitamins

Thiamine was measured with *Lactobacillus fermenti* as described by Bánhidi (1958) and riboflavine with *Lactobacillus casei* by the method of Roberts & Snell (1946). Pantothenic acid was assayed with *Lactobacillus arabinosus* in the medium of Roberts & Snell (1946) modified by the omission of pantothenate and inclusion of riboflavine. Test extracts for pantothenic acid assay were made as recommended by Neilands & Strong (1948). Nicotinic acid and vitamin B_6 were measured as described by Chapman, Ford, Kon, Thompson, Rowland, Crossley & Rothwell (1957). Vitamin B_{12} was determined by the *Ochromonas malhamensis* method of Ford (1953).

RESULTS AND DISCUSSION

Calibration of the Sephadex column

Fig. 1 shows the rates of passage of the different marker compounds through the column. Andrews (1964) investigated the correlation between elution volumes and molecular weight in a series of proteins and found, using Sephadex G75, that elution volume was proportional to log (molecular weight) in the molecular weight range 3000–35000. Marked deviations from this relationship are sometimes observed, especially among substances of lower molecular weight. Thus it is evident in Fig. 1

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

that glucagon and insulin B chain, though of closely similar molecular weight, pass through the gel at markedly different rates. Aromatic and heterocyclic compounds tend to pass more slowly down the column than would be expected from their molecular weights (Gelotte, 1960; Porath, 1960), as also do strongly basic proteins and

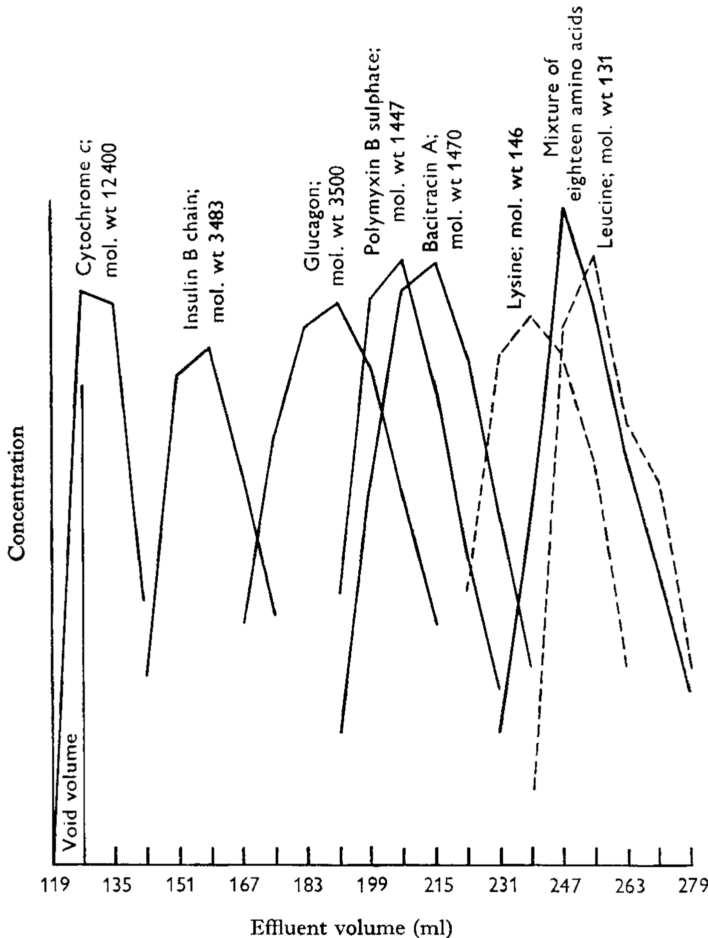

Fig. 1. Calibration of Sephadex G25 column.

peptides (Cruft, 1961). Other possible sources of error in molecular weight estimation by gel filtration are discussed by Andrews (1964) and Craig (1962). For the purposes of the work now described, it has been assumed that the proteins, peptides and amino acids in the enzymic digests appeared in the column effluent in approximate order of decreasing molecular size, as did the marker compounds.

Cod fillet preparations

Apparent digestibility in vitro. Heating the freeze-dried fish meal reduced the percentage of total nitrogen rendered soluble by the enzymic digestion, from the

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

value of 97% for the unheated meal to 73 and 84% for the steam-autoclaved and the oven-heated meals respectively.

Composition of the enzymic digests. Table 1 shows the amounts, total and available, of six of the essential amino acids in the enzymic digests, and the FDNB-lysine contents of the undigested meals. Straightforward comparison of the two estimates of available lysine is not possible, of course, because the microbiological assay values relate only to the enzymic digests from which the indigestible material had been

Table 1. *Total and available valine, histidine, arginine, methionine, leucine and lysine (g/16 g N) in enzymic digests of cod fillet preparations*

	Freeze-dried cod fillet		
Amino acid	Unheated	Autoclaved for 24 h at 121°	Heated in air for 24 h at 140°
Valine			
Total	5·7	5·6	5·9
Available	5·8	3·9	4·1
Histidine			
Total	2·0	1·6	1·8
Available	1·9	1·0	1·1
Arginine			
Total	6·1	5·6	5·7
Available	5·5	3·6	3·7
Methionine			
Total	3·3	3·0	2·9
Available	3·2	2·0	1·8
Leucine			
Total	8·0	7·8	8·2
Available	7·5	4·8	5·5
Lysine			
Total	9·1	8·5	6·7
Available	8·4	3·1	1·7
DNFB-lysine*	8·6	5·3	2·0

* Measured in the undigested materials, by reaction with 2:4-dinitrofluorobenzene (Carpenter, 1960).

removed. One might therefore have expected the FDNB-lysine values for the heated meals to be lower than the microbiological, and not higher as in fact they were. The digests were broadly similar in total amino acid composition, except that the digest of the oven-heated meal contained 26% less lysine and 12% less methionine than the control; and the digest of the autoclaved meal contained 20% less histidine. There were much greater differences in available amino acid composition. In the control meal digest, the amino acids were all highly available; in the digests of the heated meals the values for available amino acids ranged between 60 and 70% of the corresponding total values, except the available lysine values, which were considerably lower, especially for the oven-heated meal.

Fractionation of the enzymic digests on Sephadex gel. Fig. 2 shows the distribution of α-amino nitrogen in the Sephadex-gel fractions obtained from the different digests. It appears that the digest of the control meal contained predominantly small peptides,

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

with up to about twelve amino acid residues, and free amino acids, whereas the digests of the heated meals contained relatively much more material of larger molecular weight. The oven-heated meal in particular, though largely rendered soluble by the enzyme treatment, was still far from completely digested.

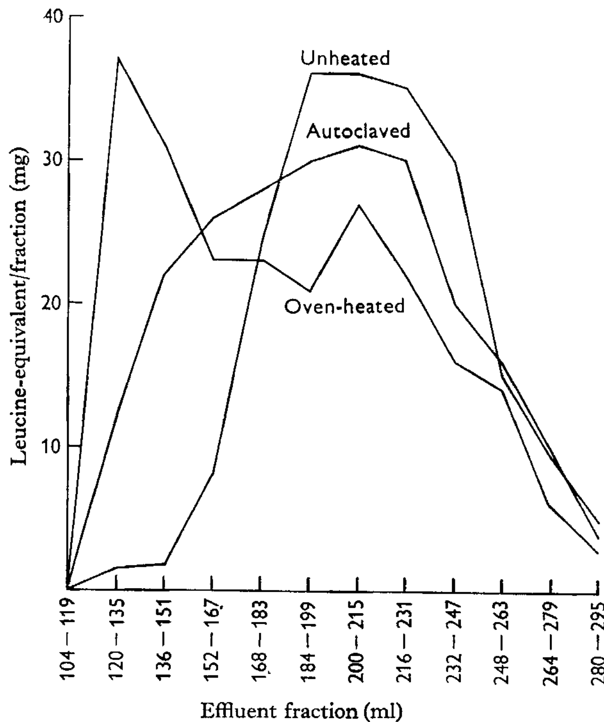

Fig. 2. Fractionation on Sephadex G25 of enzymically digested cod fillet preparations. Total amino acid content (expressed as 'leucine-equivalent') was measured by reaction with ninhydrin, after hydrolysis of the fractions with acid.

Total and available amino acids in the Sephadex-gel fractions. Fig. 3 shows the amounts of total and available lysine in the three sets of fractions assayed microbiologically with *Strep. faecalis*. The distribution of total lysine in the different digests paralleled closely the distribution of the α-amino nitrogen. In the digests of the heated meals, and especially the oven-heated meal, the preponderance of lysine in the fractions of high molecular weight was clearly evident. In contrast, the distribution of available lysine, as measured in the microbiological assays with *Strep. faecalis*, was much the same in the three sets of fractions. Very little of the lysine in the fractions of high molecular weight was available to the test micro-organism. Fig. 4 shows the corresponding distribution curves for isoleucine. The broad picture was much the same as for lysine, but there were large quantitative differences in the relative distribution of these two amino acids between the fractions of high and low molecular weight. Thus, in the digests of the heated meals the ratio of lysine to isoleucine was significantly greater in the fractions of high than in those of low molecular weight, and in this

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

respect the amino acid composition of the fractions of low molecular weight was different from that of the residual incompletely digested material. Similarly, the fractions of low molecular weight from the control meal digests were richer in lysine than the corresponding fractions from the heated meal digests.

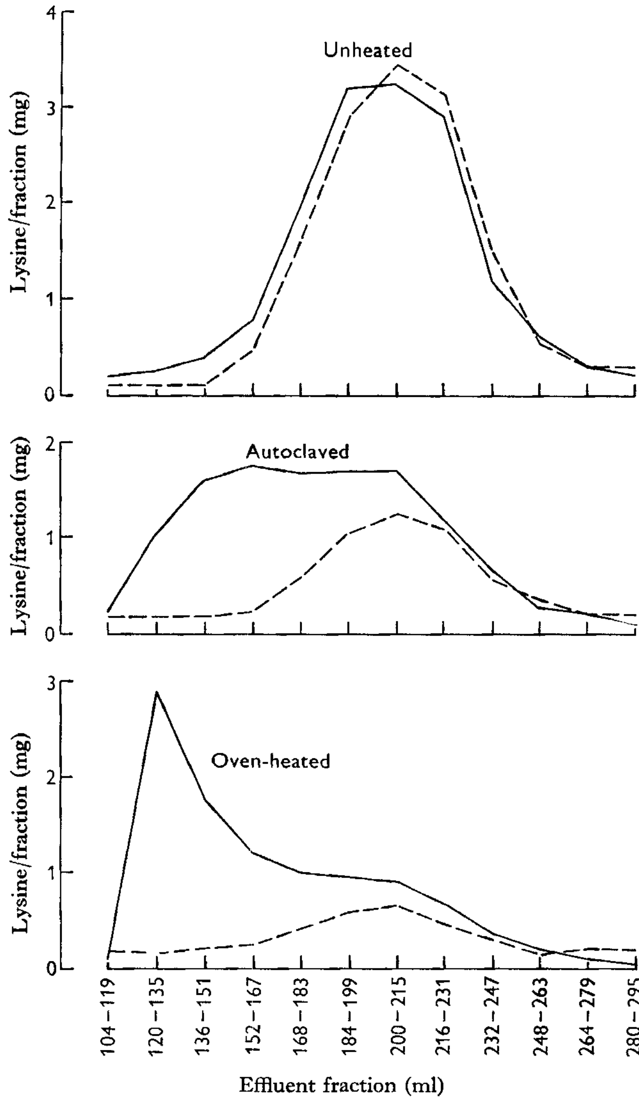

Fig. 3. Fractionation on Sephadex G25 of enzymically digested cod fillet preparations. Distribution of total and available lysine in different fractions. ——, total lysine; - - - -, available lysine.

An analysis of the distribution of total and available arginine, leucine, valine, histidine and methionine in the Sephadex-gel fractions is set out in Table 2, from which families of curves may be constructed similar to those of Fig. 3. All of these amino

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

Table 2. *Total and available arginine, leucine, valine, histidine and methionine (mg/fraction) in Sephadex-gel fractions obtained from the enzyme-digested cod fillet preparations*

Effluent fraction* (ml)	Arginine		Leucine		Valine		Histidine		Methionine	
	Total	Available	Total	Available	Total	Available	Total	Available	Total	Available
					Unheated control					
104–119	0	0	0·09	0·04	0	0	0·014	0·019	0·051	0·047
120–135	0	0	0·09	0·09	0	0	0·028	0·014	0·069	0·051
136–151	0	0	0·12	0·14	0	0	0·028	0·024	0·060	0·047
152–167	0·17	0·05	0·24	0·21	0·13	0·12	0·056	0·038	0·086	0·082
168–183	0·53	0·50	0·62	0·86	0·46	0·42	0·098	0·098	0·120	0·142
184–199	1·19	1·39	1·17	1·23	1·06	1·07	0·192	0·211	0·206	0·322
200–215	1·88	1·80	2·17	2·17	1·75	1·83	0·325	0·356	0·550	0·670
216–231	1·85	1·98	2·70	2·62	1·95	1·99	0·405	0·458	0·890	1·20
232–247	1·44	1·51	2·28	2·06	1·11	1·07	0·295	0·305	0·820	0·970
248–263	0·77	0·87	1·38	1·38	0·40	0·34	0·225	0·240	0·600	0·722
264–279	0·40	0·48	0·61	0·71	0·16	0·08	0·169	0·202	0·350	0·395
280–295	0·17	0·17	0·19	0·17	0·05	0·05	0·118	0·136	0·150	0·163
					Steam-autoclaved preparation					
104–119	0	0	0·09	0·06	0	0	0·028	0·019	0·051	0·060
120–135	0·40	0	0·67	0·16	0·29	0	0·084	0·019	0·159	0·073
136–151	0·60	0	1·02	0·16	0·55	0	0·128	0·028	0·227	0·073
152–167	0·70	0	1·25	0·30	0·81	0·17	0·150	0·038	0·279	0·069
168–183	0·92	0·35	1·30	0·59	0·86	0·35	0·173	0·070	0·335	0·150
184–199	1·02	0·70	1·40	0·83	0·94	0·68	0·164	0·097	0·279	0·236
200–215	1·11	1·20	1·50	1·3	1·15	1·10	0·178	0·168	0·335	0·353
216–231	1·21	1·11	1·71	1·65	1·14	1·29	0·220	0·233	0·430	0·520
232–247	0·81	0·88	1·38	1·33	0·72	0·80	0·210	0·233	0·430	0·545
248–263	0·49	0·57	0·96	0·97	0·40	0·38	0·118	0·121	0·279	0·382
264–279	0·35	0·32	0·54	0·62	0·20	0·25	0·084	0·084	0·241	0·296
280–295	0·13	0·10	0·28	0·21	0·10	0·05	0·070	0·080	0·138	0·138
					Oven-heated preparation					
104–119	0	0	0·16	0·10	0·04	0	0·038	0·028	0·086	0·034
120–135	1·34	0·10	2·17	0·40	1·27	0·17	0·277	0·056	0·525	0·086
136–151	0·89	0·07	1·33	0·30	0·97	0·14	0·197	0·046	0·309	0·086
152–167	0·64	0·17	0·96	0·40	0·61	0·26	0·150	0·052	0·241	0·121
168–183	0·67	0·40	1·12	0·71	0·72	0·42	0·145	0·094	0·241	0·166
184–199	0·87	0·70	1·12	1·02	0·86	0·71	0·145	0·112	0·293	0·223
200–215	1·02	0·95	1·38	1·17	1·00	0·86	0·164	0·155	0·293	0·279
216–231	1·09	1·14	1·77	1·65	1·09	1·17	0·197	0·225	0·374	0·395
232–247	0·67	0·83	1·38	1·45	0·80	0·74	0·193	0·216	0·279	0·296
248–263	0·45	0·46	1·02	0·79	0·40	0·33	0·127	0·122	0·206	0·198
264–279	0·18	0·25	0·50	0·44	0·07	0·18	0·080	0·080	0·129	0·142
280–295	0·07	0·08	0·19	0·25	0	0·07	0·052	0·060	0·099	0·108

* The first 103 ml of effluent were discarded, as they consisted only of buffer displaced from the 'void volume' (i.e. the volume of the space within the column and outside the gel granules).

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

acids, like lysine and isoleucine, were highly digestible and available in the control meal, and were partly unavailable in the heated meals.

It would be unprofitable at this stage to pursue further the comparison of the amino acid composition of the different fractions, which may have been determined very largely by the particular conditions arbitrarily chosen for the enzymic predigestion and by the selection of pepsin and papain as the digesting enzymes. It is an interesting question whether heating merely decreased the rate of in vitro enzymic hydrolysis

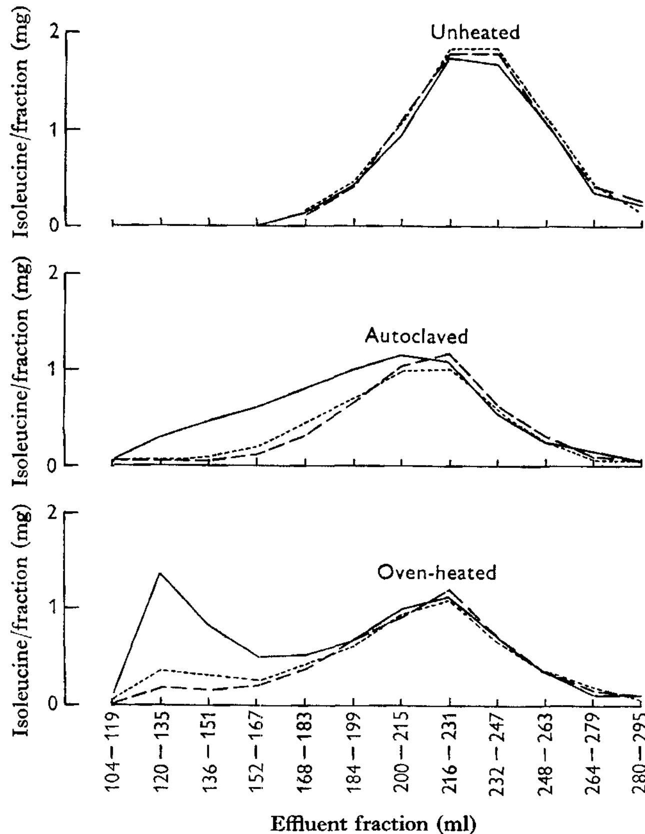

Fig. 4. Fractionation on Sephadex G25 of enzymically digested cod fillet preparations. Distribution of total and available isoleucine in different fractions. ——, total isoleucine; — — —, available isoleucine; - - - -, available isoleucine measured after reincubation of the fractions with additional papain.

without affecting the degree to which the protein was ultimately susceptible to hydrolysis. There was some apparent destruction of lysine, and perhaps also of methionine, in the oven-heated meals and to this extent at least the heating caused irrecoverable loss. But on further digestion with papain, more of the isoleucine in the unavailable fractions of higher molecular weight became available to the test micro-organism (Fig. 4). Similar increases were found for arginine, histidine and methionine, and this despite the minute further concentration of enzyme added to the fractions. With leucine and lysine the effect of this second incubation with papain was less marked, and

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

no significant increase in the 'available' values was found. The effect on valine was not measured.

Pader, Melnick & Oser (1948) found with casein that dry heating at 150° for $1\frac{1}{2}$ or 5 h progressively reduced the rate of in vitro pancreatic hydrolysis but did not reduce the ultimate digestibility. The rate of release of lysine, determined by microbiological assay of the digests with *Strep. faecalis* R, decreased with increasing time of heating, as also did the ultimate availability of the lysine. For the heated samples the relationship between the percentage liberation of lysine and time was linear up to a point at which no more lysine was released. The findings with fish meal now presented show similarly that heating retarded the enzymic release of several amino acids, but particularly of lysine. It seems, however, that the rate of liberation of amino acids from the undigested protein became progressively slower during the course of the digestion. The 'indigestible' washed residues (p. 279) were still susceptible to proteolysis, but at a slower rate than the original proteins.

Whale-meat meals and Peruvian anchovy meals

The whale-meat meals, WM 7 and WM 13, were of broadly similar amino acid composition but of widely different nutritive quality (Boyne *et al.* 1961); so also were the two anchovy meals FM 25 and FM 26.

Digestibility. The true digestibilities of nitrogen, measured with rats, of WM 7 and WM 13 were respectively 54 and 92% (Bunyan & Price, 1960). In the study now reported the percentages of the total nitrogen of WM 7 and WM 13 rendered soluble upon enzymic digestion in vitro were 56 and 87%. Similarly with FM 25 and FM 26, the true digestibilities of nitrogen were 86 and 62% (K. M. Henry, personal communication) and of the enzyme-soluble nitrogen 86 and 65%.

Fractionation of the enzymic digests in Sephadexgel. Fig. 5 shows the distribution of α-amino nitrogen in the different fractions obtained from the whale-meal digests. The digest of the poorer meal, WM 7, contained relatively much more material of large molecular weight which, though in a sense digestible, was not readily available in the microbiological test system. This picture was reflected in the content of material insoluble in 20% trichloroacetic acid in the two digests. The digest of WM 7 contained, per 100 mg N, 74 mg of material insoluble in trichloroacetic acid, compared with only 12·5 mg in the digest of WM 13.

Findings for the two anchovy meals were closely similar to those for the whale-meat meal. Again the digest of the poorer meal yielded more material of large molecular weight and poorly available to the test micro-organisms.

Available amino acids in the digests and in the undigested residues. Table 3 shows the available amounts of six amino acids in the whale-meat meal digests, and in the undigested residues. The digests were simply diluted with water before assay. The residues, however, were redigested with crystalline pepsin (at 20 mg/100 mg N; cf. Ford, 1964). Except for lysine, the amino acids in the undigested residue of WM 13 were highly available. The residue was still amenable to peptic digestion and, again excepting lysine, its available amino acid composition was similar to that of the original meal. In WM 7, the undigested residue was much poorer in available amino acids

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

than the digest, which in turn was markedly poorer in available amino acids than the digest of WM 13. Clearly, as in the heated fish-meal preparations (Figs. 2, 3 and 4), the larger peptides and soluble proteins in the digest of WM 7 (Fig. 5) were a poor source of amino acids for the test micro-organisms.

Table 4 shows a comparison of the total and available lysine contents of the undigested residues with those of the original meals. An unexpected finding was that the FDNB-lysine contents of the residues were not lower than those of the original

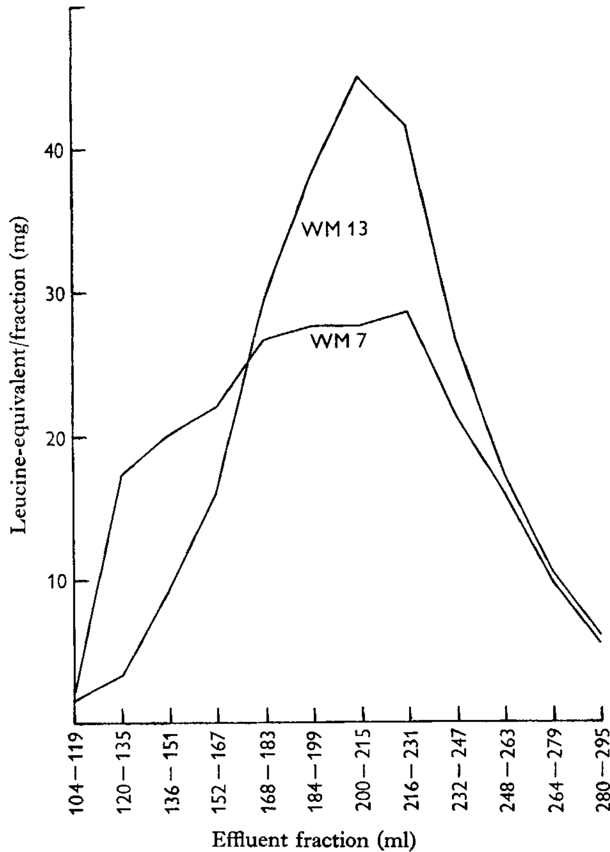

Fig. 5. Fractionation on Sephadex G25 of enzymically digested whale-meat meals of good (WM 13) and poor (WM 7) nutritive quality. Total amino acid content (expressed as 'leucine-equivalent') was measured by reaction with ninhydrin, after hydrolysis of the fractions with acid.

Table 3. *Available amino acids (g/16 g N) in whale-meat meal digests, and in the undigested residues*

Test material	Lysine*	Leucine†	Histidine†	Valine†	Arginine†	Methionine†
WM 7 digest	2·38	4·50	1·61	2·87	3·66	1·42
WM 7 residue	0·40	2·97	0·75	2·17	1·60	0·90
WM 13 digest	7·60	8·10	3·47	4·17	4·66	2·77
WM 13 residue	3·93	8·10	3·51	5·40	4·72	2·36

* Measured with *Streptococcus faecalis* R.
† Measured with *Strep. zymogenes*.

meals. Tests with *Strep. faecalis*, on the other hand, showed the residues to be much poorer than the corresponding meals. The chemical test for available lysine takes no account of rate of digestion, and it seems probable that the lysine in these residues was physiologically less readily available than was indicated. In direct feeding experiments with rats, K. M. Henry (personal communication) obtained markedly lower values for the available lysine in WM 7 than did Bunyan & Price (1960) with the chemical method.

Table 4. *Total and available lysine (g/16 g N) in whale-meat meals, and in the undigested residues*

Test material	Total lysine	Available lysine: FDNB test*	Available lysine: *Streptococcus faecalis* test
WM 7	8·1†	3·3†	2·5
WM 13	9·6†	7·3†	7·9
WM 7 (undigested residue)	8·2	4·3‡	0·4
WM 13 (undigested residue)	7·8	7·5‡	3·9

* Carpenter (1960).
† Values from Bunyan & Price, 1960.
‡ Values kindly provided by Dr D. Adair.

Many examples have been demonstrated of supplementary relationships between the different proteins in mixed diets. Thus, Henry & Kon (1946) showed that there was a marked supplementary relationship between the proteins of milk and potato, and between those of bread and cheese, when the members of each pair supplied equal amounts of protein and were given together in the diet. No supplementary relationship was exhibited, however, when the sources of protein were given separately on alternate days. Melnick, Oser & Weiss (1946) argued that time and concentration relationships might similarly determine the efficiency with which amino acids are utilized after enzymic release from an individual protein. They demonstrated that different amino acids may be released at widely different rates, and postulated that, for optimum utilization of food protein, all the essential amino acids must not only be available for absorption but must also be liberated during digestion in vivo at rates permitting mutual supplementation. Several papers seeming to support this thesis have appeared. Beuk, Chornock & Rice (1949) studied the effect of heat upon the enzymic release of amino acids from pork protein. They found that the extent of the release of all the essential amino acids on digestion with trypsin and erepsin decreased with increasing duration of the heat treatment, and that the rate of release of the different amino acids varied widely. The amounts of the individual amino acids released from pork autoclaved at 113° for only 5 min, expressed as percentages of the corresponding values for unheated pork, ranged from over 96% for arginine and leucine to less than 65% for methionine, histidine and cystine. The same relative extent of release was maintained after longer periods of heating: after 1 h the values ranged from 88% for arginine to less than 53% for methionine, histidine and cystine. Lysine was relatively highly placed in this ordering of extent of release: 89% after 5 min heat treatment and 72% after 1 h. From studies of the digestion of heated

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

casein with pepsin and pancreatin, however, Pader *et al.* (1948) identified the rate of release of lysine as critical in determining the biological value, and the indications of the study now reported could be taken to suggest that the same was true for the heated fish meals and whale-meat meals examined. But results obtained with in vitro tests of this kind must be viewed with caution, as they may reflect more the specificities of the enzymes chosen for bringing about the hydrolysis, and the abilities of the different test micro-organisms to hydrolyse the soluble peptides released during the digestion, rather than the relative susceptibilities of the different amino acids to enzymic release. For measuring the essential amino acids present in their pork digests, Beuk *et al.* (1949) employed four different test micro-organisms of which at least one, *Streptococcus lactis* R (syn. *Strep. faecalis* R and *Strep. durans*) is well endowed with peptidases whereas another, *Leuconostoc mesenteroides* P60 (syn. *Pediococcus cerevisiae* P60) makes relatively inefficient use of peptides. If methionine in the pork digests had been assayed with *Strep. faecalis* R instead of *Ped. cerevisiae* P60 it would perhaps have been judged much more highly available. Ford (1964) compared these two micro-organisms for the assay of available methionine in peptic digests and found that *Strep. faecalis* R gave uniformly higher results. For WM 13, for example, it gave a value of 2·5 g methionine/16 g nitrogen, as against 1·0 obtained with *Ped. cerevisiae* P60.

The findings presented here illustrate clearly the effects of heat processing in retarding the release of several amino acids, but they cannot readily be interpreted in terms of animal nutrition. It is not possible to reproduce in vitro the digestive milieu of the gut, in which the food protein is assailed with a full armamentarium of enzymes. Some questions concerning the unequal rates of liberation of different amino acids from damaged proteins can finally be decided only by direct feeding experiments with animals, perhaps of the kind employed by Longenecker & Hause (1959) and others, which permit the correlation of the free amino acid composition of the portal blood with the time elapsed since the meal of test protein. Meanwhile, the development of microbiological methods for assaying available amino acids proceeds empirically, guided by the results of parallel tests on laboratory animals. We must remember that the distinctions we make between 'available' and 'unavailable' are arbitrary and relate to the particular conditions of the tests: the results are valid only in the practical sense that they predict accurately the results of biological tests, and these might vary with the species of animal used, with the level of the test protein in the diet and the nature of the other proteins accompanying it, with the rate of passage of food through the gut, and with several other factors not directly related to the intrinsic availability of the amino acids in the test protein.

B-vitamin content of the test proteins

Table 5 shows the content of six vitamins of the B complex in the various test proteins. The findings confirm those of Tarr, Biely & March (1954) and of Mason & Weidner (1964), indicating as they do the general heat lability of these vitamins. In the heated cod fillet preparations all the vitamins excepting nicotinic acid were largely or completely destroyed. These laboratory preparations had of course been subjected to a much more prolonged heat treatment than would be usual in fish-meal manufacture.

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

But in WM 7 and FM 26, both commercial meals though of poor nutritive quality, the amounts of riboflavine, vitamin B_6, pantothenic acid and vitamin B_{12} were again markedly lower than in the corresponding meals of better quality. The thiamine content of all these four commercial meals was unexpectedly low, and it seems doubtful whether this was altogether the result of heating during manufacture. It is possible that thiaminase activity of gut contents present in the raw material before processing caused much of the apparent loss. Certainly in the anchovy meals, intestinal contents would comprise an important proportion of the whole, and this is probably reflected in the high level of vitamin B_{12} in this material.

Table 5. *B-complex vitamins in the test proteins*

Test material	Thiamine (µg/g)	Riboflavine (µg/g)	Vitamin B_6 (µg/g)	Nicotinic acid (µg/g)	Panto-thenic acid (µg/g)	Vitamin B_{12} (ng/g)
Freeze-dried cod fillet						
Unheated	2·0	4·0	10·0	60	10·2	64
Heated at 121° for 24 h in steam autoclave	0	0·5	0·1	59	1·2	0
Heated at 140° for 24 h in air oven	0	1·0	1·3	43	0·8	0
WM 7	0·1	3·0	1·1	74	2·2	6
WM 13	0·1	5·6	4·9	124	8·4	51
FM 25	0·1	6·1	1·7	78	10·5	160
FM 26	0·1	4·5	0·1	95	4·2	37

For methods of assay see p. 281.

In the formulation of pig and poultry diets it is customary to add riboflavine and vitamin B_{12}, and sometimes also nicotinic acid and pantothenic acid, in amounts calculated to ensure that the diet meets the accepted requirements of the animals. The supplements, however, provide only part of the requirement and the diet itself supplies the rest, together with other members of the B complex of vitamins, among them vitamin B_6 which was largely destroyed in the badly processed test proteins. Whether or not these less familiar vitamins are of practical nutritional importance, we should take into account that in protein-rich foods that have suffered heat damage during processing the content of several important vitamins may be depleted.

SUMMARY

1. Portions of a preparation of freeze-dried cod fillet were heated under controlled conditions and were then digested in vitro, first with pepsin and then with papain. Digests were similarly prepared from two commercial anchovy meals, alike in their total amino acid composition but of widely different nutritive quality, and from two whale-meat meals, also of widely different nutritive quality despite a broad similarity in amino acid composition.

2. The digests were passed through a calibrated column of Sephadex gel and were in this manner resolved into fractions containing the different molecular species graded approximately in order of size.

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press

3. The distribution of some essential amino acids in these different fractions, and their availability, were determined by microbiological and chemical assay.

4. The digests of the unheated cod fillet control, and of the better-quality commercial meals, contained predominantly small peptides with up to about twelve amino acid residues, and free amino acids. Digests of the heated cod fillet preparations, and of the poorer-quality commercial meals, contained relatively much more material of larger molecular size, which proved a poor source of available amino acids in the microbiological tests.

5. In the digests of the heated cod fillet preparations the ratio of lysine to isoleucine was higher in the fractions of high than in those of low molecular weight. Similarly, the fractions of low molecular weight from the control meal were richer in lysine than the corresponding fractions from the heated meals.

6. Six vitamins of the B complex were assayed in the various test proteins. In the heated cod fillet preparations all the vitamins except nicotinic acid were largely destroyed. Similarly, in the poorer commercial meals, the amounts of riboflavine, vitamin B_6, pantothenic acid and vitamin B_{12} were markedly lower than in the corresponding meals of better quality.

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

REFERENCES

Andrews, P. (1964). *Biochem. J.* **91**, 222.
Bánhidi, Z. G. (1958). *Acta chem. scand.* **12**, 517.
Beuk, J. F., Chornock, F. W. & Rice, E. E. (1949). *J. biol. Chem.* **180**, 1243.
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.
Bunyan, J. & Woodham, A. A. (1964). *Brit. J. Nutr.* **18**, 537.
Carpenter, K. J. (1960). *Biochem. J.* **77**, 604.
Carpenter, K. J., Hartley, A. W. & Ward, L. D. (1964). *Proc. Nutr. Soc.* **23**, xl.
Chapman, H. R., Ford, J. E., Kon, S. K., Thompson, S. Y., Rowland, S. J., Crossley, E. L. & Rothwell, J. (1957). *J. Dairy Res.* **24**, 191.
Craig, L. C. (1962). *Arch. Biochem. Biophys.* Suppl. 1, p. 112.
Cruft, H. J. (1961). *Biochim. biophys. Acta*, **54**, 611.
Ford, J. E. (1953). *Brit. J. Nutr.* **7**, 299.
Ford, J. E. (1962). *Brit. J. Nutr.* **16**, 409.
Ford, J. E. (1964). *Brit. J. Nutr.* **18**, 449.
Gelotte, B. (1960). *J. Chromat.*, **3**, 330.
Harris, R. L. & Mattill, H. A. (1940). *J. biol. Chem.* **132**, 477.
Henry, K. M. & Kon, S. K. (1946). *J. Dairy Res.* **14**, 330.
Longenecker, J. B. & Hause, N. L. (1959). *Arch. Biochem. Biophys.* **84**, 46.
Mason, V. C. & Weidner, K. (1964). *Acta agric. scand.* **14**, 87.
Melnick, D., Oser, B. L. & Weiss, S. (1946). *Science*, **103**, 326.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
Neilands, J. B. & Strong, F. M. (1948). *Arch. Biochem.* **19**, 287.
Pader, M., Melnick, D. & Oser, B. L. (1948). *J. biol. Chem.* **172**, 763.
Porath, J. (1960). *Biochim. biophys. Acta*, **39**, 193.
Rao, M. N., Sreenivas, H., Swaminathan, M., Carpenter, K. J. & Morgan, C. B. (1963). *J. Sci. Fd Agric.* **14**, 544.
Record, B. R. & Taylor, R. (1958). *Biochem. J.* **68**, 420.
Roberts, E. C. & Snell, E. E. (1946). *J. biol. Chem.* **163**, 499.
Tarr, H. L. A., Biely, J. & March, B. E. (1954). *Poult. Sci.* **33**, 242.

Printed in Great Britain

https://doi.org/10.1079/BJN19650026 Published online by Cambridge University Press