Metabolism of heat-damaged proteins in the rat

Influence of heat damage on the excretion of amino acids and peptides in the urine

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1. Freeze-dried cod muscle and casein were subjected to various conditions of heat treatment. Diets containing the different products, or the unheated materials, were given to a group of four adult male rats during successive 48 h periods, and urine was collected during the second 24 h of each 48 h period. A further collection of urine was made from the rats after they had been given protein isolated from heated skim-milk powder. The content and amino acid composition of the 'peptide' and 'free amino acids' in the urines were determined.

2. Heat damage to the cod-fillet protein increased the total urinary excretion of peptide-bound amino acids, from 18·6 to 48·8 μmol/rat. d. The composition of the peptide also changed, and in particular there was a marked increase in lysine, from 2·98 to 20·30 μmol %. Three amino acids – lysine, aspartic acid and glutamic acid – together comprised nearly 70% of the total amino acid residues. There was a corresponding increase in urinary excretion of free amino acids, from 53·7 to 114·4 μmol/rat.d. The combined losses of lysine in urinary peptide and free amino acids were 1·5% of the total lysine ingested, as against 0·3% for the unheated cod fillet.

3. The effects of similar heat treatment of casein on the composition of the urinary peptide and free amino acids were less marked. There was no increase in total urinary peptide excretion and there was a smaller increase in the lysine content of the peptide.

4. In urine of rats given protein isolated from heated skim-milk powder, the peptide hydrolysate was rich in lysine and in furosine, which together comprised 41 mol % of the total amino acid composition. These compounds were presumably formed, together with a smaller quantity of pyridosine, from lysine–carbohydrate complex in the urine. It is probable that, as compared with free lysine, the lysine–carbohydrate complex was absorbed relatively inefficiently from the rat intestine.

5. The findings are discussed in relation to the wider question of the metabolism of the 'unavailable peptide' that is released in the course of digestion of heat-damaged protein.

As is well known, severe overheating of protein foods impairs their nutritional quality, but the chemical mechanism of the heat damage, and the nutritional consequences, are not yet fully understood. A central process is the combination of free amino groups, comprised in the main of the ε-amino groups of the lysine, with other reactive groups to form enzyme-resistant inter- and intra-molecular bonds. The solubility and digestibility of the protein are thereby reduced and the time-course of digestion is prolonged. With heated cod fillet, growth tests with rats, and microbiological assays of samples digested enzymically in vitro, indicated considerable differences in the biological availability of lysine, methionine and isoleucine (Ford & Salter, 1966) and it seemed that some of the impairment of nutritive quality could be attributed to large differences in the rates of enzymic release of different amino acids. From an earlier study of chemical and nutritional changes in heated cod muscle, Miller, Carpenter & Milner (1965) had similarly concluded that the damaging effects of heat, demonstrated in growth tests with rats, were by no means explained simply

by the lower total digestibilities of the test preparations. They found that digestibility fell with increasing severity of heat treatment, but not enough to explain the fall in net protein utilization (NPU), and suggested that some of the 'digested methionine' from the heated materials may have been biologically unavailable. Donoso, Lewis, Miller & Payne (1962) reached the same conclusion from a study of the effects of heat treatment on the nutritive quality of pork-muscle protein. Bunyan & Price (1960) examined a series of whale-meat meals of similar amino acid composition but of widely different nutritive quality. They found in tests with rats that biological values and digestibilities were closely correlated. From the poorer meals, a lower proportion of the absorbed nitrogen was retained by the rats. Here again we meet the question whether lower N retention was explainable simply in terms of the poorer pattern of essential amino acids absorbed from the poorer meals.

A further possibility is that, even after absorption from the gut, some significant proportion of the amino acids might still remain locked up in indigestible peptide residues and be biologically unavailable. There is in the literature no strong evidence for this, and the accepted view is that the products of protein digestion as they appear in the portal blood consist almost entirely of free amino acids (for references, see Dawson & Porter, 1962). But the question remains, whether the situation might be different with proteins that have suffered damage during manufacture. It is of interest in this connexion that the feeding of nutritionally inadequate proteins (zein and oxidized casein) to the rat has been reported to result in a marked increase in urinary excretion of peptide-bound amino acids (Wu, 1954; Sauberlich, Pearce & Baumann, 1948), but these findings need confirmation from studies using modern and more critical analytical methods. The present paper reports on the content and composition of peptides and free amino acids in urine from rats given alternately proteins of good nutritional quality and the same proteins after heat damage.

EXPERIMENTAL

Materials

Cod fillet. A 3 kg block of frozen fillets of cod was freeze-dried and milled to form a light flocculent powder, as described by Ford (1965). A 250 g portion of this preparation was spread in a shallow (1 cm) layer in a stainless-steel tray and heated in an air oven at 135° for 20 h.

Casein. Labco vitamin-free casein (The Borden Co., New York) was used, unheated, and heat-treated as follows. Portions of the casein, weighing 250 g, were spread in a shallow (about 0·5 cm) layer in each of two stainless-steel trays; one was heated in steam at 121° for 4 h, and the other in an air oven at 135° for 20 h.

A further sample of 'casein' was prepared from heat-damaged skim-milk powder, as follows. Spray-dried skim-milk powder (500 g) was spread on trays as described above and heated in an air oven at 105° for 6 h. The heated material, which had formed a biscuit of a primrose-yellow colour, was ground coarsely and then stirred into 3 l water to form a slurry. This was acidified to pH 4·8 by addition of M-HCl and centrifuged. The supernatant fluid was decanted and brought to pH 3·8 by

further addition of M-HCl. A precipitate formed, which was separated by centrifugation and combined with the residue obtained after the centrifugation at pH 4·8. The combined residues were washed with water and then dried by extraction successively with ethanol and diethyl ether in a Buchner funnel.

Test diets

The compositions of the test diets are set out in Table 1. The experiments were essentially qualitative and designed primarily to explore the concept that unavailable peptides might be absorbed during the digestion of heat-damaged protein and excreted in the urine. No attempt was made to equalize food intake. The test diets were accepted readily and consumption was about 18 g diet (≡ 4·15 g protein) per rat per d, though generally somewhat less for the diets containing the heat-damaged proteins.

Table 1. *Composition of diets (g)*

Protein under test	46
Maize starch	80
Maize oil	30
Salts mixture*	10
Vitamin triturate†	2
Choline chloride	0·3
Rovimix E‡	0·2

* Laboratory Animal Handbook no. 2 (1968).

† Providing 200 μg biotin, 750 μg folic acid, 3 mg thiamin hydrochloride, 4 mg pyridoxine, 6 mg riboflavin, 15 mg calcium D-pantothenate, 40 mg nicotinic acid, 5 μg cyanocobalamin.

‡ Roche Products Ltd; containing 250 mg vitamin E/g.

Collection of rat urine

A group of four 6-month-old male hooded Norwegian rats, weighing about 350 g each, was kept together in a wire cage supported above a large stainless-steel funnel, so arranged as to receive all the urine and faeces and direct them on to a separator (Ackroyd & Hopkins, 1916). The urine, freed from faecal pellets, was filtered through a plug of glass wool in the neck of a filter funnel and collected into toluene in a 50 ml conical flask. The diets containing the different test proteins were given to the rats during successive 48 h periods. The rats were offered a total of 20 g diet/rat.d, in two meals, at 09.00 and 16.30 hours. For the morning meal, 10 g diet (per rat) was made to a crumbly consistency by addition of warm water and offered for 1 h. For the afternoon meal, a further 10 g diet/rat was offered, together with any uneaten food from the morning meal.

Collection of urine was begun after the morning meal on the 2nd day of each 48 h test period, and was interrupted for the hour during which the afternoon meal was eaten. Thus the '24 h collections' were in fact of 22 h, any urine voided during the feeding periods being lost. The urine was transferred from the collecting flask to a refrigerator at 16.30 hours, again at about 23.00 hours, and at 09.00 hours. The bulked collection was centrifuged in a calibrated centrifuge tube and the urine volume was recorded. The toluene layer was removed by pipette and the urine filtered through a Whatman no. 42 paper.

At least two '24 h collections' were made for each of the test proteins, and for the heated cod fillet four collections were made. Ideally, these repeated collections would have been analysed separately, but because of a large accumulation of samples awaiting amino acid analysis they were combined, and stored at −30° until needed for analysis.

Gel filtration of urine samples

Preparation of gel column. Sephadex gel filtration medium was used, type G-25 fine. Dry gel (250 g) was suspended in 2·5 l solution of pH 7·6, containing 0·02 M-sodium phosphate and 0·1 M-sodium chloride, and allowed to stand for 24 h, with occasional stirring. The column was prepared as described by Andrews (1964), in a glass tube of 2·7 cm internal diam. The settled height of the gel bed was 140 cm. Phosphate–saline solution of pH 7·6, as described above, was used for eluting the gel. It was supplied from a 5 l aspirator bottle fitted with a Marriotte constant-pressure tube, positioned to give an effective fluid level 50 cm above the top of the gel bed, at which the flow-rate was about 50 ml/h. The column was set up in a cold room at 4°.

Fractionation procedure. Portions (5 ml) of the various urine samples and marker solutions were applied in turn to the top of the column and allowed to enter the gel bed. Phosphate–saline solution (5 ml) was then applied and also allowed to enter the gel. A further 10 ml buffer were applied and the column was connected to the buffer reservoir. The buffer was now allowed to enter the gel and the effluent was collected in 150 fractions, each of 5 ml, with a fraction collector.

The urine fractions 81–92 were combined and evaporated to dryness under reduced pressure in a rotary evaporator. The residue was dissolved in 20 ml 6 M-HCl, heated for 18 h in steam at 121°, and again evaporated to dryness. This residue, designated the 'peptides' component (see Fig. 1), was redissolved in 10 ml water.

The urine fractions 93–115 were similarly combined and treated. The hydrolysate was designated the 'free amino acids' component.

Calibration. By calibrating the column with 'markers' of known molecular weight, it was possible to estimate the probable order of size of the ninhydrin-reacting components in the different fractions obtained from the urine samples (cf. Ford, 1965).

The following compounds were used: cytochrome C, from horse heart, mol. wt 12400 (Sigma Chemical Co.); polymyxin B sulphate, mol. wt 1447 (Burroughs Wellcome & Co.); a solution containing eighteen amino acids ('amino acid supplement', as used in the assay procedure described by Ford, 1962); urea (AR grade; British Drug Houses Ltd). Cytochrome C and polymyxin B sulphate in the effluent fractions were estimated photometrically by measurement of extinction at 408 and 215 nm respectively. Amino acids and urea were determined by addition of ninhydrin reagent.

Analytical procedures

Amino acid analysis. Total amino acids in the hydrolysed urinary peptides and also the free amino acids in urine were analysed by fully automated ion-exchange column procedures based on that of Spackman, Stein & Moore (1958). The preparations from urine of rats given cod fillet and casein isolated from heated skim-milk powder

were analysed with an early production model EEL (Evans Electroselenium Ltd, Halstead, Essex) automatic two column analyser, which gave poor resolution of the histidines, cystine, cysteic acid, methionine sulphone and methionine sulphoxide. Other preparations, from urine of rats given casein, were analysed with a JEOL amino acid analyser (Type JLC 5A+1; Japan Electron Optics Co. Ltd, Tokyo), which uses fine bead-form resin and gave better resolution of these amino acids.

Available methionine and leucine in the different test proteins were assessed by microbiological assay with *Streptococcus zymogenes* as described by Ford (1962) and Ford & Salter (1966). Available lysine was determined chemically by the method of Carpenter (1960).

Total-N determination. Test samples were digested with the sulphuric acid–potassium sulphate–mercuric oxide mixture recommended by Fleck & Munro (1965), and diluted to contain about 5 mg ammonia-N/100 ml. The ammonia was determined colorimetrically with a Technicon Autoanalyzer (Technicon Instruments Co. Ltd, Chertsey, Surrey), by the procedure given in the Technicon Methodology Sheet N-3b.

RESULTS

Composition of the test proteins

Miller *et al.* (1965) found that available lysine as measured chemically, and available methionine as measured microbiologically, gave a useful indication of the extent to which heating impaired the nutritive quality of cod-muscle protein for chicks, and

Table 2. *Available methionine, leucine and lysine (g/16 g N) in the different test protein preparations*

Test protein	Methionine*	Leucine*	Lysine†
Cod fillet: unheated	3·04	7·23	8·46
heated 20 h 135° in air	2·72 (90)	6·12 (84·7)	5·61 (66)
Casein L, Labco: unheated	3·07	9·70	7·84
heated 20 h 135° in air	2·72 (88)	9·33 (96·3)	6·39 (82)
heated 4 h 121° in steam	2·53 (82)	8·11 (83·6)	6·54 (83)
'Casein' from heated milk powder (see p. 312)	2·75 (90)	9·70 (100)	3·09 (40)

Figures in parentheses are 'apparent availabilities' calculated as a percentage of that of the relevant unheated protein.

* Measured microbiologically with *Streptococcus zymogenes* (Ford, 1962; Ford & Salter, 1966).

† Measured chemically by reaction with fluorodinitrobenzene (Carpenter, 1960).

Ford & Salter (1966) showed similarly that microbiological assay values for available methionine corresponded fairly closely to those obtained in growth tests with rats. In the present study the change on heating in the availability of methionine, leucine and lysine was taken as a measure of the nutritional damage suffered by the test proteins. The results of the assays are set out in Table 2.

With the cod fillet, heating in an air oven at 135° for 20 h caused a mean fall of 12·9% in the availability of leucine and methionine, and of 33·7% in that of lysine.

Similar heat treatment of the casein caused a mean fall of only 7·6% in leucine and methionine, and of 18·3% in lysine. The difference in susceptibility of casein and cod fillet to heat damage was probably more apparent than real, and attributable to poor temperature regulation in the hot air oven. Heating in steam at 121° for 4 h was more damaging to leucine and methionine, and slightly less damaging to lysine, than was the more severe heating in air. With the skim-milk powder, heating in air at 105° for 6 h caused no loss of available leucine, 10% loss of available methionine, and 60·5% loss of available lysine.

Composition of urine samples

Diets containing cod fillet. Samples (5 ml) of urine from rats given the different cod-fillet preparations were filtered in Sephadex gel G-25. A portion (0·2 ml of each fraction was hydrolysed with 6 M-HCl and made to pH 5·5 at 10 ml. A portion (1 ml) was taken and reacted with ninhydrin reagent. The relative colour yields for the different fractions are illustrated in Fig. 1, together with elution patterns obtained for the marker compounds.

The profiles for the two urine samples showed differences in content both of

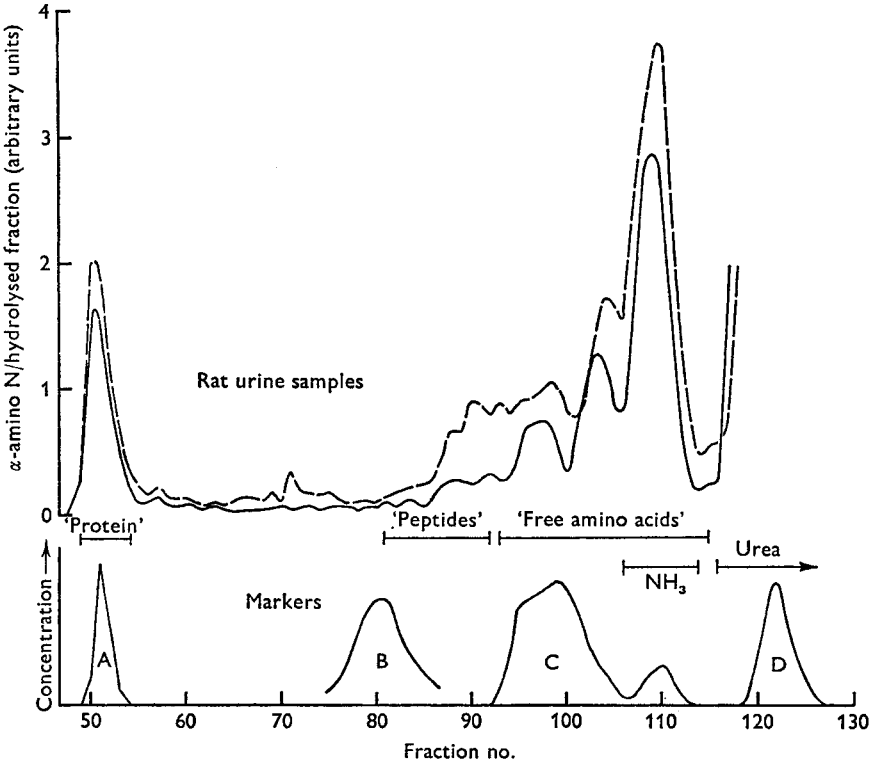

Fig. 1. Fractionation in Sephadex gel G-25 of urine of rats given unheated (———) and heated (– – –) cod fillet. α-Amino nitrogen was measured by reaction with ninhydrin, after hydrolysis of the fraction with 6 M-HCl. Markers: A, cytochrome C, mol. wt 12400, indicating void volume; B, polymyxin B sulphate, mol. wt 1447; C, mixture of eighteen amino acids; minor peak at fraction 110 is tyrosine; D, urea, mol. wt 60.

peptides and of free amino acids. For the unheated protein the urinary N excretion totalled 181 mg/rat per d, as compared with 148 mg for the heated protein. The corresponding values for peptide amino acids (fractions 81–92) were 2·6 and 7·3 mg, and for free amino acids (fractions 93–115) 11·8 and 19·8 mg. Thus, in proportion to the total urinary N, the content of N represented by the peptides + free amino acids was small – about 1·5% and 3·5% for the unheated and the heated protein respectively.

Table 3. *Amino acid content of the peptide and free amino acids in urine from rats given cod fillet: unheated and heated in an air oven for 20 h at 135°. Results are expressed as μmol/rat per d* and in parentheses as percentages of the totals*

	Unheated fillet		Heated fillet	
Total urinary N (mg/rat.d)...	181		148	
	Peptide	Free amino acids	Peptide	Free amino acids
Amino acids:				
Lysine	2·98 (16·0)	4·47 (8·3)	20·30 (41·6)	15·30 (13·4)
Aspartic acid	2·83 (15·2)	5·02 (9·3)	6·38 (13·1)	13·45 (11·8)
Glutamic acid	5·10 (27·5)	16·00 (29·8)	7·30 (15·0)	15·90 (13·9)
Glycine	3·11 (16·8)	12·60 (23·4)	3·65 (7·5)	35·30 (30·9)
Threonine	0·56 (3·0)	3·73 (6·9)	0·78 (1·6)	3·90 (3·4)
Serine	0·68 (3·7)	2·75 (5·1)	0·84 (1·7)	4·30 (3·8)
Proline	1·16 (6·2)	2·18 (4·1)	2·15 (4·4)	3·22 (2·8)
Alanine	0·50 (2·7)	1·94 (3·6)	1·25 (2·6)	5·23 (4·6)
Valine	0·44 (2·4)	1·01 (1·9)	1·69 (3·5)	4·76 (4·2)
Isoleucine	0·23 (1·2)	0·43 (0·8)	0·66 (1·4)	2·30 (2·0)
Leucine	0·30 (1·6)	0·62 (1·2)	0·93 (1·9)	3·38 (3·0)
Tyrosine	0·07 (0·4)	0·47 (0·9)	Trace —	1·09 (1·0)
Phenylalanine	0·10 (0·5)	0·22 (0·4)	Trace —	1·57 (1·4)
Arginine	0·31 (1·7)	1·36 (2·5)	1·62 (3·3)	1·18 (1·0)
Methionine	0·20 (1·1)	0·94 (1·8)	1·26 (2·6)	3·48 (3·0)
Total	18·57 (100)	53·74 (100)	48·81 (100)	114·36 (100)

Histidines, cystine and cysteic acid, methionine sulphone and methionine sulphoxide were not resolved (see p. 315).

* Urine was not collected during feeding periods (see p. 313).

The peptide component consisted in the main of small peptides, probably containing five or fewer amino acid residues. There was comparatively little peptide of mol. wt $> c.$ 700. In the free amino acids region, there was a peak at fractions 103–104, representing histidine and methyl histidine, and a further peak about fraction 109 due to ammonia.

Table 3 shows the amino acid composition of the urinary peptide and free amino acids, expressed as μmol/rat per d. It appeared that heat damage to the cod-fillet protein increased the total urinary excretion of the peptide-bound amino acids, from 18·6 to 48·8 μmol/rat per d. The composition of the peptide also changed, and in particular there was a marked increase in lysine, from 2·98 to 20·30 μmol/d in absolute terms, and from 16·0 to 41·6% when calculated as the molar percentage of the total amino acid content of the peptide. Three amino acids, lysine, aspartic acid and glutamic acid, together comprised nearly 70% of the total amino acid residues measured.

There was also a larger total content of free amino acids with the heat-damaged

protein, 114·4 mol/rat per d as against 53·7 with the unheated protein, which was contributed by increases in the urinary excretion of all the amino acids except glutamic acid, threonine and arginine. Lysine increased from approximately 4·5 to 15·3 mol/rat per d, aspartic acid from 5·0 to 13·4, and methionine from 0·9 to 3·5. The largest increase, from 12·6 to 35·3, was in glycine, which accounted for 30·9% of the total amino acids measured.

Table 4. *Amino acid content of the peptide and free amino acids in urine from rats given casein:* (1) *unheated,* (2) *heated in an air oven for* 20 *h at* 135°, (3) *heated in a steam autoclave for* 4 *h at* 121° *and* (4) *isolated from skim-milk powder that had been heated in an air oven for* 6 *h at* 105°. *Results are expressed as μmol/rat per d**

	1		2		3		4	
Total urinary N (mg/rat.d) ...	334		298		331		394	
Amino acids:	Peptide	Free amino acids	Peptide	Free amino acids	Peptide	Free amino acids	Peptide	Free amino acids
Lysine	4·06	3·71	5·37	6·46	5·17	7·70	30·40	11·20
Aspartic acid	3·69	7·10	6·07	10·53	7·95	30·80	3·75	6·12
Glutamic acid	5·67	20·20	4·32	18·40	5·15	27·80	6·47	28·40
Glycine	4·27	13·93	3·30	16·20	4·15	22·10	7·32	21·60
Threonine	1·48	3·96	1·03	3·81	1·25	5·05	3·94	7·00
Serine	1·90	3·84	1·32	4·75	1·87	10·50	2·69	3·78
Proline	3·70	5·20	2·95	7·85	4·33	50·80	2·85	2·48
Alanine	1·34	3·34	1·82	6·70	1·62	25·50	1·43	NR
Valine	1·69	1·75	1·37	3·80	1·52	9·70	1·51	1·35
Isoleucine	0·82	0·51	0·97	2·63	0·93	10·10	1·15	1·12
Leucine	1·42	1·03	0·68	1·54	0·86	2·75	0·81	0·95
Tyrosine	0·49	0·23	0·08	0·41	Trace	0·80	0·50	0·56
Phenylalanine	0·54	0·31	Trace	0·39	0·18	0·35	1·06	0·25
Histidine†	0·79	4·90	0·28	3·61	Trace	6·38	7·40	NR‡
Arginine	0·81	1·46	0·24	1·92	0·19	2·90	1·65	2·22
Methionine	0·25	0·51	0·07	0·51	0·30	7·20	6·90	3·15
Methionine sulphoxide + sulphone	0·43	1·03	0·71	2·12	1·05	5·20	*c.* 0·20‡	*c.* 0·54‡
Cystine	Trace	0·87	0·51	1·82	1·01	1·28	NR	NR‡
Cysteic acid	0·41	0·66	0·49	0·33	0·38	0·66	0·37	NR‡
Taurine	13·50	59·70	4·25	37·00	8·35	171·00	7·60	52·5
'Furosine'§	0	0	0	0	0	0	9·50	0·76

NR, not resolved.

* Urine was not collected during feeding periods.

† Total of histidine and methyl histidines.

‡ See p. 315.

§ ε-*N*-(2-furoylmethyl)-L-lysine, formed on acid hydrolysis of the lactose–lysine complex present in the heated milk powder.

Diets containing casein. Table 4 shows the amino acid composition of the peptides and free amino acids in samples of the urine from rats that had been given unheated and variously heat-treated casein with their diet. The total urinary excretion of peptide-bound amino acids with casein heated at 135° for 20 h was 24% less than with control casein, whereas with the similarly heated cod fillet there was a near threefold increase. The lysine content of the peptide increased, but only from 8·6 to 15·0 mol %, as compared with an increase from 16 to 42 with the cod fillet (the comparison is only

approximate, as fewer amino acids were measured for the tests with cod fillet). Aspartic acid increased from 7·8 to 16·9 mol %, and glutamic acid was unchanged at about 12·1. Similarly with the free amino acids, the heat treatment gave no increase in the total urinary excretion, and gave relatively small increases in the content of lysine (from 2·7 to 4·9 mol %), and aspartic acid (from 5·3 to 8·0), and a small decrease in glutamic acid (from 15·0 to 14·0).

Heat treatment in steam under pressure for only 4 h at 121° caused somewhat greater damage to the nutritional quality of the casein than did dry heating at 135° for 20 h, as judged from the evidence of the chemical and microbiological tests (Table 2). The total urinary excretion of peptide-bound amino acids was similar to that found with the unheated casein, but the molar proportions of lysine and aspartic acid were higher and broadly similar to those found with the oven-heated casein. The contents of methionine sulphoxide + methionine sulphone and of cystine were also increased.

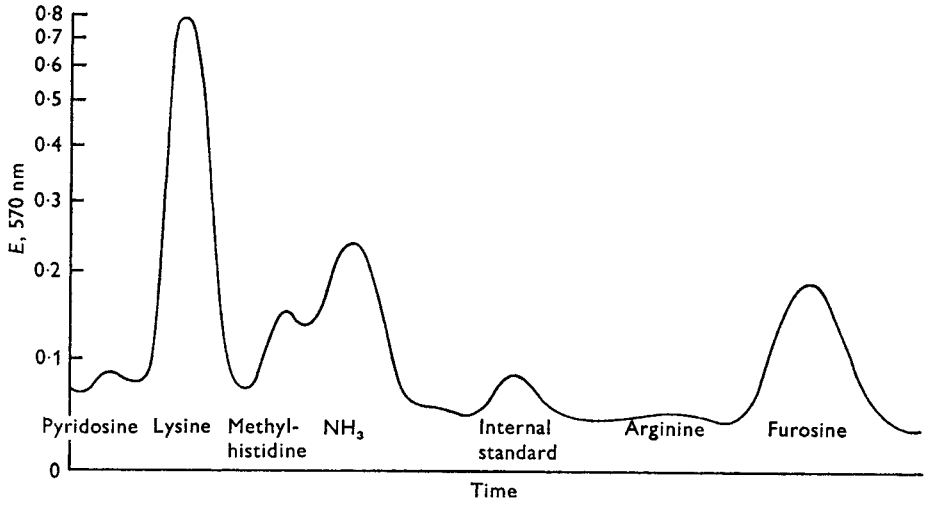

Fig. 2. Basic amino acid chromatogram of peptide fraction from urine of rats given casein from heated skim-milk powder. Internal standard, 2-amino-3-guanidino-propionic acid. Resin, Amberlite CG 120; column height, 14 cm; diam. 0·6 cm. Buffer, 0·38 M, pH 5·28. Rate, 30 ml/h. The peptide fraction was hydrolysed under reflux for 18 h with 6 M-HCl.

There was an increase in the free amino acids content, from 134 to 399 mol/rat per d. This was largely contributed by an increase in taurine excretion from 60 to 171 mol/rat per d, but there were marked increases also in the amounts of several other amino acids.

Diets containing the casein isolated from heated milk powder. Of the amino acids in the hydrolysed peptide component, 31 mol % were lysine. A further 10 mol % of the peptide amino acids was contributed by ε-*N*-(2-furoylmethyl)-L-lysine (furosine) which, together with lysine, is formed during acid hydrolysis of the lysine–lactose complex (Heyns, Heukeshoven & Brose, 1968; Finot, Bricout, Viani & Mauron, 1968) and was identified by its behaviour on ion-exchange chromatography (Fig. 2). A second derivative of the lysine–lactose complex, 1′ε-(1,4-dihydro-6-methyl-3-hydroxo-4-oxo-1-pyridyl)-L-lysine, was also present, though in comparatively small

amount. This compound was first detected and characterized by Finot, Viani, Bricout & Mauron (1969), and given the trivial name 'pyridosine'.

Besides the large content of lysine, the urinary peptide was comparatively rich in methionine, containing 7 mol % as compared with only 0·25 in urine from rats given unheated casein.

Again in the urinary free amino acids, the content of lysine and methionine (7·8 and 3·2 mol %) was higher than in the urine of rats given unheated casein (2·7 and 0·38 mol %).

DISCUSSION

The cod fillet sustained greater damage to available lysine than did the casein during heat treatment for 20 h at 135° (Table 2), and this difference may provide some explanation for the greater excretion of peptide and amino acids in the urine. With both protein sources, there was evidence for comparatively greater damage to lysine than to methionine, and this finding is supported by results obtained in growth assays with rats (Ford & Salter, 1966).

The finding with casein that the milder heating in steam is comparatively more damaging to leucine and methionine (Table 2) is called into question by the finding by E. L. Miller *et al.* (1965) that microbiological assays for available methionine gave considerably lower values for cod heated at a moisture level of 50% than at 14%, whereas chick assays failed to show any such difference. However, the same authors pointed out that heating at the higher moisture level caused greater darkening of the product, and D. S. Miller (1956) reported that heating acetone-extracted cod muscle at increasing moisture levels caused progressive lowering of the NPU of the products. The present results (Table 4) show that there were distinct differences in the composition of the urinary free amino acids as between the oven-dried and the steam-autoclaved caseins, and it seems likely that these reflect differences in the chemistry of the changes brought about by the different modes of heating, resulting in differences in the course of digestion and metabolism of the digestion products.

Results for the heated milk powder (Table 2) show that the nutritional value of the protein was considerably depressed by the 60% fall in available lysine resulting from the formation of lysine–lactose complex. The high availability of the leucine and methionine indicates that there was no corresponding fall in digestibility.

Bjarnason & Carpenter (1970) studied the chemical changes that occurred in several purified proteins during severe heating, and concluded that reaction between the ϵ-amino group of lysine and the amide groups of asparagine and glutamine constituted a major cause of lysine binding. Since these three amino acids together comprised nearly 70% of the peptide component in the urine of rats given the heated cod fillet (Table 3), it may be that biologically unavailable small peptides, based on such amide-linked asparagine or glutamine and lysine, are absorbed from the gut and excreted intact with the urine.

The corresponding increase in the free amino acids component, and its amino acid composition, suggest the further possibility that the amount and the composition of the free amino acids were determined by the influence of the peptide. This could come

about in two ways. The free amino acids might derive directly from hydrolysis of the unavailable 'peptide' within the kidney. Barman (1969) summarizes the activities of the hydrolases of *EC* group 3.5, which attack C—N bonds other than peptide bonds, and which might well attack conjugates of the constitution proposed by Bjarnason & Carpenter (1970). Thus, acyl-lysine deacylase (*EC* 3.5.1.17) occurs in rat kidney and hydrolyses a variety of ε-acyl-lysine derivatives. Other possibilities are ureidosuccinase (*EC* 3.5.1.7), β-ureidopropionase (*EC* 3.5.1.6) and aminoacylase (*EC* 3.5.1.14).

Alternatively, the presence of the peptides might lower the renal threshold for free amino acids. Buraczewski, Buraczewska & Ford (1967) studied the influence of heating of cod fillet on the course of its digestion in the rat. They found very much higher concentrations of free amino acids and small peptides in the intestinal contents of the animals that had been given severely heated protein than in controls given undamaged protein and yet, conversely, the concentrations of free amino acids in the portal blood were much lower. To explain this anomaly they postulated that the accumulation of 'unavailable' peptide material in the intestine, characteristic of heat-damaged proteins, might hinder the absorption of amino acids by saturating the sites involved in their transport across the mucosal barrier. The present findings suggest an analogous action of these peptides in hindering the reabsorption of amino acids by the renal tubules. If there is indeed such a close identity between the processes of absorption from the gut and of reabsorption from the renal tubules, then the analytical results presented in Table 3 carry an implication that is perhaps of some practical nutritional importance, namely that 'unavailable' peptide material in the intestine exerts a selective effect and may influence not only the gross amount but also the pattern of amino acids that is absorbed.

The effects of heat treatment of the cod fillet on the composition of the urinary peptides and free amino acids were clearly distinct, but those for the similarly heated casein were less convincing. There was no increase in total urinary peptide excretion such as was found with the heated cod fillet. There was, however, a small increase in lysine and aspartic acid, both in the peptide and in the free amino acid component, and to this extent the effects of heating with these different classes of protein were qualitatively similar.

In urine from rats given 'casein' isolated from heated skim-milk powder the hydrolysed peptide component was rich in lysine and in furosine, which together contributed 41 mol % of the total amino acid composition. It seems highly probable that none of this lysine was peptide-bound, and that both the lysine and furosine were formed during our acid hydrolysis of lysine–carbohydrate complex in the urine samples. Dr H. Erbersdobler (personal communication) has demonstrated that, as compared with free lysine, the lysine moiety in 1-deoxy-D-fructosyl-lysine is absorbed relatively inefficiently from the rat intestine and that the complex is for the most part broken down by the gut micro-organisms and is not recovered in the faeces. Mauron (1970) reached an opposite conclusion, namely that the complex is wholly absorbed and excreted in the urine, and seems to have discounted this possibility of destruction through the activities of the gut microflora. In the present experiments, about 225 mg unavailable

lysine per d were eaten with the test diet, and the total of lysine residues in the urinary peptide was only about 6 mg. If we assume that all of these lysine residues derived from lysine–carbohydrate complex, the urinary excretion amounted to only 2·7% of the unavailable lysine eaten with the diet. Whether the lysine–carbohydrate complex is absorbed from the gut as intact 1′ε-*N*-deoxy-lactulosyl-lysine remains to be investigated, as does the question whether all of the bound lysine that is absorbed is nutritionally unavailable and excreted with the urine.

The findings reported here show clearly that no large proportion of the N that was absorbed during the digestion of the heat-damaged proteins was excreted in the urine as unavailable peptide. If we take 90% as being the digestibility of the heated cod fillet, then of the *c.* 0·6 g N absorbed per rat per d only about 0·2% was lost in the urine as peptide, together with a further 0·6% as free amino acids. Of the total lysine ingested, the combined urinary losses in the peptide and free amino acids amounted to about 1·5% as against about 0·3% with the unheated cod fillet. At these levels, urinary losses of undigested peptide residues would seem to be of only marginal nutritional importance. It is of course possible that considerably more unavailable peptide was absorbed from the gut than was recovered in the urine, since the peptide is unavailable only in the sense that it is comparatively slowly digested. But evidence on this point is lacking, as indeed is any direct evidence that the increase in the urinary peptides was comprised of such unavailable peptide. Further work on the metabolism of unavailable peptides and their influence on amino acid absorption and excretion is in progress.

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