Reactions of proteins with oxidizing lipids

2. Influence on protein quality and on the bioavailability of lysine, methionine, cyst(e)ine and tryptophan as measured in rat assays

BY HENRIK K. NIELSEN*, P. A. FINOT AND R. F. HURRELL

Research Department, Nestlé Products Technical Assistance Co. Ltd, CH-1814 La Tour-de-Peilz, Switzerland

(*Received 8 May 1984 – Accepted 18 July 1984*)

1. The consequences of reactions between protein and oxidizing lipids on the nutritional quality of food proteins have been investigated using a whey protein–methyl linolenate–water model system.

2. In rat assays, significant reductions were observed in protein efficiency ratio, net protein ratio, net protein utilization, biological value and true nitrogen digestibility, especially when the reaction had taken place at high moisture content, high temperature and in the presence of excess oxygen.

3. The losses of bioavailable lysine and tryptophan as measured by rat assays followed a similar pattern. The chemical value of each amino acid multiplied by the true N digestibility closely resembled the rat assay value. In general, the reaction products of lysine and tryptophan formed during lipid oxidation were biologically unavailable.

4. The bioavailabilities of methionine and of 'methionine plus cyst(e)ine' were determined in separate assays. Cyst(e)ine was calculated as 'methionine plus cyst(e)ine' minus methionine. In whey protein which had reacted with oxidizing methyl linolenate, the bioavailable methionine content was not significantly reduced even though 82% of the methionine residues were present as methionine sulphoxide. In hydrogen peroxide-treated casein in which all methionine residues were oxidized to the sulphoxide, methionine sulphoxide was found to be 96% as utilizable as a methionine source to the rat. Free methionine sulphoxide was 87% utilizable.

5. Cyst(e)ine appeared to be as sensitive as lysine to reactions with lipid oxidation products. In whey protein which had reacted with oxidizing methyl linolenate, the bioavailabilities of cyst(e)ine, lysine, tryptophan and methionine were reduced by 28, 24, 11 and 8% respectively and true N digestibility by 9%. These results are discussed in relation to food products.

When foods are cooked at home, industrially processed or stored, many reactions can occur between the different food components (Hurrell, 1980, 1984). These reactions lead to browning, odour and flavour formation, loss of nutritional quality and occasionally to compounds with potential antiphysiological effects. In the previous paper (Nielsen *et al.* 1985*b*), we reported on the reactions of proteins with oxidizing lipids. Using chemical methods, we showed that the lysine, tryptophan and methionine residues in whey protein reacted with oxidizing methyl linolenate. Other works have reported similar findings (O'Brien, 1966; Gamage & Matsushita, 1973; Kanazawa *et al.* 1975). Lysine and the sulphur amino acids are most often the first limiting amino acids in food proteins (Food and Agriculture Organization, 1970) and any reduction in their content would thus reduce the nutritional quality of the protein.

In the present paper, we report the results of rat assays on the protein quality and the bioavailability of lysine, tryptophan, methionine and cyst(e)ine in the same whey protein–methyl linolenate model systems as analysed chemically in the previous study (Nielsen *et al.* 1985*b*). This necessitated the development of a rat assay to measure cyst(e)ine bioavailability and a closer study of the bioavailability of methionine sulphoxide which was extensively formed by oxidation of methionine during storage of our model system.

* Present address: Building 1K40, Novo Industri A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark.

Table 1. *Formulation and storage conditions for whey protein–methyl linolenate (1:0·5, w/w) model samples*

Treatment*	Approximate moisture content (g/kg)	Water activity (a_w)	Oxygen (mol/mol lipid)	Temperature (°)
Basal conditions	170	0·84	4	37
Low a_w	20	0·33	4	37
O_2 limitation	170	0·84	1	37
High temperature	170	0·84	4	55

* For details, see below.

EXPERIMENTAL

Preparation of test materials

Whey protein–methyl linolenate mixtures were prepared and stored in sealed tins as shown in Table 1. After 4 weeks' storage, the samples were extracted once with methanol and twice with methanol–hexane (2:1, v/v) as described by Nielsen *et al.* (1985*b*). In addition, specifically to investigate the bioavailability of protein-bound methionine sulphoxide, casein was oxidized with hydrogen peroxide as described by Nielsen *et al.* (1985*a*).

Analytical methods

The methods used for tryptophan and lysine analysis were as described by Nielsen *et al.* (1985*b*).

Animal experiments

In all experiments, 3-week-old male Sprague-Dawley rats were used, they weighed 45–55 g and were purchased from Voss, Tuttlingen, W. Germany. They were fed for 2 d on a pre-experimental diet. This was the stock laboratory diet (NAFAG No 850; Gossau, Switzerland) for the growth assays and the nitrogen-balance study and, for each amino acid availability assay, it was the basal diet low in the particular amino acid under test. After 2 d, the rats were weighed and allocated individually into cages in a one-way blocked, randomized design with six rats per treatment (diet). Each of the six horizontal rows of cages formed a single block containing one animal from each treatment. The upper block contained the heaviest rats and, going down through the blocks, the rats were progressively lighter. The blocks thus differed both in the weight of the rats at day 0 as well as the position of cages in the rack. Within each block, the treatments were allocated at random.

All diets were powder diets and were fed *ad lib.* The vitamin mixture was that of Peret *et al.* (1973) and the minerals (USP-XVII, as recommended by the Association of Official Analytical Chemists (1980)) were purchased from ICN Nutritional Biochemicals, Cleveland, Ohio, USA.

Growth assay and N-balance study. The N-free diet consisted of (g/kg): sucrose 250, arachis oil 100, cellulose 50, minerals 50, vitamins 12·5 and maize starch to 1000. The test materials were added to the N-free diet at a level of 100 g crude protein (CP; N × 6·25)/kg at the expense of maize starch. Weight gain and food intake were measured for 10 d and protein efficiency ratio (PER) and net protein ratio (NPR) (Bender & Doell, 1957) were calculated.

On day 10, the rats were put into metabolism cages and from day 11 to day 16 an N-balance study was made; faeces and urine were collected and food intake and body-weight recorded.

A group of rats was fed on a diet containing 100 g whey protein/kg from day 0 to day 6, and 25 g whey protein/kg from day 6 to day 16. When given 25 g whey protein/kg the rats just maintained their body-weight and the whey protein was assumed to be completely digestible and utilized. The faecal and urinary N of this group from day 11 to day 16 were used to estimate excreted faecal and urinary endogenous N of the test groups after correcting for food intake and mean body-weight respectively (Mitchell, 1923–24). True faecal N digestibility (TD), biological value (BV) and net protein utilization (NPU) were then calculated. The significances of differences were estimated with Students' unpaired *t* test (two-tailed).

Lysine-availability assay. This assay was a modified version of the method of Mottu & Mauron (1967). The basal diet contained (g/kg): wheat gluten 100, zein 150, L-tryptophan 1·4, L-methionine 3·3, L-threonine 1·5, L-valine 1·8, L-histidine hydrochloride monohydrate 2·0, L-arginine 5·0, sucrose 250, arachis oil 50, cellulose 20, minerals 50, vitamins 12·5 and maize starch to 1000. The basal diet was supplemented with L-lysine hydrochloride at 0, 0·8, 1·6, 2, 4 and 3·2 g/kg and the test materials were added at 9·3 and 18·6 g CP/kg at the expense of zein and maize starch to keep the diets isonitrogenous.

The animals were fed *ad lib.* for 13 d, weight gain and food consumption were recorded and food conversion efficiency (FCE; g weight gain per g food eaten) was calculated. The potencies of the test materials were calculated using the slope ratio method of Finney (1964).

Tryptophan-availability assay. The method is described by Nielsen *et al.* (1985*a*).

Bioavailability of S amino acids. Methionine can be transformed into cysteine by the rat and consequently the bioavailability of the two S amino acids cannot be determined separately. In order to measure available cyst(e)ine we have used two separate assays: (*a*) a methionine assay in which rats were fed on diets limiting in methionine but containing an excess of cystine (4 g/kg) so that their growth response depended on methionine only; and (*b*) a total S amino acid assay in which the basal diet was limiting in methionine but contained no cyst(e)ine so that the rats responded to both methionine and cyst(e)ine. Available cyst(e)ine was then calculated as the difference between available S amino acids and available methionine.

Methionine-availability assay. In this assay, the rats were fed on a diet containing an amino acid mixture as its only source of N. The composition of this mixture was based on the suggestions of Therriault as quoted by Rogers & Harper (1965). It consisted of (g/kg): L-arginine 68, L-histidine hydrochloride monohydrate 27, L-isoleucine 50, L-leucine 68, L-lysine hydrochloride 109, L-phenylalanine 71, L-tyrosine 21, L-threonine 50, L-tryptophan 10, L-valine 50, L-alanine 21, L-aspartic acid 21, L-asparagine 37, L-glutamic acid 213, glycine 142, L-proline 21 and L-serine 21. All amino acids were from Fluka AG, Buchs, Switzerland. The basal diet contained (g/kg): the amino acid mixture 224, L-cystine 4, L-methionine 0·6, calcium carbonate 8, sucrose 250, arachis oil 100, cellulose 50, minerals 50, vitamins 12·5 and maize starch to 1000. The basal diet was supplemented with L-methionine at 0·25, 0·50, 0·75 and 1·00 g/kg. Methionine sulphoxide was added at 0·41 and 0·82 g/kg. Test materials were added at the expense of the amino acid mixture, calcium carbonate and maize starch, which kept the acidity and N constant. The levels of the whey protein–methyl linolenate mixtures (untreated and O_2 limitation) were 14·8 and 29·6 g CP/kg and the levels of casein samples 11·6 and 23·2 g CP/kg. The animals were fed *ad lib.* for 13 d, weight gain and food consumption were measured and FCE values calculated. The potencies of the test materials were calculated using the slope ratio procedure of Finney (1964).

Total-S-amino-acid-availability assay. This assay was similar to the methionine assay except that cystine was not added to the diets and that methionine in the basal diet was increased to 1·6 g/kg. The basal diet was supplemented with methionine at 0·35, 0·70, 1·05, 1·40 g/kg, with methionine sulphoxide at 0·58 and 1·16 g/kg and the test materials were

Table 2. *Protein quality of whey protein stored in the presence of oxidizing methyl linolenate as measured with rats in growth and nitrogen-balance assays*

	Growth assay (days 0–10)				N-balance study (days 11–16)		
Treatment*	Wt gain (g)	Food intake (g)	PER	NPR	NPU	TD	BV
Control protein	53·8[a]	114·4[a]	4·70[a]	5·82[a]	0·944[a]	0·991[a]	0·953[a]
Basal conditions	−4·3[b]	45·2[b]	−0·97[b]	1·92[b]	0·449[b]	0·701[b]	0·640[b]
Low water activity	54·2[a]	116·9[a]	4·62[a]	5·72[a]	0·860[c]	0·969[c]	0·888[c]
Oxygen limitation	10·5[c]	58·2[c]	1·77[c]	4·00[c]	0·722[d]	0·905[d]	0·799[d]
High temperature	−4·0[b]	51·6[c]	−0·78[b]	1·70[b]	0·321[e]	0·471[e]	0·681[b]
Casein	36·7	94·9	3·86	5·21			
N-free	−12·8	49·7					
Pooled SEM	1·7	2·7	0·11	0·09	0·015	0·009	0·020

[a, b, c, d, e] Values with different superscript letters were significantly different ($P < 0·05$).

PER, protein efficiency ratio; NPR, net protein ratio; NPU, net protein utilization; TD, true faecal N digestibility; BV, biological value.

* For details, see p. 76.

added at levels which contributed 9·4 and 18·8 g crude protein/kg at the expense of the amino acid mixture, calcium carbonate and starch so as to keep the N and acidity constant. The animals were fed *ad lib.* for 13 d, weight gain and food consumption were recorded and FCE was calculated. The potencies of the test materials were calculated by the slope-ratio method of Finney (1964).

Cyst(e)ine bioavailability. Available cyst(e)ine was calculated in the stored whey protein–methyl linolenate model system ('O_2 limitation') and in the whey protein control by subtracting the available methionine from the total available S amino acids.

RESULTS

Growth assay

The results of the growth assay are shown in Table 2. Those samples incubated at high water activity (a_w), elevated temperature and in the presence of excess O_2 (the 'basal conditions' sample and the 'high temperature' sample) did not support rat growth, and slight weight losses occurred. This could partly be explained by the lower food intakes, which were less than half those recorded for the control group. For the 'low a_w' sample, weight gain, food intake, PER and NPR did not differ from those of the control group. The group fed on the 'O_2 limitation' sample had a smaller average weight gain and its food intake was half that of the control group. This resulted in a low PER (38% that of the control group). But when maintenance was taken into consideration, by including the weight loss of the group fed on a N-free diet (12·8 g) in the calculations, a NPR-value of 69% that of the control group was obtained.

N-balance study

In the N-balance study (Table 2), large reductions in NPU were observed. A significant decrease occurred even at 'low a_w'. For this sample and for the 'O_2 limitation' sample, increased urinary excretion of N, and thus reduced BV, was the major reason for the fall in NPU. Under the 'basal conditions', a reduced N digestibility and a reduced BV contributed equally to the large decrease in NPU. For the 'high temperature' sample, the N digestibility was much more extensively reduced.

Table 3. *Lysine availability of whey protein stored in the presence of oxidizing methyl linolenate**

(Pooled standard errors of the mean for FDNB-reactive lysine and for FDNB-reactive lysine multiplied by true digestibility were 1 mg/g CP. Values in parentheses represent the test protein as a percentage of the control protein)

Test supplement	Level of supplementation‡ (g/kg diet)	Wt gain‡ (g/13 d)	Food conversion efficiency‡	Calculated potency†				FDNB-reactive lysine (mg/g CP)	FDNB-reactive lysine × protein digestibility (mg/g CP)
				Absolute value (mg/g CP)		Relative to control protein (%)			
				Mean	95% confidence limits	Mean	95% confidence limits		
Control protein	10·4; 20·7	12·3; 23·7	0·146; 0·247	103	93–113	100		105 (100)	104 (100)
Basal conditions	13·4; 26·7	4·3; 6·3	0·061; 0·085	24	13–34	23	13–33	42 (40)	29 (28)
Low water activity	11·4; 22·8	12·9; 24·8	0·156; 0·244	104	94–114	101	90–113	89 (85)	86 (83)
Oxygen limitation	12·2; 24·4	9·5; 16·9	0·123; 0·195	78	69–88	76	66–87	78 (74)	71 (68)
High temperature	13·5; 27·1	3·9; 4·0	0·054; 0·060	13	2–23	12	2–22	31 (30)	15 (14)
L-Lysine hydrochloride	0; 0·8	1·4; 7·7	0·019; 0·103						
	1·6; 2·4	18·1; 30·3	0·193; 0·262						
	3·2	39·8	0·320						

FDNB, fluorodinitrobenzene; CP, crude protein (nitrogen × 6·25).

* For details, see p. 76.

† Calculations based on food conversion efficiencies.

‡ Multiple values given for each test supplement represent the different groups of rats receiving that test supplement.

Lysine-availability assay

Table 3 shows the available lysine content of the test materials. Storage under the 'basal conditions' (37°, high a_w (0·87), excess O_2) decreased the available lysine content to 23% of its original value. Increasing the temperature to 55° further increased lysine losses, but restricting the oxygen to 1 mol/mol lipid reduced losses and 76% of the original lysine remained. At low a_w, no losses were observed.

For the 'basal conditions' and the 'high temperature' samples, the bioavailable lysine was significantly lower than the fluorodinitrobenzene (FDNB)-reactive lysine. This appeared to result from reductions in protein digestibility, since FDNB-reactive lysine multiplied by TD came to within the 95% confidence limits of bioavailable lysine. For the 'O_2 limitation' sample, neither FDNB-reactive lysine nor FDNB-reactive lysine multiplied by TD was significantly different from the rat assay value. Regarding the 'low a_w' sample, both FDNB-reactive lysine and this value multiplied by TD were significantly (about 15%) below the bioavailable lysine value.

Tryptophan-availability assay

The losses of bioavailable tryptophan (Table 4) in whey protein, stored in the presence of methyl linolenate, were smaller than the losses observed for lysine, but they followed a similar pattern. High a_w and excess O_2 during storage resulted in extensive losses which were higher at 55° than at 37°; limitation of the O_2 significantly reduced the losses and, at low a_w, no changes in available tryptophan occurred. Again, the analytical method indicated smaller losses than those found by the rat assay. However, as before, this could be explained by decreased protein digestibility. Tryptophan values determined by high pressure liquid chromatography after alkaline-hydrolysis multiplied by TD were in close agreement with the bioavailable tryptophan values.

S-amino-acid-availability assay

Fig. 1 shows the standard curves for the methionine assay and for the total-S-amino-acid assays. The ratio between the slopes of the two lines is 0·57, which means that, in the presence of cystine, the amount of methionine needed to obtain a given growth response was 57% of the amount needed in the absence of cystine. In order to increase the FCE from 0·2 to 0·3, for instance, 0·63 g methionine/kg would have to be added in the absence of cystine whereas, in the presence of cystine, 0·36 g methionine/kg would be enough; i.e. on a molar basis cystine replaced 43% of the methionine.

Available methionine. The available methionine content of the whey-protein control and 'O_2 limitation' whey protein–methyl linolenate model system are shown in Table 5. In the 'O_2 limitation' sample, the methionine availability was reduced 8% compared with the control protein. This was not statistically significant. Since 82% of methionine residues in this sample were oxidized to methionine sulphoxide (Nielsen *et al.* 1985*b*), and since TD was 9% lower than in the control sample, it would appear that protein-bound methionine sulphoxide is highly bioavailable as a methionine source to the rat.

Bioavailability of methionine sulphoxide. We investigated more closely the utilization of protein-bound methionine sulphoxide since the oxidation of methionine to its sulphoxide was extensive in all the stored whey protein–methyl linolenate model systems. The bioavailability of methionine in casein oxidized with H_2O_2 was 96% that of methionine in the control protein (Table 5). In this protein, methionine was completely oxidized to its sulphoxide (Nielsen *et al.* 1985*a*). Our results show that protein-bound methionine sulphoxide is as available, or nearly as available, as protein-bound methionine. The availability of free methionine sulphoxide as a source of methionine was also assessed in

Table 4. *Tryptophan availability of whey protein stored in the presence of oxidizing methyl linolenate**

(Pooled standard errors of the mean for chemically determined tryptophan and for the chemical value multiplied by true digestibility were 0·3 mg/g CP. Values in parentheses represent the test protein as a percentage of the control protein)

Test supplement	Level of supplementation‡ (g/kg diet)	Wt gain‡ (g/14 d)	Food conversion efficiency‡	Calculated potency†				Tryptophan determined by HPLC after alkaline hydrolysis (A) (mg/g CP)	(A) multiplied by protein digestibility (mg/g CP)
				Absolute value (mg/g CP)		Relative to control protein (%)			
				Mean	95% confidence limits	Mean	95% confidence limits		
Control protein	11·9; 23·9	18·2; 39·4	0·248; 0·387	27·7	24·4–31·3	100		22·8 (100)	22·5 (100)
Basal conditions	15·4; 30·8	11·5; 19·3	0·178; 0·255	15·6	12·7–18·7	56	45–68	19·5 (86)	13·7 (60)
Low water activity	13·1; 26·2	18·0; 35·6	0·253; 0·387	27·9	24·7–31·5	101	88–115	22·9 (100)	22·1 (98)
Oxygen limitation	14·0; 28·1	12·6; 33·0	0·194; 0·373	24·5	21·4–27·9	89	77–102	21·9 (96)	19·8 (88)
High temperature	15·6; 31·2	7·2; 8·4	0·123; 0·178	8·1	5·0–11·0	29	18–40	16·9 (74)	8·0 (35)
L-Tryptophan	0; 0·16	6·3; 10·4	0·107; 0·152						
	0·32	26·1	0·319						
	0·48; 0·64	29·9; 49·5	0·330; 0·431						

CP, crude protein (nitrogen × 6·25); HPLC, high pressure liquid chromatography.

* For details, see p. 76.

† Calculations based on food conversion efficiencies.

‡ Multiple values given for each test supplement represent the different groups of rats receiving that test supplement.

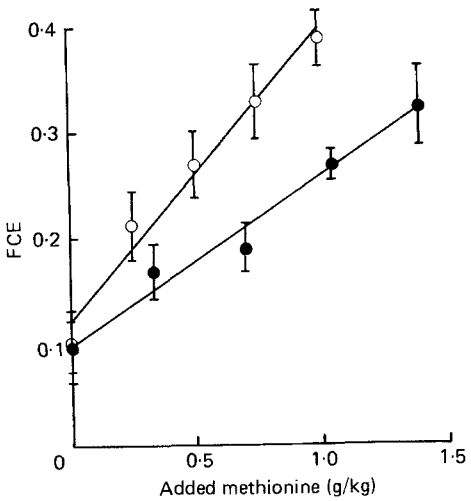

Fig. 1. Food conversion efficiency (FCE) *v.* added methionine (g/kg) for rats fed on diets containing 4 g cystine/kg (○, available-methionine assay; *r* 0·99, slope 2·80) or no cystine (●, total-sulphur–amino-acid assay; *r* 0·99, slope 1·59). For details, see p. 77. Points are mean values with their standard errors represented by vertical bars.

Table 5. *Methionine availability in whey protein, untreated or stored with oxidizing methyl linolenate* and in casein, untreated or hydrogen peroxide oxidized,* and the bioavailability of free methionine sulphoxide as a source of methionine*

Test supplement	Level of supplementation‡ (g/kg diet)	Wt gain‡ (g/13 d)	Food conversion efficiency‡	Calculated potency† (methionine equivalents)			
				Absolute value (mg/g CP)		Relative to control protein (%)	
				Mean	95% confidence limits	Mean	95% confidence limits
Methionine	0; 0·25	5·3; 13·2	0·098; 0·207		—	100	—
	0·5; 0·75	19·1; 28·6	0·268; 0·328		—	—	—
	1·0	37·5	0·388		—	—	—
Methionine sulphoxide	0·41; 0·82	15·0; 23·4	0·220; 0·283		—	83	70–96
Whey protein, control	16·8; 33·6	15·5; 24·4	0·232; 0·305	23·5	20·3–26·8	100	—
Oxygen limitation*	19·8; 39·6	13·6; 21·2	0·212; 0·295	21·5	18.3–24·8	92	77–109
Casein, control	12·9; 25·8	12·1; 27·2	0·203; 0·338	32·3	28·2–36·7	100	—
Casein, H_2O_2*	13·7; 27·6	13·2; 29·9	0·212; 0·323	31·0	26·4–32·3	96	81–113

CP, crude protein (nitrogen × 6·25).
* For details of treatments, see p. 76.
† Calculations based on food conversion efficiencies.
‡ Multiple values given for each test supplement represent the different groups of rats receiving that test supplement.

both the methionine and the total-S-amino-acid assays. In the presence of 4 g cystine/kg and 0·6 g methionine/kg, it was 83% available (Table 5) and, in the presence of 1·6 g methionine/kg, it was 90% available (Table 6). The former value was just significantly different from 100% ($P < 0·05$) whereas the latter was not.

Table 6. *Sulphur amino acid bioavailability in whey protein, stored with methyl linolenate under different conditions* and availability of free methionine sulphoxide in a diet containing no cystine*

Test supplement	Level of supplementation‡ (g/kg diet)	Wt gain‡ (g/13 d)	Food conversion efficiency‡	Calculated potency† (methionine equivalents)			
				Absolute value (mg/g CP)		Relative to control protein (%)	
				Mean	95% confidence limits	Mean	95% confidence limits
Methionine	0; 0·35	6·0; 11·3	0·025; 0·167		—	100	—
	0·7; 1·05	13·0; 21·5	0·189; 0·268		—	—	—
	1·4	29·7 —	0·322 —		—	—	—
Methionine sulphoxide	0·58; 1·76	10·2; 20·9	0·154; 0·254		—	90	77–103
Whey protein, control	10·7; 21·4	13·7; 36·0	0·190; 0·331	75·0	67·2–83·6	100	—
Basal conditions	13·8; 27·6	13·6; 23·6	0·156; 0·207	38·1	31·0–45·2	51	41–61
Low water activity	11·7; 23·5	13·6; 22·7	0·197; 0·287	64·3	56·9–72·3	86	75–97
Oxygen limitation	12·6; 25·1	11·0; 15·6	0·191; 0·268	58·6	51·3–66·3	78	68–89

CP, crude protein (nitrogen × 6·25).

* For details, see p. 76.

† Calculations based on food conversion efficiencies.

‡ Multiple values given for each test supplement represent the different groups of rats receiving that test supplement.

Total S amino acids. Significant losses were found in all stored whey–protein–methyl linolenate model systems (Table 6). Only 51% of the original total S amino acids remained in the sample stored under the 'basal conditions' compared with 86 and 78% respectively in the 'low a_w' and 'O_2 limitation' samples. Part of these losses could be explained by a fall in protein digestibility; the true N digestibilities were 0·70, 0·97 and 0·91 respectively for the 'basal-conditions', 'low a_w' and 'O_2 limitation' samples (Table 2). Since protein-bound methionine sulphoxide appeared to be bioavailable, this indicated a reduction in available cyst(e)ine in these samples of about 10–20%.

Cyst(e)ine. It was possible to quantify the reduction in cyst(e)ine availability further for the 'O_2 limitation' sample. Subtraction of the values for available methionine from the values for total-S-amino-acid availability gave 41·5 and 29·9 mg available cystine/g CP respectively for the control whey protein and the 'O_2 limitation' sample. The remaining available cyst(e)ine was thus 72% of the original value. However, because of the subtraction and division, the 95% confidence intervals were 52–92%.

DISCUSSION

The growth assay and the N-balance study (Table 2) demonstrate that protein quality can be extensively reduced when a protein reacts with oxidizing lipids, especially at high a_w and in the presence of excess O_2. A reduction in protein quality under such conditions would appear to be due to losses in availability of the essential amino acids, lysine, tryptophan and total S amino acids, together with a general reduction in N digestibility. Other workers have also reported significantly lower weight gains when proteins which had reacted with oxidizing lipids were fed to rats as the only protein source (Horigome & Miura, 1974; Horigome *et al.* 1974; Yanagita & Sugano, 1975, 1978; Horigome & Uchida, 1979). TD

and BV have also been shown to be significantly reduced (Horigome & Miura, 1974; Yanagita & Sugano, 1973; Harmuth-Hoene & Delincée, 1978). The decreases both in TD and BV observed in the present study are much greater than those previously reported. This is presumably due to the use of ethyl linoleate ($C_{18:2}$) in the earlier studies as opposed to the methyl linolenate ($C_{18:3}$) used in the present investigation.

One criticism of the present N-balance study is that the mean body-weight of the rats in the different groups at the beginning of the study and their food intake during the study were very different. It is possible that an assay with a more homogenous group of rats might have given different results. However, the N-balance assay was run immediately after the growth assay in order not to use more of the test materials for a new equilibration period.

The problems of interpreting PER values, because of their lack of proportionality with 'protein quality' (Bender & Doell, 1957; Pellet & Young, 1980), were illustrated by the 'O_2 limitation' sample. Its PER was 38% of that of the control protein whereas the NPR and NPU values of this material were 64 and 76% of their respective controls.

The losses of available lysine and tryptophan were as would be predicted from the chemical analyses and the N-digestibility values (Tables 3 and 4). The only exception was the 'low a_w' sample in which the FDNB-reactive lysine value multiplied by N digestibility was 17% lower than the bioavailable lysine. It is possible that the rat is able to utilize some of the lysine complexes formed under these conditions although, in general, the reaction products of lysine and tryptophan formed during lipid oxidation would appear to be unavailable to the rat as a source of these amino acids.

It has previously been shown that methionine is readily oxidized to its sulphoxide during reactions between protein and oxidizing lipid and that little further oxidation to methionine sulphone occurs (Cuq *et al.* 1978; Nielsen *et al.* 1985*b*). The experiments reported here demonstrate that methionine sulphoxide (free or protein-bound) is highly available as a source of methionine to the rat. A recent study has similarly indicated that methionine sulphoxide is also highly available to humans (Marable *et al.* 1980). It would appear, therefore, that the oxidation of methionine to methionine sulphoxide during food processing and storage is of little nutritional importance.

The high bioavailability of methionine sulphoxide (96% in casein and, on average, 87% for the free compound) is in agreement with most previous reports (Njaa, 1962; Slump & Schreuder, 1973; Anderson *et al.* 1976; Sjöberg & Boström, 1977; Cuq *et al.* 1978). These are, however, the first quantitative estimates of its bioavailability assayed in well-controlled tests of amino acid availability. Gjöen & Njaa (1977) found that the bioavailability of methionine sulphoxide was enhanced in the presence of cystine. Our results showing 83% availability in the presence of 4 g cystine/kg and 0·6 g methionine/kg, and 90% availability in the presence of 1·6 g methionine/kg but without cystine, do not support their findings. It is possible, however, that the presence of either methionine or cystine enhances the bioavailability of methionine sulphoxide.

Cyst(e)ine poses a special problem to the food nutritionist since the level of the different oxidation products cannot, at present, be determined in a protein and, as cyst(e)ine can be formed biologically from methionine (Finkelstein & Mudd, 1967), no assay exists in which the bioavailability of cyst(e)ine in a protein can be estimated directly. In the present study, we have developed one assay for methionine plus cyst(e)ine and one assay for methionine alone. From the ratio between the slopes of the two standard curves it appeared that 43% of methionine could be replaced by cystine. This is in agreement with the previous results of Byington *et al.* (1972), Stockland *et al.* (1973) and James & Hove (1980).

It would appear that the loss of available cyst(e)ine is important during protein–lipid reactions. Bioavailable cyst(e)ine fell by 28% in the 'O_2 limitation' sample compared with falls in available lysine, tryptophan, methionine and TD of 24, 11, 8 and 9% respectively.

The results of the total-S-amino-acid assay are also best explained by a fall in cyst(e)ine bioavailability.

No losses of total cyst(e)ine, measured as cysteic acid after performic acid oxidation and acid-hydrolysis, were observed in any of the whey protein–methyl linolenate model systems (Nielsen *et al.* 1985*b*), neither was cysteic acid detected after simple acid-hydrolysis. Cysteine and cystine might react with the secondary products of fat oxidation although it is more likely that they have been oxidized to their intermediate oxidation products such as alanine-3-sulphinic acid, or cystine monoxide and cystine dioxide (Finley *et al.* 1981). The bioavailability of cyst(e)ine oxidation products has been little studied. Cystine dioxide has been reported to be only partly bioavailable whereas alanine-3-sulphinic acid and cysteic acid are unavailable to the rat (Bennett, 1937, 1939; Anderson *et al.* 1976).

It is difficult to extrapolate from our model systems to what may happen in human food products. When O_2 uptake is restricted by the packaging, there would appear to be little or no danger of extensive protein damage due to lipid oxidation, especially in low a_w foods. With a free access to O_2, losses of lysine, cyst(e)ine, tryptophan and N digestibility could occur. Losses of lysine and N digestibility appear to result mainly from the reactions of protein with the secondary products of fat oxidation. These same products are also responsible for the adverse organoleptic changes which accompany fat oxidation, and the foodstuff would presumably be organoleptically unacceptable long before any extensive protein damage occurred. Losses of tryptophan are mainly dependent on the reduction in protein digestibility. Oxidation of methionine and cyst(e)ine take place with the primary products of fat oxidation and may well take place before any unacceptable organoleptic changes. Although methionine is readily converted to its sulphoxide by oxidizing lipids, methionine sulphoxide is almost as available as methionine itself and its formation could not be considered as nutritionally important. The oxidation of cyst(e)ine to biologically unavailable compounds might, however, be nutritionally significant. It is therefore concluded that, with the possible exception of cyst(e)ine oxidation, the reactions of proteins with oxidizing lipids are of minor practical nutritional importance during the storage of foodstuffs for humans.

The authors wish to thank Professor J. Mauron and Dr J. Löliger for valuable discussions and Miss A. Klein and Mrs M. P. Amendola for technical assistance.

REFERENCES

Anderson, G. H., Ashley, D. M. V. & Jones, J. F. (1976). *Journal of Nutrition* **106**, 1108–1114.
Association of Official Analytical Chemists (1980). *Official Methods of Analysis of the Association of Official Analytical Chemists*, p. 774 [W. Horwitz, editor]. Washington DC: AOAC.
Bender, A. E. & Doell, B. H. (1957). *British Journal of Nutrition* **11**, 140–148.
Bennett, M. A. (1937). *Biochemical Journal* **31**, 962–965.
Bennett, M. A. (1939). *Biochemical Journal* **33**, 885–892.
Byington, M. H., Howe, J. M. & Clark, H. E. (1972). *Journal of Nutrition* **102**, 219–228.
Cuq, J.-L., Besancon, P., Chartier, L. & Cheftel, C. (1978). *Food Chemistry* **3**, 85–101.
Finkelstein, J. D. & Mudd, S. H. (1967). *Journal of Biological Chemistry* **242**, 873–880.
Finley, J. W., Wheeler, E. L. & Witt, S. C. (1981). *Journal of Agricultural and Food Chemistry* **29**, 404–407.
Finney, D. J. (1964). *Statistical Methods in Biological Assay*, 2nd ed. New York: Hafner Publishing Co.
Food and Agriculture Organization (1970). *Amino-acid Content of Foods and Biological Data on Proteins*. Rome: FAO.
Gamage, P. T. & Matsushita, S. (1973). *Agricultural and Biological Chemistry* **37**, 1–8.
Gjöen, A. V. & Njaa, L. R. (1977). *British Journal of Nutrition* **37**, 93–105.
Harmuth-Hoene, A. E. & Delincée, H. (1978). *International Journal for Vitamin and Nutrition Research* **48**, 62–74.
Horigome, T. & Miura, M. (1974). *Journal of the Agricultural Chemistry Society of Japan* **48**, 437–444.
Horigome, T. & Uchida, S. (1979). *Scientific Reports of the Faculty of Agriculture, Okayama University* **54**, 43–49.
Horigome, T., Yanagida, T. & Miura, M. (1974). *Journal of the Agricultural Chemistry Society of Japan* **48**, 195–199.

Hurrell, R. F. (1980). In *Food and Health – Science and Technology*, pp. 369–388 [G. G. Birch and K. J. Parker, editors]. London: Applied Science Publishers.
Hurrell, R. F. (1984). In *Food Proteins*, vol. 3 [B. J. F. Hudson, editor]. London: Applied Science Publishers.
James, K. A. C. & Hove, E. L. (1980). *Journal of Nutrition* **110**, 1736–1744.
Kanazawa, K., Danno, G. & Natake, M. (1975). *Journal of Nutritional Science and Vitaminology* **21**, 373–382.
Marable, N. L., Todd, J. M., Korslund, M. K. & Kennedy, B. W. (1980). *Qualitas Plantarum – Plant Foods for Human Nutrition* **30**, 155–161.
Mitchell, H. H. (1923–24). *Journal of Biological Chemistry* **58**, 873–905.
Mottu, F. & Mauron, J. (1967). *Journal of the Science of Food and Agriculture* **18**, 57–62.
Nielsen, H. K., De Weck, D., Finot, P. A., Liardon, R. & Hurrell, R. F. (1985*a*). *British Journal of Nutrition* **53** (In the Press.)
Nielsen, H. K., Löliger, J. & Hurrell, R. F. (1985*b*). *British Journal of Nutrition* **53**, 61–73.
Njaa, L. R. (1962). *British Journal of Nutrition* **48**, 565–570.
O'Brien, P. J. (1966). *Biochemical Journal* **101**, 12P–13P.
Pellet, P. L. & Young, V. R. (1980). *Nutritional Evaluation of Protein Foods*. United Nations University: Tokyo.
Peret, J., Macaire, F. & Chanez, M. (1973). *Journal of Nutrition* **103**, 866–874.
Rogers, Q. R. & Harper, A. E. (1965). *Journal of Nutrition* **87**, 267–273.
Sjöberg, L. B. & Boström, S. L. (1977). *British Journal of Nutrition* **38**, 189–205.
Slump, P. & Schreuder, H. A. W. (1973). *Journal of the Science of Food and Agriculture* **24**, 657–661.
Stockland, W. L., Meade, R. J., Wass, D. F. & Sowers, J. E. (1973). *Journal of Animal Science* **36**, 526–530.
Yanagita, T. & Sugano, M. (1973). *Journal of the Agricultural Chemistry Society of Japan* **47**, 577–582.
Yanagita, T. & Sugano, M. (1975). *Agricultural and Biological Chemistry* **39**, 63–69.
Yanagita, T. & Sugano, M. (1978). *Journal of Nutritional Science and Vitaminology* **24**, 581–588.

Printed in Great Britain