Studies in rats on the nutritional value of hydrogen peroxide-treated fish protein and the utilization of oxidized sulphur-amino acids

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1. The nutritional value of fish protein oxidized with different levels of hydrogen peroxide, and the utilization of oxidized sulphur-amino acids in the rat were investigated.

2. The results showed that the biological value (BV) and protein efficiency ratio (PER) of the fish protein were significantly decreased by the treatment with H_2O_2, and although there were no significant differences between samples treated with 20, 40 or 80 g H_2O_2/kg, there was a gradual decrease of BV at higher concentrations. Digestibility was not affected by the treatment.

3. Supplementation with 1 g L-methionine/kg or 1 g L-cystine/kg significantly increased the BV for all H_2O_2-treated samples. With the exception of the sample treated with 80 g H_2O_2/kg, the BV was increased by L-methionine supplementation to the level for the untreated fish protein.

4. Treatment with H_2O_2 led to the formation of methionine sulphoxide, methionine sulphone and cysteic acid. The availability of these compounds was studied as supplements to the untreated fish protein as well as to the H_2O_2-treated fish protein. The results showed that L-methionine DL-sulphoxide was as available as methionine, but that L-methionine sulphone and cysteic acid had no supplementary effect.

5. The availability of lysine in fish protein was not affected by treatment with 20 g H_2O_2/kg as judged by its effect as a supplement to wheat flour.

6. The plasma amino acid pattern for rats given a diet with H_2O_2-treated fish protein was identical to that for rats given an untreated fish protein, with the exception of the presence of the two isomers of L-methionine DL-sulphoxide and L-methionine sulphone. The oxidized S-amino acids were also found in liver, kidney and gastrocnemius muscle from the rat.

7. Only traces of L-methionine DL-sulphoxide were found in the urine. About half the ingested L-methionine sulphone was found in the urine and almost all was present as an acid-labile conjugate.

Treatment of proteins with oxidizing agents like hydrogen peroxide is reported to decrease the nutritional value of the protein (Ellinger & Palmer (1969), Rasekh, Stillings & Sidwell (1972), Slump & Schreuder (1973), Anderson, Li, Jones & Bender (1975)).

Several explanations have been offered for the decrease in nutritional value. Ellinger & Palmer (1969) interpreted their results as a reduced availability of methionine in oxidized casein to both growing rats and to the bacteria *Streptococcus zymogenes*. Rasekh *et al.* (1972) studied H_2O_2-treated fish-protein concentrate and speculated that the observed decrease in protein efficiency ratio (PER) values on oxidation was due to breakdown of cystine or oxidation of methionine to methionine sulphoxide. Slump & Schreuder (1973) studied H_2O_2-treated casein and fish meal, and analysed the oxidized protein samples for methionine, methionine sulphoxide, methionine sulphone, cystine and cysteic acid. They suggested that methionine sulphoxide was completely available to rats and that the decrease in biological value (BV) was due to formation of cysteic acid and methionine sulphone, both unavailable to rats. Anderson *et al.* (1975) studied H_2O_2 treatment of rapeseed flour and concluded that formation of oxidized sulphur-amino acids, in particular methionine sulphone, reduced considerably the nutritional value.

Cuq, Provansal, Guilleux & Cheftel (1973) found a decreased release of methionine, or its oxidation products, from H_2O_2-treated casein on in vitro digestion with Pronase, and concluded that Pronase was unable to split methionyl-peptide bonds when methionine was oxidized to methionine sulphoxide.

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The availability of free methionine sulphoxide to rats has been studied in several laboratories. Njaa (1962) found that L-methionine sulphoxide was as effective as methionine when used as a supplement to a soya-bean-meal diet. D-methionine sulphoxide was only about 50% as active as methionine. Miller & Samuel (1968) found that methionine sulphoxide (isomer not specified) could partly replace methionine as a supplement to a casein diet. Miller, Tannenbaum & Seitz (1970) approached the problem with diets composed of synthetic amino acids. They found in rats an age-dependent adaptation to a more efficient utilization of methionine sulphoxide. By injecting labelled methionine sulphoxide and methionine sulphone into rats, Smith (1972) showed that approximately 10% of the methionine sulphoxide and about 50% of the methionine sulphone was excreted in 24 h. Both methionine sulphoxide and methionine sulphone were mainly excreted as *N*-acetyl derivatives.

The present study was undertaken to evaluate further the nutritive value of H_2O_2-treated fish protein and the availability and general turnover of peptide-bound oxidized S-amino acids. Also findings from studies on dietary supplementation with these amino acids are presented.

MATERIALS AND METHODS

Samples of fish protein were prepared from frozen blocks of cod filleting offal. Since maximum water-binding capacity and oil-emulsifying properties were aimed at, all processing steps were carried out at a maximum temperature of 50°.

Reference fish-protein sample. This was prepared as follows. The filleting offal was partially deboned and the resulting fish paste was extracted three times with azeotropic propan-2-ol at 50°, the solvent was removed and the sample was deodorized with air saturated with water, and then dried and ground. The composition by analysis was (g/kg): protein (nitrogen × 6·25) 868, fat 3, ash 120.

H_2O_2-treated fish-protein samples were produced from the same raw material as the reference sample. Fish paste was mixed with azeotropic propan-2-ol (1:1, w/v) and the temperature was increased to 50°. Sodium hydroxide was added to increase the pH to 8·5. H_2O_2 solution (350 ml/l) was then added at 20–80 g/kg dry weight of fish substance. The mixture was stirred continuously for 120 min, then additional propan-2-ol was added, and the mixture was centrifuged. The solid phase was further extracted three times with propan-2-ol, the solvent was removed and the sample was deodorized with air saturated with water, and then dried and ground. The composition by analysis was (g/kg): 20 g H_2O_2/kg: protein 851, fat 1·5, ash 133; 40 g H_2O_2/kg: protein 851, fat 1·4, ash 130; 80 g H_2O_2/kg: protein 856, fat 2·3, ash 129. The absence of free H_2O_2 in the fish-protein samples was confirmed by analysis of H_2O_2 with a modification of the thiocyanate method of Hills & Thiel (1945).

L-methionine DL-sulphoxide and L-methionine sulphone were purchased from Sigma Chemical Co., St Louis, Missouri, USA. L-methionine and L-cystine were purchased from British Drug Houses, Poole, Dorset, UK, and L-cysteic acid from Fluka AG, Buchs, Switzerland. All other chemicals were of analytical grade.

Analytical methods

N content. The N contents of fish-protein products and of samples of urine and faeces from animal experiments was determined by the Kjeldahl method, using an automatic apparatus (Kjell Foss; A/SN Foss Electric, Denmark).

Ash content. This was determined in fish-protein products by the method of the Association of Official Analytical Chemists (1970).

Lipid content. This was determined in fish-protein products by extraction with chloroform–methanol (2:1, v/v).

Amino acid composition. This was determined in fish-protein products by hydrolysis in 6 M-hydrochloric acid under vacuum at 110° for 22 h, followed by ion-exchange chromatography essentially according to the method of Stein & Moore (1954).

S-amino acids. Cysteine–cystine was determined as cysteic acid and 'total methionine' as methionine sulphone after oxidation with performic acid according to Moore (1963).

Tryptophan. This was determined according to the method of Spies & Chambers (1948, 1949).

Methionine sulphone and cysteic acid. These were determined after acid-hydrolysis and ion-exchange chromatography (as described previously) as follows.

Methionine sulphoxide was determined by two methods: (*a*) 'indirect' method, essentially according to Slump & Schreuder (1973), by alkylation with iodo-acetic acid followed by performic acid oxidation and acid-hydrolysis. Some minor modifications were introduced. To facilitate alkylation with iodo-acetic acid all samples were digested with Alcalase (Novo, Copenhagen) before the alkylation step. The protein sample (25 mg) was suspended in 4 ml 0·01 M-sodium bicarbonate, pH 8·5. Alcalase (0·25 mg) was added and the sample was shaken at 50° for 6 h. This stage was followed by alkylation with iodo-acetic acid for 40 h at 40° in darkness after adjustment of the pH to 2·0 with HCl. To remove the excess iodo-acetic acid before oxidation with performic acid, the sample was extracted with diethyl ether according to the original procedure of Neumann (1967). The samples were subsequently evaporated nearly to dryness by rotary evaporation at 40°, oxidized with performic acid according to Moore (1963) and hydrolysed with 6 M-HCl for 18 h. The amount of methionine sulphoxide present in protein samples was calculated as described by Slump & Schreuder (1973); (*b*) 'direct' method, in which the content of methionine sulphoxide in test protein samples and in rat faeces was also determined, after alkaline-hydrolysis, essentially according to Neumann (1967). To 10–15 mg sample was added 1 ml 2 M-sodium hydroxide and the mixture was hydrolysed at 100° for 18 h. Then 6 M-HCl was added to adjust the pH to approximately 2·0 followed by dilution with 10 ml of the starting buffer (pH 2·2) for ion-exchange chromatography (see p. 192).

BIOLOGICAL EVALUATIONS

Animal experiments

Biological evalutions were performed on rats in N-balance experiments and in growth experiments.

For all animal experiments male rats of the Sprague–Dawley strain (Anticimex, Stockholm) were used. The animals were housed in individual cages in a room with constant relative humidity and temperature (50% and 21° respectively) and with a 12 h light-and-dark cycle. Food and water were offered *ad lib.*

Diet composition

The diet contained (g/kg): protein 100, fat (groundnut oil) 100 (corrected for fat contribution from test protein sample), cellulose 30, mineral mixture 50 (corrected for the contribution of ash from test protein sample), vitamin mixture 20, sucrose to 1 kg. The composition of the mineral mixture (USP salt mixture XIV (US Pharmacopoeia, XIV, 1950) supplemented with trace minerals) was (g/kg): calcium carbonate 68·3, calcium citrate 308·3, calcium diphosphate 112·8, magnesium carbonate 35·2, magnesium sulphate 38·3, potassium chloride 124·7, potassium dihydrogen phosphate 218·8, sodium chloride 77·1, ferric ammonium citrate 15·281, cupric sulphate 0·077, manganese sulphate 4·200, ammonium aluminium sulphate 0·092, zinc sulphate 0·500, potassium iodide 0·740, sodium fluoride 0·507, cobalt chloride 0·023, sodium arsenite 0·009, sodium borate 0·022, sodium molybdate 0·003. The vitamin mixture, prepared in glucose, supplied the following when used at 20 g/kg diet

(g/kg diet): retinol 5·94 mg, cholecalciferol 0·05 mg, ascorbic acid 0·991, α-tocopherol 0·110, *myo*-inositol 0·110, choline chloride 1·651, menaphthone 0·049, *p*-aminobenzoic acid 0·110, nicotinic acid 0·099, riboflavin 0·022, pyridoxine hydrochloride 0·022, thiamine hydrochloride 0·022, calcium pantothenate 0·066, biotin 0·440 mg, pteroylmonoglutamic acid 1·982 mg, hydroxocobalamin 0·029 mg.

In supplementation experiments amino acids were added at the expense of sucrose.

N-balance procedure

The starting weight of the animals was 70–75 g. Seven animals were randomly assigned to each test diet. A 4 d adaptation period on the test diet was followed by a 4 d experimental period, during which weight gain and food intake were recorded, and urine and faeces were collected separately for N determination. Digestibility, BV, and net protein utilization (NPU) were calculated after correction for endogenous loss of N on a protein-free diet.

Growth experiments

The starting weight of the animals was 56–62 g. Ten rats were randomly assigned to each test diet. During a 4-week period food intake and weight gains were recorded. PER was calculated as g weight gain/g protein eaten.

Blood sampling

Blood samples for determination of plasma amino acids and plasma urea were taken from the aorta of diethyl ether-anaesthetized animals and collected into heparinized tubes. Blood samples were taken in the morning 1–2 h after withdrawal of the food. In the later part of the study blood samples for urea determination were taken 6 h after withdrawal of the food.

Urea. The urea concentration in plasma and urine was determined using a test kit (15930 THAC; Boehringer GmbH, Mannheim, West Germany).

Plasma amino acids. The concentrations were determined by ion-exchange chromatography essentially according to Jeppsson & Karlsson (1972). Before analysis the blood plasma was deproteinized with solid sulphosalicylic acid, 30 mg/ml plasma, according to Perry & Hansen (1969). Deproteinized samples were kept at −70° until required for analysis.

Methionine sulphoxide, methionine sulphone and cysteic acid. The concentrations in plasma, urine, kidney, liver and muscle were determined by a modification of the procedure (Jeppsson & Karlsson, 1972) for ion-exchange chromatography of plasma amino acids. In the Jeppsson & Karlsson (1972) procedure the elution of the amino acids starts with a lithium buffer, pH 2·80. In this procedure one isomer of methionine sulphoxide is eluted together with methionine sulphone just before threonine and the other isomer is eluted together with threonine. In the first part of this study this procedure was used to obtain a rough estimate of the content of 'oxidized methionine' in plasma and urine. This estimate assumed that the 'peak' of methionine sulphoxide in the threonine 'peak' was the same height as the 'peak' eluted before threonine. During the course of the study a new procedure was developed which gave a better resolution. A 50 cm column packed with resin (Beckman UR-30 resin; Beckman Instruments Co., Fullerton, California, USA) was used. Elution was started with 0·02 M-sodium citrate buffer, pH 3·05. The temperature was 55°. A typical elution pattern for plasma as obtained by this modified procedure is presented in Fig. 2.

Before urine analysis of methionine sulphoxide and methionine sulphone, urine samples were deproteinized with cold acetone (4:1, v/v). Since acetone precipitation of urine gave low recovery of cysteic acid, precipitation was subsequently done with solid sulphosalicylic acid (30 mg/ml). Addition of free cysteic acid before treatment showed a 10% loss on handling and, therefore, values for cysteic acid were corrected for this loss.

Kidney, liver and muscle were homogenized in cold distilled water (1:3, w/v), centrifuged and the supernatant fraction was treated with sulphosalicylic acid for protein precipitation (30 mg/ml supernatant fraction).

Thin-layer chromatography. For further identification of methionine sulphoxide and methionine sulphone in plasma, the respective fractions from the ion-exchange chromatography separation werre collected. Dansyl derivatives of the fractions were synthesized and analysed by thin-layer chromatography on silica gel according to the methods of Gray & Hartley (1963) and Gros & Labousse (1969).

The presence of acid-labile derivatives of methionine sulphone and cysteic acid (e.g. *N*-acetyl derivatives; Smith, 1972) was determined in urine, plasma liver, kidney and muscle by subjecting the material to hydrolysis with 2 M-HCl for 3 h at 110°. This procedure was shown to deconjugate N-acetyl methionine. Values were compared with results for sample not subjected to acid-hydrolysis.

The acid-hydrolysis technique was originally intended to be used also for determination of derivatives of methionine sulphoxide, since methionine sulphoxide was stable under these conditions in the test systems investigated. However, it was found that methionine sulphoxide was not stable in urine and plasma under these conditions and therefore an alkaline-hydrolysis method had to be used (Ivanetich, Bradstrom & Kaminsky (1976) reported a yield of more than 85% for methionine sulphoxide after alkaline-hydrolysis of *N*-acetylmethionine sulphoxide).

Alkali-labile derivatives of methionine sulphoxide. These derivatives were determined in plasma, kidney, liver, muscle and urine after protein precipitation with sulphosalicylic acid (for plasma and kidney determinations were also made after protein precipitation with acetone) followed by alkaline-hydrolysis (2 M-NaOH for 18 h at 110°) and ion-exchange chromatography. Values were compared with results for samples not subjected to alkaline-hydrolysis.

Statistical treatment

Values are presented as mean values with their standard errors. Statistical significance was calculated by the use of Student's t test; $P < 0.05$ was regarded as statistically significant.

RESULTS

Amino acid composition

Table 1 shows the effect of H_2O_2 treatment on the amino acid composition. As can be seen both methionine and cystine were partially oxidized. Most of the methionine sulphoxide, as well as cysteic acid, was already formed by treatment at 20 g H_2O_2/kg diet, with only a moderate successive increase at higher concentrations of H_2O_2. 'Indirect' or 'direct' methods for determination of methionine sulphoxide gave similar results, but only results from 'indirect' determination are presented. With methionine sulphone there was, however, a more pronounced progressive increase with increasing H_2O_2 concentrations. It is interesting to note the presence of methionine sulphoxide as well as cysteic acid in the reference material.

Of the other amino acids present, the contents of tryptophan, histidine and lysine seemed to be reduced by the treatment with H_2O_2.

Animal experiments

Since methionine is the first limiting amino acid in fish protein (Stillings, Hammerle & Snyder, 1969), conversion of methionine to unavailable derivatives should lead to a decrease in nutritional value.

Table 1. *Amino acid composition of fish protein treated with hydrogen peroxide*

	Amino acid content (g/kg protein (nitrogen × 6·25))			
H_2O_2 added (g/kg) ...	0 (control)	20	40	80
Amino acid				
Aspartic acid	10·12	9·87	10·15	10·22
Threonine	4·71	4·54	4·69	4·58
Serine	4·57	4·41	4·45	4·47
Glutamatic acid	14·86	14·34	14·47	14·67
Proline	4·91	4·70	4·50	4·73
Glycine	6·08	5·81	5·89	6·14
Alanine	6·29	6·11	6·20	6·29
Valine	5·54	5·40	5·50	5·40
Methionine*	3·50	3·54	3·20	3·43
Isoleucine	4·57	4·43	4·53	4·51
Leucine	8·02	7·73	7·81	7·99
Tyrosine	3·58	3·14	3·21	3·23
Phenylalanine	4·23	4·25	4·24	4·16
Lysine	8·98	8·64	8·07	6·94
Histidine	2·63	2·36	2·35	2·12
Arginine	6·54	6·39	6·25	6·29
Cystine*	1·15	1·10	1·15	1·19
Tryptophan	1·69	1·36	1·23	0·95
Methionine sulphoxide†	0·41	2·12	2·48	2·20
Methionine sulphone	0·00	0·17	0·31	0·57
Cysteic acid (calculated as cystine)	0·08	0·62	0·67	0·81

* Determined after performic acid oxidation according to Moore (1963).
† 'Indirect' method (see p. 191).

The influence of the treatment with H_2O_2 was tested in N-balance and growth experiments. Results from N-balance experiments are shown in Table 2. The results show that the BV was significantly decreased in the samples treated with H_2O_2. There was a significant decrease in BV with H_2O_2 treatment at 20 g/kg diet, and there was no significant difference between treatments (20, 40 and 80 g H_2O_2/kg diet) although there was a further gradual decrease in the BV at higher concentrations of H_2O_2. Over-all digestibility was not affected by the treatment. Corresponding values for a casein diet are: digestibility 94, BV 82, NPU 78.

As a measure of the nutritional value, values for plasma urea concentration and the amount of urea-N relative to total N in urine are included in Table 2. Plasma urea level has been shown by Eggum (1973) to be inversely correlated with the BV of proteins. As can be seen there was an increase in plasma urea-N as well as in the value for urine urea-N:total-N in urine on treatment with H_2O_2.

Results from growth experiments are presented in Table 3. The results showed a decrease in growth and food efficiency on treatment with H_2O_2. Values for a casein diet are included as a reference. The results paralleled those from the N-balance experiments except for the sample treated with 80 g H_2O_2/kg, which in the growth experiment showed a greater loss of nutritional value.

Supplementation experiments

Supplementation with 1 g L-methionine/kg diet significantly increased the BV for the H_2O_2-treated samples as well as for the reference sample (Table 2). However, the sample treated with 80 g H_2/O_2/kg still showed a lower BV than the unsupplemented reference sample and an attempt to further increase the BV by supplementation with 2 g L-methionine/kg diet was unsuccessful. The BV for the sample treated with 40 g H_2O_2/kg was not increased

Table 2. *Results from nitrogen-balance experiments* on rats given hydrogen peroxide-treated fish protein*

(Mean values with their standard errors for seven rats/treatment)

H_2O_2 added (g/kg) ...	0 (control)		20		40		80		0 (control)		20		40		80	
L-methionine added (g/kg) ...	—		—		—		—		1		1		1		1	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Diet intake (g)	43·1	2·4	40·6	2·8	37·7	1·4	35·8	1·5	41·5	4·0	42·2	1·7	44·8	1·5	39·3	2·2
Wt gain (g)	19·0	1·4	12·0	1·1	12·0	0·8	11·0	1·0	19·5	2·8	18·6	1·5	18·2	1·3	15·0	2·5
Plasma urea-N† (mg/l)	107	10	136	11	123	5	157	4	59	9	50	6	87	13	132	10
Urine urea-N:total urine N	0·509	0·0032	0·606	0·008	0·664	0·0021	0·727	0·0004	0·353	0·0026	0·346	0·0022	0·459	0·0032	0·746	0·0009
Digestibility	0·923	0·0005	0·918	0·0005	0·906	0·0003	0·923	0·0004	0·932	0·0006	0·937	0·0005	0·921	0·0005	0·918	0·0003
BV	0·948	0·0003	0·863	0·001[b]	0·854	0·0013[b]	0·835	0·0009[c]	0·970	0·0003[c]	0·961	0·0003[c]	0·940	0·001[c,d]	0·896	0·0008[c,d]
NPU	0·875	0·0007	0·790	0·0011	0·773	0·0013	0·770	0·0009	0·904	0·0008	0·901	0·0007	0·866	0·0013	0·823	0·0009

b, value significantly different from control sample ($P < 0·05$).
c, value significantly different from respective unsupplemented sample ($P < 0·05$).
d, value significantly different from supplemented control sample ($P < 0·05$).
BV, biological value; NPU, net protein utilization.
* For details, see p. 192.
† Blood sample taken 1–2 h after withdrawal of food.

Table 3. *Results from growth experiments† on rats given hydrogen peroxide-treated fish protein*

(Mean values with their standard errors for ten rats/treatment)

Diet ...	Food intake (g)		Wt gain (g)		PER	
	Mean	SE	Mean	SE	Mean	SE
Control	295·9	10·8	100·5	4·5	3·42	0·19
Control + 20 g H_2O_2/kg	270·2	6·7	73·5	3·0	2·72	0·17*
Control + 40 g H_2O_2/kg	265·9	12·6	72·7	4·3	2·75	0·13*
Control + 80 g H_2O_2/kg	210·1	10·2	45·0	2·9	2·14	0·17*
Casein	288·4	9·6	91·4	3·8	3·17	0·14

PER, protein efficiency ratio.
* Significantly different from control sample ($P < 0·05$).
† For details, see p. 192.

Table 4. *Nitrogen-balance experiments† on rats given reference fish protein supplemented with methionine, methionine sulphoxide, methionine sulphone and cysteine*

(Mean values with their standard errors for seven rats/treatment except the control diet where fourteen rats were used)

Diet ...	Control		Control							
Supplement (1 g/kg) ...	—		L-methionine		L-methionine DL-sulphoxide		L-methionine sulphone		L-cysteine	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Food intake (g)	45·4	2·2	50·2	3·2	51·2	3·2	40·3	2·7	47·2	2·3
Wt gain (g)	17·5	1·2	23·8	2·5	22·0	1·4	18·0	1·8	19·9	1·9
Plasma urea-N‡ (mg/l)	50	4	34	6	33	4	66	7	18	5
Urine urea-N:total urine N	0·496	0·0019	0·443	0·0031	0·442	0·0016	0·632	0·0038	0·364	0·0028
Digestibility	0·938	0·0003	0·933	0·0011	0·955	0·0010	0·964	0·0003	0·925	0·0007
BV	0·912	0·0006	0·933	0·0007*	0·929	0·0004*	0·903	0·0008	0·932	0·0004*
NPU	0·855	0·0007	0·87	0·0016	0·887	0·0011	0·871	0·0008	0·862	0·0009

BV, biological value; NPU, net protein utilization.
* Statistically different from the control diet ($P < 0·05$).
† For details, see p. 192.
‡ Blood sample taken 5–6 h after withdrawal of food.

by supplementation with 1 g L-methionine/kg up to the same level as supplemented reference sample.

The values for plasma urea-N and urine urea-N:total N in urine both decreased on supplementation with L-methionine, except for the diet treated with 80 g H_2O_2/kg.

Since some of the S-amino acids in the fish protein were converted during the H_2O_2 treatment to oxidized derivatives (methionine sulphoxide, methionine sulphone, cysteic acid), these derivatives together with cysteine were tested for their availability in supplementation experiments. Results from supplementation of the reference sample and the sample treated with 40 g H_2O_2/kg are presented in Tables 4 and 5.

The results showed that supplementation of the reference sample and of the sample treated with 40 g H_2O_2/kg with L-methionine DL-sulphoxide and cysteine, resulted in an increase of BV comparable to the effect of supplementation with methionine (values are compared with results from unsupplemented diets tested in parallel experiments). On the

Table 5. *Nitrogen-balance experiments† on rats given hydrogen peroxide-treated (40 g H_2O_2/kg) fish protein supplemented with methionine, cysteine and their respective oxidation products*

(Mean values with their standard errors for seven rats/treatment)

Diet ...	Control		Control									
Supplement (1 g/kg) ...	—		L-methionine		L-methionine sulphoxide		L-methionine sulphone		Cysteine		Cysteic acid	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Food intake (g)	33·2	1·7	45·3	3·5	43·3	2·2	29·8	1·9	51·6	1·7	35·7	1·4
Wt gain (g)	8·3	1·1	17·0	2·8	15·0	1·3	7·3	1·3	20·3	1·7	8·6	1·0
Plasma urea-N‡ (mg/l)	62	7	77	13	29	5	76	11	51	4	84	8
Urine urea-N:total urine N	0·637	0·0006	0·547	0·0035	0·430	0·004	0·667	0·0018	0·490	0·0026	0·655	0·0015
Digestibility	0·952	0·0011	0·944	0·0009	0·939	0·0008	0·952	0·0010	0·927	0·0004	0·947	0·0005
BV	0·817	0·0011	0·912	0·0008*	0·888	0·0021*	0·785	0·0015	0·894	0·0017*	0·814	0·0015
NPU	0·778	0·0015	0·861	0·0014	0·833	0·0017	0·747	0·0018	0·829	0·0016	0·771	0·0011

BV, biological value; NPU, net protein utilization.

* Statistically different from control diet ($P < 0·05$).

† For details, see p. 192.

‡ Blood samples taken 5–6 h after withdrawal of food.

Table 6. *Nitrogen-balance experiments* on rats given wheat flour supplemented with hydrogen peroxide-treated (20 g H_2O_2/kg) fish protein*

(Mean values with their standard errors for seven rats/treatment)

Diet ...	Wheat flour†		Wheat flour + 50 g fish protein/kg		Wheat flour + 100 g fish protein/kg	
	Mean	SE	Mean	SE	Mean	SE
Food intake (g)	27·5	2·7	40·8	2·3	43·1	3·3
Wt gain (g)	0·8	0·4	13·0	1·7	19·8	2·6
Plasma urea-N‡ (mg/l)	179	9	165	12	116	8
Urine urea-N: total urine N	0·957	0·0029	0·911	0·0016	0·756	0·0015
Digestibility	0·899	0·0013	0·872	0·0010	0·880	0·0011
BV	0·596	0·0014	0·709	0·0018	0·810	0·0022
NPU	0·536	0·0013	0·622	0·0020	0·714	0·0016

BV, biological value, NPU, net protein utilization.
* For details, see p. 192.
† Protein content 88 g/kg diet.
‡ Blood sample taken 1–2 h after withdrawal of food.

other hand, supplementation with L-methionine sulphone resulted in a slightly decreased food intake and BV. Supplementation with cysteic acid resulted in an unchanged BV.

A test in which combined supplements of 1 g L-methionine/kg and 1 g L-cystine/kg was added to the sample treated with 40 g H_2O_2/kg resulted in a smaller increase in BV than supplementation with methionine or cystine alone.

In the 'supplementation' experiments low values for both plasma urea-N and urine urea-N:total N in urine were usually associated with high values for BV.

As mentioned earlier, when fish protein is used as the only protein source in the diet, the BV is mainly a function of the availability of S-amino acids. To test the availability of lysine in the H_2O_2-treated fish protein, the fish protein was tested as a supplementary protein to wheat flour (see Table 6). In this diet lysine is the first limiting amino acid. Fish protein was added at 50 and 100 g/kg wheat flour. The results showed a good supplementary effect. There was a large increase in weight gain and BV and there was a decrease in plasma urea-N and in urea-N:total N in urine. The supplementary effect was of the same magnitude as that normally found in this laboratory for fish protein not treated with H_2O_2, and showed that lysine in this sample was essentially unaffected by the treatment with H_2O_2.

Studies of plasma and urine

The concentration of free amino acids in plasma is a measurement often studied. The free amino acid concentrations in plasma from rats given a control fish-protein diet and a diet with fish protein treated with 20 g H_2O_2/kg are presented in Fig. 1. The two patterns showed a great similarity which indicated that the treatment with H_2O_2 left most amino acids unaffected. However, there were two differences: in the H_2O_2-treated diet there was a new amino acid 'peak' appearing immediately before the threonine 'peak', and the height of threonine 'peak' was increased. This we thought would be explained by the fact that with the buffer system applied in this separation, one isomer of L-methionine DL-sulphoxide was eluted together with L-methionine sulphone immediately before threonine and that the other isomer of L-methionine DL-sulphoxide was eluted together with threonine. As described previously (p. 192), this complex could be further resolved by using a modified buffer system (see Fig. 2). As can be seen the two possible isomers of methionine sulphoxide were separated and methionine sulphone appeared in a separate 'peak'. Occasionally the second

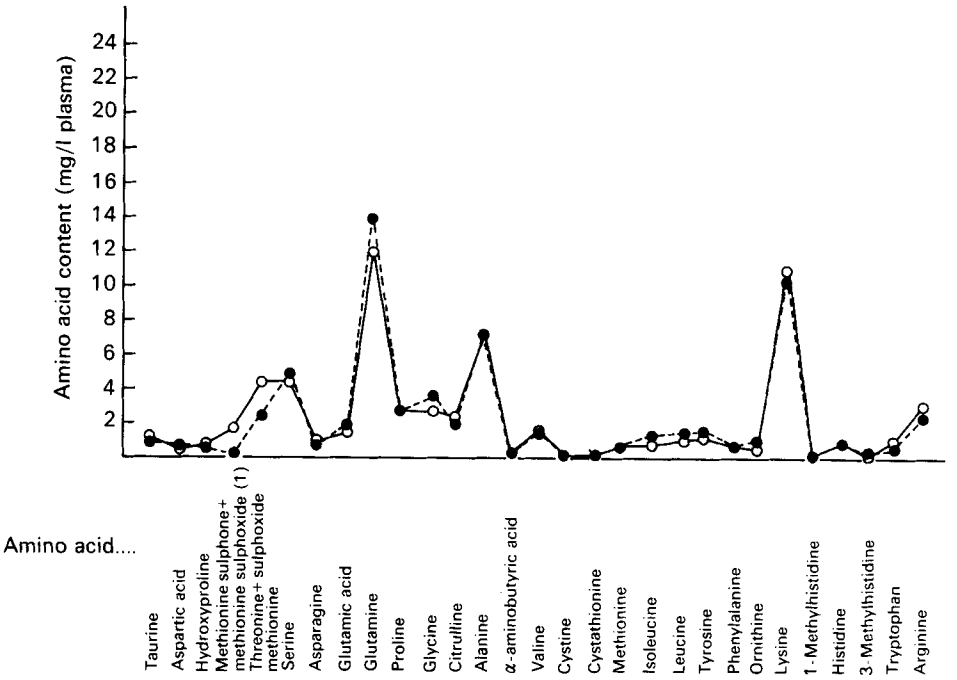

Fig. 1. Plasma 'aminogram' (obtained by ion-exchange chromatography) from rats given a diet with fish protein treated with 20 g hydrogen peroxide/kg (○—○) and a diet with reference fish protein (● - - - ●). The points represent mean values for three determinations. For details of separation procedures, see p. 191.

'peak' for methionine sulphoxide ('peak' no. 2) was higher than the first 'peak' for methionine sulphoxide ('peak' no. 1). This was due to interference by hydroxyproline present in plasma. With a standard amino acid sample, L-methionine DL-sulphoxide gave two 'peaks' of equal size.

The results obtained indicated that the new 'peaks' on the plasma 'aminogram' were methionine sulphoxide and methionine sulphone. For further identification collection was made of corresponding plasma fractions from the ion-exchange chromatography separation. Dansyl derivatives of the amino acids were analysed by thin-layer chromatography (see p. 193). The plasma fractions contained substances with the same R_t values as standard dansyl derivatives of methionine sulphoxide and methionine sulphone.

This finding of the presence of oxidized S-amino acids in plasma prompted us to investigate the concentration of these substances in various organs and fluids. Since the work of Smith (1972) showed that the rat converted methionine sulphoxide and methionine sulphone to *N*-acetylated derivatives, the possibility of the presence of acid- and alkali-labile derivatives was also investigated. The results are presented in Table 7. The results showed that in plasma methionine sulphoxide was present in higher concentrations than methionine sulphone. The value for methionine sulphoxide was approximately twice that normally found for methionine in plasma. Values for cysteic acid in plasma were probably affected by interference from other substances, because values obtained with a H_2O_2-treated fish-protein diet were of the same magnitude as values obtained with a reference fish-protein diet. However, the corresponding 'peak' obtained on chromatography of plasma appeared at the position for cysteic acid, and showed no asymmetry. This was true for other tissues also. Values for methionine sulphoxide in plasma were dependent on the protein concentration

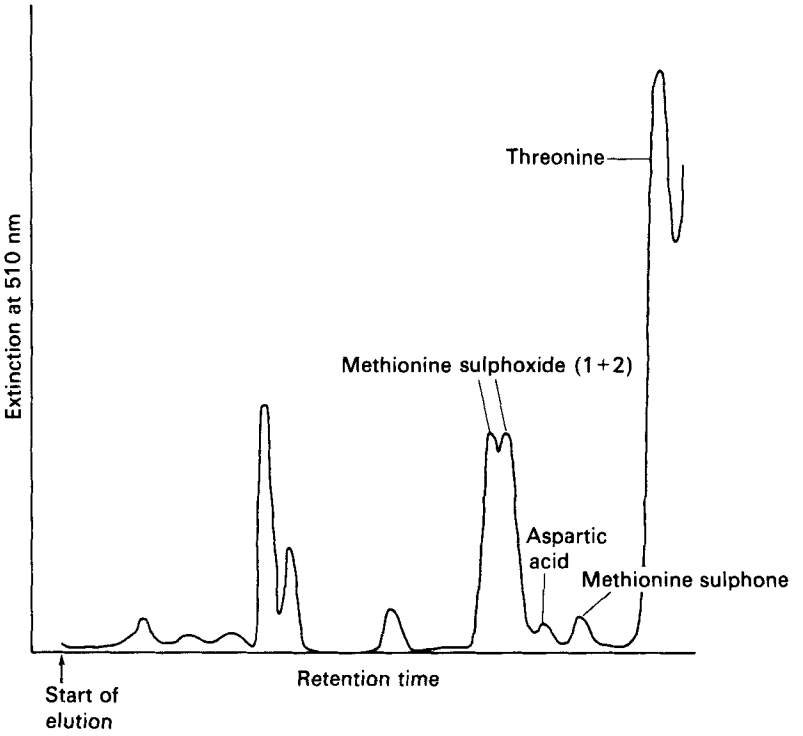

Fig. 2. A portion of a representative recorder trace obtained on ion-exchange chromatography of plasma from a rat given a diet with fish protein treated with 40 g hydrogen peroxide/kg. This indicates the pattern of elution of the oxidized sulphur-amino acids from the ion-exchange resin. For details of separation procedures, see p. 191.

in the diet. Studies with 250 g protein/kg in the diet gave, after 5 d on the diet, the following concentrations (mean ± SE; mg/ml plasma): methionine sulphoxide 16·7 ± 1·1, methionine sulphone 0·49 ± 0·02.

In urine only traces of methionine sulphoxide were found, but there were large amounts of methionine sulphone which were almost all present as an acid-labile derivative. Results from kidney, liver and muscle are at present few and uncertain due to substances interfering during chromatography of samples. This was particularly true for methionine sulphoxide and cysteic acid. The results, however, indicated the presence of methionine sulphoxide and methionine sulphone in all these organs and there was no evidence of the presence of an acid-labile derivative of methionine sulphone or alkali-labile derivatives of methionine sulphoxide. The concentration of cysteic acid seemed to be particularly high in the kidney. No evidence was found for the presence of acid-labile derivatives of cysteic acid.

The elimination rate of methionine sulphoxide and methionine sulphone from plasma was studied in an experiment where rats were maintained for 8 d on a diet with fish protein treated with 40 g H_2O_2/kg, followed by 8 d recovery on a diet with reference fish protein. Concentrations of methionine sulphoxide and methionine sulphone in plasma and urine were followed during the recovery period. The results showed that in plasma the levels of methionine sulphoxide and methionine sulphone decreased to trace amounts after only 1 d on the reference-protein diet. In urine (Table 7) only trace quantities of free methionine sulphoxide could be found. However, methionine sulphone (determined after acid-hydrolysis, which gave a value for free methionine sulphone + acid-labile derivatives of methionine sulphone) was excreted in urine during almost the whole recovery period and showed a

Table 7. *The content of oxidized sulphur-amino acids in plasma, urine, kidney, liver and muscle from rats given* (40 g H_2O_2/kg)-*treated fish protein*

(Mean values with their standard errors; no. of rats in parentheses)

		Tissue content					
		As the free compound*		After acid-hydrolysis*		After alkaline-hydrolysis*	
Tissue	Oxidized S-amino acid	Mean	SE	Mean	SE	Mean	SE
Plasma (mg/l)	Methionine sulphoxide	9·2	1·4 (4)		nd	9·0	0·4 (4)
	Methionine sulphone	3·4	0·4 (4)	3·7	0·2 (4)	4·5	0·5 (4)
	Cysteic acid	2·8	0·2 (4)	7·9	1·4 (3)		nd
Urine (mg/l)	Methionine sulphoxide	1·7	0·4 (4)		nd		†
	Methionine sulphone	1·0	0·4 (4)	58·1	9·6 (4)		†
	Cysteic acid	6·4	5·0 (4)	7·8	2·6 (4)		nd
Kidney (mg/g)	Methionine sulphoxide	3·30	0·10 (2)		nd		†
	Methionine sulphone	4·93	1·42 (3)	4·19	0·98 (3)	3·98	0·84 (3)
	Cysteic acid	4·44	0·55 (3)	4·48	0·92 (3)		nd
Liver (mg/g)	Methionine sulphoxide		†		nd		†
	Methionine sulphone	1·69	0·25 (4)	2·22	0·28 (4)	1·10	0·14 (4)
	Cysteic acid	0·49	0·06 (4)		†		nd
Muscle (mg/g)	Methionine sulphoxide	5–10† (4)			nd	8·19	0·10 (2)
	Methionine sulphone	2·40	0·40 (4)	2·58	1·05 (3)	2·74	0·56 (2)
	Cysteic acid	0·73	0·14 (4)		†		nd

nd, not determined (due to destruction).
* For details of procedures, see p. 191.
† Present but incomplete separation (see p. 192).

Table 8. *Distribution in urine and faeces of orally-ingested oxidized sulphur-amino acids from hydrogen peroxide-treated fish protein**

(Mean values with their standard errors for seven rats/treatment)

Oxidized S-amino acid ...	Methionine	Methionine sulphoxide	Methionine sulphone	Cysteic acid	Cystine
Consumed (mg)	31·9 ± 1·9	85·1 ± 5·13†	5·60 ± 0·33‡	27·0 ± 1·63‡	24·9 ± 1·5
Excreted in faeces (mg)	2·80 ± 0·31	2·15 ± 0·24†	0·46 ± 0·08‡	1·21 ± 0·15‡	5·08 ± 0·45
Excreted in urine (mg)	nd	trace ‖	2·68 ± 0·39‖	0·47 ± 0·03§	nd

nd, not determined.
* This batch of H_2O_2-treated fish protein had the following content of S-amino acids (mg/g protein (nitrogen × 6·25)): methionine 0·91, methionine sulphoxide 2·43, methionine sulphone 0·16, cystine 0·71, cysteic acid 0·77.
† Determined by 'indirect' method (see p. 192).
‡ Determined after acid-hydrolysis (see p. 191).
§ Determined by ion-exchange chromatography after acid-hydrolysis (see p. 193).
‖ Determined by ion-exchange chromatography after protein precipitation with acetone followed by acid-hydrolysis (see p. 193). Value includes free methionine sulphone as well as acid-labile derivatives of methionine sulphone.

progressive decrease in concentration from approximately 50 mg/l urine the day before the change in diet, to approximately 1 mg/l urine after the 8 d recovery period.

To obtain an over-all pattern of the utilization of S-amino acids in H_2O_2-treated fish protein, an experiment was designed in which, during a 4 d period, intake and excretion in faeces and urine of S-amino acids were determined. The results are presented in Table 8. The results showed that rats absorbed more than 90% of the peptide-bound oxidized

S-amino acids. For methionine sulphone, about 50% of that absorbed was excreted in urine. Results in Table 7 showed that most of this was excreted as acid-labile derivatives. Only about 55% of the methionine sulphone consumed could be recovered in urine and faeces. For methionine sulphoxide, only trace quantities of free substance were found in urine. A value for acid-labile derivatives of methionine sulphoxide in urine could not be determined because of the instability of methionine sulphoxide during mild acid-treatment, but the small increase of methionine after acid-hydrolysis indicated that only small amounts of methionine sulphoxide derivatives were excreted in urine.

As previously mentioned, values for cysteic acid in urine were subject to some uncertainty due to interference from unknown substances. Only approximately 6% of the cysteic acid consumed could be recovered as free cysteic acid + acid-labile derivatives in urine and faeces.

DISCUSSION

Chemical evaluation

H_2O_2 is a relatively non-specific oxidizing agent and therefore several amino acids in a protein treated with H_2O_2 would be expected to be modified. Our results showed that treatment of fish protein with H_2O_2 under alkaline conditions had oxidized mainly the S-amino acids, but at higher concentrations of H_2O_2, tryptophan and lysine were also oxidized.

According to Neumann (1967), cystine and cysteine would be more sensitive to oxidation than methionine under alkaline conditions. At acid pH the reverse would be true. Our results in comparison with the results of Slump & Schreuder (1973) seem to verify this. Slump & Schreuder (1973) oxidized fish meal at acid pH and got an almost total conversion of methionine into methionine sulphoxide but only about 25% of the cystine–cysteine was oxidized. In our study, treatment with 20 g H_2O_2/kg at alkaline pH resulted in approximately 65% oxidation of the methionine and approximately 55% oxidation of the cystine. Since all methionine and cystine could be recovered, there seems to be no actual destruction of S-amino acids. Rasekh *et al.* (1972) treated fish-protein concentrate with H_2O_2 at a neutral pH. Although they did not determine the amounts of oxidized S-amino acids, they found that treatment with 50 g H_2O_2/kg fish-protein concentrate resulted in only very small reductions in the contents of tryptophan, tyrosine and histidine. Our results (L. B. Sjöberg & S. L. Boström, unpublished results) from treatment of fish protein with H_2O_2 at a pH between 7·5 and 8 are in accordance with those of Rasekh *et al.* (1972). This indicates that treatment with H_2O_2 at alkaline pH causes a higher extent of amino acid modification than treatment at neutral pH. In this study, alkaline pH was selected since previous experiments had shown that this condition gave maximum bleaching effect and optimum functional properties of the fish protein.

One detail of interest is the presence of methionine sulphoxide in the reference fish protein. Slump & Schreuder (1973) also found methionine sulphoxide in fish protein which had not been treated with H_2O_2. Kido & Kassell (1975) reported the presence of methionine sulphoxide in native porcine pepsin and in pepsin exposed to pH 3·2 at 30° for 70 min. These findings indicate that some methionine residues in various types of protein are very easily oxidized to methionine sulphoxide during handling of the protein material.

Biological evaluation

Treatment of fish protein did not affect the over-all digestibility of the protein. These results are in accordance with the results of Slump & Schreuder (1973). Specific analysis of S-amino acids in faeces (Table 8) also showed that the uptake of S-amino acids was high. Consequently, the results from in vivo studies do not parallel the results of Cuq *et al.* (1973),

which showed a decreased digestibility of methionine sulphoxide peptide bonds during in vitro digestion with Pronase.

The results from N-balance experiments and growth experiments show that treatment with H_2O_2 gives a small decrease in nutritional value. These results together with results showing low levels of methionine sulphoxide in the faeces indicates that peptide-bound methionine sulphoxide is available to rats and that the reduced BV is at least partly due to the formation of methionine sulphone and cysteic acid. This conclusion is in accordance with the conclusion of Slump & Schreuder (1973). The results also show that the decrease in BV can almost equally well be compensated by addition of 1 g L-methionine/kg, 1 g L-cystine/kg or 1 g L-methionine DL-sulphoxide/kg, but not by methionine sulphone or cysteic acid. That free L-methionine DL-sulphoxide can be utilized by the rat has previously been shown by Njaa (1962), Miller & Samuel (1968) and Miller *et al.* (1970). That methionine sulphone and cysteic acid could not be used as a supplement to a casein diet was shown by Miller & Samuel (1968).

It has been shown that the relative amount of each S-amino acid as well as the total quantity of S-amino acids affects the growth of rats (Byington, Hoove & Clark, 1972). That cystine could partly replace methionine when the methionine level was suboptimal was shown by Womack & Rose (1941). The results presented in this study show that cystine also has a 'methionine-sparing' effect when the organic S is supplied mainly in the form of methionine sulphoxide.

If the decrease in BV is due to the formation of methionine sulphone and cysteic acid, one would expect to find a parallel decrease in BV as the concentration of methionine sulphone and cysteic acid increase. This was observed by Slump & Schreuder (1973) but was not found in our investigation, and we have no explanation for this. In the PER studies, we obtained a stepwise reduction in PER values with increasing concentration of H_2O_2. Rasekh *et al.* (1972) did not find any significant difference in PER values for fish protein treated with 12·5–50 g H_2O_2/kg.

Urea concentration in plasma was found to be a most valuable indicator of protein quality, even though the animals were fed *ad lib*, which meant different absolute protein intakes on different diets. Also the ratio, urine urea-N:total N in urine is a good indicator of protein quality.

Studies on the metabolism of oxidized S-amino acids

The presence of free methionine sulphoxide and methionine sulphone in plasma, liver, kidney, muscle and urine was established by ion-exchange chromatography after protein precipitation with sulphosalicylic acid. Methionine sulphoxide appeared as two 'peaks' on the chromatogram obtained with plasma which would be expected since oxidation of peptide-bound L-methionine gives L-methionine DL-sulphoxide. Usually, but not always, the two 'peaks' were of equal height. This indicated that the rate of disappearance from plasma of the two isomers was similar.

Miller *et al.* (1970) reported the presence of large amounts of a 'methionine' compound in plasma from rats given a diet containing free methionine sulphoxide. They could not identify the substance but concluded that it was not methionine, methionine sulphoxide, methionine sulphone or homocystine. Anderson *et al.* (1975) reported the presence of both methionine sulphoxide and methionine sulphone in plasma from rats given H_2O_2-treated rapeseed flour. Since our interpretation of our plasma 'aminograms' also indicated the presence of methionine sulphoxide, efforts were made to get further evidence of its presence. As reported this was done by thin-layer chromatography of dansyl derivatives. Efforts to get further evidence by mass spectrometry failed due to the instability of methionine sulphoxide.

Smith (1972) showed that the rat converts methionine sulphoxide into N-acetyl-methionine sulphoxide, and he speculated that this might be the substance observed by Miller *et al.* (1970) in plasma. To check that the methionine sulphoxide we found in plasma was not the result of breakdown of acetylated methionine sulphoxide during protein precipitation with strong acid, the following experiment was done. Samples of plasma were deproteinized with acetone followed either by direct ion-exchange chromatography or alkaline-hydrolysis followed by ion-exchange chromatography. The two treatments gave similar results, which further indicated that the methionine sulphoxide in plasma was at least mainly in the free form and that there was very little or no alkali-labile derivative like *N*-acetyl-methionine sulphoxide.

In the work of Miller *et al.* (1970) exceptionally high concentrations of free methionine sulphoxide (isomer unspecified) were added to the diet (62·5 mg L-methionine sulphoxide/g amino acid mixture; 180 g amino acid mixture/kg diet). In our supplementation experiment, in which 1 g free L-methionine DL-sulphoxide/kg was added, we also determined the plasma concentration of methionine sulphoxide. A reference fish protein supplemented with 1 g L-methionine DL-sulphoxide/kg gave a plasma concentration of $6{\cdot}7 \pm 1{\cdot}2$ mg/l plasma. Supplementation with 5 g L-methionine DL-sulphoxide/kg gave a concentration of $123{\cdot}3 \pm 18$ mg/l plasma. This indicated that also on a diet supplemented with free methionine sulphoxide, free methionine sulphoxide appears in plasma.

Miller *et al.* (1970) observed a time-dependent decrease in the 'methionine' level in plasma and explained this by an adaptation of the rat. In our work on H_2O_2-treated fish protein we did not observe any differences in plasma methionine sulphoxide level after 10 d or 28 d on the diet.

Apart from plasma the presence of methionine sulphoxide, methionine sulphone and cysteic acid was indicated in liver, kidney, muscle and urine. In urine almost all the methionine sulphone was excreted as an acid-labile derivative which, based on the results of Smith (1972) and Wingo, Smith & Wood (1953) could be the *N*-acetyl derivative or a conjugate with glutamic acid. It would be expected that the conjugation takes place in the kidney, but since acid-labile derivatives were not found in the kidney, our suggestion is that the conjugation takes place in connexion with the excretion of the molecule.

The over-all distribution in urine and faeces of ingested oxidized S-amino acids showed that only less than 3% of the ingested methionine sulphoxide could be recovered. This is a further indication that protein-bound methionine sulphoxide is utilized by the rat. On the other hand approximately 55% of the ingested methionine sulphone was recovered in urine and faeces. For cysteic acid the recovery was only 6% which indicates that cysteic acid is metabolized.

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