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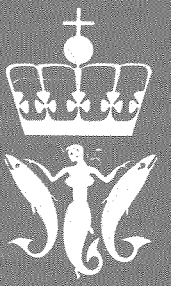

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METHIONINE SULPHOXIDE: FORMATION, OCCURRENCE AND BIOLOGICAL AVAILABILITY

A REVIEW

by

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1. INTRODUCTION

In 1922 Mueller discovered a stable sulphur containing amino acid, different from cysteine (1). Some years later, Barger and Coyne (2) isolated this new amino acid and identified the chemical structure as α-amino-γ-methylthiobutyric acid. They named it methionine because of the characteristic γ-methyl-thiol-group. Methionine was in 1943 found to be necessary for growth in mice (3) and is now known to be an essential amino acid for all higher animals.

Methionine has several biochemical functions in the organisms (Fig. 1), of which the most important is as a building block for natural proteins. Many biological metabolites are synthesized from methionine, and in this respect, S-adenosyl methionine serves as an intermediate (Fig. 1). The sulphur atom may be transferred to serine to form cysteine, and the propylamine residue in methionine may be used for spermine and spermidine synthesis. These diamines bind to DNA and stabilize its structure. Further, the methyl group of methionine may be transferred to produce some 40 different methylated compounds as creatinine, phosphatidylcholine, adrenaline and choline (4). The homocysteine formed in these transmethylation reactions may be used for methionine synthesis in which case choline or 5-methyltetrahydrofolate serves as the methyl donor (Fig. 1). Formate or formaldehyde may be reduced to form the methyl group of 5-methyltetrahydrofolate and so provide for *de novo* synthesis of methyl groups (5). Methionine therefore is not necessarily lost when it participates in transmethylation reactions. This may also be the case in synthesis of the diamines as methionine may be produced from 5'-methylthioadenosine in rat liver (6, 7).

In addition to the above metabolic pathways methionine provides some special functions in the tissues. It is the N-terminal amino acid residue in all protein synthesizing complexes, and so is necessary for the initiation of protein synthesis. The sulphur containing amino acids are the most efficient amino acids to limit the urinaty nitrogen loss when animals are fed protein-free diets, indicating that these amino acids are among the most valuable for the organism (8).

Methionine is of particular interest from a nutritional point of view as it is the limiting amino acid in many foods and feeds, cereals excepted (9). Loss of this amino acid will therefore affect the nutritional quality of the protein. Methionine is easily oxidized to methionine sulphoxide, and to methionine sulphone if strong oxidizing conditions are used (Fig. 2). Methionine

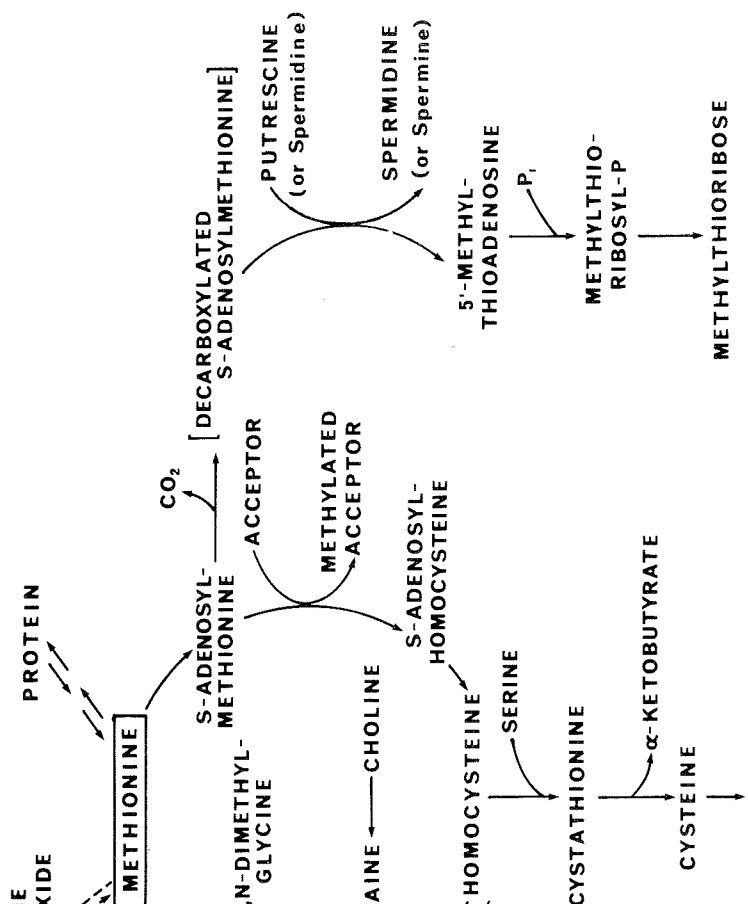


Fig. 1. Metabolic pathways for methionine. (Modified from reference 5).

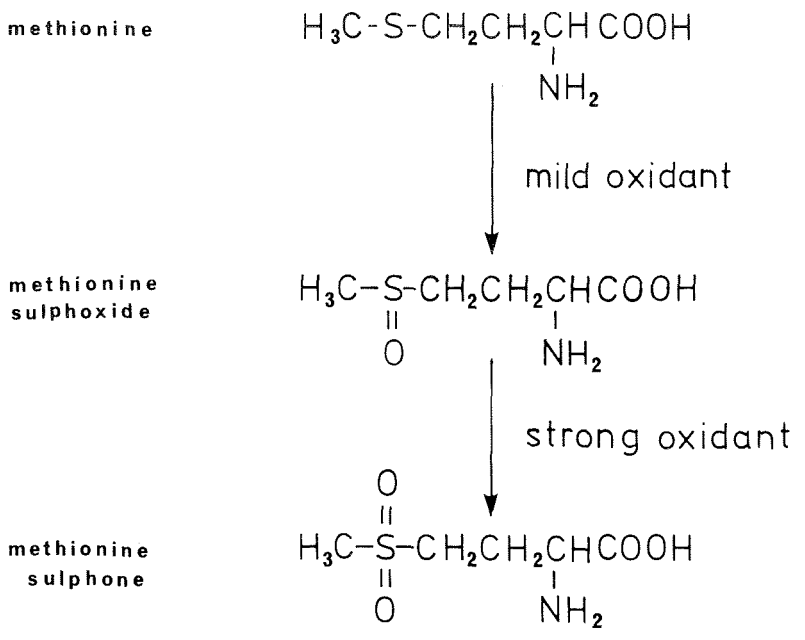


Fig. 2. Chemical structure of methionine, methionine sulphoxide and methionine sulphone.

sulphoxide is found in appreciable amounts in many protein sources. Much work has therefore been done to elucidate the reason for methionine oxidation and to investigate the biological availability of methionine sulphoxide.

It is the aim of this paper to review the work done on methionine sulphoxide, both from a chemical and nutritional point of view.

2. PROPERTIES OF METHIONINE SULPHOXIDE

The sulphur atom in methionine is a weak nucleophile, easily oxidized with formation of methionine sulphoxide (10). Methionine and its sulphoxide have different chemical properties. The pK' values for the amino groups and methionine sulphoxide are 9.2 and 6.7, and for the carboxylic groups 2.3 and 4.3, respectively (11). The sulphoxide group also is more acidic than the thioether group and the isoelectric point change from 5.7 to 4.4 upon oxidation of the amino acid (12, 13). Methionine sulphoxide is more water soluble than the unoxidized amino acid (14) and no insoluble metal complexes is formed as is the case with methionine (15).

There are two asymmetric centers in methionine sulphoxide; one at the α-carbon atom and one at the sulphur atom. The reagent employed for the oxidation determines the proportion of the two diastereomeric sulphoxides formed at the sulphur atom, possibly because of different rates of reaction (16).

The specific rotations in aqueous solution ($[\alpha]_D^{25}$) for L-methionine, L-methionine-l-sulphoxide, and L-methionine-d-sulphoxide are reported to be +14.9°, −71.6° and +99.0°, respectively (16).

Oxidation of protein bound methionine also change the properties of the protein, e.g. changes in the electrophoretic pattern and size of the proteins are found after oxidation (17–19). Furthermore, the biological activities of proteins are lost by oxidation of one or several methionine residues to methionine sulphoxide (20, for references).

Oxidation of methionine to methionine sulphone needs more drastic oxidative conditions. The solubility of this amino acid in water and the isoelectric point are between those of methionine and methionine sulphoxide, and the typical cabbagelike odour of methionine and its sulphoxide is absent in methionine sulphone (12, 21).

3. METHODS FOR THE DETERMINATION OF METHIONINE SULPHOXIDE

The methods for determination of methionine sulphoxide in proteins are often laborious and not suited for routine analyses. The main problem is that methionine sulphoxide is destroyed during acid hydrolysis in 6 N HCl, which is the usual procedure used in the amino acid analyses of proteins (22, 23). Acid hydrolysis results in a reduction of sulphoxide to methionine, but the extent of this reduction is discussed and seems to be a complicated process depending on the presence of haloacids, tryptophan and reducing agents (24–26). It must therefore be assumed that many amino acid analyses have determined methionine sulphoxide as methionine.

The problem may be solved by performing the analysis of methionine sulphoxide on the intact proteins. Thus, Neumann (27) alkylated the oxidized proteins with iodoacetamide or iodoacetic acid. This procedure alkylates sulphur in unoxidized methionine. The sulphoxide is then oxidized by performic acid and the methionine sulphoxide original present in the proteins may be determined indirectly as sulphone after acid hydrolysis. Lunder (28) used acetic anhydride and determined the methionine sulphoxide indirectly by the formaldehyde formed. Determination of methionine sulphoxide in the intact protein can also be carried out using cyanogen bromide (25, 29). This reagent will react with methionine in proteins (but not with oxidized methionine) to form homoserine (plus lactone) residues. After reduction of methionine sulphoxide to methionine and acid hydrolysis, methionine and methionine sulphoxide may be determined as homoserine (and its lactone) and methionine respectively. This method was recently modified by Bos et al. (30) to determine methionine and methionine sulphoxide in foods and feeds. Methionine was determined indirectly after reaction with cyanogen bromide by

measuring the methyl thiocyanate evolved. Methionine sulphoxide was determined similarly after reduction with $TiCl_3$. The cyanogen bromide method determines only protein bound methionine, as the cyanogen bromide does not react with free methionine.

X-ray photoelectron spectroscopy is presented as a possible technique to determine different oxidation states of sulphur in the intact protein (31). By examination of the X-ray emission spectrum the percentage of sulphur in a particular oxidation state can be determined.

Methionine, sulphoxide and sulphone are stable at high pH levels, and determination of these amino acids in proteins therefore may be carried out after hydrolysis at alkaline conditions. Potassium or barium hydroxide is best suited for this purpose as sodium hydroxide gives poor recoveries (32). Chromatography may then be used to separate the different methionine, methionine sulphoxide and methionine sulphone compounds. For instance, paper chromatography at different solvent conditions (22, 33–35) and thin-layer chromatography on cellulose (36) have been used. Direct determination of methionine may also be carried out in alkaline protein hydrolysates using ion-exchange chromatography on a weak anion exchange resin (37), as may the stereoisomers of the sulphoxide (38). This procedure will give complete separation of methionine, sulphoxide and sulphone (36, 39).

An automated colorimetric method, well suited for routine analyses of methionine and methionine sulphoxide in barium hydroxide hydrolysates was presented by Njaa (32). Methionine was measured by its bleaching effect on iodoplatinate and the interference from cysteine was eliminated by addition of cadmium acetate before hydrolysis. No other common amino acid interferred in the assay. Methionine sulphoxide was determined by difference after reduction to methionine in the hydrolysates using $TiCl_3$ as reductant. The advantage of this method is that it determines both methionine and methionine sulphoxide in the same hydrolysate. Methionine sulphone is not determined, however.

4. OXIDANTS FOR THE CONVERSION OF METHIONINE TO METHIONINE SULPHOXIDE

Oxidation of methionine to methionine sulphoxide has been of interest in several connections. The conversion is utilized to protect the sulphinyl group in methionine from reacting during peptide synthesis, and it is a useful technique in sequence analysis to prevent peptide overlapping as methionine sulphoxide is not cleaved in the cyanogen bromide reaction. Further, oxidation of methionine is used for the identification of active sites on enzymes and to prepare free and peptide bound methionine sulphoxide for investigations of biological availability of this amino acid. It is important in all these cases to

obtain a selective oxidation of methionine to methionine sulphoxide, avoiding overoxidation to methionine sulphone, and effects on other amino acids.

The most easily oxidized amino acids, besides methionine, are cysteine/cystine and tryptophan, followed by histidine and tyrosine (27). In contrast to the other nucleophilic groups in amino acid side chains, the sulphinyl group in methionine is not protonated at low pH. This brings about a possible spesific oxidation of this group (27).

Hydrogen peroxide is the most commonly used oxidant for the oxidation of methionine. At low pH (1 to 5) the reaction is rapid (17, 40). At higher pH (5.5–8.0) the reaction is slow and not complete within 48 hours using equivalent amounts of the two reactants at room temperature (41). More alkaline solutions increased the reaction rate (41), and formation of hydroxyl radical, superoxide anion and singlet oxygen from H_2O_2 was postulated to explain this increase (42). Methionine sulphoxide is the only product formed from methionine in acid solutions (17, 40, 42) although Njaa (22) reported a mixture of methionine sulphoxide and methionine sulphone when methionine applied to filter paper in hydrochloric acid solution was treated with H_2O_2. The oxidation of methionine with H_2O_2 is a first order reaction with respect to both reactants (41), and gives a racemic mixture with equal proportions of the two possible diastereomers on the sulphur atom (17). Conversion of free methionine to sulphoxide in the presence of H_2O_2 may be accelerated by Cu^{2+}, iodine and selenite (41) while molybdate addition resulted in methionine sulphone formation (21). No inhibition of the reaction was obtained with other free amino acids containing reactive side chains (41). This investigation was carried out at the pH found in fish extracts and at a composition of the amino acids related to that in fish proteins.

The situation may be different for oxidation of methionine in proteins. Wasi and Hofmann (43) reported, however, that the rate constants for H_2O_2 oxidation of the exposed methionine residue in chymotrypsinogen and α-chymotrypsin was close to that of free methionine. They concluded that the protein *per se* had no influence on the methionine reactivity. Methionine in proteins is more resistant to oxidation as it contains a nonpolar side chain which is often buried in the interior of the protein. This is well documented in the case of chymotrypsinogen which contains one exposed and one shielded methionine residue with quite different reactivities (43).

A pH below 4 and excess of H_2O_2 are the conditions usually employed for the specific oxidation of methionine in proteins (27). At alkaline conditions especially cystine, cysteine and tryptophan may be oxidized (27, 44), and methionine may be converted to the sulphone (39, 45). Exceptions are reported for purified proteins, however (46). In some reports perchloric acid is used to adjust the pH to perform the H_2O_2 oxidation at acidic conditions. This procedure was found to cause overoxidation of methionine as well as attack on

tryptophan and cystine (27). Correspondingly, appreciable amounts of methionine sulphone was found in samples treated with H_2O_2 at acidic conditions in the presence of performic acid (47).

Even at neutral conditions methionine in protein seems to have a greater reactivity than any other amino acid, and at a low H_2O_2 concentration and moderate temperature it is possible specifically to oxidize methionine to methionine sulphoxide in the proteins. This was shown in an experiment by Raksakulthai et al. (48) using minced and cooked fillets of saithe oxidized with H_2O_2. Only methionine was affected and quantitatively converted to methionine sulphoxide. All other amino acids where unaffected by the H_2O_2 treatment which was carried out at pH 6.6 and with H_2O_2 concentrations from half to double the amount of methionine present. This specific oxidation was confirmed by Chang et al. (49) who oxidized casein, egg white and soy isolate with H_2O_2. At low temperature and dilute concentrations of H_2O_2, only methionine sulphoxide was formed. At higher temperature, or at higher H_2O_2 concentrations or both, however, methionine sulphone and cysteic acid were produced. This experiment was carried out at neutral or slightly acidic conditions. Anderson et al. (50) also found appreciable amounts of methionine sulphone when rapeseed flour was oxidized with a great excess of H_2O_2 at neutral conditions.

Iodine was early found to be a powerful oxidant for methionine at both neutral and acidic conditions (17). Methionine sulphoxide was the ultimate product and the reaction mechanism took place with dehydromethionine as an intermediate (51, 52). Iodine was a more powerful oxidant for methionine than H_2O_2, and a catalytic effect of iodide in the H_2O_2 oxidation of methionine seemed possible. Iodide was oxidized by H_2O_2 to iodine which in turn would oxidize methionine. The net result was a faster oxidation of methionine (41). Tryptophan was reported to inhibit the oxidation of methionine caused by iodide, while tyrosine, histidine, phenylalanine, serine and threonine were without effect (41).

Less is known about the iodine reaction on proteins, although it is used to modify tyrosine at neutral or alkaline conditions. Rosenberg and Murray found that tyrosine inhibited the methionine oxidation caused by iodine at slightly alkaline conditions (53). In an experiment performed to gather information on factors that might influence oxidation of methionine to methionine sulphoxide in foods and feeds, iodine did not oxidize methionine in casein or in fish fillet proteins. Neither was iodide a catalyst for the H_2O_2 oxidation of methionine in these protein sources at slightly acidic conditions (41).

Periodate is a reagent known to oxidize several amino acids and carbohydrates (27, 54). However, when the reaction was performed under certain specified conditions, e.g. pH 5.0 and low temperature, or with low concentration of periodate at both moderate acidic or alkaline conditions, specific oxidation of

methionine to methionine sulphoxide could be obtained, leaving tyrosine, tryptophan, histidine and phenylalanine unaffected (55). The possibility for an overoxidation of methionine to methionine sulphone exists, however, as reported by Knowles (56).

Several reagents containing positive halogens have been used for the selective oxidation of methionine sulphoxide (24). Mostly used are N-bromosuccinimide, N-chlorosuccinimide and chloramine-T (sodium p-toluene-sulphone-chloramide). O'Brien (57) reported that methionine and tryptophan, and to a lesser extent, tyrosine and cysteine in ferricytochrome c were modified at moderate conditions with N-bromosuccinimide at pH 4 and 8.5. Chloramine-T at more severe conditions resulted in methionine sulphone formation as well as deamination (17, 25, 53). In a study comparing the specificities of compounds containing positive halogens, Shechter et al. (25) found that chloramine-T was the most specific, oxidizing only methionine and cysteine whereas N-chlorosuccinimide also oxidized tryptophan. N-bromosuccinimide was the least specific as it also was found to oxidize tyrosine, histidine and cysteine residues. This study was carried out at pH 7.5–8.5 and at mildly oxidizing conditions.

Oxygen dependent oxidants, including singlet oxygen, superoxide anion and hydroxyl radical are very powerful oxidants with low specificities. Singlet oxygen produced by dye-sensitive photooxidation in neutral or alkaline conditions is found to affect free and protein bound histidine, tryptophan and methionine (58–60). Methionine was quantitatively oxidized to methionine sulphoxide, and both exposed and buried amino acids in the protein were accessible to reaction with singlet oxygen (59). Methylene blue mediated photooxidation of cytochrome c at acidic conditions was reported to affect only methionine which was oxidized to methionine sulphoxide (61).

Hydroxyl radical will also oxidize methionine. This reagent will, however, not only attack the sulphur atom but also the α-amino group and in consequence, several degradation products from methionine are reported (62, 63). It is not clear whether superoxide anion is a potent oxidant for methionine.

Free methionine will be oxidized in solutions containing ascorbic acid at pH 6.0. The reaction mechanism is complex, involving hydroxyl radical and possibly singlet oxygen, resulting in methionine sulphoxide formation as well as deamination and demethylation reactions (64). Peptides containing methionine were oxidized to a much lower extent and neither loss of methionine, nor methionine sulphoxide were found when casein or ovalbumin were present in solutions containing autooxidizing ascorbic acid.

Lipton and Bodwell (65) reported a reversible conversion of methionine to methionine sulphoxide using dimethylsulphoxide as oxidant in the presence of a hydrogen halide. The reaction had to be carried out in strongly acidic solutions and cysteine as well as methionine were affected. A stereospecific

oxidation of methionine at neutral or acidic conditions has been obtained using gold (III) as an oxidant (66). Only one of the two possible diastereomers of methionine sulphoxide was formed but nothing was reported on the effects on other amino acids.

5. CONTENT OF METHIONINE SULPHOXIDE IN PROTEINS

As mentioned above, methionine sulphoxide cannot be determined in the usual acid hydrolysates of proteins. Moreover, as methionine is easily oxidized it is possible that any methionine sulphoxide detected was not present in the original sample, but was formed during handling of the protein (67). However, methionine sulphoxide has been detected in proteins, especially in protein sources used for foods and feeds.

The highest content of methionine sulphoxide in natural unprocessed proteins is reported by Kikuchi and Tamiya (68). They found only methionine sulphoxide and no methionine in the resilium protein of surf clams. The content of methionine sulphoxide was very high, corresponding to 12–15 mol percentages of the total amino acid content. The analysis of sulphoxide was verified as the resilium protein resisted cyanogen bromide treatment and hydrolysis in 6 N HCl resulted in detection of methionine. Ligament proteins of other bivalves contained both methionine and methionine sulphoxide with contents of the latter from 6 to 34 % of total methionine.

In an extract from red algae, Miyazawa and Ito (69) also found only methionine sulphoxide and no methionine. This is the only work where the stereoisomeric form of the sulphoxide has been studied, and it is of interest that only L-methionine-l-sulphoxide and no L-methionine-d-sulphoxide was detected.

Appreciable amounts of oxidized methionine in proteins from higher animals are reported for collagen and basement membranes where as much as 30 % of total methionine appeared as methionine sulphoxide (70, 71). A special case is the lense capsule protein from cataracts where 75 % of total methionine were found as methionine sulphoxide (72). This is not a characteristic tissue, however, as no recycling of macromolecules exists. No sulphoxide was found in normal lenses.

The contents of methionine sulphoxide in protein sources used for foods and feeds usually vary from 0 to 30 % of the total methionine content (Table 1). In some special products, for which severe handling or oxidative treatments were performed, much higher values were obtained as is the case with Promine D (Table 1). The values also vary for the same protein source, probably because of different conditions during storage and processing (see soy bean meal, fish protein concentrate and gelatin in Table 1).

Table 1. Content of methionine sulphoxide in some foods and feeds (percentage of total methionine).

Protein source	Methionine sulphoxide	Reference
Soy bean meal	0–10	30
	26	32
	12–80	73
	51	74
	59	75
Promine D	82	30
	~100	32
Wheat gluten	28	30
	16	32
Ground nut meal	33	32
Alfalfa	19	74
Textured vegetable protein	47	75
Single cell protein	26	32
Green peas	18	32
Casein	15	32
Milk powder	11	32
Gelatin	0	30
	~100	32
Fish protein concentrate	4–50	32,76
	12	45
Lyophilized fish	4–42	77
Lean beef	13	75
Milk-based infant formula	10	73
Soy based infant formula	16	73
Orange juice	ca. 50	78

Methionine sulphone has not been detected in appreciable amounts in processed proteins, although exceptions exist (73).

6. METHIONINE SULPHOXIDE FORMATION IN FOODS AND FEEDS

6.1. *Addition of oxidative agents*

Several potential oxidants for methionine may be added to protein sources for various purposes. The most frequently used is hydrogen peroxide which is employed for the sterilization and preservation of milk (79–81), for the bleaching of fish flesh, fish protein concentrate and milk products (82–84), to destroy glucosinolates in rapeseed flour (50) and to improve the functional properties of protein sources (85, 86).

As pointed out in chapter 4, methionine in proteins may be selectively and quantitatively oxidized to methionine sulphoxide at low temperature, low

concentration of H_2O_2 and in neutral or acidic conditions. It must be stressed that even low levels of H_2O_2 effectively oxidize methionine. For example, equivalent amounts of H_2O_2 and methionine in cooked, minced saithe fillets will oxidize more than half of the methionine present at slightly acidic conditions at room temperature (48). Model systems may not be useful in investigations of H_2O_2 oxidation of methionine as substances present in the sample may exhibit both stimulatory and inhibitory effects. For instance, Boonvisut et al. (41) found that selenite accelerated, and iodide inhibited the H_2O_2 oxidation of methionine in casein and fish fillets proteins.

Sulphite is used as a bleaching agent in food processing (87) and autooxidizing sulphite is reported effectively to oxidize free methionine to methionine sulphoxide in the presence of Mn^{2+} and oxygen at neutral pH (48, 88, 89). In a study of sulphite adddition to fish fillets, no oxidation of methionine or any other amino acid was observed even though a bleaching effect was obtained (48). The result is explained by a possible reduction of methionine sulphoxide under the conditions used, as hydrogen sulphite has also been reported as a powerful reducing agent for the conversion of methionine sulphoxide to methionine (11).

Ascorbic acid may be used as an antioxidant in fish fillet products (90, for references) and to improve the baking characteristics of wheat flour (91). Under certain circumstances, however, ascorbic acid may act as a prooxidant and indeed increase the oxidative rate in the material (90). The shift from an antioxidative to a prooxidative property depends on the concentration of ascorbic acid. Effects of ascorbic acid on free and protein bound methionine were reported by Aksnes and Njaa (64) who found that ascorbic acid easily autooxidized at pH 6.0 and rapidly oxidized free methionine. Peptide bound methionine was affected to a much lesser extent and purified proteins were not affected at all.

6.2 *Formation of methionine sulphoxide during storage and processing.*

Several enzymatic reactions produce potent oxidants for methionine *in vivo*. These include hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen (92). The amounts of these oxidants are kept low by the enzymes catalase, glutathione peroxidase and superoxide dismutase, respectively. Enzymatic oxidation of methionine may also occur through the action of cytochrome P-450, a nonspecific monooxygenase which was found to oxidize thioethers (93) or of dopamine-β-hydroxylase which oxidized sulphinyl groups to the corresponding sulphoxides (94). Doney and Thompson (95) looked for an enzyme in turnip leaves which would oxidize methionine to methionine sulphoxide. They concluded, however, that methionine sulphoxide was very easily formed, but that the reaction was nonenzymatic. Any methionine

sulphoxide formed *in vivo* may possibly be enzymatically reduced to methionine as a peptide methionine sulphoxide reductase is said to be present in various tissues (chapter 8).

Appreciable amounts of methionine sulphoxide may be formed during storage of biological materials, however, and Aksnes and Njaa (77) found as much as 40 % of the total methionine contents as sulphoxide in whole fish kept at −20° C for several months. There were differences among species and the content of sulphoxide correlated with the levels of glutathione peroxidase and superoxide dismutase activities in the fresh fish, indicating that high levels of these enzymes point to higher oxidation potentials in these fishes.

Autooxidizing unsaturated fatty acids are quantitatively the most important potential oxidants for methionine in stored biological material, and as the side chain of methionine is hydrophobic, the thioether group may be solubilized in the lipid layer. Hence, lipid soluble antioxidants were found to have the highest inhibitory effect on methionine oxidation in lyophilized saithe stored for two months at 30° C and high humidity (96). Scavengers for singlet oxygen (histidine and carnosine), or for hydroxyl radical (benzoic acid) and further bacteriostatics as sodium azid, antibiotics and sodium nitrite plus formaldehyde did not inhibit the oxidation.

In a model systems where cytochrome c was exposed to peroxidizing linolenic acid, histidine was found to be most affected, followed by serine, proline, valine, arginine, methionine and cystine (97). The experiment was carried out with excess of linolenic acid and hydrolysis showed that 70 % of the fatty acid was bound to the protein. The analysis was performed after acid hydrolysis and any methionine sulphoxide present would not have been detected. Njaa et al. (98) investigated the antioxidative property of methionine compounds in a semisynthetic diet containing autooxidizing cod liver oil. In this system, containing 12 % of fat, methionine esters acted as antioxidants, and were at the same time converted to the corresponding sulphoxide.

Formation of methionine sulphoxide in contact with autooxidizing lipids was also indicated in a model system investigated by Tannenbaum et al. (99). Enzymatic hydrolysis showed that about 80 % of the original methionine present in casein was lost during storage for 113 days. This loss of methionine was partially regained after acid hydrolysis and this was taken as evidence for the presence of methionine sulphoxide. The decrease of methionine was proportional to the nonenzymatic browning of the sample, and the authors postulated that hydroperoxides formed during autooxidation of methyl linoleate reacted with methionine to form carbonyl compounds and methionine sulphoxide, respectively. The former could then react with other sites on the protein to produce brown pigments. An alternative explanation for the damaging of proteins, involving lipid radicals was postulated (100).

Tufte and Warthesen demonstrated that free methionine was quantitatively

oxidized to methionine sulphoxide in the presence of stripped corn oil, whereas the oxidation of free methionine was very slow in the presence of proteins (101). Methionine sulphone was not detected, nor any Maillard products. Even in the absence of proteins H_2O_2 is a much more effective oxidant for methionine than various hydroperoxides (102).

The experiments referred to above were performed in model systems, with addition of lipids. The oxidation of methionine in natural protein sources, caused by lipids present in the material, was recently discussed by Gulbrandsen et al. (103). In this study fish meal was prepared from mackerel because of a high content of unsaturated fatty acids in this fish species, and the oxidation of lipids and methionine was measured over time. In the presence of a lipid soluble antioxidant, no oxidation of lipids or methionine occurred during storage for two years. In the absence of an antioxidant, there was no substantial oxidation of methionine or lipids during the first month of storage. After this time, however, accelerating oxidation of both took place with lipid oxidation in advance of methionine oxidation. The oxidations were completed within one year of storage, when about 20 % of the total methionine content was present as methionine sulphoxide.

Methionine is not oxidized appreciably during processing of foods and feeds. In the experiment referred to above, only about 5 % of the total methionine content appeared as methionine sulphoxide immediately after the fish meal processing, involving heating to about 90° C, evaporation and steam drying (103). This is in accordance with the results of Marshall et al. (74) who studied the effect on sulphur amino acids during processing of legume proteins at various conditions. Processing of peanuts, dry-beans, alfalfa, and soy bean did not oxidize methionine, but in some cases methionine sulphoxide was present in the raw materials. Likewise, emulsification and cooking/smoking of frankfurters did not affect the methionine content (104), neither did tanning of milk powder or drying of fish offal (105).

In some special materials, however, methionine sulphoxide may be much more easily formed. This was the case in processing silk worm chrysalid protein in which water washing resulted in substantial amounts of methionine sulphoxide (106).

7. BIOLOGICAL AVAILABILITY OF OXIDIZED METHIONINE IN ANIMALS

Substantial levels of methionine sulphoxide may be present in protein sources used as nourishment for animals and humans (Table 1, chapter 5), and much work on the nutritional availability of this amino acid has been carried out. These results may be difficult to interprete, as other amino acids than

methionine may have been affected in the test sample, and methionine sulphone as well as methionine sulphoxide may have been present. Furthermore, different methods, test animals, isomers of the sulphoxide and the use of free and peptide bound methionine sulphoxide complicate the interpretations.

The biological availability of methionine sulphoxide is the net result of digestion of the oxidized protein in the intestine, absorption of methionine sulphoxide from the intestine and the ultimate utilization or metabolism in the body. These steps will be discussed in turn.

7.1. *Digestibility (proteolytic cleavage) of proteins containing oxidized methionine residues.*

The digestibility of oxidized proteins containing methionine sulphoxide residues has been determined by *in vitro* as well as *in vivo* methods. Cuq et al. (107) analyzed the free amino acids liberated from H_2O_2 treated casein after incubation with the broad spectered protease, Pronase. No free methionine sulphoxide was detected after the proteolysis. The authors concluded that Pronase which acts on methionyl peptide bonds, cannot split the same bond after oxidation of methionine to methionine sulphoxide. In a later study, however, using pepsin/pancreatin or proteases from *Streptomyces griseus,* Cuq et al. (39) found a 20 % release of methionine sulphoxide from oxidized casein compared with the methionine released after proteolysis of untreated casein. In both the above studies, the overall digestibility of casein, as measured by the release of all the other amino acids, remained unchanged after the H_2O_2 treatment. Similarly, in a rapid three enzyme *in vitro* technique (peptidase, trypsin, and α-chymotrypsin), Raksakulthai et al. (48) found no difference in the pH changes upon hydrolysis of saithe fillets proteins containing from 3 to 75 % of the total methionine residues as methionine sulphoxide.

Liberation of methionine sulphone from performic acid oxidized proteins was slower than the measured release of methionine from untreated protein (108) although the liberation was faster than that reported by Cuq et al. for the release of methionine sulphoxide (39). Different proteolytic enzymes were used in these studies, however.

A huge diversity of different proteolytic enzymes with both intermembrane, extracellular and intracellular locations exist in the intestine (109), and the *in vitro* methods for determination of protein digestibility may not be equivalent to the digestibility *in vivo*. Thus, *Escherichia coli* will cleave small peptides containing methionine sulphoxide (110) and this seems also to be the case for higher animals. Raksakulthai et al. (48) observed a tendency to a slightly lower digestibility in rat experiments in the samples with the highest levels of methionine sulphoxide. No significant difference in digestibility in the rat was found, however, between untreated and oxidized casein (39, 47), in silk worm

chrysalid proteins containing 37 % or 80 % of total methionine as methionine sulphoxide (106) or in fish proteins where either none or all methionine residues were present as methionine or methionine sulphone (45, 47).
residues were present as methionine sulphoxide or methionine sulphone (45,47).

7.2. *Intestinal absorption of methionine sulphoxide.*

Studies on the intestinal absorption of methionine sulphoxide have shown partly conflicting results. Higuchi et al. (111) studied the *in vitro* and *in situ* absorption of methionine and methionine sulphoxide in rat small intestine at 5 mM substrate levels. They found no difference in the absorption rate or in the kinetic parameters for the two amino acids. The absorption of sulphoxide was inhibited by leucine and methionine, while the basic amino acids, histidine and lysine and the acidic amino acids, glutamic acid and aspartic acid did not inhibit the sulphoxide absorption. They concluded that the absorption mechanism did not change upon oxidation of methionine and that the absorption efficiency of methionine sulphoxide would not reduce the nutritional availability of this amino acid. This latter conclusion was also drawn by Aksnes and Njaa (112) although they found a slower absorption of the sulphoxide than of methionine at low physiological concentrations of the amino acids. At higher concentrations (5 mM) or if glutathione or cysteine was present, no difference in absorption rates were observed. Different absorption mechanisms for the two amino acids were indicated, however, as neither of the two amino acids inhibited the absorption rate of the other. Finot et al. (113) also reported a slower intestinal absorption of sulphoxide compared to methionine, but no details of methods or results were given. In the rabbit, the transport of methionine sulphoxide into purified intestinal brush border membrane vesicles was found to be an active and Na^+-dependent process (34). The transport of methionine sulphoxide was inhibited by both acidic and basic amino acids in addition to methionine. In studying the absorption of methionine in rat intestine, Sugawa et al. (114) observed an accumulation of methionine sulphoxide in the intestinal tissue. They postulated that methionine was oxidized during absorption and was again reduced in the portal circulation, and that this oxido-reduction of methionine was coupled with its mechanism of absorption. No comparison between methionine and methionine sulphoxide absorption was made in this study.

To complete the confused picture of the mechanism for methionine sulphoxide absorption, transport of sulphoxide in bacteria was found to take place by two different mechanisms; the high-affinity methionine transport system and the glutamine transport system. The low-affinity methionine transport system did not transport methionine sulphoxide (115).

The rate and the mechanism of methionine sulphoxide absorption are still disputed, but there is agreement that the absorption rate is fast enough to

obtain complete absorption of methionine sulphoxide. This can be explained by the fast absorption of methionine compared with other amino acids (109). A reduced rate of transport relative to this amino acid may make the absorption rate of the sulphoxide comparable with that of other amino acids. A near complete absorption of free methionine sulphoxide also was confirmed in analyses of intestinal contents of rats given a synthetic amino acid diet containing methionine sulphoxide (116) or a diet containing H_2O_2 treated fish protein (45).

The main absorption of amino acids from proteins takes place as di- and tri-peptides with ultimate proteolysis to the free amino acids. Nothing is known about the absorption of peptides containing methionine sulphoxide, but as it is suggested that neutral, acidic and basic peptides share a common transport mechanism (109) it is assumed that peptides containing methionine sulphoxide will be absorbed in the intestine. Cleavage of the peptide bonds by the mucosa cells also is indicated as blood plasma from rats given oxidized proteins contain high levels of free methionine sulphoxide (39, 45, 117, 118).

7.3. *Biological utilization of methionine sulphone.*

All studies on the biological utilization of methionine sulphone conclude that this amino acid cannot be utilized as a source of methionine. This was first demonstrated by Bennett (119) who found no net positive or negative effect on the growth of rats fed a methionine deficient diet supplemented with methionine sulphone. This was later verified by Tsuchiya (120) and by Anderson et al. (121). Njaa (122) showed that methionine sulphone did not improve the nitrogen balance in rats given a soy-bean diet and therefore was unavailable to the rat. This was later confirmed by nitrogen balance experiments on rats given untreated and oxidized fish protein supplement with methionine sulphone (45). Similar results were obtained for the growing chick (123).

From these reports one must conclude that methionine sulphone cannot be utilized as a source of methionine. On the other hand, as no negative effect on growth or nitrogen balance were seen, methionine sulphone does not seem to have any noxious effect, even if this was indicated by the results obtained by Miller and Samuel (124) who found 11 % decrease in the net protein utilization of casein supplemented with methionine sulphone.

Most of the methionine sulphone given to rats is excreted in the urine. Smith (125) regained 55 % of the intraperitoneally injected methionine sulphone in the urine after 24 hours, and most of this was identified as N-acetyl-methionine sulphone. A similar percentage of consumed methionine sulphone was excreted in the urine and feces in an experiment by Sjøberg and Bostrøm (45). The main portion was found in the urine as an acid-labile compound.

7.4. *Biological utilization of methionine sulphoxide.*

As early as in 1939, Bennett (126) reported that methionine sulphoxide was equivalent to methionine in the rat, as supplementation of these two amino acids to a methionine deficient diet resulted in identical growth curves. In spite of this, some recent papers still state that methionine sulphoxide is unavailable as a source of methionine (65, 127). One of the reasons for this may be the bewildering results later obtained for the utilization of methionine sulphoxide. This section reviews the work done on biological utilization of methionine sulphoxide, and for clarity the section discusses firstly those studies using free and secondly those using peptide bound methionine sulphoxide in the biological assays.

7.4.1. Utilization of free methionine sulphoxide.

All experiments which use synthetic amino acids and L-methionine sulphoxide as the only sulphur containing amino acid in the diets, agree that methionine sulphoxide is utilized, but not as well as methionine in the rat. Among these, Miller et al. (128), Anderson et al. (121) and Gjøen and Njaa (117) found that rats given methionine sulphoxide attained about 66, 60 and 40 %, respectively, of the weight gains obtained for rats given methionine, although the feed efficiencies were somewhat higher. Feeding rats diets containing methionine sulphoxide also resulted in increased «methionine activity» (measured microbiologically) in the blood plasma (117, 128) which by chromatography was identified as methionine sulphoxide (117, 121). The content of sulphoxide in plasma increased with the level of this amino acid in the diets (117, 121).

Exchanging one half of the methionine sulphoxide content in the diet with methionine, resulted in equal growth and feed efficiency in this group compared with rats given only methionine, but the «methionine activity» in plasma temporarily increased. The rats were fed for 17 and 132 days (128). Correspondingly, when one third of the sulphur containing amino acids was given as cystine, no difference was found between the utilization of methionine and its sulphoxide in the rat (117).

Carcass analysis of rats given a synthetic amino acid diet containing methionine sulphoxide and cystine as the sulphur containing amino acids, proved that methionine sulphoxide is reduced and utilized as a source of methionine. This conclusion was drawn as 15 % of the ingested sulphoxide was incorporated as methionine into the carcasses of the rats. The total protein and methionine contents were more than doubled in this experiment (116). Compared with rats given methionine and cystine as the sulphur containing amino acids, the rats grew at the same rate for the first 14 days whereafter the methionine group grew faster. This latter group also deposited more fat, but

the difference between the lipid extracts accounted for only 25 % of the difference in the weight gains. The rest of the weight gain was accounted for by differences in the protein and the water contents (116).

Njaa and Aksnes (129) used the nitrogen sparing effect of the sulphur containing amino acids to assess any difference in the utilization of free methionine and its sulphoxide. In this experiment, the L-methionine sulphoxide was as potent as L-methionine in decreasing the urinary nitrogen loss in rats given a protein free diet, while the D-methionine sulphoxide was slightly less active. It was concluded that in such a short-term experiment, cystine was the key substance in the nitrogen sparing effect, and the authors argued therefore that methionine sulphoxide was reduced to methionine and metabolized to cystine. The rats in this experiment lost weight, and the situation is different from that during growth. This could possibly explain the different results obtained for utilization of free methionine sulphoxide in the absence of methionine or cystine at the two different conditions.

7.4.2. Utilization of free methionine sulphoxide in supplementation experiments with diets deficient in sulphur amino acids.

Different protein sources and different stereoisomers of the sulphoxide have been used in supplementation experiments to investigate the utilization of methionine sulphoxide, and the results obtained are not conclusive. Using DL-methionine sulphoxide Bennett (126) found it to be fully equivalent with DL-methionine to attain growth in rat fed a diet containing arachin as the protein source. This contrasts results later obtained by nitrogen balance experiments, which showed that DL-methionine sulphoxide was utilized only 75 % as efficiently as DL-methionine in supplementing soy bean protein (122). Further, Miller and Samuel (124) found that net protein utilization (NPU) was increased by only 6 % with DL-methionine sulphoxide supplementation to casein while DL-methionine increased the NPU by 25 %. On the other hand, L-methionine sulphoxide was as well utilized as L-methionine in rat measured by growth experiments (45, 117, 120) and by NPU (122), while D-methionine sulphoxide showed even poorer utilization than the DL-isomer (122).

Different utilization of the various stereoisomers of the sulphoxide is also reported for weight gain experiments with the growing chick (123), where the L-methionine sulphoxide was utilized only 77 % as efficiently as L-methionine. DL- and D-methionine sulphoxide were 59 % and 52 % as effectively utilized as the corresponding methionine isomers, respectively.

7.4.3. Utilization of peptide bound methionine sulphoxide.

As pointed out in chapter 4, oxidation of protein bound methionine may result in methionine sulphone formation and to destruction of other amino

acids. Great caution therefore is needed in interpreting biological assays of oxidized proteins. Relative to methionine, Rasekh et al. (83) found a reduced protein efficiency ratio (PER) for rats fed fish protein concentrates treated at elevated temperatures with a great excess of H_2O_2. Near equivalent amounts did not decrease the PER appreciable. The authors concluded that the decrease in protein quality possibly was caused by the destruction of cysteine and formation of methionine sulphoxide. However, as the amino acid analysis on acid hydrolysates showed reduced amount of methionine, the loss in PER was most likely caused by the presence of methionine sulphone in the oxidized protein. A decrease in tyrosine, tryptophan and histidine was found as well. Slump and Schreuder (47) reported decreased NPU in oxidized casein and fish protein. The oxidized samples contained appreciable amounts of methionine sulphoxide but also methionine sulphone and cysteic acid. The presence of the latter compounds was interpreted as the reason for the reduced NPU, while the peptide bound methionine sulphoxide was stated to be equivalent to peptide bound methionine. Similarly, nitrogen balance and growth experiments on rats fed H_2O_2 oxidized fish protein indicated a small decrease in nutritional value (45). This decrease was partly due to the formation of methionine sulphone and cysteic acid, while peptide bound methionine sulphoxide was regarded as fully available to the rat. The H_2O_2 treatment was carried out at pH 8.5 and 50° C and the content of tryptophan, histidine and lysine were reduced which also could have affected the biological availability.

Aksnes and Njaa (130) recently found a fairly good correlation between the methionine sulphoxide content and the protein quality of fish meals as measured by weight gain, PER, NPU and digestibility on rats. The protein quality parameters were not improved, however, by the addition of methionine to the diets, and the authors concluded that decreased protein digestibility rather than the content of methionine sulphoxide might be the reason for the decreased protein quality.

Some authors plead to have used oxidized proteins in the biological assay in which methionine was specifically oxidized to methionine sulphoxide. This include the work by Ellinger and Palmer (131) who oxidized casein by the method of Toennies and Kolb (14) using H_2O_2 in a HCl/methanol medium. Methionine was still the limiting amino acid after oxidation and the NPU of casein decreased from 71 to 58 upon oxidation. This is in conflict with the results later obtained by Gjøen and Njaa (117) and Cuq et al. (39) where the biological availability of methionine sulphoxide in «specifically» oxidized fish meal and casein was found to be only slightly lower than the unoxidized controls. These oxidized protein sources did not contain methionine sulphone. Gjøen and Njaa (117) used a method for the oxidation of the fish meal which earlier was reported to produce some cysteic acid (47) and the presence of this compound may have affected the protein quality. Furthermore, the differences

in NPU and PER between the oxidized and untreated casein observed by Cuq et al. was quite small and not always significant, especially for equal food intakes (39). Oxidized fish protein was also found to be equivalent to the unoxidized control in supporting growth in chicks (132).

Even when methionine is not selectively oxidized to the sulphoxide in the protein, utilization of the sulphoxide may be indicated by a total balance between ingested and excreted methionine sulphoxide. Sjøberg and Bostrøm (45) showed that less than 3 % of the ingested methionine sulphoxide from oxidized fish protein was recovered in urin and feces. Likewise, in a preliminary study in humans of methionine sulphoxide utilization from oxidized soy isolates, the total urinary sulphoxide excretion was only a small fraction of the totally ingested amount of this amino acid (118). These studies show that methionine sulphoxide is metabolized in rats and humans, but do not prove its utilization as a source of methionine.

Protein quality assays of proteins which have not been oxidized by addition of oxidants, but which contain appreciable amounts of methionine sulphoxide also indicate a good utilization of this amino acid. So, Njaa and Lied (76) found no correlation between available methionine measured on chicks and the content of methionine sulphoxide in 16 different fish protein concentrates. Gulbrandsen et al. (103) observed no decrease in available methionine for chicks in a fish meal with little sulphoxide (5 %) and in a corresponding meal where fatty acid autooxidation had resulted in a methionine sulphoxide content of about 20 % of the total methionine. In a study by Lin et al. (106) water washing of silk worm chrysalid protein resulted in 80 % methionine sulphoxide. As measured by PER, NPU and biological value, however, this water washing procedure only slightly affected the protein quality.

Summing up, most biological assays carried out with oxidized proteins conclude that methionine sulphoxide is as well, or nearly as well utilized as methionine. It must be pointed out, however, that because of the possible formation of other products of oxidation than methionine sulphoxide, a lower rather than a higher availability should be expected. A good availability of the sulphoxide is supported by experiments with free amino acids when cystine or methionine is present in the diet. In the absence of these amino acids the utilization of methionine sulphoxide is poor, possibly because cysteine participates in the reduction of the sulphoxide to methionine. That this reduction occurs, is confirmed in total carcass analysis, although this study does not tell to what extent methionine sulphoxide is utilized. The supplementation experiments indicate a stereospecific utilization of methionine sulphoxide as DL- and to a greater extent D-methionine sulphoxide was less well utilized than the L-isomer.

Most studies have been carried out on rats, but experiments with chicks seem to agree that methionine sulphoxide is utilized as a source of methionine in this species, as does the one preliminary study carried out in humans.

8. REDUCTION OF METHIONINE SULPHOXIDE

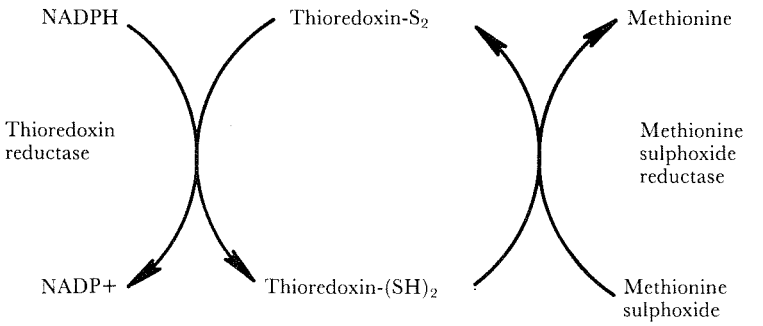
From chapter 7.4., it is clear that methionine sulphoxide is used as a source of methionine in higher animals, even if it may be discussed to what extent it is utilized. The key enzymes in the metabolism of methionine cannot accept methionine sulphoxide as a substrate. Thus, methionine adenosyltransferase was found not to transform methionine sulphoxide to S-adenosylmethionine (133). The sulphoxide further was fully inactive as a substrate in the exchange reaction involving methionyl t-RNA synthetase, the first step in the protein synthesis (134). Methionine sulphoxide must therefore be reduced to methionine before utilization in the body.

Chemical reduction of free and protein bound methionine sulphoxide is obtained by mildly reducing agents as bisulphite, cysteine and other thiol compounds (reviewed in 135). The reduction proceeds readily at room temperature, near neutral pH, but with excess of reductants.

Biological reduction of methionine sulphoxide to methionine has been demonstrated in bacteria, yeast and plant leaves, and purified enzyme systems have been obtained. In 1953 Sourkes and Trano (136) reported that about 90 % of L-methionine-l-sulphoxide was reduced to methionine within 20 min by *E. coli,* while the other stereoisomers from sulphoxide were less effectively reduced. It was pointed out that the configuration around the sulphur atom was more important than that around the α-carbon atom. Stereospecific reduction of methionine sulphoxide was later confirmed in other studies. So, Tuffnell and Payne (137) found that sulphoxide prepared from DL-methionine was reduced at a rate only half of that of L-methionine sulphoxide in *E. coli,* and Aksnes (116) found L-methionine-d-sulphoxide completely inactive for the reduction to methionine while the L-methionine-l-sulphoxide was readily reduced. The sulphoxide is taken up by the bacteria, reduced to methionine and thereafter secreted to the environment, followed by later re-absorption (116, 137). This peculiar situation indicates that the intestinal flora may be of importance for the utilization of methionine sulphoxide in higher animals. However, a 90 % reduction of the intestinal flora by antibiotics did not change the relative composition of methionine and methionine sulphoxide in the intestinal content. (116). Reduction of the sulphoxide also took place in oral microorganisms (35). The reductive system in *E. coli* was partially purified by Ejiri et al. (138, 139) and was found to be a multienzyme system requiring NADPH as reductant. This *in vitro* system was stable towards heat and showed no specifity for different configurations around the sulphur atom. The apparent K_m for the sulphoxide appeared to be about 3×10^{-7} M.

Yeast and plant leaves will also reduce methionine sulphoxide to methionine (95, 140, 141). A multienzyme system responsible for the reduction was purified from yeast. The system utilized only L-methionine-l-sulphoxide as a substrate, and had an absolute requirement for NADPH (140). The

multienzyme system for yeast was later shown to consist of thioredoxin and thioredoxin reductase which functioned as hydrogen carriers in the reduction of methionine sulphoxide (141). The following reaction mechanism was postulated:



All the assays referred to above for the reduction of methionine sulphoxide were carried out with a good yield of methionine when near equivalent amounts of the sulphoxide and reductants were used. This is not the case in assays performed to demonstrate methionine sulphoxide reduction in higher animals. Even with great excess of reductants, yields well below 10 % are usually obtained. Two different systems for reduction of sulphoxide are said to be found in animals, one reducing free and one reducing peptide bound methionine sulphoxide. The most important from a nutritional point of view, is a sulphoxide reductase which uses the free amino acid as substrate. This was first described by Aymard et al. (36). They used an assay for detecting the reductase activity which involved four times excess of reductants with respect to the sulphoxide, long incubation period (3 hours) at 25° C, and great amounts of crude extracts from rat kidney and liver (3–25 mg protein in 1.5–1.8 ml). The yields were very low; 0.9–3.7 % of the initial amounts methionine sulphoxide. In contrast to the systems from yeast and bacteria, the enzyme was thermolabile and NADH was found to be a better substrate than NADPH. A better yield was obtained by Ganapathy et al. (34) in rabbit tissues. The incubation lasted for 3 hours at 37° C with 4.5 times excess of reductants and methionine was detected by paper chromatography. The highest reductase activity was found in liver where 60–90 % of the initial amount of sulphoxide (22 nmol) appeared as methionine. High amounts of crude extracts were used (2–3 mg in 0.1 ml), however, and methionine was detected also without added reductant, at levels up to 15 % of the initial amount of sulphoxide. In the rabbit, NADPH gave a faster reduction than NADH. Using perfused rat liver, Finot et al. (113) observed such a low rate for methionine sulphoxide reduction that the authors concluded that the reduction of methionine sulphoxide had to take place in other tissues, although one of the authors later, with reference to

this first paper, stated that the sulphoxide was rapidly reduced (142). Aksnes (116) could not detect any reduction of methionine sulphoxide after incubation for 24 hours in the presence of extracts from various tissues from rats which had been given methionine sulphoxide. Several reductants were tried at different pH and conditions.

Methionine in peptides and proteins is to some extent oxidized *in vivo* and may then be associated with loss of biological activity (20). To restore these oxidized biomolecules, an enzyme was found which reduces peptide bound methionine sulphoxide. This was first demonstrated by Reiss and Gershon (143), who partially regained the catalytic effects of oxidized enzymes after incubation with extracts from rat liver. No details of preparation, characterization or conditions were given, however. Later, another group has published several papers on this subject (10, 20, 144–147). Common for the assays used were long incubation times (1–2 hours) at 37° C, hundreds to thousand times excess of reductants with respect to methionine sulphoxide and low yields. Dithiothreitol was usually used as the reductant instead of NADPH, thioredoxin and thioredoxin reductase, which is supposed to be the natural reducing system (144). Dithiothreitol is reported, however, easily to reduce both free and peptide bound methionine sulphoxide (135) although this was not found in the enzymatic assay (144). The peptide methionine sulphoxide reductase seems to be universal as it is present in quite different species and tissues (involving *E. coli* and humans) (144, 146, 147).

An enzymatic reduction of methionine sulphoxide in higher animals therefore seems to be very much slower than the corresponding reduction in yeast and bacteria, and the enzymatic reduction observed in higher animals may in some cases be disputed.

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