METHIONINE: OXIDATION STATE IN PROCESSED
FOODS AND ENZYME-CATALYZED REACTION WITH
ADENOSINE TRIPHOSPHATE

by

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INTRODUCTION

Because of increasing population, the world faces the critical
problem of increasing food production. A central aspect of this
problem is to assure an adequate supply of protein (Scrimshaw and
Young, 1977). In the developing countries of the world the need is
urgent for low cost food which can fulfill energy requirement, provide
good quality protein, and gain popular acceptance (Weisberg, 1976).
In the more affluent countries the steadily increasing cost of animal
protein is forcing the food industry to find alternate sources of good
quality protein (Johnson, 1975). Bird (1975) speculates that before
the end of this decade, more than one-half of food-grade protein may
come from plant sources.

The United Nations has proposed increased use of oilseeds and
oilseed protein concentrates for supplementing protein in the human
diet (United Nations, 1968). Among oilseeds, soybeans stand out as an
excellent source of good quality protein (Liener, 1972). The protein
content of soybeans ranges from 39 to 44% (Rackis et al., 1975). The
essential amino acid content of soybeans compares favorably with that
of whole egg. Soybeans contain high amounts of lysine and meet or
exceed the levels of most of the other essential amino acids in whole
egg. Soybeans are low, however, in the sulfur containing amino acids,
cystine and methionine, and methionine is considered to be the limiting
amino acid in soybean protein (Coppock, 1974; Wolf and Cowan, 1975).

In the Western world, soybeans are processed into a variety of
products such as full fat flour, defatted soy flour and grits, soy

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protein concentrates, isolated soy protein, texturized soy protein, and spun protein fibers (Johnson, 1975; Waggle and Kolar, 1979). In the past in the United States, these products were used in food processing primarily for their functional characteristics with nutritional value being of secondary importance. Presently, the nutritional aspect is gaining in importance; soy protein products are being used increasingly in infant foods, as a partial substitute for ground meat in the School Lunch Program in the United States, and as meat analogs and extenders in the consumer market (Johnson, 1975; Horan, 1974). It has been estimated that in the United States approximately 10% of formula-fed infants are receiving formulas made from soy protein isolate (Fomon and Ziegler, 1979). The nutritional quality of the processed soy protein in these formulas is of the utmost importance, since they provide the sole source of protein in the early weeks of life of infants fed soy formulas.

Processing of soybeans improves flavor and other functional properties but may decrease protein quality (Bressani, 1975). Isolated soy protein, a highly processed product, sometimes has a lower Protein Efficiency Ratio (PER) than defatted soy flour. Supplementation of isolated soy protein with methionine raises its PER to a value comparable to defatted soy flour (Bressani, 1975). Supplementation of soy protein isolate with methionine has also been shown to increase nitrogen retention in humans when compared to unsupplemented soy protein isolate (Zezulka and Calloway, 1976).

Since supplementation with methionine increases the quality of processed soy protein, it would seem reasonable to assume that

processing has some detrimental effect on methionine and/or cystine, a methionine sparing amino acid. There does appear to be some loss of cystine and methionine through processing; however, the evidence suggests that this reduction does not fully account for the lowered nutritive quality (Wolf and Cowan, 1975; Longnecker et al., 1964; Cogan et al., 1968; Longnecker and Lo, 1974). Thus, changes in methionine and/or cystine may be occurring during processing, reducing their nutritive value without actually destroying them. Walker and his co-workers (1974) have suggested that the procedures used to toast and defat soy flour may promote oxidation of methionine. The oxidized forms, methionine sulfoxide and methionine sulfone, are not detected by the usual method of amino acid analysis. These forms may not be as readily available or utilizable as methionine, leading to lowered nutritive quality of the protein.

Few procedures exist for the direct measurement of methionine sulfoxide in food protein and the methods for quantitating methionine and methionine sulfone are either lacking or poorly defined. This study, therefore, sought to improve the methodology for measuring methionine, methionine sulfoxide, and methionine sulfone in food proteins by ion-exchange chromatography. The methods which were developed were then used to measure methionine and its oxidized forms in a variety of processed soy products and in selected infant formulas.

Secondarily, this study attempted to develop an enzymatic method for measuring unaltered L-methionine residues in food proteins with the enzyme ATP:L-methionine S-adenosyltransferase (E.C. number: 2.5.1.6).

REVIEW OF THE LITERATURE

Although the nutritive value of soybeans has been appreciated for many years, the beany, bitter flavor of soybeans has retarded their use as a human food. This characteristic flavor is extremely difficult to remove. Oriental countries developed fermentation products which resolved the flavor problem to their satisfaction; however, these products have not been generally accepted in countries outside the Orient. In order to gain acceptance by Western countries it became necessary to develop soy products such as soy flours, concentrates, and isolates with bland flavors (Smith and Circle, 1972). These products may be used in a variety of food preparations for either their nutritive or functional properties or maybe further processed into textured soy protein (Alden, 1975; Horan, 1974; Waggle and Kolar, 1974).

Processing Procedures

Heat Treatment

The simplest processing procedure for soybeans is heat treatment. In 1917 Osborne and Mendel noted that soybeans would not support the growth of rats unless they were cooked in a steam bath for three hours. This observation was confirmed several times in later years. These later studies indicated that attainment of maximum nutritive value depends on the interaction of several variables: temperature, pressure, moisture content, and duration of heating. Klose et al., (1948) determined that moist heat was superior to dry heat and that excessive heat treatment led to lower nutritive values for soybeans.

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Klose and his co-workers (1948) as well as other investigators
(Borchers et al., 1948; Smith et al., 1964; Rackis, 1965; 1966) found
that under laboratory conditions, maximum nutritive value was attained
by treatment with live steam for about 30 minutes or by autoclaving
for 15 to 20 minutes at 15 pounds pressure. Arnold et al., (1971),
using chicks as test animals, examined the nutritive value of soybeans
as a function of time and percent moisture. Their data indicate that
as moisture increases, higher temperatures are needed for optimum feed
efficiency.

Wing and Alexander (1971) investigated heat treatment of soy-
beans with microwaves. They found that optimum nutritive values for
soymeal as measured by PER occurred after a four minute treatment
(irradiation at 2450 megaherz and an output of 1250 watts). PER
dropped sharply with a five minute treatment.

Further Processing of Soybeans

While heat treatment improves the nutritive value of soybeans,
it does little to remove the objectionable flavor. For this reason
other processing procedures have evolved to improve soy acceptability
for humans. The basic steps involved in processing the seeds for
human foods are cleaning, cracking, dehulling, tempering, flaking,
extracting the oil and desolventizing (Horan, 1974). If full fat
flour is to be produced, the oil extraction step is omitted, the
flakes are heat treated, and then ground into a flour. If defatted
grits and flour are to be produced, the full fat flakes are loaded
into an oil extraction unit. Hexane is poured on top and allowed to

percolate through the flakes. The flakes, saturated with hexane, are heat treated at low pressures and low temperatures. This evaporates the hexane, leaving white defatted flakes which can be ground into grits and flour suitable for use in human foods. The protein content of flakes, grits, and flour is about 50% (Rakosky, 1974; Alden, 1975; Wolf, 1977; Waggle and Kolar, 1979). The soy grits and flour can be used in a wide variety of foods without further treatment, or the defatted flakes and flour may be processed further to produce soy protein concentrates and soy protein isolates.

Soy protein concentrate is defined as "the product prepared from high quality, sound, clean, dehulled soybeans by removing most of the oil and water soluble non-protein components that shall contain not less than 70% protein (percent nitrogen times 6.25) on a moisture free basis" (Circle and Smith, 1972a). Three common methods are used to produce soy protein concentrate commercially. These methods differ from each other depending on the technique used to keep the protein from being dissolved and removed. The protein is immobilized with heat, acid, or alcohol (Rakosky, 1974; Alden, 1975; Wolf, 1977). All three methods yield a product with a minimum of 70% protein but differing in other properties such as flavor, color, sodium content, and amount of water-soluble protein (Rakosky, 1974).

Soy protein isolate is defined as "the major proteinaceous fraction of soybeans prepared from high quality, sound, clean, dehulled soybeans by removing a preponderance of the non-protein components, that shall not contain less than 90% protein (percent nitrogen times 6.25) on a moisture free basis" (Circle and Smith, 1972a). The

processing for soy protein isolate differs from that of concentrate in that initially both the carbohydrates and proteins are solubilized using a mildly alkaline solution. The residue (water-insoluble polysaccharides plus residual protein) are separated from the liquor by centrifugation. The protein-containing liquor is acidified to pH 4.5 to precipitate the proteins. The protein curd is washed, concentrated, and dried to yield the isoelectric form of soy isolate. The washed curd may be resolubilized by neutralizing and then spray dried to yield the proteinate form of soy isolate. This latter form is often preferred because of its increased water dispersibility (Circle and Smith 1972a; Rakosky, 1974; Horan, 1974; Alden, 1975; Wolf, 1977). A comparison of the proximate analyses of soy flour, concentrate, and isolate is presented in Table 1.

Soy flours, concentrates, and isolates are powdery high protein materials. A significant development in recent years is the technique of transforming this powdery material into a product which has a fibrous character and "chewiness." This product, textured soy protein, can be used to simulate various meats when flavoring and coloring are added. The two most commonly used processes to impart meat-like character to soy are extrusion and fiber spinning.

In the spinning process soy isolate is solubilized in 14-18% sodium hydroxide at a pH of 10 to 11. It is then passed through a spinneret to form fibers which are coagulated in an acid bath, stretched, and gathered into bundles held together by edible binders. These fiber bundles are treated with flavor, color, seasonings, and supplementary nutrients and fabricated into slices, chunks, bits, or granules (Horan, 1974; Circle and Smith, 1972; Alden, 1975).

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Table 1. Proximate analyses of soy flours, protein concentrates,
and protein isolates[1]

	Full-fat Flour	Toasted Defatted Flour	Protein Concentrate	Protein Isolate
Moisture, %	3.4	6.5	8.0	4.8
Protein (N X 6.25), %	41.0	53.0	65.3	92.0
Crude fat, %	22.5	1.0	0.3	-
Crude Fiber, %	1.7	3.0	2.9	0.25
Ash, %	5.1	6.0	4.7	4.0

[1]Table taken from Wolf, 1977.

In the extrusion method, soy flours are used as the starting materials, giving a cost advantage over spun fibers. Processing consists of forcing a thermoplastic protein material through an extruder under pre-determined conditions of temperature, moisture, time, and pressure. A die at the outlet of the extruder controls the size and shape of the product (Circle and Smith, 1972a; Horan, 1974). Temperatures may reach 138°C with pressures up to 600 psi before extrusion (Alden, 1975).

The Protein Quality of Processed Soy Products

As soybeans are processed into more refined, blander tasting products, the protein nutritive value may be lowered (Bressani, 1975; Gillberg, 1977). Bioassays of protein quality such as protein efficiency ratio (PER), net protein utilization (NPU), biological value (BV), and nitrogen balance indicate processed soy products are sometimes inferior to the soy flour, flakes, or meal from which they were made. During the last two decades many animal and human studies have been done to investigate the protein nutritive value of various processed soy products. In general, they indicate that soy isolates have lowered nutritive value compared to soy flour or soy concentrates.

Animal Studies

Data from the Central Soya Company (1978) lists the PER[1] of toasted defatted soy flour (Soyafluff 200W) and alcohol extracted

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[1]Whenever possible PER's given in this dissertation have been corrected on the basis of 2.5 for casein.

protein concentrate (Promosoy 100) as 2.3 each compared to PER's of 1.1 and 1.6 for Promine D and Promine F, respectively, two of their commercial soy isolates. Rakosky (1970; 1974) reported a PER range of 2.16 to 2.48 for defatted soy flour compared to a range of 1.98 to 2.11 for soy isolates. Supplementation with DL-methionine raised the PER of the isolates to a range comparable to that of soy flour.

Longenecker et al. (1964) compared PER's of heated and unheated soy flour, concentrates, and isolates. They found unheated soy flour had a PER of 2.03 while mild heating lowered the PER to 1.99. For the three commercial concentrates tested, the PER range was 0.28 to 1.55 unheated and 1.68 to 1.75 heated. The PER's for the four isolates ranged from 1.13 to 1.59 in the unheated state and 1.22 to 1.91 after mild heating. Supplementation of one of the soy isolates with methionine hydroxy analog raised the PER from 1.22 to 1.87.

Rackis et al. (1963) also tested soy isolates for their nutritive value. They found that three commercial isolates had a PER range of 1.65 to 1.76. A laboratory prepared soy isolate had a PER of 1.40 in the unheated state compared to 1.63 after a mild heat treatment.

Cogan et al. (1968) compared the PER's of soy meal, an extraction mixture prepared from the meal, the filtered extract, soy isolates prepared from the extract by either calcium salt precipitation or isoelectric precipitation, and one commercial soy isolate. The PER for the soy meal was 2.22 while that of the extraction mixture was 1.66. The range for the various isolates was 1.66 to 1.84 with no significant difference between the types of preparation. The PER for the commercial soy isolate was 1.76. They found, however, that the isoelectrically precipated protein which was spray dried had a higher

PER than that which was freeze dried. This difference was not noted with the calcium salt precipitated protein.

Gillberg (1977) also compared protein nutritive value of soy meal and four soy isolates prepared from the meal by different methods. NPU (net protein utilization) was 66 for the meal while the range for the isolates was about 52 to 62. Biological value (BV) for the meal was 72 compared to a range of about 53 to 62 for the isolates.

Taper et al. (1978) determined PER for a commercial soy flour and an isolate from the same company. The flour had a PER of 1.5 compared to 1.4 for the isolate. The author remarked on the low PER for the flour and suggested that since the flour had not received optimal heat treatment, factors (including trypsin inhibitor activity) could be acting to lower the flour's nutritive value.

Bressani et al. (1967) compared the nutritive value of soy iso-late, and a soy protein textured food containing other protein along with soy protein. The soy isolate had the lowest PER followed by the spun fibers. The textured food had a PER equivalent to 89% of the value of casein. After a mild heat treatment both the isolate and fibers showed a significant improvement in PER with the isolate now surpassing the fibers. The PER's, however, were still significantly lower than the textured food. Supplementation of the textured food with lysine had no significant effect on PER while supplementation with methionine had only a slight effect. Addition of both lysine and methionine, however, led to a significant improvement in PER.

Kies and Fox (1971) reported on the nutritive value of a tex-tured soy product prepared by the extrusion process. The PER for the

textured product was 2.12 compared to 2.37 for beef. Supplementation of the soy product with DL-methionine raised its PER to 2.82.

Rosenfield and Hartman (1974) of Miles Laboratories, reporting on the nutritive value of finished products containing spun soy fibers, list PER's of 2.1 to more than 2.5 for these products depending on what other ingredients are in the product. They emphasize that the nutritional properties of the end product are more important than that of the fibers themselves.

Shemer et al. (1973) compared PER's and NPU's of whole soy flakes, canned soybeans, and a soy-banana weanling food. The PER's were 2.1, 1.4, and 1.4, respectively. NPU's were 46, 33, and 36, respectively. Dramatic improvement in these parameters was seen when each product was supplemented with methionine.

A soy isolate obtained by extracting soy flour at pH 5.5 and then precipitating the proteins isoelectrically has recently been developed by Miles Laboratories. Weanling rats fed this isolate failed to gain weight (Cogan et al., 1979). Nutritive value was not improved with heat treatment of the isolate or methionine supplementation. PER did increase to the level of casein when this isolate was supplemented with methionine, cystine, threonine, and tryptophan.

Steinke et al. (1980) found that the mean PER for isolated soy protein (Supro 620) was 1.82 ± 0.15 based on 24 PER tests. This PER value was 73% of casein and the authors comment that this "corresponds well with the 75.6% for mean sulfur amino acid content of isolated soybean protein relative to casein." These workers also reported that

when the soy isolate supplies as much as 50% of the protein in combination with egg, lactalbumin, turkey, emulsified beef or pork, or tuna fish, a PER of 2.5 is achieved.

Yanez et al. (1979) determined PER's on a thermoplastic extrusion product containing mixtures of sunflower flour and soy flour in ratios of 1:1 (TSP_1) and 3:1 (TSP_2). The PER's were 1.96 and 1.63, respectively, for TSP_1 and TSP_2. Supplementation of TSP_1 with 0.15% DL-methionine raised the PER to 2.32. Supplementation of TSP_2 with 0.1% lysine raised the PER to 2.09.

The nutritive value of soy isolate based formulas has also been investigated in laboratory animals. Sarett (1976) summarized the results of many studies done in his laboratory in the 1960's. He found that weight gain in baby pigs fed soy protein isolate formulas supplemented with methionine was equivalent to that obtained with milk protein; however, the level of calories supplied by soy protein was 15% of the total calories compared to 12.4% for the milk protein. Other parameters, such as plasma proteins, calorie efficiency, and liver protein concentration indicated that soy protein isolate with added methionine had about 85% of the nutritive value of milk protein (Schneider and Sarett, 1969).

Nutritional studies were also done on two commercial soy isolate formulas at the 10% protein level using rats as test animals (Theur and Sarett, 1970). One of the formulas produced fatty livers in the rats as well as low weight gain. Serum proteins were also lower in rats fed this soy formula compared to the other commercial formula and to a laboratory prepared formula. When choline was added

to this formula, the fatty livers were prevented but weight gains and caloric efficiency were not improved. Further study showed that the trypsin inhibitors had not been completely destroyed in the formula, indicating inadequacy of the processing procedure.

Human Studies

Infants and Children

Several studies have been done investigating the efficacy of soy protein in promoting growth in infants and children. A study by Cherry et al. (1968) compared growth in infants fed a modified milk formula and infants fed a soy isolate based formula. In both formulas, 15% of the total calories was supplied by soy or milk protein and each contained about 18 kcal per fluid ounce. By the end of the first month of life and persisting throughout the six months of the study, females fed the milk formula demonstrated faster growth than the females fed the soy formula as measured by head circumference, length, and weight. Although the same tendency was noted in the male infants, the differences were not statistically significant. Since the authors did not specifically mention whether or not the soy formula was fortified with methionine, the assumption is that it was not. Weisberg (1974), in commenting on this study, speculated that the soy formula, although supplemented with vitamins and minerals may still have been deficient in calcium, zinc, magnesium, and possibly methionine.

Fomon et al. (1973) compared two groups of male infants, one group receiving soy isolate formula supplemented with L-methionine and the other receiving the same formula without methionine supplementation. They found no statistically significant difference in food

intake or growth. Serum urea nitrogen levels, however, were signifi-
cantly less in the methionine supplemented group compared to the un-
supplemented groups, suggesting improvement in protein quality of soy
isolate through methionine supplementation.

Recently, Fomon and Ziegler (1979) reported on the results of
their studies in which infants aged 8 to 112 days were fed either a
soy isolate formula (Edi-Pro A) fortified with methionine, a milk-
based formula with a comparable protein content, or human milk. The
serum concentrations of urea nitrogen, total protein, and albumin
were similar for the three groups. They also found that infants
receiving the soy formula had weight gains comparable to those fed
milk formulas or human milk.

Fomon and Ziegler (1979) also summarized additional published
and unpublished data from their laboratory on a large number of normal
infants fed soy isolate formulas fortified with DL-methionine or
L-methionine and on other groups of infants fed milk formulas. There
was no difference in weight gain for male infants although those
receiving the soy formulas had a greater energy intake than the milk-
fed infants. For female infants, those fed soy formulas had a lower
mean rate of weight gain than those fed milk formulas but the differ-
ence was not statistically significant. Mean weight gain per 100 kcal
was significantly less for both male and female infants fed soy for-
mulas compared to milk-fed infants.

Nitrogen retention has also been used to measure protein quality
of soy protein formulas in infants. Graham (1971) investigated nitrogen
retention in infants 6 to 23 months of age fed a commercial soy-based

infant formula, a soy isolate, or a full-fat soy flour with and with-
out added methionine. In all three preparations, the addition of
methionine increased nitrogen retention in the infants. In two of the
children, nitrogen retention was determined when these children were
fed soy isolate, with and without added methionine and compared to
nitrogen retention when they were fed casein. Nitrogen retention was
72% of casein for the unsupplemented soy isolate and 78% of casein for
the methionine-supplemented soy isolate.

Fomon et al. (1973) conducted six balance studies with six
infants, 114 to 118 days of age, and found no difference in nitrogen
balance whether the protein was supplied by soy isolate formulas
supplemented with methionine or from a cow's milk formula. The
researchers compared these data to infants fed human milk and found no
difference in nitrogen balance between infants fed soy isolate formula
supplemented with methionine and the infants fed human milk.

Fomon and Ziegler (1979) summarized their data from nitrogen
balance studies done on infants aged 8 to 120 days. When soy isolate
formula supplemented with methionine was fed at a level that supplied
6.5% of the calories from soy protein, nitrogen retention, when sta-
tistically adjusted for differences in nitrogen intake, was 149 mg per
kg per day compared to 162 mg per kg per day for infants fed milk-
based formulas. This difference was not significant. No significant
differences were seen when the protein level was raised to the 8-11%
range or the 12-16% range compared to cow's milk protein.

Fomon and Ziegler (1979) also reported on balance studies in
infants 121 to 727 days of age, when fed soy protein or milk protein

at the 8-11% level. When nitrogen retention values were adjusted for intake differences, retention was 111 and 95 mg per kg per day for the soy-fed and milk-fed infants, respectively. This was statistically significant at the 0.01 level.

In the early studies done by Fomon's research group, the level of supplementation of soy isolate formulas was 6 mg of DL-methionine per gram of protein. In their more recent studies the level of supplementation was 4-8 mg of L-methionine per gram of protein.

Torun (1979) reported two studies in which children, aged 19 to 44 months and nutritionally recovered from malnutrition, were fed a liquid diet containing one of two soy isolates: Supro 620 or Supro 710 at various levels. Digestibilities were comparable to milk protein and the biological values for Supro 620 and Supro 710 were 96% and 86% that of milk, respectively. The amounts of nitrogen needed to attain equilibrium for Supro 620 and Supro 710 were 90% and 107%, respectively, relative to milk.

Parathasarathy et al. (1964) studied the effect of soy flour, soy flour + DL-methionine, and soy flour + DL-methionine hydroxy analog on 8 and 9 year old children. Biological value and net protein utilization were 63.5 and 53.3, respectively, for soy flour alone compared to 74.9 and 64.7 for soy flour + methionine. The hydroxy analog was nearly as effective as the methionine. The corresponding values for skim milk were 82.6 and 72.0.

Bressani et al. (1967) fed a soybean textured vegetable food, containing other protein along with soy, to children between 22 and 72 months of age. They found that nitrogen equilibrium was achieved when

the children received about 138 mg of nitrogen from the soy product
compared to 97 mg from milk. They concluded that the soybean protein
textured food was equivalent to about 80% of the protein quality of
milk.

Graham et al. (1976) examined plasma amino acid levels in con-
valescent malnourished infants, 5 to 42 months of age who were fed
isolated soy protein at different levels with or without added sup-
plemental methionine. The range of energy provided by the soy pro-
tein was 4 to 12%. There was no difference in total plasma amino
acid concentration in any group with or without methionine; however,
the molar fraction of essential to total amino acids was lower for the
4% protein group without added methionine compared to the other
groups. They also found that the methionine to essential amino acid
ratio fell at three and four hours postprandial for infants fed a
test meal of either isolated soy protein or milk protein at less than
a 6.7% energy level when these proteins were methionine deficient.
This effect was not seen in groups fed the same test meals with sup-
plemental methionine. These authors suggested that a comparison of
methionine to essential amino acid ratio in fasting plasma and three
and four hours postprandial after a test meal could be used to judge
whether or not the dietary protein was limiting in methionine. Con-
ceivably, this method could also be used in adults to measure methi-
onine availability in test proteins.

Adolescents and Adults

Using textured vegetable protein (TVP), TVP supplemented with
DL-methionine, or beef at two different levels of nitrogen intake,

Kies and Fox (1971) determined nitrogen retention in adult men receiving these proteins. At a level of 8.8 grams per day, no significant difference was seen in nitrogen balance among the three groups. At a level of 4.8 grams nitrogen per day none of the diets kept the men in positive balance; however, beef was superior to TVP. TVP supplemented with methionine was more effective than TVP alone but not as effective as beef.

Korslund et al. (1973) determined nitrogen balance in 12- to 16-year-old boys fed extruded TVP with and without added methionine. At a nitrogen intake of 5.0 grams of nitrogen per day, balance was increased from -0.08 g/day to + 0.48 g/day with methionine fortification.

Derby et al. (1974) conducted studies using extruded textured soy to replace partially the meat protein in the diets of ten adults. Soy protein was fed at a level of 23% of total protein in the diet. Animal protein accounted for 29% of the total protein and the remainder, 37% was other vegetable protein. The subjects served as their own controls eating a normal diet one week and the test diet the second week. There were no significant differences seen in digestibility, NPU, or BV between the two diets.

Doraiswamy (1972) compared an extruded defatted soy product and also a spun concentrated product to beef and whole egg when fed to adult subjects. The products were fed at a level of 4.0 grams of nitrogen per day and nitrogen retention was measured. The soy products gave lower nitrogen retention than either the beef or whole egg.

Turk et al. (1973) fed a spun fiber soy product to adult sub-
jects. Egg albumin, used as a fiber binder, constituted 32% of the
protein in the product. The product was fed at varying levels of
nitrogen per kg of body weight for each subject. A majority of the
subjects were in positive balance at intakes of 0.06 grams of nitro-
gen per kg per day.

Morse et al. (1972) compared the protein quality of spun soy
protein and casein-lactalbumin when these proteins were adjusted so
that their essential amino acid patterns were equal to the FAO pro-
visional pattern. On the basis of nitrogen retention, the authors
concluded that spun soy protein and casein-lactalbumin supplemented
with the crystalline amino acids and fed at a level of 0.45 grams of
protein per kg of body weight per day were utilized equally well.

Taper et al. (1978) compared nitrogen retention in young women
fed either soy flour, soy isolate, or soy isolate supplemented with
amino acids such that the pattern of essential amino acids matched
that of soy flour. At a level of 4.0 grams of nitrogen no statisti-
cally significant differences in nitrogen retention were observed
among the groups.

The nutritional quality of a soy protein isolate, Supro 620, was
evaluated in a series of nitrogen balance experiments in young adult
males (Scrimshaw and Young, 1979; Young and Scrimshaw, 1979). These
workers found that the nutritive value of the soy isolates, as meas-
ured by the amount of protein necessary for balance was no different
from milk and beef protein and only slightly less than egg protein.
Methionine supplementation improved soy protein utilization when the

level of protein intake was inadequate but when the level of supple-
ment was raised to a level just slightly exceeding recommended levels
of total sulfur amino acids (WHO, 1973) an adverse effect was seen in
some subjects with deterioration in nitrogen balance. When, however,
the level of protein intake from soy was adequate to meet the dietary
allowance for total protein, methionine supplementation had no measur-
able affect.

Effects of Processing on Soy Products

Effect of Heat

Moderate heat treatment of soybeans is necessary to obtain a
product that will support growth in test animals. Raw soybeans have
been shown not only to inhibit growth, but also to depress metaboliz-
able energy and fat absorption, reduce protein digestibility, cause
pancreatic hypertrophy, stimulate hyper- and hyposecretion of pan-
creatic enzymes, and reduce amino acid, vitamin, and mineral avail-
ability in rats and chicks   (Rackis, 1974). The question of why
heating improves the nutritive value of soybeans has not been com-
pletely answered. Studies do indicate that trypsin inhibitors present
in soy are a contributing factor to the growth depressing effect of
raw soy in rats but are not the whole answer (Kakade et al., 1973a).
The mechanism by which the trypsin inhibitors were thought to reduce
nutritive value was their ability to inhibit proteolysis in vivo
(Liener, 1977). This however, does not appear to be the case since
trypsin inhibitor preparations added to pre-digested protein or free
amino acids are still capable of inhibiting growth (Desikachar and De,

1947; Khayanbashi and Lyman, 1966). Rackis (1974) discusses three possible mechanisms whereby trypsin inhibitors may lower soy nutritive value in rats -- (1) the inhibitors may stimulate biosynthesis of pancreatic enzymes leading to the endogenous loss of essential amino acids and therefore a greater need for the sulfur amino acids in the diet than soy can provide, (2) the inhibitors may form complexes with dietary proteins which resist digestion. (To explain the growth inhibition in rats fed hydolyzed protein containing inhibitors the inhibitors may complex with endogenous protein of intestinal origin.) (3) Trypsin and chymotrypsin in the intestine may control and suppress pancreatic enzyme secretions by a feedback mechanism. The inhibitors may form a complex with trypsin thereby overcoming the feedback response, enhancing protein synthesis in the pancreas as well as reducing proteolytic activity in the intestine.

In large animals such as calves, Kakade et al. (1973) found that raw soybeans caused a depression of growth and reduced digestibility of protein and fat compared to heated soybeans; however, soybean trypsin inhibitors added to heated soybeans did not differ from heated soybeans without inhibitors added in its effect on calves. Furthermore, the inhibitors did not cause pancreatic enlargement in calves nor did they cause an increase in pancreatic enzyme activities. Other investigators have found that in pigs (Hooks et al., 1965; Jensen et al., 1974) and in dogs (Patten et al., 1971) soybean trypsin inhibitors, as with calves, do not cause pancreatic enlargement. Based on the work of various investigators, Kakade et al. (1973) summarized the possible reasons for the differences seen in small and large animals:

(1) species differences, (2) lower sulfur amino acid requirement in larger animals, (3) lower pancreatic enzyme secretion in larger animals, (4) smaller size pancreas relative to body weight in larger animals.

The biological effect of raw soy in humans has not been studied extensively. One study (Lewis and Taylor, 1947) indicated that raw soy flour could support positive nitrogen balance in man but not as efficiently as autoclaved soy flour. More recent studies (Feeney et al., 1969; Travis and Roberts, 1969; Coan and Travis, 1971) indicate that the cationic form of human trypsin is only weakly inhibited by the soybean inhibitors. The anionic form, however, was found to be fully inactivated by the inhibitors (Mallory and Travis, 1973; Figarella et al., 1975). The cationic form, however, represents the major portion of human trypsin (Liener, 1977).

Liener (1977) concludes from the work done in his and other laboratories that "there must be present in raw soybeans some other factor, totally unrelated to the trypsin inhibitor, which is also capable of causing pancreatic enlargement and an inhibition."

Other anti-nutritional factors that have been identified in soybeans include hemagglutinins, a weak goitrogen whose activity is largely abolished by heat or addition of small amounts of iodine to the diet, small quantities of saponins, phytosterols, and phenolic compounds (Rackis, 1974). Rackis (1974), however, ascribes little nutritional significance to these factors.

Effects of Overheating

While moderate heating of raw soy is necessary for optimum pro-
tein nutritive value, overheating has long been known to decrease
nutritive value. Mitchell et al. (1945) demonstrated that the high
temperatures used in the commercial processing of oilseed protein
results in low nutritive value of the protein. Klose et al. (1948)
found that autoclaving defatted soy flakes for three hours at 15 lbs
pressure decreased PER to a value lower than that of raw soybeans.
Supplementation with methionine significantly raised the PER but a
higher PER occurred when the overheated flakes were supplemented with
methionine, leucine, and lysine.

Later studies confirmed that overheating soy products lowered
their nutritive value. Rios-Iriarte and Barnes (1966) found that
autoclaving soy flour, full-fat chips, and soy meal for two hours
resulted in a drastic lowering of PER compared to a mildly heat
treated control. Addition of enough cystine to replace that which had
been destroyed raised the PER but not to the level of the unsupple-
mented control. Replacement of other amino acids that had been
destroyed during processing (threonine, lysine, tryptophan, and histi-
dine) along with the added cystine did not raise the PER above that of
cystine replacement alone.

Hackler et al. (1965) demonstrated that heating soy milk at
121°C for 32 minutes or longer caused a decrease in PER. They also
found that spray drying of soy milk at inlet temperatures of 277°C or
higher also lowered PER.

Taira et al. (1969) heated soy flour at 126°C for four hours and compared it to soy flour heated at 108°C for one hour. The nutritive value of the overheated soy flour was lower compared to the mildly heated flour and supplementation with amino acids which were destroyed improved the nutritive value but did not increase it to the level of the control.

Effect of Alkaline Processing

Alkali may also contribute to the decrease in protein quality in soy products. Several commercial procedures for soy processing employ alkali. For example, during the processing of soy isolate a mild alkaline treatment may be used. In the process of spinning soy fibers for textured products, the alkaline treatment is more severe, with the pH kept at 10 to 12 for several minutes at room temperature (DeGroot et al., 1977). Pre-soaking whole soybeans in alkaline solution before processing them into soy milk can improve flavor (Badenhop and Hackler, 1970).

Several studies have been done investigating the effect of alkaline treatment on the nutritive value of soy protein. One report indicates that in the preparation of soy milk the PER drops sharply as the pH of the soaking medium rises above 8.0 (Rackis et al., 1975). Badenhop and Hackler (1970) noted that soaking soybeans in sodium hydroxide prior to preparing soy milk led to decreasing PER's as the pH increased from 6.55 to 9.18. A later study by Badenhop and Hackler (1973) confirmed the lowering effect of alkaline soaking on PER. They soaked soybeans at pH's of 6.50 to 8.97 for two hours at 50°C. The

PER of the soy milk prepared from the soybeans soaked at pH 8.97 was significantly lower than the PER's of the soy milk prepared from samples soaked at pH 7.81 or lower. The amino acid analyses indicated that cystine was the only amino acid that decreased with increasing pH in both the heat-treated and unheated samples. A decrease was also noted in tryptophan in the heat-treated samples. Supplementation of both the heated and unheated samples with 0.35% L-methionine increased PER at all pH's for both heated and unheated samples. The most dramatic increase was seen when the pH 8.97 heated sample was supplemented with L-methionine. The PER of the supplemented sample at this pH was 92% higher than that of the unsupplemented control. The authors suggest that high pH can cause both destruction and reduced availability of sulfur amino acids.

The effect of more drastic alkaline treatment on soy was studied by DeGroot and Slump (1969). They treated a commercial soy isolate with alkali varying pH, temperature, and time. At 40°C and four hours of treatment the NPU and digestibility of the isolate were lowered as the pH was raised above 10. NPU and digestibility also dropped with increasing time and temperature when the pH was held constant at 12.2.

Thus, several studies demonstrate that protein quality is reduced with severe heat treatment and high pH. The question of how these treatments reduce protein quality, however, has not been completely answered.

Possible Factors Contributing to Lowered Nutritive
Value of Processed Soy Protein

The protein quality of processed soy products is dependent on
several factors: amino acid composition, presence of residual anti-
nutritional factors, overall composition of the diet, and nutrient
requirement of the particular species (Wolf and Cowan, 1975). Addi-
tional factors which could play a part in lowering protein quality
include reduced digestibility, lowered amino acid and mineral avail-
ability, and formation of toxic compounds. Each of these factors
must be assessed in the products in order to answer the question of
why processing, at least sometimes, reduces protein quality.

Amino Acid Composition

While the pattern of essential amino acids in unprocessed soy-
beans compares favorably with the FAO reference pattern except for
the sulfur amino acids, processing may lead to the destruction or
loss of some of the amino acids, thus, reducing quality. Several
studies compare the amino acid composition of soy isolates and soy
flour (Wolf and Cowan, 1975; Mattil, 1974; Longenecker et al., 1964;
Gillberg, 1977). In general, the content of the non-sulfur essential
amino acids in soy isolates shows little variation from that of soy
flour or meal. The sulfur amino acids, which are low to begin with,
are decreased by processing in some cases.

Longenecker et al. (1964), using a microbiological technique,
compared sulfur amino acid content of three soy isolates and a soy
flour. The content was similar for two of the soy isolates and the

soy flour; however, the third isolate had a lower sulfur amino acid content compared to the other three soy products and also had the lowest PER. This isolate also contained a small amount of cysteic acid, an oxidized form of cystine, while the other two isolates did not.

Almost in a category by itself is the newly developed soy isolate, SP-55 (Miles Laboratories). It has unusual functional characteristics compared to other isolates but poor nutritive value (Cogan et al., 1979). The methionine and cystine levels in the soy isolate, as determined by ion-exchange chromatography after acid hydrolysis, were about half that of the soy flour. The levels of threonine and tryptophan were also dramatically lower in the isolate compared to the flour. The lowering of these four amino acids appears to be due to loss of certain proteins during processing rather than destruction (Cogan et al., 1979).

A notable exception to the correlation between lowered sulfur amino acid content and lowered nutritive value is seen in a study done by Shemer et al. (1973). Using acid hydrolysis followed by gas chromatography as their method of analysis, these researchers assessed the sulfur amino acid content of whole soy flakes, canned whole soybeans, and a soy-banana weanling infant food. They found that while the methionine level was decreased in the canned whole soybeans, the cystine level and the total sulfur amino acid content were higher than in the soy flakes. The PER for the canned soybeans, however, was 1.4 compared to 2.1 for the soy flakes. The authors attributed the low PER to harsh thermal processing. They did not comment on the

relatively high cystine content. Supplementation of the three soy
products with 0.5% methionine increased PER in each of the products;
the most dramatic improvement seen in the case of the canned soybeans,
which rose from 1.4 to 2.6.

Effect of Overheating on Amino Acid Destruction

Overheating has long been known to decrease nutritive value in
soy protein. In a series of studies by Evans and his co-workers
(Evans and Butts, 1949a, 1949b; Evans and McGinnis, 1948; Evans et
al., 1951) it was established that overheating soybeans destroyed
much of the cystine so that it became the first limiting amino acid
for rats. Rios-Iriarte and Barnes (1966) also noted that severe heat
treatment caused a destruction of cystine and a dramatic decrease in
PER in various soy products. Supplementation with cystine or methi-
onine increased PER but did not restore it to the level of the mildly
heated control. The control was also supplemented with cystine or
methionine. The PER was increased over the unsupplemented control
with methionine having the greater effect. The authors suggest that
the slight destruction of cystine which may occur with even mild
heating was sufficient to make cystine the first limiting amino acid,
thus accounting for the improvement of PER with cystine or methionine
addition in the control. They suggest, further, that a delicate
balance exists between the heat treatment necessary for inactivation
of growth inhibitors and that which will destroy enough cystine to
make it growth limiting for rats.

Taira et al. (1969) investigated amino acid destruction and

in vitro digestibility after soy flour had been heated for four hours

at 126°C. Significant amounts of cystine, lysine, arginine, and

tryptophan were destroyed. When the overheated flour was supplemented

with all the amino acids destroyed during the heat treatment, the

nutritive value was increased but did not reach the level of the

mildly heated control. In vitro enzymic digestion of the overheated

flour showed that the extent of release of the various amino acids

was decreased compared to the control.

Hackler and Stillings (1967) investigated amino acid destruction

in soy milk subjected to moist heating and spray drying. They found

that at 121°C some cystine and tryptophan were destroyed but at 93°C

no destruction took place. When the soy milk was spray dried using

drying temperatures of 277°C or higher, there were decreases in

lysine, histidine, arginine, threonine, serine, proline, phenylalanine,

tyrosine, and tryptophan. Their results indicate that cystine is

quite susceptible to damage under high moisture conditions but stable

to dry heat. They also suggest that there is a critical time-tempera-

ture relationship for development of optimum protein nutritive value.

Effect of Alkali on Amino Acid Destruction

Alkaline treatment of soy products has also been shown to destroy

some amino acids. DeGroot and Slump (1969) found that treatment of a

commercial soy isolate at pH 12.2 for four hours at temperatures

increasing from 20°C to 80°C led to a decrease of lysine and cystine

which was proportional to temperature and a loss of serine at 60°C and

80°C. They also subjected the alkali treated isolate and untreated

isolate to pepsin-pancreatin enzyme mixtures and measured enzymatic

release of amino acids by amino acid analysis of the enzyme digests.

No difference was seen in release of valine, arginine, alanine,

aspartic acid, and glutamic acid; however, for the other amino acids,

release from the alkali treated isolate was lower than from the un-

treated isolate. In vitro absorption by rat intestine was increased

for most amino acids in the treated isolate compared to the untreated

control except for methionine and threonine which showed lowered

absorption. The authors suggest that the alkali treatment could lead

to racemization and other changes affecting availability of threonine

and methionine, thus reducing their absorption.

Woodard and Short (1973) treated soy protein with 0.1N NaOH for

eight hours at 60°C. Decreases were seen in lysine, serine, methi-

onine, threonine, and arginine content. Badenhop and Hackler (1973)

found that alkaline soaking of soybeans prior to the preparation of

soy milk led to a decrease in cystine which was proportional to pH

with and without subsequent heat treatment. In the heat-treated

samples, tryptophan content was also decreased with increasing pH.

Amino Acid Availability

It is apparent from the studies cited above that severe heat

treatment and/or treatment with alkali can lead to destruction of

significant amounts of cystine and other amino acids. It is also

apparent that supplementing the damaged protein with the amino acids

lost during processing does not always restore the nutritive value to

the level of the properly heat-treated protein. Consequently, it can

be inferred that changes may be occurring in the amino acids that

alter them without actually destroying them but rendering these amino

acids biologically unavailable. The following studies examined not

only total amino acid content in relation to heat and alkali treat-

ments but also the bio availability of certain amino acids.

Measurement of Amino Acid Availability

Hackler et al. (1965) noted that when soy milk was heated for

increasing periods of time at 121°C or spray dried at an inlet tempera-

ture of 277°C, the PER dropped along with a concurrent drop in avail-

able lysine as measured by the method of Carpenter (1960). A similar

study done by Hackler and Stillings (1967) noted that heat processing

soy milk at 121°C caused not only a decline in cystine and tryptophan

but also a decrease in available lysine but no change in total lysine.

When the soy milk was spray dried at temperatures between 143°C and

316°C there was a decrease in both total and available lysine with no

change in cystine. In both types of treatment there was a decline in

protein quality.

Badenhop and Hackler (1971) investigated the effect of dry

roasting of soybeans on amino acid composition, PER, and available

lysine. They found that PER was inversely related to the degree of

roast and that tryptophan, cystine, total lysine, and available lysine

as measured by the method of Rexen and Christensen (1967), were also

inversely related to the degree of roast.

Although methionine is generally considered to be the first limiting amino acid in soy protein, few studies have been done to assess its availability in soy products damaged by processing. Cogan et al. (1968) assessed both available lysine and available methionine in soy meal, an extraction mixture, an extract, and several soy isolates made from the extract by either isoelectric precipitation or calcium salt coagulation. The extraction mixture was agitated for 30 minutes at 55°C with a final pH of 9.5 to 9.8. Available lysine was measured by a dinitrophenyl condensation method of Baliga et al. (1959) and also by a microbiological method. Available lysine was higher in the isolates compared to the extraction mixture while available methionine was slightly lower in the isolates. There did not appear to be any correlation between available methionine and PER, however.

Longenecker and Lo (1974) attempted to assess quantitatively available methionine in severely heat-treated soy concentrate. The technique they used was to measure plasma methionine in a human subject following ingestion of the damaged concentrate, a mildly heated control concentrate, and the damaged concentrate supplemented with varying levels of L-methionine. The amount of L-methionine needed to restore the plasma level of methionine to the level of that found after ingestion of the control was used to predict methionine availability. Their results indicated that methionine availability in the heat damaged concentrate was 54% of that in the control even though the percent of actual destruction of methionine was only 15%.

Achinewhu and Hewitt (1979) measured available methionine and lysine in soy isolate that was either untreated, steamed, or heat damaged by autoclaving. Using a chick biological assay and a microbiological technique with Streptococcus zymogenes, they found that although the autoclaving caused little change in amino acid composition, it reduced availability of methionine by about one-half. Availability of lysine was reduced by about two-thirds. Availability of methionine was not affected by steaming.

While there have been few studies on available methionine in processed soy products, there have been a number of studies on other proteins indicating that heat damaged proteins exhibit decreased methionine availability as well as decreases in the availability of other amino acids. In a series of studies on animal protein, Donoso et al. (1963) and Miller and his co-worker (Miller and Carpenter, 1964; Miller et al., 1965a, 1965b) found that although the sulfur amino acids were the limiting amino acids in meat, fish and whale-meat meals, the destruction of these amino acids did not account fully for the lowered NPU seen. Using a microbiological assay and a chick assay they found that 42% to 98% of the methionine was unavailable. There was also a decrease in the availability of tryptophan as determined by microbiological assay and of lysine determined by chick growth assay and the dinitrofluorobenzene method (Carpenter, 1960).

Rao et al. (1963) found that in a casein-glucose system even mild heating caused a slight lowering of available methionine. Heating, however, in the absence of reducing sugars resulted in nutritional damage only if heating was severe, and then the level of available methionine was as low as that of available lysine.

Varnish and Carpenter (1975), using both microbiological and
chick growth assays, determined that in heat damaged chicken muscle,
39% of the methionine was available by microbiological assay and 66%
by chick growth assay.

Pieniazek et al. (1975a) estimated available methionine in
several foods which had been thermally processed. They used the
sodium nitroprusside method of McCarthy and Sullivan (1941) for their
assay. Available methionine (i.e., nitroprusside-reactive methionine)
was reduced 15% in sweetened condensed milk and 11% in the unsweetened
condensed milk compared to unprocessed controls. For roller dried
milk, whey powders, and mackerel sterilized at 126°C, reductions in
available methionine were 22%, 14%, and 19%, respectively. They found
no difference in total sulfur amino acids in the processed products
compared to their unprocessed counterparts. The authors also esti-
mated cysteine using a spectrophotometric method based on the forma-
tion of a colored complex formed by the reaction of the free thiol
group of cyteine and 5,5'-dithiobis-2-nitrobenzoic acid (Zahler and
Cleland, 1968). Reductions of 32% were seen in the sweetened, con-
densed milk and 64 and 75% for mackerel sterilized at 115°C and 126°C,
respectively. Values for available sulfur amino acids in the food
materials were also determined by rat bioassay and, according to the
authors, showed good agreement with the in vitro assays.

In a second study by Pieniazek et al. (1975b), these workers
determined available methionine and cysteine as a function of water
content and the presence or absence of glucose when casein was heated
at 90°C for 24 hours. Using the same chemical methods described in

the previous study they found that the greatest losses in available

methionine (51%) occurred when the moisture level was high and glucose

present. Losses of 100% of the cyteine occurred when glucose was

present at both high and low moisture levels. The greatest losses in

total methionine (22%) and in total cysteine (24%) occurred when the

moisture level was high and glucose present.

Waibel et al. (1977) compared lysine and methionine availability

in steer blood which was vat dried at temperatures up to 165°C with

blood that was rapidly dried in a current of hot air at 400° to 410°C.

Bioassays indicated that the vat dried blood suffered much greater

damage with reduced digestibility and methionine and lysine avail-

ability reduced to zero. Lysine availability ranged from 80% to 97%

in the blood dried by the short time high temperature method while

available methionine stayed near 100%.

In order to be available, an amino acid must be absorbed and

retained by the organism. Several studies have investigated the effect

of various processing procedures on absorption and retention of amino

acids in processed proteins. Nitsan and Liener (1976) fed rats either

raw or heated soy flour. They collected fecal material from the rats

and, after sacrificing the rats, removed all the chyme from various

segments of the rat intestine. Feces and chyme were analyzed for

amino acid content by ion-exchange chromatography. Their results indi-

cated that the apparent digestibility (or the percent absorption of

intake) of all the amino acids was significantly lower for the raw soy

flour compared to the heated flour. The apparent net absorption was

also less from the raw flour with the "notable exception of cystine

and methionine, which were absorbed to the same extent in both groups."
Retention for the five amino acids monitored (cystine, methionine,
aspartic acid, threonine and serine) was significantly lower in the
group fed raw soy flour with cystine being markedly lower.

Goldberg and Guggenheim (1962) found that after rats were fed a
test meal of either raw, mildly heated or overheated soy flour, the
concentrations of methionine, lysine, and tryptophan in the rat intes-
tine and concentration in the portal vein was significantly lower for
groups fed raw flour and overheated flour compared to the properly
heated control flour. The technique used to determine the amounts of
the three amino acids was microbiological with Leuconostoc mesenteroides
P-60 used to assay for lysine and methionine and Lactobacillus
arabinosus to assay for tryptophan.

Achinewhu and Hewitt (1979) determined ileal digestibility of
various amino acids in chicks fed untreated or autoclaved (heat
damaged) soy isolate. After a test meal, chicks were sacrificed, the
ileal contents expressed, and this material subjected to acid hydrol-
ysis and ion-exchange chromatography. Digestibility of most of the
amino acids in the autoclaved isolate was reduced by about one-half.
The values for available methionine, as measured by chick growth and
also by microbiological assay with Streptococcus zymogenes, correlated
fairly well with methionine digestibility. Digestibility did not give
a good estimate of available lysine, however, as measured by chick
and rat growth and also by the method of Carpenter (1960).

A recent study by Shorrock and Ford (1978) investigated the
possibility that impaired absorption of amino acids of heat damaged

protein could be the result of 'unavailable' peptides interfering or

sterically blocking uptake of amino acids. An extract was isolated by

gel filtration from an enzymatic digest of heat-damaged cod fillet

containing 'unavailable' small peptides. This extract along with

radioactively labeled free leucine was added to a Krebs-Ringer solu-

tion; everted rat intestinal sacs were incubated in this medium for 30

minutes. Absorption of leucine from the incubating medium and transfer

to the serosal solution inside the sacs was determined microbiologi-

cally with Streptococcus zymogenes. Uptake of radioactive leucine

was also measured by determining the amount of labeled leucine left in

the incubating medium after 80 minutes. These values were compared to

those obtained from a control system that contained all the components

of the test model except the 'unavailable' peptides. Uptake of leucine

was decreased by about 50% and 63%, respectively, as determined by

radioactive counts and microbiological assay. Available lysine,

methionine, leucine, isoleucine and valine were determined micro-

biologically in the 'unavailable' peptides and found to be 7%, 30%,

26%, and 30%, respectively, of the total amount of these amino acids

in the peptides. Availability of these amino acids in the heat

damaged cod fillet itself was 28%, 52%, 57%, 52%, and 57%, respectively,

compared to over 90% availability for these amino acids in the freeze-

dried control.

It would appear from the study above that 'unavailable' peptides

can decrease uptake of amino acids in vitro. Evans and Bauer (1978),

however, found that in adult rats, only about 50% of the methionine

and cystine of heat damaged bean flour was absorbed, but all of the

added free methionine and cystine was absorbed. Their study also indicated that some water soluble, heat stable, low molecular weight substance(s) did interfere with methionine and cystine utilization and depressed growth in rats. This material was present in the water extract of autoclaved beans but not in the water extract of raw beans or in water from beans boiled in 0.1% sodium bicarbonate solution.

Vaughan et al. (1977) measured plasma amino acids in six adult human subjects in the fasting state and at half hour intervals after ingesting untreated lactalbumin, heated lactalbumin, or no protein. The plasma level of essential amino acids decreased after the protein-free meal, but increased after the meal of untreated lactalbumin. After ingestion of heated lactalbumin, there was either no increase or a lowered increase compared to the untreated control. The authors interpret these results as indicating a delay in the initial digestive release of the constituent amino acids was delayed. They propose that changes in postprandial plasma amino acid levels could be used for determining any adverse effect on digestibility in human subjects as a result of processing and thereby indicating the nutritive value of the protein in question.

Possible Mechanisms for Decreased Amino Acid Availability

The studies cited above indicate that many foods have decreased availability of lysine, methionine, and cystine after processing procedures. The mechanisms whereby these amino acids become biologically unavailable remain to a large extent obscure. The proposed mechanisms for rendering amino acids unavailable include: new cross-links formed by protein-protein interaction, protein-carbohydrate

interaction, protein-lipid interaction; racemization of amino acids; and oxidation of amino acids (Mauron, 1972; Cheftel, 1977).

Protein-Protein Interactions

Ford (1973) states that overheating may impair nutritional quality by the formation of a tangle of new linkages within and between peptide chains involving the functional groups of the various amino acids. These new linkages may be resistant to hydrolysis by the proteolytic enzyme in the gut and may also impair digestibility of adjacent peptide bonds.

Although few studies have been done to demonstrate abnormal crosslinks in processed soy products, several studies have been done which demonstrate the presence of these crosslinks in other foods which have been heat and/or alkali treated.

Bjarnason and Carpenter (1969), using pure proteins, demonstrated that glutamine will react with the epsilon amino group of lysine forming epsilon-(gamma glutamyl)-lysine upon heating with the liberation of ammonia. Otterburn et al. (1977) isolated two other dipeptides formed by reaction of lysine with either glutamine or asparagine from a variety of food proteins which had been overheated. These dipeptides would appear to have little significance, however, since they have been shown to be highly digestible _in vivo_ in test animals (Mauron, 1972; Hurrell et al., 1976). It is possible however, that such linkages in a polypeptide chain could reduce the rate of digestion of the protein either by insolubilization, by preventing enzyme penetration, or by masking the sites of enzyme attack (Hurrell and Carpenter, 1977).

Cross linkages can also result from the degradation of cystine. Heat can disrupt the disulfide bond yielding dehydroalanine which can then condense with cystine to form lanthionine (LAT) as with the epsilon amino group of lysine to form lysinoalanine (LAL) (Hurrell and Carpenter, 1977).

Klostermeyer and Reimerdes (1977) studied the behavior of -SH and -S-S- groups of individual milk proteins. They found polymer formation and losses of sulfur with the formation of dehydroalanine and lysinoalanine on heating β-lactoglobulin.

Severe alkali treatment can also produce crosslinks. Asquith and Otterburn (1977) present evidence to show that protein-bound cystine upon alkaline deradation yields two dehydroalanine residues which may then react with amines, thiols, or amino-thiols in proteins to form LAL, LAT, or other crosslinks depending on the kinds of functional groups available in the peptide chain and on other compounds which may be present in the food. Other unnatural amino acids that may be formed are ornithinoalanine and derivatives of histidine, arginine, and kynurinine (Gould and MacGregor, 1977).

The effect of alkali on the formation of LAL in proteins was investigated by Provansal et al. (1975). They found LAL formed even under mild alkaline conditions in sunflower isolate. The amount of LAL increased and then decreased as the severity of the alkaline treatment increased.

Aymard et al. (1978) found LAL and to a lesser extent LAT in a wide variety of processed foods. Two commercial soy isolates had values of 1300 and 20 micrograms/gram of protein of LAL plus LAT.

Heating the isolates increased the amounts to 2000 and 650 micrograms, respectively. Alkaline conditions during heat treatment enhanced LAL formation.

Sternberg and Kim (1977) also found that heat as well as alkali contributed to the formation of LAL in foods. Even mild heat treatment, such as boiling for three minutes led to detectable LAL formation. Forty-five commercial samples of soy protein isolate from two manufacturers had 0 to 370 ppm of LAL.

Raymond (1980) also analyzed a variety of foods for LAL and found that only a few of the samples he tested contained LAL. Among those that did were a strained egg yolk product used in infant feeding, two samples of evaporated milk, and one milk-based formula. In contrast to Sternberg and Kim (1977) Raymond found that boiling an egg for as long as 30 minutes did not lead to LAL formation. He cautioned that the amount of LAL present in a food protein as measured by ion-exchange chromatography could easily be overestimated because of interfering compounds such as galactosamine and any tryptophan still present in the hydrolysate after acid hydrolysis.

Finley et al. (1977) found that LAL formation in soy and other proteins could be inhibited by the addition of mercaptoamino acids such as cysteine during the isolation process. These authors, however, comment that the safety of thiol-treated proteins must also be evaluated.

Protein-Carbohydrate Interactions

In heat processed proteins where carbohydrates are present in the system, reactions can occur between the carbonyl group of aldehydes,

ketones, and sugars with amino groups present in the protein and may also react with other functional groups in the protein. This kind of reaction, commonly called Maillard browning, leads finally to the production of brown pigments called melanoidins. In this reaction some amino acids are destroyed or rendered unavailable. Most commonly, lysine is damaged to the greatest extent. Other amino acids, however, may also be damaged. Evans and Butts (1949a) found that when soy was autoclaved with sucrose the sulfur amino acids as well as the basic amino acids were damaged. Even leucine and phenylalanine were somewhat affected.

Evans and Butts (1949b) also observed, that when soy was heated with carbohydrates 97% of the methionine was inactivated but practically all of it was recoverable on acid hydrolysis. Horn et al. (1968) theorized that the thioether group of methionine could be a reactive site for reaction with carbohydrates resulting in a linkage that makes methionine biologically unavailable. To determine whether such a complex would be biologically available, they synthesized a model methionine-fructose compound (1-deoxy-L-methionino-D-fructose). The method of synthesis was that of Abrams et al. (1955) using glucose, L-methionine, and absolute methanol. Microbiological assay showed the methionine in this compound to be 80% available, while the rat assay indicated almost complete unavailability of the methionine.

Tufte and Warthesen (1979) investigated the loss of added methionine. The model systems were composed of 39.5% microcrystalline cellulose, 35.0% soy protein, 5.0% D-glucose, 20.0% deionized water, and 0.5% DL-methionine. The authors compared three methods of addition of

methionine to the system: (1) methionine added directly by dry

blending, (2) methionine added in water solution and (3) methionine and

glucose added together in water solution. All samples were heat sealed

in evacuated foil pouches and heated to 80°C for three hours. Unheated

controls were held at room temperature. Recovery of methionine was

measured by high pressure liquid chromatography (HPLC) and found to be

50% when the methionine was added in solution alone or with glucose,

but was 72% when methionine was dry blended. The losses were attri-

buted to Maillard browning reactions. Methionine sulfoxide and methi-

onine sulfone were not determined since the authors thought that these

products would not be formed in this system, without oxidized lipids

or air. The authors theorize that the higher recovery of methionine

when it was added in the dry state occurred because the methionine

exists as large crystals with few exposed amino groups; consequently,

only areas of localized Maillard browning but less overall browning.

Another type of reaction that is at least theoretically possible

between peptide-bound amino acids and sugars is the formation of

crosslinks between two peptide chains by the reaction of a hexose

molecule with the carboxyl groups in the respective chains forming an

ester linkage. Such crosslinks might sterically hinder proteolytic

enzymes and so render a portion of the peptide chains unavailable

(Mauron, 1972).

Protein-Lipid Interactions

Interactions between proteins and oxidized lipids are also known

to occur. Peptide-bound lysine becomes unavailable when the protein

is exposed to oxidized fats or fatty acids. The protein also becomes resistant to the action of trypsin (Mauron, 1972). The carbonyl group of fats and fatty acids that are undergoing autooxidation can react with the epsilon amino group of lysine in a Maillard-type reaction inactivating lysine (Lea et al., 1958).

Another reaction that may occur between proteins and oxidized lipids is the oxidation of some peptide-bound amino acids. Tufte and Warthesen (1979) found that a portion of free methionine in a model food system containing oxidized lipids was converted to methionine sulfoxide. When soy protein was present in the system a much smaller portion of free methionine was oxidized. The authors proposed that the protective influence of the protein could be due to the preferential oxidation of methionine residues in the protein rather than the free methionine.

Racemization

Measurement in processed proteins -- Processing may also cause some isomerization of L-amino acids converting them into the D or allo forms. Bjarnason and Carpenter (1970) noted that isoleucine residues in bovine plasma albumin were partially isomerized to allo-isoleucine on heating the protein. Hayase et al. (1973, 1975) found that casein, lysozyme, and poly L-amino acids which were dry heated from 180° to 300°C for 20 minutes showed isomerization. Aspartic acid, glutamic acid, alanine, and lysine were markedly inverted; also phenylalanine, leucine, isoleucine, valine, and proline were also inverted, but to a lesser extent. Quantitative analyses for isomerization of the other amino acids were unsuccessful.

The effect of alkali in inducing inversion of amino acids in food proteins has not been studied extensively. Levene and Bass (1928, 1929) treated casein, albumin, elastin, and fibrin with dilute and concentrated NaOH at room temperature for 0 to 15 days. Using changes in optical rotation as their indicator of racemization, they found that inversion to the D form did occur and was directly proportional to time and concentration of alkali.

Pollock and Fromhagen (1968) treated egg albumin, gelatin, and soil humic and fulvic acids with 0.5N NaOH for varying lengths of time. Using gas chromatography they found that in all samples the D enantiomers of aspartic acid, phenylalanine, glutamic acid, and lysine were present in significant amounts.

Geschwind and Li (1965) studied the effect of alkali on melanocyte-stimulating hormone. After exposure to 0.1N NaOH for 15 minutes in a boiling water bath, the protein was assayed microbiologically with an organism which could utilize only L amino acids. The amounts of the L forms of arginine, histidine, phenylalanine, and methionine in the protein were lowered significantly according to these authors.

The isomerization of L-lysine to D-lysine in sunflower protein isolate subjected to alkaline treatment was investigated by Provansal et al. (1975). Using both an enzymatic method and microbiological assays they found that the ratio of D to L lysine increased with the severity of the alkaline processing reaching 40% with a treatment of 0.2M NaOH for 16 hours at 80°C. Mild to moderate alkaline treatment

(less than 0.2M NaOH) did not appear to cause any significant degree of inversion.

Marable et al. (1980) found that the optical rotation changed in soy-protein isolates prepared by extracting soy flour at pH's of 9.0 to 12. This effect was not seen at pH 6.7. They inferred that inversion of some amino acid residues was taking place. The effect was enhanced as the time of exposure to alkali increased.

Gilbert (1977) examined a commercial soy flour and commercial soy isolate for the presence of D-methionine and D-valine. She hydrolyzed the soy products, collected and derivatized the valine and methionine and separated them by ion-exchange chromatography. She found D-methionine but not D-valine in the soy flour and isolate. These qualitative results suggest that some isomerization of L-methionine takes place during commercial processing of soy protein.

Utilization of D-amino acids by animals -- Since D-amino acids are not found naturally in most mammalian tissues (White et al., 1952) the question of utilization of the D isomers requires that they be converted to the L forms after absorption (White et al., 1952). The mammalian liver and kidney do contain D amino oxidases which are capable of catalyzing this conversion (Krebs, 1935).

Berg (1959) determined that rats had the ability to convert many of the D amino acids including D-methionine to the L isomer when the D amino acids were fed singly, although with varying degrees of facility depending on the particular amino acid. Wretland (1952) found that D-methionine was as effective as the L form in promoting

growth in rats when five of the essential amino acids were in the L

form but that much slower growth resulted when all eight essential

amino acids were present as the D isomer. Other studies (Phillips

and Berg, 1954; Wachter and Berg, 1960; Kamath and Berg, 1964) also

indicate that when several D amino acids are fed simultaneously to

rats a reduced growth rate resulted.

In chicks, Kuzmicky et al. (1974) found that D-methionine was

about 93% as effective as DL-methionine in promoting growth. Katz and

Baker (1975) established that chickens can utilize the D isomer of the

hydroxy analog of methionine as well as D-methionine. The work of

Burns and Baker (1977) suggests that D-homocysteine is utilized by the

chick but less efficiently than D-methionine and the D-hydroxy analog

of methionine. Baker and Harter (1978) found that it took twice as

much DL-cystine as L-cystine to achieve the same level of weight gain

in chicks. They concluded that chicks cannot utilize D-cystine. The

D isomer itself, however, was not tested.

Utilization of D-amino acids by humans -- Results of studies on

the ability of humans to utilize D amino acids are conflicting. While

there is general agreement that most of the essential amino acids

cannot be utilized by man in the D form, Rose et al. (1955) concluded

that D-methionine was utilized by humans as well as the L isomer.

Several other studies, however, concluded that D-methionine is not

utilized readily by man. Harper and Ureyam (1948) fed DL-methionine

to humans and then measured plasma levels of both isomers. They found

that L-methionine was removed from the blood at a faster rate than

D-methionine. Camien et al. (1952) found that when humans were fed DL-methionine urinary excretion of L-methionine increased slightly while excretion of D-methionine increased markedly. Their conclusion was that D-methionine is not utilized as well as the L form.

Recent studies have also suggested that the D isomer of methionine is not well utilized by humans. Kies et al. (1975) measured nitrogen retention in humans when "instant" oatmeal was unsupplemented or supplemented with either L-, DL-, or D-methionine at levels of 0.58 and 1.16 grams per day. The level of nitrogen in the diet was 4.0 grams per day. Although only subjects receiving the diet supplemented with the higher level of L-methionine were in positive balance, the retention at both the higher and lower levels of L-methionine was significantly better than those of the unsupplemented control. DL-methionine supplementation at the higher level resulted in a nitrogen retention comparable to that seen in the subjects on the lower level of L-methionine supplementation. No significant difference was seen in nitrogen retention in subjects receiving the oatmeal diet supplemented with either level of D-methionine and the unsupplemented control. Urinary excretion of methionine was significantly higher in subjects while on the higher level of supplementation with D- and DL-methionine compared to unsupplemented and to L-methionine supplemented diets. The authors conclude that D-methionine is utilized less efficiently in humans than the L- isomer.

Zezulka and Calloway (1976), using adult males as subjects, investigated the ability of D-methionine, N-acetyl-L-methionine, and sodium sulfate to replace L-methionine in a diet deficient in sulfur

amino acids when these compounds were used to supplement the diet. The supplements provided enough sulfur to be equivalent to the sulfur present in 420 mg of L-methionine, the amount added to the soy isolate diet to bring the total sulfur amino acid content to 900 mg per day. Five of six subjects were in negative nitrogen balance when the diet was supplemented with D-methionine; the mean nitrogen retention for the six subjects was not significantly different from the unsupplemented diet, suggesting that D-methionine is poorly utilized in humans. N-acetyl-L-methionine was equivalent to L-methionine as a supplement, with five of six subjects exhibiting positive nitrogen balance with L-methionine and N-acetyl-L-methionine supplements. Sodium sulfate effected positive balance in two of five subjects and caused a statistically significant increase in mean nitrogen retention for the five subjects compared to the unsupplemented diet, indicating that sodium sulfate has some value as a supplement in sulfur amino acid deficient diets.

Oxidation of Amino Acids

Another way amino acids may become unavailable is through oxidation. The sulfur amino acids are especially susceptible to oxidation (Mauron, 1972; Walker et al., 1975). In foods such as soy products where the sulfur amino acids are low, oxidation may be an especially important factor. Walker et al. (1975) state that processing procedures used for desolventizing and toasting oilseed meals at high temperatures "seem likely to create a favorable environment for sulfur amino acid oxidation." Oxidation may also occur from the use of chemicals such as hydrogen peroxide in food processing and in sterilization

of food processing equipment (Cuq et al., 1973). Auto-oxidizing fats

which may be present in the food may also cause oxidation of the

sulfur amino acids (Lea et al., 1958; Tannenbaum et al., 1969).

Methionine, when it is oxidized, may contain either one or two

oxygen atoms bonded to the sulfur atom forming methionine sulfoxide

or methionine sulfone, respectively. Cystine has a greater number of

theoretically possible oxidation products. The common derivatives

that have been prepared are cystine monoxide, cystine dioxide, cysteine

sulfinic acid, and cysteine sulfonic acid (cysteic acid) (Walker

et al., 1975). Structures for some of these compounds are shown in

Appendix 1.

Few studies have determined the oxidized sulfur amino acids

present in processed foods. Cysteic acid, as determined by ion-

exchange chromatography after acid hydrolysis, was found in a soy

isolate with an especially low PER (Longenecker et al., 1964).

Happich (1975) in a study designed to test the nutritive value of

mixtures of beef and other animal and plant proteins found methi-

onine sulfoxide in a sample of textured vegetable protein and in a

soy protein concentrate. The method used for detection of methionine

and methionine sulfoxide was ion-exchange chromatography after acid

hydrolysis.

Kehrberg (1976) found methionine sulfoxide in one of seven soy

products tested. The product was a commercial soy isolate, Promine D,

and her method of determination of methionine sulfoxide was ion-exchange

chromatography after alkaline hydrolysis using the method described by

Neumann (1967). Her results were not truly quantitative and probably

underestimated quantities, since recovery rates for free methionine

sulfoxide under the conditions used for alkaline hydrolysis were low.

Methionine sulfoxide could have been present in the other products

tested, but at levels that were too low to be detected by the procedure

used.

Kehrberg (1976) also found cysteic acid in all the products

tested. The range of cysteic acid was from 2.9 to 23.2% of total

half-cystine content. The soy isolates contained higher amounts than

the soy flours and concentrates. No methionine sulfone was found in

any of the products.

Small amounts of methionine sulfoxide have been found even in

unprocessed foods. Slump and Schreuder (1973) found the sulfoxide in

fish protein. Happich (1975) found a small amount in lean beef and

Kido and Kassel (1975) found the compound in native porcine pepsin and

also in pepsin held at pH 3.2 for 70 minutes at 30°C. Sjöberg and

Boström (1977) suggest that these findings indicate that methionine

residues are "easily oxidized to methionine sulfoxide during handling

of protein material."

Oxidized methionine in model systems -- Tufte and Warthesen

(1979) measured methionine stability in methionine-fortified model

food systems in the presence of oxidized lipids using high pressure

liquid chromatography to determine the amount of methionine recovered

and the amount of methionine sulfoxide and methionine sulfone gained.

When they stored mixtures containing free methionine with corn oil

oxidized to varying degrees, they found that after 28 days of storage,

methionine recovery in the model with the most highly oxidized oil was about 70% and gain in methionine sulfoxide was about 30% indicating almost quantitative conversion of methionine to methionine sulfoxide. No methionine sulfone was detected in any sample.

Cuq et al. (1973) treated casein and pasteurized milk with hydrogen peroxide. When a 5.0% (w/v) casein solution was treated with 0.12M hydrogen peroxide for 30 minutes at 50°C, about 94% of the methionine was converted to methionine sulfoxide. No methionine sulfone was detected. When pasteurized milk was treated with 0.018 hydrogen peroxide for 30 minutes at 50°C, about 50% of the methionine was converted to methionine sulfoxide. The methods used to determine methionine sulfoxide were that of Neumann (1967) as modified by Blackburn (1968) and also the indirect method of Neumann (1967). No information was presented as to the stability of methionine sulfoxide in their procedures nor was any correction factor used for any destruction or conversion during hydrolysis.

In the same study Cuq et al. (1973) looked at changes in the in vitro enzymatic digestion of oxidized casein and milk. They found that the release of free methionine from casein by Pronase was inversely related to the methionine sulfoxide content. In the sample treated with the highest concentration of peroxide, no free methionine was released, nor was any free methionine sulfoxide or sulfone. For milk, where the percentage of methionine converted to the sulfoxide was 50%, release of free methionine by Pronase was 70% of the untreated control. Based on these results, the authors conclude that the "proteolytic enzymes in Pronase which act on methionyl peptide bonds

cannot split the same bonds once methionine residues have been oxi-
dized into methionine sulfoxide."

In other research, Cuq et al. (1977) studied the effect of
hypochlorite on a methionine residue in a synthetic tripeptide,
glycyl-L-methionyl-glycine. They found that at a concentration of
0.01 to 0.1% sodium hypochlorite at pH 8 and 60°C, significant amounts
of methionine sulfoxide were formed; however, at 0.4% hypochlorite
and at an acid pH and 80°C there was a decrease in methionine sul-
foxide content and a marked increase in methionine sulfone compared
to treatment at the lower levels. Furthermore, one mole of glycine
was destroyed and only 40% of the original methionine could be
accounted for. The authors hypothesized that N chlorination of some
peptide bonds could modify the stability of the methionine residues
or the oxidized forms during alkaline hydrolysis.

The method used to determine methionine sulfoxide content was
a modification of the method of Neumann (1967). The tripeptide was
hydrolyzed under nitrogen with 3M NaOH for 16 hours at 110°C. An
alkaline hydrolysis was also done on a non-hypochlorite treated
sample. They found hydrolysis was complete and the yield of glycine
close to the theoretical value; however, there was a 10% loss of
methionine probably due, according to the authors, to partial destruc-
tion during alkaline hydrolysis.

Bioavailability of oxidized sulfur amino acids -- The studies
cited above indicate that oxidation of methionine and cystine does
occur to some extent even under relatively mild processing conditions.

The question, however, of the utilization of these oxidized forms has still not been resolved. Njaa (1962) demonstrated that while free methionine sulfone was without activity as a replacement for methionine, DL-methionine sulfoxide was 75% as active DL-methionine in promoting growth in rats. Studies by Miller and co-workers (1968; 1970a; 1970b) and by Anderson et al. (1976) found that methionine sulfone and cysteic acid in the free form could not be utilized for growth in rats but that methionine sulfoxide showed partial effectiveness as a replacement for methionine. Miller and Samuel (1970a) found that free methionine sulfoxide was 26% as effective as methionine in supporting growth in rats. In another study, Miller et al. (1970b) found that weanling rats did not utilize methionine sulfoxide as well as methionine in short term studies (17 days) but they found no significant difference in the utilization of methionine sulfoxide compared to methionine in long term studies (132 days). They suggested that either older rats have a lower methionine requirement or that some adaptive mechanism develops with age in rats.

A series of chick growth experiments indicated that L-cysteic acid and DL-methionine sulfone were completely unavailable to chicks. DL-, L-, and D-methionine sulfoxide were significantly less available than DL-methionine sulfoxide. DL- and D-methionine sulfoxide were significantly less available than L-methionine (Kuzmicky et al., 1974).

Ellinger and Palmer (1969) attempted to assess the bioavailability of peptide-bound methionine sulfoxide using a microbiological technique. They found that Streptococcus zymogenes did not utilize peptide-bound methionine sulfoxide as well as peptide-bound methionine.

Previously, Miller et al. (1965) reported that Streptococcus zymogenes responded equally to free methionine sulfoxide and methionine. Ellinger and Palmer (1969) concluded that utilization of methionine sulfoxide by Streptococcus zymogenes is retarded if the methionine sulfoxide is peptide bound.

Slump and Schreuder (1973) assessed availability of peptide-bound methionine sulfoxide based on NPU measurement in rats fed oxidized casein and fish meal. They found that peptide-bound methionine sulfoxide was completely available to rats even though it was peptide bound.

Pieniazek et al. (1975b) fed oxidized casein diets containing either 110g or 180g of protein per kg of diet to weanling rats. These diets were supplemented with tryptophan, tyrosine, and cysteine. They found that oxidized casein did not support growth at either diet level even when supplemented with the three amino acids. Addition of methionine to the other supplemental amino acids improved the growth rate to 30% of the unoxidized casein controls at both protein levels. When 15% of the oxidized casein in the lower protein diet was replaced by an equivalent amount of unoxidized casein and supplemented with all four amino acids, the growth rate improved to 76% of the control diet. Omission of methionine from this diet led to a failure of growth in the rats. These data suggest to the authors that oxidized casein contains linkages in the polypeptide chain that are resistant to in vivo enzymatic hydrolysis.

Anderson et al. (1975) treated rapeseed flour with hydrogen peroxide. The treatment resulted in destruction of a portion of the

tryptophan, the conversion of 85% to 90% of the methionine to methi-
onine sulfoxide and methionine sulfone, and the conversion of 26% to
67% of the cysteine to cysteic acid. Rats fed the oxidized rapeseed
flour survived but lost weight. They also had high plasma levels of
methionine sulfoxide and sulfone. Addition of methionine resulted in
weight gains in the rats. The authors concluded that the nutritional
value of the peroxide-treated rapeseed flour was reduced by the pre-
sence of methionine sulfone and cysteic acid.

Cuq et al. (1978) found that peptide-bound methionine sulfoxide
in oxidized casein was just slightly less nutritionally available
than methionine. While their 1973 and 1978 studies showed decreased
release of methionine sulfoxide in vitro for oxidized casein, the
1978 study indicated that in vivo "a large proportion of methionine
sulfoxide residues is released from casein, absorbed, converted to
methionine and used for protein synthesis" in rats.

Sjöberg and Boström (1977) also investigated the nutritional
effects of feeding oxidized proteins to rats. Using varying amounts
of hydrogen peroxide, they oxidized fish protein. They found that
PER and BV were significantly decreased compared to the unoxidized
control. With the exception of the fish meal treated with the highest
concentration of peroxide (80g per kg protein), supplementation with
L-methionine or L-cystine increased BV for all oxidized samples.
L-methionine supplementation increased BV to the level for the un-
treated fish protein except for the sample treated with the highest
concentration of hydrogen peroxide. Supplementation of the oxidized
fish protein with L-methionine-DL-sulfoxide was as effective as

supplementation with methionine but L-methionine sulfone and cysteic
acid had no supplementary effect when they were added to the oxidized
proteins.

Sjöberg and Boström analyzed the oxidized fish protein for oxi-
dized sulfur amino acids. They found that at the lowest level of
peroxide treatment used (20g hydrogen peroxide per kg protein)
approximately 65% of the methionine and 55% of the cystine were oxi-
dized. Of the oxidized methionine only about 5% was methionine
sulfone. On examining the rat plasma they found that both methionine
sulfoxide and methionine sulfone were present, with the sulfoxide
level in the plasma being higher than that of the sulfone. Only
traces of methionine sulfoxide were found in the urine, but large
amounts of an acid-labile derivative of methionine sulfone were pre-
sent in the urine. Methionine sulfoxide, methionine sulfone, and
cysteic acid were also found in the liver, kidney, and muscles.

Digestibility and absorption of the oxidized sulfur amino acids
in the oxidized fish protein were also measured by Sjöberg and Boström
(1977). The overall digestibility did not appear to be impaired.
Furthermore, over 90% of the peptide-bound sulfur amino acids were
absorbed.

Gjøen and Njaa (1977) also tested the ability of methionine
sulfoxide to function as a replacement for methionine in the growing
rat. They found that when methionine sulfoxide was the sole sulfur
amino acid in a diet of amino acids, rats grew poorly compared to
diets with methionine as the sole sulfur amino acid. Substitution of
one-third of the sulfoxide with cystine, however, improved growth so

that it approached methionine in its ability to promote growth of rats.

In the same study, Gjøen and Njaa (1977) fed rats mixtures of unoxidized and oxidized fish meal. Only when the oxidized fish meal was given alone, without added unoxidized fish meal, was there a significantly reduced rate of growth in the rats compared to the unoxidized control.

In attempting to resolve the conflict about the availability of methionine sulfoxide, Gjøen and Njaa (1977) re-examined the data of Miller and Samuel (1968, 1970a), Miller et al. (1970b), Ellinger and Palmer (1969), and Slump and Schreuder (1973). They found that in general, when cystine was not present in the amino acid mixtures, methionine sulfoxide was poorly utilized but, when cystine was present, utilization of the sulfoxide improved greatly. The data suggested to them that when methionine sulfoxide is the sole sulfur amino acid, synthesis of cystine is too slow for healthy growth. This theory, however, does not explain the results of Slump and Schreuder (1973) who reported methionine sulfoxide available even though the cystine content was low in their oxidized casein.

Slump and Schreuder (1973) suggest that the conflict about availability of methionine sulfoxide may be due, at least partially, to differences in stero configurations of the mixtures of methionine sulfoxide used by the various nutritionists.

The bioavailability of methionine sulfoxide in humans is unknown. One study (Pryzrembel et al., 1973) studying the effect of two levels of protein on urinary amino acid excretion in premature infants

reported relatively large amounts of methionine sulfoxide excreted by most subjects in the study. The group fed the higher level of protein (3.0 g per 100 ml) in a formula called "Alete initial" had a higher level of excretion than the group fed the lower protein level (1.5 g per 100 ml) in a milk-based formula (SMA). Although the authors did examine the infants' blood plasma for amino acid content, methionine sulfoxide was not listed among the amino acids found in the plasma. The authors did not comment on the excretion of methionine sulfoxide in the urine nor did they state whether or not they specifically looked for this amino acid in the plasma.

The Presence of Toxic Substances in Processed Food Proteins

Another possible explanation for the lowered nutritive quality in processed food proteins is the presence of toxic compounds. Toxic compounds may be produced during processing and/or anti-growth factors originally in the protein may not have been removed completely by the processing.

Anti-Growth Factors

Longenecker et al. (1964) demonstrated that three of four commercial soy isolates had higher PER's after heating. Bressani et al. (1967) also found PER's were improved when soy isolate and the fiber made from the isolate were heated. The improvement in nutritional quality was attributed to inactivation of residual growth inhibitors in the isolate and fiber. Liener (1975) found relatively high levels of anti-trypsin activity in a soy isolate and fiber made from the

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isolate. Meat analogs made from the fibers, however, had low levels

of anti-trypsin activity. The data of Liener (1975), Bressani et al.

(1967), and Longenecker et al. (1964) indicate that residual growth

inhibitors are, indeed, present in some soy isolates and fibers.

Heating, however, does not always restore the PER of soy isolates to

the level of the soy flour (Longenecker et al., 1964), indicating

other factors are also involved in the lowered nutritive value of soy

isolates and fibers.

Maillard Browning Compounds

Products of the Maillard browning reaction may also be toxic.

Erbersdobler (1977) attempted to measure the effect in rats of protein-

bound fructosylysine, a compound formed in the early stages of

Maillard browning; results showed that although fructosylysine is

digested and absorbed to some extent, it is not metabolized and it

does not appear to be harmful to rats.

Research by Stegink and co-workers (Stegink et al., 1974;

Stegink et al., 1975; Freeman et al., 1975) indicates that when sugar-

amino compounds are present in fluids used in venous alimentation,

mild dehydration occurs in infants as well as excessive excretion of

zinc and other trace metals in infants and adults. However, these

losses were not seen when these solutions were introduced into the

stomachs of subjects.

Cheftel (1977) reviewed the research on the toxicity of Maillard

compounds. He reported that Adrian et al. (1966) and Adrian and

Susbielle (1975) have shown that biologically active compounds (which

are capable of stimulating or inhibiting growth in rats) are produced when mixtures of free amino acids are autoclaved with reducing sugars. This is relevant only when significant levels of free amino acids and reducing sugars are present in the food system. Cheftel, in reporting on the work of Mauron (1975), stated that compounds from Maillard reactions are only partially digested and absorbed and only influence animal or growth when lysine is deficient.

Lysinoalanine

Heat and alkali have been shown to cause the formation of amino acids not found normally in food proteins. Of these "unnatural" amino acids lysinoalanine (LAL) has been studied most extensively. Studies on the toxicity of LAL are conflicting. Gould and MacGregor (1977) reviewed the recent research. They note that some investigators (Reyniers et al., 1974; Woodard, 1969; Woodard and Alvarez, 1967; Newberne et al., 1968; Newberne and Young, 1966) consistently find nephrocytomegalia in rats fed LAL either in the free form or contained in alkali-treated proteins. Studies by DeGroot and his co-workers (DeGroot and Slump, 1969; DeGroot et al., 1976; 1977) on the other hand fail to demonstrate any renal lesions in rats, mice, Japanese quail, hamsters, dogs, or Rhesus monkeys fed protein-bound LAL. These investigators did find renal lesions in rats fed free LAL and to a lesser extent in rats fed oligopeptides containing LAL (DeGroot et al., 1977).

In an attempt to resolve the conflicting results found with protein-bound LAL, Gould and MacGregor (1977) noted that the

laboratories that report renal lesions (Woodard and Short, 1973;
Newberne and Young, 1966; Karayiannia, 1976) fed rats alkali-treated
soy protein of almost identical composition, while the studies by
DeGroot and co-workers (DeGroot and Slump, 1969; DeGroot et al.,
1976a, 1976b; Van Beek et al., 1974) used a slightly different composi-
tion in their soy protein diets. The diets differed in salt concentra-
tion and carbohydrate source as well as protein. Newberne and Young
(1966) and Newberne et al. (1968) found that dietary supplementation
with methionine, choline, and vitamin B_{12} prevented the renal lesions.
Woodard and Alvarez (1967) could not confirm this finding, however.
Gould and MacGregor (1977) think that "methionine and other nutri-
tional factors may strongly modulate the expression of nephrocytomegaly."

A recent study examined the effect of protein-bound LAL on fetal
and neonatal rats. Struthers et al. (1978) fed diets containing 5 to
30% of an alkali-treated soy isolate which contained 1% LAL to female
rats during gestation and lactation. They found no teratological
effects and no significant differences in birth weight, mortality,
live births per litter, or number of pups per litter at any of the
levels LAL fed. The protein level of the milk of all females was
similar and no LAL was found in the milk. They did observe decreased
weight gains of pups of dams fed high levels of the soy isolate and
concluded that this was due to reduced milk production in the mothers.
The reduced milk supply was due, in the opinion of the authors, to the
lowered digestibility in the alkali-treated soy protein, although
they did not rule out the possibility of a toxic effect from LAL.

Recently, Struthers et al. (1979) reviewed the animal studies done on the toxicity of LAL and concluded that "not all 'protein-bound' LAL is equivalent in availability or biological effect." Factors that may contribute to the bioavailability and, thereby, to the pathological effect include: severity of alkaline treatment, type of protein, concentration of the protein in the diet, concentration of LAL in the protein, nature of the crosslink involving LAL, and stereo configuration of LAL.

As to toxicity in humans, Gould and MacGregor (1977) point out that adults probably have very low intakes of LAL compared to the relative amount needed to induce nephrocytomegaly in rats. Infants, however, are a special exception since relatively high LAL levels have been reported in some infant formulas (Sternberg and Kim, 1977) and, at least for the first few months of life, these formulas constitute the major source of nutrition.

Trace Mineral Availability

Another possible explanation for lowered protein quality in processed food proteins is the decreased bioavailability of trace minerals. Of the trace minerals, only zinc has been clearly shown to have low bioavailability when it is consumed in the presence of soy protein (O'Dell, 1979). According to O'Dell (1979), who has summarized many of the recent studies on trace mineral availability, the evidence is overwhelming that soluble phytates bind zinc and retard absorption. Furthermore, excess calcium accentuates the effect.

Rackis (1975) believes that a primary factor in the variability in PER's for various commercial soy isolates is due to unavailability of zinc in some soy isolates. Rackis (1979) points out that zinc availability in an isoelectric form of soy protein isolate, manufactured specifically for infant formulas, is high while other isolates, which have been modified by alkali processing, have low zinc availability even though the isolates have similar levels of phytic acid. It has been suggested that processing can also lead to insoluble protein-phytate-zinc complexes which may lower intestinal absorption of zinc (Lease, 1967). O'Dell (1979), however, believes that protein-phytate complexes are of little significance, nutritionally. O'Dell (1979) and Rackis (1974; 1975) suggest that processing procedures should be selected which rid the soy of phytates and promote formation of binding agents.

<u>Determination</u> <u>of</u> <u>Methionine</u> <u>and</u> <u>Cystine</u> <u>and</u>
<u>Their</u> <u>Oxidized</u> <u>Derivatives</u> <u>in</u> <u>Foods</u>

Since a major objective of this study is to develop a set of procedures for determining total methionine, methionine, methionine sulfoxide, methionine sulfone, total cystine, and cysteic acids in foods, a review of the methods which are currently in use or which have been suggested is presented.

The determination of the sulfur amino acids in foods presents many difficulties. The usual method of acid hydrolysis of the proteins followed by automated ion-exchange chromatography is unsatisfactory for determination of both methionine and cystine. Acid

hydrolysis of peptide-bound cystine in foods results in losses of cystine by reaction with tryptophan, carbohydrates, and other substances. Oxidation of cystine may also occur (Friedman and Noma, 1975).

Methionine is also subject to losses during acid hydrolysis. Jennings (1969) found losses of methionine during acid hydrolysis to be as high as 59% in plant materials. Furthermore, if any methionine sulfoxide is present, a portion of it may be reduced to methionine (Njaa, 1962; Floyd et al., 1963).

The most common method used to overcome the problems involved in acid hydrolysis of methionine and cystine is to oxidize the proteins with performic acid prior to acid hydrolysis. This converts methionine to methionine sulfone and cystine to cysteic acid, both of which are stable to acid hydrolysis (Moore, 1963). During oxidation with performic acid, methionine sulfoxide is also converted to methionine sulfone and any oxidized intermediates of cystine are converted to cysteic acid; therefore, this procedure cannot differentiate methionine from any methionine sulfoxide or methionine sulfone that may have been present originally in the food protein. The procedure does give a measurement of total methionine (methionine plus methionine sulfoxide plus methionine sulfone) and total cystine (cystine plus all oxidized derivatives of cystine).

Chemical Methods for Determination of Sulfur Amino Acids

Several methods have been devised to measure chemically oxidized sulfur amino acids in foods. Because methionine sulfone and cysteic

acid are acid stable they can be quantitated directly by ion-exchange chromatography after acid hydrolysis (Blackburn, 1968); however, the usual ion-exchange chromatography conditions would probably not separate methionine sulfone from aspartic acid. Using a modified set of elution conditions, Kehrberg (1976) found that recoveries of cysteic acid and methionine sulfone were 96% and 101%, respectively, for a mixture of pure amino acids subjected to acid hydrolysis and chromatography on a Technicon NC-2P amino acid analyzer. When pure ovalbumin was acid hydrolyzed, no methionine sulfoxide, methionine sulfone, or cysteic acid was detected indicating that the acid hydrolysis conditions used (6M HCl, 110°C 18 hours) did not lead to oxidation of methionine or cystine.

Analysis for methionine sulfoxide is more complicated. Acid hydrolysis not only reduces a portion of methionine sulfoxide to methionine but also converts small amounts to methionine sulfonium salt, homocystine, and homocysteic acid (Walker et al., 1975). To circumvent these problems, Neumann et al. (1962, 1967) suggested two different approaches to measure methionine sulfoxide. The first, called the direct method, is simply ion-exchange chromatography after alkaline hydrolysis of the proteins with either sodium hydroxide (final concentration 15%) at 110°C for 16 hours or with barium hydroxide. This method, while simple, results in the loss of some methionine sulfoxide during the alkaline hydrolysis. Neumann (1967) reported losses of from 10% to 25%. Lunder (1972) stated that on alkaline hydrolysis (conditions not given), pure methionine sulfoxide was decomposed into five ninhydrin positive peaks. Hugli and Moore (1972) reported that

protein losses occurred during removal of barium when proteins were hydrolyzed with barium hydroxide. More recently, Kehrberg (1976) using a modification of Neumann's method, found losses of approximately 60% for methionine sulfoxide when it was hydrolyzed with 15% sodium hydroxide at 110°C for 16 hours. With 2M barium hydroxide at 110°C for 16 hours, losses of up to 80% occurred, with large variations between duplicate samples. Kohler and Palter (1967) used 2M barium hydroxide to hydrolyze the protein in wheat products prior to tryptophan analysis by ion-exchange chromatography. They, also, found that their results for the amount of tryptophan in the proteins analyzed were erratic and could only be thought of as minimum values.

The second method for methionine sulfoxide determination suggested by Neumann (1962) depends on the reaction of iodoacetic acid with methionine to form a sulfonium salt. This reagent does not react with methionine sulfoxide or methionine sulfone. After alkylation of methionine, the protein is oxidized with performic acid, acid hydrolyzed, and chromatographed. If the protein is also chromatographed after acid hydrolysis of the unoxidized protein, the amounts of methionine, methionine sulfoxide, and methionine sulfone may be calculated. A diagram of the indirect procedure is given in Appendix 2. The major disadvantage, besides being a more complicated and time-consuming procedure than the direct method, is that complete alkylation of all methionine residues may not occur, resulting in erroneously low values for methionine and high values for methionine sulfoxide (Neumann, 1967; Walker et al., 1975).

Cuq et al. (1973) used both the direct and indirect method to measure methionine sulfoxide in oxidized food. They used 2M barium hydroxide in the direct analysis of oxidized casein and found that this method gave slightly higher values for methionine sulfoxide than the indirect method. The authors concluded that the direct method was more accurate. They gave no indication, however, of any corrections made for possible losses during the alkaline hydrolysis.

Sjöberg and Boström (1977) oxidized fish protein and modified the indirect method by an enzymatic digestion step prior to alkylation. The digestion was done to facilitate the alkylation of methionine residues not easily accessible in the fish protein. Sjöberg and Boström also determined methionine sulfoxide by the direct method using 2M sodium hydroxide at 100°C for 18 hours in the hydrolysis. They indicated that both methods gave similar results but only reported the results of the indirect method. As with Cuq et al. (1973), Sjöberg and Boström did not mention any corrections used to account for losses of methionine sulfoxide in the hydrolysis.

Several other chemical approaches have been suggested to measure methionine and/or methionine sulfoxide. Lunder (1972) devised a method for measuring methionine sulfoxide based on the reaction of acetic anhydride with peptide-bound methionine sulfoxide. According to Lunder (1972), this reagent acts only on sulfoxides rearranging these residues to a derivative, which, upon acid hydrolysis, liberates formaldehyde. This compound can then be determined colorimetrically to give a quantitative measurement of methionine sulfoxide. A major disadvantage appears to be the sample size needed (one to three grams

of protein). Furthermore, Lunder (1972) did not demonstrate the specificity of this reaction when it was used on intact food proteins.

Pieniazek et al. (1975a, 1975b) modified the procedure of McCarthy and Sullivan (1941) to measure methionine. In this procedure sodium nitroprusside reacts with methionine but not with methionine sulfoxide or sulfone to form a compound which, upon acidification, turns red. Prior enzymatic digestion is necessary, and specificity of the reaction is reduced by interference from other protein and non-protein components present in foods (Lipton and Bodwell, 1977).

A gas chromatographic method was reported by Ellinger and Duncan (1976) and Duncan et al. (1976) for the determination of methionine. The method utilizes the reaction of cyanogen bromide with peptide-bound methionyl residues releasing methylthiocyanate which is then measured by gas-liquid chromatography. This method does not require prior hydrolysis of the protein, but does require preliminary extraction if γ-glutamyl-S-methyl-cysteine, an interfering substance, is present in the test material. The major disadvantages are the toxicity of cyanogen bromide and the length of reaction time needed for release of methylthiocyanoate (Lipton and Bodwell, 1977).

Lipton and Bodwell (1977) also developed a gas chromatographic method for measuring methionine in proteins. Their method uses the reaction of dimethyl sulfoxide with methionine, reducing the dimethyl sulfoxide to dimethyl sulfide which is then measured by gas-liquid chromatography. The authors claim a good correlation between their chemical estimate of protein quality of a soy isolate and a textured soy preparation and PER for these products. The authors point out

that other reducing substances in food could interfere with their assay and that more work is needed to establish the usefulness of this method for foods.

Shechter et al. (1975) also used the reaction of cyanogen bromide with methionine. In this reaction, methionine is converted to homoserine but the methionine sulfoxide remains unaltered. The protein is then hydrolyzed with acid in the presence of either mercaptoethanol or dithioerythritol reducing the methionine sulfoxide to methionine. The hydrolysate is then subjected to analysis by ion-exchange chromatography. The homoserine (and its lactone) represents the methionine originally in the sample and the methionine represents the original methionine sulfoxide.

Savige and Fontana (1977) reported a method in which the protein is hydrolyzed in evacuated sealed tubes with 3N p-toluene sulfonic acid. The authors claim both methionine and methionine sulfoxide are stable in this acid and also in methanesulfonic acid. The hydrolysate may then be analyzed by ion-exchange chromatography for methionine and methionine sulfoxide directly. A disadvantage of this method is that even traces of haloacids will reduce methionine sulfoxide, and therefore the method is not suitable for food systems.

All of the previously described methods are designed for methionine or methionine sulfoxide detection but not for cystine or its oxidized forms. A method proposed by Walker et al. (1975) measured not only methionine and its oxidized forms but also cystine and its oxidized products. The method uses X-ray photoelectron spectroscopy to measure the various oxidative states of sulfur. The authors stated

that there is a need for more analyses on both model compounds and naturally occurring substances to establish its validity. It is unlikely that it is applicable to foods.

Biological Methods

Microbiological, enzymatic, and bioassay methods exist for the determination of "available" methionine. Mauron (1973) and Morrison and McLaughlan (1972) have reviewed these methods, not only for methionine, but for the "availability" of other essential amino acids. These procedures measure forms of methionine that can be utilized by a particular animal, microorganism, or enzyme. They do not necessarily give an accurate measurement of the bioavailability of these forms in humans. In fact, the availability of the various derivatives of methionine in humans has not been established. These procedures can, however, serve as estimates of the amount of damage done to methionine during processing.

Bioassays and Microbiological Techniques

Bioassays for amino acid "availability" are based on either growth of animals, recovery of amino acids in the feces, nitrogen balance, or plasma amino acid levels (Morrison and McLaughlan, 1972). In general, these methods are time consuming, tedious, and expensive (Lipton and Bodwell, 1977; Morrison and McLaughlan, 1972).

In the microbiological techniques, various organisms have been used such as Streptococcus zymogenes, Streptococcus durans, and Lactobacillus arabinosis (Morrison and McLaughlan, 1972). Recently the protozoan, Tetrahymena pyriformis has been used since its amino

acid needs mimic man's (Frank et al., 1975; Landers, 1975; Satterlee et al., 1977). When the assay with this microorganism is preceded by a protease digestion step, the assay shows high correlation with PER's of various food proteins (Frank et al., 1975; Landers, 1975; Satterlee et al., 1977). The growth of Tetrahymena, however, appears to be affected by various food additives and interpretation of amino acid "availability" and protein quality must, therefore, be done with care (Satterlee et al., 1977). Wang et al. (1979), for example, found that microbiological assays of selected food samples with Tetrahymena pyriformis and Aspergillus flavus were not correlated with rat bio-assays for protein quality. A possible explanation proposed by the authors was the presence of spices and preservatives in the food samples.

Enzymatic Techniques

Enzymatic methods for determining amino acid "availability" depend primarily on measuring the release of a given amino acid when the test material is incubated with various proteolytic enzymes. Stahmann and Woldegiorgis (1975) described the technique and its applications in detail.

The use of enzymes, which are specific for certain amino acids, to determine isomerization or other changes in a particular amino acid residue has been very limited. Provansal et al. (1975) used lysine decarboxylase to demonstrate isomerization of L-lysine in sunflower protein. McBee and Marshall (1978) developed a method for determining available lysine based on the method of Carpenter (1960) combined with the use of lysine decarboxylase. It would appear that

research into the area of specific enzymes for specific amino acids
might prove fruitful as a means of assessing isomerization and avail-
ability.

Enzymes have been used in a variety of research and industrial
operations for analytical purposes. Their specificity allows them to
be used to detect quantitatively minute amounts of compounds present
in complex mixtures. Enzymatic analyses, besides being specific, are
usually capable of being run near room temperature and at or near
neutral pH's so that determination of labile products or intermediates
is possible (Whitaker, 1974).

It is feasible that an analytic procedure for measuring avail-
able methionine in food proteins could be developed using the enzyme ATP:
S-adenosyl-L-methionine transferase (E.C. number: 2.5.1.6). This
enzyme catalyzes the transfer of the adenosyl group of adenosine
triphosphate (ATP) to L-methionine forming S-adenosyl methionine (SAM).
The reaction, shown below, was first described by Cantoni and Durell
(1957).

$$\text{L-methionine} + \text{ATP} \xrightarrow{\quad Mg^{2+} \quad} \text{SAM} + PP_i + P_i$$
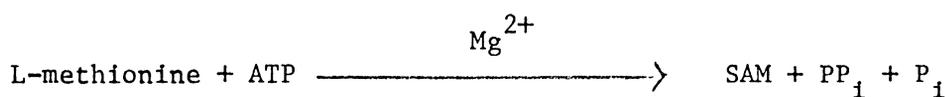
This enzyme is found in the livers of many mammalian species, in
yeast and in some bacteria (Cantoni and Durrell, 1957). The catalyzed
reaction is the first step in the transulfuration pathway whereby
methionine is converted in vivo to cystine or cysteine (Sturman et al.,
1970). It also plays a part in other physiological reactions in the
mammalian body.

Lombardini et al. (1970) investigated compounds other than L-methionine that could serve as substrates for the enzyme. They found that D-methionine, DL-methionine sulfoxide, and DL-methionine sulfone were inactive as substrates. The L-methyl and ethyl esters as well as N-formyl DL-methionine and N-acetyl DL-methionine were active as substrates though at reduced rates. Lombardini et al. (1970) concluded that the structural requirements for an active substrate were (1) a thioether sulfur located at a distance of two methylene groups from the carbon atom with a free hydrogen; (2) an amino group in the L configuration; (3) a carboxyl group or its ester; (4) a methyl group attached to the sulfur amino. Rates of reaction with the various substrates and specificity of substrates varies somewhat depending on the source of the enzyme.

No studies were found which had tested peptide-bound methionine as a substrate for this enzyme. The structural requirements for an active substrate do not seem to preclude the possibility of peptide-bound methionine acting as a substrate. If peptide-bound methionine can act as a substrate, an enzyme assay could be developed whereby unaltered methionine residues could be differentiated from methionine which had undergone some changes during processing.

OBJECTIVES

Processing of soy sometimes reduces the protein nutritive value
(Bressani, 1975). Supplementation of processed soy products with
methionine sometimes increases their nutritive value to a level compar-
able to that of soy flour (Meyer, 1967; Longenecker et al., 1964).
While some loss of methionine and cystine does occur during processing,
it is not sufficient to account for the total reduction in protein
nutritive value (Longenecker and Lo, 1974). Other changes altering
methionine and cystine thereby affecting their bioavailability may be
occurring during processing procedures. These changes may include
oxidation of methionine to forms that are physiologically unavailable
or have reduced availability. The usual methods for measuring methi-
onine such as the performic acid oxidation method of Moore (1963) do
not distinguish the oxidized forms from the reduced form of methionine.
A need exists for methods that measure not only total methionine but
also the amount of each of the forms of methionine.

The objectives of this study were:

1. To develop a standard methodology for measuring

   methionine, methionine sulfoxide, methionine sulfone,

   total cystine, and cysteic acid in food proteins by

   developing new chemical methods or refining existing

   methods.

Methodologies used in the measurement of these sulfur amino acids in-
clude:

(a) Determination of total methionine and total cystine by the

    performic acid oxidation method of Moore (1963).

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(b) Determination of methionine sulfone and cysteic acid by ion-exchange chromatography after acid hydrolysis (Spackman et al., 1958).

(c) Determination of methionine sulfoxide by ion-exchange chromatography after alkaline hydrolysis (Neumann, 1967).

(d) Determination of methionine by the difference between total methionine and the sum of methionine sulfoxide and methionine sulfone.

(Methionine = Total methionine - (methionine sulfoxide + methionine sulfone).

2. To determine methionine, methionine sulfoxide, methionine sulfone, total cystine, and cysteic acid in soy flour, soy concentrate, soy isolate, and infant formulas using the set of procedures developed for these determinations.

3. To test the feasibility of using ATP:L-methionine S-adenosyl-transferase to measure the number of unaltered methionine residues in processed food proteins.

In order to achieve objective (1) the following procedures were used:

(1) Total methionine and total cystine determinations were done essentially according to the procedure of Moore (1963). Modifications included increasing the amount of reagents added to food samples high in carbohydrates to insure complete oxidation of methionine residues.

(2) Methionine sulfone and cysteic acid were determined using the acid hydrolytic conditions essentially as described by Moore (1963) -- 6M HCl for 18 or 24 hours at 110°C. Conditions for optimal elution of methionine sulfone and cysteic acid were determined for the Technicon NC-2P Amino Acid Analyzer.

(3) Methionine sulfoxide was determined by ion-exchange chromatography after alkaline hydrolysis. Since the conditions of Neumann (1967) were intended for determination of methionine sulfoxide content in relatively pure proteins, conditions to be used for the food proteins were determined in the following manner:

    (a) Free amino acids were hydrolyzed alone and in mixtures of amino acids with 2M NaOH for 18 hours at 100°C (Sjöberg and Boström, 1977) or 3M NaOH for 16 hours at 110°C (Cuq et al., 1977). Percent recoveries of methionine, methionine sulfoxide, methionine sulfone, cystine, and cysteic acid were determined.

    (b) Model proteins were hydrolyzed with 2M NaOH for 18 hours at 100°C or 3M NaOH for 16 hours at 110°C. Percent recoveries of methionine and methionine sulfoxide were determined as a percentage of total methionine (Moore, 1963).

Conditions which resulted in greater recoveries of these amino acids were chosen for food analyses.

Since some of the products to be analyzed were high in sugar content and relatively low in protein content, the effect of sugar on oxidation of methionine and/or methionine sulfoxide during alkaline hydrolysis was also determined in the following manner:

(1) Free amino acids were hydrolyzed alone and in mixtures of amino acids with glucose. Percent recovery of methionine, methionine sulfoxide, and methionine sulfone were determined and compared to percent recoveries of the same amino acid hydrolyzed under the same conditions (2M NaOH for 18 hours at 100°C) in the absence of glucose.

(2) Model proteins were hydrolyzed in the presence of glucose, with 2M NaOH for 18 hours at 100°C and recovery of methionine, methionine sulfoxide, and methionine sulfone compared to the model proteins hydrolyzed in the absence of sugar under the same alkaline hydrolytic conditions. Casein and Promine F were also hydrolyzed in the presence of lactose and sucrose, respectively, with copper (II) and iron (II) ions in the hydrolysis mixture.

The following steps were taken to achieve objective (3):

(1) The enzyme was isolated from E. coli cells according to the procedure of Lombardini et al. (1970).

(2) An enzyme assay was developed based on the method of Tallen and Cohen (1976).

(3) Incubation procedures using soy products and the enzyme
    were developed.

(4) A spectrophotometric method for measuring increases in
    absorbance of reaction mixtures containing protein sub-
    strates was developed.

(5) Methods were developed using alkaline and acid hydrol-
    ysis of the incubation mixtures followed by ion-exchange
    chromatography to confirm enzyme activity with parti-
    cular substrates.

MATERIALS AND METHODS

Products

The processed soy products were obtained from the manufacturer,
Central Soya Company of Chicago, Illinois. These products were:
Soyafluff 200 W, a soy flour; Promosoy 100, a soy concentrate; and
Promine D and Promine F, soy isolates. The four infant formulas were
purchased locally: two milk-based formulas, powdered Similac forti-
fied with iron and concentrated liquid Similac (Ross Laboratories,
Columbus, Ohio) and two soy isolate-based formulas in concentrated
liquid form, Prosobee (Mead Johnson and Company, Evansville, Indiana)
and Isomil (Ross Laboratories). Animal Nutrition Research Council
reference casein was obtained from Humko-Sheffield Chemical Company,
Memphis, Tennessee.

Materials

All chemicals were reagent or chromatographic grade. Egg white
lysozyme, S-adenosyl methionine (SAM), disodium adenosine triphosphate
(ATP), and L-methionine, D-methionine, DL-methionine, DL-methionine
sulfoxide, L-methionine DL-sulfoxide, DL-methionine sulfone, DL-
cystine, DL-cysteic acid, and tris (hydroxymethyl) amino methane,
basic form (Tris buffer) were purchased from Sigma Company, Saint
Louis, Missouri. Piperazine-N-N'-bis (ethanesulfonic acid) monohydrate
(Pipes buffer) in the sodium salt form was purchased from Calbiochem
Company, LaJolla, California. Synthetic di- and tripeptides were
obtained from the Vega Company, Tucson, Arizona. Amino acid standards

were purchased from Pierce Chemical Company, Rockford, Illinois or were prepared from crystalline DL-amino acids purchased from Sigma Company.

Over the course of this study, ninhydrin was supplied by three different companies: Pierce Chemical Company, Rockford, Illinois; Sigma Company, Saint Louis, Missouri, and Beckman Instruments, Incorporated, Fullerton, California. Ethylene glycol monomethyl ether (methyl cellosolve) was obtained either from Fisher Scientific Company, Fairlawn, New Jersey or Pierce Chemical Company. Thiodiglycol was purchased from Pierce Chemical Company. Frozen E. coli cells, Strain B at one-half log phase grown in Kornberg medium, were purchased from the Grain Processing Corporation, Muscatine, Iowa. The anion exchange resin, AG 1-X8, 200-400 mesh, chloride form, was purchased from BioRad Company, Richmond, California, and glass beads, diameter 210-297 micrometers, were obtained from Scientific Products, McGaw Park, Illinois. Ten ml polypropylene centrifuge tubes with screw caps, products of Nalge Company, Rochester, New York, were purchased from Dynalab, Incorporated, Rochester, New York and from Fisher Scientific Company, Fairlawn, New Jersey.

Methods

Hydrolytic Studies of Free Amino Acids

Preparation of Stock Standards

Stock solutions of DL-methionine sulfone, DL-cysteic acid, DL-methionine, DL-methionine sulfoxide, DL-valine, DL-leucine, DL-phenyl-alanine, and DL-glutamic acid were prepared in deionized water with

concentrations between three and six micromoles per ml of solution. These stock solutions were diluted 1 to 25 with cartridge equilibrating buffer (Technicon, 1973), pH 1.9, before application to the NC-2P Amino Acid Analyzer.

Because of the relative insolubility of cystine in water, it was dissolved in 0.1M HCl at a concentration of 1.0 micromole per ml of solution. This solution was diluted 1 to 5 before application to the Amino Acid Analyzer.

A stock solution containing a mixture of methionine, methionine sulfoxide, methionine sulfone, cystine, and cysteic acid was also prepared in 0.1M HCl with concentrations of approximately one micromole per ml for each amino acid. This solution was diluted 1 to 5 with cartridge buffer before application to the Amino Acid Analyzer. Another stock solution of 20 common amino acids, including methionine, methionine sulfoxide, methionine sulfone, cystine, and cysteic acid, was prepared in 0.1M HCl at concentrations ranging from 0.4 to 2.8 micromoles per ml of solution for the amino acids. Appendix 3 lists the amino acids in this stock solution and the concentration of each. This solution, also, was diluted 1 to 5 before application to the Amino Acid Analyzer.

Basic Hydrolysis of Free Amino Acids

The stock solutions of amino acids were subjected to basic hydrolysis under two sets of conditions: 2M NaOH for 18 hours at 100°C (Sjöberg and Boström, 1977) and 3M NaOH for 16 hours at 110°C (Cuq et al., 1977). One ml of methionine, methionine sulfoxide,

methionine sulfone, or cysteic acid was placed in a 10 ml polypropylene centrifuge tube, 1.0 ml of either 4M or 6M NaOH was added, the contents mixed on a Vortex Mixer, the tube flushed with nitrogen for 30 seconds, and capped immediately with a polypropylene screw type cap. The final concentrations of NaOH were 2M or 3M, respectively. In the case of cystine and the amino acid mixtures, 2.0 ml of each stock solution was mixed with 2.0 ml of either 4M or 6M NaOH in the polypropylene tubes and then treated as the previous samples. All samples were placed in either a 100°C or 110°C oven for 18 or 16 hours, respectively.

After hydrolysis the tubes were allowed to cool to room temperature and the contents transferred quantitatively to a small beaker. The tube was rinsed two or three times with cartridge buffer and the rinsings transferred to the same beaker. The pH of the solution was adjusted to pH 2 with 6M HCl, stirring constantly. The solution was then transferred to either a 10.0 or 25.0 ml volumetric flask. The microelectrode was rinsed with two or three drops of cartridge buffer and these washings also transferred to the flask. The beaker was rinsed two or three times with cartridge buffer to insure quantitative transfer of the hydrolysate and these washings were also added to the volumetric flask. The contents of the flask were brought to volume with cartridge buffer, pH 1.9, mixed, and transferred to test tubes and capped with either two layers of parafilm or a screw cap. The samples were kept in the refrigerator until chromatographed.

In one set of hydrolyses, stock solutions of methionine, methionine sulfoxide, methionine sulfone, and the mixture of amino acids containing 20 amino acids were hydrolyzed with 2M NaOH for 18 hours at

100°C in the presence of D-glucose. The amount of glucose added was five times greater by weight than the amino acid(s) in the hydrolysis mixture. After hydrolysis, these samples were treated in the same manner as those hydrolyzed without glucose.

Chromatography

An aliquot of each hydrolysate was applied, via an injector system, to the ion-exchange column of the NC-2P Technicon Amino Acid Analyzer. Conditions used for elution are given in Appendix 4. Percent recovery was calculated by comparing peak area of each amino acid to that of its counterpart in an equal amount of the respective un-hydrolyzed stock solution diluted in the same manner as the hydrolysate.

Acid Hydrolysis of Free Amino Acids

The stock solutions of methionine, methionine sulfoxide, methionine sulfone, cysteic acid, and cystine and the mixture of these amino acids were subjected to acid hydrolysis. One ml of each of these solutions was added to 10 ml glass ampoules and 4.0 ml of 6M HCl added. The ampoule was flushed with nitrogen for 30 seconds and sealed under a slight vacuum with a propane torch. The samples were hydrolyzed for 18 hours or 24 hours in a 110°C oven. After hydrolysis, the samples were allowed to cool to room temperature and then filtered through Pyrex superfine glass wool. Two or 3.0 ml of the filtrate were dried in a vacuum desiccator and residue resuspended in 5.0 ml of cartridge buffer, pH 1.9. The acid hydrolysates were chromatographed, and percent recovery of the various amino acids was calculated in the same manner as for the base-hydrolyzed samples.

Hydrolytic Studies on Model Protein Systems

The model proteins used were: ANRC casein, egg-white lysozyme, oxidized egg-white lysozyme, oxidized ANRC casein, and the soy isolate, Promine F. Casein and Promine F were used in the dry form, as received from the manufacturer, and weighed directly into 10 ml polypropylene centrifuge tubes (basic hydrolysis) or glass ampoules (acid hydrolysis and performic acid oxidation). The size of the glass ampoule depended on the size of the protein sample. In general 10 ml ampoules were used when the amount of protein per sample was less than 60 mg and 20 ml ampoules when the amount was greater than 60 mg.

The other model proteins were used in solution for all hydrolytic studies. Lysozyme and oxidized lysozyme are water soluble and, while casein is quite insoluble in water, oxidized casein is soluble and could be used in solution. All the water-soluble proteins were transferred to either polypropylene tubes or glass ampoules by volumetric pipets, then treated in a manner similar to the dry products.

Preparation of Stock Solutions

Lysozyme -- Lysozyme, isolated from egg white, is a protein whose molecular weight and amino acid composition and sequence are known. A solution, in deionized water, of approximately 10 mg per ml was prepared. The exact concentration of 10.6 mg per ml was derived by measuring the absorbance of the solution on a Beckman DU Spectrophotometer at a wavelength of 280 nm and calculating the concentration using the extinction coefficient of 26.35 for a 1.0% solution of egg white lysozyme at 280 nm (Sober, 1970). This solution was used in all the hydrolytic procedures using lysozyme as the model protein.

Oxidized lysozyme -- Egg white lysozyme, in crystalline form, was oxidized according to the procedure of Cuq et al., (1973) with some modifications. A small portion (0.508 g) was weighed, transferred to a 150 ml beaker, and dissolved in 25 ml of deionized water. This solution was heated to 55°C on an electric hot plate equipped with a magnetic stirrer. Exactly 0.50 ml of 30% hydrogen peroxide was then added. This mixture was kept at 55°C ± 3° for 1.5 hours with constant stirring. About 12 mg of crystalline catalase was added and this mixture was kept at 55°C with constant stirring for an additional 30 minutes. The reaction of catalase with the unused hydrogen peroxide led to vigorous foaming so the solution was placed in the refrigerator overnight to allow the foam to dissipate. The solution was then diluted to volume with deionized water in a 50.0 ml volumetric flask, giving a concentration of about 10 mg per ml. At the time of hydrolysis the concentration was re-checked by determining the amount of glutamic acid in a given volume of the oxidized lysozyme after acid hydrolysis and back calculating sample size based on the glutamic acid content of crystalline lysozyme. The concentration determined in this manner was 9.6 mg per ml.

Oxidized casein -- Casein was also oxidized using the procedure of Cuq et al. (1973). After grinding the casein with a mortar and pestle and sieving through an 80 mesh sieve, 3.6 g of casein was transferred to a 250 ml beaker and 63 ml of deionized water was added, giving a 5% (w/v) suspension. The pH was adjusted to pH 8 with 4M NaOH giving a volume of about 80 ml. The solution was heated on an

electric hot plate (equipped with a magnetic stirrer) to 53°C ± 3° and

1.2 ml of 30% hydrogen peroxide added (0.015 ml per ml of solution).

This mixture was stirred constantly at 53°C for 45 minutes. About

72 mg of catalase was added and stirring continued at 53°C for an

additional 30 minutes, resulting in vigorous foaming. The mixture was

placed in a refrigerator overnight and diluted to volume in a 100.0 ml

volumetric flask with deionized water. The concentration of this

solution was 36 mg per ml. The oxidized casein was freeze-dried for

storage and later resuspended in deionized water. The concentration,

determined in the manner described for oxidized lysozyme, was 66 mg

per ml.

Basic Hydrolysis of Model Proteins

The model proteins were subjected to basic hydrolysis alone and

in the presence of various sugars and in some experiments in the pre-

sence of copper (II) sulfate and iron (II) sulfate. The hydrolytic

conditions were either 2M NaOH for 18 hours at 100°C (Sjöberg and

Boström, 1977) or 3M NaOH for 16 hours at 110°C (Cuq et al., 1977).

The procedure was essentially that described in the section, Basic

Hydrolysis of Free Amino Acids. The experiments are summarized in

Tables 2-6. In general, each hydrolysis was replicated 3-6 times and

the samples were chromatographed on an NC-2P Technicon Amino Acid

Analyzer. Some samples were also chromatographed on the TSM Technicon

Amino Acid Analyzer (TSM).

Table 2. Conditions used in the basic hydrolyses of oxidized lysozyme

Amount Oxidized Lysozyme Used	Amount Sugar Added	Amount of Base Added	Conditions of Hydrolysis	Conditions Used in Amino Acid Analysis
2.0 ml[1]	-	2.0 ml 4M NaOH	2M NaOH, 18 Hrs 100°C	Appendix 4
2.0 ml	50 mg of αD-Glucose	2.0 ml of 4M NaOH	2M NaOH, 18 Hrs 100°C	Appendix 4

[1]Concentration of oxidized lysozyme solution was 9.6 mg/ml deionized water.

Table 3. Conditions used in the basic hydrolysis of lysozyme.

Amount Oxidized Lysozyme Used	Amount Sugar Added	Amount of Base Added	Conditions of Hydrolysis	Conditions Used in Amino Acid Analysis
1.0 ml[1]	-	1.0 ml of 4M NaOH	2M NaOH, 18 Hrs 100°C	Appendix 4
1.0 ml	-	4.0 ml of 2.5M NaOH	2M NaOH, 18 Hrs 100°C	Appendix 4
1.0 ml	-	1.0 ml of 6M NaOH	3M NaOH, 16 Hrs 110°C	Appendix 4
1.0 ml	50 mg αD-Glucose[2]	1.0 ml of 4M NaOH	2M NaOH, 18 Hrs 110°C	Appendix 4

[1]Concentration of lysozyme solution was 10.6 mg/ml deionized water.

[2]Weight of glucose used was about 5 times greater by weight than lysozyme.

Table 4. Conditions used in the basic hydrolysis of oxidized casein.

Amount of Oxidized Casein Used	Amount of Sugar Added	Other Additions	Amount of Base Added	Conditions of Hydrolysis	Conditions Used in Amino Acid Analysis
1.0 ml[1]	-	1.0 ml deionized water	2.0 ml 4M NaOH	2M NaOH, 18 Hrs 100°C	Appendix 4
1.0 ml	300 mg α-D-Glucose	1.0 ml deionized water	2.0 ml 4M NaOH	2M NaOH, 18 Hrs 100°C	Appendix 4
1.0 ml	300 mg α-D-Glucose	1.0 ml $FeSO_4$ Solution[2]	2.0 ml 4M NaOH	2M NaOH, 18 Hrs 100°C	Appendix 4

[1]Concentration of solution of oxidized casein was 66 mg/ml deionized water.

[2]Concentration of solution was 0.05 mg Fe^{2+} per ml deionized water.

Table 5. Conditions used in the basic hydrolysis of casein.

Approximate Amount of Casein[1]	Approximate Amount of Sugar Added[1]	Other Additions	Amount of Base Added	Conditions of Hydrolysis	Conditions Used in Amino Acid Analysis
100 mg	-	3.0 ml de-ionized water	3.0 ml 4M NaOH	2M NaOH, 18 Hrs, 100°C	Appendix 4
100 mg	-	3.0 ml de-ionized water	3.0 ml 6M NaOH	3M NaOH, 16 Hrs, 110°C	Appendix 4
100 mg	500 mg α-D-Glucose	3.0 ml de-ionized water	3.0 ml 4M NaOH	2M NaOH, 18 Hrs, 100°C	Appendix 4
100 mg	500 mg lactose	3.0 ml Fe^{2+} Cu^{2+} containing solution[2]	3.0 ml 4M NaOH	2M NaOH, 18 Hrs, 100°C	Appendix 4

[1] Weights were determined exactly. Table values are rounded off.

[2] $FeSO_4 \cdot 7 H_2O$ and $CuSO_4 \cdot 5 H_2O$ solution with concentrations of 0.025 mg/ml Fe^{2+} and 0.001 mg/ml Cu^{2+}.

Table 6. Conditions used in the basic hydrolysis of Promine F.

Approximate Amount of Promine F[1]	Approximate Amount of Sugar Added[1]	Other Additions	Amount of Base Added	Conditions of Hydrolysis	Conditions Used in Amino Acid Analysis
100 mg	–	3.0 ml de-ionized water	3.0 ml 4M NaOH	2M NaOH, 18 Hrs, 100°C	Appendix 4
100 mg	500 mg sucrose	3.0 ml of $FeSO_4$ and $CuSO_4$ solution[2]	3.0 ml 4M NaOH	2M NaOH, 18 Hrs, 100°C	Appendix 4

[1] Weights were determined exactly. Table values are rounded off.

[2] The solution contained 0.024 mg Fe^{2+} per ml deionized water and 0.001 mg Cu^{2+} per ml deionized water.

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Acid Hydrolysis of Model Proteins

The model proteins were also subjected to acid hydrolysis. The
method was essentially that as described in the section, Acid Hydrol-
ysis of Free Amino Acids. The procedure differed, however, in the
amount of protein and acid used and the presence, in some cases, of
other substances in the system. All samples were hydrolyzed at 110°C
for 24 hours. Usually 6M HCl was used in the hydrolyses. In some
cases, to compensate for the liquid from the solutions of proteins,
higher molarities were used. The conditions used in the acid hydrol-
yses of these proteins are summarized in Tables 7 and 8. The number
of replications per hydrolysis varied from three to six. All samples
were chromatographed on an NC-2P. Selected samples were also chroma-
tographed on the TSM.

Performic Acid Oxidation of Model Proteins

All model proteins were analyzed for total methionine and total
cystine using the performic acid oxidation method of Moore (1963).
Five to 10 mg of protein were transferred to 10 ml glass ampoules,
2.0 ml of performic acid added (1.0 ml 30% hydrogen peroxide plus
9.0 ml 88% formic acid), and the ampoules covered with parafilm and
placed in a refrigerator. After an overnight oxidation period,
ampoules were removed from the refrigerator and 0.30 ml of 48% HBr
added. The samples were taken to dryness with a rotary evaporator
and 3.0 ml 6M HCl added. The samples were then treated in the manner
described in the section, Acid Hydrolysis of Free Amino Acids.

Table 7. Conditions used in the acid hydrolyses of casein and Promine F.

Approximate Amount of Protein[1]	Approximate Amount of Sugar Added	Amount of $FeSO_4$-$CuSO_4$ Solution[2] Added	Amount of Acid Added	Conditions Used in Amino[3] Acid Analysis
Casein				
100 mg	-	-	10.0 ml 6M HCl	Appendix 4
100 mg	500 mg lactose	1.0 ml	10.0 ml 6.6M HCl	Appendix 4
Promine F				
100 mg	-	-	10.0 ml 6M HCl	Appendix 4
100 mg	500 mg sucrose	1.0 ml	10.0 ml 6.6M HCl	Appendix 4

[1] Weights were determined exactly. Table values are rounded off.

[2] Concentration of solution: 0.048 mg Fe^{2+}/ml and 0.002 mg Cu^{2+}/ml.

[3] For all acid hydrolysates except casein hydrolyzed alone, 2.0 ml were dried and the residue resuspended in 5.0 ml of cartridge buffer, pH 1.9. In the case of casein, 1.0 ml or 2.0 ml of hydrolysate were dried and residue resuspended in 3.0 ml and 5.0 ml of cartridge buffer, respectively.

Table 8. Conditions used in the acid hydrolyses of lysozyme, oxidized lysozyme, casein, and oxidized casein.

Amount of Protein Used	Amount of Acid Added	Amount of Hydrolysate Dried	Amount of Resuspending Buffer Used[1]	Conditions Used in Amino Acid Analyzer
Lysozyme 2.0 ml[2]	3.0 ml of 10M HCl	2.0 ml	5.0 ml	Appendix 4
Oxidized Lysozyme 1.0 ml[3]	3.0 ml of 6M HCl	2.0 ml	3.0 ml	Appendix 4
Oxidized Casein 1.0 ml[4]	4.0 ml of 6M HCl	2.0 ml	2.0 ml	Appendix 4

[1] Suspending buffer was sodium citrate buffer, pH 1.9 (Cartridge Equilibrating Buffer, TSM Technicon Amino Acid Analyzer Manual)

[2] Concentration of solution was 10.6 mg/ml deionized water.

[3] Concentration of solution was 9.6 mg/ml deionized water.

[4] Concentration of solution was 66 mg/ml deionized water.

After hydrolysis samples were chromatographed on an NC-2P Technicon Amino Acid Analyzer using the conditions described in Appendix 4. Sample size was determined by measuring the glutamic acid content of the oxidized samples and comparing it to the glutamic acid content of larger unoxidized samples which had been acid hydrolyzed. Glutamic acid is one of the amino acids which Moore (1963) states is stable to the procedure and can be used for such calculations.

Analyses of Food Products

All products, except Prosobee, were analyzed in the form in which they were obtained. Prosobee liquid concentrate was freeze-dried and used in this form for all analyses. Nitrogen determination in Prosobee was done in the liquid concentrate form as well as in the freeze-dried form, however.

Nitrogen Determination

Products were analyzed for total nitrogen using a modified Kjeldahl-Gunning-Arnold method (A.O.A.C., 1970). Triplicate or quadruplicate determinations were done on each product with sample size ranging from 0.10 g to 1.00 g for the dry materials and approximately 5 g for the liquid samples. The sample size chosen was such that the estimated amount of nitrogen present in the sample was in the 15 to 45 mg range. Crude protein values were derived by multiplying the nitrogen content by the factor, 6.25.

Total Methionine and Total Cystine Determination

Total methionine and total cystine in the products were measured using the performic acid oxidation procedure of Moore (1963). Samples

containing an estimated 5 to 10 mg of protein were placed in 10 ml

glass ampoules (Scientific Products) and oxidized by adding 2.0 ml

performic acid (1.0 ml 30% hydrogen peroxide plus 9.0 ml 88% formic

acid) to the ampoules. Because it was thought that products high in

carbohydrate might use some of the oxidizing capacity of the performic

acid, 3.0 ml of performic acid were added to samples such as the

infant formulas that were high in sugars and/or corn syrup. The

ampoules were stored in the refrigerator overnight and the excess

performic acid was then removed by the addition of 0.3 ml or 0.45 ml

48% HBr, depending on the amount of performic acid added to the

samples. The samples were taken to dryness using a rotary evaporator.

Some samples which could not be dried completely on the rotary evap-

orator were taken to approximately 0.3 ml volume with this device and

then taken to dryness in a vacuum desiccator. Three ml of 6M HCl were

added to the ampoules containing the dry oxidized samples, the ampoules

flushed with nitrogen for 30 seconds, and sealed under a slight vacuum

with a propane torch. The samples were placed in a 110°C oven and

hydrolyzed for 18 hours. After cooling the samples were filtered

through Pyrex superfine glass wool and 1.0 or 2.0 ml aliquots were

dried in a vacuum desiccator and the residue resuspended in either

2.0 ml or 3.0 ml citrate buffer (pH 1.9, 0.2M Na^+; Technicon, 1970).

Samples were subjected to ion-exchange chromatography on a Technicon

NC-2P Amino Acid Analyzer (NC-2P) using the conditions described in

Appendix 4.

The samples were weighed directly into the ampoules prior to

oxidation and were in the 5 to 50 mg range depending on protein

content. Since those samples falling at the lower end of that range were subject to weighing errors, glutamic acid content was used to calculate original samples size based on the glutamic acid content of the particular product as determined by acid hydrolysis of larger-sized (50 mg to 500 mg) unoxidized samples.

Performic acid oxidation results in the conversion of methionine and methionine sulfoxide to methionine sulfone and of cystine and its intermediate oxidation product to cysteic acid. The procedure has no effect on methionine sulfone or cysteic acid present in the protein prior to performic acid oxidation (Moore, 1963); therefore, the measurement of methionine sulfone and cysteic acid by ion-exchange chromatography after performic acid oxidation and acid hydrolysis gives a value for total methionine (methionine plus methionine sulfoxide plus methionine sulfone) and total cystine (cystine plus all its oxidized forms).

Methionine Sulfone and Cysteic Acid Determination

Methionine sulfone and cysteic acid already present in the products were determined by ion-exchange chromatography after acid hydrolysis. Samples contained from about 50 mg to 100 mg of protein and actual sample sizes varied from about 50 mg to 100 mg for soy isolates to about 500 mg for freeze-dried Prosobee. Dry samples (Soyafluff, Promosoy, Promine D, Promine F, powdered Similac, freeze-dried Prosobee) were weighed directly into 10 or 20 ml glass ampoules depending on sample size and 5.0 ml or 10.0 ml of 6M HCl added, respectively. Liquid samples (Isomil liquid concentrate and Similac

liquid concentrate) were pipetted in undiluted form (2.0 ml) into
20 ml glass ampoules. Then 10.0 ml of 7M HCl were added, giving a
final concentration of 6M HCl. All samples were then flushed with
nitrogen for 30 seconds, sealed under a slight vacuum with a propane
torch, and hydrolyzed at 110°C for 24 hours. After hydrolysis the
contents were allowed to cool, filtered through Pyrex superfine glass
wool, and 2.0 ml aliquots dried in a vacuum desiccator over NaOH. The
dried residues were resuspended in 3.0, 4.0, or 5.0 ml of citrate
buffer (pH, 1.9, 0.2M Na^{+}). The samples were chromatographed on an
NC-2P using the conditions described in Appendix 4. Selected samples
were also chromatographed on the TSM Technicon Amino Acid Analyzer
(TSM). Conditions used are presented in Appendix 5.

Methionine sulfone was also measured by ion-exchange chroma-
tography after basic hydrolysis. The basic hydrolytic procedure is
detailed in the section, Determination of Methionine Sulfoxide.

Methionine Sulfoxide Determination

Methionine sulfoxide was determined by ion-exchange chroma-
tography after basic hydrolysis. The conditions used were 2M NaOH for
18 hours at 100°C. Dry samples containing an estimated 100 mg of pro-
tein were weighed directly into polypropylene centrifuge tubes. Three
ml of deionized water were added and then 3.0 ml 4M NaOH. In the case
of the liquid samples, 3.0 ml of the liquid concentrate were pipetted,
using volumetric pipets, into the polypropylene tubes and 3.0 ml of
4M NaOH added. The contents of all samples were mixed, flushed with
nitrogen for 30 seconds, capped immediately, and placed in a 100°C

oven for 18 hours. After hydrolysis the samples were treated in a manner identical to base-hydrolyzed samples of the free amino acids. The samples were chromatographed on an NC-2P Technicon Amino Acid Analyzer using the conditions described in Appendix 4. The size of sample applied was standard but each was diluted such that about 0.5 mg of protein was applied to the column. Some samples, notably the infant formulas, contained some particulate matter floating at the surface of the samples. In these cases, the samples were filtered through glass wool before application to the ion-exchange column. Selected samples were also chromatographed on the TSM Technicon Amino Acid Analyzer. Conditions of analysis are given in Appendix 5.

Methionine Determination

Methionine in the food products was determined indirectly. After methionine sulfoxide, methionine sulfone, and total methionine were determined based on the methods given previously, methionine was determined by difference using the following equation:

Methionine = Total methionine - (methionine sulfoxide +

methionine sulfone).

Methionine was also measured after acid hydrolysis and after alkaline hydrolysis. Acid hydrolysis, however, is not generally considered a reliable method since methionine sulfoxide is reduced to methionine during acid hydrolysis (Floyd et al., 1963; Cuq et al., 1973). The results of this study support these findings and also suggest that some methionine is destroyed or not completely released during alkaline hydrolysis under the conditions used in this study.

Determination of Methionine, Methionine Sulfoxide, and Methionine
Sulfone in Deproteinated Isomil

To deproteinate, two 4.0 ml samples of Isomil were each mixed
with 2.0 ml of 20% sulfosalicylic acid (w/v in citrate buffer, pH
2.0, 0.3M Li$^+$ Technicon, 1973). The samples were mixed and cooled in
an ice bath for 30 minutes. They were then centrifuged for 15 minutes
at 10,000 X g in a refrigerated Sorvall Centrifuge. The supernatant
appeared very cloudy so it was subsequently filtered through qualita-
tive filter paper. The filtrate, though still slightly cloudy, was
applied directly to the NC-2P Technicon Amino Acid Analyzer and
chromatographed using the conditions described in Appendix 4.

ATP:L-Methionine S-Adenosyltransferase Experiments

Isolation of the Enzyme

The procedure used for isolating the enzyme from E. coli cells
was that of Lombardini et al. (1971). All steps in the isolation
procedure were conducted in a refrigerated room at a temperature of
4°C unless otherwise noted.

Tris buffer, 0.05M, pH 7.0 containing 0.005M mercaptoethanol,
was prepared from Trizma Base (Sigma Company), adjusted to pH 7.0
with 6M HCl and cooled to 4°C before use. The frozen E. coli cells
were allowed to thaw partially overnight in the refrigerator. Approxi-
mately 120 g of these partially thawed cells were transferred to an
Osterizer Blender, along with 400 ml of the Tris buffer. About 400 g
of glass beads were added and the contents of the blender mixed on
low speed for one minute. The contents were then homogenized by
blending at high ("liquefy") speed for 2 minute periods five times

with 10 minute cooling intervals between blending periods. This mixture was then centrifuged in a refrigerated Sorvall centrifuge, using the GSA head, at 10,000 x g (8,000 rpm) for 10 minutes. The supernatant was decanted and saved and the precipitate was washed 3 times with Tris buffer. The washings were added to the supernatant and the precipitate was discarded. Enough $(NH_4)SO_4$ was added to bring the solution to 30% of saturation and this solution was stirred slowly overnight in a refrigerated room using a magnetic stirrer.

The next morning the solution was centrifuged at 10,000 x g for 10 minutes and the precipitate discarded. The supernatant was brought to 50% of saturation with $(NH_4)_2SO_4$ and this solution stirred for 1.5 hours. The solution was then centrifuged at 10,000 x g for 15 minutes and the supernatant discarded. The precipitate was dissolved in a minimum amount of Tris buffer (about 40 ml). The dissolved precipitate was transferred to dialysis tubing and dialyzed overnight against 0.05M Tris buffer, pH 7.0 containing 0.005M mercaptoethanol and 20% glycerol, with one buffer change after 3 hours. The dialyzed solution, containing the enzyme, was transferred in small aliquots to plastic test tubes and frozen for later use.

An estimate of the protein content of the enzyme preparation was made using the absorbance at 280 nm and 260 nm on the Beckman DU Spectrophotometer and applying the relationship:

Protein in mg/ml $= 1.45\ A_{280} - 0.74\ A_{260}$ (Bailey, 1967).

Enzyme Assay

The assay procedure was that of Tallen and Cohen (1976) with some modifications. A reaction mixture was prepared which contained all the components needed in the assay. Pipes buffer, 0.336 M, pH 6.8, was the suspending medium for the other components. A small amount (0.8268 g of Na_2ATP) was added to 7 ml of Pipes buffer and stirred until dissolved. An equimolar amount of $MgCl_2$ (0.3050 g) was added to another 7 ml portion of Pipes buffer and this was dissolved. The two solutions were then combined and the pH brought to 6.8 with 4M NaOH. An additional 1.0165 g of $MgCl_2$ was added to this solution, along with 0.373 g of KCl and 0.1492 g of L- or D-methionine. The solution was thoroughly mixed with a magnetic stirrer and the volume brought to 25.0 ml with Pipes buffer in a volumetric flask. The resulting reaction mixture was 0.060M in $MgATP^{2-}$, 0.20M in KCl, 0.20M in $MgCl_2$, and 0.04M in methionine. In some experiments, methionine was omitted from the reaction mixture and other compounds which were to be tested as substrates added at the time of the assay.

In some experiments, another reaction mixture was used. This solution was similar to that used by Chiang and Cantoni (1977). The suspending medium was 0.10M Tris buffer, pH 7.8, and the molarities of the components were 0.20M KCl, 0.20M $MgCl_2$, and 0.020M $MgATP^{2-}$.

The anion exchange resin, used to quench the reaction, was washed twice with 2M NaOH, rinsed with deionized water, washed 5 times with 6M HCl, and rinsed again about 10 times with deionized water. After settling the excess deionized water was decanted and a 50% v/v slurry of the resin and deionized water prepared. A dilute perchloric

acid solution was also prepared by diluting 8.6 ml of 70% perchloric acid to 100 ml with deionized water.

In the normal incubation procedure, 0.25 ml of reaction mixture containing L-methionine was pipetted into plastic centrifuge tubes and warmed to 37°C in a shaker-waterbath. An equal volume of enzyme solution was then added, the tops sealed with parafilm, and the tubes allowed to incubate at 37°C with gentle agitation for 10 minutes to 24 hours, depending on the particular experiment. At the end of incubation, 2.0 ml of a 50% v/v slurry of the anion exchange resin, kept in suspension by a magnetic stirrer, was pipetted into the tubes with a Gilson automatic pipetter. The resin removed the ATP, thereby, presumably, stopping the reaction. Five ml of cold deionized water was added and this mixture centrifuged at 5000 rpm for about 10 minutes. Three ml of the supernatant was transferred to another set of plastic centrifuge tubes and 1.0 ml of the dilute perchloric acid added to precipitate the protein. These tubes were centrifuged for about 10 minutes at 5000 rpm and the absorbance of the supernatant determined on a DU Beckman Spectrophotometer at 250, 260, and 280 nm against a blank and sometimes against distilled water or 6M urea. The blank (zero time) was prepared by adding reaction mixture, anion exchange resin, then enzyme solution, in the same amounts as in the samples. Samples and blanks were incubated together for the same length of time unless otherwise stated. In some instances enzyme solution was added at the end of incubation to the blanks.

The amount of S-adenosylmethionine (product) formed was deter-
mined using the molar extinction coefficient (E_m) of 15,400 for SAM in
water at 260 nm (Shapiro and Ehninger, 1966).

When the di- and tripeptides were used as substrates, these were
weighed out in crystalline form and added to the reaction mixture
prepared without substrate in Pipes buffer, pH 6.8. Enough substrate
was added to make the reaction mixture 0.04 in the dipeptide; however,
L-methionyl-L-methionyl-L-methionine was quite insoluble in the
aqueous reaction mixture and much of the crystalline matter clung to
the sides of the tube. Thus, the concentration in solution and in
suspension was uncertain for this tripeptide.

A blank was prepared for each peptide. After addition of sub-
strate to the samples and blanks, these mixtures were treated in the
same manner as those in the previous assay.

Proteins as substrates -- When proteins were used as substrates,
the assay procedure was modified. In the experiments with casein and
lyophilized oxidized casein, about 25 mg of each protein was weighed
into plastic centrifuge tubes. Then 0.20 ml of reaction mixture in
Pipes buffer, pH 6.8, was added along with 0.20 ml of enzyme solution.
Blanks were prepared by adding the respective proteins, reaction mix-
ture, and anion exchange resin to plastic centrifuge tubes. Blanks
and samples were incubated at 37°C with gentle agitation for 17 hours.
At the end of incubation, enzyme solution was added to the blanks and
2.0 ml of the 50% slurry of anion exchange resin were added to the
samples.

The procedure deviated at this point from the previous assay procedures with methionine and the di- and tripeptides. Since the product expected to be formed if the methionine in the proteins could function as a substrate would be peptide-bound SAM, the protein could not be precipitated out as in the previous assays; therefore, 10 ml of 6M urea was added to solubilize at least a portion of the protein, and to make the solution clear rather than turbid as it would have been if water were added at this point.

After addition of the urea, samples and blanks were placed in a 37°C waterbath for one-half hour to assure maximum solubilization. During this time all tubes were inverted and contents thoroughly mixed 3 times. The tubes were then centrifuged at 5000 rpm for 10 minutes and the absorbance of the supernatant of the samples and blanks measured against 6M urea at 250, 260, and 280 nm.

Promine D and Promine F as substrates -- The soy isolates, Promine D and Promine F were also used as substrates. About 25 mg of each isolate were weighed into plastic centrifuge tubes and 0.20 ml reaction mixture in 0.10M Tris, pH 7.8, along with 1.0 ml Tris buffer, pH 7.8, and 0.20 ml enzyme solution. Contents were mixed and tubes were placed in a 37°C waterbath and incubated with gentle agitation for 19 hours. Blanks were prepared for each isolate as in the case of casein and oxidized casein with the enzyme solution being added at the end of incubation.

After incubation 2.0 ml of the anion exchange resin was added to the samples and 5.0 ml of 6M urea was added to samples and blanks. The

tubes were centrifuged and the absorbance of the samples and blanks read against 6M urea at 250, 260, and 280 nm. Since the absorbance of the Promine F samples was very high, samples and blanks were diluted 1 to 4 with 6M urea before reading against a 6M urea blank. Although the supernatant of the samples and blanks was less than 6M in urea, it was thought that 6M urea could be used as a reference standard since both samples and blanks would be read against this reference standard.

As a check on the activity of the enzyme, an assay with L-methionine as a substrate was always done along with the assays using other compounds as substrates.

Amino Acid Analysis of S-adenosylmethionine

S-adenosylmethionine (2 mg/ml) was base hydrolyzed using 1.0 ml of the SAM in deionized water and 1.0 ml of 4M NaOH. The samples were flushed with nitrogen for 30 seconds, capped, and hydrolyzed in a 100°C oven for 18 hours. After hydrolysis, the samples were cooled, the pH adjusted to about pH 2, and the contents brought to 10.0 ml with cartridge equilibrating buffer, pH 1.9 (Technicon, 1973). The samples were chromatographed on an NC-2P Technicon Amino Acid Analyzer using the conditions shown in Figures 9 and 10.

The SAM solution was also subjected to acid hydrolysis. Three ml of 6M HCl was added to 0.5 ml of SAM solution (1 mg/ml) in a 10 ml glass ampoule. The ampoule was flushed with nitrogen, sealed under a slight vacuum with a propane torch, and hydrolyzed for 24 hours at 110°C. After hydrolysis, the hydrolysate was dried without removing from the ampoule and 1.0 ml cartridge buffer, pH 1.9, added to the

residue in the ampoule. An aliquot was applied to the NC-2P Technicon Amino Acid Analyzer and chromatographed using the conditions shown in Figure 13.

A solution of SAM was also subjected to performic acid oxidation. Two ml of performic acid were added to 0.10 ml of a 10 mg/ml solution of SAM in a 10 ml glass ampoule. After an overnight oxidation, the samples were treated in the same manner as other samples subjected to performic acid oxidation described in the section, Total Methionine and Total Cystine Determination. The samples were then chromatographed on the NC-2P Technicon Amino Acid Analyzer using the conditions shown in Figure 17.

Amino Acid Analysis of Enzyme-Treated Substrates

Alkaline hydrolysis -- In another experiment with Promine D and Promine F, about 30 mg of either Promine D or Promine F was placed in a 10 ml polypropylene centrifuge tube. Then 0.2 ml of the reaction mixture in 0.10M Tris, pH 7.8, 1.0 ml Tris buffer, pH 7.8, and 0.2 ml enzyme solution were added to the tubes. Blanks were prepared containing reaction mixture, soy isolate and Tris buffer. Samples and blanks were incubated for about 22 hours at 37°C with gentle agitation. After incubation 0.6 ml deionized water was added to the samples and 2.0 ml 4M NaOH. The same substances in the same amounts were added to the blanks along with 0.2 ml enzyme solution. All samples and blanks were flushed with nitrogen for 30 seconds, capped, and placed in a 100°C oven for 18 hours. After hydrolysis, samples and blanks were treated in the same manner as other alkaline hydrolysates--

pH brought to pH 1.9 and volume to 10.0 ml with cartridge buffer, pH
1.9. The samples were chromatographed on an NC-2P Technicon Amino
Acid Analyzer using the conditions shown in Figure 9. L-methionine
(0.0532 mg) was also incubated in polypropylene tubes at the same time
as the soy isolates, hydrolyzed in the same manner as the soy isolates,
and chromatographed using the same conditions.

Performic acid oxidation -- Five to 8 mg of Promine D or Promine
F were weighed directly into 10 ml glass ampoules. Then 0.20 ml,
each, of reaction mixture in 0.10M Tris buffer, pH 7.8, deionized
water, and enzyme solution were added. The blanks contained isolate,
reaction mixture, and deionized water. The ampoules were incubated
at 37°C for 20 hours with gentle agitation. To terminate the reaction,
2.0 ml of performic acid were added to samples and blanks. Enzyme
solution was then added to the blanks. The samples and blanks were
then treated according to the method of Moore (1963) for the oxidation
of methionine to methionine sulfone. Details of this procedure are
given in the section, Determination of Total Methionine and Total
Cystine. After oxidation and acid hydrolysis of samples and blanks,
1.0 or 2.0 ml of hydrolysate was dried in a vacuum desiccator and the
residue was resuspended in 3.0 ml cartridge equilibrating buffer, pH
1.9. Samples and blanks were chromatographed on an NC-2P Technicon
Amino Acid Analyzer using the conditions shown in Figure 17.

L-methionine (0.052 mg) was also incubated in a 10 ml glass
ampoule at the same time as the soy isolates, oxidized in the same
manner, subjected to acid hydrolysis, and chromatographed using the
same conditions.

Statistical Methods

Variances of a set of data were checked for homogeneity using the F_{max}-test (Sokal and Rohlf, 1969). When the variance in a set of data containing only two groups was homogeneous, Student's t-test was done to compare means of the groups. When the variances in a set of data containing three or more groups were homogeneous, one-way analysis of variance was used to analyze the data and the range simultaneous test procedure (Sokal and Rohlf, 1969) was used to detect differences between specific means.

When the variances in a set of data were not homogeneous, a modified t-test for comparing means of groups with unequal variances, described by Sokal and Rohlf (1969), was used to compare means.

Means were considered different from each other if the probability of such a difference was 0.05 or less.

RESULTS AND DISCUSSION

Hydrolytic Studies in Model Systems

In Part I of the hydrolytic studies, the percent recovery of

selected amino acids was determined in model systems by ion-exchange

chromatography after alkaline hydrolysis with 2M NaOH at 100°C for

18 hours, with 3M NaOH at 110°C for 16 hours, with 2M NaOH at 100°C

for 18 hours in the presence of sugars, and, in some cases, with 2M

NaOH at 100°C for 18 hours in the presence of sugars, copper (II) ions

and iron (II) ions. Percent recovery of the selected amino acids was

also determined after acid hydrolysis of the model systems with 6M HCl

for 18 or 24 hours at 110°C.

Free Amino Acids

Basic Hydrolysis of Selected Amino Acids

Methionine, methionine sulfoxide, methionine sulfone, cystine,

and cysteic acid were hydrolyzed individually and in combination under

both sets of alkaline hydrolytic conditions and the amount of each

amino acid recovered determined by ion-exchange chromatography. The

results are presented in Tables 9 and 10. The amount of methionine

sulfoxide recovered was significantly greater ($P < 0.001$) when hydrol-

yzed individually with 2M NaOH compared to hydrolysis with 3M NaOH.

No other significant differences ($P > 0.05$) were apparent between per-

cent recovery at 3M NaOH. No significant interconversions among

methionine, methionine sulfoxide, or methionine sulfone were observed

when these amino acids were hydrolyzed individually under either set

of conditions.

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Table 9. Percent recovery of selected amino acids hydrolyzed individually under two sets of alkaline hydrolytic conditions.

PERCENT AMINO ACID RECOVERY[1]

Amino Acid	2M NaOH, 18 Hours, 100°C	3M NaOH, 16 Hours, 110°C
Methionine	94.8 ± 3.3^a	93.7 ± 4.0^a
Methionine Sulfoxide	89.8 ± 3.6^a	67.6 ± 2.0^b
Methionine Sulfone	101.1 ± 3.8^a	100.5 ± 4.5^a
Cystine	0^2	0^3
Cysteic Acid	77.6 ± 3.8^a	76.6 ± 5.2^a

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[1] $\dfrac{\text{Area of peak in hydrolyzed sample}}{\text{Area of peak in unhydrolyzed sample}} \times 100$;

values given are means and standard deviations of three to seven determinations. Within each horizontal row means having the same letter are not significantly different at 0.05 level. See Appendix 6.

[2] 44% of the cystine was recovered as cysteic acid.

[3] 42% of the cystine was recovered as cysteic acid.

Table 10. Percent recovery of selected amino acids hydrolyzed in a
mixture of five amino acids under two sets of alkaline
hydrolytic conditions.

PERCENT AMINO ACID RECOVERY[1]

Amino Acid	2M NaOH, 18 Hours, 100°C	3M NaOH, 16 Hours, 110°C
Methionine	76.2 ± 1.3	77.8 ± 5.8
Methionine Sulfoxide	85.7 ± 3.3	83.3 ± 7.6
Methionine Sulfone	96.0 ± 4.8	97.4 ± 8.2
Cystine	0	0
Cysteic Acid	183.3 ± 28.8	150.4 ± 32.8

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[1] $\dfrac{\text{Area of peak in hydrolyzed sample}}{\text{Area of peak in unhydrolyzed sample}}$ X 100;

values given are means and standard deviations of three or four
determinations; there were no significant differences at the 0.05
level between recoveries at 2M NaOH and 3M NaOH for any amino acid
in the mixture. See Appendix 7.

No cystine was recovered as such under either set of alkaline conditions when it was hydrolyzed alone or in combination. A substantial amount of cystine was oxidized to cysteic acid under both sets of basic conditions (Table 9). The amount of cystine oxidized to cysteic acid when cystine was hydrolyzed in combination with the other four amino acids appeared to be quite variable with a greater amount of cystine apparently being converted to cysteic acid with 2M NaOH (Table 10).

The difference between recovery of methionine, methionine sulfoxide, and methionine sulfone hydrolyzed in a mixture of five amino acids with 2M NaOH compared to 3M NaOH was not significant at the 0.05 level although the variability was greater with 3M NaOH (Table 10). The shape of the methionine sulfoxide and methionine sulfone peaks on the NC-2P chromatograms after hydrolysis with 3M NaOH was broader compared to the shape of these peaks of the unhydrolyzed samples and the samples hydrolyzed with 2M NaOH (Figs. 1-3).

The effect of other amino acids in the hydrolysis mixture on the recovery of the sulfur amino acids was investigated further by hydrolyzing these amino acids under both sets of alkaline conditions in a mixture containing most of the other naturally occurring amino acids. The results are shown in Table 11. Recoveries of methionine sulfoxide, methionine sulfone, and leucine were greater ($P < 0.05$) and variability among samples lower when the mixture was hydrolyzed with 2M NaOH compared to 3M NaOH. Recoveries of methionine and valine also appeared to be higher after the 2M NaOH hydrolysis but the differences were not significant ($P > 0.05$). No cystine was recovered under either set of
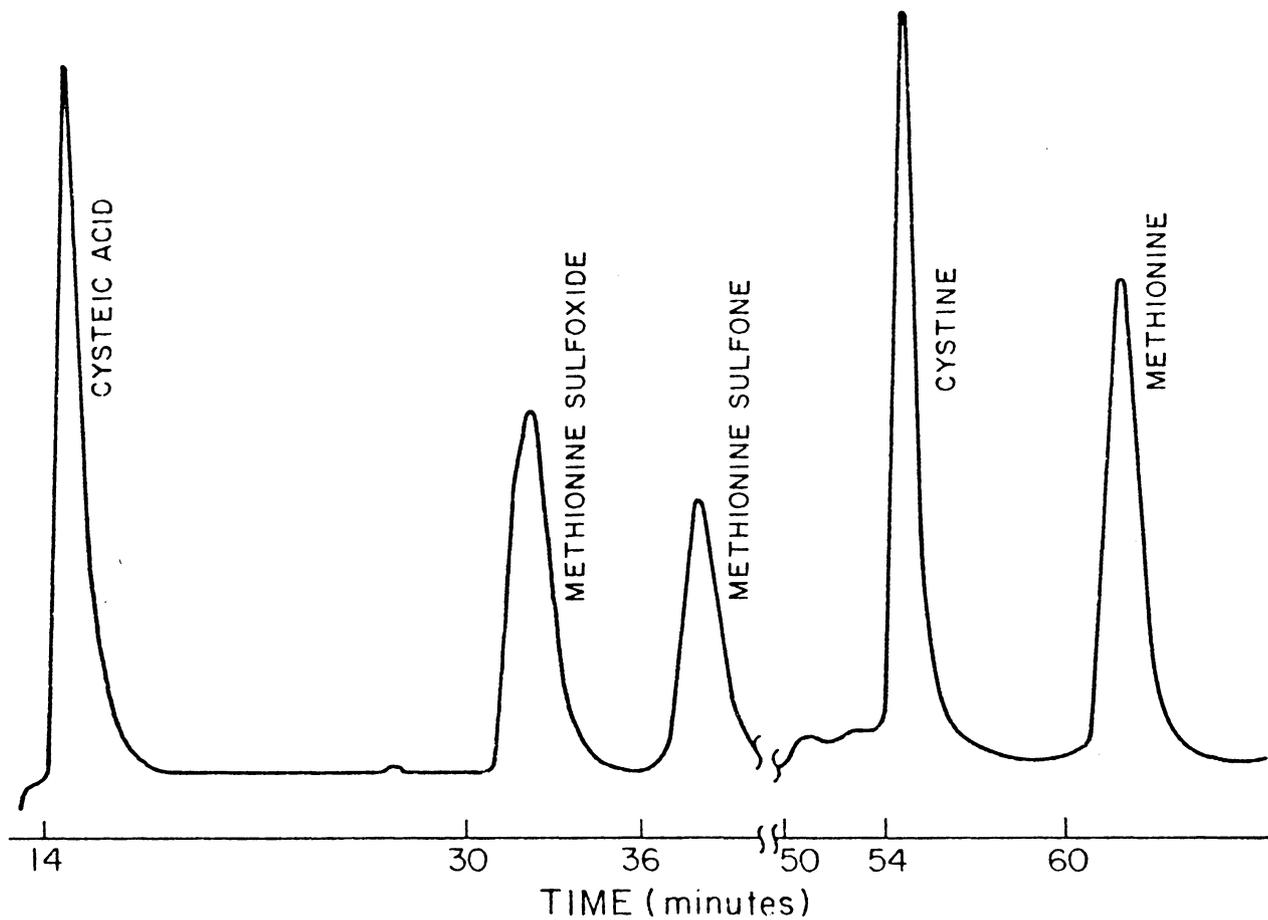
Figure 1. NC-2P chromatogram[1] of unhydrolyzed standard solution containing cysteic acid, methionine sulfoxide, methionine sulfone, cystine, and methionine.

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[1]Conditions of elution: column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 2.70 for 24 min; buffer 2, pH 4.00 for 20 min.
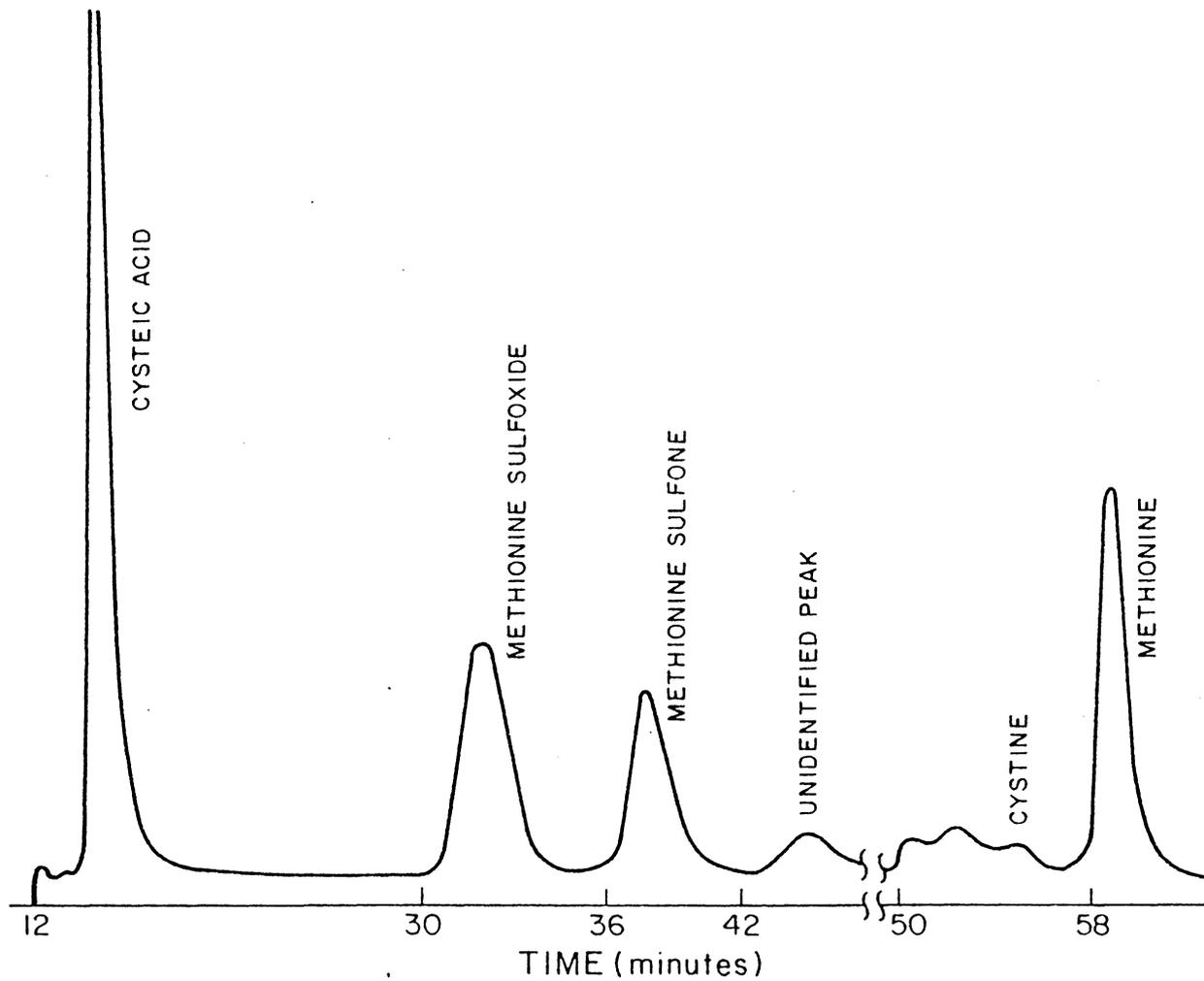
Figure 2. NC-2P chromatogram[1] of standard solution containing cysteic acid, methionine sulfoxide, methionine sulfone, cystine, and methionine hydrolyzed with 2M NaOH for 18 hours at 100°C.

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[1]Conditions of elution: column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 2.70 for 24 min; buffer 2, pH 4.00 for 20 min.
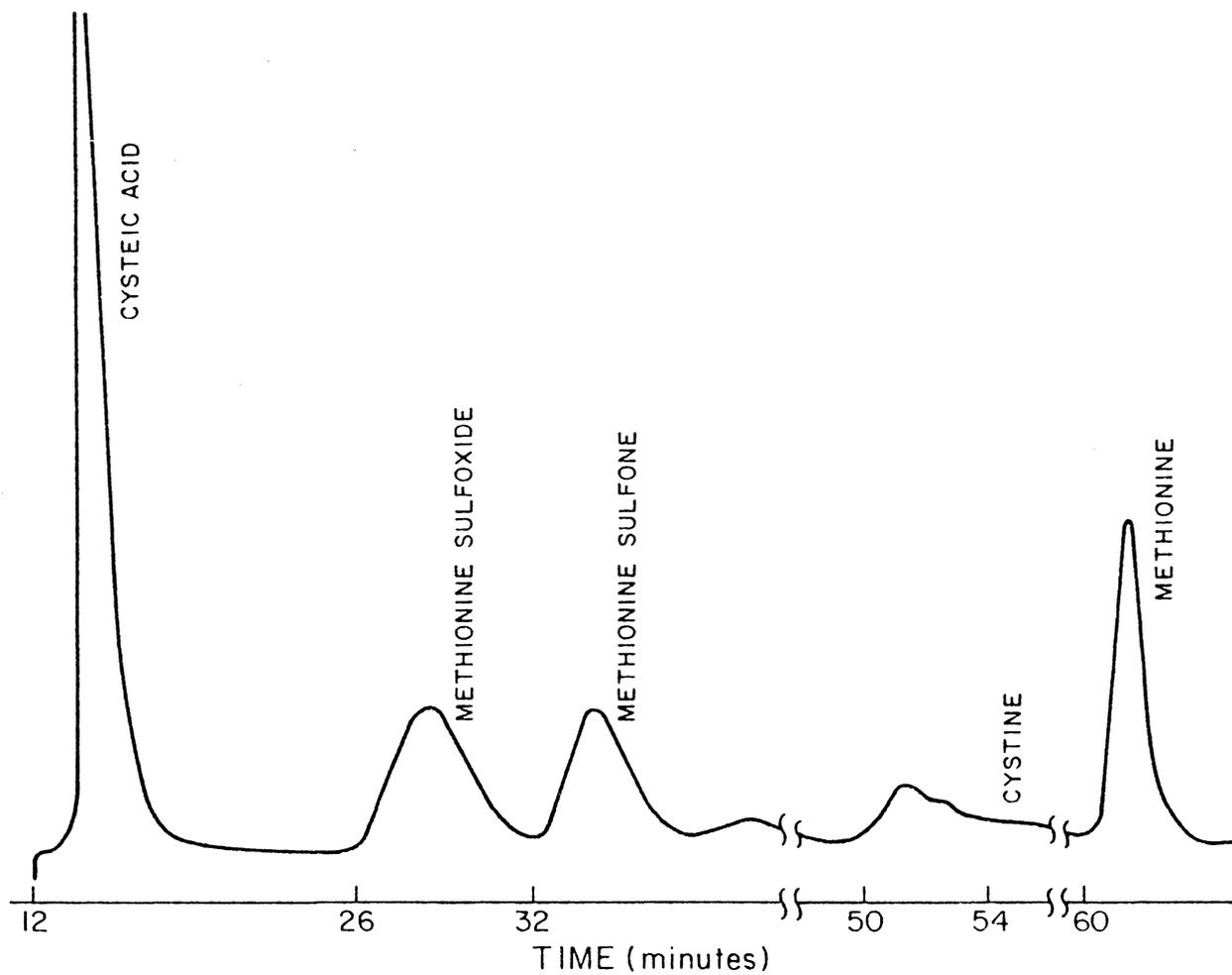
Figure 3. NC-2P chromatogram[1] of standard solution containing cysteic acid, methionine sulfoxide, methionine sulfone, cystine, and methionine hydrolyzed with 3M NaOH for 16 hours at 110°C.

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[1] Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 2.70 for 24 min; buffer 2, pH 4.00 for 20 min.

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Table 11. Percent recovery of selected amino acids hydrolyzed in a mixture of 20 amino acids under two sets of alkaline hydrolytic conditions.

PERCENT AMINO ACID RECOVERY[1]

Amino Acid[2]	2M NaOH, 18 Hours, 100°C	3M NaOH, 16 Hours, 110°C
Methionine	89.1 ± 3.2^a	83.4 ± 4.5^a
Methionine Sulfoxide	100.3 ± 2.2^a	90.5 ± 3.9^b
Methionine Sulfone	104.7 ± 2.1^a	97.8 ± 4.3^b
Cystine	0	0
Cysteic Acid	161.9 ± 22.3^a	154.7 ± 30.0^a
Valine	95.4 ± 3.6^a	93.2 ± 6.6^a
Leucine	99.7 ± 2.4^a	90.1 ± 3.1^b

[1] $\dfrac{\text{Area of peak in hydrolyzed sample}}{\text{Area of peak in unhydrolyzed sample}} \times 100$;

values given are means and standard deviations of four or five determinations; horizontal pairs of means having the same letter are not significantly different at 0.05 level. See Appendix 8.

[2] Amino acids present in the mixture are listed in Appendix 3.

conditions. The disproportionate amount of cysteic acid recovered was probably the result of oxidation of a portion of the cystine to cysteic acid.

Comparison between recoveries of amino acids hydrolyzed alone and in mixtures -- Recovery values for methionine methionine sulfoxide, and methionine sulfone were compared to see if differences existed in percent recoveries of the amino acids depending on the presence or absence of other amino acids in the hydrolysis mixture. Results from Table 9-11 are summarized in Tables 12 and 13. Results for another set of amino acids are also shown in Table 12.

When the amino acids were hydrolyzed with 3M NaOH (Table 13), recovery of methionine was greatest when it was hydrolyzed alone compared to recovery from mixtures. Recovery of methionine sulfoxide was least when hydrolyzed alone. The amount of methionine sulfone recovered when this amino acid was hydrolyzed in mixtures was not different from the percent recovery when it was hydrolyzed alone with 3M NaOH. These results suggest that hydrolysis with 3M NaOH at 110°C for 16 hours may promote some oxidation of methionine to methionine sulfoxide when a mixture of 20 amino acids are hydrolyzed but that methionine sulfoxide is not oxidized to methionine sulfone during this procedure.

When the amino acids were hydrolyzed with 2M NaOH, recovery of methionine sulfoxide and methionine sulfone was greatest when these amino acids were hydrolyzed in the mixture of 20 amino acids and least when hydrolyzed in the mixture of five amino acids. Methionine recovery was greatest when it was hydrolyzed alone, least when hydrolyzed in

Table 12. Percent recovery of selected amino acids hydrolyzed indivi-
          dually or in mixtures of amino acids with 2M NaOH for 18
          hours at 100°C.

PERCENT AMINO ACID RECOVERY[1]

Amino Acid	Individual	Mixture A[2]	Mixture B[3]	Mixture C[4]
Methionine	94.8 ± 3.3[a]	90.9 ± 6.5[ac]	76.2 ± 1.3[b]	89.1 ± 3.2[c]
Methionine Sulfoxide	89.8 ± 3.6[ab]	91.3 ± 2.2[a]	85.7 ± 3.3[b]	100.3 ± 2.2[c]
Methionine Sulfone	101.1 ± 3.8[a]	96.3 ± 4.1[a]	96.0 ± 4.8[a]	104.7 ± 2.1[b]

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[1]$\dfrac{\text{Area of peak in hydrolyzed sample}}{\text{Area of peak in unhydrolyzed sample}}$ X 100;

values shown are means and standard deviations of three to seven
determinations; within each horizontal row means having the same
letters are not significantly different at 0.05 level. See Appendices
6-8.

[2]Mixture A contained methionine, methionine sulfoxide, and methionine
sulfone.

[3]Mixture B contained methionine, methionine sulfoxide, methionine
sulfone, cystine, and cysteic acid.

[4]Mixture C contained a total of 20 amino acids including methionine,
methionine sulfoxide, methionine sulfone, cystine, and cysteic acid.
The other amino acids in this mixture are listed in Appendix 3.

Table 13. Percent recovery of selected amino acids hydrolyzed individually or in mixtures of amino acids with 3M NaOH for 16 hours at 110°C.

PERCENT AMINO ACID RECOVERY[1]

Amino Acid	Individual	Mixture A[2]	Mixture B[3]
Methionine	93.7 ± 4.0^a	77.8 ± 5.8^b	83.4 ± 4.5^c
Methionine Sulfoxide	67.6 ± 2.0^a	83.3 ± 7.6^b	90.5 ± 3.9^b
Methionine Sulfone	100.5 ± 4.5^a	97.4 ± 8.2^a	97.8 ± 4.3^a

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[1] $\dfrac{\text{Area of peak in hydrolyzed sample}}{\text{Area of peak in unhydrolyzed sample}}$ X 100;

values shown are means and standard deviations of three to five determinations; within each horizontal row means having the same letters are not significantly different at 0.05 level.

[2] Mixture A contained methionine, methionine sulfoxide, methionine sulfone, cystine, and cysteic acid.

[3] Mixture C contained a total of 20 amino acids including methionine, methionine sulfoxide, methionine sulfone, cystine, and cysteic acid. The other amino acids in this mixture are listed in Appendix 3.

the mixture of five amino acids, and intermediate in the mixture of 20

amino acids. Although little oxidation of methionine to methionine

sulfoxide and sulfone occurred when methionine was hydrolyzed alone,

a greater degree of oxidation of methionine may have occurred when it

was hydrolyzed in the mixture of 20 amino acids leading to higher

apparent recoveries of methionine sulfoxide and methionine sulfone.

This, however, does not appear to be true in the mixture of five amino

acids, since methionine recovery is much lower than in the other mix-

ture without the recoveries of methionine sulfoxide and methionine

sulfone being correspondingly higher. It appears that loss of methi-

onine during alkaline hydrolysis can occur in ways other than oxida-

tion.

Effect of glucose in the hydrolysis mixture -- The effect of

glucose on recovery of the sulfur amino acids when these amino acids

were hydrolyzed with 2M NaOH in the presence of glucose was also

investigated. The results are presented in Table 14. When the amino

acids were hydrolyzed individually with 2M NaOH in the presence of

glucose, there were no differences $(P > 0.05)$ in percent recovery of

methionine or methionine sulfone compared to hydrolysis with 2M NaOH

in the absence of glucose. Recovery of methionine sulfoxide, however,

was lower when glucose was present in the hydrolysis mixture. Some of

the loss of methionine sulfoxide may have resulted from oxidation of

methionine sulfoxide to methionine sulfone since there was a small,

though not significant, increase in the percent recovery of methionine

sulfone when glucose was in the hydrolysis mixture. Recoveries of

Table 14. Percent recovery of selected amino acids hydrolyzed with 2M NaOH for 18 hours at 100°C individually or in a mixture of amino acids in the presence of glucose.

PERCENT AMINO ACID RECOVERY[1]

Amino Acid	Hydrolyzed Individually		Hydrolyzed in a Mixture[2]	
	Without Glucose	With Glucose[3]	Without Glucose	With Glucose[3]
Methionine	94.8 ± 3.3^a	99.2 ± 4.5^a	89.1 ± 3.2^b	90.9 ± 4.3^b
Methionine Sulfoxide	89.8 ± 3.6^a	85.6 ± 2.9^b	100.3 ± 2.2^c	101.8 ± 3.0^c
Methionine Sulfone	101.1 ± 3.8^a	103.4 ± 5.2^a	104.7 ± 2.1^a	102.8 ± 6.4^a
Cysteic Acid	77.6 ± 3.8^a	–	161.9 ± 22.3^b	138.3 ± 14.4^b
Valine	91.6 ± 4.8^a	–	95.4 ± 3.6^a	92.9 ± 8.2^a
Leucine	94.1 ± 4.9^a	–	99.7 ± 2.4^a	96.4 ± 3.1^a

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[1] $\dfrac{\text{Area of peak in hydrolyzed sample}}{\text{Area of peak in unhydrolyzed sample}} \times 100$;

values shown are means and standard deviations of three to seven determinations; values within horizontal rows having the same letter are not significantly different at 0.05 level. See Appendix 6 and Appendix 8.

[2] Mixture of amino acids contains 20 amino acids which are listed in Appendix 3.

[3] The amount of glucose present in the hydrolysis mixture was five times greater by weight than the amount of the amino acids in the sample.

methionine, methionine sulfoxide, methionine sulfone, cysteic acid, valine, and leucine, hydrolyzed in a mixture in the presence of glucose did not differ from recovery of these amino acids hydrolyzed in a mixture without glucose.

When recoveries of methionine and methionine sulfoxide hydrolyzed individually in the presence or absence of glucose were compared to their counterparts hydrolyzed in the mixture, differences were apparent (Table 14). Recovery of methionine hydrolyzed individually both with and without glucose was significantly higher than methionine recovered from the mixture. The reverse was true of methionine sulfoxide with the percent recovery being lower when this amino acid was hydrolyzed individually both with and without glucose compared to recovery of methionine sulfoxide from the mixture. Recovery values appear to be influenced by the presence of other amino acids in the hydrolysis mixture and, to a lesser degree, by the presence of glucose.

Acid Hydrolysis of Selected Amino Acids

Methionine, methionine sulfoxide, methionine sulfone, cystine, and cysteic acid individually and in combination were also subjected to acid hydrolysis with 6M HCl at 110°C for 18 hours. Table 15 summarizes the results of these hydrolyses. Methionine sulfone appeared to be quite stable to acid hydrolysis. Methionine sulfoxide, on the other hand, was not as stable to acid hydrolysis with recovery ranging from 78.3% when hydrolyzed alone to 85.8% when hydrolyzed in a mixture. A substantial amount of methionine sulfoxide (15.5%) was reduced to methionine when it was hydrolyzed alone and also, apparantly,

Table 15. Percent recovery of selected amino acids hydrolyzed individually or in a mixture under acidic conditions.[1]

PERCENT AMINO ACID RECOVERY[2]

Amino Acid	Hydrolyzed Individually	Hydrolyzed in a Mixture[3]
Methionine	94.8 ± 9.7^a	104.9 ± 8.63^a
Methionine Sulfoxide[4]	78.3 ± 5.0^a	85.3 ± 3.0^a
Methionine Sulfone	97.7 ± 10.1^a	106.7 ± 4.8^a
Cystine[5]	75.6 ± 12.2^a	91.4 ± 1.7^a
Cysteic Acid	98.5 ± 6.4^a	122 ± 2.7^b

[1]Conditions used for hydrolysis were 6M HCl at 110°C for 18 hours.

[2]$\frac{\text{Area of peak in hydrolyzed sample}}{\text{Area of peak in unhydrolyzed sample}}$ X 100;

values shown are means and standard deviations for three determinations of the hydrolysis of the mixture and four to six determinations of the hydrolysis of the individual amino acids; values having the same letter in horizontal rows are not significantly different at 0.05 level. See Appendix 9.

[3]Mixture contained methionine, methionine sulfoxide, methionine sulfone, cystine, and cysteic acid.

[4]15.5% $\pm$ 2.9% of methionine sulfoxide hydrolyzed individually was recovered as methionine.

[5]Between 2 and 5% of the cystine hydrolyzed individually was recovered as cysteic acid.

when it was hydrolyzed in a mixture, since methionine recovery in the mixture was over 100%. A small amount of cystine, 2% to 5%, appeared to be converted to cysteic acid when cystine was hydrolyzed individually. This conversion, however, accounts for only a portion of the cystine lost in the individual hydrolysis. In the mixture, less cystine was lost during hydrolysis, and the loss could be accounted for through the gain in cysteic acid. Since, on a mole for mole basis, two moles of cysteic acid are produced for every mole of cystine oxidized, the conversion appears to be close to quantitative.

Summary of Results of Alkaline Hydrolytic Studies of Free Amino Acids

The primary objective of these experiments on the effect of alkaline hydrolysis on the sulfur amino acids was to determine the feasibility of using alkaline hydrolysis for measuring methionine sulfoxide. A comparison of two sets of alkaline conditions, 2M NaOH for 18 hours at 100°C and 3M NaOH for 16 hours at 110°C, indicated that, in general, whether methionine sulfoxide was hydrolyzed alone or in mixtures of amino acids, recovery of this amino acid was greater but not always significantly greater and variability usually lower when the amino acids were hydrolyzed with 2M NaOH at 100°C for 18 hours compared to the other set of hydrolytic conditions (Tables 9-11). Some oxidation of methionine to methionine sulfoxide may have occurred during alkaline hydrolysis of the mixture of 20 amino acids with 2M NaOH. Oxidation of methionine sulfoxide to methionine sulfone was negligible under both sets of alkaline hydrolytic conditions.

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The addition of glucose to the hydrolysis mixture when methionine sulfoxide was hydrolyzed alone or in mixtures of amino acids with 2M NaOH appeared to have some effect on the recovery of methionine sulfoxide. Glucose appeared to be a minor factor in promoting oxidation of methionine to methionine sulfoxide. The more important factor in promoting oxidation may have been the presence of other amino acids in the hydrolysis mixture.

Cystine was totally destroyed by hydrolysis under both sets of alkaline conditions. A portion of it, less than 50%, was converted to cysteic acid when cystine was hydrolyzed alone with 2M NaOH and with 3M NaOH. The other products from the degradation of cystine were not determined.

Model Proteins

Total methionine of several proteins was determined by the performic acid oxidation method of Moore (1963) prior to using them as model proteins in basic and acidic hydrolytic studies (Table 16). Lysozyme, oxidized lysozyme, casein, and oxidized casein were hydrolyzed with 2M NaOH at 100°C for 18 hours or with 3M NaOH at 110°C for 16 hours. These proteins were hydrolyzed under the first set of conditions with or without glucose added to the hydrolysis mixture. The proteins were also hydrolyzed with 6M HCl at 110°C for 24 hours. Recoveries of selected amino acids were determined in all hydrolysates by ion-exchange chromatography on the NC-2P Technicon Amino Acid Analyzer and, in some cases, on the TSM Technicon Amino Acid Analyzer. Conditions used for elution are shown in Appendix 4 and Appendix 5.

Table 16. Total methionine content of model proteins as determined by performic acid oxidation of the proteins.

Protein	Total Methionine[1] (millimoles/100 g)[2]	Grams of Nitrogen per 100 Grams Product[3]
Lysozyme	13.16 ± 0.84[4]	-
Oxidized Lysozyme	15.30 ± 0.60	-
Casein	19.08 ± 2.09	14.25 ± 0.50
Oxidized Casein	18.06 ± 0.76	-
Promine F	7.65 ± 1.08	14.00 ± 0.31

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[1]Values shown are means and standard deviations of three to five determinations using the performic acid oxidation method of Moore (1963).

[2]Sample size was determined by measuring glutamic acid content of the performic acid sample and calculating weight based on glutamic acid content of samples as determined by ion-exchange chromatography after acid hydrolysis of the particular protein.

[3]Nitrogen was determined by macro-Kjeldahl (AOAC, 1970).

[4]Theoretical amount of methionine in lysozyme is 13.97 millimoles per 100 grams (Sober, 1970).

Lysozyme

Basic hydrolysis without glucose -- No methionine sulfoxide or methionine sulfone was detected in lysozyme when it was hydrolyzed with either 2M NaOH or 3M NaOH. Recovery of methionine was significantly higher with 2M NaOH compared to 3M NaOH when the lysozyme was hydrolyzed in the ratio of 10 mg of protein to 2.0 ml of base. The amount of methionine recovered after hydrolysis with 2M NaOH was not different from the theoretical amount of methionine in lysozyme indicating total release of methionine without destruction (Table 17).

To determine if the ratio of protein to volume of base had any effect on recovery values after hydrolysis with 2M NaOH, 10.0 mg of lysozyme was hydrolyzed in a total volume or either 2.0 ml or 5.0 ml. The concentration of NaOH in both cases was 2M; other conditions of hydrolysis and ion-exchange chromatography were identical. The results indicate (Table 17) that there were no significant differences in recoveries of methionine, valine, leucine, or phenylalanine between the two ratios tested.

The amounts of valine, leucine, and phenylalanine recovered were compared to theoretical amounts of these amino acids in lysozyme (Sober, 1970). These three amino acids proved to be much more resistant to release from the protein after alkaline hydrolysis than methionine, with only about 28% of the theoretical amount of valine recovered when lysozyme was hydrolyzed with 2M NaOH, rising to about 51% with 3M NaOH (Table 17). For comparative purposes the results of the acid hydrolysis of lysozyme are also shown in Table 17. The amount

Table 17. Recoveries of selected amino acids from lysozyme which has been hydrolyzed under two sets of alkaline conditions at two different ratios of protein to volume of base, or hydrolyzed with acid.[1]

Amino Acid	Theoretical[2] Quantity	2M NaOH, 18 Hrs[3] 100°C	2M NaOH, 18 Hrs[4] 100°C	3M NaOH, 16 Hrs 110°C	6M HCl, 24 Hrs 110°C
		(Millimoles per 100 grams)			
Methionine[6]	13.97^a	13.32 ± 0.70^{ac} (95.3%)[5]	12.58 ± 1.51^{abc} (90.0%)	11.15 ± 1.12^b (79.8%)	12.60 ± 1.10^{bc} (90.2%)
Valine	41.94^a	11.80 ± 0.86^b (28.1%)	11.04 ± 0.25^b (26.3%)	21.31 ± 0.92^c (50.8%)	39.08 ± 2.38^d (93.2%)
Leucine	55.92^a	36.65 ± 1.75^b (65.5%)	36.67 ± 0.72^b (65.6%)	45.84 ± 2.14^c (82.0%)	56.47 ± 2.90^a (101.0%)
Phenyl-alanine	20.97^a	10.85 ± 0.38^b (51.7%)	10.72 ± 0.93^b (51.1%)	15.76 ± 1.01^c (75.2%)	20.30 ± 1.16^a (96.8%)

[1] Values shown are the means and standard deviations of four to six determinations; values having the same letters in horizontal rows are not significantly different at 0.05 level. See Appendix 10 and Appendix 11.

[2] Values shown are taken from Sober (1970).

[3] Ratio of protein to volume of base is 10 mg/2.0 ml 2M NaOH.

[4] Ratio of protein to volume of base is 10 mg/5.0 ml 2M NaOH.

[5] All values in parentheses are: $\dfrac{\text{Millimoles recovered}}{\text{Theoretical number of millimoles}} \times 100$.

[6] No methionine sulfoxide or sulfone peaks under any hydrolytic conditions.

of methionine recovered after acid hydrolysis was lower than the theoretical amount suggesting some destruction of methionine during the hydrolysis.

Basic hydrolysis with glucose -- In another experiment, lysozyme was, again, hydrolyzed with 2M NaOH at 100°C for 18 hours. At the same time glucose was added to another set of lysozyme samples, and the samples of lysozyme, with and without glucose, were hydrolyzed at the same time under the same conditions and chromatographed in the same manner. The results are shown in Table 18. The amount of methionine recovered in the samples without glucose was lower than the amount recovered in the previous experiment (Table 17). This may have resulted from greater destruction of methionine in this experiment compared to the earlier one. Since no methionine sulfoxide or methionine sulfone was detected in the hydrolysate without glucose, however, the discrepancy between the recovery of methionine in the two experiments was not due to oxidation of methionine to methionine sulfoxide. Earlier experiments with free methionine hydrolyzed in a mixture of amino acids indicated that the amount of methionine recovered was variable and much of the loss could not be accounted for by a corresponding increase in methionine sulfoxide.

The presence of glucose in the hydrolysis mixture did not decrease recovery of methionine in lysozyme. There were no significant differences in analyses done on the Technicon NC-2P analyzer compared to the Technicon TSM in measuring methionine recovery. However, there were differences in the amount of methionine sulfoxide measured

Table 18. Recovery of methionine, methionine sulfoxide, and
methionine sulfone from lysozyme hydrolyzed with 2M NaOH
for 18 hours at 100°C in the presence or absence of
glucose.[1]

Amino Acid	NC-2P Analyzer	TSM Analyzer	Nc-2P Analyzer	TSM Analyzer
	Without Glucose		With Glucose[2]	
	(Millimoles per 100 grams)			
Methionine[3]	11.41 ± 0.43^a (81.68%)[4]	11.56 ± 1.01^a (82.7%)	10.76 ± 0.44^a (77.0%)	11.10 ± 2.53^a (79.4%)
Methionine Sulfoxide[5]	0	0	4.13 ± 0.19^a (29.6%)	0.96 ± 0.30^b (6.87%)
Methionine Sulfone	0	0	0	0
Total	11.41	11.56	14.89 (106.6%)	12.06 (86.3%)

[1]Values shown are means and standard deviations of four to six deter-
minations; values in horizontal rows having the same letter are not
significantly different at 0.05 level. See Appendix 12.

[2]Amount of glucose added was five times that of lysozyme by weight.

[3]Theoretical amount of methionine in 100 grams of lysozyme is 13.97
millimoles per 100 grams (Sober, 1970).

[4]Values in parentheses are: $\dfrac{\text{Millimoles recovered}}{\text{Theoretical amount of methionine}} \times 100.$

[5]Values for methionine sulfoxide may include compounds which co-elute
with methionine sulfoxide.

by the NC-2P compared to that measured by the TSM when glucose was present in the hydrolysis mixture. In the first attempt to measure methionine sulfoxide on the NC-2P, the amount of material eluting in the position of methionine sulfoxide on the NC-2P chromatogram was very high -- about 12 millimoles per 100 grams of lysozyme when glucose was present in the hydrolysis mixture. The sum of methionine plus methionine sulfoxide was far above the amount of total methionine in lysozyme; consequently, it was apparent that other products were co-eluting with methionine sulfoxide under the conditions used. By lowering the flow rate from 0.50 to 0.45 ml per min and raising the pH of the first buffer from 2.40 to 2.70 a portion of the material which had been co-eluting with methionine sulfoxide was separated from this amino acid. The value of 4.13 millimoles per 100 grams of lysozyme for methionine sulfoxide is probably still spuriously high, however, since methionine plus methionine sulfoxide are above the value for total methionine in lysozyme as measured by performic acid oxidation (Moore, 1963) as well as that given in the literature (Chemical Rubber Co., 1970). The value of 0.96 millimoles of methionine sulfoxide per 100 grams of lysozyme as determined on the TSM Amino Acid analyzer is probably closer to the true amount of methionine sulfoxide in the hydrolysate and even it represents a maximum value since the shape of the sulfoxide peaks on the TSM chromatograms were, at times, asymmetric which suggests other compounds co-eluting with methionine sulfoxide.

Discussion of results of hydrolytic studies of lysozyme -- The
amount of methionine recovered was greater when lysozyme was hydro-
lyzed with some 2M NaOH conditions compared to 3M NaOH. The amount
recovered after hydrolysis with 2M NaOH was not different from the
theoretical amount of methionine in lysozyme (Sober, 1970). The
hydrolytic procedures did not generate any methionine sulfoxide or
methionine sulfone when lysozyme was hydrolyzed alone, but when
lysozyme was hydrolyzed in the presence of glucose a substantial
amount of an unidentified compound was eluted with what was probably
a very small amount of methionine sulfoxide on the NC-2P analyzer.
It would appear that hydrolysis with glucose leads to reactions which
generate products that may interfere with analysis of methionine
sulfoxide, especially when the analyses are done on the NC-2P Amino
Acid analyzer. The value for methionine sulfoxide of 0.96 millimoles
per 100 grams of lysozyme as measured on the TSM Amino Acid analyzer
is probably closer to the amount of methionine sulfoxide in the
hydrolysate, but it, too, may represent a maximum. On the other hand,
methionine sulfoxide may not be quantitatively recovered from hydrol-
ysis mixtures. This would be an off-setting error. It would appear
that while some methionine may be converted to methionine sulfoxide
during alkaline hydrolysis of lysozyme in the presence of glucose,
that amount is small. A major source of error in quantitating methi-
onine sulfoxide by ion-exchange chromatography in lysozyme after
hydrolysis in the presence of glucose is interfering substances which
co-elute with methionine sulfoxide.

Methionine sulfone was not detected in any of the lysozyme hydrolysates. This indicates that neither the alkaline hydrolytic process nor the presence of glucose leads to the production of methionine sulfone during hydrolysis.

Oxidized Lysozyme

Basic hydrolysis without glucose -- A portion of lysozyme was oxidized using a modification of the method of Cuq et al. (1973) as described in the section, Materials and Methods. These samples were then hydrolyzed under both sets of alkaline hydrolytic conditions (Table 19) and recovery of methionine, methionine, sulfoxide, methionine sulfone, valine, and leucine determined. About 74% of the theoretical amount of methionine was recovered as methionine and about 27% as methionine sulfoxide when oxidized lysozyme was hydrolyzed with 2M NaOH. When it was hydrolyzed with 3M NaOH the recoveries of methionine and methionine sulfoxide were, respectively, 65% and 25%. These data suggest that a small amount of methionine may have been destroyed by hydrolysis with 3M NaOH since total recovery (methionine plus methionine sulfoxide) was only 90% of the theoretical amount of methionine. No methionine sulfone was detected under either set of basic conditions. As in the case of lysozyme, release of valine and leucine from oxidized lysozyme was incomplete under both sets of alkaline conditions with a greater percentage of these two amino acids being released with 3M NaOH compared to 2M NaOH (Table 19).

Basic hydrolysis with glucose -- A second preparation of oxidized lysozyme was subjected to amino acid analysis after acid

Table 19. Recovery of selected amino acids from oxidized lysozyme after hydrolysis under two sets of alkaline hydrolytic conditions.

AMINO ACID RECOVERY[1]

Amino Acid	Theoretical Amount[2]	2M NaOH, 18 Hrs, 100°C	3M NaOH, 16 Hrs, 110°C
		(Millimoles per 100 grams)	
Methionine	13.97[a]	10.33 $\pm$ 0.35[b] (73.$\overline{9}$%)[3]	9.12 $\pm$ 0.32[c] (65.$\overline{3}$%)
Methionine Sulfoxide	0	3.80 $\pm$ 0.26[a] (27.$\overline{2}$%)[4]	3.54 $\pm$ 0.28[a] (25.$\overline{3}$%)[4]
Methionine Sulfone	0	0	0
Valine	41.94[a]	15.80 $\pm$ 0.22[b] (37.$\overline{7}$%)	25.38 $\pm$ 2.76[c] (60.$\overline{5}$%)
Leucine	55.92[a]	41.70 $\pm$ 1.18[b] (74.$\overline{6}$%)	47.56 $\pm$ 1.54[a] (85.$\overline{0}$%)

[1]Values shown are means and standard deviations of four determinations for each set of conditions; values having the same letters in horizontal rows are not significantly different at the 0.05 level. See Appendix 13.

[2]Values are for unoxidized lysozyme (Sober, 1970).

[3] $\dfrac{\text{Millimoles recovered}}{\text{Theoretical number of millimoles of methionine}}$ X 100.

[4] $\dfrac{\text{Millimoles recovered}}{\text{Theoretical number of millimoles of methionine}}$ X 100.

hydrolysis, performic acid oxidation (Moore, 1963), and 2M NaOH hydrolysis in the presence and absence of glucose. The results are shown in Tables 20 and 21. This preparation contained a greater amount of methionine sulfoxide and less methionine than the first preparation, as determined by ion exchange chromatography after alkaline hydrolysis. This resulted from a slight change in the conditions of oxidation of the lysozyme. In the second oxidation the time of reaction between hydrogen peroxide and lysozyme was increased from 1.5 hours to 1.75 hours.

The amount of methionine recovered from oxidized lysozyme after alkaline hydrolysis in the presence of glucose was not significantly different ($P > 0.05$) from that recovered after alkaline hydrolysis without glucose (Table 20). The amount of methionine sulfoxide recovered, however, appeared to be greater when glucose was present in the analyses done on the NC-2P Analyzer. This may indicate that some oxidation of methionine to methionine sulfoxide occurred during the hydrolysis in the presence of glucose. The value for methionine sulfoxide recovery determined on the TSM analyzer, however, was about the same as the hydrolysis without glucose. This suggests that, as in the case of unoxidized lysozyme hydrolyzed with glucose, other compounds may be co-eluting with methionine sulfoxide on the NC-2P analyses under the conditions used: 0.45 ml per minute, flow rate; 55°C column temperature; citrate buffer (0.2M Na^+, pH 2.69). No methionine sulfone was detected in any of the samples.

Table 20. Recovery of methionine, methionine sulfoxide, and methionine sulfone from oxidized lysozyme hydrolyzed with 2M NaOH for 18 hours at 100°C in the presence or absence of glucose.

AMINO ACID RECOVERY[1]

(Millimoles per 100 grams)

Amino Acid	2M NaOH, 18 Hours, 100°C		
	Without Glucose	With Glucose[2]	
	(NC-2P)	(NC-2P)	(TSM)
Methionine[3]	7.30 ± 0.23^a (52.2%)[5]	7.11 ± 0.37^a (50.9%)	8.17 ± 1.24^a (58.5%)
Methionine Sulfoxide[4]	5.41 ± 0.48^a (38.7%)	8.35 ± 0.35^b (59.8%)	4.49^6 (32.1%)
Methionine Sulfone	0	0	0

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[1] Values shown are means and standard deviations of three to five determinations except where noted; values having the same letters are not significantly different at 0.05 level. See Appendix 14.

[2] Amount of glucose added was five times that of oxidized lysozyme by weight.

[3] Theoretical amount of methionine in 100 grams of lysozyme is 13.97 millimoles (Sober, 1970).

[4] Methionine sulfoxide values for analyses of hydrolysates with glucose may be high because of interferring compounds co-eluting with methionine sulfoxide.

[5] Values in parentheses are percent of theoretical methionine present as that particular methionine derivative.

[6] Value is from a single analysis.

Table 21. Recovery of methionine, methionine sulfoxide, methionine sulfone, cysteic acid, and total cystine after acid hydrolysis or performic acid oxidation of oxidized lysozyme.

AMINO ACID RECOVERY[1]

(Millimoles per 100 grams)

Amino Acid	6M HCl, 24 Hours, 110°C	Performic Acid Oxidation
Methionine[2]	14.61 ± 0.64^a $(104.\overline{6}\%)$[3]	0
Methionine Sulfoxide	0	0
Methionine Sulfone	0	15.30 ± 0.60^a $(109.\overline{5}\%)$
Cysteic Acid	1.71 ± 0.05	33.64 ± 1.69

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[1]Values shown are means and standard deviations of four determinations. Values having the same letter are not significant at 0.05 level. See Appendix 15.

[2]Theoretical amount of methionine in 100 grams of lysozyme is 13.97 millimoles (Sober, 1970).

[3]Values in parentheses are percent of theoretical methionine represented by that particular methionine derivative.

<u>Acid</u> <u>hydrolysis</u> -- Methionine recovery after acid hydrolysis was
not significantly different ($P > 0.05$) from the value for total methi-
onine as determined by performic acid oxidation (Moore, 1963) or from
the literature value (Sober, 1970) for the amount of methionine in
lysozyme (Table 21). No methionine sulfoxide or methionine sulfone
was detected, indicating quantitative reduction of methionine sul-
foxide to methionine on acid hydrolysis without any loss of methionine
or methionine sulfoxide.

A small amount of cysteic acid was detected after acid hydrol-
ysis. This amount represented only about 5% of the total cystine in
the oxidized lysozyme as determined by performic acid oxidation
(Table 21).

<u>Discussion</u> <u>of</u> <u>results</u> <u>of</u> <u>hydrolytic</u> <u>studies</u> <u>of</u> <u>oxidized</u> <u>lyso-</u>
<u>zyme</u> -- Recovery of methionine plus methionine sulfoxide after hydrol-
ysis with 2M NaOH for 18 hours at 100°C was 100% of the total methi-
onine (Moore, 1963) in oxidized lysozyme but only about 90% when the
protein was hydrolyzed with 3M NaOH for 16 hours at 110°C. The loss
appeared to be in the methionine rather than the methionine sulfoxide
content of the oxidized lysozyme. The TSM analysis indicated that
the presence of glucose did not promote oxidation of methionine to
methionine sulfoxide during alkaline hydrolysis. Furthermore, no
methionine sulfone was detected in any of the samples after either
acid or alkaline hydrolysis. The addition of glucose to the alkaline
hydrolysis mixture did not lead to methionine sulfone production from
oxidation of methionine or methionine sulfoxide. As in the case of

the unoxidized lysozyme hydrolyzed with glucose, other compounds
appear to be eluting with methionine sulfoxide in the NC-2P analyses
when oxidized lysozyme is hydrolyzed in the presence of glucose with
2M NaOH. The single analysis done on the TSM analyzer of oxidized
lysozyme hydrolyzed with 2M NaOH in the presence of glucose indicated
that no additional methionine sulfoxide was generated by the process
compared to hydrolysis of oxidized lysozyme without glucose. It
appears that the more accurate values for methionine sulfoxide after
alkaline hydrolysis in the presence of glucose are derived from the
TSM analyses rather than the NC-2P, at least in the cases of lysozyme
and oxidized lysozyme.

Acid hydrolysis reduced all of the methionine sulfoxide that
was present in the oxidized lysozyme to methionine. These results
differ from those observed when free methionine sulfoxide was acid
hydrolyzed. Only a minor portion of the free methionine sulfoxide
(15.5%) was reduced to methionine. These differences reaffirm the
frequently observed fact that a given compound in the free form need
not react in the same way or to the same extent as the same compound
in peptide-bound form.

The results also indicate that the process used to oxidize lyso-
zyme with hydrogen peroxide in this study (Cuq et al. 1973) does not
produce any methionine sulfone. Furthermore, after acid hydrolysis
only a small amount of cysteic acid (about 5% of total cystine as
determined by the method of Moore, 1963) was detected indicating that
the hydrogen peroxide oxidation, under the conditions used in this
study, does not produce a significant amount of cysteic acid.

Casein

Basic hydrolysis without glucose -- Casein was hydrolyzed under
both sets of alkaline hydrolytic conditions and also subjected to acid
hydrolysis. Significantly more methionine was recovered after hydrol-
ysis with 2M NaOH compared to 3M NaOH, although recovery of methionine
in both hydrolyses was less than that recovered after acid hydrolysis
(Table 22). After hydrolysis with 2M NaOH, recovery of methionine was
74.0% that of total methionine as determined by performic acid oxida-
tion (Moore, 1963). Little more than a trace of methionine sulfoxide
was recovered under any of the conditions (Table 22). A trace of
methionine sulfone was present (< 0.2 millimoles per 100 grams of
casein).

The values for methionine content of casein as determined by
performic acid oxidation (19.08 millimoles per 100 grams) and as deter-
mined after acid hydrolysis (17.67 millimoles per 100 grams) are in
fair agreement with the values of 16.9 and 18.4 millimoles per 100
grams, respectively, for the same analyses as reported by Cuq et al.
(1973). The low values for methionine recovery after alkaline hydrol-
ysis are probably a reflection of destruction of methionine occurring
during alkaline hydrolysis or incomplete release of methionine from
the proteins. Of these two explanations, destruction of methionine
(in reactions other than oxidation) appears to be the more likely
cause, since recovery of methionine was lower under the more severe
alkaline conditions (3M NaOH, 16 hours, 110°C). Cuq et al. (1977)
also found that methionine was destroyed during alkaline hydrolysis of
a tripeptide containing methionine. Therefore, it is a reasonable

Table 22. Recovery of selected amino acids from casein subjected to acid hydrolysis or alkaline hydrolysis under two sets of alkaline hydrolytic conditions.

AMINO ACID RECOVERY[1]
(Millimoles per 100g)

Amino Acid	2M NaOH, 18 Hours, 100°C	3M NaOH, 16 Hours, 110°C	6M HCl, 24 Hours, 110°C
Methionine[2]	14.09 ± 0.38^a	10.28 ± 1.97^b	17.67 ± 2.14^c
Methionine Sulfoxide	~ 0.5	~ 0.3	~ 0.5
Methionine Sulfone	trace	trace	trace
Cysteic[3] Acid	–	–	~ 0.3
Valine	9.98 ± 0.37^a	12.26 ± 1.34^b	52.24 ± 2.18^c
Leucine	46.78 ± 4.68^a	37.15 ± 4.86^b	67.55 ± 3.56^c
Phenyl- alanine	–	–	27.74 ± 1.61

[1]Means and standard deviations of three to seven determinations; values having the same letters in horizontal rows are not significantly different at 0.05 level. See Appendices 16-19.

[2]Total methionine as determined by performic acid oxidation (Moore, 1963) was 19.08 ± 2.09 millimoles per 100 grams of casein.

[3]Total cystine as determined by performic acid oxidation (Moore, 1963) was 3.29 ± 0.29 millimoles per 100 grams of casein.

assumption that methionine can be destroyed during alkaline hydrolysis, and that this accounts for the low recovery of methionine.

Basic hydrolysis with glucose -- Two experiments were done investigating the effect of glucose on recoveries of methionine, methionine sulfoxide, and methionine sulfone from casein after alkaline hydrolysis (Table 23). The results of Experiment I suggested that a significant amount of methionine was being oxidized to methionine sulfoxide during the hydrolysis when glucose was present. In Experiment II the conditions of elution on the NC-2P analyzer were modified in an effort to separate methionine sulfoxide from other compounds which might be co-eluting with methionine sulfoxide. In addition, some of the samples were run on the TSM analyzer as well as the NC-2P. The amount of methionine sulfoxide recovered after hydrolysis of casein in the presence of glucose was higher than that recovered without glucose but not as high as in Experiment I. The amount of methionine sulfoxide determined on the TSM analyzer after hydrolysis in the presence of glucose was slightly lower than the value determined on the NC-2P analyzer. This suggests that at least a portion of the compounds co-eluting with methionine sulfoxide in Experiment I were eliminated from the methionine sulfoxide peak but does not prove that all interfering compounds have been separated; consequently, the value reported for methionine sulfoxide in Experiment II from casein hydrolyzed in the presence of glucose is a maximum and represents about 8% of the total methionine in casein as measured by performic acid oxidation (Moore, 1963). This value is

Table 23. Recovery of selected amino acids from casein hydrolyzed with 2M NaOH for 18 hours at 100°C in the presence or absence of glucose.

AMINO ACID RECOVERY[1]
(Millimoles per 100 grams)

| Amino Acid | Experiment I[2] 2M NaOH, 18 Hours, 100°C | | Experiment II[2] 2M NaOH, 18 Hours, 100°C | | | |
| | Without Glucose | With Glucose | Without Glucose | | With Glucose | |
	(NC-2P)	(NC-2P)	(NC-2P)	(TSM)	(NC-2P)	(TSM)
Methionine	14.09 ± 0.38^a	9.48 ± 0.62^b	7.71 ± 1.14^c	11.48 ± 0.82^d	8.45 ± 1.01^{bce}	9.06 ± 0.007^{bcde}
Methionine Sulfoxide	0.52 ± 0.05^a	3.62 ± 0.48^b	0.36 ± 0.07^c	0.82 ± 0.02^d	1.62 ± 0.09^e	1.48 ± 0.007^e
Methionine Sulfone	trace	trace	0	0	0	0
Valine	9.98 ± 0.37^a	5.90 ± 0.32^b	-	-	-	-
Leucine	46.78 ± 4.68^a	29.03 ± 1.89^b	-	-	-	-

[1] Values shown are means and standard deviations of three to five determinations on the NC-2P Amino Acid Analyzer and of two determinations on the TSM Amino Acid Analyzer; values having the same letters in horizontal rows are not significantly different at 0.05 level. See Appendix 17 and Appendix 18.

[2] Conditions of elution in Experiment I were: 0.50 ml/min, flow rate; 55°C column temperature; buffers; pH 2.41, 35 min; pH 3.25, 16 min; pH 3.61, 30 min. In Experiment II conditions were altered to: 0.40 ml/min, flow rate; 55°C column temperature; buffers: pH 2.69, 35 min; pH 3.25, 16 min; pH; pH 3.61, 30 min (NC-2P Analyzer).

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about the same as the amount of methionine sulfoxide recovered from lysozyme when it was hydrolyzed in the presence of glucose as determined on the TSM analyzer. The value of 8%, then, may represent a good estimate for the amount of methionine sulfoxide that is generated by the alkaline hydrolytic procedure itself.

A trace of methionine sulfone was detected in casein both with and without glucose in Experiment I and none in Experiment II. These data indicate that glucose does not promote oxidation of methionine or methionine sulfoxide to methionine sulfone during alkaline hydrolysis under the conditions used.

The amount of methionine recovered from casein after alkaline hydrolysis with 2M NaOH in the absence of glucose was markedly lower in Experiment II compared to Experiment I. This may have been the result of experimental error; however, since the TSM values for methionine recovery in Experiment II are also lower (though not at as NC-2P values) any experimental errors must have been in the hydrolytic procedure and not in calculation of peak size or application of the samples to the respective column. The decrease in methionine recovery could also have been the result of destruction of methionine during hydrolysis. It may be that even under seemingly identical conditions, a variable amount of methionine is destroyed during hydrolysis and cannot be accounted for by simple oxidation to the next oxidative state. This variability in recovery of methionine from experiment to experiment was also seen in the cases of lysozyme and oxidized lysozyme, though to a lesser degree (Tables 17-20). These results do indicate that methionine recovery after alkaline hydrolysis, even with a

substance that is over 90% protein, is not a valid measurement of the methionine present in the protein and may seriously underestimate the true amount.

The presence of glucose also had an effect on the amount of valine and leucine recovered. The amount of these amino acids recovered after alkaline hydrolysis in the presence of glucose was lower by about 40% than that recovered under the same hydrolytic conditions in the absence of glucose. As in the case of methionine, this may have occurred because of destruction of these amino acids.

Oxidized Casein

A portion of the casein used in the previous experiments was oxidized with hydrogen peroxide using a slightly modified version of the method of Cuq et al. (1973) (see Materials and Methods). The oxidized casein was then subjected to alkaline hydrolysis under both sets of conditions, to acid hydrolysis, and to performic acid oxidation. The results (Table 24) indicate that after alkaline hydrolysis, no methionine was recovered under either set of conditions, and about 70% of the total methionine as determined by performic acid oxidation (Moore, 1963) was recovered as methionine sulfoxide. No methionine sulfone was detected after alkaline hydrolysis with 2M NaOH or 3M NaOH.

There are at least three possible explanations which might account for the 25-30% of total methionine which was not recovered after alkaline hydrolysis: (1) all of the methionine in casein was oxidized to methionine sulfoxide during the oxidation with hydrogen peroxide and a portion of it was destroyed in the alkaline hydrolysis

Table 24. Recovery of methionine, methionine sulfoxide, and methionine sulfone from oxidized casein after acid hydrolysis, performic acid oxidation, and under two sets of alkaline hydrolytic conditions.

AMINO ACID RECOVERY[1]
(Millimoles per 100 grams)

Amino Acid	2M NaOH, 18 Hours, 100°C	3M NaOH, 16 Hours, 110°C	6M HCl, 24 Hours, 110°C	Performic Acid Oxidation
Methionine	trace	0	14.81 ± 0.89	0
Methionine Sulfoxide	13.46 ± 0.67	13.67 ± 0.36	trace	0
Methionine Sulfone	0	0	0	18.06 ± 0.76
Total	13.46 ± 0.67^a	13.67 ± 0.36^a	14.81 ± 0.89^b	18.06 ± 0.76^c

[1]Values shown are means and standard deviations of three to eight determinations; values having the same letter in horizontal rows are not significantly different at 0.05 level. See Appendix 20 and Appendix 21.

(2) a portion of the methionine in casein remained in the reduced state during the oxidation with hydrogen peroxide and this methionine was destroyed in the alkaline hydrolysis, or (3) the performic oxidation value for total methionine in oxidized casein is in error. The third explanation is less likely since the value for total methionine in oxidized casein (18.06 millimoles per 100 grams) compares well with the value for unoxidized casein (19.08 millimoles per 100 grams).

Upon acid hydrolysis of the oxidized casein (Table 24), only a trace of methionine sulfoxide was found, with 82% of the total methionine being detected as methionine and none as methionine sulfone. These results suggest that if methionine sulfoxide values derived from alkaline hydrolyses represent the total sulfoxide content, then acid hydrolysis almost quantitatively reduces methionine sulfoxide to methionine.

Oxidized casein was also hydrolyzed under basic conditions with glucose and with glucose plus iron (II) ions (Table 25). The amount of methionine sulfoxide recovered under these conditions was not significantly different from that recovered when oxidized casein was hydrolyzed alone (Table 25). The number of samples, however, in this experiment was small and the results should be interpreted with caution. No methionine sulfone was detected under any of the conditions used indicating that alkaline hydrolysis of oxidized casein with glucose or with glucose plus iron (II) ions does not generate methionine sulfone.

Table 25. Recovery of methionine, methionine sulfoxide, and methi-
onine sulfone from oxidized casein hydrolyzed with
2M NaOH for 18 hours at 100°C in the presence and absence
of glucose and in the presence of glucose plus iron (II)
sulfate.

AMINO ACID RECOVERY[1]
(Millimoles per 100 grams)

Amino Acid	2M NaOH, 18 Hours, 100°C		
	Without Glucose	With Glucose	With Glucose and $FeSO_4$
Methionine	Trace	Trace	Trace
Methionine Sulfoxide	13.46 ± 0.67^a	12.82 ± 0.98^a	11.82 ± 0.18^a
Methionine Sulfone	0	0	0

[1]Values shown are means and standard deviations of two or three
determinations; values having the same letter are not significantly
different at 0.05 level. See Appendix 22.

Effect of Sugars and Metal Ions on Recoveries of Selected Amino Acids from Casein and Promine F

Since the infant formulas to be tested for methionine sulfoxide content contained either lactose or sucrose, and because they contained copper (II) ions and, in some cases, iron (II) ions, two other experiments were performed: Casein and Promine F were hydrolyzed in the presence of lactose or sucrose, respectively, and copper (II) and iron (II) ions with 6M HCl for 24 hours at 110°C or with 2M NaOH for 18 hours at 100°C. After hydrolysis all samples were subjected to ion-exchange chromatography. The results are presented in Tables 26 and 27. It can be seen that for casein hydrolyzed with 2M NaOH for 18 hours at 100°C, a small increase in the amount of methionine sulfoxide was detected in the samples with lactose compared to those without lactose. The samples with lactose also had less methionine after both basic and acidic hydrolysis. These results, however, must be interpreted with caution since methionine recovery appears to be variable from experiment to experiment even in the absence of glucose (Table 23). No methionine sulfone was detected in any of the samples.

When casein was hydrolyzed with 6M HCl for 24 hours at 110°C in the presence of lactose, copper (II) ions, and iron (II) ions, no methionine sulfoxide or methionine sulfone was detected. The amount of methionine recovered from casein after acid hydrolysis in the presence of lactose and the metal ions was much lower than that recovered when casein was hydrolyzed alone. It is probable, however, that the presence of lactose and/or the ions caused some destruction of methionine in some way other than oxidation. A slight, but insignificant, increase was seen in the amount of methionine sulfoxide present

Table 26. Recovery of methionine, methionine sulfoxide, and methionine sulfone from casein hydrolyzed under alkaline and acidic conditions in the presence of lactose, copper (II) sulfate, and iron (II) sulfate and hydrolyzed alone.

AMINO ACID RECOVERY[1]
(Millimoles per 100 grams)

| Amino Acid | 2M NaOH, 18 Hours, 100°C | | 6M HCl, 24 Hours, 110°C | |
	Alone	With Lactose, $FeSO_4$, and $CuSO_4$	Alone	With Lactose, $FeSO_4$, and $CuSO_4$
Methionine	14.09 ± 0.38^a	10.68 ± 0.08^b	17.67 ± 2.14^c	10.74 ± 0.87^b
Methionine Sulfoxide	0.52 ± 0.05^a	0.77 ± 0.09^a	∿0.5	0
Methionine Sulfone	0	0	0	0

[1]Values shown for alkaline hydrolysis are means and standard deviations of four determinations for each set of conditions and for acid hydrolysis are means and standard deviations of six determinations for each set of conditions; values in horizontal rows having the same letter are not significantly different at 0.05 level. See Appendix 16, Appendix 18, and Appendix 23.

Table 27. Recovery of methionine, methionine sulfoxide, methionine sulfone from Promine F hydrolyzed alone and in the presence of sucrose, copper (II) sulfate, and iron (II) sulfate under acidic and basic conditions.

AMINO ACID RECOVERY[1]
(Millimoles per 100 grams)

Amino Acid	2M NaOH, 18 Hours, 100°C				6M HCl, 24 Hours, 110°C[2]	
	Alone		With Sucrose, $FeSO_4$, $CuSO_4$		Alone	With Sucrose, $FeSO_4$, $CuSO_4$
	(NC-2P)	(TSM)	(NC-2P)	(TSM)		
Methionine[3]	5.05 ± 0.22^a	4.70 ± 0.78^a	3.75 ± 0.27^b	3.31 ± 0.45^b	7.10 ± 0.38^c	5.47 ± 0.22^d
Methionine Sulfoxide	0.59 ± 0.12^a	0.53 ± 0.12^a	1.05 ± 0.06^b	1.02 ± 0.40^b	~ 0.5	0
Methionine Sulfone	0	0	0	0	~ 0.3	0
Cysteic Acid	0.62 ± 0.22	–	–	–	~ 0.2	~ 0.2

[1] Values shown are means and standard deviations of three to thirteen determinations for the NC-2P values and two to four determinations for the TSM values; values having the same letter in horizontal rows are not significantly different at the 0.05 level. See Appendix 24 and Appendix 25.

[2] Acid hydrolysates were analyzed only on the NC-2P Amino Acid Analyzer.

[3] Total methionine as determined by performic acid oxidation (Moore, 1963) was 7.65 ± 1.08 millimoles per 100 grams.

in the alkaline hydrolysate with lactose and metal ions compared to the other hydrolysates.

When Promine F was hydrolyzed with 2M NaOH for 18 hours at 100°C in the presence of sucrose and the metal ions, again, a small increase was observed in the amount of methionine sulfoxide recovered (Table 27). The amount of methionine sulfoxide detected rose from about 8% of the total methionine (Moore, 1963) in the absence of sucrose and metal ions to about 14% of the total methionine (Moore, 1963) in their presence. NC-2P and TSM results did not differ from each other and no methionine sulfone was detected. After acid hydrolysis with sucrose and the metal ions, no methionine sulfoxide or methionine sulfone was found. A small amount of each of these, however, was found after acid hydrolysis of Promine F alone suggesting that both these compounds are, indeed, present in Promine F and not an artifact produced solely by the hydrolytic procedures. Acid hydrolysis, in general, tends to reduce methionine sulfoxide to methionine so that any methionine sulfoxide persisting after acid hydrolysis probably represents the remains of a larger amount of the compound.

After alkaline hydrolysis in the presence of sugars, small amounts of other compounds may co-elute with methionine sulfoxide; therefore, the values reported for methionine sulfoxide in Promine F and casein, after hydrolysis of these proteins in the presence of sugars and metal ions, should probably be thought of as maximum values. The difference between the amount of methionine sulfoxide detected in the hydrolysates containing sugar and the metal ions and the hydrolysates without these components was 0.25 millimoles per 100g (NC-2P

values) for casein and 0.46 millimole/100g (NC-2P values) for Promine F
(Tables 26 and 27). This is about 7% and 2% of the total methionine
(Moore, 1963) present in Promine F and casein, respectively and
probably represents the amount of methionine sulfoxide generated by
the alkaline hydrolyses procedure. Again, it should be noted that no
methionine sulfone was generated in either protein under either acidic
or basic hydrolytic conditions in the presence of sugars, copper (II)
ions, and iron (II) ions.

After acid hydrolysis in the presence of sucrose and the metal
ions the amount of methionine recovered from Promine F was substanti-
ally lower than that recovered after hydrolysis of Promine F alone.
About 93% of the total methionine (Moore, 1963) in Promine F was
recovered as methionine after acid hydrolysis of Promine F alone com-
pared to about 72% of the total methionine in Promine F when it was
acid hydrolyzed in the presence of sucrose and metal ions. These
data at least suggest that the amount of methionine recovered after
acid hydrolysis is variable and that this is not an accurate method of
measuring methionine content of proteins, even when methionine sul-
foxide and methionine sulfone are not present in the protein.

Review of Results of Hydrolytic Studies in Model Systems

Free Amino Acids

The results of the studies on the percent recovery of selected
free amino acids after hydrolysis with either 2M NaOH for 18 hours at
100°C or 3M NaOH for 16 hours at 110°C indicated that a greater amount
of methionine sulfoxide was recovered using the first set of conditions

compared to the second set. When methionine sulfoxide was hydrolyzed in mixtures of amino acids containing methionine, methionine sulfoxide, methionine sulfone, cystine, and cysteic acid, some oxidation of methionine to methionine sulfoxide apparently occurred. This effect was greater when 3M NaOH was used compared to 2M NaOH. Little if any oxidation of methionine sulfoxide to methionine sulfone occurred under either set of conditions. Glucose added to the hydrolysis mixture had little effect on the percent recovery of methionine, methionine sulfoxide, or methionine sulfone compared to recoveries without glucose in the mixture. The presence of other amino acids in the hydrolysis mixture appeared to have a greater effect on percent recovery and oxidation of methionine to methionine sulfoxide than the presence of glucose.

Model Proteins

For the model proteins, recovery of methionine and methionine sulfoxide was greater using 2M NaOH compared to 3M NaOH. The alkaline hydrolytic process itself did not appear to generate methionine sulfoxide or methionine sulfone when the model proteins were hydrolyzed alone; however, in the presence of sugars, some methionine sulfoxide appeared to be generated by the alkaline hydrolysis procedure itself. In the case of lysozyme hydrolyzed with glucose, about 8% of the total methionine appeared to be oxidized to methionine sulfoxide and in the case of Promine F hydrolyzed with sucrose and metal ions, the amount of methionine oxidized by the procedure itself was about 7%. Thus, a good estimate for the amount of methionine which may be

oxidized to methionine sulfoxide during alkaline hydrolysis (2M NaOH for 18 hours, 100°C) when sugars are present is about 8%.

The presence of sugars sometimes led to products which co-eluted with methionine sulfoxide during ion-exchange chromatography leading to erroneously high values for methionine sulfoxide. Altering the elution conditions helped in separating at least a portion of these compounds from methionine sulfoxide but the values for this amino acid are probably maximum values when sugars are present along with the protein. Whenever sugars are present, caution must be used in interpreting the results after alkaline hydrolysis of food products.

Conclusions

The results of these studies with free amino acids and model proteins indicate that hydrolysis with 2M NaOH for 18 hours at 100°C to determine methionine sulfoxide content by ion-exchange chromatography is valid for products high in protein and relatively low in carbohydrates. Foods such as casein and the soy isolates are in this category and, probably, products such as soy concentrates and flours where the protein content is 50% or more of the total weight. When sugars are present at high levels, however, as much as 8% of the methionine may be oxidized to methionine sulfoxide during the hydrolysis procedure and this must be taken into consideration in interpreting results.

Based on the recovery values for methionine and methionine sulfoxide when these amino acids were hydrolyzed as free amino acids and

also from hydrolyzed proteins, the conditions of 2M NaOH for 18 hours

at 100°C were chosen to analyze the food products for methionine sul-

foxide content.

Because of the variability in recoveries of methionine from the

model proteins after both basic and acid hydrolysis methionine content

of the food products was determined by difference: Methionine =

"Total methionine" - (methionine sulfoxide + methionine sulfone).

<div align="center">

Analysis of Food Products for Methionine,
Methionine Sulfoxide, Methionine Sul-
fone, Cysteic Acid, and Total
Cystine

</div>

Eight food products, including four infant formulas, were ana-

lyzed in this study. The percent protein in the food products, as

measured by Kjeldahl analysis and as reported by the manufacturers,

is given in Table 28. Measured values for the dry soy products were

slightly lower than the values reported by the manufacturer. Since

the products were not dried before analysis, the moisture present may

have been responsible for the lower measured values. Measured values

and reported values for the infant formulas did not differ.

Total methionine and total cystine for all the products were

determined using the performic acid oxidation method of Moore (1963).

These values along with those reported by the manufacturers are shown

in Tables 29 and 30. For the dry products measured total methionine

is slightly higher than that reported by the manufacturer. For the

liquid infant formulas the reported values for total methionine are

about the same as the measured amounts in Isomil and Prosobee, but

Table 28.   Percent protein composition of food products as measured and as reported by the manufacturer.

Products Dry Form	Percent Protein[1]	
	Measured[2]	Reported
Soyafluff 200 W	56.5	
Promosoy	64.3	65.4
Promine D	89.0	91.3
Promine F	87.5	91.5
Powdered Similac	12.0	11.8
Concentrated Prosobee (Freeze Dried)	19.8	–
Liquid Form	Measured[3]	Reported
Concentrated Isomil	4.0	4.0
Concentrated Similac	3.1	3.1
Concentrated Prosobee	5.0	5.0

[1]Protein = Kjeldahl nitrogen X 6.25; nitrogen content determined on samples as received from the manufacturer.

[2] $\frac{\text{Grams of protein}}{100 \text{ grams of product}}$.

[3] $\frac{\text{Grams of protein}}{100 \text{ ml undiluted product}}$.

Table 29. Total methionine and total cystine in dry food products as performic acid oxidation products (methionine sulfone and cysteic acid), as methionine and cystine equivalents, and as reported by the manufacturers.

Products	Methionine Sulfone[1]	Cysteic Acid[1]	Methionine		1/2 Cystine	
			Measured	Reported	Measured	Reported
	(mmoles/100 g Protein[2])		(g/100 g Protein)		(g/100 g Protein)	
Soyafluff 200W	10.26 $\pm$ 0.57	11.61 $\pm$ 0.78	1.53	1.3	1.39	1.6
Promosoy	11.06 $\pm$ 0.45	11.26 $\pm$ 1.21	1.65	1.45	1.35	1.25
Promine D	8.80 $\pm$ 0.99	9.16 $\pm$ 0.57	1.31	1.05	1.10	1.15
Promine F	8.74 $\pm$ 1.23	9.82 $\pm$ 0.71	1.30	1.27	1.18	1.17
Powdered Similac with iron	17.63 $\pm$ 1.78	6.44 $\pm$ 0.85	2.63[3]	-	0.77[4]	-

[1] Values shown are means and standard deviations of three to eight determinations. See Appendices 24, 26, 27, 28 and 32.

[2] Protein = g N X 6.25.

[3] If formula is prepared according to directions on container, 40.4 mg methionine/100 ml formula.

[4] If formula is prepared according to directions on container, 12.0 mg cystine/100 ml of formula.

Table 30. Total methionine and total cystine in liquid infant formulas as performic acid oxidation products (methionine sulfone and cysteic acid), as methionine and cystine equivalents, and as reported by the manufacturers.[1]

Products	Methionine Sulfone[2]	Cysteic Acid[2]	Methionine Measured	Methionine Reported	1/2 Cystine Measured	1/2 Cystine Reported
	(mmoles/100 ml)		(mg/100 ml)		(mg/100 ml)	
Liquid Similac	0.23 ± 0.02	0.10 ± 0.02	34.3	45	12.0	15
Liquid Isomil	0.30 ± 0.02	0.20 ± 0.02	44.8	45	24	19
Liquid Prosobee	0.32 ± 0.04	0.21 ± 0.11	47.7	47	25.2	22

[1]Values are for formulas diluted according to directions on containers.

[2]Values are means and standard deviations of three or four determinations. See Appendices 21-31.

significantly less for liquid Similac. The measured values for total

cystine for all the products differ somewhat from reported values with

some being slightly higher and some slightly lower than the reported

amounts. Performic acid oxidation analyses were performed on the

NC-2P analyzer.

All eight products were also subjected to acid and alkaline

hydrolysis. The alkaline and acid hydrolysates were analyzed on the

Technicon NC-2P amino acid analyzer for methionine, methionine sul-

foxide, and methionine sulfone using the conditions detailed in

Appendix 4. In addition some of the hydrolysates were also analyzed

on the Technicon TSM amino acid analyzer using the conditions

described in Appendix 5. The amount of cysteic acid present in the

acid hydrolysates was also determined for all products using the

NC-2P for analysis. Results of analyses after acid and alkaline

hydrolysis and performic acid oxidation are presented in Tables 31 to

38.

Soy Products

For Soyafluff and Promosoy (Tables 31 and 32) the amount of

methionine as measured after alkaline and acid hydrolysis is consider-

ably less than total methionine as measured by performic acid oxida-

tion. About 5% of the total methionine, as measured by performic

acid oxidation, was present as methionine sulfoxide in both products

when measured after alkaline hydrolysis. No significant amount of

methionine sulfone or cysteic acid were present in either product.

Table 31. Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from Soyafluff which was subjected to alkaline or acid hydrolysis.

AMINO ACID RECOVERY[1]

| | Hydrolytic Conditions | | |
Amino Acid	2M NaOH, 18 Hours, 100°C	6M HCl, 24 Hours, 110°C	Performic Acid Oxidation
	(Millimoles per 100 grams)		
Methionine	3.24 $\pm$ 0.18 (55.9%)[2]	2.48 $\pm$ 0.20 (42.8%)	0
Methionine Sulfoxide	0.29 $\pm$ 0.03 (5.0%)	0	0
Methionine Sulfone	<0.1 (<2%)	<0.1 (<2%)	5.80 $\pm$ 0.32 (100%)
Total	3.53 (60.9%)	2.58 (44.5%)	5.80 (100%)
Cysteic Acid	-	<0.1	6.56 $\pm$ 0.44

[1]Means and standard deviations of four to six determinations as measured on Technicon NC-2P Amino Acid Analyzer. See Appendix 26.

[2]Values in parentheses are percent of total methionine (as measured by performic acid oxidation) represented by that particular methionine derivative.

Table 32. Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from Promosoy which was subjected to alkaline or acid hydrolysis.

AMINO ACID RECOVERY[1]

	Hydrolytic Conditions		
Amino Acid	2M NaOH, 18 Hours, 100°C	6M HCl, 24 Hours, 110°C	Performic Acid Oxidation
	(Millimoles per 100 grams)		
Methionine	4.18 ± 0.22 (58.8%)[2]	4.14 ± 0.34 (58.2%)	0
Methionine Sulfoxide	0.31 ± 0.05 (4.4%)	0	0
Methionine Sulfone	<0.1 (<2%)	0	7.11 ± 0.29
Total	4.49 (63.2%)	4.14 (58.2%)	7.11 (100%)
Cysteic	–	0	7.24 ± 0.78

[1]Means and standard deviations of three to six determinations as measured on Technicon NC-2P Amino Acid Analyzer. See Appendix 27.

[2]Values in parentheses are percent of total methionine (as measured by performic acid oxidation) represented by that particular methionine derivative.

These data suggest that methionine may be destroyed by alkaline and acid hydrolysis in ways other than oxidation and that quantitation after acid or alkaline hydrolysis is not an accurate measure of the amount of methionine in these products. An alternative explanation is that the performic acid oxidation values, at least for some products, are high and do not accurately represent total methionine in that product. Measured performic acid oxidation values for methionine were slightly high relative to manufacturer's reported values as has been shown in Table 29.

The values for total methionine in the two soy isolates, Promine D and Promine F, are comparable (Tables 33 and 34); however, the amounts of methionine and its oxidized forms vary markedly between the two isolates with Promine D having about 80% of its total methionine present as methionine sulfoxide and methionine sulfone while Promine F has only about 12% present in these oxidized forms. Promine D also contains an appreciable amount of cysteic acid (23% of total cystine).

<div align="center">Infant Formulas</div>

A peak with the chromatographic elution position of methionine sulfone was found in all four infant formulas. Calculated as methionine sulfone, the peaks represented a range of about 8 to 13% of total methionine (NC-2P values) after acid hydrolysis and 12 to 19% of total methionine (NC-2P values) after alkaline hydrolysis (Tables 35-38). The shape of the methionine sulfone peak after alkaline hydrolysis was asymmetric and atypical compared to the shape of the

Table 33. Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from Promine F subjected to alkaline or acid hydrolysis.

AMINO ACID RECOVERY[1]

Amino Acid	Hydrolytic Conditions			Performic Acid Oxidation
	2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C	
	(Millimoles per 100 grams)			
	(NC-2P)	(TSM)	(NC-2P)	
Methionine	5.05 ± 0.22^a	4.70 ± 0.78^a	7.10 ± 0.38^b	0
	$(66.0\%)^2$	(61.4%)	(92.5%)	
Methionine Sulfoxide	0.59 ± 0.12^c	0.53 ± 0.12^c	$\sim$0.5	0
	(7.7%)	(6.9%)	($\sim$6%)	
Methionine Sulfone	0	0	$\sim$0.3	7.65 ± 1.08^b
			($\sim$4%)	
Total	5.64	5.23	7.16	7.65
	(73.7%)	(68.4%)	(93.6%)	(100%)
Cysteic Acid	0.62 ± 0.22	-	0.2	8.59 ± 0.62

[1]Means and standard deviations of three to thirteen determinations; values having the same letters are not significantly different at 0.05 level. See Appendix 24 and Appendix 25.

[2]Values in parentheses are percent of total methionine (as measured by performic acid oxidation) represented by that particular methionine derivative.

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Table 34. Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from Promine D subjected to alkaline or acid hydrolysis.

AMINO ACID RECOVERY[1]

Amino Acid	Hydrolytic Conditions			
	2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C	Performic Acid Oxidation
	Experiment I (NC-2P)	Experiment II (TSM)	(NC-2P)	
	(Millimoles per 100 grams)			
Methionine	0.72 ± 0.06^a (9.2%)[2]	0.87 ± 0.13^b (11.1%)	6.59 ± 0.69^c (84.2%)	0
Methionine Sulfoxide	5.83 ± 0.86^a (74.4%)	4.60 ± 0.60^a (58.7%)	trace	0
Methionine Sulfone	0.31 ± 0.01^a (4.0%)	0.42 ± 0.22^a (5.4%)	0.45 ± 0.09^a (5.7%)	7.83 ± 0.88
Total	6.86 (87.7%)	5.42 (69.2%)	7.22 (92.2%)	7.83 (100%)
Cysteic Acid	0.81 ± 0.18	–	1.77 ± 0.29	8.15 ± 0.51

[1]Values shown are means and standard deviations for four to fourteen determinations; values having the same letter in horizontal rows are not significantly different at 0.05 level. See Appendix 28.

[2]Values in parentheses are percent of total methionine (as measured by performic acid oxidation) represented by that particular methionine derivative.

Table 35. Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from concentrated liquid Similac[2] subjected to alkaline or acid hydrolysis.

AMINO ACID RECOVERY[1]

	Hydrolytic Conditions				
	2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		Performic Acid Oxidation
Amino Acid	(NC-2P)	(TSM)	(NC-2P)	(TSM)	
	(Millimoles per 100 ml)				
Methionine	0.33 ± 0.02	0.31 ± 0.02	0.45 ± 0.02	0.44 ± 0.06	0
	(71.7%)[3]	(67.4%)	(97.8%)	(95.6%)	
Methionine Sulfoxide	0.04 ± 0.01	0.06 ± 0.01	∿0.01	–	0
	(8.7%)	(13.0%)	(∿2%)		
Methionine Sulfone	0.07 ± 0.01	∿0.01	0.06 ± 0.02	0.08 ± 0.05	0.46 ± 0.02
	(15.2%)	(∿2%)	(13.0%)	(17.4%)	
Total	0.44	0.38	0.52	0.52	0.46
	(95.6%)	(82.6%)	(113%)	(113%)	(100%)
Cysteic Acid	–	–	<0.01	–	0.19 ± 0.02

[1]Values shown are means and standard deviations of four to six determinations for NC-2P analyses and two or three determinations for TSM analyses. See Appendix 29.

[2]Values are for concentrated liquid samples, as withdrawn from container without dilution.

[3]Values in parentheses are percent of total methionine (as measured by performic acid oxidation) represented by that particular methionine derivative.

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Table 36. Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from concentrated liquid Isomil[2] subjected to alkaline or acid hydrolysis.

AMINO ACID RECOVERY[1]

Amino Acid	Hydrolytic Conditions				Performic Acid Oxidation
	2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		
	(NC-2P)	(TSM)	(NC-2P)	(TSM)	
	(Millimoles per 100 ml)				
Methionine	0.41 ± 0.01 (68.3%)[3]	0.37 ± 0.05 (61.7%)	0.50 ± 0.02 (83.3%)	0.46 ± 0.07 (76.7%)	0
Methionine Sulfoxide	0.07 ± 0.01 (11.7%)	0.05 ± 0.02 (8.3%)	∿0.01 (∿2%)	∿0.01 (∿2%)	0
Methionine Sulfone	0.07 ± 0.01 (11.7%)	<0.01	0.05 ± 0.01 (8.3%)	0.05 ± 0.03 (8.3%)	0.60 ± 0.02
Total	0.55 (91.7%)	0.42 (70.0%)	0.56 (93.3%)	0.52 (88.3%)	0.60 (100%)
Cysteic Acid	–	–	∿0.01	–	0.41 ± 0.02

[1] Values shown are means and standard deviations for three to six determinations for NC-2P and TSM analyses. See Appendix 30.

[2] Values are for concentrated liquid samples, as withdrawn from container without dilution.

[3] Values in parentheses are percent of total methionine (as measured by performic acid oxidation) represented by that particular methionine derivative.

Table 37. Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from freeze-dried concentrated Prosobee liquid subjected to alkaline or acid hydrolysis.

AMINO ACID RECOVERY[1]

	Hydrolytic Conditions			
Amino Acid	2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C	Performic Acid Oxidation
	(NC-2P)	(TSM)	(NC-2P)	(NC-2P)
	(Millimoles per 100 grams)			
Methionine	1.72 ± 0.07 (68.2%)[2]	1.78 ± 0.16 (70.6%)	2.24 ± 0.21 (88.9%)	0
Methionine Sulfoxide	0.43 ± 0.02 (17.1%)	0.32 ± 0.06 (12.7%)	∿0.05 (∿2%)	0
Methionine Sulfone	0.48 ± 0.08 (19.0%)	$0.05 \pm .01$ (∿2%)	0.30 ± 0.11 (11.9%)	2.52 ± 0.31
Total	2.63 (104.4%)	2.20 (87.3%)	2.59 (102.7%)	2.52 (100%)
Cysteic Acid	∿0.05	–	∿0.05	1.80 ± 0.21

[1] Values shown are means and standard deviations for four to nine determinations for NC-2P analyses and two to four determinations for TSM analyses. See Appendix 31.

[2] Values in parentheses are percent of total methionine (as measured by performic acid oxidation) represented by that particular methionine derivative.

Table 38. Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from powdered Similac fortified with iron which was subjected to alkaline or acid hydrolysis.

AMINO ACID RECOVERY[1]

	Hydrolytic Conditions			
	2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C	Performic Acid Oxidation
Amino Acid	(NC-2P)	(TSM)	(NC-2P)	(NC-2P)
	(Millimoles per 100 grams)			
Methionine	1.18 ± 0.22 (57.2%)[2]	0.94 ± 0.05 (45.6%)	1.76 ± 0.20 (85.4%)	0
Methionine Sulfoxide	0.36 ± 0.17 (17.3%)	0.65 ± 0.01 (31.6%)	~ 0.05 ($\sim 2\%$)	0
Methionine Sulfone	0.25 ± 0.06 (12.1%)	~ 0.1 ($\sim 4\%$)	0.18 ± 0.05 (8.7%)	2.06 ± 0.20
Total	1.80 (87.4%)	1.69 (82.0%)	1.99 (96.6%)	2.06 (100%)
Cysteic Acid	-	-	-	0.76 ± 0.10

[1] Values shown are means and standard deviations of four to nine determinations for NC-2P analyses and a single sample run in duplicate for the TSM analysis. See Appendix 32.

[2] Values in parentheses are percent of total methionine (as measured by performic acid oxidation) represented by that particular methionine derivative.

peak in chromatograms of standard solutions of methionine sulfone. This factor, along with the higher values for alkaline hydrolysis compared to those for acid hydrolysis, suggest that other compounds are co-eluting with methionine sulfone on the NC-2P analyzer after alkaline hydrolysis. Thus, the acid hydrolysis values are probably the more accurate of the NC-2P values for methionine sulfone.

The TSM values for methionine sulfone after acid hydrolysis are comparable to NC-2P values after acid hydrolysis. The methionine sulfone peak on the TSM chromatograms of the acid hydrolysates was poorly separated from aspartic acid, a large peak on the chromatograms. Calculation of peak area, therefore, represents a crude approximation.

The values for methionine sulfone determined from TSM chromatograms of alkaline hydrolysates (Tables 35-38) are much lower than all other values determined for methionine sulfone. These low values are difficult to explain. Early studies of free methionine sulfone indicated that it was stable to alkaline hydrolysis. Whether peptide-bound methionine sulfone is stable to alkaline hydrolysis or whether all of it is released under the conditions used was not determined.

Since the NC-2P values are approximately the same as the TSM values after acid hydrolysis and, since on NC-2P chromatograms the methionine sulfone peak appears typical of standard methionine sulfone peak, the NC-2P values for methionine sulfone probably represent the most accurate values for methionine sulfone in these experiments.

Methionine sulfoxide was also found in all the infant formulas. Amounts determined on the NC-2P analyzer ranged from about 8% of total methionine for liquid Similac to 18% for the powdered form of Similac.

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Values determined on the TSM analyzer varied by about 5% from the NC-2P values except for powdered Similac where the TSM value was almost double that of the NC-2P value.

The liquid infant formulas contain about 15% carbohydrate (w/v) in the undiluted form and about 56% by weight in the powdered Similac. Carbohydrate degradation products produced during both alkaline and acid hydrolysis interfered with the analysis of both methionine sulfoxide and methionine sulfone. On both the NC-2P analyzer and the TSM analyzer some other compound appeared to be coeluting with methionine sulfoxide after alkaline hydrolysis. Efforts to separate these compounds from the sulfoxide were only partially successful. Furthermore, earlier experiments with model proteins hydrolyzed in the presence of sugars resulted in a small increase in methionine sulfoxide over that in the same protein hydrolyzed alone. Thus, the values for methionine sulfoxide in the infant formulas should be thought of as a maximum.

After acid hydrolysis fewer interfering compounds were present in the hydrolysates; however, a peak which was not identified was characteristically present on NC-2P chromatograms and eluted between methionine sulfoxide and methionine sulfone. The peak was also present in most performic acid hydrolysates but not in alkaline hydrolysates. When this peak was unusually large, calculation of peak area for sulfone was hindered; consequently, though the NC-2P values for methionine sulfone in the chromatograms of the acid hydrolysates are probably the most accurate of the values reported for methionine sulfone, they, too must be considered approximations.

There was no appreciable amount of cysteic acid detected in any of the infant formulas.

A portion of concentrated liquid Isomil was deproteinized, filtered, and the filtrate chromatographed on the Technicon NC-2P analyzer to determine if the L-methionine added to the product by the manufacturer was oxidized to methionine sulfoxide and/or methionine sulfone. The results indicated that 2.6% of the free methionine present in the filtrate existed as methionine sulfoxide and 2.0% as methionine sulfone. These data suggest that the bulk of the methionine sulfoxide and methionine sulfone determined previously in the concentrated liquid Isomil was in the soy isolate protein and not in the added L-methionine. The number of samples, however, was small and this experiment should be repeated with a larger number of samples and include Prosobee as well as Isomil.

Summary of Results

Table 39 summarizes the amounts of methionine, methionine sulfoxide, methionine sulfone, cysteic acid, and total cystine in all the products analyzed (NC-2P values). Amounts are given in terms of millimoles per 16 grams of nitrogen for comparative purposes. Table 40 summarizes the same data in terms of percent of total methionine represented by each methionine derivative. Of the four infant formulas analyzed, the concentrated liquid Prosobee contained the greatest amount of the combined oxidized methionine derivatives (29%) and liquid Isomil the least (20%). Of the soy products Promine D by far contained the greatest amounts of sulfoxide and sulfone, together

Table 39. Summary of the sulfur amino acid content of selected soy products and selected infant formulas.

Product	Total Methionine[1]	Methionine Sulfoxide[2]	Methionine Sulfone[3]	Methionine[4]	Total Cystine[1]	Cysteic Acid[3]
		(Millimoles per 16 grams Nitrogen)				
Soyafluff	10.26	0.51	<0.2	9.55	11.61	<0.2
Promosoy	11.06	0.48	0	10.58	11.26	0
Promine D	8.80	6.55	0.50	1.75	9.16	1.98
Promine F	8.74	0.67	<0.3	8.07	9.82	<0.4
Powdered Similac	17.33	3.08	1.50	12.75	6.33	-
Liquid Similac	14.84	1.29	1.94	11.61	6.13	<0.4
Liquid Prosobee	12.73	2.17	1.52	9.03	9.09	<0.3
Liquid Isomil	15.0	1.75	1.25	12.0	10.25	<0.3

[1]Performic acid oxidation.

[2]Alkaline hydrolysis followed by amino acid analysis on Technicon NC-2P analyzer.

[3]Acid hydrolysis followed by amino acid analyses on Technicon NC-2P analyzer.

[4]Methionine = Total methionine - (methionine sulfoxide + methionine sulfone).

Table 40. Percent of total methionine, as measured by performic acid oxidation, represented by methionine, methionine sulfoxide, methionine sulfone, and methionine sulfoxide plus methionine sulfone.

Product	Total Methionine[1]	Methionine Sulfoxide[2]	Methionine Sulfone[3]	Methionine Sulfoxide + Methionine Sulfone	Methionine[4]
Soyafluff	100%	5.0%	2%	7%	93.1%
Promosoy	100%	4.3%	0	4.3%	95.7%
Promine D	100%	74.4%	5.7%	80.1%	19.9%
Promine F	100%	7.7%	4%	11.7%	92.3%
Powdered Similac	100%	17.8%	8.6%	26.4%	73.6%
Liquid Similac	100%	8.7%	13.1%	21.8%	78.2%
Liquid Prosobee	100%	17.0%	11.9%	28.9%	70.9%
Liquid Isomil	100%	11.7%	8.3%	20.0%	80.0%

[1]Performic acid oxidation.

[2]Alkaline hydrolysis followed by amino acid analysis on Technicon NC-2P analyzer.

[3]Acid hydrolysis followed by amino acid analysis on Technicon NC-2P.

[4]Methionine = Total methionine - (methionine sulfoxide + methionine sulfone).

comprising about 80% of the total methionine. Promosoy, a soy concentrate, contained the least amount of the oxidized methionine forms.

Implications of the Results of the Analyses of Food Proteins

The results of the analyses of food products illustrate the inadequacy of manufacturers' values for methionine. Promine D and Promine F have similar amounts of total methionine as determined by performic acid oxidation (Moore, 1963) and also as reported by the manufacturer (Central Soya Co., 1978). Yet, Promine D's methionine content is largely in the form of methionine sulfoxide. The availability of this form of methionine in humans has not been determined. If methionine sulfoxide is not available or has reduced availability compared to methionine, then total methionine values greatly overestimate the amount of utilizable methionine in this protein. For adults and children over one or two years old, the presence of methionine sulfoxide and methionine sulfone in great quantities in one food in a varied diet may be inconsequential to their general health. This, however, is not true for infants subsisting solely on one source of protein for weeks or months. The need for high quality protein with adequate amounts of methionine is essential to normal growth in infants (FAO, 1973). The assessment of the amount of methionine in an infant food product by the usual methods such as the performic acid oxidation method of Moore (1963) or measurement of methionine by ion-exchange chromatography after acid hydrolysis is inadequate since these methods do not discriminate among the forms of methionine. The performic acid oxidation method converts all forms of methionine to

methionine sulfoxide, and acid hydrolysis reduces much of the methionine sulfoxide in the protein to methionine. This was reported by Cuq et al. (1973) and also demonstrated with oxidized casein and oxidized lysozyme in this study.

The study reported here attempted to not only determine total methionine but to measure the amounts of methionine, methionine sulfoxide, and methionine sulfone present in four infant formulas. The results suggested that in one formula, liquid Prosobee, as much as 30% of the total methionine may be present in oxidized forms. Methionine sulfone was not detected in any of the model proteins hydrolyzed under any of the test conditions, but a compound with the elution time of sulfone was found in small amounts in all the infant formulas. Methionine sulfoxide was also detected in all the formulas. A portion of the sulfoxide seen in the formulas may have been produced during alkaline hydrolysis since experiments with casein and lysozyme hydrolyzed under the same conditions in the presence of glucose indicated that as much as 8% of the total methionine was oxidized during the hydrolysis. If 8% of the methionine sulfoxide in the hydrolysates of the formulas is a result of the hydrolytic procedure, liquid Prosobee and powdered Similac still contain an appreciable amount. Table 41 is an illustration of the difference made if methionine is adjusted so that it only reflects methionine in the reduced state. While the adjusted values for methionine plus total cystine are almost certainly an under-estimation (methionine sulfoxide and methionine sulfone values in these formulas are probably maximums for reasons discussed previously) the values reported by the manufacturers are

Table 41. Amount of methionine plus total cystine in four infant
formulas as measured by performic acid oxidation, as
reported by the manufacturers, and as adjusted to reflect
only methionine in the reduced state.

	Methionine plus Total Cystine[1]		
Product	(Milligrams per gram of protein)		
	Measured	Reported	Adjusted[3]
Liquid Similac	29.9	38.7	25.0
Liquid Isomil	34.4	32.0	29.9
Liquid Prosobee	29.2	27.6	23.6
Powdered Similac	34.0	-	27.0

[1]Based on an intake of two grams protein per kg per day the average of
suggested levels for methionine plus cystine is 29 mg/g of protein for
the period 0-6 months for human infants (FAO, 1973).

[2]Total methionine and total cystine as measured by performic acid
oxidation.

[3]The values were adjusted by multiplying total methionine, as deter-
mined by performic acid oxidation (measured) by the estimation of
percentage of methionine, in the reduced state, as determined in
this study and reported in Table 30.

probably over-estimations. This table does serve to illustrate dramatically the effect oxidation could have on the amount of reduced methionine present in infant formulas. The measured and reported values are all at or above the suggested intake levels for methionine plus cystine for infants (FAO, 1973) but all except Isomil fall below this level when they are adjusted to take into account the oxidized forms. Again, it must be stressed that these adjusted values are probably under-estimations and also, that the availability of methionine sulfoxide and methionine sulfone in humans has not been determined. However, the need to measure methionine sulfoxide and methionine sulfone in infant formulas as well as total methionine cannot be over-emphasized as a part of normal quality control.

ATP:L-Methionine S-Adenosyltransferase

Spectrophotometric Studies

ATP:L-methionine S-adenosyltransferase was isolated from E. coli cells according to the procedure of Lombardini et al. (1971). The reaction catalyzed by this enzyme is:

L-Methionine + ATP $\longrightarrow$ S-Adenosylmethionine + PPi + Pi
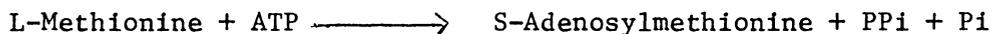
The protein content of the preparation was estimated at 4.0 mg per ml using the ratio of absorbance of the enzyme preparation at 260 nm and 280 nm (Bailey, 1967) to calculate concentration. The preparation was assayed for ATP:L-methionine S-adenosyltransferase activity at 37°C and pH 6.8 using the method of Tallen and Cohen (1976). Specific

activity, as measured by the amount of S-adenosylmethionine (SAM) produced, was 9.6 nmoles of SAM per minute per mg of protein. Lombardini et al. (1970) reported a specific activity of 83.3 nmoles of SAM per minute per mg of protein for their enzyme preparation isolated from E. coli. Tallen and Cohen (1976) found that specific activity for the enzyme isolated from human liver ranged between 3.0 and 5.0 nmoles of SAM per minute per mg of protein and between 13.5 and 21.5 nmoles of SAM per minute per mg of protein for the enzyme isolated from rat liver.

Enzyme Activity with L-Methionine

Three experiments were done measuring the amount of SAM produced at three different substrate levels over a period of time from 6 minutes to at least 60 minutes of incubation (see Figures 4-6). Blanks were prepared by adding reaction mixture, substrate, anion resin, and enzyme, in that order. The blanks were incubated with the samples. The assays were done in the manner described in the section, Methods and Materials, using the reaction mixture in Pipes buffer at pH 6.8. Table 42 gives the initial velocity at each substrate level as well as the percentage of L-methionine converted to SAM after 45 minutes of incubation. Since the amount of product formed is not greater at a substrate level of 8.0×10^{-3} mmoles (the highest substrate level) compared to a substrate level of 3.56×10^{-4} mmoles, saturation of the enzyme under the conditions used occurs between the two lowest substrate levels.
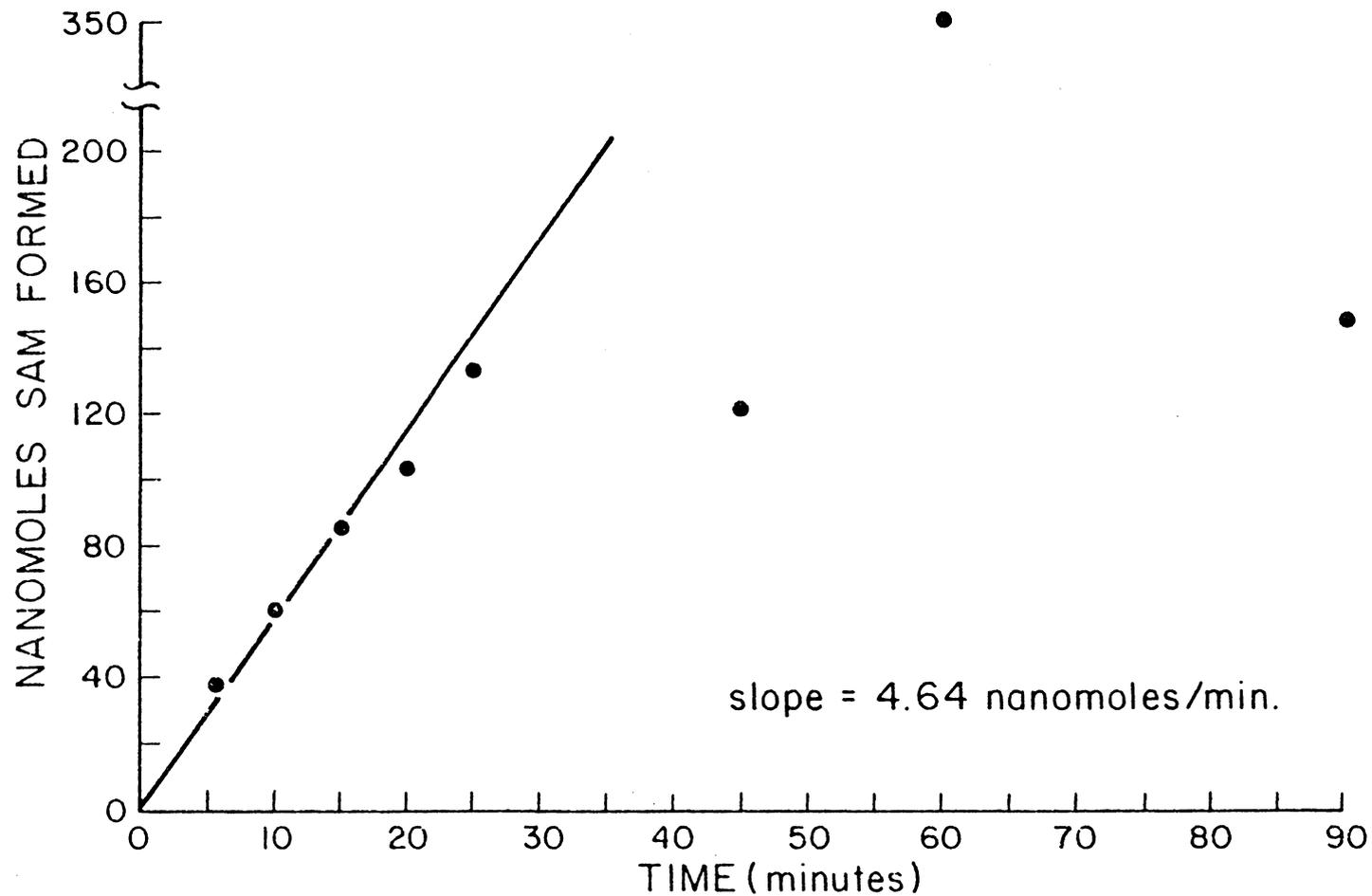
Figure 4. Formation of S-adenosylmethionine (SAM) as a function of time and substrate level.[1]

---

[1] Incubation mixture contained: Pipes buffer, pH 6.8, 0.067 mmoles; 0.040 mmoles KCl; 0.040 mmoles $MgCl_2$; 0.012 mmoles $MgATP^{2-}$; 1.77×10^{-4} mmoles L-methionine; and 0.8 mg of enzyme protein in a total volume of 0.50 ml.
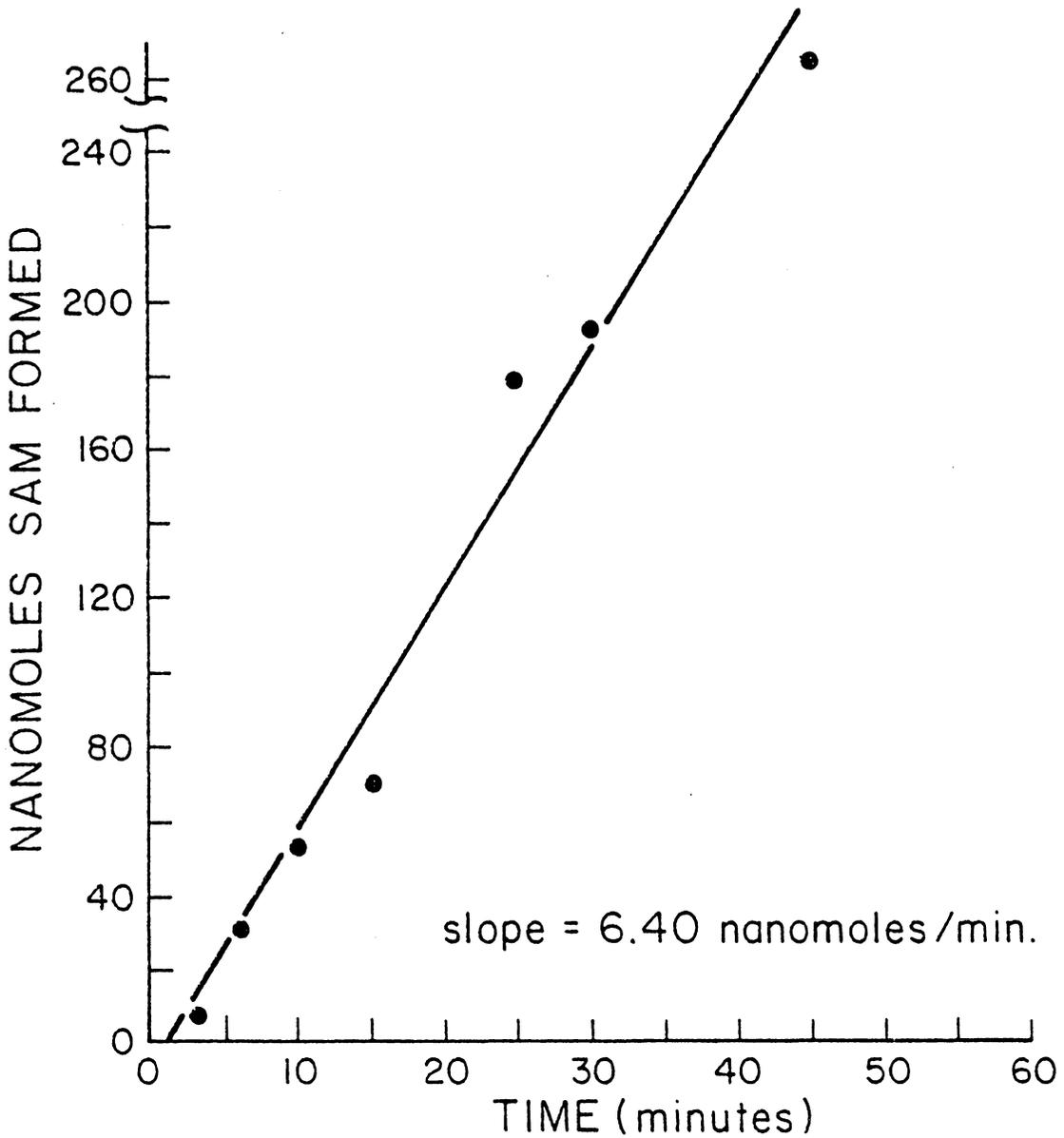
Figure 5. Formation of S-adenosylmethionine (SAM) as a function of time and substrate level.[1]

---

[1]Incubation mixture contained: Pipes buffer, pH 6.8, 0.067 mmoles; 0.04 mmoles KCl; 0.04 mmoles $MgCl_2$; 0.012 mmoles $MgATP^{2-}$; 3.56 X 10^{-4} mmoles L-methionine; and 0.8 mg of enzyme protein in a total volume of 0.50 ml.

slope = 6.24 nanomoles/min.
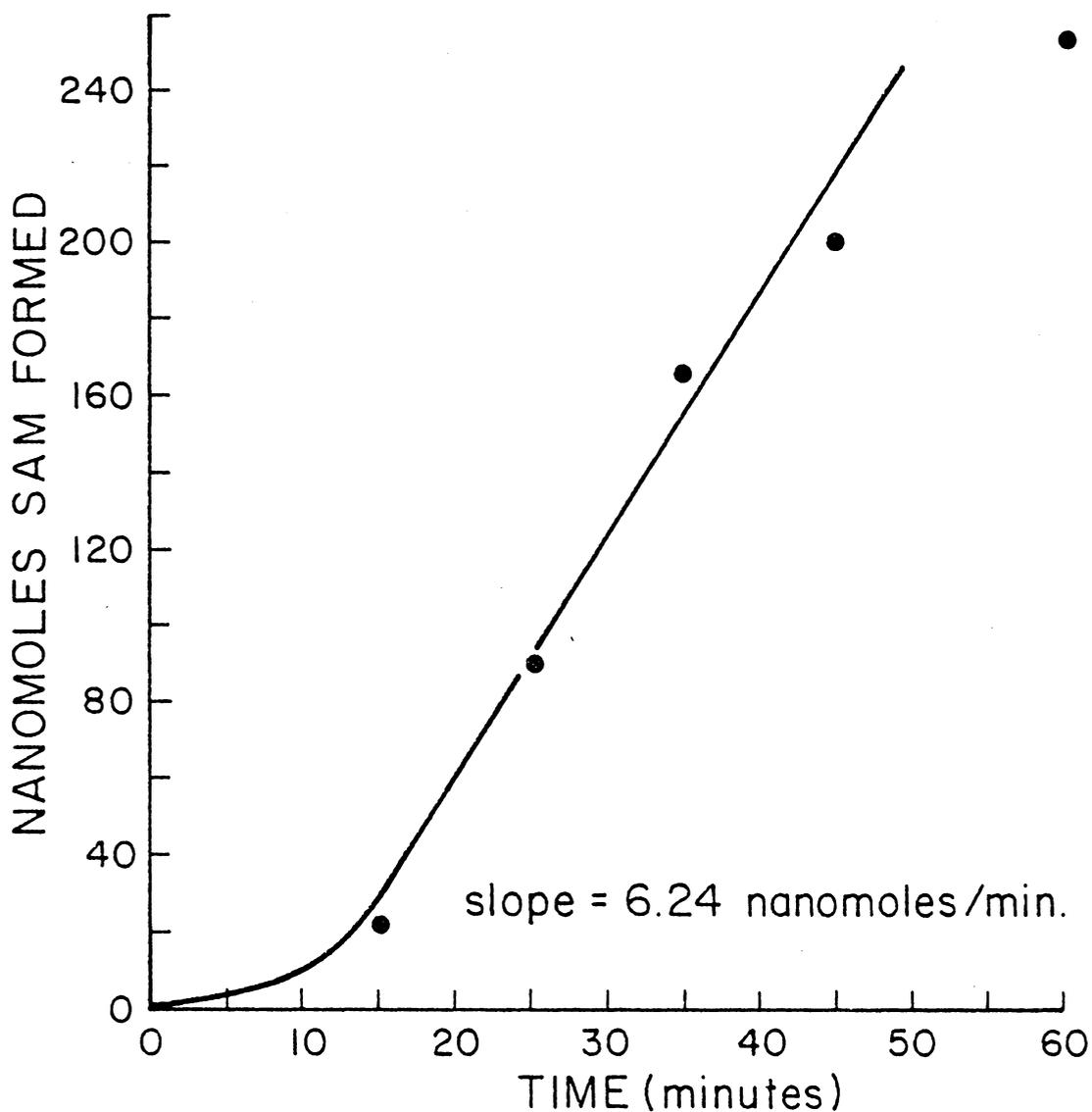
Figure 6. Formation of S-adenosylmethionine (SAM) as a function of time and substrate level.[1]

---

[1]Incubation mixture contained: Pipes buffer, pH 6.8, 0.067 mmoles; 0.040 mmoles KCl; 0.04 mmoles $MgCl_2$; 0.012 mmoles $MgATP^{2-}$; 8.0 X 10^{-3} mmoles L-methionine and 0.8 mg of enzyme protein in a total volume of 0.40 ml.

Table 42. Initial velocity and total amount of S-adenosylmethionine
           formed when L-methionine S-adenosyltransferase was
           incubated at 37°C with L-methionine at three substrate
           levels.

Substrate[1] Level (mmoles)	Initial Velocity[2] (nmoles SAM per min)	SAM Produced in 45 minutes (mmoles)	Percent Conversion[3]
1.77×10^{-4}	4.64	1.22×10^{-4}	69.0
3.56×10^{-4}	6.40	2.64×10^{-4}	74.2
8.0×10^{-3}	6.24	2.01×10^{-4}	2.5

[1] Amount of substrate (L-methionine) in incubation mixture.

[2] Initial velocity determined from slopes in Figures 1-3.

[3] $\dfrac{\text{mmoles SAM produced}}{\text{mmoles L-methionine in incubation mixture}} \times 100.$

Early experiments with the enzyme indicated that the absorbance of the blank (zero time) containing reaction mixture, substrate, anion exchange resin, and enzyme, added in that order, increased during long incubations. To determine the extent of this increase in absorbance in the blanks, a regular assay using a new enzyme preparation with an estimated concentration of 7.0 mg of protein per ml was done. The lowest substrate level (1.77×10^{-4} mmoles of L-methionine) was used. Blanks and samples were incubated for either two or 22 hours. After incubation blanks and samples were treated in the usual manner and absorbance of blanks and samples read at 260 nm on a Beckman DU Spectrophotometer. The results are shown in Table 43. Absorbance of the assay samples did not differ at 22 hours compared to two hours indicating that under the given conditions the reaction reached equilibrium within two hours. The absorbance of the blanks continued to increase such that at 22 hours there was little difference between the absorbance of the blanks and samples. This increase in absorbance in the blanks was not an effect of substances leaching out of the resin since two blanks prepared with all the elements of the regular blanks except the enzyme solution had absorbances less than 0.100 after 22 hours of incubation at 37°C. These data suggest, then, that the enzyme-substrate reaction continued to occur even in the presence of the anion exchange resin when incubated for extended periods of time. Because of these results in later experiments, to preclude this kind of effect, the enzyme solution was added to the blanks at the end of the incubation period in those experiments with extended incubation times.

Table 43. Absorbance at 260 nm of samples and blanks incubated with L-methionine S-adenosyltransferase at 37°C for two or 22 hours.

2 Hour Incubation[1]		22 Hour Incubation[1]	
Blank[2]	Sample[2]	Blank[3]	Sample[3]
0.292 ± 0.016	0.451 ± 0.026	0.414 ± 0.026	0.431 ± 0.003

[1] Protein concentration: 7 mg/ml.

L-methionine level: 1.77×10^{-4} mmoles.

[2] Mean and standard deviation of three determinations measured against distilled water.

[3] Mean and standard deviation of two determinations measured against distilled water.

Enzyme Activity with Other Substrates

D-methionine and L-methionine DL-sulfoxide were also tested as substrates. D-methionine had less than 7% of the activity of L-methionine after 35 min of incubation at 37°C and L-methionine DL-sulfoxide had no activity after one hour of incubation. After 24 hours, the sulfoxide incubated with the enzyme showed an absorbance of 0.100 compared to 0.54 for L-methionine incubated the same length of time and read against the same blank.

The slight activity seen with D-methionine as substrate could be the result of slight contamination of the D-isomer with L-methionine. It is also possible that the enzyme has some capacity to use D-methionine as a substrate. Lombardini et al. (1970) found D-methionine inactive as a substrate for this enzyme isolated from E. coli. However, Pan and Tarver (1967) reported that the D-isomer could function as a substrate for rat liver transferase, although at a reduced rate compared to L-methionine. The results of this experiment do not prove either explanation correct; however, it can be concluded that if D-methionine does act as a substrate for the enzyme it does so at a much-reduced rate compared to L-methionine.

A dipeptide, L-methionyl-L-methionine, and a tripeptide, L-methionyl-L-methionyl-L-methionine, were also tested as substrates for the enzyme. In these experiments, L-methionine was also used as a substrate for comparative purposes. Originally, the intention was to have each substrate present in the reaction mixture in equimolar amounts compared to each other; however, the tripeptide was quite insoluble in the reaction mixture. So, on a molar basis the amount of

the tripeptide was about one-half that of the other two substrates and in suspension rather than solution. A blank was prepared for each substrate containing that substrate, the reaction mixture, anion exchange resin, and enzyme solution. Samples and blanks were incubated at 37°C for various lengths of time. Upon removal from the water bath, samples and blanks were treated using the regular assay procedure. Absorbance was measured at 260 nm against the respective blanks incubated either three hours (L-methionine and L-methionyl-L-methionine) or one hour (L-methionyl-L-methionyl-L-methionine). The samples incubated for 20 hours were also read against these blanks. The results are presented in Table 44.

Proteins as Substrates

Two experiments were done exploring the possibility of the peptide bound methionine of intact protein acting as a substrate for the enzyme. In Experiment 1 casein and oxidized casein were incubated in the usual manner. Blanks were prepared using casein or oxidized casein, reaction mixture in Pipes buffer, pH 6.8, and anion exchange resin. Enzyme was added to the blanks at the end of incubation. Samples and blanks were incubated for 17 hours at 37°C and the reaction quenched with anion exchange resin. After this step the procedure deviated from the usual assay in that the protein present in the reaction tubes was not precipitated but rather 6M urea was added in order to solubilize at least a portion of the protein. Samples and blanks were read against a 6M urea blank at 250 nm, 260 nm, and 280 nm. Results are shown in Table 45. Since sample weights varied from 20

Table 44. Absorbance at 260 nm of L-methionine, L-methionyl-
L-methionine, and L-methiony-L-methionyl-L-methionine
incubated with L-methionine S-adenosyltransferase at
37°C.

Substrate	Time of Incubation[1] (hours)	Absorbance at 260 nm[2]
L-methionine	3	0.126 ± 0.002
L-met-L-met	3	0.272 ± 0.40
L-met-L-met-L-met	1	0.338 ± 0.088
L-methionine	20	1.20 ± 0.042
L-met-L-met	20	1.32 ± 0.28
L-met-L-met-L-met	20	1.18 ± 0.026

[1] Incubation mixture contained 0.067 mmoles Pipes buffer, pH 6.8; 0.04 mmoles KCl; 0.04 mmoles $MgCl_2$; 0.012 mmoles $MgATP^{2-}$; and either 0.008 mmoles of L-methionine, 0.008 mmoles L-methionyl-L-methionine in solution, or about 0.004 mmoles L-methionyl-L-methionyl-L-methionine in suspension in a total volume of 0.40 ml.

[2] Values shown are the means and standard deviations of two determinations at each time period read against the respective one or three hour blank.

Table 45. Absorbance at 250 nm, 260 nm, and 280 nm of casein or oxidized casein incubated with L-methionine S-adenosyl-transferase for 17 hours at 37°C.

Substrate[1]	Absorbance[2]		
	250 nm	260 nm	280 nm
Casein			
Blank	0.016	0.580	1.17
Sample	0.148	0.733	1.26
Oxidized Casein			
Blank	0	0.430	0.693
Sample	0.289	0.892	1.08

[1]Substrate level was 20 to 25 mg of casein or lyophilized oxidized casein in a reaction mixture of 0.067 mmoles Pipes buffer, pH 6.8; 0.04 mmoles KCl; 0.04 mmoles $MgCl_2$; 0.012 mmoles $MgATP^{2-}$; 1.4 mg protein (enzyme) in a total volume of 0.40 ml.

[2]Means of two or three determinations read against a 6M urea blank.

to 25 mg the absorbances shown are all adjusted for weight variation and are based on a sample weight of 23.0 mg.

In Experiment 2, Promine D and Promine F were incubated with L-methionine S-adenosyltransferase. The procedure was essentially the same as with casein and oxidized casein except that Promine F samples and blanks had to be diluted 1:4 with 6M urea to read absorbance on the DU spectrophotometer. The results are shown in Figure 7.

The results of the experiment with casein and oxidized casein show that both substrates when incubated with the enzyme had higher absorbances at all three wavelengths than the respective blanks suggesting some reaction of the methionine of oxidized casein and casein with ATP. If, however, the reaction involved only peptide-bound L-methionine and ATP the results are difficult to explain. Previous experiments had indicated that the oxidized casein contained little or no methionine but large amounts of methionine sulfoxide. Theoretically, the reaction should have been much greater with the unoxidized casein, which had almost all of its total methionine in the reduced form, compared to oxidized casein; yet, the extent of reaction, as measured by absorbance was approximately the same for both substrates. There is a possibility that the methionine sulfoxide in the oxidized casein was reduced to methionine during the overnight incubation. Mercaptoethanol was present in the enzyme preparation at 0.005 M and this compound has been reported to reduce methionine sulfoxide in peptides and proteins (Savige and Fontana, 1977). To investigate this possibility another experiment was done using casein and also oxidized casein as substrates and an enzyme preparation which had the
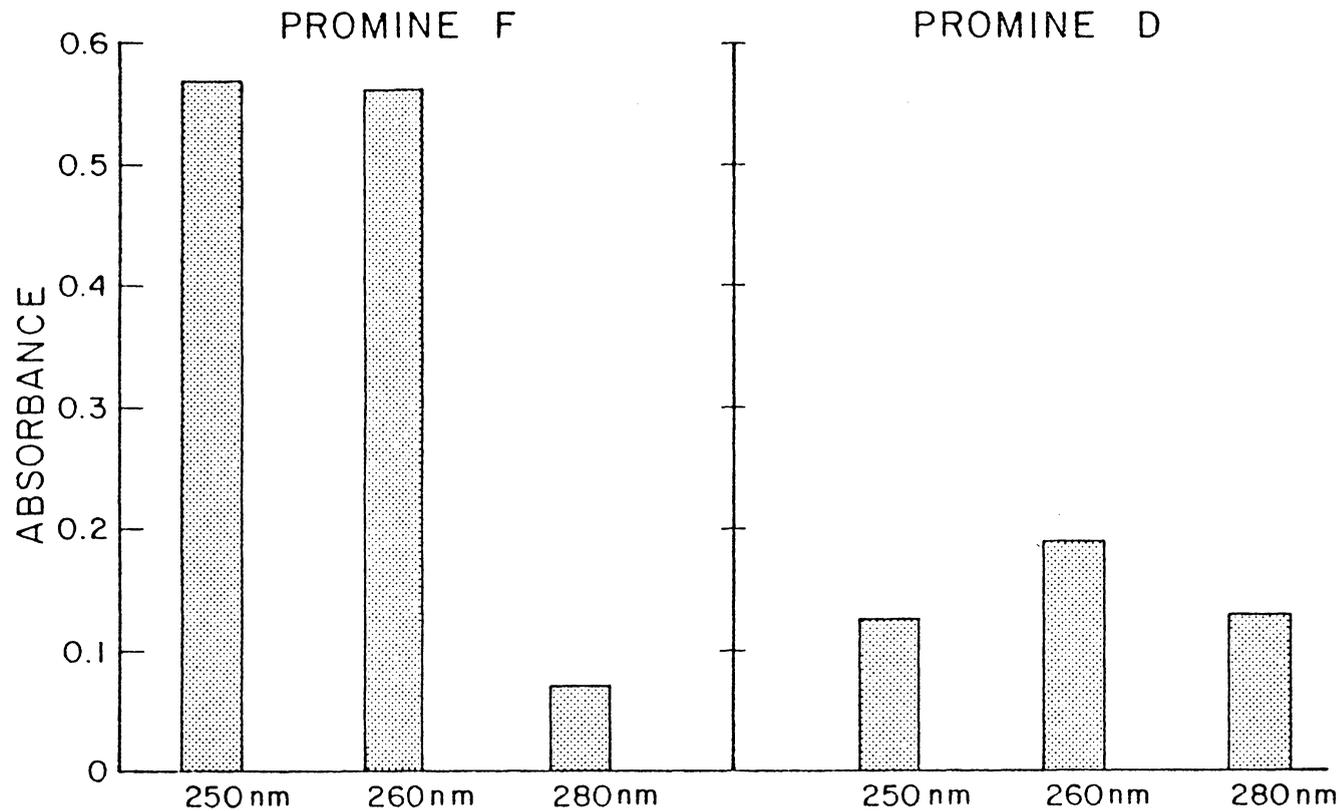
Figure 7. Absorbance of Promine D and Promine F[1] at three wavelengths on the DU Beckman Spectro-
photometer after incubation with ATP:L-methionine S-adenosyltransferase and
solubilization with 6M urea.[2]

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[1] Samples of Promine F were diluted 1:4 before measuring absorbance against a 6M urea blank. Samples
of Promine D were undiluted.

[2] Incubation mixture contained: Tris buffer, pH 7.8, 0.24 moles; 0.040 mmoles KCl; 0.040 mmoles $MgCl_2$;
29 to 34 mg of either Promine D or Promine F; and 0.3 mg of enzyme protein in a total volume of
1.4 ml.

mercaptoethanol removed by dialysis. In a two hour incubation at 37°C an increase in absorbance at all three wavelengths was still observed compared to the blanks when oxidized casein was the substrate but not when casein was the substrate (Table 46). These results do not support the supposition that the increase in absorbance is caused by the reaction of L-methionine with ATP but suggest some other reaction is occurring which increases absorbance at these wavelengths.

When Promine D and Promine F were used as substrates the absorbance was much greater at all three wavelengths for Promine F compared to Promine D. This was true even of the respective blanks. The results of this experiment are consistent with those which would be expected if a reaction were occurring between L-methionine and ATP. Promine D has large amounts of methionine sulfoxide as determined previously by ion-exchange chromatography after alkaline hydrolysis and would not be expected to react to as great an extent with ATP as Promine F which was shown to have most of its total methionine in the reduced form. The increase in absorbance is, then, presumptive evidence that peptide-bound methionine is reacting with ATP to form SAM in these isolates. It is also possible, however, that the same reaction which occurred when oxidized casein was incubated with the enzyme increasing absorbance is also occurring with the soy isolates. In an effort to confirm the presence of peptide-bound SAM in Promine F, enzyme-treated isolate was hydrolyzed for amino acid analysis.

Table 46. Absorbance at 250 nm, 260 nm, and 280 nm of casein or oxi-
dized casein incubated with L-methionine S-adenosyl-
transferase with mercaptoethanol dialyzed out of the
enzyme preparation.

Substrate[1]	Absorbance[2]		
	250	260	280
Casein			
Blank	0.608	0.780	0.802
Sample	0.552	0.716	0.762
Oxidized Casein			
Blank	0.685	0.860	0.690
Sample	0.990	1.26	0.832

[1] Substrate level was 23 mg of casein or lyophilized oxidized casein in
a reaction mixture of 0.067 mmoles Pipes buffer, pH 6.8; 0.04 mmoles
KCl; 0.04 mmoles $MgCl_2$; 0.012 mmoles MgATP; in a total volume of
0.40 ml.

[2] Mean of two determinations read against a 6M urea blank.

Amino Acid Analyses

Before analyzing the soy isolate hydrolysates, a standard solution of SAM (0.2 mg per ml in cartridge buffer, pH 1.9) was analyzed. This solution was also subjected to acid and alkaline hydrolysis as well as performic acid oxidation to determine the products formed when SAM is hydrolyzed. The solution of SAM was analyzed on the Technicon NC-2P Amino Acid Analyzer. SAM failed to be eluted when the regular NC-2P amino acid program was used. It was eluted when a borate buffer (0.1 M borate, pH 8.0 (Technicon, 1975) was substituted for the third citrate buffer, pH 6.51, usually used in the program. Figure 8 is a modified reproduction of the chromatogram indicating elution time and shape of the peak as well as the conditions used for elution.

After alkaline hydrolysis (2M NaOH, 100°C, 18 hours) the hydrolysate was analyzed on the NC-2P Analyzer under two sets of conditions (Figures 9 and 10). The chromatogram indicated that after alkaline hydrolysis, two major peaks eluted in the range where acid-neutral amino acid usually elute (buffers at pH 2.4 to 3.6) and one in the alkaline range (buffer pH 8.0). Standard solutions were also run (Figures 9-12). The amino acids in the acid-neutral range of the alkaline hydrolysate of SAM were identified as homoserine and methionine, respectively. The proportion of methionine to homoserine was approximately three to one. The third peak, in the alkaline range, remained unidentified. It did not appear to be unhydrolyzed SAM since the elution time of this peak differed from that of SAM.
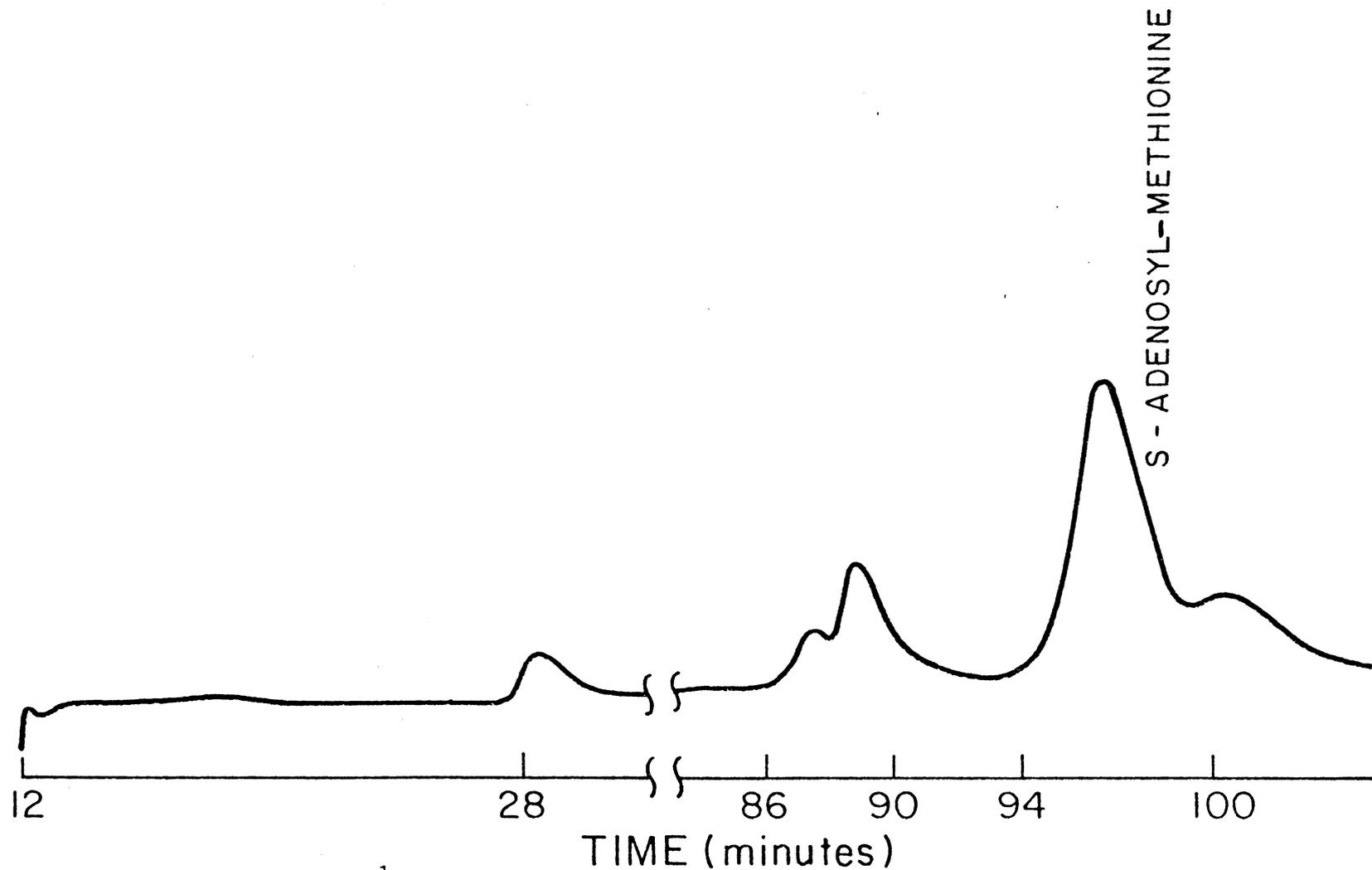
Figure 8: NC-2P chromatogram[1] of a standard solution of S-adenosylmethionine (SAM).

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 3.25 for 33 min; buffer 2, pH 4.25 for 23 min; buffer 3, pH 8.0 for 40 min.
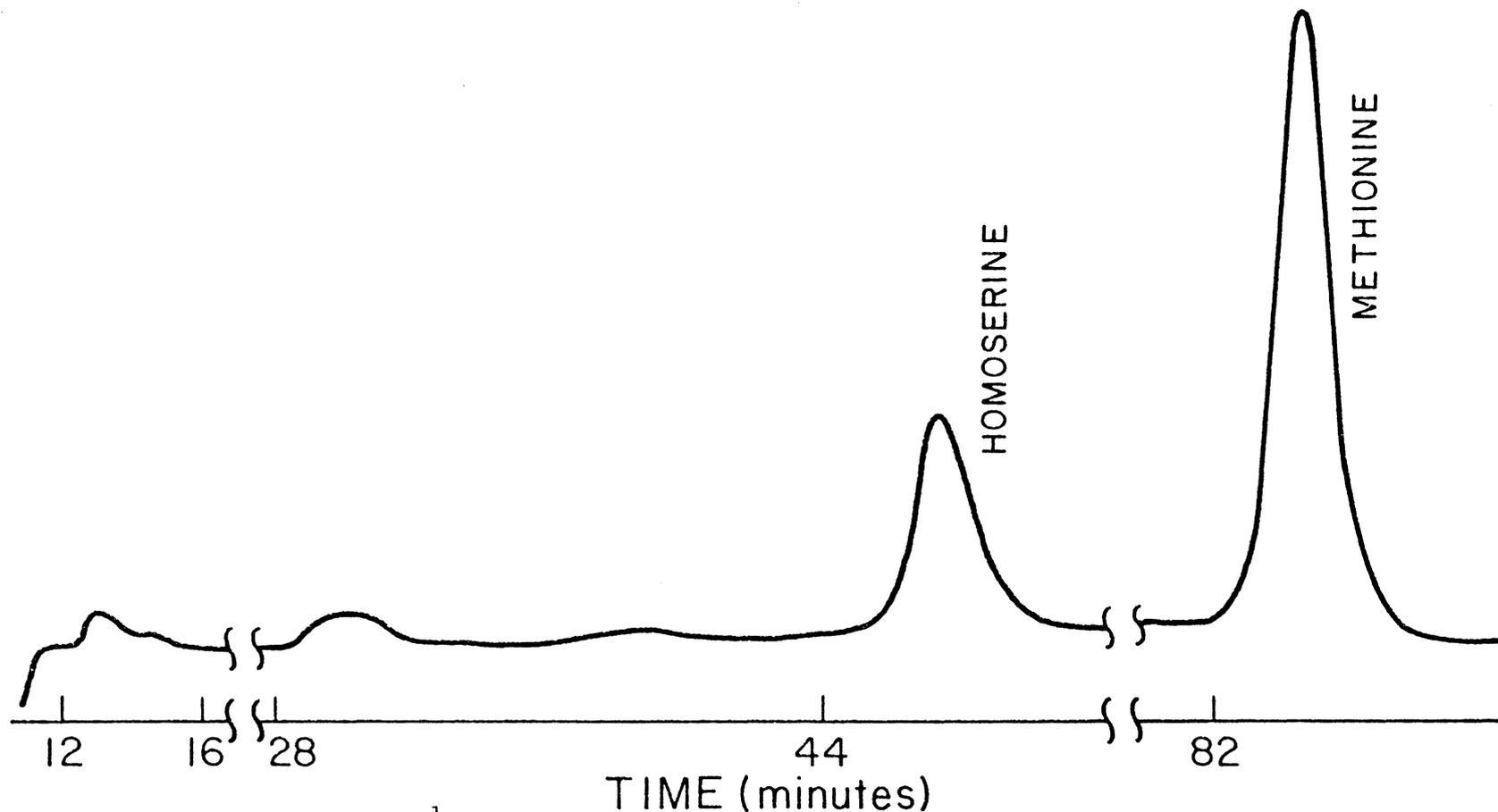
Figure 9. NC-2P chromatogram[1] of a standard solution of S-adenosylmethionine (SAM).

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.46 ml/min; buffer 1, pH 2.69 for 35 min; buffer 2, pH 3.25 for 16 min; buffer 3, pH 3.61 for 30 min.
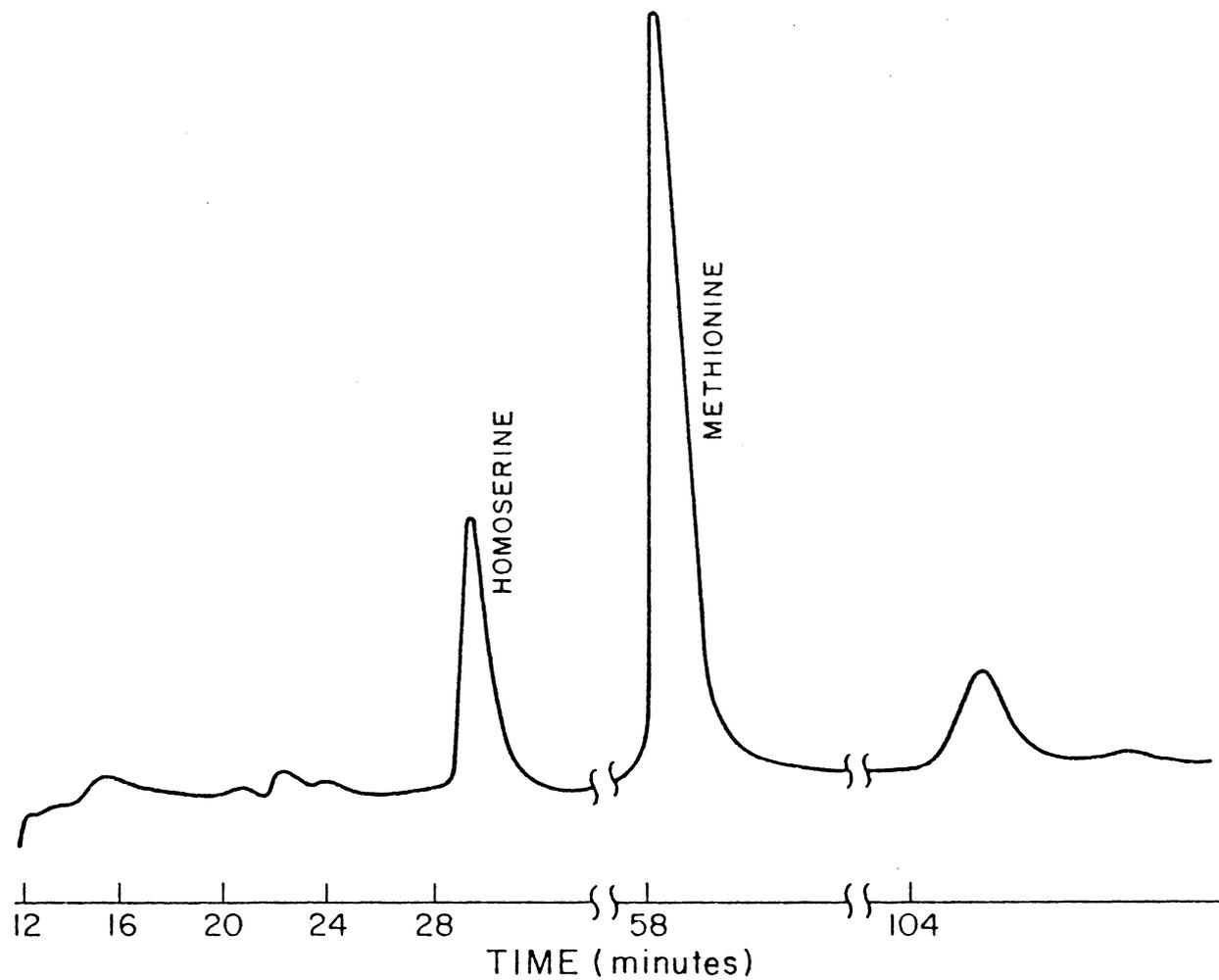
Figure 10. NC-2P chromatogram[1] of S-adenosylmethionine after hydrolysis with 2M NaOH for 18 hours at 100°C.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 3.25 for 33 min; buffer 2, pH 4.25 for 24 min; buffer 3, 8.0 for 40 min.
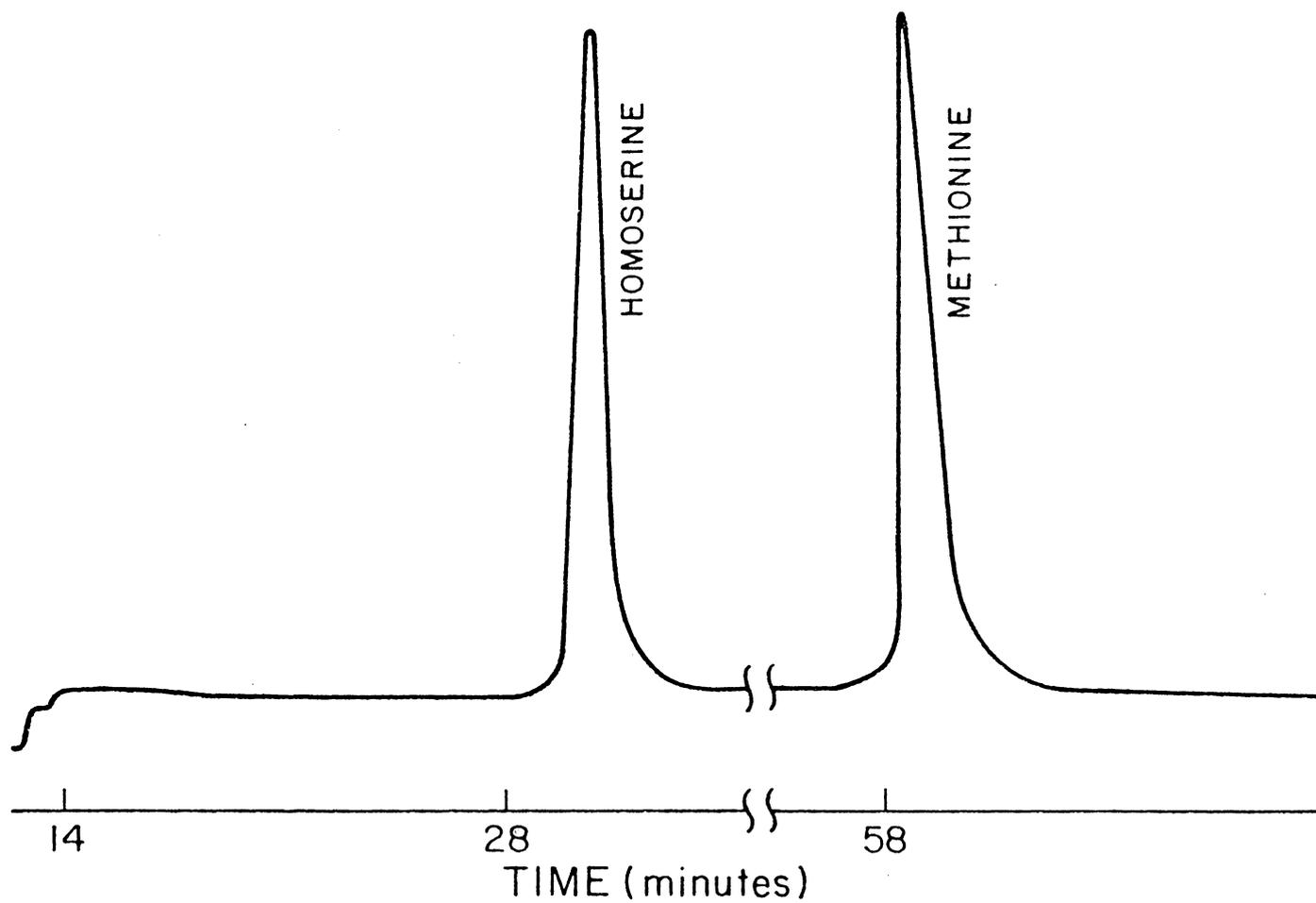
Figure 11. NC-2P chromatogram[1] of a standard solution containing homoserine and methionine.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 3.25 for 33 min; buffer 2, pH 4.25 for 24 min; buffer 3, pH 8.00 for 40 min.
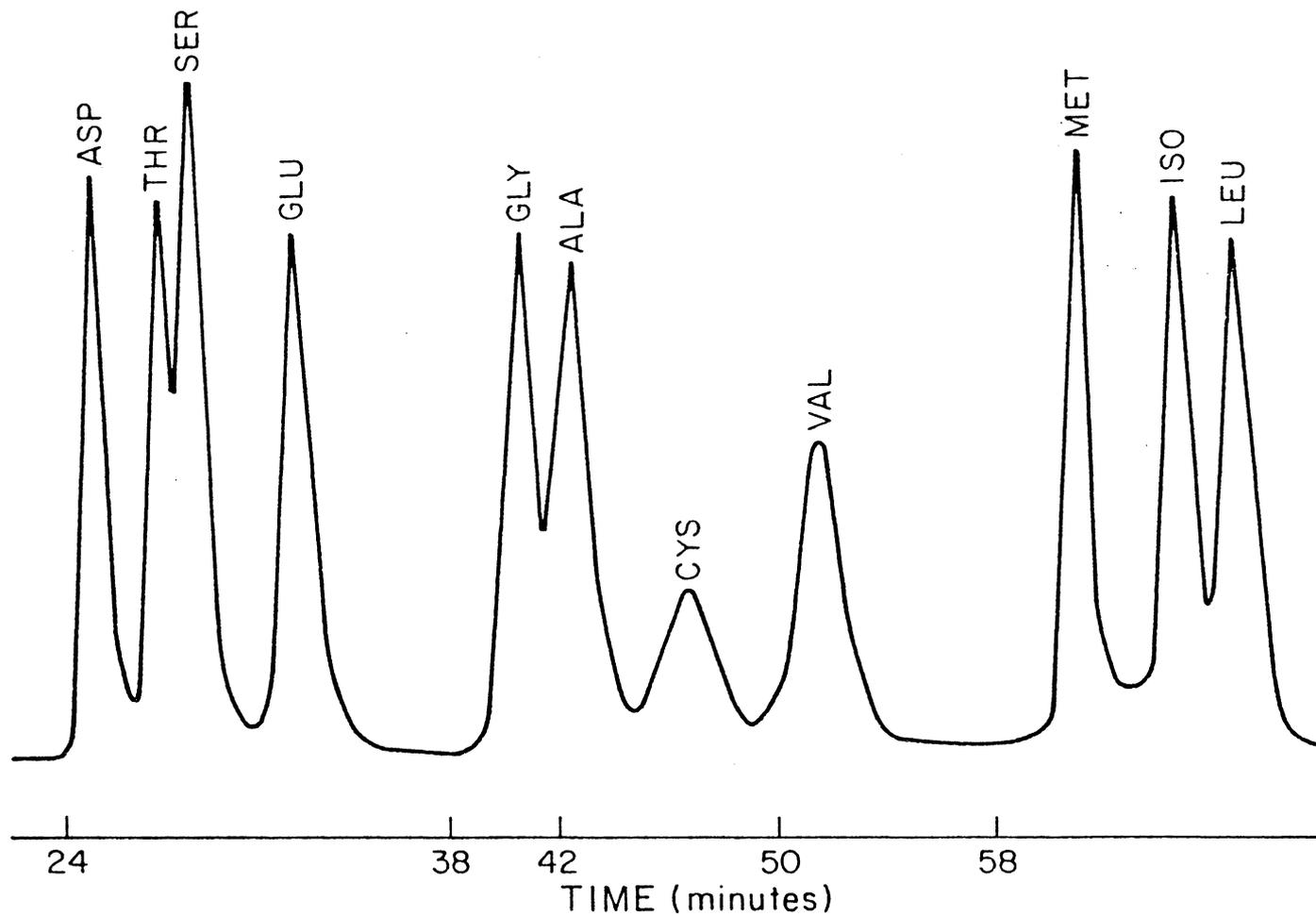
Figure 12. NC-2P chromatogram[1] of a standard solution containing aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cystine, valine, methionine, isoleucine, and leucine.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1 pH 3.25 for 33 min; buffer 2, pH 4.25 for 24 min.

After acid hydrolysis (6M HCl, 110°C, 24 hours), two major peaks were eluted in the range where neutral amino acids usually elute. A large peak was also present in the alkaline range (Figures 13 and 16). The first peak eluted in a position just preceding the position of alanine (Figure 15). The second peak was identified as methionine. Based on the structure of SAM and the fact that one of the products of acid hydrolysis was methionine, it is possible that the first peak is 2-amino butanoic acid (homoalanine). This was not confirmed, however. The large peak in the alkaline range remained unidentified but appeared to be a different compound from the alkaline peak eluted after alkaline hydrolysis based on elution time.

After performic acid oxidation of SAM followed by acid hydrolysis, no methionine sulfone was observed in the chromatograms. Again, two major peaks were noted in the neutral range. These were tentatively identified as homoalanine and methionine, respectively (Figures 17 and 18). A third unidentified peak emerged in the alkaline range. Because the conditions used for elution were different from those used on the acid hydrolysate of SAM, it was not possible to determine if the peak in the alkaline range after performic acid oxidation was the same as that in the same range after acid hydrolysis.

Acid Hydrolysis

L-methionyl-L-methionyl-L-methionine was incubated with the enzyme preparation as previously described in the section on spectro-photometric studies. After reading the absorbance of these samples on the spectrophotometer 2.0 ml from one sample which had been incubated
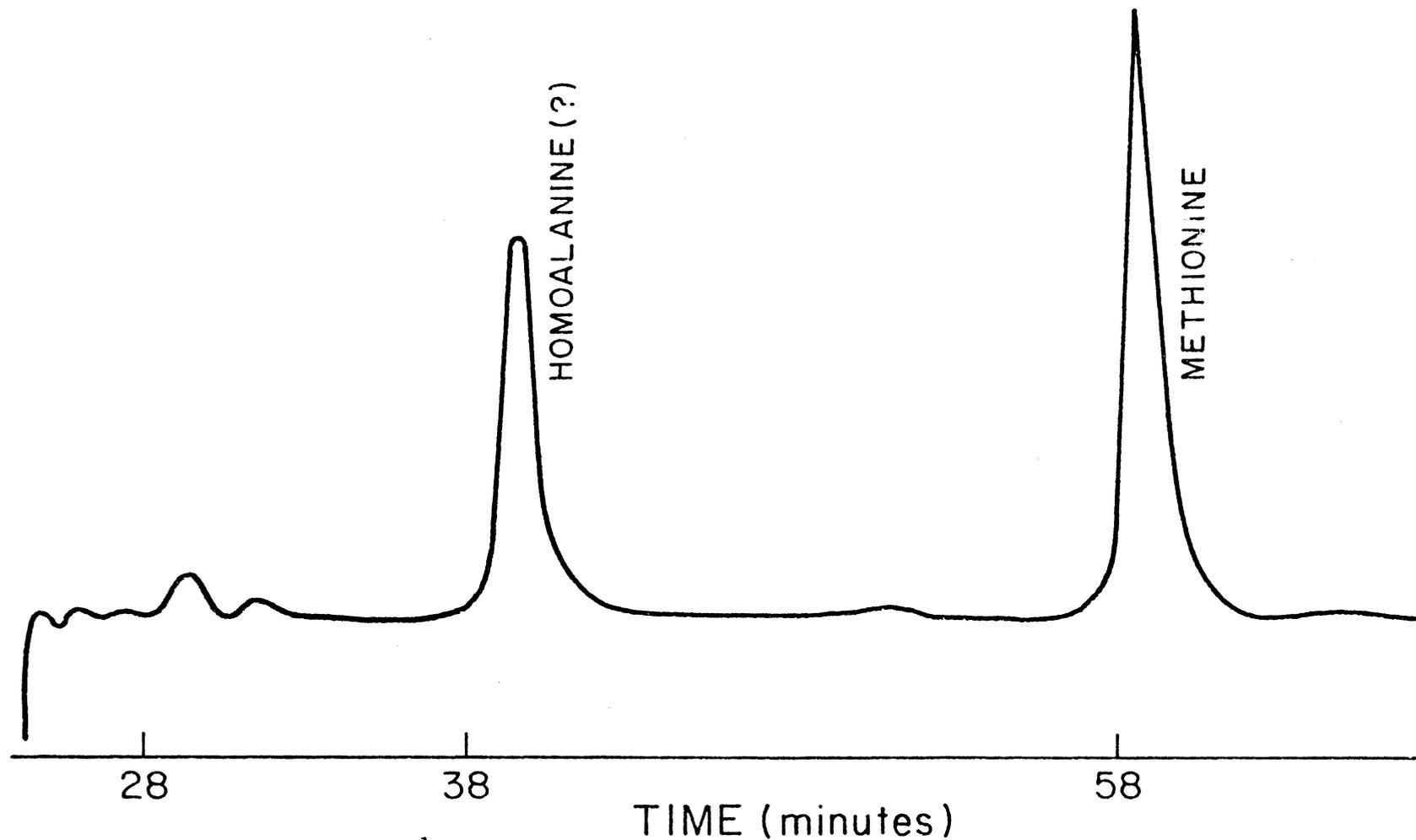
Figure 13. NC-2P chromatogram[1] of S-adenosylmethionine after acid hydrolysis with 6M HCl for 24 hours at 110°C.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 3.25; buffer 2, pH 4.25 for 24 min.
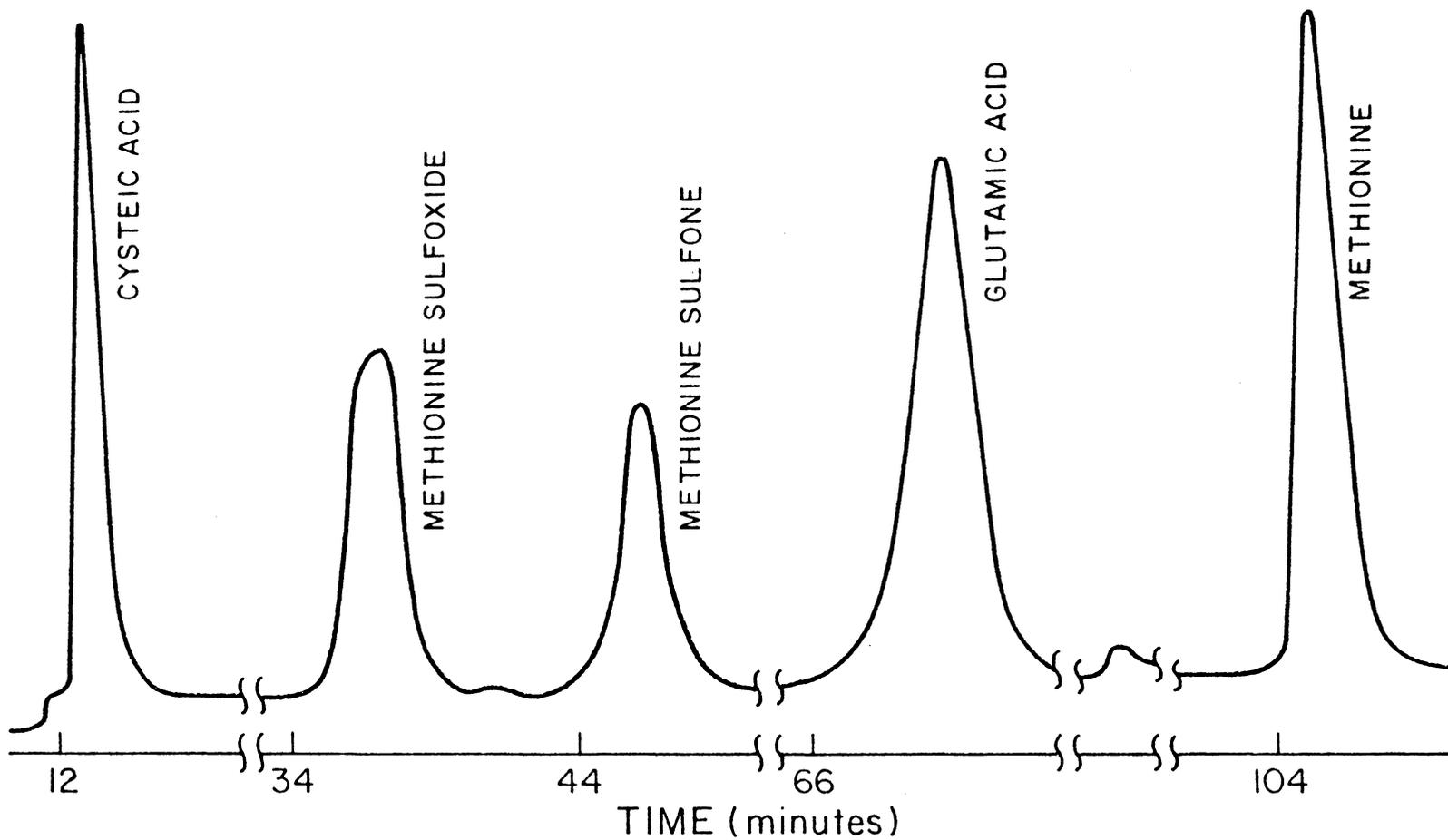
Figure 14.   NC-2P chromatogram[1] of a standard solution containing cysteic acid, methionine sulfoxide, methionine sulfone, glutamic acid, and methionine.

_____

[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.46 ml/min; buffer 1, pH 2.40 for 65 min; buffer 2, pH 3.25 for 15 min; buffer 3, pH 3.61 for 24 min.
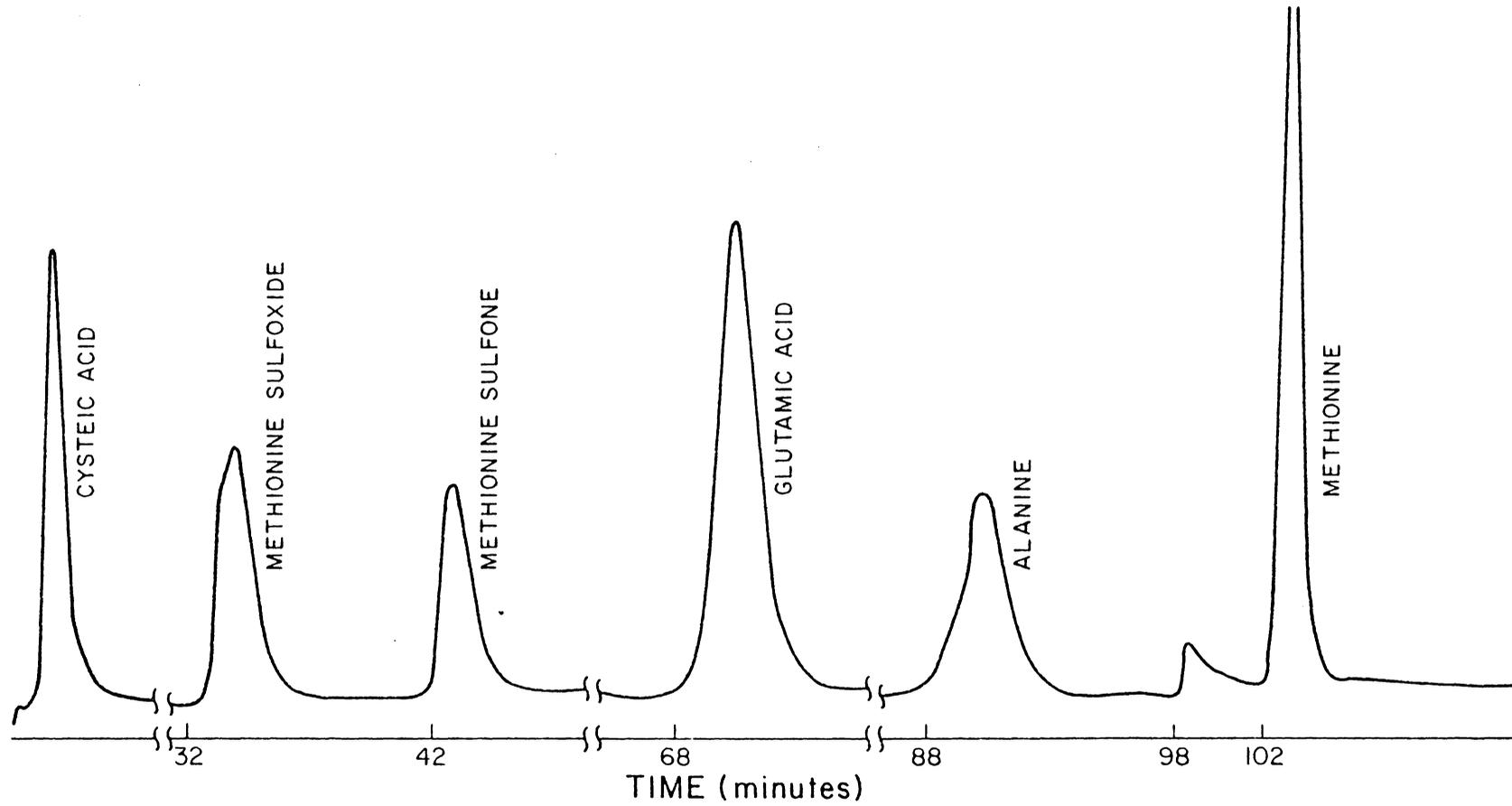
Figure 15. NC-2P chromatogram[1] of a standard solution containing cysteic acid, methionine sulfoxide, methionine sulfone, glutamic acid, alanine, and methionine.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 2.41 for 75 min; buffer 2, pH 4.25 for 22 min.
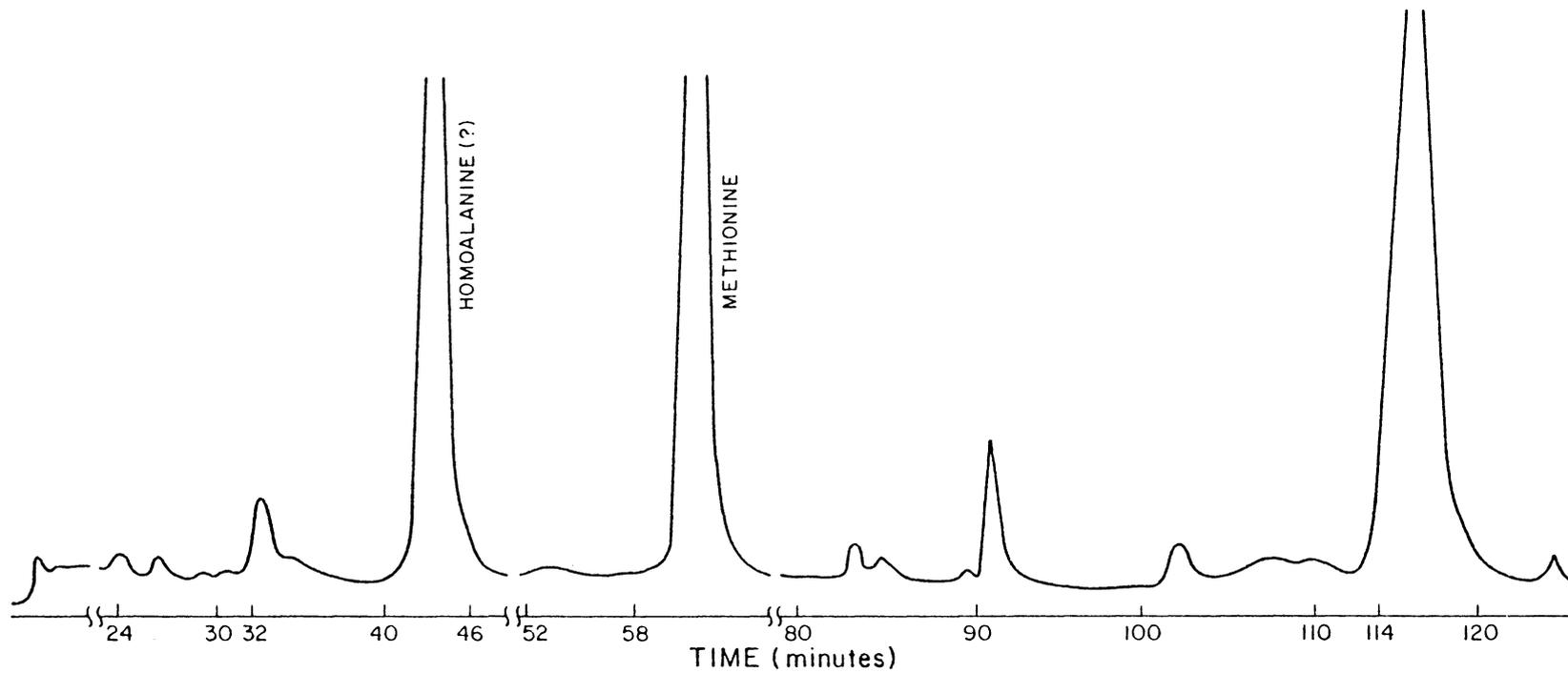
Figure 10. NC-2P chromatogram[1] of S-adenosylmethionine after acid hydrolysis with 6M HCl for 24 hours at 110°C.

[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 3.25 for 33 min; buffer 2, pH 4.25 for 24 min; buffer 3, pH 8.00 for 40 min.
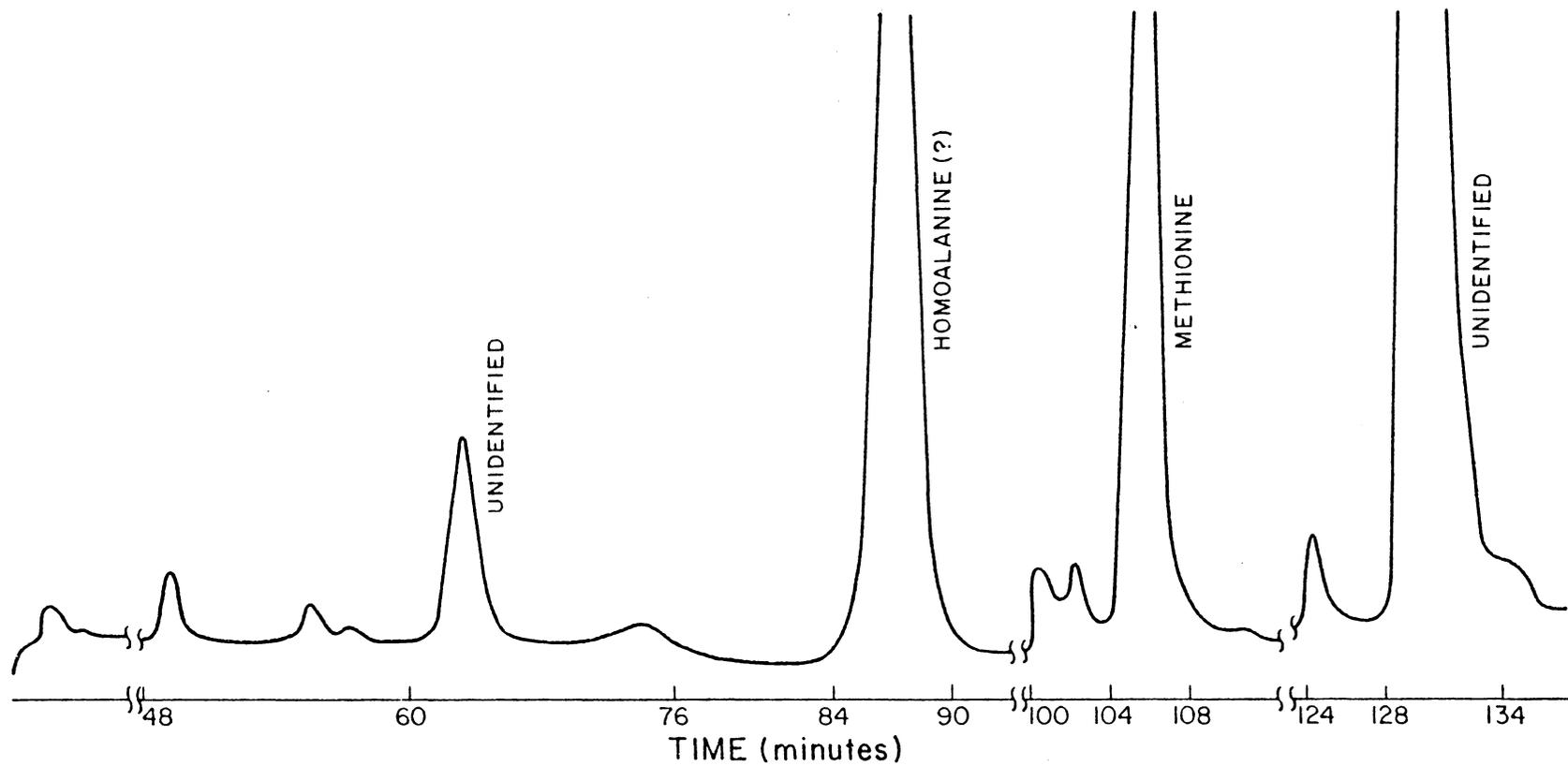
Figure 17. NC-2P chromatogram[1] of S-adenosylmethionine after performic acid oxidation followed by
acid hydrolysis with 6M HCl for 18 hours at 110°C.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min. buffer 1, pH 2.41 for 75
min; buffer 2, pH 4.25 for 22 min; buffer 3, pH 6.51 for 20 min.
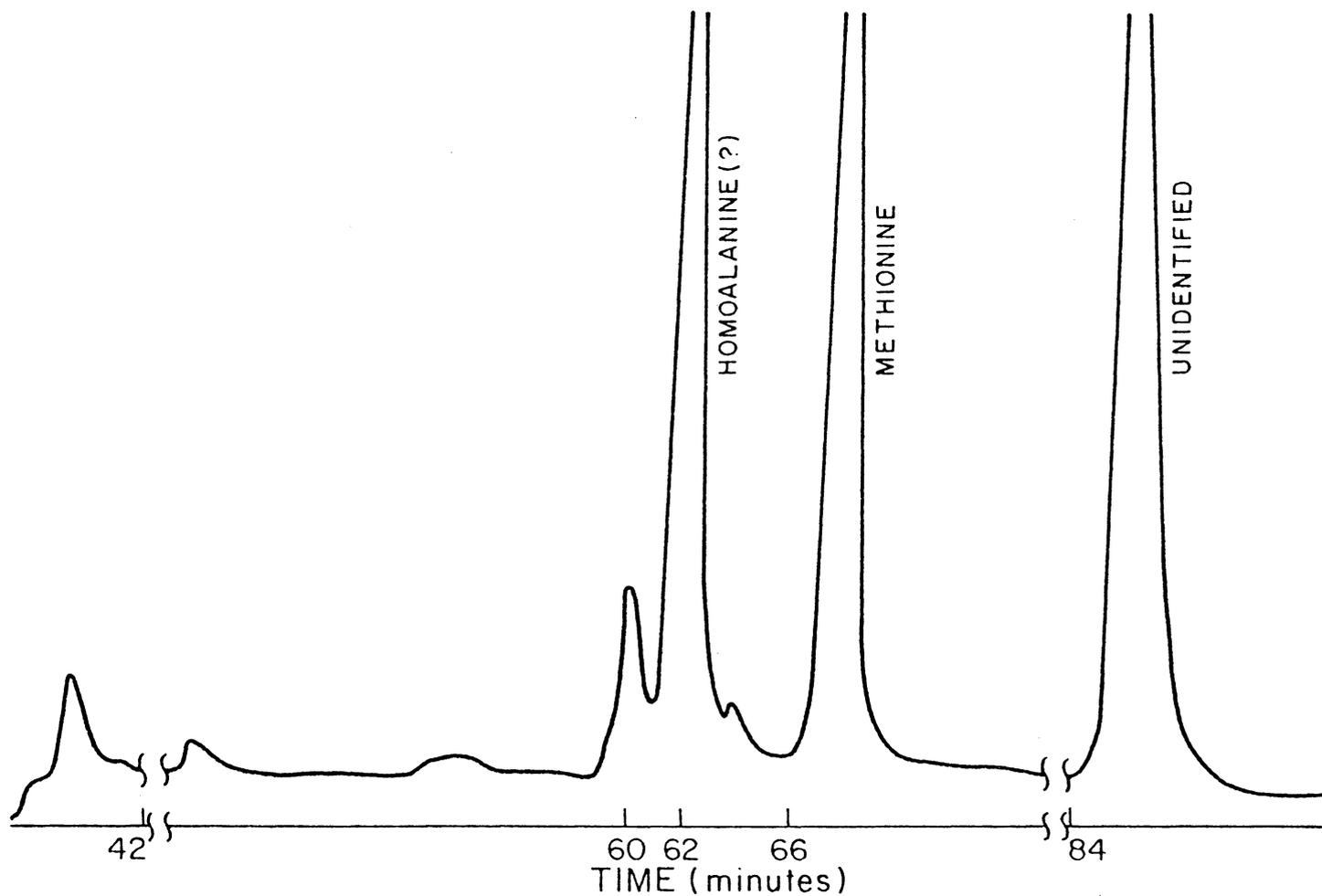
Figure 18. NC-2P chromatogram[1] of S-adenosylmethionine after performic acid oxidation followed by acid hydrolysis with 6M HCl for 18 hours at 110°C.

[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 2.41 for 33 min; buffer 2, pH 4.25 for 24 min; buffer 3, pH 9.00 for 40 min.

with the enzyme for 20 hours was transferred to a 10 ml glass ampoule

and 3.0 ml of 6M HCl added. The ampoule was flushed with nitrogen,

sealed, and hydrolyzed for 24 hours at 110°C. After hydrolysis the

entire contents of the ampoule were dried, 1.0 ml of cartridge buffer,

pH 1.9 added to the residue, and an aliquot applied to the column of

the NC-2P Technicon analyzer. Two peaks, eluted under the same condi-

tions used for the SAM hydrolysate, were superimposable upon the first

two major peaks of the SAM chromatogram (Figure 19). In addition

there was one large peak and a series of smaller peaks eluting in the

range of the acidic amino acids. These peaks roughly corresponded to

a series of small peaks in the acid hydrolysate of SAM. The presence

of a peak in the position of "homoalanine" strongly suggests that SAM

was formed in the reaction of the tripeptide with ATP. Furthermore,

the size of the "homoalanine" peak was roughly that of the methionine

peak suggesting that the reaction occurred on more than just one of

the methionine residues of the tripeptide. Other explanations, how-

ever, are possible such as the large peak emerging first being a

dipeptide which survived the relatively mild acidic hydrolytic condi-

tions, thus accounting for a large proportion of the total methionine

present in the original incubation mixture.

Alkaline Hydrolysis

L-methionine was incubated with reaction mixture and the enzyme

in polypropylene tubes for 23 hours at 37°C. The reaction mixture

used contained KCl, $MgCl_2$, $MgATP^{2-}$ in 0.10 M Tris buffer, pH 7.8. A

blank was also prepared containing all the elements of the incubation
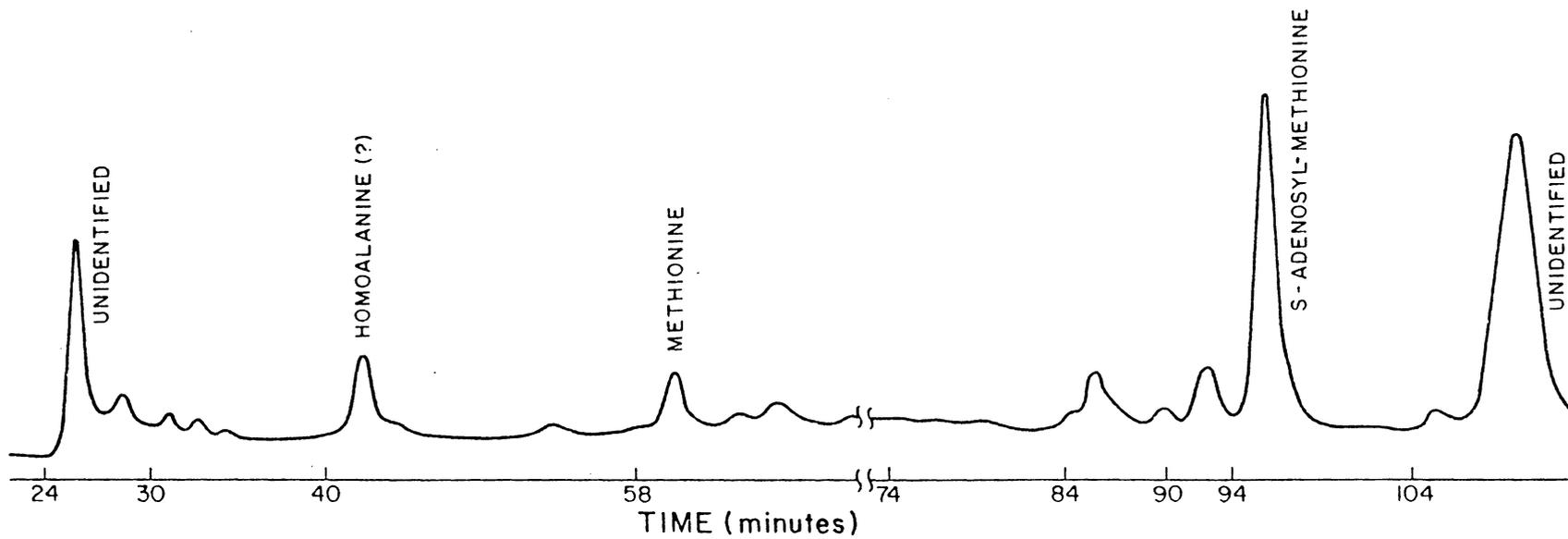
Figure 19. NC-2P chromatogram[1] of L-methionyl-L-methionyl-L-methionine after a 20-hour incubation with ATP:L-methionine S-adenosyltransferase at 37°C followed by acid hydrolysis.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.50 ml/min; buffer 1, pH 3.25 for 33 min; buffer 2, pH 4.25 for 24 min; buffer 3, pH 8.00 for 40 min.

mixture except enzyme. At the end of incubation 2.0 ml of 4M NaOH were added to all tubes. Enzyme solution was then added to the blanks. The final concentration of NaOH was 2M. The tubes were flushed with nitrogen, capped, and placed in a 100°C oven for 18 hours. After hydrolysis the tubes were allowed to cool, the contents adjusted to pH 1.9 with 6M HCl, brought to 10.0 ml volume with cartridge buffer and chromatographed on the NC-2P analyzer. Figures 20 and 21 are representations of the chromatograms for sample and blank, respectively. The chromatogram of the blank differs in several respects from the enzyme treated L-methionine. A homoserine peak appears to be present in the sample but not in the blank as would be expected if SAM were formed and then base hydrolyzed. Other differences, however, do not lend themselves to simple explanations. Virtually no methionine is evident in the blank and little in the enzyme treated sample. There appears to be a methionine sulfoxide peak in both the blank and enzyme treated sample. In addition, in the blank there is a peak in a position approximately that of methionine sulfone. This peak is much smaller in the enzyme-treated sample. Whether the methionine was oxidized to methionine sulfoxide during hydrolysis or during the incubation is open to question. In either case, however, the presence of homoserine in the enzyme-treated sample indicates that SAM was formed.

Promine D and Promine F were also incubated in polypropylene tubes with reaction mixture in Tris buffer, pH 7.8 along with the enzyme. Blanks were prepared containing Promine D or Promine F and the reactions mixture. Samples and blanks were incubated for 23 hours at 37°C. At the end of incubation 2.0 ml of 4M NaOH were added to all
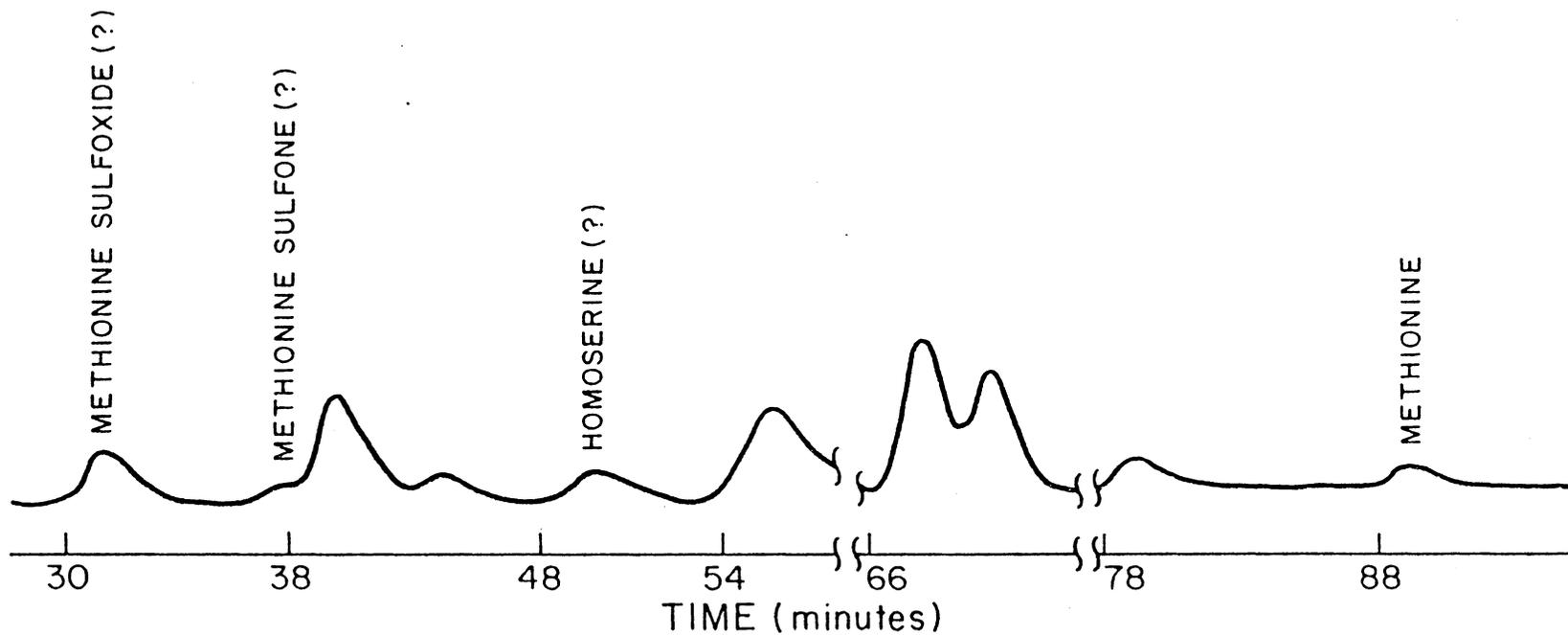
Figure 20. NC-2P chromatogram[1] of L-methionine incubated with ATP:L-methionine S-adenosyltransferase at 37°C for 22 hours followed by hydrolysis with 2M NaOH for 18 hours at 100°C.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.46 ml/min; buffer 1, pH 2.69 for 35 min; buffer 2, pH 3.25 for 16 min; buffer 3, pH 3.61 for 30 min.
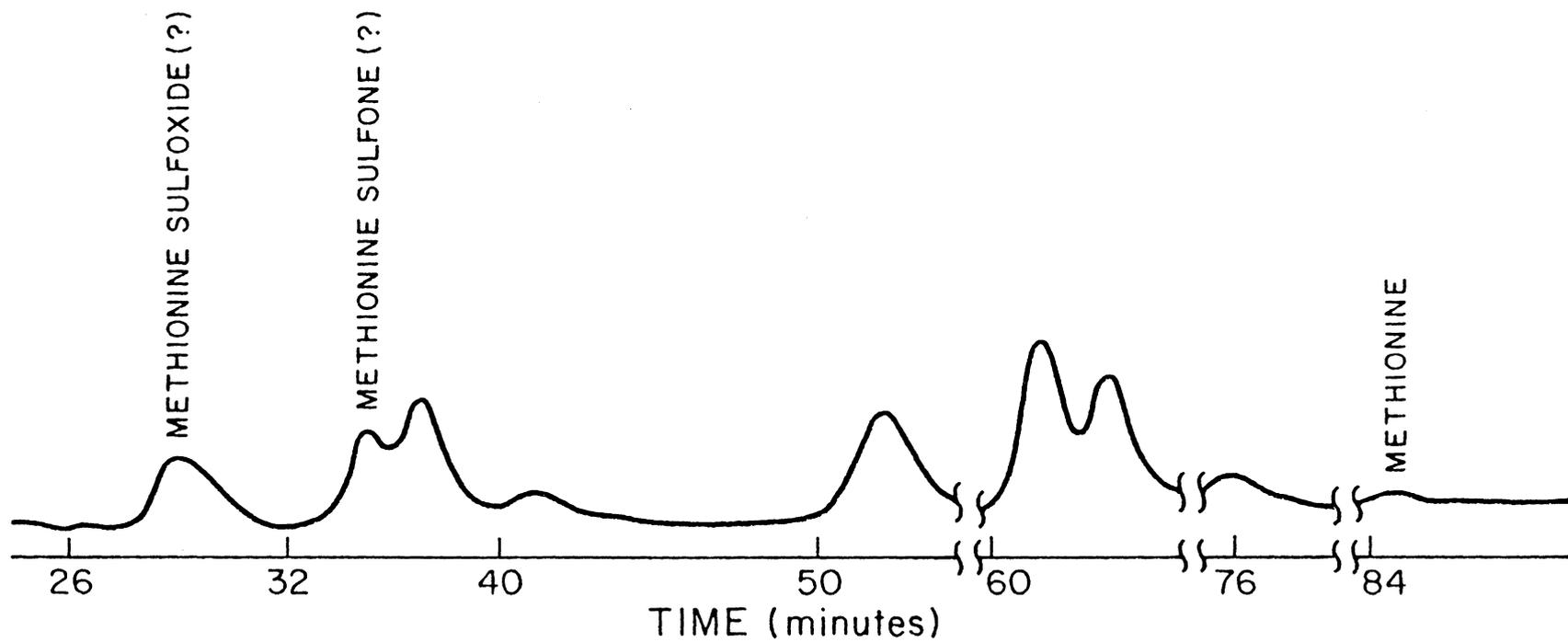
Figure 21. NC-2P chromatogram[1] of enzyme blank containing L-methionine and ATP:L-methionine S-adenosyltransferase and hydrolyzed with 2M NaOH for 18 hours at 100°C.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.46 ml/min; buffer 1, pH 2.69 for 35 min; buffer 2, pH 3.25 for 16 min; buffer 3, pH 3.61 for 30 min.

tubes giving a final concentration of 2M NaOH. The enzyme solution

was added to the blanks after the addition of NaOH. All tubes were

then subjected to alkaline hydrolysis using the procedure described

for L-methionine hydrolysis. An aliquot of the hydrolysates was

applied to the column of the NC-2P analyzer.

In comparing the chromatogram of the blank to that of the sample

for both Promine D and Promine F there appeared to be no evidence of

homoserine in any of the samples or the blanks. The size of the

methionine peak did not differ in blanks compared to samples. If

peptide-bound SAM had been formed and if it reacted to alkaline hydrol-

ysis in the same way as free SAM, homoserine should have been formed

and the methionine peak diminished in size in the enzyme-treated

samples compared to the blanks. The results of this experiment, then,

do not support the theory that ATP is reacting with peptide-bound L-

methionine forming peptide-bound SAM.

Performic Acid Oxidation

L-methionine was incubated in the usual manner with the reac-

tion mixture in Tris buffer, pH 7.8, and the enzyme preparation in

10 ml glass ampoules. A blank was also prepared with the enzyme being

added at the end of incubation. The reaction was quenched by the

addition of 2.0 ml performic acid and the samples and blanks were

treated in the usual manner for performic acid oxidation (Moore,

1963). The samples were chromatographed on the NC-2P analyzer. A

methionine sulfone peak was observed in the blank but was absent in

the enzyme treated sample. A methionine peak was present in the

chromatogram of the enzyme-treated sample but absent in the chromato-
gram of the blank (Figures 22 and 23). These results are consistent
with the expected results in that the formation of SAM would prevent
methionine sulfone formation upon oxidation with performic acid oxi-
dation and yield instead methionine and homoalanine upon acid hydrol-
ysis.

Promine F was also subjected to the same procedure. After an
incubation of 20 hours at 37°C with the reaction mixture in Tris
buffer, pH 7.8, and the enzyme preparation, the samples were treated
with performic acid in the manner of Moore (1963) and acid hydrolyzed.
A blank was also prepared with all the elements of the sample except
the enzyme. Enzyme was added after addition of the performic acid to
the blanks. After acid hydrolysis samples and blanks were chromato-
graphed on the NC-2P analyzer using the same conditions as the previous
experiment.

The results indicated a methionine sulfone peak and an absence
of methionine in the chromatograms of both the blanks and enzyme-
treated samples. The size of the methionine sulfone peak was similar
for both samples and blanks. If the peptide-bound methionine in Pro-
mine F had reacted with ATP producing peptide bound SAM, the methionine
sulfone peak in the enzyme-treated samples should have been absent or
at least smaller in size compared to the blanks and a methionine peak
should have been present in the treated samples if the peptide-bound
SAM reacted to performic acid oxidation as free SAM had done.
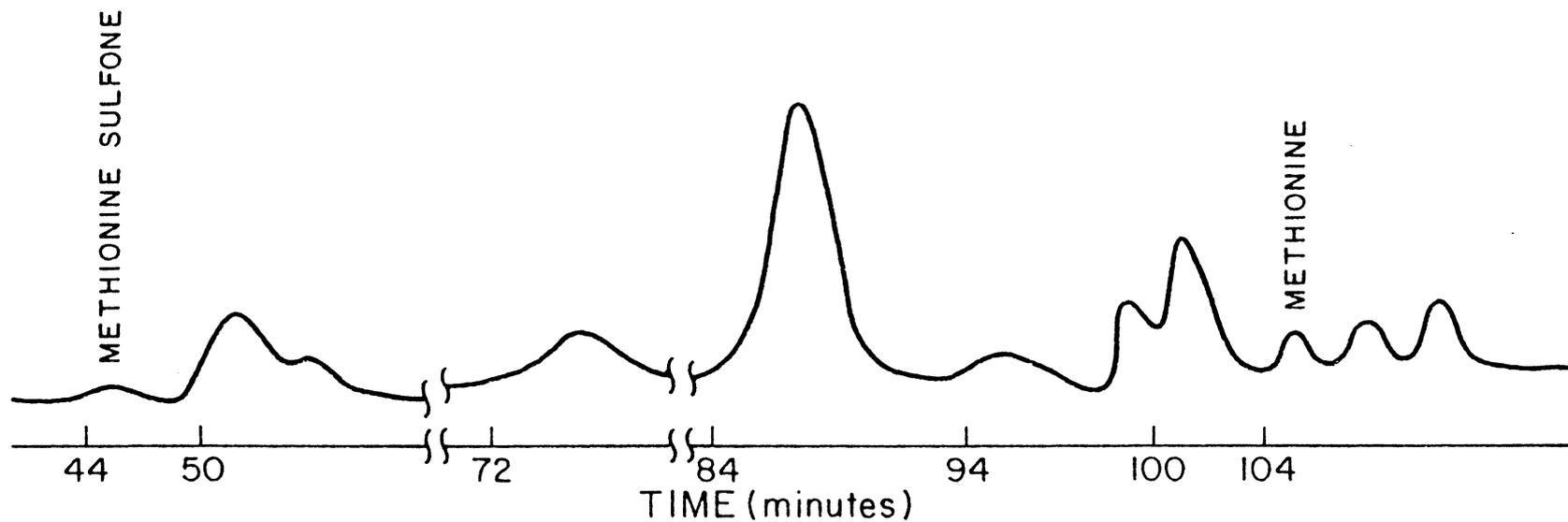
Figure 22. NC-2P chromatogram[1] of L-methionine incubated with ATP:L-methionine S-adenosyltransferase at 37°C for 20 hours followed by performic acid oxidation and acid hydrolysis.

[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.46 ml/min; buffer 1, pH 2.69 for 35 min; buffer 2, pH 3.25 for 16 min; buffer 3, pH 3.61 for 30 min.
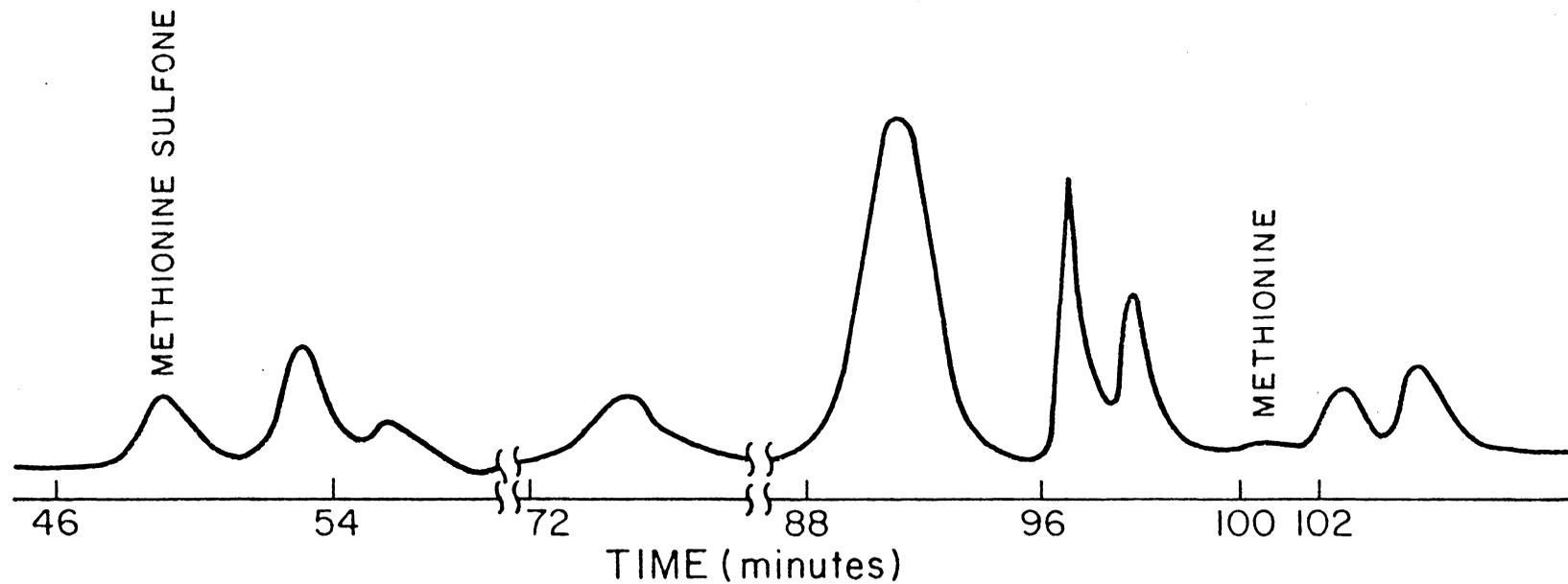
Figure 23.   NC-2P chromatogram[1] of enzyme blank containing L-methionine and ATP:L-methionine S-adenosyltransferase which was subjected to performic acid oxidation followed by acid hydrolysis.

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[1]Conditions of elution: Column temperature, 55°C; flow rate, 0.46 ml/min; buffer 1, pH 2.69 for 35 min; buffer 2, pH 3.25 for 16 min; buffer 3, pH 3.61 for 30 min.

Discussion of Results of Studies of
ATP:L-methionine S-adenosyltransferase

Evidence from both spectrophotometric studies and amino acid
analyses indicated that L-methionyl-L-methionine and L-methionyl-L-
methiony-L-methionine could function as substrates for ATP:L-methi-
onine S-adenosyltransferase. Based on absorbance at 260 nm it ap-
peared that these peptides functioned as well as L-methionine as a
substrate. The question of which residue(s) in the peptides reacted
with ATP, however, could not be answered with the data derived from
these experiments. The fact that the methionine and "homoalanine"
peaks on the NC-2P amino acid analyzer chromatogram were approximately
equal in size after acid hydrolysis of the enzyme-treated tripeptide
suggests that more than one of the three methionine residue was re-
acting with ATP. If only one residue were reacting, a methionine peak
much larger than the homoalanine peak would be expected after acid
hydrolysis. Other unidentified peaks in the chromatogram, however,
confounded the results since any one of them might be a dipeptide
containing two methionine residues.

When intact proteins were used as substrates for the enzyme, it
appeared from the spectrophotometric studies that the peptide-bound
methionine did, indeed, act as a substrate for the enzyme. The
results obtained when casein and oxidized casein were incubated with
the enzyme indicated both could act as substrates. An increase in
absorbance in the urea extract of the incubation mixture compared to
the respective blanks was observed for both proteins. Theoretically,
if the reaction which caused an increase in absorbance was the

formation of peptide-bound SAM, little or no reaction should have been observed with oxidized casein since that protein contained only methionine sulfoxide. The possibility exists that a reduction of methionine sulfoxide to methionine occurred during the incubation by some unknown mechanism. Sourkes and Trano (1953) described an enzyme from E. coli which reduced free methionine sulfoxide to methionine. It is possible that such an enzyme was isolated with the adenosyltransferase and caused a reduction in peptide-bound methionine sulfoxide; however, this is unlikely since when free methionine sulfoxide was used as a substrate with the enzyme preparation in this study very little activity was seen even after an overnight incubation with the enzyme preparation. Further studies are needed to establish whether or not the methionine sulfoxide in oxidized casein is reduced. This could be done by amino acid analysis after alkaline hydrolysis of the incubation mixture containing oxidized casein which had been incubated with the enzyme overnight.

Promine D and Promine F were also used as substrates. There was a slight increase in absorbance at the three wavelengths used for a urea extract of Promine D and a marked increase in absorbance at all three wavelengths for Promine F. The increase was more pronounced at 260 nm, a wavelength at which SAM absorbs maximally (Shapiro and Ehninger, 1966; Stekol, 1965) than at 280 nm where most proteins absorb maximally (Sober, 1970). Since previous experiments had indicated that as much as 80% of the total methionine in Promine D was present as methionine sulfoxide and only about 10% of Promine F's total methionine was present as methionine sulfoxide, the results are

reasonable. If ATP were reacting with L-methionine in the proteins forming peptide-bound SAM, absorbance of the urea extract of Promine F should be much higher than that of Promine D. Amino acid analyses of enzyme-treated Promine F, however, failed to confirm the presence of SAM in the treated isolate. The amino acid analysis of the blank was essentially the same as the enzyme-activated sample after alkaline hydrolysis, acid hydrolysis, and performic acid oxidation. Some change did occur in absorbance in these samples, however, indicating some change, physical or chemical, in the sample compared to the blank.

Various explanations can be advanced to account for the increase in absorbance. Among them are: (1) formation of peptide-bound SAM which does not react in the same way as free SAM when subjected to various hydrolystic procedures, (2) a non-enzymatic reaction taking place between the protein and ATP resulting in peptide-bound adenosyl compounds wither than SAM, (3) an enzymatic reaction, other than the one forming SAM, involving ATP and the protein, (4) some physical change taking place in the protein conformation which alters the protein's solubility or entraps ATP, (5) alteration in the charge on $MgATP^{2-}$ such that the anion exchange resin does not remove all the free ATP.

A feasible explanation must be able to account for the difference in absorbance between Promine D and Promine F. Both are soy isolates manufactured by the same company, prepared, according to the company (Central Soya Co., 1978), essentially by the same process,

with similar amino acid contents and differing chiefly in gelling
characteristics -- Promine D being a gel-forming isolate.

With the data from the experiments done in this study, it would
appear that an increase in the amount of protein solubilized is not a
feasible explanation since the maximum increase in absorbance is in
the 250-260 nm range rather than in the 280 range where most proteins
absorb maximally. It also appears that the reaction which increases
absorbance occurs maximally only in the presence of the enzyme solu-
tion since the blanks for Promine F have lower absorbances than the
sample. While this does not necessarily rule out a non-enzymatic
reaction, it at least indicates the need for the enzyme solution to be
in the incubation mixture during the incubation for maximal effect.

Alteration in charge on $MgATP^{2-}$ could be occurring but again, if
this is true, the alteration occurs maximally only in the presence of
the enzyme preparation and occurs to a far lower degree for Promine D
compared to Promine F. The same ions are present in the incubation
mixtures for both Promine D and Promine F, as well as the same enzyme
protein. The net charge or distribution of charges on the isolates
in dispersion in the incubation medium may be different, however,
causing an electrostatic binding of more $MgATP^{2-}$ to Promine F than D.
This explanation could explain the greater absorbance at 250 and 260
nm compared to 280 nm but only explains the difference in absorbance
between blank and samples if one assumes the enzyme solution is some-
how involved in altering the charge on the proteins in the isolates.

Since the enzyme ATP:L-methionine S-adenosyltransferase was iso-
lated but not purified, other enzymes almost certainly are still

present in the preparation. Another enzyme in this preparation may be catalyzing some other reaction involving ATP and other amino acids in the isolates. But the question that still remains unanswered is: why does the reaction occur to a greater extent with Promine F compared to Promine D? Both isolates have approximately the same amino acid content and probably a similar amino acid sequence since both are derived from soybean protein.

Of the various explanations advanced to explain the increase in absorbance is that SAM is actually formed but that this peptide-bound SAM does not react to the hydrolytic procedures in the manner as free SAM. This is a feasible explanation. Free compounds do not always react in the same way as peptide-bound compounds. For example, only 15% of free methionine sulfoxide was found to be reduced when it was subjected to acid hydrolysis while peptide-bound methionine sulfoxide in casein was reduced almost quantitatively under the same hydrolytic conditions. Thus, it is not unreasonable to assume that peptide-bound SAM reacts differently than free SAM under identical conditions. This possibility should be more fully explored.

Since there are differences in the amount of L-methionine in Promine F compared to Promine D and the enzyme reaction appears to discern this difference other experiments should be done to determine if the enzyme preparation "sees" differences in "available methionine" in other proteins. Perhaps the next set of experiments should consist of using some food protein which is freshly isolated and subjecting a portion of it to a severe heat treatment. Both damaged and undamaged protein should be subjected to the enzymatic assay with ATP:L-methionine

S-adenosyltransferase. At the same time these proteins should have

"available methionine" determined by some other independent means as

microbiological assay to see if the results of the two methods are

correlated. This, of course, would not indicate exactly what is

occurring but would indicate if the assay has any validity in measuring

"available methionine" in processed food proteins.

SUMMARY AND CONCLUSIONS

Methionine is the limiting amino acid in many food proteins,
including soy and milk protein. Processing of these proteins may
result in changes which reduce the amount of unaltered methionine to
even lower levels than in the unprocessed proteins. Changes which may
occur during processing procedures include oxidation of methionine to
methionine sulfoxide and/or methionine sulfone (Walker et al., 1975).
Whether these oxidized forms can replace methionine in human metabolic
processes requiring methionine has not been determined. Rats and
chicks, however, cannot utilize methionine sulfone (Miller and Samuel,
1970; Kuzmicky et al., 1974) and controversy exists as to the ability
of test animals, such as rats and chicks, to use methionine sulfoxide
(Miller and Samuel, 1968; Ellinger and Palmer, 1969; Anderson et al.,
1976; Gjøen and Njaa, 1977) as a replacement for methionine.

The usual procedures for determining methionine in food do not
distinguish among the various forms of methionine. This can result in
an imprecise picture of the amount of methionine present in the protein
in the reduced form. A need exists to establish procedures to measure
not only total methionine but also methionine, methionine sulfoxide,
and methionine sulfone. Therefore, this study attempted to establish
procedures to differentiate and quantitate methionine and its oxidized
forms in food proteins and to use these methods to analyze food pro-
ducts for these components. In addition, the feasibility of using an
enzyme, ATP:L-methionine S-adenosyltransferase (E.C. Number: 2.5.1.6),
to measure unaltered L-methionine residues in food proteins was also
explored.

The outline of procedures used in determining methionine, methionine sulfoxide, and methionine sulfone was:

(1) Total methionine by performic acid oxidation (Moore, 1963).

(2) Methionine sulfoxide by ion-exchange chromatography after alkaline hydrolysis.

(3) Methionine sulfone by ion-exchange chromatography after acid hydrolysis.

(4) Methionine by difference: Methionine = Total methionine − (methionine sulfoxide + methionine sulfone).

Neumann (1962, 1967) proposed that methionine sulfoxide could be measured by ion-exchange chromatography after alkaline hydrolysis in relatively pure proteins, but the method had not been tested thoroughly on food proteins. This study then, compared two sets of alkaline hydrolytic conditions, (1) 2M NaOH for 18 hours at 100°C (Sjöberg and Boström, 1977) and (2) 3M NaOH for 16 hours at 110°C (Cuq et al., 1977), to determine the better set of conditions for measuring methionine sulfoxide in food proteins.

The validity of the performic acid oxidation procedure and stability of methionine sulfone to acid hydrolysis had been determined previously by Moore (1963).

Evaluation of Conditions for Determination of Methionine Sulfoxide

The recovery of the free amino acids, methionine, methionine sulfoxide, and methionine sulfone, hydrolyzed under both sets of alkaline hydrolytic conditions was compared. Recovery of methionine sulfoxide was greater when it was hydrolyzed alone or in mixtures of

amino acids with 2M NaOH compared to 3M NaOH. Recovery of methionine sulfoxide was in the range of 85-90% with 2M NaOH. Oxidation of methionine to methionine sulfoxide was less using 2M NaOH compared to the other set of conditions.

The presence of glucose during hydrolysis with 2M NaOH did not appear to influence recovery of methionine sulfoxide or oxidation of methionine to methionine sulfoxide. The presence of other amino acids was a more important factor in promoting oxidation of methionine to methionine sulfoxide than the presence of glucose.

Acid hydrolysis of free methionine sulfoxide, alone, resulted in a reduction of 15.5% of the sulfoxide to methionine. Recovery of methionine and methionine sulfone hydrolyzed individually under acidic conditions resulted in recoveries of 95% and 98%, respectively.

Casein, oxidized casein, lysozyme, and oxidized lysozyme were used as model proteins and hydrolyzed under both sets of alkaline conditions. They were also hydrolyzed with glucose and 2M NaOH. In addition, casein was hydrolyzed with lactose and copper (II) and iron (II) ions and Promine F was hydrolyzed with sucrose and the same metal ions under the 2M NaOH hydrolytic conditions. The results with the model proteins indicated that hydrolysis with 2M NaOH for 18 hours at 100°C led to slightly greater recoveries of methionine from these proteins compared to 3M NaOH. The amount of methionine recovered from lysozyme hydrolyzed with 2M NaOH was 100% of the theoretical value indicating complete release of methionine from lysozyme under the conditions used; however, the release of valine, leucine, and phenyl-alanine was incomplete under these conditions. A comparison of

recoveries of methionine sulfoxide from oxidized casein and oxidized lysozyme at 2M NaOH and 3M NaOH showed no difference as a result of base strength. The 2M NaOH conditions did not appear to generate methionine sulfoxide in lysozyme or casein but the presence of sugars in the hydrolysis mixture with these proteins appeared to cause the oxidation of about 8% of the methionine in the proteins to methionine sulfoxide. The presence of glucose did not promote oxidation of methionine sulfoxide or methionine to methionine sulfone under any of the test conditions in any of the model proteins. The presence of metal ions with the sugars did not increase oxidation of methionine to methionine sulfoxide above that seen with sugars alone.

The experiments with free amino acids and model proteins hydrolyzed under two sets of alkaline hydrolytic conditions indicated:

(1) 2M NaOH for 18 hours at 100°C is better than 3M NaOH for 16 hours at 110°C for determining methionine sulfoxide.

(2) Hydrolysis of free amino acids and relatively pure model proteins with 2M NaOH for 18 hours at 100°C does not promote oxidation of methionine to methionine sulfoxide.

(3) The presence of sugars in the hydrolysis mixture promotes oxidation of about 8% of the methionine in casein and lysozyme to methionine sulfoxide.

(4) The presence of sugars does not promote oxidation of oxidation of methionine or methionine sulfoxide to methionine sulfone.

Based on these conclusions about the method it can be assumed that foods that are primarily protein in nature, such as soy isolates,

can be analyzed for methionine sulfoxide using this procedure with

recoveries of methionine sulfoxide of 85-90%. Foods with low protein

levels and high carbohydrate levels, especially simple sugars, can be

analyzed for methionine sulfoxide content using this procedure, but

the results must be interpreted with caution since the interaction of

amino acids in the protein and sugars can lead to conversion of 8%, or

possibly more, of methionine to methionine sulfoxide. Furthermore, in

the presence of sugars, alkaline hydrolysis produces substances which

can co-elute with methionine sulfoxide on ion-exchange chromatography

and this factor must be considered when assessing results.

Determination of Methionine, Methionine Sulfoxide, and Methionine Sulfone in Foods

Four soy products and four infant formulas were analyzed for

methionine, methionine sulfoxide, and methionine sulfone using the

outline of procedures given previously. The alkaline hydrolytic con-

ditions used for methionine sulfoxide determination were 2M NaOH for

18 hours at 100°C.

Three of the four soy products, Soyafluff 200W, Promosoy, and

Promine F had, respectively, methionine sulfoxide contents of 5.0%,

4.4%, and 7.7% of the total methionine in these products. There was

a slight trace or no methionine sulfone or cysteic acid in these pro-

ducts. The fourth product, Promine D, had 74% of its total methionine

in the form of methionine sulfoxide and about 4% as methionine sulfone.

There was also a substantial amount of cysteic acid (23.3% of the total

cystine). Since these products are in the category of foods that are

primarily protein, the results of the analyses for methionine sulfoxide content is probably an accurate reflection of methionine sulfoxide content in the proteins of these products.

Four infant formulas were also analyzed. Because of high sugar content and the subsequent production of compounds tending to co-elute with sulfoxide, problems were encountered in analyzing these products. These problems were at least partially resolved by altering elution conditions on the NC-2P Amino Acid Analyzer such that at least a portion of these interfering compounds were separated from methionine sulfoxide. The results of the analyses indicated that the amount of methionine sulfoxide ranged from about 9% to 18% in these formulas and methionine sulfone ranged from about 8% to 13% of total methionine as determined by performic acid oxidation. It must be stressed, however, that these values for methionine sulfoxide and methionine sulfone content in the infant formulas are maximum values and the true content of these compounds is probably somewhat lower.

Since there is difficulty in assessing the accuracy of the results in products such as the infant formulas with high sugar contents, it would be of some value to establish baseline data for infant formulas to use for comparative purposes. This baseline data might be derived from analyses of fresh human milk and fresh, unprocessed cow's milk for methionine, methionine sulfoxide, and methionine sulfone content.

Summary of ATP:L-Methionine S-adenosyltransferase Studies

The enzyme was successfully isolated from E. coli cells and an adequate assay procedure developed. It was demonstrated that L-methionine was the preferred substrate and that D-methionine had only a slight capacity to act as a substrate. Methionine sulfoxide was virtually inactive as a substrate. L-methionyl-L-methionine and L-methionyl-L-methionyl-L-methionine were tested as substrates and were found to function at least as well as L-methionine as substrates based on spectrophotometric studies. Amino Acid analysis of enzyme-treated L-methionyl-L-methionyl-L-methionine confirmed the presence of S-adenosylmethionine in the incubation mixture.

When Promine D and Promine F were tested as substrates, spectro-photometric studies suggested that peptide-bound S-adenosylmethionine formation was being catalyzed by the enzyme. Large increases in absorbance were seen in samples of Promine F compared to blanks and slight increases in absorbance of Promine D samples compared to blanks. These results were reasonable since Promine D was known to contain large amounts of methionine sulfoxide. Amino acid analyses of enzyme-treated Promine F, however, failed to confirm the presence of S-adenosylmethionine in the protein.

Further study is needed to establish unequivocally whether or not the enzyme, ATP:L-methionine S-adenosyltransferase can indeed act on unaltered peptide-bound methionine residues in proteins thereby providing a means of determining the percentage of the total methi-onine present that is there as unaltered L-methionine.

In conclusion, this study demonstrated that values for methionine, as reported by the manufacturers of food products, do not always represent the true values for the amount of unaltered methionine in the proteins in these foods. Furthermore, it illustrated the need for methods to determine not only total methionine but also methods to measure the amount of methionine sulfoxide and methionine sulfone present in the proteins. The study presented and tested a set of procedures to determine all the forms of methionine. These procedures were tested on eight food products and results indicated that they are valid in measuring methionine and its oxidized forms in products that are high in protein. Results, however, from food low in protein and high in sugar must be interpreted with care since the procedure itself may cause oxidation of methionine to methionine sulfoxide in the methionine sulfoxide determination. Furthermore, compounds caused by interactions of sugars and amino acids during alkaline hydrolysis may co-elute with methionine sulfoxide giving falsely high values for methionine sulfoxide in the hydrolysate.

The results of the experiments with the enzyme incubated with food proteins suggested that the enzyme may be of value in predicting the amount of unaltered L-methionine residues in food proteins. More research is needed, however, using other food proteins before a final judgment can be made about the value of the enzyme in this respect.

LITERATURE CITED

Achinewhu, S.C. and Hewitt, D. 1979. Assessment of the nutritional
    quality of proteins: 'The use of ileal' digestibilities of
    amino acids as measures of their availabilities. Br. J. Nutr.
    41:559.

Adrian, J. and Susbielle, H. 1975. The Maillard reaction. 10.
    Effects of premelanoidins on reproduction in rats. Ann. Nutr.
    Alimentation. 29:151. (French).

Adrian, J., Frangne, R., Petit, L., Godon, B., and Barbier, L. 1966.
    Nutritional effects of soluble products from Maillard reaction.
    Ann. Nutr. Alimentation. 20:257. (French).

Albanese, A. 1944. Utilization of tryptophan, methionine, and
    phenylalanine in man. Johns Hopkins Hosp. Bull. 75:175.

Alden, D.E. 1975. Soy processing: From beans to ingredients. J.
    Am. Oil. Chem. Soc. 52:244A.

Anderson, G.H., Li, G.S.K., Jones, J.D., and Bender, F. 1975. Effect
    of hydrogen peroxide treatment on the nutritional quality of
    rapeseed flour fed to weanling rats. J. Nutr. 105:317.

Anderson, G.H., Ashley, D.V.M., and Jones, J.D. 1976. Utilization
    of L-methionine sulfoxide, L-methionine sulfone and cysteic
    acid by the weanling rat. J. Nutr. 106:1108.

Arnold, J.B., Summers, J.D., and Bilanski, W.K. 1971. Nutritive
    value of heat treated whole soybeans. Can. J. Animal Sci. 51:57.

Asquith, R.S. and Otterburn, M.S. 1977. Cystine-alkali reactions in
    relation to protein crosslinking. In "Protein Crosslinking."
    Part B. Nutritional and Medical Consequences, M. Friedman (Ed.),
    Plenum Press, New York.

Association of Official Agricultural Chemists. 1970. Official Methods
    of Analysis. 11th Ed., Washington, D.C.

Aymard, C., Cuq, J.L., and Cheftel, J.C. 1978. Formation of lysino-
    alanine and lanthionine in various food proteins, heated at
    neutral or alkaline pH. Food Chem. 3:1.

Badenhop, A.F. and Hackler, L.R. 1973. Methionine supplementation of
    soy milk to correct cystine loss resulting from an alkaline
    soaking procedure. J. Food Sci. 39:471.

233

Badenhop, A.F. and Hackler, L.R. 1971. Protein quality of dry
    roasted soybeans: Amino acid composition and protein effi-
    ciency ratio. J. Food Sci. 36:1.

Badenhop, A.F. and Hackler, L.R. 1970. Effects of soaking soybeans
    in sodium hydroxide as pre-treatment for soy milk production.
    Cer. Sci. Today 15:84.

Baker, D.H. and Harter, J.M. 1978. D-cystine utilization by the
    chick. Poultry Sci. 57:562.

Bailey, J.L. 1967. Miscellaneous analytical methods. In "Techniques
    in Protein Chemistry," J.L. Bailey (Ed.) Elsevier Publishing
    Company, Amsterdam, New York.

Baliga, B.P., Bayliss, M.E., and Lyman, C.M. 1959. Arch. Biochem.
    Biophys. 84:1.

Berg, C. 1959. Utilization of D-amino acids. In a "Protein and
    Amino Acid Nutrition." A. Albanese (Ed.), Academic Press,
    New York.

Bird, K.M. 1975. Plant proteins: Their role in the future. J. Am.
    Oil Chem. Soc. 52:240A.

Bjarnason, F. and Carpenter, K.J. 1970. Mechanisms of heat damage
    in proteins. 2. Chemical changes in pure proteins. Br. J.
    Nutr. 24:313.

Blackburn, S. 1968. In "Amino Acid Determination," Marcel Dekker,
    Inc., New York.

Borchers, R.A., Ackerson, C.S., and Mussehl, F.E. 1948. Trypsin
    inhibitor. VI. Effect of various heating periods on the growth
    promoting value of soybean oil meal for chicks. Poultry Sci.
    27:601.

Bressani, R. 1975. Nutritional contribution of soy protein to food
    systems. J. Am. Oil Chem. Soc. 52:254A.

Bressani, R., Viteri, F., Elias, L., DeZeighi, S., Alvardo, J., and
    Odell, A. 1967. Protein quality of a soybean textured food in
    experimental animals and children. J. Nutr. 93:349.

Burns, J.H. and Baker, D.H. 1977. Methionine and cystine sparing
    activity of glutathione, homocysteine, and alpha hydroxy and
    alpha keto analogues. of DL-methionine. Fed. Proc. 36:1098.

Camien, M., Malin, R., and Dunn, M. 1951. Urinary excretion of in-
    gested L- and DL-methionine measured microbiologically. Arch.
    Biochem. Biophys. 30:62.

Cantoni, G.L. and Durell, J. 1957. Activation of methionine for transmethylation. II. The methionine activating enzyme: Studies on the mechanism of the reaction. J. Biol. Chem. 225:1033.

Cantoni, G.L. 1957. Preparation of S-adenosylmethionine. Methods in Enzymology, Vol. III. S.P. Colowick and N.E. Kaplan (Eds.). Academic Press, Inc., New York.

Carpenter, K.J. 1960. The estimation of the available lysine in animal-protein foods. Biochem. J. 77:604.

Central Soya Company. 1978. Product information sheets. Chicago, Illinois.

Cheftel, J.C. 1977. Chemical and nutritional modifications of food proteins due to processing and storage. In "Food Proteins," J.R. Whitaker and S.R. Tannenbaum (Eds.), AVI Publishing Company, Westport, Conn.

Chemical Rubber Company. 1970. "Handbook of Biochemistry: Selected Data for Molecular Biology," 2nd Edition, H.A. Sober (Ed.), Cleveland.

Cherry, F.F., Cooper, M.D., Stewart, R.A., and Platou, R.V. 1968. Cow versus soy formulas. Am. J. Diseases Children. 115:677.

Circle, S.J. and Smith, A.K. 1972a. Processing soy flours, protein concentrates, and protein isolates. In "Soybeans: Chemistry and Technology." A.K. Smith and S.J. Circle (Eds.), AVI Publishing Company, Westport, Conn.

Circle, S.J. and Smith, A.K. 1972b. Functional properties of commercial edible soybean protein products. In "Symposium: Seed proteins." G.E. Inglett (Ed.), AVI Publishing Company, Westport, Conn.

Coan, M.H. and Travis, J. 1971. Interaction of human pancreatic proteinases with naturally occurring proteinase inhibitors. In "Proceedings of the International Research Conference on Proteinase Inhibitors." H. Fritz and H. Tschesche (Eds.), Walter de Gruyter, Berlin.

Cogan, U., Yaron, A., Berk, Z., and Zimmermann, G. 1968. Effect of processing conditions on the nutritive value of isolated soybean proteins. J. Agr. Food Chem. 16:196.

Cogan, U., Fuchs, S.M., and Shemer, M. 1979. Nutritional upgrading of a new soya-protein isolate -- SP 55. J. Sci. Food Agric. 30:664.

Coppock, J. 1974. Soy proteins in foods -- retrospect and prospect. J. Am. Oil Chem. Soc. 51:59A.

Cuq, J.L., Provansal, M., Guilleux, F., and Cheftel, J.C. 1973. Oxidation of methionine residues of casein by hydrogen peroxide. Effects on in vitro digestibility. J. Food Sci. 38:11.

Cuq, J.L., Bresancon, P., Chartier, L., and Cheftel, J.C. 1978. Oxidation of methionine residues of food proteins and nutritional availability of protein bound methionine sulphoxide. Food Chem. 3:85.

Cuq, J.L., Aymard, C., and Cheftel, C. 1977. Effects of hypochlorite treatments on a methionyl peptide. Food Chem. 2:309.

DeGroot, A.P., Slump, P., Van Beek, L., and Feron, V.J. 1977. Severe alkali treatment of proteins. In "Evaluation of Proteins for Humans." C.E. Bodwell (Ed.), AVI Publishing Company, Westport, Conn.

DeGroot, A.P., Slump, P., Feron, V.M., and Van Beek, L. 1976. Effects of alkali-treated proteins. Feeding studies with free and protein-bound lysinoalanine in rats and other animals. J. Nutr. 106:1527.

DeGroot, A.P. and Slump, P. 1969. Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. J. Nutr. 98:45.

Derby, G., Poullain, B., and Bleyer, R. 1974. Study of nutritive and biological value of textured soy proteins in adults, children, and rats. J. Am. Oil Chem. Soc. 51:203A.

Desikachar, H.S.R. and De, S.S. 1947. Role of inhibitors in soybeans. Science 106:421.

Donoso, G., Lewis, O.A., Miller, D.S., and Payne, P.R. 1962. Effect of heat treatment on the nutritive value of proteins: Chemical and balance studies. J. Sci. Food Agric. 13:192.

Doraiswamy, M. 1972. Comparison of the protein nutritional value of several vegetable protein products at equal levels of intake for human adults. M.S. thesis, University of Nebraska, Lincoln.

Duncan, A., McIntosh, A., and Ellinger, G.M. 1976. Determination of methionine in seeds of legumes. Proc. Nutr. Soc. 35:149A.

Ellinger, G.M. and Duncan, A. 1976. The determination of methionine in proteins by gas-liquid chromatography. Biochem. J. 155:615.

Erbersdobler, H.F. 1977. The biological significance of carbohydrate-lysine crosslinking during heat treatment of food proteins. In "Protein Crosslinking, Part B." Nutritional and Medical Consequences, M. Friedman (Ed.), Plenum Press, New York.

Evans, R.J., Groschke, A.C., and Butts, H.A. 1951. Studies on the heat inactivation of cystine in soybean oil meal. Arch. Biochem. 30:414.

Evans, R.J. and Butts, H.A. 1949a. Inactivation of amino acids by autoclaving. Science 109:569.

Evans, R.J. and Butts, H.A. 1949b. Studies on the inactivation of methionine in soybean oil meal. J. Biol. Chem. 178:543.

Evans, R.J. and McGinnis, J. 1948. Cystine and methionine metabolism by chicks receiving raw or autoclaved soybean oil meal. J. Nutr. 35:477.

FAO. 1973. Energy and protein requirements: Report of a joint FAO/WHO ad hoc expert committee. FAO Nutrition Meetings Report Series No. 52. Food and Agriculture Organization of the United Nations, Rome.

Feeney, R.E., Means, G.E., and Bigler, J.C. 1969. Inhibition of human trypsin, plasmin and thrombin by naturally occurring inhibitors of proteolytic enzymes. J. Biol. Chem. 244:1957.

Finley, J.W., Snow, J.T., Johnston, P.H., and Friedman, J. 1977. Inhibitory effect of mercaptoamino acids on lysinoalanine formation during alkali treatment of proteins. In "Protein Crosslinking," Part B, Nutritional and Medical Consequences, M. Friedman (Ed.), Plenum Press, New York.

Floyd, N.F., Cammaroti, M.S., and Lavine, T.F. 1963. The decomposition of DL-methionine sulfoxide in 6 N HCl. Arch. Biochem. Biophys. 102:343.

Fomon, S.J., Thomas, L.N., Filer, L.J., Anderson, T.F., and Bergmann, K.E. 1973. Requirement for protein and essential amino acids in early infancy. Acta Paediat. Scand. 62:33.

Fomon, S.J. and Ziegler, E.E. 1979. Soy protein isolates in infant feeding. In "Soy Protein and Human Nutrition." H.L. Wilcke, D.T. Hopkins, and D.H. Waggle (Eds.), Academic Press, Inc., New York.

Ford, J.E. 1973. Some effects of processing on nutritive value. In "Proteins in Human Nutrition," W.G. Porter and B.A. Rolls (Eds.), Academic Press, New York.

Ford, J.E. and Shorrock, C. 1971. Metabolism of heat damaged protein in the rat. Influence of heat damage on the excretion of amino acids and peptides in the urine. Br. J. Nutr. 26:311.

Frank, O., Baker, H., Hutner, H., Rusoff, I.I., and Morck. 1975. Evaluation of protein quality with the phagotrophic protozoan Tetrahymena. In "Protein Nutritional Quality in Foods and Feeds," Part 1, M. Friedman (Ed.), Marcel Dekker, Inc., New York.

Freeman, J.B., Stegink, L.D., Meyer, P.D., Fry, L.K., and Denbesten, L. 1975. Excessive urinary zinc losses during parenteral alimentation. J. Surg. Res. 18:463.

Geschwind, T.I. and Li, C.H. 1964. Effects of alkali-heat treatment on beta-melanocyte-stimulating hormone. Arch. Biochem. Biophys. 106:300.

Gilbert, L.M. 1977. M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Gillberg, L. 1977. A study of the nutritive value of soybean meal and soybean protein isolates. Nutr. Reports Int'l. 16:603.

Gjøen, A.U. and Njaa, L.R. 1977. Methionine sulphoxide as a source of sulphur-containing amino acids for the young rat. Br. J. Nutr. 37:93.

Goldberg, A. and Guggenheim, K. 1962. The digestive release of amino acids and their concentrations in the portal plasma of rats after protein feeding. Biochem. J. 83:129.

Gould, D.H. and MacGregor. 1977. Biological effects of alkali-treated protein and lysinoalanine: An overview. In "Protein Crosslinking," Part B, Nutritional and Medical Consequences, M. Friedman (Ed.), Plenum Press, New York.

Graham, G.G. 1971. Methionine or lysine fortification of dietary protein for infants and small children. In "Amino Acid Fortification of Protein Foods," N.S. Scrimsahw and A.M. Altschul (Eds.). M.I.T. Press, Cambridge, Mass.

Graham, G.G., MacLean, W.C., Jr., and Placko, R.P. 1976. Plasma amino acids of infants consuming soybean protein with and without added methionine. J. Nutr. 106:1307.

Hackler, L.R., Van Buren, J.P., Steinkraus, K.H., El Rawi, I., and Hand, D.B. 1965. Effect of heat treatment on nutritive value of soy milk protein fed to weanling rats. J. Food Sci. 30:723.

Happich, M.L. 1975. Protein nutritive value of selected and potential meat extenders. In "Protein Nutritional Quality of Foods and Feeds," Part 2, M. Friedman (Ed.), Marcel Dekker, Inc., New York.

Harper, A. and Uyeyama,K. 1948. Plasma methionine after oral administration of DL-methionine in human subjects. Proc. Soc. Exp. Biol. Med. 68:296.

Hayase, F., Kato, H., and Fujimaki, M. 1975. Racemization of amino acid residues in proteins and poly (L-amino acids) during roasting. F. Agric. Food Chem. 23:491.

Hayase, F., Kato, H., and Fujimaki, M. 1973. Racemization of amino acid residues in proteins during roasting. Agr. Biol. Chem. 37:191.

Hightower, N.C., Jr., and Janowitz, H.D. (Eds.). 1973. Secretion and absorption in the intestine. In "Best and Taylor's Physiological Basis of Medical Practice," J.R. Brobeck (Ed.), 9th Edition, 1973. The Williams & Wilkins Company, Baltimore.

Hooks, R.D., Hays, V.W., Speer, V.C., and McCall, J.T. 1965. Effect of raw soybeans on pancreatic enzyme concentration and performance of pigs. J. Animal Sci. 24:894. (Soc. Proceedings Abstract.)

Horan, P.E. 1974. Soy protein products and their production. J. Am. Oil Chem. Soc. 51:67A.

Horan, F. 1974. Meat Analogs. In New Protein Foods. A. Altschul (Ed.), Academic Press, New York. p. 366.

Horn, M.F., Lichtenstein, H., and Womack, M. 1968. Availability of amino acids: A methionine-fructose compound its availability to micro-organisms and rats. J. Agr. Food Chem. 16:741.

Hugli, T.E. and Moore, S. 1972. Determination of the tryptophan content of proteins by ion exchange chromatography of alkaline hydrolysates. J. Biol. Chem. 247:2828.

Hurrell, R.F., Carpenter, K.J., Sinclair, W.J., Otterburn, M.S., and Asquith, R.S. 1976. Mechanisms of heat damage in proteins. 7. The significance of lysine-containing isopeptides and of lanthionine in heated proteins. Br. J. Nutr. 35:383.

Jennings, D.M. 1969. Methionine loss during protein hydrolysis of plant materials. J. Agr. Food Chem. 17:668.

Jensen, A.H., Yen, J.T., and Hymowitz, T. 1974. Effects of soybeans of different trypsin inhibitor activities on performance of growing swine. J. Animal Sci. 38:304.

Johnson, D.W. 1975. Soybean processing, products, characteristics, and uses. In Soybean Production, Protection and Utilization. Proceedings of a conference of Africa, the Middle East and South Asia, Oct. 14-17, 1974. Addis Abada, Ethiopia. D.K. Whigham (Ed.). College of Agriculture, University of Illinois at Urbana-Champaign.

Kakade, M.L., Thompson, R.D., Engelstad, W.E., Behrens, G.C., Yoder, R.D., and Crane, F.N. 1975. Failure of soybean trypsin inhibitor to exert deleterious effects in calves. J. Dairy Sci. 59:1484.

Kakade, M.L., Hoffa, D.E., and Liener, I.E. 1973. Contribution of trypsin inhibitor to the deleterious effects of unheated soybeans to rats. J. Nutr. 103:1772.

Kamath, S., and Berg, C. 1964. Antagonism of poorly invertible amino acids toward growth promotion by readily invertible D-amino acids. N. Nutr. 82:237.

Karayiannia, N. 1976. Lysinoalanine formation in alkali treated proteins and their biological effects. Ph.D. Thesis, University of California, Berkeley.

Katz, R.S. and Baker, D.H. 1975. Efficacy of D-, L-, and DL-methionine for growth of chicks fed crystalline amino acids. Poultry Sci. 54:1667.

Kehrberg, N.L. 1976. Chemical and molecular properties of processed soy proteins. Ph.D. Dissertation, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Khayanbashi, H. and Lyman, R.L. 1966. Growth depression and pancreatic and intestinal changes in rats force fed amino acid diets containing soybean trypsin inhibitor. J. Nutr. 89:455.

Kido, K. and Kassell, B. 1975. Oxidation of methionine residues of procine and bovine pepsins. Biochem. 14:631.

Kies, C., Fox, H., and Aprahamian. 1975. Comparative value of L-, DL-, and D-methionine supplementation of an oat-based diet for humans. J. Nutr. 105:809.

Kies, C. and Fox, H. 1971. Comparison of the protein nutritional value of TVP, emthionine-enriched TVP, and beef at two levels of intake for human adults. J. Food Sci. 36:341.

Klose, A.A., Hill, B., and Fevold, H.L. 1948. Food value of soybeans as related to processing. Food Technol. 2:201.

Klostermeyer, H. and Reimerdes, E.H. 1977. Heat induced crosslinks in milk proteins and consequences for the milk system. In "Protein Crosslinking," Part B, Nutritional and Medical Consequences, M. Friedman (Ed.), Plenum Press, New York.

Kohler, G.O. and Palter, R. 1967. Studies on methods for the amino acid analysis of wheat products. Cereal Chem. 44:512.

Korslund, M., Kies, C., and Fox, H.M. 1973. Comparison of the protein nutritional value of TVP, methionine-enriched TVP, and beef for adolescent boys. J. Food Sci. 39:637.

Krebs, H. 1935. Metabolism of amino acids. III. Deamination of amino acids. Biochem. J. 29:1620.

Kuzimicky, D.D., Kohler, G.O., Walker, H.G., Jr., and Mackey, B.E. 1974. Availability of oxidized sulfur amino acids for the growing chick. Poultry Sci. 53:1945.

Landers, R.E. 1975. Relationship between protein efficiency ratio of foods and relative nutritive value measured by _Tetrahymena pyriformis_ W bioassays techniques. In "Protein Nutritional Quality of Foods and Feeds," Part 1, M. Friedman (Ed.), Marcel Dekker, Inc., New York.

Lea, C.H., Parr, L.J., and Carpenter, K.J. 1958. Chemical and nutritional changes in stored herring meal. Br. J. Nutr. 12:297.

Lease, J.G. 1970. Availability to the chick of zinc-phytate complexes from oil seed meals by an _in vitro_ digestion method. J. Nutr. 93:523.

Levene, P.A. and Bass, L.W. 1929. Studies on racemization. VIII. The action of alkali on proteins: Racemization and hydrolysis. J. Biol. Chem. 82:171.

Levene, P.A. and Bass, L.W. 1928. Studies on racemization. VII. The action of alkali on casein. J. Biol. Chem. 78:145.

Lewis, J.H. and Taylor, F.H.L. 1947. Comparative utilization of raw and autoclaved soybean protein by the human. Proc. Soc. Exp. Biol. Med. 64:85.

Liener, I.E. 1977. Protease inhibitors and hemagglutinins of legumes. In "Evaluation of Proteins for Humans." C.E. Bodwell (Ed.), AVI Publishing Company, Westport, Conn.

Liener, I.E. 1975. Effects of anti-nutritional and toxic factors on the quality and utilization of legume proteins. In "Protein Nutritional Quality of Foods and Feeds," Part 2, M. Friedman (Ed.), Marcel Dekker, Inc., New York.

Liener, I.E. 1972. Nutritional value of food protein production. In Soybeans: Chemistry and Technology. Vol. 1. Proteins. A.K. Smith and S.J. Circle (Eds.), The AVI Publishing Company, Westport, Conn.

Lipton, S.H. and Bodwell, C.E. 1977. A rapid method for detecting chemical alteration of methionine. J. Agr. Food Chem. 25:1214.

Lipton, S.H. and Bodwell, C.E. 1975. Chemical approaches for estimating nutritionally available methionine. In "Protein Nutritional Quality in Foods and Feeds," Part 1, M. Friedman (Ed.), Marcel Dekker, Inc., New York.

Lombardini, J.B., Coulter, A.W., and Talalay, P. 1970. Analogues of methionine as substrates and inhibitors of the methionine adenosyltransferase reaction. Mol. Pharmacol. 6:481.

Lombardini, J.B., Burch, M.K., and Talalay, P. 1971. An enzymatic derivative double isopote assay for L-methionine. J. Biol. Chem. 246:4465.

Longenecker, J.B. and Lo, G.S. 1974. Protein digestibility and amino acid availability -- assessed by concentration changes of plasma amino acids. In "Nutrients in Processed Food, Proteins," P.L. White and D.C. Fletcher (Eds.), Publishing Sciences Group, Acton, Mass.

Longenecker, J.B., Martin, W.H., and Sarett. 1964. Improvement in the protein efficiency of soybean concentrates and isolates by heat treatment. J. Agr. Food Chem. 12:411.

Lunder, T.L. 1972. Determination of methionine sulfoxide content of intact protein based on rearrangement of sulfoxide with acetic anhydride. Anal. Biochem. 49:585.

Mallory, P.A. and Travis, J. 1973. Human pancreatic enzymes. Characterization of anionic human trypsin. Biochem. 12:2847.

Marable, N.L., Wesson, J.B., and Kennedy, B.W. 1980. Net optical rotation of hydrolyzates of alkali-treated soy flour. Qualitas Plantarum. In press.

Mattil, K.F. 1974. Composition, nutritional, and functional properties and quality criteria of soy protein concentrates and soy protein isolates. J. Am. Oil Chem. Soc. 51:81A.

Mauron, J. 1972. Influence of industrial and household handling on food protein quality. In "Protein and Amino Acid Functions," E.J. Bigwood (Ed.), Pergamon Press, Oxford.

Mauron, J. 1975. Nutritional evaluation of processed proteins. Deutsche Lebense. Rundsch. 71:27. (German).

McBee, L.E. and Marshall, R.T. 1978. Enzymatic estimation of available lysine. J. Food Sci. 43:1355.

McCarthy, R.E. and Sullivan, N.X. 1941. A new and highly specific colorimetric test for methionine. J. Biol. Chem. 141:871.

Meyer, E.W. 1967. Proc. Int. Conf. on Soybean Protein Foods, U.S. Dept. Agric. ARS-71, 142.

Miller, D.S. and Carpenter, K.J. 1964. Availability of sulfur amino acids in protein foods. 1. Total sulfur amino acid content in relation to sulfur and nitrogen balance studies with the rat. J. Sci. Food Agric. 15:810.

Miller, D.S., Carpenter, K.J., Morgan, C.B., and Boyne, A.W. 1965a. Availability of sulphur amino acids in protein foods. 2. Assessment of available methionine by chick and microbiological assays. Br. J. Nutr. 19:249.

Miller D.S. and Samuel, P. 1968. Methionine sparing compounds. Proc. Nutr. Soc. 27:21A.

Miller, D.S. and Samuel, P. 1970a. Effects of the addition of sulphur compounds to the diet on utilization of protein in young growing rats. J. Sci. Fd. Agric. 2:616.

Miller, E.L., Carpenter, K.J., and Milner, C.K. 1965b. Availability of sulphur amino acids in protein foods. 3. Chemical and nutritional changes in heated cod muscle. Br. J. Nutr. 19:547.

Miller, S.A., Tannenbaum, S.R., and Seitz, A.W. 1970b. Utilization of L-methionine sulfoxide by the rat. J. Nutr. 100:909.

Mitchell, H.H., Hamilton, T.S., and Beadles, J.R. 1945. The importance of commercial processing for the protein value of food products. J. Nutr. 29:13.

Moore, S. 1963. On the determination of cystine as cysteic acid. J. Biol. Chem. 238:235.

Morrison, A.B. and McLaughlan, J.M. 1972. Availability of amino acids in Foods. In "Protein and Amino Acid Functions," E.J. Bigwood (Ed.), Pergamon Press, Oxford.

Morse, E., Merrow, S., Keyser, D., and Clark, R. 1972. Comparative utilization of casein-lactalbumin and spun-soy protein diets by human subjects. Am. J. Clin. Nutr. 25:912.

Neumann, N.P. 1967. Analysis for methionine sulfoxides. Methods in Enzymology. Vol. XI. S.P. Colowick and N.E. Kaplan (Eds.). Academic Press, New York.

Neumann, N.P., Moore, S., and Stein, W.H. 1962. Modification of the methionine residues in ribonuclease. Biochem. 1:68.

Newberne, P.M. and Young, V.R. 1966. Effects of diets marginal in methionine and choline with and without vitamin B_{12} on rat liver and kidney. J. Nutr. 89:69.

Newberne, P.M., Rogers, A.E., and Wogan, G.N. 1968. Hepatorenal lesions in rats fed a low lipotrope diet and exposed to aflatoxin. J. Nutr. 94:331.

Nitsan, Z. and Liener, I.E. 1976. Studies of the digestibility and retention of nitrogen and amino acids in rats fed raw or heated soy flour. J. Nutr. 106:292.

Njaa, L.R. 1962a. Utilization of methionine sulfoxide and methionine sulfone by the young rat. Br. J. Nutr. 16:571.

Njaa, L.R. 1962b. Some problems related to detection of methionine sulfoxide in protein hydrolysates. Acta Chem. Scand. 16:1359.

O'Dell, B.L. 1979. Effect of soy protein on trace mineral avail-ability. In "Soy Protein and Human Nutrition." H.L. Wilcke, D.T. Hopkins, and D.H. Waggle (Eds.), Academic Press, Inc., New York.

Osborn, R.B. and Mendel, L.B. 1917. The use of soybean as food. J. Biol. Chem. 32:369.

Otterburn, M., Healy, M., and Sinclair, W. 1977. The formation, isolation, and importance of isopeptides in heated proteins. In "Protein Crosslinking," Part B, Nutritional and Medical Conse-quences, M. Friedman (Ed.), Plenum Press, New York.

Pan, F. and Tarver, H. 1967. Comparative studies on methionine, selenomethionine, and their ethyl analogues as substrates for methionine adenosyltransferase from rat liver. Arch. Biochem. Biophys. 119:429.

Parthasarathy, H.M., Doraiswamy, T.R., Panemangalore, M., Rao, M.N., Chandrasekhar, B.S., Swaminathan, M., Screenivasan, A., and Subrahmanyan, V. 1964. The effect of fortification of processed soya flour with DL-methionine hydroxy analogue or DL-methionine on the digestibility, biological value, and net protein utilization of the proteins as studied in children. Can. J. Biochem. 42:377.

Patten, J.R., Richards, E.A., and Pope, H. III. 1971. The effect of raw soybean on the pancreas of adult dogs. Proc. Soc. Exp. Biol. Med. 137:59.

Philips, W. and Berg, C. 1954. Effect upon growth of the D-isomer of synthetic mixtures of essential amino acids. J. Nutr. 53:481.

Pieniazek, D., Rakowska, M., Szkilladziowa, W., and Brabarek, Z. 1975a. Estimation of available methionine and cystein in proteins of food products by in vivo and in vitro methods. Br. J. Nutr. 34:175.

Pieniazek, D., Rakowska, M., and Kunachowica, H. 1975b. The participation of methionine and cystine in the formation of bonds resistant to the action of proteolytic enzymes in heated casein. Br. J. Nutr. 34:103.

Pollock, G. and Frommhagen, L. 1968. The extent of racemization of some amino acids in dilute alkali treated protein and soil humic and fulvic acid. Anal. Biochem. 24:18.

Poullain, B., Guisard, D., and Derby, G. 1972. Nutr. Met. 14:298.

Provansal, M.P., Cuq, J.L., and Cheftel, J.C. 1975. Chemical and nutritional modifications of sunflower proteins due to alkaline processing. Formation of amino acid cross-links and isomerization of lysine residues. J. Agric. Food Chemc. 23:938.

Przyrembel, H., Leupold, D., Toshberg, P., and Bremer, H.J. 1973. Amino acid excretion of premature infants receiving different amounts of protein. Clin. Chim. Acta 49:27.

Rackis, J.J. 1979. Discussion: Effect of soy protein on trace mineral availability. B.L. O'Dell (Author). In "Soy Protein and Human nutrition." H.L. Wilcke, D.T. Hopkins, and D.H. Waggle (Eds.), Academic Press, Inc., New York.

Rackis, J.J., McGhee, J.E., Honig, D.H., and Booth, A.N. 1975. Processing soybeans into foods: Selected aspects of nutrition and flavor. J. Am. Oil Chem. Soc. 52:249A.

Rackis, J.J. 1974. Biological and physiological factors in soybeans. Am. J. Oil Chem. Soc. 51:161A.

Rackis, J.J. 1966. Soybean trypsin inhibitors; their inactivation during meal processing. J. Food Technol. 20:102.

Rackis, J.J. 1965. Physiological properties of soybean trypsin inhibitors and their relationship to pancreatic hypertrophy and growth inhibition of rats. Fed. Proc. 24:1488.

Rackis, J.J., Smith, A.K., Nash, A.M., Robbins, D.J., and Booth, A.N. 1963. Feeding studies on soybeans. Growth and pancreatic hypertrophy in rats fed soybean meal fractions. Cereal Chem. 40:531.

Rakosky, J., Jr. 1970. Soy products for the meat industry. J. Agr. Food Chem. 18:1005.

Rakosky, J., Jr. 1974. Soy grits, concentrates and isolates in meat products. J. Am. Oil Chem. Soc. 51:123A.

Rao, N., Sreenivas, H., Swaminithan, M., Carpenter, K.J., and Morgan, C.B. 1963. J. Sci. Food Agr. 14:544.

Raymond, M.L. 1980. Studies concerning the determination of lysino-alanine in food proteins. J. Food Sci. 45:56.

Rexen, B. and Christensen, B. 1967. The determination of available lysine in foodstuffs. Automation in analytical chemistry. Proceedings of a symposium sponsored by Technicon Corp. Mediad Inc., White Plains, New York.

Reyneirs, J.P., Woodard, J.D., and Alvarez, M.R. 1974. Nuclear cytochemical alterations in alpha protein induced nephrocy-tometalia. Lab. Invest. 30:582.

Rios-Iriarte, B.J. and Barnes, R.H. 1966. The effect of over-heating on certain nutritional properties of the protein of soybeans. Food Technol. 20:131.

Rose, W.C., Coon, M.J., Lockhar, H.B. and Lambert, G.F. 1955. The amino acid requirements of man. XI. The threonine and methionine requirements. J. Biol. Chem. 215:101.

Rosenfield, D., and Hartman, W.E. 1974. Spun-fiber textured products. J. Am. Oil Chem. Soc. 51:91A.

Sarett, H.P. 1976. Soy-based infant formulas. In "World Soybean Research." L.D. Hill (Ed.), The Interstate Printers and Publishers, Inc., Danville, Illinois.

Satterlee, L.D., Kendrick, J.C., and Miller, G.A. 1977. Rapid in vitro assays for estimating protein quality. Food Technol. 31:78.

Savige, W.E. and Fontana, A. 1977. Interconversion of methionine and methionine sulfoxide. In "Methods in Enzymol." S.P. Colowick and N.O. Kaplan (Eds.). 47:453.

Schneider, D.L. and Sarett, H.P. 1969. Growth of baby pigs fed infants soybean formulas. J. Nutr. 98:279.

Scrimshaw, N.S. and Young, V.R. 1979. Soy protein in adult human nutrition: A review with new data. In "Soy Protein and Human Nutrition." H.L. Wilcke, D.T. Hopkins, and D.H. Waggle (Eds.), Academic Press, Inc., New York.

Scrimshaw, N.S. and Young, V.R. 1977. Nutritional evaluation and the utilization of protein resources, in Evaluation of proteins for humans. C.E. Bodwell (Ed.). The AVI Publishing Co., Westport, Conn. p. 2.

Shapiro, S.K. and Ehninger, D.J. 1966. Methods for the analysis of adenylmethionine and adenosylhomocysteine. Anal. Biochem. 15:323.

Shechter, Y., Burstein, Y., and Patchornik, A. 1975. Selective oxidation of methionine residues in proteins. Biochem. 14:4497.

Shemer, M., Wei, L.S., and Perkins, E.G. 1973. Nutritional and chemical studies of three processed soybean foods. J. Food Sci. 38:112.

Shorrock, C. and Ford, J.E. 1978. Metabolism of heat-damaged proteins in the rat: Inhibition of amino acid uptake by 'unavailable peptides' isolated from enzymic digest of heat-damaged cod fillet. Br. J. Nutr. 40:185.

Sjöberg, L.B. and Bostrom, S.L. 1977. Studies in rats on the nutritional value of hydrogen peroxide treated fish protein and the utilization of oxidized sulphur amino acids. Br. J. Nutr. 38:189.

Slump, P. and Schreuder, H.A.W. 1973. Oxidation of methionine and cystine in foods treated with hydrogen peroxide. J. Sci. Food Agric. 24:657.

Smith, A.K. and Circle, S.J. 1972. Protein products as food ingredients. In "Soybeans: Chemistry and Technology." AVI Publishing Company, Westport, Conn.

Sober, H.A. (Ed.). 1970. "Handbook of Biochemistry: Selected Data for Molecular Biology." 2nd edition, Chemical Rubber Company, Cleveland, Ohio.

Sokal, R.R. and Rohlf, F.J. 1967. "Biometry." W.H. Freeman and Company, San Francisco, California.

Sourkes, T.L. and Trano, Y. 1953. Reduction of methionine sulfoxides by Escherichia coli. Arch. Biochem. Biophys. 42:321.

Spackman, D.H., Stein, W.H., and Moore, S. 1958. Automatic recording apparatus for the use in the chromatography of amino acids. Anal. Chem. 30:1190.

Stahlmann, M.A. and Woldegiorgis, C. 1975. Enzymatic methods for protein quality. In "Protein Nutritional Quality of Foods and Feeds," Part 1. M. Friedman (Ed.). Marcel Dekker, Inc., New York.

Stegink, L.D., Shepherd, J.A., Fry, L.K., and Filer, L.J., Jr. 1974. Sugar-amino complexes in parenteral alimentation. Pediat. Res. 8:396.

Stegink, L.D., Freeman, J.B., Meyer, P.D., Filer, L.J., Jr., Dry, L.K., and Denbasten, L. 1975. Excessive trace metal ion excretion due to sugar-amino acid complexes during total parenteral nutrition. Fed. Proc. 34:931.

Steinke, F.H., Prescher, E.E., and Hopkins, D.T. 1980. Nutritional evaluation of isolated soybean protein and combinations of food proteins. J. Food Sci. 45:323.

Stekol, J.A. Formation and metabolism of S-adenosyl derivatives of S-alkylhomocysteine in the rat and mouse. In "Transmethylation and Methionine Biosynthesis." S.K. Shapiro and F. Schlenk (Eds.), The University of Chicago Press, Chicago, Illinois.

Sternberg, M. and Kim, C.Y. 1977. Lysinoalanine formation in protein food ingredients. In "Protein Crosslinking," Part B, Nutritional and Medical Consequences, M. Friedman (Ed.), Plenum Press, New York.

Struthers, B.J., Dahlgren, R.R., Hopkins, D.T., and Raymond, M.L. 1979. Lysinoalanine: Biological effects and significance. In "Soy Protein and Human Nutrition." H.L. Wilcke, D.T. Hopkins, and D.H. Waggle (Eds.), Academic Press, Inc., New York.

Struthers, B.J., Hopkins, D.T., Prescher, E.E., and Dahlgren, R.R. 1978. Effects of protein-bound lysinoalanine. N- DL-(2 amino-2 carboxyethyl)-L-lysine on fetal and neonatal rats. J. Nutr. 108:954.

Sturman, J.A., Gaull, G., and Raiha, N.C.R. 1970. Absence of cystathionase in human fetal liver: Is cystine essential? Science 169:74.

Tabor, H. and Tabor, C.W. 1971. Methods in Enzymology, Vol. 17, Part B. S.P. Colowick and N.E. Kaplan (Eds.). Academic Press, New York.

Taira, H., Kayanagi, T., Takanohashi, T., and Oikawa, K. 1969. Studies on amino acid contents of processed soybean. Part XI. Evaluation of nutritional losses of overheated defatted soybean flour. Agr. Biol. Chem. 33:1387.

Tallen, H.H. and Cohen, P.A. 1976. Methionine adenosyltransferase: Kinetic properties of human and rat liver enzymes. Biochem. Med. 16:234.

Tannenbaum, S.R., Barth, H., and LeRoux, J.P. 1969. Loss of methionine in casein during storage with auto-oxidizing methyl linoleate. J. Agr. Food Chem. 17:1353.

Taper, L.J., Marable, N.L., Korslund, M.K., and Ritchey, S.J. 1978. Utilization of protein from soy flour and soy isolate by adult women. J. Agr. Food Chem. 26:802.

Technicon Instrument Corporation. 1973. Operation Manual for the Technicon TSM System. Technical publication No. TA 1-0233-10, Tarrytown, New York.

Technicon Instrument Corporation. 1975. Operation Manual for the Technicon NC-1P. Technical publication No. TA 5-0341-00, Tarrytown, New York.

Theur, R.C. and Sarett, H.P. 1970. Nutritional adequacy of soy isolate infant formulas in rats: Choline. J. Agr. Food Chem. 18:913.

Thio, G.L. 1976. Small scale and home processing of soybeans with applications and recipes. Royal Tropical Institute, Amsterdam Dept. Agricultural Research. Communication 64. 1975. as quoted by Winarno and Karyadi.

Todd, J.M. 1978. Unpublished data.

Torun, B. 1979. Nutritional quality of soybean protein isolates: Studies in children of pre-school age. In "Soy Protein and Human Nutrition." H.L. Wilcke, D.T. Hopkins, and D.H. Waggle (Eds.), Academic Press, Inc., New York.

Travis, J. and Roberts, R.C. 1969. Human trypsin isolation and physical-chemical characterization. Biochem. 8:2884.

Tufte, M.C. and Warthesen, J.J. 1979. Methionine stability in methionine-fortified model food systems as influenced by method of methionine incorporation and by lipid oxidation. J. Food Sci. 44:1967.

Turk, R.E., Cornwell, P.E., Brooks, M.D., and Butterworth, C.E. 1973. J. Am. Diet Assoc. 63:519.

United Nations. 1968. International action to avert an impending protein crisis. UN publication E 68 1112. New York.

Varnish, S.A. and Carpenter, K.J. 1975a. Mechanisms of heat damage in proteins. 5. The nutritional values of heat-damaged and propiolylated proteins as sources of lysine, methionine, and tryptophan. Br. J. Nutr. 34:325.

Varnish, S.A. and Carpenter, K.J. 1975b. Mechanisms of heat damage in proteins. 6. The digestibility of individual amino acids in heated and propionylated proteins. Br. J. Nutr. 34:339.

Vaughan, D.A., Womack, M., and McClain, P.E. 1977. Plasma free amino acid levels in human subjects after meals containing lactalbumin, heated lactalbumin, or no protein. Am. J. Clin. Nutr. 30:1709.

Wachter, J. and Berg, C. 1960. Growth promotion by invertible D-amino acids in diets containing extraneous poorly invertible D-amino acids. J. Nutr. 70:31.

Waibel, P.E., Cuperlovic, M., Hurrell, R.F., and Carpenter, K.J. 1977. Processing damage to lysine and other amino acids in the manufacture of blood meal. J. Agric. Food Chem. 24:171.

Wang, Y.D., Miller, J., and Beuchat, L.R. 1979. Comparison of Tetrhymena pyriformis W, Aspergillus Flavus and rat bioassays for evaluating protein quality of selected commercially prepared food products. J. Food Sci. 44:1390.

Walker, H.G., Jr., Kohler, G.O., Kuzmicky, D.D., and Witt, S.C. 1975. Problems in analysis for sulfur amino acids. In "Protein Nutritional Quality of Foods and Feeds," Part 1. M. Friedman (Ed.), Marcel Dekker, Inc., New York.

Weisberg, S.M. 1974. Nutritional experience with infant formulas containing soy. J. Am. Oil Chem. Soc. 51:204A.

Weisberg, S.M. 1976. Food products intended to improve nutrition in the developing world in Advances in Food Research. C.E. Chichester, E.M. Mark, and G.F. Stewart (Eds.), Academic Press, New York.

Wesson, J. 1975. Racemization of alkali treated soybean protein. M.S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

White, J., Fones, W., and Sober, H. 1952. The utilization of D-valine for growth by rats. J. Biol. Chem. 199:505.

Winarno, F.G. and Karyadi, D. 1976. Nutrition and processing of soybeans. In "Expanding the Use of Soybeans," Proceedings of a conference for Asia and Oceania. R.M. Goodman (Ed.), International Agricultural Publications, University of Illinois, Urbana, Ill.

Wing, R.W. and Alexander, J.C. 1971. The heating of soybean meals by microwas radiation. Nutr. Reports Int'l. 4:387.

Wolf, W.J. 1977. Legumes: Seed composition and structure, processing into protein products, and protein properties. "In Food Proteins," J.R. Whitaker and S.R. Tannenbaum (Eds.). AVI Publishing Company, Westport, Conn.

Wolf, W.J. and Cowan, J.C. 1975. "Soybeans as a Food Source," 2nd ed., CRC Press Inc., Cleveland, Ohio.

Woodard, J.C. and Short, D.D. 1973. Toxicity of alkali-treated soy protein in rats. J. Nutr. 103:569.

Woodard, J.C. 1969. On the pathogenesis of alpha protein-induced nephrocytomegalia. Lab. Invest. 20:9.

Woodard, J.C. and Alvarez, N.R. 1967. Renal lesions in rats fed diets containing alpha protein. Arch. Path. 84:153.

Wretland, K. 1952. Effect of D-amino acids on the stereonaturalization of D-methionine. Acta. Physiol. Scand. 25:267.

Yanez, E., Vasquez, M. and Sanchez, F. 1979. Texturization of sunflower/soy flour mixtures: Chemical and nutritive evaluation. J. Food Sci. 44:1714.

Yen, J.T., Hymowitz, T., and Jensen, A.H. 1974. Effects of soybeans of different trypsin inhibitor activities on performance of growing swine. J. Animal Sci. 38:304.

Zahler, W.L. and Cleland, W.W. 1968. A specific and sensitive assay for disulfides. J. Biol. Chem. 243:716.

Zezulka, A.Y. and Calloway, D.H. 1976. Nitrogen retention in men fed isolated soybean protein supplemented with L-methionine, D-methionine, H-acetyl-L-methionine, or inorganic sulfate. J. Nutr. 106:128C.

APPENDIX 1

Structures of Sulfur Amino Acids and
Some of Their Derivatives

Methionine

$$CH_3-S-CH_2CH_2\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle COOH}{|}}{C}}-NH_2$$

Methionine Sulfoxide

$$CH_3-\overset{\overset{\displaystyle }{\underset{\underset{\displaystyle O}{||}}{S}}}{}-CH_2CH_2-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle COOH}{|}}{C}}-NH_2$$

Methionine Sulfone

$$CH_3-\overset{\overset{\displaystyle O}{\overset{||}{}}}{\underset{\underset{\displaystyle O}{||}}{S}}-CH_2CH_2-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle COOH}{|}}{C}}-NH_2$$

Cystine

$$H_2N-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle COOH}{|}}{C}}-CH_2-S-S-CH_2-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle COOH}{|}}{C}}-NH_2$$

Cysteine

$$HS-CH_2-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle COOH}{|}}{C}}-NH_2$$

Cysteic Acid

$$HO_3S-CH_2-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle COOH}{|}}{C}}-NH_2$$

S-adenosylmethionine
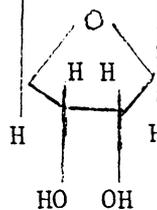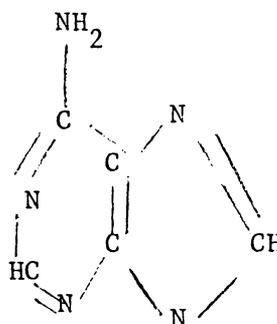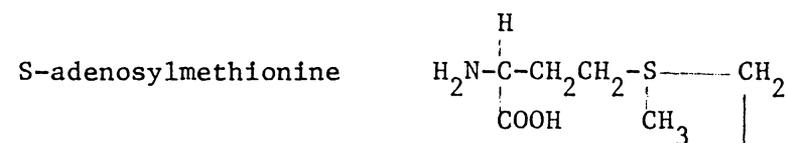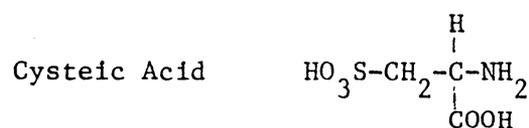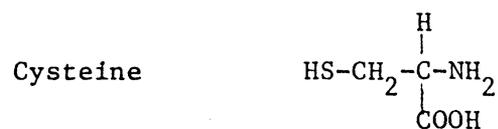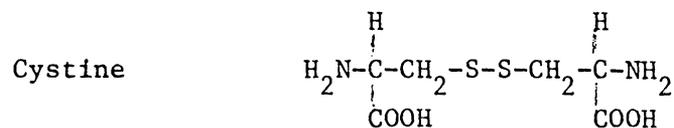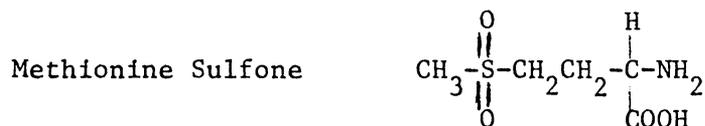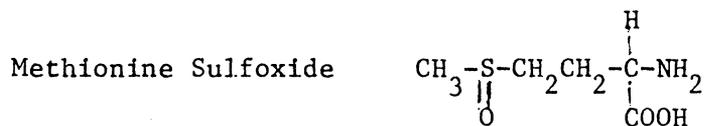
$$H_2N-\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle COOH}{|}}{C}}-CH_2CH_2-\overset{}{\underset{\underset{\displaystyle CH_3}{|}}{S}}-----CH_2$$

Indirect method for measuring methionine sulfoxide in a protein containing methionine, methionine sulfoxide, and methionine sulfone.

Procedure I

| Methionine   +   Methionine Sulfoxide   +   Methionine Sulfone |

Iodoacetic Acid

↓

| Carboxymethyl-sulfonium Salt   +   Methionine Sulfoxide   +   Methionine Sulfone |

Performic Acid
Oxidation

↓

| No Reaction   +   Methionine Sulfone   +   Methionine Sulfone |

Acid Hydrolysis

Ion Exchange Chromatography

↓

Methionine sulfone peak represents methionine sulfoxide + methionine sulfone
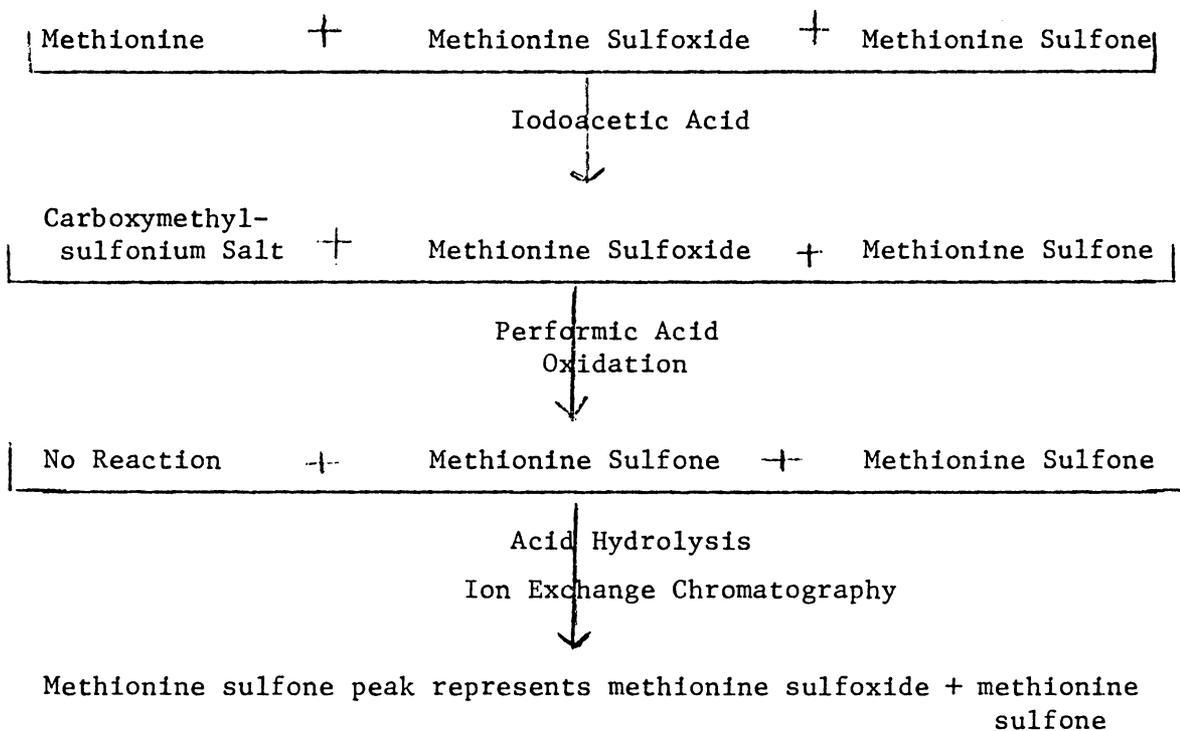
Procedure II

| Methionine   +   Methionine Sulfoxide   +   Methionine Sulfone |

Acid Hydrolysis

Ion Exchange Chromatography

↓

Methionine   +   [Methionine + Methionine Sulfoxide] + Methionine Sulfone

Methionine Sulfoxide = Methionine Sulfone (Procedure I) –

Methionine Sulfone (Procedure II)

APPENDIX 3

The amounts of amino acids present in a mixture of amino acids in 250 ml of 0.01 M HCl.

Amino Acid	Molecular Weight	Amount in Mixture (mg)	Micromoles/ml of Solution
Glycine	75.1	29.3	1.56
DL-Asparagine	132.1	52.1	1.58
DL-Alanine	89.1	∿50	∿2
DL-Valine	117.2	29.2	1.00
DL-Serine	105.1	30.5	1.16
DL-Glutamic Acid	147.1	38.0	1.03
DL-Tyrosine	181.2	27.4	0.60
DL-Proline	115.1	35.1	1.22
DL-Lysine HCl	182.7	17.0	0.37
DL-Threonine	119.1	40.0	1.34
DL-Histidine	191.6	33.6	0.70
DL-Tryptophan	204.2	∿25	∿0.5
DL-Phenylalanine	165.2	64.5	1.56
DL-Leucine	131.2	51.1	1.56
DL-Methionine Sulfoxide	165.2	46.8	1.13
DL-Cysteic Acid	169.2	25.7	0.61
DL-Methionine Sulfone	181.2	45.3	1.00
L-Methionine	149.2	104.3	2.80
L-Cystine	240.3	25.7	0.40
DL-Arginine HCl	210.7	∿30	∿0.6

Conditions used for elution of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid on the NC-2P Technicon Amino Acid Analyzer.

Samples: Dissolved in citrate buffer (pH 1.9, 0.2M Na^+).

Column: 0.5 cm X 23 cm

Resin: Type C-3 (Technicon)

Column Temperature: 55°C

Performic Acid Oxidation Determinations

   Flow Rate: 0.5 ml/minute (Flow rate may be lowered to 0.40 ml/min
                to improve separation of methionine sulfone from
                aspartic acid.)

   Buffer 1:   Citrate buffer (pH 2.40, 0.2M Na^+) -- 65 minutes. (If
                flow rate is 0.4 ml/minute, increase time to 75
                minutes.)

   Cysteic acid, methionine sulfone, and glutamic acid are eluted with this buffer, in that order. Glutamic acid may be used to back calculate sample size based on glutamic acid content of substance as determined by previous acid hydrolysis.

Chromatography of Acid Hydrolysates

   Flow Rate: 0.45 ml/minute. (If methionine sulfone is present flow
                rate may be lowered to 0.40 ml/minute to improve
                separation from aspartic acid.)

   Buffer 1:   Citrate buffer (pH 2.36 to 2.41, 0.2M Na^+) -- 35 minutes.

   Buffer 2:   Citrate buffer (pH 3.25, 0.2M Na^+) -- 16 minutes.

   Buffer 3:   Citrate buffer (pH 3.61, 0.2M Na^+) -- 30 minutes.

   Cysteic acid, methionine sulfoxide, and methionine sulfone are eluted with first buffer. Methionine is eluted with third buffer.

   If glutamic acid content is being determined, increase time of first buffer to 65 or 75 minutes depending on flow rate.

Chromatography of Alkaline Hydrolysates -- Low Carbohydrate Content

   Flow Rate: 0.45 to 0.50 ml/minute.

   Buffer 1:   Citrate buffer (pH 2.36 to 2.41, 0.2M Na^+) -- 35 minutes.

   Buffer 2:   Citrate buffer (pH 3.25, 0.2M Na^+) -- 16 minutes.

   Buffer 3:   Citrate buffer (pH 3.61, 0.2M Na^+) -- 30 minutes.

Methionine sulfoxide and methionine sulfone are eluted with the first buffer. Methionine is eluted with the third buffer and separates well from alloisoleucine which is present in alkaline hydrolysates.

Chromatography of Alkaline Hydrolysates -- High Carbohydrate Content

(1) Determination of Methionine Sulfoxide

Flow Rate: 0.40 ml/minute.

Buffer 1: Citrate buffer (pH 2.70, 0.2M Na$^+$) -- 35 minutes.

Buffer 2: Citrate buffer (pH 3.25, 0.2M Na$^+$) -- 16 minutes.

Buffer 3: Citrate buffer (pH 3.61, 0.2M Na$^+$) -- 30 minutes.

Methionine sulfoxide is eluted with first buffer. This results in improved separation from compounds which are present in alkaline hydrolysates high in carbohydrate content and which tend to co-elute with methionine sulfoxide under the usual conditions of elution. Methionine is eluted with the third buffer.

(2) Determination of Methionine Sulfone

Flow Rate: 0.40 to 0.45 ml/minute.

Buffer 1: Citrate buffer (pH 2.36 to 2.41, 0.2M Na$^+$) --

35 minutes.

Methionine sulfone is eluted with first buffer. If both methionine sulfoxide and methionine sulfone are to be determined in alkaline hydrolysates high in carbohydrates, (especially simple sugars) hydrolysate should be chromatographed twice using conditions described in (1) and (2).

Preparation of Buffers and Other Amino Acid Analyzer Solutions

Solutions and buffers were prepared according to directions in the TSM Manual (Technicon Corporation). Pentachlorophenol was used in buffers and in hydrazine sulfate as a preservative.

Conditions used for elution of methionine, methionine sulfoxide, and methionine sulfone on the TSM Technicon Amino Acid Analyzer.

Samples: Dissolved in citrate buffer (pH 1.9, 0.2M Na$^+$).

Column: 0.5 cm X 40.0 cm

Resin: Type C-3 (Technicon)

Flow Rate: 0.41 ml/minute

Column Temperature: 52°C

Buffer 1: Citrate buffer (pH 2.68, 0.3M Li$^+$) -- 23 minutes

Buffer 2: Citrate buffer (pH 2.61, 0.3M Li$^+$) -- 59 minutes

Buffer 3: Citrate buffer (pH 3.30 0.3M Li$^+$) -- 28 minutes

Buffer 4: Citrate buffer (pH 4.15, 0.3M Li$^+$) -- 55 minutes

Buffers were prepared from directions described by Technicon Corporation (TSM Manual). Standard protocol for application of the samples to the columns was used. Methionine sulfoxide and methionine sulfone were eluted with the second buffer and methionine with the fourth buffer.

Percent recovery of selected amino acids hydrolyzed individually under two sets of alkaline hydrolytic conditions and in the presence or absence of glucose.

2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C
Without Glucose	With Glucose	Without Glucose
Methionine	Methionine	Methionine

Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery
1	94.9	1	103.8	1	96.3
2	94.2	2	96.1	2	89.1
3	99.4	3	94.1	3	88.1
4	89.6	4	104.0	4	95.4
5	98.0	5	97.9	5	96.2
6	95.3			6	97.1
7	92.6				

Mean = 94.8
S.D. = 3.3

Mean = 99.2
S.D. = 4.5

Mean = 93.7
S.D. = 4.0

2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C
Without Glucose	With Glucose	Without Glucose
Methionine Sulfoxide	Methionine Sulfoxide	Methionine Sulfoxide
Sample Number — Percent Recovery	Sample Number — Percent Recovery	Sample Number — Percent Recovery
1.......... 96.3	1.......... 86.0	1.......... 67.3
2.......... 90.3	2.......... 83.4	2.......... 68.7
3.......... 89.5	3.......... 84.5	3.......... 69.2
4.......... 87.4	4.......... 91.0	4.......... 68.5
5.......... 85.8	5.......... 82.8	5.......... 64.3
6.......... 89.5	6.......... 86.1	
Mean = 89.8 S.D. = 3.6	Mean = 85.6 S.D. = 2.9	Mean = 67.6 S.D. = 2.0
Methionine Sulfone	Methionine Sulfone	Methionine Sulfone
1..........103.0	1..........110.5	1..........103.3
2.......... 99.2	2..........104.0	2.......... 95.3
3.......... 95.3	3..........100.6	3..........103.0
4..........104.0	4.......... 98.7	
5..........104.0		
Mean = 101.1 S.D. = 3.8	Mean = 103.4 S.D. = 5.2	Mean = 100.5 S.D. = 4.5

2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C
Without Glucose	With Glucose	Without Glucose
Cysteic Acid	Cysteic Acid	Cysteic Acid

Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery
1	80.0			1	70.7
2	75.9	NOT DONE		2	74.1
3	74.5			3	82.2
4	83.2			4	79.5
5	74.6				

Mean = 77.6
S.D. = 3.8

Mean = 76.6
S.D. = 5.2

2M NaOH, 18 Hours, 100°C

(Without Glucose)

Valine		Leucine		Phenylalanine	
Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery
1	99.3	1	99.6	1	97.3
2	91.1	2	97.1	2	91.6
3	86.2	3	99.6	3	92.4
4	87.0	4	93.4	4	97.9
5	92.0	5	92.0		
6	93.7	6	90.6		
		7	86.5		

Mean = 91.6
S.D. = 4.8

Mean = 94.1
S.D. = 4.9

Mean = 94.8
S.D. = 3.2

Percent recovery of selected amino acids hydrolyzed in a mixture of five amino acids under two sets of alkaline hydrolytic conditions.

2M NaOH, 18 Hours, 100°C				3M NaOH, 16 Hours, 110°C			
Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery
Methionine		Methionine Sulfoxide		Methionine		Methionine Sulfoxide	
1	74.2			1	79.5		
2	75.9	1	86.6	2	86.5	1	74.0
3	76.9	2	83.8	3	72.8	2	74.5
4	75.3	3	84.9	4	72.2	3	87.1
5	77.1	4	80.7	5	78.2	4	82.7
6	77.8	5	90.2			5	90.0
		6	87.9			6	91.3
Mean = 76.2		Mean = 85.7		Mean = 77.8		Mean = 83.3	
S.D. = 1.3		S.D. = 3.3		S.D. = 5.8		S.D. = 7.6	
Methionine Sulfone		Cysteic Acid		Methionine Sulfone		Cysteic Acid	
1	100.0	1	168.3	1	102.5	1	117.3
2	89.9	2	155.0	2	105.3	2	127.2
3	96.8	3	212.9	3	92.9	3	176.2
4	95.6	4	150.9	4	95.5	4	181.0
5	91.5	5	198.2	5	83.9		
6	102.2	6	214.5	6	104.0		
Mean = 96.0		Mean = 183.3		Mean = 97.4		Mean = 150.4	
S.D. = 4.8		S.D. = 28.8		S.D. = 8.2		S.D. = 32.8	

Percent recovery of selected amino acids hydrolyzed in a mixture of 20 amino acids under two sets of alkaline hydrolytic conditions and in the presence and absence of glucose.

2M NaOH, 18 Hours, 100°C				3M NaOH, 16 Hours, 110°C	
Without Glucose		With Glucose		Without Glucose	
Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery
Methionine		Methionine		Methionine	
1	87.0	1	88.6	1	88.9
2	85.9	2	88.2	2	85.1
3	87.8	3	95.9	3	80.2
4	93.6			4	79.2
5	91.2				
Mean = 89.1		Mean = 90.9		Mean = 83.4	
S.D. = 3.2		S.D. = 4.3		S.D. = 4.5	
Methionine Sulfoxide		Methionine Sulfoxide		Methionine Sulfoxide	
1	97.4	1	99.7	1	90.2
2	98.8	2	99.7	2	92.2
3	102.5	3	102.0	3	86.0
4	101.0	4	106.0	4	88.0
5	102.0			5	96.0
Mean = 100.3		Mean = 101.8		Mean = 90.5	
S.D. = 2.2		S.D. = 3.0		S.D. = 3.9	

2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C

Without Glucose		With Glucose		Without Glucose	
Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery
Valine		**Valine**		**Valine**	
1	92.7	1	94.8	1	102.4
2	91.3	2	81.5	2	97.7
3	97.8	3	94.3	3	90.5
4	100.0	4	100.9	4	86.3
5	95.4			5	89.1
Mean = 95.4		Mean = 92.9		Mean = 93.2	
S.D. = 3.6		S.D. = 8.2		S.D. = 6.6	
Leucine		**Leucine**		**Leucine**	
1	101.5	1	92.6	1	86.8
2	100.2	2	98.3	2	91.2
3	101.3	3	99.5	3	88.7
4	100.0	4	95.2	4	88.7
5	95.6			5	95.0
Mean = 99.7		Mean = 96.4		Mean = 90.1	
S.D. = 2.4		S.D. = 3.1		S.D. = 3.1	

2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C

Without Glucose		With Glucose		Without Glucose	
Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery
Methionine Sulfone		**Methionine Sulfone**		**Methionine Sulfone**	
1	106.8	1	102.8	1	97.3
2	102.0	2	96.1	2	101.2
3	105.7	3	101.0	3	94.5
4	106.0	4	111.4	4	92.8
5	103.0			5	103.0
Mean = 104.7		Mean = 102.8		Mean = 97.8	
S.D. = 2.1		S.D. = 6.4		S.D. = 4.3	
Cysteic Acid		**Cysteic Acid**		**Cysteic Acid**	
1	153.5	1	159.1	1	197.7
2	127.2	2	127.2	2	151.1
3	175.0	3	130.3	3	129.7
4	184.0	4	136.7	4	140.4
5	169.8				
Mean = 161.9		Mean = 138.3		Mean = 154.7	
S.D. = 22.3		S.D. = 14.4		S.D. = 30.0	

Percent recovery of selected amino acids hydrolyzed under acidic conditions individually or in a mixture of five amino acids.

6M HCl, 18 Hours, 110°C

Hydrolyzed Individually				Hydrolyzed in a Mixture			
Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery	Sample Number	Percent Recovery
Methionine		Methionine Sulfoxide		Methionine		Methionine Sulfoxide	
1	86.8	1	79.4	1	104.0	1	81.9
2	112.0	2	83.9	2	114.0	2	86.3
3	95.8	3	71.8	3	96.8	3	87.7
4	98.0	4	78.0				
5	90.0						
6	85.9						
Mean = 94.8		Mean = 78.3		Mean = 104.9		Mean = 85.3	
S.D. = 9.7		S.D. = 5.0		S.D. = 8.6		S.D. = 3.0	
Methionine Sulfone		Cystine		Methionine Sulfone		Cystine	
1	96.2	1	75.2	1	111.6	1	92.8
2	96.7	2	88.2	2	107.1	2	89.5
3	100.0	3	86.6	3	102.0	3	91.8
4	112.0	4	59.0				
5	83.7	5	68.9				
Mean = 97.7		Mean = 75.6		Mean = 106.9		Mean = 91.4	
S.D. = 10.1		S.D. = 12.2		S.D. = 4.8		S.D. = 1.7	

6M HCl, 18 Hours, 110°C

Hydrolyzed Individually		Hydrolyzed in a Mixture	
Sample Number	Percent Recovery	Sample Number	Percent Recovery
Cysteic Acid		Cysteic Acid	
1.............	99.2	1.............	119.0
2.............	95.0	2.............	124.3
3.............	102.4	3.............	122.8
4.............	106.0		
5.............	87.7		
6.............	101.0		

Mean = 98.5   Mean = 122.0

S.D. = 6.4   S.D. =   2.7

Recovery of selected amino acids after alkaline hydrolysis of lysozyme under two sets of conditions and two ratios of protein to base.

2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C
10 mg Lysozyme/2.0 ml Base	**10 mg Lysozyme/5.0 ml Base**	**10 mg Lysozyme/2.0 ml Base**
Sample Number mmoles/100g	Sample Number mmoles/100g	Sample Number mmoles/100g
Methionine	**Methionine**	**Methionine**
1.............. 12.71	1.............. 13.66	1.............. 10.85
2.............. 13.92	2.............. 14.10	2.............. 11.32
3.............. 12.71	3.............. 11.26	3.............. 11.08
4.............. 13.92	4.............. 11.32	4.............. 11.38
		5.............. 9.38
		6.............. 12.88
Mean = 13.32	Mean = 12.58	Mean = 11.15
S.D. = 0.70	S.D. = 1.51	S.D. = 1.12
Valine	**Valine**	**Valine**
1.............. 11.08	1.............. 11.36	1.............. 21.11
2.............. 12.97	2.............. 10.85	2.............. 20.75
3.............. 11.25	3.............. 11.10	3.............. 22.40
4.............. 11.91	4.............. 10.84	4.............. 22.50
		5.............. 20.25
		6.............. 20.88
Mean = 11.80	Mean = 11.04	Mean = 21.31
S.D. = 0.86	S.D. = 0.25	S.D. = 0.92

| | 2M NaOH, 18 Hours, 100°C | | 3M NaOH, 16 Hours, 110°C |

<table>
<tr><td colspan="2">2M NaOH, 18 Hours, 100°C</td><td>3M NaOH, 16 Hours, 110°C</td></tr>
<tr><td>10 mg Lysozyme/2.0 ml Base</td><td>10 mg Lysozyme/5.0 ml Base</td><td>10 mg Lysozyme/2.0 ml Base</td></tr>
<tr><td>Sample mmoles/100g
Number</td><td>Sample mmoles/100g
Number</td><td>Sample mmoles/100g
Number</td></tr>
<tr><td>Leucine
1............. 34.60
2............. 35.85
3............. 38.44
4............. 37.71</td><td>Leucine
1............. 36.79
2............. 36.11
3............. 37.65
4............. 36.13</td><td>Leucine
1............. 44.69
2............. 44.69
3............. 45.75
4............. 43.88
5............. 49.88
6............. 46.12</td></tr>
<tr><td>Mean = 36.65
S.D. = 1.75</td><td>Mean = 36.67
S.D. = 0.72</td><td>Mean = 45.84
S.D. = 2.14</td></tr>
<tr><td>Phenylalanine
1............. 10.38
2............. 10.81
3............. 11.32
4............. 10.88</td><td>Phenylalanine
1............. 10.40
2............. 12.10
3............. 10.31
4............. 10.07</td><td>Phenylalanine
1............. 15.92
2............. 14.50
3............. 15.92
4............. 15.12
5............. 17.50
6............. 15.62</td></tr>
<tr><td>Mean = 10.85
S.D. = 0.38</td><td>Mean = 10.72
S.D. = 0.93</td><td>Mean = 15.76
S.D. = 1.01</td></tr>
</table>

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Recoveries of selected amino acids from lysozyme which has been acid hydrolyzed or subjected to performic acid oxidation prior to acid hydrolysis.

6M HCl, 24 Hours, 110°C				Performic Acid Oxidation	
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Expt. I		Expt. II			
Methionine		Methionine		Methionine Sulfone	
1	12.74	1	13.49		
2	14.03	2	14.03	1	13.99
3	11.20	3	13.29	2	11.99
4	13.56	4	13.25	3	13.25
5	12.50			4	13.40
6	11.56				
Mean = 12.60		Mean = 13.52		Mean = 13.16	
S.D. = 1.10		S.D. = 0.36		S.D. = 0.84	
Valine		Glutamic Acid		Cysteic Acid	
1	35.53				
2	37.74	1	40.71	1	35.93
3	38.09	2	40.58	2	33.93
4	41.74	3	39.08	3	28.91
5	40.09	4	37.72	4	29.30
6	41.27				
Mean = 39.08		Mean = 39.52		Mean = 32.02	
S.D. = 2.38		S.D. = 1.41		S.D. = 3.46	

6M HCl, 24 Hours, 110°C		Performic Acid Oxidation		
Sample Number	mmoles/100g	Sample Number	nmoles/sample	Weight of Sample[1] (mg)
Expt. I				
Leucine		**Glutamic Acid**		
1...............	59.08	1...............	3298.5...............	8.34
2...............	56.01	2...............	4024.1...............	10.18
3...............	57.55	3...............	4024.1...............	10.18
4...............	56.96	4...............	5622.4...............	14.23
5...............	50.94			
6...............	58.25			

Mean = 56.47
S.D. = 2.90

Phenylalanine	
1...............	18.28
2...............	21.22
3...............	19.58
4...............	20.64
5...............	21.22
6...............	20.87

Mean = 20.30
S.D. = 1.16

[1] Weight of performic acid samples backcalculated based on glutamic acid content of samples compared to glutamic acid content of lysozyme which had been acid hydrolyzed.

Recovery of methionine, methionine sulfoxide, and methionine sulfone from lysozyme which was hydrolyzed with base in the presence or absence of glucose.

2M NaOH, 18 Hours, 100°C

Without Glucose		With Glucose	
NC-2P Analyzer	TSM Analyzer[1]	NC-2P Analyzer	TSM Analyzer
Sample mmoles/100g Number	Sample mmoles/100g Number	Sample mmoles/100g Number	Sample mmoles/100g Number
Methionine	Methionine	Methionine	Methionine
1............ 10.78	1............ 12.02	1............ 10.26	1............ 12.91
2............ 11.63	1............ 10.85	2............ 10.98	2............ 7.58
3............ 11.49	4............ 12.06	3............ 11.05	4............ 10.92
4............ 11.73	4............ 12.05		5............ 12.97
	5............ 12.53		
	6............ 9.85		
Mean = 11.41 S.D. = 0.43	Mean = 11.56 S.D. = 1.01	Mean = 10.76 S.D. = 0.44	Mean = 11.10 S.D. = 2.53
Methionine Sulfoxide	Methionine Sulfoxide	Methionine Sulfoxide	Methionine Sulfoxide
None Present	None Present	1............ 4.22	1............ 1.12
		2............ 4.26	2............ 0.53
		3............ 3.92	4............ 0.80
			5............ 1.07
			6............ 1.30
		Mean = 4.13 S.D. = 0.19	Mean = 0.96 S.D. = 0.30

[1] Sample numbers for the NC-2P and TSM runs identify the same sample.

Without Glucose		With Glucose	
NC-2P Analyzer	TSM Analyzer	NC-2P Analyzer	TSM Analyzer
Sample mmoles/100g Number	Sample mmoles/100g Number	Sample mmoles/100g Number	Sample mmoles/100g Number

Methionine
Sulfone

    No methionine sulfone detected in any samples on either analyzer

Valine	Valine	Valine	Valine
1.......... 11.51 2.......... 11.25 3.......... 11.04 4.......... 10.41	Not Determined	1.......... 11.79 2.......... 12.73 3.......... 9.84	Not Determined

Mean = 11.05
S.D. = 0.47

Mean = 11.45
S.D. = 1.47

Leucine	Leucine	Leucine	Leucine
1.......... 42.13 2.......... 45.92 3.......... 44.60 4.......... 46.95	Not Determined	1.......... 41.83 2.......... 40.76 3.......... 42.26	Not Determined

Mean = 44.90
S.D. = 2.08

Mean = 41.62
S.D. = 0.77

Recovery of selected amino acids from oxidized lysozyme after hydrolysis under two sets of alkaline hydrolytic conditions.

2M NaOH, 18 Hours, 100°C				3M NaOH, 16 Hours, 110°C			
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine		Methionine Sulfoxide		Methionine		Methionine Sulfoxide	
1	10.78			1	9.27		
2	10.41	1	3.6	2	9.46	1	3.34
3	9.96	2	3.81	3	9.01	2	3.28
4	10.16	3	3.64	4	8.72	3	3.63
		4	4.17			4	3.90
Mean = 10.33		Mean = 3.80		Mean = 9.12		Mean = 3.54	
S.D. = 0.35		S.D. = 0.26		S.D. = 0.32		S.D. = 0.28	
Valine		Leucine		Valine		Leucine	
1	15.75	1	43.11	1	29.26	1	49.76
2	16.09	2	41.96	2	24.38	2	47.02
3	15.82	3	41.49	3	22.80	3	47.27
4	15.56	4	40.25	4	25.06	4	46.18
Mean = 15.80		Mean = 41.70		Mean = 25.38		Mean = 47.56	
S.D. = 0.22		S.D. = 1.18		S.D. = 2.76		S.D. = 1.54	

Recovery of methionine, methionine sulfoxide, and methionine sulfone from oxidized lysozyme hydrolyzed with 2M NaOH for 18 hours at 100°C in the presence or absence of glucose.

2M NaOH, 18 Hours, 100°C					
Without Glucose		With Glucose			
(NC-2P)		(NC-2P)		(TSM)[1]	
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine		**Methionine**		**Methionine**	
1...............	7.54	1...............	7.60	1...............	7.73
2...............	7.37	2...............	7.08	1...............	8.32
3...............	7.31	3...............	7.24	3...............	6.85
4...............	6.98	4...............	6.57	4...............	9.79
		5...............	7.04		
Mean = 7.30		Mean = 7.11		Mean = 8.17	
S.D. = 0.23		S.D. = 0.37		S.D. = 1.24	
Methionine Sulfoxide		**Methionine Sulfoxide**		**Methionine Sulfoxide**	
1...............	5.17	1...............	8.71	3...............	4.49
2...............	6.00	2...............	8.33		
3...............	4.91	3...............	8.01		
4...............	5.57				
Mean = 5.41		Mean = 8.35			
S.D. = 0.48		S.D. = 0.35			

No methionine sulfone in any samples on either analyzer.

[1] Sample numbers for the NC-2P and TSM runs identify the same sample.

APPENDIX 15

Recovery of methionine, methionine sulfoxide, methionine sulfone, cysteic acid, and total cystine
after acid hydrolysis or performic acid oxidation of oxidized lysozyme.

	6M HCl, 24 Hours, 110°C				Performic Acid Oxidation			
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine		Cysteic Acid		Methionine Sulfone		Cysteic Acid	
1...........	15.04			1...........	14.85		
2...........	14.10	1...........	1.78	2...........	15.98	1...........	35.76
3...........	15.28	2...........	1.65	3...........	14.76	2...........	34.19
4...........	14.03	3...........	1.70	4...........	15.63	3...........	32.60
		4...........	1.71			4...........	32.00
Mean = 14.61		Mean = 1.71		Mean = 15.30		Mean = 33.64	
S.D. = 0.64		S.D. = 0.05		S.D. = 0.60		S.D. = 1.69	

No methionine sulfoxide or methionine sulfone detected in acid hydrolysates.

Recovery of selected amino acids from casein subjected to acid hydrolysis or alkaline hydrolysis under two sets of alkaline hydrolytic conditions.

Experiment I

2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C		6M HCl, 24 Hours, 110°C	
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine		**Methionine**		**Methionine**	
1	14.09	1	8.38	1	15.26
2	13.64	2	12.14	2	16.69
3	14.64	3	11.82	3	18.57
4	14.20	4	8.80	4	17.16
5	13.87			5	16.83
				6	22.0
				7	17.18
Mean = 14.09		Mean = 10.28		Mean = 17.67	
S.D. = 0.38		S.D. = 1.97		S.D. = 2.14	

Methionine Sulfoxide and **Sulfone** ≤ 0.5 mmoles per 100 grams in all samples; peaks too small to be quantitatively measured.

Valine		Valine		Valine	
1	10.00	1	12.01	1	51.13
2	10.53	2	13.60	2	52.58
3	9.65	3	12.91	3	55.14
4	9.62	4	10.50	4	50.11
5	10.10				
Mean = 9.98		Mean = 12.26		Mean = 52.24	
S.D. = 0.37		S.D. = 1.34		S.D. = 2.18	

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2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C		6M HCl, 24 Hours, 110°C	
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Leucine		**Leucine**		**Leucine**	
1...............	49.98	1...............	35.06	1...............	66.25
2...............	53.33	2...............	41.94	2...............	72.12
3...............	42.46	3...............	40.28	3...............	68.16
4...............	44.75	4...............	31.33	4...............	63.66
5...............	43.36				
Mean = 46.78		Mean = 37.15		Mean = 67.55	
S.D. = 4.68		S.D. = 4.86		S.D. = 3.56	
Phenylalanine		**Phenylalanine**		**Phenylalanine**	
Not Determined		Not Determined		1...............	26.88
				2...............	26.51
				3...............	30.08
				4...............	27.48
				Mean = 27.74	
				S.D. = 1.61	

APPENDIX 17

Recovery of selected amino acids from casein hydrolyzed with 2M NaOH for 18 hours at 100°C in the presence or absence of glucose.

2M NaOH, 18 Hours, 100°C

Without Glucose		With Glucose	
(NC-2P)	(TSM)[1]	(NC-2P)	(TSM)
Sample Number / mmoles/100g	Sample Number / mmoles/100g	Sample Number / mmoles/100g	Sample Number / mmoles/100g

Experiment II

Methionine

Without Glucose (NC-2P)	(TSM)	With Glucose (NC-2P)	(TSM)
1.......... 7.91	2.......... 10.90	1.......... 7.74	2.......... 9.07
2.......... 6.48	2.......... 12.06	2.......... 9.93	3.......... 9.06
3.......... 8.73		3.......... 8.27	
		4.......... 7.87	
Mean = 7.71	Mean = 11.48	Mean = 8.45	Mean = 9.06
S.D. = 1.14	S.D. = 0.82	S.D. = 1.01	S.D. = 0.007

Methionine Sulfoxide

(NC-2P)	(TSM)	(NC-2P)	(TSM)
1.......... 0.43	2.......... 0.80	1.......... 1.48	2.......... 1.47
2.......... 0.26	2.......... 0.83	2.......... 1.66	3.......... 1.48
3.......... 0.38		3.......... 1.65	
4.......... 0.39		4.......... 1.68	
Mean = 0.36	Mean = 0.82	Mean = 1.62	Mean = 1.48
S.D. = 0.07	S.D. = 0.02	S.D. = 0.09	S.D. = 0.007

[1]Sample numbers for the NC-2P and TSM runs identify the same sample.

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Recovery of selected amino acids from casein hydrolyzed with 2M NaOH for 18 hours at 100°C in the presence or absence of glucose.

2M NaOH, 18 Hours, 100°C

Without Glucose	With Glucose
(NC-2P) Experiment I	**(NC-2P)**
Sample mmoles/100g	Sample mmoles/100g
Number	Number

Methionine

Without Glucose	With Glucose
1.............14.09	1............. 9.63
2.............13.64	2............. 8.60
3.............14.64	3............. 9.30
4.............13.87	4............. 9.54
5.............14.20	5.............10.33
Mean = 14.09, S.D. = 0.38	Mean = 9.48, S.D. = 0.62

Methionine Sulfoxide

Without Glucose	With Glucose
1............. 0.48	1............. 3.06
2............. 0.53	2............. 3.43
3............. 0.50	3............. 3.96
4............. 0.60	4............. 3.38
5............. 0.50	5............. 4.25
Mean = 0.52, S.D. = 0.05	Mean = 3.62, S.D. = 0.48

Trace(<0.05) of methionine sulfone present with and without glucose

Valine

Without Glucose	With Glucose
1.............10.00	1............. 5.73
2.............10.53	2............. 5.52
3............. 9.65	3............. 6.05
4............. 9.62	4............. 5.84
5.............10.10	5............. 6.35
Mean = 9.98, S.D. = 0.37	Mean = 5.90, S.D. = 0.32

Leucine

Without Glucose	With Glucose
1.............49.98	1.............30.56
2.............53.33	2.............29.75
3.............42.46	3.............30.40
4.............44.75	4.............25.98
5.............43.36	5.............28.48
Mean = 46.78, S.D. = 4.68	Mean = 29.03, S.D. = 1.89

Recovery of selected amino acids after acid hydrolysis of casein and after performic acid oxidation of casein.

6M HCl, 24 Hours, 110°C		Performic Acid Oxidation			
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Glutamic Acid		Methionine Sulfone		Cysteic Acid	
1............128.61		1............21.94		1............3.79	
2............135.89		2............20.41		2............3.03	
3............133.51		3............16.63		3............3.22	
		4............18.17		4............3.28	
		5............18.24		5............3.14	
Mean = 132.67		Mean = 19.08		Mean = 3.29	
S.D. = 3.71		S.D. = 2.09		S.D. = 0.29	

Methionine	Glutamic Acid	nmoles/sample	Weight of Sample[1] (mg)
	1............17835.3		............13.44
	2............ 8015.85		.... 6.04
	3............12607.65		.... 9.5
	4............16920.9		............12.75
	5............14886.0		............11.22

[1]Weight of samples backcalculated based on glutamic acid content of sample compared to glutamic acid content of casein as determined by acid hydrolysis.

APPENDIX 20

Recovery of methionine, methionine sulfoxide, and methionine sulfone
from oxidized casein hydrolyzed under two sets of alkaline hydrolytic
conditions.

2M NaOH, 18 Hours, 100°C		3M NaOH, 16 Hours, 110°C	
Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine		**Methionine**	
Trace		None Detected	

Methionine Sulfoxide		**Methionine Sulfoxide**	
1......................13.99		1.....................13.98	
2......................12.67		2.....................13.26	
3......................13.92		3.....................13.37	
4......................13.68		4.....................13.68	
5......................13.23		5.....................14.06	
6......................12.40			
7......................13.48			
8......................14.34			

Mean = 13.46 ± 0.67   Mean = 13.67 ± 0.36

Methionine Sulfone		**Methionine Sulfone**	
None Detected		None Detected	

APPENDIX 21

Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid after acid hydrolysis of oxidized casein and after performic acid oxidation.

6M HCl, 24 Hours, 110°C		Performic Acid Oxidation	
Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine		**Methionine**	
1....................14.28		None Detected	
2....................15.84			
3....................14.32			
Mean = 14.81, S.D. = 0.89			
Methionine Sulfoxide		**Methionine Sulfoxide**	
0.2 mmoles/100g (estimate)		None Detected	
Methionine Sulfone		**Methionine Sulfone**	
None Detected		1....................18.93	
		2....................17.53	
		3....................17.71	
		Mean = 18.06, S.D. = 0.76	
Cysteic Acid		**Cysteic Acid**	
Not Determined		1.................... 2.88	
		2.................... 2.66	
		3.................... 1.96	
		Mean = 2.50, S.D. = 0.48	

APPENDIX 22

Recovery of methionine, methionine sulfoxide, and methionine sulfone from oxidized casein hydrolyzed with 2M NaOH for 18 hours at 100°C in the presence or absence of glucose and in the presence of glucose plus iron (II) sulfate.

2M NaOH, 18 Hours, 100°C					
Without Glucose		With Glucose		With Glucose Plus FeSO$_4$	
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/100g
Methionine		Methionine		Methionine	
See Appendix 20		Trace		Trace	
Methionine Sulfoxide		Methionine Sulfoxide		Methionine Sulfoxide	
See Appendix 20		1..........12.13 2..........13.51		1..........11.95 2..........11.70	
		Mean = 12.82 S.D. = 0.98		Mean = 11.82 S.D. = 0.18	
Methionine Sulfone		Methionine Sulfone		Methionine Sulfone	
See Appendix 20		None Detected		None Detected	

Recovery of methionine, methionine sulfoxide, and methionine sulfone from casein hydrolyzed under alkaline and acidic conditions in the presence of lactose, copper (II) ions and iron (II) ions.

2M NaOH, 18 Hours, 100°C	6M HCl, 24 Hours, 110°C
With Lactose, $FeSO_4$, and $CuSO_4$	With Lactose, $FeSO_4$, and $CuSO_4$

Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine		Methionine	
1..................10.74		1..................9.88	
2..................10.56		2..................10.26	
3..................10.74		3..................10.70	
4..................10.70		4..................12.02	
		5..................10.04	
		6..................11.56	

Mean = 10.68   Mean = 10.74
S.D. = 0.08   S.D. = 0.87

Methionine
Sulfoxide
    1.................. 0.90
    2.................. 0.74
    3.................. 0.74
    4.................. 0.70

Methionine
Sulfoxide

        None Detected

Mean = 0.77
S.D. = 0.09

Methionine
Sulfone

    None Detected

Methionine
Sulfone

        None Detected

Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from Promine F hydrolyzed alone and in the presence of sucrose, copper (II) sulfate, and iron (II) sulfate and basic conditions.

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2M NaOH, 18 Hours, 100°C							
Alone				With Sucrose, $FeSO_4$, and $CuSO_4$			
(NC-2P)		(TSM)[1]		(NC-2P)		(TSM)	
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine		Methionine		Methionine		Methionine	
1	4.83	1	4.94	1	3.63	4	3.64
2	5.02	2	3.79	2	3.86	4	3.48
3	4.98	2	5.64	3	4.13	4	2.80
4	5.38	2	4.45	4	3.40		
5	5.06			5	3.91		
6	5.47			6	3.56		
7	5.09						
8	4.99						
9	5.34						
10	4.80						
11	4.93						
12	4.77						
13	5.02						
Mean = 5.05 S.D. = 0.22		Mean = 4.70 S.D. = 0.78		Mean = 3.75 S.D. = 0.27		Mean = 3.31 S.D. = 0.45	

[1] Sample numbers from NC-2P and TSM runs identify the same sample.

Alone				With Sucrose, FeSO$_4$, and CuSO$_4$			
(NC-2P)		(TSM)		(NC-2P)		(TSM)	
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine Sulfoxide		Methionine Sulfoxide		Methionine Sulfoxide		Methionine Sulfoxide	
1	0.68	2	0.53	1	0.99	3	0.74
2	0.62	2	0.64	2	1.11	3	1.30
3	0.65	2	0.41	3	0.97		
4	0.68			4	1.08		
5	0.71			5	1.11		
6	0.72			6	1.03		
7	0.47						
8	0.45						
9	0.46						
10	0.44						
Mean = 0.59		Mean = 0.53		Mean = 1.05		Mean = 1.02	
S.D. = 0.12		S.D. = 0.12		S.D. = 0.06		S.D. = 0.40	

No methionine sulfone detected in any samples

Cysteic Acid			
1	0.35		
2	0.53	Cysteic acid not determined in any other samples	
3	0.83		
4	0.78		
Mean = 0.62			
S.D. = 0.22			

Recovery of methionine, methionine sulfoxide, and methionine sulfone from Promine F hydrolyzed alone and in the presence of sucrose, copper (II) sulfate, and iron (II) sulfate under acidic conditions or subjected to performic acid oxidation.

6M HCl, 24 Hours, 110°C		Performic Acid Oxidation
Alone	With Sucrose, $FeSO_4$, and $CuSO_4$	

Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine		Methionine		Methionine	
1	7.08	1	5.15	None Detected	
2	7.06	2	5.40		
3	7.41	3	5.51		
4	7.19	4	5.79		
5	7.53	5	5.58		
6	6.81	6	5.37		
7	7.68				
8	6.43				
9	6.77				
10	7.07				

Mean = 7.10	Mean = 5.47	
S.D. = 0.38	S.D. = 0.22	

Methionine Sulfoxide	Methionine Sulfoxide	Methionine Sulfoxide
0.3 mmoles/100g (estimate)	None Detected	None Detected

6M HC1, 24 Hours, 110°C

Alone		With Sucrose, FeSO$_4$, and CuSO$_4$		Performic Acid Oxidation	
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine Sulfone		Methionine Sulfone		Methionine Sulfone	
0.3 mmoles/100g (estimate)		None Detected		1................7.24 2................7.49 3................7.03 4................9.55 5................6.93	
				Mean = 7.65 S.D. = 1.08	
Cysteic Acid		Cysteic Acid		Cysteic Acid	
0.2 mmoles/100g (estimate)		0.2 mmoles/100g (estimate)		1................8.18 2................8.76 3................8.52 4................9.55 5................7.94	
				Mean = 8.59 S.D. = 0.62	

Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from Soyafluff 200W which was subjected to alkaline or acid hydrolysis and performic acid oxidation.

2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		Performic Acid Oxidation	
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine[1] (570 nm)		Methionine		None Detected	
1	2.99	1	2.35		
2	3.29	2	2.80		
3	3.07	3	2.37		
4	3.02	4	2.43		
5	3.11	5	2.66		
6	3.10	6	2.27		
(440 nm)					
1	3.31				
2	3.49				
3	3.29				
4	3.42				
5	3.46				
6	3.36				
Mean = 3.24		Mean = 2.48			
S.D. = 0.18		S.D. = 0.20			

[1] The amount of methionine in the alkaline hydrolysate was determined from absorbance at both 570 nm and 440 nm. Sample numbers identify the same sample for 570 nm and 440 nm.

2M NaOH, 18 Hours, 100°C	6M HCl, 24 Hours, 110°C	Performic Acid Oxidation
Sample mmoles/100g Number	Sample mmoles/100g Number	Sample mmoles/100g Number

2M NaOH, 18 Hours, 100°C   Sample Number   mmoles/100g

Methionine
Sulfoxide
```
1...............0.24
2...............0.36
3...............0.31
4...............0.30
5...............0.27
6...............0.28
7...............0.28
8...............0.29
```

Mean = 0.29
S.D. = 0.03

Methionine
Sulfone
```
  trace
  <0.1 mmole/100g
```

Cysteic Acid
```
  Not Determined
```

6M HCl, 24 Hours, 110°C   Sample Number   mmoles/100g

Methionine
Sulfoxide
```
  None Detected
```

Methionine
Sulfone
```
  trace
  <0.1 mmole/100g
```

Cysteic Acid
```
  trace
  0.1 mmole/100g
```

Performic Acid Oxidation   Sample Number   mmoles/100g

Methionine
Sulfoxide
```
  None Detected
```

Methionine
Sulfone
```
1...............5.82
2...............5.56
3...............6.25
4...............5.57
```
Mean = 5.80
S.D. = 0.32

Cysteic Acid
```
1...............7.10
2...............6.41
3...............6.70
4...............6.05
```
Mean = 6.56
S.D. = 0.44

Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from Promosoy which was subjected to alkaline or acid hydrolysis and performic acid oxidation.

2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		Performic Acid Oxidation	
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine		Methionine		Methionine	
1................4.31		1................4.61		None Detected	
2................3.85		2................4.24			
3................4.26		3................3.67			
4................4.31		4................3.91			
		5................4.39			
		6................3.99			
Mean = 4.18		Mean = 4.14			
S.D. = 0.22		S.D. = 0.34			
Methionine Sulfoxide		Methionine Sulfoxide		Methionine Sulfoxide	
1................0.34		None Detected		None Detected	
2................0.32					
3................0.23					
4................0.35					
5................0.31					
Mean = 0.31					
S.D. = 0.05					

2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		Performic Acid Oxidation	
Sample Number	mmoles/100g	Sample Number	mmoles/100g	Sample Number	mmoles/100g
Methionine Sulfone		Methionine Sulfone		Methionine Sulfone	
trace 0.1 mmole/100g		None Detected		1..............6.77 2..............7.29 3..............7.26	
				Mean = 7.11 S.D. = 0.29	
Cysteic Acid		Cysteic Acid		Cysteic Acid	
Not Determined		None Detected		1..............6.48 2..............7.19 3..............8.04	
				Mean = 7.24 S.D. = 0.78	

Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from Promine D subjected to alkaline or acid hydrolysis and to performic acid oxidation.

2M NaOH, 18 Hours, 100°C				6M HCl, 24 Hours 110°C		Performic Acid Oxidation	
Experiment I (NC-2P)		Experiment II (TSM)					
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine		Methionine		Methionine		Methionine	
1	0.71	1	0.74	1	6.22	None Detected	
2	0.78	2	0.72	2	6.28		
3	0.73	3	0.93	3	5.90		
4	0.78	4	0.82	4	6.50		
5	0.68	5	0.98	5	6.34		
6	0.70	6	1.04	6	6.45		
7	0.77			7	7.65		
8	0.59			8	5.55		
9	0.76			9	6.30		
				10	7.36		
				11	7.72		
				12	6.89		
				13	7.36		
				14	5.80		
Mean = 0.72		Mean = 0.87		Mean = 6.59			
S.D. = 0.06		S.D. = 0.13		S.D. = 0.69			

2M NaOH, 18 Hours, 100°C				6M HCl, 24 Hours 110°C		Performic Acid Oxidation	
Experiment I (NC-2P)		Experiment II (TSM)					
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine Sulfoxide		**Methionine Sulfoxide**		**Methionine Sulfoxide**		**Methionine Sulfoxide**	
1	5.55	1	4.05				
2	5.79	2	4.91	trace		None Detected	
3	5.16	3	5.11				
4	4.91	4	4.81				
5	5.96	5	3.66				
6	5.38	6	5.06				
7	5.69						
8	7.87						
9	6.17						
Mean = 5.83 S.D. = 0.86		Mean = 4.60 S.D. = 0.60					
Methionine Sulfone		**Methionine Sulfone**		**Methionine Sulfone**		**Methionine Sulfone**	
1	0.24	1	0.23	1	0.59	1	7.51
2	0.32	2	0.27	2	0.40	2	8.81
3	0.38	3	0.78	3	0.42	3	8.00
		4	0.46	4	0.39	4	6.49
		5	0.27			5	8.32
		6	0.26				
		7	0.64				
Mean = 0.31 S.D. = 0.07		Mean = 0.42 S.D. = 0.22		Mean = 0.45 S.D. = 0.09		Mean = 7.83 S.D. = 0.88	

2M NaOH, 18 Hours, 100°C				6M HCl, 24 Hours, 110°C		Performic Acid Oxidation	
Experiment I (NC-2P)		Experiment II (TSM)					
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Cysteic Acid		Cysteic Acid		Cysteic Acid		Cysteic Acid	
1	0.76	Not Determined		1	1.56	1	8.66
2	0.82			2	1.52	2	8.04
3	1.05			3	1.72	3	8.52
4	0.61			4	1.55	4	8.19
				5	1.61	5	7.35
				6	1.62		
				7	1.72		
				8	2.24		
				9	1.95		
				10	2.13		
				11	2.24		
				12	1.42		
Mean = 0.81				Mean = 1.77		Mean = 8.15	
S.D. = 0.18				S.D. = 0.29		S.D. = 0.51	

Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from concentrated liquid Similac subjected to alkaline or acid hydrolysis and performic acid oxidation.

2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		Performic Acid Oxidation
Sample mmoles/ Number 100 ml (NC-2P)	Sample mmoles/ Number 100 ml (TSM)	Sample mmoles/ Number 100 ml (NC-2P)	Sample mmoles/ Number 100 ml (TSM)	Sample mmoles/ Number ml
Methionine	Methionine	Methionine	Methionine	Methionine
1.......0.33	1.......0.30	1.......0.46	1.......0.39	None Detected
2.......0.33	2.......0.34	2.......0.46	2.......0.44	
3.......0.31	3.......0.30	3.......0.47	3.......0.50	
4.......0.36		4.......0.42		
5.......0.33		5.......0.42		
Mean = 0.33 S.D. = 0.02	Mean = 0.31 S.D. = 0.02	Mean = 0.45 S.D. = 0.02	Mean = 0.44 S.D. = 0.06	
Methionine Sulfoxide	Methionine Sulfoxide	Methionine Sulfoxide	Methionine Sulfoxide	Methionine Sulfoxide
1.......0.04	1.......0.05	trace <0.01	None Detected	None Detected
2.......0.04	2.......0.07			
3.......0.04	3.......0.07			
4.......0.04				
5.......0.05				
Mean = 0.04 S.D. = 0.01	Mean = 0.06 S.D. = 0.01			

	2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		Performic Acid Oxidation
	Sample Number (NC-2P) — mmoles/100 ml	Sample Number (TSM) — mmoles/100 ml	Sample Number (NC-2P) — mmoles/100 ml	Sample Number (TSM) — mmoles/100 ml	Sample Number — mmoles/100 ml

2M NaOH, 18 Hours, 100°C

(NC-2P) — Methionine Sulfone

1.......0.076
2.......0.075
3.......0.082
4.......0.070
5.......0.067

(TSM) — Methionine Sulfone

1.......0.023
2.......0.002
3.......0.012

6M HCl, 24 Hours, 110°C

(NC-2P) — Methionine[1] Sulfone

570 nm
1.......0.04
2.......0.05
3.......0.04
4.......0.05
5.......0.06
6.......0.06

440 nm
1.......0.08
2.......0.10
3.......0.06
4.......0.06

(TSM) — Methionine Sulfone

1.......0.047
2.......0.115

Performic Acid Oxidation — Methionine Sulfone

1.......0.46
2.......0.46
3.......0.45
4.......0.44
5.......0.49

	2M NaOH (NC-2P)	2M NaOH (TSM)	6M HCl (NC-2P)	6M HCl (TSM)	Performic Acid Oxidation
Mean	0.07	0.01	0.06	0.08	0.46
S.D.	0.01	0.01	0.02	0.05	0.02

Cysteic Acid

- 2M NaOH (NC-2P): Not Determined
- 2M NaOH (TSM): Not Determined
- 6M HCl (NC-2P): trace <0.01 mmole/100 ml
- 6M HCl (TSM): Not Determined
- Performic Acid Oxidation:
  1.......0.18
  2.......0.17
  3.......0.17
  4.......0.20
  5.......0.22

  Mean = 0.19
  S.D. = 0.02

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Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from concentrated liquid Isomil subjected to alkaline or acid hydrolysis and performic acid oxidation.

2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		Performic Acid Oxidation
Sample Number (NC-2P) / mmoles/100 ml	Sample Number (TSM) / mmoles/100 ml	Sample Number (NC-2P) / mmoles/100 ml	Sample Number (TSM) / mmoles/100 ml	Sample Number / mmoles/100 ml
Methionine[1]	**Methionine**	**Methionine**	**Methionine**	**Methionine**
1.......0.41	1.......0.41	1.......0.51	2.......0.38	None Detected
2.......0.41	1.......0.41	2.......0.53	2.......0.50	
3.......0.40	1.......0.32	3.......0.51	2.......0.54	
4.......0.41	4.......0.38	4.......0.49	5.......0.46	
5.......0.40	4.......0.31	5.......0.47	5.......0.40	
	4.......0.42	6.......0.50		
Mean = 0.41 S.D. = 0.01	Mean = 0.37 S.D. = 0.05	Mean = 0.50 S.D. = 0.02	Mean = 0.46 S.D. = 0.07	
Methionine Sulfoxide	**Methionine Sulfoxide**	**Methionine Sulfoxide**	**Methionine Sulfoxide**	**Methionine Sulfoxide**
1.......0.077	2.......0.062	trace <0.01 mmole/ 100 ml	trace <0.01 mmole/ 100 ml	None Detected
2.......0.063	2.......0.032			
3.......0.063	2.......0.037			
4.......0.062	5.......0.038			
5.......0.097	5.......0.032			
	5.......0.072			
Mean = 0.066 S.D. = 0.01	Mean = 0.046 S.D. = 0.02			

[1] Sample numbers on NC-2P and TSM runs identify the same sample.

2M NaOH, 18 Hours, 100°C		6M HCl, 24 Hours, 110°C		Performic Acid Oxidation
Sample mmoles/ Number 100 ml (NC-2P)	Sample mmoles/ Number 100 ml (TSM)	Sample mmoles/ Number 100 ml (NC-2P)	Sample mmoles/ Number 100 ml (TSM)	Sample mmoles/ Number 100 ml
Methionine[1] Sulfone	Methionine Sulfone	Methionine Sulfone	Methionine Sulfone	Methionine Sulfone
1.......0.082	3.......0.008	1.......0.041	2.......0.106	1.......0.57
2.......0.072	5.......0.007	2.......0.046	3.......0.050	2.......0.61
3.......0.081		3.......0.046	3.......0.031	3.......0.62
4.......0.064		4.......0.042	4.......0.046	4.......0.62
5.......0.058		5.......0.055	4.......0.038	
Mean = 0.07 S.D. = 0.01	Mean = 0.0075	Mean = 0.046 S.D. = 0.01	Mean = 0.054 S.D. = 0.03	Mean = 0.60 S.D. = 0.02
Cysteic Acid	Cysteic Acid	Cysteic Acid	Cysteic Acid	Cysteic Acid
Not Determined	Not Determined	trace 0.01	Not Determined	1.......0.42 2.......0.42 3.......0.39 4.......0.42
				Mean = 0.41 S.D. = 0.02

[1] Sample numbers on NC-2P and TSM runs identify the same sample.

Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from freeze-dried concentrated Prosobee liquid subjected to alkaline or acid hydrolysis and performic acid oxidation.

2M NaOH, 18 Hours, 100°C				6M HCl, 24 Hours, 110°C		Performic Acid Oxidation	
(NC-2P)		(TSM)		(NC-2P)			
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine[1]		Methionine		Methionine		Methionine	
1	1.63	2	1.92	1	2.29	None Detected	
2	1.77	2	1.89	2	2.08		
3	1.78	3	1.58	3	1.94		
4	1.71	3	1.75	4	2.14		
				5	2.44		
				6	2.13		
				7	2.38		
				8	2.17		
				9	2.62		
Mean = 1.72 S.D. = 0.07		Mean = 1.78 S.D. = 0.15		Mean = 2.24 S.D. = 0.21			
Methionine Sulfoxide		Methionine Sulfoxide		Methionine Sulfoxide		Methionine Sulfoxide	
1	0.42	2	0.40	<0.05 mmole/ 100g		None Detected	
2	0.43	2	0.35				
3	0.41	3	0.27				
4	0.46	3	0.27				
Mean = 0.43 S.D. = 0.02		Mean = 0.32 S.D. = 0.06					

[1]Sample numbers on NC-2P and TSM runs identify the same sample.

| 2M NaOH, 18 Hours, 100°C | | 6M HCl, 24 Hours, 110°C | Performic Acid Oxidation |
| (NC-2P) | (TSM) | (NC-2P) | |

Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine[1] Sulfone		Methionine Sulfone		Methionine Sulfone		Methionine Sulfone	
1	0.57	2	0.04	1	0.57	1	2.82
2	0.47	2	0.06	2	0.33	2	2.48
3	0.50	3	0.05	3	0.25	3	2.10
4	0.38			4	0.26	4	2.66
				5	0.21		
				6	0.18		
				7	0.31		
				8	0.28		
				9	0.32		

| Mean = 0.48 | Mean = 0.05 | Mean = 0.30 | Mean = 2.52 |
| S.D. = 0.08 | S.D. = 0.01 | S.D. = 0.11 | S.D. = 0.31 |

Cysteic Acid	Cysteic Acid	Cysteic Acid	Cysteic Acid
0.05 mmole/100g	Not Determined	< 0.05 mmole/100g	1 .... 1.82
			2 .... 1.43
			3 .... 1.93
			4 .... 1.87
			5 .... 1.96

| | | | Mean = 1.80 |
| | | | S.D. = 0.21 |

[1] Sample numbers on NC-2P and TSM runs identify the same sample.

Recovery of methionine, methionine sulfoxide, methionine sulfone, and cysteic acid from powdered Similac fortified with iron which was subjected to alkaline or acid hydrolysis and performic acid oxidation.

2M NaOH, 18 Hours, 100°C				6M HCl, 24 Hours, 110°C		Performic Acid Oxidation	
(NC-2P)		(TSM)		(NC-2P)			
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine		**Methionine**		**Methionine**		**Methionine**	
1	1.24	1	0.90	1	1.59	None Detected	
2	0.91	2	0.97	2	1.55		
3	1.43			3	1.89		
4	1.14			4	2.07		
				5	1.75		
				6	1.68		
Mean = 1.18 S.D. = 0.22		Mean = 0.94 S.D. = 0.05		Mean = 1.76 S.D. = 0.20			
Methionine Sulfoxide		**Methionine Sulfoxide**		**Methionine Sulfoxide**		**Methionine Sulfoxide**	
1	0.61	1	0.66	<0.05 mmole/ 100g		None Detected	
2	0.25	2	0.64				
3	0.24						
4	0.53						
5	0.20						
6	0.32						
Mean = 0.36 S.D. = 0.17		Mean = 0.65 S.D. = 0.01					

2M NaOH, 18 Hours, 100°C (NC-2P)		(TSM)		6M HCl, 24 Hours, 110°C (NC-2P)		Performic Acid Oxidation	
Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g	Sample Number	mmoles/ 100g
Methionine Sulfone		**Methionine Sulfone**		**Methionine Sulfone**		**Methionine Sulfone**	
1	0.23	1	0.061	1	0.14	1	1.79
2	0.33	2	0.067	2	0.16	2	1.93
3	0.32			3	0.14	3	1.89
4	0.20			4	0.16	4	2.08
5	0.20			5	0.13	5	1.88
6	0.20			6	0.22	6	2.13
				7	0.26	7	2.21
				8	0.20	8	2.43
				9	0.22	9	2.16
Mean = 0.25		Mean = 0.064		Mean = 0.18		Mean = 2.06	
S.D. = 0.06		S.D. = 0.004		S.D. = 0.05		S.D. = 0.20	
Cysteic Acid		**Cysteic Acid**		**Cysteic Acid**		**Cysteic Acid**	
Not Determined		Not Determined		Not Determined		1	0.70
						2	0.62
						3	0.72
						4	0.70
						5	0.80
						6	0.85
						7	0.74
						8	0.96
						Mean = 0.76	
						S.D. = 0.10	

METHIONINE: OXIDATION STATE IN PROCESSED
FOODS AND ENZYME-CATALYZED REACTION WITH
ADENOSINE TRIPHOSPHATE

by

Jeanne Marie Todd

(ABSTRACT)

Two conditions of alkaline hydrolysis of proteins, (1) 2M NaOH,
18 hours, 100°C and (2) 3M NaOH, 16 hours 110°C, prior to ion-exchange
chromatography were tested on free amino acids and model protein sys-
tems to determine the better set of conditions for measurement of
methionine sulfoxide in food proteins. Recoveries of methionine,
methionine sulfoxide, and methionine sulfone from base-hydrolyzed
amino acid mixtures were, respectively, 89, 100, and 105% with the
2M NaOH conditions and 83, 90, and 98% with the 3M NaOH conditions.
The percentages of total methionine, determined by performic acid
oxidation, recovered as methionine, methionine sulfoxide, and methi-
onine sulfone after hydrolysis with 2M NaOH were, respectively 101, 0,
and 0% in lysozyme, 68, 25, and 0% in oxidized lysozyme, 74, 3, and
0% in casein and 0, 74, and 0% in oxidized casein. The presence of
glucose in the hydrolysis mixture with the model proteins caused as
much as 8% oxidation of methionine to methionine sulfoxide. The pres-
ence of copper (II) and iron (II) ions along with sugars did not
increase the amount of methionine generated in casein and a soy iso-
late. Methionine sulfone was never generated in any of the model
systems. These results suggested that determination of methionine

sulfoxide after basic hydrolysis with 2M NaOH in foods low in car-
bohydrates is valid but in foods high in carbohydrates the procedure
may slightly overestimate the methionine sulfoxide content.

Acid hydrolysis of free methionine sulfoxide reduced 15% of the
methionine sulfoxide to methionine while acid hydrolysis of oxidized
lysozyme and oxidized casein led to reduction of all the methionine
sulfoxide in these proteins.

Eight food products were analyzed for methionine, methionine
sulfoxide, and methionine sulfone. Total methionine was measured by
the performic acid oxidation method, methionine sulfone by ion-exchange
chromatography after acid hydrolysis, methionine sulfoxide by ion-
exchange chromatography after hydrolysis with 2M NaOH for 18 hours at
100°C, and methionine by the difference between total methionine and
the sum of methionine sulfoxide and sulfone. Only a trace of methi-
onine sulfone and less than 6% of total methionine was present as
methionine sulfoxide in a soy flour and a concentrate. Two soy iso-
lates contained 74 and 8% of total methionine as sulfoxide and 6 and
4%, respectively, as sulfone. Two soy-based infant formulas contained
17 and 12% of total methionine as the sulfoxide and 12 and 8%,
respectively, as sulfone. Two milk-based formulas contained 18 and
9% as sulfoxide and 8 and 13%, respectively, as sulfone.

The feasibility of using ATP:L-methionine S-adenosyltransferase
to determine the number of unaltered methionine residues in food pro-
teins was also explored. Di- and tripeptides composed of methionine
appeared to be able to function as well as L-methionine as substrates.

Spectrophotometric studies suggested that the enzyme could act on methionine residues in two soy isolates; however, these results could not be confirmed by amino acid analyses of the isolates after incubation with ATP and the enzyme.