Studies of Methionine Sulfoxide. III. A New Colorimetric Method for the Determination of Methionine Sulfoxide and Its Derivatives

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In a previous paper of this series,[1] the author reported that a rearrangement reaction occurred when methionine hydantoin sulfoxide (MHSO) was treated with acetic anhydride giving the corresponding α-acetoxy-thio-ether quantitatively. In the present work, a new photometrical method for the determination of methionine sulfoxide (MSO) and its derivatives based on this rearrangement reaction will be established. The method is based on the facts that formaldehyde is easily released from the products of the rearrangement reaction by hydrolysis and that it can be determined photochemically by using chromotropic acid.[2]

Though one is needed for biochemical studies, no direct method for the determination of MSO in the presence of methionine (Met) has yet been reported except that of polarography.[3] Recently, Neumann et al. developed an indirect procedure for the chromatographic determination of MSO residues in ribonuclease,[4] but their method cannot be applied to the routine analysis of MSO as it is very complicated. On the other hand, the present method is very simple and suitable for routine determinations of MSO, although it is impossible to use it to analyze the MSO residues in proteins accurately.

Results and Discussion

The Colorimetric Determination of the Formaldehyde Released from MSO and MHSO.— The formaldehyde released from the products of the above-mentioned rearrangement reaction can be effectively determined by the photometrical method of MacFadyen using chromotropic acid.[2] As the chromotropic acid reagent is strongly acidic, its use could eliminate the necessity of treating the reaction mixture with acid or alkali to release the formaldehyde. The procedure for the determination is given below (see "Recommended Procedure"). The experiments reported in this paper were carried out essentially by means of this procedure.

Reaction Conditions. — As is summarized in Table I, various conditions for the rearrangement were investigated. Thirty minutes boiling with acetic anhydride was found to be the most practical condition.

The Reaction Period of the Rearrangement. —To find the time taken for the rearrangement reaction, the formaldehydes released from

1) K. Morihara, This Bulletin, **37**, 1785 (1964).
2) D. A. MacFadyen, *J. Biol. Chem.*, **158**, 107 (1945).
3) K. Morihara, 13th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1960.
4) N. P. Neumann, S. Moore and W. H. Stein, *Biochem.*, **1**, 68 (1960).

TABLE I. REACTION CONDITIONS

Condition	O. D. (observed)
Reflux for 30 min. (bath temp. 140°C)	0.391
Reflux for 30 min. (bath temp. 170~180°C)	0.382
97°C for 30 min.	0.057
97°C for 120 min.	0.357
97°C for 150 min. and then room temp. for 17 hr.	0.344
97°C for 20 hr.	0.332

MHSO and from MSO were successively analyzed by the colorimetric method. The results obtained are shown in Fig. 1; they confirm the results previously reported. The liberation of formaldehyde from MHSO began more rapidly than that from MSO without any lag in time, and the optical density became constant after the first 10 min. On the other hand, the liberation of formaldehyde from MSO was rather slow for the first 5 min. In this period, an azlactone ring may be formed prior to the rearrangement. The optical density became a little less after about 20 min., as the reaction period became longer, indicating that some unkown decomposition reaction took place. Twenty to 30 min. was a suitable reaction period for either reaction. In all the experiments described below, therefore, the reaction period was 30 min.

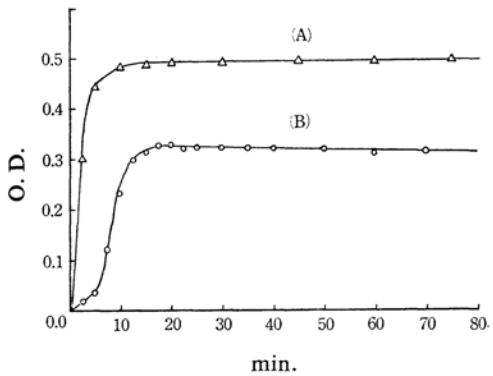

Fig. 1. Reaction period.
(A) MHSO 31.2 μ mol. in 1 ml. of acetic acid
(B) MSO 29.3 μ mol. in 1 ml. of acetic acid

The Optimal Ratio of Acetic Anhydride to Acetic Acid.—It was observed experimentally that the recovery of formaldehyde was affected by the amount of acetic anhydride relative to that of the sample solution, though the acetic anhydride used was stoichiometrically sufficient for the reaction. The results are shown in Fig. 2.

Thus, in standard determinations 2 ml. of acetic anhydride and 1 ml. of an acetic acid solution of the sample were used.

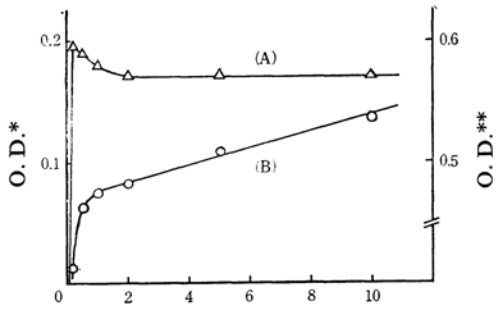

Fig. 2. Ratio (volume of acetic anhydride/volume of acetic acid solution of substrate).
(A) MSO (B) MHSO
* For curve A ** For curve B

The Period of Heating with a Chromotropic Acid Reagent.—In the usual procedure for the determination of formaldehyde with a chromotropic acid reagent, full color development was obtained by heating the solution at 100°C for 30 min. In this case, however, the chromotropic acid reagent acted in two ways: as a hydrolytic reagent to liberate formaldehyde on the one hand, and as a color reagent for the resulting formaldehyde on the other. Thus the optimal heating period had to be tested experimentally. As is shown in Table II, the optical density of the color increased with the heating period, but that of an authentic formaldehyde solution also increased at almost the same rate. Therefore, heating at 100°C for 30 min. was adopted as the standard procedure. However, in the presence of a large amount of an interfering substance, heating for 30 min. was not always sufficient, as will be mentioned below. In such cases, the period had to be prolonged until the optical density became the maximum value.

TABLE II. PERIOD OF HEATING WITH CHROMOTROPIC ACID REAGENT

Substrate	Heating period min.	O. D. (observed)
Diluted reaction mixture*	30	0.592
	60	0.612
	120	0.635
	180	0.641
	240	0.655
	300	0.670
Standard formaldehyde solution**	30	0.962
	60	0.981
	120	0.988
	180	1.009
	240	1.021
	300	1.043

1 ml. of sample solution was mixed with 10 ml. of chromotropic reagent.
* 6.06×10^{-1} μ mol. per ml.
** 6.55×10^{-1} μ mol. per ml.

Standard Curves of MHSO and MSO.—As is shown in Fig. 3, good linear standard curves of MHSO and MSO were obtained in the range between 1.0×10^{-1} μ mol. per ml. and at least 8.0×10^{-1} μ mol. per ml. It was found that, as the standard curve of MHSO was more constant and was reproducible in different determinations than that of MSO, the standard curve in Fig. 3 (C) could be used with a good accuracy. However, the standard curve of MSO shown in Fig. 3 (B) was more variable in different determinations, though it was always linear, so a parallel solution of MSO of a known concentration must be treated under the same conditions in order to determine the exact amount of MSO in a given sample. However, the variation in the standard curve of MSO was so slight that in practice MSO could be determined with a maximal error of only 5 per cent even when curve B in Fig. 3 was used. Moreover, when a ratio of 1 ml. of acetic anhydride to 1 ml. of MSO acetic acid solution was used, almost the same linear standard curve was obtained (curve A in Fig. 3.) However, this standard curve was more variable in different determinations than that obtained with 2 ml. of acetic anhydride (curve B in Fig. 3).

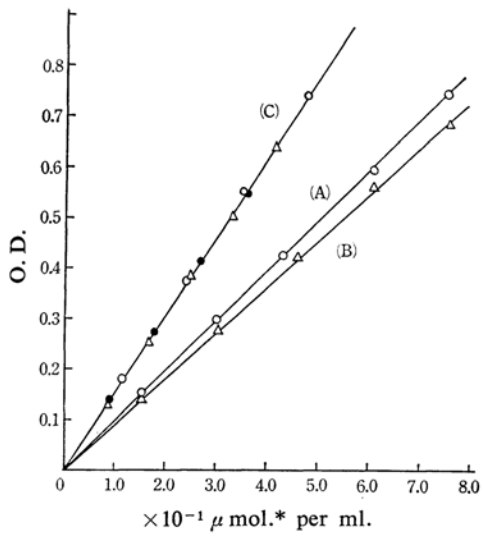

Fig. 3. Standard curves of MSO and MHSO.
(A) MSO 1 ml. of acetic anhydride and 1 ml. of an acetic acid sample solution were used.
(B) MHSO 2 ml. of acetic anhydride and 1 ml. of the sample solution were used.
(C) MHSO -○- and -●- 1 ml. of acetic anhydride and 1 ml. of the sample solution were used. The determinations were independently carried out.
-△- 2 ml. of acetic anhydride and 1 ml. of the sample solution were used.
* Final concentration of substrate when the reaction mixture was diluted to 100 ml.
The procedure used is described in "Recommended Procedure."

The Recovery of the Formaldehyde Released from MHSO and MSO. — It has previously been reported that the quantitative liberation of formaldehyde may be confirmed by iodometric titration.[5] This result was confirmed by the photometrical method. An exact, known weight of hexamethylene tetramine which had been twice purified by sublimation was dissolved in 100 ml. of 3 N sulfuric acid. To hydrolyze the hexamethylenetetramine to formaldehyde, this solution was incubated at 80°C until the aliquots gave a constant value for optical density when treated with a chromotropic reagent. Using this hydrolyzate as the standard formaldehyde solution, an optical density of 0.498 was obtained for 31.2 μ mol. of formaldehyde and an optical density of 0.648, for 41.1 μ mol. of MHSO. Thus the optical density per 1 μ mol. of the two was identical; the recovery was quantitative. The recovery of formaldehyde from MSO was determind indirectly by a comparison with that from MHSO, which was reacted in parallel; it was always found to be about 60 per cent of that of MHSO.

The Recovery of Formaldehyde from Other Derivatives of MSO and Their Standard Curves. —To ascertain why the recovery of formaldehyde from MSO was considerably lower than that from MHSO, the relationship between the structure of the sulfoxide and the recovery of formaldehyde was investigated by studying the

Table III. Recoveries of MSO derivatives

Substrate*	Recovery** %
Acetyl methionine sulfoxide (AcMSO)	57.0
Benzoyl methionine sulfoxide (BzMSO)	66.5
Carbobenzoxy methionine sulfoxide (CbzMSO)	87.5
Carbobenzoxy methionine sulfoxide ethyl ester (CbzMSO-OEt)	59.5
Carbobenzoxy *S*-oxo-methionyl methionine sulfoxide ethyl ester (CbzMSO-MSO-OEt)	90.5
2,4-Dinitrophenyl methionine sulfoxide (DNP-MSO)	43.0

* Substrates in 1 ml. of acetic acid were mixed with 2 ml. of acetic anhydride in the standard manner.
** Recoveries were measured by comparison of their O. D. with the O. D. of a sample of MHSO treated in parallel.

5) L. H. Donnally, *Ind. Eng. Chem. Anal. Ed.*, **25**, 91 (1933).

TABLE IV. INTERFERENCE BY ACYL AMINO ACID AND PREVIOUSLY TREATED AMINO ACID

Substrate (S)	Interfering substance* (I)	(I)/(S) (by mol.)	O. D. (corrected)	Recovery %
20.05 μ mol. MHSO	45 mg. glycine	30	299	96.8
20.05 μ mol. MHSO	75 mg. acetyl glycine	30	299	96.8
20.05 μ mol. MHSO	90 mg. methionine	30	288	90.3
20.05 μ mol. MHSO	114 mg. acetyl methionine	30	287	90.0
20.05 μ mol. MHSO	(Previously treated)**			
20.05 μ mol. MHSO	60 mg. methionine	20	309	97.5
20.05 μ mol. MHSO	90 mg. methionine	30	305	96.3
20.05 μ mol. MHSO	120 mg. methionine	40	292	92.1

* Interfering substances were added to the sample solution which were then treated in the standard manner.

** Methionine suspended in 1 ml. of acetic acid was treated with 2 ml. of acetic anhydride for 30 min. The resulting reaction mixture was added to that of MHSO and diluted to 100 ml. with water, and the resulting solution was measured as usual. O. D. was corrected by drawing 2 times of "blank."

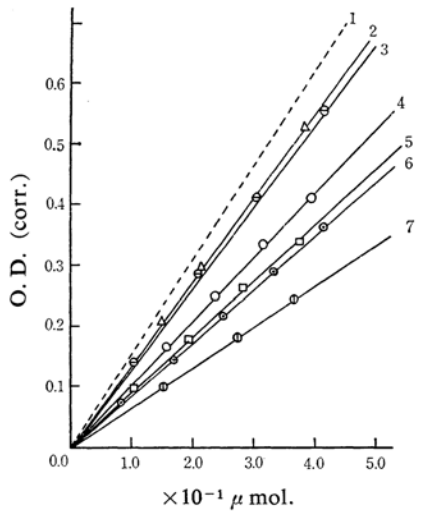

Fig. 4. Standard curves of derivatives of MSO.
1 MHSO 2 Cbz-MSO-MSO-OEt
3 Cbz-MSO 4 Bz-MSO
5 Cbz-MSO-OEt 6 AcMSO
7 DNP-MSO

standard curves of their recoveries. The results are shown in Table III and Fig. 4, but no clear conclusion can be deduced from them. The recovery from AcMSO* was almost identical with that from MSO, indicating that azlactone formation occurred prior to the rearrangement reaction. This conclusion was also supported by the curve in Fig. 1 mentioned above. Thus, it seems unlikely that steric hinderance due to the hydantoin ring decides the direction of the rearrangement to give a quantitative liberation of formaldehyde, because the steric effect due to the azlactone ring seems similar to that due to the hydantoin ring. This is also supported by the fact that the recovery of formaldehyde from Cbz-MSO,* Cbz-MSO-OEt* and Cbz-MSO-MSO-OEt* was not quantitative, though there is more steric hinderance in these compounds. Moreover, it is impossible to ascribe the lower recovery of formaldehyde from MSO than from MHSO to the consumption of sulfoxide by the oxidation of the amino group, because the recovery of formaldehyde from AcMSO, which had no amino group to be oxidized, was identical with that from MSO. Another possibility is that the azlactone formed from MSO strongly inhibited the color reaction of chromotropic acid. Although this possibility was indirectly disproved by the fact that an equivalent molarity of Met scarcely interfered at all with the determination of MSO and MHSO, this possibility was examined directly. If interference occurred, the addition of acyl amino acids, which easily form azlactones, to the substrate solution, or the addition of the acyl

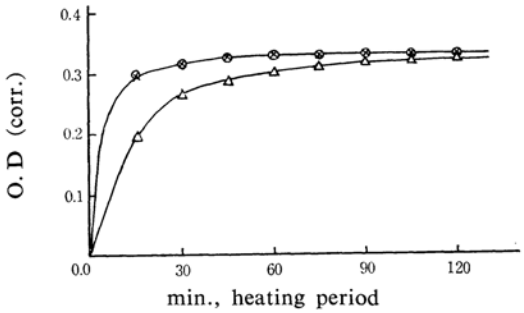

Fig. 5. Development of the color under copresence of interfering substances.
-○- 20.05 μ mol. MHSO
-×- 20.05 μ mol. MHSO plus 800 μ mol. glycine
-△- 20.05 μ mol. MHSO plus 800 μ mol. methionine

* Each abbreviation is explained in the "Experimental" Section.

amino acid to the reaction mixture of MHSO after they have been treated with acetic anhydride should markedly inhibit the color developed with a chromotropic acid reagent.

As is shown in Table IV, this did not hold true. Furthermore, when large amounts of Met were present, the inhibition observed could be overcome by prolonging the heating period with a chromotropic acid reagent, as may be seen in Fig. 5. Thus, the cause of the relatively lower recovery of formaldehyde from MSO than from MHSO is uncertain. Perhaps the hydantoin stabilizes the sulfoxide group in the molecule, since it acts on hydrogen peroxide as a stabilizer.

Table V. Interference by other substances

Substrate (S)	μ mol.	Interfering subsance (I)	mg.	Approximate molar ratio, I/S	Recovery %
MSO	39.9	30 methionine		5	96
		60 methionine		10	100
		120 methionine		20	92
		180 methionine		30	81
MSO	39.9	35 glycine		10	98
		52 glycine		15	99
		44 threonine		10	95
		59 glutamic acid		10	102
		44 cystine		5	96
		97 cystine		10	86
		23 serine		5	79
		39 serine		10	76
MSO	39.9	32 histidine		5	67
		59 histidine		10	61
		48 cysteine hydrochloride		10	17
		66 ascorbic acid		10	56
		42 trimethylamine oxide		10	530
		45 dimethyl sulfoxide		10	805
		39 tryptophane		5	34 (?)
		78 tryptophane		10	45
		36 tyrosine		5	64 (?)
		72 tyrosine		10	79
		31 dimethyl formamide		10	100
MHSO	21.3	17 glycine		10	100
		34 glycine		20	92
		10 serine		5	99
		22 serine		10	104 (?)
		24 threonine		5	96
		35 glutamic acid		10	96
		26 cystine		5	94
		48 cystine		10	94
		48 leucine		10	98
		19 histidine		5	89
		39 histidine		10	84
		15 cysteine		5	5
		20 tyrosine		5	75
		36 tyrosine		10	66

Interference by Other Substances. — For the practical application of the method it is necessary to know what kinds of substances interfere with the present determination. As may be seen in Table V, MSO and MHSO were each determined in the presence of various substances. In the case of the inhibition of methionine, which is required in biochemical studies, the presence of 10 times the molarity of MSO scarcely affected the results at all, but more than 20 times the molarity reduced the value to some extent. However, even this does not decrease the usefulness of the present method much.

Substances that interfere markedly can mainly be divided into four groups. The first group are reducing substances, e. g., cysteine hydrochloride and ascorbic acid. These substances are thought to reduce the sulfoxide prior to the rearrangement. The second group (e. g., trimethylamine oxide and dimethyl sulfoxide) are substances that release formaldehyde when treated with acetic anhydride and subsequent hydrolysis. Here, it should be noted that trimethylamine oxide also released formaldehyde under these conditions. It seems possible that methyl-*N*-oxide might be determined by this method.

The third group (e. g., tryptophan) are substances that are decomposed so as to cause complications when treated with a strong acid.

The forth group (e. g., tyrosine) are substances that compete with chromotropic acid in combination with formaldehyde.

The last case was studied as follows. The substance to be tested, tyrosine in the present case, was added to a reaction mixture containing MHSO treated with acetic anhydride; the mixture was then boiled with a chromotropic

Table VI. Inhibition of chromotropic acid development with tyrosine

Substrate	Tyrosine added, mg.	O. D. (corrected)
Diluted reaction mixture* (1 ml.)	0	0.300**
Diluted reaction mixture* (1 ml.)	5	0.232
Diluted reaction mixture* (1 ml.)	10	0.207
Diluted reaction mixture* (1 ml.)	20	0.194

* Containing 2.13×10^{-1} μ mol. of MHSO per ml.

** Average of five measurements. Tyrosine was added to the diluted reaction mixture and the resulting suspension was depeloped with chromotropic acid in the standard way.

TABLE VII. DETERMINATION OF MIXTURES OF MHSO AND MSO

Substrate	O. D. observed (corrected)	O. D.* calcd.	O. D. observed/ O. D. calcd., %
MSO plus MHSO			
0 μ mol. +21.3 μ mol.	0.335	0.330	98.5
10.1 +15.9	0.332	0.338	98.2
20.2 +10.6	0.342	0.347	98.5
30.3 + 5.3	0.359	0.356	100.5
40.4 + 0	0.365	0.365	100.0

* The O. D. calculated is O. D. (MSO) plus O. D. (MHSO), here O. D. (MSO) and O. D. (MHSO) are obtained independently from calibration curves obtained in parallel.

reagent in the standard way. As may be seen in Table VI, tyrosine inhibited the color developed with the chromotropic acid reagent.

Determinations were made on a mixture of MHSO and MSO. No mutual interference was observed, and the recoveries were additive in the range of concentrations studied, as may be seen in Table VII. As is shown in Table VIII, the protein ovoalbumine does not seem to interfere with the determination of MSO or MHSO.

TABLE VIII. INTERFERENCE BY PROTEIN

Substrate	Ovoalbmin added, mg.	O. D. (corrected)	Recovery %
40.4 μ mol. MSO	10	0.370	103.6
40.4 μ mol. MSO	20	0.360	100.2
21.3 μ mol. MHSO	10	0.315	97.3
21.3 μ mol. MHSO	20	0.322	99.3

The Determination of MSO and MHSO in an Aqueous Solution. — As the method was to be applied to biochemical measurements of samples of MSO or its derivatives, the present method had to be used on aqueous mixtures of the substrates. When acetic anhydride was present in more than five times the molarity of water, the water in the aqueous solution was completely converted to acetic acid. Subsequent boiling resulted in the rearrangement, and satisfactory results were obtained, as is shown in Fig. 6. In these experiments, the ratio of 7.5 ml. of acetic anhydride was used to 0.5 ml. of MSO and MHSO aqueous solutions.

Recommended Procedure.—When the sample to be analyzed is in the solid state, dilute it exactly with purified acetic acid (see "Experimental" section) in a measuring flask to about 10 to 60 μ mol. of the substrate per ml. Pipette 1 ml. of this sample solution into a test tube attached to a suitable reflux condenser, and then add 2 ml. of purified acetic anhydride (see "Experimental" section). After stirring, heat the mixture under reflux for 30 min. on an oil bath.

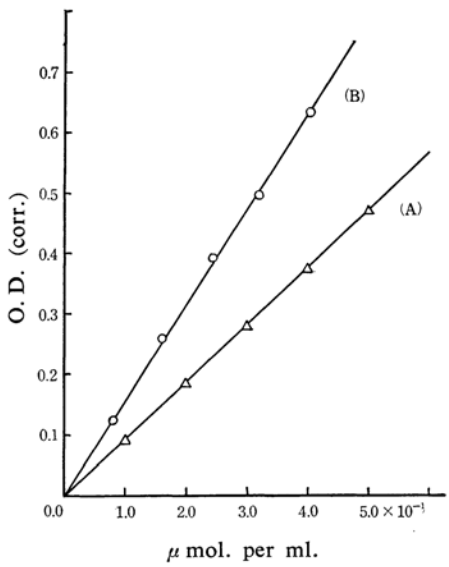

Fig. 6. Standard curves of MSO and MHSO in aqueous solution.
(A) MSO (B) MHSO

A standard solution of the sulfoxide which contains a known amount of the substrate (10 to 60 μ mol. per ml.), as a control, and 1 ml. of the purified acetic acid, as a blank, should be treated in the same way. When the sample to be analyzed is in an aqueous solution, dilute it with purified acetic acid to about 10 to 60 μ mol. per ml. Add 7.5 ml. of purified acetic anhydride to 0.5 ml. of the sample solution, and heat the mixture as described above. The standard aqueous solution of the sulfoxide and 0.5 ml. of water, as a blank, should be treated in parallel. Cool the reaction mixtures with water, and dilute them to 100 ml. with distilled water. Transfer 1 ml. of the resulting solution to a test tube, and then add 10 ml. of a chromotropic acid reagent; after stirring, heat the mixture in a boiling water bath for 30 min. When many other substances are present, it is desirable to prolong the heating period until a constant optical density is observed, since a heating period of 30 min. is

not always sufficient, as has been described above. After the mixture has been cooled to room temperature, measure the color developed with a photometer at 580 mμ. The optical density of the blank should then be subtracted from that of the sample. The optical density obtained here is O. D. (corrected).

As Beer's law holds exactly in the present concentration range, the sulfoxide can be determined by comparison with the optical density of the standard solution. For methionine sulfoxide the accuracy is within a range of error of 3 per cent.

Experimental

The Preparation of Acetyl Methionine Sulfoxide (AcMSO). — This material was obtained by the oxidation of acetyl-L-methionine, m. p. 101~103°C, prepared by the Schotten-Baumann reaction in the standard way. To 1.9 g. of acetyl-L-methionine dissolved in a mixture of 2 ml. of methanol and 2 ml. of water, 2 ml. of a 30% hydrogen peroxide solution was added while the mixture was cooled on ice; the solution was then allowed to stand for 1.5 hr. at room temperature. The reaction mixture was concentrated to a syrup under reduced pressure at below 40°C, and the syrup was dehydrated three times with benzene by the azeotropic procedure. The residual syrup was dissolved in a small amount of acetone and stored in a refrigerator for a long time, with occasional scratching until crystallization occurred. Crystals were collected, washed thoroughly with cold ethanol, and recrystallized from hot acetone. Yield, 500 mg.; m. p. 181~183°C.

Found: C, 40.60; H, 6.28; N, 6.72. Calcd. for $C_7H_{13}O_4NS$: C, 40.50; H, 6.36; N, 6.82%.

The Preparation of Benzoyl-L-methionine Sulfoxide (BzMSO). — Benzoyl-L-methionine (0.6 g.) (m. p. 85~90°C), prepared by the stndard Schotten-Baumann reaction was dissolved in 5 ml. of acetone, and 0.3 ml. of 30% hydrogen peroxide was added to the solution while the mixture was cooled with water. Then the mixture was allowed to stand for an hour at room temperature. When the solution was concentrated under reduced pressure at 40°C, a powder was obtained. The crystalline powder was collected by filtration and recrystallized with acetic acid containing 30 per cent water. The yield was 0.1 g.; m. p. 184~185°C.

Found: C, 53.72; H, 5.58; N, 5.21; S, 11.90. Calcd. for $C_{12}H_{15}O_4NS$: C, 54.09; H, 5.31; N, 5.60; S, 11.98%.

The Preparation of Carbobenzoxy-L-methionine Sulfoxide (Cbz-MSO).—To 5.67 g. (0.02 mol. of Cbz-Met in 50 ml. of methanol was added 2.2 ml. of a (30%) hydrogen peroxide solution with mechanically stirring in for 20 min. at 0~5°C. The solution was then allowed to stand for a further 2 hr. at room temperature. Fifteen milliliters of water was added to the mixture, which was then concentrated to about 20 ml. under reduced pressure at 35°C to remove the methanol. The resulting aqueous mixture was extracted four times with 25 ml. of ethyl acetate. The ethyl acetate extracts were combined and washed with a small amount of a saturated aqueous sodium chloride solution, and then dried over sodium sulfate overniglt. The dried ethyl acetate solution was concentrated under reduced pressure at below 40°C until the residue became slightly viscous. When seeded with Cbz-MSO (see below), the solution became cloudy, and on storage in a refrigerator crystallization occurred. The crystalline product was collected, washed with a mixture of ether and petroleum benzine, and dried in vacuo. The yield was 4.8 g., or 80%. The material was then recrystallized from methanol-water. M. p. 134°C.

Found: C, 52.44; H, 5.64; N, 4.61. Calcd. for $C_{13}H_{17}O_5NS$: C, 52.16; H, 5.72; N, 4.68%.

The Cbz-MSO used for seeding was obtained as follows: The ethyl acetate extract mentioned above was concentrated to a syrup and dissolved in a minimum amount of 1 N sodium hydroxide. The alkaline solution was titrated drop by drop with 6 N sulfuric acid until it became cloudy. One drop of 1 N sodium hydroxide was added to make the solution clear, and then the mixture was stored in a refrigerator for a few days. Fine needle-like crystals were obtained. Yield, 38%.

The Preparation of the Carbobenzoxy Methionine Sulfoxide Ethyl Ester (Cbz-MSO-OEt).— This compound was obtained by the esterification of Cbz-MSO in ethanol containing thionylchloride. To 10 ml. of ethanol at below −10°C was added, drop by drop, 0.65 ml. (9 mmol.) of thionylchloride. After 15 min. 2.14 g. of Cbz-MSO (7.15 mmol.) was added with strring and then the mixture was allowed to stand for about 24 hr. at room temperature. The ethanol was distilled off under reduced pressure, and the residual syrup was washed thoroughly with ether by decantation. The syrup, covered with ether, was kept in deep-freeze for about two weeks. Aggregated white crystals were deposited. The crystals were filtered off, washed thoroughly with ether, and dried in vacuo. The yield was 1.37 g. (58%). On the addition of petroleum benzine to the combined ether extracts, a second crop of crystals was obtained. These were recrystallized from ethyl acetate-petroleum benzine. M. p. 53.5°C.

Found: C, 55.21; H, 6.46; N, 4.96. Calcd. for $C_{15}H_{21}O_5NS$: C, 55.04; H, 6.47; N, 4.28%.

The Preparation of the Carbobenzoxy-*S*-oxomethionyl Methionine Sulfoxide Ethyl Ester (Cbz-MSO-MSO-OEt). — Cbz-Met-Met-OEt, prepared by the mixed anhydride method, was easily oxidized to Cbz-MSO-MSO-OEt with a hydrogen peroxide solution. To Cbz-Met-Met-OEt (2.21 g.; 5 mmol.) in a mixture of 30 ml. of water and 300 ml. of methanol, 1.2 ml. of (30%) hydrogen peroxide solution was added; the mixture was then allowed to stand overnight. The mixture was concentrated under reduced pressure to obtain a solid white product. The yield was 0.6 g., and the crystals melted at 185~186°C.

From the mother liquor 0.7 g. of the product was obtained. The second crop of crystals melted at 165~171°C, and from the mother liquor of the second crop, 0.8 g. of the product was obtained (m. p. 110~112°C). 0.6 g. of the first crop was

recrystallized from a methanol-ether mixture. Yield, 0.45 g.; m. p. 189~190°C.

Found: C, 50.52; H, 6.40; N, 5.75. Calcd. for $C_{20}H_{30}O_7N_2S_2$: C, 50.61; H, 6.37; N, 5.90%.

The Preparation of 2, 4-Dinitrophenyl-L-methionine Sulfoxide (DNP-MSO).—This compound was prepared according to the directions of Levy et al.; [6] m. p. 195°C (Ref. 184.5°C).

Found: C, 39.91; H, 4.15; N, 12.47. Calcd. for $C_{11}H_{13}O_7N_3S$: C, 39.88; H, 3.96; N, 12.68%.

The Purification of Acetic Acid and Acetic Anhydride.—The usual methods for the purification of acetic acid, and acetic anhydride, were not satisfactory for the present photometrical method. Acetic acid was, however, purified effectively as follows. A solution of 5 per cent phenylhydrazine (by weight) in 10 per cent (by volume) of acetic anhydride and 800 ml. of acetic acid was boiled under reflux for several hours. The acid was then distilled, and the fraction boiling at 115~117°C was collected. Distillation was repeated two or three times. The purified acid was kept in a brown bottle. When it was diluted 10 times (by volume) with distilled water, 1 ml. of the resulting solution showed an optical density of 0.005~0.008 when treated with 10 ml. of a chromotropic acid reagent.

Acetic anhydride was purified as follows: 800 ml. of acetic anhydride was mixed with a few drops of concentrated sulfuric acid, and the mixture was boiled under reflux for several hours. The mixture, which turned dark, was distilled, and the fraction boiling at 137~139°C was collected. One milliliter of the anhydride had an optical density of about 0.025 when treated with 10 ml. of a chromotropic reagent.

6) A. L. Levy and D. Chung, *J. Am. Chem. Soc.*, **77**, 2899 (1955).

Chromotropic Acid Reagent.—A chromotropic acid, disodium 1,8-dihydroxynaphthalene-3,6-disulfonate, commercially available, was recrystallized from a mixture of water and ethanol. One gram of the purified salt was dissolved in 100 ml. of distilled water, and 400 ml. of 24 N sulfuric acid was added to this solution.

Summary

1) A new colorimetric method for the determination of methionine sulfoxide and its derivatives on the basis of the rearrangement of sulfoxide by the action of acetic anhydride has been established.

2) From about 10 μ to 80 μ mol. of methionine sulfoxide can be determined with an experimental error of 3 per cent.

3) The interference with the reaction by various amino acids and related substances has been investigated.

4) Several compounds related to methionine sulfoxide have been prepared and analyzed by the present method.

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