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A MICROCHEMICAL MODIFICATION OF THE McCARTHY AND SULLIVAN METHOD FOR THE DETERMINATION OF METHIONINE

by

Emilia Rodelas Joson

A Thesis Submitted to the Graduate Faculty

of

South Dakota State College of Agriculture and Mechanic Arts

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in Chemistry

July 1954

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A MICROCHEMICAL MODIFICATION OF THE McCARTHY AND SULLIVAN METHOD FOR THE DETERMINATION OF METHIONINE

By
Emilia Rodelas Joson

This thesis is approved as a creditable independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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The Author

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INTRODUCTION

Methionine was discovered in 1922 by Mueller (33) and was first known as Mueller's acid. In 1928 Barger and Coyne (6) found that it was a methylated sulfur compound known as alpha-amino-gamma-methyl thio-butyric acid and later gave it the present shorter name of methionine. Rose and his associates (34) have shown that it is the essential sulfur amino acid and not cystine as was formerly thought. Methionine plays an important role in the growth of plants and animals: first, as a methylating agent; second, as an amino acid essential for the building of tissue proteins; third, as a source of sulfur for the synthesis of cysteine, cystine, and glutathione; and fourth, as a part of sulfhydryl-dependent enzymes which are vital in animal metabolism. Its labile methyl group, which is held by a covalent bond to sulfur, constitutes, together with the methyl groups from betaine and choline, a dietary "pool" of physiologically interchangeable methyl groups (18). It also provides the N-methyl group of the hormone adrenalin (22), prevents fatty infiltration of the liver, prevents hemorrhagic degeneration of the kidneys (13), and promotes the metabolic synthesis of creatine, phosphocreatine, choline, and acetylcholine. Its sulfur group prevents the high blood cholesterol level (41) which is associated with atherosclerosis. It also aids in the healing of bone fractures (42), in the healing of wounds (40), and in the clotting of blood (43). Methionine increases the reten-

tion of nitrogen and sulfur in the bodies of dogs and rats (1) and (19). It alleviates the growth inhibition effect produced by ethionine when added to the diet (36) and affords protection against poisoning by pyridine (7).

Methionine has been found to exhibit a greater number of functions than any of the other essential amino acids in biochemistry and nutrition. One commercial company is at present producing it on a large scale for poultry feeds. Since the future of methionine is bright with the prospects of new discoveries and new applications, a convenient and simple chemical means of detecting it with as much accuracy and sensitivity as possible is desirable.

It was the aim of this experimental work to develop a chemical micro-method for the analysis of methionine by a modification of the McCarthy-Sullivan method. This method has been applied with satisfactory results to extracts of tissues and hydrolysates of casein, blood and liver of rat, and corn.

REVIEW OF METHODS

Chemically, methionine is largely demethylated by sulfuric acid but is inert toward phosphoric acid (2). It is decomposed by hot 50% sulfuric acid into homocystine and methionine methylsulfonium hydrogen sulfate (29). It is readily deaminized enzymatically, which suggests that alpha-keto-gamma-methyl mercapto butyric acid may be the only product of methionine in the presence of tissue slices (12). The methyl group is readily oxidized by the animal organism to carbon dioxide and water (31). Its sulfur content, unlike that of cystine and cysteine, is not converted to sulfate by oxidation with nitric acid (23). It is oxidized to sulfate only if the methyl group is accepted by another compound and if there is no available thio group acceptor (12). Oxidation takes place in the liver as found by Forker, et al. (21). It is not destroyed by acid hydrolysis (30). It forms sulfonium compounds when it reacts with an excess of methyl bromide or methyl iodide in a mixture of equal parts of 85% formic acid and acetic acid (37). It increases the dephosphorylation of adenosinetriphosphate by the catalytic action of the enzyme system known as nicotinamide methylkinase. This system catalyzes the over-all transmethylation of methionine (14). Lavine (28) showed that among common amino acids methionine is distinguished by a reversible reaction with iodine. It reacts with sodium nitroprusside in alkaline medium to give a yellow color which turns red on

acidification (32).

Based on the chemical properties of methionine, various quantitative methods were introduced. The earliest method was devised by Baernstein (4) who hydrolyzed the protein with hydriodic acid and thereby caused the demethylation of methionine. Then the methionine was indirectly determined either as the volatile methyl iodide (4) or by the titration of the resulting homocysteine (5). Toennies with Callan (38) and with Kolb (27) made use of the destructive effect of hydrogen peroxide. Methionine is selectively oxidized by hydrogen peroxide in 60% perchloric acid, and then the excess hydrogen peroxide is back-titrated with sodium thiosulfate. A gravimetric method was employed by Beach and Teague (8) in which methionine was demethylated by hydriodic acid to give a thiolactone which could be separated from cysteine by cuprous oxide. Tutiya (39) employed the methyl sulfide procedure where methionine is fused with sodium hydroxide to yield methyl sulfide. The latter is aerated into a solution of isatin, and the resulting green compound is determined colorimetrically. Tutiya claimed that with the exception of a mixture of cystine and betaine, other amino acids do not give any color under these conditions. Lavine (28) based his determination on the reversible reaction with iodine. A modification of this method was made by Bakay and Toennies (3) when they devised a spectrophotometric microdetermination of iodine instead of the thiosulfate titration.

A color reaction was used by Sofin, Rosenblum, and Schultz (35), wherein methionine reacted with anhydrous cupric sulfate in concentrated sulfuric acid to give a yellow color similar to that obtained by Kolb and Toennies (27) when they treated a solution of methionine with cupric chloride in concentrated hydrochloric acid. Tryptophane and tyrosine responded to this color reaction. Recent colorimetric and microbiological methods appear to be more accurate and convenient. McCarthy and Sullivan (32) based their colorimetric method on the reaction of methionine with sodium nitroprusside in an alkaline medium. They applied this method to casein hydrolysates and confirmed the results as reported by Baernstein (4). They used strong sodium hydroxide (14.3 N), a 1% solution of glycine to eliminate the interference of histidine, and a mixture of hydrochloric and phosphoric acids for acidification to give a clearer color. The author claimed this method to be sensitive to 50 p.p.m. in the Duboscq colorimeter, but it did not follow Beer's law. Modifications of this method have been made by Hess and Sullivan (25), Csonka and Denton (16), Chitre (15), Horn, Jones, and Blum (26), and Bolling (10).

Hess and Sullivan (25) hydrolyzed the protein for analysis with 5N sodium hydroxide for two hours at 110°C. It was claimed that this short hydrolysis period minimized the destruction of methionine in alkali. A lower concentration of sodium nitroprusside (20 mg. instead of 30 mg.) was used.

Csonka and Denton (16) precipitated histidine with

phosphotungstic acid to avoid the use of glycine, using a 1% solution of sodium nitroprusside instead of 10% as used by original authors, and using only 20% hydrochloric acid for acidification.

Chitre (15) introduced 1 mg. of methionine per determination if the protein was low in methionine, and he used only phosphoric acid for acidification.

Horn, Jones, and Blum (26) subjected the protein to acid hydrolysis or to digestion by the enzyme papain, using a 3% solution of glycine, 5N sodium hydroxide, and 85% phosphoric acid for acidification. These authors reported excellent agreement in microbiological assay values when proteins were hydrolyzed by acid and by the enzyme papain.

Stekol (10) made the following comment on the McCarthy-Sullivan methionine method: "Cystathionine, methionine sulfoxide, S-beta-carboxyethyl-cysteine, and homocystine gave no color at all concentrations employed. On the other hand, ethionine, S-n-propyl-homocysteine, S-isopropyl-homocysteine, bimethionine, N-benzoyl-methionine, S-benzyl homocysteine, S-carboxymethyl-homocysteine, S-beta-carboxy-ethyl-homocysteine gave colors in intensities of 12% to over 200% of that given by methionine calculated on molar basis."

The McCarthy-Sullivan method was applied to the estimation of methionine in blood and urine by Bennett, et al. (9). They made a stepwise precipitation of protein with acetic acid, heat, and trichloroacetic acid in order to lessen the

loss of methionine. They noted that urea caused the methionine color to fade in urine analysis, and so readings were taken immediately after development of color.

At the present time the useful range of the method is between 80 and 400 micrograms. The extension of the range from 80 to 0 micrograms would represent a distinct advancement due to the small amounts of methionine in biological tissues and the advantages of using small weights of tissues. This has been accomplished by a careful analysis of the method, which now measures between 10 and 360 micrograms with accuracy and precision.

EXPERIMENTAL PROCEDURES AND RESULTS

I. Quantitative Determination of Methionine.

(a) Principle: This method is a modification of the colorimetric method of McCarthy and Sullivan (32). They used a strong concentration of NaOH (14.3N), a 1% solution of glycine to eliminate interference of histidine, a 10% solution of sodium nitroprusside, and then kept the temperature of the mixture at 35°C - 45°C to obtain a clearer color. The authors then cooled the reaction mixture in ice water for about 2 minutes before adding an HCl-H_3PO_4 mixture (9 volumes of conc. HCl and 1 volume of 85% H_3PO_4) to prevent the interference of tryptophane.

(b) Reagents:

Standard methionine solution in 0.1N HCl (200 p.p.m.): Dissolve 50 mg. of methionine in 250 ml. of 0.1N HCl.

5N NaOH: Dissolve 20 gms. of NaOH pellets in distilled water. Dilute to 100 ml., or dilute a concentrated NaOH solution.

1% glycine: Dissolve 1 gm. of glycine in water and dilute to 100 ml.

1% sodium nitroprusside: Dissolve 1 gm. of sodium nitroprusside in 100 ml. of water and store in a glass stoppered brown bottle away from light.

85% H_3PO_4: Reagent grade.

0.1N HCl: Dissolve 3.65 gms. of HCl in water, dilute to 1000 ml. and standardize, or by the dilution of a known amount of constant boiling point HCl.

(c) Analysis of the Method:

1. Effect of Sodium Hydroxide Concentration. Sodium hydroxide is essential in order to bring about the yellow color of sodium nitroprusside with methionine. The solution later turns pinkish red on acidification. Concentrations of 14.3N and 5N, as used by previous investigators, were first used. The greater concentration gave a higher transmission, i.e., a lighter color. Then varying quantities of 5N NaOH were added separately to each of the calibrated test tubes containing 1.0 ml. of standard methionine. Data, as presented in Table I, show that a basicity between 0.1N and 1.0N is satisfactory. It is essential that the medium be basic before the addition of sodium nitroprusside. The amount of 5N NaOH required depends on the acidity of the sample, or if the amount of NaOH required by the procedure is constant, the sample should be brought to a pH of 4.5-5.0 before analysis.

2. Effect of Sodium Nitroprusside Concentration. Sodium nitroprusside in the presence of methionine produces a yellow color in an alkaline medium and a red color on acidification. Different concentrations of sodium nitroprusside ranging from 40% to 1% were used in the first trial. In the second trial the concentrations used ranged from 1% to 0.1%. Separate blanks were made for each determination. Results, as shown in Table II, show that a high concentration of sodium nitroprusside gave a very deep color which is difficult to read in the colorimeter, while a low concentration

gave a very faint color. A 1% concentration was found suitable.

3. Effect of Glycine. Glycine may be eliminated in the procedure as in (d_1) (see section d), if the histidine has been precipitated or its absence is known. Histidine gives the same color reaction with nitroprusside as methionine does, but its interference is prevented by glycine. Different concentrations of glycine ranging from 10% to 1% were used. A high concentration of glycine increases the transmission, i.e., decreases the intensity of the red color produced by methionine. A concentration of 1% was found to give the least decrease in transmission. When there is a large amount of histidine present, the concentrations of glycine, sodium hydroxide, and sodium nitroprusside may be increased, with the corresponding changes being made in the blanks and standards.

4. Effect of Phosphoric Acid Concentration. As has been stated by Andrews (2), methionine is stable in phosphoric acid. The pH of the solution before the addition of acid must be basic or else the red color will not appear. Phosphoric acid is better than hydrochloric acid because it gives an almost colorless blank and a pure pink color in the presence of methionine. Hydrochloric acid gives an orange color with methionine and a slight color to the blank. Less acid gives less color. A greater acid concentration results in more color due, apparently, to the greater solution density. A greater concentration of acid tends to stabilize color

against time. Results are shown in Table III.

5. Effect of Temperature. Heating to 35°C - 40°C as required by the McCarthy-Sullivan method has been omitted because it does not affect the transmission. Cooling, which was also required, was omitted because the modification is always applied to acid hydrolysates where tryptophane is absent, since tryptophane is destroyed by strong acid. It is essential, however, that this fact be kept in mind when making the hydrolysates.

6. Effect of Adding Water in the First or Last Part of the Procedure. Adding water to the methionine solution or to the mixture before reading in the colorimeter does not affect the per cent of transmission. So the addition of water as the last addend, was preferred.

7. Effect of Order of Addition of Reagents. Sodium hydroxide must be added to the methionine solution before the addition of sodium nitroprusside, or else no yellow color will be produced. Phosphoric acid should be added after the addition of the nitroprusside, in order that a red color will be produced. If phosphoric acid is added before the nitroprusside, no color reaction of methionine will take place.

Table I

Effect of Sodium Hydroxide Concentration on Color Reaction of Methionine

Test tube No.		1	2	3	4	5	6	7	8	9	10
Standard Methionine	ml.	1.0	1.0	1.0	1.0	1.0*	1.0	1.0	1.0	1.0	1.0
5N Sodium Hydroxide	ml.	0.4	0.3	0.2	0.1	0.0	0.5	0.6	0.8	0.9	1.0
1% Sodium Nitroprusside	ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
85% Phosphoric Acid	ml.	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Water	ml.	3.0	3.1	3.2	3.3	3.4	2.9	2.8	2.6	2.5	2.4
Total Volume	ml.	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6
Per Cent Transmission		78	78	78	78	100	78	78	78	78	77
Normality of Mixture prior to Addition of Acid		0.45	0.34	0.23	0.11	Acid pH	0.57	0.68	0.91	1.0	1.13

*No yellow color developed in acid medium and no red color developed on addition of acid. Blanks were run on each determination using 0.1 N HCl.

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Table II

Effect of Varying Concentrations of Sodium Nitroprusside on the Color Reaction of Methionine

Test Tube No.		1	2	3	4	5	6	7	8
Standard Methionine	ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
5N Sodium Hydroxide	ml.	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
% Sodium Nitroprusside		40	30	20	10	1.0	0.5	0.25	0.1
Sodium Nitroprusside	ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
85% Phosphoric Acid	ml.	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Water	ml.	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Total Volume	ml.	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6
Per Cent Transmission		7	12	23	85	78	80	79	90

The data show that 1% sodium nitroprusside gives the best result.

Table III

Effect of Concentration of Phosphoric Acid on Color Reaction of Methionine

Test Tube No.		1	2	3	4	5	6	7	8	9	10	11
Standard Methionine	ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
5N Sodium Hydroxide	ml.	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
1% Sodium Nitroprusside	ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
85% Phosphoric Acid	ml.	2.0	1.5	1.0	0.8	0.6	0.4	0.2	0.1	0.0	2.5	3.0
Water	ml.	3.0	3.5	4.0	4.2	4.4	4.6	4.8	4.9	5.0	2.5	2.0
Total Volume	ml.	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6
Per Cent Transmission		78	79	80	80	80	81	81	100	100	76	76
Per Cent Transmission (after 2 hours)		81	81	82	82	82	83	83	100	100	77	76
Final Normality of Acid Mixture		3.57	2.61	1.64	1.26	0.87	0.55	0.09	0.09	0.29	4.57	5.5

Columns 8 and 9 give a basic reaction and so no red color was produced.

Columns 10 and 11 give a darker color due perhaps to a higher concentration of acid, which produces a greater solution density.

Greater concentrations of acid tend to stabilize color against time.

(d) The Modified Procedure (d_1).

Use from 0.1 ml. to 1.80 ml. of standard methionine, prepared as in section (b), and place each known volume in calibrated test tubes. Add to each test tube 0.2 ml. of 5N NaOH and 0.1 ml. of 1% sodium nitroprusside. Shake after each addition and allow to stand for 10 minutes. Then add 1.0 ml. of 85% H_3PO_4 and shake vigorously for about one minute. Add water to make the volume to 3.30 ml. Let stand for 15 minutes before reading in the Cenco colorimeter, using a green filter. Record the reading as per cent of transmission. Include a blank for every determination. See Table IV for the experimental plan of the determination and Figure 1 for the standardization graph.

The Modified Procedure (d_2).

This method is applicable to analysis of hydrolysates or samples where histidine is present. This is a modification of the above procedure, (d_1), in that 0.2 ml. of 1% glycine solution is added after the addition of 5N NaOH. The rest of the procedure is the same as before. The results are tabulated in Table V and plotted in the graph of Figure 2.

Table IV

Per Cent Transmission of Varying Amounts of Methionine

Test Tube No.		1	2	3	4	5	6	7	8	9	10	11	12
Micrograms of Methionine		10	20	40	60	80	120	160	200	240	280	320	360
Standard Methionine	ml.	0.05	0.1	0.2	0.3	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8
5N Sodium Hydroxide	ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
1% Sodium Nitroprusside	ml.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
85% Phosphoric Acid	ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Water	ml.	1.95	1.9	1.8	1.7	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2
Total Volume	ml.	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Per Cent Transmission		97	94	90	85	81	73	66	59	53	47	43	38

Blanks are run on each determination using 0.1N HCl.
Readings made after mixture had stood for 15 minutes at room temperature.

Table V

Per Cent Transmission of Varying Amounts of Methionine with Glycine

Test Tube No.		1	2	3	4	5	6	7	8	9	10	11	12
Micrograms of Methionine		10	20	40	60	80	120	160	200	240	280	320	360
Standard Methionine	ml.	0.05	0.1	0.2	0.3	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8
5N Sodium Hydroxide	ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
1% Glycine	ml.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
1% Sodium Nitroprusside	ml.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
85% Phosphoric Acid	ml.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Water	ml.	1.75	1.7	1.6	1.5	1.4	1.2	1.0	0.8	0.6	0.4	0.2	0.0
Total Volume	ml.	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Per Cent Transmission		99	97	95	92	90	85	81	77	72	70	65	63

Blanks are run on each determination using 0.1N HCl.
Readings taken after mixture had stood for 15 minutes at room temperature.

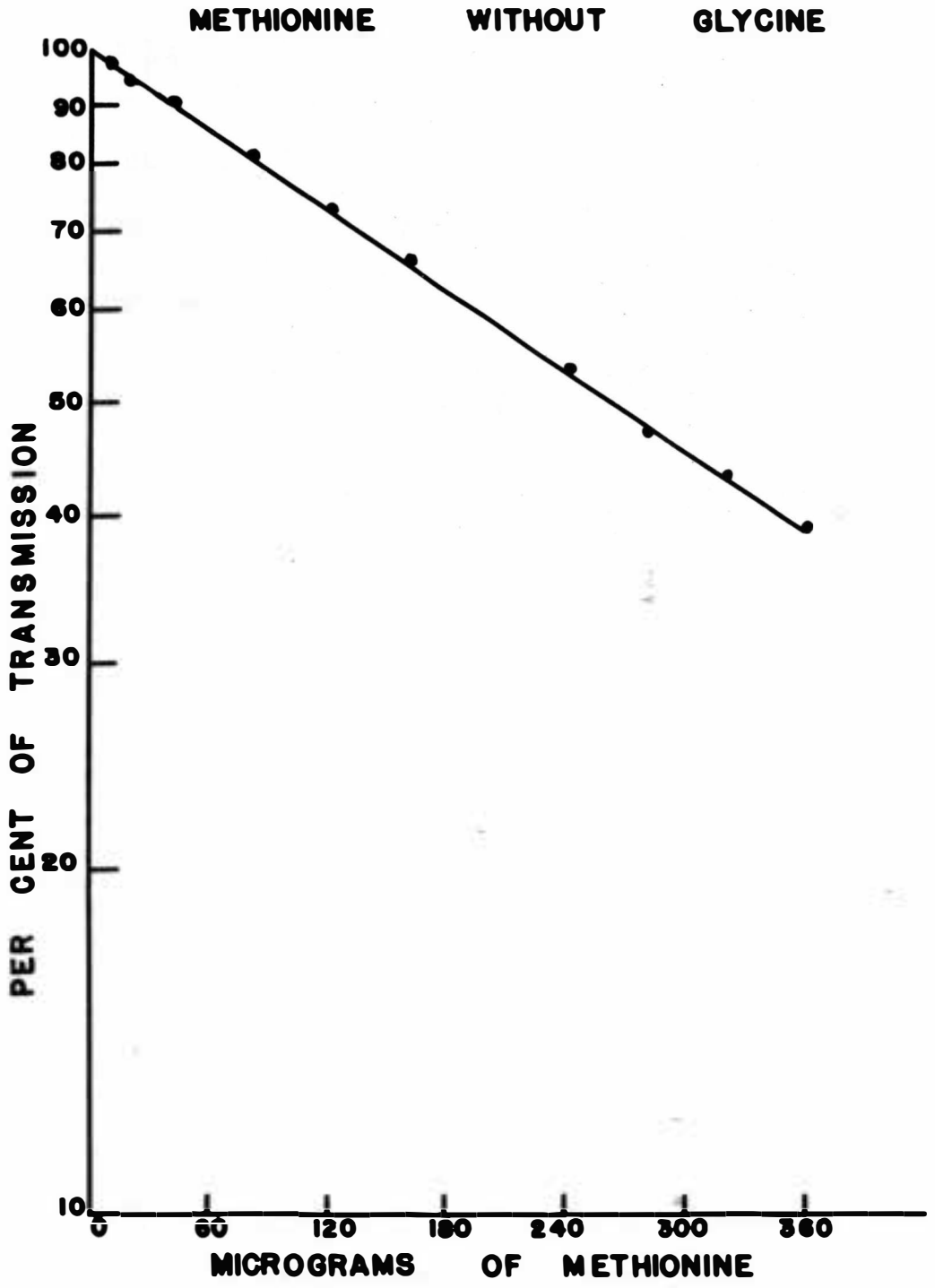

Figure 1. Standardization graph of methionine without glycine according to the modification of the McCarthy and Sullivan method. Readings were made in the Cenco colorimeter, using a green filter.

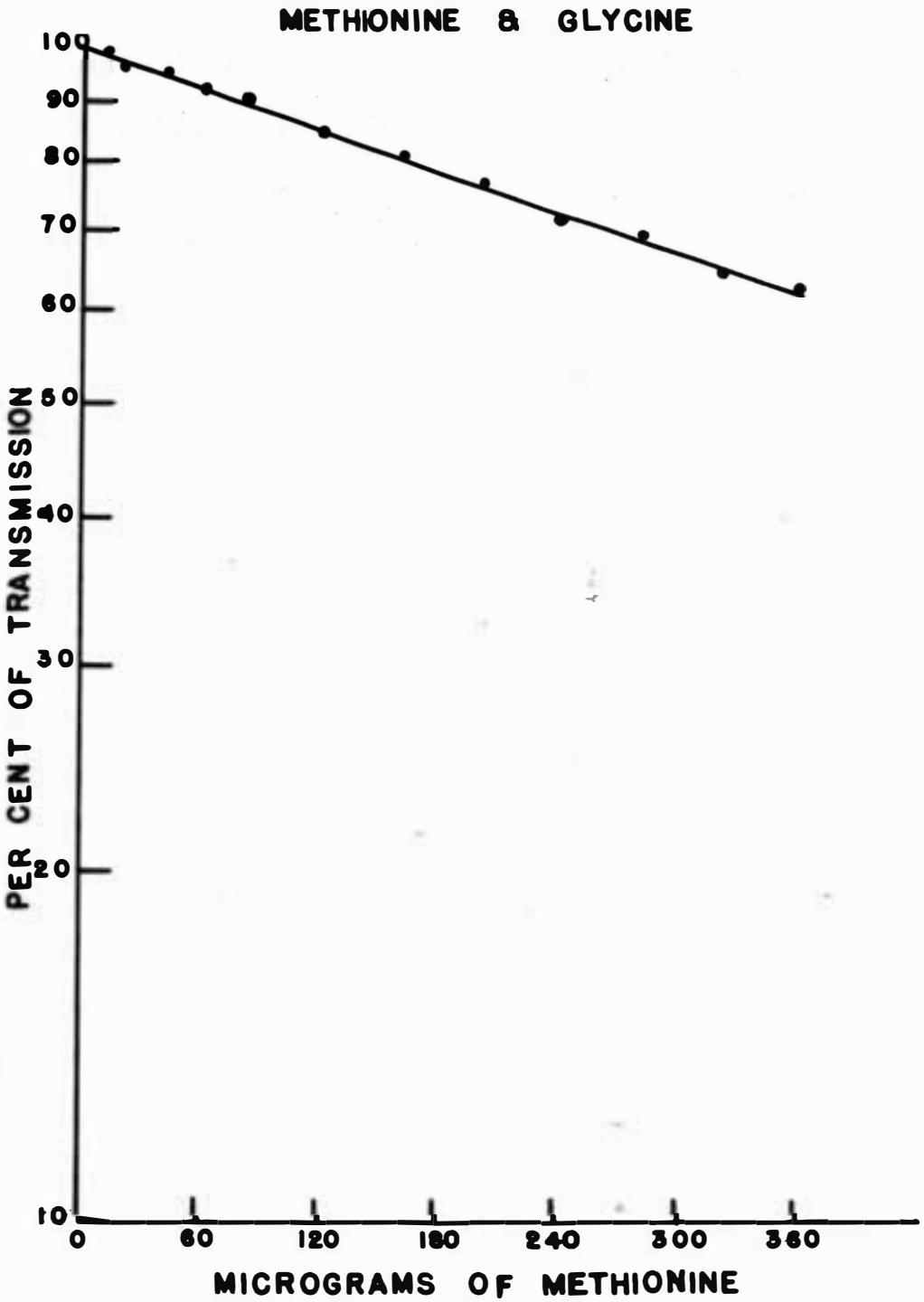

Figure 2. Standardization graph of methionine with glycine according to the modification of the McCarthy and Sullivan method. Readings were made in the Cenco colorimeter, using a green filter.

(e) Specificity of Reaction.

The method (d_1) was tried on the following compounds with the results as indicated:

1. alanine	—	13. hydroxyproline	—
2. arginine monochloride	—	14. isoleucine	—
3. aspartic acid	—	15. lysine monochloride	—
4. cysteine	—	16. norleucine	—
5. cystine	—	17. phenylalanine	—
6. ethionine	+	18. proline	—
7. glutamic acid	—	19. serine	—
8. glutathione	—	20. threonine	—
9. glycine	—	21. tryptophane	+
10. glycyl glycine	—	22. tyrosine	—
11. histidine monochloride	+	23. valine	—
12. homocysteine	—		

All of these compounds gave negative results with the exception of histidine, tryptophane, and ethionine. The interference of histidine is eliminated by the addition of glycine as in procedure (d_2); tryptophane is destroyed by acid hydrolysis; and ethionine is not a physiological compound.

(f) Recovery of Methionine.

Two mixtures (a) and (b) of amino acids which gave negative reactions with sodium nitroprusside were prepared as follows: Mixture (a) consisted of 10 mg. each of the following: alanine, arginine monohydrochloride, glutamic acid, leucine, lysine monohydrochloride, and threonine. This was dissolved in 50 ml. of 0.1N HCl. Mixture (b)

consisted of 10 mg. each of the following: phenylalanine, proline, serine, tyrosine, and valine. This was also dissolved in 50 ml. of 0.1N HCl. To 1.0 ml. of each of the mixture solutions was added from 0.1 ml. to 1.0 ml. of standard methionine (equivalent of 20 µg to 200 µg). Then the amount of methionine was determined as in procedure (d_1). Results as shown in Table VI show that there was a complete recovery of the added methionine.

The trichloroacetic acid filtrate of rat blood was analyzed for methionine and gave negative result. To 1.5 ml. of this filtrate, 0.2 ml. of standard methionine (equivalent to 40 µg) was added, and again there was a complete recovery of the added methionine as shown in Table VII. Procedure (d_2) was followed.

(g) Reaction of Methionine with other Iron Compounds:

One per cent solutions of potassium ferrocyanide, potassium ferricyanide, ferrous sulfate, and ferric chloride were each substituted for sodium nitroprusside in the procedure. No red color was developed in each case, showing that sodium nitroprusside is the only one forming a chromogen. This work confirmed and extended the results obtained by McCarthy and Sullivan (32).

(h) Effects of Some Compounds on the Intensification of the Methionine-Nitroprusside Color Reaction:

One per cent solutions of ferric chloride, ferrous sulfate, potassium ferrocyanide, dimethyl-amino-azo-benzene, dinitrosalicylic acid, diphenyl amine, orcinol, and potassium

ferricyanide were added separately after the addition of sodium nitroprusside. The first five compounds did not alter the color produced by sodium nitroprusside with methionine. Diphenyl amine turned the whole mixture into a violet color, while orcinol and potassium ferricyanide produced a fainter color. This was done to find a means of intensifying the color produced by sodium nitroprusside with methionine. None of these tested compounds proved satisfactory.

(i) Effect of Arsenic and Selenium Compounds on the Color Reaction of Methionine:

One per cent solutions of sodium salts, of selenite, selenate, arsenite, and arsenate were prepared. Aliquot portions ranging from 0.1 ml. to 1.0 ml. of each were added separately to 1.0 ml. of standard methionine solution. The amount of methionine was determined. The results as shown in Table VIII showed that these compounds even in concentrations equal to that of methionine did not interfere with the color reaction of methionine.

Table VI

Recovery of Methionine in a Mixture of Amino Acids

Test Tube Number		1	2	3	4	5	6
Mixture (a)	ml.	0.0	1.0	0.0	0.0	1.0	0.0
Mixture (b)	ml.	0.0	0.0	1.0	0.0	0.0	1.0
Methionine	ml.	1.0	1.0	1.0	0.1	0.1	0.1
5N Sodium Hydroxide	ml.	0.2	0.2	0.2	0.2	0.2	0.2
1% Sodium Nitroprusside	ml.	0.1	0.1	0.1	0.1	0.1	0.1
85% Phosphoric Acid	ml.	1.0	1.0	1.0	1.0	1.0	1.0
Water	ml.	1.0	0.0	0.0	1.9	0.9	0.9
Total Volume	ml.	3.3	3.3	3.3	3.3	3.3	3.3
Per Cent Transmission		59	59	59	97	97	97

Blanks were run for every determination using 0.1N HCl.
Mixture (a) - alanine, arginine monohydrochloride, glutamic acid, lysine monohydrochloride, leucine, and threonine.
Mixture (b) - phenylalanine, proline, serine, tyrosine, and valine.

Table VII

Recovery of Added Methionine
in Trichloroacetic Acid Filtrate of Rat Blood

Test Tube Number		1	2	3	4	5
Trichloroacetic Acid Filtrate of Rat Blood	ml.	0.0	1.5	1.5	1.5	1.5
Standard Methionine	ml.	0.2	0.2	0.2	0.2	0.2
5N Sodium Hydroxide	ml.	0.2	0.2	0.2	0.2	0.2
1% Glycine	ml.	0.2	0.2	0.2	0.2	0.2
1% Sodium Nitroprusside	ml.	0.1	0.1	0.1	0.1	0.1
85% Phosphoric Acid	ml.	1.0	1.0	1.0	1.0	1.0
Water	ml.	1.6	0.1	0.1	0.1	0.1
Total Volume	ml.	3.3	3.3	3.3	3.3	3.3
Per Cent Transmission		95	94	95	94	95

Blanks were run for each determination using 0.1N HCl.

Table VIII

Effect of Selenium and Arsenic Compounds on Color Reaction of Methionine

Test Tube Number		1	2	3	4	5
Standard Methionine	ml.	1.0	1.0	1.0	1.0	1.0
1% Sodium Selenite	ml.	0.0	1.0	0.0	0.0	0.0
1% Sodium Selenate	ml.	0.0	0.0	1.0	0.0	0.0
1% Sodium Arsenite	ml.	0.0	0.0	0.0	1.0	0.0
1% Sodium Arsenate	ml.	0.0	0.0	0.0	0.0	1.0
5N Sodium Hydroxide	ml.	0.2	0.2	0.2	0.2	0.2
1% Sodium Nitroprusside	ml.	0.1	0.1	0.1	0.1	0.1
85% Phosphoric Acid	ml.	1.0	1.0	1.0	1.0	1.0
Water	ml.	1.0	0.0	0.0	0.0	0.0
Total Volume	ml.	3.3	3.3	3.3	3.3	3.3
Per Cent Transmission		59	59	59	59	59

Blanks were run for every determination using 0.1N HCl.

II. Application of Test for Determination of Methionine:

(a) Casein.

A 0.5 gm. sample of casein (vitamin free) was hydrolyzed from 1.5 to 2 hours with 5 ml. of 20% HCl in an oil bath at 125°C. Then 5 ml. of water was added, the solution was decolorized with 50 mg. of animal charcoal and warmed. The mixture was filtered and washed with 5 ml. of hot 1N HCl and 5 ml. of cold 1N HCl. Combined washings and filtrate were neutralized with 5N NaOH added dropwise

with stirring to pH 3.5 - 4.0. The mixture was diluted to 50 ml. with 0.1N HCl. Aliquot portions, using from 0.2 ml. to 1.0 ml., were analyzed for methionine using procedure (d_2). Procedure (d_1) was not used due to the interference of histidine, which interference is prevented by the glycine in procedure (d_2). Nitrogen content was determined by the Kjeldahl method and protein content was calculated from it. Results are given in Table IX.

(b) Yellow Corn.

A 0.5 gm. sample of ground yellow corn was treated the same way as casein in II (a). Nitrogen content was determined by the Kjeldahl method, and protein content was calculated from it. The amount of methionine was calculated to 16.0 gms. of nitrogen. Results are given in Table IX.

(c) Blood of Rat.

Three ml. of blood from the vena cava of an anesthetized 280 gm. male rat were taken by means of a syringe moistened with a solution of sodium citrate. Two ml. of the sample were hydrolyzed and treated as was casein. The remaining portion (1 ml.) was used for the determination of nitrogen by the Kjeldahl method. The percentage of protein was calculated from the nitrogen content. The amount of methionine present was calculated to 16.0 gms. of nitrogen. Results are given in Table IX.

Two ml. of blood taken from a 265 gm. anesthetized female rat were diluted with 8.0 ml. of 0.85% saline solution. Then 10 ml. of 10% trichloroacetic acid was added as protein

precipitant. Filtrate was analyzed for methionine using procedure (d_2). Results as indicated in Table IX showed that there is no free methionine in the blood.

(d) Liver of Rat.

The liver of a female rat weighing 265 gms. was immediately removed after killing the animal. Then 1.1018 gms. of it was treated in the same way as was casein for determination of methionine. The nitrogen content was determined by the Kjeldahl method, and from it the protein content was calculated. Results are given in Table IX.

A 20% homogenate was prepared from a 1.0 gm. sample of the same liver and treated with equal volume of 16% trichloroacetic acid to precipitate the protein. The mixture was filtered and the filtrate was analyzed for methionine. Data as shown in Table IX show that there is no free methionine in the filtrate.

Table IX

Methionine Content of Various Samples

Samples	Protein per cent	Nitrogen per cent	Methionine in gms.	Literature Values	Reference
Casein (vitamin free)	84.68	13.29	3.61	3.6	Bolling (11) Grau (24)
Yellow Corn (whole)	7.99	1.27	3.15	3.1	Evans (20)
Blood (rat)	.114*	1.79*	5.58*		
Blood TCA Filtrate (rat)			none		
Liver (rat)	19.78	3.07	4.5	4.2-4.8	Dent (17)
Liver TCA Filtrate (rat)			none		

*Values for blood are expressed in gms./100 ml.
Values for methionine in gms. were calculated to 16.0 gms. of nitrogen.
These values are not calculated on moisture free basis.
As shown in data above, blood and liver filtrates from trichloroacetic acid (TCA) do not contain free methionine.

Discussion of Results

This modified method is adapted to a smaller quantity of liquid (3.3 ml.) in the colorimeter tube, and there is no major special apparatus required. Since heating and cooling of the tubes have been eliminated, the modification is more convenient and time-saving than the original method.

The values obtained by the Cenco colorimeter follow Beer's law, that is they follow a straight line from 0 to 360 micrograms. This affords a definite advantage, because the corresponding methionine content of any small amount of sample can be read directly from the calibration curves of Figure 1 or Figure 2 without use of standard solution of methionine.

The method has been thoroughly reworked, and the optimum concentration of each reagent has been determined. The order of addition of the reagents has been determined. It is important to emphasize that the solution must be alkaline before the addition of sodium nitroprusside and that it should be allowed to stand 10 minutes before the addition of the acid. Interfering amino acids have been observed and identified. Acid hydrolysis destroys tryptophane, and strong alkali plus glycine prevents the interference of histidine. Ethionine gives a positive color reaction, but it is not a physiological compound. With these precautions, the method is highly specific.

The wave length of minimum transmission was found to be 520 millimicrons in the Beckman spectrophotometer.

Sodium nitroprusside is the only iron compound among those tried which forms a chromogen with methionine. This indicates that the red color produced with sodium nitroprusside on acidification is probably due to an iron complex of methionine. The reaction between sodium nitroprusside and methionine is empirical, but it appears that the NO group of the nitroprusside is the one linked with a group of methionine. Whether it is linked with the sulfur, methyl, or amino group of methionine is still unknown.

Several compounds which show color reactions were added separately with an aim of intensifying the color produced by nitroprusside, but no positive result was obtained. Should such a chromogen be discovered, the method would be greatly improved because methionine represents a very small portion of the total amino acids formed on hydrolysis, regardless of its wide distribution.

Selenium and arsenic compounds have been found not to interfere with this color reaction.

The validity of the reaction for methionine was tested by addition of a known quantity of methionine to a mixture of amino acids or to samples which showed negative reaction. There was a complete recovery of the methionine.

This test was applied for the determination of methionine content of casein, corn, rat blood, and rat liver. The period of hydrolysis was shortened to 1.5 - 2 hours, for investigation showed that shorter periods gave as reliable results as longer

periods. Values obtained for these samples were in close agreement with those reported by previous investigators.

Suggestions for Further Work

Since methionine is becoming so important biochemically, much attention should be given to this essential amino acid. In this method the addition of glycine prevents the interference of histidine, but at present no one has reported the manner in which glycine reacts with histidine. The same is true of methionine and sodium nitroprusside. All these reactions are empirical and research should be done on them. Further studies should be made on finding a reagent which will produce a more intense color reaction with methionine than does sodium nitroprusside. Another area of research is to determine to what extent this method measures bound methionine, e.g. in peptides.

An increase of methionine content of the blood was noted when a rat received 10 p.p.m. of arsenic in the drinking water. This observation was made on only one rat and further research is necessary to confirm and extend this observation.

Methionine added to soil planted with corn cannot be recovered in the soil or in the germinated corn. When the corn seeds begin to germinate, a strong odor is produced. Where the methionine goes and what that odor is is not known.

Summary

A quantitative microchemical modification of the McCarthy and Sullivan method for the determination of methionine has been established and applied with satisfactory results to the analysis of biological materials.

Attempts to intensify the nitroprusside-methionine chromogen or to discover a new chromogen have not yet been successful.

Sodium salts of arsenate or arsenite and the corresponding forms of selenium have been shown not to interfere in this test.

The significance of the modification relative to the biochemistry of methionine has been discussed.

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