A MICRO-METHOD FOR THE DETERMINATION OF DISULPHIDE-AND SULFHYDRYL-COMPOUNDS.[1] I.

By Hideo TOYODA.

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Introduction.

Biochemistry of sulphur has been paid much attention by various workers. And a number of methods for the determination of sulfhydryl (R—SH) and disulphide (R—S—S—R) compounds have been proposed. However, as it is well known, every one of these methods is subject to its own disadvantage, and none can be accepted as entirely dependable.

It is well known that sulphur plays an important rôle in its variously combined forms such as sulfhydryl (—SH), disulphide (—S—S—), sulfinate (—S(=O)—OH), sulfoxide (—S(=O)—), and sulphone (—S(=O)(=O)—) groups, in the field of physiology of living tissue, especially in the process of proliferative growth mechanism. Therefore, in order to carry out further investigation of this problem in the realm of biochemistry, there is an acute necessity of establishing a dependable method for the determination of sulfhydryl (R—SH) compounds and their oxidation products, the disulphides (R—S—S—R), that will give accurate results.

In 1929, I. St. Lorant[2] published his investigation on a micro-colorimetric method for the determination of hydrogen sulphide. Lorant's method is based upon Caro's methylene blue reaction.

In order to find out applicability of this principle of methylene blue reaction for the determination of R—SH and R—S—S—R compounds, and also to establish a new method thereby, the following experiments were carried out. It was found that dimethyl-*p*-phenylenediamine hydrochloride gave a distinct blue colour quite identical with that of methylene blue, with sulfhydryl compounds in acid solution of proper concentration in presence of ferric ammonium sulphate. The experimental results proved

(1) Read before the Monthly Meeting of the Chemical Society of Japan, March 10, 1934. H. Toyoda, "Observation on the new colour reaction of sulfhydryl compounds, and its application to the quantitative determination of cystine." *J. Chem. Soc. Japan*, **55** (1934), 272.

(2) I. St. Lorant. "Über eine neue colorimetrische Mikro-methode zur Bestimmung des Schwefels in Sulfiden, Sulfaten u.s.w.", *Z. physiol. Chem.*, **185** (1929), 245.

that the new method is available for the micro-determination of R—SH and R—S—S—R, i.e. cystine, cysteine, glutathione and thiocresol in blood, tissue, urine, and other biological substances. Tyrosine, tryptophane, uric acid, creatin and acetone do not interfere the test.

Experimental.

Reagents. (1) **Dimethyl-*p*-phenylenediamine hydrochloride solution.** Half a gram of dimethyl-*p*-phenylenediamine hydrochloride (Merck) was placed in a 1000 c.c. volumetric flask together with 100 c.c. distilled water, and 50 c.c. of concentrated sulphuric acid (sp. g. 1.84) was added directly, the temperature being kept low. Then, the mixture was diluted to the mark with distilled water also under cooling.

(2) **Ferric ammonium sulphate solution.** Twenty-five grams of ferric ammonium sulphate (Merck) was dissolved with 5 c.c. of concentrated sulphuric acid (sp. g. 1.84) in a 200 c.c. volumetric flask, and diluted to the mark with distilled water.

(3) **Cystine solution.** 1/2000 Molar solution of cystine was prepared by dissolving 0.12 g. of cystine (Merck) in 20 c.c. of 2 *N* HCl, and making up to 1000 c.c. with sulphur-free water. One c.c. of this solution contained 0.12 mg. of cystine.

(4) **Methylene blue solution.** In a 1000 c.c. volumetric flask, 0.032 g. of methylene blue (Merck) was placed, and dissolved in 20 c.c. of 2 *N* HCl, and then the whole was diluted to the mark with distilled water.

(5) **Normal HCl.** The solution was standardized against the normal NaOH.

(6) **Preparation of sulphur-free water.** The ordinary distilled water was made a little alkaline, and then distilled over potassium permanganate.

Procedure. The different amounts of the cystine solution of known concentration were placed in 50 c.c. graduated cylinders. Then, about 50 mg. of pure metallic zinc powder was introduced into each cylinder, and exactly 0.50 c.c. of the *N* HCl was added. Immediately after the HCl was added, 7.5 c.c. of dimethyl-*p*-phenylenediamine hydrochloride solution was introduced slowly into each cylinder. Finally, 0.5 c.c. of ferric ammonium sulfate was added with care.

The resulting solutions were diluted to 20, 25, and 50 c.c. with distilled water, according to the concentration of cystine, for the colorimetric read-

ings. The cylinders were well stoppered and set aside at room temperature for, at least, twelve hours before taking colorimetric readings.

The standard for the colorimetric readings was prepared with the cystine solution of known concentration. For this purpose 0.30 mg. or 0.60 mg. in 20 c.c. was found to be convenient concentration.

The Study of Establishing Proper Acidity of Test Solution. Since concentration of hydrochloric acid had much influence on the development of colour, a study was made to determine the concentration that would give maximum intensity. A series of experiments, using hydrochloric acid of varied concentrations, were carried out. Into two series of cylinders, 0.60 mg. and 1.20 mg. of cystine were introduced respectively, and 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95, and 1.00 c.c. of the normal HCl were added to them

Table 1.

No. of cylinders	Volume of *N* HCl added c.c.	(A) Value of 20/R.	(B) Value of 20/R.
1	0.10	1.000	1.000
2	0.15	1.111	1.064
3	0.20	1.124	1.130
4	0.25	1.176	1.298
5	0.30	1.197	1.309
6	0.35	1.235	1.316
7	0.40	1.266	1.333
8	0.45	1.724	1.504
9	0.50	1.923	1.739
10	0.55	1.639	1.562
11	0.60	1.538	1.351
12	0.65	1.481	1.333
13	0.70	1.428	1.290
14	0.75	1.143	1.280
15	0.80	1.124	1.227
16	0.85	1.099	1.111
17	0.90	1.081	1.093
18	0.95	1.010	1.075
19	1.00	0.990	1.053

(A) Series. 0.60 mg. of cystine was used. Dilution volume····20 c.c. The cylinder No. 1 was used as standard, set at 20 mm.

(B) Series. 1.20 mg. of cystine was used. Dilution volume····20 c.c. The cylinder No. 1 was used as standard, set at 20 mm.

in the order of the cylinders, other conditions being exactly the same as described in procedure. At the end of 5, 6, 7, 12, and 24 hours, the colour intensity of each solution was studied, having cylinder No. 1 as the standard. The average values from a number of experiments are listed in Table 1. From these figures, it was obvious that the colour intensity varied with the different concentrations of hydrochloric acid, and it developed best in its intensity and shade when 0.50 c.c. of the normal HCl was added.

The Study of Colour Intensity with Varied Concentrations of Cystine. In order to prove the proportionality of the colour intensity with the concentration of cystine, the following experiments were carried out. Into a series of cylinders, different amounts of cystine were introduced, and they were treated according to the method described in procedure. The figures are listed in Tables 2 and 3. In the tables the experimental, and also the theoretical, ratios of the colour intensity are listed. The ratios obtained by the actual colorimetric comparison are listed in the column, "Value of 20/R." The theoretical ratio means the value $\frac{\text{mg. weight of cystine used}}{\text{mg. weight of cystine used as St.}}$ As the standard for Table 2, 0.36 mg. of cystine was used, and 1.32 mg. of cystine was used as the standard for Table 3. In both series, the standards were set at 20 mm. for the colorimetric readings. The volumes of the final dilution with distilled water were 20 and 50 c.c. for Tables 2 and 3 respectively.

From the figures in the tables, it was shown that there was direct proportionality between the colour intensity and the concentration of cystine. Figs. 1 and 2 were plotted on the basis of Tables 2 and 3 respectively.

Table 2.

No. of cylinders	Weight of cystine in mg.	Value of 20/R.	Theoretical ratio
1	0.24	0.64	0.66
2	0.36	1.00	1.00
3	0.48	1.33	1.30
4	0.60	1.66	1.67
5	0.72	2.04	2.00
6	0.84	2.35	2.33
7	0.96	2.63	2.67
8	1.08	2.94	3.00
9	1.20	3.33	3.33

Standard—0.36 mg. cystine set at 20 mm. Dilution volume····20 c.c.

Intensity of the colour with varied concentrations of cystine.

Standard—0.36 mg. of cystine set at 20 mm. on the colorimeter. Volume of the final dilution····20 c.c.

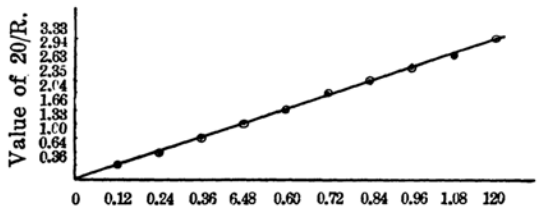
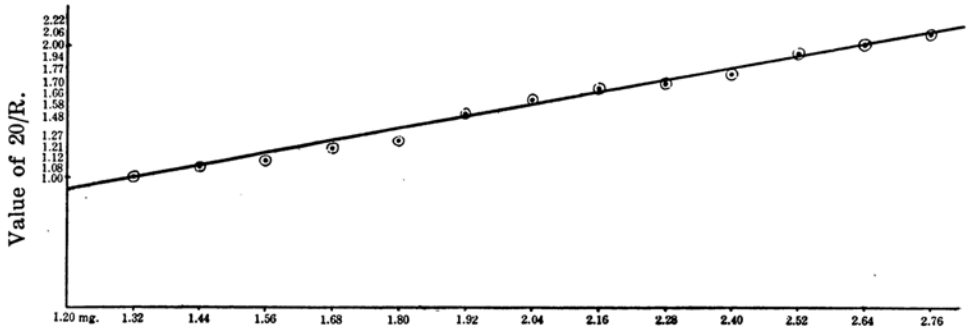


Fig. 1. (see Table 2).

Intensity of the colour with varied concentrations of cystine.
Standard—1.32 mg. of cystine set at 20 mm. in the colorimeter. Volume of the final dilution····50 c.c.



Concentration of cystine in mg. Fig. 2. (see Table 3).

Table 3.

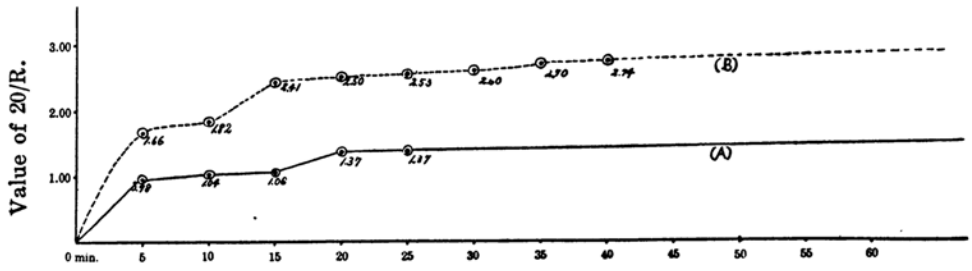
No. of cylinders	Weight of cystine in mg.	Value of 20/R.	Theoretical Ratio
1	1.44	1.08	1.09
2	1.56	1.12	1.18
3	1.68	1.21	1.27
4	1.80	1.27	1.36
5	1.92	1.48	1.45
6	2.04	1.58	1.55
7	2.16	1.66	1.64
8	2.28	1.70	1.71
9	2.40	1.77	1.82
10	2.52	1.94	1.91
11	2.64	2.00	2.00
12	2.76	2.06	2.09
13	2.88	2.22	2.10
14	3.00	2.30	2.27

Standard—1.32 mg. cystine set at 20 mm. Dilution volume····50 c.c.

Study of Colour Intensity and its Relation to Time. In order to find the time required for development of the colour with the varied concentrations of cystine solution, a number of experiments were carried out. On the basis of average values obtained, at the different time intervals, Fig. 3 was drawn. For obtaining colorimetric readings, the present author used the stock methylene blue solution as the standard, treating it exactly same as the cystine solution. As it was seen from the Fig. 3, the reaction of the colour development was obviously slow, and quite gradual.

The results of the further investigations on the colour reaction will be reported in a separate paper.

Relation between colour intensity and time intervals.
——— (A) 0.60 mg. ····· (B) 1.20 mg.
Dilution volume····20 c.c. for both series. Methylene blue solution was used as the standard. Standard was set at 20 mm.on the colorimeter.



Time intervals in minutes. Fig. 3.

Summary.

A new micro-method for the determination of R—S—S—R (Cystine) and R—SH (Cysteine) is described, based on the fact that dimethyl-*p*-phenylenediamine hydrochloride gives a distinct methylene blue colour with R—SH compounds when the proper concentration of acid and of ferric ammonium sulphate are used. The colour thus obtained is permanent.

The advantages of this method are ease in determination, lack of interfering substances under normal biological conditions, and relative equivalence of R—SH and R—S—S—R groups.[3]

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Department of Chemistry,
New York Post Graduate Medical School and Hospital,
Columbia University,
New York, N. Y.
U. S. A.

(3) The water extract of yeast gave a certain colour reaction to the test, and it suggested that vitamin B_1 in the yeast might have contained sulphur, of which chemical property is somewhat similar to that of R-SH or R-S-S-R compounds, in its complex chemical body.