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Summary

Dimethanesulphonylthioalkanes and analogous compounds were synthesized by the reaction of corresponding sodium thiosulphonate and appropriate dibromide to test the carcinostatic activity compared with Myleran. It was found that some of these compounds inhibited pronouncedly the growth of the solid tumor produced by Ehrlich ascites carcinoma cells.

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175. Masahiro Nakadate, Takatsune Maki, and Michiya Kimura : Fundamental Studies on Clinical Chemistry. VIII.*1 A New Method for the Colorimetric Determination of Urinary Creatinine with 2,2′,4,4′-Tetranitrobiphenyl.

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The Folin-Wu's procedure,[1] that is based upon the Jaffé's reaction[2] using picric acid and alkali, is one of the most commonly employed methods for the determination of urinary creatinine at present. Although this procedure is characteristic with regard to its higher sensitivity, it seems that reproducible results are difficult to obtain for several reasons as follows : First, the absorbances should be measured inevitably at the wave lengths of between 510 mμ and 520 mμ where the absorption curve shows a remarkably steep inclination, in order to avoid a extremely higher blank value at 480 mμ of maximum absorption in the reaction mixture; secondly, since picric acid can be reduced in an alkaline solution into picramic acid[3] which shows higher light absorption at the wave lengths around 515 mμ, the presence of reducing substances such as, for instance, glucose and ascorbic acid in the urine sample can affect some errors on the determination of creatinine; thirdly, the color produced is sensitive for the temperature of reaction mixture as well as even at the moment of absorbance measurement within spectrometer.[4] Although some improvements have been made on this method,[5~7] most of them seemed to be scarcely satisfactory in respects to some of the sensitivity, specificity, and stability.

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*1 Part Ⅶ : This Bulletin, **12**, 1138 (1964).

*2 Kita-12-Jo, Nishi-5-Chome, Sapporo (中舘正弘, 真木孝常, 木村道也).

1) O. Folin, H. Wu : J. Biol. Chem., **38**, 81 (1919).
2) M. Jaffé : Z. physiol. Chem., Hoppe-Seyler's **10**, 399 (1886).
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3,5-Dinitrobenzoic acid[8] was employed as an alternative reagent but the defect has been seen that the color produced with creatinine showed a photosensitivity.[9]

In the previous paper*1 of this series the Janovsky reactions of fifteen polynitrodiphenyl compounds were examined and it has been observed that they were able to give sensitive and stable coloration with active methylene compounds such as acetone and cyclohexanone under the Canbäck condition*3 and that creatinine, digitoxin, and cyclohexanone were able to give intensive colors with 2,2′4,4′-tetranitrobiphenyl (TNBP) under the Zimmermann condition,*3 though acetone and dehydroepiandrosterone did not show any considerable sensitivities under the latter condition.

The present paper, on the basis of these results, describes a new method for the colorimetric determination of urinary creatinine as one of the applications of color reaction by TNBP.

Experimental

Reagents and Apparatus——2,2′,4,4′-Tetranitrobiphenyl solution : TNBP was synthesized according to Gull, *et al.*,[10] purified by means of recrystallization from acetic acid to give pale yellow needles, m.p. 165°, and was used as a methylcellosolve-EtOH (2.7:2.3) solution (0.6%) in the standard procedure. Both crystalls and solution should be kept in brown bottles.
Potassium hydroxide : KOH of reagent grade was dissolved into pure water to make 4% solution.
Creatinine : Creatinine of reagent grade was used without any purification.
Methylcellosolve : Methylcellosolve of first grade was treated with 2,4-dinitrophenylhydrazine (500 mg./L.) and concentrated sulfuric acid (0.1 ml./L.) in a boiling water bath for 6 hr. and was distilled twice under reduced pressure ($b.p_{15}$ 35~36°). This solvent should be kept in a brown bottle.
Ethanol : EtOH was purified by refluxing with *p*-nitrophenylhydrazine (500 mg./L.) and conc. H_2SO_4 (0.1 ml./L.) for 6 hr. and distilled twice.
Spectrophotometer : Absorbance measurements were made with a Hitachi EPU-2A Spectrophotometer and absorption curve was obtained by a Hitachi Recording Spectrophotometer, using matched 10 mm. glass cells.

Standard Procedure——Place 0.5 ml. of urine (diluted into 1/10~1/100 in volume) in a test tube. Make a blank solution with 0.5 ml. of pure water in an another test tube. Add 5 ml. of TNBP solution

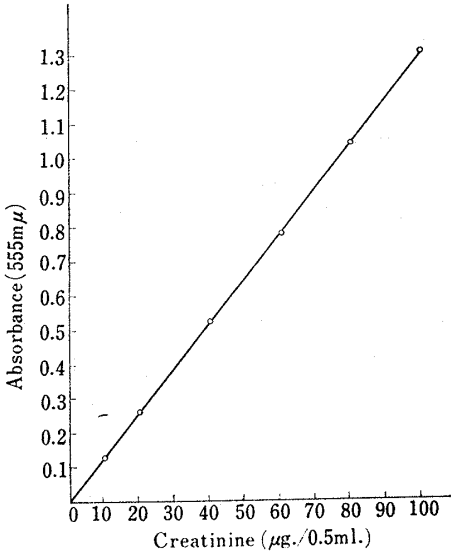

Fig. 1. Calibration Curve

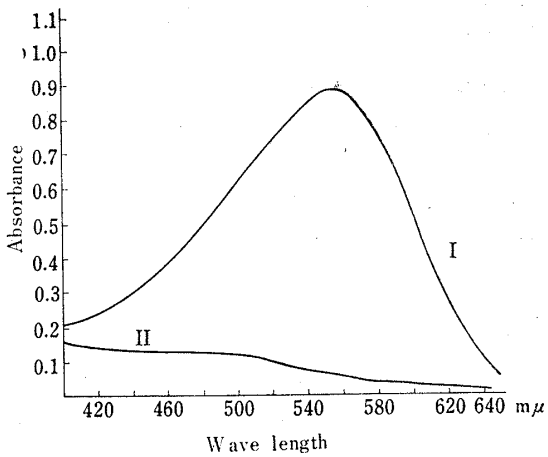

Fig. 2. Absorption Spectra

I : Color produced by creatinine (70 μg./0.5 ml.) under standard procedure.
II : Blank

*3 Norymbersky classified the Janovsky reaction according to the relative concentrations of reagents; C. S. Corker, J. K. Norymbersky, R. Thow : Biochem. J., 83, 586 (1962). See also the foot-note in the preceding paper, Part VII.

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10) H. C. Gull, E. E. Turner : J. Chem. Soc., **1929**, 491.

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and 0.1 ml. of KOH to each tube. Keep the tube at 40° for 10 min. after shaking thoroughly. Transfer the content of the tube to a colorimetercuvette after cooling and measure the absorbance at the wave length of 555mμ against the blank solution within 40 min. Read the concentration of creatinine from calibration curve (Fig. 1).

Results and Discussion

Absorption Spectrum

The absorption curve given by the standard procedure is shown in Fig. 2 which indicates that the maximum absorption is located at the wave length of 555 mμ and the reagent blank shows considerably smaller absorbance. The measurement of absorbanece in this method was, therefore, undergone at this wave length.

Solvent

Methylcellosolve was a suitable solvent for TNBP as has been described in the previous paper*[1] and it was observed that the presence of ethanol made some increase in the absorbance which was retained in a constant value at the ratio of methylcellosolve to ethanol of between 2.5:2.5 and 2.8:2.2 (in volume) as shown in Fig. 3. The ratio of 2.7:2.3 was, therefore, selected as the optimum for this procedure.

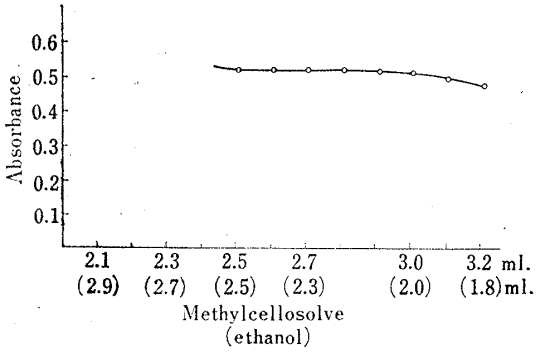

Fig. 3. Changes in Absorbance with Combinations of Methylcellosolve and Ethanol

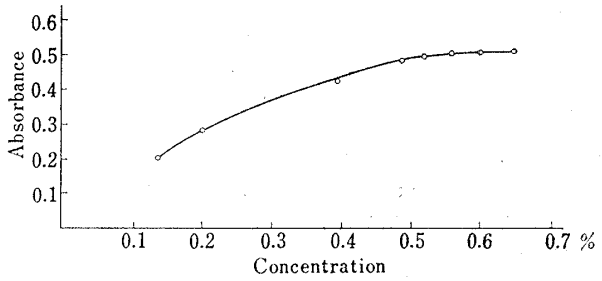

Fig. 4. Changes in Absorbance with Concentrations of 2,2′,4,4′-Tetranitrobiphenyl

Concentration of TNBP

Although higher absorbance was observed with increase in the concentration of TNBP, almost constant extinction value was obtained at the concentration of between 0.5 and 0.7% as shown in Fig. 4. The optimum concentration was consequently settled on 0.6%.

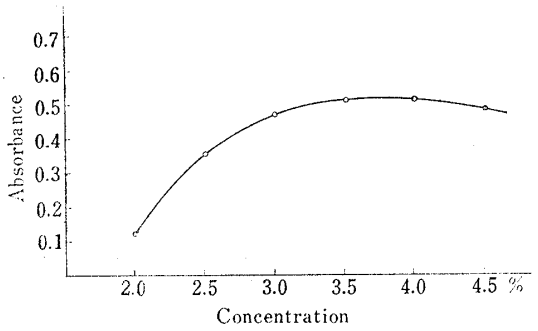

Fig. 5. Changes in Absorbance with Concentrations of Potassium Hydroxide

Alkali

Potassium hydroxide, sodium carbonate, sodium bicarbonate, and tetramethylammonium hydroxide were examined and none of them except potassium hydroxide was shown to be suitable to give an intensive coloration. It was observed that potassium hydroxide made gradual increase in absorbance by the concentration of 3.5% and showed almost constant extinction value at the concentration of between 3.5 and 4.2% as is shown in Fig. 5. The concentration of 4% was, therefore, settled as an optimum one.

More increase in the concentration of potassium hydroxide showed some fading of the color produced during the lapse of time, when the maximum absorption at 555 mμ

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showed rapid blue shift to the new maxima at 460 mμ and 360 mμ. This underwent proportionally to the increase in concentration of alkali. The studies on this phenomena are in progress at present and will be reported at the later date.

Effects of Time and Temperature

The most rapid, intensive, and stable coloration was given at the temperature of between 35° and 45° as is shown in Figs. 6 and 7. It was concluded consequently to be optimum for this color reaction to keep the reaction mixture at 40° for ten minutes. As in the cases of higher concentrations of alkali, similar fading in color was observed with increase in time and temperature as is shown in Figs. 6 and 7.

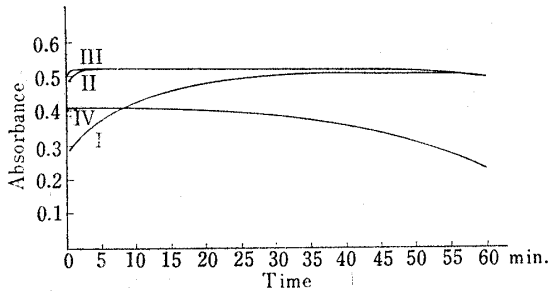

Fig. 6. Effect of Temperature on the Color Development

I: 12° II: 35°
III: 40°, 45° IV: 60°

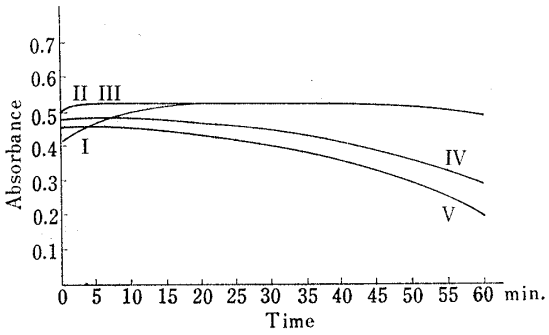

Fig. 7. Effect of Heating Time at 40° on the Color Development

I: 5 min. II: 10 min. III: 15 min.
IV: 20 min. V: 25 min.

Photosensitivity

In the standard procedure presented here, nothing was obsered on the photosensitivity which has been shown by the color reaction with 3,5-dinitrobenzoic acid.[9]

Specificity

Since the standard procedure is essentially based upon the reaction of active methylene compound with *m*-dinitrobenzene derivative, some of the disturbing factors should be, on the one hand, in the side of active methylene compound and, on the other hand, in the side of nitrobenzene derivative which can be reduced to give colored substance under some conditions. The ordinary components in urine samples, which might reasonably be expected to interfere this color reaction, are acetonebodies, reducing sugars, and ketosteroids. As to acetone, the absorbance was too small in this procedure to give practically any disturbing effects as has been shown in the previous paper*[1] and in Table I. Glucose and ascorbic acid were also indifferent even at the concentration of 100 γ/ml. as is shown in the same Table. As to urinary 17-ketosteroids, which have active

Table I. Specificity of this Method

Compounds	Normal urine (μg./ml.)	Concn. (μg./ml.)[a]	Found (μg./ml.)[b]
Acetone	74	99.4	2.0
Pyruvic acid	2.5	300	4.1
Ascorbic acid	5~55	100	—
Glucose	43	100	—
Dehydroepiandrosterone	15	100	—

a) Initial concentrations in the standard procedure.
b) Calculated as creatinine from calibration curve when abnormally large excess (shown in *a*)) of these compounds were added.

NII-Electronic Library Service

methylene group in their molecules and are usually present in smaller concentrations, the color development was shown to be remarkably slow under this procedure and nothing was observed on the disturbing effect. Table I shows that considerably larger amounts of urinary ketosteroid, represented by dehydroepiandrosterone, can not give any coloration in this standard procedure.

Recovery Test

Three different amounts of creatinine were added to each of three samples of urine and the standard procedure was carried out on these nine samples and three intact origins. The mean recovery value of 100.61% ($\sigma=0.99$) was obtained as is shown in Table II.

Table II. Recovery of Creatinine added to Urine

Creatinine (μg./0.5 ml. urine) Added	Found	Recovery (%)	Creatinine (μg./0.5 ml. urine) Added	Found	Recovery (%)
0	21.3	—	0	24.2	—
10	31.5	100.4	10	34.6	101.0
20	41.3	100.0	20	45.8	103.6
30	51.5	100.4	30	54.1	99.9
0	23.5	—			mean 100.6
10	33.6	100.2			$\sigma=0.99$
20	43.6	100.2			
30	53.7	100.3			

Precision

The determinations of urinary creatinine by the standard procedure were carried out five times on each of three urine samples and the percentages of the standard deviation to the mean value were obtained as is shown in Table III which seems indicate that this method is in a satisfactory precision.

Table III. Standard Deviation of Determination

Urine	Mean value (μg./0.5 ml.)	Standard deviation
I	21.3	0.2
II	23.5	0.3
III	24.2	0.2

Table IV. Perallel Test of Creatinine

Urine	This method (mg./dl.)	Folin-Wu's method (mg./dl.)
I	85.2	82.4
II	93.8	95.1
III	191.2	188.8
IV	69.6	67.9

Parallel Test with Other Method

On each of four urine samples were carried out the two methods, the standard procedure presented here and an improved method of Folin-Wu's[11] and almost similar results were given as is shown in Table IV.

Conclusion

The standard procedure presented in this paper is based upon the color reaction between creatinine and 2,2′,4,4′-tetranitrobiphenyl,*1 which belongs essentially to the wellknown reaction of active methylene compounds with *m*-dinitrobenzene derivatives in the presence of alkali. It was observed on the color produced by this method that

11) R. W. Bonsnes, H. H. Taussky: J. Biol. Chem., **158**. 581 (1945).

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it was stable for fourty minutes, nothing was shown on the photo- and temperature-sensitivity, the presence of some kinds of active methylene compound and reducing substance in reasonable amounts affected negligibly on the absorbances as is shown in Table I, and that 0 to 200 μg./ml. of creatinine were able to be determined with accuracy as is shown in Fig. 1 and Table Ⅲ. When different amounts of creatinine were added to the urine samples, the recovery of added material has been ranged from 99.9% to 103.6% as is shown in Table Ⅱ. An inspection of Table Ⅳ indicated that the results given by the present method was in good agreement that by the Folin-Wu's method.

It seemed most reasonable to conclude that the standard procedure presented in this paper was suitable for the practice of the clinical test.

This work was supported in part by a Grant-in-aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.

Summary

A sensitive and accurate colorimetric determination for urinary creatinine is described. The principle of this method is based on the reaction of creatinine with 2,2,′4,4′-tetranitrobiphenyl in the presence of potassium hydroxide to give a stable color which shows no photo- and temperature-sensitivity and is measured spectrometrically at 555 mμ. The applicable concentration range is 0 to 200 μg./ml. of creatinine. No interferences were shown by considerably larger amounts of acetone, glucose, ascorbic acid, pyruvic acid, and dehydroepiandrosterone.

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UDC 612.015.3 : 615.782.54

176. Toshihiko Ariyoshi and Eigo Takabatake*1 : Biochemical Studies on the Drug Metabolism. III.*2 Inductive Effect of Malonic Acid Derivatives on Cyclobarbital-Metabolizing Enzyme in Rat Liver.*3

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Many drugs are metabolized by NADPH-dependent enzyme system in liver microsomes.[1,2] Activity of this drug-metabolizing enzyme is affected by various kinds of condition : age, sex, strain, species, or neutritional status of animal,[3~6] the administra-

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*3 Presented at the 32nd Meeting of Kyushu Branch, Pharmaceutical Society of Japan, in May, 1963.

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