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87. Morizo Ishidate and Toshio Nambara : Naphthoresorcinol Picrate for the Determination of Glucuronic Acid in Physiological Materials.

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The naphthoresorcinol reaction[1] for the qualitative determination of glucuronic acid is commonly used in metabolic studies due to the comparatively selective reaction for uronic acid.

The reagent however is considerably unstable even in crystalline state and the solution is not quite valid on storage as well as on a series determination. For this reason various attempts have been made in which naphthoresorcinol-carboxylic acid[2] was alternatively used or the reagent solution was previously oxidized with oxygen,[3] persulfate or ferricyanate.[5] However, such modified methods still lacked consistency and reproducibility in the determination.

The present paper proposes naphthoresorcinol picrate (NRP) as the reagent which is easily obtainable in pure crystalline state from each mole of the two components.

The new reagent is practically stable for one year so far observed when it was kept in an ampule in dry state, excluding light and oxygen. Its solution in dehydrated ethanol was fairly stable as a stock solution at least for one week. Moreover, the absorbance at λ_{max} 570 mμ obtained from NRP reagent and glucuronic acid in the presence of sulfuric acid was about 30% higher than in the case of naphthoresorcinol. It is assumed therefore that picric acid in the reagent plays a rôle in oxidizing action in favor of the formation of the characteristic dye[6] in this determination.

The procedure employing the new reagent was carried out according to the description of Fishman, *et al.*,[7] with a slight modification. The calibration curve with glucuronic acid quite satisfied the Beer's law in comparatively large magnitude of the amount (10~80 γ/4 cc.) with good reproducibility. A satisfactory result was also obtained in the recovery test with human urine mixed with a definite amount of glucuronic acid.

Although the new reagent possesses the advantage in some points over naphthoresorcinol reagent, the indispensable difficulties due to the unstable nature of the complicated pigments formed in the reaction are not solved.

Reagents and Apparatus

Naphthoresorcinol Picrate (NRP) Solution**—1% solution of NRP (see below) in dehyd. EtOH. It should be kept in a cold and dark place, and is valid for 1 week.

Sulfuric acid solution**—21.6*N* solution obtained from 3 vols. of H_2SO_4 (reagent grade) and 2 vols. of water.

Ethanol—95%; J. P. VI.

Toluene—First grade J. I. S.

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** A new curve should be drawn whenever a new batch of NRP or sulfuric acid is used.

1) B. Tollens, F. Rorive : Ber., **41**, 1783(1908).
2) A. Ogata, T. Yamanouchi : J. Pharm. Soc. Japan, **49**, 554(1929).
3) N. E. Artz, E. M. Ogman "Biochemistry of Glucuronic Acid," Academic Press Publishers, Inc. N. Y., 7(1950).
4) K. Meyer *et al.* : Federation Proc., **1**, 125(1942).
5) N. G. Bisset *et al.* : Biochem. J. (London), **42**, 366(1948).
6) T. Momose, Y. Ueda, M. Iwasaki : This Bulletin, **4**, 49(1956).
7) W. H. Fishman *et al.* : J. Biol. Chem., **215**, 527(1955).

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A standard Solution of Glucuronic Acid—Prepared from crystalline glucuronolactone(recrystallized from MeOH : m.p. 174°).

Spectrophotometer—In this work both Shimadzu Model QB-50 spectrophotometer and Hirama photometer model II were used.

Procedure

In a glass-stoppered test tube (30~35 cc.), 4 cc. of a test solution (not less than 10 γ as total glucuronic acid), 1 cc. of NRP reagent, and 2 cc. of 21.6*N* H_2SO_4 are placed. The mixture is agitated and heated in a boiling water bath for 90 mins. After cooling the tube in a tap-water for 15~20 mins. the produced pigment is dissolved by addition of 10 cc. of 95% EtOH and then extracted with 8 cc. of toluene under vigorous agitation. The toluene layer is separated with a pipette, filtered rapidly through cotton-wool, and the absorbance of the filtrate is measured at 570 mμ or by using 562 mμ-filter of photometer with 1-cm. cell. The blank determination with the reagent used is undertaken. In the case of urine, 50~200 times diluted test solution is employed.

Experimental

Naphthoresorcinol Picrate (NRP)—A mixture of naphthoresorcinol (0.48 g.), freshly prepared from barium naphthoresorcinolcarboxylate, and pure picric acid (0.69 g.) were dissolved in hot water (30 cc.) and rapidly filtered. On cooling, deep red crystals separated; m.p. 178.5~179°(decomp.); yield, 0.82 g. *Anal.* Calcd. for $C_{16}H_{11}O_9N_3$: N, 10.80. Found : N, 10.52.

Absorption Spectrum—The absorption curve of the colored solution produced from 80 γ of glucuronic acid in 4 cc. according to the above procedure is shown in Fig. 1. The instrument used was Shimadzu Spectrophotometer Model QB 50.

Comparison of the Absorbance obtained with NRP and NR—The absorbance at 570 mμ obtained by using NRP (0.49%) on one hand and the same mole of naphthoresorcinol (NR) on the other hand was compared, employing 18*N* and 24*N* H_2SO_4, the other conditions being the same as above. The results are shown in Table I and Fig. 2.

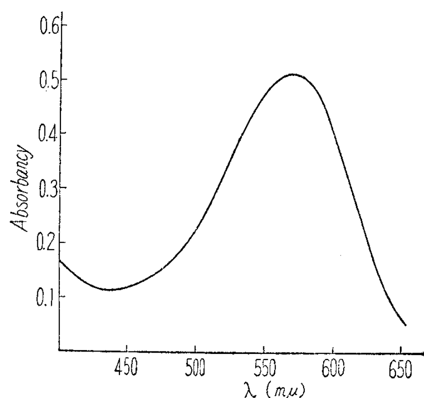

Fig. 1. Absorption Spectrum of the Colored Solution produced from NRP and 80 γ Glucuronic Acid in 4 cc. under the Standard Procedure

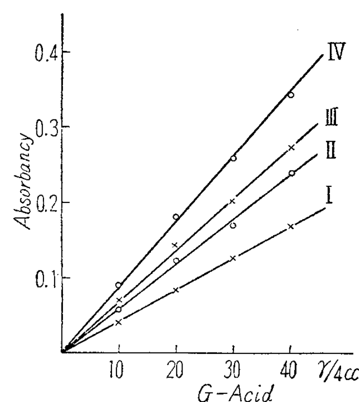

Fig. 2. Comparison of the Absorbancy obtained with NRP and NR

—o—o— 0.49% NRP —×—×— 0.2% NR

I, II : 18 *N* H_2SO_4 III, IV : 24 *N* H_2SO_4

Table I. Comparison of the Absorbancy 0.49% NRP and 0.2% NR with 18*N* and 24*N* sulfuric acid

H_2SO_4	Acid (γ/4 cc.)	0.49% NRP (E)	0.2% NR (E)
18*N*	10	0.059	0.040
	20	0.124	0.083
	30	0.170	0.124
	40	0.239	0.167
24*N*	10	0.089	0.070
	20	0.180	0.147
	30	0.259	0.201
	40	0.344	0.275

Relationship between Absorbance and Concentration of NRP—The aqueous solution of NRP was not stable enough for longer storage than 48 hrs., whereas dehyd. EtOH solution stood longer use (1 week at least).

The effect of concentration (1%, 1.2%, and 1.6% in dehyd. EtOH) of NRP on the absorbance

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under the same condition as the standard procedure is shown in Fig. 3. It indicates that the values of absorbance through each run linearly change continuously with the concentration of the reagent. From the practical view point it is recommended therefore that when 1% NRP-solution is employed the test solution should be diluted as far as possible.

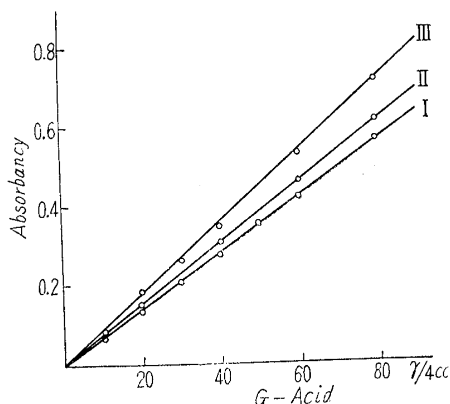

Fig. 3.

Effect of the Concentration of NRP

I : 1% NRP (dehyd. EtOH soln.)
II : 1.2% NRP (dehyd. EtOH soln.)
III : 1.6% NRP (dehyd. EtOH soln.)

Effect of Reaction Time and the Concentration of Sulfuric Acid—Employing 1% NRP and 24*N* H_2SO_4, the influence of heating time at 100° on the absorbance was studied. As shown in Fig. 4, the value of absorbance increased with prolongation of reaction time. A more powerful effect was observed in the case of H_2SO_4 concentration used. Fig. 5 shows the relationship between absorbance and H_2SO_4 concentration of 18*N*, 21.6*N*, and 24*N* under the standard condition.

In consideration of practical application in biological material, the standard procedure adopted the reaction time of 1.5 hrs. and 21.6 *N* H_2SO_4.

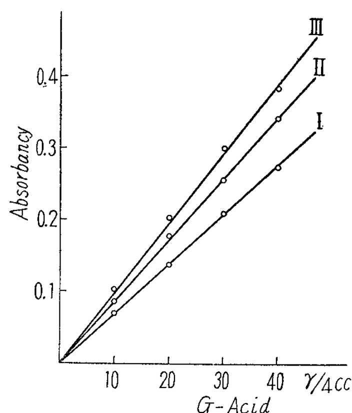

Fig. 4. Effect of Reaction Time
I : 1 hr., II : 1.5 hrs., III : 2 hrs.

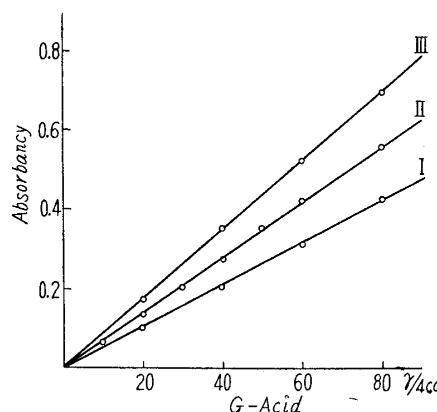

Fig. 5. Effect of the Concentration of Sulfuric Acid
I : 18*N* H_2SO_4, II : 21.6*N* H_2SO_4, III : 24*N* H_2SO_4.

The nature of the reaction was greatly influenced by acid concentration, temperature, and length of time employed for color development. Therefore, the conditions cited above should be followed strictly in order to obtain reproducible results.

Recovery Test for Glucuronic Acid added to Diluted Urine—A known amount of glucuronic acid which was added to diluted human urine (1 : 50 and 1 : 100) was measured using the calibration curve by the standard procedure. The calculated and found values for glucuronic acid are given in Table II and the results are quite satisfactory.

Table II. Recovery Test for Glucuronic Acid added to Diluted Urine

	Acid in urine (γ/4 cc.)	Acid added (γ/4 cc.)	Total Acid Calcd. (γ/4 cc.)	Total Acid Found (γ/4 cc.) (%)
Urine I (1 : 100)	11.1	20	31.1	30.7 (98.8)
		30	41.1	40.6 (98.7)
Urine II (1 : 100)	11.6	20	31.6	31.0 (98.0)
		30	41.6	41.4 (99.4)
		40	51.6	49.9 (96.6)
Urine III (1 : 50)	18.5	20	38.5	37.8 (98.0)
		30	48.5	47.2 (97.3)
		40	58.5	57.6 (98.5)

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Estimation of Free and Conjugated Glucuronic Acid—Fishman, *et al.*[7] demonstrated the determination of glucuronide in presence of glucuronic acid which is based on the prior oxidation of the acid and the other interfering substances with alkaline hypoiodide solution, the remaining glucuronides being determined by the NR reaction. This procedure provided a valuable method for the estimation of alkali-stable glucuronides in physiological fluids.

The new reagent (NRP), however, was found to be not applicable to the above procedure. Iodide produced by the prior oxidation was oxidized to I_2 during the reaction with the new reagent and interfered in the following color estimation. This difficulty was tolerably well overcome by employing an alkaline Br_2 solution instead of I_2 solution. An example of simultaneous estimation of free and conjugated glucuronic acids (borneol glucuronide) is presented in Table III. Further investigation is being continued.

TABLE III. Estimation of Free and Conjugated Glucuronic Acid (Borneol Glucuronide)

Total acid (T)		Acid (C) in borneol glucuronide		Free Acid (T−C)	
Calcd. (γ/5 cc.)	Found (γ/5 cc.) (%)	Calcd. (γ/5 cc.)	Found (γ/5 cc.) (%)	Calcd. (γ/5 cc.)	Found (γ/5 cc.) (%)
120	118.2 (98.5)	80	78.1 (97.6)	40	40.1 (100.3)
120	119.9 (99.9)	40	39.4 (98.5)	80	80.5 (100.0)
100	101.7 (101.7)	60	61.7 (102.8)	40	40.0 (100.0)
140	143.8 (102.7)	60	60.3 (100.5)	80	83.5 (104.4)

Summary

Naphthoresorcinol picrate, 1:1 molecular compound, is recommended for the microdetermination of glucuronic acid. The new reagent is advantageous over the usual naphthoresorcinol in the stability on storage, both in crystalline and solution state, and it exhibits higher absorbancy and better reproducibility. The standard procedure was established. It has been found that the measurement of glucuronide with this reagent in the presence of glucuronic acid is possible by employing an alkaline bromine method instead of hypoiodide procedure described by Fishman, *et al.*

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88. **Keiichiro Hayashi** : Studies on the Microanalysis of Essential Oil Components. V.[1] Detection of Essential Oil Components by Paper Electrophoresis.

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In the present work, a new method was tried for the electrophoresis of some essential oil components which were regarded hitherto to be difficult to treat by paper chromatography or electrophoresis, since most of them are usually insoluble in water and therefore, it could not be developed on paper by aqueous electrophoresis. A reversed-phase method by silicone-treated or method of chromatography with a paper impregnated with sodium hydrogen sulfite had already been made by the author with special reference to aldehyde compounds.[2] In this respect somewhat similar procedures were applied also in the paper electrophoresis of essential oil components, by

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1) Part IV. K. Hayashi, Y. Hashimoto : This Bulletin, 5, 618(1957).
2) K. Hayashi, Y. Hashimoto : *Ibid.*, 5, 74(1957).

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