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Reaction of Guanidines with α-Diketones. I. A New Colorimetric Method for the Determination of Guanidine and Monosubstituted Guanidines with 9,10-Phenanthraquinone and 3,5-Dihydroxybenzoic Acid[1]

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A new colorimetric method for the determination of non- and mono-substituted guanidine compounds was established, by using 0.04% 9,10-phenanthraquinone in dioxane–EtOH (1:4) and 2% 3,5-dihydroxybenzoic acid in EtOH with the addition of 2 N KOH aqueous solution, and measuring the absorbance at 615 nm. The color intensity becomes constant after standing for 90 min in the case of guanidine, and for more than 100 min in the case of mono-substituted guanidine at room temperature. This method is recommended for the determination of non- and mono-substituted guanidines. Beer's law holds in the range of 2.5×10^{-3} to 6×10^{-2} μmol/ml in the final solution of various guanidine compounds such as salts of guanidine (**25**—**28**), glycocyamine (**32**), agmatine (**33**), and arginine (**34**). Guanidines with a large substituent or with an electronegative group, showed less color intensity than methylguanidine (**29**), or did not show any coloration.

On the other hand, 1-naphthol method can be used only for the detection of mono-substituted guanidines. Limit of identification of these compounds was in the range from 0.3 to 2 μg.

In the preceding paper,[3] we reported that only CH_3COCOR-type α-diketones underwent coloration in the Voges-Proskauer reaction using N,N-disubstituted guanidines and 1-naphthol in the presence of alkali, and its mechanism was confirmed by the isolation of three kinds of pigments.[4–8] The present paper describes a novel colorimetric determination based on the new color reaction for non- and mono-substituted guanidine compounds, using 9,10-phenanthraquinone (phenanthraquinone) and 3,5-dihydroxybenzoic acid or 1-naphthol, under the condition of Voges-Proskauer reaction.

Experimental

Materials——Guanidine compounds such as agmatine, glycocyamine, guanidine, arginine, streptomycin, creatine, and creatinine were highly purified or reagent grade and obtained from commercial source. Urea, thiourea, formamidine, and benzamidine were also obtained commercially in a high grade of purity. Other materials (Tables I and II) were synthesised according to the literature.[9,10]

Instruments——Absorption spectra and color intensity were measured with a Hitachi EPS-3T recording spectrophotometer and a Hitachi 101 spectrophotometer, respectively, by the use of 10-mm cells.

1) This work was presented at the 92nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 1972.
2) Location: *1–33 Yayoi-cho, Chiba, 280, Japan.*
3) T. Sakaguchi, S. Tanabe, U. Nonaka, M. Takeuchi, Y. Onuki, and M. Ishizaki, *Yakugaku Zasshi*, **91**, 695 (1971).
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9) T. Nishimura, S. Tanabe, H. Toku, Y. Sakabe, T. Kono, and T. Sakaguchi, *Chem. Pharm. Bull.* (Tokyo), **17**, 639 (1969).
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Detection of Guanidines with Phenanthraquinone and 1-Naphthol——The reagent for color development (solution A) was prepared freshly at each experiment by dissolving 40 mg of phenanthraquinone and 3 g of 1-naphthol in 100 ml of dioxane-EtOH mixture. When 1 drop of each solution A and 3 N KOH solution were added with stirring at room temperature, to 1 drop of the test solution of guanidines, blue color appeared immediately.

Results

Reactivity of the Reagents

1. Reactivity of α-Diketones and Phenols and Naphthols with Guanidines——The reactivity of α-diketones with 1-naphthol in the case of guanidines having a free amidino group

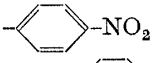
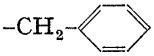
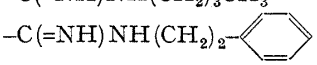
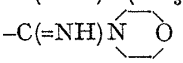
Table I. Reactivity of α-Diketones and Phenols and Naphthols with Guanidines

Materials / Compd. No.	Guanidines: Guanidine, Color of the blank	Guanidine, Colored solution	Methylguanidine, Color of the blank	Methylguanidine, Colored solution	N,N-Dimethylguanidine, Color of the blank	N,N-Dimethylguanidine, Colored solution
A) α-Diketones[a]						
1. phenanthraquinone	pale yellow	blue (###)	pale yellow	blue (##)		—
2. β-naphthoquinone		—		—		—
3. benzil		blue (+)		blue (+)		—
4. 1,2-cyclohexanedione		—		—		—
5. diacetyl	pale yellow	pink (+)	pale yellow	pink (##)	pale yellow	pink (###)
6. 3,4-hexanedione		—		—		—
7. 4,5-octanedione		—		—		—
8. 1-phenylpropane-1,2-dione	pale yellow	violet (+)	pale yellow	violet (##)	pale yellow	violet (###)
B) Phenols and Naphthols[b]						
9. 1-naphthol	pale yellow	blue (###)	pale yellow	blue (##)		—
10. 2-naphthol		—		—		—
11. sodium 1-naphthol-2-sulfonate		—		—		—
12. potassium 1-naphthol-4-sulfonate		—		—		—
13. 2-chloro-1-naphthol	pale yellow	blue (+)	pale yellow	blue (+)		—
14. 4-chloro-1-naphthol	pale yellow	blue (+)	pale yellow	blue (+)		—
15. 2,4-dichloro-1-naphthol		—		—		—
16. 1,2-dihydroxynaphthalene		—		—		—
17. 2,3-dihydroxynaphthalene		—		—		—
18. 1,5-dihydroxynaphthalene		—		—		—
19. 1,7-dihydroxynaphthalene		—		—		—
20. resorcinol[c]	yellowish green	green (###)	yellowish green	green (##)		—
21. 2-methylresorcinol		—		—		—
22. 4-hexylresorcinol[c]	reddish orange	violet (##)	reddish orange	violet (+)		—
23. 5-methylresorcinol[c]	orange	violet (##)	orange	violet (+)		—
24. 3,5-dihydroxybenzoic acid	pale yellow	blue (###)	pale yellow	blue (##)		—

a) To 1 ml of 0.01% sample, 1 ml of 0.5 mM guanidines solution and 1 ml of 1% 1-naphthol alkaline solution (10% KOH) were added.
b) To 1 ml of 1% sample (10% KOH soln.), 1 ml of 0.05% phenanthraquinone-EtOH solution and 1 ml of 0.5 mM guanidines solution were added.
c) Blank's color gradually developed and color of the reaction mixtures faded in a short time.
###: strong, ##: medium, +: weak, —: negative

is summarized in Table I. From the results shown in Table I, diacetyl (Compd. No. **5**) and 1-phenylpropane-1,2-dione (No. **8**) of CH_3COCOR type gave positive reaction as was expected,[4] and benzil (No. **3**) and phenanthraquinone (No. **1**) gave a positive reaction with guanidine and methylguanidine. The color intensity was less in benzil than in phenanthraquinone. Cyclic (No. **2** and **4**) and aliphatic (No. **6** and **7**) α-diketones did not show any coloration. From these results, it was found that phenanthraquinone may be applicable as a new color reagent for mono- and non-substituted guanidines instead of CH_3COCOR-type α-diketones. The reactivity of phenols and naphthols was then tested when phenanthraquinone was used. Results shown in Table I, indicated that resorcinols (No. **20**—**24**) and 2-chloro-(No. **13**) and 4-chloro-1-naphthol (No. **14**) showed the same color reactions as 1-naphthol (No. **9**). 3,5-

Table II. Detection of Guanidines and Related Nitrogen Compounds with Phenanthraquinone and 1-Naphthol in Alkaline Medium

Compd. No.	Formula	Limit of identification (μg)	
A)	$H_2N-C(=NH)-NH_2$	HX	
25	$H_2N-C(=NH)-NH_2$	H_2CO_3	0.3
26	$H_2N-C(=NH)-NH_2$	$1/2HClO_4$	0.3
27	$H_2N-C(=NH)-NH_2$	HNO_3	0.3
28	$H_2N-C(=NH)-NH_2$	HCl	0.3
B)	$H_2N-C(=NH)-NHR$	HX	
29	$-CH_3$	$1/2H_2SO_4$	1
30	$-NH_2$	H_2CO_3	2
31	$-NO_2$		—
32	$-CH_2COOH$		1
33	$-(CH_2)_4NH_2$	HCl	2
34	$-(CH_2)_3CH(NH_2)COOH$	HCl	1
35	Streptomycin		—
36		HNO_3	—
37		$1/2H_2SO_4$	—
38		$1/2H_2SO_4$	100
39	$-C(=NH)-NH_2$	HCl	1
40	$-C(=NH)NH(CH_2)_3CH_3$	HCl	—
41		HCl	—
42	$-C(=NH)N(CH_3)_2$	HCl	—
43		$1/2H_2SO_4$	—
C)	$H_2N-C(=NH)-NR_1R_2$	HX	
44	$-CH_3, -CH_3$	$1/2H_2SO_4$	—
45	$-(CH_2)_5-$	$1/2H_2SO_4$	—
46	$-CH_3, -CH_2COOH$	H_2O	—
D)	$R_1HN-C(=NH)-NHR_2$	HX	
47	$-CH_3, -CH_3$	HI	—
E)	$R_1R_2N-C(=NH)-NR_3R_4$	HX	
48	$-CH_3, -CH_3, -CH_3, -CH_3$	HI	—
F)	$R_1R_2N-C(=NR_5)-NR_3R_4$	HX	
49	$-CH_3, -CH_3, -CH_3, -CH_3, -CH_3$	HI	—
G)	$H_2N-C(=NH)R$	HX	
50	$-H$	HCl	—
51		HCl	—
H) 52	$H_2N-(C=O)-NH_2$		—
I) 53	$H_2N-(C=S)-NH_2$		—

Dihydroxybenzoic acid (No. **24**) gave a good result as resorcinols but required longer time (about 30 min) than 1-naphthol (immediately or after 5 min) to reach the maximum intensity.

2. Reactivity of Guanidines with Phenanthraquinone and 1-Naphthol——The reactivity of guanidines under the conditions described in the experimental part is summarized in Table II.

The blue coloration occurred only with non-substituted guanidines (Compd. No. **25**—**28**) and mono-substituted guanidines (No. **29**, **30**, **32**—**34**, and **39**). N,N-Disubstituted guanidines (No. **44**—**46**), and mono- and di-substituted biguanides (No. **40**—**43**), which were positive both in Voges-Proskauer and Sakaguchi reactions, did not show any coloration in the reaction of this method.

Guanidines substituted with a free amidino group (Compd. No. **47**, **48**, and **49**), urea (No. **52**), thiourea (No. **53**), and amidines (No. **50** and **51**) did not exhibit any color reaction. Hydrazines, such as phenylhydrazine, semicarbazide, etc., which reacted with phenanthraquinone to give reddish orange coloration in alkaline solution, interfered with this reaction of guanidines.

Limit of identification of guanidine and mono-substituted guanidines was ranging from 0.3 to 2 μg (Table II).

Colorimetric Determination for Guanidine and Mono-substituted Guanidines using Phenanthraquinone and 3,5-Dihydroxybenzoic Acid

As shown in Table II, salts of guanidine (Compd. No. **25**—**28**), arginine (No. **34**), glycocyamine (No. **32**), and agmatine (No. **33**), which are biologically important compounds, gave positive results in this color reaction. In order to know the best condition for the determination of these compounds by this method, the reaction was examined by using guanidine carbonate and the following colorimetric method was established. Reagent 1: 0.04% phenanthraquinone dioxane–EtOH (1: 4). Reagent 2: 2N KOH aqueous solution. Reagent 3: 2% solution of 3,5-dihydroxybenzoic acid in EtOH. The sample solution of 1 ml, added to 2 ml of reagent 1, 1 ml of reagent 3 and 1 ml of reagent 2 was shaken vigorously, and the color

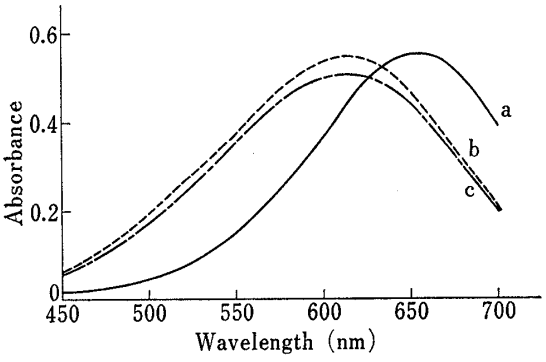


Fig. 1. Absorption Spectra of Guanidine and Arginine with 1-Naphthol-phenanthraquinone and 3,5-Dihydroxybenzoic Acid-Phenanthraquinone in Alkaline Medium

a: reaction mixture of guanidine and 1-naphthol-phenanthraquinone
b: reaction mixture of guanidine and 3,5-dihydroxybenzoic acid-phenanthraquinone
c: reaction mixture of arginine and 3,5-dihydroxybenzoic acid-phenanthraquinone

1-naphthol-phenanthraquinone method: To 2 ml of 0.2 mM guanidine or arginine, 5 ml of 0.03% phenanthraquinone dioxan-EtOH mixture, 2 ml of 5% 1-naphthol EtOH solution, and 1 ml of 3N KOH were added. 3,5-Dihydroxybenzoic acid-phenanthraquinone method: To 1 ml of 0.2 mM guanidine or arginine, 2 ml of 0.04% phenanthraquinone dioxan-EtOH mixture, 1 ml of 2% 3,5-dihydroxybenzoic acid-EtOH solution, and 1 ml of 2N KOH were added.

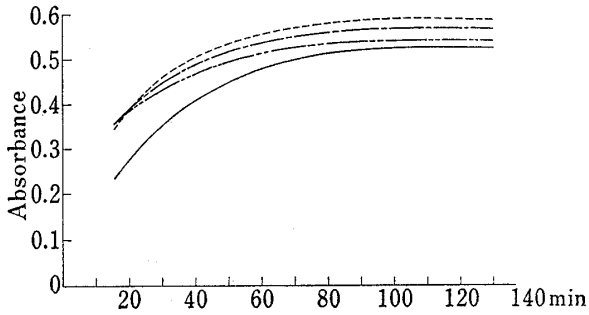


Fig. 2. Effect of Standing Time on Color Development for the Various Concentrations of KOH with 3,5-Dihydroxybenzoic Acid-Phenanthraquinone Method at 615 nm

———: 1N KOH, – – – – –: 2N KOH,
— · — · —: 3N KOH, — — — —: 5N KOH

To 1 ml of sample, 2 ml of 0.04% phenanthraquinone dioxane-EtOH mixture, 1 ml of 2% 3,5-dihydroxybenzoic acid EtOH solution, and 1 ml of KOH were added. sample: 0.2 mM guanidine carbonate

intensity was measured after standing for 90 min (Fig. 7) at 615 nm (Fig. 1). A linear relationship was obtained in the range from 2.5×10^{-3} to 6×10^{-2} μmol/ml of guanidine in the final solution (Fig. 6).

1. Determination of Guanidine——a) Phenanthraquinone-3,5-Dihydroxybenzoic Acid Method: Guanidine gave an absorption maximum at 615 nm when treated with phenanthraquinone and 3,5-dihydroxybenzoic acid, as shown in Fig. 1-b.

Fig. 2 shows the effect of standing time on color development when the concentration of KOH was varied, while other conditions being kept constant. The developed color became stable after standing for about 90 min in all concentrations examined and the maximum intensity was observed when 1 ml of 2N KOH was used.

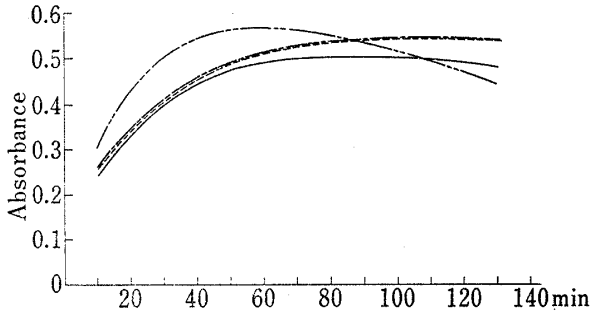


Fig. 3. Effect of Standing Time on Color Development for the Various Concentrations of Phenan thraquinone with 3,5-Dihydroxybenzoic Acid-Phenanthraquinone Method at 615 nm

———: 0.02%, – – – – –: 0.03%,
———: 0.04%, – · – · – ·: 0.05%

To 1 ml of sample, 2 ml of phenanthraquinone dioxane-EtOH mixture, 1 ml of 2% 3,5-dihydroxybenzoic acid EtOH solution, and 1 ml of 2N KOH were added.
sample: 0.2 mM Guanidine carbonate

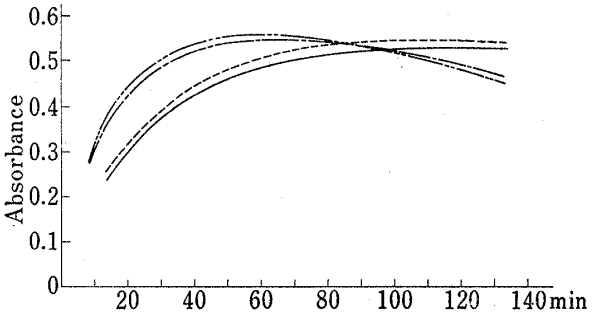


Fig. 4. Effect of Standing Time on Color Development for the Various Concentrations of 3,5-Dihydroxybenzoic Acid with 3,5-Dihydroxybenzoic Acid-phenanthraquinone Method at 615 nm

———: 1%, – – – – –: 2%,
———: 3%, – · – · – ·: 4%

To 1 ml of sample, 2 ml of 0.04% phenanthraquinone dioxane-EtOH mixture, 1 ml of 3,5-dihydroxybenzoic acid EtOH solution, 1 ml of 2N KOH were added.
sample: 0.2 mM guanidine carbonate

The maximum absorbance was found when the concentration of phenanthraquinone was more than 0.03%, and 0.04% was regarded as the best concentration when the color development in the blank is taken into account, as shown in Fig. 3.

Finally, the effect of color development on the concentration of 3,5-dihydroxybenzoic acid was examined as shown in Fig. 4. The concentration of 3,5-dihydroxybenzoic acid required for the optimum sensitivity was found to be more than 2%. Taking into consideration of the color developed in the blank, 2% of 3,5-dihydroxybenzoic acid was adopted, where the developed color became stable after standing for about 90 min.

b) Phenanthraquinone-1-Naphthol Method: As shown in Table I, 1-naphthol can also be used as a reagent for guanidine with phenanthraquinone. Guanidine showed absorption maximum at 655 nm (Fig. 1-a). Phenanthraquinone is only slightly soluble

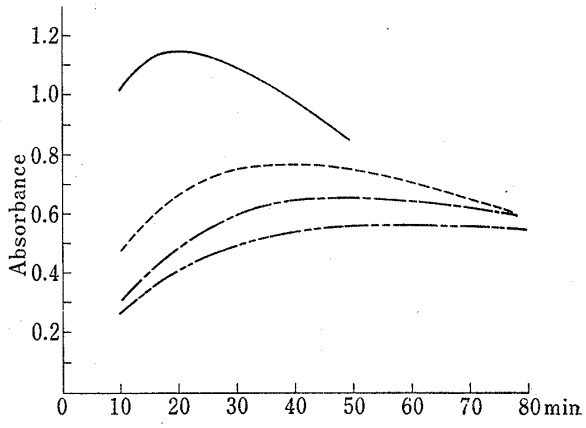


Fig. 5. Effect of Standing Time on Color Development for Various Concentrations of Hydroquinone as a Stabilizing agent with 1-Naphthol-phenanthraquinone Method at 655 nm

———: none, – – – – –: 0.05%,
———: 0.01%, – · – · – ·: 0.02%

To 1 ml of sample, 2.5 ml of 0.03% phenanthraquinone-dioxane-EtOH mixture, 0.5 ml of 10% 1-naphthol EtOH solution, 0.5 ml of hydroquinone EtOH solution, and 0.5 ml of 3N KOH were added.
sample: 0.4 mM guanidine carbonate

in ethanol and insoluble in water, but very soluble in dimethylformamide and dioxane. However, phenanthraquinone reacts in dimethylformamide to give red coloration in the presence of alkali, and further dioxane separates into two layers when alkali is added to the reaction mixture. Accordingly, dioxane–EtOH mixture (1: 4) was found to be the most suitable solvent as in the previous method. However, the developed color gradually faded after 20 min in various concentrations of the reagents. Therefore, the addition of some stabilizing agent was needed.

Only hydroquinone gave a good result as the stabilizing agent in the reaction with 1-naphthol, among the reagents tested such as sodium metabisulfite, sodium sulfite, sodium hydrosulfite, hydroxylamine hydrochloride, ascorbic acid, glucuronolactone, and hydroquinone. Increase in the concentration of hydroquinone enhanced the stability of the coloring matter, but lowered the color intensity, as shown in Fig. 5. High concentration of hydroquinone had to be avoided, because the blank solution gradually turned brown. Therefore, 0.01% concentration of hydroquinone was adopted (the curve of odd broken line in Fig. 5). The color was stable for about 50 min.

A calibration curve shown in Fig. 6 was obtained by the use of 0.03% phenanthraquinone in dioxane–EtOH mixture (1: 4) and 5% 1-naphthol EtOH solution in 3N KOH aqueous solution with the addition of hydroquinone in the concentration of 0.01%. Figure 6 indicates that in 1-naphthol-hydroquinone method, color intensity was about one half of that in 3,5-dihydroxybenzoic acid method, and further strong coloration was observed in the blank solution. 3,5-Dihydroxybenzoic acid method was regarded as the method to be recommended on the whole, although this method requires longer time to reach to the maximum intensity than 1-naphthol method.

2. Determination of Arginine, Glycocyamine, and Agmatine with Phenanthraquinone and 3,5-Dihydroxybenzoic Acid——The method of the determination of arginine, glycocyamine, and agmatine was investigated based on the results described above. Absorption maxima of these compounds under the condition mentioned above were observed at 615 nm similarly to the case of guanidine (Fig. 1-c), and the color intensity became stable after standing for about 100—120 min, as shown in Fig. 7. The color intensity of these compounds increased in the order, arginine, glycocyamine, and agmatine, and a linear relationship between the absorbance

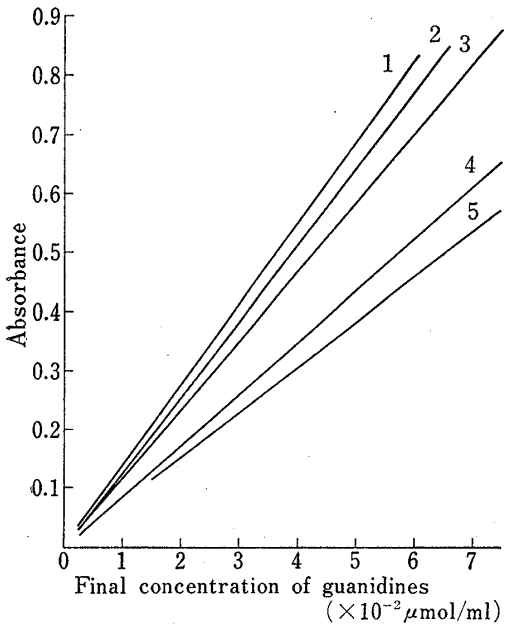


Fig. 6. Calibration Curves of Guanidines

1: guanidine, 2: arginine, 3: glycocyamine, 4: agmatine, 5: guanidine
1—4: 3,5-dihydroxybenzoic acid-phenanthraquinone method at 615 nm
5: 1-naphthol-phenanthraquinone-hydroquinone method at 655 nm

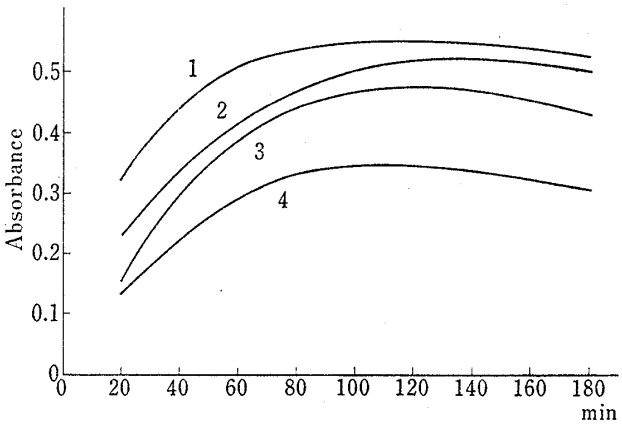


Fig. 7. Effect of Standing Time on Color Development of Guanidine and Monosubstituted Guanidines with 3,5-Dihydroxybenzoic Acid-Phenanthraquinone Method at 615 nm

(1) guanidine, (2) arginine, (3) glycocyamine, (4) agmatine

and the concentration was observed in the range from 2.5×10^{-3} to 6×10^{-2} μmol/ml in the final solution, similarly to the case of guanidine (Fig. 6).

Discussion

With respect to the method of the analysis of guanidines, Feigl's spot test[11] for guanidine carbonate and the fluorometric analysis with phenanthraquinone for mono-substituted guanidines reported by Yamada and Itano[12] have been presented. A new method described here is based on the color reaction of non- and mono-substituted guanidines with phenanthraquinone.

Guanidines with a large substituent or with an electronegative group, *e.g.*, streptomycin (Compd. No. **35**), phenylguanidine (No. **36**), nitroguanidine (No. **31**), *p*-nitrophenylguanidine (No. **37**), benzylguanidine (No. **38**), and mono-substituted biguanides (No. **39**—**43**), except biguanide (No. **39**), showed less color intensity than methylguanidine (No. **29**), or did not show any coloration, as shown in Table II. Whereas, in Voges-Proskauer reaction,[9] or in the reaction reported by Yamada,[12] such dependence of coloration on the structure of guanidine derivatives has not been observed. Phenols and naphthols, resorcinols (Compd. No. **20**—**24**) gave positive reaction as in Voges-Proskauer reaction,[6] but these compounds except for 3,5-dihydroxybenzoic acid were not useful as the reagent, because strong coloration was observed in the blank, and the color of the reaction mixture was unstable in the cases of these compounds. The discoloration was considered to be due to the oxidation of the coloring matter in the presence of phenanthraquinone, and 1-naphthol or resorcinols in the presence of alkali, being considered from the fact that potassium ferricyanide showed the similar effect on the reaction mixture, including the reaction with 3,5-dihydroxybenzoic acid. Consequently, a new method with 3,5-dihydroxybenzoic acid is recommended for the determination of mono- and non-substituted guanidines (Fig. 6). On the other hand, 1-naphthol-phenanthraquinone method can be recommended as shown in Table II only for the detection of mono-substituted guanidines.

Table III. Color Intensity of Guanidines in 3,5-Dihydroxybenzoic Acid-Phenanthraquinone Method and Other Color Reactions

Compd. No.	Guannidines	Color reaction		
		3,5-Dihydroxybenzoic acid-phenanthraquinone method (ε')	Voges-Proskauer reaction (ε')	Sakaguchi reaction (ε')
25—**28**	guanidine	1.38×10^4	1.79×10^3	6.38
29	1-methyl guanidine	1.11×10^4	4.84×10^3	1.77×10^4
33	glycocyamine	1.11×10^4	5.01×10^3	1.55×10^4
34	arginine	1.30×10^4	3.65×10^3	1.55×10^4
46	creatine	—	1.68×10^4	1.79×10^2

a) J.F. Van Pilsum, *J. Biol. Chem.*, **222**, 225 (1956).

In comparison with other color reactions, the color intensity for guanidine is the highest in 3,5-dihydroxybenzoic acid method, but for mono-substituted guanidines (especially arginine), both the 3,5-dihydroxybenzoic acid method and Sakaguchi reaction gave nearly the same color intensity, as shown in Table III. Furthermore, 3,5-dihydroxybenzoic acid method does not show any coloration for N,N-disubstituted guanidines (Tables II and III).

11) F. Feigl, "Spot Tests in Organic Analysis," 6th English ed., Elsevier, London, 1960, p. 345.
12) S. Yamada and H.A. Itano, *Biochim. Biophys. Acta*, **130**, 538 (1966).