Studies on the Voges-Proskauer Reaction. I. Structure of Positive Substance in the Diacetyl Reaction^{1,2)}

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The structural limitation of positive compounds in the Voges-Proskauer Reaction was investigated with guanidines, ureas, thioureas, formamidine and formaldoxime, using the α -naphthol alkaline and diacetyl solution.

Only guanidino derivatives showed positive reaction among compounds tested above. Strongly positive compounds were *asym*-1,1-dialkylguanidines.

Especially, 1,1-dimethylguanidine, 1-methyl-1-phenylguanidine and 1,1-pentamethyleneguanidine had the highest absorbance at 535 m μ .

Creatine which was usually used in this reaction, showed second higher absorbance. Monosubstituted guanidine gave always moderately positive reaction, and unsubstituted one was weakly positive.

sym-1,3-Dialkylguanidines such as 1,3-dimethylguanidine was negative, suggesting that a free amidino group ($\text{H}_2\text{N}-\overset{\text{NH}}{\underset{\text{||}}{\text{C}}}-$) would be necessary for the positive V-P reaction.

This is further supported by the fact that tri-, tetra- and pentamethylguanidine also gave negative reaction.

8-Hydroxyquinoline could be used in place of α -naphthol in this reaction.

Conditions for color development, stability of colored solution and the effect of pH on the wave length of λ_{max} were studied.

Voges and Proskauer⁴⁾ reported in 1898 the production of an eosin-like coloration in glucose peptone cultures of certain organisms to which was added 10% potassium hydroxide. The reaction is generally referred to as the V-P reaction and has been utilized to differentiate microorganisms. Harden⁵⁾ showed that the coloration was due to the presence in the culture medium of acetylmethylcarbinol, which is a product of the growth of certain bacteria on sugars, and that acetylmethylcarbinol was oxidized to diacetyl in the course of the reaction. Subsequently, Harden and Norris⁶⁾ tested about seventy substances by the V-P test and found that arginine, agmatine, dicyandiamide, creatine and guanidinoacetic acid were positive in the V-P reaction.

They concluded that this diacetyl reaction for proteins was attributed to the presence in the protein of the group $\text{NH}: \text{C}(\text{NH}_2)\text{NHR}$. As the coloration is faint and indistinct, many attempts have been made to improve the test by increasing the rate of oxidation of

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acetoin to diacetyl.⁷⁻⁹ O'Meara¹⁰ could intensify the coloration by employing creatine in combination with 40% KOH for the purpose of increasing the amount of the reacting guanidine group.

Barritt¹¹ led the V-P reaction to strikingly sensitive reaction by addition of α -naphthol. Eggleton,¹² and Westerfeld¹³ employed Barritt's method to determine acetoin, diacetyl and creatine. Mold, *et al.*¹⁴ reported that the most intense coloration in the diacetyl reaction for some methyl substituted guanidines was given by 1,1-dimethylguanidine.

The authors found, however, that in all the preceding reports, little seemed to have been clarified about determining the range of functional groups concerning the reaction structure of V-P positive substances.

In the present paper, we wish to give a full detail of the structural relation among various guanidinoderivatives and others.

Experimental

Reagents—All of the reagents used were of analytical grade, and α -naphthol was recrystallized from EtOH and H₂O.

α -Naphthol: A 1% solution was prepared by dissolving 0.25 g of α -naphthol in 25 ml of an alkaline stock solution, which was prepared by dissolving 12 g of NaOH and 32 g of Na₂CO₃ in redistilled water and diluting to 200 ml.

This α -naphthol solution was prepared before use.

8-Hydroxyquinoline¹⁵: A 0.1% solution was prepared shortly before use by dissolving 0.025 g of this reagent in 25 ml of the alkaline stock solution described above.

8-Mercaptoquinoline: A 0.1% solution was prepared freshly each day by dissolving 0.025 g of this reagent in 25 ml of the alkaline stock solution.

Diacetyl: A 0.05% aqueous solution was prepared by diluting 20 times the diacetyl stock solution (1%) with water. This stock solution was kept in a refrigerator.

Procedure—Each sample solution (1 ml) was taken in a 10 ml test tube with stopper.

One ml of 0.05% diacetyl solution and 2 ml of 1% α -naphthol alkaline solution were introduced and diluted to 10 ml with distilled water. The tubes were stored in dark after thorough shaking. After 15 min visible absorption spectra were determined to know absorption maxima of the colors given by various compounds by using a Hitachi recording spectrophotometer EPS-2.

The change in the color intensity relative to time at $\lambda_{\max}$ was then measured, 0.1% 8-hydroxyquinoline and 0.1% 8-mercaptoquinoline being also used instead of 1% α -naphthol. A mixture prepared by diluting 1 ml of 0.05% diacetyl and 2 ml of 1% α -naphthol alkaline solution to 10 ml with distilled water was run as a blank.

For measurement of absorption spectra at different pH values, 5 ml of a sample solution (0.5 μ mole/ml), 5 ml of 0.05% diacetyl solution, and 10 ml of 1% α -naphthol solution were mixed and the mixture was diluted to 50 ml with distilled water. After 10 min the solution was adjusted to a desired pH by adding 1N HCl and its spectrum was determined on the photometer.

Material—The following material were prepared according to the methods described in the literature: 1-methyl-2-thiourea,¹⁶ mp 119—121°; 1,1,3-trimethyl-2-thiourea,¹⁶ mp 77—78°; 1,1,3,3-tetramethyl-2-thiourea,¹⁷ mp 76—77.5°; 2,3-dimethyl-2-thiopseudourea hydroiodide,^{18a} mp 133—134°; 1,2,3-trimethyl-2-thiopseudourea hydroiodide,^{18b} mp 208—210°; 1-methylguanidine sulfate,¹⁹ mp 237.5°; 1-phenylguanidine

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TABLE I. Structural Formula of New Guanidino Compounds

R ₁	R ₂	Formula	Analysis (%)						mp (°C)	Appearance	
			Calcd.			Found					
C	H	N	C	H	N	C	H	N
H	C ₈ H ₁₁ N ₃ ·½H ₂ SO ₄ ^{a)}	48.47	6.10	21.20	48.48	5.99	21.17	200	—201.5	colorless prisms (EtOH)
H	C ₇ H ₈ O ₂ N ₄ ·½H ₂ SO ₄ ^{a)}	36.68	3.96	24.44	36.76	4.05	24.55	266		pale yellow powder (EtOH-H ₂ O)
C ₅ H ₁₁ ON ₃ ·½H ₂ SO ₄ ^{a)}	33.70	6.79	23.58	33.41	6.46	23.33	>280		colorless prisms (50% EtOH)
		C ₅ H ₁₁ ON ₃ ·HCl	36.26	7.31	25.37	35.84	7.53	25.33	161.5	—162.5	colorless plates (EtOH-ether)
C ₇ H ₁₇ N ₃ ·HCl ^{b)}	46.78	10.09	23.38	46.88	9.60	23.15	157.5	—159	white powder (EtOH-ether)
		C ₁₃ H ₂₀ O ₇ N ₆ ^{c)}	41.93	5.41	22.57	41.99	5.28	22.81	217	—218	yellow needles (EtOH)
C ₇ H ₁₇ N ₃ ·HCl ^{b)}	46.78	10.09	23.38	46.85	9.76	23.21	281		white powder (EtOH-ether)
		C ₁₃ H ₂₀ O ₇ N ₆ ^{c)}	41.93	5.41	22.57	42.18	5.35	22.41	234.5	—235.5	yellow needles (MeOH)
C ₉ H ₁₃ N ₃ ·HCl ^{b)}	54.13	7.07	21.64	53.91	6.96	21.11	112	—113	colorless plates (EtOH-ether)
		C ₁₅ H ₁₆ O ₇ N ₆ ^{c)}	45.92	4.11	21.42	46.28	4.29	21.32	188	—190	yellow needles (MeOH)
C ₈ H ₁₀ O ₂ N ₄ ·HCl ^{b)}	41.65	4.81	24.29	41.41	4.71	24.12	243.5	—244	pale yellow powder (EtOH-ether)
		C ₁₁ H ₁₃ O ₉ N ₇ ^{c)}	39.72	3.09	23.17	39.59	3.15	22.85	227	—228	yellow needles (75% MeOH)
C ₉ H ₁₂ O ₂ N ₄ ·HCl ^{b)}	44.17	5.35	22.90	44.65	5.61	22.74	187	—188	colorless plates (EtOH-ether)
		C ₁₃ H ₁₅ O ₉ N ₇ ^{c)}	41.19	3.46	22.42	41.43	3.58	22.48	234	—234.5	yellow needles (MeOH-EtOH)

nitrate,²⁰) mp 120—121°; 1-*n*-butylguanidine sulfate,²¹) mp 214°; 1,1-dimethylguanidine sulfate,¹⁹) mp 291°, hydrochloride, mp 140—148°; 1,1-pentamethyleneguanidine sulfate,²²) mp 286° (decomp.), hydrochloride,²³) mp 183—184°; 1,1-anhydrobis (2'-hydroxyethyl)biguanide hydrochloride,²³) mp 188.5—191°; 1,1-dimethylbiguanide hydrochloride,²⁴) mp 220—221.5°; 1,3-dimethylguanidine hydroiodide,²⁵) mp 92—96°, picrate, mp 177—177.5°; 1,2,3-trimethylguanidine hydroiodide,²⁵) mp >305°; 1,1,3-trimethylguanidine hydroiodide²⁵) mp 200—201°; 1,1,2,3-tetramethylguanidine hydroiodide,²⁵) mp 106—110°, picrate, mp 160—161.5°; 1,1,2,3,3-pentamethylguanidine hydroiodide,²⁵) mp 118.5—119.5°, picrate, mp 162—163°; 1,1-diethylguanidine hydrochloride,^{26,27}) mp 148—150°, picrate, mp 220°; 1,1-di-*n*-butylguanidine hydrochloride,²⁷) mp 104—105°, picrate, mp 153—155°; 1-methyl-2-phenylguanidine hydrochloride,²⁸) mp 213—213.5, picrate, mp

TABLE II. Absorption Maxima of Colored Solution produced from Various Nitrogen Compounds

Nitrogen compound	α -Naphthol		8-Hydroxyquinoline		8-Mercaptoquinoline	
	$\lambda_{\max}$	m μ	$\lambda_{\max}$	m μ	$\lambda_{\max}$	m μ
A. Guanidino compounds						
guanidine hydrochloride	525		510		—	
arginine hydrochloride	525		520		505	
glycocyamine	525		510		(495)	
agmatine sulfate	525		—		(453)	
streptomycin	535		515		(453)	
1-nitroguanidine	—		—		—	
1-aminoguanidine bicarbonate	(480)		—		—	
creatine	535		515		520	
creatinine	535		522		530	
B. Ureas and ureido compounds						
urea	—		—		—	
citrulline	—		—		—	
allantoin	—		—		—	
alloxane	—		—		—	
biuret	—		—		—	
sodium 5,5'-diethyl barbiturate	—		—		—	
1,3-diphenylurea	—		—		—	
1-phenyl semicarbazide	—		—		—	
semicarbazide hydrochloride	—		—		—	
hydantoin	—		—		—	
hydantoinic acid	—		—		—	
C. Thiourea compounds						
thiosemicarbazide	—		—		—	
1-methyl-2-thiourea	—		—		—	
1,3-dimethyl-2-thiourea	—		—		—	
1,3,3-trimethyl-2-thiourea	—		—		—	
1,1,3,3-tetramethyl-2-thiourea	—		—		—	
2,3-dimethyl-2-thiopseudourea	—		—		—	
hydroiodide	—		—		—	
1,2,3-trimethyl-2-thiopseudourea	—		—		—	
hydroiodide	—		—		—	
D. Amidoxime compound						
formaldoxime hydrochloride	—		—		—	
E. Amidine compound						
formamidine hydrochloride	—		—		—	

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Fig. 1. Absorption Spectra of Colors produced by Positive Compounds

— α -naphthol-diacetyl
 - - - 8-hydroxyquinoline-diacetyl
 - - - 8-mercaptoquinoline-diacetyl
 concentration (μ mole/ml):
 — 0.05 - - - 0.2 - - - 0.2
 Measured after 30 min.

Fig. 2. Effect of Standing Time on Absorbance at $\lambda_{\max}$ (α -Naphthol-diacetyl)

a,b) concentration (μ mole/ml): 0.1 c,d) concentration (μ mole/ml): 0.05
 The numbers in the figure indicate the compound number.

Fig. 3. Effect of Standing Time on Absorbance at $\lambda_{\max}$ (α -Naphthol-diacetyl)

concentration (μ mole/ml): 0.05.

The numbers in the figure indicate the compound number.

Fig. 4. Effect of pH on Wave Length of Absorption Maximum

—○— creatine (20)
 —○— 1,1-dimethylguanidine sulfate (22)
 —●— 1,1-pentamethyleneguanidine sulfate (24)

TABLE III. Absorption Maxima of Colored Solutions produced from Guanidino Compounds

Comp. No.	$\begin{array}{c} R_3 \\ \diagup \\ N-C-N \\ \diagdown \quad \\ R_4 \quad NR_5 \end{array} \cdot HX$						8-Mercaptoquinoline	
	R ₁	R ₂	R ₃	R ₄	R ₅	HX	λ_{max} m μ	λ_{max} m μ
I-1	H	H	H	H	H	HCl	525	520
II-2	H	-(CH ₂) ₃ CH(NH ₂)COOH	H	H	H	HCl	525	515
3	H	-CH ₂ COOH	H	H	H		525	510
4	H	-(CH ₂) ₄ NH ₂	H	H	H	H ₂ SO ₄	525	495
5	H	streptomycin	H	H	H		535	453
6	H	-NO ₂	H	H	H			453
7	H	-NH ₂	H	H	H	H ₂ CO ₃	480 ^(a)	
8	H	-N=C(CH ₃)-	H	H	H			
9	H	-N=C(CH ₃)CH=CH-	H	H	H	HCl		
10	H	-N=CH-	H	H	H			
11	H	-N=CH-CH=CH-	H	H	H			
12	H	-N=CH-CH=CH-	H	H	H			
13	H	-C(=NH)-N	H	H	H	HCl		
14	H	-CH ₃	H	H	H	HCl	560	540
15	H	-CH ₂ -	H	H	H	1/2 H ₂ SO ₄	525	545
16	H	-	H	H	H	1/2 H ₂ SO ₄	535	570
17	H	-C(=NH)N(CH ₃) ₂	H	H	H	HNO ₃	550	570
18	H	-(CH ₂) ₃ CH ₃	H	H	H	HCl	550	550
19	H	-	H	H	H	1/2 H ₂ SO ₄	525	510
III-20	-CH ₃	-COOH	H	H	H	1/2 H ₂ SO ₄	535	515
21	-CH ₃	-CH ₃	H	H	H	1/2 H ₂ SO ₄	535	515
22	-CH ₃	-CH ₃	H	H	H	HCl	535	515
23	-CH ₂ CH ₂ CH ₂ CH ₂ CH ₂ -	-CH ₂ CH ₂ CH ₂ CH ₂ CH ₂ -	H	H	H	1/2 H ₂ SO ₄	535	515
24	-CH ₂ CH ₂ CH ₂ CH ₂ CH ₂ -	-CH ₂ CH ₂ CH ₂ CH ₂ CH ₂ -	H	H	H	HCl	535	515
25	-CH ₂ CH ₂ CH ₂ OCH ₂ CH ₂ -	-CH ₂ CH ₂ CH ₂ OCH ₂ CH ₂ -	H	H	H	1/2 H ₂ SO ₄	550	515
26	-CH ₂ CH ₂ OCH ₂ CH ₂ -	-CH ₂ CH ₂ OCH ₂ CH ₂ -	H	H	H	HCl	550	510
IV-27	H	-CH ₃	H	-CH ₃	H	HI		500
28	H	-CH ₃	H	-CH ₃	H	picrate		500

V-29	H	-CH ₃	HI	530
VI-30	H	-NH ₂	HI	
31	H	-N=CH-	HI	
32	H	-N=(CH ₃)CHC=CH-	HI	
33	H	-N=(CH ₃)C-	HI	522
34	H	-CH ₃	HI	
35	H	-COCH ₂ -	HI	535
VII-36	H	-CH ₃	HI	
37	H	-CH ₃	picrate	
VIII-38	-CH ₃	-CH ₃	HI	
39	-CH ₃	-CH ₃	picrate	

a) positive by Barritt's Method

224.5—225°; 1,1-dibenzylguanidine hydrochloride,²⁹ mp 191—192°, picrate, 174—174.5°; 1,1-tetramethyleneguanidine sulfate,³⁰ mp >305°, picrate,³¹ mp 214—215°. Synthesis of nine amidinohydrazones (8—12, 30—33) will be published elsewhere.

Eight new guanidino compounds synthesized by reacting the corresponding secondary amines with 2-methyl-2-thiopseudourea of cyanamide are summarized in Table I.

Results

Results of the test conducted at a concentration of 0.1 mm of substances, using 1% α -naphthol alkali, 0.1% 8-hydroxyquinoline and 0.1% 8-mercaptoquinoline, with 0.05% diacetyl solution, and the wave lengths of absorption maxima of the colored solution produced from various compounds are summarized in Table II (A, B, C, D, E).

The compounds tested are classified into five groups; A(guanidines), B(ureas), C(thioureas), D(formaldoxime), and E(formamidine).

From the results of Table II, we found that only guanidino compounds showed positive reaction. Therefore, we have classified further the guanidino compounds to the eight classes as listed in Chart 1.

Chart 1

And we tested 39 compounds including unsubstituted (I-1), monosubstituted (II-2—19), disubstituted (II-20—25, IV-27—28), trisubstituted (V-29, VI-30—35), tetrasubstituted (VII-36, 37), and pentasubstituted (VIII-38, 39) as described in Table III.

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TABLE IV. Color Intensity of V-P Reaction Positive Compounds

Comp. No.	α -Naphthol (ϵ')	8-Hydroxyquinoline (ϵ')	8-Mercaptoquinoline (ϵ')
I-1	1.79×10^3	2.40×10^2	
II-2	3.65×10^3	6.20×10^2	2.60×10^3
3	5.01×10^3	5.10×10^2	2.80×10^3
4	1.63×10^3		1.90×10^3
5	5.20×10^3	1.90×10^2	0.90×10^3
13	1.57×10^3	4.20×10^2	0.20×10^3
14	4.84×10^3	1.45×10^2	0.13×10^3
15	2.97×10^3	2.90×10^2	5.90×10^2
16	2.77×10^3	2.50×10^2	0.53×10^3
17	2.21×10^3	4.70×10^2	0.28×10^3
18	2.36×10^3	2.05×10^2	0.48×10^3
III-20	1.68×10^4	1.60×10^3	7.65×10^2
	$(2.84 \times 10^3)^a$		
21	2.24×10^4	1.70×10^3	5.50×10^2
	(4.50×10^3)		
22 ^b	1.68×10^4	2.10×10^3	4.70×10^2
23	2.54×10^4	2.40×10^3	7.25×10^2
	(6.60×10^3)		
24	2.10×10^4	2.14×10^3	6.35×10^2
25	2.10×10^4	1.61×10^3	3.15×10^2
	(4.30×10^3)		
26	2.30×10^4	2.44×10^3	5.09×10^2
VI-35	3.04×10^3	4.20×10^2	0.25×10^2
II-7	0.36×10^2		

All data are measured at maximum absorptivity.

a) 0.1% α -naphthol

b) This material is considered to be impure, as judged from the molar absorptivity, melting point and elemental analyses.

TABLE V. Relationship of Chemical Structure with Color Intensity

Comp. No.	R ₁	R ₂	HX	$\lambda_{\max} \text{ m}\mu$	$\epsilon'_{\max}{}^a (\times 10^4)$
20	CH ₃ -	-CH ₂ COOH	H ₂ O	535	1.68 (0.01)
21	CH ₃ -	CH ₃ -	$\frac{1}{2}\text{H}_2\text{SO}_4$	535	2.22 (0.04)
40	C ₂ H ₅ -	C ₂ H ₅ -	HCl	525	1.67 (0.00)
41	<i>n</i> -C ₃ H ₇ -	<i>n</i> -C ₃ H ₇ -	HCl	525	1.41 (0.02)
42	iso-C ₃ H ₇ -	iso-C ₃ H ₇ -	HCl	525	0.80 (0.00)
43	<i>n</i> -C ₄ H ₉ -	<i>n</i> -C ₄ H ₉ -	HCl	525	1.25 (0.00)
44	CH ₃ -	C ₆ H ₅ -	HCl	540	2.70 (0.00)
45	CH ₃ -	C ₆ H ₅ CH ₂ -	HCl	535	1.99 (0.00)
46	C ₆ H ₅ CH ₂ -	C ₆ H ₅ CH ₂ -	HCl	540	1.85 (0.00)
47	CH ₃ -	<i>p</i> -NO ₂ -C ₆ H ₄ -	HCl	590	2.15 (0.00)
48	CH ₃ -	<i>p</i> -NO ₂ -C ₆ H ₄ CH ₂ -	HCl	535	1.63 (0.00)
49		-(CH ₂) ₄ -	$\frac{1}{2}\text{H}_2\text{SO}_4$	535	1.55 (0.00)
23		-(CH ₂) ₅ -	$\frac{1}{2}\text{H}_2\text{SO}_4$	535	2.55 (0.10)
25		-CH ₂ CH ₂ OCH ₂ CH ₂ -	$\frac{1}{2}\text{H}_2\text{SO}_4$	550	2.30 (0.00)

a) the mean of two independent experiments

The figures in parentheses represent the difference between the two experimental values.

Color intensity of eleven positive representative compounds are shown in Table IV and relationships of chemical structure with the color intensity of 1,1-dialkylguanidines (**21**, **40—43**), 1,1-alkyleneguanidines (**23**, **49**), and 1-methyl-1-benzylguanidines (**45**, **48**) are shown in Table V.

The curves of absorption spectra of the colors produced by the main, positive compounds are shown in Fig. 1.

Effect of standing time on absorbance at $\lambda_{\max}$, using α -naphthol and diacetyl is shown in Fig. 2a, 2b, 2c, and in Fig. 3a, 3b.

Finally, the effect of pH on wave lengths of absorption maximum of creatine, 1,1-dimethylguanidine sulfate and 1,1-pentamethyleneguanidine sulfate is shown in Fig. 4.

Discussion and Conclusion

1. Nature of Functional Group

As demonstrated in Table III, only unsubstituted, mono-, and *asym*-1,1-disubstituted guanidines gave the positive reaction with the exception of 1-nitro-(II-6), 1-amino-(II-7, VI-30), substituted 1-amino-(II-8—12, VI-31—33) and 1-*p*-nitrophenyl-(II-19) guanidine. As indicated in Table IV and Table V the compounds which gave the most intense coloration were *asym*-1,1-disubstituted guanidines (III-groups in Table IV and **40—49** compounds in Table V).

On the other hand, it was a very important evidence that 1,3-dimethylguanidine was negative, suggesting that a free amidino group would be necessary for the positive V-P reaction.

This is further supported by the fact that tri-(VI-34, V-29), tetra-(VII-36, 37), and penta-(VIII-38, 39)methylguanidines gave also negative reaction (Table III). From these results it was concluded that in the guanidino compounds, the presence of a free amidino group whose carbon is attached to an amino-nitrogen, should be necessary for the positive V-P reaction.

In the unsubstituted formamidine (E in Table II), however, this reaction was negative, because it has hydrogen directly on the carbon of amidino group.

For the exact reason of this structural relations would be necessary more detailed experiments.

2. $\lambda_{\max}$ of Positive Substances

Unsubstituted and mono substituted guanidines, if positive, gave the colors having absorption maximum at 525—535 m μ when α -naphthol was used.

Also *asym*-1,1-dialkylguanidines (**20**, **21**, **40—43**), *asym*-1,1-alkyleneguanidines (**23**, **49**), and 1-methyl-1-benzylguanidines (**45**, **48**) gave the color, having almost the same $\lambda_{\max}$ at 525—535 m μ .

The $\lambda_{\max}$ was slightly longer (540—550 m μ) in 1-methyl-1-phenyl-(**44**), 1,1-dibenzyl-(**46**), and 1,1-anhydrobis (2'-hydroxyethyl)-(**25**) guanidines, while guanidine derivatives having 1,1-anhydrobis (2'-hydroxyethyl) amidino-(**13**), 1,1-dimethylamidino-(**17**), and phenyl-(**16**) substituted ion gave the colors having absorption maxima at 550—560 m μ .

3. Comparison of the Color Intensities with the Length of Alkyl Side Chain in 1,1-Dialkylguanidines (**21**, **40—43**) (Table V)

It was revealed that the intensity was highest with shortest side chain compound, 1,1-dimethylguanidine, and decreased with diethyl, di-*n*-propyl and di-*n*-butyl derivatives in the order of longer length of side chain.

And di-isopropyl derivatives (**42**) gave the color of lower intensity than di-*n*-propyl derivative (**41**). As is obvious from the comparison of 1,1-dimethylguanidine with methyl-

phenylguanidine, replacement of phenyl group for a methyl group of the former compound resulted in the marked increase 10 times in the intensity of color produced.

Benzyl substituted derivative (45) gave the color of less intensity than that produced by phenyl substituted guanidine (44). Dibenzylguanidine (46) produced the color of almost the same intensity as that given by monobenzyl derivative (45).

4. Comparison of the Color Intensities among 1,1-Dialkyleneguanidines

Pentamethylene derivatives (23) gave the color of higher intensity than tetramethylene-guanidine (49) containing less carbon atoms.

This result is of a marked difference from that obtained in 1,1-dialkylguanidines.

5. Introduction of Nitro Group into the Benzene Ring of V-P Positive Guanidine Derivatives

Introduction of a nitro group into the benzene ring showed the effect of reducing the color intensity as revealed by the comparison of 1-methyl-1-phenylguanidine with 1-methyl-1-*p*-nitrophenylguanidine, and of 1-methyl-1-benzyl- with 1-methyl-1-*p*-nitrobenzylguanidine (Table IV).

In accordance with finding this, moderately V-P positive 1-phenylguanidine (16) was converted into an entirely negative 1-*p*-nitrophenylguanidine (19) (Table III, IV).

6. Introduction of $-N=C<$ Group to the Aminonitrogen of the Guanidino Compounds

Introduction of this group either to the aminonitrogen of guanidine directly (8—12 in Table III) or to the same nitrogen of monosubstituted guanidines (31—33 in Table III) showed an entirely negative V-P reaction.

Also introduction of amino and nitro group directly to the aminonitrogen of guanidine led to the negative results.

7. The Applicability of 8-Hydroxy- and 8-Mercaptoquinoline for α -Naphthol in V-P Reaction

As it was impossible to prepare 1% solution of 8-hydroxy- and 8-mercaptoquinoline respectively, color intensities given by 0.1% solutions of these reagents were compared with several 1,1-disubstituted guanidines (20, 21, 23, 25). As a result, it was found that α -naphthol, 8-hydroxy- and 8-mercaptoquinoline give the colors having the decreasing intensity and with the shift of $\lambda_{\max}$ to shorter wave lengths in the order named (Table III).

8. Conditions for Color Development and Color Stability

When α -naphthol was used, the intensities of colors produced by unsubstituted and mono-substituted guanidines (1—5, 13—18) reached to maxima in 50—70 min except for monophenylsubstituted one and slowly decreased except for monomethyl one (Fig. 2a, 2b).

In the case of creatine (20), however, the rapid decrease was not observed. Creatinine (35) developed the color very slowly (Fig. 2c). This might be ascribed to the conversion of creatinine to creatine in alkaline medium. As is obvious from Fig. 3a, 3b, the color intensities produced by di-*n*-butyl-(43), and di-isopropyl-(42) guanidines reached to a maximum in 20—40 min and gradually decreased thereafter.

The color intensities in the cases of dimethyl-(21), methylphenyl-(44), dibenzyl-(46), methyl-*p*-nitrophenyl-(47), methyl-*p*-nitrobenzyl-(48), pentamethylene-(23), and anhydrobis (2'-hydroxyethyl)-(25) derivatives gave the peak in 5—15 min and then showed a relatively rapid decrease (Fig. 2c, 2d, Fig. 3b). Diethyl-(40), di-*n*-propyl-(41), methylbenzyl-(45), and tetramethylene-(49) guanidines produced the color, of which intensity reached to a maximum in 10—25 min, and decreased rapidly in 10—30 min, and then very slowly (Fig. 3a, 3b). Methylbenzyl-, tetramethylene- and diisopropylguanidines gave remarkably stable colors during the period from 30 to 100 min.

9. Effect of pH on the Wave Length of $\lambda_{\max}$ of the Colored Solutions

This effect is shown in Fig. 4. At a pH range of 10—13, red color was produced from 1,1-dimethylguanidine, 1,1-pentamethyleneguanidine, and creatine.

These three compounds had almost the same maximum absorption of 535 $m\mu$ at these pH range of 10—13.

The $\lambda_{\max}$ in the case of 1,1-pentamethyleneguanidine increased from 535 $m\mu$ to 610 $m\mu$ with decreasing pH from 10 to 9, and became constant at pH_a 4 or lower.

The colors produced from creatine and 1,1-dimethylguanidine showed maximum absorption at pH 6 in the former and at pH 4 in the latter.

Calculated from these curves, we obtained approximate pK_a values of 9.4, 9.9 and 9.2 in the case of creatine, 1,1-pentamethyleneguanidine and 1,1-dimethylguanidine respectively.

It was emphasized that these experiments appeared to be useful in explaining the mechanism of a guanidino structure and might be of further value in the design of specific functional group whose use may help to relate these correlations to the chemical details of the structure of the amidino compounds.