[Chem. Pharm. Bull. 16(4) 626—633 (1968)]
UDC 615.754-011 : 543.422.5 : 546.171.1.08

Mechanism of a New Color Reaction of Aminopyrine and Its Application to Determination of Ammonia

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(Received May 4, 1967)

The mechanism of a new color reaction of aminopyrine has been studied. Aminopyrine gives orange red coloration by the reaction with phenol and potassium ferricyanide in ammonium acetate or ammonium chloride buffer. Colored substance of this reaction has been isolated and determined to be 4-(1',4'-benzoquinone-4'-imino)antipyrine, which is the same as the reaction product obtained from 4-aminoantipyrine. But in the reaction of aminopyrine it has been found that presence of ammonium ion is essential to the coloration, contrary to the reaction of 4-aminoantipyrine, and in presence of excess aminopyrine intensity of color is proportional to the concentration of ammonium ion. Further investigation of the role of ammonium ion in the color reaction of aminopyrine supports the suggestion that in the first step ammonium ion and phenol are oxydatively condensed to produce 1,4-benzoquinone imide type intermediate, which reacts with aminopyrine to produce 4-(1',4'-benzoquinone-4'-imino)antipyrine as the consequence of transdimethylaminaton. On the basis of these experiments, a new colorimetric determination method of ammonia has been established.

In our previous paper[2] it was reported that aminopyrine (dimethylaminoantipyrine) gives orange red coloration with phenol in presence of potassium ferricyanide, and showed that this colored substance is the same as that of 4-aminoanitpyrine, which is known as Emerson's color reaction.[3]

But during the course of an investigation of colorimetric determination of aminopyrine in pharmaceutical preparation by this color reaction, it was found that composition of buffer solution much affected the coloration of aminopyrine and that in some buffer solution, aminopyrine did not give coloration at all.

The main purpose of this work described below was to clarify the mechanism of this color reaction and to elucidate the difference between the color reaction of aminopyrine and that of 4-aminoantipyrine.

As the result of an investigation of the mechanism of this color reaction, it was found that presence of ammonium ion is essential to the coloration of aminopyrine and in presence of excess aminopyrine, intensity of color is proportional to the concentration of ammonium ion. Application of this fact to a colorimetric determination of ammonia was also described.

Mechanism of Color Reaction:

The mechanism of color reaction of 4-aminoantipyrine is assumed to be oxydative condensation of 4-aminoantipyrine with phenol, but there has been no chemical proof. As the first step of an investigation of the mechanism, it appeared of importance to isolate and determine the structure of the colored substance of 4-aminoantipyrine and that of aminopyrine. pH 8.0 ammonium acetate buffer solution of 4-aminoantipyrine was allowed to stand for thirty minutes with aqeuous solution of phenol and potassium ferricyanide according to the procedure for the determination of aminopyrine.[2] The colored substance was extracted from the reaction mixture with chloroform. After washed with 1 N sodium hydroxide and water, and then dried with anhydrous sodium sulfate, the chloroform extract was sumbitted to

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[1] Location: *Juso, Higashiyodogawa-ku, Osaka.*
[2] S. Ono, R. Onishi, M. Tange, K. Kawamura, and T. Imai, *Yakugaku Zasshi*, **85**, 245 (1965).
[3] E. Emerson, *J. Org. Chem.*, **8**, 417 (1943).

thin–layer chromatography over silica gel developed by methyl alcohol, in which two spots were detected.

The main-spot of *Rf* 0.58 was accompanied with the minor one of *Rf* 0.23. The chloroform solution was then chromatographed over alumina column and each fraction successively eluted by chloroform was examined by thin–layer chromatography described above. From the fraction giving a single spot of the main product, brownish orange crystalline powder (I), mp 162.1°, was obtained by recrystallization from the mixture of ether and chloroform (10:1), which gave analytical figure consistent with $C_{17}H_{15}O_2N_3$.

Table I. *Rf* Values of Color Reaction Products obtained from 4–Aminoantipyrine, 4–Methylaminoantipyrine and Aminopyrine

	Solvent		
	(1)	(2)	(3)
I in $CHCl_3$	0.58	0.68	0.61
II in $CHCl_3$	0.56	0.70	0.62
4–Aminoantipyrine	0.59	0.69	0.62
4–Methylaminoantipyrine	0.58	0.70	0.60
Aminopyrine	0.58	0.71	0.62

(1): MeOH (2): Benzene-EtOH (1:1) (3): Benzene-Pyridine (1:1)

pH 8.0 ammonium acetate buffer solution of aminopyrine was treated as in 4–aminoantipyrine, and brownish orange crystalline powder (II), mp 162.5°, was obtained, which gave analytical figure consistent with $C_{17}H_{15}O_2N_3$.

I and II did not show any melting point depression by admixture with each other.

As shown in Fig. 1 and Table I, absorption curve and *Rf* values in thin–layer chromatography of the chloroform solution of I and II were in good agreement with that of the colored solution obtained by the colorimetric procedure of aminopyrine.

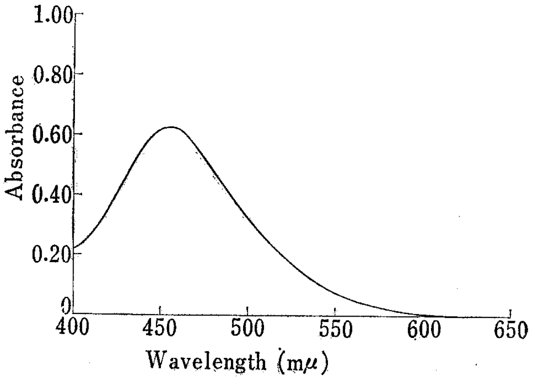

Fig. 1. Absorption Spectrum of the Colored Substance obtained from Aminopyrine and 4–Aminoantipyrine

Nuclear magnetic resonance spectra of I and II in deuterochloroform showed absorption at $\tau=7.57$ and 6.70 ppm, suggesting the presence of one C–CH_3 and one N–CH_3 respectively. Analytical figures and nuclear magnetic resonacne spectra confirm that the structure of the colored substance obtained by the colorimetric procedure of aminopyrine is 4–(1′,4′–benzoquinone–4′–imino)antipyrine (I, II) and that the reaction proceeds as shown in Chart 1.

In the course of an investigation of color reaction of aminopyrine, it was found that aminopyrine does not give coloration at all in phosphate, borate Clark–Lubs' buffer and sodoium bicarbonate solution, when aminopyrine is submitted to the color reaction with phenol in presence of potassium ferricyanide, whereas it gives similar coloration in ammonium acetate and ammonium chloride buffer solution, and shows optimal color development at pH 8.0.

Concerning this fact, Nambara4) reports that in the color reaction of 4–aminoantipyrine, intensity of color is more than twice as strong in Clark–Lubs' buffer solution as in Michaelis' buffer solution, Lacoste5) reports that the intensity of color varies inversely with ionic strength.

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4) T. Nambara and T. Urakawa, *Yakugaku Zasshi*, **80**, 1663 (1960).

5) R.J. Lacoste, S.H. Venable, and J.C. Stone, *Anal. Chem.*, **31**, 1246 (1959).

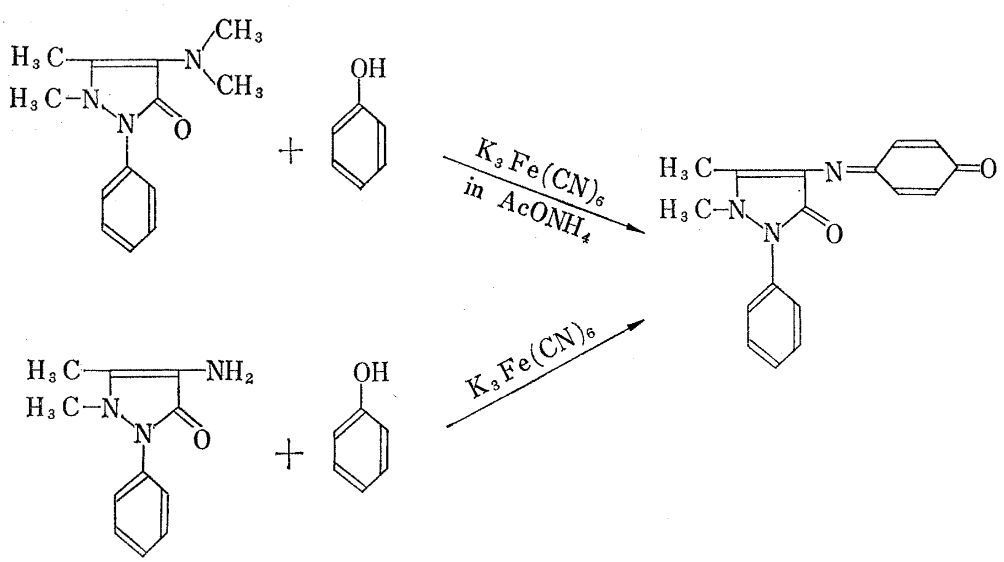

Chart 1

But in the color reaction of aminopyrine, intensity of color depends on the composition of buffer solution more distinctly. In order to make clear the effect of the components of buffer solution on this color reaction, color development in various buffer solutions was investigated when aminopyrine was submitted to the color reaction with phenol in presence of potassium ferricyanide according to the colorimetric procedure of aminopyrine.[2)]

The results given in Table II indicate that aminopyrine does not give coloration at all in absence of ammonium ion, on the contrary to 4–aminoantipyrine.

Table II. Absorbances of Chloroform Solution obtained by the Determination Procedure of Aminopyrine in Various pH 8.0 Buffer Solutions

Buffer solution	Absorbance (E 458 mμ)[a)]	Buffer solution	Absorbance (E 458 mμ)[a)]
Sodium borate • HCl	0.014	Phosphate	0.035
Soidum borate. • boric acid	0.010	Ammonium acetate	0.672 × 10
Veronal	0.016	Ammonium chloride	0.579 × 10
Citrate	0.054	Antipyrine[b)] + ammonium chloride	0.006
Tris aminomethane	0.021		

[a)] 0.005 M aminopyrine soln. was used as sample soln. (ten times as concentrated as in usual colorimetric procedure).
[b)] 0.005 M antipyrine soln. was used as sample soln.

In view of the above facts, it is assumed that ammonium ion takes part in the color reaction of aminopyrine. In order to confirm the effect of ammonium ion on this color reaction, relationship between the intensity of color and the concentration of ammonium ion in presence of excess aminopyrine has been studied according to the colorimetric procedure of aminopyrine.[2)]

To 1.0 ml of aqueous solution of ammonium chloride, which contains 200—2000 μg of ammonium chloride per ml, 10.0 ml of pH 8.0 Clark–Lubs' buffer solution, 2.0 ml of 0.5% aqueous solution of aminopyrine, 1.0 ml of 0.2% aqueous solution of phenol and 2.0 ml of 1.0% aqueous solution of potasssium ferricyanide were added, and the mixture was allowed to stand for 30 minutes. The reaction mixture was extracted with 20.0 ml of chloroform.

Absorbance of the chloroform extract was determined at the wave length of 458 mμ against the reagent blank solution obtained by treating 1.0 ml of water instead of aqueous solution of ammonium chloride according to the colorimetric procedure described above.

Relationship between the intensity of the color and the concentration of ammonium ion is shown in Fig. 2, which obeys Beer's law to the absorbance of 0.4, that is 400 μg of ammonium chloride or 104.7 μg of nitrogen. When 1.0 ml of 1% aqueous solution of phenol and 2.0 ml of 5% aqueous solution of potassium ferricyanide were used instead of 0.2% phenol solution and 1.0% potassium ferricyanide solution, and the mixture was treated as described above, relationship between the intensity of color and the concentration of ammonium ion obeys Beer's law as far as the absorbance of 0.8, that is 1000 μg of ammonium chloride or 261.7 μg of nitrogen, as shown in Fig. 2. In the former case, intensity of reagent blank was 0.01, but in the latter case that of reagnet blank was 0.140. This is probably because demethylation of fair portions of aminopyrine occurred in rather high concentration of potassium ferricyanide solution to produce 4-aminoantipyrine, which is oxydatively condensed with phenol.

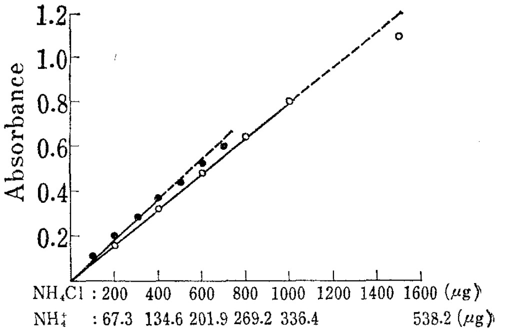

Fig. 2. Relationship between Absorbance and Concentration of Ammonium Ion in Presence of Excess Aminopurine

—○—○— : 1% phenol, 0.5% aminopyrine, 5% $K_3Fe(CN)_6$
—●—●— : 0.2% phenol, 0.5% aminopyrine, 1% $K_3Fe(CN)_6$

On the basis of these experiments, it seems reasonable to assume that ammonium ion takes part in the color reaction of aminopyrine. In an effort to determine the role of ammonium ion in the color reaction of aminopyrine, production of 4-aminoantipyrine and/or antipyrine as intermediate of the reaction, and the order of reagent addition were studied.

To the aqueous solution of aminopyrine (100 μg/ml), pH 8.0 Clark-Lubs' buffer solution and aqueous solution of potassium ferricyanide were added and allowed to stand for thirty minutes in the room temperature. After the reaction mixture was extracted with chloroform, concentrated solution of this chloroform extract was submitted to the thin-layer chromatography over silica gel, developed by chloroform-methyl alcohol (2:1). It was found that neither 4-aminoantipyrine nor antipyrine was produced and only aminopyrine was detected by thin-

Table III. Relation between Order of Reagents Addition and Absorbance

(1)	(2)	(3)	(4)	(5)
NH_4Cl+buffer phenol	NH_4Cl+buffer aminopyrine	NH_4Cl+buffer $K_3Fe(CN)_6$	NH_4Cl+buffer phenol $K_3Fe(CN)_6$	NH_4Cl+buffer aminopyrine $K_3Fe(CN)_6$
↓	↓	↓	↓	↓
		60 min		
↓	↓	↓	↓	↓
aminoyprine $K_3Fe(CN)_6$	phenol $K_3Fe(CN)_6$	phenol aminopyrine	aminopyrine	phenol
↓	↓	↓	↓	↓
		30 min		
↓	↓	↓	↓	↓
		chloroform extraction		
↓	↓	↓	↓	↓
0.226	0.216	0.214	0.220	0.114

Amount of reagnet:	
NH_4Cl (120 μg/ml)	5.0 ml
pH 8.0 Clark-Lubs' buffer	10.0 ml
0.2% phenol soln. in buffer	1.0 ml
0.5% aminopyrine soln. in buffer	2.0 ml
1% $K_3Fe(CN)_6$ soln. in buffer	2.0 ml

layer chromatography. From the above experiment and the fact that antipyrine did not give coloration when it was submitted to the color reaction of aminopyrine according to the colorimetric procedure, it might be concluded that neithter 4-aminoantipyrine nor antipyrine was produced as intermediate, and that oxydation of aminopyrine was not a cause of coloration. This interpretation was strengthen by the fact that aminopyrine gave less coloration when potassium ferricyanide was added to the solution of aminopyrine before the addition of phenol.

Aqueous solution of 5% chloramine T could also be used as the oxidizing agent, which gave the same color intensity as in the case of 1% aqueous solution of potassium ferricyanide. Absorption spectra of colored solution and thin-layer chromatogram over silica gel developed by the solvents mentioned above suggested that the colored substances obtained by potassium ferricyanide and chloramine T as the oxidizing agent were the same ones.

According to Bolleter⁶⁾ mechanism of color reaction of ammonia by production of indophenol is as follows: in the first step ammnoia is oxydized to chloramine in hot weak acidic medium, and then chloramine is oxydatively condensed with phenol to produce 1,4-benzoquinone-4-chlorimide intermediately, and indophenol is produced as the consequence of further reaction with phenol, which becomes blue in alkaline medium, as shown in Chart 2.

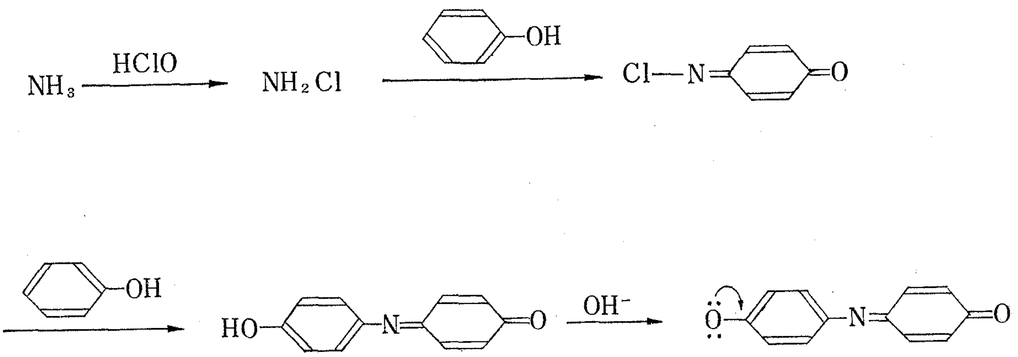

Chart 2

To pH 8.0 aqueous solution of ammonium chloride (600 μg/ml), 1% aqueous solution of potassium ferricyanide or 5% aqueous solution of chloramine T and 0.2% aqueous solution of phenol were added, and after allowed to stand for thirty minutes, the mixture was shaken with chloroform. But any colored substance was not obtained. Result of this experiment indicates that indophenol is not produced in the condition of color reaction of aminopyrine. This was also supported by the fact that strong alkaline medium was essential for the production of indophenol.

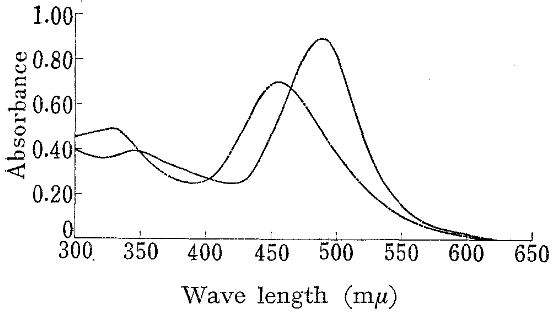

Fig. 3. Absorption Spectra of 4-(1′,4′-Benzoquinone-4′-imino)antipyrine and the Colored Substance obtained from Aminopyrine and 2,6-Dibromoquinone-4-chlorimide

– – – – : 4-(1′,4′-benzoquinone-4′-imino)antipyrine
——— : Colored substance obtained from aminopyrine and 2,6-dibromoquinone-4-chlorimide

In view of these facts, color reaction of aminopyrine was supposed to proceed through quinone imide type intermediate as in the case of indophneol production.

A strong basis for this postulation was found in the reactions of aminopyrine with 2,6-dibromoquinone-4-chlorimide in pH 8.0 Clark-Lubs' buffer solution. As was expected, reaction of aminopyrine with 2,6-

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6) W.T. Bolleter, C.J. Bushman, and P.W. Tidwell, *Anal. Chem.*, 33, 592 (1961).

dibromoquinone–4–chlorimide in pH 8.0 Calrk–Lubs' buffer solution produced orange red coloration, wkich was extracted with chloroform and showed absorption maximum at the wave length of 485 mμ (Fig. 3).

The colored substance obtained by this reaction was assumed to be 4–(2′,6′–dibrom–1′,4′–benzoquinone–4′–imino)antipyrine.

In view of these experiments the most reasonable conclusion to be drawn from available data might be that in the first step ammonia and phenol were oxydatively condensed to produce 1,4–benzoquinone imide type intermediate, which reacted with aminopyrine to produce 4–(1′,4′–benzoquinone–4′–imino)antipyrine as shown in Chart 3.

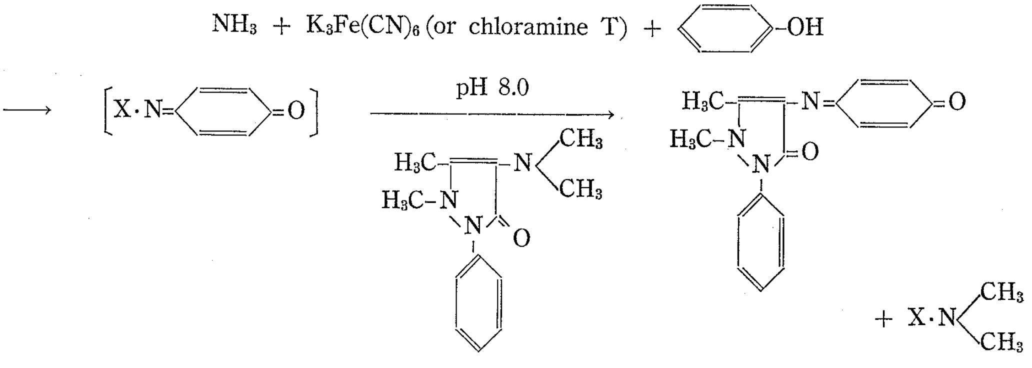

Chart 3

When aminopyrine was submitted to the reaction with phenol in presence of ammonia and potassium ferricyanide according to the colorimetric procedure, yield of 4–(1′,4′–benzoquinone–4′–imino)antipyrine from aminopyrine was found to be sixty per cent.

Application to the Colorimetric Determination of Ammonia:

During the course of an investigation of the mechanism of color reaction of aminopyrine, as was described above it was found that ammonia takes part in this reaction, and intensity of the color is proportional to the concentration of ammonia in presence of excess aminopyrine. This reaction was expected to be applicable to the determination of ammonia in nearly neutral solution. As the chromogen produced is extracted with chloroform quantitatively, sensitivity of this color reaction can be more increased by extracting with samller amount of chloroform. Although increase of the concentration of aminopyrine, phenol and potassium ferricyanide cause high reagent blank absorbance, liniarity of calibration curve is more satisfactory in high concentration of phenol, aminopyrine and in 5% of potassium ferricyanide solution rather than in low concentration of reagents. From the consideration of these facts, concentration and amount of reagents were determined as described in colorimetric procedure of ammonia. Calibration curve obtained by carrying out according to the colorimetric procedure is shown in Fig. 4. The results of colorimetric determination of ammonia from Kjeldahl digestion are shown in Table IV.

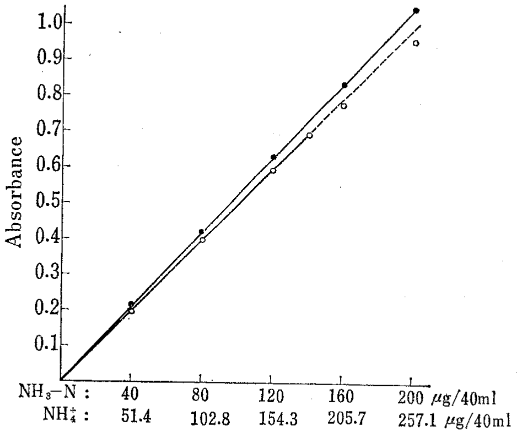

Fig. 4. Calibration Curve of Ammonia

—●—●— : 1% phenol, 1% aminopyrine, 5% $K_3Fe(CN)_6$ soln.

—○—○— : 0.2% phenol, 0.4% aminopyrine, 5% $K_3Fe(CN)_6$ soln.

The effect of substances which might interfere in the analysis for ammonia was determined by adding 1 ml of 43 mmole solution of the substance to 40.0 ml of the solution containing 0.214 mmole of ammonia (N: 3 μg/ml).

Table IV. Colorimetric Determinatnion of Ammonia

Sample	Taken			Found		
	sample (mg)	nitrogen (mg)	calculated (%)	sample (mg)	nitrogen (mg)	found (%)
Ethoxybenzamide	35.61	3.02	8.48	35.72	3.03	8.50
Glycine	16.36	3.05	18.66	16.28	3.04	18.57
Acetanilide	28.92	3.00	10.36	30.08	3.12	10.78
Phenacetin	39.29	3.07	7.82	41.60	3.25	8.27
Sulfanilamide	18.72	3.04	16.27	19.47	3.17	16.92

Hydroxylamine, urea and ethanolamine were found not to interfere in this amount. But cyan ion and large amount of reducing agent were found to interfere.

Comparison of this method with other colorimetric procedure for ammonia indicates that aminopyrine method is superior to indophenol[6] and bispyrazolone method[7] in its reproducibility, simplicity and stability of the reagents. But as far the sensitivity concerned, aminopyrine method is almost same as indophenol method and rather inferior to bispyrazolone method. Reprodubility of this method was determined from ten times replicate analysis on a given ammonia solution. Standrad deviation was 1.86% at a level of 3 μg/ml of nitrogen in the sample solution. As previously described, 5% aqueous solution of potassium ferricyanide gave fairly higher intensity of reagent blank than that obtained by 1% aqueous solution of potassium ferricyanide. But because of liniarity of the calibration curve, former solution was recommended.

Table V. Comparison of Colorimetric Determination Method of Ammonia

Method	Accuracy Std. dev. (%)	Sensitivity	
		nitrogen (μg)	Absorbance
Aminopyrine method	1.86 ($n=10$)	112	0.233
Indophenol method[a]	4.88 ($n=10$)	82	0.222
Bispyrazolone method[b]	2.71 ($n=10$)	8.2	0.305

a) W. T. Bolleter, C.J. Bushman, and P.W. Tidwell, *Anal. Chem.*, **33**, 592 (1961).
b) L. Procházková, *Anal. Chem.*, **36**, 865 (1964).

Colorimetric Procedure:

Add to a separatory funnel 40.0 ml of sample solution containing 0.5 to 5 μg/ml of nitrogen (as ammonia), which was previously adjusted to pH 8.0.

Add 4.0 ml of pH 8.0 Clark-Lubs' buffer solution of 1.0% phenol and 1.0% aminopyrine, 3.0 ml of pH 8.0 Clark-Lubs' buffer solution of 5% potassium ferricyanide and allow to stand for thirty minutes in the room temperature. Extract the reaction mixture with 10.0 ml of chloroform. Dry the chloroform extract with anhydrous sodium sulfate, and then read the absorbance of chloroform solution in 1 cm cell at 458 mμ against a reagent blank. Calibration data are obtained by adding aliquots of dilution of standard stock ammonia solution to a separatory funnel and carrying out the color developing procedure. Standard solution is recommended to carry out at each determination.

Experimental

Instruments——Beckmann spectrophotometer model DU, Shimadzu pen-recording spectrophotometer model IV-50A, Beckmann pH meter model G and Varian's HA 100 NMR spectrometer were used.

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[7] Lidmila Procházková, *Anal. Chem.*, **36**, 865 (1964).

Materials——Aminopyrine, phenol, 2,6–dibromoquinone–4–chlorimide and 4–aminoantipyrine were obtained commercially.

Ammonium acetate buffer, ammonium chloride buffer, ammonium acetate-ammonium chloride buffer: Ammonia water (10%) was added to 1 M $AcONH_4$ soln., 1 M NH_4Cl soln., 0.5 M $AcONH_4 \cdot 0.5$ M NH_4Cl soln. respectively to adjust the pH value 8.0.

pH 8.0 sodium borate·HCl buffer: To 28.75 ml of 0.05 M sodium borate soln. 0.1 N HCl was added to make 50.0 ml.

pH 8.0 veronal buffer: To 35.80 ml of 0.1 M barbital soln. 0.1 N HCl was added to make 50.0 ml.

pH 8.0 citrate buffer: To a solution of citric acid (0.1 M, 1.1 ml), 0.2 M Na_2HPO_4 soln. was added to make 50.0 ml.

pH 8.0 tris aminomethane buffer: To 25.0 ml of tris–(oxymethyl)aminomethane soln. (24.23 mg/ml), 27.5 ml of 0.1 N HCl and water were added to make 100.0 ml.

pH 8.0 sodium borate–boric acid buffer; To 35.0 ml of 0.2 M boric acid·0.05 M NaCl soln., 15.0 ml of 0.05 M sodium borate was addd.

pH 8.0 phosphate buffer: To a solution of Na_2HPO_4 (1/15 M, 47.5 ml), 2.5 ml of 1/15 M KH_2PO_4 soln. was added.

Isolation of Colored Substance obtained from 4-Aminoantipyrine——4–Aminoantipyrine (1.0 g) was dissolved in 240 ml of pH 8.0 $AcONH_4$ buffer and 120 ml of 0.2% phenol soln., and 240 ml of 1% potassium ferricyanide soln. was added. After the mixture was allowed to stand for 30 min, the reaction mixture was shaken with four 200 ml portions of $CHCl_3$ successively. The combined extract was evaporated to 200 ml under reduced pressure. The concentrated extract was washed with 50 ml of 1 N NaOH, then three 100 ml portions of water, dried with anhydrous sodium sulfate, submitted to column chromatography(45 mm diameter, 100 mm length, alumina) eluted with $CHCl_3$. The fractions, which gave a main spot of the colored substance by TLC, was combined and evaporated to dryness. Recrystallization of the residue from ether–$CHCl_3$ (10:1) gave brownish orange crystalline powder. $\lambda_{max}^{CHCl_3}$ 458 mμ, mp 162.1°. *Anal.* Calcd. for $C_{17}H_{15}O_2N_3$: C, 69.62; H, 5.15; N, 14.31. Found: C, 69.06; H, 5.08; N, 13.94.

Isolation of Colored Substance obtained from Aminopyrine——To a solution containing 1.0 g of aminopyrine in 240 ml of pH 8.0 $AcONH_4$ buffer, 120 ml of 0.2% phenol soln. and 240 ml of 1% potassium ferricyanide was added. The mixture was allowed to stand for 30 min and treated as in 4–aminoantipyrine. Brownish orange crystalline powder was obtained. $\lambda_{max}^{CHCl_3}$ 458 mμ, mp 162.5°. *Anal.* Calcd. for $C_{17}H_{15}O_2N_3$: C, 69.62; H, 5.15; N, 14.31. Found: C, 69.87; H, 5.20; N, 14.57.

Color Reaction of Aminopyrine with 2,6-Dibromoquinone-4-chlorimide——To 10.0 ml of aminopyrine soln. (100 μg/ml), 10.0 ml of pH 8.0 Clark–Lubs' buffer and 0.5 ml of 10% 2,6–dibromoquinone–4–chlorimide EtOH soln. were added. After the mixture was allowed to stand for 30 min, the reaction mixture was extracted with 20.0 ml of $CHCl_3$, and the extract was dried with 2 g of anhydrous sodium sulfate. The $CHCl_3$ soln. shows absorption maximum at the wave length of 485 mμ (Fig. 4). Reagent blank soln. was obtained by treating as described above, but aminopyrine soln. was replaced with 10.0 ml of water.

Acknowledgement The author expresses his deep gratitude to Professor Z. Tamura, Faculty of Pharmaceutical Sciences, University of Tokyo, for his kind guidacne throughout this work, and Mr. E. Yamazaki, Director of this plant, Dr. I. Nakayama, Messrs. S. Ono, M. Kuriyama and R. Onishi for their constant encouragement. Thanks are also due to the memberes of the Elementary Analysis Laboratory, Takeda Chemical Industries, Ltd. Author is deeply indebted to Miss Y. Negoro for her technical assistance.