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# Colorimetric Determination of Ammonia. IV.[1] A New Method with *o*-Chlorophenol and 2,6-Dichloroisocyanurate[2]

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Use of *o*-chlorophenol instead of salicylate improved the sensitivity of salicylate-2,6-dichloroisocyanurate method[4] for the determination of ammonia. By the use of 2,6-dichloroisocyanurate, the stored oxidant solution became more stable than sodium hypochlorite, which had been commonly used up to now. The presence of a micro-amount of sodium nitroprusside was necessary. Apparent molar extinction coefficient on the basis of nitrogen atom and coefficient of variation at 15.15 $\mu$g/ml ammonium sulfate were $3.14 \times 10^4$ and 1.83%, respectively. Absorbance of the reagent blank at 665 m$\mu$ was as low as $0.058 \pm 0.005$.

In 1966, Searcy, *et al.*[4] proposed a new colorimetric method for the determination of ammonia by the use of salicylate-2,6-dichloroisocyanurate (abbr. Sal-DCIC). It improved the stability of stored oxidant solution, and made the reagent blank value as low as 0.015. Poor sensitivity and interference by proteins and amines[5] were the shortcomings of this method.

In order to raise its sensitivity, attempts were made to use *o*-chlorophenol which was explored in our laboratory as a hopeful reagent for the determination of ammonia. Consequently, some improvements were obtained as described below. Sensitivity was made over twice that of Sal-DCIC method; reproducibility at the nitrogen concentration of 0.264 $\mu$g/ml in final colored solution was fairly good at 1.83% (coefficient of variation); reagent solutions were stable at least a month, when they were stored in a refrigerator; this color reaction was indifferent to the interval between addition of phenol and of oxidant, contrary to the case of *m*-cresol-,[1] thymol-,[6] and naphthol–hypochlorite[7] methods; it gave sufficiently low absorbance of the reagent blank as $0.058 \pm 0.005$. Thus, present method is recommended as one of the sensitive and stable methods for the determination of ammonia.

## Experimental

**Apparatus and Reagents**——1) Apparatus: Shimadzu RS-27 type autorecording spectrophotometer. Shimadzu QV-50 type spectrophotometer. Tōa Dempa HM-5 type glass electrode pH-meter.

2) Reagents: All aqueous solutions were prepared by the use of deammonized water.

a) Standard Solution of $(NH_4)_2SO_4$: Accurately weighed 202.02 mg of $(NH_4)_2SO_4$, recrystallized twice from deionized water and dried for more than a week over a concentrated $H_2SO_4$ in a vacuum desiccator, was dissolved in water to make one liter of "stock standard solution." Standard solution of any designated concentration was prepared by dilution of the stock solution with water before use.

b) EDTA Buffer Solution: One gram of disodium EDTA·$2H_2O$ was dissolved in 75 ml of water and pH of this solution was adjusted by the addition of 1N HCl or 1N NaOH.

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[1] Part III: R. Yamaguchi, T. Machida, and M. Ueki, *Yakugaku Zasshi*, **89**, 1534 (1969).

[2] This work was presented at the 90th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, July, 1970.

[3] Location: *2-Ebara, Shinagawa-ku, Tokyo.*

[4] J. Reardon, J.A. Foreman, and R.L. Searcy, *Clin. Chim. Acta*, **14**, 403 (1966).

[5] J.H. Seely, J.C. Petitclerc, and L. Benoiton, *Clin. Chim. Acta*, **18**, 85 (1967).

[6] H. Hashitani and H. Yoshida, *Bunseki Kagaku*, **17**, 1136 (1968).

[7] Y. Morita and K. Ogure, *Nippon Kagaku Zasshi*, **84**, 816 (1963).

c) Methanolic Solution of *o*-Chlorophenol: Commercially available reagent grade *o*-chlorophenol was fractionally distilled and the distillate in the range of 175—176° was collected, and it was dissolved in distilled MeOH.

d) Aqueous Solution of Sodium Nitroprusside: Recrystallized once from water, dissolved in water, and stored 1 week or more before use.

e) Alkaline Solution of 2,6-Dichloroisocyanurate (DCIC): Sodium salt of DCIC was weighed and dissolved in sodium hydroxide solution to make the solutions of desired alkalinity. Available chlorine in this solution was determined by the usual iodometry.

f) Diluent: Dimethylformamide was mixed with water in the ratio of 2:1 (v/v). Because of exothermic heat of mixing, large quantity of this diluent was prepared in advance. Use of this diluent was necessary to make the reactant mixture a clear solution.

All the reagent solutions were stored in a refrigerator.

**Standard Procedure**——To 0.5 ml of sample solution pipetted into a glass-stoppered test tube of 20 ml capacity, 0.5 ml of EDTA buffer solution (pH 6.5), 1.0 ml of 3.25% (v/v) *o*-chlorophenol solution, 0.1 ml of 0.15% sodium nitroprusside solution, and 1.0 ml of 0.15% DCIC/0.4N NaOH solution are added successively, mixed thoroughly, warmed at 37° for 20 min, cooled under running water, and finally 3.0 ml of diluent is added. This mixture is shaken vigorously until the whole solution becomes entirely homogeneous, and its absorbance is measured at 665 mμ against the reference of reagent blank. If required, absorbances of both the sample and the blank against the reference of deionized water are read out separately.

## Result and Discussion

### Examination of Analytical Conditions

The absorbance spectrum of the final colored solution, which was prepared by the standard procedure from the sample solution of 0.264 μgN/ml, was measured by the autorecording spectrophotometer. Only one absorption band appeared at 665 mμ in the visible region in both the sample and the blank. Consequently, in all subsequent experiments, this wave length was employed, and also concentration of ammonium sulfate was set at 10.10 μg/ml.

**(1) Order of Addition and Indispensability of Nitroprusside**——In view of our preceding work,[1,8] order of adding the reagents was carefully examined in this case and it was found that the best order was sample→*o*-chlorophenol→DCIC. In contrast, the order of sample→DCIC→*o*-chlorophenol scarecely gave the blue coloration even though 20 μg/ml ammonium sulfate was added. The presence of at least a catalytic amount of nitroprusside was necessary. These behaviors resembled the case of *o*-phenylphenol.[8a] Considering the reproducibility of various orders of addition, the order given in the standard procedure was selected.

**(2) Concentration of Nitroprusside**——Fig. 1 shows the correlation between the absorbance and volume of 2% sodium nitroprusside solution added, with correction to a unit concentration. All the samples showed almost same value of absorbance at above 0.005 ml, though absorbance of the blank tended to increase. This fact indicated that nitroprusside itself participated in the blue coloration, provided only a micro-quantity of ammonia was present.

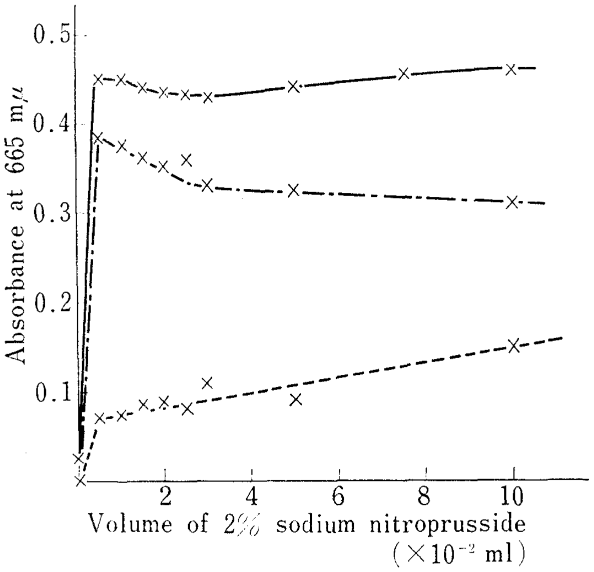

Fig. 1. Effect of Quantity of Sodium Nitroprusside

——×——: 10.10 μg/ml $(NH_4)_2SO_4$ ---×---: blank
·—·×—·: difference

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[8] *a*) R. Yamaguchi and T. Machida, *Yakugaku Zasshi*, **88**, 1383 (1968); *b*) *Idem, ibid.*, **89**, 804 (1969).

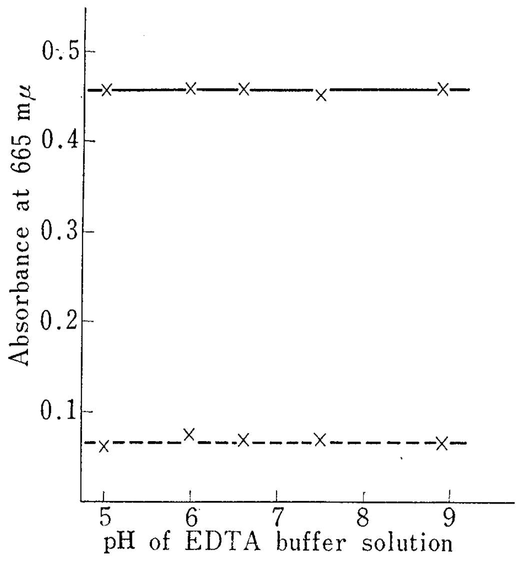

Fig. 2a. Effect of pH at the First Reaction Stage

——×——: 10.10 μg/ml $(NH_4)_2SO_4$
---×---: balnk

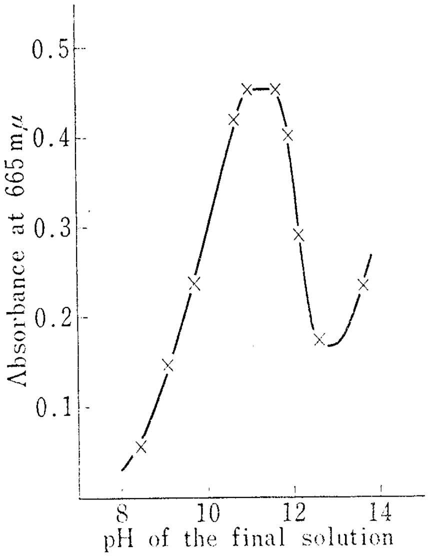

Fig. 2b. Effect of pH of the Final Solution

——×——: 10.10 μg/ml $(NH_4)_2SO_4$

The maximum difference in the absorbances was found near 0.007 ml. Consequently, 0.1 ml of 0.15% sodium nitroprusside was employed for later experiments.

(**3**) **pH at Two Addition Stages**——a) Fig. 2a shows the effect of pH at the first stage, *i.e.*, varying pH of EDTA buffer solution from 5.0 to 8.9. In case of the sample, all the values were quite identical, while a negligible slight variation ranging from 0.060 to 0.074 was found with the blank. This fact seems to mean that pH of buffer in this range does not affect the reaction or its rate in neutral medium. Thus pH of buffer was set at 6.5.

b) Fig. 2b shows the effect of pH at the second stage, *i.e.*, varying pH of DCIC/NaOH from 8.4 to 13.6. A marked change of the value was found. An upward curve from pH 8.4 to 11.0 may be explained as the change in the degree of an acid–base dissociation of indophenol molecule produced. The concave curve from 11.6 to 13.6 may perhaps be attributed to the change of the reaction mechanism under the influence of $OH^-$ concentration. Within only a narrow pH range (11.0—11.6), constant absorbance maximum for genuine blue was sustained, otherwise, yellow mingled more or less. The pH of DCIC/NaOH was therefore set at 12.9 (0.4N NaOH) so that the pH of the final solution might become 11.4.

(**4**) **Concentration of Sodium Salt of DCIC**——Fig. 3 shows the effect of quantity of sodium salt of DCIC. Since about the same value was obtained above a concentration of 1.0 g/liter DCIC, its concentration was set at 0.15% in 0.4N NaOH solution.

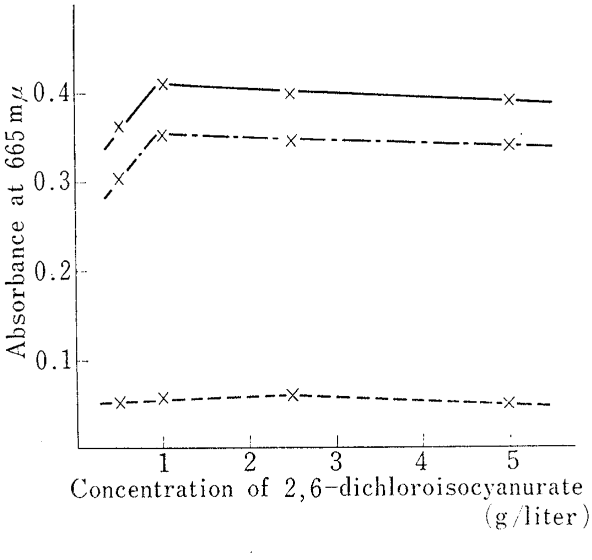

Fig. 3. Effect of Quantity of Sodium 2,6-Dichloroisocyanurate

——×——: 10.10 μg/ml $(NH_4)_2SO_4$ ---×---: blank
-·—·×—·-: difference

**(5) Concentration of *o*-Chlorophenol and Molar Ratio between *o*-Chlorophenol and Sodium Hydroxide**——Fig. 4a shows the effect of concentration of *o*-chlorophenol under two kinds of alkalinity of DCIC/NaOH. Similar but discrete convex curves were obtained. The molar ratios between *o*-chlorophenol and NaOH showing maximum absorbances were determined as 0.773 in 0.375N NaOH and as 0.787 in 0.4N NaOH, and it seemed necessary to set the ratio to about 0.78, because otherwise remarkable decrease of absorbance became inevitable.

Fig. 4b shows the effect of concentration of *o*-chlorophenol at the fixed molar ratio of 0.780. Over a wide concentration range of *o*-chlorophenol, absorbance showed about the same value of 0.43, except a little higher value (0.464) at a point where the final concentration of *o*-chlorophenol was set near $3.12 \times 10^{-4}$M. Therefore, the concentration of *o*-chlorophenol was hereafter set at 1.0 ml of 3.25% (v/v), and the molar ratio of *o*-chlorophenol *vs.* NaOH naturally became 0.780.[9]

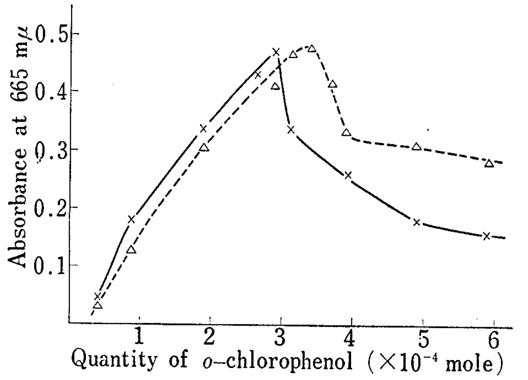

Fig. 4a. Effect of Quantity of *o*-Chlorophenol

—×—: 10.10 μg/ml $(NH_4)_2SO_4$, 0.375N NaOH
--△--: 10.10 μg/ml $(NH_4)_2SO_4$, 0.40N NaOH

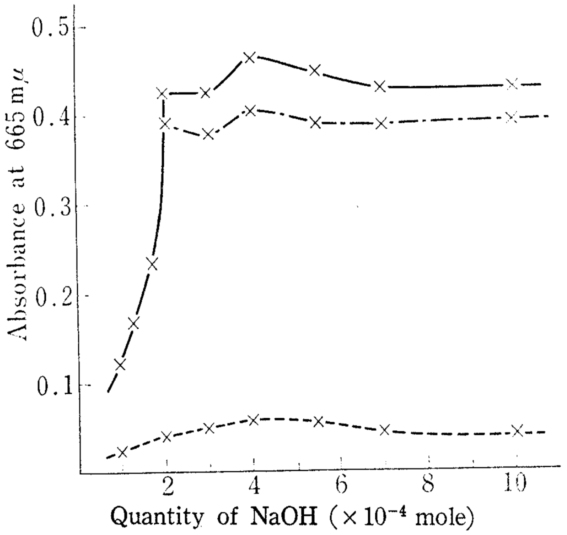

Fig. 4b. Effect of Quantity of *o*-Chlorophenol at a Constant Molar Ratio of *o*-Chlophenol: NaOH at 0.780

—×—: 10.10 μg/ml $(NH_4)_2SO_4$ ---×---: blank
—·×—·: difference

It may be assumed that this constant molar ratio is necessary because the molar ratio of undissociated *o*-chlorophenol *vs.* dissociated *o*-chlorophenol in alkaline $H_2O \cdot MeOH \cdot$dimethylformamide mixture may have an important bearing on the reaction rate and mechanism.

**(6) Time Course of the Reaction**——Fig. 5 shows the periodical variation in the absorbance at a constant temperature of 37±0.5°. In 20 min immersion in water bath, absorbance reached a maximum value, then declined gradually during 50 min, and then became constant. The reaction time was therefore set at 20 min, even though 15 min seemed almost sufficient.

Temperature above 37° was not examined this time.

## Calibration Curve

Measurements were made with concentration of ammonium sulfate from 2.02 to 20.20 μg/ml, three times for each concentration, and mean values were plotted *vs.* concentration of ammonium sulfate. From Fig. 6, the apparent molar extinction coefficient (based on nitrogen atom in the final solution) was found to be $3.14 \times 10^4$, or 2.24, expressed with alternative dimension of "(mean value of absorbance)/(μgN/ml of final solution)". This implies

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[9] Specific gravity and molecular weight of *o*-chlorophenol are 1.241 and 128.56, respectively.

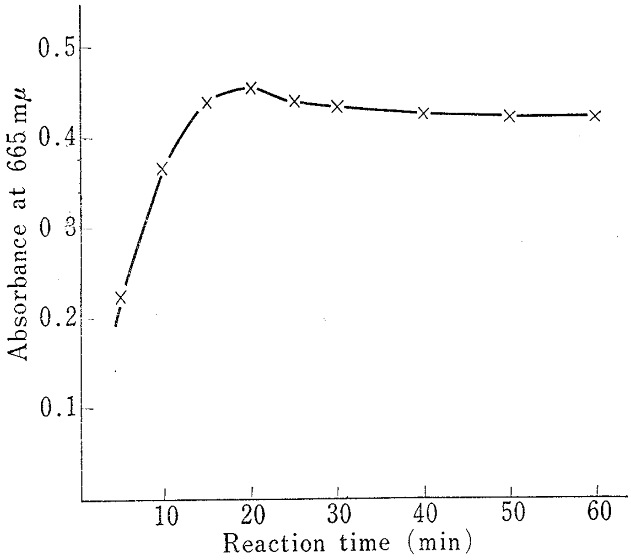

Fig. 5. Effect of the Reaction Time at 37°

—×—: 10.10 μg/ml $(NH_4)_2SO_4$

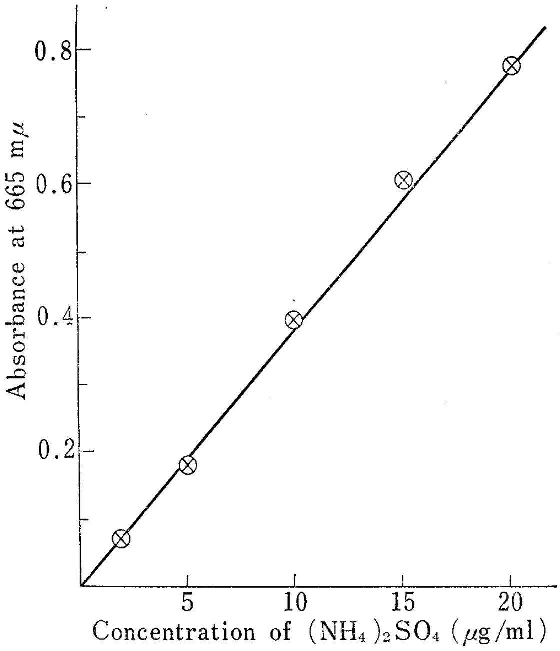

Fig. 6. Calibration Curve for Ammonium Sulfate

that sensitivity of the present method is comparable to that of *o*-phenylphenol method which is 2.23,[8b] and is better than that of Sal-DCIC method, which is 1.05.[4]

## Reproducibility and Its Evaluation

Table I shows the reproducibility of the present method resulting from ten repeated procedures at a concentration of ammonium sulfate at 15.15 μg/ml. Standard deviation and coefficient of variation were found to be satisfactorily small at $1.08 \times 10^{-2}$ and 1.83%, respectively. This value means that reproducibility of the present method is better than that of *o*-phenylphenol–NaOCl (2.4%),[8a] and guaiacol–NaOCl (2.3%),[8b] and far exceeding that of phenol-(4.88%)[10] or thymol–NaOCl (3.6%),[6] though is somewhat inferior to that of *m*-cresol–NaOCl method (1.60%).[1]

Table I. Reproducibility of the Present Method

| Expt. No. | Absorbance at 665 mμ<br>15.15 μg/ml $(NH_4)_2SO_4$ | Blank | Difference | Statistical values |
|---|---|---|---|---|
| 1 | 0.650 | 0.062 | 0.588 | • mean of difference ($\bar{x}$) 0.591 |
| 2 | 0.645 | 0.060 | 0.585 | |
| 3 | 0.640 | 0.061 | 0.579 | |
| 4 | 0.645 | 0.056 | 0.589 | • standard derviation ($\sigma$) $1.08 \times 10^{-2}$ |
| 5 | 0.672 | 0.060 | 0.612 | |
| 6 | 0.640 | 0.057 | 0.583 | • coefficient of variation 1.83% |
| 7 | 0.640 | 0.056 | 0.584 | |
| 8 | 0.660 | 0.053 | 0.607 | |
| 9 | 0.658 | 0.060 | 0.598 | |
| 10 | 0.645 | 0.057 | 0.588 | |

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

The reason for this improvement seems to be the stability of the reagents as is described below and the mild reaction condition due to the use of DCIC.

**Stability of Color and of Stored Reagents**

Color of the final solution remained almost constant for 30 hr. When all the reagents were tightly stoppered and stored in a refrigerator, ammonium sulfate, nitroprusside, and *o*-chlorophenol solutions remained unchanged at least for two months, and DCIC/NaOH solution for one month, but over that period, the capability of color developement gradually diminished, especially at a higher concentration of ammonium sulfate, this tendency increased so much that it could not be regarded as negligible (up to about 3%). Therefore, it is recommended that DCIC/NaOH be prepared fresh every one month.

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