ANALYSIS OF BIURET

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Introduction

Previously, on the equilibrium of urea-water system the formation of biuret was not considered[1,2], but in the investigation of the formation of melamine from urea was recognized the existence of biuret in the early product of the decomposition of urea[3,4]. In this case, the biuret was analysed by using the so-called biuret reaction, but the author reexamined the biuret reaction because of the inconsistent values of the analysis of biuret in urea as fertilyzer.

Generally, the color of the biuret reaction which is the reaction of biuret with copper in alkaline solution was used as the method of quantitative analysis of biuret. Several papers were reported on the biuret reaction[5], about the composition of colored substances, the values of copper/biuret were decided as 1/2 in the case of red color and 1/1 in reddish violet color from the results of the analysis of the colored substances which were precipitated by alcohol from the colored solution[6]. Moreover, about the composition of colored substance in solution, the values of copper/biuret were the same as in the above case of analysing the precipitate, by subtracting the precipitated copper hydroxide which was considered not used to coloring from the quantity of the copper used[7]. The color of the copper-biuret complex changed with the quantity of copper used[7], with the concentration of alkali and with temperature, and as the color is fairly unstable, so the biuret reaction was studied by measuring the wave-length and absorbance of the reaction to search the competent conditions for analysis.

Experimentals

Coloring is as follows: the water solution of $CuSO_4$ is added on alkaline solution of biuret. The concentrations of $CuSO_4$, biuret and NaOH are usually 0.0120, 0.0071 mole/l and 0.25 N (or 0.20 N) respectively and when it is necessary to change the concentrations of $CuSO_4$ and NaOH, the concentrations of $CuSO_4$ are 0.0006～0.0120 mole/l and those of NaOH are 0.05～0.50 N. As there exists the precipitate of copper hydroxide in colored solution the precipitate is removed by centrifuging or by natural sedimentation. In the case of the former, it is centrifuged immediately after coloring (it is necessary for 1～2 min setting in a centrifuge) or centrifuged at 30 min after coloring. The time of centrifuging is 5 min at 3000 r. p. m. The stability of color is measured

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1) R. Kiyama and H. Kinoshita, *This Journal*, **21**, 9 (1951)
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4) H. Kinoshita, *ibid.*, **25**, 34 (1955)
5) H. Jesserer, *Biochem. Z.*, **287**, 71 (1936)
6) M. M. Rising *et al.*, *J. Biol. Chem.*, **89**, 1 (1930)
7) Kato *et al.*, *J. Chem. Soc. Japan*, **75**, 1134 (1954)

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for 1～5 hr and 2～10 days. The temperatures of the experiment are 7～20°C. The absorption of color is measured by the Beckman Model D. U. spectrophotometer at the range of 375～1000 mμ and the path length of cell is 10 mm.

Results

Relation between the value of copper/biuret and absorption In the cases of the concentration of copper 0.0006～0.0120mole/l are changed to a definite quantity of biuret 0.0071mole/l, the absorption diagrams are shown in Fig.1. The wave-length of maximum absorption becomes longer and the absorbance becomes larger as the concentration of copper becomes higher. The relations between the value of copper/biuret and maximum absorption are shown in Fig. 2. In this figure the wave-length becomes longer continuously as the value of copper/biuret becomes larger, but there exists a limit and there is no change as the value of copper/biuret becomes larger than 1/1. Also the absorbance becomes larger continuously as the value of copper/biuret becomes larger, but the change is very small when the value of copper/biuret becomes larger than 1/1. In the case

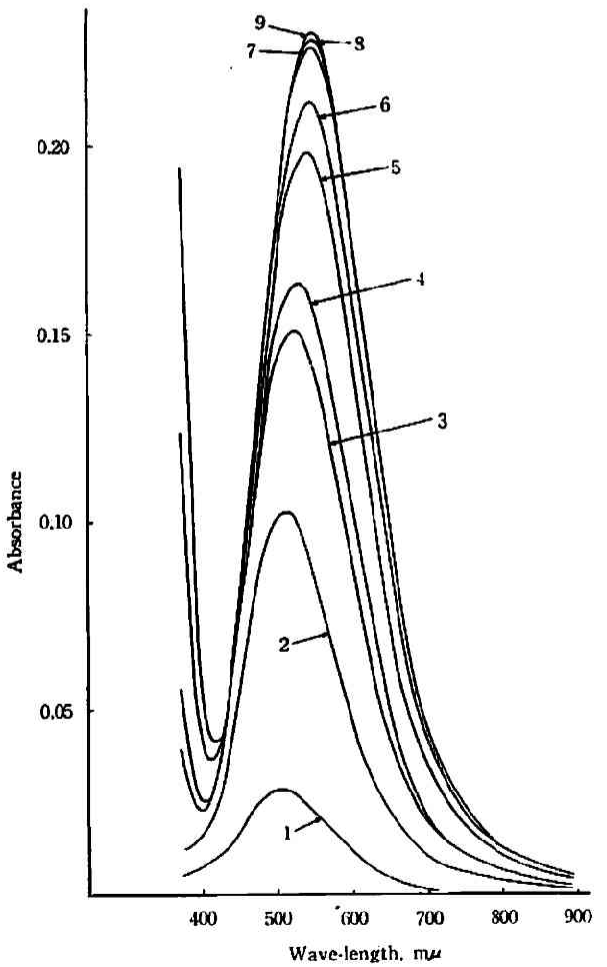

Fig. 1 Relations between the value of copper/biuret and absorption

No. of curves	$CuSO_4$	
1	0.0006	mole/l
2	0.0024	〃
3	0.0038	〃
4	0.0045	〃
5	0.0054	〃
6	0.0060	〃
7	0.0075	〃
8	0.0090	〃
9	0.0120	〃
$NH_2CONHCONH_2$	0.0071	mole/l
NaOH	0.25	*N*
Temp.	7～8	°C

centrifuged at 30 min after coloring
absorption measured at 60 min after coloring

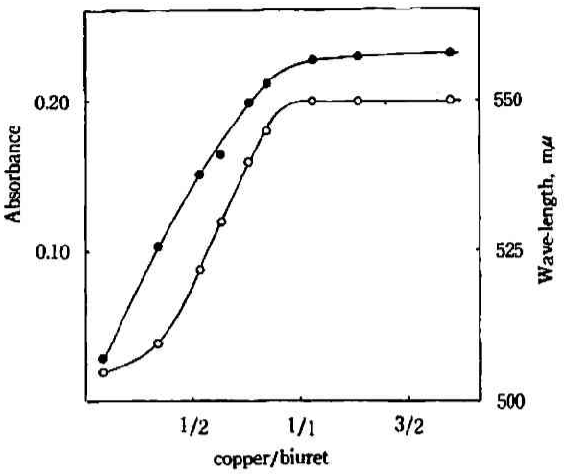

Fig. 2 Relations between the value of copper/biuret and absorption (wave-length and absorbance at their maxima)

●: absorbance, ○: wave-length

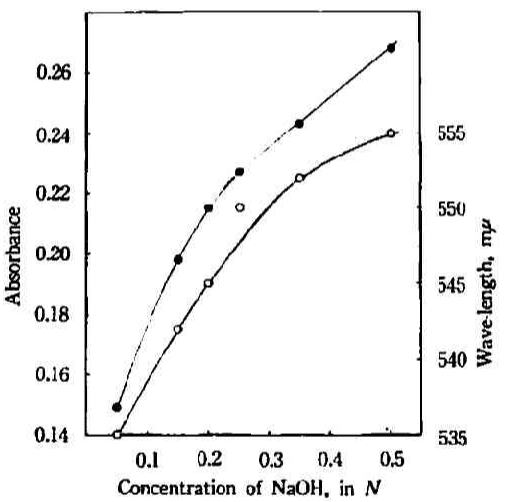

Fig. 3 Relations between the concentration of NaOH and absorption (wave-length and absorbance at their maxima)

●: absorbance, ○: wave-length

$CuSO_4$ 0.0120 mole/l
$NH_2CONHCONH_2$ 0.0071 〃
Temp. 11~12 °C

centrifuged at 30 min after coloring
absorption measured at 60 min after coloring

of coloring, there is no appearance of the precipitate of copper hydroxide until the value of copper/biuret comes near 1/1 and the precipitate appears at the value of over 1/1.

Effect of the concentration of NaOH When the concentration of NaOH is changed from 0.05 to 0.50 *N*, the change of the wave-length and absorbance at maximum absorption are shown in Fig. 3. The wave-length becomes longer and absorbance becomes larger as the concentration of NaOH becomes higher. The stability of color is slightly good as the concentration of NaOH is higher.

Effect of temperature The absorptions of colored substance at 12 and 20°C are compared from curves 1, 4 in Fig. 4. The bell shapes of absorption of the two cases are coincident with each other during several hours after coloring, except that the maximum absorbance is larger as the temperature is lower.

Stability of color At 1 hour after coloring the absorption of the three different cases

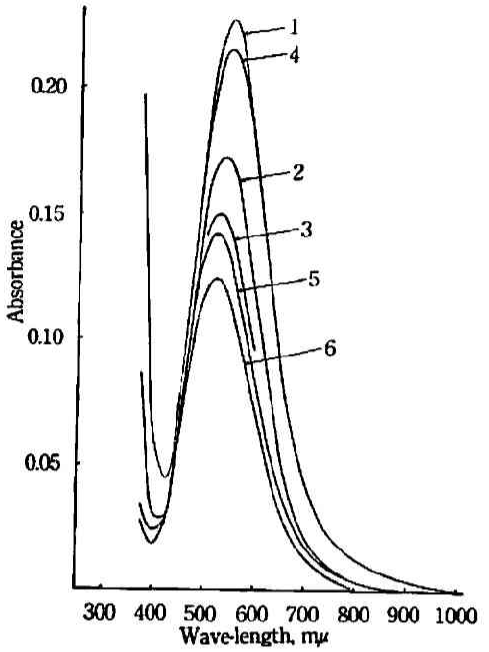

Fig. 4 Effects of temperature and time after coloring on absorption

$CuSO_4$	0.0120	mole/l
$NH_2CONHCONH_2$	0.0071	〃
NaOH	0.25	N

not centrifuged

	time after coloring (hr)	Temp.(°C)
1	1~3	12
2	72	12
3	240	12
4	1	20
5	72	20
6	240	20

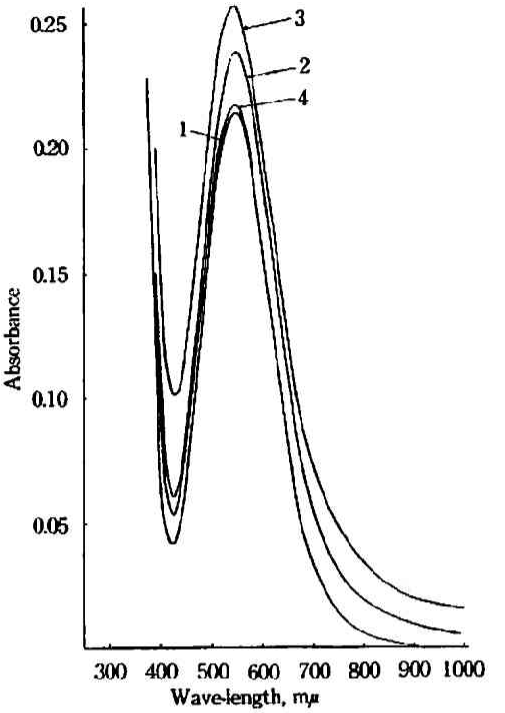

Fig. 5 The increase of absorption

$CuSO_4$	0.0120	mole/l
$NH_2CONHCONH_2$	0.0071	〃
NaOH	0.20	N
Temp.	7~8	°C

	time before centrifuging (min)	time before absorption measurement (hr)
1	30	1~24
2	1~2	3
3	1~2	7
4	1~2	7

recentrifuged during 30 min before absorption measurement

of the separation of precipitate, centrifuged the copper hydroxide immediately after coloring, centrifuged at 30 min after coloring and naturally sedimented are as follows: the wave-length are 550, 545, 545 mμ and absorbance are 0.228, 0.215, 0.212 respectively in the three cases at the conditions of copper/biuret=1.7/1. NaOH=0.20 N. Namely, when the precipitate is centrifuged immediately after coloring, the wave-length is slightly longer and the absorbance is also slightly larger as compared with the other two cases. The color of copper-biuret complex changes after separating of copper hydroxide as time passes, the change of color is traced until 10 days at the conditions that the concentrations of NaOH are 0.05~0.50 N and temperatures are 12 and 20°C. From these experiments, the stability of color is good as the concentration of NaOH is higher and as the temperature is lower. In the case of copper/biuret=1.7/1, the concentration of NaOH is 0.25 N and naturally sedimented, the results are shown in Fig. 4. When the time after coloring

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are 1, 72, 240 hr respectively, the curves of absorption are 1, 2, 3 at 12°C and are 4, 5, 6 at 20°C. The wave-length at maximum absorption becomes shorter and the absorbance becomes smaller as time passes. But on centrifuging, the time after coloring has a considerable effect on the stability of color. When the precipitate is centrifuged at 30 min after coloring the color is most stable, namely the change of color is negligible within 5 hr after coloring and the change of color is smaller than the other two cases at 10 days. In the case of centrifuging immediately after coloring the colored solution becomes turbid with time after centrifuging and absorbance becomes larger. In Fig. 5 by comparing the curves 3 and 1 or 2 and 1, the increase of absorbance is almostly parallel at each wave-length. Consequently the increase of absorbance must be due to the white turbidity. The increase of absorbance happens in the case of centrifuged at 15 min after coloring. The white turbidity is not removed perfectly by centrifuging the sample during 5 min, but is almost removed by centrifuging during 30 min (curves 4 and 3). The turbid sample becomes transparent in the next day by natural sedimentation and subsequently the wave-length of maximum absorption becomes shorter and the absorbance of it becomes smaller by separating copper hydroxide. The change of color with time is similar to the first experiment in which the corresponding value of copper/biuret.

As mentioned above, when the value of copper/biuret becomes larger, the wave-length at maximum absorption becomes larger and absorbance becomes larger continuously until the value of copper/biuret becomes 1/1, and there is no change as the value of copper/biuret becomes larger than 1/1. Accordingly there is possibility of the existence of the compound of copper/biuret= 1/1, but the existence of the compound of 1/2 is hard to understand from this experiment. However, the existence of the compound of copper/biuret=1/1 is contradicted by the experiment that the wave-length and absorbance of maximum absorption are gradually changed with the concentration of NaOH.

From these experiments the following condition may be the best one of the quantitative analysis of biuret. At coloring it is necessary to use the copper as the value of copper/biuret is over 1/1, namely the precipitate of copper hydroxide exists slightly. The following conditions are the most suitable to separation that the precipitate is sedimented naturally or it is centrifuged at 30 min after coloring and that the absorption is measurable within 5 hr after coloring. As to the concentration of NaOH because of the inconvenience of handling of concentrated NaOH solution, 0.2～0.3 *N* NaOH (the inflection point of the curve in Fig. 3) is suitable. As to the temperature, the change of color is rapid and it becomes easily turbid over 20°C, so that it is suitable below 20°C. According to the above conditions it is capable in the 10 mm path length of absorption cell to determine 4 and 16 mg/*l* biuret in the cases of centrifuging at 30 min after coloring and natural sedimentation respectively.

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