UDC 543.544 : 547.854.4

131. Satoru Kuwada, Toru Masuda, Toyokazu Kishi, and Mitsuko Asai: Application of Chromatography. XLI.*1 Behavior of Diaminouracil Derivatives.

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Formerly the authors obtained a green fluorescent substance from the mycelium of *Eremothecium ashbyii*[1] and elucidated its structure by total synthesis.[2] In the course of this synthesis, 5-amino-6-ribitylaminouracil (I) was isolated as its hydrochloride and sulfite in crystalline form, and it was found that when the aqueous solutions of these salts were left standing at room temperature or heated in a water-bath, they began to show bluish green fluorescence, becoming negative to Ehrlich reaction. This fact seemed to suggest their structural change.

All attempts to find whether (I) is contained in the mycelium of *Er. ashbyii* or not were unsuccessful. This may be due to the absence of (I) or to its variability during the isolation process, but probability of the latter is strong from the above-mentioned finding. Accordingly, it became necessary to investigate the behavior of diaminouracil derivatives and model experiments were carried out using the hydroxyethyl (II) and butyl (III) derivatives instead of the rather costly ribityl derivative. Heating of the aqueous solutions of the three diaminouracil derivatives, (I), (II), and (III), under the same conditions changed their ultraviolet spectra as shown in Fig. 1, but the resemblance of the spectra hinted that the new products all have the same skeleton.

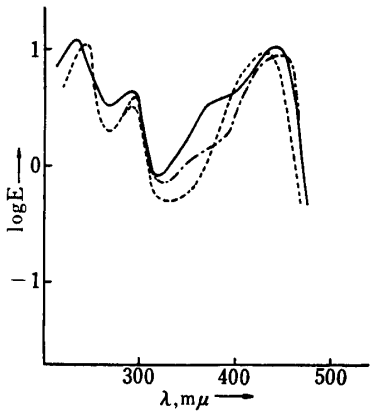
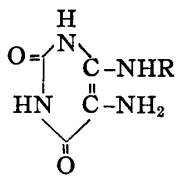


Fig. 1. Ultraviolet Spectra
(After heating of a 0.5% aqueous solution)
——— (I)
- - - - - - - (II)
—·—·— (III)



Diaminouracil derivatives

(I) R=ribityl
-CH(OH)-CH(OH)-CH(OH)-CH(OH)-CH_2OH

(II) R=hydroxyethyl
-CH_2-CH_2-OH

(III) R=butyl
-CH_2-CH_2-CH_2-CH_3

On heating the aqueous solution of the diaminouracil derivative (II), it began to show a bluish green fluorescence in a short time. After heating for one hour, the solution was acidified with acetic acid, the precipitated orange-yellow substance was dissolved in water,

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1) T. Masuda: This Bulletin, 4, 71, 375(1956).
2) T. Masuda, T. Kishi, M. Asai, S. Kuwada: *Ibid.*, 7, 361, 366(1959).

and the solution, after being filtered to remove a sparingly soluble green fluorescent substance, was concentrated to separate yellow crystals, which crystallized from *N* hydrochloric acid to needles, m.p. above 360°. At first the product was considered to be lumazine (IV), produced by ring formation between the hydroxyl and amino groups by elimination of water, followed by oxidation, but the melting point and Rf value of the product were not identical with those of lumazine. Further, the ring closure is unthinkable from the fact that the butyl derivative, which cannot undergo such a ring closure, also gives a product having the same skeleton.

Heating of the aqueous solution of (II) evolved ammonia detectable by the Nessler reagent and the condensation shown in Chart 1 was assumed. On the other hand, Korte, *et al.*[3] reported of late that 5,6-diaminouracil, when left as a diluted aqueous solution, readily condensed into bisalloxazine (VII).

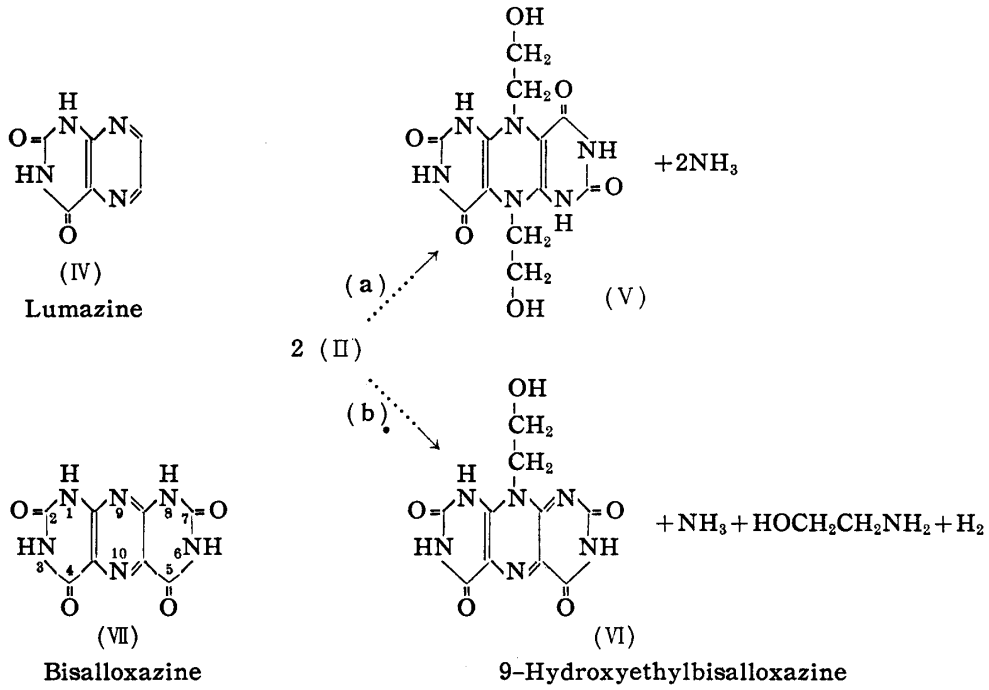


Chart 1.

Table I. Paper Partition Chromatography of the Products obtained by heating Aqueous Solutions of 5-Amino-6-hydroxyethylaminouracil at various pH's

pH of heated solutions	Rf value			
	EtOH-BuOH-H_2O (15:50:35)		Benzyl alcohol-H_2O	
0.1*N* NaOH	0.19 G +			0.23 G +
pH 9	0.19 G ++	0.22 B +	0.02 B ±	0.23 G ++
pH 7	0.19 G ++	0.22 B +	0.02 B +	0.22 G ++
H_2O	0.19 G ++	0.22 B ++	0.02 B +	0.22 G +
pH 3	0.19 G +	0.22 B ++	0.02 B ++	0.22 G +
0.1*N* HCl		0.22 B ++	0.02 B ++	

Color of fluorescence : G—green, B—blue
Intensity : + ⟶ ++

Therefore 9-hydroxyethylbisalloxazine (VI) was synthesized by condensation of 5-nitroso-6-hydroxyethylaminouracil and barbituric acid, utilizing Timmis' method[4] for the synthesis of pyrimidopyrazine and also by the condensation of (II) with alloxane

3) F. Korte, W. Paulus, K. Störiko : Ann., **619**, 63(1959).
4) G. M. Timmis : Nature, **164**, 139(1949); U. S. Pat. 2,581,889 (1952).

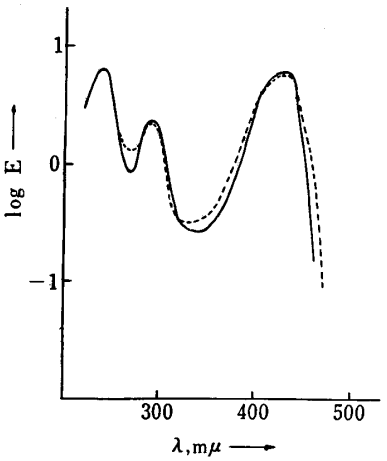


Fig. 2. Ultraviolet Spectra

——— 9-Hydroxyethylbisalloxazine synthesized

– – – – The blue fluorescent substance obtained by heating an aqueous solution of 5-amino-6-hydroxyethylaminouracil

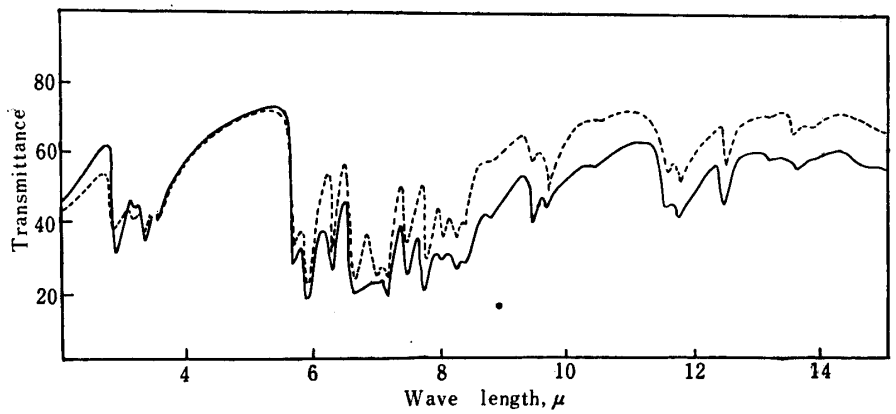


Fig. 3. Infrared Spectra

——— 9-Hydroxyethylbisalloxazine synthesized

– – – – The blue fluorescent substance obtained by heating an aqueous solution of 5-amino-6-hydroxyethylaminouracil

Table II. Rf Values in Paper Partition Chromatography

Solvent	Product from heating of (II) in water		Synthesized 6-hydroxyethyl-bisalloxazine
AcOH-BuOH-H_2O (1:4:5)	0.01	0.03	0.02
EtOH-BuOH-H_2O (15:50:35)	0.19		0.19
Pyridine-BuOH-H_2O (3:4:7)	0.49		0.49
(4:6:3)	0.23		0.23
Migration distance (cm.) in paper ionophoresis (400 v, 3 hrs. pH 7.0) (Theorell), $\mu=0.1$	+ 4		+ 4

Table III. Paper Partition Chromatography of the Product obtained by heating 5-Amino-6-butylaminouracil in Neutral Water

Solvent	Rf	
AcOH-BuOH-H_2O (1:4:5)	0.35 B ±	0.82 G ##
EtOH-BuOH-H_2O (15:50:35)	0.42 B ±	0.84 G ##
Pyridine-BuOH-H_2O (3:4:7)	0.72 B ±	0.87 G ##
Benzyl alcohol-H_2O	0.31 B ±	0.89 G ##

according to the method of Wieland, *et al.*[5] Comparison of (VI) with the product obtained by heating the aqueous solution of (II) showed that they are in complete agreement in every respect as shown in Figs. 2 and 3, and in Table II, and therefore the product derived from (II) must have been formed through the course (b) in Chart 1.

As stated above, heating of an aqueous solution of (II) produced a sparingly soluble and green-fluorescent yellow substance besides the above-mentioned bisalloxazine derivative. Recrystallization of the yellow substance from water acidified with hydrochloric acid converted it to a more soluble, needle-like crystals of the same color, and the aqueous solution of the product showed blue fluorescence. This substance is nothing but 9-hydroxyethylbisalloxazine. Question remains yet as to the identity of the green fluorescent substance before the acid treatment.

Attempts to clarify the structure of this substance resulted in failure because its analytical values fluctuated and the substance did not agree with compound of (XIII) type derivable from the butyl derivative. Since the cause of the phenomenon was assumed to be due to hydroxyl group in the hydroxyethyl group, already mentioned 6-butylaminouracil (X) was prepared from 6-chlorouracil (VIII) and butylamine (IX), and the 5-nitroso derivative (XI) of the product was reduced with hydrosulfite to 5-amino-6-butylaminouracil (III) in order to carry out the reactions shown in Chart 2.

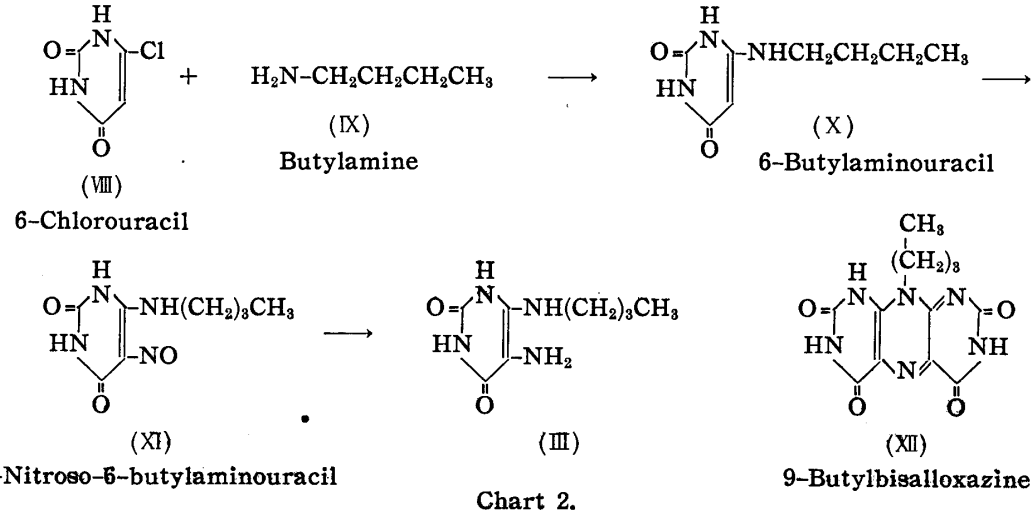


Chart 2.

On heating the aqueous solution of (III), the color gradually turned from yellow to red and then again to yellow, and at the same time a yellow crystalline precipitate separated out. Recrystallization of the precipitate from 0.5*N* hydrochloric acid gave yellow needles (A), m.p. above 360°, the aqueous solution of which showed green fluorescence. Acidification with hydrochloric acid of the filtrate from the crystals (A) separated yellow crystals and recrystallization of the product from *N* hydrochloric acid afforded a substance (B), the aqueous solution of which also showed blue fluorescence. As (B) is identical

Table IV. Paper Partition Chromatography of the Product obtained by heating 5-Amino-6-butylaminouracil in *N* HCl

Solvent	Rf	
AcOH-BuOH-H_2O (1:4:5)	0.82 G	0.37 B
EtOH-BuOH-H_2O (15:50:35)	0.84 G	0.44 B
Pyridine-BuOH-H_2O (3:4:7)	0.87 G	0.73 B
Benzyl alcohol-H_2O	0.89 G	0.33 B

5) H. Wieland, A. Tartter, R. Purrmann : Ann., **545**, 209(1940); E. C. Taylor, C. K. Cain, H. M. Loux : J. Am. Chem. Soc., **76**, 1874(1954).

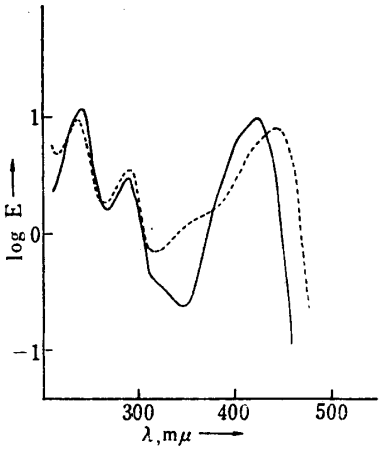


Fig. 4. Ultraviolet Spectra
-------- Product (A)
——— Product (B)

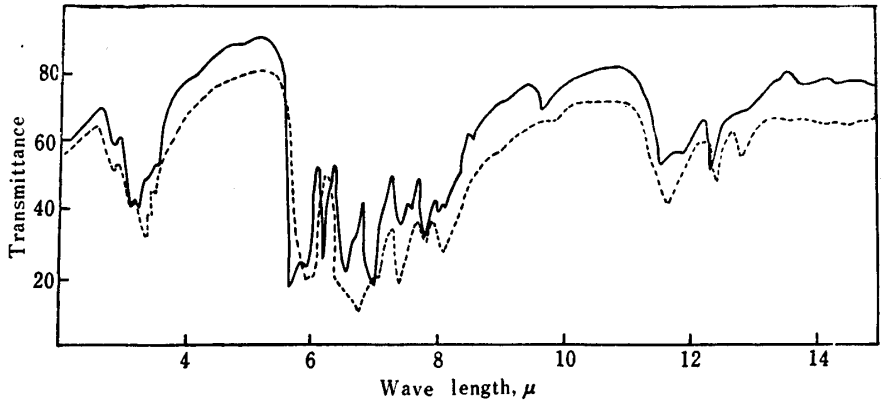


Fig. 5. Infrared Spectra
-------- Product (A) ——— Product (B)

with 9-butylbisalloxazine (XII) in every respect as shown in Figs. 6 and 7, and in Table V, its structure is self-evident. In the next experiment, a solution of the butyl derivative of diaminouracil in *N* hydrochloric acid was heated in a water-bath for about 2 hours and the resulting precipitate was recrystallized from *N* hydrochloric acid to yellow crystals, m.p. above 360°. The product was also identical with 9-butylbisalloxazine and its aqueous solution showed blue fluorescence.

Table V. Rf Values in Paper Partition Chromatography

Solvent	Synth. 9-butyl-bisalloxazine	Compd. (B), produced from (III)
AcOH-BuOH-H_2O (1:4:5)	0.36 B	0.37 B
EtOH-BuOH-H_2O (15:50:35)	0.44 B	0.44 B
Pyridine-BuOH-H_2O (3:4:7)	0.72 B	0.73 B
Benzyl alcohol-H_2O	0.34 B	0.33 B

Thus, heating of a solution of (III) in neutral water yielded two substances, (A) and (B), showing green and blue fluorescence, respectively, whereas the same treatment of a solution of the same substance in acid water gave only one product (B) as shown in Table V. The same phenomenon was also observed with the 6-hydroxyethyl derivative of diaminouracil as shown in Table I, as well as with the 6-ribityl derivative as shown in Tables VI and VII. Besides the above-mentioned difference, (A) differs from (B) in ultraviolet and infrared spectra.

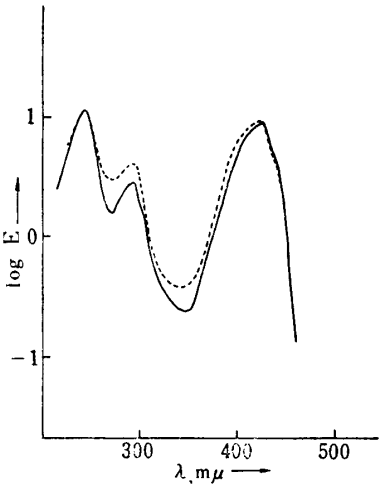


Fig. 6. Ultraviolet Spectra

——— The product (B) obtained from 5-amino-6-butylaminouracil

- - - - - - - - 9-Butylbisalloxazine synthesized

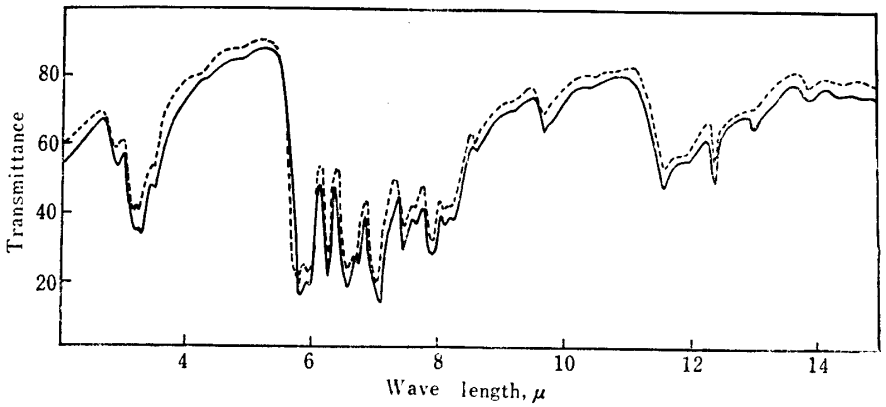


Fig. 7. Infrared Spectra

——— 9-Butylbisalloxazine synthesized

- - - - - - - - The product (B) obtained from 5-amino-6-butylaminouracil

Table VI. Paper Partition Chromatography of the Products obtained by heating 5-Amino-6-ribitylaminouracil in Neutral Water

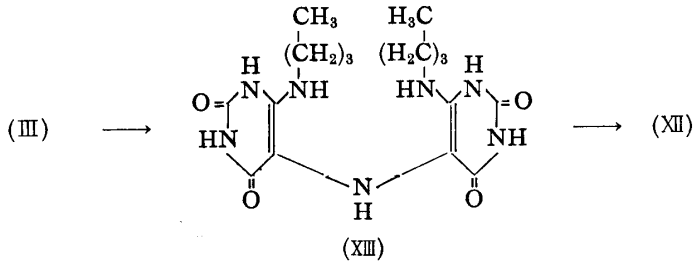
Solvent	Rf	
AcOH-BuOH-H_2O (1:4:5)	0.02 G ++	0.03 B +
EtOH-BuOH-H_2O (15:50:35)	0.08 G ++	0.13 B +
Pyridine-BuOH-H_2O (3:4:7)	0.40 G ++	0.43 B +
Benzyl alcohol-H_2O	0.02 B +	0.25 G ++

Table VII. Paper Partition Chromatography of the Products obtained by heating 5-Amino-6-ribitylaminouracil in 0.1*N* HCl

Solvent	Rf	Solvent	Rf
AcOH-BuOH-H_2O (1:4:5)	0.03 B ++	Pyridine-BuOH-H_2O (3:4:7)	0.43 B ++
EtOH-BuOH-H_2O (15:50:35)	0.13 B ++	Benzyl alcohol-H_2O	0.02 B ++

An aqueous solution of the compound (A) was acidified with hydrochloric acid and heated, and a part of the solution was subjected to paper chromatography. The resulting chromatogram is shown in Table IV, which indicates that (A) is converted into (B) in acid water. Purification of (A) under the conditions described in the experimental part yielded a substance of m.p. above 360°, corresponding to $C_{16}H_{25}O_4N_7$, and this composition is in complete agreement with that of (XIII), which may be produced by the condensation of 2 moles of (III) under elimination of 1 mole of ammonia from the two amino groups

at 5-position. Formation of the blue fluorescent 9-butylbisalloxazine (XIII) must have been effected by further progress of the above condensation with elimination of butylamine, especially in the presence of hydrochloric acid.



(XIII)

The same condensation is considered to take place with the diaminouracil derivative having a ribityl or hydroxyethyl group in the amino group at 6-position but, while the bisalloxazine derivative, i.e. (B)-type substance, was produced in experiment with the hydroxyethyl derivative, green fluorescent (A)-type substance was not obtained pure in the first stage of the reaction. This may be due to the formation of a hardly separable by-product owing to the presence of the hydroxyl group.

At any rate, the resemblance of ultraviolet spectra shown in Fig. 1 can be explained only by assuming that the above condensation probably occurs also with 5-amino-6-ribitylaminouracil.

Table VIII. Rf Values in Paper Partition Chromatography

Solvent	Product from heating of (I)	Synthesized 9-ribityl-bisalloxazine
AcOH-BuOH-H_2O (1:4:5)	0.02	0.02
EtOH-BuOH-H_2O (15:50:35)	0.13	0.13

Experimental

5-Nitroso-6-hydroxyethylaminouracil—A mixture of 5 g. of 6-chlorouracil, 7.5 cc. of 2-aminoethanol, and 50 cc. of water was heated in an autoclave at 140° for 3 hr. at 10 atm. pressure. After concentration, the reaction mixture was nitrosated with 5 g. of $NaNO_2$ and 5 cc. of AcOH, and left standing, whereupon a product separated out as red needles, m.p. 232°(decomp.). Yield, 3 g. *Anal.* Calcd. for $C_6H_8O_4N_4$: C, 36.00; H, 4.03; N, 27.99. Found: C, 36.26; H, 3.95; N, 28.04.

5-Amino-6-hydroxyethylaminouracil (II)—$NaHSO_3$ was added to a suspension of 100 mg. of the above-mentioned nitroso compound in 5 cc. of water at 90°, when red color of the suspension disappeared immediately and white crystals, m.p. 230°(decomp.), separated out on standing. The formation of this compound was ascertained by the production of the 8-hydroxyethyl derivative of 6,7-dimethyllumazine by the action of diacetyl.

Heating of the Aqueous Solution of 5-Amino-6-hydroxyethylaminouracil (II)—About 5 g. of (II) was dissolved in 1 cc. of 0.1*N* NaOH, 0.1*N* HCl, or phosphate buffer of various pH. The solution was heated in a water-bath at 100° for 2 hr. and subjected to paper partition chromatography. The chromatogram is shown in Table I and it shows that formation of the green fluorescent substance increases with increasing basicity of the solvent, and that of the blue fluorescent substance, with increasing acidity.

When 100 mg. of (II) was heated with 10 cc. of water on a water-bath, the solution turned yellow and showed bluish green fluorescence. After heating for 1 hr., the solution was acidified with AcOH, the resulting orange-yellow substance was collected, and dissolved in about 200 cc. of water. The solution, after being filtered to remove an insoluble green fluorescent substance, was shaken several times with benzyl alcohol to transfer the remaining green fluorescent substance into the benzyl alcohol layer. The aqueous solution was treated with Et_2O, concentrated under a reduced pressure, and the substance that separated was recrystallized from *N* HCl to yellow crystals, m.p. >360°. *Anal.* Calcd. for $C_{10}H_8O_5N_6 \cdot H_2O$: C, 38.71; H, 3.25; N, 27.09. Found: C, 38.83; H, 3.69; N, 26.98.

The UV and IR spectra, and Rf values of this compound are shown in Figs. 2 and 3, and in Table II.

Synthesis of 9-Hydroxyethylbisalloxazine (IV)—According to the method of Timmis,[4] 2 drops of conc. HCl was added to a mixture of 2 g. of 5-nitroso-6-hydroxyethylaminouracil, 2 g. of barbituric acid, and 60 cc. of AcOH, and the whole was refluxed for 2 hr. The reaction mixture once became clear, but a yellow precipitate separated out immediately, which was recrystallized from *N* HCl to 800 mg. of yellow needles, m.p. >360°. *Anal.* Calcd. for $C_{10}H_8O_5N_6 \cdot H_2O$: C, 38.71; H, 3.25; N, 27.09. Found : C, 38.41; H, 3.78; N (Micro-Kjeldahl), 27.11.

The UV and IR spectra, and Rf values of this compound are shown in Figs. 2 and 3, and in Table II. They are in agreement with those of the blue fluorescent substance produced by heating of (II) in acid water.

6-Butylaminouracil (X)—A mixture of 3 g. of 6-chlorouracil and 20 g. of butylamine was heated in an autoclave at 140° for 3 hr. at 10 atm. pressure. The reaction mixture was concentrated under a reduced pressure and the resulting colorless needles (3.6 g. or 96%) were recrystallized from water, m.p. 265° (decomp.). *Anal.* Calcd. for $C_8H_{13}O_2N_3$: C, 52.44; H, 7.15; N, 22.94. Found : C, 52.44; H, 7.16; N, 23.01.

5-Nitroso-6-butylaminouracil (XI)—To a solution of 3 g. of (X) in 150 cc. of water, 1.5 g. of $NaNO_2$ and then 3 cc. of AcOH were added dropwise, when the solution turned red. After heating for 30 min. on a water-bath, the reaction mixture was concentrated and the red prisms that separated (2.4 g. or 66%) were recrystallized from water, m.p. 198° (decomp.). *Anal.* Calcd. for $C_8H_{12}O_3N_4$: C, 45.28; H, 5.70; N, 26.40. Found : C, 45.40; H, 6.03; N, 26.21.

5-Amino-6-butylaminouracil (III)—To a solution of 300 mg. of the nitroso compound (XI) dissolved in 30 cc. of water with warming, 0.6 g. of $NaHSO_3$ was added, when the red color faded and changed to a light yellow clear solution. The solution was concentrated under a reduced pressure and left standing, whereupon colorless prisms, m.p. 228° (decomp.), separated out (194 mg. or 95%). The product gradually colored yellow on standing and converted to a bisalloxazine derivative when recrystallized from water. Therefore, it was impossible to purify the product by recrystallization. *Anal.* Calcd. for $C_8H_{14}O_2N_4$: C, 48.47; H, 7.12; N, 28.27. Found : C, 48.64; H, 7.27; N, 28.50.

Change of 5-Amino-6-butylaminouracil (III) in Aqueous Solution—1) Change by Heating a Neutral Aqueous Solution : A 5% neutral aqueous solution of (III) was heated at 100° for 1 hr. and then subjected to paper partition chromatography to give the spots shown in Table III.

A solution of 2 g. of (III) dissolved in ca. 30 cc. of hot water, after being filtered, was heated at 90° on a water-bath, when the color of the solution turned from yellow to red and again to yellow, and yellow needle-like crystals separated gradually. After 2 hr. the crystals were collected, washed thoroughly with water to remove a blue fluorescent substance, and dissolved in 0.1*N* NaOH. The solution was decolorized with activated carbon, acidified with HCl while hot, and the resulting yellow substance was recrystallized from 0.5*N* HCl to yellow needles (A), m.p. >360°. The aqueous solution of the product showed green fluorescence. *Anal.* Calcd. for $C_{16}H_{25}O_4N_7$: C, 50.60; H, 6.60; N, 25.85. Found: C, 50.90; H, 6.71; N, 25.45.

That the product (A) is a little different in structure from the substance (B) to be described later was easily presumed from its UV spectrum shown in Fig. 4 as well as from its IR spectrum shown in Fig. 5.

The product (A) which exhibited only one green fluorescent spot on paper chromatogram was heated in *N* HCl at 100° for 2 hr., when the solution gradually showed blue fluorescence. It is obvious from the chromatogram shown in Table IV that the new product is nothing but the substance (B). The UV and IR spectra of the product are shown in Figs. 6 and 7.

2) Change by Heating in Aqueous Acid Solution : A solution of 2 g. of (III) in 30 cc. of *N* HCl was heated, when the solution turned light yellow, and yellow needles separated simultaneously. After 2 hr., the product was collected and dissolved in 0.5 *N* NaOH, and the solution, after being decolorized with activated carbon, was acidified with HCl to separate crystals, which were purified by recrystallization from *N* HCl. The purified product was further dissolved in hot conc. HCl and the solution was diluted with water while hot so as to contain 5% of HCl, whereupon pretty yellow crystals, m.p. >360° (decomp.), separated out. From its analytical values and from the blue fluorescence of its aqueous solution, the product was found to be identical with the substance (B), that is, 9-butylbisalloxazine (XII). *Anal.* Calcd. for $C_{12}H_{12}O_4N_6$: C, 47.37; H, 3.98; N, 27.62. Found : C, 47.39; H, 4.16; N, 27.76.

3) Synthesis of 9-Butylbisalloxazine (XII) : A mixture of 0.8 g. of 5-nitroso-6-butylaminouracil, 0.7 g. of barbituric acid, and 120 cc. of AcOH was refluxed for 2 hr., when the red color of the nitroso compound disappeared and the solution showed bluish green fluorescence. AcOH was distilled off under a reduced pressure, the yellow residue was dissolved in *N* HCl with warming, and the solution was concentrated, giving 1.0 g. (82%) of yellow crystals, m.p. >360° (decomp.). *Anal.* Calcd. for $C_{12}H_{12}O_4N_6$: C, 47.37; H, 3.98; N, 27.62. Found : C, 47.09; H, 3.98; N, 27.65.

As shown in Figs. 6 and 7, and in Table V, the UV and IR spectra, and Rf values of the pro-

duct are in complete agreement with those of the substance (B) which was produced on treatment of (III) in water, especially in acid water.

Change of 5-Amino-6-ribitylaminouracil (I) by Heating in Water—About 0.5% solution of 5-amino-6-ribitylaminouracil, prepared by the method described in Part XXXVII[6] of this series, in neutral or acid water was heated at 100° for 1 hr. and the reaction mixture was subjected to paper partition chromatography. The chromatograms are shown in Tables VI and VII. As seen in these tables, only the blue fluorescent substance was produced in the acid solvent, as observed in the model experiments with the hydroxyethyl and butyl derivatives. The Rf values of the above fluorescent substance was in complete agreement with that of the crude 9-ribitylbisalloxazine prepared from 5-nitroso-6-ribitylaminouracil and barbituric acid according to the method of Timmis (Table VIII).

In the present work, measurement of various spectra was conducted by Messrs. T. Shima and H. Nakamachi, elementary analysis by members of the analytical section, and high-pressure experiments by Mr. I. Uchida. The authors thank them for their kind cooperation.

Summary

5-Amino-6-hydroxyethylaminouracil was heated in neutral water, and a sparingly soluble, green fluorescent substance and a more soluble, blue fluorescent substance were obtained. The latter was identified with 9-hydroxyethylbisalloxazine.

A similar result was obtained with 5-amino-6-butylaminouracil, yielding a green and a blue fluorescent substances. The latter was found to be 9-butylbisalloxazine and the former was concluded to have been produced by the condensation of two moles of the diaminouracil derivative under elimination of one mole of ammonia.

A similar phenomenon was assumed to take place with 5-amino-6-ribitylaminouracil, an intermediate in the synthesis of 6,7-dimethylribolumazine.

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6) T. Masuda, T. Kishi, M. Asai, S. Kuwada : This Bulletin, **7**, 361(1959).