72. Tokunosuke Kanzawa: Application of the Countercurrent Distribution Method. III[1]. Homogeneity Test of Agroclavine.

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In the previous paper[1], the partition coefficients and stability of agroclavine were described. Based on those results, 19-plate countercurrent distributions were carried out. The results were not so simple as anticipated before the experiments. A few similar experiments are found in the isolation or homogeneity test of alkaloids from *Veratrum viride*,[2,3] where the experimental distribution curves occasionally do not show good agreement with the theoretical one and give a tailing portion following a maximum in the curve.

An aqueous solution of agroclavine, during standing at a room temperature for some days, becomes brown and comes to emit blue or greenish yellow fluorescence under ultraviolet rays. It would be an interesting problem too, to find whether or not these fluorescent substances, which are probably decomposition products, can be separated from agroclavine by the distribution method.

Experimental

Materials—The source and purity of agroclavine used in this work are as mentioned previously.[1] Purification of the organic solvents and preparation of the buffer solution were carried out in the same way as described before.[1]

Apparatus—A 20-tube instrument previously described[4] was used.

Procedure—Before the experiments started, the organic solvent ($CHCl_3$ or AcOBu) and 0.1 *M* phosphate buffer were mutually saturated at a room temperature by shaking in a separatory funnel. The amount of the sample to be distributed was about 50 mg. Stepwise countercurrent distribution was conducted as described previously[4]. Time of shaking and standing was 1 and 4 minutes. Upon completion of a run, the content in each tube of the instrument was pipetted out into a glass-stoppered tube. The organic layer was extracted twice with 3 cc. of 1% H_3PO_4 and the extracts were combined with the aqueous layer. The volume of the combined solution was measured with a measuring cylinder. One cc. of the aqueous solution was diluted with a suitable volume of distilled water and 1 cc. of the diluted solution was analysed colorimetrically according to the method previously mentioned.[1] From the values thus obtained the total amount of agroclavine in each tube was calculated. The recovery of the original sample was about 90%, or 80% in poor cases. In case the melting point or ultraviolet spectrum of the content in a tube was necessary to be measured, the solution was made alkaline with 5% NH_4OH solution and the precipitated crystalline substance was filtered, dried after washing with distilled water, and subjected to the measurement. The ultraviolet spectra were taken with the Hilger quartz spectrograph.

Results

Distribution in Chloroform-Buffer System—A pure sample (m.p. 206~208°(decomp.)) was subjected to 19-plate distributions in a system composed of $CHCl_3$ and 0.1 *M* phosphate buffer at pH 3.2. A typical example of the results is shown in Fig. 1. From the partition coefficient previously determined (0.24 in this system), it is expected that the position of the maximum of the distribution curve will be located at tube 17 since aqueous layer migrates. The distribution curve shows 2 maxima, the positions of which are respectively at tubes 6 and 12. This discrepancy might be due partly to the change of partition coefficients because of the use of a higher concentration of agroclavine than in the previous experiments, or to the salting out effect. In spite of this unexpected result, the same

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1) Part II: This Bulletin, **2**, 312(1954).
2) J. Fried, P. Numerof, N. Coy: J. Am. Chem. Soc., **74**, 3041 (1952).
3) M. W. Klohs, *et al.*: *Ibid.*, **74**, 5107(1952).
4) Part I: This Bulletin, **2**, 308(1954).

system was used in the following experiments, because it was desirable to prevent the decomposition of agroclavine in the solution of a higher acid concentration. The part having the maximum at tube 6 in the curve agreed fairly well with the theoretical one calculated according to the method of Williamson and Craig.[5] The acid solutions in tubes 5, 6, and 7 gave each a faint greenish yellow flouresсence, and in tubes 17, 18, and 19, a faint blue one under the ultraviolet rays. The ultraviolet absorption spectra of the contents in tubes 6, 11, and 15 are shown in Fig. 2. The spectra of the

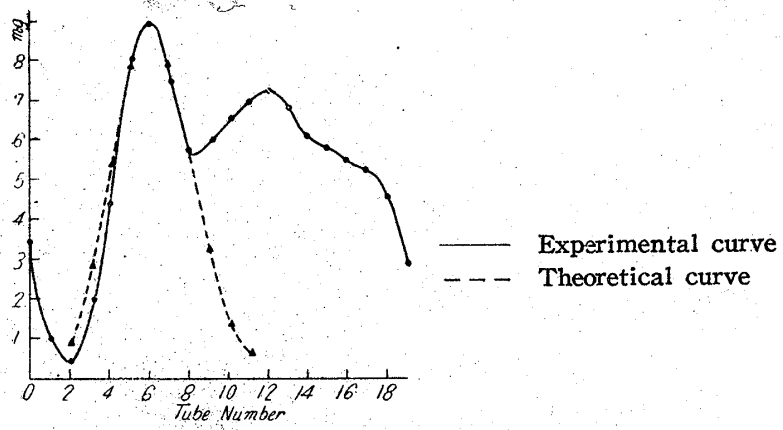

Fig. 1. 19-Plate Distribution of Agroclavine in a Chloroform–0.1 *M* Phosphate Buffer System at pH 3.2

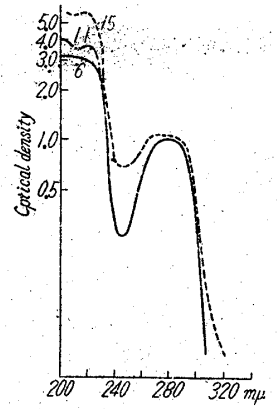

Fig. 2. Absorption Spectra of Contents in Tubes 6, 11, and 15

contents in tubes 6 and 11 resemble each other very closely, but that of tube 15 is different from the other two, especially at about the absorption minimum of 240 mμ.

After the analysis, the aqueous solution in a few tubes was made alkaline with NH_4OH and crystalline substances were obtained. Their melting points are listed in Table I. From the Table it is

Table I.

Tube No.	m.p. (°C)	Tube No.	m.p. (°C)
4	195	10	200
6	197	12	201
7+8	197	13~19	203

evident that the crystalline substances from the tubes of higher tube numbers have a melting point close to that of the orginal sample. The filtrate was extracted three times with ether. The ethereal and residual aqueous solutions respectively showed blue and greenish yellow fluorescence and the latter did not color with *p*-dimethylaminobenzaldehyde.

Successive Distributions in Chloroform-Buffer System—In order to confirm the results described above a series of successive distributions in the same system were run as shown in Table II. In Experiment 1, 200 mg. of agroclavine was subjected to 4-plate distribution in separatory funnels containing 10 cc. each of both solvents. In this case it is expected from calculation that the funnels 3

Table II.

Agroclavine Expt. 1. 4-plate
(0, 1, 2) and (3, 4)

Expt. 5. 19-plate max. No. 17

Expt. 2. 19-plate max. No. 6 and 11
(0~9) and (10~19)

Expt. 3. 19-plate max. No. 15

Expt. 4. 19-plate max. No. 15

and 4 contain a mixture of 60% of the total amount of the substance corresponding to the maximum at tube 6 in the previous experiment and 20% of that corresponding to the maximum at tube 11, and funnels 0, 1, and 2 contain the residual portion. The contents in the separatory funnels thus divided into two groups were combined respectively and extracted with $CHCl_3$. The extracts were subjected to respective 19-plate distributions in Experiments 2 and 5.

The distribution curve of Experiment 2 is shown in Fig. 3, curve 2. As in the previous experiment there appears two maxima in the curve. The content in each tube showed a very faint blue fluorescence. The white crystalline substances obtained from tubes 11 and 19 melted at 201° and 203°.

5) B. Williamson, L. C. Craig: J. Biol. Chem., **168**, 687(1947).

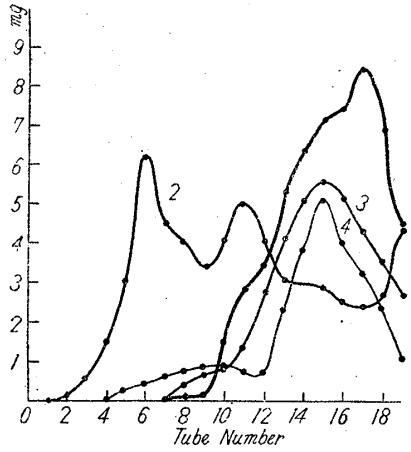

Fig. 3.
Successive 19-Plate Distribution of Agroclavine. Chloroform–0.1 *M* phosphate buffer at pH 3.2. (Curve number represents experimental number)

The contents in the tubes were combined into 2 groups (tubes 0 to 9 and tubes 11 to 19), which were subjected to next 19-plate distribution after extraction.

The distribution curve of Experiment 3 has a maximum at tube 15 as shown in Fig. 3. This migration of the position of maximum from tube 6 or 11 to tube 15 was entirely an unexpected result. The crystalline substances obtained from tubes 8 to 12 and tubes 14 to 19 showed m.p. 201° and 204°, respectively.

Experiment 4 gave a maximum at tube 15 like Experiment 3. The fluorescence in each tube became more intense than in the cases of Experiments 2 and 3, as shown in Table III.

Table III.

		Tube No. 0	2	4	6	8	10	12	14	16	18
Expt. 3.	aq.					+	+	+	−	−	−
Expt. 4.	org.				+	+	++	+++	++		
	aq.				−	−	−−	−−−	−−	−	−−
Expt. 5.	org.	++	+	+	+	−	−	−−−	−−		
	aq.		+	+	+	+	+	+++	++	−	−−
Expt. 7.	org.								+	++	+++
	aq.				−	−−	−		−	−−	−−−

aq. : Aqueous layer org. : Organic layer
fluorescence : greenish yellow, weak (+) medium (++) strong (+++)
blue, 〃 (−) 〃 (−−) 〃 (−−−)

Experiment 5 was conducted on a combined acid solution of tubes 0, 1, and 2 of the Experiment 1, after it was allowed to stand for 4 days at a room temperature. The $CHCl_3$ layer colored pink and the aqueous layer showed a greenish yellow fluorescence. The shape of the distribution curve is differernt from that of pure substance as shown in Fig. 3. The fluorescence of each tube is shown in Table III. From this Table it appears that there are two fluorescent substances, one of which is in the tubes of lower numbers and the other in the tubes of higher numbers, than in those of the tube number at the maximum of the distribution curve.

Distributions in Butyl Acetate-Buffer System — 19-Plate distribution curve of agroclavine (m.p.

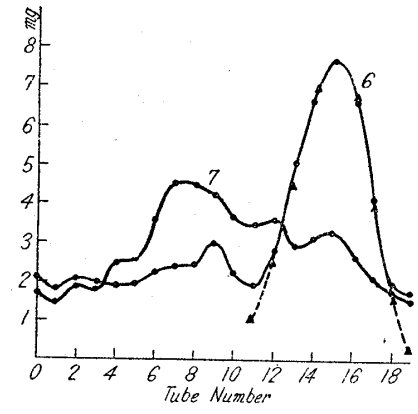

Fig. 4.
19-Plate Distribution of Agroclavine in a Butyl Acetate–0.1 *M* Phosphate Buffer System
——— Experimental curve
– – – – Theoretical curve
curve 6 : at pH 5.8
curve 7 : at pH 4.5

205~208°(decomp.)) in a system of AcOBu and 0.1 *M* phosphate buffer at pH 5.8 is shown in Fig. 4, curve 6. The curve has a maximum at tube 15, instead of 2 maxima in Experimnt 2 in Fig. 3, which is in fair agreement with the theoretical one. The position of the maximum agrees with the one expected from the partition coefficient previously determined[1]. The contents in the tubes 0 to 10 show the presence of a substance colored by *p*-dimethylaminobenzaldehyde, but no definite maximum. This indicates that agroclavine is not so stable a compound in this system.

Further experiment was run at pH 4.5 with a slightly impure sample of m.p. 203°, which gives a yellow solution in AcOBu (Fig. 4, curve 7). As shown in Fig. 4, the distribution curve does not show a definite maximum but a broad one. Also, in this case the fluorescent substances were separated into two fractions.

Discussion of the Results

The above results are summarized as follows: 1) Agroclavine which had been thought to be a pure compound showed two maxima in the distribution curve in a system of chloroform and phosphate buffer; 2) in a series of experiments the amounts of the fluorescent substances increased as the experiments were repeated and the two maxima in the earlier experiment were reduced to one in the later one; 3) there were two kinds of fluorescent substances; 4) a maximum appeared in distribution curve in a system of butyl acetate and phosphate buffer.

The reason why two maxima appear in the distribution curve in a chloroform-buffer system, the position of which is different from the the one expected from the measured partition coefficient, is not interpreted as due to the change of pH of buffered solution, because the shift of the position is too large and the presence of two maxima cannot be explained.

Secondly, the decomposition of agroclavine during distribution procedure should be taken into consideration. There are similar experiments about the decomposition of alkaloids reported by other authors. For example, Beroza[6] described that in distribution of wilforine the experimental curve consistent with theoretical one was obtained by using 1% HCl instead of 10% HCl, and in the use of the latter disagreement between both curves was attributed to decomposition. Although it has been confirmed in the previous experiment that agroclavine does not decompose so much within five hours in an acid solution, actually there was an evidence of decomposition, as shown in Experiments 3 and 4, that the greenish yellow and blue fluorescence was observed at both sides of a maximum of the distribution curve. Their intensities increase than that observed in Experiment 2. In addition there is another fact that even a pure substance, which initially shows no fluorescence, exhibits a faint blue fluorescence in each tube after a 19-plate distribution.

The assumption that one of the two maxima originates from a decomposition product is not sufficient to explain the fact that there is only one marked maximum at the expected position when system of butyl acetate and phosphate buffer is used.

However, this is interpreted as follows in connection with the fact that in Experiment 6 there is another small maximum at tube 9 and only one maximum is observed in Experiments 3, 4, and 5 on fairly decomposed samples. That is, it can be assumed that the decomposition of agroclavine proceeds through several stages and the rate of the decomposition is greater in a system including chloroform than in the one with butyl acetate. Therefore, there appear two maxima in the distribution curves in both systems and the one at a tube of lower number may be due to the decomposition products. The amount of the decomposition product is small in the latter system, while relatively large in the former. In Experiments 3, 4, and 5, further decomposition results in giving a single maximum.

6) M. Beroza: J. Am. Chem. Soc., **74**, 1585 (1952).

There is also another interpretation that in addition to decomposition, agroclavine is essentially a mixture of compounds having similar and labile structures. Some difference in a part of agroclavine molecule affecting its ionization constant may give the two maxima in the distribution curve. If this is true, the ratio of the amounts of the two compounds is accounted to about 4:6 in the system of Experiment 2 and 1:4 in the system of Experiment 6, but the former may be partly contaminated by some decomposition products.

Although which of above two explanations is correct cannot be determined from these results alone, it is certain that agroclavine partly decomposes in these distribution experiments. Recently, Abe[7] found the presence of a minute amount of dihydroagroclavine in the ordinary preparation of agroclavine. Although the fact has not been confirmed in the present work, it should be taken into consideration in future experiment.

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Summary

The homogeneity of agroclavine was tested by a 19-plate countercurrent distribution. The distribution curves indicated that agroclavine decomposed faster in a system containing chloroform than in the one containing butyl acetate, forming two kinds of fluorescent substances. The decomposition appeared to proceed through several stages.

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7) M. Abe, S. Yamatodani : J. Agr. Chem. Soc. Japan (Communication), **28**, 501 (1954).