ON THE VIOLET-FLUORESCENT SUBSTANCE, PRODUCED BY *EREMOTHECIUM ASHBYII*.

SYOICHI SHIMIZU

*Department of Industrial Chemistry, School of Engineering,
University of Kyoto, Sakyo-ku, Kyoto.*

(Received September 24, 1954)

When *Eremothecium ashbyii* was cultured on a rice germ medium or in a culture fluid added with rice germ extract, the author and collaborators (1) found a new substance which appeared as a violet-fluorescent spot on paper chromatogram. This substance will be referred to as "V substance" henceforth.

Katagiri *et al.* (2) likewise observed by paper chromatography that *Clostridium acetobutyricum*, one of the riboflavin producing bacteria, when grown in a synthetic medium, produced a blue-fluorescent substance which might be identified as 2-amino-4-hydroxypteridine-6-carboxylic acid (P-6-COOH) because of its similar R_F value and fluorescence after developing with *n*-butanol-acetic acid-water. Considering that the nuclei of flavin and of pteridine have a common part, they presumed this substance to be a precursor of riboflavin.

The two substances, independently reported, have fairly approximate R_F values by using the developing solution above mentioned, their fluorescent colors are similar each other and both are produced commonly by riboflavin producing microorganisms. The above findings are of much interest, but whether or not the two substances are identical remains to be decided, as no direct comparison of the two has hitherto been made.

The author has compared the V substance with pteridine derivatives and found them to be evidently different substances.

From the fact that some of the chemically decomposed substances of riboflavin present a fluorescence very similar to that of the V substance, the latter may be related with the formation of riboflavin in one way or another. The author has therefore studied the significance and mechanism of the production of the V substance, and has so far found several characteristics of this substance which will be reported below.

EXPERIMENTAL

1. Preparation of the Sample.

As the separation of a crystalline V substance has not yet been established, the sample used in this report is a solution obtained by eluting the spot corresponding to V substance of the paper chromatogram as follows :

A germ-culture of *E. ashbyii* was extracted with five volumes of water and the extract was applied on a straight line, running parallel with, and at a distance of about 7 cm from, one side of 40 × 40 cm Toyo filter paper No. 50. After having developed with 5 % $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$, the violet-fluorescent zone around R_F 0.55, was cut off in a belt-form. As in the case of the descending method, the cut-off strip was hung, subjected to diffusion of water, and the drippings from the other end were collected up to about 2 ml.

The liquid thus obtained presented an intense violet fluorescence under ultra-violet ray, manifesting paperchromatographically a single violet-fluorescent spot by using various developing solutions and is presumed to be almost free from impurity. Its R_F values are given in Table I.

Table I
 R_F Values of V Substance and Pteridine Derivatives.

- a, *n*-Butanol-acetic acid-water (4 : 1 : 5)
- b, *n*-Butanol-pyridine-water (4 : 3 : 7)
- c, iso-Propylalcohol-collidine-water (1 : 1 : 2)
- d, *n*-Butanol-water (1 : 1)
- e, Benzyl alcohol-ethanol-water (3 : 1 : 1)
- f, 5 % (w/v) $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ aq. solution

Developing solution	V Substance	2-NH ₂ -4-OH-Pteridine		
		6-COOH	6-CH ₃	7-CH ₃
a	0.14	0.12	0.46	0.46
b	0.55	0.33	0.67	0.65
c	0.50	0.31	0.63	0.62
d	0.01	0.00	0.33	0.37
e	0.03	0.01		
f	0.56	0.44	0.44	0.44

2. Comparison of R_F Values of V Substance with those of Pteridine Derivatives.

With Toyo filter paper No. 50, paper chromatography was performed by the ascending method at a constant temperature of 25°. After drying in the air, the fluorescent spot was observed by the mercury lamp.

As shown in Table I, both V substance and P-6-COOH have very approximate R_F values in (a), but it is not the case with the other developing solutions. Especially, in (f), their fluorescent spots lie distinctly apart, and the fluorescence of the V substance is fairly violet tone in color, while the spots of pteridine derivatives appear blue. Even from this point alone, it can be presumed that the latter are quite different substances from the V substance. Further, as has been stated in another report (3), all the pteridine derivatives exhibit no precursor effect on *E. ashbyii*. It might thus be concluded that the substance found by Katagiri *et al.* is different from V substance, or that, if the two were identical, their assumption of the substance to be P-6-COOH may be erroneous.

3. Relationship between Fluorescence Intensity and pH.

After adding the same volume of 2 *N* H₂SO₄ to V substance solution, the fluorescence disappeared entirely. The analogous quenching phenomenon was observed by adding the same volume of 2 *N* NaOH. It was not due to the decomposition, for it gave a nearly original fluorescence after neutralization. Being presumed from this fact that the V substance is an amphoteric substance, the relationship between the intensity of fluorescence and pH was investigated. Each solution of various pH was added to 1 ml of V substance solution up to 10 ml. The fluorescence of each solution was compared with a series of standard solutions prepared by diluting the V substance. The results are shown in Table II.

Table II
Fluorescence Intensity and pH.

pH	Relative Intensity of Fluorescence (%)			
	V Substance	Riboflavin	P-6-COOH	Thiochrome
0		0	0	
1		20	5	
2	0	50	30	
3	20	88	52	0
4	30	98	64	5
5	50	100	60	20
6	78	100	50	55
7	98	100	44	80
8	100	96	78	85
9	100	80	100	92
10	98	50	98	95
11	95	20	93	100
12	90	8	85	100
13	75	5	70	100
14	45	0		100

As can be seen from Table II, the fluorescence was most intensive on the neutral to alkaline side, while it suddenly decreased on the acid side. Some changes in the tone of the fluorescence seemed to occur at pH 5.0–6.4 and pH 12.5–13.0 and there was also some difference, though slight, in the intensity of fluorescence according to the kinds of buffer solutions. The values shown in Table II can not therefore be considered to be very exact, yet, a general trend can be supposed. Thus, in the V substance, similarly to riboflavin, the neutral molecule seems to be the centre of fluorescence, while the ionized form, at least the cationic form, has obviously no fluorescence. Calculating a basic dissociation constant K_b from the pH-fluorescence curve, the following value was obtained. $K_b \approx 1 \times 10^{-9}$. As the basic dissociation constant of riboflavin is 0.5×10^{-12} (4), it is considered that the V substance has a stronger basic group, provided the above assumption is correct.

The results of the similar experiments on riboflavin, P-6-COOH, and thiochrome are also given in Table II for the sake of comparison. The V substance shows a similar curve to that of riboflavin, with a deviated fluorescence maximum. The pH of fluorescence maximum of P-6-COOH agreed almost with

that of V substance, but the curve for the former differed in having another peak around pH 4. Thiochrome loses, similarly as the V substance, a fluorescence suddenly on the acid side. Nevertheless, as no complete agreement is established, it is clear that the V substance is quite different from those mentioned above.

4. Thermostability.

To each 10 ml of *N* NaOH, water and *N* HCl, respectively, 10 ml of the V substance solution was added and each resulting solution was divided into 3 parts. The first part was boiled and the other parts were placed in a thermostat at 37° and 80°, respectively. After 1, 10 and 24 hours, an aliquot of 1 ml was taken out, neutralized exactly to pH 7.0 and made up to 10 ml in order to estimate the degree of decomposition. As a standard solution, the original V substance solution preserved in a refrigerator was employed. The result were shown in Table III. In general, the V substance can be said to be thermostable.

Table III
Thermostability of the V Substance Solution.
Remaining Fluorescence (%)

Medium	Alkaline			Neutral			Acidic		
Temperature	37°	80°	100°	37°	80°	100°	37°	80°	100°
After { 1 hr	100	88	40	100	100	100	100	100	100
10 hr	100	70	8	100	100	90	100	100	92
24 hr	90	43	0	100	95	88	100	100	88

Further, the production of some new substances was investigated by paper chromatography. The reaction product, 0.01 ml, was applied on the paper and after development, the fluorescent spot was examined in the ultra-violet ray. No other fluorescent spot than that of V substance was detected, showing that the decomposed products are non-fluorescent.

5. Photostability.

As photolysis is one of the most remarkable characteristics of riboflavin, the photolyzability of V substance was investigated. Photolysis was performed at a distance of 30 cm from a 200-W lamp for 2 hours under ice-cooling. Neither new substance was detected by paper chromatography nor any decrease of fluorescence intensity was observed, showing that the V substance, unlike riboflavin, is photostable and can be dealt with in light.

6. Effect of Oxidizing Agents.

H₂O₂ and KMnO₄ were used as oxidizing agents. As in the case of thermostability test, the V substance solution was first diluted with an equal amount of *N* NaOH, water, *N* HCl, respectively. To 5 ml of this diluted solution 1 ml of 5 % H₂O₂ or 1 ml of 4 % KMnO₄ was added and the whole was kept standing for 5 hours. It was tested by paper chromatography, and at the same time the remaining fluorescence was examined. In the case of

KMnO₄, regardless of pH, the fluorescence disappeared entirely and no spot was observed on a paper chromatogram, indicating the decomposition to have taken place. In case of H₂O₂, the intensity of fluorescence remained to a certain extent, though it was fairly decreased compared with a control. Consequently, the substance is considered to have little resistance to oxidation.

7. Effect of Reducing Agents.

The V substance solution loses fluorescence when Na₂S₂O₄ is added, but by bubbling, it restores fluorescence quantitatively. That is, it composes a reversible redox system, its reduced form being non-fluorescent. This is a property worthy of note, in common with riboflavin, lumiflavin, and quinoxaline derivatives.

8. Absorption Spectrum.

An absorption spectrum of V substance was estimated, using Beckman spectrophotometer with the result given in Fig. 1. Absorption maxima were observed at 260, 282 and 338 m μ , and the minima at 248, 275 and 315 m μ , respectively, without showing any sharp maximum. It might probably be interpreted to contain a nucleus different from those of flavins or pteridines.

SUMMARY

It is confirmed by paper chromatography that the violet-fluorescent substance (V substance) produced by *Eremothecium ashbyii* is different from three 2-amino-4-hydroxy-pteridine derivatives, i.e. 6-methyl-, 7-methyl-, and 6-carboxylic acid. It is thermostable and photoresistant, especially on neutral to acid side. It is decomposed by oxidizing agents and becomes a non-fluorescent substance by reduction, which restores fluorescence by aeration, indicating it to be a reversible redox substance. The relationship between pH and fluorescence intensity, as well as absorption spectrum furnished an important basis for presuming its structure.

ACKNOWLEDGEMENT

A sincere acknowledgment with many thanks is due to Prof. R. Takata who has kindly directed in performing this investigation and to Dr. S. Kuwada, Director of the Takeda Laboratory, who has kindly furnished with pteridine derivatives.

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Fig. 1 Absorption Spectrum of V Substance.