carried out rapidly and accurately. Its principle is to measure the intensity of a blue color that is developed by adding a stannous chloride solution to a solution of the combined substance made from benzalkonium chloride and phosphomolybdic acid.

(Received August 5, 1955)

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85. **Toru Masuda**: Application of Chromatography. XXVIII. On the Formation of FAD in the Culture of *Eremothecium ashbyii**

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In the previous paper[1] the author and co-workers reported on the formation of riboflavin and flavin-adenine dinucleotide (FAD) in the culture of *Eremothecium ashbyii*, and added that studies on the flavin compounds in the mycelium and the culture broth by paper chromatography were under way. In the present paper are announced detailed explanation of such studies and a consideration about the formation of FAD.

E. ashbyii was cultivated under the same conditions as in the previous work and the result was about the same. Samples were collected at 20, 30, 40, 51, 66, 72, and 90 hours after the start of the culture and numbered from one to seven, and various measurements were conducted on them (Table I).

The culture broth separated from the mycelium was applied, as such or after concentrating *in vacuo* to one-tenth its volume, on the starting line of a paper strip and developed with butanol-ethanol-water or with 5% Na_2HPO_4 solution, and also subjected to paper ionophoresis to give the chromatogram and the pherogram shown in Table II. In this test, spots other than that of riboflavin were also detected somewhat clearly in the sample collected after some progress of the culture, but in general, none were so distinct as the spot of riboflavin. It is noteworthy, however, that adenosine was detected in the broth collected after 20 hours but was already absent in the broth collected after 30 hours.

The wet mycelium was extracted with pyridine-methanol (1 : 1) with warming and the extract was developed with butanol-acetic acid-water (4 : 1 : 5) or with 5% Na_2HPO_4 solution, or examined by paper ionophoresis. The resulting spots of flavin compounds were observed by naked eye or under ultraviolet rays and phosphorus was detected by molybdic acid reagent.

On the other hand, an aqueous extract of the mycelium, after addition of ammonium sulfate, was extracted with phenol, the phenol layer was shaken with ether and water, and the aqueous layer was tested as above. However, since in these cases flavin compounds seemed to have been lost considerably during the procedures, the mycelium was extracted with hot water (80°), and after filtering off the separated riboflavin, the filtrate was developed with butanol-ethanol-water (50: 15 : 35) or with 5% Na_2HPO_4 solution, or subjected to paper ionophoresis (Table III). As a result, flavin compounds were hardly lost and all of them could be detected on the paper, and butanol-ethanol-water was found to be the most suitable solvent.

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* This constitutes a part of a series entitled "Application of Chromatography" by Satoru Kuwada.

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1) This Bulletin, **3**, 375(1955).

In parallel with the tests, authentic samples of FAD, adenosine triphosphate (ATP), adenosine, etc. were subjected to paper chromatography and ionophoresis under the same conditions as before to get control values of their Rf and migration distance in ionophoresis (Table IV).

It is interesting to find that in the present experiment, besides riboflavin and FAD, other flavin compounds formed clear spots. Of these spots the one with Rf 0.00~0.02, formed when the sample was developed with butanol–ethanol–water, was in accord with the spot of ATP in ultraviolet absorption image, but the spot overlapped with that of FAD when the sample was developed with butanol–acetic acid–water.

To identify the spot more clearly, a sample obtained from the mycelium collected from a 40 hour culture was applied in a line on a large sheet of filter paper and developed with butanol–ethanol–water, and the spot which appeared to be for ATP was cut out and extracted with water. The ultraviolet spectrum of the extract (Fig. 1) was found to be identical with that of ATP.

A bluish purple fluorescent substance (V) and a green fluorescent substance (G) in the above sample were also respectively isolated, and developed with various solvents to settle their respective positions in the chromatogram mentioned before. The identity of the two substances and the meaning of their presence will be discussed in a separate paper.

Next, the chromatogram was printed on a printing paper with ultraviolet light of 2537 Å (Fig. 2). According to this method, not only FAD and riboflavin but also ATP and other substances which are hardly pervious to ultraviolet light were also clearly detected. Also from the picture thus obtained it was found that ATP appeared 20 hours after the start of the culture and disappeared after 90 hours, with 40th hour as the peak, and adenosine appeared after 20 hours but disappeared already after 30 hours.

In the present study the controversial problem as to whether or not FMN is present in the culture was another important subject. However, FMN was detected neither in the mycelium nor in the broth obtained by the culture under the above conditions. The suspicion that either the green fluorescent or the bluish purple fluorescent spot mentioned above may be for FMN was also dispelled because in the ionophoresis the former migrated in the opposite direction to FMN and the latter, though migrated in the same direction, lacked phosphorus reaction.

Schrecker and Kornberg[2] previously stated that the enzyme in yeast produced FAD and pyrophosphoric acid from FMN and ATP, and Yagi[3] reported that he prepared FAD in a test tube from FMN and ATP in the presence of the kidney or liver of the rat, but that the synthesis was unsuccessful with riboflavin and ATP. On the other hand, Watarai and Itadani[4] announced that ^{32}P–FAD was synthesizable from ^{32}P–FMN and ATP in the presence of liver homogenate of the rat.

Although FMN could not be detected despite detailed investigations in the samples collected at various stages of the culture of *E. ashbyii*, a fairly large amounts of riboflavin and adenosine were found in the early stage of the culture. In addition, it was discovered that in the mycelium ATP increased gradually and then decreased with the increase of FAD, while the latter, though kept on increasing even after disappearance of ATP, decreased with the autolysis of the mycelium. In the present study the author detected for the first time ATP and adenosine in

2) A. W. Schrecker, A. Kornberg : J. Biol. Chem., **182**, 795(1950).
3) K. Yagi : Igaku to Seibutsugaku (Japan), **19**, 305(1951).
4) Watarai, Itadani : Vitamins (Japan), 8, 331(1955).

the mycelium and adenosine in the broth. This seems to offer a glimpse of the formation mechanism of FAD the author hitherto pictured.

The evidence by Trufanov[5] of the formation of FAD from riboflavin in the presence of thin slices of the liver, brain, kidney or heart of the rat, and the synthesis by Ito and Ota[6] of FAD from riboflavin and ATP in the presence of the acetone powder of the kidney of the pig and phosphoric acid also appears to affirm the biosynthesis of riboflavin+ATP ⟶ FAD.

To make the relation between FAD and ATP more clear, study was made on their formation in the mycelium. The mycelium was extracted with hot water, the extract was subjected to ionophoresis, and the spots of FAD and ATP were detected under ultraviolet rays. Each spot was cut out, extracted with water, and the FAD in the extract was determined from its extinction at 450 mμ and ATP from its extinction at 260 mμ. The result of one of the experiments is shown in Fig. 3. This figure also seems to show the soundness of the author's views on the biosynthetic mechanism of FAD in the mycelium of *E. ashbyii*.

The author is grateful to Mr. Juzo Kaneko for his performance of the culture and to Mr. Yoichi Sawa and Miss Mitsuko Asai for their cooperation in the present work.

Experimental

I. Increase of the Mycelium and Change of pH of the Broth during Culture—A 72-hour seed culture of *E. ashbyii* was cultivated on 30 L. of a nutrient medium consisting of polypeptone, glucose, $MgSO_4$, KH_2PO_4, NaCl, and soy-bean oil. Three or four liters of the culture was separated as a sample at 20, 30, 40, 51, 66, 72, and 90 hours after the start of the culture and numbered from 1 to 7, and each of them was divided into the mycelium and broth by filtration. The weight of mycelium (g./L.) in Table I means the amount of the wet mycelium in 1 L. of the culture. As is seen from Table I, the pH of the broth changed from acidic to neutral as the culture proceeded.

Taele I.

Sample No.	Duration of culture (hr.)	Amt. of mycelium in culture (g./L,)	pH of broth
1	20	15	6.4
2	30	27.5	6.3
3	40	30.7	6.1
4	51	34	6.0
5	66	33	6.2
6	72	33	6.3
7	90	29	7.1

II. Paper Chromatography of Broth—The above broth was applied as such on a paper strip and developed with BuOH•EtOH•H_2O (50 : 15 : 35) or with 5% Na_2HPO_4 solution, and the resulting chromatogram was observed under ultraviolet light. Since, however, no clear spots were detected owing to too small a content of the substances, the broth was concentrated *in vacuo* to 1/10 its volume and processed as above to give the result shown in Table II. The paper was Toyo Roshi No. 5B. The numbers in Table II correspond to those in Table I.

III. Paper Chromatography of the Mycelium—(i) Paper chromatography of pyridine-methanol extract: Five grams of the mycelium was extracted with 15 cc. of pyridine-MeOH (1:1) at 80° for 10 mins., and the filtered extract was developed with solvents of various systems. However, as the number of the detected spots was smaller than in the case of the aqueous extract, description of the result is omitted here.

(ii) Paper chromatography of components of the aqueous extract which transferred to phenol: Fifteen grams of the mycelium was extracted twice with 100 cc. of hot water (80°) and the extract, after addition of ca. 100 g. of $(NH_4)_2SO_4$, was shaken with 20 cc. of phenol. The phenol layer was shaken with 100 cc. of ether and 5 cc. of water, and the aqueous layer was subjected to paper chromatography. In this case, too, the number of the detected spots was small, as compared with the case in which the aqueous extract was developed as such.

5) A. V. Trufanov: Biokhimiya, **7**, 188 (1942).
6) F. Itō, K. Ōta: Vitamins (Japan), **3**, 27 (1950).

Table II. Rf Values of the Bands in the Chromatogram of Broth

Sample No.	Detection	Developing Solvent: BuOH·EtOH·H_2O (50 : 15 : 35)							Developing Solvent: 5% Na_2HPO_4 Soln.						Electrophoresis: Acetate buffer (u=0.05 pH=5.3) 300 V. 3 hr.			
1	U. V.					0.35 Y+	0.45 Ab+	0.00 ?		0.38 Y+					(−) 2.5 B‖	(−) 1.5 Y+		
	P	0.15																
2	U. V.				0.22 ?	0.35 Y‖		0.00 ?		0.38 Y‖					(−) 2.5 B+	(−) 1.5 Y+		
	P	0.15																
3	U. V.			0.20 G+	0.23 V+	0.35 Y‖		0.00 ?		0.38 Y‖		0.58 ?			(−) 2.5 G±	(−) 1.5 Y‖	(+) 5.5 V+	
	P	0.15																
4	U. V.	0.00 ?		0.20 G+	0.23 V+	0.35 Y‖		0.00 ?		0.38 Y‖		0.58 ?	0.68 G		(−) 2.5 G+	(−) 1.5 Y‖	(+) 5.5 V+	
	P	0.15																
5	U. V.	0.00 ?		0.20 G+	0.23 V+	0.35 Y‖		0.00 ?	0.07 ?	0.38 Y‖	0.44 Y±	0.57 V+	0.67 G+		(−) 2.5 G+	(−) 1.5 Y‖	(+) 5.5 V+	(+) 7 ?
	P	0.15																
6	U. V.	0.00 ?	0.10 Y±	0.20 G+	0.23 V+	0.35 Y‖		0.00 ?	0.07 ?	0.38 Y‖	0.44 Y±	0.57 V+	0.67 G+		(−) 2.5 G+	(−) 1.5 Y‖	(+) 5.5 V+	(+) 7 Y±
	P	0.15																
7	U. V.	0.00 ?	0.10 Y±	0.20 G+	0.23 V+	0.35 Y‖		0.00 ?	0.07 ?	0.38 Y‖	0.44 Y±	0.57 V+	0.67 G+		(−) 2.5 G‖	(−) 1.5 Y‖	(+) 5.5 V‖	(+) 7 Y±
	P	0.01	0.10	0.15														

U. V.=Observation under ultraviolet light.
P=Positive phosphorus reaction by molybdic acid reagent.
Ab=Absorption of ultraviolet light (2537Å).
Y=Yellow fluorescence. V=Violet fluorescence.
G=Green fluorescence. B=Blue fluorescence.
± + ‖=Intensity of fluorescence.

TABLE III. Rf Values of the Bands in the Chromatogram of the Aqueous Extract of Mycelium

Sample No.	Detection	Developing Solvent: BuOH·EtOH·H_2O (50 : 15 : 35)								Developing Solvent: 5% Na_2HPO_4 Soln.								Electrophoresis: Acetate buffer (u=0.05 pH=5.3) 300 V. 3 hrs.				
1	U. V.	0.01~0.05 Ab‖				0.20 G?		0.33 Y‖	0.43 Ab+	0.00~ ?		0.38 Y‖	0.45 ?					(−)1.5 Y±				(+)10 Ab
	P	0.01																				(+)10
2	U. V.	0.01 Ab+		0.10 Y±		0.20 G+	0.23 V+	0.33 Y‖		0.00~ ?		0.38 Y‖	0.46 Y±.	0.58 V+	0.69 G+	(−)2.0 G	(−)1.5 Y	(+)5.5 V	(+)7 Y	(+)10 Ab
	P	0.01		0.10	0.15																	(+)10
3	U. V.	0.02 Ab+	0,05 B?	0.10 Y+		0.20 G+	0.23 V+	0.33 Y‖		0.00~ ?		0.38~ Y‖	0.46 Y+	0.58 V+	0.68 G+	(−)2.5 G	(−)1.5 Y	(+)5.5 V	(+)7 Y	(+)10 Ab
	P	0.02		0·10	0.15																	(+)10
4	U. V.	0.02 Ab+	0.05 B?	0.10 Y+		0.20 G+	0.25 V+	0.35 Y‖		0.00~ ±		0.38~ Y‖	0.46 Y+	0.58 V+	0.68 G+	(−)2.5 G	(−)1.5 Y	(+)5.5 V	(+)7 Y	(+)10 Ab
	P	0.02		0.10	0.15																	(+)10
5	U. V.	0.01 Ab+	0.05 B?	0.10 Y+		0.22 G+	0.25 V+	0.35 Y‖	0.60 B?	0.00 ±	0.05 B±	0.38~ Y‖	0.46 Y+	0.58 V+	0.68 G+	(−)2.5 G	(−)1.5 Y	(+)5.5 V	(+)7 Y	(+)10 Ab
	P	0.01		0.10	0.15																	(+)10
6	U. V.	0.01 Ab+	0.05 B?	0.10 Y+		0.22 G+	0.25 V+	0.35 Y‖	0.60 B?	0.00 ±	0.05 B±	0.38~ Y‖	0.46 Y+	0.58 V+	0.68 G+	(−)2.5 G	(−)1.5 Y	(+)5.5 V	(+)7 Y	(+)10 Ab
	P	0.01		0.10	0.15																	(+)10
7	U. V.	0.01 Ab+	0.05 B+	0.10 Y+		0.22 G+	0.25 V+	0.35 Y‖	0.60 B?	0.00 ±	0.05 B±	0.38~ Y‖	0.46 Y+	0.58 V+	0.68 G+	(−)2.5 G	(−)1.5 Y	(+)5.5 V	(+)7 Y	(+)10 Ab
	P	0.01		0.10	0.15																	(+)10

(iii) Paper chromatography of the aqueous extract: Five grams of the mycelium was extracted with 10 cc. of distilled water at 80° for 10 mins., and the extract, as such or after filtration if riboflavin separated, was developed with several solvents, to give the results shown in Table III. The remarks in Table III have the same significance as those in Table II.

(iv) Paper chromatography of authentic samples for control: Authentic samples of riboflavin, FAD, ATP, AMP, FMN, and adenosine were subjected to paper chromatography under the same conditions as above. The V and G in Table IV represent unidentified substances, but their Rf values were obtained by separating them in somewhat large amounts on a large sheet of filter paper and developing the aqueous extracts of the spots with various solvents.

Table IV.

Sample \ Developing Solvent	BuOH·EtOH·H_2O (50 : 15 : 35)	Na_2HPO_4 (5% Soln.)	Electrophoresis Acetate buffer pH 5.3
Riboflavin	0.35	0.38	(−) 1.0~(−) 1.5
FMN	0.28	0.58	(+) 2.0~(+) 2.5
FAD	0.10	0.45	(+) 6 ~(+) 7
ATP	0.02	0.83	(+)10 ~(+)11
AMP	0.15	0.71	(+) 2.0~(+) 2.5
Adenosine	0.43	0.52	(−) 1.5~(−) 2.0
V	0.25	0.58	(+) 4 ~(+) 5
G	0.22	0.68	(−) 1.5~(−) 2.0

IV. Evidence of ATP in the Aqueous Extract of the Mycelium—As stated in the general description, the aqueous extract of the mycelium was developed with BuOH·EtOH·H_2O on a large sheet of filter paper, the spot with Rf 0.00~0.02 which absorbed ultraviolet light was cut out and extracted with water, and extinction of the extract was measured with the Beckman spectrophotometer to give the result in Fig. 1. As a result, it was found that the ultraviolet spectrum was in close agreement with that of ATP given in the literature.

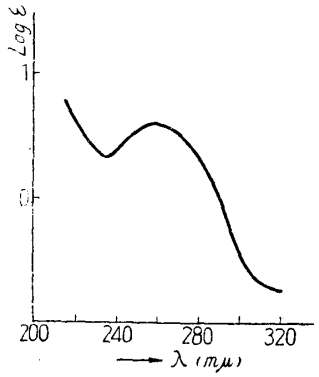

Fig. 1.

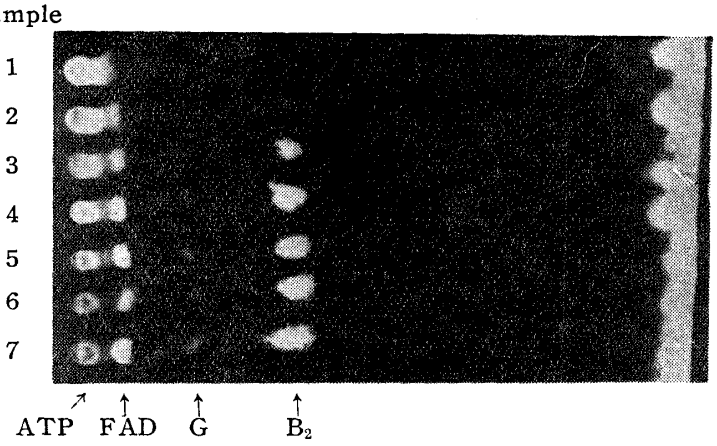

Fig. 2.

V. Weight Increase and Decrease of Riboflavin, FAD, and ATP in the Mycelium—(i) Qualitative test: Aqueous extracts of various samples of the mycelium were developed with BuOH·EtOH·H_2O and the resulting chromatograms were printed on a sensitized paper with ultraviolet light of 2537Å, when the spots hardly pervious to the light emerged on the paper as white spots. From the picture, increase or decrease of the afore-mentioned substances was easily observed.

(ii) Quantitative test: Five grams of the mycelium was extracted with 10 cc. of water in a test tube at 80° for 15 mins., and 0.1 cc. of the filtered extract was applied in a line on a paper strip 8 × 45 cm. and subjected to paper ionophoresis for 3 hours (both electrolytes, pH 5.3 acetate buffer of 0.05 ionic strength; 300 V.)

The spots of FAD and ATP detected under ultraviolet light of 2537Å were cut out, and the former was extracted with 5 cc. of water at 60° for 15 mins. and extinction at 450 mμ of the extract was measured with the Beckman spectrophotometer, while the latter was extracted with 10 cc. of water at 60° for 15 mins. and extinction at 260 mμ of the extract was measured likewise. Since pure FAD and ATP show the same pherograms with those of the corresponding products and extinctions of 100 γ/cc.=1.435 and 476.2 γ/cc.=15.9, respectively, the amount of both compounds in the mycelium were calculated from the E values obtained and further into the corresponding amounts in the mycelium in 1 L. of the culture.

When the content of FAD was too small to measure its extinction, 2 cc. of the extract of the FAD spot was heated with 2 cc. of 0.5 *N* HCl at 80° for 15 mins. to liberate riboflavin. The mixture was neutralized with *N* NaOH using phenolphthalein as the indicator and after addition of 3 cc. of 10% AcOH, diluted with water to 10 cc., and the fluorescence of the free riboflavin was measured. On the other hand, as a control 2 cc. of 2 γ/cc. solution of pure riboflavin was treated as above, and from both fluorescence values thus obtained the amount of riboflavin in the former test was calculated and further converted into the amount of FAD by multiplying the value of riboflavin by 785.566(FAD)/376.36(riboflavin)=2.08 (cf. Table V and Fig. 3).

Table V.

Sample / Time: Duration of Culture (hr.)	ATP: Amt. of ATP in wet mycelium (mg./g.)	ATP: Amt. of ATP in culture (mg./L.)	FAD: Amt. of FAD in wet mycelium (mg./g.)	FAD: Amt. of FAD in culture (mg./L.)
20	1.92	28.8	0.052	0.785
30	3.24	89	0.168	4.6
40	3.68	113	0.910	28
51	3.30	112	1.230	41.5
66	2.94	97	1.990	65.5
72	2.64	87	2.230	73.5
90	2.16	62.5	2.650	77

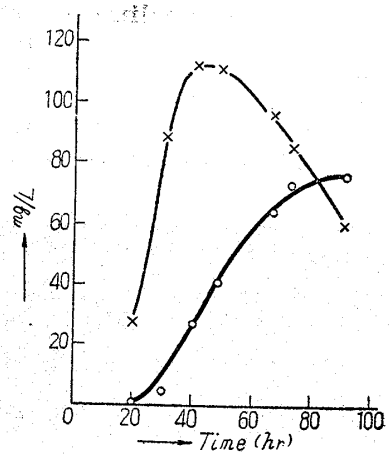

Fig. 3.

○——○——○ FAD

×——×——× ATP

Summary

Culture samples collected at regular intervals during the tank culture of *Eremothecium ashbyii* were separated into the mycelium and broth. The mycelium was extracted with various solvents and the extracts were examined by paper chromatography and paper electrophoresis. The broth, as such or after concentration, was also investigated by the same methods. Development with the solvent system of butanol–ethanol–water afforded detection of the greatest number of substances in the mycelium and gave the best separation. From the spots fixed by various methods it was found that adenosine was formed besides riboflavin and FAD, in the early stage of the culture and then ATP was produced gradually. Despite many experiments FMN was never detected.

On the other hand, formation process of ATP and FAD in the mycelium was also observed, and the conclusion was reached that in the culture of *E. ashbyii* FAD was produced from riboflavin and ATP.

(Received August 13, 1955)