und an Hand der spektrographischen Untersuchung der Des-N-base sowie durch die Zinkstaubdestillation der Des-N-Base sichergestellt.

(Eingegangen am 16. Juni, 1955)

74. Toru Masuda, Yoichi Sawa, and Mitsuko Asai: Application of Chromatography. XXVII. On the Formation of FAD in the Culture of *Eremothecium ashbyii*.*

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The authors[1,2] previously obtained flavin-adenine dinucleotide (FAD) of high purity by extracting the mycelium of *Eremothecium ashbyii* and purifying the resulting crude FAD through its reduced form. In the present study relationship of FAD to riboflavin (vitamin B_2), another important product of *Eremothecium ashbyii*, in the culture of the microörganism was investigated.

First of all, simultaneous determination of free riboflavin and conjugated riboflavin was studied. In this regard, there have so far been reported three methods; one is that of Burch, Bessy, and Lowry,[3] and of Bessy, Lowry and Love,[4] which utilizes fluorescence method, another is that of Yagi,[5] which is a combination of lumiflavin method and paper chromatography, and the third is that of Peel,[6] which is a combination of three processes, namely adsorption by Florisil, paper ionophoresis, and measurement of extinction. However, some of them lack in accuracy and some are too time-consuming, which prompted the authors to develop a more reliable and more rapidly practicable method. In this case, foremost was the method for determining riboflavin by Ariyama and Hoshino.[7]

They treated a sample containing riboflavin with potassium permanganate in an acetic acidic medium to oxidize substances other than riboflavin and, after decolorizing the excess permanganate, determined the riboflavin colorimetrically under monochromatic light of 445 mμ.

In the authors' hands, experiments were performed as follows: When the material of the determination was the culture broth it was used as such, and when the mycelium, it was extracted with a 1 : 1 mixture of pyridine and methanol to prepare a sample. The total of riboflavin in the sample thus obtained was determined by the method of Ariyama and Hoshino. On the other hand, the same sample was completely freed from free riboflavin by shaking with benzyl alcohol and FAD in it was determined by the same method. The difference between these two values accounts for the amount of the free riboflavin. After repeated experiments, the authors became confident that this method is reproducible and practicable with reasonable rapidity.

Next, mention is made of experiments conducted on the samples collected at various stages of the culture of *Eremothecium ashbyii* in peptone containing nutrient medium in a 50-L. tank. Needless to say, the result of a culture varies with the conditions

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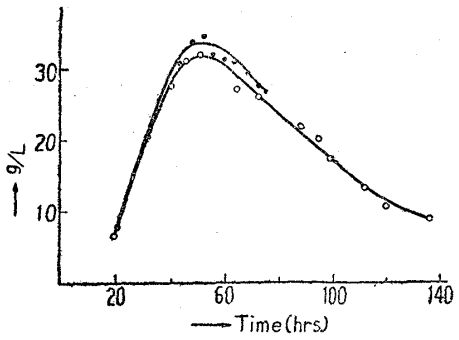

Fig. 1. Amount of the Mycelium

•–•–• First experiment

o–o–o Second experiment

employed. In the present experiments, increase in the weight of the mycelium reached maximum 50~60 hours after start of the culture (Fig. 1). During this period, a part of the culture was collected at definite intervals and separated into the mycelium and the culture broth. Riboflavin and FAD in the broth were determined by the method mentioned before (Figs. 2 and 3). In these figures the amount of each component is shown converted into the corresponding amount in 1 L. of the culture liquid for convenience.

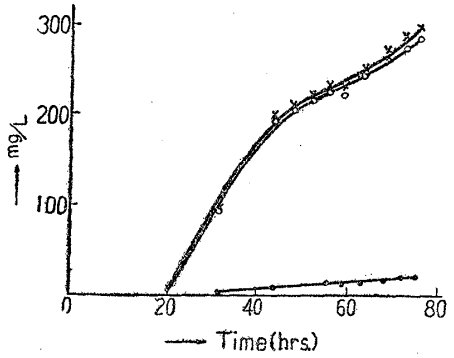

Fig. 2. Component of Broth in the First Experiment

•–•–• FAD

x–x–x Total riboflavin

o–o–o Riboflavin

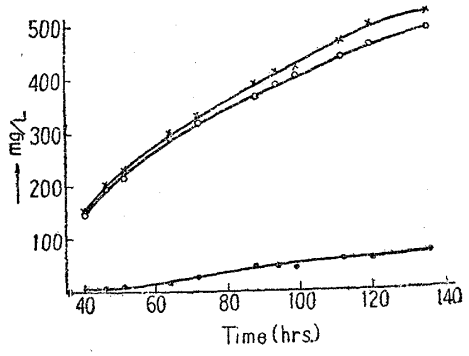

Fig. 3. Component of Broth in the Second Experiment

•–•–• FAD

x–x–x Total riboflavin

o–o–o Riboflavin

As the figures show, the total riboflavin in the broth suddenly increased in weight from about 20 hours after the start of the culture and showed an upward trend even after the mycelium began decreasing in weight. However, in all the experiments, not to speak of the two cases shown in the figures, there was found no maximum within the observation period. As for the FAD in the broth, it occurred gradually from

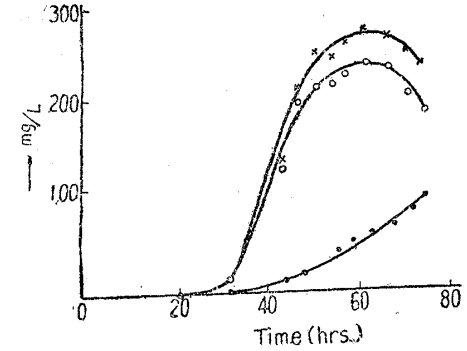

Fig. 4. Component of the Mycelium in the First Experiment

•–•–• FAD

x–x–x Total riboflavin

o–o–o Riboflavin

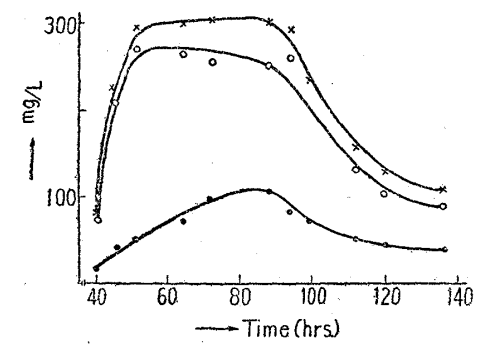

Fig. 5. Component of the Mycelium in the Second Experiment

•–•–• FAD

x–x–x Total riboflavin

o–o–o Riboflavin

about 30 hours after the start of the culture and also showed no peak.

Contrary to this, relation between formations of riboflavin and FAD in the mycelium showed an interesting contrast (Figs. 4 and 5). As is evident from these figures the amount of riboflavin in the mycelium showed a sort of maximum in all the experiments, and though FAD in the mycelium also showed a peak, it occurred about 40 hours later than riboflavin in the case of Fig. 5.

Although the large-scale experiment is yet small in number, the result of the culture may be summed up as follows:

Free riboflavin appearing in the broth in a comparatively early stage of culture, suddenly increases in weight and shows no indication of decreasing even after the mycelium begins autolysis, whereas FAD in the broth gradually increases in weight after the mycelium starts autolysis, though the amount is very small as a whole. On the contrary, riboflavin and FAD in the mycelium increase in proportion in weight as the mycelium increases and decrease in proportion as the autolysis of the mycelium proceeds, each showing a maximum. However, while riboflavin keeps a definite level during 50 to 80 hours after the start of the culture, FAD is formed gradually and shows a high content even at the time the autolysis of the mycelium begins, gradually decreasing thereafter with riboflavin.

Simultaneously with the above determinations the authors also investigated the broth and the mycelium at various stages of the culture by paper partition chromatography to make clear the mechanism of formation of FAD. Since, however, there remain some problems to be solved, the detailed results will be reported at a later opportunity.

The authors thank Mr. Juzo Kaneko for his carrying out the tank culture.

Experimental

Increase in Weight of the Mycelium during the Culture of *Eremothecium ashbyii*—A 72-hr. seed culture of *Eremothecium ashbyii* was cultivated on a peptone medium in a 50-L. tank. After 21 hrs., 1 L. of the culture was collected at definite intervals and filtered by suction. The weight of the wet mycelium was taken as the amount of the mycelium in 1 L. of the culture (g./L.) (Fig. 1).

Determination of Riboflavin and FAD—(i) Culture broth as the sample: Five cc. of the broth, as such or diluted with water to 4 times its volume, was treated with 3 cc. of glacial AcOH and 3 cc. of 4% $KMnO_4$ solution at 0° for 10 mins., and after decolorizing the excess $KMnO_4$ with 3% H_2O_2 it was made up to 20 cc. and its extinction at 445 mμ was measured with the Beckman spectrophotomer, using a 1-cm. cell. On the other hand, about 100 cc. of the same sample was shaken repeatedly (5~6 times) with benzyl alcohol until the benzyl alcohol layer became colorless. The aqueous layer was washed with ether to eliminate the remaining benzyl alcohol, and after driving off the ether by warming, 2.5~5.0 cc. of it was treated as before and the extinction was measured to find the amount of FAD. Since 100 γ/cc. of riboflavin shows $E^{1\,cm}_{445\,m\mu}=3.10$ and 100 γ/cc. of FAD $E^{1\,cm}_{445\,m\mu}=1.490$, the amounts of riboflavin and FAD in the broth were calculated from the values obtained. The amount corresponding to the difference between both E values shows, of course, the amount of free riboflavin.

(ii) Mycelium as the sample: Five g. of the wet mycelium was extracted 8 or 9 times with a mixture of pyridine and MeOH (1 : 1) at 60°, when the yellow color of the mycelium disappeared. The combined extracts was placed in a graduated flask, made up to 300 cc. with the same solvent, and 2.5~5.0 cc. of the diluted solution was treated with $KMnO_4$ as above and subjected to the extinction measurement. From these values the amount of riboflavin in the mycelium in 1 L. of the culture was calculated. On the other hand, 100~200 cc. of the same pyridine-MeOH extract was evaporated to dryness, the residue was dissolved in water, placed in a graduated flask, made up to 100 cc., and treated according to the FAD determination in the broth.

Progress of the Culture—One liter of culture was collected at definite intervals during the mycelium increasing and decreasing periods set by the method mentioned before and separated into the mycelium and the broth. The values of riboflavin and FAD in the broth and in the mycelium are shown in Tables I and II, which can be rewritten into Figs. 2 and 3, and Figs. 4 and 5, respectively.

Table I.

Sample	Mycelium							Broth		
Time of culture (hr.)	Wt. of wet mycelium (g./L.)	Total riboflavin (γ/g.)	FAD (γ/g.)	Riboflavin (γ/g.)	Total riboflavin (mg./L.)*	FAD (mg./L.)*	Riboflavin (mg./L.)*	Total riboflavin (mg./L.)	FAD (mg./L.)	Riboflavin (mg./L.)
21	7.5	24.6	11.7	18.7	0.2	0.1	0.14	5.1	—	5.1
32	20.4	800	67.3	765.9	16.3	1.4	15.6	96.8	3.8	94.9
44	29.0	4760	420	4550	137.5	12.6	134	204	9.0	199.5
48	33.8	6200	650	5875	228	20.9	198	208	—	208
52	35.9	7440	2040	6420	274	75,0	230	224	6.5	220.8
55.5	30.6	7840	1680	7400	255	51.4	226	236.8	13.6	230.0
59	30.8	8800	2180	7710	276	67.2	238	232	13.0	225.5
63	30.8	9400	2300	8250	295	70.7	254	256	15.5	248.2
68	29.4	9600	2460	8370	282	72.2	246	278.4	18.1	268.4
72	27.7	9100	2840	7680	252	78.6	213	292.8	23.2	293.2
75	27.2	9200	3840	7280	251	105.4	198	302.4	20.8	292.0

* The values were obtained by converting the values in 1 g. of the mycelium into those in the mycelium contained in 1 L. of the culture.

Table II.

Sample	Mycelium							Broth		
Time of culture (hr.)	Wt. of wet mycelium (g./L.)	Total riboflavin (γ/g.)	FAD (γ/g.)	Riboflavin (γ/g.)	Total riboflavin (mg./L.)*	FAD (mg./L.)*	Riboflavin (mg./L.)*	Total riboflavin (mg./L.)	FAD (mg./L.)	Riboflavin (mg./L.)
20	7.0	—	—	—	—	—	—	—	—	—
40	27.6	2992	540	2697	82.8	16.6	74.6	149.6	5.2	147.0
46	31.2	7360	1270	6725	228	39.3	209.1	198.6	6.4	195.4
51	32.0	9200	1610	8395	294	51.5	268.3	220.8	9.0	216.3
64	27.0	11100	2680	9760	299	72.4	263.7	297.6	16.8	289.3
72	26.6	11360	3600	9560	302	95.7	254.2	329.6	27.0	316.1
88	22.5	13320	4800	11120	300	108.0	250.0	384.0	47.2	360.4
94	20.8	14000	4040	11980	291	84.0	259.0	403.2	48.0	379.2
99	17.8	13400	4100	11350	235	73.0	202.0	412.8	44.6	390.5
112	13.4	11700	3840	9780	157	51.3	131.0	459.2	60.6	428.9
120	10.7	12120	4360	9940	129	46.6	106.2	488.0	63.6	455.2
136	9.0	12000	4360	9820	108	39.2	88.3	512.0	69.6	477.2

* Same as above.

Summary

Eremothecium ashbyii was cultivated on a peptone medium by tank culture and increase and decrease in weight of the mycelium were investigated on the samples collected at various stages of the culture. At the same time, amount of riboflavin and FAD in the mycelium and the broth were determined. As a result it was found that the amount of riboflavin in mycelium showed a maximum during 50~90 hours after the start of the culture, but the maximum of FAD occurred several ten hours later than the former. On the other hand, riboflavin in the broth suddenly increased in weight at a comparatively early stage of the culture and showed no sign of decreasing even after the mycelium began autolysis, while FAD in the broth gradually increased in weight after the mycelium began autolysis, though the total amount was very small.

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