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75. Toru Masuda : Application of Chromatography. XXX.* Studies on Tryptophan Metabolism in the Culture of *Eremothecium ashbyii*.

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The author previously reported the isolation of a green fluorescent substance, G compound, from mycelium of *Eremothecium ashbyii*.[1]

In order to purify the substance, the crude product was developed on a column of powdered cellulose with benzyl alcohol and the eluate containing G compound was concentrated. As a result it was found that the first separated yellow crystals were not the desired substance, the mother liquor giving the desired substance.

As will be detailed in the experimental part the yellow crystals were established as those of *l*-3-hydroxykynurenine.

Since the peculiar production of riboflavin by *Er. ashbyii* seemed to have a close relation with the metabolism of amino acids, the author investigated the production of amino acids by the strain. A 42-hour-old peptone culture was divided into the mycelium and the broth. The mycelium was extracted repeatedly with hot water and the broth was evaporated to a suitable concentration to make respective samples, which were developed by two-dimensional method on Toyo filter paper No. 51 with butanol•ethanol•water (50 : 15 : 35), and with 80% phenol to give the results listed in Table I.

Table I.

	Aspartic Acid	Glutamic Acid	Serine	Lysine	Glycine	Threonine
Mycelium	++	+++	+	++	+	+
Broth	++	+++	±	++	?	+

	Alanine	*l*-Hydroxy-kynurenine	Tyrosine	Tryptophan	Histidine	Phenylalanine, leucine, isoleucine
Mycelium	++	+	+	+	++	−
Broth	?	+	+	+	++	++

In this case, besides the spots of the above amino acids, some other unidentifiable spots probably of a peptide were detected from the mycelium, as well as from the broth. Strange enough, however, despite the detection of tryptophan and *l*-hydroxykynurenine, the intermediate kynurenin could never be detected.

The author first studied the formation of tryptophan, *l*-hydroxykynurenine, nicotinic acid, and riboflavin in the mycelium by paper partition chromatography and after their determination, came to the conclusion that the peculiar production of riboflavin by the strain might be due to the abnormal metabolism of tryptophan.

To endorse the conclusion, the above result was compared with that obtained on a pigmentless strain of *Er. ashbyii* which was produced by successive culture of the yellow strain. Although it is well known that the production of riboflavin in such a leuco strain is very poor, the proportional low yield of *l*-hydroxykynurenine seems to support the above conclusion.

Experimental

Separation of *l*-Hydroxykynurenine from the Mycelium of *Er. ashbyii*—A 500-g. portion of the

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1) T. Masuda : This Bulletin 4, 374(1956).

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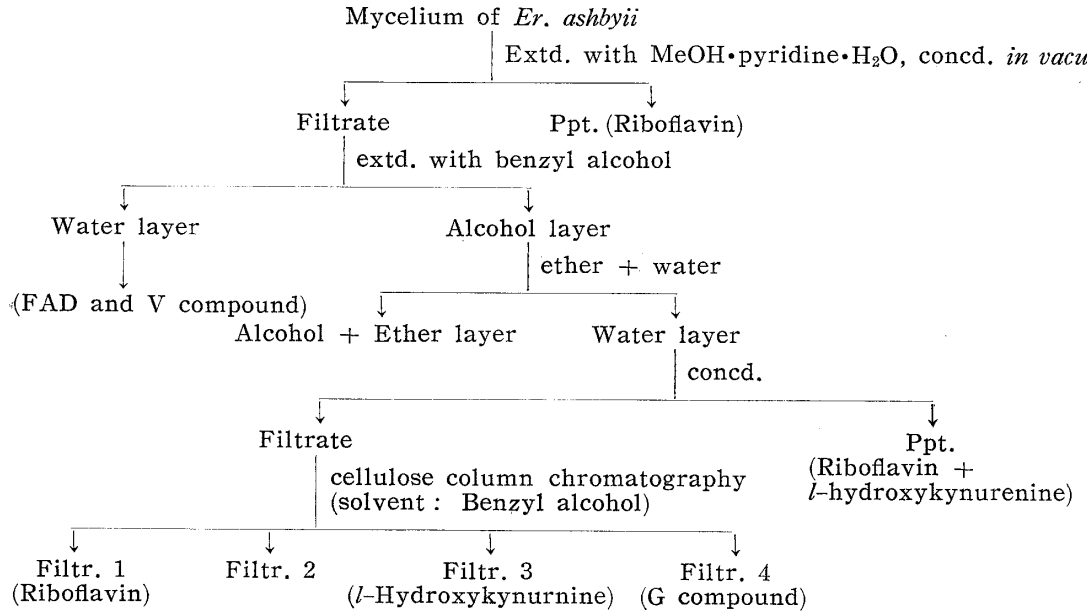
mycelium was extracted twice with 2 L. of 70% MeOH containing 5% of pyridine at 80° for 15 mins. The combined extract was concentrated to about 500 cc. under reduced pressure, the separated riboflavin (VB_2) was filtered off, and the filtrate was shaken with the same volume of benzyl alcohol, when a K-derivative, together with G compound and the remaining VB_2, transferred into the benzyl alcohol layer. The same procedure was repeated 3~4 times, the combined alcohol extract was shaken with the same volume of ether and a little water, and the aqueous layer was concentrated *in vacuo*. The precipitated VB_2 was filtered off, the filtrate was concentrated to ca. 20 cc. *in vacuo*, and after filtering while hot, the filtrate was cooled with ice water, whereupon the K-derivative precipitated with VB_2. The crude product, together with active carbon, was recrystallized from hot water to yellow crystals.

As the mother liquor from the crude product still contained a considerable amount of the K-derivative and some G compound, it was applied on a column of powdered cellulose (150 g.) impregnated with water saturated with benzyl alcohol (45 cc.) and developed with benzyl alcohol. The first 85 cc. of the eluate was set aside and the next 145 cc. was collected and shaken with a large amount of ether and a little water in a separating funnel. When the aqueous layer was concentrated *in vacuo*, an additional crop (ca. 50 mg.) of the yellow crystals was obtained. Of the eluate, the first 20 cc. fraction contained a brown impurity, 20~85 cc. fraction VB_2, and 230~350 cc. fraction the K-derivative and G compound. Therefore, the last fraction was concentrated *in vacuo* and, after filtering off the separated K-derivative, evaporated to dryness, and the residue was recrystallized from EtOH to obtain G compound. The diagram of the separation is shown in Chart 1.

Chart 1. Separation of *l*-Hydroxykynurenine and G Compound



***l*-3-Hydroxykynurenine**—When the afore-mentioned yellow crystals were recrystallized repeatedly from water, the melting point settled at 223~224°(decomp.) and the purified product showed $[\alpha]_D^{20}$ —43.2°. *Anal.* Calcd. for $C_{10}H_{12}O_4N_2$: C, 53.56; H, 5.39; N, 12.49. Found : C, 53.25; H, 5.39; N, 12.28.

The aqueous solution of this substance gives purple coloration with ninhydrin, orange yellow with *p*-dimethylaminobenzaldehyde, blood red with $FeCl_3$, and orange red with diazotized sulfanilic acid. This substance reduces Fehling reagent and is positive to silver mirror test. When left standing in the air the aqueous solution turns brown and deposits a precipitate, and when observed under ultraviolet rays the solution applied on filter paper shows pale bluish white fluorescence.

Comparison between the Extracted *l*-Hydroxykynurenin and Synthetic *dl*-Hydroxykynurenin (m.p. 217~223°(decomp.))—

(1) Rf values on filter paper obtained by development with various solvent systems (Table II).

Table II. Rf Values in Various Solvent Systems

Sample \ Solvent	BuOH•EtOH•H_2O (50:15:35)	BuOH•AcOH•H_2O (4:1:5)	$PhCH_2OH$ •H_2O	Phenol	BuOH•Pyridine•H_2O (4:3:7)
l-3-Hydroxykynurenine	0.37	0.30	0.10	0.57	0.45
Synth. *dl*-3-Hydroxy-kynurenine	0.35 0.37	0.30 0.32	0.11 0.12	0.55 0.57	0.44 0.47

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TABLE IV. Chromatogram of the Mycelium Extract

Duration of culture (hr.)	Method of detection*	Solvent System : Benzyl alcohol satd. with water						Solvent System : BuOH•EtOH•H_2O (50:15:35)							
	F	0.00 B‖					0.20 Y±					0.35 Y±			
20	E					0.13 P ?									0.40 P+
	N	0.00 P‖	0.02 P‖	0.04 BP‖	0.10 BP±	0.13 BP±	0.20 BP+								
	F	0.00 B‖	0.02 V ?		0.10 G ?		0.20 Y+	0.04 B±	0.07 Y±	0.12 B+	0.22 G ?	0.25 V ?	0.35 Y+		
25	E				0.10 O±	0.13 P±								0.37 O±	0.40 P±
	N	0.00 P‖	0.02 P‖	0.04 BP‖	0.10 BP±	0.13 BP±	0.20 BP+								
	F	0.00 Y	0.02 V+		0.10 G+		0.20 Y‖	0.04 B±	0.07 Y+	0.12 B+	0.22 G±	0.25 V±	0.35 Y‖		
42	E				0.10 O‖									0.37 O+	0.40 P+
	N	0.00 P±	0.02 P‖	0.04 BP‖	0.10 BP‖		0.20 BP+								
	F	0.00 Y+	0.02 V+		0.10 G+		0.20 Y‖	0.04 B±	0.07 Y‖	0.12 B ?	0.22 G+	0.25 V+	0.35 V‖		
52	E				0.10 O‖									0.37 O+	0.40 P+
	N	0.00 P±	0.02 P‖	0.04 BP‖	0.10 BP‖		0.20 BP+								
	F	0.00 Y‖	0.02 V+		0.10 G+		0.20 Y‖	0.04 B±	0.07 Y‖	0.12 ?	0.22 G+	0.25 V+	0.35 Y‖		
66	E				0.10 O‖									0.37 O+	0.42 P+
	N	0.00 P±	0.02 P‖	0.04 BP‖	0.10 BP‖	0.18 BP±									
	F	0.00 Y‖	0.02 V+		0.10 G+		0.20 Y‖		0.07 Y‖		0.22 G+	0.25 V+	0.35 Y‖		
76	E				0.10 O‖									0.37 O+	0.42 P+
	N	0.00 P±	0.02 P‖	0.04 BP‖	0.10 BP‖	0.18 BP±									
	F	0.00 Y‖	0.02 V+		0.10 G+		0.20 Y‖		0.07 Y‖		0.22 G+	0.25 V+	0.35 Y‖		
84	E				0.10 O‖									0.38 O+	0.42 P+
	N	0.00 P±	0.02 P‖	0.04 BP‖	0.10 BP‖		0.20 BP‖								

* F : Fluorescence, E : Reaction with Ehlrich's reagent, N : Ninhydrin Reaction.
Color of Fluorescence : B=blue, Y=yellow, V=violet, G=green.
Color of Reactions : O=orange, P=purple, Bp=bluish purple, Rp=reddish purple. ‖, ‖, +, ±, ? =intensity of color.

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TABLE V. Chromatogram of the Concentrated Broth

Duration of culture (hr.)	Method of detection*	Solvent System : Benzyl alcohol satd. with water								Solvent System : BuOH•EtOH•H_2O (50:15:35)										
20	F	0.00 B‖		0.05 B‖						0.05~0.20 B+		0.35 Y ?		0.40 B ?						
	E				0.10 O ?								0.37 O ?	0.40 P +						
	N	0.00 RP‖	0.02 RP‖	0.04 BP‖	0.10 BP+	0.13 BP+	0.20 BP‖			0.05~0.20 RP‖			0.37 P +	0.40 BR‖	0.45 BP‖	0.49 BP‖				
25	F	0.00 B‖		0.05 B‖						0.05~0.20 B+		0.35 Y ±		0.40 B ?						
	E				0.10 O ±								0.37 O ±	0.40 P ±						
	N	0.00 RP‖	0.02 RP‖	0.04 BP‖	0.10 BP+	0.13 BP	0.20 BP‖			0.05~0.20 RP‖			0.37 P +	0.40 BP‖	0.45 BP‖	0.49 BP‖				
42	F	0.00 B‖	0.02 BV+		0.10 B ?		0.20 Y +		0.50 V +	0.05 B+		0.35 Y+		0.40 B ±			0.60 B ±	0.70 V +	0.80 ?	
	E				0.10 O+								0.37 O+							
	N	0.00 RP‖	0.02 RP‖		0.10 BP‖		0.20 BP‖			0.05~0.20 RP			0.37 P‖		0.46 BP‖					
52	F	0.00 BY‖	0.02		0.10 G ±			0.23 Y‖	0.50 V +	0.05 B+	0.08 Y ±		0.35 Y+		0.40 B ±			0.60 B ±	0.70 V +	0.80 ?
	E				0.10 O+								0.37 O+							
	N	0.00 RP‖	0.02 RP‖		0.10 BP‖		0.20 BP‖			0.05~0.20 RP‖			0.37 P‖		0.45 BP+	0.48 BP‖				
66	F	0.00 Y +	0.02 V +		0.10 G +			0.23 Y‖	0.50 V +	0.03 B+	0.08 Y+	0.20 G ?	0.23 V ?	0.35 Y‖	0.40 B ±			0.60 B ±	0.07 V +	0.80 ?
	E				0.10 O+									0.37 O+						
	N	0.00 RP‖	0.02 RP‖		0.10 BP‖		0.20 BP+			0.05~0.20 RP‖				0.37 P‖	0.45 BP+	0.48 BP‖				
76	F	0.00 Y +	0.02 V +		0.10 G +			0.23 Y‖	0.50 V +	0.02 B+	0.08 Y+	0.20 G ±	0.23 V ±	0.35 Y+	0.40 B ±			0.60 B ±	0.70 V +	0.80 ?
	E				0.10 O+									0.37 O+						
	N	0.00 RP‖	0.02 RP‖		0.10 BP‖		0.20 BP+			0.05~0.20 RP‖				0.37 P‖	0.45 BP ±	0.48 BP‖				
84	F	0.00 Y +	0.02 V +		0.10 G +			0.23 Y‖	0.50 V +	0.03 B+	0.08 Y+	0.20 G+	0.23 V+	0.35 Y+	0.40 B ±			0.60 B ±	0.70 V +	0.80 ?
	E				0.10 O+									0.37 O+						
	N	0.00 BP‖	0.02 BP‖	0.04 BP‖	0.10 BP‖		0.20 BP‖			0.02~0.20 RP‖				0.37 P +	0.40 BP‖	0.46 BP+	0.50 BP‖	0.53 BP‖		

* Same as in the Table IV.

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(2) Each of the samples was hydrolyzed with 1*N* NaOH and the hydrolyzate was developed with various solvent systems. The Rf values of the spots detected with *p*-dimethylaminobenzaldehyde are shown in Table III.

(3) Comparison of the ultraviolet spectra of the extracted *l*-hydroxykynurenin and synthetic *dl*-hydroxykynurenine (Fig. 1).

Table III. Rf Values of Hydrolyzed Products

Sample \ Solvent	$BuOH \cdot H_2O$	$BuOH \cdot EtOH \cdot H_2O$ (50:15:35)	$BuOH \cdot AcOH \cdot H_2O$ (4:1:5)
from extd. *l*-3-Hydroxyknurenine	0.80	0.85	0.83
from synthd. *dl*-3-Hydroxykynurenine	0.80	0.85	0.83

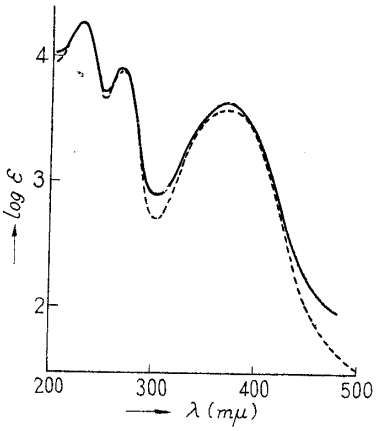

Fig. 1.

——— Extracted *l*-hydroxykynurenine

····· Synthetic *dl*-hydroxykynurenine

Quantitative Change of Tryptophan and *l*-Hydroxykynurenine in the Culture of *Er. ashbyii*— A 72-hr.-old seed culture was cultivated on a peptone medium, 1~2 L. of the culture was collected at definite intervals, and they were respectively divided into the mycelium and broth. A 5-g. portion of the wet mycelium was extraced with two 50-cc. portions of hot water and the extract was concentrated to 5 cc. The broth was concentrated to 1/10 the original volume. About 0.03 cc. of each of the samples thus obtained was applied on Toyo filter paper No. 5B and developed with benzyl alcohol saturated with water as well as with BuOH·EtOH·water in a dark room. The chromatograms, after being observed under ultraviolet rays, were colored with *p*-dimethylaminobenzaldehyde and ninhydrin to give the results shown in Tables IV and V. The Rf values of tryptophan and *l*-hydroxykynurenin and their sensitivity to the detecting reagents are shown in Table VI.

Table VI. Rf Values and Sensitivity

	Solvent-System				Sensitivity to	
	$PhCH_2OH$ satd. with H_2O	Rf	$BuOH \cdot EtOH \cdot H_2O$ (50:15:35)	Rf	Ninhydrin γ	Ehrlich's Reagent γ
Tryptophan	0.13		0.40		1	10 P
l-3-Hydroxykynurenine	0.10		0.37		1	5 O

P : Purple O : Orange

Relation between VB_2 and *l*-Hydroxykynurenine in the Culture of *Er. ashbyii*— A definite amount (1~2 L.) of the culture was collected at definite intervals and divided into the mycelium and the broth, and the weight of the former and the volume of the latter were measured. A 5-g. portion of the mycelium was extracted with pyidine·MeOH, and VB_2 in the extract was determined by the method mentioned before.[2] The broth was concentrated to 5 cc. or to 2.5 cc. and subjected to the same determination.

For the determination of *l*-hydroxykynurenine, 5 g. of the mycelium was extracted with hot water, the extract was concentrated to 5 cc., and 0.05 cc. of the concentrated extract was spotted in straight line on the starting position of a paper strip, 4×44 cm., and developed with $BuOH \cdot EtOH \cdot H_2O$ (50:15:35).

On the other hand, standard test was conducted as follows : One mg. of crystalline *l*-hydroxykynurenine was weighed into a measuring flask of 2-cc. capacity and dissolved in water to make a 2-cc. solution, 0.05 cc. of this solution was applied on a filter paper and developed as above.

The location of *l*-hydroxykynurenine in the chromatograms of the sample and the standard

2) T. Masuda, Y. Sawa, M. Asai : This Bulletin, **3**, 375(1955).

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substance was detected by observing them under ultraviolet rays. Each of the bands was cut out and heated for 25 mins. with 1 cc. each of water, pyridine, ascorbic acid solution (0.05%), and ninhydrin reagent (1% aqueous solution) according to the method of Yamagishi *et al.*[3] From their optical density at 570 mμ the corresponding amount of *l*-hydroxykynurenine was calculated.

As the amount of nicotinic acid in the mycelium was very small, it was impossible to separate it by paper chromatography and determine. So the extract was subjected to bioassay with *L. arabinosus* and the substance responding to the assay was taken as nicotinic acid.

The above results are summarized in Tables VII and VIII. Since the quantitative relation among these substances in the mycelium is very important in the present study, it is shown by curves in Fig. 2.

Table VII. Riboflavin, *l*-Hydroxykynurenine, and Nicotinic Acid in the Mycelium

Duration of culture (hr.)	Mycelium g./L.	Riboflavin mg./g.	Riboflavin mg./L.	*l*-hydroxykynurenine γ/g.	*l*-hydroxykynurenine mg./L.	Nicotinic acid γ/g.	Nicotinic acid mg./L.
20	6.3			380	2.4	60	0.38
25	10.1			460	4.6	60	0.60
42	29.0	3.8	110	1000	29.0	180	5.22
52	42.0	6.5	278	1200	50.5	160	5.72
66	35.0	8.8	308	1440	50.5	100	3.50
76	37.0	8.5	314	1880	69.5	100	3.70
84	35.0	8.3	290	2200	77.0	100	3.50

Table VIII. Riboflavin and *l*-Hydroxyknyurenine in the Broth

Duration of culture (hr.)	Riboflavin mg./L.	*l*-Hydroxykynurenine mg./L.
20	—	—
25	2	13
42	134.4	70
52	194.0	91
66	251.0	123
76	280.0	139
84	412.0	155

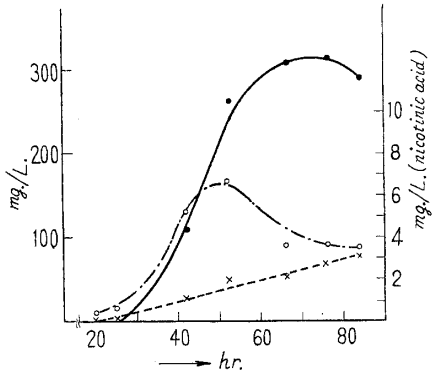

Fig. 2.
•——• Total riboflavin
×——× *l*-Hydroxykynurenine
o-·—·-o Nicotinic acid factor

Formation of VB_2 and *l*-Hydroxykynurenine in the Leuco Strain of *Er. ashbyii*—By the successive culture of *Er. ashbyii* a yellowish white or nearly white strain was obtained and it was cultivated under the same conditions as in the case of the afore-mentioned yellow strain. The mycelium and broth were developed as before to give the chromatograms shown in Table IX. Of the bands thus obtained those of riboflavin and *l*-hydroxykynurenine were processed as before to obtain the results given in Table X.

Discussion

There is no doubt that the yellow crystals, m.p. 223~224° (decomp.), $C_{10}H_{12}O_4N_2$, $[\alpha]_D^{20}$ −43.2°, separated along with G compound is *l*-3-hydroxykynurenine (*l*-HK). This

3) M. Yamagishi, T. Yoshida: J. Pharm. Soc. Japan, **73**, 675(1953); M. Yamagishi: *Ibid.*, **74**, 1004(1954).

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TABLE IX.

Solvent System : BuOH•EtOH•H_2O (50:15:35)

Strain	Duration of culture (hr.)	Method of detection	Mycelium							Broth						
White	42	F		0.07 Y+	0.22 G+	0.25 V+	0.35 Y卄		0.40 Y ?	0.55 B ?	0.00～0.18 B卄		0.35 Y卄		0.40 B ?			0.70 V±
		E						0.37 O±	0.40 V±			0.20 V+		0.37 O+	0.40 V+	0.43 Y+		
	63	F	0.04 B+	0.07 Y+	0.22 G卄	0.25 V卄	0.35 Y卄		0.40 Y±	0.55 B±	0.00～0.15 B-BV卄	0.18 YG±	0.35 Y卄		0.40 B+		0.55 ?	0.70 V卄
		E			0.20 V卄							0.20 V+		0.37 O+	0.40 V+	0.43 Y+		
Yellowish white	42	F	0.04 B+	0.07 Y+	0.22 G+	0.25 V卄	0.35 Y卄		0.40 Y ?	0.55 B ?	0.00～0.15 B-BV卄	0.18 YG±	0.35 Y卄		0.40 B ?		0.55 ?	0.70 V+
		E						0.37 O±	0.40 V±			0.20 V+		0.37 O+	0.40 V+	0.43 Y+		
	63	F	0.04 B+	0.07 Y+	0.22 G卄	0.25 V卄	0.35 Y卄		0.40 Y±	0.55 B ?	0.00～0.15 B-BV卄	0.18 YG±	0.35 Y卄		0.40 B ?		0.55 B±	0.70 V+
		E			0.20 V+			0.37 O±				0.20 V+		0.37 O+	0.40 V+	0.43 Y+		

* Same as in Teble IV.

TABLE X.

Strain \ Duration of culture (hr.)	Mycelium g./L.			Riboflavin γ/g.			1-Hydroxykynurenine γ/g.		
	42	63	88	42	63	88	42	63	88
Yellow	29	35	—	3780	8800	—	1000	1440	—
Yellowish-white	17.8	29	—	307	2590	—	285	312	—
Leuco (I)	15.7	26.4	—	85	750	—	290	160	—
〃 (II)	21	—	40	20	—	200	121	—	110

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substance was isolated for the first time by Kikkawa[4] from silk-worm eggs and named +chromogen by him, and thereafter its structure was established by Butenandt *et al.*[5] This substance is considered to be a metabolite of tryptophan and to take an important position in the hereditary biochemistry of insects, like kynurenine (K) which was discovered by Kotake[6] and clarified by Butenandt.[7]

Hasikins *et al.*[8] evidenced that in the metabolism of tryptophan in *Neurospora*, tryptophan changes into nicotinic acid, and Dalgliesh *et al.*[9] discussed the necessity of riboflavin in the conversion of K to HK in the metabolism of tryptophan.

In the present study tryptophan was found in all samples of the mycelium, but *l*-hydroxykynurenine was detected clearly only after about 40 hours from the start of the culture. These amino acids were detected in the broth as well, but as it was difficult to judge whether they were excreted from the mycelium or orginally formed in the broth, the result was described in the experimental part as a mere reference material (cf. Table V).

The amounts of ribofiavin (VB_2), *l*-hydroxykynurenine (HK), and nicotinic acid were measured at definite intervals during the culture. The results are shown in Table V and Fig. 2. VB_2 was determined by the method reported earlier[2] and *l*-hydroxykynurenine was separated by paper chromatography and determined by the ninhydrin reaction of Yamagishi *et al.*[3] As for nicotinic acid, it was determined directly by bioassay with *Lactobacillus arabinosus* because its amount was too small to be separated by paper chromatography. Therefore, the nicotinic acid thus determined should rather be called nicotinic acid factor.

As is evident from Fig. 2, the increase in the amount of VB_2 was proportional to that of *l*-hydroxykynurenine, but the formation of nicotinic acid had no relation with them. Setting aside the Kikkawa's idea[10] that VB_2 might be derived from K, it seems reasonable to think that tryptophan probably changes into *l*-hydroxykynurenine through K, and that quickly, and the resulting *l*-hydroxykynurenine is gradually excreted out of the mycelium together with its subsequent metabolites, nicotinic acid and VB_2 due to other enzymes.

The metabolism of K → HK is effected by oxidation in the living body, and if in this case the presence of VB_2 is necessary as Dalgliesh *et al.*[9] pointed out, it would be produced in proportion to the amount of the matabolism of K → HK.

The fact may serve as an endorsement to the above consideration, that the production of VB_2 and HK in the leuco strain of *Er. ashbyii* is remakably low, compared with the production in the yellow strain. If the metabolism of tryptophan, especially K → HK, is effected normally, it would proceed at the same level whether in the leuco or yellow strain, irrespective of the production of VB_2, but the fact remains that the metabolism is closely related to the production of VB_2. Further, in the study of the leuco strain, other unidentifiable amino acids giving the color reaction of tryptophan series were detected, and the appearance of G and V compounds was distinct. These facts seem to have an interesting relation with the metabolism of amino acids, but their clarification would require a considerable further study.

The author is grateful to Mr. Siroo Senoh for his generosity in giving the valuable sample of

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6) Y. Kotake : Z. physiol. Chem, **243**, 237(1938).
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synthetic *dl*-3-hydroxykynurenine, to Mr. Yutaka Shiraishi for his cooperation in the culture, to Mr. Takeji Hasegawa for the separation of strains, and to Mr. Minoru Goto for carrying out the bioassay of nicotinic acid factor. Thanks are due also to Mr. Yoichi Sawa and Mrs. Mitsuko Asai who helped the author througout the present work.

Summary

In isolating a green fluorescent substance (G compound) from the mycelium of *Er. ashbyii*, yellow crystals, m.p. 223~224°(decomp.), $[\alpha]_D^{20}$ −43.2°, were also separated and this substance was established as *l*-3-hydroxykynurenine. Study of the mycelium and broth of *Er. ashbyii* by two-dimentional paper chromatography detected only tryptophan and *l*-hydroxykynurenine as amino acid of tryptophan series, kynurenine being never detected. The two compounds were found from a comparatively early stage of the culture and they were readily distinguished by the color reaction with *p*-dimethylaminobenzaldehyde (Ehrlich reagent).

Formation of riboflavin, *l*-hydroxykynurenine, and nicotinic acid was also investigated at intervals during the culture and it was found that riboflavin increased in proportion to the increase of *l*-hydroxykynurenine, but the formation of nicotinic acid factor seemed to have no quantitative relation to them.

A leuco strain of *Er. ashbyii* obtained by successive culture of the yellow strain was also cultivated on the same medium and it was found that in addition to a slightly smaller production of the mycelium, the production of riboflavin and *l*-hydroxykynurenine was extremely low, compared with their production in the yellow strain. From such a fact it was presumed that the metabolism of kynurenine to *l*-hydroxykynurenine in the yellow strain is very active and that this oxidative process in the living body requires a large quantity of riboflavin.

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