[Chem. Pharm. Bull. 17(6)1175—1182(1969)]

UDC 577.15 : 576.882.821.2.095.3

Biochemical Studies on the Metabolism of *Penicillium islandicum* Sopp. II.[1] Acetyl-CoA Carboxylase and Related Enzymes

Yoshio Ueno[2a] and Takashi Tatsuno[2b]

Laboratory of Chemical Microbiology, Faculty of Pharmaceutical Sciences, Science Universtiy of Tokyo[2a] and the Insititute of Physical and Chemical Research[2b]

(Received September 11, 1968)

In order to disclose the enzymatic mechanism of the biosynthesis of anthraquinoid pigment of *P. islandicum* Sopp. an investigation was made on acetyl-CoA carboxylase and related enzymes with the following results. 1) With the stationary culture of *P. islandicum* Sopp. on Czapek medium, luteoskyrin and other quinoid pigments were produced in the late stage of fungal growth; 2) Sodium arsenate, monoiodoacetate, 2,4-dinitrophenol and monofluoroacetate inhibited the fungal growth and the pigment formation, and malonate stimulated the latter. 3) With the crude and partially purified enzyme preparations of mechanically-disruptured mycelium, the formation of malonyl-CoA, the first intermediate of anthraquinoid pigments, was demonstrated. 4) With the crude extract of the mycelium, malonyl-CoA was decarboxylated to yield acetyl-CoA. 5) With the supernatant of the crude extract, the carboxyl group of pyruvate was exchanged with $^{14}CO_2$. 6) In the early phase of the fungal growth, activities of aldolase and citrate synthetase were relatively high, and in the later stage activities of malate dehydrogenase and glucose-6-phosphate dehydrogenase were remarkably high.

Penicillium islandicum Sopp. produces more than dozen quinoid pigments in which luteoskyrin is proved to be a hepatotoxic mycotoxin.[3] With the aid of labeled precursors, it is assumed that the anthraquinoid molecule of pigments is derived *in vivo* by the condensation of acetate and malonate.[4] Experiments carried out with avidin indicated that biotin-containing enzyme, acetyl-CoA carboxylase, catalysed the first step of the synthetic reaction and malonyl-CoA produced was presumably the first intermediate of the pigment biosynthesis.[5]

In the present report, as a step to disclose the enzymatic mechanism of the biosynthesis of pigment *in vitro*, the authors investigated acetyl-CoA carboxylase and related enzymes in the cell-free system of the mycelium, and the results indicated that activities of acetyl-CoA carboxylase, malonyl-CoA decarboxylase and pyruvate dependent fixation of CO_2 were demonstrated in the cell-free extract and that in the early phase of fungal growth aldolase and citrate synthetase were highly active and in the later phase dehydrogenase such as malate dehydrogenase and glucose-6-phosphate dehydrogenase were active.

Materials and Methods

Fungus——Strain E of *P. islandicum* Sopp. which was isolated from "Yellowed Rice" by Dr H. Tsunoda, the Food Research Institute, was used throughout the experiment, and the fungus was stored at 4° after an inoculation on Czapek-agar (sucrose 30 g, $NaNO_3$ 2 g, K_2HPO_4 1 g, $MgSO_4$ 0.5 g, $FeSO_4$ 0.01 g, agar 15 g, in one liter of deionized water) for two weeks at 27°

1) Part I: Y. Ueno, S. Suzuki, I. Ueno and T. Tatsuno, *Chem. Pharm. Bull.* (Tokyo), **12**, 479 (1964).
2) Location: *a*) *12, Ichigaya-funagawara-cho, Shinjuku-ku*, Tokyo; *b*) *4775, Shimoniikura, Yamato-cho, Saitama.*
3) K. Uraguchi, T. Tatsuno, F. Sakai, M. Tsukioka, Y. Sakai, O. Yonemitsu, H. Ito, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiko, *Jap. J. Exp. Med.*, **31**, 19 (1961).
4) S. Gatenbeck, *Acta Chem. Scand.*, **16**, 1053 (1962).
5) Y. Ueno, *J. Bacteriol.*, **94**, 786 (1967).

Culture——Fifty ml of sterile Czapek medium in 200 ml of Ehlenmeyer flask were inoculated with spores. The flask was incubated at 27° and after appropriate intervals of the stationary culture, the mycelium was harvested and washed with deionized water to remove fungal metabolites around the cells.

Preparation of the Cell-Free Extract——Ten g of the mycelium, which were previously cut into a small piece, were suspended in 10 ml of 0.1 M potassium phosphate buffer (pH 7.4)–0.001 M K-EDTA, and after adding of 10 g of acid-washed glass beads (0.5 mm in diameter), were agitated in the cell homogenizer of Merkenschlager, *et al.*[6] for 2 min at 1 to 3°. The fluid was decanted from the beads and the turbid solution adjusted at pH 7.4 with 2 N KOH was centrifuged for 10 min at $3000 \times g$. The turbid brown supernatant was used as the crude extract (S–3), and for some experiments the extract was dialysed against the same buffer for 4 hr in a cold room (S–3D), centrifuged at $10000 \times g$ for 30 min (S–10), or fractionated with ammonium sulfate. All operations were conducted at 4° with precooled materials and equipments.

Measurements of Enzyme Activity——Components of incubation mixtures and condition of the incubation were illustrated in the appropriate Tables and Figures. Incorporation of acetate-1-^{14}C into a lipid fraction and a carboxylation of acetyl-1-^{14}C-CoA were conducted in the presence of supplemented fatty acid synthetase of yeast, and the extraction of labeled lipid was conducted by the method of Numa, *et al.*[7] Malonyl-CoA decarboxylase was measured by the enzymatic determination of the remaining malonyl-CoA in the reaction mixture at the various time of the incubation. After the incubation, 0.1 ml of 2 N H_2SO_4 was added to the mixture to stop the reaction, and malonyl-CoA remained was measured by the decreased amounts of NADPH in the presence of fatty acid synthetase and acetyl-CoA, according to the method of Wakil and Gunguly.[8]

Fixation of radioactive CO_2 was measured by the incorporation of $^{14}CO_2$ in the presence of pyruvate. The assay was conducted at 37° in stoppered 10×100 mm test tubes. After incubation for 30 min, 0.2 ml of 20% H_2SO_4 was added and the remaining $^{14}CO_2$ was completely removed by passing a stream of N_2 gas through the ice-cold mixture for 15 min, or by adding a small piece of solid CO_2 into the mixture. After centrifuged, the supernatant was adjusted to pH 7 and 0.4 ml was mixed with 10 ml of a scintillation solution of Bray[9] to count the radioactivity.

The activity of glucose-6-phosphate dehydrogenase, malate dehydrogenase and aldolase were measured photometrically by observing the change in the optical density at 334 mμ of NADH or NADPH. Citrate synthetase was measured by coupling the synthetase and malate dehydrogenase according to the method of Berg.[10] Pyruvate kinase was measured by coupling the kinase and lactate dehydrogenase according to Bucker and Peleidered.[11]

Chemicals——Fatty acid synthetase was prepared from baker's yeast according to Lynen[12] and the second ammonium sulfate fraction was employed for experiments. Acetyl-CoA and acetyl-1-^{14}C-CoA were synthesized from CoA and acetic anhydride according to Ochoa,[13] and malonyl-CoA was prepared from CoA snd S-malonyl-N-caprylcysteamine according to Eggerer and Lynen.[14] NAD, NADP, their reduced forms, CoA, phosphoenolpyruvate, ATP, ADP, malate dehydrogenase, lactate dehydrogenase, glyceroaldehyde-3-phosphate dehydrogenase and citrate synthetase were the product of Boeringer and Soehne, Mannheim. $Ba^{14}CO_3$ (20.3 mC/mM) and acetate-1-^{14}C (29.0 mC/mM) were purchased from the Radiochemical Centre.

The content of total pigments in the mycelium was determined as followed; the mycelium was dried at 60° overnight, and total pigments were extracted with acetone after removal of the mycelial lipids with *n*-hexane. The amounts of pigments were determined by weighing the residue after evaporation of the solvent. The content of protein in the fungal extract was measured by the method of Lowry, *et al.*[15] "Oxalated silica plates" for a thin-layer chromatography of pigments were prepared from a slurry of Kieselgel G with 0.1 M oxalic acid, and acetone–*n*-hexane–water (6:3:1.5) was used as a developer.

6) M. Merkenschlager, K. Schlossmann and W. Kunz, *Biochem. Z.*, **329**, 332 (1957).
7) S. Numa, M. Matsuhashi and F. Lynen, *Biochem. Z.*, **334**, 203 (1961).
8) S.J. Wakil and J.J. Gunguly, *J. Am. Chem. Soc.*, **81**, 2597 (1959).
9) P. Bray, *Anal. Biochem.*, **1**, 279 (1960).
10) P. Berg, "Methods in Enzymology," Vol. V, ed. by S.P. Colowik and N.O. Kaplan, Academic Press Inc., New York, 1962, p. 466.
11) J. Bucker and G. Peleidered, "Method in Enzymology," Vol. I, ed. by S.P. Colowik and N.O. Kaplan, Academic Press Inc., New York, 1955, p. 435.
12) F. Lynen, "Method in Enzymology," Vol. V, ed. by S.P. Colowik and N.O. Kaplan, Academic Press Inc., New York, 1962, p. 443.
13) S. Ochoa,"Methods in Enzymology," Vol. I, ed. by S.P. Colowik and N.O. Kaplan, Academic Press Inc., New York, 1955, p. 688.
14) H. Eggerer and F. Lynen, *Biochem. Z.*, **335**, 540 (1962).
15) O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Results

Fungal Growth and Pigment Formation

Production of the fungal mycelium and pigment was investigated at appropriate intervals of the cultivation. As shown in Fig. 1, the fungus started to produce a white mycelium after 3 days of the cultivation, and the increase in the mycelial weight was significant during 5—7 days of the culture. While, the increase in total pigments, which was represented by the acetone-soluble materials of the dried mycelium, was remarkable during 8—10 days of the culture. Thin-layer chromatography of the extracted pigments indicated that the relative amount of luteoskyrin, skyrin, islandicin, iridoskyrin and rubroskyrin was scarcely varied during the growth phases of the mycelium.

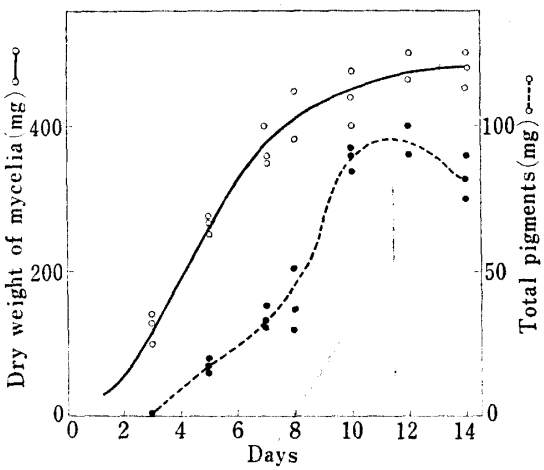

Fig. 1. Daily Changes of Fungal Growth and Pigments Formation

Effects of Metabolic Inhibitors on the Mycelium Growth and the Pigment Formation

Effects of metabolic inhibitors for citric acid cycle and glycolysis on the fungal growth and pigmentation were examined, as shown in Table I. Arsenate, monoiodoacetate, 2,4-dinitrophenol, monofluoroacetate and azide inhibited both the fungal growth and pigment formation at the concentration given in the Table. On the other hand, malonate, an inhibitor of succinate dehydrogenase in many biological systems, had a pronounced stimulatory

Table I. Effects of Metabolic Inhibitors on the Mycelial Growth and Pigment Production

Inhibitor	Concentration M	Mycelium mg	Pigments mg	Pigments %
Sodium arsenate	0	308	47	15.4
	10^{-3}	343	52	13.1
	10^{-2}	0	—	—
Monoiodoacetate	0	320	35	11.1
	10^{-6}	319	32	9.7
	10^{-5}	306	29	9.7
	5.10^{-5}	225	6	2.8
	10^{-4}	209	7	3.2
	10^{-3}	0	—	—
2,4-Dinitrophenol	10^{-5}	264	35	14.2
	10^{-5}	0	—	—
Monofluoroacetate	0	231	19	12.0
	10^{-5}	39	0	—
Sodium azide	10^{-4}	0	—	—
Malonic acid	0	345	43	12.5
	2.10^{-2}	427	97	22.4
	4.10^{-2}	484	99	20.1

The spores of the fungus were inoculated on 200 ml Ehlenmeyer flask which contained 50 ml of Czapek medium, and cultivated at 27° for two weeks. Each value in the Table represented the mean of three flasks.

effect on the pigment formation without influencing the fungal growth at the concentration of 0.02 and 0.04 M.

Aldolase and Citrate Synthetase

As shown in Fig. 2 and 3, the specific activities of aldolase and citrate synthetase in the crude extract (S–3) were highly active in the early phase of the fungal growth and gradually decreased in proportional to the time of cultivation.

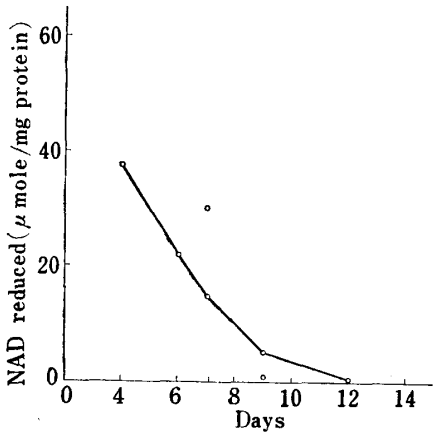

Fig. 2. Activity of Aldolase in the Crude Extract of Mycelium

Reaction mixture contained the following components (in μmoles); Tris-HCl buffer (pH 7.5) 50, hexose-1,6-diphosphate 3.3, glycine 2.6, Na_2HAsO_4 0.5, NAD 0.1, and glyceroaldehyde-3-phosphate dehydrogenase 3 μg, in total volume of 1.0 ml. The mixture was incubated at 25°, and the optical density at 334 mμ was measured.

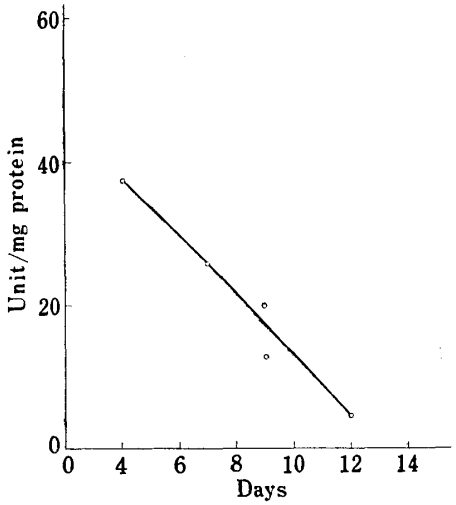

Fig. 3. Activity of Citrate Synthetase in the Crude Extract

Reaction mixture contained the following components (in μmoles); Tris-HCl buffer (pH 8.1) 200, K-malate 12, acetyl-CoA 0.1, NAD 0.3, and malate dehydrogenase 3 μg, in total volume of 1.0 ml. The mixture was incubated at 25°, and the optical density was measured at 334 mμ.

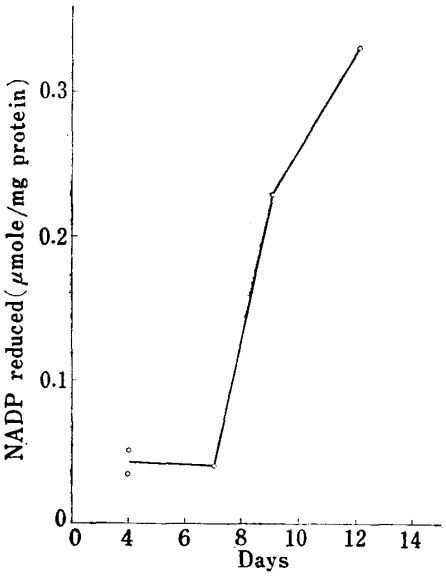

Fig. 4. Activity of Glucose-6-phosphate Dehydrogenase

Reaction mixture contained the following components (in μmoles); glycylglycin (pH 7.5) 25, glucose-6-phosphate 0.5, $MgCl_2$ 20, NADP 0.13. Total volume was 1.0 ml, and the mixture was incubated at 25°.

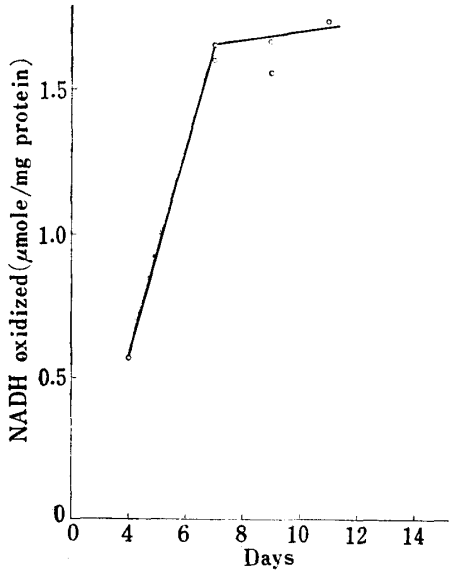

Fig. 5. Activity of Malate Dehydrogenase

Reaction mixture contained the following components (in μmoles); Tris-HCl buffer (pH 7.4) 25, oxaloacetate 2.23, NADP 0.043, in total volume of 1.0 ml. The mixture was incubated at 25°.

Glucose-6-phosphate Dehydrogenase and Malate Dehydrogenase

In contrast to the cases of aldolase and citrate synthetase, the specific acitvities of glucose-6-phosphate and malate dehydrogenase in the crude extract (S–3) were low in the initial phase of the fungal growth and gradually increased in the log phase, as shown in Fig. 4 and 5.

Acetyl-CoA Carboxylase

The activity of acetyl-CoA carboxylase was examined with acetate-1-^{14}C or acetyl-1-^{14}C-CoA as a substrate. In the case of acetate-1-^{14}C, as shown in Table II, the labeled precursor was incorporated into a lipid fraction by the extract of 4 days-old mycelium in the presence of supplemented fatty acid synthetase of yeast. This uptake was reduced when $KHCO_3$, CoA, citrate or ATP was omitted from the complete reaction system, and was increased when the crude extract (S–3) was replaced by the dialysed one (S–3D).

Table II. Incorporation of Acetate-1-^{14}C into Lipid Fraction by the Crude Extract of the 5 Days–old Mycelium

Enzyme	Reaction system	Acetate-1-^{14}C incorporated cpm/mg protein
(a) Crude extract (S-3)	complete	480
(b) Dialysed crude extract (S-3D)	complete	1640
	minus $KHCO_3$	980
	minus G-6-P and G-6-P dehydrogenase	920
	minus CoA	286
	minus citrate	522
	minus ATP	140
	minus fatty acid synthetase	180

The complete system contained the following components (in μmoles unless otherwise specified); potassium phosphate buffer (pH 7.3) 10, $MgCl_2$ 10, $MnCl_2$ 0.03, $KHCO_3$ 5, glucose-6-phosphate 0.5, CoA 0.05, ATP 2, NADP 0.5, NAD 1, cysteine 5, acetate-1-^{14}C 0.5 (5 μC), citrate 5, K-EDTA 5, glucose-6-phosphate dehydrogenase 10 μg and fatty acid synthetase 10 μg. Total volume was 0.6 ml, and the mixture was incubated at 25° for 30 min.

In case of acetyl-1-^{14}C-CoA, as shown in Table III, the activity of carboxylase was not demonstrated in the crude extract (S–3) and its first ammonium sulfate fraction. However, when the dialysed supernatant of 0.8 saturation was refractionated with the same salt, the

Table III. Acetyl-CoA Carboxylase in the 7 Days–old and 12 Days–old Mycelia

Enzyme fraction	Acetyl-1-^{14}C-CoA incorporated cpm/mg protein	
	7 days–old	12 days–old
Crude extract (S-3)	6	8
1st ammonium sulfate fraction		
0.4 saturation	2	6
0.8 saturation	11	14
0.8 saturation supernatant	23	23
2nd ammonium sulfate fraction		
0.4 saturation	—	49
0.8 saturation	8	40
0.8 saturation supernatant	1.760	1.40

Reaction mixture contained the following components (in μmoles unless otherwise specified); potassium phosphate buffer (pH 7.5) 20, ATP 2, $MgCl_2$ 10, Mg.K_2-EDTA 8, NADP 0.2, cysteine 5, $KHCO_3$ 10, acetyl-1-^{14}C-CoA 0.149, serum albumine 0.3 mg, fatty acid synthetase 10 μg and glucose-6-phosphate dehydrogenase 10 μg, in total volume of 0.8 ml. The reaction mixture was incubated at 27° for 30 min.

uptake of acetyl-1-^{14}C-CoA into the lipid fraction was demonstrated only with the supernatant of the second 0.8 saturation in the presence of supplemented fatty acid synthetase. This activity was remarkably higher in 7 days-old mycelium than in 12 days-old one.

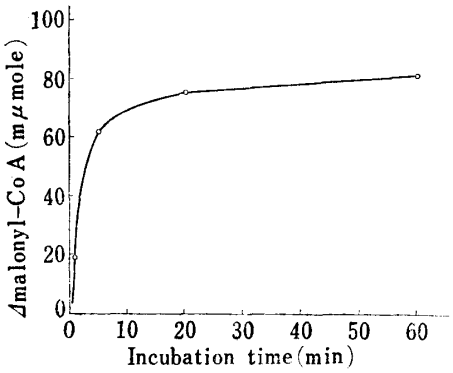

Fig. 6. Activity of Malonyl-CoA Decarboxylase

Reaction mixture contained the following components (in μmoles); Tris-HCl buffer 200, $MgCl_2$ 10, malonyl-CoA 0.1 and the crude extract (S-3) of the 8 days-old mycelium. Total volume was 1.0 ml. At appropriate intervals of the incubation at 37°, 0.1 ml of 2N H_2SO_4 was added to the reaction mixture. After adding 0.4 ml of 1M $KHCO_3$ to the acidified mixture, the malonyl-CoA remained was measured by fatty acid synthetase system as followed; potassium phosphate buffer (pH 7.4) 50 μmoles, NADPH 0.1 μmole, acetyl-CoA 0.04 μmole, serum albumine 0.6 mg, fatty acid synthetase 10 μg and the malonyl-CoA solution, in total volume of 1.0 ml, were incubated at 25°, and the decrease of NADPH was measured at 334 mμ.

Malonyl-CoA Decarboxylase

Decarboxylation of malonyl-CoA was investigated with the crude extract of 8 days-old mycelium, which was in actively-synthesizing phase of the pigments. As shown in Fig. 6, the extract (S-3) decarboxylated rapidly malonyl-CoA to yield acelyl-CoA, and nearly 90% of the added substrate were disappered from the reaction solution after 20 min incubation. The amount of malonyl-CoA disappeared was proportional to that of the extract added, and this reaction did not occured when the crude extract was replaced by the supernatant of the second ammonium sulfate fraction.

Fixation of $^{14}CO_2$

Enzymatic fixation of $^{14}CO_2$ was demonstrated in the extract of the mycelium. As shown in Table IV, the dialysed crude extract (S-3D) catalysed the fixation of $^{14}CO_2$ in the presence of pyruvate, and the addition of ATP and acetyl-CoA to the reaction mixture stimulated the amount of fixed $^{14}CO_2$. The stimulatory effect of supplemented ATP and acetyl-CoA were remarkable when the extract (S-3D) was centrifuged at $10000 \times g$ for 30 min. The fixation did not occured when pyruvate was replaced with oxaloacetic acid or phosphoenolpyruvate.

Table IV. Pyruvate-dependent Fixation of $^{14}CO_2$

Enzyme	Reaction system	$^{14}CO_2$ fixed cpm/mg protein
(a) S-3D	enzyme alone	1220
	plus ATP and pyruvate	2960
	plus ATP, pyruvate and acetyl-CoA	1990
	plus acetyl-CoA	990
	plus acetyl-CoA and pyruvate	804
(b) S-10D	enzyme alone	956
	plus pyruvate	2000
	plus pyruvate and ATP	13500
	plus pyruvate, ATP and acetyl-CoA	16000

The complete system contained the following components (in μmoles); Tris-HCl buffer (pH 7.4) 200, ATP 5, $MgCl_2$ 5, $KH^{14}CO_3$ 0.1 (2 μC), and (a) the dialysed crude extract (S-3D) or (b) the dialysed supernatant (S-10D) of the 6 days-old mycelium. Total volume was 1.0 ml, and the mixture was incubated at 37° for 30 min.

Pyruvate Kinase

The activity of pyruvate kinase in the crude extract (S-3) was measured in the following conditions; Tris-HCl buffer (pH 7.5) 60 μmoles, KCl 200 μmoles, NADH 0.15 μmole and lactate dehydrogenase 10 μg, in total volume of 1.0 ml, were incubated at 25°, and the optical

density at 334 mμ was measured. The activity was 0.4, 0.7 and 0.4 μmoles/mg protein in NADH oxidation in the mycelial extracts of the 6th, 7th and 9th day of the cultivation, respectively.

Discussion

The polyacetate structure of many naturally occuring substances has been confirmed by labeling studies with acetate-^{14}C, and many of these compounds are derived by condensation of acetate and malonate units, as suggested by Lynen and Tada.[16] The suggested mechanism is similar to that for fatty acid synthesis except that during the steps of condensation of acetate and malonate units intermedial polyketothiolester is remained partially unreduced. The incorporation of acetyl-CoA or malonyl-CoA into 6-methyl salicylic acid,[17] patulin[18] and alternariol[19] supports the above-mentioned mechanism.

In the case of anthraquinoid pigments of *P. islandicum* Sopp. the nucleus of the molecule is assumed to be derived by the same mechanism,[4] though no *in vitro* experiments were reported.

As shown in Fig. 1, the rate of biosynthesis of pigments in *P. islandicum* Sopp. is significantly high in the late stage of the fungal growth, and as shown in Table I, the pigment formation was proved to be dependent closely upon glycolysis and citric acid cycle. Furthermore, aldolase and citrate synthetase, which were selected as parameters for the activities of glycolysis and citric acid cycle, respectively, were highly active in the early stage of fungal growth, as shown in Fig. 2 and 3, and dehydrogenases such as malate dehydrogenase and glucose-6-phosphate dehydrogenase, which were selected as indicators for the dehydrogenation reaction, were remarkably high in the late stage, as shown in Fig. 4 and 5. These findings strongly indicate that the metabolic pattern of the mycelium and the enzymatic system for the pigment formation differ in the stage of fungal growth.

In order to demonstrate the formation of malonyl-CoA, the activity of acetyl-CoA carboxylase was examined with acetate-^{14}C or acetyl-^{14}C-CoA as substrate. The dependency of the incorporation on supplemented CoA and fatty acid synthetase indicates that this uptake represents the carboxylation of acetate, as shown in Table II. However, when acetyl-CoA was used as the substrate, the incorporation was observed only with the second ammonium sulfate fraction, as shown in Table III. One of the reason why acetyl-CoA was not incorporated by the crude extract was assumed to be due to the destruction of malonyl-CoA formed by the contaminated decarboxylase. As shown in Fig. 6, the crude extract of 8 days mycelium decarboxylated malonyl-CoA and this decarboxylation was not demonstrated with the supernatant of the second ammonium fraction. The higher activity of acetyl-CoA carboxylase in 7 days mycelium than in the case of 12 days one was coincident with the actively synthesizing phase of the pigment.

In the preceding paper,[1] the fixation and incorporation into pigments of $^{14}CO_2$ were observed with the *in vivo* experiments. In this respect, the authors investigated the *in vitro* fixation of $^{14}CO_2$ using the extract of the mycelium. Among pyruvate, oxaloacetate and phosphoenolpyruvate, the fixation of $^{14}CO_2$ was demonstrated only with pyruvate, and fixed amount of $^{14}CO_2$ was remarkably higher when the supernatant of the dialysed crude extract was used as the enzyme source to indicate that pyruvate is the acceptor of CO_2 and the produced compound was utilized further by contaminated enzyme(s) of the extract. Pyruvate-dependent fixation of CO_2 is considered to be catalysed by two enzymatic reactions; one is the carboxylation of pyruvate to form oxaloacetate, and the other is the exchange of carboxyl

16) F. Lynen and M. Tada, *Angew. Chem.*, **73**, 513 (1961).
17) R.J. Light, *J. Biol. Chem.*, **242**, 1880 (1967).
18) E.W. Bassett and S.W. Tanenbaum, *Biochim. Biophys. Acta*, **59**, 524 (1962).
19) S. Gatenbeck and S. Hormodsson, *Acta Chem. Scand.*, **19**, 65 (1965).

group of pyruvate with $^{14}CO_2$. No reduction of fixed radioactive material in the reaction mixture by the addition of aniline[20] and the activation of the fixation by added acetyl-CoA[21] indicate that the observed fixation of $^{14}CO_2$ in the presence of pyruvate is proceeded *via* the exchange of carboxyl group of pyruvate, as in case of anaerobic bacterium.[22]

Summing up the above discussion, it is concluded as follows: 1) Luteoskyrin and other anthraquinoid pigments of *P. islandicum* Sopp. were synthesized in the late stage of fungal growth; 2) With various enzyme preparations, acetate and acetyl-CoA were carboxylated to yield malonyl-CoA, and malonyl-CoA formed was actively decarboxylated to acetyl-CoA; 3) In the pigment formation stage of the mycelium, the activity of dehydrogenases was comparatively high.

Acknowledgement This investigation was partly carried out in the Max Planck Institute für Zellchemie, München (1965). The authors wish to express gratitude to Prof. F. Lynen and Dr. H. Eggerer for their helpfull suggestion and cooperation.

20) D.E. Green, L.F. Leloir and N. Nocito, *J. Biol. Chem.*, **161**, 559 (1945).
21) C.W. Shuster and F. Lynen, *Biochem. Biophys. Res. Comm.*, **3**, 350 (1960).
22) R.S. Wolfe and D.J. O'Kane, *J. Biol. Chem.*, **215**, 637 (1955).