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Utilization of Malonyl-CoA for the Biosynthesis of β-Carotene and Ergosterol in Cell-free Preparations from *Blakeslea trispora*

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The incorporation of labelled acetate, acetyl-CoA, and malonyl-CoA into β-carotene, ergosterol, and fatty acids was studied using cell-free preparations from *Blakeslea trispora*.

The results indicate that both, acetyl-CoA and malonyl-CoA, can be utilized as substrates for the isoprenoid synthesizing system(s). The formation of malonyl-CoA is not a limiting step for the biosynthesis of β-carotene.

A number of investigations with various systems have shown that acetyl-CoA is the substrate for synthesis of isoprenoids or their precursors (for recent reviews, see Refs. 1–2). It is uncertain, however, whether the initial step is a condensation of two acetyl-CoA molecules or whether one of them is first carboxylated to malonyl-CoA, analogously to what happens during the biosynthesis of fatty acids. The "direct" condensation is thermodynamically unfavourable, but it could be demonstrated in various coupled enzyme systems.[3–6] The incorporation of malonyl-CoA in HMG-CoA * and in mevalonate has been demonstrated with preparations of fatty acid synthetase from pigeon liver.[7–10]

There are also indications of the participation of malonyl-CoA in the sterol biosynthesis by dispersed rat liver cells [11] as well as in the biosynthesis of squalene by rat liver fractions.[12–13]

Other results, however, point against the involvement of malonyl-CoA in isoprenoid synthesis. Thus, *e.g.*, biotin deficiency does not affect the biosynthesis of cholesterol in yeast [14] and in rat tissues,[15] whereas, under similar conditions, fatty acid synthesis is significantly decreased. Other negative evidence of the malonyl-CoA involvement comes from studies on the synthesis of cholesterol [16] and mevalonate [17] by rat liver preparations and from studies on HMG-synthesis by preparations from sweet potatoes.[18]

* Hydroxy-methyl-glutaryl-coenzyme A.

The initial stages of carotene synthesis have not been studied in great detail. It is generally recognized that they follow the pathway common to all isoprenoids. During studies on β-carotene production by the mould *Blakeslea trispora* we have observed stimulatory effects of pyruvate and members of the tricarboxylic acid cycle.[19] Since citrate and isocitrate are known to activate the enzyme acetyl-CoA carboxylase (EC 6.4.1.2) from various sources,[23] our observation raised the question whether this enzyme and thus the "malonate pathway" may be involved in β-carotene synthesis in the mould under study.

The present paper reports results of studies on the incorporation of labelled acetate, acetyl-CoA, and malonyl-CoA into β-carotene, ergosterol, and fatty acids using cell-free preparations from *B. trispora* and also how this incorporation is affected by inhibitors and activators of acetyl-CoA carboxylase. The results indicate that both the thiolase pathway and the malonate pathway are operating in the biosynthesis of β-carotene in our system.

EXPERIMENTAL

Materials, organism and conditions of culture. 1-^{14}C-acetate was obtained from The Radiochemical Centre, Amersham, England; 1-^{14}C-acetyl-CoA and 1,3-^{14}C-malonyl-CoA were products from New England Nuclear Corp., USA. GSH, ATP, CoA, TPP, G-6-P, NADH, NADPH, and acetyl-CoA were purchased from Sigma Chemical Co., avidin and malonyl-CoA from Nutritional Biochemicals Corp., USA.

Organism and growth conditions were essentially as described previously,[20] but the hydrocarbon and β-ionone components were omitted from the growth medium and the lipid content was decreased to 3 %. After 2 days in mated culture the mycelium was filtered off and washed three times with one litre 0.2 M Tris-HCl (pH 8.0).

Preparation of cell-free homogenates. The washed mycelium was suspended in a small volume of Tris-HCl as above, but containing 0.02 M niacinamide and 0.002 M GSH. Portions of the suspension were homogenized in an Elvehjem-Potter homogenizer for 60 sec at 300 rpm. The homogenate was filtered through four layers of gauze tissue and centrifuged. The procedure was repeated three times in succession. All operations were carried out at +4°C. The final homogenate was free from live mycelium as judged by microscopic examination and by a test for viable cells. Protein content of the homogenate was determined according to Lowry *et al.*[21]

Incorporation of labelled precursors. 8 ml of the cell free suspension containing 80—120 mg protein was mixed with 1 ml of a solution containing (in μmoles): Tris-HCl (pH 8.0) 400; $MgCl_2$ 20; ATP 20; CoA 3; TPP 10; G-6-P 40; NADH 4; NADP 6; NADPH 10; pH (adj. with KOH) 8.0. The incubation was carried out in 50 ml Erlenmeyer flasks, stoppered with plugs containing Ascarite (Arthur H. Thomas Co., USA) to collect CO_2. The flasks were shaken for 5 h at 28°C. The reaction was initiated by addition of labelled and non-labelled precursors as stated in Tables 1 and 2. In certain experiments the incubation mixture was further supplemented after 30 min with an additional 1 ml of the cofactor solution. The reaction was stopped by addition of 10 ml acetone. The mixture was acidified with H_2SO_4 to pH 1 and all coloured matter was extracted using 5 ml portions of ether (6—10 extractions). The combined ether extracts were washed 3—4 times with 50 ml H_2O. Unlabelled β-carotene (0.2 mg) was added and β-carotene, ergosterol, and fatty acids were isolated essentially as described by Olson and Knizley.[22] β-Carotene and ergosterol were determined from absorption spectra in hexane in the range 250-650 nm. We used the peak at 450 nm and E(1 %, 1 cm) = 2560 for β-carotene. We used 283 nm and E(1 %, 1 cm) = 320 for ergosterol. Fatty acids were determined gravimetrically. The completeness of all extractions, washings, etc. was tested by radioactivity measurements. Countings were carried out in Packard Tri-Carb Liquid Scintillation Spectrometer, using standard techniques. The highly coloured samples were bleached by UV-light to diminish quenching.

RESULTS AND DISCUSSION

Table 1 shows the effect of avidin and activators of acetyl-CoA carboxylase on the incorporation of labelled acetate into β-carotene, ergosterol, and fatty acids in cell-free homogenates.

Table 1. The effect of avidin and activators of acetyl-CoA carboxylase on the incorporation of 1-^{14}C-acetate (20 μC, 20 μmoles) into β-carotene, ergosterol, and fatty acids in cell-free homogenates (80—120 mg protein) from *B. trispora*. Pre-incubation with avidin was carried out for 1 h at 4°C. Other tested substances were added immediately before the addition of the labelled precursor. Incubation for 5 h at 28°C.

Experiment No.	Addition to the standard incubation mixture (pH 8.0)	Activity (cpm) incorporated into β-Carotene	Ergosterol	Fatty acids
1	None	18	100	530
	$KHCO_3$ 200 μmoles	12	104	564
	$KHCO_3$ 200 μmoles, avidin 65 units	18	100	330
2	None	18	86	478
	Avidin 65 units	18	84	344
3	None	18	100	1140
	Avidin 65 units	22	92	730
4	None, pH 7.5	14	54	416
	K-citrate 20 μmoles, pH 7.5	14	58	492

Bicarbonate and citrate are two substances known to stimulate the activity of acetyl-CoA carboxylase [23] and thus the formation of malonyl-CoA. It appears from Table 1 that they are without effect on the incorporation of labelled acetate into β-carotene and ergosterol, whereas they significantly stimulate this incorporation into fatty acids. Avidin, which specifically binds the biotin moiety in acetyl-CoA carboxylase, significantly decreases the incorporation into fatty acids, whereas it is essentially without effect on the incorporation into β-carotene and ergosterol. These data indicate that the formation of malonyl-CoA is not a limiting step in the biosynthesis of isoprenoids as it is in the biosynthesis of fatty acids.

The data summarized in Table 2 indicate, however, that both acetyl-CoA and malonyl-CoA can act as substrates for isoprenoid synthesis (*cf.* mix 1 and 2). The much higher incorporation into carotene and ergosterol of the label from malonyl-CoA (mix 2) as compared to that from acetyl-CoA (mix 1) may be due to the inhibition of acetyl-CoA carboxylase by avidin. This strengthens the conclusion that malonyl-CoA can be utilized for biosynthesis of isoprenoids.

The possibility that malonyl-CoA is to any significant extent decarboxylated prior to the incorporation of its acetyl-CoA moiety into the isoprenoids does not seem probable considering the results obtained with malonyl-CoA as the only added precursor (mix 2). If this were the case one would expect

Table 2. Incorporation of 1-^{14}C-acetyl-CoA and 1,3-^{14}C-malonyl-CoA into β-carotene, ergosterol, and fatty acids in cell-free homogenates (110 mg protein) from *B. trispora*. The reaction mixture without precursors was incubated with avidin (3 units/ml), after which 1 ml of precursor solution was added to 9 ml of the mixture. The same batch of homogenate and of cofactor solution was used in all four experiments. Incubation for 5 h at 28°C. The specific activity of malonyl-CoA is given for the total molecule.

Mix No.	Precursors	Activity (cpm) incorporated into β-Carotene	Ergosterol	Fatty acids
1	Acetyl-CoA 3.9 μmoles 1.2 μC/μmole	23	44	485
2	Malonyl-CoA 5.8 μmoles 0.8 μC/μmole	145	744	62 100
3	Acetyl-CoA 0.08 μmoles 61 μC/μmole, malonyl-CoA 5.3 μmoles unlabelled	62	1804	29 600
4	Acetyl-CoA 3.8 μmoles unlabelled malonyl-CoA 0.5 μmoles 9.3 μC/μmole	270	534	97 600

an incorporation that was equal to or lower than that obtained with acetyl-CoA as the only precursor (mix 1). Assuming a complete decarboxylation of the malonyl-CoA present in mix 3 and 4, one would obtain a pool of acetyl-CoA with a specific activity of 0.84 μC/μmole in mix 3 and 0.53 μC/μmole in mix 4. One could thus, in both cases, expect a lower incorporation of the label into the isoprenoids in comparison to that in mix 1 with its specific activity of 1.2 μC/μmole acetyl-CoA. In contrast, this incorporation is several times higher in mix 3 and 4 than it is in mix 1. Also the amount of the label in fatty acids in mix 3 and 4 indicates that no extensive decarboxylation of malonyl-CoA takes place. On the other hand, the incorporation obtained in mix 2 and 4 points against the possibility that malonyl-CoA is the only substrate for isoprenoid synthesis. Considering the much higher specific activity of malonyl-CoA in mix 4 as compared to that in mix 2, one would then expect a correspondingly higher incorporation of the label in mix 4 than in mix 2 and this is not the case.

There is a good agreement between the incorporation of the label into fatty acids and the specific activity of the labelled precursor (acetyl-CoA) in mix 3 as compared to mix 1.

The incorporation of the label into β-carotene increases when labelled malonyl-CoA is supplied instead of labelled acetyl-CoA, both precursors being present in comparable amounts (mix 3 and 4). The incorporation in ergosterol (mix 4) is only apparently low, considering the differences in the specific activity of the precursors.

Our results indicate the occurrence in *B. trispora* of either two different isoprenoid synthesizing systems or of one such system capable of utilizing both acetyl-CoA and malonyl-CoA as substrates. The relative importance of the two biosynthetic pathways *in vivo* remains, however, to be evaluated.

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REFERENCES

1. Lynen, F. *Pure Appl. Chem.* **14** (1967) 11.
2. Porter, J. W. and Anderson, D. G. *Ann. Rev. Plant. Physiol.* **18** (1967) 197.
3. Lynen, F., Wessley, L., Wieland, O. and Rueff, L. *Angew. Chem.* **64** (1952) 687.
4. Lynen, F., Henning, V., Bublitz, C., Sörbo, B. and Kröplin-Rueff, L. *Biochem. Z.* **330** (1958) 269.
5. Rudney, H. *J. Biol. Chem.* **227** (1957) 363.
6. Rudney, H. and Ferguson, J. J., Jr. *J. Biol. Chem.* **234** (1959) 1076.
7. Brodie, J. D. and Porter, J. W. *Biochem. Biophys. Res. Commun.* **3** (1960) 173.
8. Brodie, J. D., Wasson, G. W. and Porter, J. W. *Biochem. Biophys. Res. Commun.* **8** (1962) 76.
9. Brodie, J. D., Wasson, G. W. and Porter, J. W. *J. Biol. Chem.* **238** (1963) 1294.
10. Brodie, J. D., Wasson, G. W. and Porter, J. W. *J. Biol. Chem.* **239** (1964) 1346.
11. Ishihara, A., Koyama, E. and Takeda, Y. *J. Biochem. (Tokyo)* **58** (1965) 480.
12. Knauss, H. J., Porter, J. W. and Wasson, G. W. *J. Biol. Chem.* **234** (1959) 2835.
13. Higgins, M. J. P. and Keckwick, R. G. O. *Biochem. J.* **113** (1969) 36P.
14. Bloomfield, E. K. and Bloch, K. *J. Biol. Chem.* **235** (1960) 337.
15. Dakshinamurti, K. and Desjardins, P. R. *Can. J. Biochem.* **46** (1968) 1261.
16. Foster, D. W. and Bloom, B. *Biochim. Biophys. Acta.* **70** (1963) 341.
17. Fimognari, G. M. and Rodwell, V. W. *Abstr. 6th Intern. Congr. Biochem.* New York, *VII-49* (1964) 573.
18. Oshima, K. and Uritani, I. *J. Biochem. (Tokyo)* **63** (1968) 617.
19. Björk, L. and Neujahr, H. Y. *Acta Chem. Scand.* **23** (1969) 2908.
20. Cederberg, E. and Neujahr, H. Y. *Acta Chem. Scand.* **23** (1969) 957.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
22. Olson, J. A. and Knizley, H., Jr. *Arch. Biochem. Biophys.* **97** (1962) 138.
23. Gregolin, C., Ryder, E. and Lane, M. D. *J. Biol. Chem.* **243** (1968) 4227.

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