ACTA CHEMICA SCANDINAVICA 25 (1971) 3634–3640

Biosynthesis of Mevalonic Acid in *Blakeslea trispora*

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Radioactivity from ^{14}C-labelled malonate added to the substrate in cultures of *Blakeslea trispora* was detected in intracellular fatty acids and mevalonic acid. Degradation and determination of the distribution of radioactivity in the molecules suggest that the malonate essentially was metabolized without any decarboxylation. The results indicate that malonyl-coenzyme A is of no or little significance for biosynthesis of mevalonic acid *in vivo* in *Blakeslea trispora*.

The biosynthesis of isoprenoids has eversince Lynen's and Rudney's fundamental studies *in vitro* generally been considered to start with a condensation between two acetyl-coenzyme A molecules to acetoacetyl-coenzyme A. (For recent reviews, see Lynen [1] and Porter and Anderson [2].) Another route, where the initial step is a condensation between one acetyl-coenzyme A and one malonyl-coenzyme A has been postulated and supported by experimental evidence.[3–9] Other results disfavour such a possibility.[10–14]

Contributory to the different opinions of the presumptive participation of malonyl-coenzyme A seems to be if the experiments have been carried out *in vivo* or *in vitro*. No paper including both *in vivo* and *in vitro* studies of, *e.g.*, the mevalonate biosynthesis on the same organism has so far appeared.

Results from studies of the incorporation of ^{14}C-labelled acetyl-coenzyme A and malonyl-coenzyme A into ergosterol and β-carotene *in vitro*, using homogenates of *Blakeslea trispora*, indicate that malonyl-coenzyme A is incorporated into these isoprenoids.[9] The present paper reports *in vivo* studies of the biosynthesis of mevalonic acid with *Blakeslea trispora*.

Sodium malonate-1,3-^{14}C was added to submerse cultures from which mevalonic acid and fatty acids were extracted. The mevalonic acid was lactonized and the fatty acids methylated. After purification by chromatographic techniques and control of the constancy of specific radioactivities the distribution of radioactivity in the molecules was determined.

Wet combustions [16] were used to get average values for the radioactivity of all carbon atoms in mevalonolactone and fatty acid methyl esters, respectively. Chromic acid oxidations [15] facilitated isolation of carbon atoms C-3 and C-6 in mevalonolactone and the C-terminal carbon atoms of the fatty

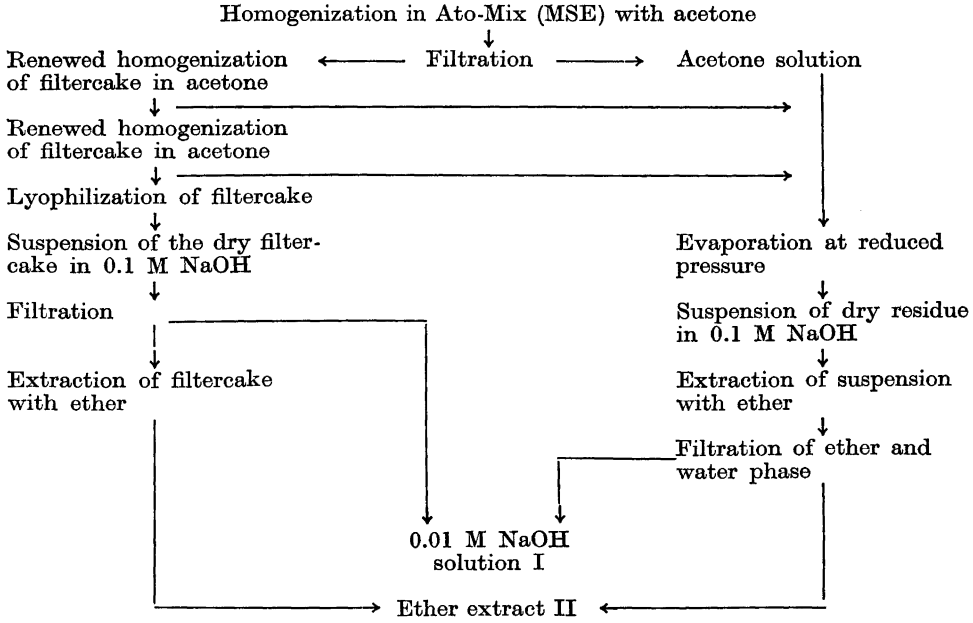
acid methyl esters. The carbon dioxide evolved during the oxidations was collected and used for determination of the radioactivity in the other atoms. The radioactivity of the carbonyl group in the mevalonolactone was determined after preparation of the barium salt and subsequent pyrolysis. In each case the carbon atoms were finally collected as barium carbonate, the radioactivity of which was determined in a liquid scintillation spectrometer. The study of fatty acids was performed to determine to what extent malonate was metabolized without any preceding decarboxylation.

EXPERIMENTAL

Mevalonolactone from Fluka AG, Schweiz. Sodium malonate-1,3-^{14}C from the Radiochemical Centre, England. Thixotropic gel powder, CAB-O-SIL, PPO and dimethyl-POPOP from the Packard Instrument Co., USA. Ato-Mix homogenizer from the MSE, England. Packard Tri-Carb liquid scintillation spectrometer model 3375 from the Packard Instrument Co., USA. Aerograph 600 D converted for preparative use by a micro-collector splitter assembly from Varian Aerograph, USA.

Procedure. Cultures of *Blakeslea trispora* NRRL 2456(+) and 2457(−) were separately grown in acid hydrolyzed corn meal (23 g/l) and soybean meal (47 g/l) on a rotary shaker as described by Ciegler *et. al.*[17] After 48 h fermentation the cultures were brought together in the ratio 2 : 1 (2456 : 2457 wet volumes) and homogenized for 9 sec in an Ato-Mix homogenizer. The mixture was used as inoculum (10 % v/v) in fresh substrate supplemented with 1 % cotton oil of known fatty acid composition. After 44 h growth malonate-1,3-^{14}C was aseptically added. The mycelium was harvested 4 h later by filtration and washed several times with 1 % sodium chloride in water. The washed mycelium was extracted as shown in Scheme 1. From the extracts mevalonolactone and fatty acids were isolated as described in Schemes 2 and 3, respectively.

Scheme 1. Extraction of mycelium of *B. trispora*.



Scheme 2. Isolation of mevalonolactone.

0.01 M NaOH solution I
↓
Acidification with H_2SO_4 to pH 1
↓
Extraction with petroleum ether (Petroleum ether extract III)
↓
Lactonization 15 min at 38°C
↓
Addition of Na_2SO_4 and ether extraction [18]
↓
Evaporation of the ether phase and dissolving in 5 M H_2SO_4
↓
Column chromatography on Celite 535 [18,20]
↓
Drying with Na_2SO_4 and evaporation of solvent
↓
Preparative gas chromatography [21]
↓
Collection of mevalonolactone → An aliquot analyzed for specific radioactivity
↓
An aliquot run on thin-layer chromatography [22]
↓
Determination of specific radioactivity.

Scheme 3. Isolation of fatty acids.

Evaporation of solvents from extract II and III
↓
Refluxing with 20 % KOH in methanol for 6 h
↓
Dilution with two volumes of water
↓
Extraction with ether
↓
Acidification of methanol-water phase with H_2SO_4 to pH 1 in ice bath
↓
Filtration
↓
Extraction of precipitate and methanol-water phase with ether
↓
Drying of ether extract over Na_2SO_4
↓
Evaporation of ether
↓
Refluxing with 5 % H_2SO_4 in methanol for 6 h
↓
Dilution with two volumes of water
↓
Extraction with ether
↓
Drying over Na_2SO_4
↓
Evaporation of ether
↓
Preparative gas chromatography using 6′ × 1/8″ stainless steel column with 20 % DEGS on Chromosorb W 60 – 80 mesh. Injection temperature was 220°C and column oven temperature 195°C. Nitrogen was used as carrier gas.

Scheme 3. Continued.

↓
Collection of methyl esters of palmitic, oleic and linoleic acid
| ⟶ An aliquot of each analyzed for specific radioactivity
↓
An aliquot of each run on thin-layer chromatography as mercury adducts [23]
↓
Decomposition of adducts and isolation of esters
↓
Determination of specific radioactivity.

Prior to determinations of specific activities each substance was oxidized [16] to CO_2, which was precipitated as $BaCO_3$. The precipitate was filtered off by suction and immediately washed with boiling water and acetone and then dried for 2 h at 105°C. After weighing the carbonate was ground in liquid scintillation vials. To the finely disaggregated carbonate was added CAB-O-SIL (0.4 g/vial) and the mixture dispersed in a toluene solution (12 ml/vial) of PPO (4 g/l) and dimethyl POPOP (0.050 g/l). The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. The amounts of substances oxidized were in all cases calculated to give about 40 mg of $BaCO_3$.

Determination of the distribution of radioactivity in mevalonolactone. The different carbon atoms are referred to with numbering as shown in Fig. 1.

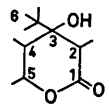


Fig. 1. Mevalonolactone.

Wet combustion [16] and determination of the specific radioactivity of the evolved CO_2 as described above gives an average value for all carbon atoms (C-1 – C-6).

Chromic acid oxidation [15] gives in the same way an average value of the specific radioactivities of C-1, C-2, C-4 and C-5. The acetic acid, produced in this reaction, was isolated as sodium acetate by steam distillation and neutralization with 0.01 M NaOH. The water was evaporated and the sodium acetate dried 2 h at 125°C. The specific radioactivity of the acetate was determined after wet combustion as described above, giving an average value of C-3 and C-6.

Barium salt of the mevalonic acid was prepared.[18] By pyrolysis of the salt at 500°C for 1 h at reduced pressure, C-1 was obtained as $BaCO_3$. The carbonate was decomposed with HCl and the released CO_2 reprecipitated as $BaCO_3$. An alternative way of isolation of C-1, *i.e.* decarboxylation according to Schmidt,[24] was tested but gave values of lower reproducibility.

Determination of the distribution of radioactivity in methyl esters of fatty acids. Analysis of distribution of radioactivity was performed on methyl esters of palmitic, oleic, and linoleic acid, obtained from different cultures. The esters were totally oxidized [16] to get an average value of the radioactivity in all carbon atoms as described for mevalonolactone. By chromic acid oxidation [15] groups originating from malonyl-coenzyme A and methanol were obtained as CO_2, whose specific radioactivity was determined. To get a yield of about 90 % 8 h were needed for the oxidation of palmitic acid ester and 6 h for oleic and linoleic acid esters. Other samples were oxidized for 12 h and CO_2 driven off with nitrogen. The C-terminal groups originating from acetyl-coenzyme A were isolated as acetic acid from the reaction mixtures and analyzed for their specific activities as shown. Values reported in Table 2 are corrected for contributions of the methoxyl group in the methyl esters of the acids.

RESULTS AND DISCUSSION

Table 1 shows the measured specific radioactivities of CO_2 from different C-atoms of mevalonic acid and the corresponding calculated values in the mevalonic acid molecule. Figures from the determinations on the fatty acids are shown in Table 2.

Table 1. Specific radioactivities for different C-atoms of mevalonic acid. Added: I. 0.1 mC sodium malonate-1,3-^{14}C (specific radioactivity 14.5 mC/mmol), IIa,b 0.5 mC sodium malonate-1,3-^{14}C (specific radioactivity 6.6 mC/mmol).

Atom	Experiment No.	cpm/mmol CO_2	cpm/mmol mevalonic acid
C-1 – C-6	I	183	1098
	IIa	4027	24162
	IIb	4126	24756
C-1, C-2, C-4, C-5	I	187	748
	IIa	3738	14952
	IIb	3351	13404
C-3, C-6	IIa	4106	8212
	IIb	4483	8966
C-1	IIa	8400	8400
	IIb	7875	7875

Table 2. Specific radioactivities for different C-atoms of fatty acids. Added: Ia,b 0.1 mC sodium malonate-1,3-^{14}C (specific radioactivity 14.5 mC/mmol), IIa,b 0.5 mC sodium malonate-1,3-^{14}C (specific radioactivity 6.6 mC/mmol).

Atom and acid	Experiment No.	cpm/mmol CO_2	cpm/mmol fatty acid
Palmitic acid			
C-1 – C-16	Ia	10 649	170 384
	b	11 248	179 997
	IIa	83 300	1 332 800
	b	85 300	1 364 800
C-1 – C-14	Ia	12 053	168 750
	b	12 233	171 260
	IIa	67 800	949 200
	b	75 000	1 050 400
C-15 – C-16	Ia	2 610	5 219
	b	2 490	4 980
	IIa	18 000	36 000
	b	19 300	38 600
Oleic acid			
C-1 – C-18	Ia	5 404	97 272
	b	5 667	102 010
	IIa	17 644	317 600
	b	18 043	324 800
C-1 – C-16	Ia	5 781	92 500
	b	6 587	105 390

Table 2. Continued.

	IIa	17 100	273 600
	b	19 500	312 000
C-17 – C-18	Ia	1 164	2 328
	b	1 337	2 673
	IIa	6 030	12 060
	b	7 010	14 020
Linoleic acid			
C-1 – C-18	Ia	4 166	74 988
	b	4 295	77 310
C-1 – C-16	Ia	4 569	73 100
	b	4 806	76 900
C-17 – C-18	Ia	318	637
	b	332	664

It appears from Table 2 that the specific radioactivities of CO_2 from the carbon atoms of the fatty acids incorporated as acetyl-coenzyme A do not exceed one third of the corresponding values for CO_2 originating from malonyl-coenzyme A incorporation. This indicates that most of the metabolized malonate has not passed acetyl-coenzyme A as an intermediate step. Participation of malonyl-CoA in the biosynthesis of mevalonic acid could thus be expected to give about three times lower specific radioactivity of carbon atoms derived from acetyl-coenzyme A. As seen in Table 1 the radioactivity is equally distributed in the C_2-units, C-1 – C-2, C-3 – C-6 and C-4 – C-5 of the mevalonic acid, why an incorporation of malonyl-coenzyme A must be considered less probable. As recently reported, evidence from *in vitro* studies of the isoprenoid synthesis suggests the possibility of a malonyl-CoA incorporation into isoprenoids in *B. trispora*.[9] In the light of the evidence presented above the participation of malonyl-coenzyme A, however, is likely an artifact, existing only in disorganized cells and not in the intact organism.

Acknowledgements: This work was made possible through a scholarship from *Bengt Lundquist Memorial Fund* and was supported by a graduate student fellowship from the *Royal Institute of Technology*, Stockholm.

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Received April 14, 1971.