Distribution of β-Carotene in Subcellular Fractions of *Blakeslea Trispora*

Very little is known about the physiological function of carotenoids in non-photosynthetic microorganisms. Their intracellular distribution has not been studied to any great extent. The mould under study here, *Blakeslea trispora*, is a very active producer of carotenoids, of which more than 80% is β-carotene. This paper describes an attempt to determine the subcellular distribution of carotenes by differential centrifugation of disrupted mycelium.

Organisms, growth media and techniques were as described by CEDERBERG and NEUJAHR[1], but the lipid content of the growth medium was decreased to 4% or 1%.

Harvest and disruption of the mycelium. The mycelium was separated from the growth medium by filtration under suction. The filter-cake was resuspended and washed 3–4 times with ice-cold distilled water using some surfactant in the first wash. The washed suspension, half the original culture volume, was disrupted in an X-press[2] at −15 °C. The frozen homogenate was stored at 4 − 15 °C until thawed immediately before fractionation.

Differential centrifugation of the disrupted mycelium. The homogenate was diluted to the initial culture volume and centrifuged in a Spinco Preparative Ultracentrifuge Model L 50. The homogenate was first centrifuged at 600 *g* and the 600 *g* supernatant at 75,000 *g*. The 75,000 *g* sediment was then centrifuged at 4,900 *g* and the 4,900 *g* supernatant again at 75,000 *g*. The 75,000 *g* sediment was then refractionated at lower speeds and sediments were collected also at 10,000, 25,000 and 75,000 *g*. All runs were made for 30 min. The fractions were washed once with ice-cold distilled water and recentrifuged. The supernatant liquid after each centrifugation was filtered through gauze to removed the pools of fat forming at the surface.

Analyses. The respective fractions were resuspended in water and duplicate samples were taken for each analysis. Dry solids were determined after drying at 105 °C to constant weight. Protein was measured according to LOWRY et al.[3]. Carotenes and lipids were extracted with acetone and light petroleum. The carotenes were determined as β-carotene at 452 nm using $E^{1\%}_{1cm} = 2560$. Total lipids were determined gravimetrically after evaporation of the solvent.

Results and discussion. Unless otherwise stated all results are mean values of 4 different experiments. The Table shows the relative distribution of carotene, lipids, protein and dry solids in the various fractions. As can be seen, using mycelium grown with 4% lipids, about 59% of the protein, but only 39% of the lipids and 51% of the carotene, are recovered in the 600 × *g* sediment. This fraction is composed of undisrupted cells, together with some cell debris. The figures thus indicate that some of the lipid content of the cells is thrown out of the whole cells during centrifugation and that some of the carotene is dissolved in this lot. A single determination was made of the lipid pool gathering on the surface of the supernatant (Table).

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The relative distribution of carotene, total lipids, protein and dry solids in subcellular fractions of *B. trispora*, grown in media containing 4 or 1% lipids

Fraction	Content of lipids in growth medium %	Content in mycelium of			
		Carotene ‰	Lipids ‰	Protein ‰	Dry solids ‰
Whole homogenate		1000	1000	1000	1000
600 × *g*	4	510	390	590	540
sediment	1	400	260	660	660
4,900 × *g*	4	21	9.6	14	11
sediment	1	2.3	4.8	6.0	4.2
10,000 × *g*	4	1.2	1.1	1.6	1.3
sediment	1	0.04	0.36	0.41	0.26
25,000 × *g*	4	0.40	0.75	1.3	1.0
sediment	1	0.01	0.12	0.12	0.12
75,000 × *g*	4	0.14	0.37	3.6	2.1
sediment	1	0.01	0.05	0.14	0.11
75,000 × *g*	4	6.4[a]	6.6[a]	130	100
supernatant	1	27	90	160	170
Lipid pool	4	240[a]	370[a]	–[b]	190[a]
Not recovered	4	220	220	260	150
	1[c]	570	640	170	170

[a] One single determination. [b] Not determined. [c] This includes the lipid pool.

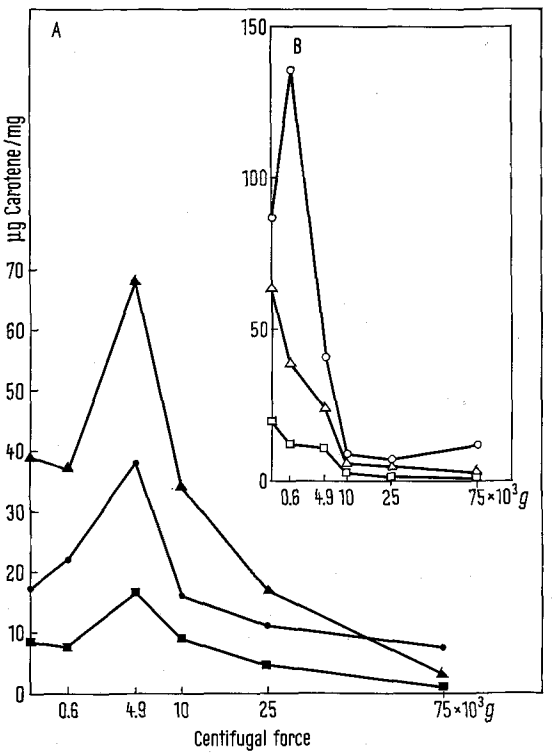

Fig. 1. The distribution of carotene in relation to dry solids (□—□), protein (△—△) and total lipids (○—○) in subcellular fractions of *B. trispora*. (A) Growth medium containing 4% lipids (solid symbols). (B) Growth medium containing 1% lipids (single experiment).

The fraction with the second largest accumulation of carotene is the 4,900×*g* sediment. There was a similar relative distribution of carotene, total lipids, protein and dry solids when the mycelium was grown with 1% lipids in the medium. However, comparatively less lipid and more protein and dry solids sedimented in the 600×*g* fraction (Table). It seems that the mycelium grown at the lower lipid level is more resistant to disruption, which may be a reflection of differences in proportions of carbohydrates and lipids in the mycelium cell wall.

The distribution of carotene in relation to dry solids, protein and total lipids using mycelium grown with 4% or 1% lipids in the medium is shown in Figure 1. In the first mentioned case all 3 parameters have a distinct peak at 4,900×*g*. The pattern is quite different in fractions prepared from mycelium grown with 1% lipids in the medium. The peaks at 4,900×*g* are missing, while the carotene : lipid ratio in the 600×*g* sediment is very high (Figure 1B).

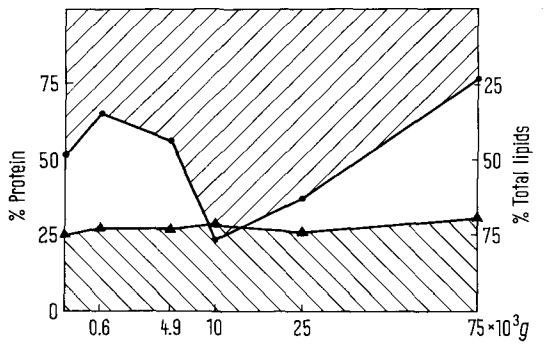

Fig. 2. Contents of protein (▲—▲) and total lipids (●—●) expressed as a percentage of dry solids, in subcellular fractions of *B. trispora*. Medium containing 4% lipids.

Figure 2 shows the contents of total lipids and protein in relation to dry solids in the various sediments. There is a minimum in the lipid content of the 600×*g* sediment which may be explained by the aforesaid ejection of 'free' lipids during centrifuging. The large content of lipids in the 10,000×*g* fraction probably corresponds to light mitochondria. The pattern was essentially similar using mycelium grown with 1% lipids, but the lipid content of all fractions was lower.

It appears from these data that there are at least 2 pools of β-carotene in the mycelium of *B. trispora*. One of them is associated with the fraction sedimenting at 4,900×*g*, presumably consisting mainly of cell walls and heavy mitochondria. There also seems to be a large pool of free β-carotene, dissolved in fat globules suspended in the cytoplasm. Similar fat inclusions are considered to be the main pool of carotenoids in fungi[4]. The magnitude of this pool in *B. trispora* may be related to the excessive synthesis of β-carotene which, as pointed out above, is substantially increased in the presence of exogenously supplied lipids[5].

Résumé. On a obtenu 2 pools majeurs de β-carotène dans l'homogénat du mycélium de *B. trispora*. L'un d'eux est associé à la fraction sédimentable à 4,900×*g*, l'autre aux globules de matière grasse, dans le cytoplasme.

EVA CEDERBERG and HALINA Y. NEUJAHR

The Department of Pure and Applied Biochemistry, The Royal Institute of Technology, S-100 44 Stockholm 70 (Sweden), 6 October 1969.

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Microbiological Assay of Cyclic 3′, 5′-AMP

Cyclic 3′, 5′-AMP is present in many tissues of various animals, where it may act as a 'second messenger'[1]. Several biochemical assays for cyclic 3′, 5′-AMP are available. A microbiological assay for qualitative and quantitative determination of cyclic 3′, 5′-AMP is described in this report using myxamoebae which move towards sources containing this compound. This method shows high specificity and sensitivity. An additional advantage is that all kinds of extracts may be assayed for cyclic 3′, 5′-AMP without previous purification procedures.

Materials and methods. The preparation of myxamoebae, a hydrophobic agar surface and the measurement of its rigidity and hydrophoby have been described previously[2,3]. Using a hydrophobic agar of a suitable rigidity the cells stayed inside the drops unless an active attractant outside the drops induces them to pass the boundary (Figure 1). 100–150 drops (each 0.1 µl) of a myxamoebae suspension were placed on such an agar. Every responding population (drop diameter ca. 0.6 mm) contained 500–1000 cells.

Myxamoebae were most sensitive to attractants at the onset of aggregation[4,5]. Small populations of myxamoebae, incubated at 22 °C in darkness, aggregated 8–10 h after deposition. The sensitivity of the cells in the various drops was better synchronized when they were incubated at 22 °C for 2–3 h, stored overnight at 5–6 °C, and used the next day. A sudden change in temperature may sometimes lead to withdrawal of pseudopods, especially at 25 °C or higher. Incubation at 16 °C for 30–60 min before exposure to room temperature prevented rounding-up of the cells. Drops (0.1 µl) of an active extract were placed near (100–500 µm away) the responding drops. The attraction was observed 30 or 45 min after deposition, through a phase contrast microscope (× 80). The cells in the responding populations should not be aggregating at the time of observation. A response was scored positive

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