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INTRACELLULAR LOCATION OF FLEXIRUBINS IN *FLEXIBACTER ELEGANS* (CYTOPHAGALES)

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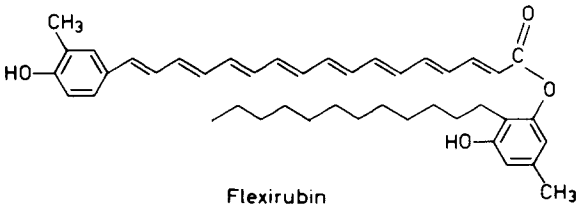
Summary

The inner and outer membranes of 2 strains of Gram-negative *Flexibacter elegans*, *Fx* e1 and *Fx* 3/4, could be separated on sucrose density gradients after the cells had been converted into spheroplasts, and the spheroplasts had been lysed in presence of EDTA and the detergent Brij 58. The light fraction (ρ = 1.14 g · cm^{-3}) contained the components of the respiratory chain in high concentrations, but only low amounts of the lipopolysaccharide component, 2-keto-3-deoxyoctonic acid, and was thus mainly material from the inner membrane. The heavy fraction (ρ = 1.175 g · cm^{-3}) contained only traces of respiratory chain enzymes, but the majority of the 2-keto-3-deoxyoctonic acid, and was thus mainly material from the outer membrane. The flexirubin pigments were found almost quantitatively in the latter fraction. Strain *Fx* 3/4 produced carotenoids in addition to flexirubins; in this case the flexirubins were located in the outer, and the carotenoids in the inner membrane.

Introduction

Flexibacter elegans is a Gram-negative bacterium. Taxonomically it belongs into the order Cytophagales [1]. Two of its properties are particularly remarkable: (1) during growth the cells undergo a dramatic shape change. While in young cultures there are long (15—25 μm), flexible, and very agile thread cells, the population consists during later growth stages of short (2—3 μm) immotile rods, which finally may become almost coccoid [2—4]. (2) The bacterium produces yellow pigments, which were found to be chemically of a novel type [5—7]. The new pigments have been named 'flexirubins' (I). In addition to flexirubins, some *Flexibacter* strains contain carotenoids.

The function of the flexirubins is not yet known. One way to approach this problem is to study the control mechanisms of biosynthesis. It has been found



Flexirubin

(I) The chemical structure of flexirubin.

that flexirubins are synthesized by *F. elegans* only during growth, and that a low pH as for example produced by the bacteria when cultivated in sugar-containing media, reduces the rate of synthesis. Light, which stimulates carotenoid biosynthesis in flexibacteria and many other organisms, leads to a slightly lowered specific flexirubin content of the cells [2].

Also, the intracellular distribution of flexirubins could give hints as to their possible function. The lipophilic character of the molecules suggests two possible sites for their deposition: the cytoplasmic membrane and the outer membrane of the cell envelope. The complex structure of the cell envelope of Gram-negative bacteria makes the separation of the two membranes difficult, although it has been achieved with several organisms [8—18]. The cells were first desintegrated by treatment with lysozyme [8,10,12,14—16,18], by use of the French pressure cell [9,13,17], or by growing the organism in presence of penicillin [11]. In some cases the detergent Brij 58 was added [11,15]. The membranes were then separated on the basis of their different buoyant densities on a sucrose gradient. A separation of the membranes of *Flexibacter* or related bacteria has not been described before. In fact, none of the methods mentioned above were successful with our organism. Only with a modified lysozyme method, and after application of Brij 58, the outer and inner membranes of *Flexibacter* could be separated. The membrane fractions were characterized by measuring enzymatic activities and the concentration of typical membrane components. As will be shown, the flexirubins are located in the outer membrane, while the carotenoids, when present, are found in the cytoplasmic membrane.

Methods

Strains and culture conditions. Experiments were carried out with *F. elegans* strain *Fx* e1 [2], or with *Flexibacter* strain *Fx* 3/4. The latter strain produces carotenoids in addition to flexirubins and was isolated in 1967 from soil collected on Tutuila (Samoa). Cultures were grown in Erlenmeyer flasks as has been described before [2]. Cultures of strain *Fx* 3/4 were illuminated with blue light from Philips TL 40W/18 tubes to stimulate carotenoid synthesis.

Production and desintegration of spheroplasts. The cells were harvested in the upper logarithmic growth phase on a cold centrifuge at 16 000 × *g* and washed once with cold 0.01 M Tris · HCl buffer, pH 7.6. The sediment from 200 ml of culture was resuspended in 4.5 ml of Tris buffer containing 6 M glycerol and 1 mg of lysozyme. After incubation at room temperature for 8 min, the suspension was injected with a syringe into 23 ml of cold Tris

Protein concentrations were determined according to Lowry et al. [24].

Membrane fractions were prepared for electron microscopy according to Oltmann and Stouthamer [11]. A Zeiss electron microscope EM 10B was used.

Chemicals. Lysozyme, RNAase, DNAase and NADH were purchased from Boehringer (Mannheim), 2,6-dichlorophenolindophenol, Coomassie Brilliant Blue R250, and Brij 58 from Serva (Heidelberg), 2-keto-3-deoxyoctonic acid from Sigma Chemie (München).

Results

All efforts to separate the 2 membranes of the cell envelope of *F. elegans Fx* e1 failed if the cells had been broken up with ultrasound or in the French pressure cell. Neither did we obtain spheroplasts by treating the cells with lysozyme and EDTA in sucrose solution so that lysis by osmotic shock could not be applied, a method often useful for the preparation of membranes. When we modified the method, however, as outlined above, i.e., by replacing sucrose with glycerol, and by omitting EDTA, did we obtain spheroplasts within a few minutes and with nearly the total cell population. Such spheroplasts would burst when treated with EDTA; the membranes, however, still did not separate well. Only when we added also the detergent Brij 58 to the lysing suspension, the inner and outer membrane could be brought apart on a sucrose gradient (Fig. 1). There appeared 2 bands with the buoyant densities of 1.14 $g \cdot cm^{-3}$ (L fraction) and 1.175 $g \cdot cm^{-3}$ (H fraction), respectively. Light absorbance at 280 nm was taken as a measure for protein concentrations. A comparison of the peak areas showed that the L fraction contained 40%, the H fraction 60% of the total protein. The ratio $A260/A280$ indicated that the fractions were nearly free of nucleic acids (below 5%) [25]. To further characterize the 2 fractions the distribution of respiratory chain enzymes and of typical membrane compounds was determined. Succinate dehydrogenase and NADH-oxidase are both typical for the inner membrane [26]. Their specific activities with respect to protein were found both to be 25 to 30 times higher in the L fraction than in the H fraction (Table I). In accordance with this, cytochromes could be

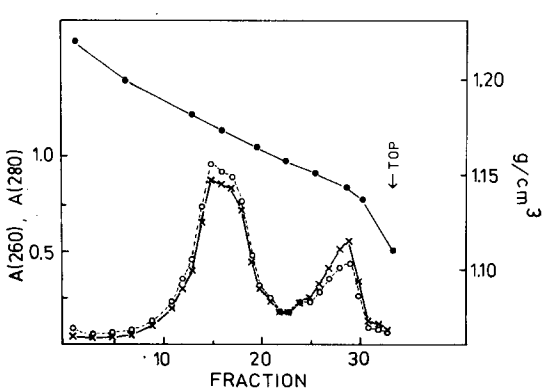


Fig. 1. Separation of a crude membrane preparation from *Flexibacter elegans Fx* e1 on a continuous sucrose gradient (27 to 50% sucrose, w/v). Centrifugation: 17 h at 208 000 × *g* in rotor SW41 (Beckman). X———X, Extinction at 280 nm; o- - - - -o, extinction at 260 nm; •———•, specific density ($g \cdot cm^{-3}$).

buffer to which were added 40 μg/ml each of DNAase and RNAase. About 10 min later more than 90% of the cells had become spheroplasts. The suspension was then shaken on a water bath at 12°C, and 0.3% Brij 58 and 1.35 mM EDTA were added. Upon this the suspension became quickly transparent. The mixture was held in an ice bath for 20 min and then treated with ultrasound for 10 s (MSE Ultrasound Power Unit).

Density gradient centrifugation. Cells and cell debris were removed from the homogenized lysate by centrifugation at 10 000 × *g* for 10 min on a cold centrifuge. The supernatant was centrifuged at 120 000 × *g* for 1 h, the pellet suspended in 0.01 M Tris · HCl buffer, pH 7.6, containing 0.01% Brij 58 and 1.35 mM EDTA, centrifuged again and resuspended as before. This crude membrane suspension was then layered on top of a linear sucrose gradient (27—50% sucrose, w/v, in Tris buffer) and centrifuged on a Beckman ultracentrifuge equipped with the SW 41 rotor at 208 000 × *g* for 17 h. The 2 bands which appeared were sucked off separately. The material was centrifuged down and washed once with Tris buffer and, if needed for sugar determinations, a second time with distilled water.

Disintegration of bacteria with French pressure cell. Washed bacteria were passed twice through a French pressure cell at 16 000 lb/sq. inch. Crude membranes were harvested and purified as described above, with the exception that Brij 58 and EDTA were omitted from the buffer.

Succinate dehydrogenase (EC 1.3.99.1) and NADH oxidase activities were measured as described recently [19].

Determination of membrane-bound cytochromes was done according to Osborn et al. [10]. As the type of cytochrome present in our bacteria was not known, an extinction coefficient of $E = 20$ [$mM^{-1} \cdot cm^{-1}$] was used, which is the one for cytochrome *c*. That of cytochrome *b* is $E = 19$ [$mM^{-1} \cdot cm^{-1}$] [20].

Total sugar content was measured as described by Hodge and Hofreiter [21] using phenol-H_2SO_4 reagent and glucose as a standard. The membranes were first precipitated with 7% trichloroacetic acid and then washed twice with distilled water.

2-Keto-3-deoxyoctonic acid was determined according to Osborn et al. [10] with 2-keto-3-deoxyoctonic acid as a standard. The red pigment which appeared during the reaction was extracted with butanol plus 5% HCl, and the extinction measured at 548 nm.

Pigments were extracted with acetone, transferred to dichloromethane, and separated on silica gel thin layers using the solvent system benzene/light petroleum (b.p. 60—80°C)/acetone (50 : 50 : 16, v/v) [2]. The separated pigments were then dissolved in acetone, and their quantities determined photometrically using as extinction coefficients: for flexirubin $E = 99.4$ [$mM^{-1} \cdot cm^{-1}$] [2], for zeaxanthin $E = 134$ [$mM^{-1} \cdot cm^{-1}$] [22].

Gel electrophoresis of proteins was performed according to Laemmli [23] with the modifications of Oelze et al. [13]. To dissociate the proteins from the membranes, samples were incubated for 7 min at 75°C. Molecular weights were estimated using a test set of protein standards (Boehringer, Mannheim). Protein bands were stained with 0.25% Coomassie Brillant Blue R250 in 50% methanol + 7% acetic acid (all w/w). Gels were destained in 30% methanol + 7.5% acetic acid.

TABLE I

ENZYME ACTIVITIES IN DIFFERENT MEMBRANE FRACTIONS FROM *FLEXIBACTER ELEGANS*

Figures are obtained from 3 independent experiments.

Bacterial strain	Method of cell disintegration and type of membrane fraction tested	Succinate-dehydrogenase (μmol 2,6 dichlorophenol-indophenol reduced/mg protein per min)	NADH-oxidase (μmol NADH oxidized/mg protein per min)
Fx e1	French pressure cell:		
	Crude membrane	0.09—0.15	n.t.
	Lysozyme/EDTA/Brij 58:		
	Crude membrane	0.19—0.3	0.02—0.03
	L Fraction	0.44—0.55	0.08—0.10
	H Fraction	0.01—0.02	0.003—0.004
Fx 3/4	Lysozyme/EDTA/Brij 58:		
	L Fraction	2.2—2.6	n.t.
	H Fraction	0.09—0.2	

n.t., not tested.

demonstrated only in the L fraction (Table II). The carbohydrate content with respect to protein was 3 times higher and the content of 2-keto-3-deoxyoctonic acid, a component typical for lipopolysaccharide, was as much as 13 times higher in the H fraction than in the L fraction (Table II). Thus, it was clear that the L fraction contained mainly material from the cytoplasmic membrane, while the H fraction consisted of material from the outer membrane.

The two fractions differed also considerably in their protein patterns, as revealed by gel electrophoresis (Fig. 2). Typical for the L fraction were the bands no. 10, 11, 13, 17 and 18, and especially 2, 6, 14, 15, with molecular weights of 114, 75, 28 and $25 \cdot 10^3$. Characteristic for the H fraction were the bands no. 1, 3—5, 7—9, 12 and 16, with no. 1 and 7 as the main proteins (with molecular weights of 120 and $70 \cdot 10^3$, respectively). The two fractions differed

TABLE II

COMPONENTS OF DIFFERENT MEMBRANE FRACTIONS FROM *FLEXIBACTER ELEGANS*

Figures are obtained from 3 independent experiments.

Bacterial strain	Method of cell disintegration and type of membrane fraction tested	Cytochrome content (nmol/mg protein)	Carbohydrate content (μg/mg protein)	2-Keto-3-deoxy-octonic acid (nmol/mg protein)	Flexirubin content (μmol/mg protein)	Cartenoic content (μmol/m protein)
Fx e1	French pressure cell:					
	Crude membrane	n.t.	n.t.	n.t.	2.6—4.4	
	Lysozyme/EDTA/Brij 58:					
	Crude membrane	n.t.	n.t.	n.t.	3.2—4.3	
	L Fraction	0.65—0.68	28—30	0.25—0.27	trace	
	H Fraction	n.d.	82—100	3.0—3.2	5.9—6.8	
Fx 3/4	Lysozyme/EDTA/Brij 58:					
	L Fraction	0.61—0.87	n.t.	n.t.		2.8—3.3
	H Fraction	0.0—0.03	n.t.	n.t.	10.7—13.3	

n.d., not detectable; n.t., not tested.

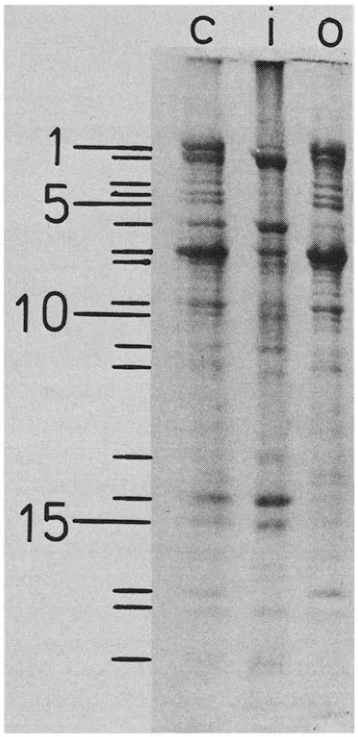
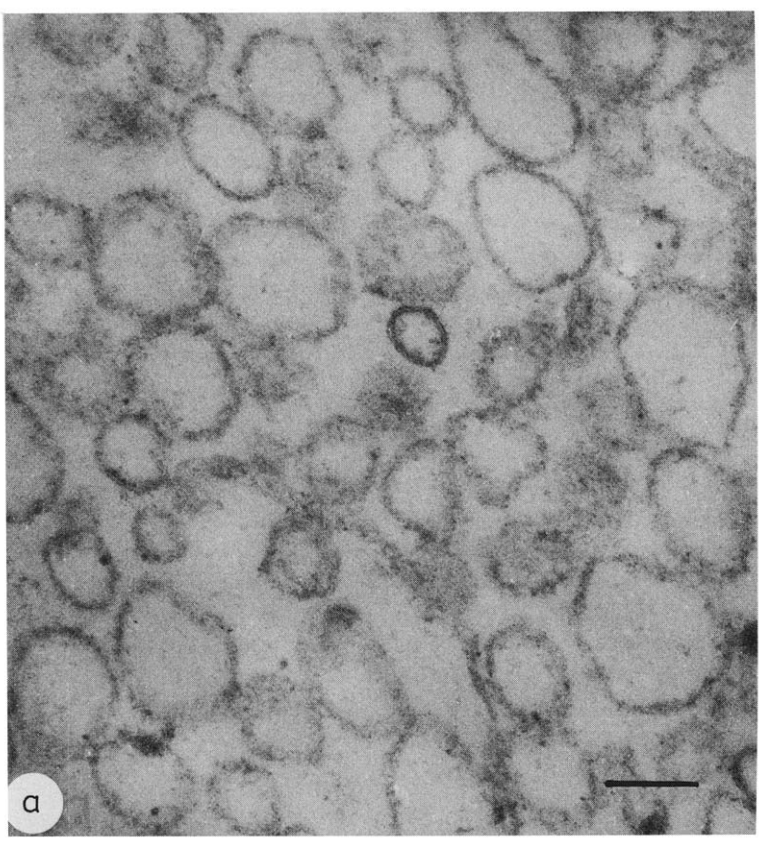


Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (according to Laemmli [23]) of membrane preparations from *Flexibacter elegans Fx* e1. Each track was loaded with 20 μg of protein. Start is at the top. c, crude membrane; i, inner membrane; o, outer membrane.



further in their electron microscopic appearance (Fig. 3). Both fractions consisted of vesicles, but the surface of the H fraction vesicles was distinct and of high contrast, while that of the L fraction vesicles was hazy and of low contrast. The pigment flexirubin was found predominantly in the H fraction (Table II).

To rule out an interference between Brij 58 and the lipophilic pigments, cells were disintegrated with a French pressure cell and crude membranes isolated without application of Brij 58. These membranes were compared with crude membranes, obtained by treatment with lysozyme-EDTA-Brij 58. There was no significant difference in flexirubin content nor in succinate dehydrogenase activity (Tables I and II).

Flexibacter strain *Fx* 3/4 contains carotenoids, mainly zeaxanthin, in addition to flexirubins. The membranes of this organism could be separated with the same methods. The L fraction had a buoyant density of 1.13 $g \cdot cm^{-3}$, the H fraction 1.175 $g \cdot cm^{-3}$. The typical membrane components separated with the 2 bands as before (Tables I and II). For unknown reasons succinate dehydrogenase activity of the L fraction of *Fx* 3/4 was 5—6 times higher than that in the corresponding fraction of *Fx* e1. With *Fx* 3/4 the flexirubins were

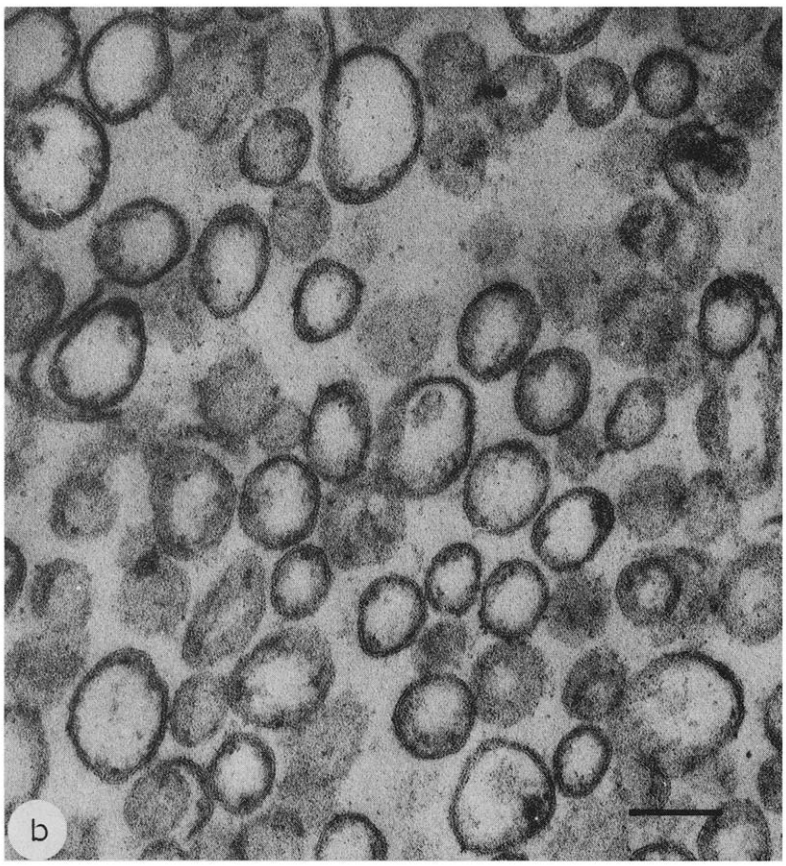


Fig. 3. Electron micrographs of membranes from the L (a) and from the H fraction (b) of *Flexibacter elegans Fx* e1. Preparation for electron microscopy was according to Oltmann and Stouthamer [11]. Micrographs were taken with a Zeiss EM 10 B at a magnification of 40 300×. Bar is 100 nm.

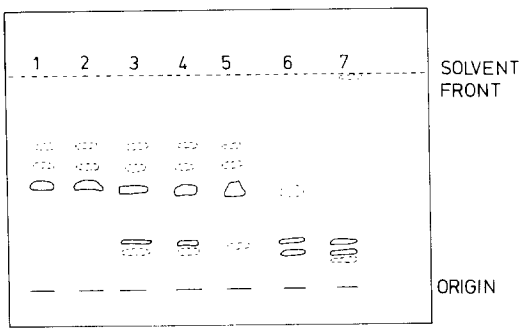


Fig. 4. The pigments from whole cells and from membrane fractions of *Flexibacter Fx* 3/4. Pigments were extracted with acetone and separated on a silica gel thin layer (Kieselgel 60 on aluminium foil, Merck, Darmstadt). Solvent system: benzene/light petroleum (b.p. 60—80°C)/acetone, (50 : 50 : 16, v/v). Pigments came from: 1, *Fx* e1, whole cells; spots are (from top): chlorflexirubin, mixture of several minor flexirubin pigments, flexirubin (main component). 2, *Fx* 3/4, whole cells grown in the dark, i.e. (almost) carotenoid free. 3, *Fx* 3/4, whole cells grown in the light; the additional spots at bottom are *cis*- and *trans*-zeaxanthin. 4, *Fx* 3/4, crude membranes, from cells grown in the light. 5, *Fx* 3/4, H fraction from cells grown in the light. 6, *Fx* 3/4, L fraction from cells grown in the light. 7, Zeaxanthin, standard containing as main components *trans*- and *cis*-zeaxanthin and, in addition, traces of a second *cis*-isomer and carotene hydrocarbons (near front).

also found predominantly in the H fraction, whereas the carotenoids were found mainly in the L fraction (Fig. 4).

Discussion

The membranes of several Gram-negative bacteria have recently been isolated, separated and characterized [8—18]. The buoyant density of the inner membranes of these organisms was generally around 1.14 $g \cdot cm^{-3}$, that of the outer membrane about 1.22 $g \cdot cm^{-3}$. The specific density of the L fraction of *F. elegans Fx* e1 was 1.14 $g \cdot cm^{-3}$ and, thus, fell neatly into the range known for the cytoplasmic membrane of other bacteria. The H fraction, however, showed a considerably lower density (1.175 $g \cdot cm^{-3}$) than expected. This low density cannot be explained by a contamination of the H fraction by the material from the inner membrane, for while the protein ratio between the H and the L fraction was 60 : 40, 95% of the succinate dehydrogenase activity was found in the L fraction, so that contamination of the outer membrane by the inner membrane can be assumed to be only 5%.

Equally, 95% of the 2-keto-3-deoxyoctonic acid was found in the H fraction: therefore, contamination of the inner membrane by outer membrane is also only 5%.

The specific density of the outer membrane of *Rhodospirillium rubrum* is high as compared with other microorganisms and it is assumed that this is due to a low phospholipid content of the *R. rubrum* outer membrane [13]. The lipophilic flexirubin appeared in a very high concentration in the outer membrane of *Flexibacter*: viz. about 4 mg of flexirubin per mg of protein (the molecular weight of flexirubin is 634). The flexirubin concentration is thus about 10 times as high as the phospholipid content in the outer membrane of ordinary Gram-negative bacteria [10,12,13]. This could explain the unusually

low density of the outer membrane of our *Flexibacter* strains.

Cytoplasmic membrane and outer membrane differed also in their protein patterns: as is usual with Gram-negative bacteria there were only a few main proteins (viz. nos. 1 and 7) in the outer membrane. However, the cell wall protein of molecular weight approx. 40 000, which is characteristic for many Gram-negative organisms [9—18], was lacking in *Flexibacter*.

The intracellular localization of pigments in Gram-negative bacteria has been determined for halophilic [27] and for photosynthesizing bacteria [28]. Carotenoids and bacteriochlorophylls were found in cytoplasmic and intracytoplasmic membranes. Flexirubin, in contrast, was retrieved almost completely from the H fraction, i.e., the outer membrane of *F. elegans Fx* e1. With *F. Fx* 3/4 which contained both flexirubins and carotenoids, we found the carotenoids in the inner, the flexirubins in the outer membrane. The small amounts of either pigment observed in the 'wrong' membranes were probably due to cross contaminations. While carotenoid synthesis by *Fx* 3/4 was stimulated by light, flexirubin synthesis was not (Fig. 4). Flexirubin synthesis is closely correlated with growth and can be influenced only in very narrow limits [2,29].

Our data make it unlikely that flexirubins function as photoprotective compounds, as do the carotenoids, nor can they be involved in the respiratory chain. Their high concentration in the outer membrane suggests that they may have a structural function, but definite proof of this is still lacking.

Acknowledgments

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