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MEMBRANE DIFFERENTIATION IN PHOTOTROPHICALLY GROWING *RHODOSPIRILLUM RUBRUM* DURING TRANSITION FROM LOW TO HIGH LIGHT INTENSITY

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SUMMARY

1. The effects of the transference of cells cultivated at low light intensities to high light intensity (8000 lux) on membranes of phototrophically growing *Rhodospirillum rubrum* were investigated.

2. For at least two generations no bacteriochlorophyll is formed. Thus the pigment contents of whole cells and intracytoplasmic membranes as well as the cellular contents of intracytoplasmic membranes decrease.

3. Radioactive label incorporated into intracytoplasmic membranes is not transferred into the cytoplasmic membrane fraction during pulse chase. However, there is a direct incorporation of label into both types of membranes under high light intensity.

4. Protein patterns of crude fractions composed of cytoplasmic and intracytoplasmic membranes reveal an increase of a protein (Zone B) typical for cytoplasmic membranes. Within purified intracytoplasmic membranes there is no such increase. Incorporation of labeled amino acids demonstrates a synthesis of all of the membrane proteins except those associated with bacteriochlorophyll synthesis (Zone G).

5. KCN-sensitive NADH oxidase increases in crude membrane fractions on a bacteriochlorophyll basis, it stays constant on a cellular protein basis. In purified intracytoplasmic membranes there is only a very slight increase on a bacteriochlorophyll basis and a decrease of the oxidase activity on a membrane protein basis.

6. Photophosphorylation activity increases slightly on a bacteriochlorophyll basis within crude membranes and purified intracytoplasmic membranes.

7. The results are compared with results obtained previously under aerobic conditions. Unlike aerobiosis, high light intensity and anaerobiosis result obviously in a conservation of the intracytoplasmic membranes and their functions. Cytoplasmic membranes are formed independent from intracytoplasmic membranes. Intracytoplasmic membranes are differentiated, structurally and functionally, but are not converted to cytoplasmic membranes.

INTRODUCTION

When growing anaerobically in the light the nonsulfur purple bacterium *Rhodospirillum rubrum* contains intracytoplasmic membranes in addition to cyto-

plasmic membranes, whereas cells cultivated under appropriate O_2 partial pressures lack the intracytoplasmic membranes[1].

Intracytoplasmic membranes are sites for photochemical electron transport and, in at least some phototrophic bacteria, they show low, but measurable respiratory chain activities[1,2]. Respiration is a typical function of cytoplasmic membranes. It has been demonstrated previously that intracytoplasmic and cytoplasmic membranes are not principally different types of membranes, but rather represent modifications which can be transformed into each other by a process of differentiation[3–5]. Differentiation of cytoplasmic membranes to intracytoplasmic membranes starts when aerobic dark-grown cells are transferred to anaerobic conditions in the light (or to semiaerobiosis)[3,6,7]. It continues preferentially within the invaginating intracytoplasmic membranes[3]. Conversely intracytoplasmic membranes are differentiated to cytoplasmic membranes upon transfer of anaerobic cells to aerobic conditions. The differentiation process can be measured by following the incorporation of proteins typical for the membrane being formed and by following the changes in specific physiological activities[4,5]. Such typical activities are photophosphorylation in the case of intracytoplasmic membranes[2] and the NADH oxidase system in the case of cytoplasmic membranes[8,9]. During differentiation of intracytoplasmic membranes to cytoplasmic membranes, intracytoplasmic membranes disappear as the cells continue to grow aerobically[4].

Since a decrease of the cellular contents of intracytoplasmic membranes can also be demonstrated when anaerobic cultures are transferred from low to high light intensities[10,11], it might be assumed that the light-induced and the oxygen-induced changes of the membrane contents are brought about by the same mechanisms. That this is apparently not the case will be reported by the present paper.

MATERIAL AND METHODS

R. rubrum, strain FR 1, was precultivated as before[5]. Anaerobically light-grown cells from the late phase of logarithmic growth were used as inoculum throughout the experiments. Cells in this phase are actually growing under low light intensity, due to a high degree of self-shadowing.

To adjust low light cultures to high light intensities a small inoculum (30–40 μg protein per ml) was transferred to fresh culture medium. Illumination was provided by Osram Opalin lamps arranged in a circle around the incubation vessel. The light intensity measured within the cylindrical culture vessel was adjusted to 8000 lux after inoculation. The culture was kept at 30 °C under continuous stirring and bubbling with N_2. Samples were withdrawn under sterile conditions by increasing the N_2 stream. For membrane isolation cells were sedimented, washed and homogenized by means of a French pressure cell two times at 16000 lb/inch^2. Homogenates were freed from cell debris at 15900 $\times g$ for 20 min. Supernatants were centrifuged at 313900$\times g$ for 60 min. The resulting sediment was used as the crude membrane fraction. Membrane purification on linear Ficoll gradients (Ficoll, Pharmacia, Uppsala, Sweden) was done according to published methods[12]. After centrifugation in Ficoll gradients two membrane-containing bands were obtained. The heavy one contains purified intracytoplasmic membranes and the light one is enriched in cytoplasmic membranes[12]. Whenever protein

compositions, radioactive labels and bacteriochlorophyll contents of membranes were determined, all steps were performed in Tris–HCl (pH 7.6, 0.02 M) *plus* 0.1% EDTA. Phosphate buffer (pH 7.8, 0.05 M) was used in all steps when NADH oxidase activity was estimated. In the case of photophosphorylation, membranes were isolated in glycylglycine buffer (0.1 M, pH 7.5) containing 2 mM $MgCl_2$, 0.25 mM sodium succinate and 10% sucrose.

Photophosphorylation was measured according to Cusanovich and Kamen[13]. KCN-sensitive NADH oxidase was determined as the difference of activity in the absence and presence of 0.01 M KCN[8,14].

Cultures used for pulse chase experiments under high light intensities were preincubated for about 20–24 h in normal culture medium supplemented with 0.05% acetate and 30 μCi [2^{14}-C]acetate per 50 ml. Acetate was shown to be incorporated preferentially into membrane phospholipids[15,16]. The experiments were started by inoculating radioactive cells into fresh culture medium containing 0.05% acetate. Membranes were labeled directly under high light conditions by incubating cells either with 60 μCi[2^{14}-C]acetate per 1.5 l of medium *plus* 0.05% acetate or with 50 μCi[U-^{14}C]-labeled protein hydrolyzate per 1.5 l of culture medium. Radioactive compounds were obtained from the Radiochemical Centre, Amersham, Great Britain.

Solubilization and gel electrophoretic separation of membrane proteins as well as techniques for protein localization and radioactive measurements within gels were carried out as previously described[4]. Bacteriochlorophyll concentrations were estimated in methanol according to Smith and Benitez[17]. Protein was determined by the Folin–phenol assay[18].

RESULTS

Bacteriochlorophyll contents of intracytoplasmic membranes

During the first two to three generations of adaptation from low to high light intensities, cells of *R. rubrum* grow without any synthesis of bacteriochlorophyll. Thus the amount of bacteriochlorophyll per cell protein decreases within two generations to about 25%, whereas in purified intracytoplasmic membranes the bacteriochlorophyll content decreases to about 50% of the initial values (Table I).

TABLE I

DECREASE OF BACTERIOCHLOROPHYLL CONTENTS OF WHOLE CELLS AND ISOLATED INTRACYTOPLASMIC MEMBRANES OF *R. RUBRUM* DURING TWO GENERATIONS OF GROWTH AT 8000 lux

For precultivation see Material and Methods.

t (h)	*Cell protein (mg/ml)*	*Bacteriochlorophyll (μg/mg)*	
		Per cell protein	*Per membrane protein*
0	0.024	18.3	94.7
6	0.1	4.7	44.7

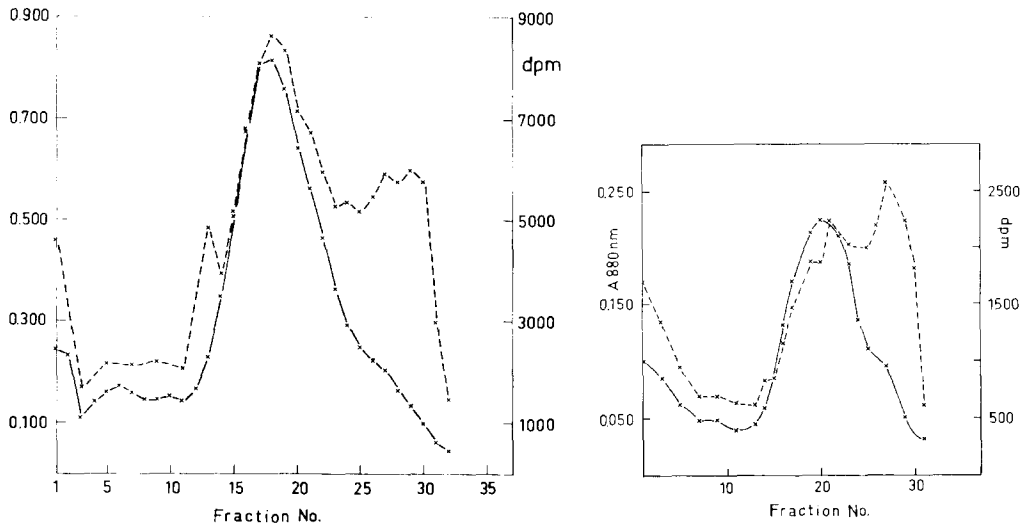


Fig. 1. Pulse chase of [2-^{14}C]acetate incorporated into membrane fractions of *R. rubrum*. Cultures were incubated for 20 h in the presence of 30 μCi [2-^{14}C]acetate per 50 ml. After this, one part of the culture was broken (A) and the second part was used as an inoculum for fresh culture medium (1.8 l) supplemented by 0.05% acetate. This culture was grown anaerobically for two generations at high light intensity (8000 lux) and then homogenized in buffer for membrane isolation (B). After separation of the membranes by means of Ficoll gradient centrifugation gradients were fractionated. Bacteriochlorophyll ($A_{880\,nm}$) and the radioactivity of each fraction was recorded. (×—×) $A_{880\,nm}$ for bacteriochlorophyll-containing membranes; (×– – –×), dpm of membrane-bound radioactivity.

Pulse chase with labeled membranes

The results of pulse chase experiments in which prelabeled low light intensity grown cells were transferred to high light intensity for 6 h are seen in Figs 1 A and B. The distribution of label and bacteriochlorophyll in the fractionated membranes of the original inoculum (Fig. 1A) and of cells after 6 h of high light intensity growth (Fig. 1B) was not significantly different. In both cases 67% of the membrane-bound label is found in the heavy band, while 33% is found in the light band. These values were calculated on the assumption that the distribution of radioactivity is symmetric in the heavy band. Since the two peaks in Fig. 1B are closer together, the lighter band has an apparent proportionately higher count than the heavy band, however, when the overlapping counts from the heavy band were subtracted, the 33% figure was obtained. This phenomenon was observed in every case and while the causes are not clear, it is assumed that there is a change in the density of the heavy band material during the 6 h of growth at high light intensity.

Labeling membranes with [2-^{14}C]acetate

To determine membrane synthesis under high light intensity cells were incubated in the presence of [2-^{14}C]acetate. After 6 h of cultivation membranes were isolated. According to Fig. 2, the label is incorporated into both cytoplasmic membranes (light band) and intracytoplasmic membranes (heavy band).

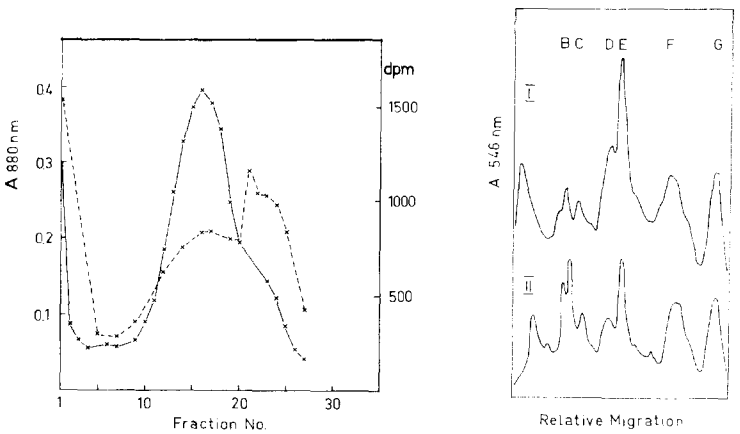


Fig. 2. Pulse labeling of membrane fractions of *R. rubrum* with [2-^{14}C]acetate. Cultures were precultivated for 24 h in the medium supplemented with 0.05% acetate. Fresh culture medium containing 0.05% acetate *plus* [2-^{14}C]acetate (50 μCi per 1.5 l) was inoculated with these cultures. The cells were grown anaerobically for two generations at 8000 lux, after which they were harvested and fractionated as before. For symbols see Fig. 1.

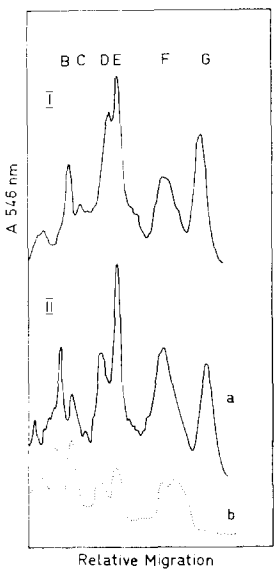
Fig. 3. Protein patterns of crude membrane fractions of anaerobically grown *R. rubrum*. Membrane fractions containing both cytoplasmic and intracytoplasmic membranes were isolated before (I) and after (II) two generations of growth under high light intensity (8000 lux). Protein zones were determined densitometrically after staining with amido Black.

Patterns and synthesis of membrane proteins

The protein patterns of the crude membrane fractions of low and high light intensity grown cells are shown in Fig. 3. Besides some minor changes within the patterns, Zone B clearly exhibits an increase relative to the other membrane proteins. In previous publications it has been reported that Zone B is typical for cytoplasmic membranes[1]. Although no increase of Zone B protein can be registered among proteins of purified intracytoplasmic membranes, Zone C increases whereas Zone G decreases (Fig. 4). Radioactive tracing of gels of purified intracytoplasmic membranes isolated from a high light culture grown in the presence of U-^{14}C-labeled protein hydrolyzate shows that all the typical proteins are synthesized except for those of Zone G (Fig. 4b). The proteins of Zone C are preferentially labeled under these conditions. It seems to be noteworthy that compared with the other proteins synthesis of Zone D and especially Zone E proteins is significantly reduced (Fig. 4b).

Activities of NADH oxidase and photophosphorylation

When cells are transferred from low to high light intensities, KCN-sensitive NADH oxidase activity increases on a bacteriochlorophyll basis in crude membrane fractions (Fig. 5). As calculated on a cellular protein basis, however, NADH oxidase activity remains constant. Photophosphorylation on the other hand increases significantly correlated to bacteriochlorophyll of the crude membranes (Fig. 6). On a cellular basis photophosphorylation decreases as the bacteriochlorophyll content decreases. Measurements with purified intracytoplasmic membranes



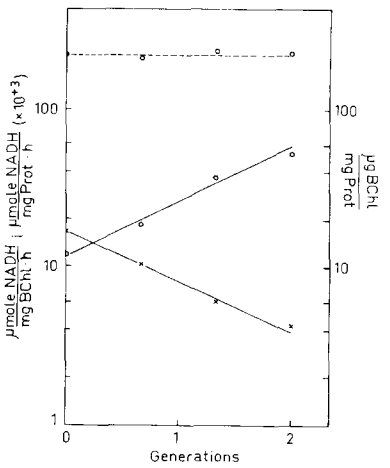


Fig. 4. Protein patterns of purified intracytoplasmic membranes before (I) and after (IIa) cultivation of *R. rubrum* for two generations anaerobically at high light intensity (8000 lux). Synthesis of membrane proteins was determined by incubating 1.5 l of culture in the presence of 50 μCi U-^{14}C-labeled protein hydrolyzate. After gel electrophoresis of membrane prroteins radioactivity was estimated within the gels by autoradiography (IIb). Protein patterns and the distribution of radioactivity were measured densitometrically.

Fig. 5. The effect of anaerobic growth under high light intensity (8000 lux) on KCN-sensitive NADH oxidase in crude membranes of *R. rubrum*. KCN-sensitive NADH oxidase: ○—○, μmoles NADH oxidized/mg bacteriochlorophyll per h; ○---○, μmoles NADH oxidized/mg of cell protein per h. Bacteriochlorophyll per cell protein (×—×) in μg per mg.

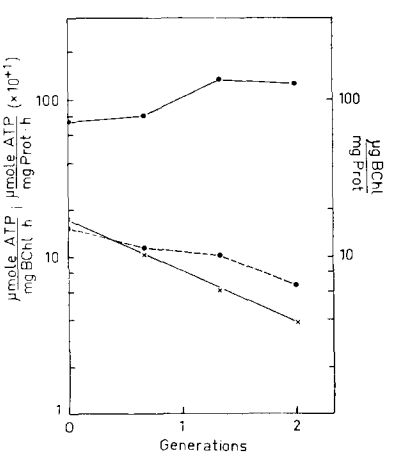


Fig. 6. Photophosphorylative activity of crude membranes of *R. rubrum* during anaerobic growth under high light intensity (8000 lux). Photophosphorylation: ●—●, μmoles ATP formed/mg bacteriochlorophyll per h; ●- - -● μmoles ATP formed/mg of cell protein per h. Bacteriochlorophyll per cell protein (×—×) in μg per mg.

show that NADH oxidase activity stays nearly constant per membrane protein (Table II). According to a decrease of the membrane-bound bacteriochlorophyll, NADH oxidase activity increases in intracytoplasmic membranes per unit of bacteriochlorophyll.

Photophosphorylation of intracytoplasmic membranes exhibits a small, but significant, increase on a bacteriochlorophyll basis during growth under high light intensity (Table II).

TABLE II

THE EFFECT OF HIGH LIGHT INTENSITY (8000 lux) ON ACTIVITIES OF ELECTRON TRANSPORT CHAINS BOUND TO INTRACYTOPLASMIC MEMBRANES OF *R. RUBRUM*

For precultivation at low light intensity see Material and Methods.

t(h)	*NADH oxidation*		*Photophosphorylation*
	nmole NADH/μg bacteriochlorophyll per min	*μmole NADH/mg protein per min*	*μmoles ATP/mg bacteriochlorophyll per min*
0	0.33	0.033	2.18
6	0.49	0.026	3.3

DISCUSSION

The intracytoplasmic membrane content of photosynthetically grown *R. rubrum* cells has been shown to decrease both when cultures are exposed to aerobic conditions[4] and when they are transferred from low to high light intensities under anaerobic conditions[10,11]. In the first case, O_2 inhibits bacteriochlorophyll synthesis and the formation of the photosynthetic apparatus ceases[19]. In the second case, bacteriochlorophyll synthesis stops until a critical level commensurate with the light intensity employed, is reached (Table I). Following this, bacteriochlorophyll synthesis begins again.

Previous investigations have shown that when anaerobically grown cells are transferred to aerobic conditions the following changes occur within intracytoplasmic membranes: (1). The bacteriochlorophyll content is lowered[4,5]: (2) there is a pronounced shift of label from the intracytoplasmic membranes to cytoplasmic membranes during pulse chase experiments[4]: (3) there is a predominant synthesis of the membrane proteins typical for cytoplasmic membranes[5]: (4) the NADH oxidase level, relative to both protein and bacteriochlorophyll increases[19]: (5) photophosphorylation remains constant per bacteriochlorophyll and thus declines relative to membrane protein[19].

These results have been interpreted in terms of a differentiation of intracytoplasmic membranes into cytoplasmic membranes[1].

In this study it has been shown that although the bacteriochlorophyll content decreases under high light intensity, the magnitude of change is different from that under aerobic conditions. Under aerobic conditions both cellular and membrane-bound bacteriochlorophyll decline by a factor of two during one generation[5].

However, under high light intensity the same decrease takes two generations (Table I). A further difference in the effects of aerobiosis and anaerobiosis under high light intensity is shown by the fact that in the presence of O_2 half of the label is transferred from the intracytoplasmic membrane fraction to the cytoplasmic membrane fraction[4], whereas there is no transfer of label between the two membrane fractions under high light intensity (Fig. 1A and 1B). Since direct labeling of the membrane phospholipids shows that both membrane systems are synthesized under high light conditions it is clear that a *de novo* synthesis of both membranes takes place under high light conditions.

Gel electrophoresis of solubilized membrane proteins is one of the most sensitive methods of registering changes in membrane composition. Several methods of separating intracytoplasmic membrane proteins have been applied successfully in the last few years[1,20–22], but in all cases, except for one protein[23], insight into possible functions of the different proteins has been obtained only by indirect means. Proteins of Zone B (Fig. 3) are typical for cytoplasmic membranes[5], accordingly, their quantity increases when NADH oxidase, one of the typical activities of the cytoplasmic membranes, increases. Crude membrane fractions are composed mainly of intracytoplasmic *plus* cytoplasmic membranes. When these fractions are investigated for changes in the protein pattern during growth at 8000 lux mainly proteins of Zone B exhibit an increase (Fig. 3). However, with purified intracytoplasmic membranes no increase in Zone B is seen (Fig. 4). In the latter case, all of the typical proteins except those within Zone G incorporate radioactivity from the protein hydrolyzate. Proteins of Zone G have been correlated before with bacteriochlorophyll or its synthesis[24], thus the lack of synthesis of G proteins under the present conditions when no bacteriochlorophyll is formed is in agreement with this interpretation. On the other hand, this result together with the fact that radioactive acetate is incorporated into the lipid framework of intracytoplasmic membranes opens a new aspect concerning regulation of intracytoplasmic membrane formation. Until now experimental data led to the conclusion that intracytoplasmic membranes could only be built up if bacteriochlorophyll was syntnesized[22,24]. Under high light intensity as described in this paper this is obviously not necessary. In this case the essential constituents of membranes are formed without any bacteriochlorophyll synthesis.

A considerable synthesis of Zone C proteins occurs which has not yet been correlated with any function. On the other hand synthesis of Zone D and E proteins is relatively low. It should be noted that under high light conditions the proteins of Zone B are not synthesized to a remarkably greater extent than the other intracytoplasmic membrane proteins as was the case for aerobic conditions. Under aerobic conditions the decrease of intracytoplasmic membrane-bound bacteriochlorophyll can be correlated with a preferential increase in Zone B proteins[5], however, under high light conditions this decrease must be due to a synthesis of nearly all of the proteins (Fig. 4).

Measurements of functions of the two membrane fractions support the structural results. Fig. 5 demonstrates the increase of KCN-sensitive NADH oxidase activity in crude membrane fractions. Different results on NADH oxidation in *R. rubrum* membranes are published in the literature[8,14]. However, there is agreement that most of the NADH is oxidized *via* an electron transport chain containing

cytochromes. There is further agreement that KCN-sensitive NADH oxidase represents the respiratory chain of aerobic dark cells. Calculated on a cell-protein basis NADH oxidase activity stays constant at 8000 lux (Fig. 5). On a bacteriochlorophyll basis it increases as the pigment content decreases. The same results were reported with cells growing aerobically either in dark or in light[19,25]. Therefore, the conclusion can be confirmed that in *R. rubrum* the respiratory activity remains unchanged regardless of cultural conditions and what is even more interesting, regardless of the intracytoplasmic membrane contents[14,19]. Since the NADH oxidase activity of isolated intracytoplasmic membranes does not increase after growth of two generations at high light intensity (Table II), it must be concluded that the increase observed in crude membrane preparations is due to an increase in the cytoplasmic membrane moiety. This is contrary to aerobic conditions where NADH oxidase activity increases in both membrane fractions[5,19].

Photophosphorylation shows a slight increase relative to bacteriochlorophyll in crude membranes as well as in intracytoplasmic membranes (Fig. 6). According to Aagaard and Sistrom[26] the ratio of reaction center to bulk bacteriochlorophyll is always a constant in *R. rubrum*. This implies that the increase of photophosphorylation cannot be due to either a transformation of bulk bacteriochlorophyll into reaction center bacteriochlorpohyll or to an unidentified small, but preferential, synthesis of reaction center bacteriochlorophyll. The effect can be explained by the investigations of Nishimura[27,28] who showed that the rate-limiting step for photochemical electron transport lies between cytochrome *b* and cytochrome *c*. Synthesis of all membrane proteins, except those of Zone G, makes it possible that rate-limiting constituents are formed under high light intensity even though no bacteriochlorophyll is synthesized (Fig. 6). This implies that the changes in photochemical activity depend on the concentration of specific electron transport catalysts. Moreover, it indicates that the photochemical apparatus is not quantitatively fixed in its composition.

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