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ELECTRON TRANSPORT IN THE MEMBRANE OF LUTOIDS FROM THE LATEX OF *HEVEA BRASILIENSIS*

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SUMMARY

1. An antimycin-insensitive NADH-cytochrome *c* oxidoreductase (E.C. 1.6.99.3) activity can be demonstrated in the membrane of lutoids isolated from the latex of *Hevea brasiliensis*. This electron transport system can also use ferricyanide as an electron acceptor, but is unable to oxidize NADPH.

2. Two *b*-type cytochromes are present in the membranes. Cytochrome b_{563} is partially reduced by NADH and ascorbate, but is not reducible by NADPH. It shows a double peak at 555 and 561 nm at 77 °K. A second cytochrome, cytochrome b_{561}, seems to be reducible by hydrosulfite only.

3. In the reduced state, these cytochromes do not combine with CO. The occurrence of cytochrome *P*-450 could not be demonstrated.

4. The role of the NADH oxidation system is considered in relation to the biosynthesis of polyisoprene compounds in the latex.

INTRODUCTION

The latex of *Hevea brasiliensis* can be considered as the true cytoplasm of the latex vessels [1–3], whose main function is to carry out isoprene and rubber synthesis. Its major inclusions are rubber particles, but latex also contains various cell organelles, amongst which lutoids dominate. Lutoids are small, vacuole-like organelles with a single membrane. They bear some analogy with the lysosomes of animal cells [4, 5].

Most studies on lutoids have been concerned with their morphological identification, ultrastructural features and enzymic activities [4–11]. Very few studies, however, have been devoted to the lutoid membrane itself [11,12] and, so far, no clear relation has been established between the presence of these particles and the particular biochemistry of the laticiferous cell or the biosynthesis of isoprene compounds [4–9].

Thus, a better knowledge of the metabolic role of these cell particles can be gained by studying the biochemical properties of the lutoid membrane itself together with those of the whole organelle. In this paper, we report on the presence of an electron transport system which is able to oxidize NADH.

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MATERIALS AND METHODS

Isolation of lutoids and preparation of lutoid membranes. The latex of *H. brasiliensis* L. (clones PR 107 and PB 86) was collected at the I.R.C.A. plantation, Languédédou (Ivory Coast). Fresh latex was collected, from *Hevea* trees, in glass vessels held in melting ice. The latex was either immediately processed or sent by air to Paris in containers kept at 0 °C.

Lutoids can be isolated by density gradient centrifugation [8, 9]. For immediate study, latex (2 ml) was laid on the top of 40-ml centrifuge tubes containing seven layers of sucrose solutions with concentrations ranging from 0.8 to 2.0 M by 0.2-M steps. The tubes were then spun at 80 000 × *g* for 120 min (Beckmann L3-50 Centrifuge, rotor SW 27). The lutoid particles were collected from each density interface, appropriately diluted and immediately used for enzymes assays.

In Paris, where larger amounts of lutoids were needed for spectrophotometric studies, another isolation technique was used [8]. The latex was first diluted four times with 20 mM phosphate buffer (pH 7.2) containing 0.3 M mannitol, to make the medium slightly hypertonic with respect to the inner compartment of the lutoids. The diluted latex was then centrifuged at 6000 × *g* for 10 min. The pellet was suspended in five times its volume of the same buffer and centrifuged at 4000 × *g* for 10 min. This operation was repeated four times in order to get rid of most of the rubber particles. The final pellet consisted most exclusively of intact lutoids; the other latex organelles, plastid-like "Frey-Wyssling particles" [3], being present in very small amounts [8].

Lutoid membranes were prepared by breaking lutoids by osmotic swelling. Intact lutoids were added to 10 times their volume of 3 mM phosphate buffer (pH 7.2), gently stirred at 4 °C for 30 min and then centrifuged at 90 000 × *g* for 30 min. The pellet was washed in the same medium and centrifuged again in the same way. The final suspension was made in 10 mM phosphate buffer (pH 7.2).

Preparation of cytoplasmic and lutoid sera. The cytosol phase of the latex, known as the "cytoplasmic serum", was prepared by centrifuging pure latex at 38 000 × *g* during 90 min. The clear supernatant (serum) was collected, lyophilized and kept at −30 °C.

The inner phase of the lutoids, known as the "lutoid serum", was prepared from the lutoid pellet of the above 38 000 × *g* centrifugation. The pellet was washed with 300 mM mannitol and resedimented at 4 000 × *g* for 10 min. It was then suspended in 0.1 % Triton X-114, stirred for 30 min and centrifuged at 18 000 × *g* for 15 min. The sediment was discarded and the supernatant lyophilized and kept at −30 °C.

Enzyme assays. Acid phosphatase was measured by following the increase in absorbance at 400 nm when *p*-nitrophenyl phosphate is hydrolyzed [13].

NAD(P)H-cytochrome *c* oxidoreductase activities were measured by following the reduction of cytochrome *c* at 550 nm [14] in a medium containing 300 mM mannitol, 10 mM phosphate buffer (pH 7.2), 50 μM cytochrome *c*, 1 mM NADH or 100 μM NADPH, 1 mM KCN and 50–100 μg of protein (in a volume of 3 ml). The reaction was started by adding NADH or NADPH.

NAD(P)H-ferricyanide oxidoreductase activities were assayed in the same medium as above except that 500 μM ferricyanide was substituted for cytochrome *c*. The decrease in absorbance at 420 nm was measured [14].

O_2 measurements. NAD(P)H oxidase activities were measured at 20 °C using a Clark oxygen electrode and the following medium: 300 mM mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate buffer (pH 7.2) and 1 g/l bovine serum albumin [15].

Cytochrome identification. Cytochromes were identified from reduced minus oxidized difference spectra at room or liquid nitrogen (77 °K) temperature (Aminco-Chance dual wavelength spectrophotometer). NADH, ascorbate or hydrosulfite were used as reductants. Cytochrome spectra were also made at room temperature, using the dual wavelength mode of the spectrophotometer. One wavelength was set at 575 nm, the other exploring the 540–575 nm region. The base line was carefully checked on the oxidized preparation, before addition of reductants.

For these studies intact lutoids or lutoid membranes were suspended in 10 mM phosphate buffer (pH 7.2). Sera were used by dissolving 50 mg of dry powder per ml of 10 mM phosphate buffer (pH 7.2).

Proteins were measured by mineralization and nesslerization [15].

NADH, NADPH and beef heart cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and *p*-nitrophenyl phosphate from Calbiochem, Los Angeles, Calif., U.S.A.

RESULTS

Evidence for the presence of an NADH-cytochrome c oxidoreductase activity

Lutoids prepared from fresh latex are distributed over the whole sucrose density gradient (Fig. 1). The distribution is highly dependent on the origin of the latex, indicating wide variations in the density of these organelles. However, the major fractions are found on top of the 1.4, 1.6 and 1.8 M sucrose layers, as shown by the distribution of acid phosphatase activity (Fig. 1), which is used as a marker enzyme for these organelles [8]. The lutoid fractions suffer no contamination by mitochondria as evidenced by the absence of cytochrome $a+a_3$ (cf. spectra of Fig. 3).

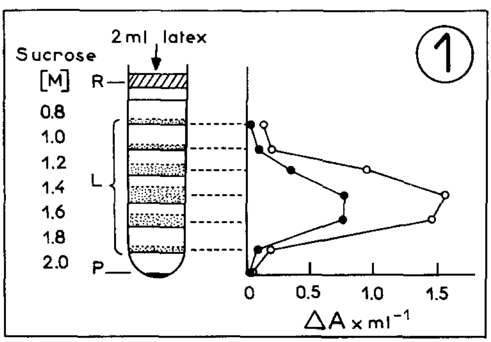

Fig. 1. Distribution of lutoids and of various enzyme activities after centrifugation of *H. brasiliensis* latex on a sucrose density gradient. See Materials and Methods for technical data. ○—○, acid phosphatase; ●—●, NADH-cytochrome *c* reductase. R, rubber layer on top of the centrifuge tube after centrifugation; L, lutoids; P, cell debris pellet; ΔA, increase in absorbance as measured for each enzyme activity. Lutoid fractions were removed and diluted to identical volumes. ΔA refers to 1 ml of the diluted lutoid suspension.

On the other hand, microsomes and plastid-like Frey-Wyssling particles do not sediment beyond the 0.8 M sucrose layer [8, 10]. Lutoids are thus satisfactorily purified, with the restriction that small rubber particles may still adhere to their membranes. This introduces rather large variations in their density, resulting in a loose distribution over the density gradient.

The distribution of NADH-cytochrome *c* oxidoreductase activity closely parallels that of acid phosphatase (Fig. 1). The same remark applies to the distribution of NADH-ferricyanide oxidoreductase activity, which has not been represented in Fig. 1. From these results it can be concluded that these reductase activities are closely associated with the lutoid particles.

In order to gain more information on the precise location of these activities, lutoids were osmotically lysed and membranes and lutoid serum collected separately. More than 70 % of the original NADH-cytochrome *c* oxidoreductase activity of intact lutoids were recovered in the membrane fraction whereas only about 20 % were present in the serum. The recovery of the activity after disruption appeared satisfactory, indicating that the osmotic treatment had only a slight influence on the measurement of the activity. However, it could not be excluded that the activity found in the serum did not result from a partial solubilization during osmotic treatment.

Since the reductase activity appears to be essentially located in membranes, all further experiments were carried out exclusively on purified lutoid membranes (cf. Materials and Methods).

Fig. 2 shows the kinetics of cytochrome *c* and ferricyanide reductions by these membranes. No reduction occurs unless NADH is added. Initial velocities are proportional to the amounts of lutoid membranes used in the assays. The NADH-cytochrome *c* oxidoreductase activity can use ferricyanide as an electron acceptor three times more efficiently than cytochrome *c* itself (Fig. 2). The enzyme activity was strongly resistant to inhibition by antimycin (less than 10 % inhibition), further indicating the absence of mitochondrial contamination. It could not use NADPH as an electron donor and did not react directly with oxygen.

It should be noticed that the reduction of cytochrome *c* in the presence of NADH is typically biphasic. However, contrary to what is observed with similar

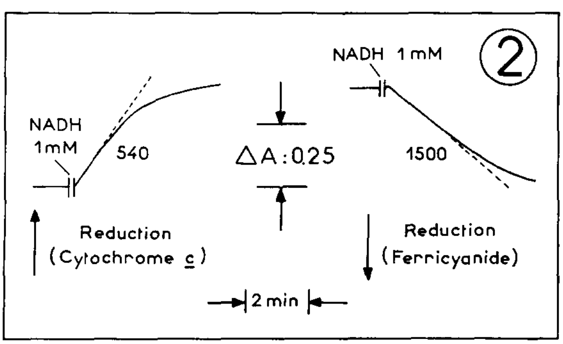

Fig. 2. Kinetics of cytochrome *c* and ferricyanide reductions by lutoid membranes from the latex of *H. basiliensis*. See Materials and Methods for technical data. Figures are specific activities in nmol · min^{-1} · mg $protein^{-1}$.

mitochondrial or microsomal enzyme activities, the kinetics are linear for only a short period of time and reach a plateau well before the total amount of cytochrome *c* present in the medium has been reduced. This could indicate that the accessibility of cytochrome *c* to the enzyme sites decreases as the reaction progresses. Such a behaviour could eventually be explained by the existence of strong interactions between exogenous cytochrome *c* and the lutoid membranes which are highly electronegative [7].

Evidence for the presence of cytochromes

At room temperature, after reduction by NADH (Fig. 3A), the reduced minus oxidized difference spectrum is typical of a *b*-type cytochrome with an α band at 563 nm, a β band at 534 nm and a γ band at 431 nm [16]. An identical difference spectrum can be obtained when ascorbate is used as a reductant. On the other hand, if the membrane preparation is reduced by hydrosulfite (Fig. 3B), the difference spectrum shows three similar absorption peaks whose intensities are greatly enhanced and positions shifted by 1–2 nm towards the ultraviolet. The difference spectrum between a hydrosulfite-reduced and an NADH-reduced preparation shows the same type of spectrum with α, β and γ peaks at 561, 530 and 429 nm, respectively (Fig. 3C). No presence of cytochrome $a+a_3$ or cytochrome *c* is detectable in the α or γ regions of these spectra, as an indication that no contamination by mitochondrial membranes occurs.

At liquid nitrogen temperature (77 °K) all peaks in difference spectra are

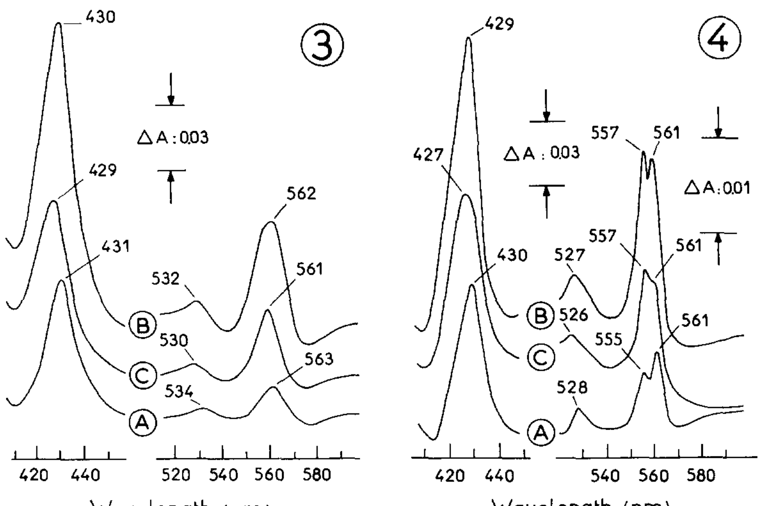

Fig. 3. Room temperature difference spectra of cytochromes in lutoid membranes from the latex of *H. brasiliensis*. 3-ml quartz cuvettes containing 3 mg/ml of membranes in 10 mM phosphate buffer (pH 7.2). Optical path, 10 mm. Oxidation is brought about by bubbling O_2 and reduction by addition of excess NADH or a few hydrosulfite crystals. A, NADH reduced minus O_2; B, hydrosulfite reduced minus O_2; C, hydrosulfite reduced minus NADH reduced.

Fig. 4. Low temperature (77 °K) difference spectra. Same conditions as in Fig. 3 except that 1-ml plexiglass cuvettes of 2 mm optical path were used. A, NADH reduced minus O_2; B, hydrosulfite reduced minus O_2; C, hydrosulfite reduced minus NADH reduced.

shifted by 1–2 nm towards the ultraviolet. A splitting of the α peaks is also observed. The absorption maxima are at 555 and 561 nm when NADH (or ascorbate) is used as a reductant (Fig. 4A), but at 557 and 561 nm in hydrosulfite-reduced preparations (Fig. 4B). Moreover, there is a difference in the relative heights of the peaks depending on the reducing conditions. In NADH (or ascorbate)-reduced preparations the peak at 561 nm is higher than the peak at 555 nm. By contrast, reduction by hydrosulfite brings about an increase in the heights of both peaks but also an inversion of their relative heights. A difference spectrum between a hydrosulfite-reduced and an NADH (or ascorbate)-reduced preparation shows an α peak at 557 nm with a shoulder at 561 nm (Fig. 4C).

These observations are confirmed by studies at room temperature using the dual wavelength technique (Fig. 5). Following reduction by NADH (or ascorbate), two absorption bands are found in the α region of the spectrum, one at 563 nm and the other as a shoulder around 556 nm (Fig. 5A). If the same preparation is then reduced by adding a small crystal of hydrosulfite, an asymmetrical peak of higher intensity appears with a maximum at 562 nm (Fig. 5B). The calculated difference spectrum (Fig. 5C) shows a single peak at 561 nm and corresponds to the fraction that is not reduced by NADH, but is reducible by hydrosulfite only. These spectra can be compared with those of Fig. 4. The absorption bands at 556 and 563 nm at room temperature (Fig. 5A) are the same as those at 555 and 561 nm at low temperature (Fig. 4A). Similarly, the peak at 561 nm at room temperature (Fig. 5C) must be identical with the composite peak at 557 nm at low temperature (Fig. 4C).

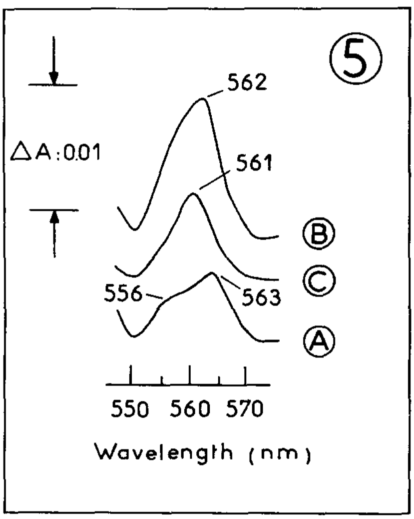

Fig. 5. Room temperature identification of *b*-type cytochromes in lutoid membranes from the latex of *H. brasiliensis*. A single 3-ml quartz cuvette containing 3 mg/ml of membranes in 10 mM phosphate buffer (pH 7.2) is used. Optical path, 10 mm. One fixed wavelength is set at 575 nm while the other wavelength varies from 540 to 575 nm. The membrane preparation is oxidized by bubbling O_2 and the base line is drawn under these conditions. The preparation is first reduced by addition of 20 μl of 150 mM NADH (spectrum A) and then further reduced by addition of a few hydrosulfite crystals (spectrum B). Spectrum C is the calculated difference spectrum between spectra B and A.

All these results suggest the presence of at least two *b*-type cytochromes. They can be named cytochromes b_{563} and b_{561} from the positions of their α peaks at room temperature (Figs 3 and 5) corresponding to α peaks at 561 and 557 nm at low temperature (Fig. 4). Among several explanations which can be offered concerning the properties of these cytochromes, we would favor the following interpretation. Cytochrome b_{563} shows a double α peak at 555 and 561 nm at 77 °K (Fig. 4A) and is reducible by NADH. However, the reduction of this cytochrome is not complete as shown by the extents of reduction in the α region between NADH or hydrosulfite-treated preparations (Figs 3–5). Reduction by hydrosulfite would bring about an extra-reduction of cytochrome b_{563} and a specific reduction of b_{561} (with a single peak at 557 nm at 77 °K). This could explain the shape of spectrum C in Fig. 4. Other explanations, however, could be provided: both cytochromes could be reduced by NADH to different extents and then be fully reduced by hydrosulfite. Whatever the true explanation, any interpretation of Figs 3–5 implies the presence of two *b*-type cytochromes in lutoid membranes.

A rough estimation of the concentrations of these cytochromes can be made, using the data of Figs 3 and 5 and the extinction coefficients generally accepted for cytochrome *b* [17]. The figures would be 0.21 and 0.33 nmol · mg protein^{-1} for cytochrome b_{563} and cytochrome b_{561}, respectively. These concentrations are in the range of cytochrome concentrations generally observed in mitochondrial or microsomal membranes [17, 18].

Difference spectra made at room or low temperature between two hydrosulfite-reduced preparations, after treatment of one of them by CO according to the technique of Omura and Sato [19] did not show the presence of any cytochrome able to combine with CO. Consequently, none of the two *b*-type cytochromes found in the lutoid membranes can be thought of as cytochrome *P*-450. Moreover, these cytochromes could not be reduced by NADPH, a fact in accordance with the absence of an NADPH-cytochrome *c* reductase activity in these membranes.

Difference spectra obtained with cytoplasmic or lutoid sera did not show the presence of any soluble cytochrome. This observation confirms the location of both *b*-type cytochromes in the lutoid membrane exclusively.

DISCUSSION

The occurrence of an NADH dehydrogenase activity (NADH-cytochrome *c* oxidoreductase, EC 1.6.99.3) in the membrane of lutoids from the latex of *H. brasiliensis* has been demonstrated. The properties of this enzyme complex can be compared with those of similar oxidation systems found in microsomal membranes from plant or animal cells [20, 21]. However, the system present in lutoid membranes is more specific since it cannot use NADPH as an electron donor.

Two *b*-type cytochromes are present in lutoid membranes. A cytochrome, whose α band at room temperature is located at 561 nm (cytochrome b_{561}), seems to be similar to cytochrome b_{561} found by Sichi and Hackett [22] in mung bean seedlings. The mung bean cytochrome, however, whose intracellular location has not been clearly established [16, 22], shows a single α peak at 77 °K and is ascorbate reducible, whereas cytochrome b_{561} from lutoid membranes seems to be mostly reducible by hydrosulfite only and shows a peak at 557 nm at low temperature. This adds new confusion to the problems of the nature and role of *b*-type cytochromes reduced by hydrosulfite only [23, 24].

A second cytochrome, whose α peak at room temperature is at 563 nm (cytochrome b_{563}) displays some properties in common with those of cytochrome b_5 (b_{555}) from microsomes of plant or animal cells [16, 23]. It is reduced by NADH and ascorbate; it does not bind CO in its reduced state; it is not oxidizable by O_2 and shows a double α peak at 77 °K. However, the peak positions are not identical in both types of cytochrome and, in addition, the latex cytochrome is not reducible by NADPH.

The existence in the lutoid membrane of two *b*-type cytochromes with rather specific characters can be related to the metabolic peculiarities of the latex vessels in which they are exclusively encountered. In this respect cytochrome b_{563}, and possibly cytochrome b_{561}, could play a key role in the biosynthesis of isoprene compounds by virtue of their being NADH reducible.

In the laticiferous cell, acetate, an essential precursor in rubber synthesis, originates from pyruvate or acetaldehyde produced by the glycolytic pathway. In the presence of an excess of NADH, lactate or alcohol are produced from these substrates, at the expense of acetate synthesis [24], thus impairing the synthesis of *cis*-polyisoprene. Therefore, a high level of rubber synthesis in the laticiferous cell implies that NADH produced by glycolysis be removed. In the latex vessel, one of the control mechanisms is the production of malate by reduction of oxaloacetate in the presence of a soluble malate dehydrogenase [24]. The NADH reductase activity found in the lutoid membrane could play a similar role provided a suitable, but so far unknown, electron acceptor is found.

In addition, in the laticiferous cell, the presence of NADPH is also required for the activity of β-hydroxymethylglutaryl-CoA dehydrogenase (EC 1.1.1.34) which produces mevalonic acid and regulates isoprene synthesis [25]. This requirement can also be correlated to the inability of the reductase system of the lutoid membrane to use NADPH as an electron donor. Therefore, it appears that the biochemical properties of this enzyme system are well fitted for its integration into the metabolism of the laticiferous cell and for its participation in the regulation of rubber synthesis.

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