Porphyrsynthesis in Isolated Particles from Tissue Cultures of Tobacco

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Particles from green and white tissue cultures of Nicotiana tabacum were fractionated by differential and density gradient centrifugation. Besides mitochondria, microbodies and (broken) chloroplasts particles were found able to metabolize 5-amino-levulinate to porphobilinogen and porphobilinogen to uroporphyrinogen. Succinyl-CoA was not synthetized by them but by mitochondria. The porphyrin forming particles had a density of about 1.21 g/cm³ and were accumulated in fractions better II, mitochondria (1.19 g/cm³) and microbodies (1.23 g/cm³). Small differences in density were found between particles from white and green tissue cultures. The data available suggest that the porphyrin synthetizing particles are proplastids or leucoplasts.

In the last few years various experiments with isolated organelles confirmed suggestions that chloroplasts and also etioplasts contain enzymes to synthetize early precursors of chlorophylls and porphyrins. The bulk of the enzymes are formed during maturation of these organelles which is controlled by light and development of the cell. We have no information, however, where porphyrsynthesis takes place before maturation. The enzymes involved in porphyrin synthesis are present in dark-grown, non-pigmented cells as well as in green ones. This is also the fact in white tissue cultures of Nicotiana tabacum in which the development of plastids has not reached the point of protochlorophyllide containing etioplasts with a prolamellar body. For that reason tissue cultures of Nicotiana tabacum were used to get informations about localization of porphyrin synthetizing enzymes before maturation of plastids.

Methods

Material and conditions of culture: The experiments were performed with colourless and green tissue cultures of Nicotiana tabacum var. "Samsun", which were grown at 25°C on the medium of MURASHIGE and SCOOG with 10⁻³ g/l naphtylactic acid and solidified by 0.8% of agar.

Fractionation: After harvesting the cells were washed on a Büchner funnel, suspended in buffered sucrose (1 : 1, w/v) which was composed as described by BREIDENBACH et al. and broken in a Potter-Elvehjem homogenizer. Particles were then separated by sucrose density gradient centrifugation (5 hours, 23x10⁵ rpm, Spinco SW 25.1 rotor) following differential centrifugation steps. Gradients were prepared from 30 to 60% sucrose.

Up to 28 fractions of about 1 ml of volume were removed from the bottom of the centrifuge tube by a puncture. After determination of the refraction index of sucrose the different particles in these fractions were detected by enzymes associated with these particles. (Density and refraction index (n) of sucrose are correlated by the formula: Density = 2.6314 n – 2.5800.)

Biochemical assays: Catalase was assayed by the method of LÜCK, succinic dehydrogenase according to HIATT with the use of extinction coefficients of 6.7 x 10⁴ cm²/mole for H₂O₂ (240 nm) and 1.56 x 10⁴ cm²/mole for dichlorphenol indophenol (600 nm), respectively. For succinyl-CoA-synthetase the method of KAUFPANN was used and the yield estimated by a standard curve. Porphobilinogen and uroporphyrin were assayed according to MAUZERALL and GRANICK and BOGORAD, respectively. The reactions mixtures used for the determination of 5-aminolevulinate dehydratase and the porphobilinogens deaminase/uro-

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phyrinogen-III-cosynthetase system (Porphobilinogenase) \(^{21}\) are identical with those described in a previous paper \(^{10}\).

**Determination of protein and chlorophyll:** Protein was determined by a modification of the method of LOWRY et al. \(^{22}\). Aliquots of the fractions were diluted with \(\text{H}_2\text{O}\) and precipitated by TCA to remove interfering sucrose. After centrifugation the supernatant was discarded and the precipitate solubilized by 0.2 ml of 1% sodium dodecyl sulfate.

Fractions containing chlorophyll were diluted (1 : 5) with 60% acetone and brought to 80% by pure acetone. Optical density was determined at 663 and 645 nm and chlorophyll estimated by means of a nomogram \(^{23}\).

**Results**

Particles from white and green tissue cultures separated by differential centrifugation exhibited very similar patterns of distribution after density centrifugation on sucrose gradients. Besides enzymatic activities in the supernatant (concentration of sucrose less than 30%) of the density gradient, three peaks of activity could be detected owing to three different enzymes (Figs. 1 and 2). In the region between mitochondria and microbodies which were usually characterized by succinic dehydrogenase and

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**Fig. 1.** Distribution of enzymatic activities, protein and chlorophyll from a particulate fraction of green tobacco tissue cultures after isopycnic density centrifugation on a continuous sucrose gradient. 100% correspond with 15 nmole of porphobilinogen formed per ml and hour (5-Aminolevulinate dehydratase), 40 nmole of succinohydroxamic acid formed per ml and hour (Succinyl-CoA-synthetase), 22 nmole of fumarate formed per ml and min (Succinic dehydrogenase), 48 \(\mu\)mole of \(\text{H}_2\text{O}_2\) destroyed per ml and min (Catalase), 1.5 mg of protein per ml gradient, 69.0 pg of chlorophyll per ml gradient.

**Fig. 2.** Distribution of enzymatic activities and protein from a particulate fraction of white tobacco tissue cultures after isopycnic density centrifugation on a continuous sucrose gradient. 100% correspond with 3.2 nmole of porphobilinogen formed per ml and hour (5-Aminolevulinate dehydratase), 64 nmole of fumarate formed per ml and min (Succinic dehydrogenase), 40 \(\mu\)mole of \(\text{H}_2\text{O}_2\) destroyed per ml and min (Catalase), 0.77 mg of protein per ml gradient.

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catalase, respectively, fractions were found which synthetized porphobilinogen from 5-aminolevulinate as substrate. The very same fractions metabolized porphobilinogen which was added to the samples to uroporphyrinogen (Fig. 3).

Fig. 3. Distribution of 5-aminolevulinate dehydratase and porphobilinogen deaminase/uroporphyrinogen-III-cosynthetase from a particulate fraction of green tobacco tissue cultures after isopycnic density centrifugation on a continuous sucrose gradient. 100% correspond with 1.9 nmole porphobilinogen/ml gradient × hour and 0.41 nmole uroporphyrinogen/ml gradient × hour, respectively.

Succinyl-CoA-synthetase, an enzyme possibly furnishing succinyl-CoA for 5-aminolevulinate synthetase, the first enzyme in the porphyrin synthesis chain, was found in the same fractions as succinic dehydrogenase. Only low activity of succinyl-CoA synthetase was present in the supernatant. Thus it may be inferred that succinyl-CoA synthetase is localized in mitochondria as it is known for animal tissues²⁴. Similar conclusions were drawn recently by STOBART and PINFIELD²⁵, which contradict the finding of NANDI and WAYGOOD²⁶ that succinyl-CoA synthetase is associated with chloroplast fractions.

In a great number of experiments the distribution graph of catalase activity shows a shoulder or a second peak in the region of fractions containing 5-aminolevulinate dehydratase. In some experiments carried out with white tissue cultures the peak of catalase even coincided with the peak of 5-aminolevulinate dehydratase. Particles from some non-green tissues, including ricinus endosperm of a species not known in detail, showed similar patterns of distribution. Therefore it cannot wholly excluded that low catalase activity is present in the porphyrin synthetizing particles.

Experiments with aged tissue cultures resulted in a very low yield of all particles. This may be in accordance with low protein content in these cultures.

The greatest portions of protein were accumulated in the fractions of the supernatant and the mitochondria. So far as the fractions containing chlorophyll were separated from the supernatant another protein peak or shoulder appeared in this region. The activity peaks of 5-aminolevulinate dehydratase and catalase were often situated on the slope of the mitochondria maximum without a distinct protein peak or a shoulder.

The chlorophyll peak always corresponds with the minimum of 5-aminolevulinate dehydratase activity on the gradient. Chloroplasts from tobacco tissue cultures scarcely can be isolated without damage in aqueous media⁹. Therefore it is not surprising that the peak of chlorophyll contains only broken chloroplasts. The porphyrin synthetizing particles which were accumulated in the fractions between mitochondria and microbodies should therefore be different from wholly differentiated chloroplasts, although a flat second maximum of chlorophyll could sometimes be detected in these fractions. 42% of 5-aminolevulinate dehydratase activity applied to the gradient accumulated in these fractions, but only 15% of the chlorophyll (these numbers correspond with about 6% activity and 2 to 3% chlorophyll of the whole homogenate). Moreover the position of 5-aminolevulinate dehydratase from white and green tissues is almost the same.

Besides the fact that microbodies are more stable when isolated from white tissue, another point is noticeable. In differential centrifugation experiments a smaller part of the over all activity of 5-aminolevulinate dehydratase is particulate using chlorophyll containing tissue cultures. This is understandable, if the higher activity of 5-aminolevulinate dehydratase of green tissues is localized in the

The distribution curve of this enzyme covers a wide range of densities and often exhibits a shoulder or a second peak in the region of the porphyrin synthesizing particles. On the other hand there is no difference in stability of porphyrin synthesizing particles which are already isolated by differential centrifugation. 44.8 and 42.0%, respectively, were recovered in density gradient experiments (Tab. 1).

The densities of mitochondria, microbodies and 5-aminolevulinate dehydratase particles are different with respect to their origin from white or green tissue cultures. Although the statistic significance is not high in all cases, mitochondria and 5-aminolevulinate dehydratase particles from white tissue cultures are to all appearances heavier than those from green ones. Microbodies show an inverse effect. They are heavier in green cultures. Parallel to increasing densities of particulate catalase, there was the tendency to a lower yield of these particles.

The densities of mitochondria from tobacco tissue cultures are in good agreement with those from ricinus endosperm and other tissues assayed in our laboratory. Microbodies and 5-aminolevulinate dehydratase particles from tobacco tissue cultures, however, can only be compared with microbodies (specific density 1.25) and proplastids (spec. d. 1.23) from ricinus endosperm with respect to their relative position on the sucrose density gradients.

**Discussion**

Tissue cultures of *Nicotiana tabacum* which were grown in light show metabolic activities and growing rates different from dark-grown cultures. To all appearances these differences extend also to the cell organelles of these cultures. Isopycnic centrifugation experiments show that the densities of particles are not identical in white and green tissues. Comparable effects are reported from young and aged spadix appendices of *Arum* and *Sauromatum*. The cell organelles seem to be formed and provided with enzymes according to cell development and necessity. Even within the cultures the densities of the particles are not wholly constant, especially those of microbodies as characterized by catalase. The distribution curve of this enzyme covers a wide range of densities and often exhibits a shoulder or a second peak in the region of the porphyrin synthesizing particles. On the other hand, the densities of mitochondria are less variable.

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**Table 1. Densities of particulate fractions from tobacco tissue cultures.**

<table>
<thead>
<tr>
<th>Enzyme/Particle</th>
<th>White</th>
<th>Green</th>
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<tbody>
<tr>
<td>Succinic dehydrogenase (mitochondria)</td>
<td>80.0 ± 0.00a</td>
<td>80.0 ± 0.00a</td>
</tr>
<tr>
<td>5-aminolevulinate dehydratase (catalase)</td>
<td>33.4 ± 2.4</td>
<td>33.4 ± 2.4</td>
</tr>
<tr>
<td>5-aminolevulinate dehydratase (proplastids)</td>
<td>29.1 ± 2.5</td>
<td>29.1 ± 2.5</td>
</tr>
<tr>
<td>Microbodies</td>
<td>27.6 ± 7.5</td>
<td>27.6 ± 7.5</td>
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**Note:** The densities of mitochondrial and catalase (microbodies) enzymes correspond to the activity gradient centrifugation. n = number of experiments, n = number of density gradients.
Except mitochondria, particles isolated from cells are very fragile. Therefore, the percentage of particulate 5-aminolevulinate dehydratase in non-pigmented tissues is surely higher than one third, as it was found after differential centrifugation. But until now there are no criteria to distinguish between soluble and particulate 5-aminolevulinate dehydratase on an enzyme level. In addition it is a problem, wether the particles characterized by ALA dehydratase and porphobilinogenase are inactive enzyme compartments or active stations in the biosynthesis of porphyrins for organelles like mitochondria and microbodies which do not possess these enzymes. Although the early stages of porphyrin-synthesis take place in these particles, succinyl-CoA-synthetase is localized in the mitochondria. Considering the relative position on density gradients and the particulate 5-aminolevulinate dehydratase activity in gradients of rizinus endosperm porphyrin synthetizing particles can best be compared with the proplastids isolated and identified by BREIDENBACH et al., although plastids from white tissue cultures of tobacco are different in morphology and do not show red fluorescence. Similarity of these two kinds of particles would mean that the ability to synthesize porphyrins is already present in proplastids and leucoplasts. Moreover 5-aminolevulinate dehydratase and porphobilinogenase could serve as markerenzymes for these organelles, hitherto not characterized by biochemical assays.

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Über den Katabolismus von Hamamelose [2-C-(Hydroxymethyl)-D-ribose]

I. Die Oxidation von Hamamelose durch Pseudomonas

On the Catabolism of Hamamelose [2-C-(hydroxymethyl)-D-ribose]

I. The oxidation of Hamamelose by Pseudomonas

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Free hamamelose [2-C-(hydroxymethyl)-D-ribose] occurs in almost all higher plants, but except a reduction to the corresponding sugar alcohol (hamamelitol), no metabolism of this branched chain hexose could be detected in plants until now. Therefore we tried to find microorganisms which would allow us to study the catabolism of hamamelose. A strain of Pseudomonas ("H 1"), showing dependence between growth and hamamelose concentration in the medium (Fig. 1), was isolated from soil on which Primula clusiana was grown (this plant contains large amounts of hamamelose and hamamelitol). However, this organism needs citrate besides hamamelose for growth. When "H 1" was incubated in a phosphate buffer containing only 14C-labelled hamamelose, hamamelonic acid was the sole radioactive product formed (Tables 2, 3, 4).

As no further degradation of hamamelonic acid by "H 1" could be detected, we conclude that this organism uses hamamelose as a hydrogen source only. Thus it becomes reasonable that "H 1" needs an additional carbon source (citrate) for growth.

Untersuchungen von SELLMAIR und VAN SCHERPENBERG et al. haben es wahrscheinlich gemacht, daß freie Hamamelose [2-C-(hydroxymethyl)-D-Ribose] in allen höheren Pflanzen gebildet wird. In den meisten der 560 untersuchten Arten ist jedoch der Pool dieser Hexose so klein, daß ein sicherer Nachweis nur mit der Isotopenmethode möglich war. Primulaceen allerdings synthetisieren Hama-