Studies Supporting the Concept of Glyoxyperoxisomes as Intermediary Organelles in Transformation of Glyoxysomes into Peroxisomes

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Intermediary forms of microbodies, glyoxyperoxisomes, are most likely synthesized at the stage of replacement of glyoxysomes by peroxisomes during the greening of fatstoring cells of cucumber cotyledons. This hypothesis is supported by three lines of results: 1. The levels of both glycollate oxidase and malate synthase are increased in the microbody fractions upon illumination of cotyledons. 2. Both peroxisomal and glyoxysomal marker enzymes are localized in organelles which exhibit the same buoyant densities. 3. *De novo* synthesis of malate synthase takes place even when irradiation already leads to a drastic increase of glycollate oxidase. The amount of malate synthase synthesized is almost identical in the light-induced system and in the dark control.

Introduction

In cases of epigeous germination cotyledons mobilize their storage compound and the plant grows heterotrophically in the dark. Some time later when the cotyledons come up to zones with light, metabolism and cellular organization in the cotyledons are switched to photoautotrophic conditions. During this transition, the fat-metabolizing glyoxysomes [1–4] disappear while other forms of microbodies, peroxisomes, can be detected. Finally, a stage is reached where in a green leaf, with respect to microbodies, only leaf-peroxisomes function and participate in the photomediated glycollate synthesis and metabolism [5, 6].

On the basis of electron micrographic investigations Schopfer *et al.* [7] concluded that peroxisomes of greening cotyledons ultimately originate as products of the gradual replacement of glyoxysomes by microbodies of intermediary character (glyoxyperoxisomes) and then by organelles of predominantly peroxisomal properties. According to that proposal, peroxisomes should not represent an independent population which abruptly originates *de novo*, and should also not be formed by loss of one set of enzymes and the insertation of other enzymes while the envelope of the organelle remains intact. This new concept of glyoxyperoxisomes contrasts with 2 other hypotheses, put forward earlier, that peroxisomes originate either (a) directly from glyoxysomes by specific exchange of enzymes [8] or (b) by gradual replacement, an immediate degradation of one population glyoxysomes and a concomitant *de novo* assembling of a new population, peroxisomes [9].

In this communication we demonstrate, by means of biochemical methods, that during the early stage of peroxisome formation synthesis of glyoxysomal enzymes continues. This would not be expected if, upon light-treatment, the synthesis of glyoxysomes is switched off immediately and microbodies are repacked with peroxisomal proteins ("one population hypothesis") or if one form of microbodies is substituted by another ("two population hypothesis"), that involves a prompt stop of the synthesis of the first population and its specific and continuous degradation by the lytic compartment.

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Abbreviations: EDTA, Na_2-ethylenediaminotetraacetate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-glycine; SDS, sodium dodecyl sulfate.

Materials and Methods

Plant material and growth conditions

Seeds of *Cucumis sativus* (Chinesische Schlangengurken) were soaked for one hour, surface-sterilized with NaOCl, rinsed with demineralized water and spread on moist filter paper in Petri dishes. The seeds were germinated in the dark at 27 °C. When indicated, 3 day old seedlings were exponsed to continuous white light (7,000 lx).

Preparation of cell-free extracts

Fifty cotyledons, selected for uniformity, were homogenized (4×15 s) in 30 ml of grinding me-

dium using an Ultraturrax blendor at half-maximal speed (10,000 min^{-1}). The grinding medium contained 100 mM Tricine, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 0.3 M sucrose. The pH was adjusted to 7.5. The crude homogenate was squeezed through two layers of miracloth and centrifuged at 10 000 x*g* for 30 min. After centrifugation, the pellet was suspended in 5 ml of grinding medium, and both, supernatant and resuspended pellet, used for enzyme assays.

Isolation of microbodies

One hundred cotyledons, either etiolated or green, were finely chopped with a razor blade for 20 min in 5 ml of a grinding medium containing 150 mM Tricine (pH 7.5), 10 mM KCl, 1 mM EDTA and 0.4 M sucrose. The crude homogenate was squeezed through miracloth and the filtrate layered on top of a discontinuous gradient consisting of the following sucrose solutions prepared in 50 mM Tricine (pH 7.5) and 1 mM EDTA: 3 ml 2.3 M, 5 ml 2.1 M, 5 ml 1.8 M, 6 ml 1.6 M, 2 ml 1.4 M, 4 ml 1.2 M, 3 ml 1.0 M and 5 ml 0.6 M sucrose. The gradients were centrifuged for 3 h at 27,000 min^{-1} using a SW-27 rotor (Beckman), and subsequently fractionated by collecting 0.6 ml samples with an Isco Model 680 density gradient fractionator.

Preparation of antiserum

Rabbit antiserum was prepared against malate synthase purified as described elsewhere [10]. The antigen was obtained from glyoxysomes of 5 day old etiolated cotyledons of *Cucumis sativus*. The homogeneous enzyme (200 µg) was emulsified with complete Freund's adjuvant (1.0 ml) and injected subcutaneously. At intervals of one week the injection was repeated three times with 100 µg of enzyme. After another week, blood was taken from an ear vein. A γ-globulin fraction was obtained by fractionation with ammonium sulfate. The titre was 1.0 ml serum per 1 mg antigen. Double immunodiffusion was used to demonstrate that we were dealing with a monovalent serum.

Application of [^{3}H]glycine

Seedlings of *C. sativus* were grown in the dark for 3 days and then exposed to white light (7,000 lx) for 8 h. After this preillumination period, one hundred cotyledons were separated from the hypocotyls and placed into 5 ml of water containing 0.5 mCi [^{3}H]glycine (1 Ci/mmol). The application was performed under continuous light for a period of 16 h. A dark-control was treated in the same way, but without preillumination and continuous illumination.

Isolation of malate synthase

Organelles from the labelled cotyledons were prepared as described above. Samples from sucrose gradients containing microbodies were pooled and diluted with the double volume of a 0.20 M KCl solution. After 6 h incubation, the preparation was centrifuged at 40,000 min^{-1} (Beckman, Ti-60 rotor) for 60 min. The supernatant was mixed with antiserum sufficient for quantitative precipitation and left standing overnight at 2 °C. The resulting precipitate was isolated by centrifugation at 40,000 rpm (Beckman Ti-60 rotor) for 60 min.

SDS-polyacrylamide gel electrophoresis

The immunoprecipitate was dissolved in a SDS-mixture according to Laemmli [11]. Electrophoresis was performed in a slab gel apparatus using the discontinuous system, described by Laemmli [11]. After staining and destaining, the traces of the frozen gel-slabs were cut into 1.5 mm pieces. The zones, except those containing the subunits of malate synthase, were covered with 1 ml of a 10 mM SDS-solution and shaken overnight. For determination of absolute radioactivity, 10 ml of Quickszint 212 (Zinsser, Frankfurt) were added. Malate synthase-containing slices were dried on filter papers and then oxidized in a Searle-Combustor model 6550.

Enzyme assays and other determinations

Malate synthase (EC 4.1.3.2) was assayed as described previously [10]. Glycollate oxidase (EC 1.1.3.1) was determined according to Feierabend and Beevers [12]. Chlorophyll was estimated according to Arnon [13] Absorbance at 280 nm was monitored by an Isco UA-4 absorbance monitor and sucrose density was determined refractomatrically. Radioactivity was measured in a scintillation spectrometer (Berthold-Friesecke, BF 5001) equipped with an Alphatronic computer (Diehl). Absolute radioactivity was calculated by a program based on channel ratio methods.

Results

Before *de novo* synthesis of intermediary microbody species could be studied, experiments were performed to clarify whether cucumber cotyledons behave similar as greening cotyledons of sunflower [14, 15] or watermelon [9]. This first set of experiments also assumes importance in view of the required knowledge about the time periods when the transformation of heterotrophic to autotrophic metabolism takes place under the conditions employed. The second part of experiments deals with the incorporation of precursor amino acid into a glyoxysomal enzyme, malate synthase, at a time when cotyledons were already greened. Basic knowledge of the properties of malate synthase from 5 day old or 3 day old cucumber seedlings was already available [10, 16].

Appearance of peroxisomal activities prior to and after irradiation

A survey of changes in enzyme activities measured in homogenates from cotyledons is given in Fig. 1. When 3 day old dark grown seedlings were illuminated, the activity of malate synthase was not changed significantly while the activity of glycollate oxidase increased markedly upon illumination, after a lag period of 6 h.

Furthermore, we analyzed cell structures of cotyledons by isopycnic gradient centrifugation. Firstly, when seedlings were germinated and grown for 3 days in the dark (Fig. 2), and secondly, after transferring 3 day old seedlings into light and thus exposing them during a period of time – 12 h (Fig. 3) or 24 h (Fig. 4) – under continuous light.

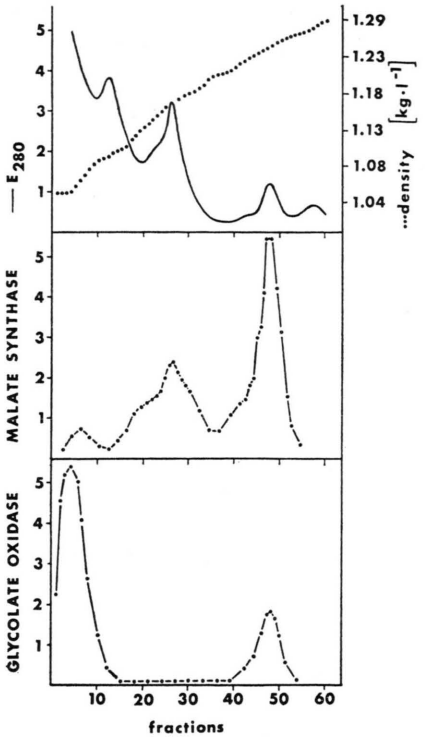

Fig. 2. Sucrose density gradient separation of a crude homogenate obtained from cotyledons (3 days dark). 1 arbitrary unit: 0.25 (E_{280}), 1875 pkat ml^{-1} (malate synthase), 250 pkat ml^{-1} (glycollate oxidase). The profiles represent the recovery from 100 cotyledons.

The experiments show that activity of peroxisomal enzymes, as examplified by glycollate oxidase, is already present when cells function under heterotrophic conditions. A sinificant proportion of the enzyme was confined to glyoxysomes buoying at density of 1.245 kg/l (Fig. 2).

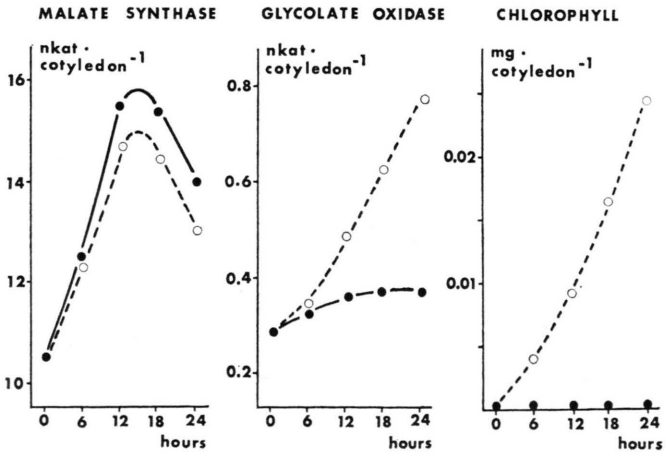

Fig. 1. Developmental changes in activity of malate synthase and glycollate oxidase in cotyledons of 3 day old dark grown seedlings transferred to continuous light. ○—○ light, ●—● dark.

Figs. 3 and 4 demonstrate that during these early stages of transition of glyoxysome-containing cells into fully autotrophic cotyledons the mean equilibrium densites of peroxisomal and glyoxysomal microbody forms are apparently the same. As to the total enzyme activities we emphasize the different scales on the ordinate in the various figures.

To survey the developmental changes in cell structures the percentage of malate synthase and glycollate oxidase attributable to microbodies are summarized in Table I. The Table includes data from one gradient not shown and also, for comparison, total activities obtained from homogenates. The data reveal that the proportion of glycollate oxidase which is located at the position of microbodies is significantly increased.

Table I. Time course of enzyme activities during illumination. Three day old dark grown seedlings were transferred to continuous light for the period indicated. Total activities were determined in cell-free extracts after a 1-min homogenation of cotyledons. Percentage values were calculated from data obtained by isopycnic sucrose density separations.

Illumination [h]	Malate synthase		Glycollate oxidase	
	Total activity [nkat/100 cotyledons]	Percentage bound to microbodies	Total activity [nkat/100 cotyledons]	Percentage bound to microbodies
0	363	55	23	23
6	410	56	30	29
12	489	61	48	32
18	477	64	65	40
24	430	65	88	43

Synthesis of malate synthase in a time period 8 – 24 h after light-induced change of metabolism

Seedlings of cucumber were grown in the dark for 3 days and then exposed to continuous white light for a period of 8 h. Subsequently, the cotyledons were excised and incubated, for 16 h in the presence of light, with ^{3}H-labelled glycine. Then, organelles were isolated according to their equilibrium density, and the respective malate synthase-containing fractions were subjected to immuno-

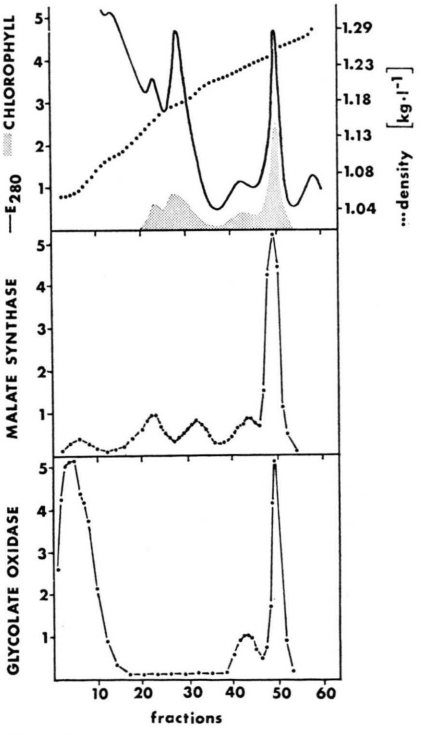

Fig. 3. Sucrose density gradient separation of a crude homogenate obtained from cotyledons (3 days dark + 12 h light). 1 arbitrary unit: 0.25 (E_{280}), 0.04 mg ml^{-1} (chlorophyll), 6250 pkat ml^{-1} (malate synthase), 420 pkat ml^{-1} (glycollate oxidase). The profiles represent the recovery from 100 cotyledons.

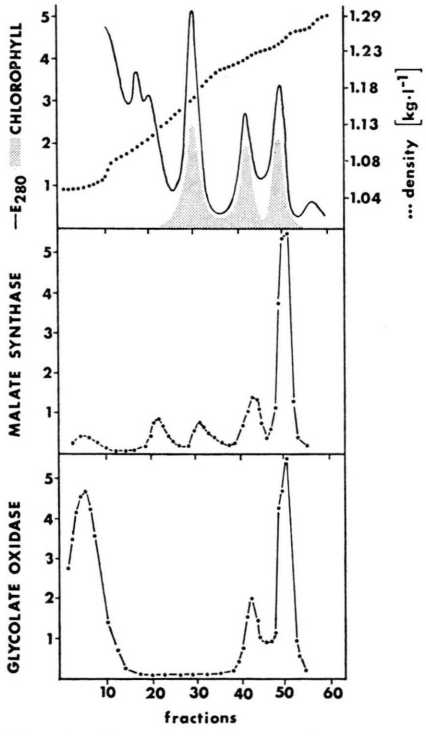

Fig. 4. Sucrose density gradient separation of a crude homogenate obtained from cotyledons (3 days dark + 24 h light). 1 arbitrary unit: 0.25 (E_{280}), 0.06 mg ml^{-1} (chlorophyll), 4200 pkat ml^{-1} (malate synthase), 550 pkat ml^{-1} (glycollate oxidase). The profiles represent the recovery from 100 cotyledons.

precipitation by antibodies raised against malate synthase from glyoxysomes of 5 day old etiolated cotyledons.

Figs. 5 and 6 give the radioactivity profiles of gradients obtained by centrifugation of homogenates from etiolated cotyledons – for reason of comparison – and from cotyledons after 8 h of light adaption. In both cases, the time for incorporation of radioactivity, either in the presence or absence of light, was 16 h. Two positions in the gradients, labelled I and II, were chosed to be analyzed for radioactive malate synthase. We set great store by to demonstrate unequivocally that the immunoprecipitate contains only radioactivity which is attributable to the 63 kd subunit of malate synthase [10]. This is shown in Fig. 7 which describes the electrophoretic analysis of the immunoprecipitates.

Upon quantitative comparison of radioactive malate synthase formed in light-treated cotyledons *versus* etiolated cotyledons the data give evidence that (a) malate synthase is formed also during the middle of the greening phase, and (b) the

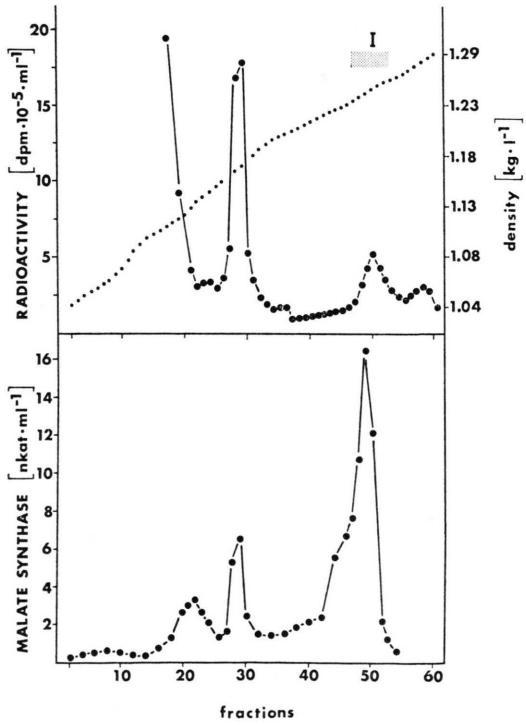

Fig. 5. Profiles of radioactivity and malate synthase after sucrose density separation of a crude homogenate obtained from cotyledons labelled with [^{3}H]glycine in the dark.

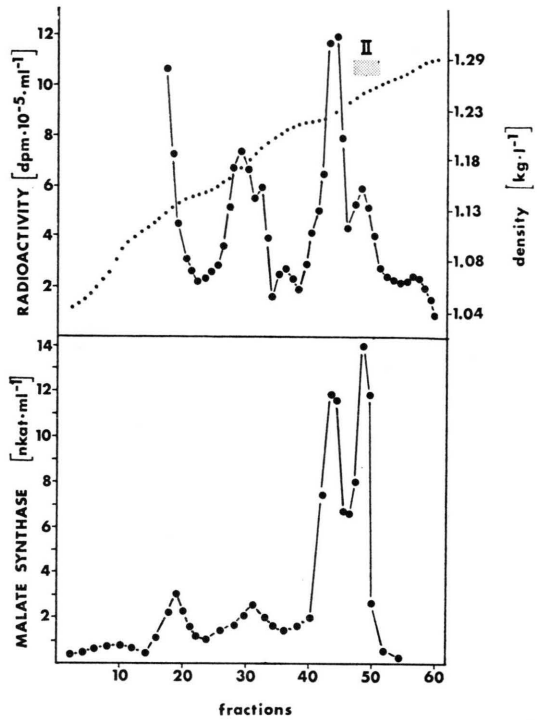

Fig. 6. Profiles of radioactivity and malate synthase after sucrose density separation of a crude homogenate obtained from cotyledons labelled with [^{3}H]glycine under continuous light.

amount of newly synthesized malate synthase (Fig. 7) is only slightly smaller relative to the one in the stage of maximal glyoxysomal activity. But when we related the radioactivity to the enzyme activity measured prior to immunoprecipitation we found almost exactly the same values 68.8 dpm/nkat for malate synthase from etiolated cotyledons (fraction I in Fig. 5) and 70.1 dpm/nkat for the enzyme of greened cotyledons (fraction II in Fig. 6). As to the level of total enzyme activity in the microbody-containing fractions, it should be emphasized that the amount of malate synthase is even slightly higher in the case of green cotyledons (55 nkat) compared the 53 nkat found in the gradient after analysis of etiolated cotyledons.

Discussion

Kagawa *et al.* [17] showed that the level of malate synthase drastically dropped when 4 day old watermelon seedlings were exposed to continuous light. Schnarrenberger *et al.* [18] found only slight variations in specific activity of malate synthase

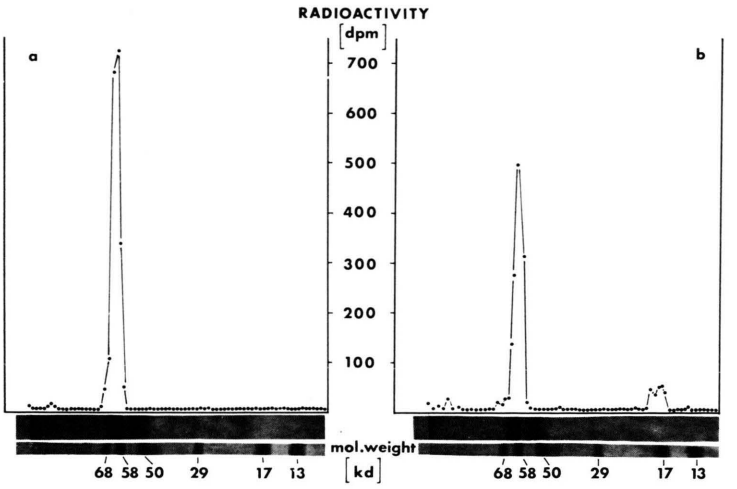

Fig. 7. Distribution of radioactivity after SDS-gel electrophoresis of immunoprecipitated malate synthase bound to microbodies. a) Fraction I (Fig. 5) after labelling in the dark; b) fraction II (Fig. 6) after labelling under continuous light. Fraction II represents only a part of the malate synthase migrated into the gradient.

when cotyledons of sunflowers were compared after 6 days dark germination and germination 4 days in the dark plus 2 days in the light. With cucumber cotyledons we did not experience a significant decrease of the glyoxysomal enzyme level during the first 24 h of irradiation. The same tendency was observed when cotyledons were gently treated with razor blades or homogenized more rigorously in a blendor and then the enzymes were analysed in homogenates or when we compared the enzyme profiles in the gradients.

This may be interpreted that light has no effect on glyoxysome biosynthesis, a conclusion which was also drawn on the basis of time courses of isocitrate lyase in mustard cotyledons in the presence and absence of far-red light [7].

Glycollate oxidase was found to be the more associated with microbodies the longer the cotyledons had been irradiated. Feierabend and Beevers [5] observed a similar phenomenon when they investigated the development of microbodies in wheat leaves.

Gradient profiles obtained from homogenates of greened cotyledons indicate an association between chloroplasts and microbodies. The question as to whether the phenomenon is due to an artefact of isolation or reflects the existance of adhering structures in the cell remains open (*cf.* [19]).

Beevers proposed for the greening of cotyledons that glyoxysomes (population A) functioning during the heterotrophic phase of germination are selectively degraded and replaces by peroxisomes (population B) newly synthesized *in toto*. This simple version of the two-population hypothesis is supported by a light-mediated incorporation of choline into microbody membranes [20].

A second hypothesis, the one-population hypothesis, requires an organelle whose envelope remains intact during the transformation, the proteins being replaced, set by set. The arguments are largely based on information from electron micrographs [4]. Especially the cytochemical demonstration of both malate synthase and glycollate oxidase in microbodies was taken as support of this hypothesis [8].

Our results show that during appearance of peroxisomal proteins also a glyoxysomal enzyme is synthesized *de novo*. This cannot be reconciled with either of the two hypotheses mentioned in their simple version. Our data prove that peroxisomal and glyoxysomal proteins are synthesized at the same time during the phase of greening; they also indicate that these proteins are housed in the same organelle. The results are most easily interpreted in terms of gradually changing microbody forms with long transient periods.

Our data would also be in agreement with a modified two-population hypothesis but which must include a distinct, relatively long period of transition where the population A is still *de novo* synthesized while population B is assembled, at a different rate. Arguments for glyoxyperoxisomes and against the other possibility may come from ultrastructural analysis – data on association of microbodies to oleosomes or plastids [7] or even results of Burk and Trelease [8] may be interpreted in this direction – or may be raised by the fact that both species obviously exhibit the same over all

density. But this is extremely improbable as peroxisomes posses, unlike the glyoxysomes with their heavily coated membrane [10], a less protein-rich limiting membrane.

For a continuous, and not a discontinuous change in protein synthesis during the replacement of glyoxysomes by peroxisomes speak the findings of Theimer *et al.* [21] who did not find a drastic *de novo* synthesis of microbodies during greening of fat-storing cotyledons.

Experiments with proteins which are housed in glyoxysomes by perotisomes speak the findings of the idea that the same type of enzyme protein is continuously used for the glyoxysomes and the peroxisomes. *E. g.* peroxisomal malate dehydrogenase was reported to cross-react immunologically with anti-malate dehydrogenase from glyoxysomes [22, 23] and we found a very close immunological relationship between glyoxysomal and a distinct form of peroxisomal catalase (Schiefer and Kindl, unpublished). Also experiments with isoenzymes separated on starch gels [24] are not in disagreement with the hypothesis of a continuous but gradually different utilization of information supplied for microbody assembling.

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