DENSITY-LABELING EVIDENCE AGAINST A DE NOVO FORMATION OF PEROXISOMES DURING GREENING OF FAT-STORING COTYLEDONS

Roland R. THEIMER, Gisela ANDING and Barbara SCHMID-NEUHAUS

Botanisches Institut der Universität, Abt. Zellphysiologie, Menzinger Straße 67, D-8000 München 19, G.F.R.

Received 10 June 1975

1. Introduction

During greening of fat-storing cotyledons, the functional properties of the microbody population changes from that of glyoxysomes to that of leaf peroxisomes. The amount of microbody protein per cell is assumed to be substantially reduced [1,2,3]. Little is known about the mechansim of this change of microbody function. Recently, data obtained from labeling of microbody membranes with C^{14}- and H^3-choline were interpreted as evidence that light triggers both a selective destruction of glyoxysomes and a concomitant de novo formation of leaf peroxisomes [4]. In contrast, electron microscopic examinations [5,6] as well as studies on the change of microbody isoenzyme spectra [2,7] had previously led to the conclusion that in greening cotyledons leaf peroxisomes do arise from glyoxysomes. Direct evidence for a de novo synthesis of proteins is provided by density labeling with stable heavy isotopes [8]. In this paper the lack of light-dependent density labeling of leaf peroxisomes is contrasted with light-dependent density labeling of plastids, and with density labeling of glyoxysomes in early stages of germination of sunflower seeds.

2. Methods

Sunflower seeds (*Helianthus annuus,* var. 'Von Dognuslowski's Frühe') were dehusked, surface sterilized with 0.2% 8-hydroxy-chinoline sulfate–potassium sulfate (Merck, Germany) solution and placed on filter paper moistened either with pure D_2O (99.7%, Merck, Germany) or demineralized H_2O. Cucumber seeds (*Cucumis sativus,* var. 'Delikatess') were sown on filter paper moistened with H_2O. The seeds were germinated in the dark for 3 (sunflower) or 4 (cucumber) days at 28°C. Then pure D_2O was applied by vacuum infiltration either to intact seedlings (sunflower) or excised cotyledons (cucumber). Controls were treated identically with H_2O instead of D_2O. Subsequently the plant material was placed into plastic boxes lined with filter paper, moistened with the infiltrated isotope solution, and illuminated for 24 (cucumber) or 48 (sunflower) hours at 28°C. The light source was a Philips fluorescent tube TL 15 W/32 Warmton de Luxe (incident light intensity appr. 1000 lux).

Homogenization of the cotyledons and separation of the cell organelles by linear sucrose density gradient centrifugation was performed essentially as reported previously [9]. Enzyme activities were assayed as described in the literature: fumarase [10], catalase [11], isocitrate lyase [12], hydroxypyruvate reductase [13], glycolate oxidase [14], and fructose-1,6-diphosphate aldolase [15]. Chlorophyll content was measured by the method of Arnon [16]. Sucrose density was estimated from the refractive index of the sucrose solution.

3. Results and discussion

Etiolated sunflower or cucumber cotyledons illuminated in the presence of D_2O produced 30–50% less chlorophyll than the H_2O controls. But development of microbody enzyme activities was not affected

significantly by the D_2O-treatment (data not presented). When crude homogenates of sunflower cotyledons which had been illuminated in the presence of D_2O were resolved by linear sucrose density-gradient centrifugation the major chlorophyll peak (thylakoids) was recovered from the gradients at a density of 1.20 g/cm^3 while the bulk of thylakoids of the H_2O controls sedimented at a buoyant density of 1.17 g/cm^3 (fig.1). Moreover, whole chloroplasts indicated by the peak of fructose-1,6-diphosphate aldolase activity showed a remarkable increase in equilibrium density which was found to rise from 1.215 (H_2O controls) to 1.24 g/cm^3 in D_2O treated cotyledons. These data clearly demonstrate that D_2O indeed was incorporated in proteins or other macromolecules newly synthesized after illumination of the seedlings. But the distribution pattern of leaf peroxisomal and glyoxysomal enzyme activities in the gradients revealed no significant shift of peroxisomal equilibrium density after D_2O treatment of the cotyledons as expected if leaf peroxisomes were not newly synthesized. Also, mitochondria showed no change of equilibrium density which probably reflects both a lack of de novo production and a slow turnover rate of their components [4]. Almost identical data were obtained when crude homogenates of etiolated cucumber cotyledons illuminated after application of D_2O were centrifuged in linear sucrose-density gradients (fig.2).

When sunflower seeds were imbibed and germinated in the presence of pure D_2O the development of glyoxysomal enzyme activities was not affected (data not shown) but density-labeled glyoxysomes were produced. As illustrated in fig.3 a major portion of the microbody fraction of the cotyledons sedimented in the gradients with a buoyant of 1.26–1.27 g/cm^3 which exceeds that of the glyoxysomes from the H_2O controls by 0.01–0.02 g/cm^3.

The results with D_2O labeling clearly indicate that during early stages of seed germination in the dark a

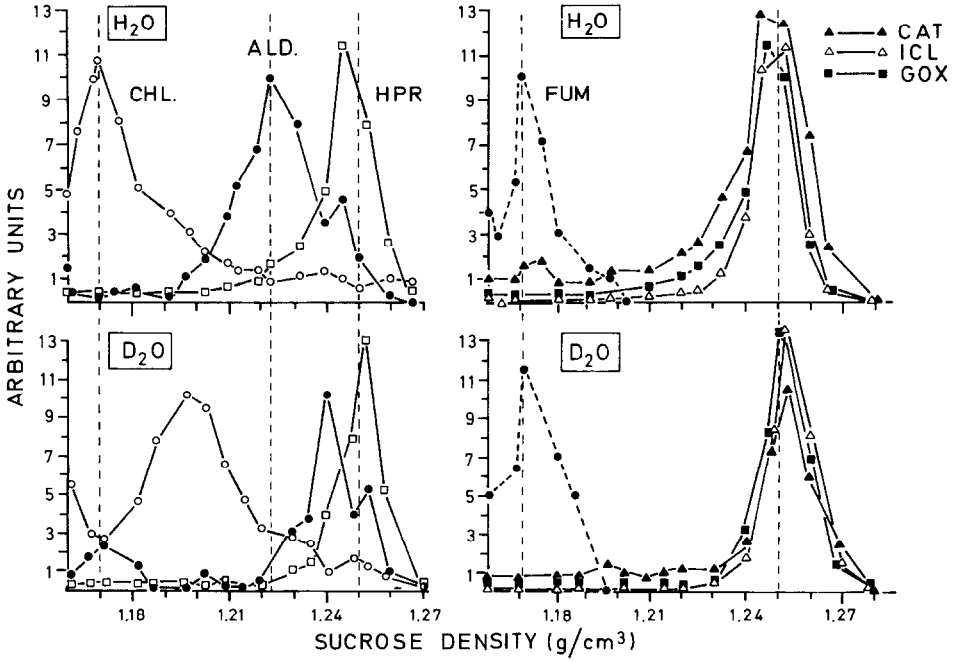

Fig.1. Distribution of chlorophyll (broken plastids) and the activities of the marker enzymes of whole cholorplasts (ALD), mitochondria (FUM), glyoxysomes (ICL, CAT), and leaf peroxisomes (HPR, GOX) after sucrose density gradient separation of crude homogenates of 5 day old sunflower cotyledons illuminated at day 3 for 48 hr in the presence of H_2O or D_2O. CHL, chlorophyll; ALD, fructose-1,6-diphosphate aldolase; HPR, hydroxypyruvate reductase; FUM, fumarase; CAT, catalase ICL, isocitrate lyase; GOX, glycolate oxidase.

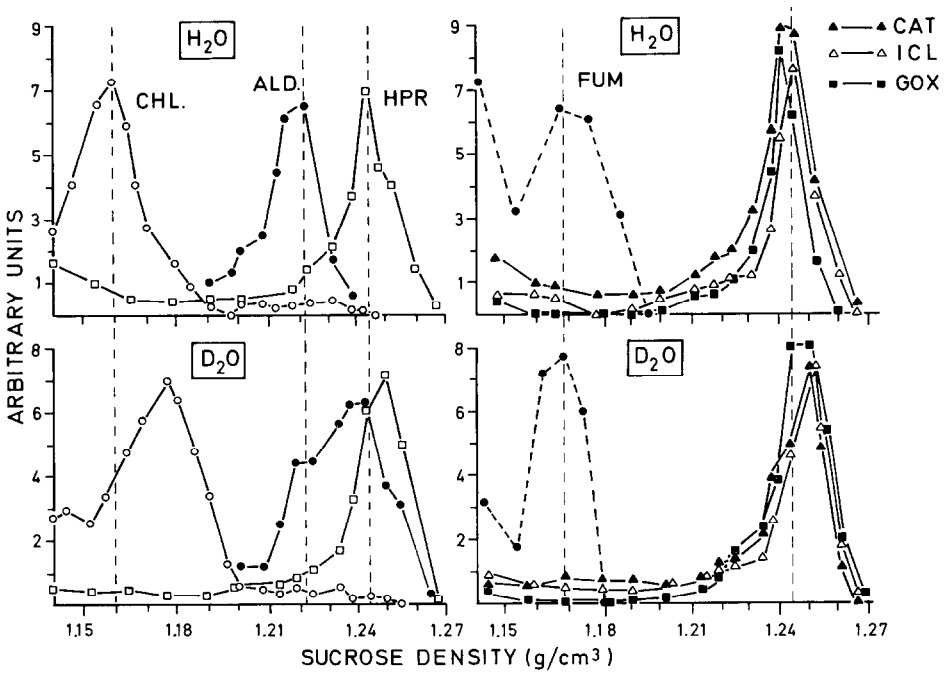

Fig.2. Distribution of chlorophyll and particulate marker enzyme activities after sucrose density gradients centrifugation of crude homogenates of 5 day old cucumber cotyledons illuminated at day 4 for 24 hr in the presence of H_2O or D_2O.

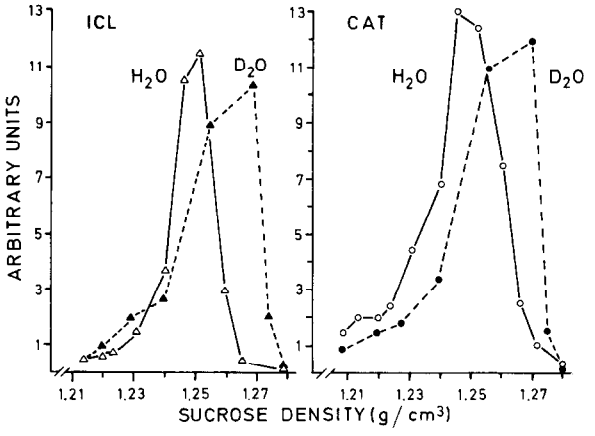

Fig.3. Distribution of the activities of the glyoxysomal marker enzymes isocitrate lyase (ICL) and catalase (CAT) after sucrose density gradients separation of crude homogenates of the cotyledons of 3 day old sunflower seedlings germinated in the presence of H_2O of D_2O. The fractions of two typical gradients representing the peaks of glyoxysomal enzyme activities are superimposed.

portion of the microbody population is formed de novo. This does not occur when seedlings are exposed to light in the presence of D_2O. In conclusion, our findings are interpreted to provide direct evidence against a de novo formation of leaf peroxisomes during the light-dependent change of microbody function in sunflower or cucumber cotyledons. This is in contrast to the hypothesis put forward by Beevers and coworkers [3,4]. Their proposal suggesting a de novo production of leaf peroxisomes in illuminated cucurbit cotyledons is based essentially on data showing an increased incorporation of C^{14}-choline into membrane material of the microbody fraction separated on density gradients. However, no information is presented [4] which excludes possible contamination of the microbody fraction by organelles such as whole plastids [17] (see also fig.1) which could have incorporated substantial amounts of choline in the developing membrane system. It is also possible that the incorporation of labeled choline into microbody membranes may not reflect formation of new organelles, but rather an elaboration

of the membrane of existing microbodies. This might explain the pleomorphism observed in microbody structure during the dark to light transition [5,6,18].

References

[1] Schnarrenberger, C., Oeser, A. and Tolbert, N. E. (1971) Plant Physiol. 48, 566.
[2] Gerhardt, B. (1973) Planta 110, 15.
[3] Kagawa, T. and Beevers, H. (1975) Plant Physiol. 55, 258.
[4] Kagawa, T., Lord, J. M. and Beevers, H. (1975) Arch. Biochem. Biophys. 167, 45.
[5] Gruber, P. J., Trelease, R. N., Becker, W. M. and Newcomb, E. H. (1970) Planta 93, 269.
[6] Trelease, R. N., Becker, W. M., Gruber, P. J. and Newcomb, E. H. (1971) Plant Physiol. 48, 461.
[7] Drumm, H. and Schopfer, P. (1974) Planta 121, 13.
[8] Anstine, W., Jacobsen, J. V., Scandalois, J. G. and Varner, J. E. (1970) Plant Physiol. 45, 148.
[9] Theimer, R. R. and Beevers, H. (1971) Plant Physiol. 47, 246.
[10] Racker, E. (1950) Biochem. Biophys. Acta 4, 211.
[11] Lück, H. (1965) in: Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) p. 885, Academic Press, New York.
[12] Dixon, G. H. and Kornberg, H. L. (1959) Biochem. J. 72, 3 p.
[13] Beevers, H., Theimer, R. R. and Feierabend, J. (1973) in: Biochemische Cytologie der Pflanzenzelle (Jacobi, G. ed.) p. 127, Thieme Verlag, Stuttgart.
[14] Baker, A. L. and Tolbert, N. E. (1966) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 9, p. 338, Academic Press, New York.
[15] Bruns, F. H. and Bergmeyer, H. U. (1962) in: Methoden der enzymatischen Analyse (Bergmeyer, H. U., ed.) p. 724, Verlag Chemie, Weinheim.
[16] Arnon, D. I. (1949) Plant Physiol. 24, 1.
[17] Schnarrenberger, C., Oeser, A. and Tolbert, N. E. (1972) Plant Physiol. 50, 55.
[18] Vigil, E. L. (1973) Sub-Cell. Biochem. 2, 237.