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Use of Dextran Gradient for the Study of the Development of Mitochondria and Glyoxysomes During the Germination of Pine Seeds

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An isotonic gradient of dextran-sucrose was used for the separation of glyoxysomes and mitochondria. By this method, these organelles were easily separated from pine seeds on the first day of germination, with densities of 1.110 and 1.175 g/ml for mitochondria and glyoxysomes, respectively. Nevertheless, these sedimented closely at a density of 1.075 g/ml when coming from 30-day stratified seeds; and both organelles highly increased their densities as germination proceeded. A mild treatment of the organelles from 30 days stratified seeds with SDS modified their sedimentation pattern, being similar after this treatment to that of the particles from the seeds on the first day of germination.

Previous investigations have shown the presence of glyoxysomes in germinating pine seeds by means of fractionation through sucrose density gradients (3, 8). The separation of the mitochondria and glyoxysomes became more apparent as germination progressed; the specific activity of glyoxysome isocitrate lyase increased during the process, thus suggesting a progressive differentiation of the organelles. However, the glyoxysomes do not vary their buoyant density in the sucrose gradient throughout the germination. This fact suggests that such a gradient was not suitable for the *in vitro* study of the developing stages of these particles.

STORRIE and ATTARDI (14), and SOLOMOS *et al.* (12) have shown that the equilibrium density for subcellular particles may vary when solutes of low osmotic potential are used for generating the gradient. Under these conditions, separation would be based on the true density of the organelles. Although the isolation of glyoxysomes and mitochondria has usually been carried out by a stepwise (3)

or continuous sucrose gradients (4, 6, 8), occasionally other gradients generated by solutes of low osmotic potential, such as Ficoll, have been used (13).

In the present study we have used a dextran-sucrose gradient which does not affect the osmotic behaviour of the particles, this being an approach to the development of mitochondria and glyoxysomes during the germination of pine seeds.

Materials and Methods

Pinus pinea seeds from Valladolid (Spain), kindly supplied by the *Instituto Forestal de Madrid*, were stratified in moistened vermiculite for 30 days at 4° C and germinated under a permanent fluorescent light at 25° C. These were then collected at different stages of germination, as described under «Results». The vermiculite and glassware were previously sterilized to prevent the contamination of the seeds.

Isolation of glyoxysomes. Chopped megagametophytes from 50 seeds were ground in a mortar with 10 ml of 0.4 M sucrose in 10 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM cysteine, and 50 mM Tris at pH 7.5, for 20 seconds as previously described (8). The 13,000 × *g* pellet was resuspended in 2 ml of 0.25 M sucrose in the same buffer and layered on the top of a dextran-sucrose gradient consisting of five 2 ml steps of 5, 10, 15, 20 and 25 % (w/w) T 40 Dextran (Pharmacia Fine Chemicals). In order to maintain the dextran steps of the gradient under isotonic conditions for the organelles, the solution were prepared in 0.25 M sucrose in the same extraction buffer. The two bands at the bottom of the gradient consisted of 2 ml steps of 45 and 60 % (w/w) sucrose in the same buffer.

The gradient was then centrifuged at 22,500 rpm for two and a half hours in a SW 27.1 rotor of a Beckman L5-50 Ultracentrifuge and fractioned into the different 2 ml bands by pumping out. All these operations were carried out at 4° C.

Densities were calculated experimentally according to the refraction index of the different bands obtained after the centrifugation.

Enzymatic Assays. These were carried out spectrophotometrically at 37° C. Isocitrate lyase was determined according to DIXON and KORNBERG (5) catalase according to AEBI (1), fumarase according to RACKER (11) and malate dehydrogenase according to BERGMEYER's method (2). Cytochrome c oxidase was assayed as described by TURNER (15). Protein was determined by LOWRY's method (10).

Results and Discussion

Separation of glyoxysomes and mitochondria in a dextran-sucrose gradient. The separation of glyoxysomes and mitochondrias from megagametophytes of pine seeds in a sucrose gradient showed a marked improvement the day following the radicle appearance (8). We, therefore, first assayed the separation of both particles from this material in a dextran gradient.

Isocitrate lyase and catalase activities were used as enzymatic markers for glyoxysomes, since both of them are specifically located in these organelles in the megagametophytes of pine seeds (3, 8). Fumarase, a specific enzyme of matrix, and cytochrome c oxidase, located in the inner membrane (3, 4), were used as mitochondrial markers. Malate dehydrogenase is common to both organelles (3, 4, 8).

The distribution, in a dextran-sucrose gradient, of the activities from seeds collected on the first day after the appearance of the radicle (G1), is shown in figure 1. The highest amount of isocitrate lyase activity corresponded to a 25 % dextran fraction (D 25). A similar pattern was found for catalase, with a peak activity also at this fraction.

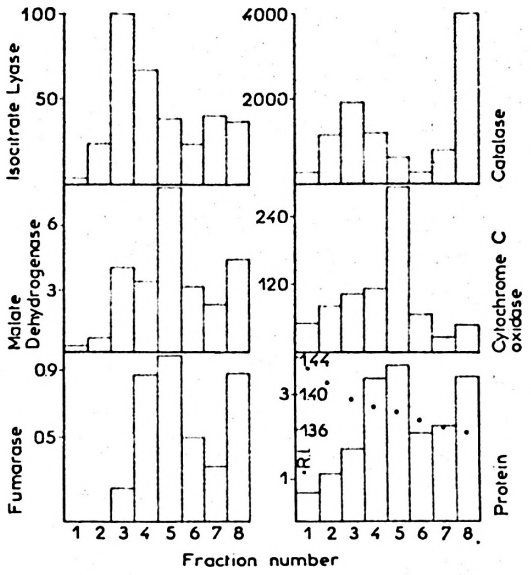

Fig. 1. *Distribution of enzyme markers and protein in a dextran-sucrose gradient for a crude particulate from the endosperm of pine seeds germinated for one day.*
Enzymatic activities are expressed as nmol × $min^{-1} \times ml^{-1}$. Protein is expressed as $mg \times ml^{-1}$. Fraction numbers corresponding to 60 and 45 % sucrose; 25, 20, 15, 10 an 5 % dextran, and supernatant bands, are represented on abscissa by 1, 2, 3, 4, 5, 6, 7 and 8 respectively. R. I. represents values of refraction index.

Fumarase and cytochrome c oxidase showed their highest activity in the 15 % dextran step (D 15). Malate dehydrogenase was mostly distributed among the supernatant and the D 15 and D 25 bands.

All the specific activities assayed have been summarized in table I. The highest values for isocitrate lyase and catalase were found in the D 25 band, and for fumarase and cytochrome c oxidase in the D 15 band. The densities of the D 15 and D 25 bands were 1.110 g/ml and 1.175 g/ml respectively.

These results thus indicate that glyoxysomes sedimented at a density of 1.175 g/ml in a dextran-sucrose gradient, while their equilibrium density in a sucrose gradient was that of 1.25 g/ml (8). This different pattern can be explained by the differences in the osmolarity of each gradient. In a sucrose gradient, the organelles are subject to hypertonic conditions and they sediment partially in a dehydrated state. Under these circumstances, no significant differences will appear at the successive stages of differentiation.

In the dextran gradient, where sucrose is isotonic to the organelles (0.25 M), the glyoxysomes sediment in a relaxed state similar to that of the *in vivo* situation, thus decreasing their buoyant density and allowing to determine a slight modification in their true density.

The high values for catalase activity at the top gradient was also found by COOPER and BEEVERS (4), in castor bean endosperm, and by CHING (3) in pine megagametophyte in a sucrose gradient probably due to glyoxysomes broken during organelle preparation. However, the highest specific activity found for catalase in the

Table 1. *Specific enzyme activities of the fractions of fig. 1.*
Values are expressed as nmol of substrate transformed per minute per mg of protein.

	Fraction number							
	1	2	3	4	5	6	7	8
Isocitrate lyase	10	16	60	19	11	9	17	10
Catalase	285	1,071	1,254	398	144	125	355	966
Fumarase	0	0	0.10	0.25	0.27	0.24	0.15	0.29
Cytochrome C oxidase	70	70	60	40	90	40	10	10
Malate Dehydrogenase	0.18	0.57	2.64	1.11	2.40	1.53	1.14	1.37

D 25 fraction, showed preference for a subcellular location.

On the other hand, the presence of fumarase and cytochrome c oxidase in the D 15 band (1.110 g/ml), agrees well with the results by STORRIE and ATTARDI (14) in Hela cells, who found values of 1.10 and 1.11 g/ml for mitochondria in a dextran gradient.

The conclusion at this stage is that the enzymatic markers for glyoxysomes and mitochondria showed a clear difference in their sedimentation pattern in the dextran gradient.

Differentiation of the organelles during germination. The organization of the glyoxysomes and mitochondria throughout germination was studied by following the distribution of particles in the dextran-sucrose gradient with isocitrate lyase and cytochrome c oxidase used as markers (figure 2).

Stratified seeds (E 30) showed the same distribution for both enzymes, mostly located in the D 10 fraction. The fact that mitochondria and glyoxysomes sedimented together at this very early stage of differentiation, may be explained by a common subcellular origin, possibly from the endoplasmic reticulum. Membranes of mitochondria and glyoxysomes contain lecithin as a major structural phospholipid. This lecithin is formed by reticulum membranes, as has been shown by LORD *et al.* (9), KAGAWA *et al.* (7) thereby suggesting that the endoplasmic reticulum could thus carry out the synthesis of the phospholipic content of mitochondrial and glyoxysomes membranes. Electron micrographs have also shown direct continuities between the endoplasmic reticulum, the microbodies and the outer membrane of mitochondria (8, 16).

A mild treatment with sodium dodecyl sulphate (SDS) before centrifugation along the gradient, makes the glyoxysomes and mitochondria sediment at different steps of the gradient, in the D 25 and D 15

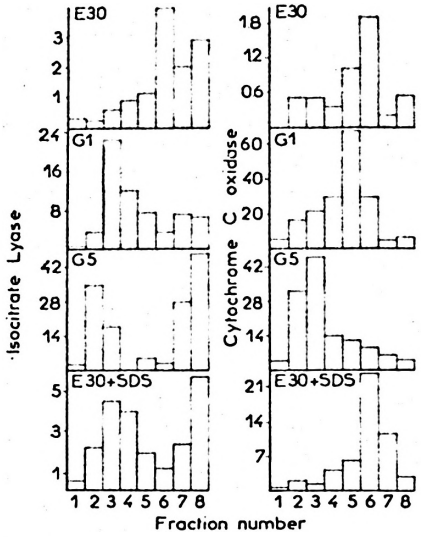

Fig. 2. *Distribution of isocitrate lyase and cytochrome C oxidase activities in a dextran-sucrose gradient at different stages of stratification and germination of pine seeds.* E 30: 30 days stratified seeds. G 1: one day of germination. G 5: five days of germination. E 30-SDS: the 13,000 × *g* pellet from thirty days stratified seeds was resuspended in 2 ml of 0.25 M sucrose, 0.05 % sodium dodecyl sulphate and centrifuged in a dextran-sucrose gradient. Enzymatic activities are expressed as nmol × min^{-1} × g of fresh $weight^{-1}$. Fractions numbers are the same as in figure 1.

bands respectively (fig. 2, SDS). Although this effect could be due to a partial separation of micelle from organelle membranes, it admits another explanation. This treatment could release both organelles from the connecting membranes and, therefore, make possible the separation of the particles through the gradient. This suggestion is in agreement with the poor separation of both organelles by a sucrose gradient at the same period of stratification (8). The release of both organelles from the reticulum membranes must take place *in vivo* on the first day of germination, with similar results to those obtained *in*

vitro by the treatment of E 30 organelles with SDS.

The results obtained after the appearance of the radicle (G 1) have been explained above. That was the only stage in which both particles were clearly separated. Five days after the radicle appearance (G 5), the isocitrate lyase sedimented at the 45 % sucrose band and cytochrome c oxidase at the D 25 band.

The location of the isocitrate lyase at the 45 % sucrose band suggests a density for glyoxysomes of 1.20 g/ml at this period of germination, which is apparently in disagreement with that of 1.25 g/ml in which organelles buoy up in a continuous sucrose gradient (8) However, since the following band is a 60 % sucrose solution (d = 1.28 g/ml), the glyoxysomes would never be able to penetrate this final step.

The evolution of glyoxysome and mitochondrial sedimentating densities in a dextran-sucrose gradient are summarized in figure 3. Densities increased progressively for both organelles as germination proceeded, but the glyxysomes became denser than the mitochondria after the appearance of the radicle.

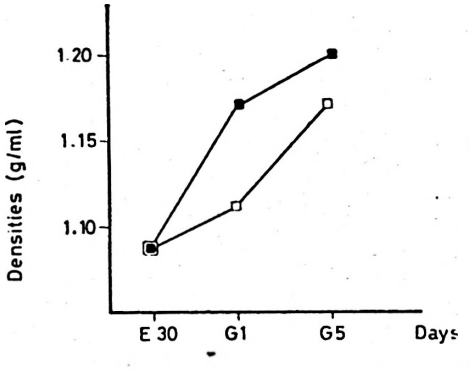

Fig. 3. *Evolution of the buoyant densities in dextran-sucrose gradient of glyoxysomes (■—■) and mitochondria (□—□) throughout the germination of pine seeds.*
The points represent the density of the band in which the highest amount of isocitrate lyase and cytochrome c oxidase were found, respectively, excluding the supernatant fraction.

At any rate, we have thus observed from the evolution of the densities from the first to the fifth day of radicle appearance that the glyoxysomes appeared to develop faster than the mitochondria.

As a conclusion to these results, we may state that centrifugation in a stepped dextran gradient constitutes an easy and reliable method for the study of the development of organelles during germination.

Resumen

Se utiliza un gradiente isotónico de dextrano-sacarosa para la separación de glioxisomas y mitocondrias. Mediante este método, ambos orgánulos se separan fácilmente con densidad de 1,110 y 1,175 g/ml para mitocondrias y glioxisomas, respectivamente, cuando proceden de semillas al primer día de germinación. Ambos orgánulos sedimentaban conjuntamente a una densidad de 1,075 g/ml cuando se obtuvieron de semillas estratificadas treinta días e incrementaban sus densidades a medida que avanzaba la germinación. Un tratamiento suave con SDS de los orgánulos obtenidos a partir de semillas estratificadas treinta días modificaba su sedimentación, siendo en este caso similar a la de las partículas obtenidas el primer día de germinación.

References

1. AEBI, H.: In «Methoden der enzymatischen Analyse», vol. 1 (Bergmeyer, H. U., ed.). Verlag Chemie. Weinheim, 1974, pp. 713-721.
2. BERGMEYER, H. U., GAWEHU, K. and GRASE, M.: In «Methoden der enzymatischen Analyse», vol. 1 (Bergmeyer, H. U., ed.). Verlag Chemie, Weinheim, 1974, pp. 516-517.
3. CHING, T. M.: *Plant Physiol.*, 46, 475-482, 1970.
4. COOPER, T. G. and BEEVERS, H.: *J. Biol. Chem.*, 244, 3507-3513, 1969.
5. DIXON, G. H. and KORNBERG, H. L.: *Biochem. J.*, 72, 3p, 1959.

6. GERHARDT, B. P. and BEEVERS, H.: *Plant Physiol.*, 44, 1475-1477, 1969.
7. KAGAWA, T., LORD, J. M. and BEEVERS, H.: *Plant Physiol.*, 51, 61-65, 1973.
8. LÓPEZ-PÉREZ, M. J., GIMÉNEZ-SOLVES, A., CALONGE, F. D. and SANTOS-RUIZ, A.: *Plant Sci. Letters*, 2, 377-386, 1974.
9. LORD, J. M., KAGAWA, T., MORE, T. S. and BEEVERS, H.: *J. Cell. Biol.*, 57, 659-667, 1973.
10. LOWRY, O. H., ROSEBROUHG, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, 193, 265-277, 1951.
11. RACKER, E.: *Biochim. Biophys. Acta*, 4, 211-214, 1950.
12. SOLOMOS, T., MALHOTRA, S. S. and SPENCER, M.: *Plant Physiol.*, 51, 807-809, 1973.
13. ST. ANGELO, A. J. and ORY, R. L.: *Biochim. Biophys. Res. Com.*, 40, 290-296, 1970.
14. STORRIE, B. and ATTARDI, G.: *J. Biol. Chem.*, 248, 5826-5834, 1973.
15. TURNER, G.: *Eur. J. Biochem.*, 40, 201-206, 1973.
16. VIGIL, E. L.: *J. Cell. Biol.*, 46, 435-454, 1970.