Biochem. J. (1976) **154**, 501–506
Printed in Great Britain

The Cellular Origin of Glyoxysomal Proteins in Germinating Castor-Bean Endosperm

By LINDA BOWDEN and J. MICHAEL LORD
Postgraduate School of Biological Sciences, University of Bradford, Bradford, Yorkshire BD7 1DP, U.K.

(*Received* 21 *August* 1975)

The capacity of castor-bean endosperm tissue to incorporate [^{35}S]methionine into proteins of the total particulate fraction increased during the first 3 days of germination and subsequently declined. At the onset of germination 66% of the incorporated ^{35}S was found in the separated endoplasmic-reticulum fraction, with the remainder in mitochondria, whereas at later developmental stages an increasing proportion of ^{35}S was recovered in glyoxysomes. The kinetics of [^{35}S]methionine incorporation into the major organelle fractions of 3-day-old endosperm tissue showed that the endoplasmic reticulum was immediately labelled, whereas a lag period preceded the labelling of mitochondria and glyoxysomes. When kinetic experiments were interrupted by the addition of an excess of unlabelled methionine, incorporation of [^{35}S]methionine into the endoplasmic reticulum rapidly ceased, but incorporation into mitochondia and glyoxysomes continued for a further 1 h. Examination of isolated organelle membranes during this period showed that the addition of unlabelled methionine resulted in a stimulated incorporation of [^{35}S]-methionine into the endoplasmic-reticulum membrane for 30 min, after which time the ^{35}S content of this fraction declined, whereas that of the glyoxysomal membranes continued to increase slowly. The ^{35}S-labelling kinetics of organelles and fractions derived therefrom are discussed in relation to the role of the endoplasmic reticulum in protein synthesis during glyoxysome biogenesis.

The endoplasmic-reticulum and glyoxysomal membranes of castor-bean endosperm are strikingly similar in their polypeptide composition when examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Bowden & Lord, 1976). Although this is of interest in itself and is consistent with a possible precursor–product relationship between the endoplasmic reticulum and glyoxysomes, such a model is not established without additional data implying a direct developmental relationship between these organelles. The purpose of the present paper is to provide evidence for this relationship from the kinetics of [^{35}S]methionine labelling of organelle proteins in developing castor-bean endosperm.

Materials and Methods

Preparation of cellular organelles

Growth of seedlings, [^{35}S]methionine labelling, homogenization and organelle separation were exactly as described previously (Bowden & Lord 1976), except that crude homogenates were centrifuged at 20000*g* for 15 min. The pellet (designated as the total particulate fraction) was gently resuspended in grinding medium before either sucrose-density-gradient centrifugation or determination of ^{35}S content. In [^{35}S]methionine pulse–chase experiments, 10μl of 2mM-methionine was added to each endosperm half 2 h after the application of radioactivity.

Fractionation of isolated organelles

Sucrose gradients containing separated organelles from [^{35}S]methionine-labelled endosperm tissue were collected as 1.0 ml fractions. A sample (0.1 ml) of each fraction was counted for radioactivity in 10 ml of scintillation fluid (Bray, 1960) as described previously (Bowden & Lord, 1976).

Peaks of radioactivity across the gradient, which corresponded to the mean buoyant densities of various organelles, were pooled. An equivalent volume of 50mM-Tricine [*N*-tris(hydroxymethyl)-methylglycine], pH 7.5, containing 0.2M-KCl was added to each pooled fraction to achieve osmotic disruption of the organelles during a 30 min incubation at 25°C. The organelle fractions were recovered by centrifuging at 100000*g* and 0–2°C for 30 min. The resultant supernatants were collected and contained the organelle soluble fraction (matrix proteins); the organelle-membrane pellets were resuspended in water. The ^{35}S content of these fractions was determined.

Results and Discussion

[^{35}S]Methionine applied to the excised endosperm halves was actively incorporated into the total particulate fraction. As shown in Fig. 1, this capacity increased markedly during the first 3 days of germination and subsequently declined. This result was

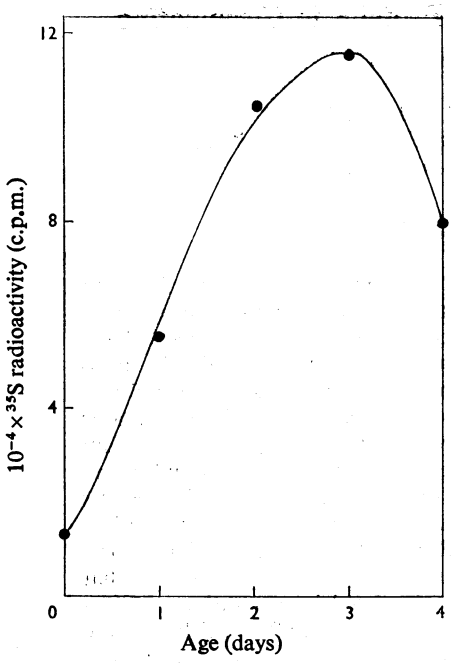

Fig. 1. *Effect of seedling age on the incorporation of* [^{35}S]-*methionine into the crude organelle pellet of castor-bean endosperm*

[^{35}S]Methionine (5μl, containing 5μCi) was applied to each of ten endosperm halves removed from seedlings at various developmental stages. The tissue was incubated for 3h at 30°C, homogenized and the homogenate centrifuged for 15min at 20000g. The crude organelle pellet was washed once with grinding medium and its radioactivity content determined.

expected, since rapid organelle synthesis occurs during this time (Beevers, 1969).

Interesting developmental changes occurred, however, in the rates of incorporation into the separated organelles constituting the total particulate fraction (Fig. 2). Endosperm halves excised at different developmental stages were incubated with [^{35}S]-methionine for 3h, homogenized, and the organelles isolated. In imbibed seedlings (day 0) most of the particulate radioactivity was present in the endoplasmic reticulum, the remainder being in mitochondria, whereas the glyoxysomes were unlabelled. The rate of incorporation of [^{35}S]methionine into endoplasmic reticulum increased strikingly during the first 2 days of seedling development. In contrast the rate of incorporation into glyoxysomes was slow during the first 24h and then increased rapidly to reach a peak on the third day. Mitochondrial labelling increased over the first 2 days, but, unlike that of the endoplasmic reticulum, did not show a marked decline between days 2 and 3 (Fig. 2*a*). These

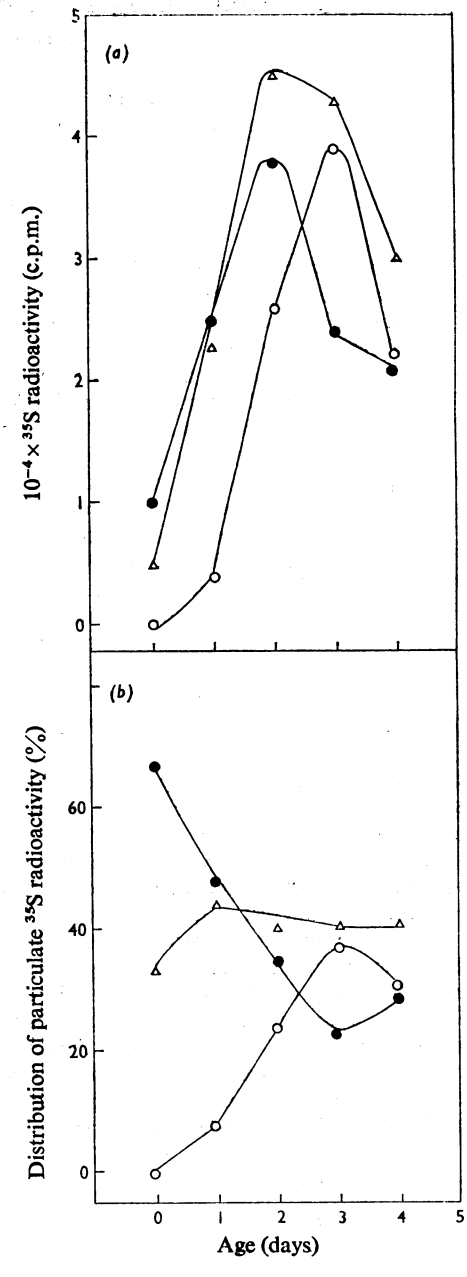

Fig. 2. *Effect of seedling age on the incorporation of* [^{35}S]*methionine into the cellular organelles isolated from castor-bean endosperm*

[^{35}S]Methionine (5μl, containing 5μCi) was applied to each of ten endosperm halves removed from seedlings at various developmental stages. The tissue was incubated for 3h at 30°C and homogenized. Cellular organelles were isolated by sucrose-density-gradient centrifugation and their radioactivity content was determined. ●, Endoplasmic reticulum; △, mitochondria; ○, glyoxysomes.

data are expressed in Fig. 2(*b*) as percentages of the total label recovered in the three cellular fractions. During early stages of development an increasing proportion of the total radioactivity is incorporated into glyoxysomes, whereas that incorporated into the endoplasmic reticulum declines in a reciprocal manner. This indicates that the synthesis of endoplasmic reticulum may precede that of glyoxysomes in the developing seedlings. Previous studies (Bowden & Lord, 1975) have shown that the capacity of the endoplasmic reticulum to synthesize phospholipids, required as structural components of organelle membranes, also reaches a maximum before the cellular glyoxysome content does so. Together these data suggest that a proliferation of the endoplasmic reticulum immediately precedes glyoxysome formation in developing seeds, and is consistent with a precursor–product relationship between these two cellular fractions. A possible mechanism by which proteins synthesized on ribosomes bound to the endoplasmic reticulum could be transferred into both the glyoxysomal membrane and matrix has been discussed by Bowden & Lord (1976). In contrast with glyoxysomes, which apparently do not have protein-biosynthetic activity, mitochondria are known to be capable of synthesizing some of their inner-membrane proteins. Thus, although mitochondria contain the genetic information and transcriptional and translational machinery that effects the synthesis of some of their constituent proteins (Mason *et al.*, 1972; Mahler *et al.*, 1974) and are enzymically capable of a degree of phospholipid synthesis (Hostetler & van den Bosch, 1972; Douce *et al.*, 1972), other proteins and lipids are cytoplasmically synthesized and must be transferred from their site of synthesis, possibly on the endoplasmic reticulum, into the mitochondria (Kadenbach, 1967, 1971; Gonzalez-Cadavid & Campbell, 1967). This partial autonomy of mitochondria may well account for the intermediate behaviour, with respect to that of endoplasmic reticulum and glyoxysomes, of the rate of [^{35}S]-methionine incorporation into this organelle fraction during seedling development (Fig. 2).

The kinetics of [^{35}S]methionine incorporation into the various organelles separated from 3-day-old seedlings provided further evidence of a precursor role for the endoplasmic reticulum. Before discussing these data it is worth emphasizing that the homogenization technique used here is highly reproducible, in that good agreement is found between the recovery of total organelle protein from separate samples of carefully selected seedlings (Kagawa *et al.*, 1973). In addition the gradients cleanly separated organelles from crude homogenates, so that reasonably accurate estimates of the radioactivity present in separated organelles, and particularly in the washed membrane fractions, can be made (Lord *et al.*, 1973). [^{35}S]-Methionine was applied to excised 3-day-old endosperm tissue. At intervals over an 8h period, samples were taken and organelles were isolated on sucrose density gradients. The total [^{35}S]methionine content of the separated organelles was determined; the organelles were then osmotically disrupted so that the distribution of ^{35}S between membrane and matrix proteins could be established. Fig. 3 shows the kinetics of labelling of total organelle, solubilized matrix proteins and organelle membrane fractions (Figs. 3*a*, 3*b* and 3*c*). The most significant feature of these data is that during the first hour the total endoplasmic reticulum (Fig. 3*a*) and the soluble and membrane fractions derived from it (Figs. 3*b* and 3*c*) became clearly labelled, whereas there was a distinct lag in the labelling of equivalent glyoxysomal and mitochondrial fractions. After this initial lag period of approx. 30min, rapid labelling of glyoxysomes and mitochondria occurred for several hours.

The differential rate of incorporation into the organelles and derived fractions becomes more apparent when the data are expressed as a percentage of the total radioactivity (Figs. 3*d*, 3*e* and 3*f*). In the first sample taken most of the radioactivity is present in the endoplasmic reticulum, but this proportion subsequently decreases as that in the glyoxysomes, and to a lesser extent the mitochondria, increases.

This reciprocal behaviour suggests that a developmental relationship may exist between the endoplasmic reticulum and the glyoxysomes and mitochondria, in that proteins initially present in the endoplasmic reticulum ultimately appear in these other organelles. An alternative explanation could be offered if mitochondria and glyoxysomes were capable of independently incorporating [^{35}S]methionine into their proteins, but initially did so less rapidly than the endoplasmic reticulum. In the mitochondria this may be true to a limited extent, owing to the protein-biosynthetic machinery of this organelle. Our present understanding of the biochemical capacity of microbodies and related particles, however, suggests that they are completely dependent on biosynthetic activity elsewhere in the cell to provide their protein components. Thus in interpreting the reciprocal behaviour observed for the various cellular fractions during methionine incorporation (Figs. 3*d*, 3*e* and 3*f*), we suggest that the results from endoplasmic reticulum and glyoxysomes indicate that proteins of the glyoxysomal membrane and matrix are initially synthesized on rough endoplasmic reticulum. The less pronounced lag for the labelling of mitochondria, particularly the membranes (Fig. 3*f*), may be explained by the partial autonomy of this organelle.

Further evidence that the endoplasmic reticulum had a precursor role during the synthesis of organelle proteins was obtained from pulse–chase experiments. Endosperm halves were incubated with [^{35}S]methionine and a 1000-fold excess of unlabelled meth-

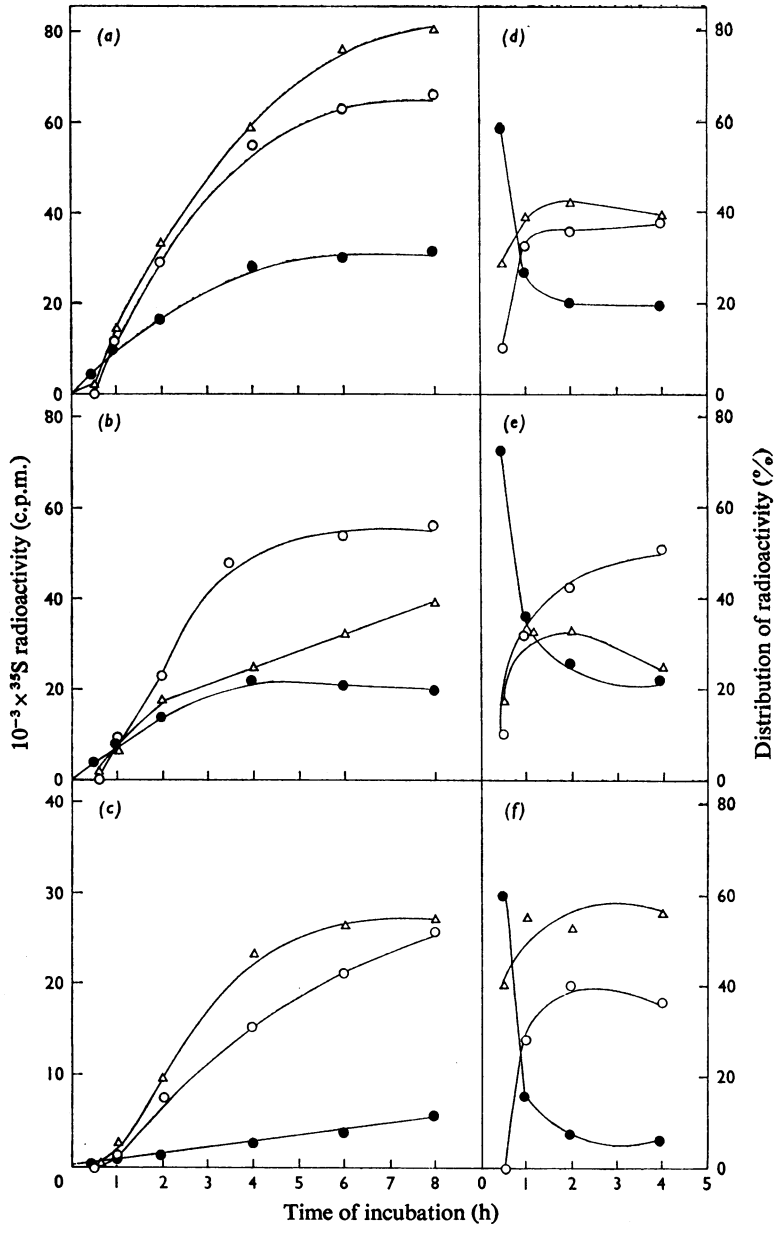

Fig. 3. *Progress of* [^{35}S]*methionine incorporation into cellular organelles isolated from castor-bean endosperm*

Endosperm halves removed from 3-day-old seedlings were incubated at 30°C after application of 5 μl of [^{35}S]methionine (containing 5 μCi) to each half. At various times ten endosperm halves were homogenized; the organelles were separated by sucrose-density-gradient centrifugation and the radioactivity content was determined in: (*a*) the whole organelles; (*b*) the soluble fraction released after osmotic disruption; (*c*) the membrane fraction. The data in (*a*), (*b*) and (*c*) are replotted in (*d*), (*e*) and (*f*) respectively to show the variation with time of the percentage of the total radioactivity present in the various organelles and derived soluble and membrane fractions. ●, Endoplasmic reticulum; △, mitochondria; ○, glyoxysomes.

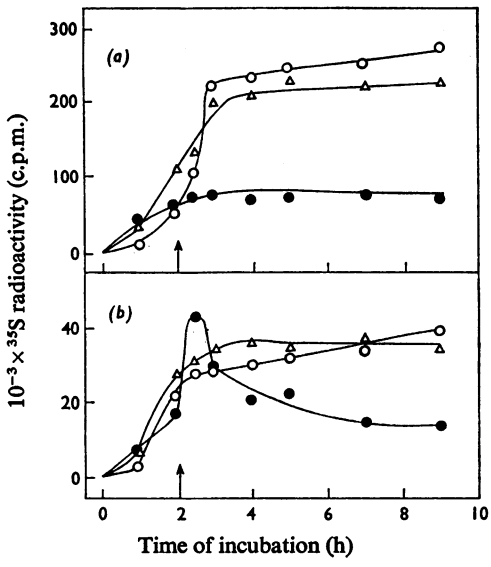

Fig. 4. *Effect of excess of unlabelled methionine on the progress of [^{35}S]methionine incorporation into cellular organelles isolated from castor-bean endosperm*

Endosperm halves removed from 3-day-old seedlings were incubated at 30°C after application of 5μl of [^{35}S]-methionine (containing 5μCi) to each half at zero time. Samples of ten halves were removed after 1 and 2h incubation and then a 1000-fold excess of unlabelled methionine was added, as indicated by the arrows. Further samples were then taken, homogenized, the organelles separated by sucrose-density-gradient centrifugation and the radioactivity content was determined in: (*a*) the whole organelles; (*b*) the organelle membranes. For ease of presentation, the radioactivity in the endoplasmic-reticulum membrane (*b*, ●) has been multiplied by 5. ●, Endoplasmic reticulum; △, mitochondria; ○, glyoxysomes.

ionine was added after 2h. Organelles were isolated at various intervals and ^{35}S content determined (Fig. 4*a*). The addition of unlabelled methionine effectively stopped the incorporation of [^{35}S]-methionine into the endoplasmic reticulum, whereas incorporation into mitochondria and glyoxysomes continued for a further 1h. A comparison of Fig. 4(*a*) with Fig. 3(*a*) shows that the chase with unlabelled methionine appears to stimulate the labelling of glyoxysomes during this time. When the behaviour of the organelle membrane was examined the chase with unlabelled methionine stimulated the incorporation of ^{35}S into the endoplasmic-reticulum membranes for up to 30min and then the ^{35}S content of this fraction declined for several hours, whereas incorporation of ^{35}S continued slowly into the glyoxysomal-membrane fraction (Fig. 4*b*).

The kinetics of [^{35}S]methionine incorporation into organelle proteins presented here is consistent with the model for glyoxysome biogenesis discussed in the preceding paper (Bowden & Lord, 1976). Recent studies have established that during the early stages of castor-bean germination, several enzymes which are markers for the glyoxysomal matrix are also recovered from sucrose gradients in the endoplasmic-reticulum fraction (Gonzalez & Beevers, 1974; L. Bowden & J. M. Lord, unpublished work). The presence of such enzymic activities in endoplasmic-reticulum-derived vesicles isolated from the tissue at a developmental stage when active glyoxysome formation is occurring may indicate that proteins destined for the glyoxysomal matrix are synthesized on ribosomes bound to the endoplasmic reticulum and then discharged intracisternally (Redman *et al.*, 1966; Redman, 1967, 1969).

Further, when [^{14}C]choline is supplied to intact castor-bean endosperm halves as a precursor of membrane phosphatidylcholine, phosphatidyl[^{14}C]-choline first appears in the endoplasmic-reticulum membranes and subsequently appears in the glyoxysomal membrane (Kagawa *et al.*, 1973). Thus the synthesis of both glyoxysomal phospholipid and protein is preceded by the synthesis of these compounds in the endoplasmic reticulum.

In conclusion, the similarity in polypeptide composition between the endoplasmic reticulum and glyoxysomal membranes (Bowden & Lord, 1976) and the [^{35}S]methionine-labelling kinetics of these organelles strengthen current concepts that the endoplasmic reticulum has a precursor role during microbody biogenesis.

We thank Dr. L. K. Evans, Croda Premier Oils Ltd., Hull, U.K., for supplying the castor-bean seeds and the S.R.C. for financial support through Grant B/RG 66629.

References

Beevers, H. (1969) *Ann. N.Y. Acad. Sci.* **168**, 313–324
Bowden, L. & Lord, J. M. (1975) *FEBS Lett.* **49**, 369–371
Bowden, L. & Lord, J. M. (1976) *Biochem. J.* **154**, 491–499
Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
Douce, R., Mannella, C. A. & Bonner, W. D. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1504–1509
Gonzalez, E. & Beevers, H. (1974) *J. Cell Biol.* **63**, 115a
Gonzalez-Cadavid, N. F. & Campbell, P. N. (1967) *Biochem. J.* **105**, 443–450
Hostetler, K. Y. & van den Bosch, H. (1972) *Biochim. Biophys. Acta* **260**, 380–386
Kadenbach, B. (1967) *Biochim. Biophys. Acta* **134**, 430–442
Kadenbach, B. (1971) in *Autonomy and Biogenesis of Mitochondria and Chloroplasts* (Boardman, N. K., Linnane, A. W. & Smillie, R. M., eds.), pp. 360–371, North-Holland, Amsterdam

Kagawa, T., Lord, J. M. & Beevers, H. (1973) *Plant Physiol.* **51**, 61–65

Lord, J. M., Kagawa, T., Moore, T. S. & Beevers, H. (1973) *J. Cell Biol.* **57**, 659–667

Mahler, H. F., Feldman, F., Phan, S. H., Hamill, P. & Dawidowicz, K. (1974) in *The Biogenesis of Mitochondria* (Kroon, A. M. & Saccone, G., eds.), pp. 432–441, Academic Press, London and New York

Mason, T., Ebner, E., Poyton, R. O., Salzgalier, J., Wharton, D. C., Mennucci, L. & Schatz, G. (1972) in *Mitochondria—Biomembranes and Biogenesis* (van den Berg, S. G., Borst, P. & Slater, E. C., eds.), pp. 53–69, North-Holland, Amsterdam

Redman, C. M. (1967) *J. Biol. Chem.* **242**, 761–768

Redman, C. M. (1969) *J. Biol. Chem.* **244**, 4308–4315

Redman, C. M., Siekevitz, P. & Palade, G. E. (1966) *J. Biol. Chem.* **241**, 1150–1158