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# Aggregated Forms of Malate and Citrate Synthase are Localized in Endoplasmic Reticulum of Endosperm of Germinating Castor Bean[1]

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## ABSTRACT

**The endosperm of 3-day germinated seedlings of *Ricinus communis* was homogenized in the presence or absence of $Mg^{2+}$. When the $Mg^{2+}$-containing homogenate was fractionated on linear, 20 to 40% sucrose gradients, the endoplasmic reticulum (ER) reached equilibrium at a density of 1.146 grams per cubic centimeter. Absence of $Mg^{2+}$ in the grinding medium resulted in displacement of the ER in the gradient from a density of 1.146 to 1.138 grams per cubic centimeter. At either density, the activities of both malate and citrate synthase were found to overlap the activity of NADH-cytochrome *c* reductase (an ER marker) in the gradient. Furthermore, this overlap of activities was observed whether the gradients were centrifuged for 3 or 19 hours. An analysis of sedimentation characteristics of the solubilized enzymes revealed that they exist, predominantly, as a 5.2S ($s_{20,w} \times 10^{-13}$) form (malate synthase) and a 6.8S form (citrate synthase) in the glyoxysomes and cytosol. When the two enzymes were released from the ER, they appeared as aggregate forms of 70S and 55S, respectively. These results support the conclusion that the synthases are associated with the ER.**

The hypothesis formulated by Beevers and others that glyoxysome formation involves differentiation and vesiculation of the ER is supported by several lines of evidence (1). Accordingly, the origin of the glyoxysomal membrane is believed to be, in whole or in part, the ER. An extension of this hypothesis results in the prediction that the ER is also involved as the site of synthesis and accretion of glyoxysomal matrix proteins. This hypothesis has been attractive in view of the findings of González and Beevers (6), González (5), and Bowden and Lord (2) who have demonstrated the association of as much as 50% of the glyoxysomal malate and citrate synthase activities with ER during early germination of castor bean. The implications of these observations were further emphasized by the recent report of Kagawa and González (9) that the citrate synthase activity associated with the ER is strictly due to the glyoxysomal isozyme.

Bowden and Lord (2) reported confirmation of the results of González and Beevers (6). Lord and Bowden (14) found that the malate synthase associated with ER was protected by the membranes from added protease. Loss of malate synthase activity in the presence of protease was observed only when the ER membranes were deliberately solubilized by detergent. The implication of these observations as well as of the developmental study of González and Beevers (6), prompted Lord and Bowden to study the physiological significance of the presence of malate synthase in the ER. The progress of [$^{35}$S]methionine incorporation into anti-malate synthase immunoprecipitates of ER and glyoxysome extracts was followed. A precursor to product relationship was shown to exist between the ER-associated malate synthase and the mature glyoxysome. Their findings supported their conclusion that synthesis of malate synthase takes place on RER followed by vectorial discharge into the ER cisternae prior to sequestration into glyoxysomes.

The work of Kindl and co-workers (10–13), however, has supported a different view of the intracellular pathway of malate synthase delivery. Köller and Kindl (12) concluded that in cucumber cotyledons, malate synthase activity is not actually associated with the ER but is localized in a structure which is neither ER nor glyoxysome. According to Kindl *et al.* (10), this structure identified as a 100S particle is not an intermediate in the biogenesis of glyoxysomes. Kindl *et al.* (13) also reported the cytosol to be the site of synthesis of malate synthase. Köller *et al.* (11) found two forms of malate synthase, a minor 6S form and a predominating 19S form. The latter form was reported to be associated with the glyoxysomal membrane.

The present study provides evidence from castor bean seedlings to support the conclusion that the glyoxysomal enzymes, malate synthase and citrate synthase, are localized in the ER.

## MATERIALS AND METHODS

Seeds of castor bean (*Ricinus communis* cv. Hale; McNair Seed Co., Plainview, TX) were soaked in running tap water for 24 h. The imbibed seeds were transferred to moist vermiculite and incubated at 30°C in darkness in a Percival incubator. RH was maintained at 90%.

Seedlings were removed from vermiculite after 3 days. Thirty-six endosperm halves were minced with a razor blade in 10 ml grinding medium (5).

To examine membrane-associated enzyme activities, endosperm were homogenized in 20% (w/w) sucrose grinding medium (100 mM Tricine, pH 7.5; 10 mM KCl; 1 mM EDTA) in the presence or absence of 2 mM $MgCl_2$. These homogenates were filtered through one layer of Miracloth and layered on 20 to 40% (w/w) linear sucrose gradients (26 ml). Gradients were centrifuged in a Beckman SW 27 rotor at 20,000 rpm. Fractions (0.9 ml) were collected as described previously (5).

For sedimentation analyses of solubilized enzymes, endosperm halves were minced with a razor blade in 5% (w/w) sucrose grinding medium plus 2 mM $MgCl_2$. The filtered homogenates were centrifuged at 600*g* for 10 min. The 600*g* supernatant was subjected to three strokes of a tissue homogenizer and further centrifuged at 30,000*g* for 20 min. The 30 *kg* supernatant (2 ml) was layered on a 5 to 40% (w/w) linear sucrose gradient (10 ml).

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Gradients were centrifuged at 40,000 rpm ($\omega^2 = 17.55 \times 10^6$) in a Beckman SW 41 Ti rotor at 5°C. Fractions (0.44 ml) were collected from the top of the tube.

To determine the organellar location of the aggregate forms of the enzymes, the tissue was minced in 20% (w/w) sucrose grinding medium. The filtered homogenate was subjected to differential centrifugation after an initial spin of 600*g*. The pellets resulting after centrifugations at 10,000*g* (10 min) and 30,000*g* (20 min) were resuspended in 5% sucrose grinding medium and subjected to three strokes of a tissue homogenizer. These preparations were subjected to centrifugation at 20,000*g* (10 min) to remove membranes. The 30 kg supernatant was diluted to 5% sucrose while maintaining all other components at the same concentration. To avoid dilution an alternate procedure was employed. Tissue was minced in 5% sucrose and subjected to centrifugation at 30 kg for 20 min to effect removal of membranes and organelles. The enzyme extracts (2 ml) representing cytosolic contents as well as, possibly, some contamination from leaking organelles, were layered on 5 to 40% sucrose gradients.

All gradient solutions contained 100 mM Tricine, pH 7.5, 10 mM KCl, and 1 mM EDTA. In addition to the preceding ingredients grinding medium contained 20% (w/w) sucrose and 2 mM $MgCl_2$ (except where otherwise indicated).

The activities of NADH-Cyt *c* reductase (5), catalase (16), malate synthase and citrate synthase (7) were assayed as described. Sucrose concentrations were determined refractometrically.

Catalase (bovine liver) was obtained from Sigma Chemical Co.

## RESULTS

Tissue minced in the presence of 2 mM $MgCl_2$ yields an ER fraction which sediments to a relatively high density (1.146 g/cc) in sucrose gradients. In the absence of $Mg^{2+}$, however, ribosomes are lost from the ER and the membranes band to a lower density (1.138 g/cc) (15, Fig. 1). It is therefore assumed that any non-ER membranes and their associated enzymes would not exhibit the displacement expected for RER in the presence and absence of $Mg^{2+}$. As the experiment of Figure 1 clearly shows, malate synthase and citrate synthase co-sedimentated with the ER membrane marker regardless of the $Mg^{2+}$ concentration in which the tissue was minced. The association of the synthases with the ER was also retained when gradients were subjected to prolonged centrifugation (Fig. 2).

The possible contribution from a rapidly sedimenting, highly aggregated, non-membrane-bound, form of the synthases to the synthase activity detected in the ER was also examined. The question was whether a sufficiently aggregated form of the enzyme existed such that centrifugation at 20,000 rpm for 3 h ($\omega^2 = 4.39 \times 10^6$) would result in a detectable migration into the ER region of the gradient. Therefore, the sedimentation coefficients of malate synthase and citrate synthase in sucrose gradients were determined. This was done under the exact conditions of composition and concentration of the grinding and gradient solutions used to analyze synthase association with the ER. Mechanical disassociation of the synthases from the membranes was considered more appropriate than the use of 0.2 M KCl washes which have been used in previous studies (2, 3, 10–12). This was done to avoid possible salt effects on the aggregation status of the enzymes. The results reveal that each of the two enzymes exists in at least two aggregation forms. The least aggregated form of the synthases comprised the predominant form of the enzyme under these conditions (Fig. 3). The largest aggregates of either synthase had sedimentation coefficients ($s_{20,w}$) calculated (17) to be between 55 and 70S.

The sedimentation coefficient of castor bean catalase calculated (17) from the data of Figure 2 was 11.4S and agrees well with the value of 11.2S reported for bovine liver catalase (4). Under these conditions, castor bean and bovine liver catalases exhibited identical sedimentation characteristics (Fig. 4). Using a $Z_0$ value of −20 for the gradients used, and assuming a protein density of 1.3 g/cc, the formulae of McEwen (17) yield a sedimentation coefficient ($s_{20,w} \times 10^{-13}$) of 5.2 for the slowest sedimenting form of malate synthase and 6.8 for the slowest sedimenting form of citrate synthase.

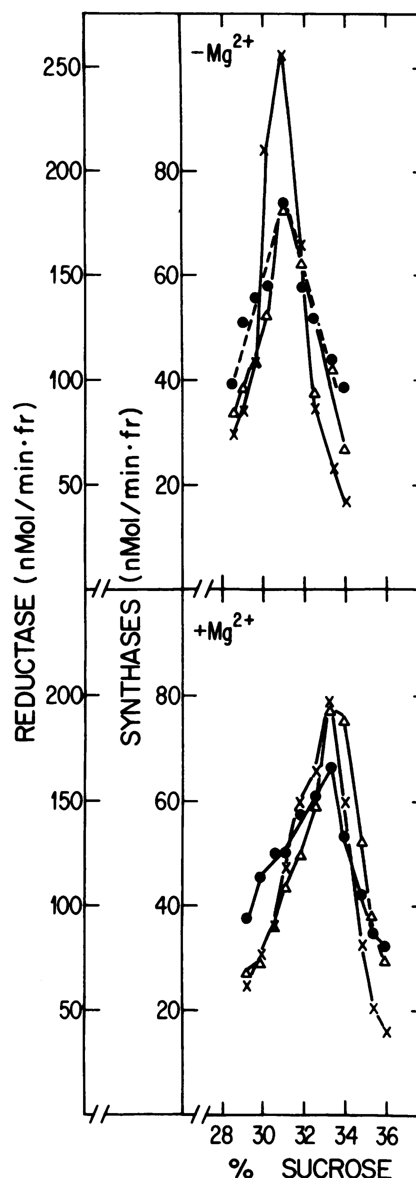


FIG. 1. The effect of 2 mM $MgCl_2$ on the buoyant density of the ER and its associated enzymes. Tissue was minced in 20% sucrose grinding medium ± 2 mM $MgCl_2$. Centrifugation through 20 to 40% sucrose gradients was for 4 h at 20,000 rpm in the SW 27 rotor. Fractions (0.9 ml) were collected from the top. (●), malate synthase; (△), citrate synthase; (×), NADH-Cyt *c* reductase.

The organelle localization of the aggregate forms was determined in the following way. The endosperm was minced in 20% sucrose grinding medium and the resulting homogenate submitted to differential centrifugation. The extracts of pellets representing glyoxysomes (*inter alia*), and ER, 10,000*g* and 30,000*g*, respectively, were analyzed on 5 to 40% sucrose gradients (Fig. 5). Aggregate forms of malate synthase (70S) and citrate synthase (55S) were recovered only from ER-derived extracts. The glyoxysomal extracts yielded, predominantly, the 5.2S and 6.8S forms of malate and citrate synthases, respectively. The situation in the cytosol is more complex. When tissue was minced in 5% sucrose

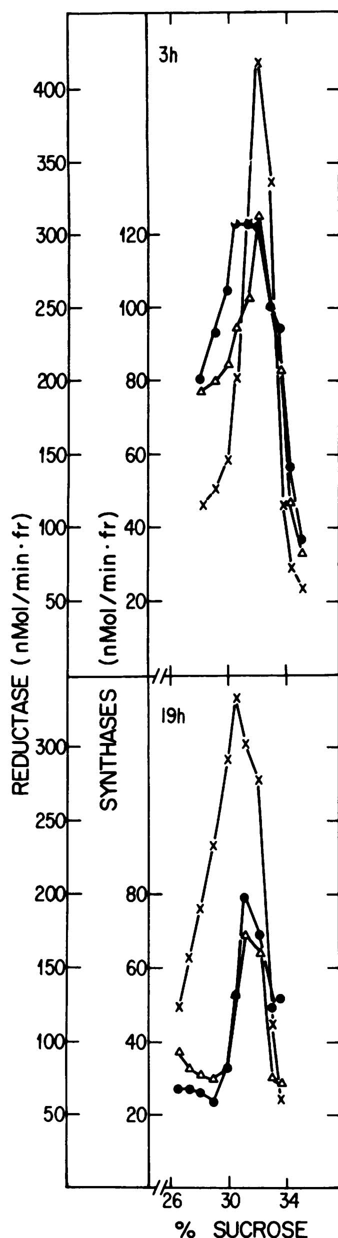


FIG. 2. Extended centrifugation of the ER in sucrose gradients. Tissue was minced in 20% sucrose grinding medium plus 2 mM $MgCl_2$. Centrifugation through 20 to 40% sucrose gradients was at 20,000 rpm in the SW 27 rotor. (●), malate synthase; (△), citrate synthase; (×) NADH-Cyt *c* reductase.

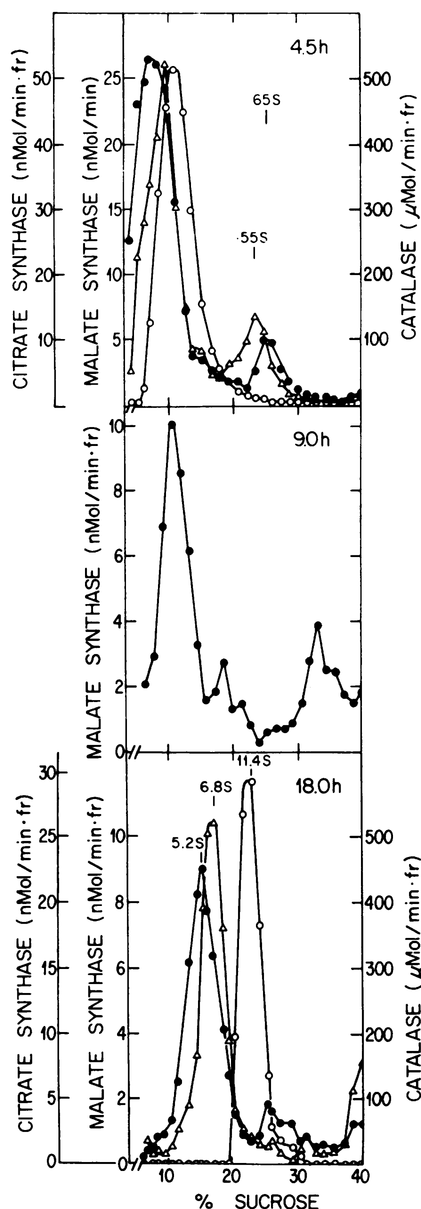


FIG. 3. Sedimentation characteristics of solubilized enzymes. Linear, 5 to 40% sucrose gradients were centrifuged at 40,000 rpm in the SW 41 Ti rotor. Fractions (0.44 ml) were collected from the top. (●), malate synthase; (△), citrate synthase; (○) catalase.

and the 30 kg supernatant examined in 5 to 40% gradients no aggregates were observed (Fig. 6A). Nevertheless, aggregates of the cytosolic enzyme were, apparently, easily generated. The addition of KCl during dilution of the 20% sucrose, 30 kg supernatant did not result in total prevention of aggregation (Fig. 6B).

## DISCUSSION

The association of malate and citrate synthase to glyoxysomal membranes was first described by Huang and Beevers (8). These enzymes were shown to be peripheral proteins whose interaction with the glyoxysomal membrane could be disrupted by increasing the ionic strength of the wash buffer. The ER membranes also have a similar association to malate and citrate synthase (6). Results presented here clearly demonstrate that the association of synthases with ER is quite real and at the same time emphasizes that this relationship is easily disrupted not only by high ionic strength buffers but also by mild mechanical force. In comparing the results of Köller and Kindl (12) to those presented here, one must emphasize that the putative separation of malate synthase from ER seen by them was only obtained after gradient-purified ER was recentrifuged through a second sucrose gradient. Under those conditions, the association of malate synthase to ER would not be expected to remain intact (compare Fig. 3 to 6A, this paper).

The association of the synthases to the ER is not the result of a coincidental, fortuitous juxtaposition of conditions of preparations and sedimentation. This position is supported by the following evidence: (a) extended centrifugation of the ER in gradients does not result in a change in the mobility of the synthases relative to the ER markers; (b) membrane-free extracts loaded on 20 to 40% gradients and centrifuged under the same conditions ($\omega^2 = 4.39 \times 10^6$; $t = 3$ h) did not allow penetration of malate synthase into

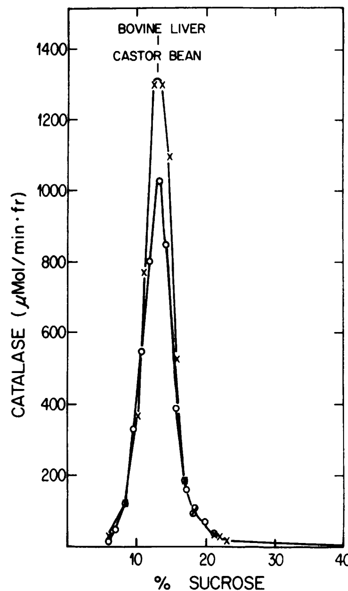


FIG. 4. Sedimentation characteristics of catalase. Bovine catalase (1.6 mg) was dissolved in 2.0 ml 5% sucrogrinding medium and layered on the 5 to 40% sucrose gradient. Centrifugation for 4.5 h at 40,000 rpm in the SW 41 Ti rotor.

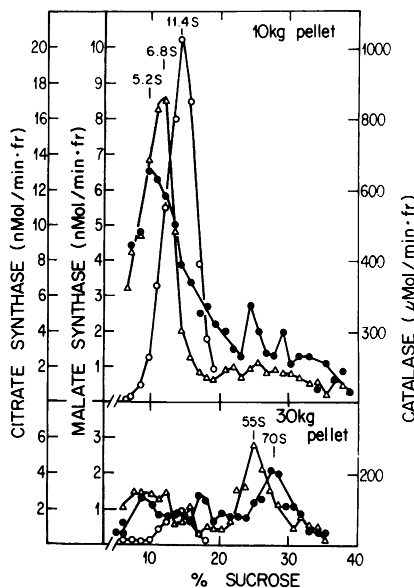


FIG. 5. Sedimentation characteristics of organelle-derived, solubilized enzymes. Tissue was minced in 20% sucrose grinding medium. Organelles were obtained by differential centrifugation. The 5 to 40% gradients were centrifuged as for Figure 4.

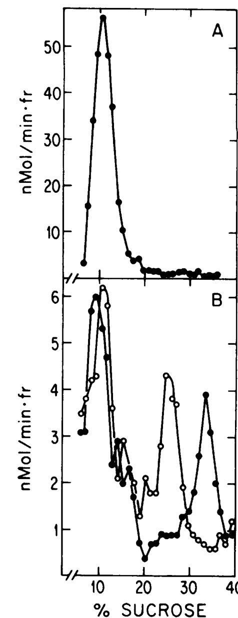


FIG. 6. Sedimentation of cytosolic malate synthase. A, tissue was minced in 5% sucrose grinding medium and membranes and organelles removed by centrifugation at 30 kg for 20 min; B, tissue was minced in 20% sucrose grinding medium, spun at 30 kg for 20 min and the supernatant diluted to 5% sucrose and a final KCl concentration of 0.2 M (○) or 0.01 M (●). Gradients and centrifugation as for Fig. 4.

gradient regions greater than density 1.112 g/cc; (c) the most highly aggregated form of malate synthase (70S) detected is, according to calculations, still too slow to reach the buoyant density of ER (1.146 g/cc) under the conditions of sedimentation. It should be noted that conditions of pH, ionic strength, salt concentration, etc., remained constant for membrane-associated and for deliberately solubilized proteins in gradients.

The sedimentation values assigned to malate synthase by: Briedenbach (3), 16S; Köller *et al.* (11), 6 and 9S; Kindl *et al.* (10), 20 and 100S are somewhat conflicting, particularly with values reported here. In most of the cases cited above, it is difficult or impossible to reconstruct the experiments and the method of calculation used to arrive at the published values. The present study found the predominant form of malate synthase to be 5.2S: this form was present in both cytosol and glyoxysomes. Under conditions where no aggregates were detectable in the cytosol, aggregates of malate and citrate synthase (70 and 55S, respectively) were recovered from isolated ER fractions. In spite of the relative ease with which aggregates could be generated by dilution of homogenates, no genuine aggregates appeared to be present in the cytosol when conditions of homogenization were carefully controlled.

The calculated, apparent mol wt of the 5.2S form of malate synthase is 71,600 daltons when catalase is used as the reference standard (4, 18, 19). Since the subunit mol wt of malate synthase has been reported (14) to be 63,000 daltons, the calculated mol wt of the 5.2S form is closest to that of the monomeric species. On the basis of a 6.8S value for citrate synthase, calculations (4, 18, 19) result in an apparent mol wt of 107,000 daltons. The glyoxysomal species of this enzyme has a subunit mol wt of 51,000 daltons (Gonzalez, unpublished). The subunit mol wt of castor bean mitochondrial citrate synthase is 47,000 daltons (Kagawa and González, unpublished). Thus, the castor bean 6.8S form of citrate synthase is, very probably, a dimer.

Although, evidence now points to a cytosolic site of synthesis for most glyoxysomal enzymes (10, 13; González, unpublished), it is clear that any model for glyoxysome biogenesis will have to take into account the significant presence of the synthases in the ER during early germination.

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## LITERATURE CITED

1. BEEVERS, H 1979 Microbodies in higher plants. Annu Rev Plant Physiol 30: 159–193
2. BOWDEN, L, JM LORD 1978 Purification and comparative properties of microsomal and glyoxysomal malate synthase from castor bean endosperm. Plant Physiol 61: 259–265
3. BREIDENBACH RW 1969 Characterization of some glyoxysomal proteins. Ann NY Acad Sci 168: 342–347
4. EDSALL JT 1953 The size, shape and hydration of protein molecules *In* H

Neurath, K Bailey, eds, The Proteins, Vol I, Part B. Academic Press, New York, p 637
5. GONZÁLEZ E 1978 Effect of gibberellin $A_3$ on the endoplasmic reticulum and on the formation of glyoxysomes in the endosperm of germinating castor bean. Plant Physiol 62: 449–553
6. GONZÁLEZ E, H BEEVERS 1976 Role of the endoplasmic reticulum in glyoxysome formation in castor bean endosperm. Plant Physiol 57: 406–409
7. HOCK B, H BEEVERS 1966 Development and decline of the glyoxylate-cycle enzymes in watermelon seedlings (*Citrullus vulgaris* Schrad). Z Pflanzenphysiol 55: 405–414
8. HUANG AHC, H BEEVERS 1973 Localization of enzymes within microbodies. J Cell Biol 58: 379–389
9. KAGAWA T, E GONZÁLEZ 1981 Organelle specific isozymes of citrate synthase in the endosperm of developing *Ricinus* seedlings. Plant Physiol 68: 845–850
10. KINDL H, W KÖLLER, J FREVERT 1980 Cytosolic precursor pools during glyoxysome biosynthesis. Z Physiol Chem 361: 465–467
11. KÖLLER W, J FREVERT, H KINDL 1979 Albumins, glyoxysomal enzymes and globulins in dry seeds of *Cucumis sativus*: qualitative and quantitative analysis. Z Physiol Chem 360: 167–176
12. KÖLLER W, H KINDL 1978 The appearance of several malate synthase-containing cell structures during the stage of glyoxysome biosynthesis. FEBS Lett 88: 83–86
13. KÖLLER W, H KINDL 1980 19S Cytosolic malate synthase: A small pool characterized by rapid turnover. Z Physiol Chem 361: 1437–1444
14. LORD JM, L BOWDEN 1978 Evidence that glyoxysomal malate synthase is segregated by the endoplasmic reticulum. Plant Physiol 61: 266–270
15. LORD JM, T KAGAWA, TS MOORE, H BEEVERS 1973 Endoplasmic reticulum as the site of lecithin formation in castor bean endosperm. J Cell Biol 57: 659–667
16. LUCK H 1965 Catalase. *In* HU Bergmeyer, ed, Methods in Enzymatic Analysis. Academic Press, New York, p 885
17. MCEWEN CR 1967 Tables for estimating sedimentation through linear concentration gradients of sucrose solution. Anal Biochem 20: 114–149
18. MARTIN RG, BN AMES 1961 A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J Biol Chem 236: 1372–1379
19. SCHROEDER WA, JR SHELTON, JB SHELTON, B ROBBERSON, G APELL 1969 The amino acid sequence of bovine liver catalase: a preliminary report. Arch Biochem Biophys 131: 653–655