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The Induction of Enzyme Activity in the Endosperm of Germinating Castor-Bean Seeds

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1. Endosperm extracts were prepared at various times during germination from intact castor-bean seeds and from seeds from which the embryos had been removed. The sterilized seeds were incubated either on solid water agar or on agar containing 0.3mM-gibberellic acid. 2. Isocitrate lyase and 3-hydroxyacyl-CoA dehydrogenase had very low activities in the mature seeds, but increased 44-fold and 27-fold respectively during germination. In contrast, the extracts of mature seeds had considerable acid and alkaline lipase activity and this only increased two- to three-fold during the incubation period. 3. Incubation of the seeds with gibberellic acid accelerated the rate of appearance of isocitrate lyase and 3-hydroxyacyl-CoA dehydrogenase. It also increased the total activity attained. However, the application of hormone had, in comparison, little effect on the development of lipase activity. 4. The removal of the embryo had little influence on the development of enzyme activity in the endosperm tissue; only with isocitrate lyase was a decrease in activity observed in the absence of the embryo.

The biochemical pathways involved in the mobilization of the storage lipid in the endosperm of germinating castor-bean seeds have been well characterized (Beevers, 1961; Canvin & Beevers, 1961). The stored triglycerides are converted into sucrose, and a substantial part of this, β-oxidation and the glyoxylate cycle, takes place in single membrane-bounded organelles termed glyoxysomes (Breidenbach *et al.*, 1968; Cooper & Beevers, 1969*a*,*b*; Hutton & Stumpf, 1969). These organelles are absent from mature castor beans, but their number and characteristic enzyme activity increases during germination and subsequently declines in phase with fat utilization (Gerhardt & Beevers, 1970).

We investigated the breakdown of storage lipid in germinating castor beans (Marriott & Northcote, 1975). Incubation of sterilized seeds on agar containing 0.3mM-gibberellic acid stimulated the rate of fat breakdown without altering the relative proportions of triglyceride species or the fatty acid composition. The removal of the embryo initially had no effect on the mobilization of lipid reserves; only after 6 days of germination was the rate of lipid breakdown decreased in the absence of the embryo.

We now report experiments that examined the influence of gibberellic acid and the removal of the embryo on the enzymes involved in the utilization of endosperm reserves. Isocitrate lyase (EC 4.1.3.1) was chosen as a marker for glyoxylate-cycle activity and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) as a marker for β-oxidation. Castor-bean endosperm contains two lipases (EC 3.1.1.3): an acid lipase with optimal activity at pH 5, characterized by Ory and colleagues (Ory *et al.*, 1960, 1962; Ory, 1969; Noma & Borgström, 1971), and an alkaline lipase, located chiefly in the glyoxysomes (Muto & Beevers, 1974). As these enzymes are required for the initial breakdown of the storage triglycerides, the development of lipase activity may be an important control point in the mobilization of reserves.

There are different findings on the importance of the embryo in the development of isocitrate lyase activity in various oleaginous seeds. Marcus & Feeley (1964) found that in peanut (*Arachis hypogaea*) seeds the enzyme formation proceeded at approximately the same rate in intact cotyledons as in those dissected free from the embryo. However, in squash (*Cucurbita maxima*) seeds, over half the increase in isocitrate lyase activity was under the control of the axial tissue (Penner & Ashton, 1967). Gibberellic acid was incapable of restoring this stimulus, but benzyladenine was active in partially restoring the effect of the embryonic axis. Nevertheless, exogenous application of gibberellic acid to germinating hazel (*Corylus avellana*) seeds was found to promote isocitrate lyase activity (Pinfield, 1968).

In the aleurone tissue of germinating wheat (*Triticum vulgare*) and barley (*Hordeum vulgare*), the induction of glyoxylate-cycle activity is dependent on the presence of the embryo, and in this case gibberellic acid is probably the principal inducing factor (Doig & Laidman, 1972; Jones, 1972). In contrast, the development of lipase activity in the wheat aleurone cells, although under the control of the embryo, could not be induced by gibberellic acid and was stimulated by glutamine together with indolylacetic acid (Taverner & Laidman, 1972).

Materials and Methods

Materials

Castor bean (*Ricinus communis* var. Zanzibariensis mixed) seeds were purchased locally (Sanders, Cambridge, U.K.). Gibberellic acid, containing not less than 90% gibberellic acid 3, was obtained from BDH (Poole, Dorset, U.K.), DL-isocitric acid (allo-free) from Calbiochem (San Diego, Calif., U.S.A.) and *p*-nitrophenyl decanoate from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Acetoacetyl-CoA was prepared by the method of Wieland & Rueff (1953).

Germination of seeds

All manipulations were carried out under sterile conditions on a sterile air bench. For each time-point, seeds of a similar weight (±0.5g) were selected and surface-sterilized by immersion in ethanol for 2min. The testa was removed and the seeds were then soaked for a further 2min in Milton solution (Richardson–Merell Ltd., London W1X 2AN, U.K.). After thorough rinsing with sterile water, the seeds were placed either intact or with the embryos removed on 60ml of solid agar in polypropylene jars of 700ml capacity. Noble agar (1%; Difco Laboratories, Detroit, Mich., U.S.A.) in glass-distilled water was used and for some experiments contained 0.3mM-gibberellic acid sterilized by Seitz filtration. The seeds were allowed to germinate in the dark at 26±2°C.

Preparation of endosperm extract

At selected time-intervals the endosperm material was carefully removed from the cotyledons and embryonic axis and weighed. The tissue was then homogenized in an MSE homogenizer at top speed for 4min in a suitable volume (1g of endosperm/4ml of buffer) of chilled 0.2M-phosphate buffer, pH7.5, containing 0.01M-EDTA, 0.01M-KCl, 0.001M-$MgCl_2$ and 0.001M-dithiothreitol. After homogenization, 5% (v/v) Triton X-100 (one-tenth the volume of homogenizing buffer used) was added to the tissue extract and this was left, with occasional shaking, for 30min. The homogenate was centrifuged at 1080*g* for 20min and the resultant supernatant used for enzyme assays. The floating fat layer was carefully removed and resuspended in a suitable volume of buffer for lipase assays. All procedures were carried out at 4°C.

Enzyme assays

All enzyme assays were carried out spectrophotometrically by using a Beckman DB GT recording spectrophotometer.

Isocitrate lyase. This was assayed by the method of Hock & Beevers (1966). The reaction mixture contained, in a final volume of 3ml: 10μmol of KH_2PO_4 adjusted to pH7.1 with NaOH, 15μmol of $MgCl_2$, 10μmol of phenylhydrazine hydrochloride (prepared fresh each day), 6μmol of EDTA and 1ml of diluted endosperm extract. The reaction was started by the addition of 10μmol of DL-isocitric acid, adjusted to pH7.1 with KOH. The change in absorbance at 324nm was recorded against a reference cell containing water in place of the substrate. The molar extinction coefficient was taken to be 1.70×10^4 litre·mol^{-1}·cm^{-1}.

3-Hydroxyacyl-CoA dehydrogenase. This activity was determined by a modification of the procedure of Overath & Raufuss (1967). In a final volume of 0.5ml, the assay mixture contained: 25μmol of KH_2PO_4 adjusted to pH6.8 with NaOH, 1μmol of EDTA, 0.125μmol of NADH, 16μmol of KCN, 0.5mg of defatted serum albumin and 0.1ml of diluted endosperm extract. The reaction was started by the addition of 0.125μmol of acetoacetyl-CoA and the change in absorbance at 366nm measured against a water blank. Control assays, identical except for the replacement of the substrate with water, were carried out. Calculations were made by assuming a molar extinction coefficient of 6.25×10^3 litre·mol^{-1}·cm^{-1}.

Lipases. Determination of lipase activity was made by measuring the enzymic release of *p*-nitrophenol from the substrate *p*-nitrophenyl decanoate (Desnuelle & Savary, 1963; Hayase & Tappel, 1970) by a modification of the method of Teng & Kaplan (1974). The assay mixture contained, in a final volume of 0.5ml, either 40mM-sodium acetate buffer, pH5, or 0.1M-Tris–HCl buffer, pH8.5, together with 0.25μmol of *p*-nitrophenyl decanoate, 2.5μmol of EDTA and Triton X-100 (3.75g/litre). Samples of the substrate, *p*-nitrophenyl decanoate in chloroform, were dried down and, after addition of the buffer, the tubes were warmed to 60°C, the contents mixed thoroughly and cooled. The reaction was started by the addition of 0.1ml of the diluted endosperm extract and allowed to proceed at 37°C. After 15min, 50μl of 3M-trichloroacetic acid was added to stop the reaction and the pH adjusted by the addition of 2.5ml of 0.2M-Tris–HCl buffer, pH8.5. The absorbance at 410nm was measured against a reference cell to which water had been added instead of the enzyme solution. These values were compared with those obtained without the addition of the substrate. The molar extinction coefficient was taken to be 1.83×10^4 litre·mol^{-1}·cm^{-1}.

Results

Germination of seeds and treatment of results

The seeds incubated on agar containing gibberellic acid were found to have a more swollen appearance than equivalent seeds grown on water agar. The fresh weight of the endosperm tissue from these

swollen seeds was higher at each time-point during germination (Fig. 1).

It was not possible to express satisfactorily the results of the enzyme activities as a total amount for each endosperm, since although similar weights of seeds were taken for each time-point in duplicate samples for each experiment, these weights were not always the same for samples for different points of the same time-course. The weights of the seeds varied from 0.7 to 1.1g, and for seeds of 0.7g the fresh weight of the endosperm was 0.4g. This fresh weight increased during germination as shown in Fig. 1.

Because of the variation in the weights of the seeds and endosperms each point of any experiment was obtained from duplicate samples and, where significant differences are claimed, two or three experiments were made. The bars on the graphs indicate the variability of the results obtained. The general pattern of the induction of the enzymes for the various treatments was always the same.

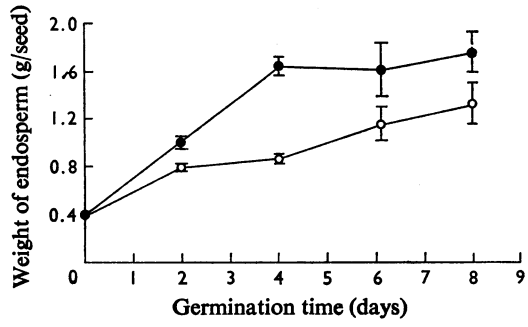

Fig. 1. *Changes in the weight of the endosperm tissue during germination*

Intact seeds weighing 0.7g were selected and germinated on water agar (○) or on agar containing 0.3mM-gibberellic acid (●). The range of weights obtained is indicated by bars.

Development of enzyme activity

Isocitrate lyase. Extracts of the endosperm tissue from mature castor-bean seeds contained very low isocitrate lyase activity, but after 24h it began to rise and reached a maximum at 6 days of germination (Fig. 2). When the embryo was removed from the seed there was a slower rise, and the peak observed with intact seeds was not attained. Incubation of

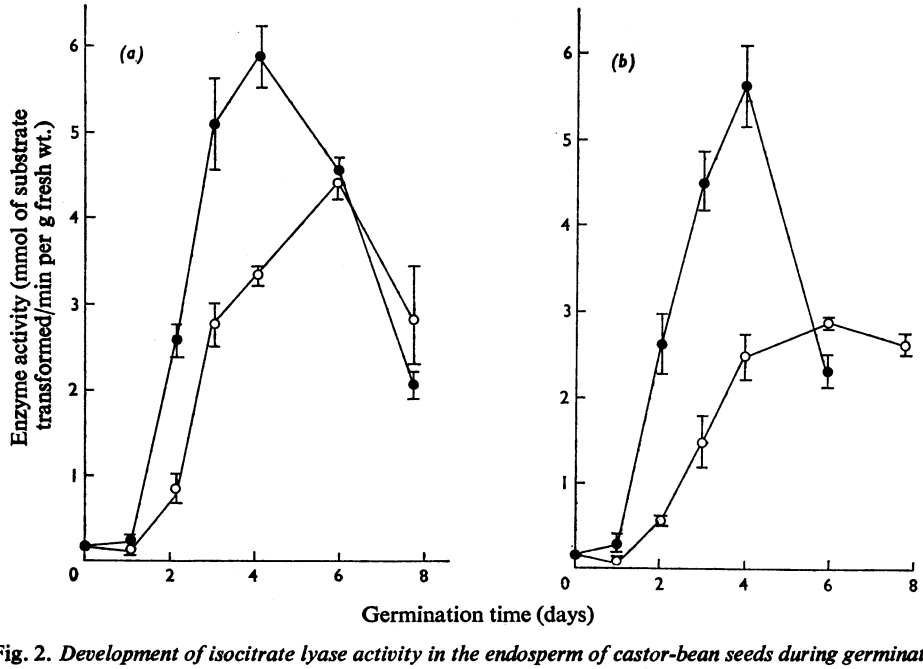

Fig. 2. *Development of isocitrate lyase activity in the endosperm of castor-bean seeds during germination*

Seeds were germinated for different time-periods either intact (*a*) or with the embryo removed (*b*) on either water agar (○) or on agar containing 0.3mM-gibberellic acid (●). An extract of the endosperm tissue was prepared, and isocitrate lyase activity assayed as described in the text. Each value is the mean for duplicate, and in some cases quadruplicate, experiments. The range of activity obtained is indicated by bars.

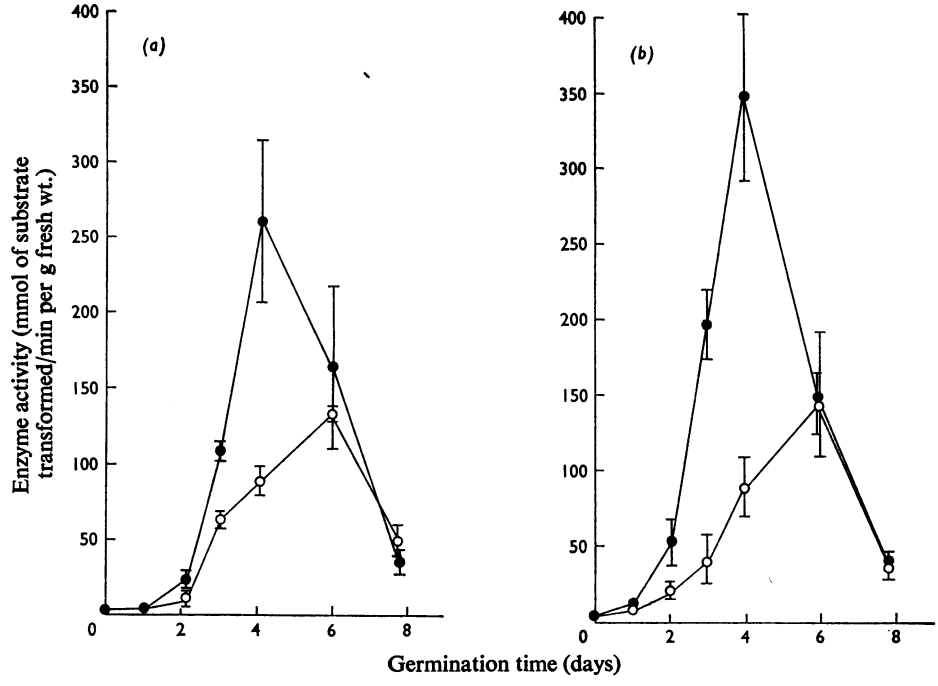

Fig. 3. *Development of 3-hydroxyacyl-CoA dehydrogenase activity in the endosperm of castor-bean seeds during germination*

Seeds were germinated for different time-periods either intact (*a*) or with the embryo removed (*b*) on either water agar (○) or on agar containing 0.3 mM-gibberellic acid (●). An extract of the endosperm tissue was prepared and 3-hydroxyacyl-CoA dehydrogenase activity assayed as described in the text. Each value is the mean for duplicate, and in some cases quadruplicate, experiments. The range of activity obtained is indicated by bars.

both intact and embryoless seeds with gibberellic acid caused a marked stimulation in the rate of development of isocitrate lyase activity. A peak of maximum activity was found after 4 days of germination and was significantly higher than that reached in seeds incubated on water agar.

3-Hydroxyacyl-CoA dehydrogenase. The endosperm extract from mature seeds showed very little 3-hydroxyacyl-CoA dehydrogenase activity, but as with isocitrate lyase, after 24h of incubation it began to rise and reached a peak at 6 days of germination (Fig. 3). In this case the absence of the embryo had little effect on the development of enzyme activity. However, with both intact and embryoless seeds, the application of gibberellic acid increased the rate of appearance of 3-hydroxyacyl-CoA dehydrogenase activity and also the activity attained, which increased twofold to a maximum after 4 days of germination.

Lipases. The endosperm tissue from mature castor beans showed both acid and alkaline lipase activity. Fig. 4 shows that the acid lipase was most active during the first 2 days of germination and it subsequently declined. It was difficult to collect quantitatively the fat layer floating on the supernatant after centrifuging at 1080*g* and hence the values of the enzyme activity in this fraction have not been included in Fig. 4. The maximum value of acid lipase activity in this fat layer was found at the first day of germination, when it reached 20% of that of the supernatant. After this time it decreased to a negligible amount. Nevertheless, it was possible to examine the influence of the embryo and the application of gibberellic acid on the pattern of development of acid lipase activity. The removal of the embryo did not alter the development of enzyme activity in the endosperm tissue. Incubation of both embryoless and intact seeds with gibberellic acid had little effect on the rise in acid lipase activity, although it possibly hastened its decline. Ory & St. Angelo (1971) also reported that the disappearance of acid lipase activity from castor beans was accelerated by treatment with gibberellic acid. The apparent fall in acid lipase activity at 12h of germination (Fig. 4) was due to uptake of water during this period, leading to a rapid increase in fresh weight of the endosperm.

The alkaline lipase was most active during the later stages of germination when the acid lipase activity was declining (Fig. 5). However, once

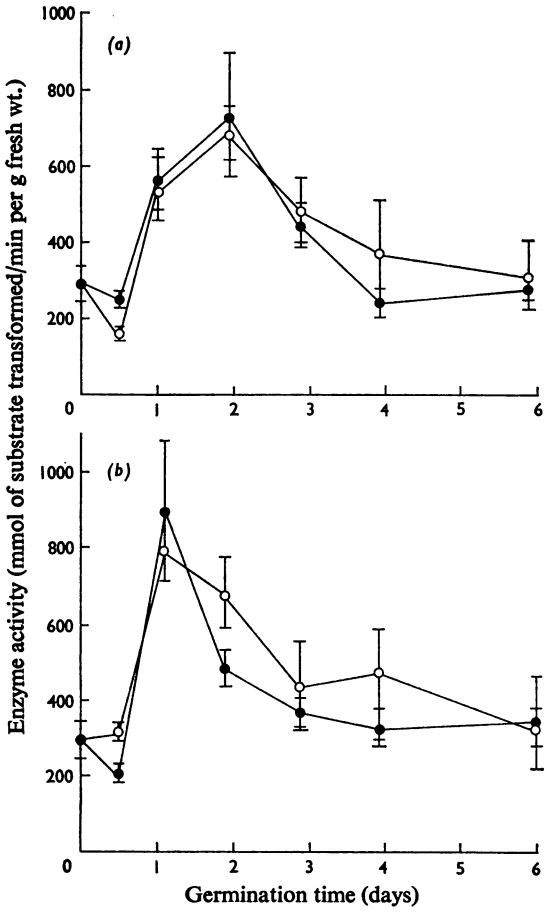

Fig. 4. *Development of acid lipase activity in the endosperm of castor-bean seeds during germination*

Seeds were germinated for different time-periods either intact (*a*) or with the embryo removed (*b*) on either water agar (○) or on agar containing 0.3mM-gibberellic acid (●). An extract of the endosperm tissue was prepared and acid lipase activity assayed as described in the text. Each value is the mean for duplicate, and in some cases quadruplicate, experiments. The range of activity obtained is indicated by bars.

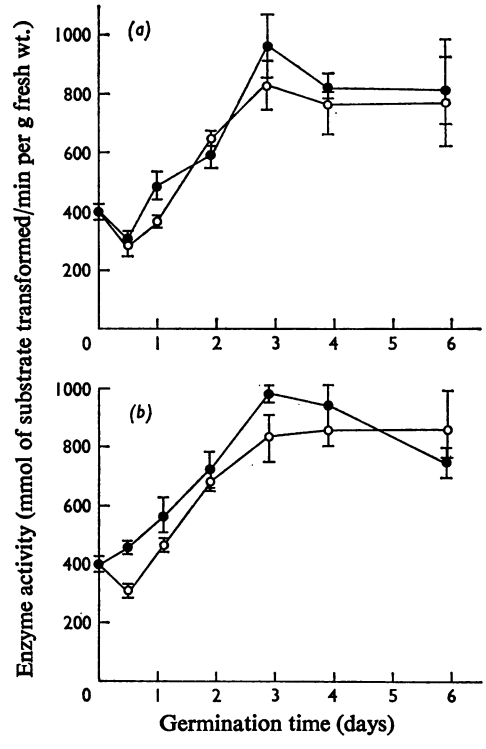

Fig. 5. *Development of alkaline lipase activity in the endosperm of castor-bean seeds during germination*

Seeds were germinated for different time-periods either intact (*a*) or with the embryo removed (*b*) on either water agar (○) or on agar containing 0.3mM-gibberellic acid (●). An extract of the endosperm tissue was prepared and alkaline lipase activity assayed as described in the text. Each value is the mean for duplicate, and in some cases quadruplicate, experiments. The range of activity obtained is indicated by bars.

again, the embryo exerted little control over the enzyme activity. Gibberellic acid slightly stimulated the development of alkaline lipase activity, but not to the extent of its effect on isocitrate lyase and 3-hydroxyacyl-CoA dehydrogenase.

Discussion

The application of gibberellic acid has a marked stimulatory effect on the development of isocitrate lyase and 3-hydroxyacyl-CoA dehydrogenase, although the hormone has no such action on the activities of acid and alkaline lipases. The lipases were considerably active in extracts from mature seeds, but during germination this only increased two- to three-fold. The pattern of development of the acid and alkaline lipase activities in intact seeds agrees with the results of Muto & Beevers (1974). In contrast, both isocitrate lyase and 3-hydroxyacyl-CoA dehydrogenase had very low activities in the endosperm tissue of dry seeds, but after 6 days of germination these had risen 44-fold and 27-fold respectively.

It is improbable that the high lipase activity found in the endosperm extracts was functional in the intact endosperm tissue of the resting seed, as this would lead to a rapid turnover of storage triglycerides. Perhaps the enzyme was not in contact

with its substrate or its action was blocked by a localized inhibitor which was removed on preparation of the tissue extracts. Hydration of the endosperm tissue on germination of the seed may allow the enzyme to become operative. Nevertheless, lipase activity was not limiting the mobilization of storage reserves during germination.

The marked stimulation of glyoxylate-cycle and β-oxidation activity by gibberellic acid could account for its effect in increasing the rate of lipid breakdown (Marriott & Northcote, 1975). The removal of the embryo had little significant effect on the development of enzyme activity in the endosperm; only for isocitrate lyase was any difference observed. Although isocitrate lyase activity increased in the endosperm of embryoless seeds during germination, the high activity normally found in intact seeds after 6 days of germination was not attained. This is very similar to the influence of the embryo on the mobilization of lipid during germination (Marriott & Northcote, 1975). Initially little difference was found in the presence or the absence of the embryo; only after 6 days of germination was the rate of fat breakdown decreased in embryoless seeds.

Huang & Beevers (1974) have studied the activity of a number of enzymes in the endosperm of mature castor beans and compared the activity (at a single time during germination) in intact seeds germinated for 5 days with those from which the embryo or embryo and cotyledons had previously been removed. In these experiments the removal of the embryo was found to decrease β-oxidation (fatty acyl-CoA dehydrogenase) activity but not that of isocitrate lyase. Huang & Beevers (1974) also concluded that the embryonic axis did not directly control the developmental changes in the endosperm. The present experiments, however, have investigated the time-course for the development of enzyme activity in the presence and absence of the embryo and attempted to relate this to the depletion of storage reserves in the endosperm tissue. During germination of castor beans the enzymes (apart from the lipases) involved in the utilization of storage reserves are induced in a co-ordinated manner in the endosperm tissue, and the embryonic axis is not necessary for the production of the stimulus. Application of gibberellic acid has a marked stimulatory effect on the development of enzymes located in the glyoxysomes, and it would be interesting to discover if there was a concomitant increase in the number of these organelles.

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