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THE EFFECTS OF SUGARS ON THE DEVELOPMENT OF HEXOSE PHOSPHORYLATING ENZYMES IN THE CASTOR BEAN COTYLEDONS

E. MARRÈ, M. P. CORNAGGIA and R. BIANCHETTI

Laboratory of Plant Physiology, Institute of Plant Sciences, University of Milan, Italy

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Abstract—The rate of development of soluble glucokinase and fructokinase activities in cotyledons isolated from castor bean seeds germinated for 36–48 hr is markedly increased by the presence of glucose, fructose or sucrose in the incubation medium. A similar effect is found for galactose on galactokinase activity. A certain degree of specificity in the effects of the different sugars on the three enzymes is observed. The rates of development of other enzyme activities such as phosphoglucomutase, glucose-6-phosphate dehydrogenase, aldolase, and NADP-dependent isocitrate dehydrogenase are not affected by the sugars. It is suggested that the effects of the sugars on kinase activities are due to enzyme induction of the type described in bacteria and lower organisms.

INTRODUCTION

STUDIES on micro-organisms demonstrate the capacity of substrates to induce or repress the synthesis of enzymes. Knowledge of similar phenomena in higher plants is extremely limited. In particular no evidence is available concerning possible control of the biosynthesis of key enzymes of respiratory metabolism by their substrates. On the other hand, wide differences are recognizable, among the different organs of a same plant, in both the concentration of the various free sugars and the level of enzymes involved in their metabolism.[1] This might suggest that the level of free sugars influences the synthesis of the enzymes which catalyse sugar utilization, and thus the development of the various pattern of carbohydrate metabolism characteristic of the different plant organs. It was thought that the problem might be studied successfully in organs where the transition from quiescence to activity is accompanied by a rapid increase of both the concentration of substrates and the levels of enzymes. In the cotyledons of castor bean seeds, germinated from 24–48 hr, both the concentration of soluble carbohydrates and the activity of hexokinase are very low; respiration is mainly supported by lipids (the RQ is *ca.* 0·6). Only later do the carbohydrates increase as the result of the transformation of stored lipids.[2] Simultaneously a marked increase of sugar phosphorylating enzymes is observed.[3] These findings suggest a causal relationship between the increase of substrates and that of enzyme activities. Inhibitors of protein synthesis, such as actinomycin, puromycin and cycloheximide, prevent the rise of enzyme activity during germination in the castor bean endosperm[4, 5] as well as in the cotyledons,[6] indicating that the increase in enzyme activity may be due to *de novo* synthesis.

[1] R. BIANCHETTI and E. MARRÈ, *Giorn. Bot. Ital.* **69**, 299 (1962).
[2] H. BEEVERS, *Respiratory Metabolism in Plants*. Row Peterson, Evanston (1961).
[3] E. MARRÈ, M. P. CORNAGGIA, F. ALBERGHINA and R. BIANCHETTI, *Biochem. J.* **97**, 20p (1965).
[4] F. ALBERGHINA, *Giorn. Bot. Ital.* **71**, 385 (1964).
[5] F. ALBERGONI, P. LADO, G. MARZIANI and E. MARRÈ, *Giorn. Bot. Ital.* **71**, 469 (1964).
[6] P. LADO and M. SCHWENDIMANN, *Giorn. Bot. Ital.* **72**, 319 (1965).

The present report deals with the effects of feeding glucose, fructose and galactose on the levels of hexokinase, fructokinase and galactokinase activities in cotyledons isolated from germinating seeds.

RESULTS AND DISCUSSION

Hexokinase and Fructokinase Activities

Crude homogenates of castor bean (*Ricinus communis*) phosphorylate both glucose and fructose. The enzymatic procedure employed for the determination of activity shows that the products are glucose-6-P and fructose-6-P, respectively. The activities are found both in the supernatant and in the sediment from centrifugation at 20,000 × *g* (Fig. 1).

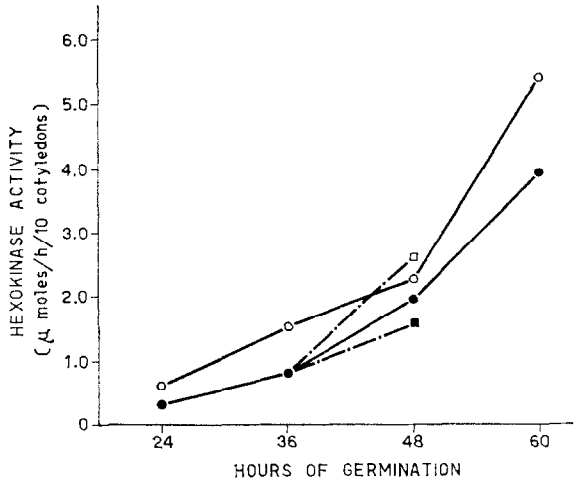

FIG. 1. DEVELOPMENT OF SOLUBLE AND PARTICULATE GLUCOKINASE ACTIVITIES IN COTYLEDONS LEFT IN THE SEED OR ISOLATED AND INCUBATED IN CULTURE MEDIA.
Open circles: particulate activity. Full circles: soluble activity in cotyledons in the intact seed. Open squares: soluble activity of cotyledons isolated from the seed at the 36th hr of germination and incubated in 0·1 M glucose. Full squares: soluble activity of isolated cotyledons incubated in water.

A 2 hr centrifugation at 100,000 × *g* of the 20,000 × *g* supernatant did not sediment appreciable activity. The ratio of the activity with fructose to that with glucose was close to 3 in the soluble fraction, and close to 1·4 in the particulate fraction. At least two kinases are present in the soluble fraction. One of them, active only on fructose, can be obtained free from the glucose phosphorylating activity by means of ammonium sulphate fractionation (Table 1). A similar kinase specific for fructose has been described in peas.[7] The activity precipitated by 30 per cent saturated ammonium sulphate (Fraction I, Table 1) might be due either to a single hexokinase, acting on both substrates, or to two (or more) different enzymes. All of the particulate activity which can be solubilized with Triton X 100 is precipitated at 30 per cent saturation of ammonium sulphate. The dialysed, or Sephadex filtered, 20,000 × *g* supernatants show also a significant galactokinase activity. In the cotyledons isolated from seeds germinated 36 hr the activity with galactose is about 1/3 of that with glucose and 1/12 of that with fructose. These activity ratios markedly change when the isolated cotyledons

[7] A. MEDINA and A. SOLS, *Biochim. Biophys. Acta* **19**, 378 (1956).

TABLE 1. FRACTIONATION BY AMMONIUM SULPHATE OF THE SOLUBLE ACTIVITY

Fraction (% saturation)	Substrate	
	Glucose activity (μmoles/hr/10 cotyledons)	Fructose
I 0–30 precipitate	1·50	0·74
II 30–50 precipitate	0·78	4·30
III 50 supernatant	0·77	6·05
IV 60–70 precipitate from III	0·00	2·80

Fractionation of the 20,000 × *g* supernatant was effected by dialysis against solutions containing the desired conc. of ammonium sulphate in 0·05 M Tris, 0·005 mercaptoethanol, 0·005 M KCl and 0·01 M $MgSO_4$, pH 7·5. Precipitates were resuspended in 0·05 M Tris, 0·01 M mercaptoethanol, pH 7·3. Fraction IV was obtained by overnight dialysis of fraction III against ammonium sulphate-free buffer, centrifugation at 10,000 × *g* and fractionation of the supernatant as described above.

are incubated in the presence of the various sugars. This suggests that the galactose kinase activity here observed is due to a specific enzyme; quite probably, a galactokinase corresponding to the enzyme found in bean extracts by Neufeld *et al.*[8]

Sugar Induced Changes of Hexose Phosphorylating Activities

In the experiments dealing with the effects of sugars on the development of kinase activities, these have been determined under conditions under which the activities phosphorylating glucose, fructose, and galactose were present together. Thus the values found for the phosphorylation of fructose depend on the activity of fructokinase as well as on that of hexokinase (an hexokinase specific for both glucose and fructose is widely diffused in higher plants). Similarly, the activity with glucose might depend on more enzymes than a non-specific hexokinase. In the results given here, however, for sake of conciseness the activity measured with glucose, fructose and galactose as substrates will be referred to as "hexokinase", "fructokinase" and "galactokinase" activities, respectively.

As shown in Fig. 1, both the soluble and particulate hexokinase activities increase in the cotyledons during germination. In isolated cotyledons incubated in water the increase of the soluble activity is of the same order as that observed in the cotyledons left for the same period in the intact seed. The presence of glucose or of fructose in the incubation medium markedly increased the soluble hexokinase and fructokinase activities (Figs. 1 and 2; Tables 2 and 3). No effect of the hexoses on the particulate activity was observed.

The effect of the sugars on the development of soluble kinase activities is already evident after 10 hr and is most evident, on a percentage basis, after 15 hr of incubation of the cotyledons in the sugar solutions (Fig. 2); at longer incubation periods the development of glucose and fructose kinase activities becomes very rapid also in the control cotyledons incubated in water, which makes more uncertain the detection of the sugar effect.

Table 2 shows that the reproducibility of the effect of glucose on the development of the activities phosphorylating glucose is satisfactory, and that its value remains unchanged when measured on dialysed extracts. This seems to rule out the possibility that the observed differences in activity are due to the presence of activators or inhibitors in the crude extracts.

[8] E. F. NEUFELD, D. S. FEINGOLD and W. Z. HASSID, *J. Biol. Chem.* **235**, 905 (1960).

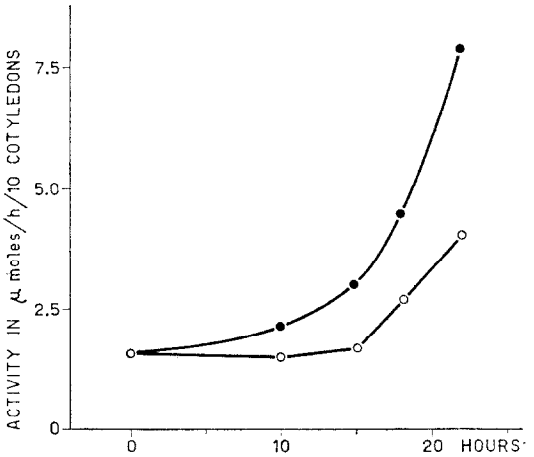

FIG. 2. THE EFFECTS OF 0·1 M FRUCTOSE ON THE DEVELOPMENT OF FRUCTOKINASE ACTIVITY IN COTYLEDONS ISOLATED AT THE 32nd hr OF GERMINATION.
Full circles: fructose; open circles: water.

TABLE 2. THE EFFECT OF GLUCOSE ON THE DEVELOPMENT OF SOLUBLE HEXOKINASE ACTIVITY IN ISOLATED COTYLEDONS

	Activity in μmoles/10 cotyledons/hr			Effect of glucose on kinase development
		H_2O	0·1 M glucose	
Exp. No.	At removal from seed	[Increase in activity (ΔH_2O)]	[Increase in activity (Δgluc)]	$\frac{\Delta gluc - \Delta H_2O}{\Delta H_2O} \times 100$
I	1·1	0·8	2·0	150
II	1·7	2·0	3·5	75
III	1·0	0·8	2·5	212
IV	1·0	0·6	2·0	233
V	1·1	1·7	2·8	62
VI	1·6	1·7	3·2	88
VI bis	1·5	1·6	3·1	93

Activity was determined on the supernatant from centrifugation at 20,000 × *g*. Cotyledons were removed from seeds germinated 34 hr and incubated 13 hr in water or in 0·1 M glucose. In Experiment VI bis the activity was determined on the same extracts of Experiment VI, but after extensive dialysis against 0·05 M Tris, pH 7, containing 0·01 M $MgSO_4$ and 0·05 M KCl.

The same conclusion is suggested by the finding that the kinase activity of a mixture of extracts from cotyledons incubated in water or in glucose and respectively in water closely corresponds to the sum of the separately determined activities of the two extracts.

The data of Table 3 suggests a certain degree of specificity in the effect of glucose and of fructose on the development of the hexokinase and of the fructokinase activities: fructose appears most effective in increasing the development of fructokinase and glucose in increasing that of the glucose phosphorylating activity. The specificity of induction might be higher than apparent from the data, as glucose and fructose are rather rapidly interconverted in the tissues.

TABLE 3. THE EFFECTS OF VARIOUS SUGARS ON INCREASE OF HEXOKINASE, FRUCTOKINASE AND GALACTOKINASE ACTIVITIES

Culture medium	% increase from the initial value		
	Hexokinase	Fructokinase	Galactokinase
Water	90	103	58
0·1 M Glucose	185	145	—
0·1 M Fructose	122	205	—
0·01 M Fructose	—	—	58
0·05 M Fructose	—	210	60
0·01 M Galactose	—	103	129
0·05 M Galactose	—	—	141

Cotyledons were removed from seed after 36 hr of germination and incubated 12 hr in the culture media indicated. The initial activities (at removal from seeds) were: 1·0, 2·8 and 0·29 μmoles/hr/10 cotyledons respectively for hexokinase, fructokinase and galactokinase.

As shown by Fig. 3 the response of fructokinase is already evident for concentrations of fructose lower than 3×10^{-3} M, which are, presumably, well within the physiological range. The effect of fructose appears maximal for the 10^{-2} M concentration and it decreases for the higher concentrations. Galactose has no effects at concentrations up to 10^{-2} M; the relatively modest effect at higher concentrations can be tentatively explained by some conversion of this sugar to glucose and fructose.

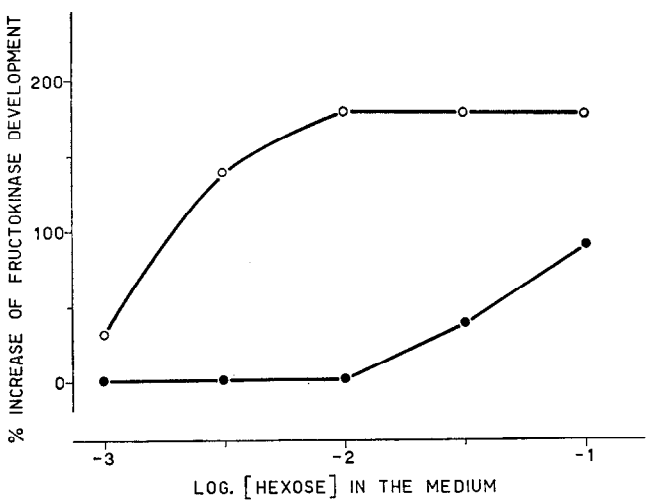

FIG. 3. THE EFFECT OF INCREASING CONC. OF FRUCTOSE AND GALACTOSE ON THE DEVELOPMENT OF FRUCTOKINASE ACTIVITY IN COTYLEDONS ISOLATED AT THE 32nd hr OF GERMINATION AND INCUBATED IN FRUCTOSE (OPEN CIRCLES) OR IN GALACTOSE (FULL CIRCLES) FOR 13 hr.

The development of galactokinase activity in the isolated cotyledons appears quite significantly stimulated by the presence in the medium of concentration of galactose too low to affect the enzymes phosphorylating glucose and fructose, while concentrations of fructose inducing the maximum effect on fructokinase leave the rate of development of galactokinase unchanged.

This suggests that galactokinase synthesis can also be specifically stimulated by the presence of galactose.

Specificity of the Effect of the Hexoses

The presence of sugars in the medium where the isolated cotyledons are incubated has been shown to induce a number of physiological changes, such as a stimulation of water uptake and a sharp change of the respiratory quotient, due to the substitution of lipids with carbohydrates as respiratory substrate.[9] This might suggest that the above-described changes of the rate of kinase development are due to a largely non-specific effect of the hexoses on the general metabolic pattern of the cotyledons. To test this possibility, the effects of the hexoses on several other commonly occurring enzymes were investigated.

TABLE 4. LACK OF STIMULATING EFFECTS OF GLUCOSE ON THE DEVELOPMENT OF SOME ENZYMATIC ACTIVITIES IN ISOLATED COTYLEDONS

Enzyme activity	Initial activity (μmoles/10 cot./hr)	Increase of activity after 12 hr incubation in		% effect of glucose on the increase of activity
		Water	0·1 M Glucose	
Aldolase	19·1	9·6	8·3	−13
Isocitrate dehydrogenase	5·2	14·1	14·9	+ 5
Glucose-6-P dehydrogenase	4·0	4·1	4·3	+ 5
6-P-Gluconate dehydrogenase	2·6	3·3	3·5	+ 6
FDP (1)phosphohydrolase	3·7	1·6	0·6	−62

Cotyledons were isolated at the 32nd hr of germination. Activities were determined in the undialysed 20,000 × *g* supernatant, prepared as described in "Methods".

The data of Table 4 show that the presence of glucose did not significantly stimulate the rate of the development of any of the five enzymes investigated; actually, in the case of the fructose diphosphatase, glucose inhibited the increase of enzyme activity. A clear-cut inhibitory effect of glucose on the synthesis of this enzyme has been shown in the germinating wheat embryos.[10] It appears likely, therefore, that the inhibition of its development in the cotyledons incubated with glucose is due to repression of its synthesis by glucose or some of its derivatives. Also fructose and galactose did not induce any significant changes of the rate of increase of aldolase, isocitrate dehydrogenase, 6-P-gluconate dehydrogenase and glucose-6-P dehydrogenase.

These results are therefore in agreement with the view that the hexose induced stimulation of the development of the respective kinases is a specific effect, and that it does not involve a generic activation of the synthesis of enzymatic proteins.

A fundamental question which arises by the above observations is whether the observed, apparently specific stimulation of kinase development depends on *de novo* synthesis or on activation of some pre-existing inactive forms of these enzymes. Several considerations support the former hypothesis. Parallel investigations carried out in this laboratory[11] show that protein synthesis inhibitors strongly inhibit the development of enzyme activities in the isolated castor bean cotyledons. It appears, therefore, that the increase of enzyme activities

[9] F. ALBERGHINA and E. MARRÈ, *Acc. Naz. Lincei fasc.* **2**, serie VIII, vol. XXXVIII (1965).
[10] E. BIANCHETTI and M. L. SARTIRANA, *Biochem. Biophys. Res. Commun.* **27**, 378 (1967).
[11] E. MARRÈ, *Current Topics in Developmental Biology*. In press (1967).

in this material depend on *de novo* synthesis, and not on activation. In the case of fructokinase activity, the experiments of Table 5 show that actinomycin D, at the concentrations required for the almost complete inhibition of RNA synthesis in this material,[11] markedly inhibit the development of fructokinase, the effect of the inhibitor appearing larger, on a percentage basis, in the presence of the inducer fructose.

TABLE 5. THE EFFECT OF ACTINOMYCIN ON THE DEVELOPMENT OF FRUCTOKINASE AND OF G1-6-P-DEHYDROGENASE

	Activity in μmoles/hr/10 cotyledons			
	Fructokinase		G1-6-P-dehydrogenase	
14 hr incubation in:	Increase of activity	Inhibition of increase by actinomycin	Increase of activity	Inibition of increase by actinomycin
Water	0·43	—	5·0	—
Actinomycin (100 μg/ml)	0·27	35%	1·1	78%
0·1 M fructose	1·05	—	5·0	—
0·1 M fructose plus actinomycin	0·57	45%	1·3	74%

The cotyledons were isolated from seeds germinated 33 hr. The activities at the 0 time of incubation were: 0·71 μmoles/10 cotyledons/hr, for fructokinase and 4·9 μmoles for Glucose-6-P-dehydrogenase.The data are averages from three different experiments.

These results are consistent with the concept that kinase induction by fructose requires *de novo* synthesis of messenger RNA.

Discussion

The evidence reported here indicates that the synthesis of hexose phosphorylating enzymes can be specifically influenced by changes in the concentrations of their substrates.

Similar observations have recently been made in animal tissues: the rate of synthesis of glucokinase[12, 13] and galactokinase[14] in the rat liver, and that of hexokinase in cultured rat kidney[15] and human[16] cells have been reported to be enhanced by a rise in the level of their substrates.

In regard to the mechanism of kinase induction, two main points are open to discussion: (*a*) the nature of the metabolite acting as an inducer; (*b*) the step of the synthetic mechanism at which it operates.

Some recent reports show that in several cases of induction of enzyme synthesis a product of the inducible enzyme, rather than a substrate, can be the inducer (e.g. in the case of glycerol kinase[17] and of β-galactosidase in bacteria.[18]) Thus, in our material, the synthesis of the kinases might be stimulated either by the free hexoses or by their phosphorylated derivatives.

[12] D. S. WALKER, *Advances in Enzyme Regulation*, Vol. 3, pp. 163–184. Pergamon Press, Oxford (1965).
[13] M. SALAS, E. VIÑUELA and A. SOLS, *J. Biol. Chem.* **238**, 3535 (1963).
[14] P. CUATRECASAS and S. SEGAL, *J. Biol. Chem.* **240**, 2382 (1965).
[15] R. LICHERMAN and A. N. HUNT, *Biochim. Biophys. Acta* **81**, 612 (1964).
[16] H. M. KATZEN, D. D. SODERMAN and H. M. NITOWSLAY, *Biochem. Biophys. Res. Commun.* **19**, 377 (1965).
[17] S. HAYASHI and E. C. LIN, *J. Molec. Biol.* **14**, 515 (1965).
[18] B. MULLER-HILL, H. V. RICKENBERG and K. WALLENFELS, *J. Molec. Biol.* **10**, 303 (1964).

The evidence till now available is strongly in favour of the former hypothesis. In fact, the (glucose-6-P)/(fructose-6-P) ratio in the castor bean cotyledons is very close to the theoretical equilibrium constant of the isomerase reaction. This ratio is not significantly changed when the cotyledons are incubated in 0·1 M glucose or fructose, in spite of the fact that the concentrations of the two hexose-6-phosphates increase, under this condition, by *ca.* 300 per cent.[19] This indicates that most of the hexose-6-phosphates belong to a single pool in the cell, where the interconversion of the two compounds is very rapid; while the interconversion between fructose and glucose is much slower. Thus, the finding that fructose is more effective in stimulating the synthesis of fructokinase, and glucose that of hexokinase, is more easily explained by assuming that the free hexoses, rather than the hexose-6-phosphates, are the true inducers.

The present evidence does not allow a clear understanding of the mechanism of kinase induction. The finding that the rate of synthesis of several other enzymes is not accelerated by an increase of the hexoses seems to rule out the possibility of a general stimulation of protein synthesis. In this connexion, it is also pertinent to mention that the presence of hexoses in the incubation medium does not affect, in our material, either the rate of O_2 uptake or its response to 2,4-dinitrophenol.[9] According to the accepted schemes, hexoses could specifically influence kinase synthesis by stimulating either the rate of synthesis or the activity of the respective messenger RNA. That is to say that this "induction" could operate at the transcription or at the translation step. The inhibition of enzyme rise by actinomycin is in favour of the view that the hexose acts rather at the transcription than at translation level. This conclusion, however, is open to the usual criticism suggested by the possibility of secondary effects of RNA synthesis inhibitors on other sections of cell metabolism.

In conclusion, our results, together with other observations made in this laboratory on the repression by phosphate of phytase formation,[20,21] and by glucose of specific fructose diphosphatase[10] and isocitrate lyase synthesis[22] in germinating seeds, indicate that specific induction and repression of enzyme synthesis by metabolites is of general importance in the control of the development and the differentiation of the enzyme pattern of the plant cell.

MATERIALS AND METHODS

Castor bean seeds (*Ricinus communis* var. *sanguineus*) were sterilized by treatment with 1 per cent "Ceresan". After removal of the seed coats the seeds were germinated on three layers of water-saturated filter paper at 27°, in the darkness, in large Petri dishes. At the end of the period of germination indicated for each experiment, the cotyledons were carefully isolated from the endosperm and from the embryo. The isolated cotyledons appearing undamaged by microscopic observation were selected, randomized, and incubated, under agitation, either in distilled water or in the sugar solution, in large Petri dishes containing *ca.* 0·5 ml of liquid per cotyledon. All operations were carried out under sterile condition and no appreciable bacterial or fungal growth was observed under our experimental conditions. At the end of incubation, the cotyledons were washed and homogenized for the determinations of enzyme activities. Homogenates were prepared by grinding the tissue in 0·05 M Tris buffer, pH 7·3, containing 0·01 M mercaptoethanol and filtering through cheese-cloth.

[19] E. MARRÈ, R. BIANCHETTI and S. COCUCCI, *Ital. J. Biochem.* **15**, 135 (1966).
[20] M. L. SARTIRANA and R. BIANCHETTI, *Physiol. Plantarum.* In press.
[21] R. BIANCHETTI and M. L. SARTIRANA, *Biochim. Biophys. Acta.* In press.
[22] P. LADO, M. SCHWENDIMANN and E. MARRÈ, *Giorn. Bot. Ital.* In press.

The homogenates were then centrifuged at 20,000 × *g* for 20 min. The supernatants in some experiments were used without further purification; in other cases after extensive dialysis against 0·05 M Tris, 0·05 M KCl, 0·01 M $MgCl_2$, or after filtration through Sephadex G-25. The kinase activities in the 20,000 × *g* sediment ("insoluble activity") was measured after solubilization obtained by repeated extraction of the pellets with Tris buffer 0·05 M, pH 7·5, containing 1 per cent Triton-X-100. Higher concentrations of this detergent, as well as the use of deoxycholate or digitonin, led to lower recoveries of enzyme activity. Hexokinase activity was measured spectrophotometrically as the increment of absorptivity at 340 nm in a reaction medium containing 0·1 M Tris buffer, pH 7·3; $1{\cdot}5 \times 10^{-4}$ M EDTA; $3{\cdot}3 \times 10^{-2}$ M $MgCl_2$; $2{\cdot}5 \times 10^{-4}$ M ATP; 10^{-4} M NADP; 0·1 M glucose; glucose-6-phosphate dehydrogenase in large excess and an aliquot of the enzyme extract corresponding to 1 cotyledon (*ca.* 15 mg fresh weight). Final volume of the mixture was 3 ml. Fructokinase was measured in the same way, except for the substitution of glucose with fructose, and the addition of a large excess of phosphohexoseisomerase (obtained from Boehringer).

Aldolase, glucose-6-P dehydrogenase, 6-P-gluconate dehydrogenase, isocitrate dehydrogenase, phosphoglucomutase and fructose diphosphatase activities were measured spectrophotometrically.[23, 10] For the determination of the galactokinase activity the incubation mixture contained, in a volume of 1 ml, 0·1 ml of the 20,000 × *g* supernatant (filtered through Sephadex G-25) ATP, 30 μmoles; galactose-1-C^{14} (0·05 μc/μmole),10 μmoles; $MgCl_2$, 30 μmoles; histidine, 20 μmoles; Tris-HCl, pH 7·3,100 μmoles; the incubation temperature was 25°. At the end of the desired incubation periods (0·15 and 30 min) the reaction was stopped by adding 1 ml of 10 per cent trichloroacetic acid and the mixture centrifuged; 1 ml of 25 per cent Ba acetate and 5 μmoles of glucose-1-P were added to the clear supernatant, the pH was adjusted to 8·2 and 4 vol. of ethanol were added. After 10 hr at 0–3° the precipitate formed, containing the labelled galactose-1-P synthesized during incubation, was collected by vacuum filtration on Whatman No. 2 paper disks and the radioactivity counted and corrected for self-absorption.

Under our experimental conditions 1 μmole of galactose-1-P corresponded to *ca.* 25,000 counts. The linearity of the values obtained as a function of the time of incubation and of the amount of enzyme present was taken as an indication of the validity of this procedure.

[23] S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vols. I–VI. Academic Press, New York (1955–1966).