Phytochemistry, 1980, Vol. 19, pp. 1637–1642. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0801-1637 $02.00/0

A REGULATORY INVERTASE FROM SUGAR CANE LEAF-SHEATHS

ANTONIO RODOLFO SAMPIETRO, MARTA AMELIA VATTUONE and FERNANDO EDUARDO PRADO

Instituto de Química Biológica, Universidad Nacional de Tucumán, Chacabuco 461, 4000-S.M. de Tucumán, Argentina

(*Received* 15 *December* 1979)

Key Word Index— *Saccharum officinarum*; Gramineae; sugar cane; β-fructofuranosidase; invertase; inhibition, enzyme kinetics.

Abstract— A soluble β-fructofuranosidase was isolated from sugar cane leaf-sheaths. The enzyme attacks sucrose with an activation energy of 5700 cal/mol above 30° and 17 000 cal/mol below 30°. The enzyme was inhibited by the reaction products. Glucose is a simple non-competitive inhibitor, but fructose is a competitive inhibitor. Kinetic studies using double reciprocal plots and replots of $1/K_i$ slope vs inhibitor concentration showed that fructose binds to two interacting sites of the enzyme. Per cent residual activity plotted against inhibitor concentration, and Hill plots confirmed the regulatory properties of the invertase. n was found to be close to 2, the number of binding sites established with the double reciprocal method. The tissue and cellular levels of sucrose, fructose and glucose were measured. Fructose was found at inhibitory concentrations confirming that the activity of the enzyme is probably modulated by the hexose pool of the leaf-sheaths.

INTRODUCTION

The examination of the mechanism of sucrose accumulation in sugar cane storage tissues has led to important conclusions about the physiological role of the invertases (EC 3.2.1.26) in the plant. Free space sucrose cannot move across sugar cane cell membrane, and a cell-wall invertase hydrolyses the sugar to fructose and glucose prior to uptake [1–3]. Sucrose is resynthesized in the metabolic compartment of the cell and stored in the storage compartment, the vacuoles [1, 2]. Sucrose synthetase, an enzyme able to break down sucrose, is not present in significant amount in the parenchyma cells. Instead, an active invertase is present, called vacuolar invertase [1, 4]. As the photosynthate and reserve sugar is mainly sucrose, vacuolar invertase controls the energetic and biosynthetic metabolism of the cell. This acid-soluble invertase is regulated by a mechanism of induction and repression affecting the synthesis of the enzyme [5]. The important role of invertase suggests that there may be a control mechanism in the cell determined by its level of activity.

Other tissues besides the stalk parenchyma have seldom been studied for invertase activity [6–8]. Leaf-sheaths are anatomically and histologically different from leaf-blades. Physiologically, leaf-blades are where sucrose is produced by photosynthesis, while leaf-sheaths are the conducting and supporting organs through which the disaccharide passes to the storage parenchyma in the stalk. Thus leaf-sheaths may have invertases adapted to the functional characteristics of this part of the plant and invertases as present in storage tissues. Indeed a bound invertase has already been found in these plant parts [9]. The activity of this bound invertase, in contrast to other invertases from sugar cane, is inhibited by excess sucrose. This may represent a specialization in the leaf-sheaths to avoid excessive breakdown of sucrose. This paper reports a soluble acid invertase in leaf-sheaths which is similar to the vacuolar invertase of storage parenchyma. Some enzyme properties and a control mechanism, new for plant invertases, are also described.

RESULTS

The purification of invertase is depicted in Table 1. The separation of the two invertases was possible by gel filtration (Fig. 1). Attempts at further purification using DEAE-cellulose, CM-cellulose, organic solvents, heating, or precipitation by acids were unsuccesful because of a total loss of enzyme activity. However, gel electrophoresis of the soluble invertase stained for enzyme activity [10] showed a single band. The MW was 127 500 as determined using Sephadex G-150. The optimum pH was *ca* 5–5.5 at 37°. Subsequent experiments were run at pH 5.5 because this was the pH in leaf-sheaths of 2–3 month-old sugar cane and also the pH found in vacuoles, the most probable localization of the enzyme.

Progress curves were straight lines at least up to 1 hr, even at the most dilute concentrations of sucrose used. The plot of 5–50 μl of extract vs initial velocity was a straight line. Thus there are no endogenous dissociable inhibitors in the enzyme preparation, as has been described in potato [11], maize [12], sweet potato, red beet and sugar beet [13].

The extract was able to hydrolyse sucrose, raffinose and stachyose but not melibiose (Table 2), so that the enzyme is a β-fructofuranosidase. Raffinose was simultaneously attacked by a β-fructofuranosidase and by an α-galactosidase with production of sucrose, melibiose, fructose and galactose. Thus the enzyme contains an α-galactosidase able to attack raffinose but not melibiose. A β-galactosidase activity is also present. The extract, as

Table 1. Purification of soluble acid invertase from leaf-sheaths

Fraction	Total activity (Units)*	Total protein (mg)	Specific activity (Units × 10^{-2}/mg)	Purification factor	Recovery (%)
100% $(NH_4)_2SO_4$ ppt.	4.00	734.35	0.54	1	100
$Ca_3(PO_4)_2$ adsorption	2.97	289.62	1.02	1.9	74.25
Sephadex G-150†	0.70	5.31	13.18	24.41	17.50

* One unit is 1 μmol substrate used per min at 37°.

† Two peaks of enzymic activity were separated. Invertase determined by gel filtration gave a particulate and a soluble enzyme.

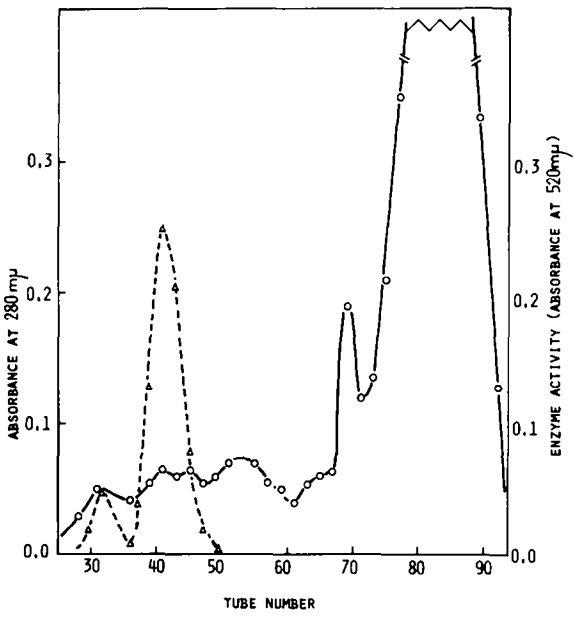

Fig. 1. Elution profile of invertase from Sephadex G-150. Fractions of *ca* 2 ml were collected from commencement of sample loading. Each fraction was then assayed to determine invertase activity (△ -△) and protein content (○ ○).

Table 2. Enzyme activities found in leaf-sheath extracts

Substrate	Units/g fr. tissue
Sucrose	3.4×10^{-2}
Raffinose	1.4×10^{-3}
Stachyose	6.4×10^{-4}
β-Phenyl-D-galactoside	1.6×10^{-3}
Inulobiose	0
Inulin	0
α-Methyl-D-galactoside	0
α-Methyl-D-glucoside	0
β-Methyl-D-glucoside	0
Melibiose	0
Levan	0

deduced from reducing power measurements, was not able to attack glucose, fructose or galactose. Invertase did not hydrolyse levan or inulin. Further, these polysaccharides were not inhibitors for invertase.

Initial velocity was not modified by 0.05×10^{-3}–3×10^{-1} M NaCl (Table 3). This assay was run with an enzyme prepared as described in the Experimental but using K^+ salts in place of the Na^+ salts. KCl had no effect. 1.25×10^{-4} M Ca^+ was an inhibitor, but not 5×10^{-3} M Mg^{2+} or Ba^{2+}. These determinations relate to the concentrations calculated for the respective ions from sugar cane juices [14]. Leaf-sheaths are reported [15] to contain *ca* 1.5×10^{-2} M Ca^{2+} supporting a possible regulatory role for this ion. Tris (50 mM) inhibited leaf-sheaths invertase *ca* 36% and stalk invertase *ca* 25% at the same pH [16].

Table 3. The effect of enzyme inhibitors assayed under standard conditions

Inhibitor	Concentration (mM)	Inhibition (%)
Zn^{2+}	1	40.0
Zn^{2+}	10	88.0
Co^{2+}	1	3.0
Co^{2+}	10	63.6
Tris	4	4.5
Tris	50	35.9
F^-	10	9.1
EDTA	5	9.1
MoO_4^{2-}	2	7.7
NH_4^+	2	7.7
NH_4^+	12	12.3
I_2	0.16	30.1
$Mo_7O_{24}^{6-}$	3	46.9
Urea	200	65.8
Hg^{2+}	2.5	100
Hg^{2+}	5	100
Ca^{2+}	0.125	13.05
Ca^{2+}	0.25	21.75
p-Chloromercuribenzoate	0.24	100
Na^+	0.05×10^{-3}–300	0
K^+	0.05×10^{-3}–300	0
BO_3^{3-}	0.064–1.6	0
PO_4^{3-}	0.1	0
Mg^{2+}	2.5 5	0

I_2, Hg^{2+} and *p*-chloromercuribenzoate were strong inhibitors, suggesting the presence of sulphydryl groups on the enzyme. Ammonium heptamolybdate is also an inhibitor of the enzyme [17].

The activation energy of the purified enzyme was determined at various temperatures between 10 and 40°. A sharp change in activation energy was observed at a temperature near 30° (Fig. 2). At a temperature below 30° the energy for the activation of the enzyme was calculated as 17 000 cal/mol and over 30° the energy for the activation was 5700 cal/mol.

A K_m of 2.84 ($\pm$0.24) $\times$ 10^{-2} M (average and s.d. of 16 determinations) was found. Fructose and glucose, the reaction products, were inhibitors of leaf-sheath invertase. Glucose was a simple non-competitive inhibitor, K_i = 37 mM (Fig. 3). By contrast, fructose was a competitive inhibitor (Fig. 4). The replot of slopes of the double reciprocal plot vs fructose concentration indicates

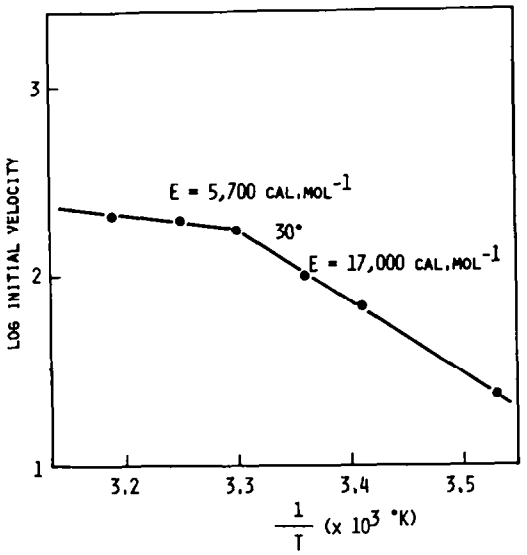

Fig. 2. Arrhenius plot of the invertase-catalyzed reaction. Assays were conducted at various temperatures as described in the Experimental.

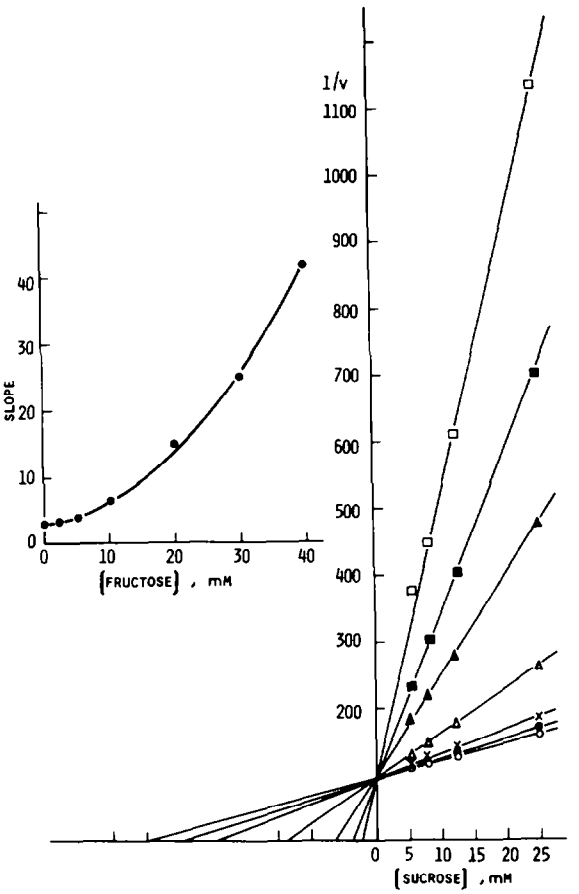

Fig. 4. Competitive inhibition of soluble acid invertase by fructose and replot of slope vs the concentration of fructose. The reactions were measured at 37° and pH 5.5. ○——○, Without inhibitor; ●——●, 2.5 mM fructose; X——X, 5 mM fructose; △——△, 10 mM fructose; ▲——▲, 20 mM fructose; ■——■, 30 mM fructose and □——□, 40 mM fructose. Each point represents the mean of four determinations.

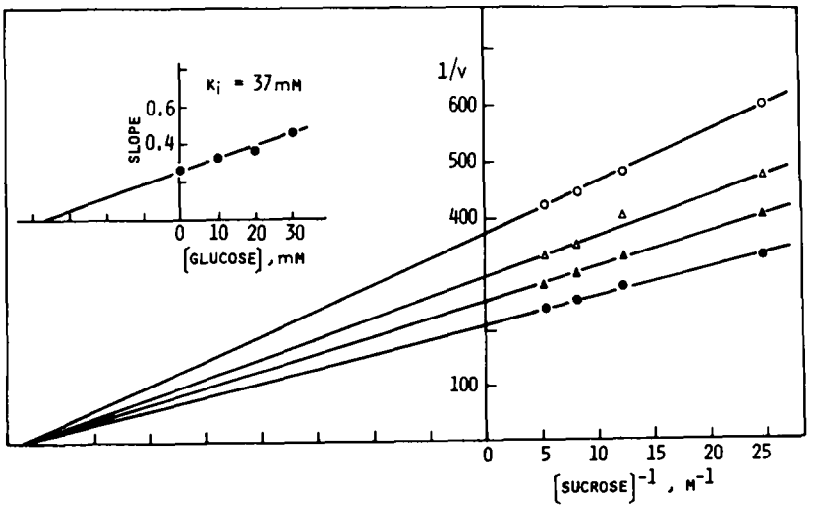

Fig. 3. Non-competitive inhibition of soluble acid invertase by glucose and replot of the slopes vs the concentration of glucose. The reactions were measured at 37° and pH 5.5. ●——●, Without inhibitor; ▲——▲, 10 mM glucose; △——△, 20 mM glucose and ○——○, 30 mM glucose. Each point represents the mean of four determinations.

that fructose acts through a more complex mechanism than glucose. A straight line was obtained when the reciprocal of K_i slope was plotted against fructose concentration (Fig. 5). K_i slopes were the apparent values of K_i calculated from the slopes of the double reciprocal plot. The difference in the value of K_i calculated from the intersection of the curve on the axis $1/K_i$ slope and from the intersection on the axis of fructose concentration indicates that a second modified aK_i is involved. According to these results the inhibition obeys the equation [18]:

$$v = \frac{V[S]}{K_s\left[1 + \frac{2[I]}{K_i} + \frac{[I]^2}{aK_i^2}\right] + [S]}$$

where K_i = the intrinsic dissociation constant for the EI complex, K_s = dissociation constant for the ES complex, a = the interaction factor, S = the substrate concentration, V = the maximum velocity for the system and v = actual velocity. This velocity equation corresponds to an enzyme model with two interacting binding sites for fructose [18] that would be represented by the equilibrium:

$$\begin{array}{ccccc} IE & \overset{K_i}{\rightleftharpoons} & I + E + S & \overset{K_s}{\rightleftharpoons} & ES \overset{k_p}{\rightarrow} E + P \\ + & & + & & \\ I & & I & & \\ \downarrow aK_i & & \downarrow K_i & & \\ IEI & \overset{aK_i}{\rightleftharpoons} & I + EI & & \end{array}$$

K_i was found to be *ca* 32.25 m M, aK_i 4.16 m M. Consequently a was *ca* 0.129.

This regulatory property was confirmed by plotting per cent residual activity vs fructose concentration (Fig. 6). The plot gave a sigmoidal inhibition curve typical for a regulatory enzyme. Furthermore, n calculated from a Hill plot was *ca* 1.85 supporting the previous interpretation.

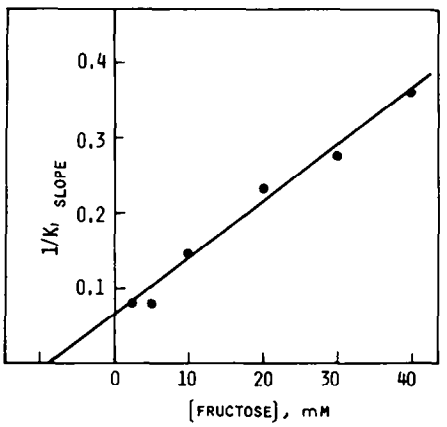

Fig. 5. Replot of the reciprocal of K_i slope vs inhibitor concentration. The K_i slope values were calculated from each curve of the double reciprocal plot of Fig. 4.

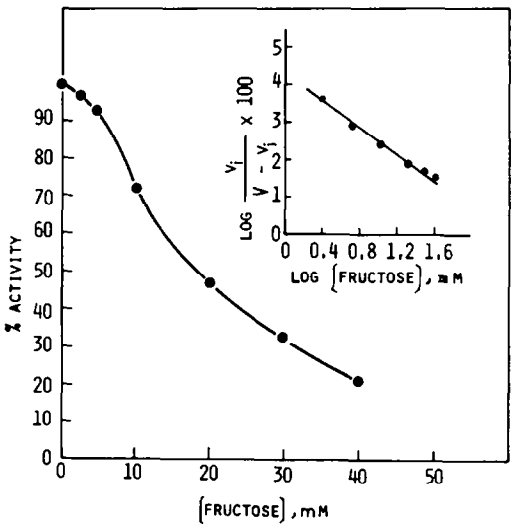

Fig. 6. Per cent of residual invertase activity vs fructose concentration and Hill plot.

Preparations of the cell membrane did not show invertase activity. Invertase activity in the 48 000 g supernatant was 6.1×10^{-2} total units. Complete and washed tissue contained fructose at inhibitory concentrations for invertase (Table 4). These sugars may have higher concentrations if they are compartmentalized and variations according to the physiological state may be expected. Assuming that the observed concentrations in washed tissues are true intracellular concentrations, the enzyme is inhibited *ca* 21% and, at the sugar concentrations of whole tissues, the inhibition is *ca* 60%.

DISCUSSION

Sugar cane leaf-sheaths have an acid β-fructofurano-stachyose but not fructans. The invertase has an activation energy of 5700 cal/mol above 30° and of 17 000 cal/mol below 30°. Currently, temperatures above 30° are observable at the field during the period October–March, and consequently the changes of the activation energy act as a system of control avoiding sucrose breakdown by the enzyme when the temperature rises above 30°. A similar temperature effect has been recently reported for an invertase (optimum pH 7.5) from chicory roots [19] and the same physiological interpretation is possible in this case.

The kinetics of inhibition by fructose of the soluble invertase supports an inhibitory mechanism with two interacting sites for the inhibitor. The kinetic results based on reciprocal plots were further confirmed using Hill plots

Table 4. Sugar concentration of sugar cane leaf-sheaths

Material (sheaths)	Fructose (mM)	Glucose (mM)	Sucrose (mM)
Whole tissue	18.08 ± 2.03	5.4 ± 0.27	39.10 ± 5
Washed tissue	5.76 ± 3.04	2.05 ± 0.25	36.60 ± 3.5

and plotting per cent residual activity versus fructose concentrations. According to Atkinson *et al.* [20], when there are strong interactions between sites, *n* equals the number of sites. In the case of soluble acid invertase, *n* was found to be *ca* 1.85, a value close to 2, the number of interacting sites previously calculated for fructose. The concentration of tissue and cellular fructose was found to reach inhibitory levels, supporting the physiological value of the regulatory properties of the enzyme. Thus, the fructose tissue pool modulates the activity of the soluble acid invertase. Plant invertases are known to be regulated by an auxin- and glucose-mediated control system [5] and at the activity level by interactions of an invertase–protein inhibitor [1–3]. Consequently, the present regulatory mechanism is new for the invertases. The mechanism of this inhibition and the breaks in the Arrhenius plot suggest a structurally complex enzyme. Regulatory enzymes are generally formed by subunits, and inflections of the Arrhenius plot are interpreted as changes in enzyme conformation. The limited data available show that other sugar cane invertases may be oligomeric enzymes [7, 8].

Apparently, the acid invertase is not a membrane enzyme, since the activity is in a soluble fraction from tissues and not in membrane preparations. A parallelism between growing stalk and leaf-sheath tissues is observable. Growing stalks have a soluble invertase, pH optimum 5–5.5, and a cell wall bound invertase. In the case of leaf-sheaths a similar pattern was observed, with a soluble activity which consisted of two enzymes, one minor component of optimum pH 2 and a more important component of optimum pH 5–5.5. Furthermore, a cell wall bound invertase from leaf-sheaths has also been observed in this laboratory (unpublished results). Consequently, the enzyme of pH 5.5 from leaf- sheaths is at least related to the vacuolar invertase of the growing stalk and the same vacuolar localization may be assumed. The vacuolar occurrence of acid invertase in *Beta vulgaris* [21] strengthens this supposition. Sucrose is supposed to be stored in the vacuoles of sugar cane [22] and this was shown also for *B. vulgaris* [21]. The present mechanism explains how sucrose can exist together with invertase. Since the inhibition by fructose is competitive, high sucrose concentrations must suppress partially or completely the regulation of acid invertase by fructose, and this may explain the change from an acid to a neutral or alkaline invertase at maturity.

EXPERIMENTAL

Plant material. 2- to 3-month-old sugar cane, cv CP 48–103, cultivated in the field at the Estación Experimental Agricola de Tucumán, was used throughout this work.

Enzyme preparation. Sugar cane acid invertase was prepared according to ref. [17].

MW determination. The MW of the invertase was determined using Sephadex G-150 filtration [24]. The column (2.5 × 45 cm) was prepared with 10 mM NaOAc buffer, pH 5.5, and 1 mM 2-mercaptoethanol. Trypsin (MW 23 800), pepsin (MW 35 500), serum albumin (bovine) (MW 70 000) and glucose oxidase (MW 152 000) were used as standards.

Determination of protein. Protein was determined by the method of Lowry *et al.* [23] using BSA as standard.

Enzyme assays. Reaction mixture contained 15 μl extract, 10 μl 0.6 M sucrose, 25 μl 0.2 M NaOAc buffer, pH 5.5 and 50 μl dist. H_2O. Incubations were performed at 37° and the reactions were stopped by a Cu alkaline reagent [25]. The reducing power released was measured by the method of ref. [25].

Enzyme kinetics. The reaction mixtures consisted of 15 μl enzyme, 4–18 μl 0.5 M sucrose and 10 μl 12.5–200 mM fructose or glucose in a final vol. of 50 μl. Incubations were made at 37°. Then, 0.1 ml H_2O were added to the mixtures containing 20–40 mM fructose or glucose and 50 μl of the dilution were taken for reducing power determinations [25] with double strength reactants. Absorbances are proportional at least up to 4 μmol reducing sugars, and the mixtures used are below 2.2 μmol. This technique was applied to row apex invertase of sugar cane and to leaf invertase from *Ricinus communis*, in order to show that the results are reliable.

Cell membrane preparation. These preparations were made according to ref. [26].

Tissue sugars. Content of H_2O was determined by weighing 20 g leaf-sheaths and desiccating at 100° to constant weight. Another 20 g sample of leaf-sheaths was killed in boiling H_2O. Two min later the mixture was homogenized for 3 min. Cheesecloth was used for separating the fibrous material which was extracted with dist. H_2O. The cheesecloth was carefully squeezed and the extracts were pooled. Sucrose and fructose were measured by the method of ref. [27]. Washed tissues were prepared cutting leaf-sheath strips 1–2 mm wide. The strips were weighed and washed under tap water until the efflux of sugar was constant. This procedure eliminated the sugar content of the free space. The strips were dropped in boiling H_2O. Two min later the procedure described for leaf-sheaths was followed.

Acknowledgements—This work was supported by grants from the Consejo Nacional de Investigaciones Cientificas y Técnicas y el Consejo de Investigaciones de la UNT.

REFERENCES

1. Hawker, J. S. and Hatch, M. D. (1965) *Physiol. Plant.* **18**, 444.
2. Sacher, J. A., Hatch, M. D. and Glasziou, K. T. (1963) *Plant Physiol.* **38**, 348.
3. Bowen, J. E. and Hunter, J. E. (1972) *Plant Physiol.* **49**, 789.
4. Slack, C. R. (1966) *Phytochemistry* **5**, 397.
5. Sacher, J. A. and Glasziou, K. T. (1962) *Biochem. Biophys. Res. Commun.* **8**, 280.
6. Alexander, A. G. (1965) *J. Agric. Univ. P. R.* **49**, 287.
7. Maretzki, A. and Alexander, A. G. (1967) *Enzymology.* **33**, 299.
8. Alexander, A. G. (1967) *J. Agric. Univ. P. R.* **51**, 39.
9. Prado, F. E., Vattuone, M. A. and Sampietro, A. R. (1978) *Proc. Int. Soc. Sugar-Cane Technol.* **16**, 1683.
10. Billet, E. E., Billet, M. A. and Burnett, J. H. (1977) *Phytochemistry* **16**, 1163.
11. Pressey, R. (1967) *Plant Physiol.* **42**, 1780.
12. Jaynes, T. A. and Nelson, O. E. (1971) *Plant Physiol.* **47**, 629.
13. Pressey, R. (1968) *Plant Physiol.* **43**, 1430.
14. Binckley, W. W. and Wolfrom, M. L. (1953) *Adv. Carbohydr. Chem.* **8**, 291.
15. Bowen, J. E. (1974) *Trop. Agric.* **52**, 131.
16. Hatch, M. D., Sacher, J. A. and Glasziou, K. T. (1963) *Plant Physiol.* **38**, 338.
17. Prado, F. E., Sampietro, A. R. and Vattuone, M. A. (1979) *Phytochemistry* **18**, 1799.
18. Segel, I. H. (1975) *Enzyme Kinetics.* John Wiley, New York.
19. Gordon, A. J. and Flood, A. E. (1979) *Phytochemistry* **18**, 405.
20. Atkinson, D. E., Hathaway, J. A. and Smith, E. C. (1965) *J. Biol. Chem.* **240**, 2682.

21. Leigh, R. A., ap Rees, T., Fuller, A. and Banfield, J. (1979) *Biochem. J.* **178**, 539.
22. Gayler, K. R. and Glasziou, K. T. (1972) *Physiol. Plant.* **27**, 25.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
24. Andrews, P. (1965) *Biochem. J.* **96**, 595.
25. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
26. Sinensky, M. and Strobel, G. (1976) *Plant Sci. Letters* **6**, 209.
27. Cardini, C. E., Leloir, L. F. and Chiriboga, J. (1955) *J. Biol. Chem.* **214**, 149.