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SUCROSE METABOLISM DURING TOBACCO CALLUS GROWTH

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; *Daucus carota*; Umbelliferae; carrot; callus tissue culture; invertase; sucrose synthetase.

Abstract—Activities of soluble and insoluble invertases and sucrose synthetase in tobacco callus increased significantly within the first 3 days of culture. After this period soluble invertase activity declined, while the activities of the insoluble invertase and the sucrose synthetase were relatively unchanged.

INTRODUCTION

CULTURES of plant tissues *in vitro* usually require an exogenous source of carbon and energy. This is normally supplied in the medium as sucrose.[1] Despite the extensive use of this sugar for well over 25 yr, we are not aware of any comprehensive study on the activities of the enzymes involved in its degradation. Sucrose breakdown occurs by two pathways: either hydrolysis by invertase (β-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26) to glucose and fructose, or the formation of UDP-glucose or ADP-glucose and fructose by sucrose synthetase (UDP-glucose: fructose-2-glucosyl transferase, E.C. 2.4.1.13).[2]

In plants both soluble and insoluble (cell wall) invertases, having optimum activity at acid pHs and neutral to alkaline pHs, have been observed.[3–6] The presence of invertase in cell wall fractions of several plant tissue cultures have been reported[7] and changes in activity of the invertases during cultures have been found in sycamore cells.[6]

In this paper the activities of the invertases and sucrose synthetase during callus growth are examined. Earlier work showed that there was a strong correlation between starch accumulation and shoot formation in tobacco callus,[8,9] and the rate of respiration in the tissue.[10] Since in most of the work the tissue was grown in the dark, the ultimate source of glucose would be from the sucrose supplied to the medium.

RESULTS

Culture medium containing sucrose was autoclaved or sucrose, sterilized by filtration, was added to autoclaved medium. Traces of glucose and fructose could be detected chromatographically in extracts of the autoclaved sucrose medium without inoculum as used

[1] H. E. STREET, in *Plant Physiology* (edited by F. C. STEWARD), Vol. VB, p. 3, Academic Press, New York (1969).
[2] J. S. HAWKER, *Phytochem.* **10**, 2313 (1971).
[3] D. VAUGHAN and I. R. MACDONALD, *Plant Physiol.* **42**, 456 (1969).
[4] C. P. P. RICARDO and T. AP REES, *Phytochem.* **9**, 239 (1970).
[5] R. L. LYNE and T. AP REES, *Phytochem.* **10**, 2593 (1971).
[6] L. G. COPPING and H. E. STREET, *Physiol. Plant.* **26**, 346 (1972).
[7] J. STRAUS, *Plant Physiol.* **37**, 342 (1962).
[8] T. A. THORPE and T. MURASHIGE, *Science* **160**, 421 (1968).
[9] T. A. THORPE and T. MURASHIGE, *Can. J. Bot.* **48**, 711 (1970).
[10] T. A. THORPE and D. D. MEIER, *Physiol. Plant.* **27**, 365 (1972).

in this study but only sucrose was observed in filter sterilized medium, confirming the finding of Ball.[11] After 14 days in culture, the medium whether autoclaved or filter-sterilized contained large amounts of fructose and glucose, in addition to sucrose. Soluble invertase activity could be detected in the medium during the first 10–12 days of culture. Fourteen-day-old and 10-week-old tobacco callus contained sucrose, as well as glucose and fructose.

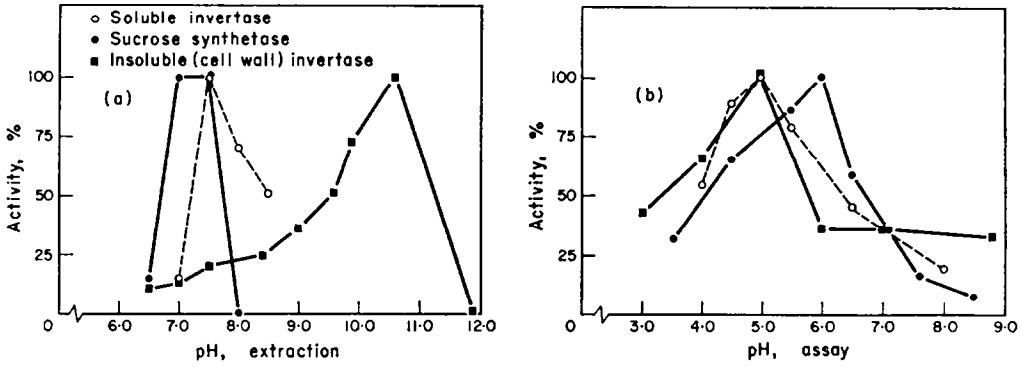

FIG. 1. pH OPTIMA OF EXTRACTION (a) AND ASSAY (b) OF ENZYMES FROM TOBACCO CALLUS. Details in Experimental.

The effect of pH of the extracting medium on the activity of invertase, insoluble invertase and sucrose synthetase is shown in Fig. 1(a). Both soluble invertase and sucrose synthetase have extraction optima around pH 7·5, whereas the optimum pH for extracting insoluble invertase is pH 10·6. However, so that a single extraction could serve as a source of all 3 enzymes pH 7·5 was chosen, since extracts made at pH 8·0 or above contained no sucrose synthetase activity. In the enzyme assays both soluble and insoluble invertase had a pH optimum at 5·0, while sucrose synthetase was most active at pH 6·0 (Fig. 1b). Accordingly measurements were made at these pHs. No neutral to alkaline invertases were detected.

The growth of tobacco callus in both light and dark during 35 days in culture is shown in Fig. 2(a). Corresponding changes in enzyme activity during culture for soluble invertase, insoluble invertase and sucrose synthetase are shown in Fig. 2(b), (c) and (d) respectively. On subculture, activity of soluble invertase increased sharply with a maximum after 2 days in culture (Fig. 2b). This increase is in excess of 10-fold and occurs in both dark-grown and light-grown tissue. Subsequently, there was a loss in activity with very low, but detectable levels being observed after 10 days in culture. In the case of the insoluble cell wall invertase (Fig. 2c) for light-grown tissue the level of activity remained about the same throughout the culture period. No significant initial rise in activity was observed in this tissue. For dark-grown tissue, there was an initial 3-fold rise in activity at day 2 after which a steady level was maintained. For sucrose synthetase (Fig. 2b), there was an initial rise in activity in both light- and dark-grown tissue; however, the rise was greater for the light-grown tissue, being over 4-fold. After day 15, there was an increase in the activity of this enzyme in the dark-grown tissue, while a steady decline in activity occurs in the light-grown tissue. This difference in activity perhaps reflected the difference in growth rate of the callus in the light and dark. No sucrose synthetase activity was detected in the culture medium.

[11] E. BALL, *Bull Torrey Bot. Club* **80**, 409 (1953).

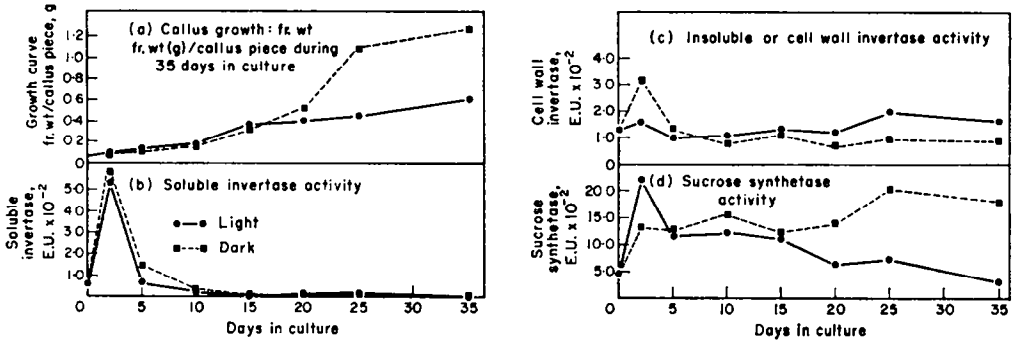

FIG. 2. GROWTH CURVE AND ENZYME ACTIVITY OF TOBACCO CALLUS DURING CULTURE IN LIGHT AND DARK.

Enzyme activities are expressed in enzyme units (E.U.) $\times 10^{-2}$ per g fr. wt. See Experimental for details. The data presented is from a single experiment. Each point in Fig. 2(b, c and d) is the average of three independent extractions and duplicate enzyme assays per extraction. A duplicate experiment gave similar results.

The comparative results for tobacco and carrot tissues after 27 days in culture in the dark and fresh explant tissue are shown in Table 1. The level of soluble invertase is much higher in tobacco pith explant tissue than in callus, whereas the activities of insoluble invertase and sucrose synthetase are of the same order. On the other hand the levels of activities of these enzymes in carrot callus is several fold higher than in tobacco callus, or in carrot root. Carrot roots also contain alkaline invertase,[4] however, this enzyme is absent in tobacco tissue.

TABLE 1. ACTIVITY OF SUCROSE-DEGRADING ENZYMES FROM DIFFERENT SOURCES

Material	Enzyme activity (E.U.) Sol. invertase ($\times 10^{-2}$)	C.W. invertase ($\times 10^{-2}$)	Sucrose synthetase ($\times 10^{-2}$)
Tobacco pith explant	4·86	1·71	16·60
Tobacco callus	0·51	1·36	13·57
Carrot root explant	0·84	2·08	8·43
Carrot callus	1·74	6·45	49·87

Activity expressed as enzyme units (EU) per g fr. wt of tissue. Callus tissue 27-days-old and grown in darkness.

DISCUSSION

Sucrose supplied in the medium can probably be taken into the cell as the disaccharide, since tissue cultures grown on a sucrose-containing medium contain high levels of this sugar. However, since an enzyme capable of degrading sucrose to fructose and glucose is present in the cell wall, a portion of the sucrose is probably degraded to hexoses as it is taken into the cell. The release of soluble invertase into the culture medium during the early culture period is probably a similar phenomenon to that observed in other cultured cells in which enzymes and metabolites are released into the medium.[12,13]

[12] H. E. STREET, in *Cells and Tissues in Culture, Methods, Biology and Physiology* (edited by E. N. WILLMER), Vol. 3, p. 591, Academic Press, New York (1966).
[13] A. C. OLSON, J. J. EVANS, D. P. FREDERICK and E. F. JANSEN, *Plant Physiol.* **44**, 1594 (1969).

Soluble and insoluble invertase and sucrose synthetase are found in the callus as well as the explant material from which the callus is derived. The presence of the invertases in tobacco callus has been reported by Straus.[7] On the other hand, we are aware of no reports on the presence of the invertases in carrot callus (as opposed to discs of carrot roots) or of sucrose synthetase in either tobacco or carrot callus.

During the early phase of growth of tobacco callus, before cell division begins in the tissue, changes in the levels of all three enzymes were observed, a more than 10-fold increase in soluble invertase activity within 2 days of culture, followed by a reduction in activity by day 5. Such fluctuations in enzyme level early in culture could be a consequence of wounding. Similar changes in the level of phenylalanine ammonia lyase were observed in citrus fruit tissue cultures.[14]

After 10 days in culture very little soluble invertase can be detected in the tissue. Consequently this enzyme may play a very small role in sucrose hydrolysis during the period of most rapid growth. Similarly, the level of insoluble cell wall invertase remains fairly constant throughout the culture period and thus shows no correlation with the growth of the tissue. This finding is in agreement with that of Straus,[7] who found no relationship between invertase activity with growth rate or nitrogen content of the cell walls of the tissue cultures studied. On the other hand, Copping and Street[6] found that soluble and insoluble invertase activity paralleled the growth rate of sycamore cells grown in cell suspension. In tobacco callus, there seems to be a greater degree of correspondence between the activity of sucrose synthetase and the growth rate of the tissue, particularly for the dark-grown tissue. Sucrose synthetase is localized intracellularly, and would consequently be closer to sites of metabolic activity than the invertase localized in the cell wall. It is possible that growing tissue utilizes both sets of enzymes in normal cell growth. The insoluble invertase could supply a steady level of glucose and fructose, but still allow some sucrose to build up in the cells. With increased need for the degradation products of sucrose for intermediary metabolism, intracellular breakdown of sucrose by sucrose synthetase could then become of greater importance. However, since the sucrose accumulates in the cell in the presence of the intracellular enzyme, the enzyme and its substrate are probably spatially separated.

EXPERIMENTAL

Plant material. Tobacco callus isolated from stem pith segments of *Nicotiana tabacum* L. CV Wisconsin 28 in the summer of 1971, was maintained on a 3/4 strength M.S. salt medium,[15] supplemented with White's organics,[16] inositol (100 mg/l.) indole-3-acetic acid (IAA) (2 mg/l.), kinetin (0·5 mg/l.), Difco Casamina acids (1 g/l.) and sucrose 2·5% (w/v). For experimental purposes, the tissue was transferred to a medium with full strength M.S. salts, the same levels of White's organics, IAA, kinetin, and inositol, but with 3% sucrose (w/v). The carrot (*Daucus carota* L.) callus was initiated from root-phloem in early 1972, and treated similarly. Inocula from 5- to 6-week-old callus (*ca.* 50 mg each) were planted in 125 ml conical flasks containing 50 ml of media, solidified with 0·9% agar (Difco Bacto Agar). Varying numbers were planted per flask, depending on the period of incubations. The cultures were grown in the dark or the light (A_{250} lx, 16 hr photoperiod).

Identification of sugars. Flasks containing medium or tissue removed from the medium were frozen at −70° and lyophilized 24 hr later. The freeze-dried material was extracted with 95% EtOH. The extracts were concentrated to 1 ml and 1 ml of hot 95% EtOH was added to dissolve sugars not in solution. PC was developed with BuOH–HOAc–H_2O (4:1:5) and sprayed with aniline diphenylamine.[17]

[14] T. A. THORPE, V. P. MAIER and S. HASEGAWA, *Phytochem.* **10** (1971).
[15] T. MURASHIGE and F. SKOOG, *Physiol. Plant.* **15**, 473 (1962).
[16] P. R. WHITE, *Growth* **7**, 53 (1943).
[17] F. PERCHERON, in *Chromatography* (edited by E. HEFTMAN), 2nd Edn, p. 573, Reinhold, New York (1967).

Preparation of enzyme extracts. Tissue (4–6 g) was homogenized with 20 ml Tris-phosphate buffer pH 7·5 (0·05 M) and 1 g Na_2SO_3 for 3 min at 2/3 top speed in a Lourdes model MM-1B blender, then further treated in a Potter–Elvejhem homogenizer, to assure complete cell breakage. All operations were carried out between 0 and 4°. Cysteine was added to 10 mM and the pH adjusted to 7·5 by adding a few drops of 1 N HCl. The homogenate was centrifuged for 15 min at 24 000 *g*. Both supernatant and pellet were retained for enzymatic preparation. 10 ml of the untreated supernatant was used for soluble invertase determination. Sucrose synthetase was precipitated from the remaining crude extract with $(NH_4)_2SO_4$ at 50% saturation. The precipitate was collected by centrifugation and dissolved in 5 ml dist. H_2O. Both enzyme extracts were dialyzed overnight against running dist. H_2O at between 0 and 4°. The pellet was washed 3× with 20 ml portions of cold dist. H_2O. The residue was suspended in 10 ml dist. H_2O and used as insoluble, cell wall invertase.

Measurement of enzyme activities. Enzyme units. All enzymes were assayed at 35°. One unit of enzyme activity is defined as the amount which will synthesize 1 μmol of product/min at 35°. Enzyme units are expressed in terms of fr. wt of tissue. *Invertase.* Both soluble and insoluble invertase were assayed by the method of Hasegawa and Smolensky[18] with slight modifications. The enzymatic reaction mixture consisted of 2 ml acetate buffer, pH 5·0 (0·1 M), $2·5 \times 10^{-2}$ M sucrose and enzyme extract (2 ml soluble or 1 ml insoluble). A 1-ml aliquot of the reaction mixture was removed after a 30-min incubation period and the reaction stopped by the addition of 0·1 ml phosphate buffer (1·0 M), pH 5·5 and heating at 100° for 5 min. The glucose liberated was assayed by the glucose oxidase/peroxidase method with a commercial reagent (Glucostat, Worthington). *Sucrose synthetase.* Sucrose synthetase was determined by the sucrose cleavage reaction adopted from the method of Pressey.[19] The reaction mixture contained 250 μmol sucrose, 10 μmol UDP, 10 μmol NaF, 50 μmol Tris-phosphate buffer at pH 6·0 and 0·2 ml enzyme in a total vol. of 1 ml. After incubation for 60 min the reaction was stopped by heating at 100° for 2 min. Fructose was assayed by the arsenomolybdate method of Nelson.[20]

Acknowledgements—The chromatographic studies were carried out in cooperation with Mr. R. L. Wample. This investigation was supported by NRC of Canada grant No. A-6467 to T.A.T.

[18] S. Hawegawa and D. Smolensky, *J. Agric. Food Chem.* **18**, 902 (1970).
[19] R. Pressey, *Plant Physiol.* **44**, 759 (1969).
[20] G. Ashwell, in *Methods of Enzymology*, Vol. 3, p. 85, Academic Press, New York (1957).