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Determination of Specific Growth Stages of Plant Cell Suspension Cultures by Monitoring Conductivity Changes in the Medium

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Summary. Conductivity changes in the medium of cultured soybean (*Glycine max* L.) cells were shown to be strictly correlated with nitrate uptake and growth of the cultures. A continuous record of the conductivity was used as a simple and reliable method of determining specific growth stages and concomitant peaks in the activities of nitrate reductase and phenylalanine ammonia-lyase.

Introduction

Plant cells in suspension culture have, in recent years, gained in importance as a research tool in biochemical and physiological studies. The principal assets (Street, 1973a, b) are the rapid growth rates, the easily controlled supply of nutrients (particularly when defined synthetic media are used), the high degree of uniformity between individual cells, and the lack of contamination by microorganisms. In addition, in intact plants, changes in metabolic activity are often associated with particular developmental stages of high complexity. Experiments designed to investigate the specific effector reactions or molecules are therefore often difficult with a whole-plant system. With cell suspension cultures, however, such experiments are more feasible. For example, the enzymes involved in flavonoid biosynthesis in parsley plants (*Petroselinum hortense*) are only recovered from very young leaves and to a lesser extent from young cotyledons (Hahlbrock *et al.*, 1971a). By contrast, the activities of all the enzymes of this pathway can be recovered from parsley cells in suspension cultures at all stages of growth a few hours after exposure of the cells to light (Hahlbrock *et al.*, 1971b).

In cell suspension cultures from soybean (*Glycine max*), some of these same enzymes reach high specific activities only during a short and

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distinct period of growth (Hahlbrock *et al.*, 1971c). Similarly, high specific activities of nitrate reductase in suspension cultures of tobacco (Filner, 1966), sycamore (Street, 1973b), and soybean cells (Bayley *et al.*, 1972a, and Oaks, unpublished) are also confined to a particular growth stage.

Thus when large amounts of cell material are required for the purification of such enzymes, a simple and precise method to monitor the growth of cultured plant cells would be useful. Recently, we have measured the conductivity of the medium in cell suspension cultures of soybean and parsley and have shown that it is inversely correlated with increases in cell fresh weight (Hahlbrock and Kuhlen, 1972). In this communication we report on a strict correlation between changes in the conductivity, in the growth rate, and in the activities of enzymes involved in nitrogen and phenylpropanoid metabolism of soybean cells in suspension culture.

Materials and Methods

Cell Suspension Cultures. Soybean cells (*Glycine max* L.) were propagated at 27° in the dark either in Erlenmeyer flasks on a rotary shaker (110 rpm) or in fermenters of various capacities (10–12, 30 or 300 l). The B5 medium originally reported by Gamborg *et al.* (1968) was slightly modified by using 5×10^{-5} M $FeSO_4$-EDTA instead of sequestrene as source of ferrous iron. The following conditions of incubation in fermenters were used.

Capacity	Agitation	Aeration
10–12 l	200 rpm	2 l/min
30 l	400 rpm	15 l/min
300 l	400 rpm	150 l/min

Inoculation (10%) was either with rotary shake cultures (small fermenters) or with a 30-l-fermenter (large fermenter).

Conductivity was measured using a conductivity meter LF 39 (Wissenschaftlich-Technische Werkstätten, Weilheim, Obb., Germany).

Nitrate of the Medium. Samples of 10 ml each were taken aseptically at the times indicated. The cells were removed from the medium by centrifugation for 5 min at about $5000 \times g$. Nitrate was determined with a nitrate ion electrode (model 92–07, Orion Research Inc., Cambridge, Mass., USA).

Nitrate Reductase and Intracellular Nitrate. Samples of 100–250 ml each were taken from a 12-l-fermenter at the times indicated in Fig. 3, and the cells were stored at −20° C. Nitrate reductase was assayed (according to Bayley *et al.*, 1972a) in extracts obtained by homogenizing 0.5 g of cells with 1 ml of 5×10^{-2} M Tricine-HCl, pH 7.5, containing 5×10^{-4} M EDTA, in an Ultra-Turrax homogenizer (Hahlbrock *et al.*, 1971c) and removing insoluble material by centrifugation. Nitrate was measured as nitrite after enzymatic reduction. The source of enzyme was a partially purified preparation of nitrate reductase from nitrate-adapted *Candida utilis* (Steward, 1972, personal communication).

Assay of Phenylalanine Ammonia-Lyase. Samples of cells were harvested at times indicated in Fig. 4 and stored at 20° C after removal of culture medium by vacuum filtration. The enzyme was extracted by stirring 0.5 g of cells with 4 ml of 0.1 M sodium borate, pH 8.8, for 60 min at 0° C. The mixture was cleared by centrifugation for 10 min at 20000× *g*. Enzyme activity was measured in the supernatant as described previously (Hahlbrock *et al.*, 1971 b). One unit of activity is defined as the amount of protein required for the formation of 1 nmol of cinnamate in 1 min at 30° C.

Results and Discussion

Culture Conditions

In previous investigations of the enzymes of the phenylpropanoid pathway (Hahlbrock *et al.*, 1971 c; Lindl *et al.*, 1973; Ebel and Grisebach, 1973) we have used rotary shake cultures of soybean cells originally derived from roots by Gamborg for his studies on the shikimic acid pathway (Gamborg, 1966). In this study fermenters of various capacities were used so that we could obtain large cell yields. A conductivity cell was mounted to each fermenter before sterilisation, projecting about 5–10 cm into the medium. The cell was then connected with a conductivity meter which recorded the results continuously throughout the incubation. Conditions which best supported high yields of enzyme activity rather than cell fresh weight were established. The rates of agitation and aeration employed in the fermenters caused considerably more whirling of cells and cell aggregates than that obtained by rotary shaking cultures in Erlenmeyer flasks. As a precaution, an antifoaming agent was added to the fermenter cultures.

All of the cultures propagated under these conditions in fermenters consisted of very fine suspensions of single cells and of small cell aggregates (Fig. 1 a). By contrast, the cells grown in Erlenmeyer flasks on a rotary shaker formed mostly clumps of up to more than 1 mm in diameter (Fig. 1 b). The results in Table 1 show that highest specific activity in phenylalanine ammonia-lyase was associated with the fraction of single cells and small cell aggregates (<0.3 mm). The specific activities in larger cell clumps were considerably lower. The growth of cells in smaller aggregates is, therefore, another advantage of the use of fermenters as compared with cultures agitated on a rotary shaker.

Loss of Nitrogen from the Medium

Gamborg *et al.* (1968), Gamborg (1970), Gamborg and Shyluk (1970), and Bayley *et al.* (1972 a, b) have shown that soybean cells can be grown on a simple synthetic medium which includes sucrose as a carbon source and potassium nitrate as the major nitrogen source. However, small amounts of ammonium ions or L-glutamine, L-alanine or putrescine are required for good growth (Bayley *et al.*, 1972 b). They found (Bayley *et al.*,

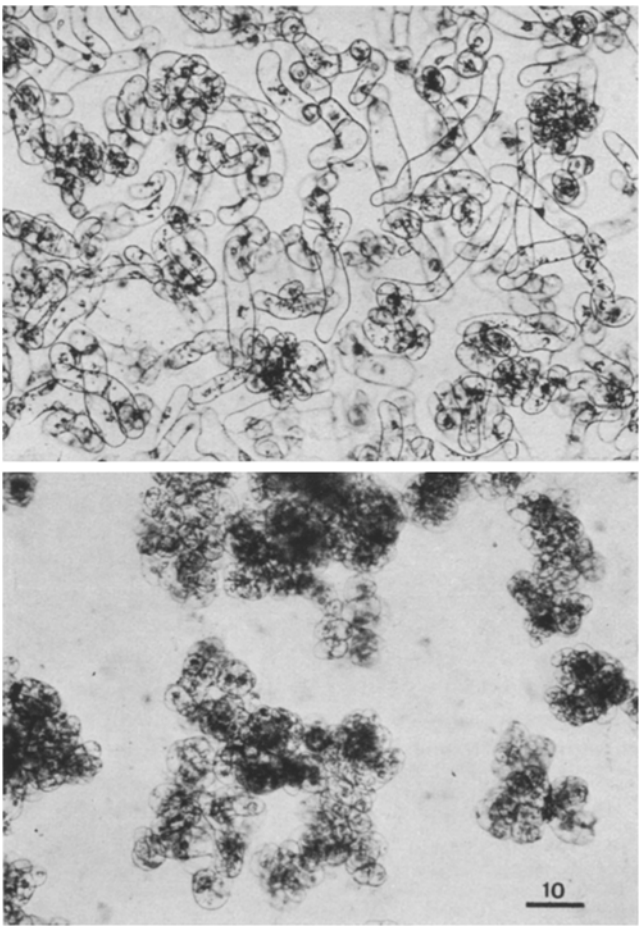

Fig. 1. Difference in size of 7-day-old cells and cell aggregates from suspension cultures of soybean which were propagated either, *a*, in a large fermenter of 300 l or, *b*, in a small rotary shaking culture of 40 ml (cf. Hahlbrock *et al.*, 1971c). Scale mark: 10 μm

1972a) and our results confirm (data not shown) that ammonia is taken up by the cells within the first 60 h of incubation. Nitrate decreased slowly during this period of time in their experiments (Bayley *et al.*, 1972a) or in ours (Fig. 2) and then declined rapidly in a manner identical with a concomitant decline in conductivity of the medium (Fig. 2). The pH of the medium did not change significantly throughout the periods of increases in cell fresh weight (Hahlbrock and Kuhlen, 1972; Gamborg *et al.*, 1968). This rules out a major contribution of protons to the con-

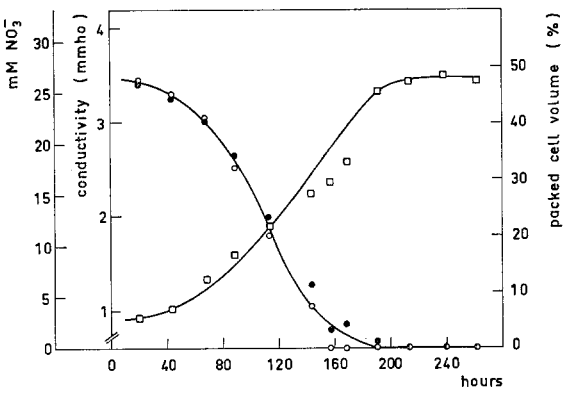

Fig. 2. Changes with incubation time of cultured soybean cells in packed cell volume (□) and in conductivity (●) and nitrate (○) in the medium. The cells were propagated in an Erlenmeyer flask (2 l) containing 400 ml of a suspension culture

Table 1. Phenylalanine ammonia-lyase activity in cultured soybean cells and cell aggregates of different size

The cells were propagated in a fermenter containing 10 l of a suspension culture. The cells were passed through metal sieves of the appropriate pore size and then assayed for enzyme activity (see Methods). When viewed under a microscope, the aggregates appeared of intermediate average size as compared with those from large fermenters (Fig. 1 a) or small rotary shaking cultures (Fig. 1 b), respectively

Size of cells or cell aggregates (mm)	Enzyme activity	
	units/mg protein	units/g cell fresh weight
<0.3	5.3	32
0.3–0.5	3.5	24
>1.0	1.9	15

ductivity changes. Although changes in cations were not measured, it is concluded from the composition of the medium that the observed conductivity changes are mainly due to the uptake of potassium and nitrate ions.

Conductivity Changes Reflect Growth Curves

Cell fresh weight increased in a manner inversely correlated with nitrate consumption and with decreases in conductivity. No further conductivity changes were observed during the subsequent 60 h following depletion of nitrate from the medium. Likewise, increases in cell fresh weight ceased concomitantly with exhaustion of exogenous nitrogen

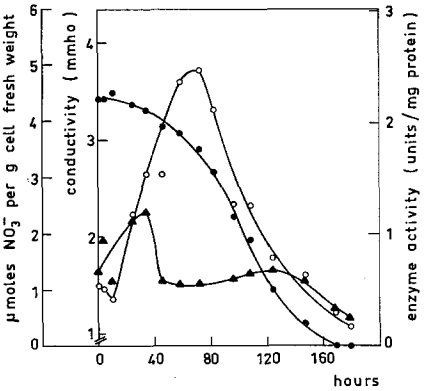

Fig. 3. Changes with incubation time in conductivity (●) of the medium (representing levels of nitrate) and in nitrate reductase activity (○) and nitrate concentration (▲) in cultured soybean cells. The cells were propagated in a fermenter containing 12 l of a suspension culture

sources. The curves obtained for changes in the conductivity are, within the limits of experimental error, precise mirror-images of the growth curves of the cells (Figs. 2 and 4).

The inverse correlation between growth and conductivity changes has also been observed with cell suspension cultures of *Petroselinum hortense* and *Haplopappus gracilis* in the modified B5 medium. Hence we assume that conductivity measurements generally reflect successive stages of the growth cycle of cells propagated in similarly simple synthetic media. Accurate recording of changes in conductivity could, therefore, be used to predict times of maximum enzyme activity as shown by the following examples.

Nitrate Reductase

In view of the striking correlation between cell growth and uptake of nitrate from the medium we investigated some further aspects of nitrogen metabolism in soybean cells (Fig. 3). In contrast to previous reports (Bayley *et al.*, 1972a), the activity of nitrate reductase (E.C. 1.6.6.1), the first enzyme involved in the conversion of nitrate to organic nitrogen, increased rapidly in the cells during the first 3 days of incubation. This pattern of induction is similar to that reported previously by Filner (1966) for tobacco cells (*Nicotiona tabacum* L.) which were grown in nitrate-containing but ammonium-free medium and by Street (1973a) for sycamore cells (*Acer pseudoplatanus*). The activity reached maximum values when the nitrate consumption began to proceed rapidly, and it declined as the nitrate content of the medium decreased (Fig. 3). The results

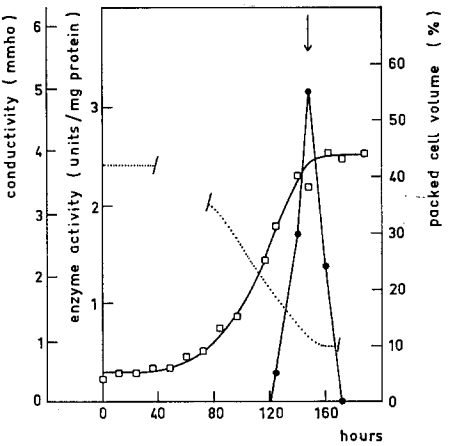

Fig. 4. Changes in phenylalanine ammonia-lyase activity (●) during the growth of cultured soybean cells. □, Packed cell volume. Continuous recording of the conductivity in the medium during hours 0 through 38 and hours 76 through 166 of incubation is shown by the dotted line. The cells were propagated in a fermenter containing 300 l of a suspension culture

show that when maximum amounts of nitrate reductase are required the cells should be harvested just as the conductivity begins to decline rapidly.

Nitrate in the cells reached a peak at 40 h just prior to the peak in nitrate reductase activity. From 50 h to 120 h, a time when the enzyme was increasing and declining in specific activity, the level of internal nitrate remained fairly constant. This suggests that changes in nitrate reductase activity are correlated with changes in the level of nitrate in the medium rather than with fluctuations in internal nitrate.

Phenylalanine Ammonia-Lyase

High activities of several enzymes specifically related to phenylpropanoid metabolism (phenylalanine ammonia-lyase, E.C. 4.3.1.5; cinnamic acid 4-hydroxylase; p-coumarate:CoA ligase) were always observed at a distinct period in the growth cycle of soybean cell cultures (Hahlbrock *et al.*, 1971c; Hahlbrock and Kuhlen, 1972, and unpublished results). This is shown in Fig. 4 for the initial enzyme in the pathway, phenylalanine ammonia-lyase. The results show that the increase in enzyme activity coincides with the beginning of the progressive deceleration phase of growth and with the exhaustion of nitrate from the medium. In order to determine this growth stage of the cell cultures we now routinely record conductivity changes in the medium throughout the growth cycle. The introduction of such a method for the accurate de-

termination of the specific *growth stages* which is independent of the time of incubation is extremely useful, because the *growth time* varies considerably from culture to culture even when conditions of incubation, such as temperature and rates of agitation and aeration, are apparently uniform.

The coincidence of the period of maximum phenylalanine ammonia-lyase activity with the exhaustion of nitrogenous nutrients suggests a switch in cellular metabolism to reactions involving predominantly the formation of nitrogen-free products. Among such compounds would be several products of phenylpropanoid metabolism, such as lignin and flavonoids, whose metabolic precursors, glucose and acetate, would continue to be available from excess sugar of the medium. For example, the accumulation of the flavone apigenin coincides with high levels of phenylalanine ammonia-lyase activity in both illuminated (Hahlbrock, 1972) and in dark-grown (unpublished) soybean cells. Similar studies on the formation of lignin by these cultures under our conditions of cell propagation are in progress (J. Ebel and H. Grisebach). Basically, the occurrence of lignin in a variety of different cultured plant cells has been well established (Hasegawa *et al.*, 1960; Koblitz, 1962; Bergmann, 1964; Jeffs and Northcote, 1966; Gamborg, 1967; Venverloo, 1969; Rubery and Fosket, 1969; Carceller *et al.*, 1971; Moore, 1973). These studies also included cultured soybean cells (Rubery and Fosket, 1969; Moore, 1973).

Results essentially similar to some of our observations with soybean cells were also reported on changes in phenylalanine ammonia-lyase activity (Davies, 1971) and on the accumulation of phenolic compounds (Nash and Davies, 1972) during the late exponential growth phase of *Rosa* cell suspension cultures. This might indicate that the observed correlation between increases in phenylpropanoid metabolism and changes in the metabolic state of the cells is not restricted to soybean cells, but is a more general phenomenon.

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