Planta (Berl.) 124, 311—318 (1975)

Further Studies on the Relationship between the Rates of Nitrate Uptake, Growth and Conductivity Changes in the Medium of Plant Cell Suspension Cultures

Klaus Hahlbrock

Biologisches Institut II der Universität, Schänzlestr. 9–11, D-78 Freiburg, Federal Republic of Germany

Received 12 April; accepted 24 April 1975

Summary. The changes in packed cell volume and in nitrate content and conductivity of the medium during the growth cycle of cell suspension cultures from *Petroselinum hortense* Hoffm., *Glycine max* Merr., and *Haplopappus gracilis* A. Grey in a chemically defined medium were compared. In all three cases sigmoidal curves obtained for large decreases in the conductivity were paralleled by similar curves for the rates of nitrate depletion from the medium until this nutrient was completely exhausted. Further decreases in the conductivity subsequent to nitrogen starvation proceeded at relatively slow rates and ceased when the cultures entered into the stationary phase of the growth cycle. Thus the previously reported method of deriving growth curves indirectly from conductivity measurements (Hahlbrock and Kuhlen, Planta **108**: 271–278, 1972; Hahlbrock *et al.*, Planta **118**: 75–84, 1974) might be generally applicable for this particular medium. The method seems to be based on a continuous uptake by the cells of ionic constituents throughout all stages of actual growth, even beyond the stage of nitrate exhaustion.

Cell suspension cultures from *Cicer arietinum* L. and *Acer pseudoplatanus* L. in two different, more complex media were used for similar experiments, in which the changes in packed cell volume and in the conductivity of the medium were recorded. As with the results obtained with the fully synthetic medium, the mirror-images of the curves obtained for the decline in conductivity *initially* paralleled the growth curves. However, the two curves became incongruous after a certain growth stage was reached. These results are discussed with respect to the composition of the media used and to the apparent limitations of the method of determining specific growth stages by monitoring conductivity changes in the medium.

Introduction

A method for the determination of specific growth stages of plant cell suspension cultures by monitoring conductivity changes in the medium has been described recently (Hahlbrock and Kuhlen, 1972; Hahlbrock *et al.*, 1974). Using the fully synthetic medium I (see Table 1), it was shown that the growth rates of *Petroselinum hortense* and *Glycine max* cell cultures were inversely correlated with the rates of decline in the conductivity of the medium. The continuous and automatic recording of the conductivity as a rapid indication of growth rates was particularly useful when the cell cultures were propagated in fermenters (Hahlbrock *et al.*, 1974; Zimmermann and Hahlbrock, 1975).

As an explanation for the relationship between conductivity changes and growth rates, it was found that the curves obtained for the rates of conductivity

decreases closely corresponded to the curves determined for the rates of nitrate depletion from the medium. This correspondence was observed for cell cultures of *Petroselinum hortense* (Hahlbrock, 1974) and *Glycine max* (Hahlbrock *et al.*, 1974). Furthermore, the inverse correlation between the growth curve and the curve determined for the rate of nitrate depletion has also been reported for a cell suspension culture of *Haplopappus gracilis* (Hahlbrock, 1974).

In the present study, the previous investigations are further extended, and the data for *Petroselinum hortense*, *Glycine max*, and *Haplopappus gracilis* cell cultures in the chemically defined medium are compared with corresponding data obtained for cell cultures of *Cicer arietinum* and *Acer pseudoplatanus* in more complex media.

Materials and Methods

Cell Suspension Cultures. Table 1 gives the composition of the three media used for the present investigations. Medium I was derived by minor modifications from B5 medium (Gamborg *et al.*, 1968), medium II had been developed by Hösel *et al.* (1972) and Wengenmayer *et al.* (1974) from a medium originally used for cultured *Cicer arietinum* cells by Sayagaver *et al.* (1969), and medium III was a modification (H. Sandermann, personal communication) of that described by Torrey (1954). The cell suspension cultures (400 ml, 10% inoculum) were propagated at 26° C in the dark in 2-l Erlenmeyer flasks on a shaker rotating at 110 rpm. *Petroselinum hortense*, *Glycine max*, *Haplopappus gracilis*, and *Cicer arietinum* cells were subcultured every 7 days and had been grown in liquid suspensions for several years in the same media as in the present study. The *Acer pseudoplatanus* cells had been grown as callus cultures and in liquid suspension for several years, but in various different media and with subculturing periods of different lengths.

The *growth* of the cell cultures was determined indirectly by measuring the packed cell volume. Samples of 5 ml each were taken aseptically from the cultures and centrifuged for 5 min at about $5000 \times g$ at the times indicated in the figures.

Conductivity was measured in the supernatant medium after centrifugation, using a conductivity meter LF 39 (Wissenschaftlich-Technische Werkstätten, Weilheim Obb., West Germany).

Nitrate was determined with a nitrate ion electrode (model 92-07, Orion Research Inc., Cambridge, Mass., USA).

Results

Growth of Cell Cultures

The three media used in this study differed greatly with respect to both the total concentration of inorganic ions and the nature and relative amounts of nitrogen sources. The molar concentrations of the inorganic macronutrients of the media are compared in Table 2. While nitrate, and to a much lesser extent ammonia, were the only nitrogen sources of the fully synthetic medium I, both media II and III contained in addition to nitrate considerable amounts of organic nitrogen, supplied in the form of yeast extract and N-Z-amine. All media also contained sucrose as a source of carbon, the growth substance 2,4-dichlorophenoxyacetic acid (2,4-D), and a number of vitamins and micronutrients. Thus all three media were capable of supporting the growth of plant cell suspension cultures. Accordingly, about ten-fold increases in packed cell volume were obtained for the various cultures used despite the large differences in composition and complexity of the media.

Table 1. Composition of the media used for cell suspension cultures. Brackets indicate compounds combined in stock solutions

Medium	I	II	III
	Amount/l		
Sucrose	20 g	20 g	40 g
Yeast extract	—	2.5 g	1 g
N-Z-Amine	—	2 g	—
$Ca(NO_3)_2 \cdot 4H_2O$	—	200 mg	242 mg
KNO_3	2.5 g	1.6 g	85 mg
$MgSO_4 \cdot 7H_2O$	250 mg	360 mg	42 mg
KCl	—	60 mg	61 mg
KH_2PO_4	—	—	20 mg
$NaH_2PO_4 \cdot H_2O$	150 mg	90 mg	—
$Na_2HPO_4 \cdot 2H_2O$	—	30 mg	—
$(NH_4)_2SO_4$	134 mg	—	—
Na_2SO_4	—	200 mg	—
$FeSO_4 \cdot 7H_2O$	13.9 mg	13.9 mg	—
Na_2EDTA	18.6 mg	18.6 mg	—
$FeCl_3$	—	—	1.5 mg
KI	750 µg	750 µg	—
$MnSO_4 \cdot H_2O$	11.2 mg	11.2 mg	4.5 mg
$ZnSO_4 \cdot 7H_2O$	3 mg	3 mg	1.5 mg
H_3BO_3	3 mg	3 mg	1.5 mg
$CuSO_4 \cdot 5H_2O$	390 µg	390 µg	40 µg
$Na_2MoO_4 \cdot 2H_2O$	250 µg	250 µg	25 µg
$CoCl_2 \cdot 6H_2O$	250 µg	250 µg	—
m-Inositol	100 mg	100 mg	—
Thiamine·HCl	10 mg	10 mg	1 mg
Nicotinic acid	1 mg	1 mg	5 mg
Pyridoxine·HCl	1 mg	1 mg	—
2,4-D	1 mg	1 mg	220 µg
Calcium pantothenate	—	2.5 mg	—
pH (before sterilization)	5.5	5.8	6.1

Cell Cultures in Medium I

Fig. 1 shows the time courses of increases in packed cell volume and of decreases in nitrate content and conductivity of the medium during the growth cycle of *Petroselinum hortense*, *Glycine max*, and *Haplopappus gracilis* cell suspension cultures in medium I. In all three cases a progressively rapid depletion of nitrate from the medium during the first 150 to 200 h of incubation was precisely paralleled by an approximately two to three fold decline of the conductivity from an initial value of about 3.4 mmho. Subsequent to the complete exhaustion of nitrate, the conductivity decreased further at a relatively slow, but significant rate, until a minimum value was reached after about 200 to 250 h of incubation. Coincident with the lowest level in conductivity, the cell cultures reached the

Table 2. Molar concentrations of inorganic macronutrients (round figures) and conductivity of the three media used for cell suspension cultures

Values are given only for those components which were supplied in the form of defined chemicals. A question mark indicates that an unknown amount of the respective ion might be present in the yeast extract and the casein hydrolysate "N-Z-amine". For the same reason, the values given for total concentrations of inorganic macronutrients in media II and III are considered to be minimal values.

Medium	I	II	III
		Cations (mM)	
K^+	25	17	2
Na^+	1	4	?
NH_4^+	2	?	?
Ca^{2+}	1	1	1
Mg^{2+}	1	1	?
		Anions (mM)	
NO_3^-	25	18	2
HSO_4^-	2	3	?
$H_2PO_4^-$	1	1	?
Cl^-	2	1	1
		Total ions (mM)	
Absolute	60	46	6
Relative to medium I	100%	76%	10%
		Conductivity (μmho)	
Complete medium[a]	3400	3150	615
Yeast extract plus N-Z-amine alone	—	700	—
Yeast extract alone	—	—	220
Difference due to defined chemicals	—	2450	395
Difference relative to medium I	100%	72%	12%

[a] After inoculation at time zero.

stationary phase of the growth cycle. Although this coincidence is not quite as convincingly demonstrated for the *Haplopappus gracilis* cell culture by the data presented in Fig. 1, results from separate experiments (not shown here, because nitrate was not determined) clearly support this interpretation.

Cell Cultures in Media II and III

The time courses of increases in packed cell volume and decreases in the conductivity of the medium for *Cicer arietinum* and *Acer pseudoplatanus* cell cultures during growth in media II and III, respectively, are shown in Fig. 2. With respect to the two parameters measured, both cultures behaved differently from the three cultures depicted in Fig. 1. The growth curve for the *Cicer arietinum* cell culture is an almost precise mirror-image (the broken line in Fig. 2A) of the curve obtained for a conductivity decline in the medium from 3.15 to 1.56 mmho during the first 400 h of incubation. During the subsequent stationary phase, however, the conductivity increased rapidly and exceeded the initial value within

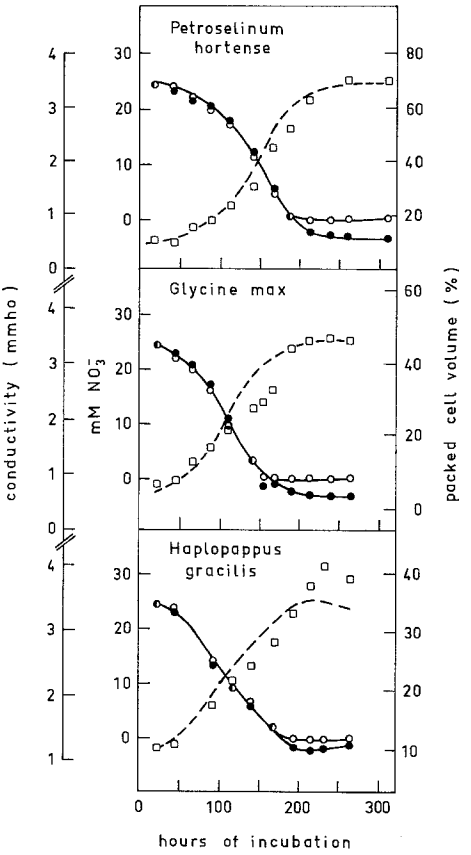

Fig. 1. Time courses for changes in packed cell volume (□), conductivity (●) and nitrate (○) in medium I during the growth of various cell suspension cultures. The broken lines are the mirror-images of the curves for changes in the conductivity. The data were plotted in such a way that (a) the broken lines matched the growth curves at the times of maximum and minimum conductivity levels and (b) the curves for nitrate and conductivity changes paralleled each other until nitrate was exhausted

less than 200 h after the lowest level had been reached. Minor variations in the packed cell volume during this period of time were within the limits of experimental error.

When medium III was used for the propagation of *Acer pseudoplatanus* cells, the conductivity only changed significantly during the exponential growth phase (Fig. 2B). A sigmoidal decline from 613 to 67 μmho during the first 300 h of incubation was accompanied by an increase in packed cell volume to less than one half of that recorded during the stationary phase. Thus, in contrast to the results obtained for all of the other cell cultures investigated, the mirror-image of the curve for conductivity changes parallels the growth curve only during the early stages of this culture.

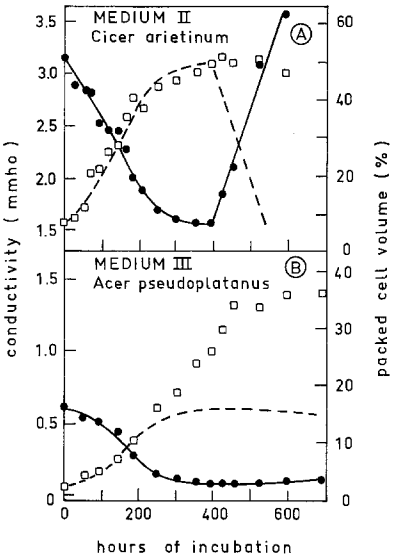

Fig. 2. Time courses for changes in packed cell volume (□) and conductivity (●) during the growth of *Cicer arietinum* and *Acer pseudoplatanus* cell suspension cultures in medium II and medium III, respectively. The data are mean values from three separate experiments. See legend of Fig. 1 for further details

Discussion

The similarity between the results obtained with the three cell cultures in medium I becomes apparent when the data are compared as in Fig. 1. The inverse correlation between the growth curves and the curves for the decline in conductivity confirms and extends previous observations (Hahlbrock and Kuhlen, 1972; Hahlbrock, 1974; Hahlbrock *et al.*, 1974). The close correspondence between the two curves had been explained by assuming that the growth of the cultures was strictly correlated with the utilization of nitrogen, thus causing a depletion of the major ionic constituent of the medium, potassium nitrate, concomitant with the increase in packed cell volume. However, it should be noted in this connection that the previous way of plotting the data (Hahlbrock *et al.*, 1974) was slightly different from the one used in the present communication, suggesting a coincidence of the rates of change of nitrate level and conductivity throughout the entire growth cycle. Fig. 1 clearly shows that nitrate was exhausted from the medium of all three cultures somewhat prior to the cessation of the decline in conductivity. Hence, as already reported for *Petroselinum hortense* cell cultures (Hahlbrock, 1974), the mirror-images of the curves for nitrate depletion match the growth curves very precisely, albeit only until the end of the linear growth phase. By contrast, the complete growth curves including the stationary phase correspond reasonably well to the mirror-images of the curves representing the conductivity changes. At least in the case of the *Glycine max* cell cultures, this close correspondence continues even when the cell fresh weight decreases subsequent to the stationary phase (Ebel *et al.*, 1974).

The results of Fig. 1 imply an uptake by the different cell cultures of small amounts of ions other than nitrate during the progressive deceleration phase of growth. This is in agreement with a similar observation by Nash and Davies (1972) who reported an uptake of phosphate beyond the point of nitrate exhaustion for Paul's scarlet rose cells cultured in another chemically defined medium.

Even in the case of the *Cicer arietinum* cell culture in the rather complex medium II, the mirror-image of the curve for conductivity changes corresponds very closely to the growth curve during all stages of net increase in packed cell volume. This seems to be due mainly to a similar relationship between the growth rate and the rate of uptake of inorganic ions as observed for the cultures in medium I. Table 2 shows that the contribution of the yeast extract and the protein hydrolysate to the conductivity of medium II is relatively small. Furthermore, the relative differences in that portion of the conductivity which is due to the inorganic ions of the media closely correspond to the relative differences in the total concentration of these ions, about 80% of which are made up of potassium nitrate in both media (Table 2). Thus the results depicted in Fig. 2A seem to indicate that the rate of growth in this culture was closely related to the rate of utilization of *nitrate nitrogen* by the cells, despite the fact that the amount of *organic nitrogen* in the medium (approximately 37 mM) was approximately double the amount of nitrate nitrogen.

The reason for the large increase in the conductivity during the stationary phase of the *Cicer arietinum* cell culture is unknown. In any case, Fig. 2A shows clearly that for this culture the method of determining specific growth stages by recording conductivity changes is limited to stages prior to the stationary phase.

In comparison with the cell cultures propagated in media I and II, the *Acer pseudoplatanus* cell culture in medium III grew at by far the slowest rate and gained the least total packed cell volume. The stationary phase was not reached until about 600 h of incubation, as compared with values of 200 to 250 h for the cell cultures in medium I and about 400 h for the cell culture in medium II. Since many factors may have influenced the growth rate (Street, 1973), it is not clear whether or not the slow rate of growth of the *Acer pseudoplatanus* cell culture was due to the limited amount of nitrate present in medium III. However, the incongruity of the curves obtained for conductivity changes and for growth of this culture suggests that either all or at least a major portion of the relatively small amounts of inorganic macronutrients, including nitrate, had been depleted from the medium during the exponentional growth phase.

This result clearly demonstrates the limitations of the method by which the various growth stages can be determined through monitoring conductivity changes in the medium. Obviously a prerequisite for the application of this method is the presence of one or more ionic constituents which contribute, at least to a large part, to the conductivity of the medium and whose rate of uptake by the cells corresponds directly to the growth rate of the culture. The example of the *Cicer arietinum* cells culture shows that the additional presence of large amounts of organic material, such as yeast estract and N-Z-amine, does not necessarily prevent the feasibility of deriving growth curves from conductivity measurements.

The author gratefully acknowledges the support by the Deutsche Forschungsgemeinschaft (SFB 46) and the technical assistance of Mrs. E. Kuhlen and Mr. H. Kostyra. The cell cultures not used previously for these studies were donated by H. E. Street to H. Sandermann (*Acer pseudoplatanus*) and by H. Wengenmayer (*Cicer arietinum*).

References

Ebel, J., Schaller-Hekeler, B., Knobloch, K. H., Wellmann, E., Grisebach, H., Hahlbrock, K.: Coordinated changes in enzyme activities of phenylpropanoid metabolism during the growth of soybean cell suspension cultures. Biochim. biophys. Acta (Amst.) **362**, 417–424 (1974)

Gamborg, O. L., Miller, R. O., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151–158 (1968)

Hahlbrock, K.: Correlation between nitrate uptake, growth and changes in metabolic activities of cultured plant cells. In: Tissue culture and plant science 1974, p. 363–378 (Street, H. E., ed.). London: Academic Press 1974

Hahlbrock, K., Ebel, J., Oaks, A., Auden, J., Liersch, M.: Determination of specific growth stages of plant cell suspension cultures by monitoring conductivity changes in the medium. Planta (Berl.) **118**, 75–84 (1974)

Hahlbrock, K., Kuhlen, E.: Relationship between growth of parsley and soybean cells in suspension cultures and changes in the conductivity of the medium. Planta (Berl.) **108**, 271–278 (1972)

Hahlbrock, K., Zimmermann, A.: Light-induced changes of enzyme activities in parsely cell suspension cultures. Purification and some properties of phenylalanine ammonia-lyase (E.C. 4.3.1.5). Arch. Biochem. Biophys. **166**, 54–62 (1975)

Hösel, W., Shaw, P. D., Barz, W.: Über den Abbau von Flavonolen in pflanzlichen Zellsuspensionskulturen. Z. Naturforsch. **27** b, 946–954 (1972)

Nash, D. T., Davies, M. E.: Some aspects of growth and metabolism of Paul's rose cell suspensions. J. exp. Bot. **23**, 75–91 (1972)

Sayagaver, B. M., Mulhandani, N. B., Narayanaswamy, S.: Isolation of formononetin from tissue cultures of *Cicer arietinum*. Lloydia **32**, 108–109 (1969)

Street, H. E.: Plant tissue and cell culture. Oxford: Blackwell 1973

Torrey, J. G.: The role of vitamins and micronutrient elements in the nutrition of the apical meristem of pea roots. Plant Physiol. **29**, 279–287 (1954)

Wengenmayer, H., Ebel, J., Grisebach, H.: Purification and properties of a S-adenosylmethionine: isoflavone 4'-O-methyltransferase from cell suspension cultures of *Cicer arietinum L.* Europ. J. Biochem. **50**, 135–143 (1974)