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Substrate Utilization by Suspension Cultures and Somatic Embryos of *Daucus carota L.* Measured by ^{13}C NMR

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ABSTRACT

The uptake and utilization of sucrose by embryogenic suspension cultures of carrot (*Daucus carota L.*) growing in the presence of 2,4-D and by somatic embryos derived from these cultures was monitored using ^{13}C nuclear magnetic resonance. The exogeneously supplied sucrose was completely hydrolyzed before cell entry; glucose was taken up preferentially when the cells were cultured in the presence of 2,4-D, while glucose and fructose were utilized at similar rates by somatic embryos in the absence of 2,4-D. Both suspension cells and somatic embryos accumulated high intracellular levels predominantly of glucose and sucrose, the latter being resynthesized intracellularly from the constitutive hexoses. Initially, fructose was converted mainly into glucose and sucrose rather than being catabolized directly through glycolysis or the pentose phosphate pathway. Carbohydrate supply that exceeded cellular demand resulted in intracellular accumulation of mono- or disaccharides. The capacity of cultured carrot cells to produce somatic embryos appeared to be positively correlated with high intracellular levels of glucose.

Plant cells grown *in vitro* in liquid or on semisolid media are known to condition their culture medium. Therefore, the cells and their surrounding media can be considered as a continuous system in which complex interrelations exist that are of prime importance to growth, regeneration, and secondary metabolism (33). One aspect of the conditioning phenomenon is the uptake and utilization of carbon- and energy sources, which in plant tissue culture media are usually supplied as sucrose: the major translocatable sugar in higher plants.

Numerous observations on plant cell suspension cultures suggest that before entry into the cell, sucrose is first hydrolyzed into its constituent hexoses, glucose, and fructose (2, 10, 11), by a cell wall-associated invertase (18, 21, 32). However, total hydrolysis of sucrose does not seem to be necessary for the *in vitro* growth of plant cells (11), and the degree of hydrolysis varies between plant species (6, 15, 20, 32). Sucrose uptake without prior hydrolysis has been shown to occur in some intact plant tissues (6, 13), solid callus (9), and protoplasts (11), the latter obviously lacking cell wall invertase. These observations have led to the idea that in plant tissue sucrose functions as a protected form of glucose, to be released as glucose in actively growing cells (12). Although a positive correlation was observed between glucose utilization and the presence of high levels of kinetin (25), mechanisms controlling the carbohydrate flow and utilization at the plant level remain poorly understood. Likewise, the initial metabolic events that glucose and fructose undergo upon entry into the cell are also still a subject of debate (11).

Carrot (*Daucus carota L.*) is used extensively as a model system for somatic embryogenesis. Embryogenic suspension cultures, capable of massive production of somatic embryos, can be readily initiated from all parts of seedlings or adult plants and maintained in easy to prepare, well-defined media with the synthetic phytohormone 2,4-D as the sole growth regulator (29). Somatic embryos derived from these cultures pass through the distinct developmental stages observed in their zygotic counterparts, *i.e.* globular, heart, and torpedo shaped embryos, and finally develop into plantlets. The somatic embryos are invariably derived from clusters of small, highly cytoplasmic, meristematic cells that are called pro-embryogenic masses (27). So far, limited data are available concerning the regulation of carbohydrate uptake and metabolism in both unorganized cell clusters in suspension cultures and in the structurally highly developed somatic embryos derived from embryogenic suspensions of carrot (16).

In vivo ^{13}C NMR is an attractive technique to study plant metabolism (22) and has already been successfully applied to fungi (8, 22), algae (24), plant cells (1, 14), and their protoplasts (14, 23). Also, the storage of sugars in whole carrot tap roots has been studied by high-resolution ^{13}C NMR (31).

In this paper, we present an investigation of the basic sugar metabolism of intact suspension cells and somatic embryos employing high-resolution ^{13}C NMR. The results show that carbohydrate uptake and utilization are highly regulated and appear to depend on the level of cellular organization present in the cultures.

MATERIALS AND METHODS

Plant Material and Culture Conditions. *Daucus carota* cv Flakkese FG766 seeds (generously supplied by Zaadunie B. V., Enkhuizen, Netherlands) were used as the starting material to obtain embryogenic, hypocotyl derived, high-density suspension cultures. These cultures, referred to as suspension cultures, were maintained at 25°C under an 18 h light period on a gyrotary shaker at 100 rpm by subculturing every 2 weeks 2 mL of packed cells in 50 mL of Gamborg's B5 medium, containing 2% w/v (57 mM) of sucrose supplemented with 2 μM 2,4-D. This resulted in an initial density of about 10^6 cells mL^{-1}. To induce embryogenesis, cells taken from a suspension culture 7 d after the last subculture were sieved to obtain cell clusters between 50 and 125 μm diameter, washed extensively in basal B5 medium without 2,4-D, resuspended at 5×10^4 cells mL^{-1} in basal B5 medium and grown as described above. These cultures are referred to as embryo cultures. Callus control cultures were inoculated identically at 5×10^4 cells mL^{-1} in B5 medium supplemented with 2 μM 2,4-D. Cell concentrations were measured by the chromic-acid dispersion method of Sung (26); for culture terminology see Sung *et al.* (27, 28). For NMR analysis samples were withdrawn

aseptically from the cultures, immediately cooled on ice to slow down metabolic processes and centrifuged at 4°C for 5 min at 200*g*. Cell-free medium samples (3 mL) were made up to 5% v/v D_2O and stored at −20°C. Suspension-, callus-, and embryo-cell samples, containing 2 ± 0.2 mL of packed cell volume, equivalent to 5×10^7 cells, were washed at least 5 times with a 10- to 15-fold excess of ice-cold B5 medium without sucrose, made up to 10% v/v D_2O, and transferred immediately to NMR sample tubes kept at 0°C. This sampling procedure yielded spectra which corresponded to the metabolic state of the cells at the time of sample withdrawal. This was verified by sequential recording of spectra in time blocks of 1 h under identical acquisition conditions for periods up to 12 h after sample preparation. No change was observed in the spectra with time.

To study fructose utilization at a 20-fold higher sensitivity, carrot suspension cells were washed extensively in B5 medium without sucrose and subsequently incubated in B5 medium with 9 mM 20% ^{13}C-enriched fructose. Cell and medium samples were then prepared as described above. Twenty percent randomly ^{13}C-enriched fructose, isolated from leaves of tobacco plants grown in a $^{13}CO_2$ atmosphere (17), was generously supplied by Dr. B. J. M. Harmsen from the Department of Biophysics of the University of Nijmegen, the Netherlands.

NMR. ^{13}C NMR spectra were obtained at 75.46 MHz on a Bruker CXP-300 NMR spectrometer equipped with a 10 mm internal diameter ^{13}C probe. Broadband proton-noise decoupling was applied throughout all experiments. The measuring temperature was kept close to 0°C. High levels of mobile endogenous sugars in the cultures allowed natural abundance ^{13}C NMR to be applied. Spectra of cells, embryos, and callus controls were measured during 1 h; 7200 transients (delay time of 0.5 s between pulses of 12 μs (60°) duration) were accumulated in 8,000 data points; a line-broadening of 5 Hz was used. Contents of the media were measured for 30 min; 1,800 transients (delay time 1 s between pulses of 9 μs [45°] duration) were accumulated in 16,000 data points; a 1 Hz line-broadening was used. Chemical shifts were measured using the α-C-1 resonance of glucose (93.0 ppm) as a reference.

RESULTS

Figure 1 depicts a typical growth curve of an embryogenic carrot suspension culture maintained at high cell density. The culture grew logarithmically, without a notable lag period, up to d 7 to 8. Cell division ceased between d 9 and 10 after subculture, and further increase in biomass after d 10 was due to cell expansion rather than division. Cells used for initiating embryo cultures (without 2,4-D) or callus control cultures (with 2,4-D) were taken from 7 d old suspension cultures which had maximum embryogenic potential. The growth curve of a callus control culture is also included in Figure 1 and indicates that cells inoculated at low density showed a slight lag period followed by rapid logarithmic growth. The increase in cell number of an embryo culture was difficult to quantify, but dry weight measurements (data not shown) equaled the values obtained for the callus cultures.

Substrate Utilization in Embryogenic Cell Suspension Cultures. Figure 2, A and B, presents the changes in sugar composition of both media and cells during the 14 d culture period of an embryogenic cell suspension culture. Note that the data for cells and media at any particular time cannot be compared because a fixed number of cells was measured. For instance, at d 0 the entire population of cells present in a 50 mL culture was collected for NMR measurement, whereas at d 9 only about 20% of the cells present were used. This would result in an underestimate of the total amount of sugars present intracellularly compared to the total amount present extracellularly.

Figures 2 and 3 reveal several interesting features:

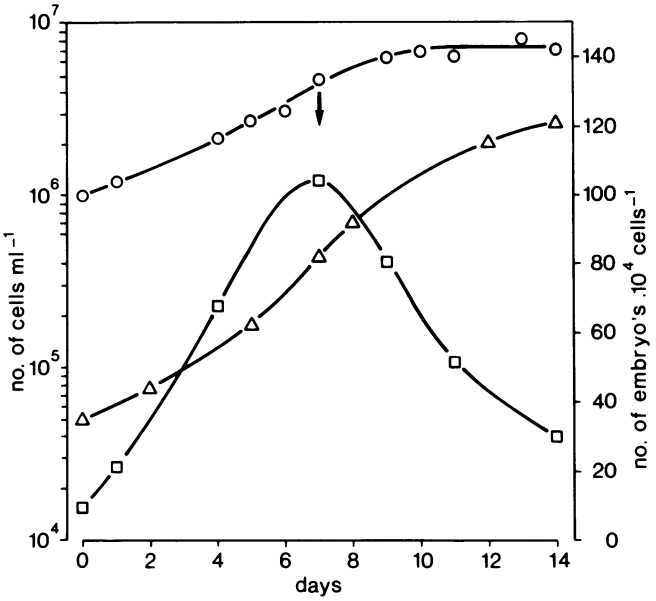

FIG. 1. Growth curves of an embryogenic suspension culture during the 14 d culture cycle expressed in cells mL^{-1} (○), the embryogenic potential of this culture expressed in numbers of embryos per 10^4 cells (□) and the growth curve of a callus control culture obtained from cells at d 7 (↓) that were sieved between 50 and 125 μm (△). The growth curve of the embryo culture that was obtained similarly to the callus controls after omitting 2,4-D is not presented since the number of cells in embryos cannot be measured with sufficient accuracy.

1. Hydrolysis of sucrose into its constitutive sugars, glucose and fructose, started immediately after cells were transferred into a fresh medium and was completed within 24 h. The rate of hydrolysis was dependent on cell density; cultures inoculated at 5×10^6 cells mL^{-1} converted all sucrose within 3 h. Conditioned media from which cells had been removed, did not hydrolyze sucrose. This indicates that the invertase responsible for hydrolysis is not released into the medium (data not shown).

2. Glucose was preferentially taken up during the period of active growth; at d 7 the medium was essentially depleted of glucose, while nearly all fructose was still present. During the first 5 d only low intracellular levels of both glucose and sucrose were found, which indicates rapid utilization of most of the glucose taken up. After d 7 the remaining fructose was rapidly taken up, and the medium became essentially sugar-free by d 9. During this period, which marked the end of the logarithmic growth phase, internal levels of free glucose and to a lesser extent of free sucrose rose rapidly while only a relatively small amount of free fructose was found. This indicates that the bulk of the incoming fructose is not catabolized directly but is converted mainly into glucose and sucrose. After d 9, when the culture enters the stationary growth phase, intracellular sugar levels declined until at the end of the subculture cycle, at d 14, the cells were essentially devoid of free sugars. Throughout the subculture cycle cell viability remained more than 96%.

3. To confirm that, independent of the growth phase, fructose is predominantly converted into glucose and sucrose after uptake, sucrose-grown suspension cells of 3 and 10 d were washed extensively in sugar-free medium and incubated with 20% randomly ^{13}C-enriched fructose. The results for cells of a 3 d culture in exponential growth phase presented in Figure 3, show that nearly all the ^{13}C label (originally present in fructose) is found in glucose and sucrose after 13 h of incubation; similar results were obtained with cells of a 10 d culture in stationary growth phase (data not shown).

To verify that the accumulation of free intracellular sucrose

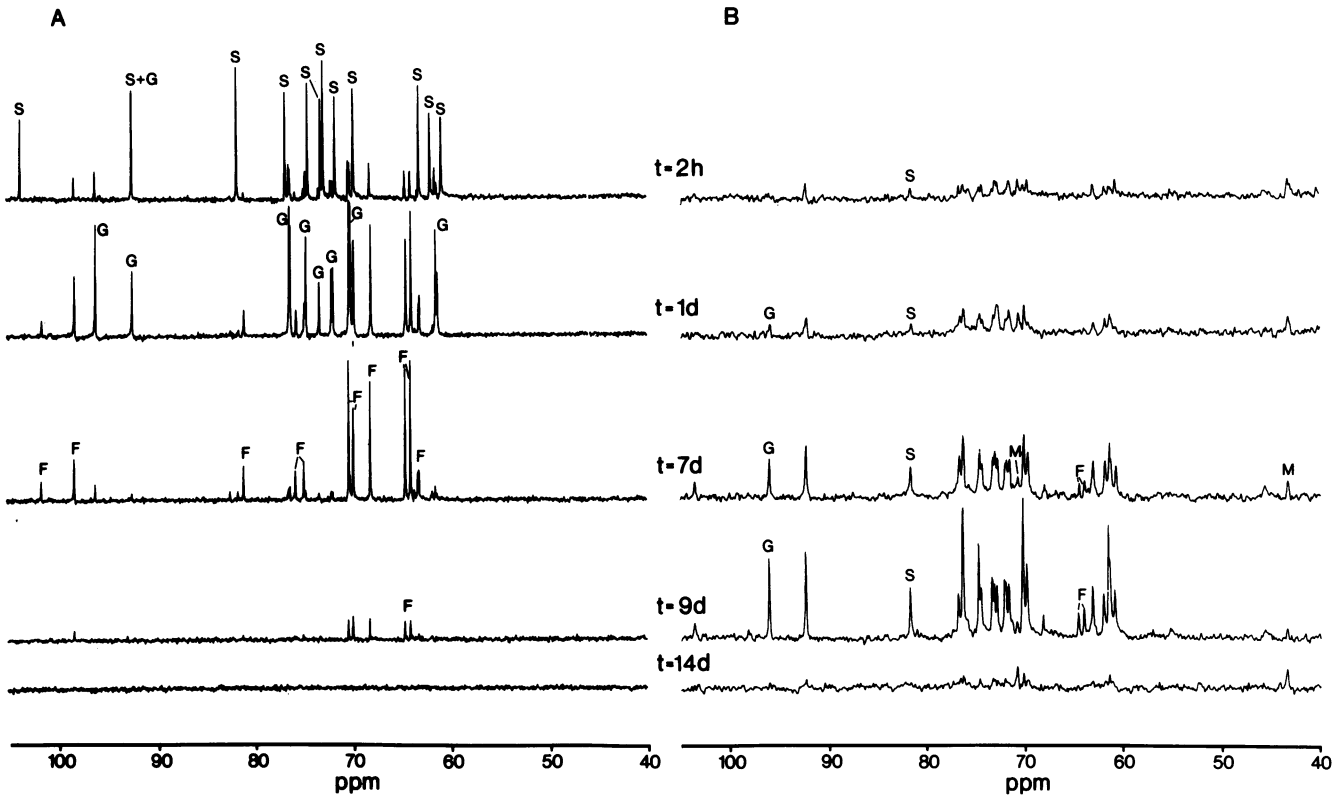

FIG. 2. Natural abundance ^{13}C NMR spectra obtained from 3 mL of medium (A) and 2 mL packed cells (B) during the 14 d culture cycle of an embryogenic suspension culture. A, For reasons of clarity, only the 12 sucrose resonances are indicated by S for $t = 2$ h, for $t = 1$ d only the 12 glucose resonances are indicated by G; for $t = 7$ d only the 12 fructose resonances are indicated by F. B, Sucrose (S) and glucose (G) are indicated each by only one single characteristic resonance; two fructose resonances are indicated by F. M indicates the malate resonances.

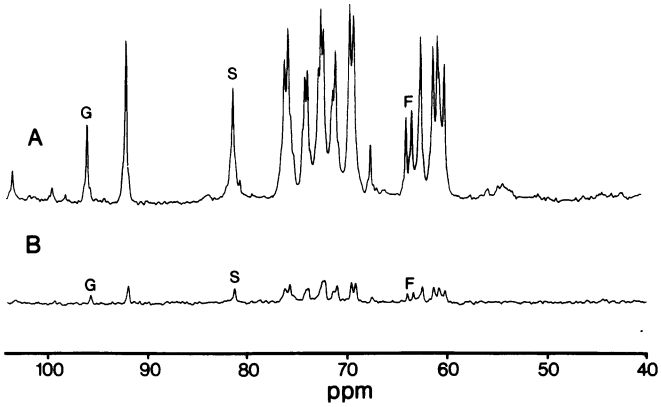

FIG. 3. ^{13}C NMR spectra obtained from 2 mL packed cells of a 3 d embryogenic suspension culture incubated with ^{13}C-enriched fructose for 13 h (A) and 0 h (B). For labels S, G, and F, see legend of Figure 2.

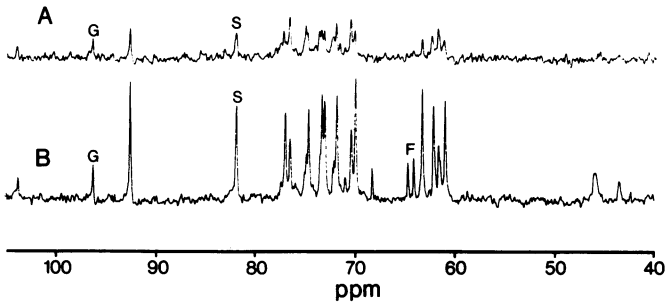

FIG. 4. Natural abundance ^{13}C NMR spectra obtained from 2 mL packed cells of an embryogenic suspension culture 9 d after transfer to a medium containing 114 mM of glucose (A) or 7 d after transfer to a medium containing 114 mM of fructose (B). For labels S, G, and F, see legend of Figure 2.

and glucose between d 7 and 9 with fructose medium was due to the presence of this ketose rather than to the stationary phase of the cells, suspension cultures comparable to the one used in the experiments of Figure 2 were grown on either glucose (Fig. 4A) or fructose (Fig. 4B). After growth on glucose for 9 d the intracellular levels of free glucose and sucrose, though lower in an absolute sense, were comparable to those observed in the sucrose-grown culture at d 7 (Fig. 2B), which by that time had predomantly utilized glucose. After growth on fructose for 7 d, the majority of intracellular sugar present was sucrose with glucose and fructose at lower and roughly equal levels. This demonstrates that accumulation of intracellular glucose and sucrose is due to the presence of extracellular fructose and is not the result of a particular growth phase (see also Fig. 2B, 9 d).

No tricarboxylic acid cycle or ppp related intermediates or derived compounds were detected by ^{13}C NMR, except for a moderate accumulation of malate. Accumulation of malate is considered to be indicative of anaerobic metabolism (7).

The suspension cultures reached maximum embryogenic potential at d 7 after subculturing, which corresponded to the late logarithmic growth phase (Fig. 1). At this point glucose in the medium was depleted and the cells had started to take up fructose (Fig. 2). No clear correlation was found between the levels of intracellular free sugars and embryogenic capacity. However, it is of interest to note that suspension cells subcultured for 6 months accumulated considerably more sucrose as compared to a newly initiated suspension culture of the same cultivar (Fig. 5, A and B). After 6 months the embryogenic capacity of this cell line was reduced approximately 10-fold and had disappeared

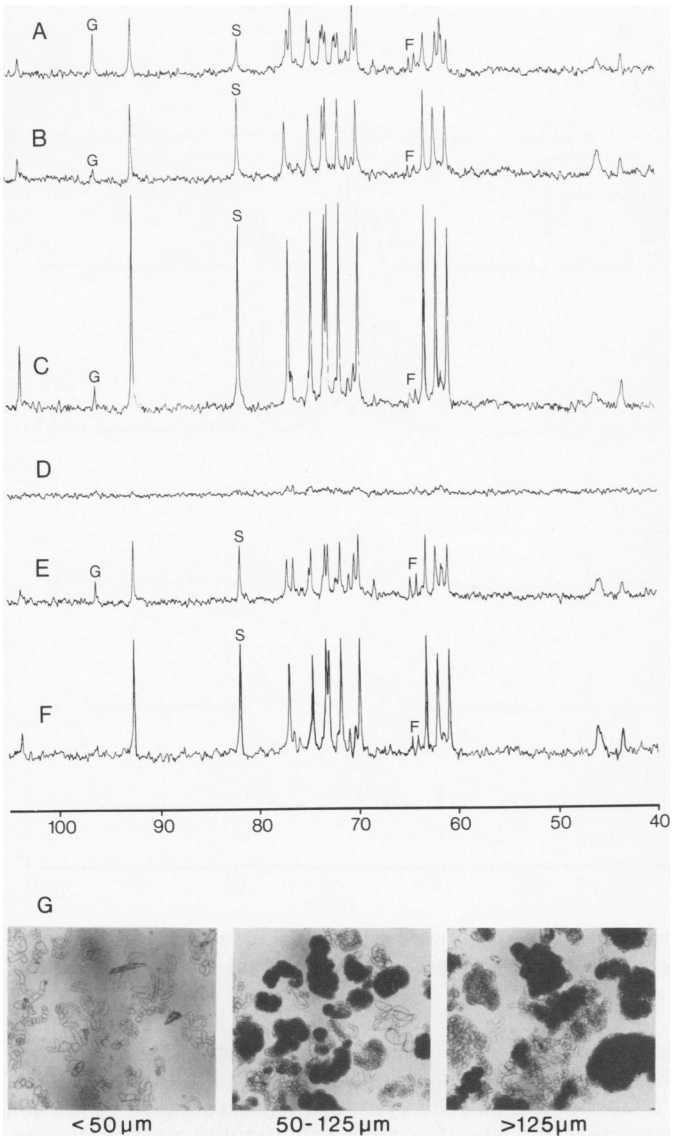

FIG. 5. Natural abundance ^{13}C NMR spectra obtained from 2 mL packed cells of suspension cultures 7 d after transfer, and subcultured for 1 month (A), 6 months (B), and 18 months (C). A suspension culture that had been subcultured for 6 months was fractionated 7 d after transfer into single cells smaller than 50 μm (D), embryogenic clusters between 50 and 125 μm (E), and large aggregates over 125 μm (F). The inserted picture shows the morphology of the different fractions (G). For labels S, G, and F, see legend of Figure 2.

completely after 18 months in culture. At this time, the predominant intracellular sugar was sucrose, while only very small amounts of free glucose were detected (Fig. 5C).

When the cells of a 6-month old suspension culture were separated by size, the remaining free intracellular glucose was found predominantly in the proembryogenic mass fraction having a cluster diameter between 50 and 125 μm (Fig. 5, D–F). This fraction contained about 10% of the cells. Nonembryogenic cells collected in the fraction below 50 μm comprised 5% of total cell number and were mainly single, highly vacuolated cells that did not accumulate sugars to a detectable level. Aggregates over 125 μm in size that consisted of more than 100 vacuolated cells included most of the cells. These cells contained mainly sucrose.

These observations suggest a positive correlation between the presence of proembryogenic masses and free intracellular glucose.

Substrate Utilization in Embryo and Callus Control Cultures. Figure 6, A and B, shows the changes in sugar composition of media and cells during the development of somatic embryos in the absence of 2,4-D, and growth of callus control cells in the presence of 2,4-D, respectively. The spectra obtained from a 7-d-old embryogenic suspension culture (Fig. 2, A and B, d 7) can be considered as a reference representing d 0 of the embryo and callus cultures. Whereas hydrolysis of sucrose by high-density suspension cultures was complete within 24 h, the much lower initial cell density used here resulted in complete sucrose hydrolysis by d 7. The embryo culture, in which globular stage embryos were already detectable at d 2, seemed slightly more efficient in the hydrolysis of sucrose when compared to cells grown in the presence of 2,4-D. Apparently, due to the limited amount of hexoses available and the rapid growth at this stage, intracellular free-sugar levels were almost zero in the embryo cultures while in the callus control cells both sucrose and glucose were still present at d 2 (compare with Fig. 2B, d 7). Seven days after the initiation of embryogenesis, by which time torpedo stage embryos had developed, the embryos contained a small amount of fructose in addition to glucose and sucrose. In contrast, the cells growing in the presence of 2,4-D did not contain any intracellular fructose, but contained only sucrose and a decreased level of glucose. This difference was most pronounced at d 14, when the embryos had reached the plantlet stage and started turning green. The sucrose concentration increased both in embryos and callus control cells, whereas the glucose concentration reached a constant level in embryos but decreased to a very low level in callus control cells. The accumulation of fructose in embryos between d 7 and 14, to an extent comparable to glucose, suggested fructose uptake since intracellular fructose in suspension cells only occurred when all glucose in the medium was depleted (Fig. 2, A and B), or when the cells were grown in medium containing only fructose (Fig. 4B). This is confirmed by the medium spectra of embryo cultures which indicated that the levels of both glucose and fructose decreased simultaneously rather than sequentially (Fig. 6A; embryos, d 14). Therefore, we conclude that the preferential uptake as observed in cells growing in the presence of 2,4-D (Fig. 2A, d 7 and Fig. 6A, callus, d 14) is not exhibited by somatic embryos.

DISCUSSION

In carrot suspension cultures grown at high cell density on sucrose, this substrate was present in the medium for only a few hours after subculture (Fig. 2A). Therefore, these cultures did not grow on sucrose but on a mixture of glucose and fructose for the majority of the growth period.

The utilization pattern of both sugars appeared to be strikingly different. During the rapid logarithmic growth phase, reflected by low intracellular levels of free sugars, glucose was utilized nearly exclusively for biomass and possibly also stored as starch. Biomass and starch are not visualized by high-resolution ^{13}C NMR because both are immobile, high-molecular weight compounds. This conversion of glucose into predominantly NMR-invisible compounds explains the low intracellular levels of free sugars during the logarithmic growth phase. By d 7 when the culture entered the stationary phase, glucose was exhausted almost completely. At this point, the pattern of utilization shows marked changes and within 2 d virtually all the initial fructose was rapidly taken up and converted intracellularly mainly into glucose, sucrose, and probably also stored as starch. Preferential uptake of glucose rather than fructose is at the moment not well understood and relies on features as, for example, competition of both substrates for the same carrier. The observed lower uptake rate of fructose compared to that of glucose in carrot cells in liquid culture (15, 20) is consistent with a higher affinity for glucose. Also, the higher affinity of hexose kinase for glucose

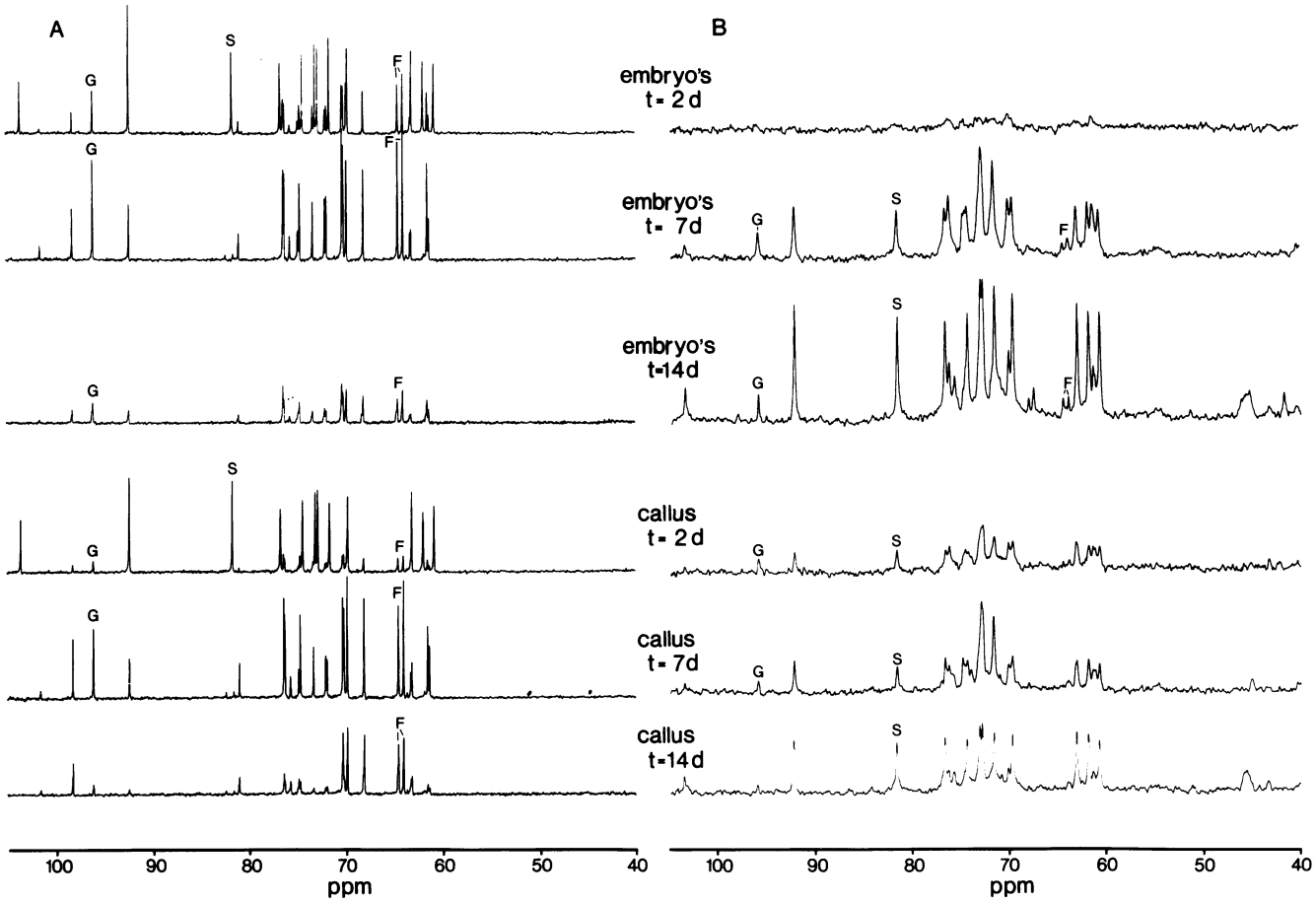

FIG. 6. Natural abundance ^{13}C NMR spectra obtained from 3 mL of medium (A) and 2 mL packed cell material (B) after transfer of washed cells, fractionated between 50 and 125 μm, into 2,4-D free medium (embryos) or 2,4-D containing medium (callus control). For labels S, G, and F, see legend of Figure 2.

(11) provides a more efficient entry into intermediary metabolism for glucose.

The accumulation of nonphosphorylated sugars is characteristic of plant cells which behave quite differently with respect to hexose metabolism as compared to cell cultures of lower eukaryotes (*e.g.* fungi). Fungi do not accumulate free substrates, but instead accumulate large amounts of polyols once the glycolytic substrate has been taken up and subsequently reduced (8). These polyols are not observed in spectra of cell suspensions of carrot, neither by monitoring natural abundance ^{13}C hexoses nor by enhancing the sensitivity 20-fold by using 20% randomly ^{13}C-enriched hexoses (Fig. 3). The accumulated sugars are utilized by the cells once the substrate in the medium is exhausted by d 9 to 10. This results in a gradual decrease of the endogenous sugar levels which become negligible by d 14.

Carrot cells catabolize fructose via fructose-6-phosphate to only a minor extent, whereas the major part of this substrate is isomerized via glucose-6-phosphate into glucose and sucrose (20). The apparent inability to catabolize fructose directly is presently unexplained, but can be interpreted when taking into account the observations made by Thom *et al.* (30). These authors showed that in addition to an ATP dependent sucrose transport system, sucrose uptake into intact red beet vacuoles operates via an UDP-glucose dependent group-translocation mechanism. In this mechanism, both fructose and glucose moieties of the vacuolar sucrose are supplied exclusively by UDP-glucose, since fructose-6-P added simultaneously does not even enter into the vacuoles. If a similar mechanism is operative in carrot cells this would explain the observed rapid conversion of fructose into glucose and sucrose, the latter compound most probably being stored in the vacuole. Employing carrot root callus cultures supplied with ^{14}C labeled sugars, Bender *et al.* (2) arrived at a similar conclusion concerning the fate of exogeneously supplied sucrose, glucose, and fructose. In that study it was also observed that only a limited amount of the supplied hexoses were immediately catabolized, whereas over 95% were resynthesized into sucrose intracellularly, depending on the growth rate of the tissue under investigation.

No fermentation products like ethanol and lactate were observed, neither in the cells nor in the media. Instead, the dicarboxylic acid malate accumulated which indicates hexose catabolism under conditions of annoxia (7). Malate is formed as a result of the NADH-dependent reduction of cytoplasmic oxaloacetate, derived from PEP via PEP-carboxylase (3). This NADH-dependent reduction of oxaloacetate provides a way in which a proper NADH/NAD cofactor balance can be maintained in the cytoplasm when reduced NADH, generated in glycolysis, is not oxidized during ethanol or lactate biosynthesis. Malate biosynthesis is also assumed to play a role in pH control of the cytoplasm (4), whereas malate accumulation can take place under anaerobic conditions by transport into the vacuole (19).

At first glance there is no obvious correlation between the level of intracellular free sugars and the capability to produce somatic embryos. However, it is interesting to note that embryogenic cells in a suspension culture are characterized by the presence of free glucose (Fig. 5E), a phenomenon also apparent when cultures with different embryogenic potential are compared. This indicates that high levels of immediately mobilizable carbohydrates

must be available in the meristematic cell types that eventually will yield somatic embryos. Initial stages of somatic embryo development are known to be accompanied by a rapid increase in metabolic activity.

The elevated metabolic activity during the first stages of somatic embryogenesis is also apparent from the total depletion of free intracellular sugars 2 d after the initiation of embryogenesis. At later stages of embryo development, when all sucrose is hydrolyzed and glucose and fructose are taken up at an equal rate, there is again a rise in the level of free intracellular sugars. When the plantlet stage is reached, predominantly sucrose is observed and the spectra are now strikingly similar to what is observed in nonfibrous tap roots (31).

The observation that regenerated somatic embryos and plantlets do not exhibit the preferential uptake of glucose over fructose could indicate an important difference in the mode of sugar uptake in organized plant tissues versus unorganized *in vitro* cells grown in the presence of nonphysiological levels of phytohormones. Callebaut *et al.* (5) recently reported that a nonembryogenic suspension culture of cucumber exhibited the same preferential uptake of glucose as demonstrated in this work. In contrast, its embryogenic counterpart utilized both glucose and fructose at equal rates. In view of the results presented here, this can be interpreted as being the result of partly organized tissue present in the embryogenic cell line.

This study shows that ^{13}C NMR is a useful and reliable technique to investigate carbohydrate metabolism in cultured plant cells. Although not as sensitive as methods employing ^{14}C labeled substrates, NMR can rapidly demonstrate which metabolic routes are operative and has a definite advantage in being nondestructive. The use of ^{13}C NMR thus circumvents laborious procedures to measure particular metabolites in plant cells, especially when more unusual sugar substrates are used (11). It may also provide a means to discriminate between plant cell cultures with different regeneration capabilities, and indicate preferable medium compositions.

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