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Regulation of Glutamate Dehydrogenase Activity in Relation to Carbon Limitation and Protein Catabolism in Carrot Cell Suspension Cultures[1]

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ABSTRACT

Glutamate dehydrogenase (GDH) specific activity and function have been studied in cell suspension cultures of carrot (*Daucus carota* L. cv Chantenay) in response to carbon and nitrogen supply in the culture medium. The specific activity of GDH was derepressed in sucrose-starved cells concomitant with protein catabolism, ammonium excretion, and the accumulation of metabolically active amino acids. The addition of sucrose led to a rapid decrease in GDH specific activity, an uptake of ammonium from the medium, and a decrease in amino acid levels. The extent of GDH derepression was correlated positively with cellular glutamate concentration. These findings strengthen the view that the function of GDH is the catabolism of glutamate, which under conditions of carbon stress provides carbon skeletons for tricarboxylic acid cycle activity.

The enzyme GDH[4] is reported to be present in all higher plants examined, and often high levels of activity are present in tissues such as roots and senescing leaves (15, 16). Until recently, controversy existed as to its metabolic role, and two apparently conflicting functions have been proposed. The enzyme might function in ammonium assimilation (11, 16, 19), or, alternatively, it could catalyze the oxidation of glutamate and thus provide carbon skeletons to the TCA cycle (18). Recent studies in this laboratory with suspension cultured cells of carrot have demonstrated that GDH is active in the oxidation of glutamate but not in the reductive amination of 2-oxoglutarate (12). Evidence was presented showing that GDH is not involved in ammonia assimilation, which occurs solely via the GS/GOGAT cycle. It was hypothesized that the primary role of GDH is the catabolism of glutamate to provide carbon skeletons for TCA cycle function under conditions of carbon limitation, and that, consequently, this enzyme fulfills an important anapleurotic function linking carbon and nitrogen metabolism in higher plants.

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[4] Abbreviations: GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; TCA, tricarboxylic acid.

If this view is correct, an inverse correlation between GDH activity and carbohydrate supply would be predicted. Here we report metabolic studies of the effect of carbohydrate supply on GDH activity and nitrogen metabolism in cultured carrot cells.

MATERIALS AND METHODS

Growth Conditions

Suspension cultures of carrot (*Daucus carota* L. cv Chantenay) were maintained on Murashige and Skoog (8) medium supplemented with 2,4-D (0.2 mg/L), kinetin (0.1 mg/L), and sucrose (2%) in 250-mL Erlenmeyer flasks at 25°C. Cells were subcultured at 14-d intervals by inoculating 7 mL suspension into 70 mL of fresh medium, or in some experiments at 7-d intervals by increasing the inoculum to 14 mL.

Biochemical Determinations

Cells were collected by vacuum filtration for fresh weight determinations, and the activities of GS, GOGAT, and GDH were determined as described previously (12) and expressed as nkat (nmol·s^{-1})/mg protein. Total cell protein was determined after alkaline hydrolysis, and the soluble amino acids were determined by HPLC analysis of the *o*-phthaldialdehyde derivatives following methanol extraction. Cellular ammonia was determined with methanol extracts, and medium sucrose, nitrate, and ammonium levels were determined directly on filtered medium. All techniques were as described previously (12). In addition, 2-oxoglutarate was determined by the method of Burlina (3).

RESULTS AND DISCUSSION

Phasing of Nitrogen Metabolism during One Culture Period

During the course of a growth passage in suspension culture, the initially nutrient-rich culture medium is progressively depleted by cell growth until one or more nutrient factors become limiting, and growth ceases on entry into stationary phase. Changes in fresh weight; specific activities of GS, GOGAT, and GDH; and total cellular protein, cellular ammonia, and medium concentrations of sucrose, nitrate, and ammonia over a single culture passage are shown in Figure 1, A to D.

After transfer of cells to fresh medium, growth as deter-

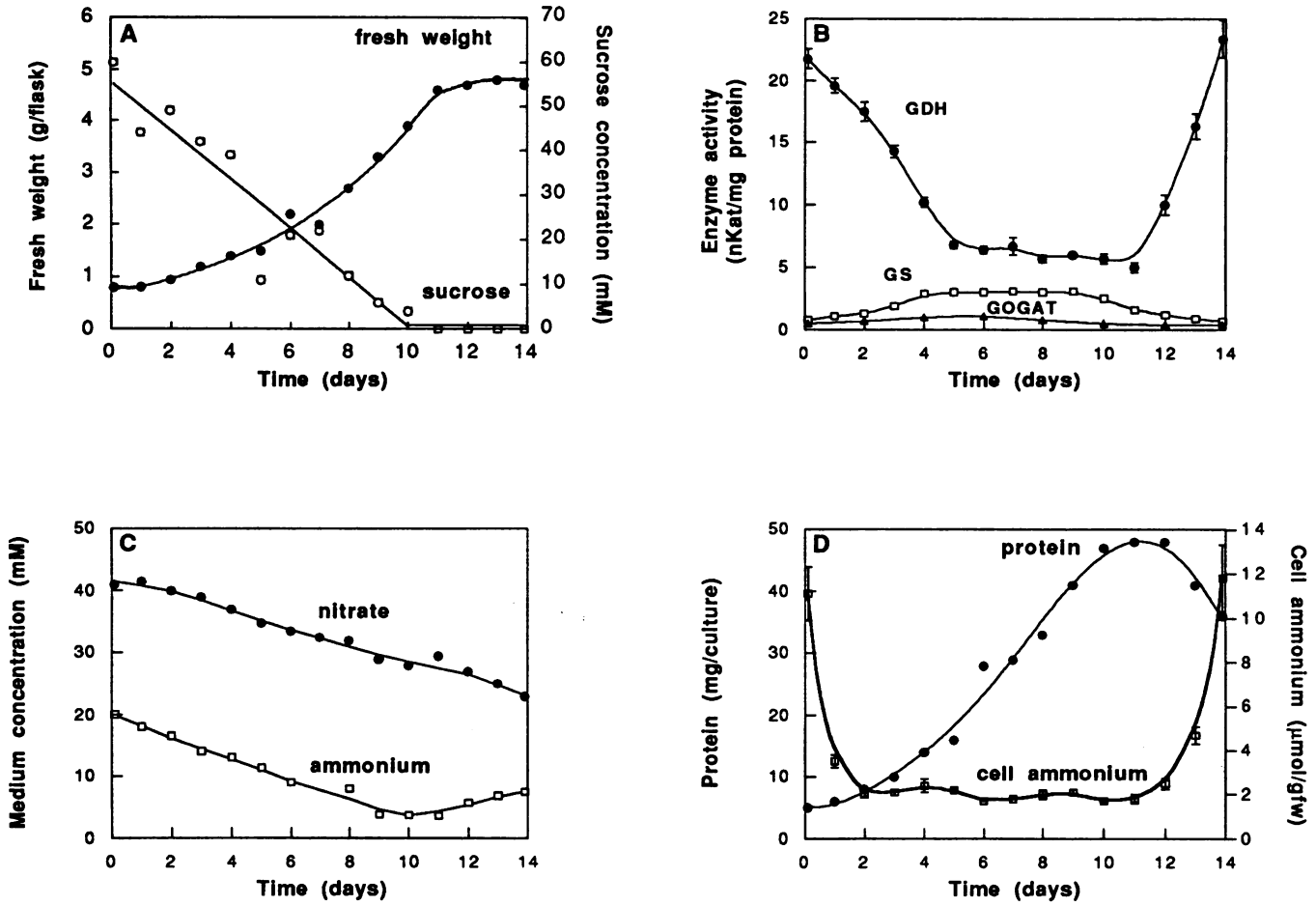

Figure 1. Changes in cellular and medium parameters throughout one 14-d culture passage. A, Medium sucrose concentration (mM), cell fresh weight (g/culture flask); B, specific activities of GDH, GS, and GOGAT (nkat/mg protein); C, medium concentrations of nitrate and ammonium (mM); D, total protein (mg/culture), cellular ammonium concentration (μmol/g fresh weight). Means of three determinations ± SEM.

mined by fresh weight increased after a brief lag period with exponential and linear growth phases followed by the onset of stationary phase after 11 to 12 d. The cessation of growth coincided with depletion of medium sucrose at about 11 d (Fig. 1A). Specific activities of GS and GOGAT were low at the beginning and end of the 14-d culture period, when growth rates were low, and high during the period of rapid growth, as would be expected for nitrogen assimilatory enzymes (Fig. 1B). This pattern is strikingly reversed for GDH activity, which reaches maximum levels in stationary phase and falls progressively during the initial growth phase to minimum levels between d 5 and 10 (Fig. 1B).

Cell growth was never restricted by nitrogen supply; free nitrate and ammonium ions remained in the culture medium in concentrations above 4 mM up to 14 d (Fig. 1C). An increase in medium ammonium from 4 mM to 7 mM between d 11 and 14 was consistently observed, and can only be accounted for by excretion of ammonium from the cells at this stage. The sixfold increase in cellular ammonium at the onset of stationary phase (Fig. 1D) supports this view. The source of the released ammonia can be inferred from the change in total protein per culture (Fig. 1D), which declined from a maximum of 46.8 mg/culture on d 10 to 36.0 mg/culture on d 14. Catabolism of this quantity of protein would release approximately 120 μmol ammonium. A total of approximately 300 μmol ammonium are released to the medium between d 11 and 14 (Fig. 1D); the balance can be accounted for by the conversion of nitrate via nitrate reductase, since nitrate uptake continues throughout this period (Fig. 1C).

These data are compatible with the following scheme, which accounts for changes in GDH activity and nitrogen metabolism. In the presence of adequate nitrogen (ammonia, nitrate) and carbon (sucrose), nitrogen assimilation proceeds via the GS/GOGAT pathway, leading to protein synthesis and growth. Depletion of the carbon source around d 11 restricts protein synthesis due to a lack of carbon skeletons, and simultaneously induces large increases in GDH activity. Continuing protein catabolism releases glutamate, which is oxidized by GDH to 2-oxoglutarate with the concomitant release of ammonia. The oxoglutarate preferentially enters the TCA cycle, while the ammonium produced by this process accumulates both within the cell and in the external medium. When the cells are placed in fresh medium containing sucrose, the activity of GDH declines, net protein catabolism ceases,

and the ammonium level within the cell is reduced. At the same time, the enzymes of ammonium assimilation, GS, and GOGAT increase in activity, and the active assimilation of ammonium and nitrate is initiated. This scheme provides a mechanism for supplying carbon skeletons to the TCA cycle for essential maintenance activities during periods of carbon starvation. This model may have wider applicability than simply accounting for the behavior of carrot cell suspension cultures. Our observations are in accord with those made earlier by Sahulka and Lisa (13), who showed that GDH levels in excised pea roots increased in response to sucrose limitation. It is evident that the increases in GDH activity seen in senescing leaves could, in terms of our model, also be related to the decline in photosynthesis and hence the availability of carbon. Similarly, the so-called ammonia induction of GDH activity seen in leaf discs and detached leaves (1) could be mediated by a depletion of carbon skeletons. A role for GDH in maintaining the supply of carbon to the TCA cycle would account for the ubiquitous occurrence of this enzyme.

Qualitative and quantitative changes in individual amino acids throughout the 14-d culture period support the view that there is extensive protein catabolism induced by carbon limitation (Table I). The total pool size dropped by 71% over the first 4 d and then increased steadily until the end of the culture period.

However, examination of the changes in individual amino acids reveals two clearly distinct subgroups, especially in terms of changes towards the end of the growth period. Those amino acids generally regarded as being metabolically active, *i.e.* glutamine, glutamate, γ-aminobutyric acid, and alanine, are most abundant during the middle phase of culture, and decline sharply at the onset of stationary phase. Those less metabolically active such as arginine, phenylalanine, and histidine are at low concentration during the growth phase and accumulate to high levels in stationary phase. These findings can be explained if we assume that the first group of amino acids is readily metabolized within the cell to provide carbon skeletons for respiration. The release of the amino group as ammonium can occur through the action of amino acid oxidases or dehydrogenases. The enzyme GDH occupies a central role in this process catalyzing the deamination of glutamate to 2-oxoglutarate and ammonium. The catabolism of the less metabolically active amino acids is more complex and therefore they are more likely to accumulate following their release from protein.

Observations that GDH activity increases during leaf senescence also fit the model we propose for GDH. The metabolic status of senescing leaves is comparable in certain respects to that of stationary phase carrot cells. As photosynthesis declines, the senescencing leaf becomes limited with respect to carbon supply, and catabolism of leaf protein produces amino acids, some of which accumulate, whereas others such as glutamate are rapidly catabolized. Thomas (18) proposed a scheme in which nitrogen from protein catabolism was converted to amide nitrogen for transport from the senescing leaf to other parts of the plant. The observation that glutamate and aspartate do not accumulate during protein catabolism suggests that they are readily metabolized on their release from protein, and their nitrogen is transferred to the amide pool. Thomas (18) suggested that glutamate was catabolized to ammonium via GDH and that this ammonium was then reassimilated via GS into glutamine and transported out of the leaf. Towards the end of senescence, there is a large increase in tissue ammonium accompanied by a continuing

Table I. *Changes in Soluble Amino Acid Concentrations with Time in Standard Culture Medium*

Daily samples were analyzed, but data only from alternate days are shown for clarity. In all cases, the omitted data interpolate between alternate data points.

Amino Acid	Concentration							
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
	μmol/g fresh wt							
Glutamate	1.38	0.66	0.73	1.75	2.17	1.42	1.17	0.78
Glutamine	1.09	3.43	1.33	2.21	2.72	2.43	1.10	0.37
GABA	1.65	1.52	1.97	1.58	0.87	0.43	0.41	0.48
Alanine	0.40	1.40	1.46	2.19	2.10	1.44	0.43	0.27
Arginine	8.38	2.92	0.92	0.25	1.94	4.01	6.12	6.14
Serine	2.75	0.81	0.49	0.69	0.84	0.76	0.66	1.17
Methionine	0.60	0.29	0.15	0.17	0.20	0.23	0.25	0.45
Glycine	0.41	0.30	0.31	0.34	0.33	0.30	0.27	0.27
Valine	4.79	1.30	0.60	0.91	1.22	1.39	1.25	1.58
Aspartate	1.28	0.26	0.19	0.30	0.33	0.27	0.42	0.54
Leucine	1.54	0.44	0.23	0.32	0.38	0.42	0.41	0.49
Isoleucine	2.93	0.58	0.33	0.41	0.45	0.43	0.58	0.83
Threonine	3.25	1.25	0.66	0.72	0.70	0.59	0.90	1.20
Phenylalanine	2.91	0.21	0.14	0.16	0.18	0.19	0.57	1.22
Asparagine	3.63	1.02	0.51	0.48	0.86	0.93	1.37	1.38
Tyrosine	1.35	0.38	0.18	0.19	0.20	0.17	0.32	0.45
Histidine	6.29	2.83	2.77	2.75	2.79	2.30	3.06	3.24
Lysine	0.57	0	0	0	0	0	0.26	0.23
Total	45.20	19.60	12.97	15.42	18.28	17.68	19.55	21.09

increase in GDH activity. This was attributed (18) to ammonium induction of GDH as reported by Barash *et al.* (1), however according to our model the continued increase in GDH activity occurs in response to carbon limitation.

Evidence that GDH has a catabolic function during senescence is widespread (2, 4, 7), and Kar and Feierabend (7) suggest that GDH represents the major path for the liberation of ammonium from amino acids in senescing wheat leaves since no L-amino oxidase activity was detected. Cammaerts and Jacobs (4) noted a pronounced activation of NAD-GDH during senescence in *Arabidopsis thaliana* leaves and suggested that the oxidation of glutamate could provide the cells with reduced nucleotides and carbon for carbohydrate metabolism in circumstances where the chloroplasts are inactivated. Berger *et al.* (2) showed that the GS activity was sufficient to account for reassimilation of all photorespiratory ammonium produced during senescence, and demonstrated that when ^{15}N-ammonium was fed to senescing wheat leaves it was incorporated first into the amide of glutamine. In the same study, the senescing wheat leaves were also incubated with ^{15}N-glutamate. This produced a slight enrichment of the ammonium pool, suggesting that mitochondrial deamination of ^{15}N-glutamate via GDH had occurred (2).

In senescing tissues, GDH activity enables a mobilization of carbon and nitrogen from protein into forms that can be utilized or transported from the leaf. A role in glutamate deamination implies that the high levels of ammonium accumulated in the senescing leaf are a product of GDH activity, rather than that the enzyme is being induced by the elevated ammonium levels. The fate of the products of glutamate oxidation depend on the status of GS in these tissues; if GS is active, then ammonium is reassimilated into glutamine and transported from the leaf. However, if GS is inactive, the ammonium released from glutamate accumulates in the leaf; this seems to occur both during the latter stages of senescence (18) and if senescing leaves are kept in the dark (7). The 2-oxoglutarate and reduced nucleotides would be utilized within the leaf for carbohydrate metabolism.

High GDH activity is also found in root tissue, especially the mature regions of the root where there are high levels of ammonium (10). Such observations have been interpreted as indicating that GDH was active in the assimilation of ammonium, however the addition of sucrose to these roots brought about a reduction in GDH activity (10), suggesting that the initial derepression of GDH was caused by carbon limitation. It seems possible that these results could equally be interpreted by the catabolic model for GDH activity.

Response to Sucrose Concentration

The involvement of carbohydrate supply in modulating GDH levels was inferred from the coincident timing of sucrose depletion and the onset of changes in GDH activity and nitrogenous metabolites. Such correlative evidence does not exclude the possibility that other factors associated with nutrient depletion or metabolite accumulation in late-phase cultures are involved in triggering changes in enzyme activity. To test these possibilities, cells at the early growth phase were exposed to different sucrose regimes (details in Fig. 2).

In carbon-replete cultures, GDH specific activity was initially low, and in cells maintained on sucrose, GDH activity remained low until d 6 (Fig. 2A) and then increased sharply on entry into stationary phase. Medium ammonium concentration declined linearly until d 6, and then increased concomitant with the increase in GDH activity (Fig. 2B). In contrast, when cells were transferred to sucrose-free medium, GDH activity increased after a lag of only 1 d (Fig. 2A) and continued to increase until d 6. The subsequent decline in activity in these sucrose-starved cells was associated with browning and senescence, and cells were dead by d 9. Medium ammonium concentration remained high throughout in cells deprived of sucrose, and even increased above the initial value as terminal senescence approached (Fig. 2B).

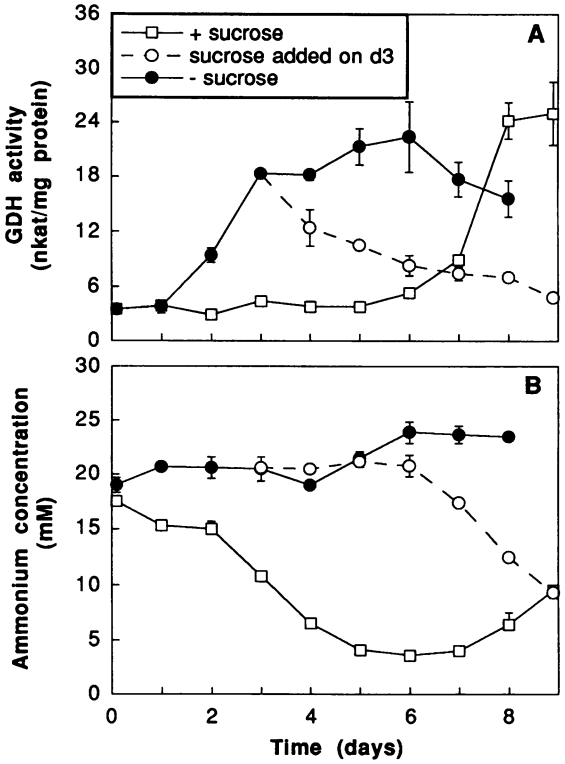

Figure 2. Changes in GDH specific activity (A) and medium ammonium concentration (B) in medium without sucrose (−sucrose) or with 2% sucrose (+sucrose). Cells grown for 4 d in standard medium were aseptically harvested, washed with sucrose-free medium, and resuspended at twice the original density in fresh standard medium with or without sucrose. After 3.5 d −sucrose cultures were injected with either sterile sucrose solution to give 2% final concentration or with the same volume of sterile distilled water. Solid lines, −sucrose; broken lines, +sucrose. All determinations in triplicate ± SEM.

However, the addition of sucrose between the 3rd and 4th d to sucrose-free cultures led to a rapid decrease in GDH activity, which continued to fall until the end of the experiment (Fig. 2A). Ammonium uptake from the medium was apparent after 2 d and continued at a rate comparable with that of sucrose-replete cultures (Fig. 2B).

These results show that GDH activity is down-regulated specifically by the presence of sucrose, independent of other medium or metabolite factors associated with aging cultures.

Table II. *Changes in GDH Specific Activity, Medium Ammonium, and Cellular Glutamate Concentrations after a 24-h Incubation in Media Supplemented with Various Nitrogen Sources*

Cells grown for 4 d in standard medium were harvested, washed, and resuspended in the same volume of treatment medium. Initial concentration of nitrogen was 10 mM in all cases. oxo = 10 mM 2-oxoglutarate.

Treatment Medium	GDH Activity[a]	Medium Ammonia Increase	Glutamate[b]
	% increase	*mM*	*μmol/g fresh wt*
−Sucrose			
NH_4Cl	+53	1.3[c]	2.79
KNO_3	+47	0.96[d]	2.04
Gln	+55	0.40[d]	4.02
Glu	+100	0.85[d]	3.22
Gln + oxo	+111	0.70[d]	4.29
NH_4Cl + oxo	+113	0.71[c]	1.98
+Sucrose			
Glu	−10	0[d]	1.29
KNO_3	+4	0[d]	0.92
Gln	−9	0[d]	0.97

[a] Initial activity = 4.51 nkat/mg protein. [b] Initial concentration = 1.73 μmol/g fresh weight. [c] Initial concentration = 10.9 mM. [d] Initial concentration = 0 mM.

When cultures were grown in the presence of 1% sucrose, the results (data not shown) were virtually identical with those for 2%.

Cellular concentrations of 2-oxoglutarate were determined in sucrose-starved and sucrose-replete cultures. The increase in GDH activity in sucrose-starved cells (*cf.* Fig. 2A) was not accompanied by an increase in free 2-oxoglutarate concentrations, which varied between 0 and 0.4 μmol/g fresh weight, as compared with 1.2 to 2.1 μmol/g fresh weight in sucrose-replete cultures. Similar levels were also found in stationary phase as compared with logarithmic phase cultures during standard growth passages. Such low levels of 2-oxoglutarate under carbon-limited conditions suggest that the demand for carbon skeletons in starved cultures is too great to permit its accumulation.

Glutamate Levels and GDH Induction

A priori considerations suggest that the increase in GDH activity following carbon depletion may be mediated either by accumulation of the substrate (glutamate) or by depletion of the product (oxoglutarate) of the glutamate oxidation reaction. To test this proposal, the effects of sucrose depletion in media containing various nitogen sources in the presence and absence of 2-oxoglutarate were determined.

Table II shows the changes in GDH specific activity, medium ammonium concentration, and cell glutamate concentration after a 24-h treatment. In sucrose-depleted cultures, GDH activity increased independently of the nitrogen source, and ammonia was excreted into the culture medium in all cases. The simultaneous presence of 2-oxoglutarate did not suppress the increase in GDH activity; in fact, the highest specific activities were observed in those treatments. In the presence of sucrose, increased GDH activities and ammonia production were not observed, irrespective of variation in nitrogen source (Table II). No data are presented for cultures supplied with ammonium chloride in the presence of sucrose since cell death occurred within 24 h accompanied by a drop in medium pH to 3.6. In all other treatments, cells remained healthy for at least 3 d, approximately linear increases in GDH activity and medium ammonia concentration were maintained over this period in sucrose-depleted cultures, and sustained fresh weight increases occurred in sucrose-supplemented cultures.

Concentrations of cellular glutamate were up to fourfold higher in sucrose-depleted as compared with sucrose-supplemented cultures (Table II). When GDH activities are plotted against the corresponding glutamate concentration for each of the nine treatments, a positive relationship is evident (Fig. 3) with a correlation coefficient of $r = 0.73$. These observations show that the increased GDH activity resulting from carbon starvation is independent of the nitrogen source and is not reversed by exogenously supplied 2-oxoglutarate. The very low concentration of 2-oxoglutarate in carbon-depleted cells indicates that this organic acid undergoes rapid metabolism and does not accumulate in cells exogenously supplied with this compound. The correlation between cellular glutamate levels and GDH activity suggests that the enzyme is regulated through an interaction of carbon supply and glutamate concentrations.

In summary, the present results indicate that GDH activity in carrot cell suspension cultures is altered in response to shifts in carbon rather than nitrogen metabolism. It is possible to reinterpret much of the published literature on the regulation of this enzyme in higher plants as being consistent with the hypothesis that the primary function of the enzyme is the oxidative deamination of glutamate to produce 2-oxoglutarate. Several reports support this interpretation; in maize and pea roots, GDH activity was found to increase under carbon-depleted conditions, and this effect was reversed by addition of various sugars (10, 13). GDH activity increased up to eightfold in pea shoots floated on tap water, and while this

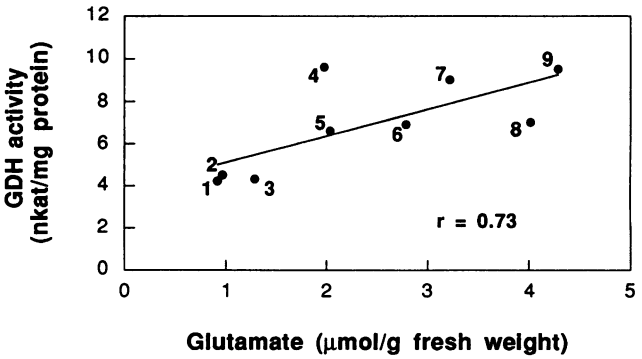

Figure 3. Correlative plot of GDH specific activities against cellular glutamate concentrations after incubation for 24 h in nine individual treatment media (see Table II). +Sucrose: 1, +KNO_3; 2, +glutamine; 3, +glutamate. −sucrose: 4, +NH_4Cl + 2-oxoglutarate; 5, +KNO_3; 6, +NH_4Cl; 7, +glutamate; 8, +glutamine; 9, +glutamine + 2-oxoglutarate.

increase was suppressed by the addition of sugars, ammonia and other metabolites had no effect (9, 17). In senescing leaf tissue, increased GDH activity is linked to carbohydrate limitation and protein catabolism (18). There are several reports (*e.g.* 1, 6, 14) showing that the addition of high concentrations of ammonium induce increases in GDH activity; our interpretation of these results is that these tissues are depleted in carbon skeletons and that it is carbon limitation rather than the presence of high cellular ammonium concentrations that promotes increased GDH activity. Our present results provide supportive evidence for the hypothesis advanced in an earlier paper (12) that the function of GDH is the catabolism of glutamate. This is of course a role that has been well established for GDH in animal cells (5).

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