Plant Physiol. (1985) 77, 374–378
0032-0889/85/77/0374/05/$01.00/0

Glucose and Glycine Metabolism in Regenerating Tobacco Protoplasts

FOLLOWED NONDESTRUCTIVELY BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Received for publication July 10, 1984

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ABSTRACT

The metabolic states and the uptake and metabolism of [1-^{13}C]glucose, [2-^{13}C]glycine, and [^{15}N]glycine in intact *Nicotiana tabacum* L. (cv Xanthi) mesophyll protoplasts were measured by ^{13}C and ^{15}N nuclear magnetic resonance spectroscopy. Changes in the concentration of metabolites during the first two days of culture in darkness were followed. Protoplasts isolated in 0.55 molar mannitol medium showed a drop in the concentration of all the intracellular metabolites during the first 28 hours of culture. Uptake of glucose and synthesis of glucose-derived metabolites were observed, indicating activity of glycolysis and the tricarboxylic acid cycle. Addition of glycine caused the accumulation of serine in dark cultured protoplasts, via the photorespiratory pathway. Glutamate dehydrogenase and glutamine synthetase activities in photorespiratory NH_4^+ assimilation were observed. Glucose uptake and metabolism and cell division were inhibited by 3 millimolar glycine, suggesting that the accumulating serine or the release of ammonia during serine synthesis had toxic effects in this system.

Plant protoplasts can be isolated and cultured in a well defined nutrient medium on a large scale (16). These advantages enhanced their use in studies of plant physiology and biochemistry (4). It is, however, important to note that the protoplasts are not wall-free replicates of the original tissue cells. This is due to different processes that occur after the isolation, such as the degeneration of chloroplasts, causing the loss of photosynthetic activity, and the initiation of cell division in leaf mesophyll protoplasts (11).

Several studies focused on the regeneration of the cell wall which is essential for cell division (18). For example, the regeneration of cell walls in *Vinca rosea* protoplasts was followed by Takeuchi and Komamine (17). The regenerated wall consisted of β 1→>4 glucan and β 1→>3 glucan. The time course of cell wall development was found to vary with the conditions of protoplast isolation. In tobacco protoplasts, fibers were observed by scanning electron microscope after 10 h of culture (3).

The transport of sugars and amino acids across the plasmalemma was studied in protoplasts by Guy *et al.* (6). Selective transport was observed using optical isomers and amino acid analogs. Changes in the rate of metabolite uptake during protoplast culture were observed by Robinson and Mayo (14). In general, an increase in uptake was observed during the first 8 h of culture followed by a decrease in uptake after 12 h.

CO_2 fixation was followed in freshly isolated spinach protoplasts (13) and enhanced photorespiration was followed in tobacco *su* mutant protoplasts (15). The measurement of both CO_2 fixation and photosynthesis in leaf protoplasts, from various species, is now a routine procedure in several laboratories.

We present here ^{13}C and ^{15}N NMR studies of intact tobacco protoplasts during the first 48 h of culture. The uptake and metabolism of ^{13}C-labeled glucose and ^{13}C- and ^{15}N-labeled glycine were monitored and the main pathways were characterized.

MATERIALS AND METHODS

Plant Material. *Nicotiana tabacum* L. (cv Xanthi) plants were grown at 22 to 27°C and 65 to 75% humidity in a greenhouse.

Media. The following media were used in our studies: N&M medium (12), CPW (16), and W5 (10) which was modified by deleting the glucose (W5/no glucose). MIH is a modification of CPW obtained by the addition of 0.55 mM meso-inositol, 29 μM thiamine HCl, 53 μM glycine, 16 μM naphthaleneacetic acid, and 4.4 μM benzylaminopurine.

Labeled Compounds. [1-^{13}C]Glucose (90%), 90% [2-^{13}C]glycine, and 99% [^{15}N]glycine were purchased from Merck. All other chemicals used were of analytical grade.

Protoplast Preparation. Protoplasts were isolated by the method of Zelcer and Galun (19). Full-size young leaves of 50- to 60-d-old plants were washed with detergent and sterilized for 10 s in 70% ethanol and for 10 min in a dilute hypochlorite solution followed by rinsing several times in sterile distilled H_2O. The sterile leaves were then cut into 1-mm strips and placed overnight (approximately 16 h) in 9-cm Petri dishes with a 10-ml solution of 0.25% Cellulase, 0.125% Driselase, 0.05% Macerozyme, in CPW. The protoplasts were then collected by centrifugation (40*g* 5 min), and broken cells were separated by suspension in a high sucrose medium (N&M) followed by centrifugation (80*g* 5 min). The cleaned, floating protoplasts were washed from the sucrose in MIH by centrifugation (40*g* 5 min) and approximately 1×10^6 cells in 10 ml medium (usually MIH), were plated in each 9-cm diameter disposable tissue culture plate. Cells were grown at 25°C in the dark for the indicated time until harvested for measurement. For measurements at $t = 0$ the protoplasts were harvested immediately after their separation in N&M. After 48 h of culture in MIH medium, about 80% of the protoplasts undergo the first cell division. To obtain further cell divisions, the protoplasts should then be transferred to a more nourishing medium.

Preparation of NMR Sample. Five to ten $\times 10^6$ cells were collected by centrifugation (40*g* 5 min). The cells were washed three times in W5-no glucose (60 ml) and suspended in 1.5 ml W5-no glucose (30% ^2H_2O for field lock in the NMR spectrometer) in 10-mm NMR tubes. The final sample volume was 2.5

ml. The protoplasts were kept at 4°C during the NMR measurement to slow down metabolic processes. Under these conditions, 90% of the protoplasts remained intact for 2 h and about 50% remained intact for 7 h.

The number of cells was determined by counting. The error in this measurement is about 20%, but the variation in cell number between different samples of the same experiment was less than 5%.

Soluble extracts were prepared in the cold from cells broken by freezing and thawing, and centrifuged for 5 min in an Eppendorf microfuge. In several experiments the extracts were dried by lyophilization, and resuspended in the desired water volume. The area of all the ^{13}C NMR signals of the soluble metabolites was the same in the intact protoplasts and in their soluble extract.

NMR Spectroscopy. NMR measurements were performed with Bruker WH-270 and Bruker CXP-300 pulse-FT spectrometers.

^{13}C spectra were obtained using 1-w broadband proton decoupling, 30° pulses, a total delay of 1 between pulses, 16,000 data points, and a spectral width of 17,000 Hz. Spectra were recorded using a line broadening of 5 Hz. An external reference containing 50 μl of a 5-M methanol + 40 mM $MnCl_2$ solution was used for calibration of chemical shift and concentration (δ = 49.90 ppm). $MnCl_2$ was added for relaxation enhancement and cancellation of the nuclear Overhauser effect enhancement.

The concentrations of metabolites were calculated from the area of each metabolite signal and of the methanol reference signal in the ^{13}C spectra by using the computer integration mode. The signal area was corrected for the ^{13}C enrichment in ^{13}C-labeling experiments, and for overlapping signals from identical nuclei in the same molecule. The signal of the reference corresponds to 1.2×10^{-4} mol. Correction factors for the nuclear Overhauser effect were obtained by recording spectra of the same sample using both the normal acquisition parameters, and the gated decoupling sequence in which the broadband proton decoupler was turned on only during data acquisition and off during a 30-s delay between 90° pulses.

Tentative identification of the ^{13}C signals was reached by comparison with published data (2) and confirmed by comparison with spectra of the pure metabolite dissolved in W5-no glucose (30% ^2H_2O) medium, and by analyzing proton coupled and off resonance decoupled ^{13}C spectra of extracts.

Due to the low sensitivity in detection of ^{13}C at natural abundance, a very long measurement time is needed (around 7 h). For such experiments, extracts were prepared from parallel samples and their spectra were compared to spectra of whole cells, to make sure that the samples did not change during the acquisition.

^{15}N spectra were obtained using broadband proton decoupling of 1 w, a tip angle of 30°, 2 s delay between pulses, 16,000 data points, and a spectral width of 6,000 Hz. Spectra were recorded using a line broadening of 5 Hz. Chemical shift was measured using an external reference containing 50 μl of 5.0 M CH_3NH_3Cl (95% ^{15}N) + 1.0 M HCl (δ = 24.5 ppm downfield from NH_3[liquid] at 30°C).

^{15}N resonances were identified by comparison with published chemical shift data (9).

RESULTS

Natural Abundance ^{13}C Studies. Changes in the pattern of free metabolite pools in regenerating protoplasts were detected by natural abundance ^{13}C measurements (Fig. 1). The various metabolites with a concentration above 1 pmol per cell could be observed. NMR signals of intracellular mannitol can be observed in all the spectra. It appears that mannitol was taken up during the protoplast isolation period, and remained inside the cells during further culture. The concentration of nearly all the metabolites (including glucose and glycine) decreased during 27 h of culture in MIH medium that has no available carbon source. To follow specifically the fate of glucose and glycine, we added ^{13}C- and ^{15}N-labeled metabolites to the growth medium.

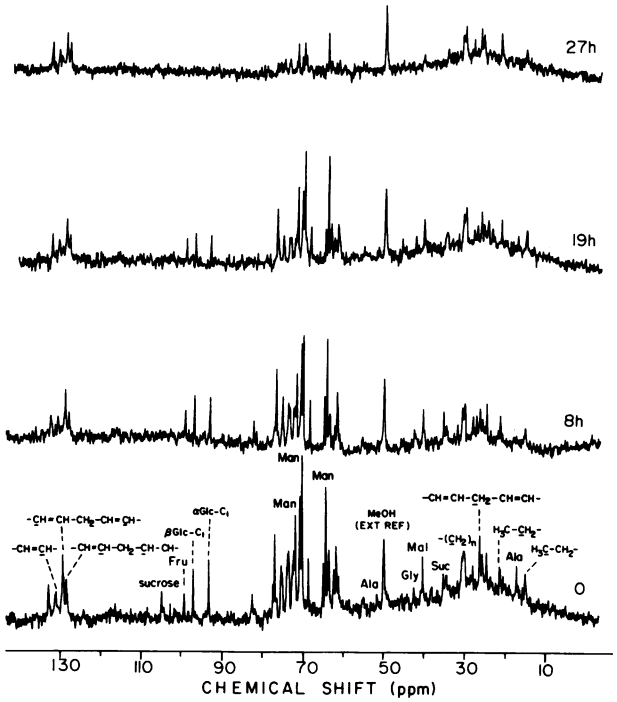

FIG. 1. The drop in intracellular metabolite pools, during culture of protoplasts in MIH medium, observed by natural abundance ^{13}C NMR. 67.89 MHz $^{13}C\{^1H\}$ NMR spectra of protoplasts isolated in CPW medium and cultured in MIH medium for the indicated periods; 5×10^6 protoplasts were harvested for each measurement. About 25,000 scans were accumulated. Abbreviations: EXT REF, external reference; MAN, mannitol; MAL, malate; SUC, succinate; CIT, citrate; αKG, alpha ketoglutarate.

Glucose Metabolism. Glucose uptake and metabolism were followed by adding 10 mM 90% [1-^{13}C]glucose to the MIH medium immediately after protoplast preparation (t = 0). A rise in the signal intensity of the different glucose-derived metabolites was observed during the first 45 h of culture (Fig. 2).

The labeling of citric acid was most intense with little labeling of amino acids. The large signals at 64.8 and 62.2 ppm are probably due to phosphate esters of glucose and fructose. The ratio of α:β glucose is about 2:1 in all the spectra, and not 2:3 as expected at chemical equilibrium. Since mutarotation rate is much slower at 4°C than the measurement time, we concluded that this ratio is the α:β ratio in the cultured cells.

Cellulose synthesized from the labeled glucose should give a broad resonance, around 103 ppm (5). Such a line appears in the spectra and its intensity increased 8-fold during the first 45 h of culture.

Glycine Metabolism. The carbon and nitrogen glycine metabolism were followed in protoplasts. ^{13}C and ^{15}N spectra of protoplasts cultured in MIH media containing 3 mM glycine enriched with either ^{13}C or ^{15}N are shown in Figures 3 to 5. During the first 10 h of culture, only ^{13}C-labeled serine was synthesized from [2-^{13}C]glycine (Fig. 3). The intracellular concentration of glycine remained constant, while the concentration of serine rose with time.

The NMR signals of intracellular metabolites are broadened by the restricted mobility of the molecules and the inhomogeneity of the sample. The more resolved ^{13}C spectrum of the soluble extract prepared from protoplasts cultured for 27 h in

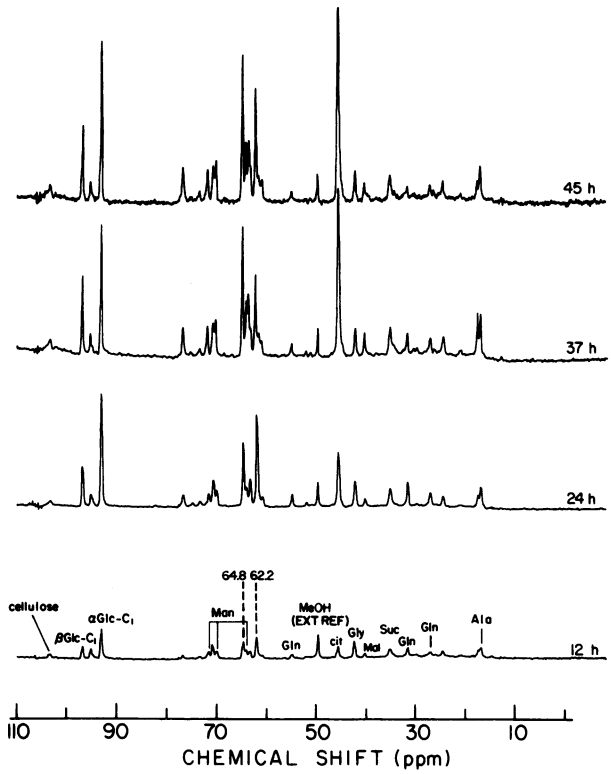

FIG. 2. Uptake and metabolism of [^{13}C]glucose; 75.47 MHz ^{13}C{^{1}H} spectra of protoplasts isolated in CPW and cultured for the indicated periods in MIH + 10 mM 90% [1-^{13}C]glucose; 6 × 10^{6} cells were harvested for each measurement. About 1000 scans were accumulated (approximately 15 min of acquisition). Note the high α:β glucose ratio, and the labeling of citrate, glycine, and cellulose. Abbreviations: see legend to Figure 1.

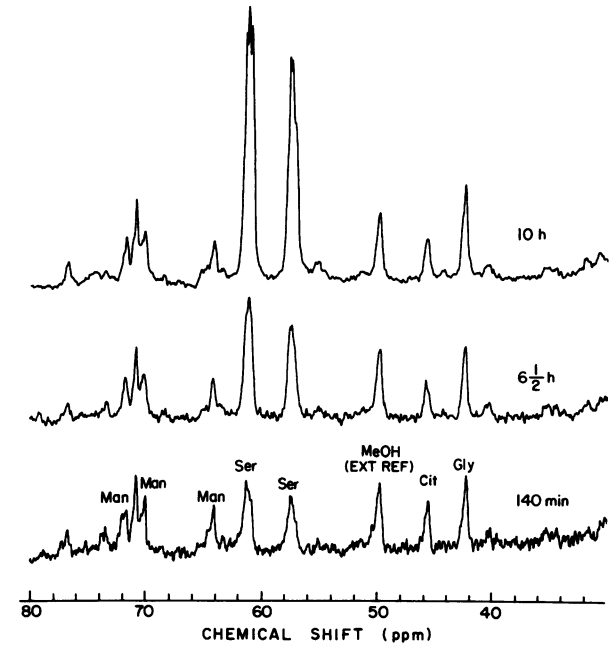

FIG. 3. Biosynthesis of [2,3-^{13}C]serine from [2-^{13}C]glycine. 75.47 MHz ^{13}C{^{1}H} spectra of protoplasts isolated in CPW and cultured for the indicated periods in MIH + 3 mM 90% [2-^{13}C]glycine; 15 × 10^{6} cells were harvested for each measurement, and 5000 scans were accumulated. Abbreviations: see legend to Figure 1.

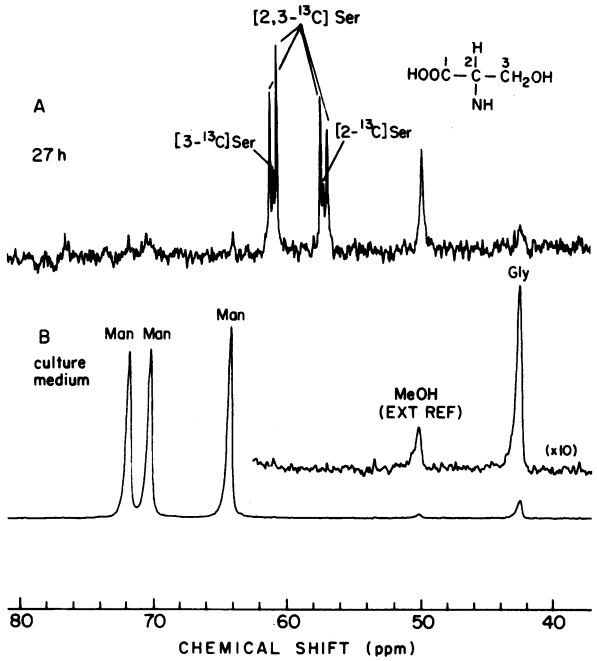

FIG. 4. 75.47 MHz ^{13}C{^{1}H} spectra of: (A) the soluble extract of 5 × 10^{6} protoplass isolated in CPW and cultured for 27 h in MIH + 3 mM 90% [2-^{13}C]glycine showing the AB pattern of the freshly synthesized double-labeled [2,3-^{13}C]serine. (B) The filtered culture medium after the protoplasts were centrifuged down showing that intracellular serine did not leak out to the medium. About 2000 scans were accumulated for each spectrum. Abbreviations: see legend to Figure 1.

Table I. *Serine Concentration and ^{13}C Enrichment in Protoplasts Cultured in MIH + 3 mM [2-^{13}C]Gly*

P_2 and P_3 are the atomic fraction of ^{13}C in carbons 2 and 3 of serine, respectively.

Time	P_2	P_3	
			pmol serine/ cell ± 0.01
140 min	0.76	0.72	0.08
6.5 h	0.76	0.71	0.15
10 h	0.84	0.84	0.44
27 h	0.87	0.85	0.52

MIH + 3 mM [2-^{13}C]glycine (Fig. 4) revealed that most of the freshly synthesized serine is double-labeled [2,3-^{13}C]serine giving an AB spectrum ($J_{C_2C_3}$ = 35.5 Hz). The peaks at the center of each doublet arise from serine molecules labeled in a single carbon. The serine was synthesized in the cells and accumulated there, as can be seen from the absence of a serine signal in the culture medium (Fig. 4B).

The ^{13}C enrichment of each carbon in serine (P_2, P_3) was calculated from the ratio of doublet to singlet in the well resolved spectra of extracts. The ^{13}C enrichment and the concentration of serine are summarized in Table I. A very large ^{13}C enrichment of the serine was observed after 140 min of culture, indicating that nearly all the newly synthesized serine is produced from the external labeled glycine, and does not involve the intracellular pool of unlabeled glycine.

The synthesis of serine from two glycine molecules involves the release of one amino group. The ^{15}N spectrum (Fig. 5) indicated a transfer of the amino group from [^{15}N]glycine to serine, glutamine, asparagine, and perhaps also alanine. Both the amide group of glutamine (114.6 ppm), and the α-amine of either glutamate or glutamine (42.7 ppm), were highly labeled.

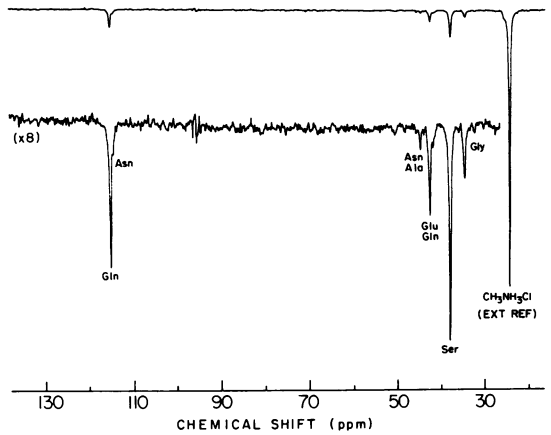

FIG. 5. The transfer of ^{15}N-labeled amine from [^{15}N]glycine to serine, amide of glutamine, and the α amino group of glutamate and/or glutamine; 30.415 MHz $^{15}N\{^1H\}$ NMR spectrum of 20×10^6 protoplasts isolated in CPW and cultured for 14 h in MIH + 3 mM 99% [^{15}N]glycine. 10,000 scans were accumulated. Abbreviations: see legend to Figure 1.

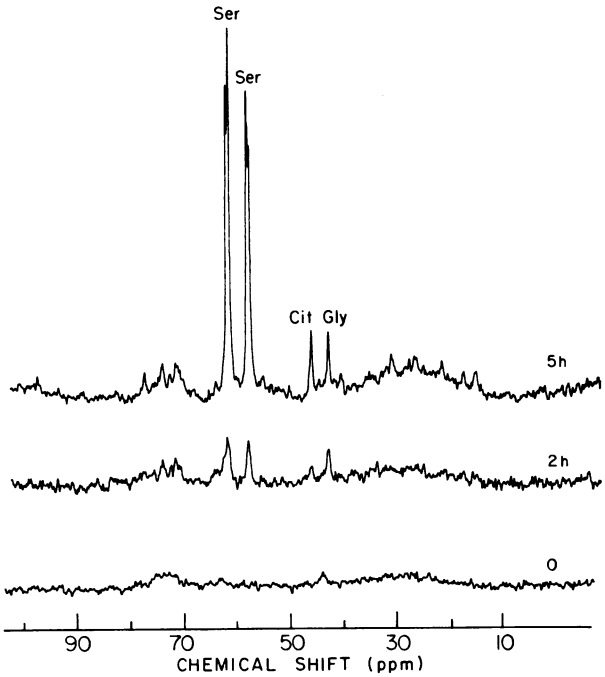

FIG. 6. The accumulation of serine synthesized from [^{13}C]glycine in leaf disks; 75.47 MHz $^{13}C\{^1H\}$ spectra of leaf disks immersed in a 3-mM 90% [2-^{13}C]glycine water solution for the indicated periods. About 5000 scans were accumulated. Abbreviations: see legend to figure 1.

The α-amine of aspartate could also contribute to the signal at 42.7 ppm. No attempt was made to better resolve this signal.

To see if the accumulation of serine in the protoplasts was caused by the high osmotic pressure of the medium, leaf disks (9 cm in diameter) were cut into 1-mm strips and floated either in a 3 mM water solution of [^{13}C]glycine (Fig. 6), or in a medium (MIH) containing 3 mM [^{13}C]glycine (data not shown). ^{13}C spectra of both leaf preparations were very similar and indicated that under both conditions the leaves accumulated serine. The concentration of citrate also increased slightly with time.

The Effect of Glycine on Glucose Metabolism and Cell Division. The accumulation of serine which was observed only when glycine was added to the growth medium indicated serine degradation was blocked by the externally applied glycine. We checked whether other processes, namely glucose metabolism and cell division were also inhibited. Parallel protoplast samples were cultured in a medium that contained 10 mM 90% [2-^{13}C] glucose with and without 3 mM 90% [2-^{13}C]glycine. In the presence of glycine, almost all the glucose uptake and metabolism were inhibited, and only the synthesis of doubly labeled serine could be observed (data not shown). The effects of glycine and glycine + glucose on the first cell division, after 3 d of culture, were followed with a light microscope. Protoplasts that were cultured in MIH, with and without 10 mM glucose, showed a high degree of cell division. When glycine was added at concentrations of 1 to 10 mM, the protoplasts did not divide.

DISCUSSION

During the protoplast isolation period, mannitol accumulated in the cells and remained there during further culture. The drop in the intracellular concentration of all the soluble metabolites during culture could be due either to their exhaustion in respiration or to their incorporation into macromolecules which, due to immobilization, exhibit very broad and hard to detect NMR signals (Fig. 1). Indeed, it is known that synthesis of proteins, DNA and cell wall occur during this period (4), and could therefore contribute to the use of the soluble metabolite pools. We assume that the cell membranes were intact and metabolites did not leak out. This assumption is verified by the fact that intracellular ^{13}C-labeled serine does not leak out to the growth medium (Fig. 4B).

We could observe cellulose synthesis in the protoplasts cultured with ^{13}C-labeled glucose (Fig. 2), probably because loose ends of the freshly synthesized polymer are more mobile and their NMR signals are narrow. Thus, the levels we measured are nonindicative of the total cellulose levels.

Glucose enters the cells via a specific α glucose pump (6). The metabolic pathways involving glucose utilize the α isomer. The high α:β glucose ratio in the cells implies, therefore, that the uptake of glucose exceeds the sum of intracellular glucose metabolism and the mutarotation rate (at 25°C).

Respiration (including glycolysis and the tricarboxylic acid cycle) emerged as the most active pathway involved in glucose metabolism, and caused the transfer of label from glucose to citrate, malate, and succinate during the first 45 h of protoplast culture (Fig. 2). The labeling of amino acids such as glutamine, glycine, and alanine was probably by synthesis through intermediates of the tricarboxylic acid cycle. The large number of changes in the cells, including massive biosynthesis of new enzymes and structural macromolecules, cell wall synthesis, and cell division, require a considerable expenditure of energy. As photosynthesis was not active the cells had to switch over to respiration.

Tobacco leaves and protoplasts cultured without the addition of glycine and in leaves do not accumulate serine, as observed in the natural abundance ^{13}C spectra (Fig. 1 and Fig. 6, lower trace, respectively). However, when 3 mM [^{13}C]glycine was added to the culture medium, very intense synthesis and accumulation of doubly labeled serine occurred (Fig. 3). The double labeling is consistent with the mitochondrial part of the photorespiratory pathway as suggested by Keys (7). Two glycine molecules condense to form one serine molecule, in which C_2 is derived from C_2 of one glycine and C_3 is derived from C_2 of the second glycine molecule. This pathway has not been shown *in situ*, only in submitochondrial particles after the addition of NAD and Pi (1). The reaction is light independent in our case because of the externally applied glycine.

The accumulation of serine can be explained either by complete inhibition of the serine degradation pathway, specifically of the peroxisomal enzyme 'serine dehydrase' which degrades serine in the photorespiration cycle, or by inhibition of the transport of serine from the mitochondria to the peroxisome.

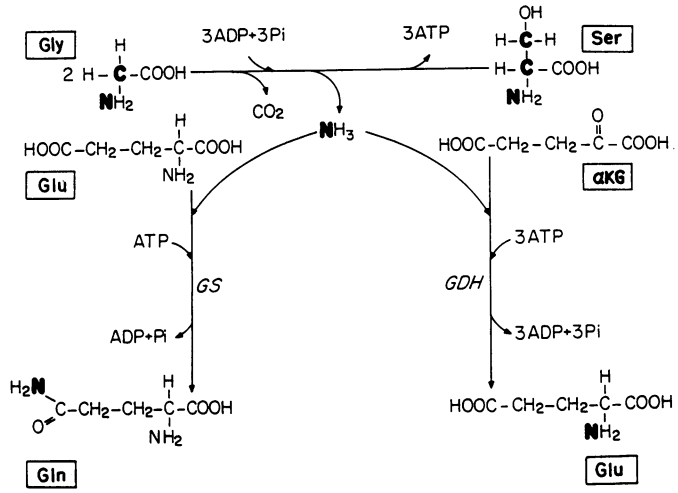

FIG. 7. Glycine metabolism in protoplasts as observed by ^{13}C and ^{15}N NMR. Boldface letters denote the transfer of label from glycine. Abbreviations: see legend to Figure 1.

Serine accumulation can also be observed in leaf disks, both under high osmotic pressure and in a water solution of glycine (Fig. 6). Thus, serine accumulation is not caused by the high osmolarity of the medium. The inhibition of serine degradation could be caused by the high intracellular glycine concentration, which can prevent the continuation of the cycle through 2-phosphoglycerate and 2-glycolate back to glycine, although preliminary results indicate that the accumulation of serine is irreversible, and serine is not degraded when the cells are transferred back to a normal growth medium. The labeling of citrate, which can be seen when leaf disks are incubated in 3 mM glycine (Fig. 6), could arise through oxidation of serine, but it is not efficient enough to prevent the accumulation of serine.

In plants, the CO_2 released from glycine in the first step of mitochondrial serine synthesis, can account for all the CO_2 released in photorespiration; however, the fate of the amino group released in the cells is not clear (8). NH_4^+ is toxic and is probably reassimilated by cytoplasmic GS[1] and/or by GDH. A third pathway catalyzed by chloroplastic 'glutamate synthase' requires photoreduced ferredoxin and would be inactive in dark grown protoplasts.

The mechanism of NH_4^+ assimilation associated with the conversion of glycine to serine was determined from the ^{15}N spectrum of protoplasts cultured in the presence of 3 mM ^{15}N-labeled glycine (Fig. 5). Activity of GDH caused the labeling of the α-amino group of glutamate while activity of GS resulted in the labeling of the amide group of glutamine. Figure 7 summarizes the pathway for the conversion of glycine to serine and the accompanying NH_3 assimilation. Boldface letters denote the transfer of ^{13}C and ^{15}N label from glycine to the other metabolites.

The transfer of ^{15}N label from glycine to glutamate can also be explained by transamination of glycine (via glutamic acid-glyoxylate aminotransferase) and the conversion of glycine to glyoxylate, but this possibility is ruled out by the result of our ^{13}C experiment, namely that glycine is converted solely to serine.

The inhibition of glucose uptake and metabolism, and the inhibition of cell division, could be due to some secondary (toxic) effect of either glycine or serine or both.

The results demonstrate that ^{13}C and ^{15}N NMR can be extremely useful in metabolic studies of plant cells and tissues. At the present, the low sensitivity limits the use of the method to processes involving metabolites present in the millimolar range.

[1] Abbreviations: GS, glutamine synthetase; GDH, glutamate dehydrogenase.

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