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Metabolic Changes Associated with Adaptation of Plant Cells to Water Stress[1]

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DAVID RHODES*, SANGITA HANDA, AND RAY A. BRESSAN
Department of Horticulture, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

Suspension cultured cells of tomato (*Lycopersicon esculentum* Mill. cv VFNT Cherry) adapted to water stress induced with polyethylene glycol 6000 (PEG), exhibit marked alterations in free amino acid pools (Handa *et al.* 1983 Plant Physiol 73: 834–843). Using computer simulation models the *in vivo* rates of synthesis and utilization and compartmentation of free amino acid pools were determined from ^{15}N labeling kinetics after substituting [^{15}N]ammonium and [^{15}N]nitrate for the ^{14}N salts in the culture medium of cell lines adapted to 0% and 25% PEG. The 300-fold elevated proline pool in 25% PEG adapted cells is primarily the consequence of a 10-fold elevated rate of proline synthesis via the glutamate pathway. Ornithine was insufficiently labeled to serve as a major precursor for proline. Our calculations suggest that the rate of proline synthesis only slightly exceeds the rate required to sustain both protein synthesis and proline pool maintenance with growth. Mechanisms must operate to restrict proline oxidation in adapted cells. The kinetics of labeling of proline in 25% PEG adapted cells are consistent with a single, greatly enlarged metabolic pool of proline. The depletion of glutamine in adapted cells appears to be a consequence of a selective depletion of a large, metabolically inactive storage pool present in unadapted cultures. The labeling kinetics of the amino nitrogen groups of glutamine and glutamate are consistent with the operation of the glutamine synthetase-glutamate synthase cycle in both cell lines. However, we could not conclusively discriminate between the exclusive operation of the glutamine synthetase-glutamate synthase cycle and a 10 to 20% contribution of the glutamate dehydrogenase pathway of ammonia assimilation. Adaptation to water stress leads to increased nitrogen flux from glutamate into alanine and γ-aminobutyrate, suggesting increased pyruvate availability and increased rates of glutamate decarboxylation. Both alanine and γ-aminobutyrate are synthesized at rates greatly in excess of those simply required to maintain the free pools with growth, indicating that these amino acids are rapidly turned over. Thus, both synthesis and utilization rates for alanine and γ-aminobutyrate are increased in adapted cells. Adaptation to stress leads to increased rates of synthesis of valine and leucine apparently at the expense of isoleucine. Remarkably low ^{15}N flux via the aspartate family amino acids was observed in these experiments. The rate of synthesis of threonine appeared too low to account for threonine utilization in protein synthesis, pool maintenance, and isoleucine biosynthesis. It is possible that isoleucine may be deriving carbon skeletons from sources other than threonine. Tentative models of the nitrogen flux of these two contrasting cell lines are discussed in relation to carbon metabolism, osmoregulation, and nitrogenous solute compartmentation.

Recent investigations of osmoregulation in tomato suspension cultures adapted to water stress induced with PEG[2], indicate that the accumulation of free amino acids may play a significant role in this process (10). The accumulation of the amino acid proline may be particularly important in osmotic adjustment, since proline represents over 50% of the total free amino acid pool of cells adapted to 30% PEG (10). Other amino acids such as alanine, GABA, and valine accumulate markedly in the water stress adapted cells (10) indicating dynamic adjustments in nitrogen metabolism. In many respects these amino acid accumulations resemble those widely reported to occur in response to water deficits at the whole plant level (12, 35). Although it is not possible to draw strict analogies between the water relations and nitrogen budgets of PEG adapted cells and whole plants experiencing field water deficits, water stress adapted cells in culture offer a convenient system for exploring metabolic fluxes in a much more controlled fashion than is possible at the whole plant level. In particular, experimentation on the steady state rates of synthesis and utilization of free amino acid pools becomes feasible using the approaches developed by Folkes and Sims to explore amino acid biosynthesis in the yeast *Candida utilis* (9, 30, 31). In principle, rates of synthesis and utilization of each free amino acid can be deduced from the observed ^{15}N labeling kinetics, total pool size, and known growth parameters using a series of kinetic equations describing the theoretical labeling kinetics of primary, secondary, and tertiary products of [^{15}N] ammonium assimilation (9, 31). These equations can be approximated by the use of iterative computer simulation models (27). The latter approach has advantages in considering some of the complexities of labeling kinetics introduced by compartmentation of pools (23, 27).

In this paper, we describe some initial data on the nitrogen budgets of control (unadapted) cell lines of tomato (*Lycopersicon esculentum* Mill. cv VFNT-Cherry) and water stress (25% PEG) adapted cultures of the same variety transferred to their respective, fresh media substituting [^{15}N]ammonium and [^{15}N]nitrate for the ^{14}N salts as sole nitrogen sources. The labeling kinetics of the major free amino acid pools were monitored using GC-MS (26) and labeling data analyzed by computer simulation. The models that we have developed to account for observed ^{15}N flux, although tentative, reveal alterations of several metabolic processes associated with the adaptation of cultured cells to growth on media of low water potential.

MATERIALS AND METHODS

Cultures and Growth Conditions. Cell cultures of tomato (*Lycopersicon esculentum* Mill. cv VFNT Cherry) were grown and maintained as stock cultures as described previously (10). Growth

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[2] Abbreviations: PEG, polyethylene glycol 6000; GABA, γ-aminobutyrate; N-HFBI, *N(O,S)*-heptafluorobutyryl isobutyl; 2-P5CA, 2-pyrrolidone-5-carboxylate; GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; gfwt, gram fresh weight; amu, atomic mass units.

of cells was determined by measuring dry weight and fresh weight gain (10). Cell lines tolerant to PEG induced water stress were obtained by transferring the cells to successively higher concentrations of PEG (10). In the present investigation cell lines adapted to control media (0% PEG) and 25% PEG were used. These cell lines were maintained for over 250 generations on their respective media before use. Details of the growth characteristics, water relations, and solute compositions of these cell lines have been reported previously (10).

Administration of ^{15}N-Labeled Precursors. Cell lines adapted to 0% PEG and 25% PEG were transferred at mid-exponential growth stages to their respective fresh media, substituting [^{15}N] $H_4[^{15}N]O_3$ (0.825 g L^{-1}) and $K[^{15}N]O_3$ (0.95 g L^{-1}) for the corresponding ^{14}N salts as nitrogen sources in the stock medium (10). [^{15}N]Ammonium, [^{15}N]nitrate (99%), and potassium [^{15}N] nitrate (99%) were obtained from MSD isotopes (St. Louis, MO). Transfer of cultures to the ^{15}N medium was performed by methods which minimized transfer shock and associated perturbations of amino acid pool sizes. Cultures were poured onto a sintered glass funnel and medium was allowed to drain slowly without applying suction. At the point of incipient dryness the unadapted cells were resuspended in 35 ml of ^{14}N medium (0% PEG) and diluted into 250 ml of ^{15}N medium; the water stress adapted cells were resuspended in 65 ml of ^{14}N medium and diluted into 500 ml of ^{15}N medium (25% PEG). This led to isotope dilution of the medium to 87% ^{15}N in the unadapted cultures and to 88% ^{15}N in the water stress adapted cultures. Zero time is defined as the time of dilution into the ^{15}N media. Inoculum densities were 85 gfwt L^{-1} for the unadapted cultures and 145 gfwt L^{-1} for the water stress adapted cultures.

Culture Harvest and Amino Acid Extraction. Aliquots of 25 ml of the suspension cultures were removed at various time intervals; cells were filtered on Whatman No. 4 filter paper in a Büchner funnel and the medium was collected in a glass tube. The cells were then washed three to four times with 2.5 ml of mannitol solutions of osmotic strength equivalent to that of the medium (30.3 g mannitol L^{-1} for unadapted cultures and 135.9 g mannitol L^{-1} for water stress adapted cultures). Cells were immediately weighed for fresh weight determination. One-half of the harvested cells was transferred and weighed into a scintillation vial and 10 ml methanol was immediately added. The remaining cells were weighed into a tared glass scintillation vial and dried for 2 d at 70°C for determination of dry weight/fresh weight ratios and microkjeldahl analysis (26). The methanol extracts were filtered through glass fiber filter paper and cell debris was washed with an additional 20 ml methanol. The 30 ml methanol extracts were stored at −20°C prior to amino acid analysis.

Aliquots of 2 ml of the methanol extracts were phase separated by addition of 1 ml chloroform and 1 ml H_2O. The upper aqueous layer was rotary evaporated to dryness and redissolved in 1 ml H_2O. The aqueous extracts were processed for amino acid analysis as described previously (26). Amino acids were derivatized as N-HFBI esters as described previously (26) and redissolved in 50 μl ethyl acetate:acetic anhydride (1:1 v/v) prior to amino acid determination by GLC and MS. No internal standard was used for the present investigations (pipecolic acid [26] is unsuitable as an internal standard for tomato cells because of the presence of pipecolic acid as a component of the free amino acid pool; see "Results and Discussion").

Gas Chromatography and Mass Spectrometry. Aliquots of 1 μl of the amino acid derivatives were subjected to GLC as described previously (26), using an external standard amino acid mixture (Sigma AA-S-18) to derive response factors required for quantitation of amino acid levels. Separate analyses were performed on mixtures of nonprotein amino acids such as GABA, β-alanine, pipecolic acid, tyramine, and ornithine to obtain response factors and retention times of these components of the free amino acid pool of tomato cells. Amino acid levels are expressed as μmol/gfwt. Each component of the free amino acid pool was identified by chemical ionization and electron impact MS (see below).

Aliquots of 1 μl of the amino acid derivatives were analyzed by in-beam chemical ionization MS using procedures described by Jamieson *et al.* (14). The aliquots of the amino acid derivatives were applied to a polyimide-coated fused silica probe (14) which is inserted into the mass spectrometer in precise alignment with the electron beam, with ammonia reagent gas flowing both through and around the probe. Bombardment of the sample at the probe tip with electrons at a source temperature of 250°C yields the $M + H^+$ and $M + NH_4^+$ ions of each amino acid derivative in the mixture with little fragmentation (where M = the molecular weight of the amino acid derivative). Multiple scans of each sample (over the mass range 300 amu to 650 amu) were acquired for 30 s for each sample. The fused silica probe (30 m × 0.22 mm) is threaded through a stainless steel housing (14). After each sample the terminal 1 cm of the probe was excised to minimize memory effects. The following is a list of the molecular ions of the amino acid derivatives identified in the tomato cell extracts: glycine ($M + NH_4^+$) = 345, alanine and β-alanine ($M + NH_4^+$) = 359, valine ($M + NH_4^+$) = 387, leucine and isoleucine ($M + NH_4^+$) = 401, GABA ($M + NH_4^+$) = 373, proline ($M + NH_4^+$) = 385, aspartate and asparagine-amino N ($M + NH_4^+$) = 459, glutamate and glutamine-amino N ($M + NH_4^+$) = 473, pipecolic acid and 2-P5CA (a deamidation/cylization product of glutamine arising during derivatization) ($M + NH_4^+$) = 399, serine ($M + NH_4^+$) = 571, threonine ($M + NH_4^+$) = 585, phenylalanine ($M + NH_4^+$) = 435, tyrosine ($M + NH_4^+$) = 647, tyramine ($M + NH_4^+$) = 547, lysine ($M + NH_4^+$) = 612, ornithine ($M + NH_4^+$) = 598. Ion ratios of $M + NH_4^+$: $M+1+NH_4^+$ were used to derive preliminary estimates of ^{15}N abundance. More precise isotope ratio measurements were obtained by electron impact GC-MS (see below).

Amino acid derivatives in ethyl acetate:acetic anhydride (1:1 v/v) were diluted 10-fold with ethyl acetate prior to analysis of 2 μl aliquots by electron impact GC-MS (26). The following conditions were adopted for the present investigations: column = 30 m × 0.22 mm fused silica capillary (SE 52) (J & W Scientific, Rancho Cordova, CA); carrier gas = H_2 at 2 ml/min; split ratio at injector port = 20:1; temperature program = 100 to 300°C at 5°C per min; source temperature = 200°C; interface temperature = 275°C; mass range = 200 to 400 amu; electron multiplier voltage = 2400; ion energy = 70 eV; mass spectrometer = HP 5985A (Hewlett-Packard, Palo Alto, CA). Selected ion ratios used to derive ^{15}N abundance of individual amino acids were as follows; alanine (240:241), glycine (226:227), valine (268:269), leucine (282:283), isoleucine (282:283), GABA (282:283 or 226:227), proline (266:267), pipecolic acid (280:281), 2-P5CA (280:281 or 252:253) aspartate or asparagine amino-N (284:285), phenylalanine (316:317), ornithine (226:227 or 266:267), glutamate or glutamine amino-N (280:281 or 298:299), lysine (226:227 or 280:281), serine (239:240) and threonine (253:254). Separate analyses were performed on the acidic and neutral amino acid fractions to distinguish between glutamate and glutamine-amino N and between aspartate and asparagine-amino N as described previously (26). Amide ^{15}N abundance of glutamine and asparagine was not determined.

Total Soluble Protein Determination. Total soluble protein was extracted and determined as described previously (10). Aliquots of the protein extracts were precipitated with TCA (10%) and protein pellets washed with absolute ethanol twice and the protein hydrolyzed in 6 N HCl at 100°C for 18 h. Amino acids in the hydrolysates were determined by GLC as for the free amino acid pools.

Computer Simulation of ^{15}N Labeling Data. Computer simulations were performed as described previously (27) using a TRS 80 Model II computer with 64K expansion interface, a single disk drive and NEC printer. Programs were written in BASIC and used a 0.1 h iteration interval. Where single pool models failed to provide a good fit to the observed labeling data, a second "storage" pool was invoked. Compartmentation between metabolic and storage pools, rate of exchange between metabolic and storage pools, and rate of synthesis of the metabolic pool were progressively varied until a close match between observed and simulated labeling kinetics was accomplished for each metabolite.

RESULTS AND DISCUSSION

Amino Acid Pool Sizes. The results of Tables I and II report the free amino acid pool sizes observed in unadapted cell cultures and 25% PEG adapted cell cultures through the time courses of incubation in ^{15}N medium. The pool sizes observed in the present investigations were very similar to those reported previously for these cell lines (10). Notably the pools of proline, GABA, alanine, and valine were markedly elevated in the 25% PEG adapted cells relative to unadapted cells. Glutamine was the only amino acid to exhibit a statistically significant decline in level in response to adaptation to 25% PEG. In the present investigations we were unable to detect high levels of methionine in the 25% PEG adapted cells (*cf.* 10), but several nitrogenous constituents were identified (β-alanine, ornithine, pipecolic acid, and tyramine) (Tables I and II) which were not previously reported (10). The mean pool sizes of Tables I and II were used for the computer simulation studies which follow, assuming a steady state, *i.e.* that synthesis rates approximately equaled utilization rates for each pool during the 48 h labeling periods. The midexponential growth phase was chosen for these investigations because this can be treated as an expanding steady state system (15).

^{15}N Labeling of Free Amino Acid Pools. Tables III and IV list the ^{15}N abundances of several of the free amino acids of unadapted and 25% PEG adapted cells, respectively, where levels were sufficient to obtain accurate isotope ratios from GC-MS electron impact fragmentation data. The most heavily labeled amino acid at all times in both cell cultures was glutamate (Tables III and IV). Proline was not rapidly labeled in the 25% PEG adapted cells, reaching a ^{15}N abundance of only 20% after 24 h (Table IV), suggesting that the greatly enlarged proline pool in water stress adapted cultures is not actively turned over. This contrasts markedly with alanine and GABA in 25% PEG adapted cells. Both alanine and GABA became heavily labeled, indicating that these pools are rapidly turned over (Table IV). Since the 99% [^{15}N]ammonium and 99% [^{15}N]nitrate supplied suffered approximately 10% dilution by ^{14}N salts in the original medium during transfer, the maximum ^{15}N labeling of any nitrogen moiety should be 87 to 88% ^{15}N if the free endogenous ammonium pool rapidly equilibrates with the exogenous supply, if protein turnover is negligible and if the free amino acids exist only as single metabolic pools. The maximum labeling of any amino acid monitored was 84% ^{15}N after 48 h (Tables III and IV). For the purposes of initial model building we will assume that protein turnover is negligible, that glutamate exists as a single metabolic pool and serves as nitrogen donor in unidirectional reactions, and receives ^{15}N label from an endogenous ammonium pool which achieves a maximum of 84% ^{15}N abundance. In the present studies we do not attempt to distinguish between ^{15}N derived from [^{15}N]H_4^+ or [^{15}N]O_3^-. These initial

Table I. *Amino Acid Pool Sizes of Tomato Suspension Cultures Adapted to Control Medium Transferred to ^{15}N Control Medium (0% PEG) at Zero Time*

Amino Acid	0.03 h	0.5 h	1.0 h	2.0 h	4.5 h	8.0 h	24.5 h	48.5 h	
					μmol/gfwt[a]				$\bar{X}$
Alanine	2.01	1.80	3.12	1.62	1.41	1.20	1.20	3.77	2.020
β-Alanine	0.29	0.09	0.14	0.10	0.28	0.17	0.09	0.15	0.164
γ-Aminobutyrate	0.55	0.66	4.02	0.54	0.46	0.78	1.13	1.70	1.23
Arginine	0.02	0.02	0.05	0.04	0.15	0.12	0.02	0.06	0.06
Asparagine	0.08	0.12	0.08	0.18	0.18	0.17	0.10	0.20	0.14
Aspartate	0.05	0.07	0.08	0.07	0.09	0.06	0.07	0.07	0.070
Glutamate	0.98	1.05	0.85	0.94	0.72	0.75	0.64	0.82	0.844
Glutamine	7.63	7.97	7.23	7.34	7.03	5.21	2.70	3.57	6.77[b]
Glycine	0.30	0.27	0.41	0.33	0.30	0.33	0.32	0.49	0.344
Histidine	0.05	0.06	0.09	0.06	0.03	0.07	0.03	0.08	0.059
Homoserine	0.01	0.02	0.06	0.05	0.04	0.06	0.05	0.09	0.048
Isoleucine	0.05	0.06	0.13	0.06	0.07	0.08	0.07	0.08	0.080
Leucine	0.09	0.08	0.14	0.09	0.07	0.08	0.11	0.14	0.100
Lysine	0.21	0.18	0.12	0.26	0.24	0.27	0.30	0.46	0.260
Methionine	0.01	0.01	0.02	0.02	0.02	0.03	0.04	0.05	0.025
Ornithine	0.04	0.04	0.04	0.03	0.04	0.04	0.02	0.03	0.035
Phenylalanine	0.07	0.07	0.11	0.08	0.14	0.15	0.11	0.15	0.11
Pipecolic acid	0.07	0.06	0.09	0.08	0.10	0.09	0.12	0.21	0.100
Proline	0.07	0.09	0.19	0.11	0.10	0.10	0.14	0.15	0.120
2-P5CA	1.04	0.75	0.86	0.77	0.97	0.35	0.35	0.39	0.684
Serine	0.73	0.66	1.06	0.71	0.61	0.56	0.40	1.54	0.780
Threonine	0.06	0.07	0.13	0.09	0.11	0.10	0.04	0.06	0.083
Tyramine	0.15	0.30	0.03	0.42	0.69	0.82	1.54	1.46	0.676
Tyrosine	0.06	0.05	0.03	0.05	0.06	0.06	0.09	0.09	0.062
Valine	0.52	0.52	1.04	0.68	0.53	0.48	0.44	1.01	0.65
Total	14.91	15.05	20.15	15.04	14.47	12.44	10.13	16.87	14.88
Dry wt/fresh wt ratio	0.089	0.094	0.100	0.088	0.059	0.094	0.103	0.093	0.090

[a] Each value represents the average of two independent analyses. [b] The mean glutamine pool represents the sum of both glutamine and 2-P5CA.

Table II. *Amino Acid Pool Sizes of Tomato Suspension Cultures Adapted to 25% PEG Medium Transferred to ^{15}N 25% PEG Medium at Zero Time*

Amino Acid	0.03 h	0.5 h	1.0 h	2.0 h	4.0 h	8.0 h	24.0 h	48.0 h	
				μmol/gfwt[a]					$\bar{X}$
Alanine	6.59	4.87	6.56	5.72	8.15	4.37	8.08	5.75	6.26
β-Alanine	0.90	0.61	0.63	0.71	0.83	0.99	1.35	1.11	0.891
γ-Aminobutyrate	12.37	9.17	11.10	12.85	17.63	16.67	15.03	9.20	13.00
Arginine	0.28	0.32	0.33	0.23	0.24	0.29	0.20	0.16	0.256
Asparagine	0.22	0.20	0.14	0.30	0.21	0.24	0.22	0.18	0.213
Aspartate	0.15	0.13	0.12	0.18	0.14	0.17	0.12	0.13	0.143
Glutamate	1.05	1.12	0.94	0.87	1.53	1.38	1.62	1.21	1.215
Glutamine	2.02	2.14	1.02	0.75	1.20	2.95	4.92	2.49	2.711[b]
Glycine	1.03	0.84	0.83	0.92	0.80	0.62	1.10	0.99	0.89
Histidine	0.41	0.44	0.43	0.37	0.44	0.39	0.52	0.31	0.414
Homoserine	0.59	0.45	0.39	0.39	0.44	0.50	0.56	0.40	0.465
Isoleucine	0.88	0.59	0.64	0.71	0.75	0.55	0.74	0.58	0.680
Leucine	4.17	2.76	2.71	2.53	3.06	2.49	2.94	1.73	2.80
Lysine	0.68	0.57	0.53	0.40	0.72	0.65	0.63	0.45	0.58
Methionine	0.05	0.03	0.06	0.12	0.10	0.11	0.08	0.06	0.076
Ornithine	0.03	0.01	0.02	0.07	0.03	0.02	0.28	0.04	0.063
Phenylalanine	0.23	0.15	0.17	0.16	0.18	0.24	0.27	0.22	0.203
Pipecolic acid	1.11	0.74	0.77	0.80	0.94	0.90	1.15	0.92	0.916
Proline	38.30	28.42	27.35	25.81	32.73	28.48	37.69	30.84	31.20
2-P5CA	0.40	0.49	0.32	0.37	0.48	0.72	0.86	0.56	0.525
Serine	4.36	3.39	3.10	3.11	3.45	2.55	5.02	4.05	3.63
Threonine	0.80	0.72	0.72	0.70	0.38	0.69	0.93	0.74	0.71
Tyramine	2.29	1.93	1.75	0.58	1.37	1.41	1.69	1.28	1.538
Tyrosine	0.42	0.32	0.34	0.23	0.50	0.44	0.32	0.22	0.349
Valine	20.95	15.52	15.69	14.71	18.81	13.56	14.91	8.37	15.315
Total	100.30	75.91	76.65	73.57	96.11	81.38	101.01	71.96	84.611
Dry wt/fresh wt ratio	0.225	0.226	0.221	0.275	0.200	0.212	0.221	0.212	0.224

[a] Each value represents the average of two independent analyses. [b] The mean glutamine pool represents the sum of both glutamine and 2-P5CA.

Table III. *^{15}N Abundance of Major Amino Acid Pool Constituents of Tomato Suspension Cultures Adapted to Control Medium Transferred to ^{15}N Control Medium (0% PEG) at Zero Time*

Amino Acid	0.03 h	0.5 h	1.0 h	2.0 h	4.5 h	8.0 h	24.5 h	48.5 h
					atom % excess			
Alanine	0	9.1	29.2	25.5	32.7	41.5	76.0	82.8
γ-Aminobutyrate	0.2	5.0	25.1	16.8	27.5	46.8	71.5	77.0
Asparagine-amino N	0	2.3	0.5	23.0	30.8	28.2	45.5	66.2
Aspartate	0	3.8	11.5	35.8	48.5	53.0	73.5	78.0
Glutamate	1.0	11.8	33.0	35.6	58.0	72.0	79.5	84.0
Glutamine-amino N	0	5.0	8.5	20.0	23.5	24.8	65.3	81.0
Glycine	0	4.1	10.1	10.8	24.6	25.7	42.9	65.4
Isoleucine	0.1	5.5	10.8	26.7	35.4	41.8	64.8	76.0
Leucine	0	6.5	5.3	30.5	37.0	54.0	70.5	73.2
Lysine (α)	0.1	0.7	2.0	4.5	6.0	5.5	14.0	25.4
Ornithine (α)	0	0.4	5.8	4.3	7.4	23.5	36.0	61.2
Phenylalanine	0	0.4	6.0	17.0	33.5	45.0	66.2	65.8
Pipecolic acid	0	2.5	0.8	2.0	1.8	1.8	15.5	46.0
Proline	0	7.6	12.0	16.8	29.6	33.0	39.9	55.1
2-P5CA	0	4.0	8.5	15.8	20.0	22.5	60.0	79.0
Serine	0	4.9	10.5	18.0	21.2	27.0	43.0	56.5
Threonine	0	1.5	9.3	18.0	14.0	19.9	30.5	40.8
Valine	0	2.6	15.2	14.5	20.3	30.7	65.2	74.3

assumptions are simplifying ones designed to allow us to arrive at a first approximation to the nitrogen budgets of the two cell lines. At later stages in the modeling process we will consider the complications which can arise from protein turnover and reverse transamination reactions leading to isotope dilution of the free amino acid pools, particularly glutamate. The potential contributions of these latter variables to the ^{15}N labeling kinetics cannot be addressed without first undertaking a detailed analysis of N flux in their absence.

(a) Net N Assimilation Rates. Total reduced N contents and cell doubling times of the cell cultures (Table V) can be used to derive estimates of the minimum NH_4^+ assimilation rates required to sustain exponential growth. For unadapted cell cultures this minimum rate of NH_4^+ assimilation represents 4.89 μmol/h·gfwt whereas in 25% PEG adapted cells this rate corresponds to 10.39 μmol/h·gfwt (Table V). Since the 25% PEG adapted

Table IV. *^{15}N Abundance of Major Amino Acid Pool Constituents of Tomato Suspension Cultures Adapted to 25% PEG Medium Transferred to ^{15}N 25% PEG Medium at Zero Time*

Amino Acid	0.03 h	0.5 h	1.0 h	2.0 h	4.0 h	8.0 h	24.0 h	48.0 h
				atom % excess				
Alanine	0.5	8.0	17.5	46.0	61.2	62.8	78.1	84.0
γ-Aminobutyrate	0.2	4.5	12.0	21.2	30.8	41.1	60.0	69.5
Asparagine-amino N	0.1	4.0	2.2	12.0	11.5	20.5	43.0	56.5
Aspartate	0.1	8.3	17.3	32.8	47.5	58.4	63.2	75.4
Glutamate	2.3	31.5	48.0	58.9	73.5	80.2	82.4	84.6
Glutamine-amino N	1.5	20.0	28.5	34.2	53.0	59.8	72.5	76.0
Glycine	0	1.5	2.6	5.5	8.2	17.5	38.4	53.6
Isoleucine	0.2	2.8	5.9	7.0	10.5	18.5	48.3	69.0
Leucine	0	1.8	3.8	3.9	10.5	20.7	47.0	63.5
Lysine (α)	0	0.2	0.7	1.0	2.5	3.0	7.5	16.0
Ornithine (α)	0	0.5	8.0	11.2	35.3	36.8	54.5	57.5
Phenylalanine	0	1.5	1.8	1.3	20.5	23.0	45.0	54.3
Pipecolic acid	0	0.1	0.5	0.3	0.3	0.6	3.8	12.0
Proline	0	0.8	1.3	1.1	2.6	4.0	20.2	40.4
2-P5CA	0	16.8	22.5	21.5	48.0	57.0	70.5	73.0
Serine	0	0.5	2.6	2.5	7.5	16.0	46.2	53.0
Threonine	0	0.8	2.2	4.2	4.2	7.0	21.0	35.8
Valine	0	0.8	1.2	1.8	4.5	9.6	30.7	50.1

Table V. *Summary of Growth and N Assimilation Rates of Unadapted (0% PEG) and Adapted (25% PEG) Cultures*

	0% PEG	25% PEG
Cell doubling time (h)	57.215	62.985
Dry wt/fresh wt ratio	0.090	0.224
Total reduced N (μmol N/g dry wt)	4500	4214
Total reduced N (μmol N/gfwt)	404	944
Rate of N assimilation (μmol N/h·gfwt)	4.893	10.387
Total soluble protein (mg/g dry wt)	88	64
Total soluble protein (mg/gfwt)	7.916	14.336

cells exhibit a 2-fold higher dry weight/fresh weight ratio (Table II) in comparison to unadapted cultures (Table I), these N assimilation rates are similar on a per gram dry weight basis for the two cell lines. These parameters place important constraints on the modeling of ^{15}N flux especially with regard to glutamate and glutamine biosynthesis (see below).

(b) Glutamate and Glutamine Synthesis. If glutamate is synthesized via the GDH pathway in unadapted cultures, then the observed ^{15}N labeling kinetics of glutamate are consistent with a rate of glutamate synthesis of approximately 1.5 μmol/h·gfwt (Fig. 1A). This rate is just sufficient to sustain the rate of labeling of glutamine-amino N but leaves no glutamate available for other glutamate dependent reactions (Fig. 1A). The combined rate of glutamine and glutamate synthesis (3.0 μmol/h·gfwt) is clearly insufficient to meet the N requirements for growth (4.89 μmol/h·gfwt). It follows that the GDH pathway cannot be the primary pathway of ammonia assimilation in unadapted cell cultures. In contrast, when NH_4^+ assimilation is envisaged to occur via the GS-GOGAT cycle (20, 21, 27) in unadapted cultures a rate of NH_4^+ assimilation of 5.5 μmol/h·gfwt can be accommodated (Fig. 1B), provided that 77% of the free glutamine pool is envisaged to be a metabolically inactive storage pool, slowly equilibrating with a small metabolically active pool of glutamine serving as both precursor and product of glutamate.

In water stress adapted cultures a GDH model accounts for a rate of assimilation of NH_4^+ into glutamate of 3.0 μmol/h·gfwt (Fig. 1C); only 29% of the NH_4^+ assimilatory requirements of 25% PEG adapted cells and again just adequate to sustain the labeling of glutamine-amino N (Fig. 1C), but leaving no glutamate available for other glutamate dependent reactions. Assuming the operation of a GS-GOGAT cycle however, a rate of NH_4^+ assimilation of 10.0 μmol/h·gfwt is accommodated (Fig. 1D) provided that the storage pool of glutamine is only 37% of the total in 25% PEG adapted cells. These results indicate that adaptation to 25% PEG may entail a selective depletion of a metabolically inactive (storage) pool of glutamine from 5.23 μmol/gfwt in unadapted cells to 1.0 μmol/gfwt in 25% PEG adapted cells. Although a GS-GOGAT cycle adequately accounts for observed ^{15}N flux, yet, a 10 to 20% contribution to NH_4^+ assimilation via the GDH pathway cannot be eliminated for both cell lines. An equally good fit to the observed data was obtained when GDH was considered to contribute 10% to NH_4^+ assimilation with the remainder assimilated via the GS-GOGAT cycle (results not shown).

It should be noted that in the GS-GOGAT models (Fig. 1, B and D) some of the glutamine is envisaged to be removed in reactions other than glutamate synthesis (*e.g.* nucleic acid and protein synthesis) so that the rate of glutamate synthesis is slightly less than twice the rate of glutamine synthesis. In unadapted cells the rate of glutamate synthesis is caculated to be (4.8 × 2) μmol/h·gfwt of which 5.5 must be reused for glutamine synthesis, leaving 4.1 μmol/h·gfwt available for all other glutamate dependent reactions. In 25% PEG adapted cells the rate of glutamate synthesis is calculated to be (9.5 × 2) μmol/h·gfwt of which 10 must be reused to sustain glutamine synthesis, leaving 9μmol/h·gfwt available for all other glutamate dependent reactions. These parameters again place important constraints on further model development since the combined rates of glutamate utilization in aspartate, alanine, GABA, proline, valine, leucine, isoleucine (etc.) syntheses should not exceed 4.1 and 9 μmol/h·gfwt for unadapted and 25% PEG adapted cells, respectively.

(c) Proline and Ornithine Synthesis. When glutamate was envisaged to donate N directly to proline (*i.e.* via negligible pools of intermediates γ-glutamyl phosphate, glutamic-γ-semialdehyde and Δ¹pyrroline-5-carboxylate [1, 37]) the labeling kinetics of proline were consistent with a net rate of proline synthesis of 0.043 μmol/h·gfwt in unadapted cells (Fig. 2A) and 0.43 μmol/h·gfwt in 25% PEG adapted cells (Fig. 2B), indicating that a 10-fold increase in rate of proline synthesis is associated with adaptation to water stress. Whereas the labeling kinetics of proline in 25% PEG adapted cells were consistent with a single, large pool

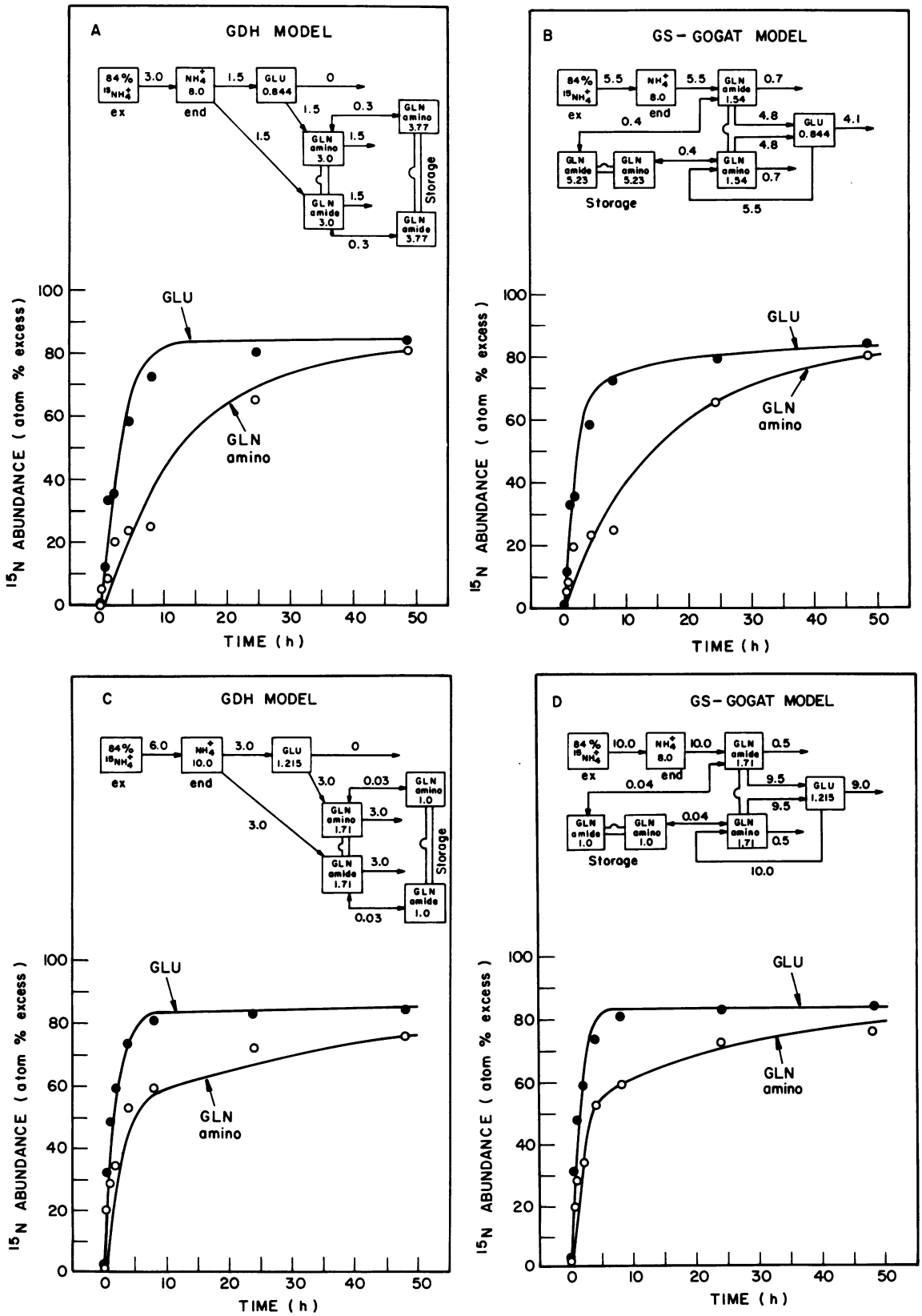

FIG. 1. A, Observed and simulated labeling kinetics of glutamate and glutamine-amino N in unadapted cultures assuming the operation of a GDH pathway of ammonia assimilation as the sole source of glutamate; B, observed and simulated labeling kinetics of glutamate and glutamine-amino N in unadapted cultures assuming the operation of a GS-GOGAT cycle of ammonia assimilation as the sole source of glutamate; C, observed and simulated labeling kinetics of glutamate and glutamine-amino N in 25% PEG adapted cultures assuming the operation of a GDH pathway of ammonia assimilation as the sole source of glutamate; D, observed and simulated labeling kinetics of glutamate and glutamine-amino N in 25% PEG adapted cultures assuming the operation of a GS-GOGAT cycle of ammonia assimilation as the sole source of glutamate. Observed labeling data, glutamate (●) and glutamine-amino N (○); simulated labeling curves (——) using the parameters of flux and compartmentation specified in each panel. Values within the boxes represent pool sizes (μmol/gfwt); values adjacent to arrows are rates (μmol/h·gfwt). Subsequent figures assume the GS-GOGAT models of glutamate synthesis.

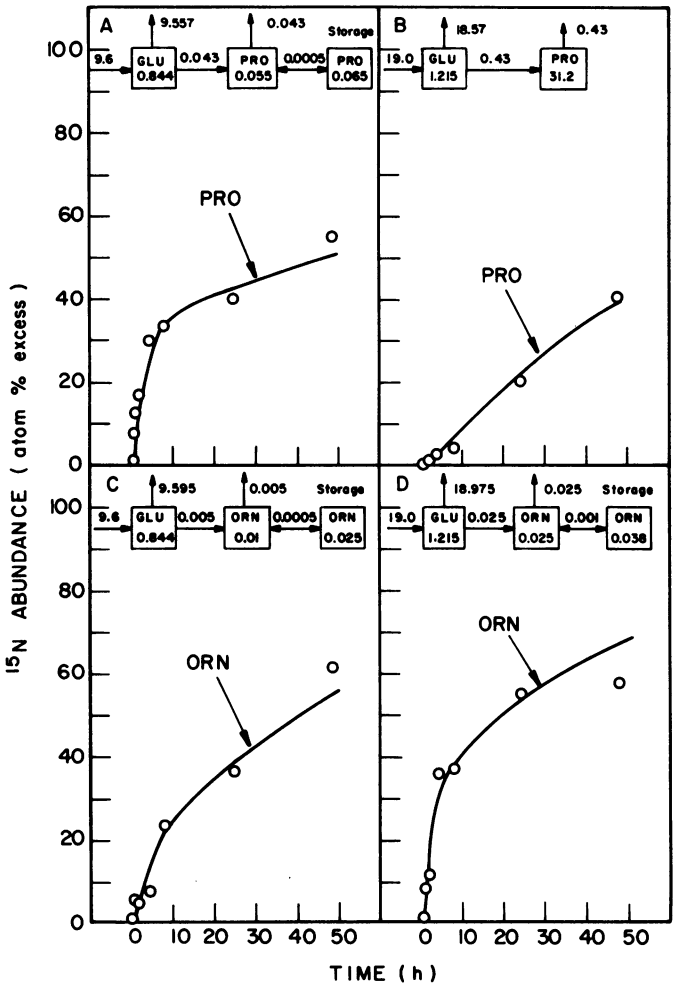

FIG. 2. A, Observed and simulated labeling kinetics of proline in unadapted cultures; B, observed and simulated labeling kinetics of proline in 25% PEG adapted cultures; C, observed and simulated labeling kinetics of the α-amino group of ornithine in unadapted cultures; D, observed and simulated labeling kinetics of the α-amino group of ornithine in 25% PEG adapted cultures. Observed labeling data (O); simulated labeling curves (——) using the parameters of flux and compartmentation specified in each panel; units as in Figure 1.

of proline (Fig. 2B), the labeling kinetics of proline in unadapted cell cultures indicated that about 64% of the free proline pool is in a metabolically inactive, storage compartment. Preliminary efflux studies have indicated that in 25% PEG adapted cells the vast majority of the free proline pool is rapid effluxing (cytoplasmic) whereas in unadapted cells approximately 50% of the proline pool is slow effluxing (vacuolar) (11). It seems likely that the single large pool of proline in 25% PEG adapted cells corresponds to the cytoplasmic pool of proline, and the metabolically inactive pool of proline in unadapted cells corresponds to the vacuolar pool (11, 24, 28). However, since our models of ^{15}N flux reveal compartmentation as kinetic rather than physical entities it is premature to assign strict subcellular localizations to the pools deduced from the labeling data; we will continue to refer to these pools as metabolically active (metabolic) and inactive (storage) pools rather than as cytoplasmic and vacuolar pools, respectively. Although a single pool model provides an adequate simulation of the observed labeling of proline in 25% PEG adapted cells, we wish to emphasize that this is not a unique solution. Alternative models in which ≥70% of the proline is in a metabolically active pool in very rapid equilibrium with a storage pool of proline, provide an equally good fit to the observed data.

The labeling kinetics of the α amino group of ornithine appear consistent with rates of synthesis of ornithine from glutamate of 0.005 and 0.025 μmol/h·gfwt for unadapted and water stress adapted cells, respectively (Fig. 2, C and D). These rates are 8.6- and 17.2-fold lower than the rates of proline synthesis, respectively (Fig. 2, A and B). Thus, ornithine does not carry sufficient ^{15}N flux to serve as a major precursor for proline in either unadapted or adapted cells. It follows that a pathway of proline synthesis via ornithine (*e.g.* the N-acetyl ornithine pathway [1, 37]) is probably not a major contributor to stress-induced proline accumulation in tomato cells. Any appreciable flux of N via ornithine → arginine → ornithine → proline (1, 37) would have been revealed by heavy ^{15}N incorporation into ornithine since the pool sizes of ornithine and arginine are relatively small in comparison to proline in 25% PEG adapted cells. The results appear consistent with the operation of a glutamate pathway of proline synthesis which exhibits a 10-fold increased activity in comparison to unadapted cells on a gfwt basis, but only a 5-fold increased activity on a per gram dry weight basis. This is a surprisingly small increase to account for a 300-fold increase in pool size of proline. As will be discussed in later sections, this is possible only because proline is not rapidly catabolized and 25% PEG adapted cells have a significantly reduced rate of cell expansion (and hence pool dilution rate) (10).

(d) Alanine and γ-Aminobutyrate Synthesis. Alanine became heavily labeled at early time points in both unadapted and water stress-adapted cultures. Assuming that alanine receives N directly from glutamate in the glutamate-pyruvate transaminase reaction the rates of synthesis (and utilization) of alanine represent 1.45 and 5.0 μmol/h·gfwt for unadapted and water stress-adapted cultures, respectively (Fig. 3, A and B). In unadapted cells it was necessary to envisage that about 50% of the alanine pool was located in a metabolically inactive pool, whereas in 25% PEG adapted cells only 20% of the alanine pool was located in a metabolically inactive (storage) compartment.

The above calculations suggest that alanine synthesis represents a major metabolic fate of glutamate; over 30% of the glutamate available for amino acid biosynthesis (other than glutamine) is used to sustain alanine synthesis in unadapted cells whereas over 55% of the available glutamate is used to sustain alanine synthesis in water stress-adapted cells. The larger metabolically active pool size of alanine plus the higher flux via alanine in water stress-adapted cells suggests a much higher availability of pyruvate in stress-adapted relative to control cells, assuming operation of glutamate-pyruvate transaminase (28):

Glutamate + pyruvate ↔ alanine + 2-oxoglutarate.

Since the glutamate pool sizes are similar in unadapted and 25% PEG adapted cells (0.844 μmol/gfwt and 1.215 μmol/gfwt, respectively) a 5-fold increase in pyruvate to 2-oxoglutarate ratio might be sufficient to cause a 5-fold increase in free alanine pool. Note that in the models of Figure 3 glutamate is envisaged to donate N to alanine unidirectionally; in an equilibrium reaction alanine can be transaminated back to glutamate leading to isotope dilution of the free glutamate pool. In the unidirectional models considered so far, the rate of glutamate synthesis could be underestimated since we have not accommodated this reverse flux.

GABA also exhibits increased synthesis rates in 25% PEG adapted cells (Fig. 3, C and D). Assuming that GABA is derived from glutamate by decarboxylation (25, 36, 38). then the rates of synthesis of GABA correspond to 0.8 and 2.4 μmol/h·gfwt, respectively, for unadapted and water stress-adapted cells (Fig. 3, C and D). About 76% of the GABA appears to be located in a metabolically inactive pool in unadapted cells but only 38% in

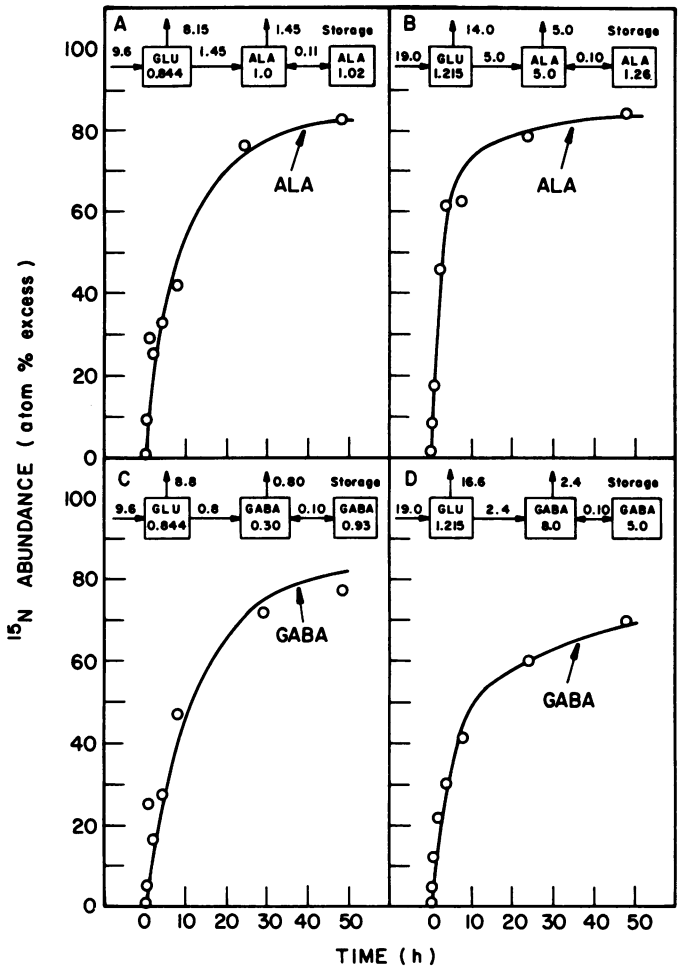

FIG. 3. A and B, Observed and simulated labeling kinetics of alanine in unadapted cultures (A) and 25% PEG adapted cultures (B); C and D, observed and simulated labeling kinetics of GABA in unadapted cultures (C) and 25% PEG adapted cultures (D). Other details as in Figure 1.

water stress-adapted cells. Thus, the major increase in free GABA pool associated with stress adaptation appears to be in the metabolically active rather than storage component. It is pertinent to note that GABA does not carry sufficient ^{15}N flux to be the sole precursor of alanine assuming the operation of a GABA-pyruvate transaminase (36). Nevertheless, it is possible that some of the GABA is utilized to augment the glutamate-pyruvate transaminase reaction. In the latter case rates of glutamate utilization for alanine synthesis may be overestimated.

(e) Branched Chain Amino Acid Synthesis. The labeling kinetics of valine, leucine, and isoleucine are consistent with rates of synthesis from glutamate of 0.10, 0.07 and 0.05 μmol/h·gfwt in unadapted cells, respectively (Fig. 4, A, C, and E) and rates of synthesis of 0.30, 0.10, and 0.025 μmol/h·gfwt in water stress-adapted cells (Fig. 4, B, D, and F). Thus, there appears to be a preferential increase in flux via the valine and leucine branches of the pathway over the isoleucine branch of the pathway. This again is consistent with an increased availability of pyruvate in 25% PEG adapted cells since pyruvate is the carbon precursor for valine and leucine but not isoleucine. Valine and isoleucine are synthesized by common enzymes utilizing different organic acid intermediates, *e.g.* 2-oxoisovalerate and 2-oxo-3-methylvalerate, respectively, at the last transamination step (3, 20). A 3-fold increase in valine synthesis rate associated with a 2-fold decrease in isoleucine biosynthesis rate would require at least a 6-fold increase in 2-oxoisovalerate:2-oxo-3-methylvalerate ratio at the site of action of valine amino transferase. It is difficult to rationalize the 24-fold accumulation of valine and 28-fold accumulation of leucine in PEG adapted cells apparently involving 2- to 3-fold increased synthesis rates, with models of cooperative feedback inhibition of acetolactate synthase by valine and leucine (3, 19, 20). Accumulations of valine and leucine should, in principle, inhibit their own syntheses and prevent such accumulations. Altered feedback inhibition characteristics of acetolactate synthase associated with adaptation to water stress needs to be explored. In 25% PEG adapted cells the valine, leucine, and isoleucine pools behave kinetically as single, metabolically active pools. It is possible that acetolactate synthase is located in the plastid (3, 20) and is thus spatially separated from the pools of valine and leucine deduced here.

(f) Aspartate Family Amino Acid Biosynthesis. In unadapted cells the rate of synthesis of aspartate from glutamate was calculated to be 0.3 μmol/h·gfwt (Fig. 5A). Adapted cells exhibit an aspartate synthesis rate of 0.4 μmol/h·gfwt (Fig. 5B). Thus, there appears to be little increase in the net flux via the aspartate pathways. Examination of individual branches of the aspartate pathway (threonine, asparagine, and lysine) (Fig. 5, C to H) appears to confirm this observation. Rates of synthesis of threonine, asparagine, and lysine were in fact lower in adapted cells than in unadapted cells, even though the pool sizes of these amino acids were larger. A major discrepancy emerges from these results; the rates of threonine synthesis deduced for both 25% PEG adapted and unadapted cells are insufficient to sustain the estimated rates of isoleucine biosynthesis. It is possible that isoleucine derives carbon skeletons (*e.g.* 2-oxobutyrate [20]) from sources other than threonine in tomato cells.

Estimated rates of lysine synthesis were extremely low (Fig. 5, G and H) and apparently inadequate to account for pipecolic acid labeling (35). For pipecolic acid we have assumed that aspartate is the immediate precursor (Fig. 5, I and J). It is possible that there is an extremely small metabolic pool of lysine serving as precursor to pipecolic acid. In this case the rates of synthesis of lysine could be substantially underestimated. Such a situation cannot yet be eliminated for threonine as an intermediate in isoleucine biosynthesis. However, since ^{15}N/^{14}N isotopic ratios can be detected down to a level of about 1% ^{15}N (26) such minor metabolic pools would have to be less than 1% of the total free pools of these amino acids in order to escape detection as separate kinetic entities in the ^{15}N labeling curves.

(g) Glycine and Serine Synthesis. Rates of synthesis of serine (assuming glutamate to be the N donor) were calculated to be 0.30 and 0.14 μmol/h·gfwt, respectively, for unadapted and water stress adapted cells (Fig. 6, A and B). These rates greatly (>3-fold) exceeded the observed rates of glycine synthesis (Fig. 6, C and D) again assuming glutamate to be the precursor of glycine. For the operation of a photorespiratory nitrogen cycle, rates of glycine synthesis should exceed those of serine by a factor of 2 since two glycines generate one serine (16, 20). Glycine synthesis rates are clearly inadequate to account for those of serine assuming a photorespiratory pathway. The absence of such a pathway in tomato suspension cultures is not surprising since the cultures are nonphotosynthetic. However, we may have slightly underestimated glycine synthesis rates if glutamate, alanine, and serine all donate N to glycine (16, 22).

(h) Aromatic Amino Acids. Of the aromatic amino acids, complete ^{15}N labeling data were acquired only for phenylalanine which appeared to be synthesized at rates of 0.025 and 0.02 μmol/h·gfwt in unadapted and adapted cells, respectively (Fig. 6, E and F). It seems likely that the amine, tyramine, observed in the free amino acid pools (Tables I and II) is derived from tyrosine by decarboxylation. The selected ions monitored for tyramine (316:317) and tyrosine (360:361) during the GC-MS analyses appeared not to contain the N moiety since no signifi-

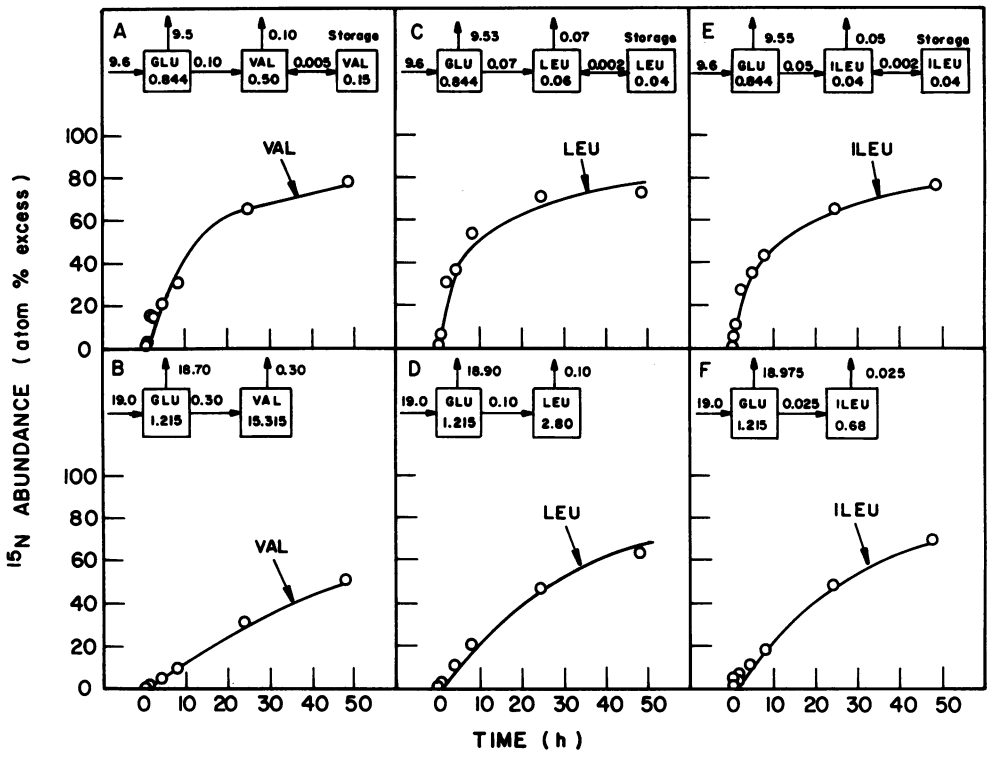

FIG. 4. A, C, and E, Observed and simulated labeling kinetics of valine (A), leucine (C), and isoleucine (E) in unadapted cultures; B, D, and F, observed and simulated labeling kinetics of valine (B), leucine (D), and isoleucine (F) in 25% PEG adapted cultures. Other details as in Figure 1.

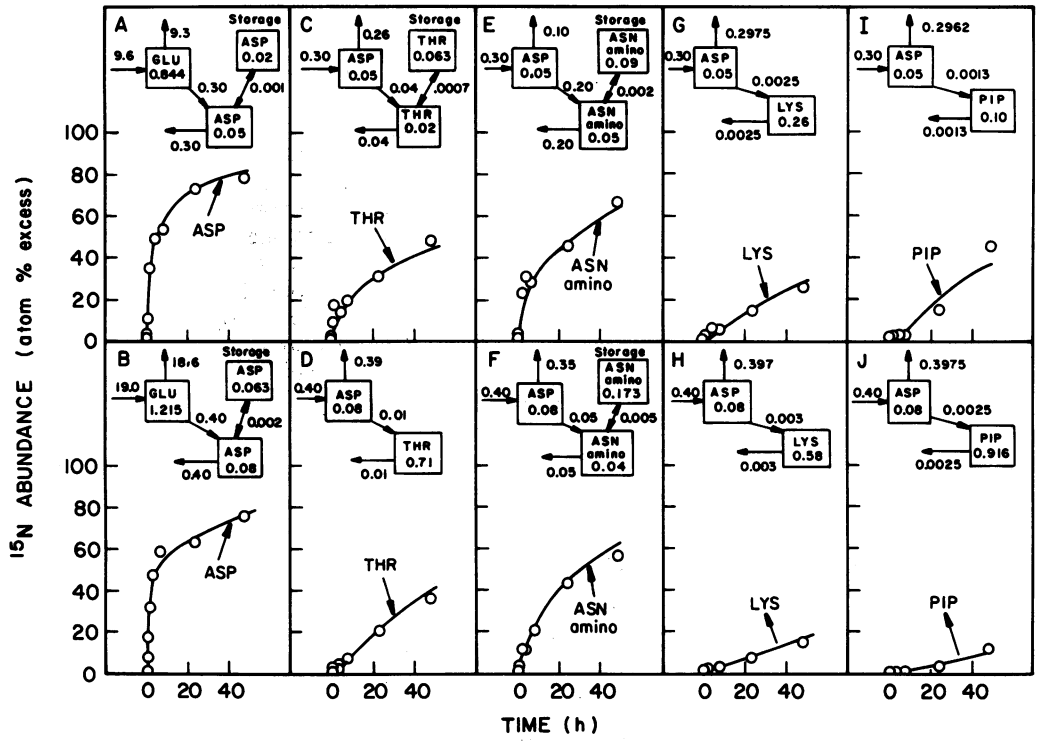

FIG. 5. A and B, Observed and simulated labeling kinetics of aspartate in unadapted (A) and 25% PEG adapted (B) cultures. C and D, Observed and simulated labeling kinetics of threonine in unadapted (C) and 25% PEG adapted (D) cultures; E and F, observed and simulated labeling kinetics of asparagine-amino N in unadapted (E) and 25% PEG adapted (F) cultures; G and H, observed and simulated labeling kinetics of the α-amino group of lysine in unadapted (G) and 25% PEG adapted (H) cultures; I and J, observed and simulated labeling kinetics of pipecolic acid in unadapted (I) and 25% PEG adapted (J) cultures. Other details as in Figure 1.

cant changes in these ion ratios occurred during the 48 h labeling periods in either culture (results not shown). Estimates of tyrosine and tyramine synthesis rates were therefore not possible.

(i) Net Rates of Glutamate Utilization in Amino Acid Biosynthesis. As noted earlier, glutamate utilization rates should not exceed glutamate synthesis rates. For unadapted cells, the combined rate of glutamate utilizaton in proline, ornithine, valine, leucine, isoleucine, GABA, alanine, aspartate family, glycine, serine, and phenylalanine syntheses is 3.243 μmol/h·gfwt *versus* 4.1 μmol/h·gfwt available for all glutamate dependent reactions other than glutamine synthesis (see section [b] above). Thus, in the case of unadapted cells the calculated rate of glutamate synthesis appears adequate to sustain all glutamate dependent reactions with 0.9 μmol/h·gfwt remaining for amino acid synthesis such as tyrosine which have not been considered. Obviously, some of this remaining glutamate must also be used for protein synthesis.

For 25% PEG adapted cells the combined rate of glutamate utilization in proline, ornithine, valine, leucine, isoleucine, GABA, alanine, aspartate, glycine, serine, and phenylalanine

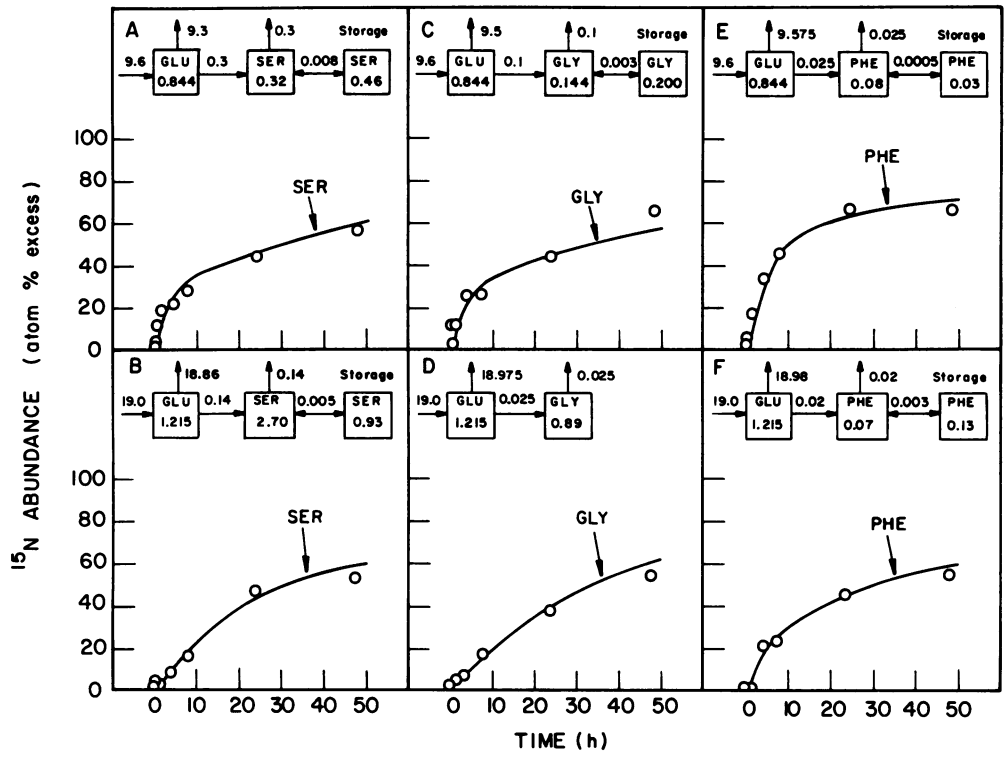

FIG. 6. A and B, Observed and simulated labeling kinetics of serine in unadapted (A) and 25% PEG adapted (B) cultures; C and D, observed and simulated labeling kinetics of glycine in unadapted (C) and 25% PEG adapted (D) cultures; E and F, observed and simulated labeling kinetics of phenylalanine in unadapted (E) and 25% PEG adapted (F) cultures. Other details as in Figure 1.

syntheses is 8.865 μmol/h·gfwt which is only slightly less than the 9.0 μmol/h·gfwt calculated to be available for this purpose (see section [b] above). Considering that the rate of glutamate synthesis in adapted cells could be underestimated if alanine is reversibly transaminated to glutamate and that glutamate utilization rates could be over estimated if GABA augments alanine synthesis (see section [d] above), then these balance sheets of glutamate synthesis and utilization do not appear grossly incompatible with one another. This represents an important test of the models developed so far, since if glutamate utilization rates had greatly exceeded the estimated rate of glutamate synthesis, then this would have invalidated the underlying assumptions. Further independent tests of the models become possible when we consider amino acid pool maintenance and protein synthesis requirements of the cell cultures (see below).

Amino Acid Utilization. In a steady state the rate of synthesis of an amino acid should be equal to the rate of utilization of the amino acid (31). Utilization rates can be partitioned into three discrete components: pool maintenance with growth (*i.e.* the expansion flux [15]), protein synthesis requirements, and catabolism (the latter including other biosynthetic reactions involving the specific amino acid, such as decarboxylation or transamination). The pool maintenance requirements can be calculated from the free amino acid pool sizes (Tables I and II) and doubling times (Table V), and protein synthesis requirements can be calculated (assuming negligible protein turnover) from doubling times, total soluble protein content, and protein amino acid composition (Table VI). Catabolism rates can then be estimated by difference:

$$\begin{array}{cccccc} \text{Amino acid} & & \text{amino acid} & & \text{amino acid} & & \text{protein} \\ \text{biosynthesis} & = & \text{catabolism} & + & \text{pool maintenance} & + & \text{synthesis} \\ \text{rate} & & \text{rate} & & \text{requirement} & & \text{requirement} \\ \text{(A)} & & \text{(B)} & & \text{(C)} & & \text{(D)} \end{array}$$

$$\therefore B = A - (C + D)$$

Calculations of these utilization components are summarized in Tables VII and VIII for unadapted and 25% PEG adapted cells, respectively. A negative catabolism rate indicates that the observed synthesis rate was inadequate to sustain pool maintenance and/or protein synthesis and such negative values define obvious inadequacies in our model(s) (*i.e.* underestimation of steady state synthesis rate). In unadapted cells the only discrepancies were observed for phenylalanine and lysine (Table VII). In 25% PEG adapted cells, however, we appear to have underestimated rates of synthesis of asparagine, glycine, isoleucine, leucine, lysine, phenylalanine, pipecolic acid, and threonine (Table VIII). Note that five of these matabolites are in the aspartate family (including isoleucine). One possible explanation for these discrepancies could be unaccounted compartmentation of the aspartate family amino acids in adapted cells where the true metabolic pools are undetected because their size is too small (less than 1% of the free pools) to be recognized as separate components in the ^{15}N labeling curves. The occurrence of protein turnover (which we have assumed to be negligible) would lead to isotope dilution of all free amino acids (the smaller the free pool size, the larger the dilution) and hence underestimation of synthesis rates (6). However, this would also lead to underestimation of protein synthesis requirements. It is possible that protein amino-N is specifically channeled into the aspartate pathway leading to selective underestimation of flux via the aspartate family amino acids.

Despite these discrepancies it is clear that the rates of proline synthesis in both unadapted and water stress adapted cells are just sufficient to sustain pool maintenance and protein synthesis requirements. In 25% PEG adapted cells the pool maintenance requirement for proline is large; 0.343 μmol/h·gfwt (Table VIII). There is little evidence for any major increase in rate of proline catabolism (*e.g.* proline oxidation) associated with adaptation to water stress. Proline catabolism rates represent 8% of synthesis rate in unadapted cells and 4.7% in adapted cells (Tables VII and VIII). Control of proline accumulation in response to water stress may reside primarily at the level of synthesis in these cells, although it is clear that regulatory mechanisms must also operate to restrict proline oxidation (12, 13, 29, 34, 37).

Valine also exhibits a large pool maintenance requirement in 25% PEG adapted cells (Table VIII). The estimated catabolism

Table VI. *Protein Amino Acid Composition of Unadapted (0% PEG) and Adapted (25% PEG) Cultures*

Amino Acid	0% PEG		25% PEG	
	μg/100 μg protein	*μmol/gfwt*[a]	*μg/100 μg protein*	*μmol/gfwt*[a]
Alanine	5.1	4.531	5.9	9.485
Arginine	3.3	1.498	2.5	1.432
Aspartate[b]	6.9	4.050	5.65	6.086
Asparagine[b]	6.9	4.050	5.65	6.086
Cystine	5.2	1.715	2.4	2.217
Glutamate[c]	7.9	4.252	6.05	5.897
Glutamine[c]	7.9	4.252	6.05	5.897
Glycine	5.3	5.588	5.6	10.697
Histidine	3.1	1.637	2.4	3.559
Hydroxyproline	<1.0	<0.602	<1.0	<1.095
Isoleucine	3.2	1.929	3.8	4.155
Leucine	7.4	4.467	7.9	8.630
Lysine	11.1	6.006	9.8	9.611
Methionine	1.8	0.952	1.3	1.247
Phenylalanine	5.0	2.397	5.5	4.770
Proline	4.6	3.162	4.9	6.097
Serine	3.4	2.560	6.5	8.869
Threonine	3.2	2.124	5.2	6.254
Tyrosine	4.5	1.965	4.5	3.559
Valine	4.2	2.834	4.2	5.139

[a] Calculated from amino acid mol wt and total soluble protein contents shown in Table V. [b] Asparagine and aspartate were not resolved; the two amino acids are assumed to be present in equimolar amounts in the total asparagine + aspartate measured in the protein hydrolysates. Glutamine and glutamate were not resolved; the two amino acids are assumed to be present in equimolar amounts in the total glutamine + glutamate measured in the protein hydrolysates.

Table VII. *Summary of Estimated Rates of Synthesis and Utilization of Amino Acids in Unadapted Cultures*

N-Compound	Assumed N-Donor	Metabolic Pool Size[a]	Storage Pool Size[a]	Rate of Exchange between Metabolic and Storage Pools[a]	Rate of Synthesis[a]	Pool Maintenance[b]	Protein Synthesis[b]	Rate of Catabolism[c]
		μmol/gfwt	*μmol/gfwt*	*μmol/h·gfwt*	*μmol/h·gfwt*	*μmol/h·gfwt*	*μmol/h·gfwt*	*μmol/h·gfwt*
NH_4^+ (end)[d]	84% $[^{15}N]H_4^+$ (ex)[d]	8.0	0	0	5.5[e]			
Glutamine-amide N	NH_4^+ (end)	1.54	5.23	0.40	5.5	0.082	0.0515	5.37
Glutamine-amino N	Glutamate	1.54	5.23	0.40	5.5	0.082	0.0515	5.37
Glutamate	Glutamine-amide N and Glutamine-amino N	0.844	0	0	(4.8) × 2	0.0102	0.0515	9.538
Alanine	Glutamate	1.0	1.02	0.11	1.45	0.0244	0.055	1.371
Proline	Glutamate	0.055	0.065	0.0005	0.043	0.0015	0.038	0.0035
Valine	Glutamate	0.50	0.15	0.005	0.10	0.0079	0.034	0.0581
Leucine	Glutamate	0.06	0.04	0.002	0.07	0.0012	0.054	0.0148
Isoleucine	Glutamate	0.04	0.04	0.002	0.05	0.00097	0.023	0.026
GABA	Glutamate	0.30	0.93	0.10	0.80	0.0149	0	0.785
Glycine	Glutamate	0.144	0.200	0.003	0.10	0.004	0.0677	0.028
Serine	Glutamate	0.32	0.46	0.008	0.30	0.0094	0.031	0.2596
Phenylalanine	Glutamate	0.08	0.03	0.0005	0.025	0.00133	0.029	−0.005
Ornithine	Glutamate	0.01	0.025	0.0005	0.005	0.0004	0	0.0496
Aspartate	Glutamate	0.05	0.02	0.001	0.30	0.00085	0.049	0.2502
Asparagine-amino N	Aspartate	0.05	0.09	0.002	0.20	0.0017	0.049	0.1493
Threonine	Aspartate	0.02	0.063	0.0007	0.040	0.001	0.026	0.013
Lysine	Aspartate	0.26	0	0	0.0025	0.003	0.073	−0.0735
Pipecolic	Aspartate	0.10	0	0	0.0013	0.0012	0	0.0001

[a] Derived from Figures 1 to 6. [b] Derived from Tables I, V, and VI. [c] Estimated by difference as described in text. [d] end, Endogenous; ex, exogenous. [e] Rate of uptake of NH_4^+ (μmol/h·gfwt).

Table VIII. *Summary of Estimated Rates of Synthesis and Utilization of Amino Acids in 25% PEG Adapted Cultures*

N-Compound	Assumed N-Donor	Metabolic Pool Size[a]	Storage Pool Size[a]	Rate of Exchange between Metabolic and Storage Pools[a]	Rate of Synthesis[a]	Pool Maintenance[b]	Protein Synthesis[b]	Rate of Catabolism[c]
		μmol/gfwt	*μmol/gfwt*	*μmol/h·gfwt*	*μmol/h·gfwt*	*μmol/h·gfwt*	*μmol/h·gfwt*	*μmol/h·gfwt*
NH_4^+ (end)[d]	84% $[^{15}N]H_4^+$ (ex)[d]	10.0	0	0	10.0[e]			
Glutamine-amide N	NH_4^+ (end)	1.71	1.0	0.04	10.0	0.0298	0.065	9.9052
Glutamine-amino N	Glutamate	1.71	1.0	0.04	10.0	0.0298	0.065	9.9052
Glutamate	Glutamine-amide N and Glutamine-amino N	1.215	0	0	(9.5)×2	0.0134	0.065	18.922
Alanine	Glutamate	5.0	1.26	0.10	5.0	0.069	0.104	4.827
Proline	Glutamate	31.20	0	0	0.43	0.343	0.067	0.020
Valine	Glutamate	15.315	0	0	0.30	0.1685	0.057	0.0745
Leucine	Glutamate	2.80	0	0	0.10	0.0308	0.095	−0.0258
Isoleucine	Glutamate	0.68	0	0	0.025	0.0075	0.046	−0.0285
GABA	Glutamate	8.0	5.0	0.1	2.4	0.143	0	2.257
Glycine	Glutamate	0.89	0	0	0.025	0.0098	0.118	−0.103
Serine	Glutamate	2.70	0.93	0.005	0.14	0.0399	0.098	0.0021
Phenylalanine	Glutamate	0.07	0.13	0.003	0.020	0.0022	0.052	−0.0342
Ornithine	Glutamate	0.025	0.038	0.001	0.025	0.0007	0	0.0243
Aspartate	Glutamate	0.08	0.063	0.002	0.40	0.0016	0.067	0.3314
Asparagine-amino N	Aspartate	0.04	0.173	0.005	0.05	0.0023	0.067	−0.0193
Threonine	Aspartate	0.71	0	0	0.01	0.0078	0.069	−0.0668
Lysine	Aspartate	0.58	0	0	0.003	0.0064	0.106	−0.1094
Pipecolic	Aspartate	0.916	0	0	0.0025	0.0101	0	−0.0076

[a] Derived from Figures 1 to 6. [b] Derived from Tables II, V, and VI. [c] Estimated by differences as described in text. [d] end, endogenous; ex, exogenous. [e] Rate of uptake of NH_4^+ (μmol/h·gfwt).

rates for valine are 0.058 μmol/h·gfwt in unadapted cells (Table VII) and 0.0745 μmol/h·gfwt in adapted cells (Table VIII).

GABA, which has no protein synthesis requirement, is clearly actively catabolized in both unadapted (0.785 μmol/h·gfwt) (Table VII) and water stress adapted cells (2.257 μmol/h·gfwt) (Table VIII). A possible metabolic fate of GABA could be transamination to alanine (as mentioned in section [d] above), or alternatively GABA could be transaminated back to glutamate. A GABA shunt which is activated by PEG stress has been reported for *Sinapsis alba* (38). The latter would essentially establish a glutamate ↔ GABA cycle (Fig. 7) in which the N flux appears futile. The carbon flux, however, could theoretically augment the tricarboxylic acid cycle flux of 2-oxoglutarate to succinate via succinyl-CoA. The occurrence of a GABA transaminase generating glutamate at a rate of 2.257 μmol/h·gfwt in adapted cells could lead to significant isotope dilution of the free glutamate pool and hence underestimation of glutamate synthesis rates. Any selective channeling of relatively unlabeled glutamate so generated into aspartate would lead to underestimation of flux via the aspartate family amino acids. It becomes clear that our models do not fully account for all potential variables in N metabolism and must be regarded as tentative estimates of the N budgets.

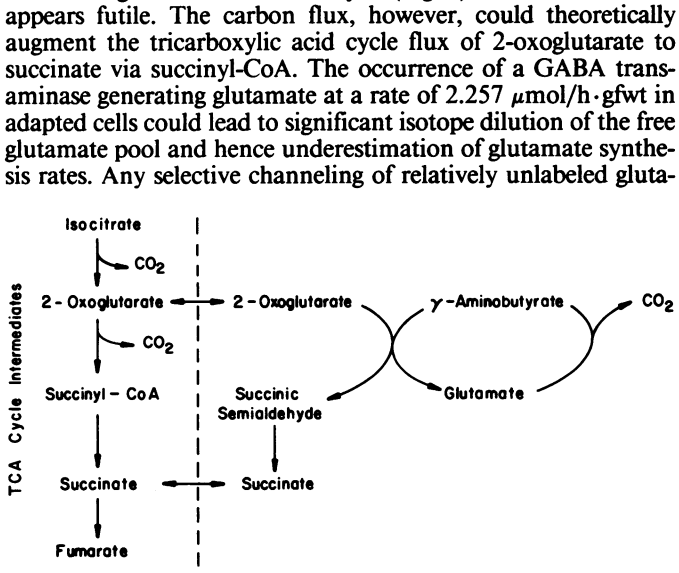

FIG. 7. A possible metabolic fate of GABA.

The solutions to the observed ^{15}N labeling data presented in Tables VII and VIII are by no means unique. For example, models involving three compartments and/or two independent sites of amino acid synthesis could provide equally good simulations of the observed labeling patterns. We have adopted a strategy of invoking the simplest possible model for each labeling curve, introducing a second compartment only when a single compartment model fails to provide an adequate simulation of the observed labeling kinetics. When these criteria fail to meet the calculated growth requirements for protein synthesis and pool maintenance (*e.g.* the aspartate family amino acids in 25% PEG adapted cells) then this clearly defines needs for additional experiments and revised assumptions.

CONCLUSIONS

Tables VII and VIII summarize the models developed to account for ^{15}N flux in unadapted and water stress adapted cell cultures of tomato. Although there are certain unresolved discrepancies in the N balance sheets, particularly with regard to the aspartate family amino acids in 25% PEG adapted cells and with regard to threonine synthesis being inadequate to sustain isoleucine biosynthesis, several important conclusions can be drawn:

1. N assimilation occurs primarily via the GS-GOGAT cycle in both adapted and unadapted cells.

2. Proline synthesis via the glutamate pathway is increased 10-fold in response to adaptation to water stress on a per gfwt basis. About 8% of the newly synthesized proline is catabolized

in unadapted cells but only 4.7% in adapted cells. Rigorous control of proline oxidation under water stress is implied by these and other results (13, 29, 34). Most of the proline synthesized is used for pool maintenance in stress adapted cells. This renders proline a highly effective osmotic solute since lack of turn over allows the metabolite to accumulate at minimal cost of synthesis. A 300-fold elevated proline pool is achieved from a 10-fold increase of synthesis rate.

3. Valine and leucine accumulate as a result of 2- to 3-fold increases in synthesis rates at the expense of isoleucine.

4. GABA and alanine exhibit marked increases in both synthesis and utilization rates, implying substantial alterations in carbon flux via pyruvate and succinate.

Further work is required to investigate the possible role of cytoplasmic pH in these amino acid accumulations and alterations of N flux and whether certain metabolic adjustments (*e.g.* GABA and alanine accumulation) are a direct result of osmotic stress or an indirect result of partial anaerobiosis induced by PEG (17, 36). Direct determinations of the O_2 concentrations of unadapted and 25% PEG adapted cell suspensions in midexponential growth suggest that the 25% PEG adapted cells are not limited by O_2; observed O_2 concentrations were 123 μM and 107 μM for unadapted and adapted cells + medium, respectively (results not shown). Modulation of the biochemical pH stat (5, 7, 33) towards a more acidic cytoplasm in water stress adapted cells might tend to decrease flux via the oxaloacetate derived amino acids (*i.e.* aspartate family) and increase flux via the pyruvate derived amino acids (valine, leucine, and alanine) as observed. However, note that acetolactate synthase is sensitized to cooperative feedback by valine and leucine at low pH (19). We would not expect valine and leucine to accumulate in response to acidification in view of this property of the enzyme *in vitro*. Amino acid decarboxylation reactions (*e.g.* glutamate decarboxylation [7, 25], tyrosine decarboxylation and polyamine synthesis [8]) may be under the control of pH and may themselves serve a role in intracellular pH control (25). But note that the biochemical pH stat may only be a fine-tuning device in relation to the biophysical pH stat regulating transport of ions in relation to growth (33). Much work remains to be done to integrate these N budgets with C budgets and ion transport characteristics.

The models developed to account for ^{15}N flux suggest that adaptation to water stress in tomato cells leads to a decrease in metabolically inactive, storage pools of the majority of the amino acids as percentages of their total pools. It is tempting to speculate that these storage pool depletions reflect decreases in vacuolar pool sizes relative to cytoplasmic pool sizes (24, 28), which would have obvious implications for cytoplasmic osmotic adjustment required for growth on media of low water potential (39). To address the amino acid compartmentation characteristics of these cell lines in more detail it would be useful to determine the ^{15}N labeling kinetics of the amino acid residues of protein. This would provide insight as to the specific isotopic abundance of the cytoplasmic amino acid pools serving as precursors for protein (31), as well as information concerning rates of protein turnover (6).

Although these investigations shed light on the cellular mechanisms of adaptation of tomato cells to water deficits induced with PEG, it should be cautioned that these mechanisms may be implemented, especially to the degree that we observe, only because the cells in suspension culture have unrestricted supplies of both C and N. At the whole plant level both C and N inputs may be substantially curtailed under water deficits (12) and may be specifically channeled to certain cells within the plant (*e.g.* the meristems) (2). It would not be unexpected that such metabolic responses in whole plants may be both attenuated and restricted to specific tissues. It remains to be seen whether regenerated plants from water stress-adapted cells exhibit increased tolerance to osmotic stress at the whole plant level. As a model system, however, these suspension cultures offer an excellent source of material to investigate the enzymology of adaptation to water stress. The models of N flux suggest that the 25% PEG adapted cells may exhibit a 10-fold increased activity of one or more of the enzymes of the proline pathway, for example. It has yet to be determined whether or not γ-glutamyl kinase exhibits altered feedback inhibition characteristics by proline in the water stress-adapted tomato suspension cultures (*cf.* bacteria [1, 4, 32]) and how such altered feedback inhibition might be induced.

It has been suggested that restricted cell expansion growth may be a prerequisite for osmotic adjustment under water deficits in plants (18). The present results emphasize the importance of considering expansion fluxes as key components of metabolism in expanding steady state systems (15). A decrease of growth rate without impairing N assimilation rate would obviously decrease all expansion fluxes and protein synthesis requirements, facilitating potentially large pool size increases of free amino acids especially for those amino acids that are not actively catabolized. The present analyses apply only to the midexponential growth-phase of these cell cultures and do not address the changes in metabolism that may occur in the transition from exponential to stationary phase during the growth cycle (10).

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