

Inactive and Protein Precursor Pools of Amino Acids in the Soybean Hypocotyl¹

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Summary. There are at least 2 amino pools for leucine and for valine in the soybean hypocotyl, a small protein precursor pool and a large inactive pool. The precursor pool decreased in size during incubation of excised hypocotyls presumably because the cotyledonary sources of amino acids had been removed. The precursor pool was subject to expansion by supplying the amino acid externally at high concentrations. After the transfer of tissue to unsupplemented media, the expanded pool was rapidly depleted.

In studies of protein synthesis in excised soybean hypocotyls, the rate of incorporation of exogenously supplied ¹⁴C-amino acid into protein was found to increase with the time of tissue pre-incubation. As a possible explanation, changes in the sizes of the amino acid pools were considered. Using the method of Britten and McClure (3), the change in the relative size of a protein precursor pool was determined and compared with the change in the total amount of a particular amino acid. There is evidence for a compartmentalization of metabolites into active and inactive pools in plant cells (1, 2, 4, 5, 11, 12, 14, 15). Our results show that active and inactive amino pools exist in the soybean hypocotyl.

Materials and Methods

Soybean seeds (*Glycine max*, var. Hawkeye 63) were germinated as previously described (9). After 3 days, a 1 cm section of hypocotyl was excised between 0.5 and 1.5 cm below the cotyledons and placed in a 1% sucrose solution at 2 to 4°. Sections were then randomized by agitation in the sucrose solution, collected in a mesh strainer, and rinsed with deionized water. After blotting, tissue was weighed as needed.

Unless otherwise stated, 1.5 g of tissue were incubated in a 50 ml culture flask containing 5 ml of incubation medium (1% sucrose and 0.01 M KH₂PO₄ brought to pH 6.0 with NH₄OH). On the average there were 48 hypocotyl sections/1.5 g

of tissue. All pretreatments involved incubating 10 g lots of tissue in 20 ml of medium in a 250 ml flask. The pretreated tissue was thoroughly rinsed over a mesh strainer and sections representing 1.5 g initial fresh weight were transferred to a 50 ml culture flask kept at 30°. All treatments were duplicated. The flasks were shaken in a darkened water bath maintained at 30°. After treatment, tissue was collected in a mesh strainer, rinsed, blotted, and frozen. In experiments involving a determination of lag-time, the tissue was rinsed with ice-cold deionized water followed by rapid freezing in pulverized dry ice.

Tissue was homogenized in a VirTis 45 homogenizer at a setting of 40 for 1 minute with 0.01 N Tris (pH 7.4) to give a final volume of 12 ml. The homogenate was filtered through glass wool, and a 6 ml portion of the filtrate was made to 10% trichloroacetic acid. After 1 hour at 2°, the samples were centrifuged 10 minutes at 500 × g. After dissolving the pellet in 3 ml of 0.5 N NaOH (containing 200 μg/ml each of ¹²C-leucine and ¹²C-valine), cold trichloroacetic acid was added to a final concentration of 9.5%. The precipitate was pelleted by centrifugation, washed twice with 5% trichloroacetic acid and then dissolved in 2 N NH₄OH. Radioactivity and total protein determinations were made using portions of the NH₄OH solution. Protein was measured by the method of Lowry et al. (10) using purified bovine serum albumin as a standard. Soluble radioactivities were obtained as the difference between the total radioactivity (measured by plating a portion of the Tris homogenate) and the protein radioactivity after corrections for differences in counting efficiencies. Wax-circled, one-eighth × 1 and one-fourth inch aluminum planchets were used for counting in a Nuclear Chicago thin window gas flow counter. All uniformly labeled ¹⁴C-amino acids were supplied

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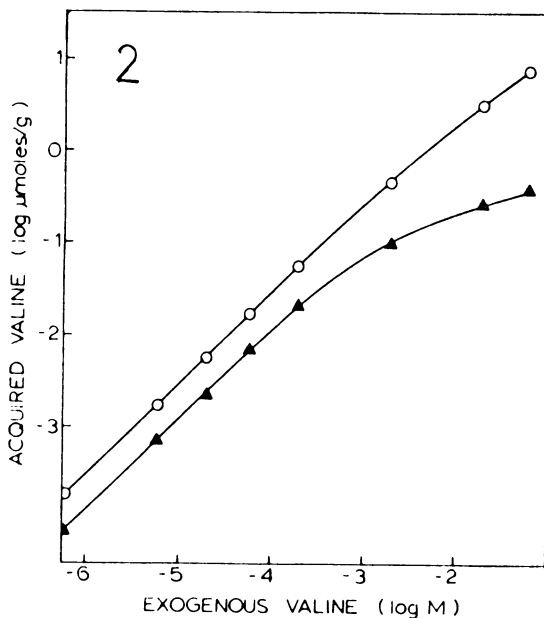
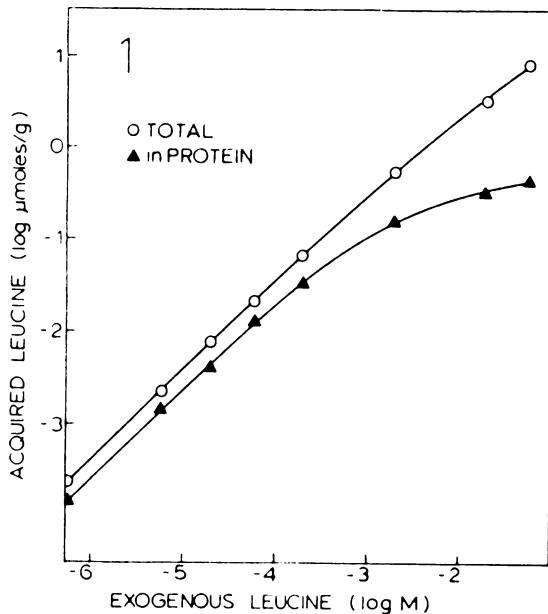


FIG. 1. Relationship between the external concentration and the uptake and incorporation of leucine. Tissue incubated for 90 minutes with no pre-incubation. Acquired leucine refers to the leucine taken up by the tissue. Amounts of acquired leucine calculated from the specific activities of exogenous leucine ($0.5 \mu\text{C}$ -leucine in each 50 ml flask together with the indicated concentration of ^{12}C -leucine). The term "g" in the above expression " $\mu\text{mole/g}$ " and in all subsequent expressions in tables and figures represents grams initial fresh weight of excised hypocotyl sections.

by Schwarz Bioresearch, Incorporated. Samples were paper chromatographed and confirmed to be radio-chemically pure. In all experiments involving tracers, $0.5 \mu\text{C}$ of amino acid ($168 \mu\text{C}/\mu\text{mole}$) was added to each 50 ml flask unless otherwise stated.

For an analysis of soluble amino acids, tissue was homogenized in 70% (v/v) ethanol and the homogenate centrifuged at $2000 \times g$ for 10 minutes at room temperature. The pellet was washed 3 times with 50% (v/v) ethanol, and all supernatant fractions were pooled. Quantitative determinations of individual amino acids were made with a Technicon Auto Analyzer. Amino acid composition of protein was determined after hydrolysis of the trichloroacetic acid precipitable protein with $6 \times \text{HCl}$ in sealed tubes for 14 hours at 122° . The hydrolysates were evaporated in vacuo over NaOH pellets and the residue dissolved in water for amino acid determination.

Definition of Terms

Total Pool (TP): that amount of an amino acid, not present in protein, which is extractible from the tissue by the above methods.

Precursor Pool (PP): that amount of an amino acid which is readily available for incorporation into protein. It is calculated by multiplying the PP output rate by the time which elapses prior to achieving a linear incorporation into protein of exogenously supplied amino acid (7). The PP output rate is equated with an experimentally measured rate of amino acid incorporation into protein.

Inactive Pool (IP): that amount of an amino acid which is not readily available for incorporation into protein. It is equal to TP minus PP.

Results

Relationship Between the External Concentration of Leucine and Valine and Their Uptake and Incorporation. The uptake and incorporation of ^{14}C -leucine and ^{14}C -valine were followed using a constant amount of radioactive amino acid and varying amounts of the corresponding ^{12}C -amino acid up to 60 mM in the external medium (figs 1, 2). Hypocotyl growth was depressed only at the highest amino acid concentration (7). Total uptake of the amino acids was proportional to the external concentration up to 2 mM. The incorporation into protein of acquired amino acid (i.e.

FIG. 2. Relationship between the external concentration and the uptake and incorporation of valine. The experiment was run concurrently and performed in an identical manner to that presented in figure 1 for leucine. Symbols explained in figure 1.

Table I. Rates of Incorporation into Protein of Leucine and Valine

Pre-incubation period (hr)	Tracer period (min)	¹⁴ C		mμmoles/g/min		
		Total	Protein	Rate of leucine incorporation***	Proportional rate of valine incorporation†	
0	30	3740	245	437	3.29	2.73
	90	6790	682			
3.0	30	5110	213	481	3.62	3.01
	90	9710	694			
3.0*	30	5350	224	493	3.70	3.07
	90	10200	717			
4.5	30	5500	249	514	3.87	3.21
	90	10250	763			

* Tissue incubated in large lots (10 \times /20 ml medium) and rinsed before the tracer period. This treatment is identical to that for control pretreated tissue.

** Values represent averages of 2 experiments with duplicate samples in each experiment. The external leucine concentration during the tracer period was 2×10^{-2} M. The symbol "g" in this expression and in all subsequent expressions in tables and figures represents grams initial fresh weight of excised hypococtyl sections.

*** Calculation based on the assumption that the specific activity of the leucine incorporated into protein was the same as that of the external leucine (1 μ C/100 μ moles).

† Calculated using the proportionality between valine incorporation and leucine incorporation at 2×10^{-2} M (figs 1, 2).

amino acid which had been taken up from the medium) was proportional to the external concentration only up to 0.06 mM for leucine and 0.2 mM for valine. Above these concentrations there was a proportionally smaller increase in the incorporation of exogenously supplied amino acid. This trend suggests a swamping of the PP with acquired amino acid at high external concentrations of amino acid. Therefore the specific activity of the endogenous amino acid should approach that of the exogenous amino acid at high external concentrations. Knowledge of the specific activity of the PP permits calculation of an absolute rate of amino acid incorporation into protein. Calculated rates for valine and leucine incorporation under various conditions are presented in table I. Incorporation rates increased slightly with increasing times of incubation.

Calculation of Protein Turnover. The rate of protein turnover was calculated using: A) the rate of incorporation of leucine and valine into protein (table I), B) the constant amount of protein in the tissue (11.4 mg/g) (7), and C) the leucine and valine content of the protein (9.3 μ mole leucine/g and 7.3 μ mole valine/g) (7). In this calculation the composition of the newly synthesized protein was assumed identical to the total protein. Likewise, the specific activities of the precursor and of the exogenous amino acids were assumed identical. With regard to leucine and valine, the first assumption is supported because the amounts of these 2 amino acids in the protein did not change during incubation of the tissue (7). Determination

of the protein turnover rate based on leucine incorporation with no pre-incubation involved the following calculations:

$$\frac{0.197 \mu\text{mole/g/hr}}{9.30 \mu\text{mole/g}} \times 11.4 \text{ mg/g} = 0.240 \text{ mg/g hour}$$

$$\frac{0.240 \text{ mg/g/hr}}{11.4 \text{ mg/g}} \times 100 = 2.1 \% \text{ hour.}$$

Similarly, turnover rates of 2.4%/hour based on valine incorporation (no pre-incubation) and of 2.5 and 2.6%/hour using the rates of leucine and valine incorporation, respectively, after a 5-hour pre-incubation were calculated.

Effect of Pre-Incubation on the Accumulation and Incorporation of Externally Supplied Amino Acid. The uptake and incorporation of ¹⁴C-leucine and ¹⁴C-valine over 30-minute periods at 1-hour intervals are shown in table II. Total uptake was enhanced about 250% by a 5-hour pre-incubation, and the ratio of protein to soluble radioactivity was about tripled for ¹⁴C-leucine and about doubled for ¹⁴C-valine. The rates of accumulation and incorporation are shown in figures 3 and 4. The lag-time was obtained by extrapolating back to the abscissa from the linear phase of incorporation (3). The lag-times for valine were 25 and 9 minutes, whereas those for leucine were 12.5 and 2 minutes after the 30-minute and 4-hour pre-incubation periods, respectively. In further experiments lag-times were determined after a 0.5-, a 2- and a 5-hour pre-incubation period (table III). The incorporation curves for exogenously supplied ¹⁴C-amino acid (figs 3, 4) followed the pattern expected

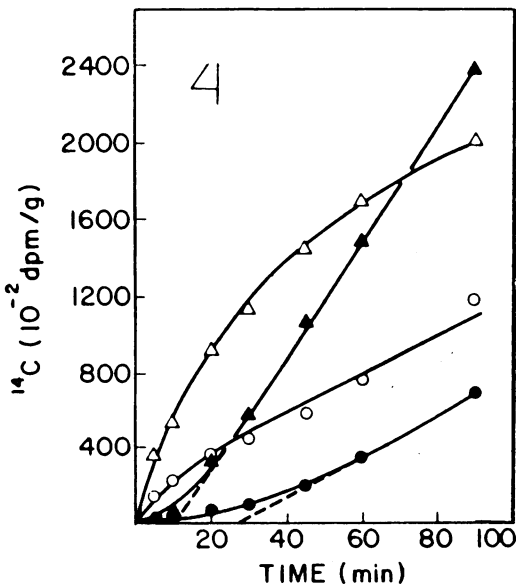
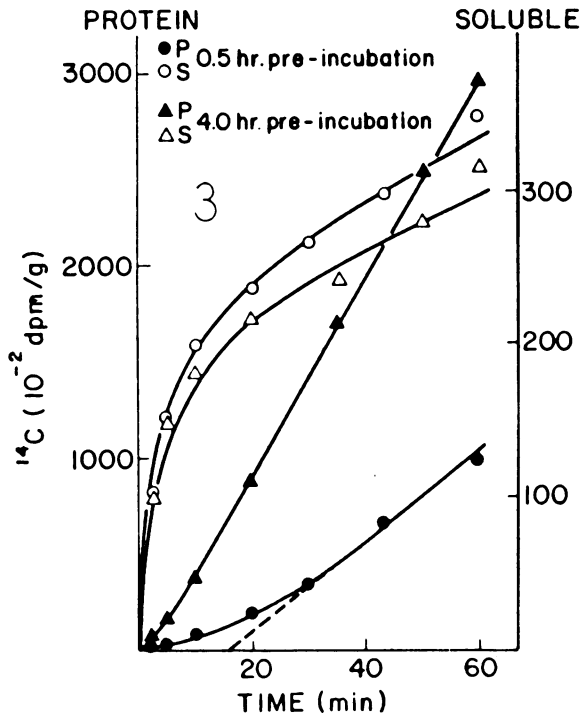


FIG. 3. Accumulation and incorporation of ^{14}C -leucine after a 30-minute and a 4-hour pre-incubation period. Protein (P) and soluble (S) radioactivities are shown.

FIG. 4. Accumulation and incorporation of ^{14}C -valine after a 30-minute and a 4-hour pre-incubation period. Symbols explained in figure 3.

if amino acids in the precursor pool (PP) were selected at random for incorporation into protein (3,7).

Since the approximate rates of total leucine and valine incorporation into protein were known (table I), the specific activity of the newly incorporated amino acid could be calculated from radioactivity measurements of the protein (figs 3,4). Using this specific activity, together with the amounts of total free leucine and valine (table III), calculations were made of the total soluble radioactivities expected if the total pools (TP's) supplied the amino acids for protein synthesis (7). For ^{14}C -leucine the expected radioactivities were 4- to 4.5-fold greater than the averages of the experimental values. Calculated radioactivities were 30 to 35 times too large in the ^{14}C -valine treatments. These calculations indicated that the pool which supplied amino acids for protein synthesis was only a part of the total pool.

The Effect of Pre-Incubation of Tissue on the Approximate Size of the Leucine and the Valine Precursor Pools. The absolute size of the PP is the PP output rate in $\mu\text{mole}/\text{min}$ times the lag-time in minutes (7). The previously determined rates of leucine and valine incorporation (table I) represent the PP output rates if it is assumed that virtually the entire PP output involves amino acid incorporation into protein. Using these rates, the approximate PP sizes were calculated (table III). Decreases in both the TP and the PP from the 0.5- to the 2-hour pre-incubation are contrasted to those from the 0.5- to the 5-hour pre-incubation. In both situations there was a relatively greater depletion of the PP than the TP.

Expansion of Pools. Lag-times were determined for tissue pretreated either with 5×10^{-3} M leucine or 1×10^{-2} M valine (table IV). The relative expansion of the PP with amino acid pretreatment was much greater than that of the TP, particularly for leucine. Approximate sizes and turnover times of the PP's are presented in table IV.

Pool Specificity. The effect of pretreatment with leucine or valine on the subsequent uptake and incorporation of the corresponding ^{14}C -amino acid and of ^{14}C -lysine is shown in table V. Valine pretreatment had no effect on the utilization of lysine in protein synthesis, whereas ^{14}C -valine incorporation was greatly depressed. Leucine pretreatment, however, slightly reduced the subsequent incorporation of lysine, although by a much smaller amount than that of ^{14}C -leucine incorporation.

Depletion of Expanded Pools. Since the ratio of protein to soluble radioactivity was higher when the tracer period was delayed until 60 minutes after pretreatment with ^{12}C -amino acid, compared to that of zero time (table V), it appeared that the PP sizes were changing. Therefore lag-times and amino acid concentrations were determined at zero and 60 minutes after pretreatment (table VI). Both in the depletion of expanded pools in pre-

treated tissue and in the depletion of non-expanded pools (table III), there was a proportionally greater decrease in the PP size than in the TP size. However, expanded pools were depleted more rapidly than non-expanded ones.

The Subsequent Fate of the Amino Acid Acquired During the Pretreatment Period. In order to follow the movement of amino acid acquired during the pretreatment period, a ^{14}C -amino acid was included in the pretreatment media. Thus, in

Table II. *Effect of Pre-incubation on the Accumulation and Incorporation of Externally Supplied ^{14}C -Leucine and ^{14}C -Valine*

After pre-incubation of tissue without added amino acid for the times indicated, 0.003 μmoles labeled amino acid was added to the appropriate flasks and the tissue collected after 30 min. Data represent averages of 2 experiments with duplicate samples in each experiment.

Pre-incubation period (hr)	^{14}C (10 ⁻² dpm/g)				Valine			
	Total	Protein	Soluble	Protein/soluble	Total	Protein	Soluble	Protein/soluble
0	367	162	105	1.54	190	51	139	0.37
1	353	211	142	1.49	295	80	215	0.37
2	549	391	158	2.47	423	128	295	0.43
3	709	536	173	3.10	557	199	358	0.56
4	838	653	185	3.53	611	233	378	0.62
5	923	750	178	4.21	693	289	404	0.72

Table III. *Effect of Pre-incubation on the Sizes of the Total and the Precursor Pools in Soybean Tissue*

Amino acid	Pre-incubation period (hr)	Total free amino acid ($\mu\text{mole/g}$)	% Depletion of TP*	Lag-time (min)	Approximate PP size		
					($\mu\text{mole/g}$)**	% Depletion*	% TP
Leucine	0.5	0.479	16	15.0	49.5	53	10.0
	2.0	0.404		6.5	23.5		5.8
	0.5	0.479	35	15.0	49.5	94	10.0
	5.0	0.313		0.8	3.1		1.0
Valine	0.5	4.05	6	27.0	74.0	27	1.8
	2.0	3.80		18.0	54.0		1.4
	0.5	4.05	24	27.0	74.0	53	1.8
	5.0	3.09		11.0	35.5		1.2

* Pool depletion refers to the percentage loss of amino acids initially present in the pool.

** Values obtained by multiplying the appropriate rate of total amino acid incorporation into protein (table I) by the lag-time.

Table IV. *Effect of an Amino Acid Pretreatment on the Size of the Total and Precursor Pools*
Tissue was pretreated for 3 hours.

Amino acid	Pre-treatment concentration (mM)	Total free amino acid ($\mu\text{mole/g}$)*	Relative expansion of the TP	Lag-time (min)*	Approximate PP size			Rate of PP turnover (pool equivalents/hr)***
					($\mu\text{mole/g}$)**	Relative expansion	% of the TP	
Leucine	0	0.43		0.3	1.1		0.25	200.0
	5	1.19	1.80	19.0	70.0	64	5.9	3.2
Valine	0	3.94		6.0	18.5		0.47	10.0
	20	4.88	0.25	33.0	101.0	5.5	2.1	1.8

* Values represent averages of 2 determinations.

** See table III for method of calculation.

*** A pool equivalent is that amount of material which is present in a pool at any particular time. Values above obtained by dividing the appropriate rates of amino acid incorporation (table I) by the approximate PP sizes.

Table V. *Effect of an Amino Acid Pretreatment of the Subsequent Uptake and Incorporation of Exogenously Supplied ¹⁴C-Amino Acid*

Amino acid pre-treatment*	Tracer period (min)	¹⁴ C (10 ⁻² dpm/g)								
		Leucine			Valine			Lysine		
		Protein	Soluble	Protein/soluble	Protein	Soluble	Protein/soluble	Protein	Soluble	Protein/soluble
None	0-15	434	327	1.33	187	481	0.39	753	727	1.04
	60-75	536	362	1.48	298	550	0.58	776	689	1.13
Leucine	0-15	25	483	0.05	468	672	0.70
	60-75	173	471	0.37	653	647	1.01
Valine	0-15	33	523	0.06	734	666	1.10
	60-75	129	600	0.22	782	728	1.08

* The 4-hour pretreatment period involved adding 100 μ moles amino acid per 20 ml medium initially and again after 3.5 hours.

Table VI. *The Depletion of Expanded Amino Acid Pools*

Tissue was pretreated with either 5 mM leucine or 20 mM valine for 3 hours.

Amino acid	Time after pre-treatment (min)	Total Free amino acid (μ mole/g)*	% Depletion of the TP	Lag time (min)*	Approximate PP size		
					(μ mole/g)**	% Depletion	% of the TP
Leucine	0	1.19		19.0	70.0		5.9
	60	0.89	25	1.3	4.8	93	0.54
Valine	0	4.88		33.0	101.0		2.1
	60	4.30	12	13.4	41.5	59	1.0

* Values represent the averages of 2 determinations.

** See table III for method of calculation.

contrast to previous experiments, the ¹⁴C-amino acid was present during the pretreatment period and not during the treatment period. Changes in the protein and the soluble radioactivities for 2.5 hours following the 3-hour pretreatment period are shown in figures 5 and 6 for leucine and valine, respectively. For all tissue pretreated with a ¹²C-amino acid together with the ¹⁴C-amino acid, there was a marked increase in the protein radioactivity over the following 2.5 hours. For tissue incubated only with the ¹⁴C-amino acid (control), there was an increase in the protein radioactivity following pretreatment with ¹⁴C-valine, but not following pretreatment with ¹⁴C-leucine even though the soluble radioactivity decreased. In experiments using ¹⁴C-valine, the only other detectable labeled compound in the soluble and protein fractions was ¹⁴C-leucine. This valine-derived leucine was much more abundant in the protein than in the soluble fraction (7). The only labeled amino acid present in protein following ¹⁴C-leucine feeding was leucine (7).

Discussion

Evidence is presented for 2 distinct amino acid pools in the soybean hypocotyl. Changes in the

size of a protein precursor pool (PP) were compared with changes in the total amount of the soluble amino acid (TP). For leucine and valine, the PP's were depleted more than the TP's during incubation of excised tissue. The leucine and valine PP's represented only a small percentage of the TP's. After a 0.5-, a 2- and a 5-hour preincubation period, leucine in the PP comprised about 10%, 6% and 1% of the total free leucine, respectively. The valine PP comprised 1.8% to 1.2% of the valine TP. Calculated rates of PP turnover (table IV) show that the leucine pool, in a statistical sense, turned over about 20 times faster than the valine pool. After removal of exogenous sources of labeled amino acid from control treatments, the rapidly turning-over leucine PP ceased supplying ¹⁴C-leucine for protein synthesis, whereas incorporation of ¹⁴C-valine from the valine PP continued (figs 5, 6).

After pretreatment of tissue with high concentrations of leucine or valine, the lag-times were increased proportionally more than were the amounts of free amino acid. In contrast to the 2.8- and 1.25-fold expansions of the TP's, the leucine and valine PP's were enlarged about 65- and 6.5-fold, respectively. After removal of exogenous ¹⁴C-leucine, the expanded leucine PP continued to

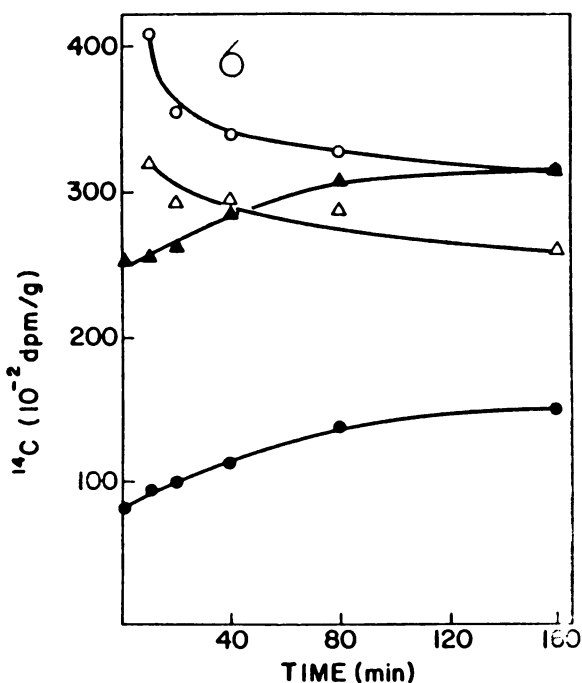
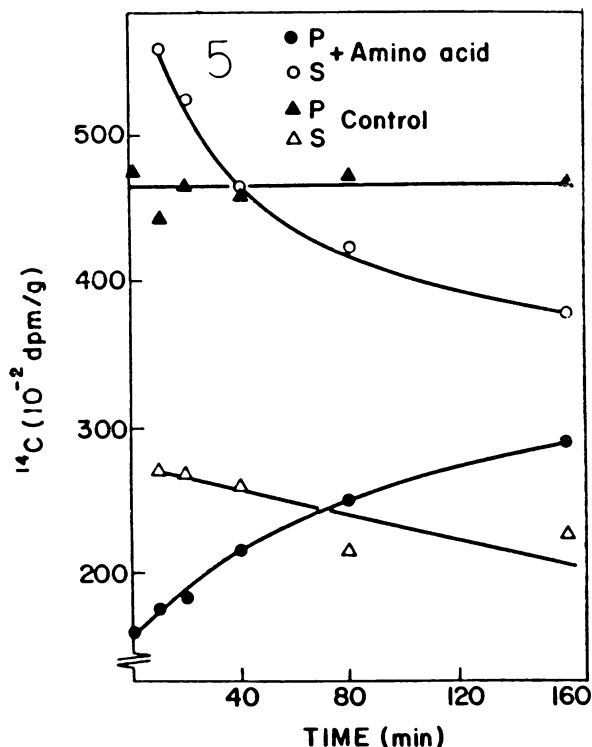


FIG. 5. The subsequent fate of ^{14}C -leucine acquired during pretreatments with or without 5×10^{-3} M leucine. Protein (P) and soluble (S) radioactivities are shown.

FIG. 6. The subsequent fate of ^{14}C -valine acquired during pretreatments with or without 1×10^{-2} M valine. Symbols explained in figure 5.

supply labeled amino acids for protein synthesis, whereas the non-expanded pool did not (fig 5). The non-expanded leucine PP turned over about 50 times faster than the expanded pool (table IV). Expansion of endogenous pools by amino acid pretreatment has been reported for *E. coli* (3) and for maize root tips (15).

After transfer of pretreated tissue to unsupplemented media, the PP size decreased more rapidly than the TP size. Moreover, these expanded PP's depleted much faster than native PP's with increasing times of incubation. Tables III and IV show that whereas the native leucine and valine PP's were depleted 94% and 53%, respectively, over a 4.5-hour incubation period, expanded pools were depleted by almost these same percentages during a 1-hour incubation. Suggested reasons for the rapid depletion of expanded pools are: A) leucine and valine pretreatment depressed biosynthesis of these amino acids by end product inhibition as shown by Oaks for maize root tips (13), and B) the extensive distilled water rinsing of pretreated tissue leached out intercellular solutes and created a diffusion gradient wherein amino acids moved out of the cytoplasm and into the tissue free space. This possible effect of the distilled water rinsing might explain the extremely small size of PP's in tissue pretreated without amino acid.

The presence of PP's in the cytoplasm is a reasonable inference since the sites of protein synthesis are in the cytoplasm (18). In the light of Cowie's suggestion (4) that the PP is composed of protein forming templates, the total μmoles of leucine and valine present in non-expanded PP's at 30 minutes were calculated and found to be about 12 times greater than the total amount of soluble RNA in soybean tissue (7). This difference strongly suggests that the PP in soybeans is not composed of amino acyl *t*-RNA. The inactive pool of amino acids (IP) may be located in the vacuole. This concept of the vacuole as a sink for inactive substances in plant cells was suggested by Stiller (17), MacLennan et al. (11), and Baker and Ray (1).

Without an exogenous source of amino acids, the PP's in excised soybean hypocotyl could be supplied with newly biosynthesized amino acids and/or amino acids exported from other pools. The conversion of ^{14}C -valine to ^{14}C -leucine shows that the terminal enzymes needed for leucine and valine biosynthesis were active in hypocotyl tissue (7); thus amino acid biosynthesis could be providing amino acids directly to the PP. Considering the proportionally greater depletions of the PP's than of the IP's during incubation, movement of amino acid from IP to the PP appears to occur very slowly if at all.

The movement of amino acids into the hypocotyl may have been considerable prior to its severance from the cotyledons. Studies on seedlings of oat (6), cucumber (16) and corn (8) suggest that

amino acids are transported to the plant axis at a rate about equal to that of hydrolytic release of amino acids in the storage organs. Starting about 2.5 days after soybean germination, there was a doubling in the amount of soluble α -amino nitrogen over the next 12 hours in the apical portion of the hypocotyl, while the protein level increased by 55% (7). Although no protein or amino acid estimations were made on the senescing cotyledons, considering the work cited above, there probably was a decrease in the amount of storage amino acid (both free and in the protein) correlating with the increase in amino acids in the hypocotyl.

Oaks (14) reported that transport amino acids were readily incorporated into the protein of the root tip. From this observation, it was inferred that a transported amino acid directly enters the PP. Thus, we suggest that the soybean hypocotyl is deprived, after excision, of this source of amino acids and that such deprivation may partially explain the subsequent rapid depletion of the leucine and valine PP's during hypocotyl incubation.

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