The Soluble Leucine Pool in Maize Root Tips [1, 2]

Ann Oaks [3]

Department of Biological Sciences, Purdue University, Lafayette, Indiana

Experiments with excised maize root tips have shown that an exogenous supply of amino acids can effectively inhibit the synthesis of threonine, valine, leucine, lysine, arginine, and proline from acetate-2-C^{14} (18). In addition it has been suggested that the metabolites in the root tip region are normally maintained by the supply from other regions of the embryo (3, 18, 26). If this is true, it is possible that the ready supply of amino acids from older regions of the embryo could control the endogenous synthesis within the tip region as the exogenous amino acids do. This prediction would require a reduction of the pool level followed by an increased rate of synthesis of the amino acids after the removal of the transport system. It was therefore the aim of this investigation to define the nature of the soluble pool for a model amino acid in the maize root tip. Since leucine is one of the amino acids potentially supplied in large amounts by the maize endosperm (21), it was chosen for this purpose.

Methods

Maize seed (hybrid var. Wf 9×38-11) was soaked overnight in water, and allowed to germinate on damp filter paper for an additional 48 hours at 30°. At this time the roots were 4 to 5 cm long.

Leucine-U- or -1-C^{14} and acetate-2-C^{14}, of specific activities 242 mc/mmole, 4.68 mc/mmole and 7.3 mc/mmole, respectively were supplied from commercial sources. A final concentration of about 10^{-8} M was generally used.

In experiments with intact roots the tracer was applied to a specific region in agar strips (1.5 %) that were 2 mm wide. Excised root tips were routinely treated for 3 hours in a 1 % surcrose-salts solution (17) to allow the amino acid pool to stabilize. They were then washed, and placed in 50 ml Erlenmeyer flasks with 2 ml of fresh sucrose-salts solution and the C^{14}-compound. Since preliminary experiments showed that the uptake and utilization of substrate was the same from agar or liquid, the results with intact and excised roots are comparable. Twenty root tips were used per sample, and similar concentrations of inorganic salts and radiotracer were used.

After the experiment, the sections were washed with a solution of leucine-C^{12} when C^{14}-leucine had been used, and water. They were then homogenized in 80 % (v/v) ethyl alcohol. The alcohol insoluble residue was washed several times with a solution of leucine-C^{12} in ethyl alcohol. Hydrolysis was performed in a closed tube with 6 N HCl for 12 hours at 100°. The extracts containing the soluble amino acid or amino acids of the residue were taken to dryness in vacuo at 40°, and the amino acids were isolated with Dowex-50 (H^+) resin (15). Aliquots of the amino acid samples were taken for total activity, or were chromatographed 1-dimensionally on Whatman No. 1 paper in an *n*-butanol: acetic acid: water (3: 1: 1) solvent, or 2-dimensionally with water-saturated phenol as the second solvent. The radioactive areas were located on X-ray film, and the material contained was eluted for the determination of radioactivity and α-amino nitrogen content. When leucine-C^{14} was fed, only 1 radioactive area corresponding to leucine was detected on the chromatogram. When the residue was exposed to trypsin, or was hydrolyzed with 1 N HCl for 48 hours at 25°, several radioactive areas were prominent, while the region corresponding to leucine was scarcely visible on the X-ray film. Thus adsorption of C^{14}-leucine to the residue was not an important source of error.

A Kjehldahl digestion followed by nesslerization was used for the nitrogen determinations (11). The α-amino nitrogen was determined with ninhydrin (28).

A balance sheet for the utilization of leucine-U-C^{14} by excised root tips is shown in table I. Leucine was either supplied continuously for 2 hours, or was pulse fed for 10 minutes, followed by an additional 2 hours in a 1 % sucrose-salts solution. In both cases the release of C^{14} as CO_2 was minor. In addition very little tracer-leucine was lost to the medium in the pulse experiment. When leucine was present all the time, about 70 % of the tracer was recovered in the alcohol insoluble residue; in the pulse experiment the proportion in the residue was less. Thus leucine is not extensively metabolized in maize roots, and is, therefore, a good amino acid to use in studying the protein-precursor relationship.

Results

Kinetics of the Incorporation of Exogenously-Fed Leucine into Root Tip Protein. Tracing the flow of carbon over very short time intervals has proved to be

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[3] Present address: Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Table I. *Utilization of Leucine by Maize Root Tips*

Leucine-U-C^{14} (specific activity 242 $\mu c/\mu mole$; 10^{-8} M final concentration) was present either over a 2-hour period (continuous), or was pulse fed for 10 minutes followed by a 2-hour period in a 1% sucrose-salts solution. Twenty excised roots were used per sample.

	Continuous C^{14}		Pulse C^{14}	
	cpm	% Distribution of leucine recovered	cpm	% Distribution of leucine recovered
Initial	76128	...	6890*	...
CO_2	3690	5.7	173	2.4
Soluble	13683	21.2	3178	44.3
Residue	47376	73.0	3498	48.7
Medium	...	...	306	4.3
Total recovered	64749	100	7155	100

* The initial C^{14}-content in the pulse experiment was the total C^{14} recovered from the root tips at 10 minutes.

a useful technique in dissociating the various soluble pools of a metabolite, and in showing which of the pools leads directly to the macromolecule (1, 4, 5). In such a kinetic study, the precursor pool is saturated with the tracer. When a steady state has been established the tracer enters and leaves the pool at an equal rate and the increase of tracer in the product is linear with time. If the precursor pool is very small a linear rate of increase in the product is observed from the earliest times; if the pool is large, there is a lag before the linear increase is observed (1).

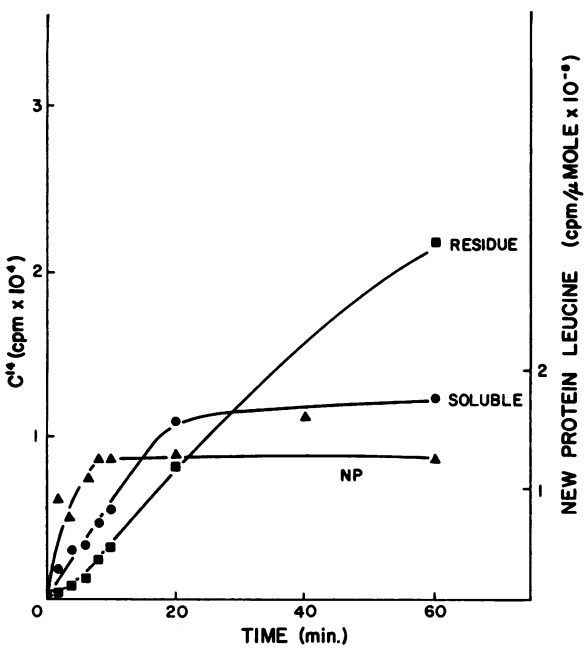

FIG. 1. The incorporation of exogenously fed leucine into root tip protein. Leucine-U-C^{14} (242 mc/mmole) with a final concentration of 10^{-8} M was fed to the root tips from agar strips. Samples of 20 five-mm tips were examined at the times indicated. The curve NP represents the specific activity of the new protein formed (increase of C^{14} in the residue relative to the increase in μmoles of leucine in the residue).

When leucine-U-C^{14} was fed locally to maize root tips (fig 1), the increase of C^{14} in the soluble fraction was linear over the first 20 minutes. After this time increases were minor. The incorporation of C^{14} into protein showed a lag of about 6 minutes before a linear increase was attained. According to Britten and McClure (1), this lag represents the time required to saturate the pool leucine that is the precursor for protein. This pool which leads to protein was saturated some 14 minutes before the total soluble pool.

When leucine-C^{14} was pulse fed to intact roots for 20 minutes, there was, subsequently a transfer of tracer from the soluble to the insoluble fraction (fig 2). Some 40 minutes after the removal of the tracer, a new equilibrium was established. Although the times involved in the pulse experiments were variable, there was always a transfer of soluble C^{14} to the insoluble residue, and when the new equilibrium was reached from one-third to one-half of the original, soluble leucine remained in the soluble fraction. Similar trends have been obtained with excised roots. Moreover, with excised roots there was essentially no change when 10^{-5} M C^{12}-leucine was administered immediately after the pulse. Similar chase experiments with resting *Escherichia coli* (10) or cultured animal cells (6) resulted in a loss of protein C^{14}.

If leucine were entering a protein precursor pool and a general soluble pool that was relatively inaccessible to protein synthesis, a new steady state would be reached when the C^{14} in the precursor pool was exhausted. Since the kinetics were not altered in the chase experiments, this interpretation rather than one involving protein turnover (8) is favored here.

Incorporation of Leucine by Excised Root Tips. A critical question which arises from these kinetic experiments concerns the fate of the soluble pool when one of the sources of soluble leucine, the transport system, is removed. Kinetic experiments with excised roots which had been aged for 3 hours in a sucrose-salts solution showed that, as with intact roots

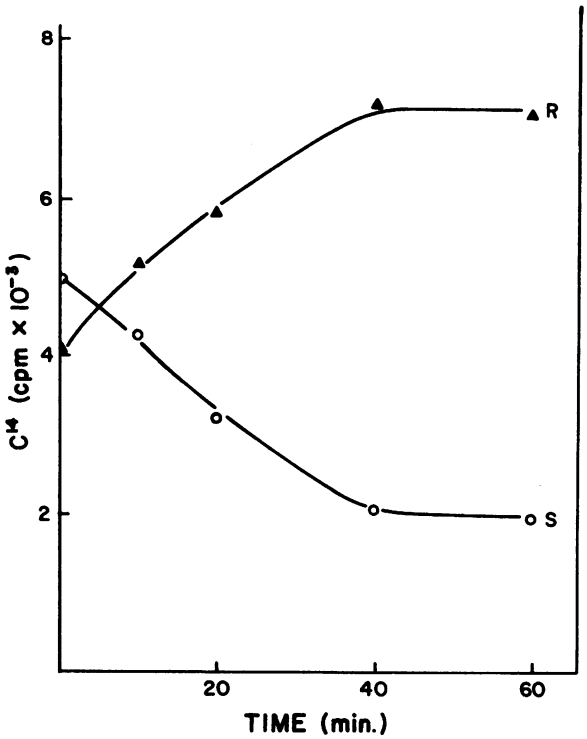

FIG. 2. The transfer of leucine from the soluble to alcohol insoluble residue: leucine-1-C^{14} (4.68 mc/mmole, final concentration 10^{-7} M) was given to intact roots in agar strips for 20 minutes. Time is in minutes after the removal of the tracer. R is the C^{14} recovered in the residue, S the C^{14} in the alcohol soluble fraction.

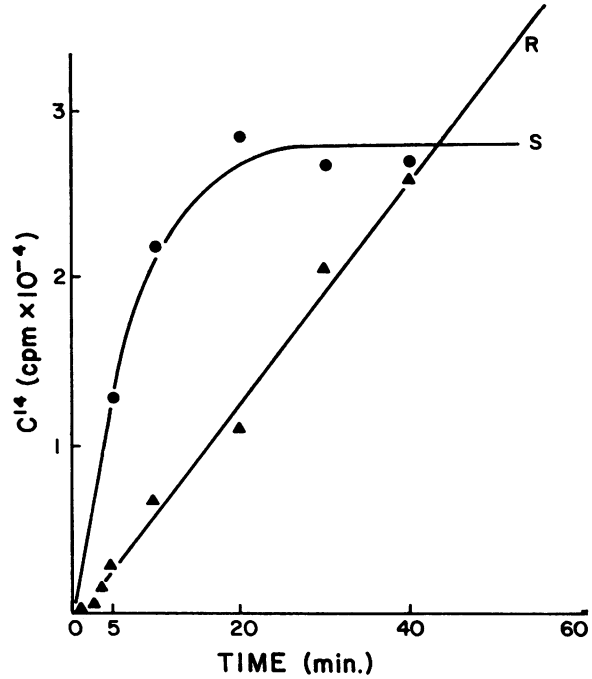

FIG. 3. The incorporation of exogenously fed leucine by excised root tips. R is the C^{14} in the residue, S the C^{14} in the alcohol soluble fraction. Leucine-U-C^{14} (242 mc/mmole; 10^{-8} M) was given after a 3-hour treatment in a sucrose-salts solution.

with the same concentration of tracer, 20 minutes were required to saturate the soluble pool (fig 3). However something less than 1 minute was required to saturate the protein precursor pool. Apparently the pool through which exogenous leucine must pass on its ways to protein was much smaller after excision. The results in table II compare the levels of soluble leucine in intact and excised maize root tips. The total pool was determined analytically with an amino acid analyzer. Then assuming that the time of saturation was proportional to the pool size, the size of the protein precursor pool could be estimated. In the intact tips 30 % of the soluble pool was found in the protein precursor pool; in the excised tips on the other hand only 10 % of the total pool was in this fraction. The removal of the transport system invoked a drastic reduction of protein precursor pool, while the effect on the remaining pool leucine was slight.

Movement of Leucine to the Root Tip. Characteristically about two-thirds of the total nitrogen in the 5 mm tip of maize roots was found in the alcohol insoluble residue (table III, column 4). In other regions of the root, the soluble nitrogen accounts for the greater part of the total nitrogen. It was therefore possible to visualize the transport of soluble nitrogen from regions of relatively high concentration to a region of relatively low concentration. When tracer-leucine was given to the scutellum or to a region of the root 4 cm from the tip, most of the tracer remained in the region of application (table IV,

Table II. *Effect of Excision on the Soluble Pool*

All values are given in μmoles/20 tips. The experimental procedure is described in figure 1 and figure 3.

	Intact μmoles	Excised μmoles	Excised/intact %
Total pool	0.189	0.114	60.5
Metabolic pool*	0.057	0.011	19.4
Residual pool	0.132	0.103	78.1

* Metabolic pool $= \frac{\text{Equilibration of metabolic pool (min)}}{\text{Equilibration of total pool (min)}} \times$ total pool (μmoles). This calculation assumes that the equilibration time is directly proportional to the size of the pool.

Table III. *Distribution of Leucine in the Maize Root*

Known amounts of soluble and insoluble material were digested in concentrated H_2SO_4 for nitrogen determination by nesslerization (11). The α-NH_2 nitrogen was measured with ninhydrin (27).

cm from tip	2.5–5.0	1.5–2.5	0.5–1.5	0–0.5
	1	2	3	4
	Nitrogen content (μg/section)			
Alcohol insoluble	53.2	11.8	10.4	17.8
Alcohol soluble	89.0	22.2	16.6	12.8
Soluble α-NH_2-N	34.0	9.0	7.0	5.2
Ratio soluble : insoluble	1.67	1.88	1.60	0.72

column 1). There was also a general movement from the point of application to the growing region, as has been found with other tracers (16, 22, 27). The specific activity of the leucine recovered in the tip (the receiver) was higher than in the 1 cm adjacent to the tip (the donor). This was observed at various time intervals and with various radiotracers. Presumably most of the leucine which moved to the tip was in an isolated system. Upon extraction the specific activity of the tracer leucine was diluted by the nonlabelled leucine in the cells. Consequently neither a concentration gradient from older to younger regions nor the rate of transport could be established.

Transport Leucine vs. Leucine Synthesized from Acetate-2-C¹⁴. The kinetics of the incorporation of the transport leucine into tip protein, when leucine was fed to the root 4 cm from the tip, is shown in figure 4. The increase in tracer-leucine in the alcohol insoluble residue was essentially linear after the first 60 minutes. There was also an increase in the alcohol soluble C^{14} throughout the experimental time. This shows that the transport leucine was saturating a particular protein precursor pool before all of the soluble leucine was saturated with tracer.

Likewise when leucine was made from acetate-2-C^{14} (fig 5) the protein-precursor leucine appeared to be distinct from the bulk of the soluble leucine. In this case the active pool may be vanishingly small since

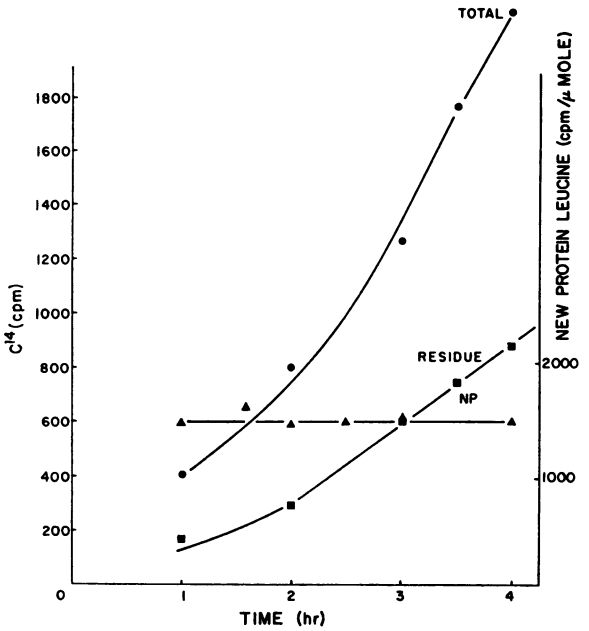

FIG. 4. The incorporation of transport leucine into root tip protein. Leucine-1-C^{14} (4.68 mc/mmole) was given locally to the root at a region 4 cm from the tip. The experimental conditions are described in figure 1.

very little tracer-leucine was recovered in the soluble fraction.

Rate of Protein Synthesis. If the transport leucine does influence the endogenous synthesis, the pools for synthesis and transport cannot be completely isolated. Thus, if there is 1 protein precursor pool, the rates of protein synthesis (increase in protein-C^{14}/specific activity of the precursor) should be the same in the situations described in figures 4 and 5. Usually the rate of protein synthesis or turnover is calculated from the rate of change of tracer in the protein relative to the specific activity of the precursor. If the specific activity of the pool leucine were the same as the specific activity of the precursor, the rate of protein synthesis for leucine made from acetate

Table IV. *Transport of Leucine to the 5-mm Root Tip*

Leucine-1-C^{14} was given to the scutellum, or a region of the root for 2 hours. After this time the critical regions of the root and application were excised and extracted as described in the Methods.

Leucine-1-C^{14} applied at:	Region of application of tracer	Leucine-1-C^{14} recovered in the alcohol soluble fraction cpm/20 sections	
		Regions of root (cm from tip)	
		0.5–1.5	0–0.5
Scutellum	63025	1203	1967
Root : 4 cm from tip	20950	865	1020
		Specific activity	(cpm/μg α-NH_2N)*
Scutellum	...	59.5	90.1
Root : 4 cm from tip	...	36.0	65.0

* No correction was made for the isoleucine present.

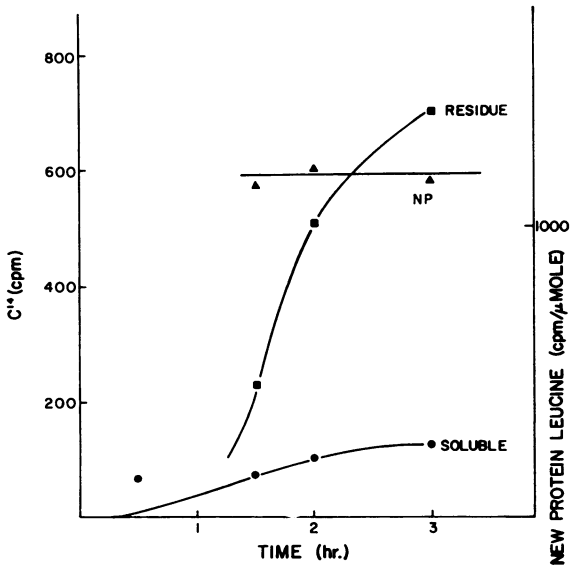

FIG. 5. The incorporation into protein of leucine made from acetate-2-C^{14}. Acetate-2-C^{14} (7.3 μc/μmole; 10^{-7} M) was fed to the root tips from agar strips. Experimental conditions are described in figure 1.

would be 5 times greater than for leucine supplied by the transport system (table V, column 5). However the kinetic data in figure 5 showed that very little of the leucine made from acetate was trapped in the soluble fraction. Thus the dilution by nonlabelled leucine would be relatively large and the rate of incorporation of leucine into protein would be correspondingly higher than the real rate. In addition, the similarity between the rate of incorporation of transport-leucine into protein and the net increase of leucine in the root tip protein was probably fortuitous, since the specific activity of the soluble leucine was still increasing at 4 hours.

The problem was, then, to establish the specific activity of the real protein precursor. In growing roots there is a reproducible net synthesis of alcohol insoluble nitrogen in the tip region (fig 6), which under the experimental conditions used here was 43 μg nitrogen/hour for 20 tips. Since 4.4 % of the alcohol insoluble nitrogen in the 5-mm tip was leucine (unpublished), this rate could be converted to 0.136 μmole leucine/hour for 20 tips. If it is assumed that a specific precursor pool is donating leucine to protein, and that little of the newly incorporated leucine is lost by protein degradation, the specific activity of the precursor should be similar to the specific activity of the new protein formed after a steady state is reached. Calculations for the specific activity of the new protein (increment of protein-C^{14} per unit time relative to the increase in protein-leucine per unit time) are shown in figures 1, 4 and 5 by the curve NP. When a linear increase of C^{14} into protein was observed, the specific activity of the new protein leucine was constant (fig 1). The coincidence of the 2 curves, when leucine was fed directly to the tip, suggests that the calculation for the specific activity of the new protein, or protein precursor is valid. When leucine was sup-

Table V. *Protein-Precursor for Leucine in Maize Root Tips*

Data from the experiments described in figures 1 (transport), 2 (synthesis intact), 4 (exogenous leucine: intact), and 5 (exogenous leucine: excised) were used. Thus transport represents leucine supplied by the transport system; synthesis, leucine made from acetate-2-C^{14}, and exogenous, leucine administered directly to the root tip. The specific activity values represent the steady state values where possible; for the transport system the specific activity of the total soluble pool at 4 hours was used; for endogenous synthesis a time of 2 hours for both excised and intact roots was used. The specific activity for the protein was determined at 2 hours, with the exception of the transport system, which was determined at 4 hours. The increase in C^{14}/hour in the residue is the average obtained after the rates were constant. The net increase of protein-leucine was 0.136 μmole/hour in 20 tips.

Source of leucine	Specific activity cpm/μmole			Increase in residue	Rate of incorporation of C^{14}-leucine into the residue* (μmoles/hr in 20 tips)	
	1 Total pool	2 New protein	3 Total protein	4 cpm/hr	5 SA_{sol}*	6 $SA_{N.P.}$*
Transport	3370	1579	362	412	0.122	0.261
Synthesis						
Intact	442	1178	222	284	0.643	0.242
Excised	702	...	595	...	...	...
Exogenous						
Intact	59300	136900	14187	1891	0.032	...
Excised	110000	...	...	39150	0.356	...

* The rate is the increase in C^{14}/hour in the residue relative to the specific activity of the precursor: i.e., SA_{sol} is the rate relative to the specific gravity of the total soluble pool; $SA_{N.P.}$ the rate relative to the specific activity of the new protein.

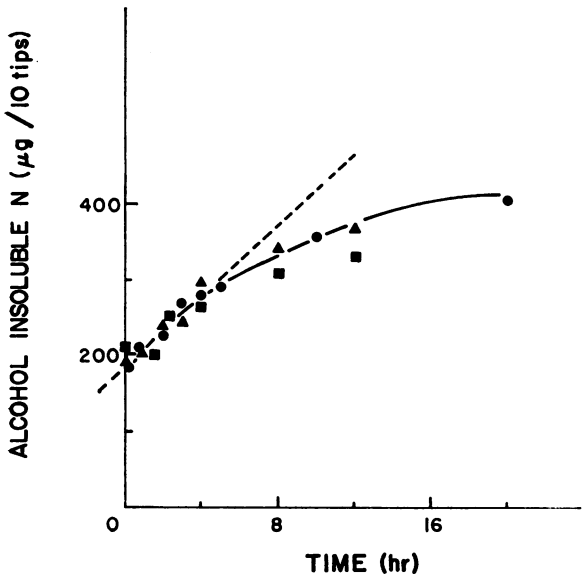

FIG. 6. The increase in alcohol-soluble nitrogen in the root tip region. Twenty tips representing the original 5-mm tip, which was marked at time zero, and the new growth were taken at the times indicated and extracted with 80% (v/v) ethyl alcohol. The alcohol-insoluble residue was used for the nitrogen determinations. The seedlings were grown on moist filter paper; the concentration of inorganic salts was similar to that used in the experiments described in figures 1, 4, and 5. The circles, triangles, and squares represent experiments performed at different times; each point is the mean of triplicate determinations. The solid curve is the observed increase in alcohol-insoluble nitrogen for 10 root tips; the dashed line, an extrapolation of the initial linear increase from which the rate of increase was calculated.

plied by the transport system (fig 4) or by endogenous synthesis (fig 5) the specific activity of the new protein leucine had already reached a constant value with the earliest samples. When the specific activity of the new protein was used to calculate the rate of protein synthesis, the rates were the same for the 2 sources of leucine (table V, column 6). The results, therefore, suggest that a common precursor pool is involved at some stage in the synthesis of protein. In addition this calculated rate of incorporation is higher than the net increase of protein leucine, and protein turnover is indicated. Protein turnover in this instance is thought of as a development of new enzyme complements with differentiation as proposed by Brown (2) rather than a protein cycle supplying carbon for respiration (24).

Synthesis of Leucine from Acetate-2-C^{14}. Comparisons of the rate of synthesis of leucine itself in intact and excised root tips are difficult because of the fact that much more radioactive acetate was taken up by the excised roots. Thus, although the specific activity of the protein-leucine was higher in the excised roots (table V, column 3), this increase relative to an internal standard, such as malic or glutamic acids, was erased. Perhaps it is relevant that the specific activity of the protein leucine relative to the specific activity of the soluble leucine increased from about 0.5 in the intact tips to about 0.8 after excision after a 2-hour exposure to acetate-2-C^{14}. Such a shift would be expected even if only part of the protein precursor were supplied by the transport system.

Discussion

Two major sources of amino acids can supply the precursors necessary for the net increase of protein in the root tip region: the transport from senescing tissue and the endogenous synthesis by the young plant cells. Both mechanisms have been demonstrated for leucine, the model amino acid used to define the protein-precursor relationship in the maize root tip. In addition, amino acids derived from protein degradation may also serve as precursors for the new proteins being formed. These reactions are illustrated in figure 7. The existence of more than 1

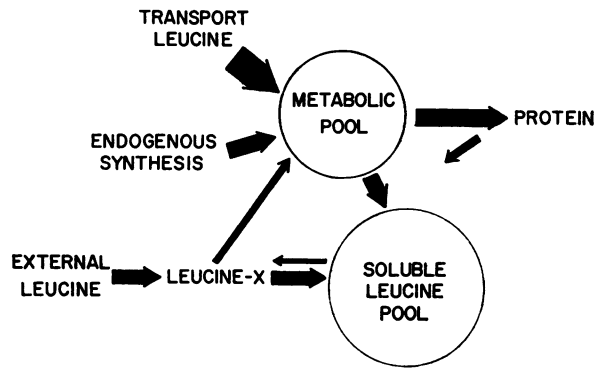

FIG. 7. The soluble leucine pool in maize root tips.

soluble pool is indicated by the kinetics of incorporation of exogenously fed leucine into root tip protein (fig 1). For simplicity these have been designated the metabolic and soluble leucine pools. In addition, the unequal dilution of leucine found in the total soluble pool and in the new protein (table V) suggests that exogenous leucine is entering each pool independently, possibly via a carrier, leucine-X. Leucine made from acetate, endogenous synthesis, appears to pass selectively through the metabolic pool on its way to protein. Two observations support this suggestion: A) The amount of tracer-leucine recovered in the soluble fraction was small, and B) the specific activity of the leucine in the new protein was almost 3 times that of the soluble leucine. Leucine supplied by the transport system is believed to be the dominant source of leucine in the maize root tip. Half the transport-leucine in the 5-mm tip was recovered in the soluble fraction (fig 4). Thus its contribution to the soluble pool is potentially greater than that of leucine supplied by endogenous synthesis. When leucine was supplied by the transport system, the specific activity of the new protein leucine was less than the specific activity of the total pool. Thus this

form of leucine is not strictly confined to the metabolic pool, as leucine made from acetate appears to be. In addition this observation suggests that a supply of nonlabelled leucine was selectively entering the protein-precursor pool. This other leucine could be coming from endogenous synthesis, from protein turnover, or from both sources.

When the incorporation of leucine into protein, relative to the specific activity of the protein precursor pool (specific activity of the new protein leucine) was calculated, the rates were similar for leucine supplied by endogenous synthesis and by the transport system (table V, column 6). That is the same protein precursor is involved at some stage in the incorporation of leucine into protein, and the same general proteins are made. Therefore the possibility that the transport leucine exerts a control over the endogenous synthesis is real. Similar arguments may be proposed for leucine derived from protein turnover; however, the appearance and fate of this leucine is unknown, and the possible controls exerted by the amino acid level are obscure (9, 13).

The localization of pools in the vacuoles or other cytoplasmic inclusions has been discussed by Folkes and Yemm (7) and Steward and Bidwell (25). Accordingly a portion of the soluble pool (fig 7) could represent leucine localized in the vacuole. This interpretation is supported by the results of MacLennan et al. (12) who found that a large part of the acids of the tricarboxylic acid cycle were in an inactive pool, and that the proportion of the acids in this pool increased with vacuolation. The metabolic pool or pools of leucine may be located in less discrete units within the cytoplasm or they may be chemically distinct species of leucine.

If the transport leucine is the dominant form, its removal should elicit a reduction in the level of the soluble pool. The decrease in the total pool leucine after excision is real, but not large. More significant, however, is the fact that the protein precursor or metabolic pool, which is only a fraction of the total pool, was severely reduced by excision (table II), and that in excised roots it is expandable by the addition of exogenous leucine (20). A second criterion for the control of leucine production by the transport system would be an enhanced rate of synthesis from endogenous substrates, or an enhanced supply from the degradation of existing proteins in the excised root tips. A higher rate of synthesis of leucine from acetate-2-C^{14} was not unequivocally demonstrated. However in excised roots, where the size of the leucine pool can be altered without appreciably affecting the rate of incorporation of radioactive acetate, leucine does specifically inhibit its own biosynthesis (20).

The elegant experiments by Matchett and DeMoss show that tryptophan derived from indole was confined to a distinct protein precursor pool in *Neurospora crassa* (14). This pool, like the metabolic pool in *Escherichia coli* (5), but unlike the metabolic pool defined here in maize root tips, was not expandable. In *Neurospora* the tryptophan metabolic pool is thought to contribute tryptophan to protein; the expandable pool, to influence the level of enzymes active in tryptophan catabolism (14). Likewise Sercarz and Gorini have dissociated the functions of 2 arginine pools in a mutant strain of *Escherichia coli* (23). Thus discrete leucine pools in maize root tips may be important in controlling specific metabolic functions and the interactions between the pools take on a new significance.

Summary

The soluble leucine pool in maize root tips may be divided for simplicity into 2 pools: a metabolic pool and a storage pool. Leucine supplied by the transport system or by endogenous synthesis enters the metabolic pool. After the removal of the transport system, both pools are reduced in size, however the metabolic pool is most severely affected. Therefore the transport system is considered to be responsible for maintaining the level of leucine in the metabolic pool.

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The Effect of Leucine on the Biosynthesis of Leucine in Maize Root Tips [1, 2]

Ann Oaks [3]

Department of Biological Sciences, Purdue University, Lafayette, Indiana

The root tip may be divided arbitrarily into regions of cell division, radial enlargement, elongation and maturation. Characteristic protein, RNA and carbohydrate contents on a per cell basis have been observed in each of these regions (13, 7, 27). The results suggest that protein and RNA synthesis is favored in the regions of radial enlargement, and elongation, and that as the cell matures reactions favoring the synthesis of carbohydrates and cell wall components become more prominent. Thus in the meristematic region the proportion of dry matter recovered as protein is relatively high. Approximately 50 % of the dry matter in the 5-mm maize root tip, for example, is protein, whereas only 20 % of the dry weight is protein in a region 4 cm from the tip. In addition Jensen (12) and Clowes (4) have shown by radioautography that the rate of protein synthesis is highest in the root tip region. However in those experiments where the radiotracers of glucose or acetate have been fed to root tips only a fraction of the total C^{14} was recovered as protein (10, 16, 23, 30). These opposing observations raise a critical question concerning the efficiency with which carbohydrate is metabolized in the root tip region. Thus if the sugars transported to the root are used primarily as precursors for carbohydrates and as an energy source, and the amino acids transported to the root represent the major precursors for protein synthesis some control limiting drainage of sugar carbon to amino acids must be active in the growing roots. Observations with excised maize embryos show in fact that an adequate supply of amino acids is required in addition to a carbohydrate source for a normal increase in protein nitrogen (22).

Two possible control mechanisms present themselves from the extensive work with bacterial systems: end-product (allosteric) inhibition of the first enzyme

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[3] Present address: Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee.