SOME ASPECTS OF THE METABOLISM OF PROLINE DURING *ZEA MAYS* GERMINATION

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by

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SCOPE AND CONTENTS: The experiments described in this thesis were undertaken to investigate:

1. the role of proline in the developing maize embryo.
2. the system regulating proline level during growth.

When ^{14}C-proline was fed to intact root tips, approximately fifty percent of the label was found in protein proline. The remainder was metabolized to other products. Principal among these were ^{14}C-glutamate and ^{14}C-malate.

^{14}C-Acetate was found to be a good precursor in the synthesis of ^{14}C-proline. Although there was difficulty getting ^{14}C-glutamate into the cell, glutamic acid was also shown to be a proline precursor. Increased proline synthesis could be obtained by extending the culture period preceeding ^{14}C-acetate application to 3 or 6 hours. A gradual inhibition of this synthesis resulted when ^{12}C-proline was added to the media over an increasing concentration range of 2-10 X 10^{-4} M.

Deductions were made from these results as to the nature of the metabolic control of proline in the intact maize root.

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INTRODUCTION

Folkes and Yemm (1) have shown that the barley embryo obtains a wide range of amino acids from hydrolysis of endosperm storage proteins. A number of these amino acids such as tyrosine, valine and the leucines are present in the endosperm in concentrations sufficient to supply the needs of the embryo for a 10 day growing period. Aspartate, arginine, lysine and alanine however are deficient in the endosperm and synthesis must take place to meet embryo requirements for these amino acids. A balance sheet for proline indicates that proline loss from the endosperm is much greater than embryo gains in this same amino acid. This probably means that proline, along with asparagine and glutamate is being metabolized and its nitrogen used in the synthesis of the new amino acids. In fact decreases in total glutamate, proline and asparagine account for over ninety percent of the nitrogen required for new synthesis.

The very small amounts of free amino acids which accumulate in the endosperm during protein degradation indicate that the transport of these amino acids may be geared to embryo demands (2). In corn, zein is the main endosperm storage protein. A mixture of amino acids similar in composition to zein delays the release of nitrogen from the endosperm (3). In the absence of proline from this mixture,

however, nitrogen is lost at its normal rate. The omission of valine, typical of other amino acids in its effects on nitrogen loss permitted release of only 32 percent of the endosperm nitrogen. In the case where three amino acids were omitted together, the loss was still only 88 percent of that without the amino acid mixture present. Proline therefore seems to play a unique role in controlling the release of endosperm nitrogen. It may be that depletion of the proline content in the endosperm is sufficient to initiate protein degradation in that tissue.

If proline is the amino acid responsible for integrating the release of endosperm nitrogen with embryo requirements, it might be expected that proline plays a major role in embryo development. Free proline exists in large quantities (comparable only to glutamate and the amides) in the intact embryos of young seedlings (4). Seventy-two hours after excision from the endosperm, however, this proline pool is greatly depleted. Other neutral amino acids show a much smaller drop or remain constant. Aspartate, glutamate and asparagine actually increase as does lysine, histidine and γ-amino butyric acid. Further evidence for a major role of proline in embryo development is illustrated when proline alone is omitted from an amino acid mixture required to support the growth of excised embryos. This omission restricts normal increase in protein nitrogen by eighty-five percent. Omission of other amino acids has a less severe effect. Glycine omission

restricts protein increase by fifty-five percent and the omission of glutamate and aspartate together results in no restriction at all (4). Again proline, of all the amino acids tested, appears to be unique in its effects during germination.

The foregoing work indicates that proline synthesis is deficient in developing embryos. In fact, Oaks (5) has shown by feeding ^{14}C-acetate to excised root tips that very little ^{14}C-proline is made. It is reasonable to assume therefore, that the major proline requirement of the embryo is met by transport from the endosperm. The delay in embryo growth when proline is omitted from the amino acid culture media supports this view. The following system is a model suggesting how proline requirements in the embryo might be integrated with proline release from the endosperm: a metabolic pool could exist in the embryo which mediates in protein synthesis and possibly proline degradation. As proline is used, this pool is depleted and a concentration gradient is set up in the tissue. This gradient could result in a transport of proline from the endosperm. The exit of proline from the endosperm in turn, would result in the depletion of an endosperm pool of proline. Degradative enzymes may be triggered when the proline reaches a critically low level and more amino acids are released. When transport proline fails to meet metabolic requirements, the metabolic proline pool shrinks and the internal biosynthesis of proline increases helping to meet metabolic demands. In the presence

of external amino acid sources, such a proline pool would be expanded preventing internal proline biosynthesis and the establishment of a diffusion gradient. Thus no endosperm hydrolysis would take place and little ^{14}C-acetate would be incorporated into proline.

The foregoing model has much of its basis in the work of Oaks dealing with leucine regulation (6). She has shown that leucine made from ^{14}C-acetate entered protein from a small protein precursor pool (PPP), distinct from other stores of free leucine. External sources of leucine were shown to expand this pool. Concurrent with this expansion there was an inhibition of leucine biosynthesis. The PPP was depleted rapidly however, when external sources of leucine were removed. Previous work had established end-product inhibition of leucine biosynthesis in corn embryos (7). Therefore Oaks suggested that the level of leucine in the leucine PPP was responsible for regulating the amount of amino acid to be synthesized. In the intact system therefore, endosperm leucine would act as the amino acid supplements used experimentally, supporting protein synthesis and regulating leucine biosynthesis.

Many precedents can be found in the literature where pools participate in regulating metabolic and developmental processes. External sources of Δ^1pyrroline-5-carboxylic acid, or ornithine will satisfy the requirement for proline in a Neurospora mutant. Yet ornithine produced internally as an intermediate in the synthesis of arginine does not contribute to proline synthesis. Vogel and Bonner (8) suggest that the

ornithine pathway is not significant in proline synthesis. Ornithine may be maintained in a pool associated with arginine biosynthesis remote from the proline producing machinery. Thus pool arrangement may determine which of several possible reactions will occur in the cell.

In some cases, different pools may be important for the efficient operation of two separate functions requiring the same metabolite. On the basis of mutant studies in Escherichia coli, Sercarz and Gorini (9) have suggested the following pool arrangements for arginine in the wild type cells: Arginine produced internally is maintained in a pool supplying protein synthesis. If arginine is produced in excess, some arginine will be transferred to a second pool maintained in partial equilibrium with the protein precursor pool. The second pool, which also accepts arginine from the external media, participates in the formation of arginine repressor. The level of arginine in the repressor pool determines the amount of arginine biosynthetic enzyme produced. The significance of these pools might be explained in terms of enzyme affinities (9). If the enzymes involved in repressor formation have a stronger affinity for arginine than those of protein synthesis, the repression of arginine biosynthesis would prevent sufficient arginine production for protein synthesis. If on the other hand, only arginine in excess of that required for protein formation reaches the repressor system, the strong affinity of repressor enzymes for substrate would stringently control the production of excess arginine, and at the same time

spare production of wasteful biosynthetic enzymes.

Further aspects of pool influence in metabolic production are demonstrated by the tryptophan system in Neurospora mycelium (10). One tryptophan pool which is produced internally supplies tryptophan for protein synthesis. The second pool is involved in tryptophan degradation processes. This second pool accumulates tryptophan from the protein precursor pool and from external sources. When it is thus expanded, the formation of some enzymes of the tryptophan biosynthetic pathway is repressed while enzymes concerned with tryptophan degradation are induced.

Although the pool system is clearly an important factor in determining which of alternate routes will operate, in maintaining optimum substrate levels for various reaction systems, and in controlling the biosynthesis of pool components, little is known about the physical structure and arrangement of the pool compartments. In a number of cases, however, it is evident that enzyme distribution determines pool location of intermediates and products.

For example, in corn root tips a pool of malate formed from $^{14}CO_2$ is found to be depleted much more slowly after a pulse label than a malate pool synthesized from H^3-acetate. These two pools apparently mix when the pH is lowered or metabolic inhibitors are applied since both malates are then metabolized at an equal rate (11). It seems apparent that the malate formed from CO_2 is normally confined to the vacuole or cytoplasm where the enzymes involved in its formation are

located (12). Only slowly does it reach the mitochondria where acetate malate is formed and metabolized. In fatty seedlings a third malate pool is formed by glyoxylate enzymes located in particles known as glyoxysomes (13). This malic pool, resulting from fatty acid oxidation does not enter the tricarboxylic acid cycle but is diverted into the carbohydrate biosynthetic pathway.

It has been shown that a large proline pool accumulates in the cytoplasm of inactive blow-fly flight muscle. At the onset of flight, this proline reserve is rapidly depleted (14). Work designed to investigate the interconversion of glutamic acid and proline has indicated that enzymes associated with proline oxidation are firmly attached to the mitochondria (15). Thus, when the flight muscle is stimulated, demands by the mitochondria for the Kreb's cycle intermediates may bring these enzymes into full operation. A concentration gradient would thus be established drawing proline into the mitochondria. The enzymes producing proline on the other hand are located in the cytoplasm (16). When the muscle is at rest Kreb's cycle intermediates in excess of current cell demands may accumulate as the cycle by-product glutamic acid. Diffusing into the cytoplasm, glutamic acid would be converted by the proline synthesizing enzymes to proline, a convenient storage form.

Pool organization is not static. New metabolic patterns may be demanded during the course of development provoking extensive rearrangements in the pool system.

Using the electron microscope, Israel and Steward (17)

obtained a direct view of pool reorganization. The large storage pools so characteristic of non-growing carrot cells disappeared when cocoanut milk was added to the culture. The hormonal stimulus promoted cytoplasmic streaming, thus facilitating the fast turnover of much smaller pools of free metabolites. The net result was a vigoriously growing population of young cells.

Pool redistribution is also implicated in the processes of senescence and death. Eilam (18), by measuring leakage of potassium ions and the alterations in apparent free space in senescing cotyledons from Phaseolus multiflorus, found that permeability changes occurred as the first detectable step in the aging process. Consequently, the respiration rate changed and deterioration ensued. Sacker (19) also found that alterations in cellular compartmentation accompanied the ripening processes in bananas. The size and composition of amino acid pools were altered at sites of protein synthesis. These changes were followed by an increased ethylene synthesis which accelerated deterioration of cellular membranes and absolute disruption of the internal organization.

The current investigation was undertaken as a step toward determining:

1. the role of proline in growing tissue,
2. the system regulating the proline level during growth, and
3. the significance of pools in cellular events.

Corn root tips were used in all investigations. The

respiration rate in this tissue is high, a net synthesis of protein takes place, and the cells are uniform. Accordingly, short incubations with ^{14}C-tracers are sufficient for the evaluation of metabolic events. Because the excised tip is isolated from transport materials from other regions of the seedlings, the effects of specific metabolite concentrations can be tested and their metabolic and regulatory significance assessed.

CHAPTER I

METHODS

Germination Conditions: Maize seeds (hybrid var. WF9 X 38-11) were sterilized briefly in Javèx, rinsed well in sterile distilled water, and germinated at 30° in the dark on petri plates containing a thin layer of 0.8% agar. When seedling roots had grown to a length of 2-3 cm, the 5 mm root tips were harvested and thoroughly rinsed in sterile deionized water.

Corn Root Culture: Forty 5 mm root tips were either extracted immediately with 80% ethyl alcohol or transferred to flasks containing 5 ml corn media, with or without one percent sucrose. Incubation was carried out at room temperature for periods of 1, 3 or 6 hours. Samples of 40 roots each were then extracted with 80% alcohol for proline determinations.

Root cultures for hydroxyproline determinations were grown on a solid medium (1% agar base) containing standard corn salts and 2% glucose. Incubations were carried out in the dark at 30° for periods of 4, 6 and 18 hours.

Quantitative Cholorometric Assays: Assays for proline and hydroxyproline were carried out on the amino acid fractions obtained after the alcohol soluble and the hydrolyzed alcohol insoluble fractions were passed through Dowex-50(H^+) and Dowex-1 acetate columns. Proline was measured using the

quantitative colorimetric assay of Chinard (20). Because ornithine and lysine add to the color reaction by this method, approximately one gram of permutit was shaken with each assay sample. Ornithine and lysine absorb to the permutit which can then be removed by centrifugation. Determination of hydroxyproline was according to the method of Neuman and Logan (21). P-dimethylamino benzaldehyde used in the assay was purified using the techniques of Adams and Coleman (22).

Radioactive Tracers: L-proline UL-^{14}C, acetate-2-^{14}C, and glutamic acid UL-^{14}C of specific activities 202 mc/μM, 1.46 mc/μM and 187 mc/μM respectively were obtained from the New England Nuclear Corporation. Final concentrations of approximately 4.1 X 10^{-5} μM proline, 1.67 μM acetate, and 3.9 X 10^{-4} μM glutamic acid were usually used.

The purity of the ^{14}C-proline and ^{14}C-glutamate was tested by subjecting concentrated samples to two-dimensional chromatography. Exposure for 4 weeks to X-ray film (method to be described) indicated that only one radioactive substance was present in each case. Further tests for purity were carried out by eluting known quantities of radioactive tracer from Dowex-50 and Dowex-1-acetate columns. Eluants were tested for amounts of radioactivity recovered (Table I, Page 12). By this method, 68% of the glutamic-UL-^{14}C was in fact glutamic acid, whereas proline-UL-^{14}C was 75% pure. The ^{14}C-glutamic used in tracer experiments was purified by collecting the glutamic acid fraction eluted from a Dowex-1-acetate column by the method described on Page 17.

TABLE I

To test the purity of ^{14}C-proline and ^{14}C-glutamic acid, known quantities of the radioactive tracers were added to Dowex columns and the recovery determined in the various eluants.

		^{14}C-Glutamic Acid		^{14}C-Proline	
		Counts/ min	% Recovery	Counts/ min	% Recovery
Initial Sample		34,100	100	38,500	100
Column Type	Wash				
Dowex-50	H_2O	496	1.46	148	0.385
	NH_4OH	29,100	85.5	33,300	86.5
Dowex-1-acetate	H_2O	188	0.54	29,100	75.5
	Acetic acid	23,200	68.0	142	0.369

Pulse Labelling of Intact Roots: ^{14}C-proline was added to a solution of 1.5% agar made from standard corn salts solution. This agar was then moulded into strips 2 mm in width and placed on glass slides (5). The 5 mm tips of the corn seedling roots were held in contact with these strips (10 seedlings/strip) using sections of unlabelled agar. Exposure was carried out at room temperature in a closed humid chamber.

Since the object of the experiment was to trace the fate of ^{14}C-proline, the pulse period had to allow maximum uptake of ^{14}C-proline yet be brief enough that very little metabolism of the tracer would occur. A one-half hour pulse was chosen on the basis of a preliminary experiment (Table II, Page 14), which indicated that at that time 80% of radioactivity taken up remained in the soluble fraction (mostly as free proline).

Following the pulse, the seedlings were washed briefly with a solution of ^{12}C-proline (10^{-7}M) and sterile distilled water. The tips were removed and again rinsed briefly as above. Forty tips were killed immediately in 80% alcohol. The others were distributed in Warburg flasks (40/flask) containing 2.2 ml of corn salts (23) in the main chamber and 0.3 ml of 10% NaOH in the centre well. Flasks were then placed on manometers and immersed in a shaking Warburg bath at 26°C for periods of 1/2, 1 1/2, or 3 hours. At appropriate times the flasks were removed and sampled for counts remaining in the media. The roots were again rinsed as above, and

TABLE II

^{14}C-Proline Incorporation by Intact Roots

Intact corn root tips applied to ^{14}C-proline-containing agar were excised after exposures of 1/2 and 1 hour, rinsed with ^{12}C-proline solution and water and ground in 80% ethanol. Incorporation of ^{14}C into the insoluble fraction was used as a crude indication of the extent of ^{14}C-proline metabolism. Because 80% of the counts remained in the soluble fraction at 1/2 hour, this was chosen as a good pulse period.

Time of Exposure (Hours)	Distribution of Radioactivity					
	X 10^3 CPM Alcohol Soluble	% of Total Uptake	X 10^3 CPM Alcohol Insoluble	% of Total Uptake	10^3 CPM Total	%
1/2	48.0	80	11.9	20	59.9	100
1	51.0	52	48.0	48	99.0	100

extracted in 80% alcohol.

Excised Roots: Five mm root tips were cut under sterile conditions and rinsed with deionized water. They were then placed in 25 ml Erlenmyers which contained 2 ml of corn salts and ^{14}C-proline. Forty tips were used per sample. An initial sample was saved for proline determinations. When experiments required a longer exposure to ^{14}C-proline, the roots were placed in Warburg flasks in the manner described above for intact roots except that the main chamber now contained ^{14}C-proline in the appropriate concentration.

Synthesis of Proline:

A. In a preliminary experiment designed to determine conditions favourable for proline synthesis, 5 mm roots were placed in corn salts with or without 1% sucrose for the various preincubation times of 0, 1, 3 or 6 hours. Following the preincubation treatment, carried out at room temperature on a shaker, the roots were transferred to Warburg flasks and exposed for 2 hours to ^{14}C-acetate on the waterbath shaker at 26°C. The incubation media in every respect was similar to that for preincubation except for its tracer content.

Following incubation, the roots were placed in a Buchner funnel and the incubation media was drawn off by suction.

B. Competition Experiments: Roots were preincubated for 3 hours in corn salts media containing 1% sucrose. They were then transferred to Warburg flasks containing 2 ml of similar media, this time supplemented with ^{14}C-tracer - either ^{14}C-acetate or ^{14}C-proline. After two hours of incubation in this media,

the media was tested for bacterial contamination, the media was drawn off, and the roots fractionated.

Competition amino acids when used were added in equal concentrations to both preincubation and tracer medias.

Fractionation Procedures:

Roots which had been killed in 80% ethanol were crushed in a ground glass hand homogenizer. The slurry was placed in a conical centrifuge tube and subjected to 700 rpm on a clinical centrifuge. The alcohol insoluble residue after several ethanol rinsings was subjected to hydrolysis in 6N HCl by autoclaving for 12 hours at 15 pounds pressure. The supernatant solutions obtained from each rinsing were pooled and are considered here as the total alcohol soluble material. The hydrolyzed insoluble extracts were evaporated to dryness at 40° under reduced pressure.

Using ion exchange resins, the extracts were separated into three fractions: the acidic containing organic acids, the neutral containing sugars, and the basic containing amino acids and amides (24).

An extract was first applied to a 1 X 6 cm column of Dowex 50 (H^+) resin. The effluent was collected and passed through a Dowex-1-formate column. The water effluent from this column contained sugars. Forty ml of 4N formic acid eluted the organic acids.

The amino acids and amides were eluted from Dowex-50 using 40 ml of 2N NH_4OH. Further separation of this fraction was obtained using a 1 X 6 cm Dowex-1-acetate column. Neutral

and basic amino acid and amides appeared in the effluent while 6N acetic acid served to elute the acidic amino acids. Amides were separated from the other amino acids in the water effluent by hydrolysing the fraction for 4 hours at 100°C in 2N HCl. The neutral and basic amino acids could then be washed from the Dowex-1-acetate resin with water while the amides, now as dicarboxylic amino acids remained on the resin until eluted by acetic acid.

To determine particular radioactive organic acids, an aliquot of the organic acid fraction together with known standard organic acids (0.2-0.4M) were placed on a 1 X 20 cm column of Dowex-1-formate and were eluted with an increasing gradient of formic acid obtained by the gradual mixing of solvent from two reservoirs; 8N formic acid in one reservoir and deionized water in a second reservoir. The resulting mixture was passed through the column. Two-three ml samples were collected, dried in an air stream and made up to 2 ml with water. Titration with 0.01N NaOH using phenolphthalein as an indicator served to detect standard organic acids. Samples were also taken for radioactivity measurements. Succinic, malic and citric acid peaks were detected in this manner (See Figure 1, Page 19).

Similarly the acidic amino acid fractions were added to a 1 X 20 cm Dowex-1-acetate column, together with standard glutamic and aspartic acids and were eluted sequentially using a concentration gradient established with 200 ml of acetic acid and 200 ml H_2O in the reservoirs. After the first 40 ml were

Figure 1: Separation of Radioactive Organic Acids using Gradient Elution from Dowex-1 (formate form).

To determine particular radioactive organic acids an aliquot of the sample organic acid fraction together with known standard organic acids were placed on a 1 X 20 cm column of Dowex-1-formate. Acids were eluted using a formic acid gradient obtained by the gradual mixing of solvent from two reserviors; 8N formic acid in one reservoir and deionized water in a second reservoir. Two ml samples were collected using a fraction collector and dried in an air stream. The samples were then made up to 2 ml with water, the radioactivity measured, and the location of standard organic acids detected by titration with 0.01N NaOH using phenolphthalein as indicator. Succinic, malic and citric acid peaks were detected in this manner.

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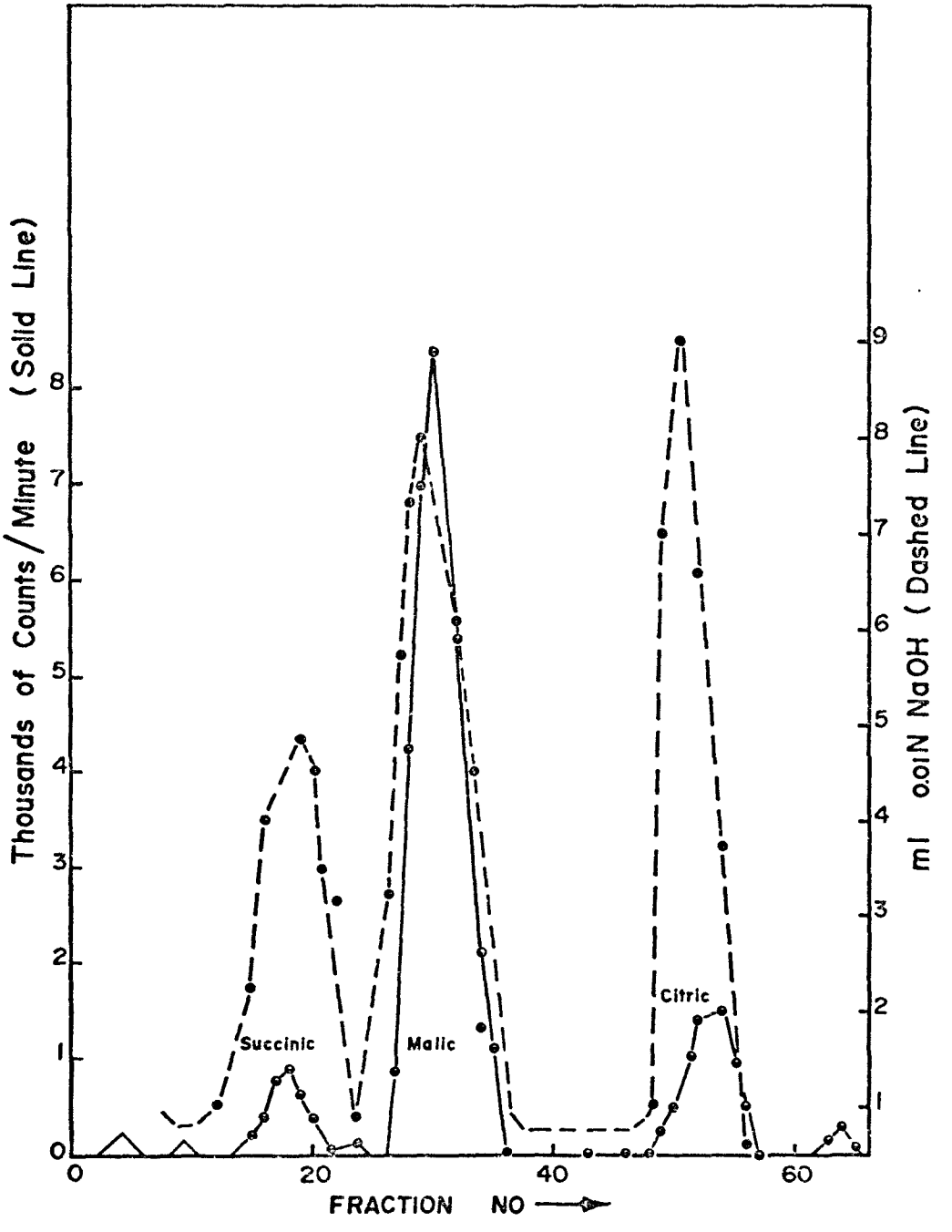

Thousands of Counts / Minute (Solid Line)
ml 0.01N NaOH (Dashed Line)
Succinic
Malic
Citric
FRACTION NO

eluted, 2 ml fractions were collected. The tubes containing glutamic acid were detected by spotting a drop from each tube on filter paper. Spots from tubes containing glutamic or aspartic acids gave a colour response with a solution of 0.5% ninhydrin in acetone. These tubes were then tested for radioactivity. Where ^{14}C-acetate, ^{14}C-proline or ^{14}C-glutamate were the radioactive tracers used, no other peaks containing appreciable radioactivity were present.

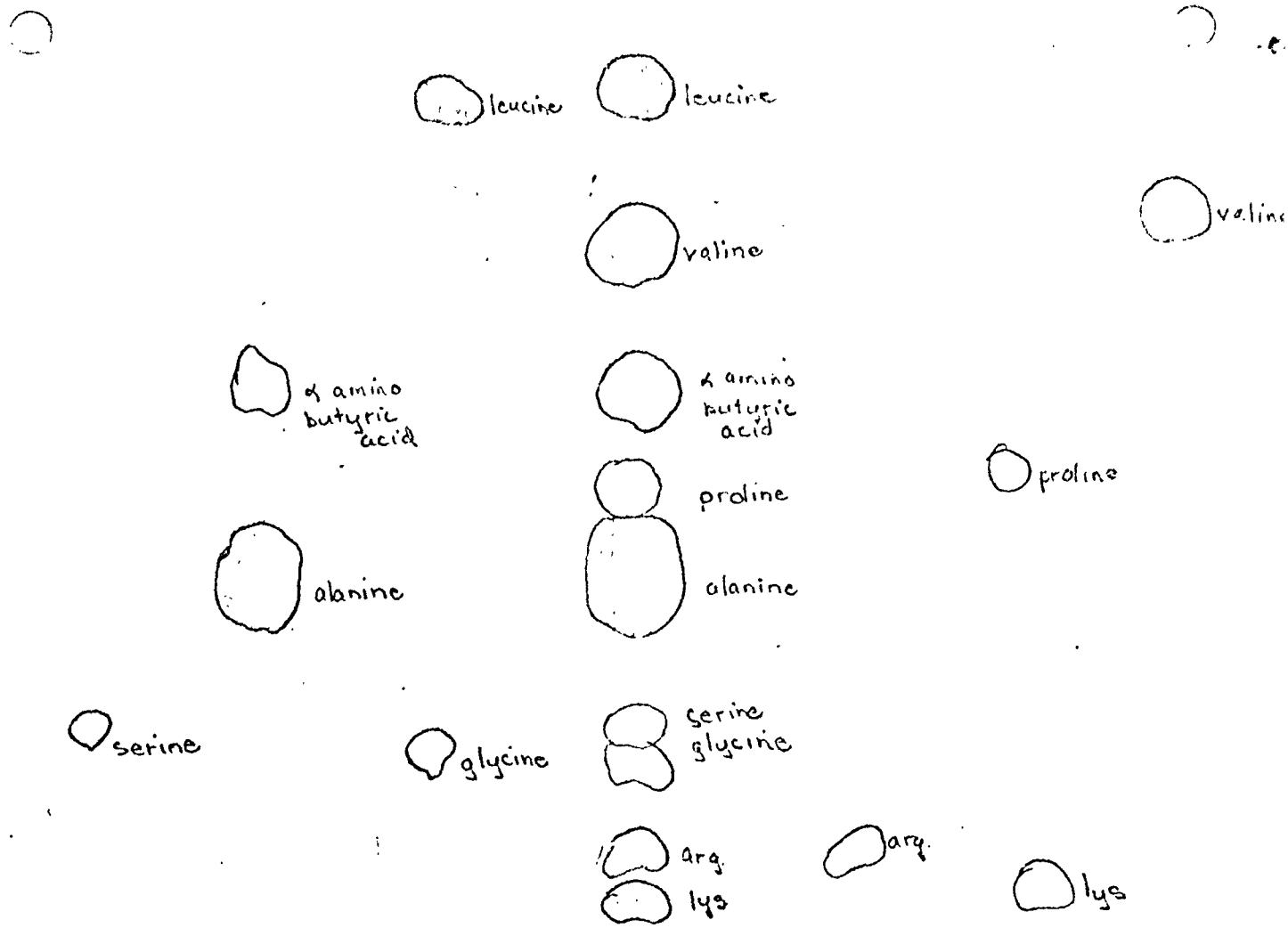
Quantitative Determination of Radioactivity: The total radioactivity of the various fractions of a sample was determined by bringing each fraction to 10 ml with water, and pipetting duplicate aliquots onto aluminum planchets. The planchet contents were distributed evenly using a few drops of 80% methyl alcohol. After the planchets were dried in a hot air oven at 100°C, they were counted under a thin window continuous gas-flow detector (Nuclear Chicago Model DHT). All ^{14}C determinations were expressed as counts/min. Corrections were made for background.

Alternatively samples were pipetted onto Whatman #3 paper discs and placed in scintillation vials containing 10 ml of water-free toluene, PPO and dimethyl POPOP (5.5 g of Packard Pre-Mix "M" in 1 litre of spectroquality toluene). This scintillation fluid proved satisfactory since essentially all the radioactivity remained in the paper. Radioactive determinations were made using a liquid scintillation counter (Nuclear Chicago Model 720).

The $^{14}CO_2$ evolved during an experiment was collected

Figure 2: Distribution of Standard Neutral and Basic Amino Acids.

0.1% solutions of amino acids were spotted on Whatman #1 chromatography paper. The solvent n-Butanol: Acetic Acid:Water (3:1:1) was passed up the paper twice during ascending chromatography. The relative distribution is the same as that obtained by descending chromatography.



serine

alanine + α amino butyric

leucine + glycine

lys + pro + ala + leu + arg + ser + α amino butyr + valine + glu

arg

lys + pro

val

in NaOH (10%). This was quantitatively transferred to a 5 ml solution containing 10% barium chloride and 1% NH_4Cl and sufficient $NaCO_3$ to give 10 mg cold $BaCO_3$. The $BaCO_3$ which precipitated was collected under suction on a millipore filter. The filter was glued to a planchet, dried and counted. Although the efficiency of the counter detection is lowered by the thickness of the $Ba^{14}CO_3$ precipitate filter, this method served to indicate that $^{14}CO_2$ did not account for a substantial fraction of metabolized ^{14}C-proline. Therefore, no attempt was made to quantitate $^{14}CO_2$ more realistically.

Chromatography: The quantitative analysis of neutral and basic amino acid fractions was obtained by the following procedure. A sheet of Whatman #1 chromatography paper 22 X 57 cm was ruled into seven columns of 3 cm width. On a base line drawn 10 cm from the top, spots containing 10, 25 and 50 µl of the neutral and basic radioactive samples (concentrated in 1 ml of water) were applied to the columns using 10 and 25 microlitre capillary tubes. One strip was used to run a standard mixture of neutral and basic amino acids. Descending chromatography was then carried out twice using butanol, acetic acid and water (3:1:1 by volume) as solvent. A run of 15 hours gave the best separation.

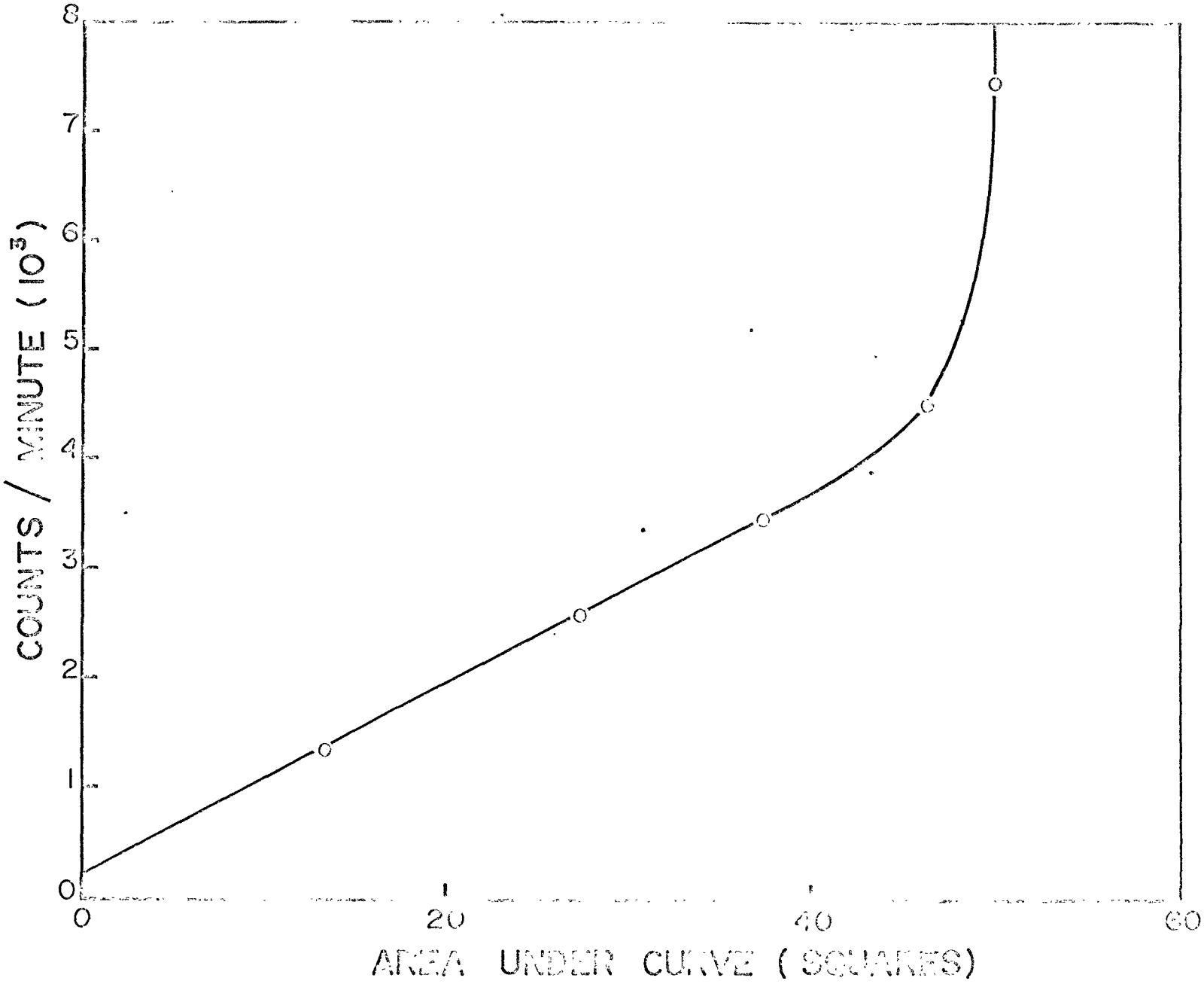
Two methods were used to identify and measure radioactive areas:

Method I:

Strip Counting: After drying, the chromatograms were cut into strips and the strips bearing the radioactive samples

Figure 3: Standardization Curve for 2π, Radiochromatogram Scanner (Packard Model 7201).

Standard concentrations of ^{14}C-proline (as measured by a thin window continuous gas-flow detector Nuclear Chicago Model DHT) were subjected to chromotography. The radioactive areas were then run through the 2π Radiochromatogram Scanner. The area under the tracing curves were correlated with CPM of ^{14}C-proline in the area.


COUNTS / MINUTE (10^3)
0
1
2
3
4
5
6
7
8
0
20
40
60
AREA UNDER CURVE (SQUARES)

passed through a 2π radiochromatogram scanner (Packard Model 7201). Peaks appearing on the tracing were identified by correlation of their positions with those of the standard amino acids (Figure 2, Page 22).

On a number of occasions, several of the amino acids (γ-amino butyric acid, alanine, proline, and threonine) were checked for more positive identification. The section of strip containing these radioactive amino acids was sewn near the top of a second chromatography sheet. Standard amino acids were applied to one area of the strip. Descending chromatography was again carried out using water-saturated phenol as solvent. The radioactive areas in question were distinctly separated. Strip counting and comparison with standard amino acids permitted reliable identification.

When the strip counter was set at linear range 300, time constant 30, speed 1 cm per minute, the area under each peak was found to be linearly related to the amount of radioactivity in that area provided the radioactivity did not exceed 3600 CPM as shown in Figure 3, Page 25.

Method II:

Scintillation Counting: After chromatography, the amino acids were located by fluorescence under the ultra violet light in the presence of 1,2-naphthaquinone-4-sulfonate (25). The fluorescent areas were identified through correlating their positions with the standard amino acid strip. The spots were then excised, cut up and placed in scintillation vials for counting (see Page 20). Recovery of radioactivity by this

method equalled the radioactivity applied to the chromatogram.

Two Dimensional Radioautography: In some instances, 2-dimensional chromatography was also used in identifying the radioactive areas of the neutral and basic amino acids. Solvent systems used were n-butanol:acetic acid:water (3:1:1) and water-saturated phenol. Radioactive areas were detected by stapling the chromatograms to X-ray film (Ilford-Ilfex). Radioactive areas could then be eluted and rechromatographed with standard amino acids for positive identification or could be cut out and used directly for radioactive determinations with the liquid scintillation counter.

CHAPTER II

RESULTS

I. Fate of Proline in Corn Root Tips:

Changes in Proline Content Resulting from Excision of Corn Roots: Corn roots were excised to prevent transport of metabolites into this region from other tissues. The proline content was measured immediately following excision and after varying incubation periods as described in the Methods.

The results in Table IIIA, Page 29, show that there is a loss of total proline from the tips. This seems to indicate that proline is being metabolized to other products and is not made in amounts that are adequate to maintain the proline level in that region.

When the culture media contains no sucrose, there is a loss of 118 μM of proline during the first 3 hours in the soluble fraction with only a 4.2 μM loss in the following three hours. By contrast, 81 μM were lost in the first 3 hours and 31 μM in the ensuing period when sucrose was in the media (Table IIIB, Page 29). Thus sucrose seems to delay the loss of proline from the soluble fraction.

The insoluble proline level is also influenced by the presence of sucrose. Whereas the sucrose free culture showed a fairly continuous drop over the six hours (54 μM in

TABLE III

Proline Content in 5 mm Root Tips

A. Excised 5 mm corn root tips were placed either in corn salts solution for 1, 3 or 6 hour periods, or extracted immediately with 80% alcohol. The insoluble fraction was hydrolyzed with 6N HCl for 12 hours at 115°C. The amino acids were isolated using Dowex-50 (H^+) resin and Dowex-1-acetate. Proline estimation was carried out as described in methods.

Time after Excision (Hours)	- Sucrose					
	Alcohol Soluble		Alcohol Insoluble		Total Proline	
	µmoles/ 40 tips	*% Intact	µmoles/ 40 tips	% Intact	µmoles/ 40 tips	% Intact
0	183	100	540	100	723	100
1	135	74	504	94	639	88.3
3	65.0	35.5	486	90	551	76.3
6	60.8	33.8	456	84.5	517	71.5

B. Experimental procedure as in A but with 1% sucrose included in the media.

Time after Excision (Hours)	+ Sucrose					
	Alcohol Soluble		Alcohol Insoluble		Total Proline	
	µmoles/ 40 tips	*% Intact	µmoles/ 40 tips	% Intact	µmoles/ 40 tips	% Intact
0	183	100	540	100	723	100
1	144	78.7	463	86.0	607	84.0
3	102	55.7	540	100.0	642	88.7
6	64	35.0	576	107.0	640	88.5

* Intact value refers to proline content at time 0 when roots were first excised.

in the first 3 hours and 30 μM in the next three hours) the culture containing sucrose had no net change in protein proline after 3 hours and actually showed a 36 μM increase by 6 hours.

Both systems show the same net decrease in soluble proline after six hours in culture. Therefore the greater amount of proline in the sucrose cultured roots (123 μM) is contained in the protein fraction. Whereas the sucrose roots showed a 36 μM net increase in protein proline during collecting, the sucrose-free roots showed a 84 μM loss.

Because the decrease in total proline is less in the presence of sucrose, sucrose may either be sparing proline (i.e. preventing proline degradation) or be increasing proline synthesis.

Fate of External Proline (^{14}C-Proline, pulse-fed) and Endogenous Proline:

The conversion of proline to hydroxyproline can account for only a very small percent of the net loss in proline (Table IV, Page 31). By using ^{14}C-proline, it should be possible to trace the fate of the remaining metabolized proline.

In order to determine whether proline from an external source would receive treatment similar to that of native proline, ^{14}C-proline was fed to intact tips for thirty minutes. The ^{14}C-content of the neutral and basic insoluble and soluble fractions were measured at the time of excision and again after three hours. These values were assumed approximate to ^{14}C-proline content although radiochromatograms of the 3 hour

TABLE IV

Hydroxyproline Content of 5 mm Root Tips

Excised roots were cultured on corn salts agar containing 2% glucose. Extractions and determinations were carried out as described in the Methods, Page 11.

Time of Culture (Hours)	Soluble µmoles/40 tips	Insoluble µmoles/40 tips
0	.04	.22
4	.04	.23
6	.04	.27
18	.05	.29

TABLE V

Fates of Proline Supplied Externally (as ^{14}C-Proline) and of Endogenous Proline (as measured colorometrically)

	Endogenous		*External	
	Proline contents µM/40 tips	% of initial proline content after 3 hours	CPM	% of initial CPM remaining after 3 hours excision
Soluble				
***Initial proline	183		25,500	
After 3 hours	65	35.4	5,060	19.9
Insoluble				
***Initial	540		11,440	
After 3 hours	486	90.0	18,360**	160.0
Total Proline				
***Initial	723		36,900	
After 3 hours	551	76.3	23,420	63.5

* External ^{14}C-proline was taken into intact roots from radioactive agar for 1/2 hour before excision.

** At 3 hours, not all counts in insoluble are ^{14}C-proline; some of radioactivity is in hydroxyproline and in the area of glycine, arginine, serine. Activity is predominantly ^{14}C-proline, however.

*** Initial proline refers to the proline level at the time of excision.

25,000 CPM is equivalent to 0.31 µM proline.

insoluble fraction indicated minor amounts of radioactivity in two other amino acids.

It is shown in Table V, Page 32, that the percent decrease in soluble ^{14}C-proline after three hours is greater than the percent decrease in the total proline content of the roots. These results may indicate that more than one proline pool exists in the roots. ^{14}C-Proline may be taken into a pool where metabolic turnover and protein incorporation is high. (About thirty percent of the ^{14}C-proline enters protein.) Another more static pool may be relatively inaccessible to external proline.

An increase of 60 percent in the ^{14}C-proline indicates that much of the external proline is being incorporated into protein. While a ^{14}C increase indicates a synthesis of new protein is occurring, the net loss in insoluble protein proline seems to mean that protein breakdown is more rapid than the synthesis of new protein in excised roots.

It cannot be said that externally supplied proline is more accessible for protein synthesis, although it does appear to be somewhat more readily available to cellular machinery than the total native pool.

Comparison of proline metabolism in excised and intact roots:

In experimental procedure, it is more convenient to work with excised roots than intact roots. It is expected however, that the excision process might in some ways disrupt the normal pathways of metabolism. Therefore, to discover how the proline pathway was being affected by

the excision process, ^{14}C-proline was fed to excised roots for 30 minutes and to intact roots for the same period.

If label appearing in fractions other than protein is considered to represent oxidized proline, it is apparent that intact roots degrade more proline than excised roots. On the other hand, excised roots incorporate more ^{14}C-proline into protein. The contrast in balance of these two activities is indicated by the ratio of percent of proline oxidized to percent of proline incorporated into protein by the two systems. That for intact roots is 1.90 while that for excised roots is 0.73. The oxidation rate of proline then is curtailed by excision, perhaps because proline is not as available to the oxidation machinery, but more likely because general cellular processes are disrupted.

Increased incorporation of proline into protein by excised roots may indicate that ^{14}C-proline is more accessible to the protein precursor pool in excised roots, or it may show that the rate of protein synthesis is increased. In intact roots, of course, the proline synthesizing machinery and/or the transport system may be preferentially supplying the proline required for protein synthesis. Accordingly, although the rate of protein turnover may remain the same as that in excised roots, the ^{14}C-proline content would be reduced.

While the balance of proline oxidation and proline incorporation into protein is different in excised roots, the

TABLE VI

To Compare Fate of ^{14}C-Proline in Intact and Excised Roots

^{14}C-Proline was presented to corn root tips (5 mm) in two different ways:

1. intact roots were placed on agar strips containing tracer,
2. excised roots took up tracer from a corn salts media.

Exposure time in both cases was 1/2 hour.

Distribution of ^{14}C	Intact		Excised	
	CPM (X100)	% of total ^{14}C	CPM (X100)	% of total ^{14}C
Soluble acidic amino acids, amides	143.0	43.2	75.8	30.0
Sugars	4.1	1.2	3.9	1.5
Organic acids	45.7	13.6	18.0	7.1
Protein Proline	114.0	34.5	146.0	57.7
Protein acidic amino acids, amides	22.3	6.7	8.4	3.3
Other insoluble materials	3.4	1.0	1.3	0.5
Total	332.5	100.0	253.4	100.0
% ^{14}C-proline oxidized		65.7		42.4
% ^{14}C-proline in protein		34.5		57.7
$\frac{\%\text{ proline oxidized}}{\%\text{ proline in protein}}$		$\frac{65.7}{34.5} = 1.9$		$\frac{42.4}{57.7} = 0.73$

These calculations do not include $^{14}CO_2$ and are therefore somewhat low.

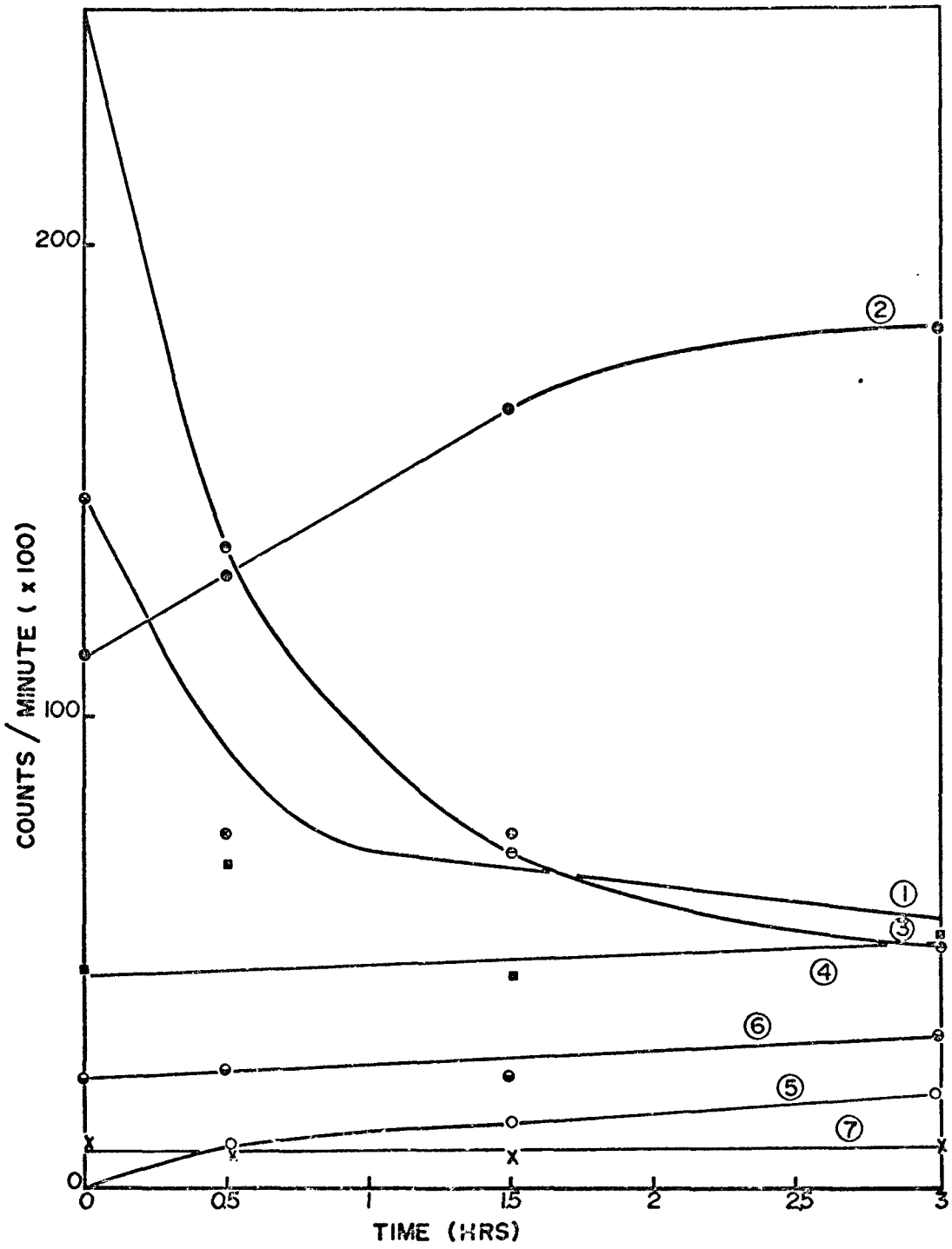
Figure 4: Metabolism of ^{14}C-Proline by Root Tips.

^{14}C-Proline incorporated into agar strips was presented to intact corn root tips over a pulse period of 30 minutes. Following this pulse, the 5 mm tips were excised and placed for varying periods of time in tracer-free corn salts medium.

The figure opposite shows the radioactive content of the various fractions obtained from the root tips by extraction initially (immediately after excision) and the changes in these fractions over the ensuing three hours.

The lines in Figure 4 represent ^{14}C content in:

1. Glutamic acid
2. Insoluble neutral and basic amino acids (mainly proline)
3. Soluble proline in cells and media
4. Organic acids
5. CO_2
6. Insoluble acidic amino acids and amides
7. Sugars


200
100
0
COUNTS / MINUTE (x 100)
0
0.5
1
1.5
2
2.5
3
TIME (HRS)
①
②
③
④
⑤
⑥
⑦

same metabolic processes are apparently being carried out. It seems justifyable therefore, to use excised roots.

Distribution of Label from ^{14}C-Proline:

Figure 4 shows the distribution of label from ^{14}C-proline after a pulse feeding of one-half hour. Immediately following the pulse, most of the ^{14}C (other than that as free soluble ^{14}C-proline) is found in the soluble acidic amino acids and their amides. Analysis of this fraction indicates that only traces of counts appeared as aspartic acid, the remainder all being in glutamic acid and glutamine. The relative proportions of acid and amide were not determined.

There is a rapid loss of ^{14}C from glutamic acid during the first half hour after excision. From this time onward the rate of loss is very slow.

Organic acids account for a substantial proportion of ^{14}C. These may arise if glutamic acid is further metabolized by entering the tricarboxylic acid cycle via α-keloglutarate. Analysis of the relative proportions of three organic acids (see Figure 1) indicates that upon excision almost all ^{14}C was in the malic pool. By 1.5 hours a steady state distribution is reached showing most activity to be in malic acid. As indicated in Table VII, Page 39, the distribution of organic acids in these three fractions is similar to the relative amounts of these individual acids found in the tricarboxylic acid turnover pools of 3 mm root tips as estimated by MacLennan et al. (24). Where the turnover pool is large, cycle intermediates

TABLE VII

Organic Acids

		Relative % of acids made from ^{14}C-Proline				Relative % in turnover pools*
Time after Excision (Hours)		0	1/2	1 1/2	3	
Organic Acids	Succinic		5.8	4.1	6.0	6.4
	Malic	100	74.7	57.0	58.0	73.0
	Citric		19.3	39.0	36.0	20.7

* The relative % of amounts of individual acids estimated to be in turnover pools as calculated from Table 12 in Ref. (24). Also see text, Page 38.

accumulate; thus the high activity of malic acid. Specific activity of this acid could be quite low.

The large amounts of glutamic acid present initially indicate that a substantial amount of proline is being oxidized to glutamate. During the first thirty minutes after excision, the rate of oxidation of ^{14}C-proline to ^{14}C-glutamic acid is apparently not as rapid as the conversion of ^{14}C-glutamic acid since the level of ^{14}C-glutamate declines. The constant level of this acid after the initial period indicates a steady state has been reached between proline oxidation and glutamic acid oxidation.

Figure 4, Page 37, indicates that 11,400 counts per minute are incorporated into protein after the 30 minute pulse. Over the following two hours incorporation continues. After that time the ^{14}C content remains constant. This may indicate that: 1. ^{14}C-proline has become limiting, 2. the rate of protein degradation equals the rate of protein formation, or 3. proline produced internally is acting as protein precursor. Major amounts of ^{14}C-proline could be inaccessable to protein synthesis because it is held in vacuoles.

The balance sheet for ^{14}C-proline disappearance and reworking (Table VIII) shows that a substantial number of counts lost from the soluble fraction remain unaccounted for. The method of hydrolysis does not permit 100% recovery of the insoluble fraction but cannot account for this large loss. The rapid drop in glutamic acid during the first half hour indicates a rapid metabolizing of proline at this time. Much $^{14}CO_2$ could be lost during this time because Warburg vessels are being

TABLE VIII

Balance Sheet: ^{14}C-Proline Metabolism

Intact roots were given a 1/2 hour pulse of ^{14}C-proline in agar, excised and placed in corn salts media without tracer. Net changes in various fractions during 3 hours of incubation in tracer free solution are presented.

	CPM
*Neutral and Basic A.A. (sol.)	-20,500
*Acidic A.A. (sol.)	- 8,900
Sugars	0
Organic Acids	+ 800
*Neutral and Basic A.A. (insol.)	+ 6,960
*Acidic A.A. (insol.)	+ 920
Other Insoluble Materials	+ 110
CO_2	+ 2,000

(-) Counts lost during 3 hours.

(+) Counts gained.

* A.A. = Amino Acids

equilibrated. $^{14}CO_2$ values are not reliable compared to other values in any case because the $BaCO_3$ precipitate was not at infinite thinness. Since the main purpose of the experiment was to determine whether proline was extensively metabolized, and this has been adequately demonstrated, a proper calibration of $Ba^{14}CO_3$ was not carried out.

II. Proline Biosynthesis:

A. Establishing Conditions for Proline Biosynthesis:

(1) The Effect of Preincubation on Amino Acid Synthesis from ^{14}C-Acetate:

A two hour incubation of excised tips in ^{14}C-acetate was preceded by varying periods of incubation in a tracer-free medium. The ^{14}C content of the various amino acids was examined to determine the effect of this preincubation on amino acid synthesis.

Figure V, Page 44, indicates that the amount of amino acids being synthesized from ^{14}C-acetate can be increased by extending the preincubation period to three hours. Threonine is presented as typical of several neutral and basic amino acids (i.e. alanine, proline and the slow running amino acid group which includes lysine, serine, glycine and arginine). One hour of preincubation does not influence the amount of these amino acids synthesized from acetate. An increase in the preincubation time to three hours however, results in a 2 to 3 fold increase in the amount of amino acid synthesized. Proline increasing thirteen fold is most responsive to the three-hour preincubation period. An increase of the preincubation

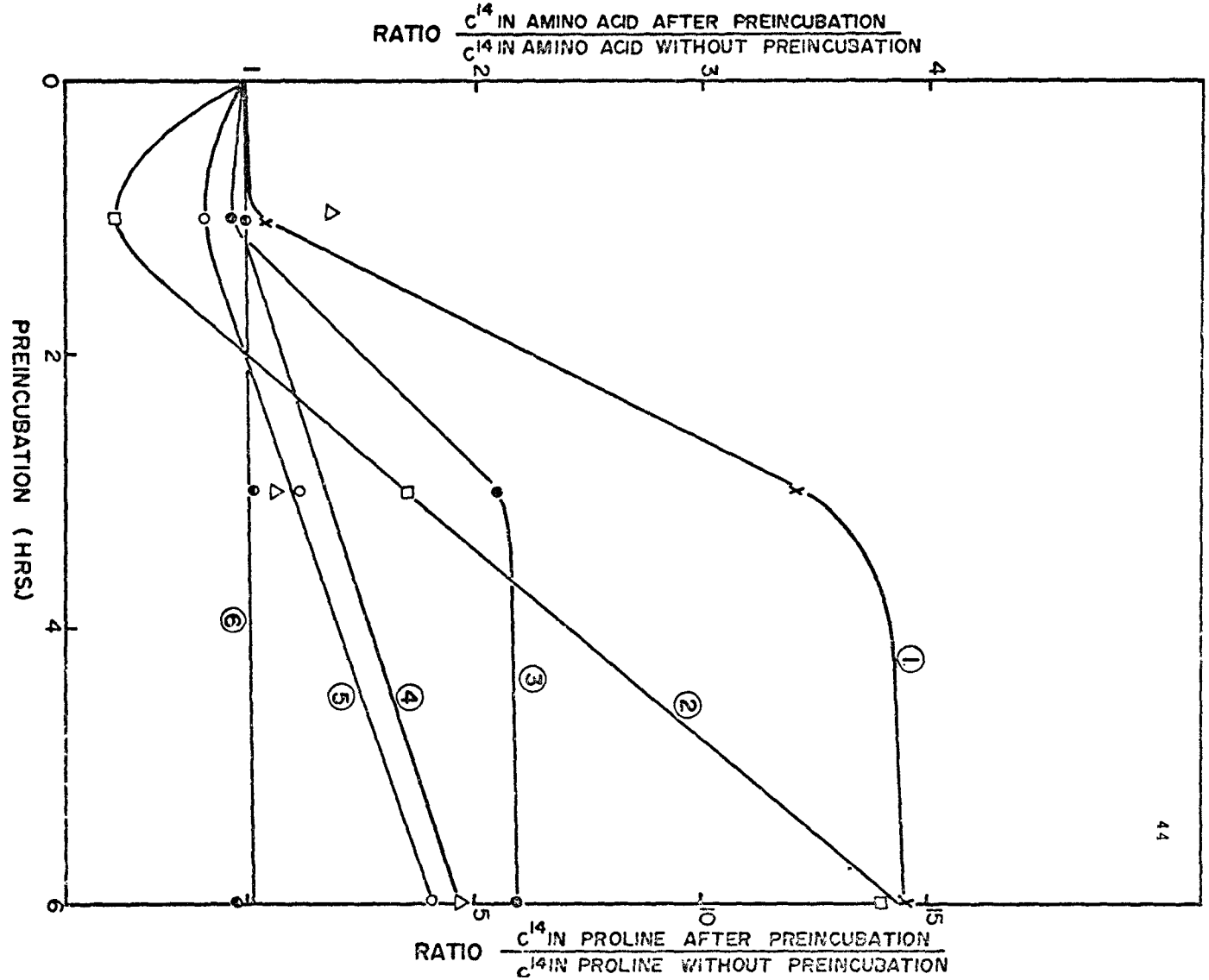
Figure 5: Preincubation - Its effect on the incorporation of ^{14}C from ^{14}C-acetate into amino acids.

Excised root tips were preincubated for varying periods of time in a tracer free medium containing 1% sucrose and corn salts. Roots were then transferred to similar media containing ^{14}C-acetate for 2 hrs. The amino acid fractions were extracted as described in Methods. Radioactivity of individual neutral and basic amino acids was measured using a Packard Strip Counter.

Figure 4 shows the content of ^{14}C-amino acid synthesized from ^{14}C-acetate without a preincubation period, and the change in the ^{14}C content of these acids as a preincubation period is given and extended.

The lines in Figure 5 represent ^{14}C content in:

1. Proline (note that proline is read from right hand scale)
2. γ-Amino butyric acid
3. Threonine
4. Valine
5. Glutamic acid
6. Leucine


RATIO C14 IN AMINO ACID AFTER PREINCUBATION / C14 IN AMINO ACID WITHOUT PREINCUBATION
1
2
3
4
0
2
4
6
PREINCUBATION (HRS.)
5
10
15
RATIO C14 IN PROLINE AFTER PREINCUBATION / C14 IN PROLINE WITHOUT PREINCUBATION
①
②
③
④
⑤
⑥
44

beyond three hours does not result in a substantial increase in the synthesis of these amino acids.

Both γ-amino butyric acid and glutamic acids show a slight decrease in radioactivity after a one hour preincubation period. However, upon longer preincubation these amino acids begin to increase linearly and continue this increase up to six hours when preincubation is stopped.

Leucine synthesis does not increase in response to preincubation. The rate of valine synthesis is irregular but there appears to be a greater increase in the rate of synthesis after a six hour preincubation.

The thirteen-fold increase in proline after three hours of preincubation does not represent a remarkable production of ^{14}C-proline. Rather, it indicates that the rate of proline synthesis was very low at the time of root excision (i.e. no preincubation period). Proline in fact showed the lowest ^{14}C content of all the amino acids at that time.

The increased production of proline after a lengthy preincubation period may indicate that some time is required to synthesize enzymes for proline synthesis. The experiment summarized in Table III, Page 29 indicates that the proline content of excised tips decreased considerably during the first hour of excision. Possibly the depletion of this proline is required before proline synthesis is initiated.

Examination of Table 4B of the Appendix shows that most of the ^{14}C-proline produced after the three hour preincubation is contained in protein. Very little of the

proline is trapped in the soluble fraction. By contrast, alanine which shows an increase in biosynthesis similar to that of proline remains mainly in the soluble fraction with very little incorporation of ^{14}C-alanine into protein.

These observations may mean that the pool which receives ^{14}C-alanine is fairly large. The large content of native alanine which is in the pool would dilute the ^{14}C-alanine as it enters. In turn, alanine entering protein from this pool would have a low ^{14}C activity.

A large pool of soluble proline has been shown to exist in corn roots even after extended incubation (Table III, Page 29). Much more labelled ^{14}C-proline would be expected to become trapped in this soluble fraction. The low activity in the soluble fraction and the high rate of ^{14}C-proline incorporation into protein seem to indicate that newly formed proline enters protein through a pool apart from the main proline pool.

There is very little increase in the rate of synthesis for a number of amino acids - alanine, proline, threonine and the lysine, glycine, arginine, serine group after three hours of preincubation. This may indicate that the rate of synthesis has become adequate to meet tissue requirements and a control mechanism is regulating the rate at this constant level.

(2) The Sucrose Effect:

Table IX shows that sucrose in the culture preincubation and incubation media causes increased production of many amino acids from ^{14}C-acetate. The synthesis of valine, aspartate and

TABLE IX

Effect of Sucrose on ^{14}C-Acetate Incorporation into Amino Acids

Roots were preincubated for 3 hours in corn media with or without 1% sucrose before transfer to media of similar composition containing ^{14}C-acetate for 2 hours.

Amino Acid(s)	-Sucrose cpm/40 tips	+Sucrose cpm/40 tips	Ratio $\frac{+\text{sucrose}}{-\text{sucrose}}$
Ala	11.7	15.0	1.28
Thr	4.3	8.9	2.07
Pro	4.3	10.1	2.35
Val	4.6	2.3	0.50
Leu	9.8	10.9	1.10
*Slow	12.7	23.3	1.81
Total Neut & Basics	49.9	80.8	1.77
GlN	177.6	204.8	1.15
AsN	8.5	4.9	0.58
Glu	335.8	432.7	1.29
Asp	33.0	28.4	0.86

*Slow refers to the group of amino acids (arginine, lysine, serine, glycine, etc.) which move slowly on a chromatogram with B.A.W. as solvent.

asparagine however is inhibited while leucine and glutamine synthesis remains relatively unaffected.

^{14}C-Proline, as indicated in Table IX, increases 2.4 times in the presence of sucrose. This dramatic increase occurs only in insoluble proline; free ^{14}C-proline actually decreases. It was shown in Table III, Page 29 that roots cultured for three hours in sucrose lost much less of their total proline content than those without sucrose treatment. These two observations then, appear to concur.

The manner in which sucrose acts to increase proline synthesis is not clear. Table IX indicates that sucrose may be exerting a general effect on the synthesis of many amino acids. Alanine, threonine, glutamic acid and the 'slow running' group of amino acids as well as proline show increases in ^{14}C content. Possibly sucrose is stimulating protein synthesis. If this is so, increases in biosynthetic enzymes would result in greater production of ^{14}C-amino acids, and a general increase in protein synthesis would cause a greater incorporation of amino acid into protein.

If such a theory applies in this situation, it is obvious that valine and asparagine biosynthetic enzymes must operate by an independant control. In the presence of sucrose ^{14}C-valine is reduced by 50 percent and asparagine by 43 percent. Perhaps the biosynthetic enzymes of these amino acids are repressed in the presence of sucrose. On the basis of the information available however, no conclusion can be reached as to the mode of sucrose action in amino acid synthesis.

B. Competition Experiments:

The incorporation of ^{14}C-acetate into amino acids provides a measure of the relative synthesis of these amino acids in the tissue. The amino acid composition of the root tip region is fairly constant (see Table XB, Page 53). Supplements of proline, arginine, glutamate and leucine were tested for their competitive effects by culturing the roots in the presence of these amino acids and ^{14}C-acetate. The results obtained are expressed as the percent of radioactivity of a given amino acid relative to the control. Expressed another way, this means counts per minute of the amino acid per forty root tips from the competition experiment divided by the counts per minute of the amino acid per forty root tips from the control experiment multiplied by one hundred (method adapted from Abelson (26)).

(1) Effect of Proline Concentration on the Synthesis of Proline from ^{14}C-Acetate:

The foregoing experiments established that a preincubation period of three hours and a culture medium containing one percent sucrose gave greatest proline synthesis from ^{14}C-acetate. Using these conditions the incorporation of ^{14}C-acetate into proline was measured when varying concentrations of ^{12}C-proline were present in the culture media. Figure VI, Page 51 shows that ^{14}C-proline decreased as concentrations of the ^{12}C-amino acid increased. The statistical significance of this result is calculated using the distribution of t (43). The standard deviation is estimated from the data as

Figure 6: Effect of ^{12}C-Proline on the Synthesis of Proline from ^{14}C-Acetate.

Roots were preincubated for 3 hours in a tracer free medium containing 1% sucrose, corn salts, and varying concentrations of ^{12}C-proline. Roots were then transferred to a media, identical in every respect to the preincubation medium but containing in addition ^{14}C-acetate. After 2 hours of incubation in tracer, the roots were extracted and fractionated. The scintillation counter was used to measure CPM in ^{14}C-proline.

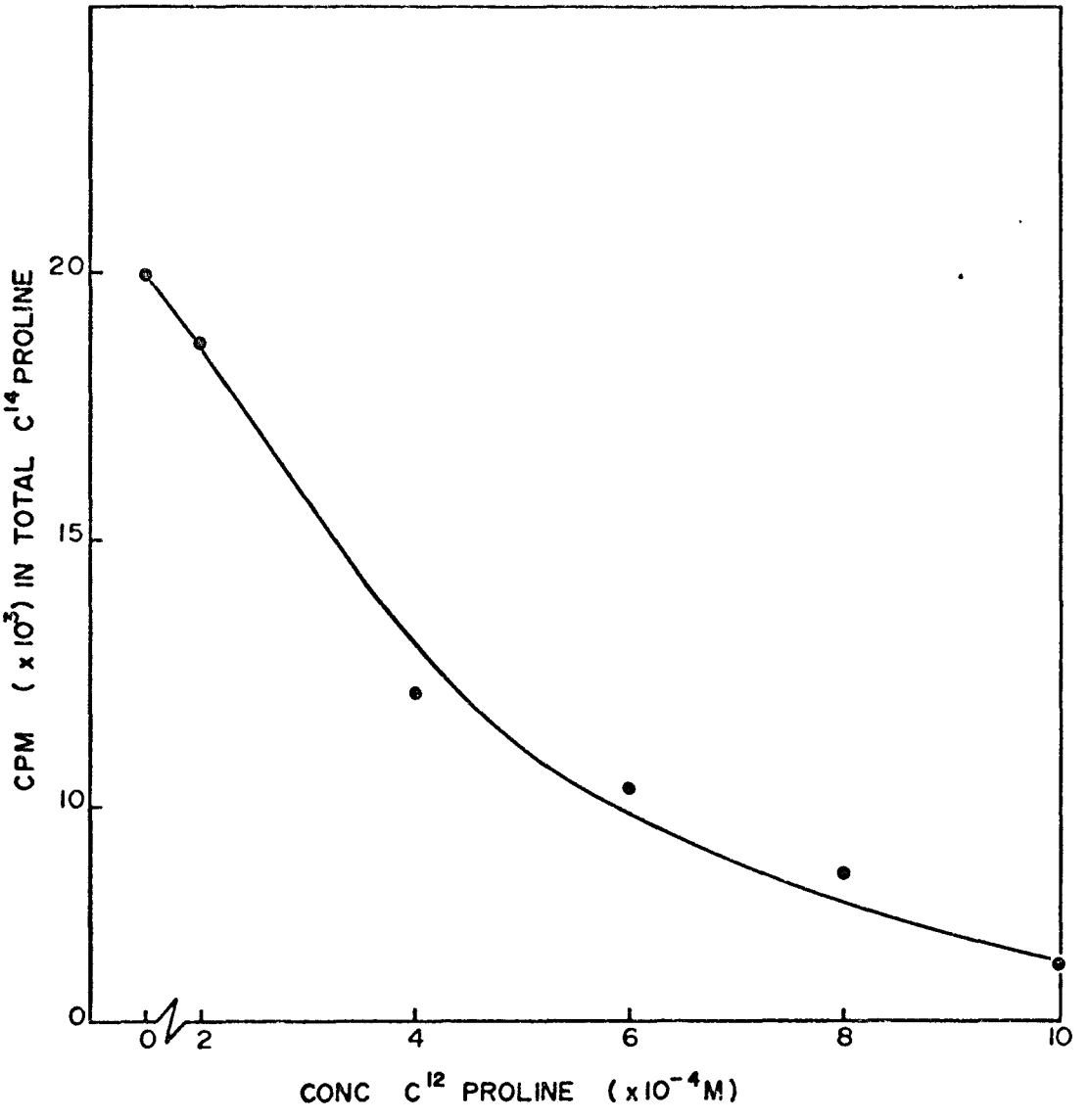

CPM (x10³) IN TOTAL C¹⁴ PROLINE
20
15
10
0
0
2
4
6
8
10
CONC C¹² PROLINE (x10⁻⁴M)

TABLE X A

Statistical Analysis to Show the Significance of Changes in Amino Acid Synthesis in the Presence of Competitors

Variations due to sampling error arise when working with a relatively small population (40 root tips). During each experiment which involved the synthesis of amino acids from ^{14}C-acetate in the presence of competitors, one or more control flask were necessary to observe synthesis when no competitor was present. The values obtained for individual amino acids in counts per minute from these 5 control runs were subjected to statistical analysis. The standard deviation is estimated from this data as shown on page 53.

Number of controls examined	N
Counts/min in amino acid	X
Arithmetic mean	$\frac{\sum X}{N} = \bar{X}$
Individual deviation from mean	$X - \bar{X}$
Squared	$(X - \bar{X})^2$
Summed	$\sum (X - \bar{X})^2$
Standard deviation	$\sqrt{\frac{\sum (X - \bar{X})^2}{N - 1}} = S$

TABLE X B

Determination of Standard Deviation Arising Due to Experimental Technique (Includes Sampling Error)

For use in 't' Distribution Test of Significance

N = 5

Amino Acid	X CPM X 10^{-3}	$\bar{X}$	$\sum(X - \bar{X})^2$	S
Slow Running Group (lys, arg, gly, ser)	27.8 30.3 42.9 41.2 30.0	34.4	199	7.05
Thr	12.3 17.9 20.0 12.9 13.6	15.3	36.5	3.02
Ala	19.6 25.1 24.0 27.3 18.8	22.9	54.8	3.7
Prol	17.4 18.4 18.6 24.7 16.7	19.1	41.0	3.2
Val Met	6.1 5.0 7.5 10.5 5.5	6.9	18.6	2.16
Leu Iso	31.9 29.7 32.3 24.9 30.1	29.8	34.6	2.94

TABLE X B (continued)

Amino Acid	X CPM X 10^{-3}	$\bar{X}$	$\sum(X - \bar{X})^2$	S
γAB	13.2 19.3 18.4 14.0 12.6	15.5	38.8	3.5

shown on Page 53. In Table XI, it is shown that 10^{-4}M proline reduces CPM to 8.8 X 10^{-3}. The mean deviation as calculated on Page 53 is 19.1 X 10^{-3}. t is the deviation (19.1 - 8.8) divided by the estimated standard deviation found to be 3.2 for proline. According to the table for distribution of t (Table XB), the probability for obtaining such a deviation by chance is about 1.5%. Other amino acids are not affected by the presence of proline. Threonine for example has a 55% probability of deviating 15.3 - 13.3 through chance alone when proline is present. A probability greater than 0.05 is not considered significant. There is a possibility that glutamic acid is also affected by proline. Glutamic acid accounts for a very large ^{14}C fraction of metabolized ^{14}C-acetate. Therefore the increase in radioactivity by twenty-five percent for this acid may be quite significant. Refer also to Table 5 in the Appendix.

In this type of experiment a source of error may arise if amino acids formed from ^{14}C-acetate exchange with the ^{12}C-amino acid in the medium. Although the media in these experiments were not tested for ^{14}C-proline, Oaks (27) has shown in similar experiments with leucine that there is no exchange between acetate-formed ^{14}C-leucine and ^{12}C-leucine in the media.

It is concluded therefore that proline specifically intereferes with proline synthesis from ^{14}C-acetate. Fifty percent inhibition is obtained using a concentration of 6 X 10^{-4}M proline.

(2) Competition Experiments with Other ^{12}C-Amino Acids:

To determine whether proline was unique in its capacity to control proline biosynthesis, other amino acids were tested.

TABLE XI

Glutamic acid, leucine, arginine and proline were added in the concentrations indicated to the preincubation media and the media used for tracer feeding. Otherwise, the experiment was carried out using the conditions described for the proline competition experiment (see Figure III).

Effect of ^{12}C Amino Acids on the Incorporation of ^{14}C from Acetate into Amino Acids

Competitor Conc. (M)	Control CPM	Proline 10^{-4} CPMX10^{-3}	Proline *% control	Arginine 5 X 10^{-3} CPM	Arginine *% control	Glutamic 10^{-3} CPM	Glutamic *% control	Leucine 10^{-4} CPM	Leucine *% control
Lys	13.3	12.1	91	14.1	106	10.8	81	14.8	111
Arg	12.8	13.3	104	2.6	20	10.8	85	21.3	166
Gly, Ser	2.1	2.5	119	1.7	81	2.4	114	3.3	157
Thr	13.6	13.3	98	15.6	115	14.0	103	16.8	124
Ala	21.7	24.5	113	24.9	115	27.0	124	29.7	137
Pro	17.7	8.8	50	24.0	136	20.5	116	27.9	156
γAB	18.8	23.6	124	21.4	114	27.4	144	24.3	126
Meth, Val	6.1	5.5	90	5.7	95	5.8	95	3.7	62
Iso, Leu	31.9	32.6	102	34.3	107	28.6	90	4.3	14
**Total N&B A.A.	147.9	136.1	92	145.5	98	147.8	100	146.1	99
Acidic A.A.	1,155.0	1,344.0	116	1,279.0	111	1,190.0	103	1,479.0	128
Amides	659.0	727.0	111	488.0	73	528.0	80	618.0	94

* See text, page 49 for description of this calculation.

**N&B A.A. = Neutral and Basic Amino Acids.

Arginine, glutamic acid, and leucine were added to the incubation media at concentrations of 5 X 10^{-3}M, 10^{-3}M, and 10^{-4}M respectively.

Arginine was chosen as a competitor because other work (8) has indicated that the biosynthetic step:

Glutamic acid ⟶ Glutamic γ-semialdehyde

is common to both the arginine and proline biosynthetic pathways. It was of interest to learn therefore, if the control of these two pathways was also related. Arginine inhibited its own biosynthesis specifically. At the same time a 36% increase in ^{14}C-proline also occurred. These observations may indicate that arginine exerts its control on the arginine biosynthetic pathway at a step following Δ^1pyrroline-5-carboxylic acid (PCA) so that this intermediate accumulates in the presence of excess arginine. Some ^{14}C-PCA so spared might then be diverted to the proline biosynthetic machinery. If arginine controlled the step:

Glutamate ⟶ Glutamic γ-semialdehyde,

^{14}C-glutamic acid would show some accumulation. Very little increase was observed in the acidic amino acid fraction. However, these amino acids account for a very large proportion of ^{14}C counts. Subsequently any decrease or increase in content of these amino acids would have to be substantial to appear significant.

Leucine, used to represent an example of the neutral amino acids, inhibited its own biosynthesis and to a lesser

TABLE XII

Rate of ^{14}C-Glutamic Acid Uptake and Metabolism

5 mm corn root tips were preincubated for 30 minutes in a corn salts solution. They were then transferred to Warburg flasks containing two ml of corn salts and ^{14}C-glutamic acid. At intervals of 15, 30, 45 and 60 minutes the roots were harvested and fractionated.

Time of Exposure to tracer (min)	15	30	45	60
		CPM X 100		
Total Soluble	340.7	532.1	852.2	1130.0
Organic Acids	16.0	57.1	182.0	158.0
Sugars	0.8	0.9	1.8	2.6
N & B A.A.	10.9	21.9	44.7	54.5
Acidic A.A.	284.2	390.3	565.1	880.0
Total Ins.	23.8	43.8	63.0	95.5
N & B A.A.	3.9	5.1	8.3	11.6
Acidic A.A.	16.1	29.3	33.3	49.4

Organic Acids

Time of Exposure to tracer (min)	15	30	45	60
Organic Acids Measured		*Relative %		
Succinate	16.3	8.5	10.7	7.4
Malate	77.5	71.5	72.0	71.3
Citrate	6.3	20.0	17.2	20.6

* $\frac{\text{Radioactivity in the individual organic acid}}{\text{Radioactivity in malate + citrate + succinate}} \times 100$

N & B A.A. = Neutral and Basic Amino Acids

degree that of valine. Resulting from this inhibition, there was a general increase in other amino acids, possibly a sparing effect.

Glutamic acid has been shown by Roberts et al. (28) to be on the proline biosynthetic pathway of E. coli. If such a pathway operates in corn root tips, the addition of ^{12}C-glutamate would be expected to expand the soluble glutamic acid pool, dilute the ^{14}C-glutamate entering this pool, and as a result lower the activity of metabolites derived from glutamic acid.

Since no decrease appeared either in proline or arginine, it appears that external glutamic acid is not entering the glutamate pool which serves as precursor for these amino acids in amounts sufficient to have an observable effect. Joy and Folkes (29) found that the acidic amino acids were not readily taken up from the media. Furthermore in an experiment in which ^{14}C-sucrose was fed to barley embryos grown either on nitrate or on an amino acid mixture, all amino acids except glutamic acid were shown to have lower relative activities in the amino acid fed embryos. By referring to Table XII where ^{14}C-glutamate has been fed to root tips, it is clear that glutamate entering the tissue is extensively metabolized. It is therefore accessible to the mitochondria and a number of amino acid biosynthetic enzymes. When ^{14}C-proline and ^{14}C-glutamate were provided to corn roots for thirty minutes, it was found that roots receiving ^{14}C-glutamate had only fifty percent as much ^{14}C content in their

TABLE XIII A

Metabolism of ^{14}C-Glutamic Acid

5 mm corn roots were placed in 5 ml corn salts media containing 1% sucrose for 3 hours. Following this, roots were transferred to 2 ml of similar media which contained ^{14}C-glutamate.

Tracer	^{14}C Glu
Fraction	(X1000) CPM
Total Soluble	1,253.8
Amino Acids	910.6
Neutral & Basic A.A.	33.4
Acidic A.A.	345.3
Amides	316.6
Organic Acids and Sugar	319.1
Total Insoluble	122.5
Total A.A.	117.7
Neutral & Basic A.A.	32.3
Acidic A.A.	81.5
Neutral Fraction	6.7

Amino Acids	Soluble		Insoluble	
	X 1000 CPM Sol	*Ratio % of N & B	X 1000 CPM Ins.	**% of Total Ins
Total	34.47	100	32.27	100
Lys	.84	2.4	4.80	14.90
Arg	.22	.6		
Unknown			6.74	20.90
Gly) Ser)	.97	2.8	2.92	9.10
Thr	1.92	3.5	3.45	10.70
Ala	5.05	14.6	2.88	8.80
Prol	0.35	1.0	8.60	26.60
γAB	24.10	70.0		
Meth) Val)	.03	.9	1.13	3.50
Leu) Isol)	.00	.0	1.75	5.43

* $\frac{\text{Counts in soluble amino acid X}}{\text{Counts in soluble neutral and basic amino acids}}$

** $\frac{\text{Counts in insoluble amino acid X}}{\text{Counts in insoluble neutral and basic amino acids}}$

For further information consult the text (Page 61).

TABLE XIII B

Metabolism of ^{14}C-Glutamic Acid Competition with Proline

Experimental procedure as described for Table XIIIA but medias contained proline at concentration 10^{-4}M

Tracer	^{14}C-Glu	
Competitor	^{12}C-proline (10^{-4}M)	
Fraction	CPM (X1000)	Ratios of fraction $\frac{\text{with proline}}{\text{without proline}} \times 100$ = % of fraction without competitor
Total Soluble	439.5	35
Amino Acids	342.7	37
Neutral & Basic A.A.	36.6	111
Acidic A.A.	136.3	39
Amides	84.7	29
Organic Acids and Sugars	96.5	30
Total Insoluble	34.4	28.3
Total A.A.	33.7	29
Neutral & Basic A.A.	8.4	26
Acidic A.A.	25.2	31
Neutral Fraction	2.2	30

Amino Acids	Soluble X 1000 CPM Sol.	Soluble *Ratio % of N & B	Insoluble CPM Ins.	Insoluble **Ratio % of N & B
Total	36.34	100	8.39	100
Lys	.82	2.3	1.4	16.7
Arg	.20	.6		
Unknown			1.68	20.0
Gly, Ser	1.36	2.7	1.40	16.7
Thr	1.98	5.5	1.46	17.4
Ala	4.75	13.2	.87	10.4
Prol	.25	0.7	.34	4.2
γAB	27.0	74.3		
Meth, Val	.27	.7	.37	4.4
Leucines	.00	0	.87	10.3

* and ** See TABLE XIIIA.

soluble fraction as roots fed equal amounts of ^{14}C-proline.

C. Glutamic Acid as Proline Precursor:

(1) Synthesis of Proline:

^{14}C-glutamate was fed to corn root tips under conditions for maximum proline biosynthesis. Table XIIIA shows that glutamic acid may be a precursor for proline. Whereas it is conceivable that ^{14}C may enter proline by some other pathway since ^{14}C-glutamate is extensively metabolized, no reasonable alternatives can be suggested.

(2) Effects of ^{12}C-Proline on the Synthesis of Proline from Glutamic Acid:

Because proline inhibited the synthesis of proline from ^{14}C-acetate, it was important to know if a similar concentration of proline would inhibit proline synthesis from ^{14}C-glutamic acid.

Table XIIIB shows that ^{14}C-glutamic acid uptake is greatly reduced by the presence of 10^{-4} M of proline in the media. Proline may be competing with glutamic acid for entry into the tissue.

The interpretation of this experiment becomes somewhat difficult because it is hard to distinguish the effects of proline at the level of ^{14}C-glutamate uptake from those at the level of ^{14}C-glutamate metabolism. The difficulty is augmented because the distribution of label in the various fractions is not proportionately reduced by the presence of proline. For example, proline has not affected ^{14}C incorporation into the soluble neutral and basic amino acid fraction but has

reduced ^{14}C content of the insoluble fraction by seventy percent.

Consequently the following calculations were made in an attempt to discover whether proline was affecting individual amino acid biosynthesis: the total amount of activity entering the neutral and basic fractions was considered as 100%. Each amino acid was considered as a relative percent of this fraction. Thus, in the absence of proline, ^{14}C-proline from glutamic constitutes one percent of the soluble neutral and basic fraction and 26.6 percent of the protein fraction. In the presence of proline, ^{14}C-proline is 0.7 percent of the soluble fraction and 4.2 percent of the insoluble fraction. It appears that proline biosynthesis from ^{14}C-glutamic acid is inhibited by the presence of proline in the media - particularly since no other amino acid shows this proportionate decrease. Glycine, serine, threonine and leucines show rather marked increases by this ratio method. Whereas a ratio decrease in a fraction is expected to be accompanied by a compensating increase in other fractions, these amino acids have increased in excess of the proportion expected if actual increases in synthesis had not occurred. Proline may therefore, be sparing glutamic acid for incorporation into these acids.

From these results it appears that:

1. proline does inhibit proline synthesis from ^{14}C-glutamic, but
2. ^{14}C-glutamate is not a good precursor for <u>in vivo</u> studies of proline synthesis because uptake of this amino acid by the tissues is poor. This uptake is further hindered when proline is present in the medium.

CHAPTER III

DISCUSSION

A. Control of Proline Synthesis

(1) In Corn Root Tips:

When ^{14}C-acetate was fed to newly excised corn root tips over a two hour period, little label was found in proline. The synthesis of ^{14}C-proline increased 13-fold, however, if a three hour incubation period preceded the application of radioactive precursor. This synthesis, whether from ^{14}C-glutamate or ^{14}C-acetate was sensitive to concentrations of external proline greater than 2×10^{-4}M. Fifty percent inhibition of proline synthesis was obtained using 6×10^{-4}M of proline.

It has been demonstrated in other plant tissues that proline can exert an influence on its own biosynthesis. A sixty percent decrease in soluble ^{14}C-proline was obtained in tobacco leaf discs metabolizing ^{14}C-glutamate when a concentration of 2×10^{-3}M of exogenous proline was added to the media (30). Dougall could prevent the incorporation of ^{14}C-proline into the protein of cultured Paul's scarlet rose cells by adding 5×10^{-3}M of proline to the culture medium containing ^{14}C-glucose as radioactive precursor (31). In these instances, control of proline synthesis was attributed to feedback mechanisms. In the root tip, where proline synthesis is somewhat sensitive to proline inhibition (10^{-4}M), where inhibition is seen to be gradual over a

range of concentrations and where total proline synthesis has indicated inhibition, there would seem to be adequate basis for suggesting that a feedback and/or an induction mechanism operates in the normal control of proline biosynthesis.

(2) Control Mechanisms in Other Systems:

The proline biosynthetic pathway as established for several organisms (28, 32) has two reductive steps:

1. Glutamic Acid $\xrightarrow{\text{NADH} \quad \text{NAD+}}$ Glutamic γ-semialdehyde
2. Glutamic γ-semialdehyde $\rightleftharpoons$ Δ^1pyrroline-5-carboxylic acid $\xrightarrow{\text{NADPH} \quad \text{NADP+}}$ Proline

These enzymes are located in the supernatant cell fraction (16).

Several mechanisms for the control of this sequence have been suggested. When proline is omitted from a suspension culture of L-cell mouse fibroblasts, five times as much Δ^1pyrroline-5-carboxylic acid reductase is formed as is produced when the cells are grown with proline (33). In Neurospora, the specific activity of partially purified Δ^1pyrroline-5-carboxylic acid reductase was shown to be decreased when mycelium was grown in minimal media supplemented with 8.5×10^{-3}M of proline. Some inductive control of proline synthesis may occur therefore at the second reductive step.

In Escherichia coli, Baich and Pierson (35) observed no feedback inhibition of Δ^1pyrroline-5-carboxylic acid reductase by proline, nor was the synthesis of this enzyme repressed by 8.8×10^{-3}M of proline in this growth media.

Glutamic acid fed in high concentration to the wild

type E. coli strain, resulted in no excretion into the media. A mutant E. coli however excreted considerable amounts of this amino acid. When glutamic γ-semialdehyde was fed to these bacteria at the same molar concentration as glutamic acid had been fed, both bacteria excreted proline. These observations were interpreted to mean that control of proline synthesis is at the level of glutamic acid reductase.

In support of this theory, a mutant which could not use glutamic γ-semialdehyde accumulated high amounts of this intermediate when fed glutamic acid. In the presence of proline however, the glutamic γ-semialdehyde in the media was reduced. Furthermore, the synthesis of glutamic γ-semialdehyde reductase appeared to be curtailed in the presence of chloramphenicol. This information seems to indicate that in some systems at least the first reductive enzyme controls proline biosynthesis. The control at work in corn root tips is yet to be elucidated.

B. Proline Degradation:

(1) Degradative Products:

Gains in protein proline and hydroxyproline cannot account for all the loss in soluble proline during the period following corn root excision. Folkes and Yemm (1), noting such decrease in the net proline content of barley seedling during germination, suggested that proline was being interconverted to other amino acids and nitrogen products.

By feeding tracer ^{14}C-proline to intact corn roots, it was possible to follow the fate of this amino acid after

root tip excision. It was found that about one-half of the label entered fractions other than protein. Most of this ^{14}C from metabolized proline was found in glutamic acid, intermediates of the tricarboxylic acid cycle and in carbon dioxide. It is proposed therefore that proline is being oxidized by way of glutamic acid and α-ketoglutarate to intermediates of the Krebs cycle and their derivatives.

(2) The Oxidative System:

Evidence for such a proline oxidative system has been described for Escherichia coli (36), Neurospora crassa (37), and rat liver tissue (15). As described by Johnston and Strecker for rat liver the pathway consists of two enzymes: the first step is aerobic and involves a cytochrome c linked oxidation of proline to Δ'pyrroline-5-carboxylic acid (PCA). The second step is pyrridine nucleotide-linked dehydrogenation of PCA to glutamic acid (15). Both enzymes are firmly associated with the mitochondria.

(3) Control of the Oxidative System:

Frank (36) found that if E. coli were grown in the presence of proline, the specific activity of proline oxidase increased to fourteen times that when no proline was in the media. No other oxidase system showed such an increase. He concluded that proline oxidase was induced in E. coli by the presence of proline.

C. A Control Scheme for Proline Metabolism in Corn Seedlings:

The normal development of growing tissue may be

strongly dependant on the proper integration of proline supply with proline degradation and proline incorporation into protein. Growth of excised corn embryos is delayed when proline is limiting (4). There is an inhibition of seedling development if the proline analogue azetedine-2-carboxylic acid is incorporated into protein during germination (38). In addition, an accumulation of proline is often associated with the uncontrolled growth typical of tumors (39, 40).

Therefore it is important to consider a control system which would govern all aspects of proline metabolism.

In this respect the scheme suggested by Matchett and DeMoss for the control of tryptophan metabolism in Neurospora may be considered (10). Tryptophan is subject to a cyclic synthesis and degradation by way of the intermediate anthranilic acid. The tryptophan arising endogenously from tryptophan synthetase is thought to proceed directly to an inner pool used for the synthesis of protein. This pool appears to mediate the end product regulation of enzymes of the biosynthetic sequence. A larger outer pool receives tryptophan from the external media. When this pool is expanded, critical enzymes of the tryptophan cycle appear to be induced. Exchange between the two pools takes place.

An analogous system could be visualized for the control of proline in corn root tips. For example, Oaks (6) has presented evidence for a protein precursor pool in root tips similar to the inner pool of tryptophan described above. This pool mediates in leucine biosynthesis. She has shown

that external leucine expands this pool more rapidly than the total pool. Since it has been shown that newly synthesized proline does not seem to equilibrate with total proline in the root tip before incorporation into protein, it is assumed, without very good experimental evidence that a similar pool may exist for proline.

In the intact root, transport proline from other areas of the embryo would keep the large 'external' proline pool expanded (5). This expansion in turn may ensure continued production of the proline oxidative enzymes through a proline induction mechanism. Biosynthetic enzymes on the other hand may be repressed by the presence of exogenous transport proline entering the inner protein precursor pool from the external pool. During preincubation of excised roots, when transport proline is not available, the depletion of the general proline pool would curtail the induction of oxidative enzymes. At the same time, incorporation of proline into protein from the precursor pool would lower the level of proline there, thus ending feedback repression of proline biosynthetic enzymes.

Proline oxidative enzymes appear to be very high in intact root tissue (Table VI, Page 35). In E. coli it has been shown that the proline oxidizing system severely limits the amount of exogenous proline available for protein synthesis in a prolineless mutant (41). The question arises then, as to how transport proline is spared for uptake into the protein precursor pool during corn germination.

Two answers may be possible within the present scheme. Firstly, the release of transport proline from endosperm sources appears to be a regulated process, governed by proline demands in the embryo (3). Since proline does not arrive in the root tip all at once the amino acid cannot be oxidized during the initial germination stages, depriving the internal pool of a continued supply for proline synthesis.

Secondly, proline may be transported in a form not readily available for oxidation. Stone and Hoberman (41) have found that the growth of prolineless bacteria is proportional to the amounts of proline which escapes from degradation by proline oxidation. Growth is prolonged by feeding proline in peptide form. In this respect, a study has been made of the amino acid composition of peptide fractions in germinating barley (42). Endosperm peptides had an amino acid content similar to storage protein. Embryo peptides also had the composition of mobilized storage protein. Thus it is possible that proline is transported in peptide form. If this is the case, then the ^{14}C-proline fed to intact roots may not have truly represented the fate of transport proline entering the root tip region during the normal germination process.

CHAPTER IV

SUMMARY

Proline is used by maize root tips in two ways: it is oxidized to glutamic acid and thus becomes available for metabolism by the tricarboxylic acid cycle, and it is incorporated into protein.

The synthesis of proline is controlled by the level of free proline in the tissue. This was shown by the increase in proline biosynthesis when the proline content in tissue was depleted, and by the inhibition of proline biosynthesis when an external source of proline was supplied.

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APPENDIX

Table 1

Utilization of ^{14}C-Proline by Intact Corn Roots

^{14}C-Proline incorporated into agar was presented to corn root tips during a 30 minute pulse. Following this roots were excised and placed for varying periods in tracer-free corn salts media.

Time after Exposure to tracer	0 Hours	1/2 Hours		1 1/2 Hours		3 Hours	
Fraction	CPM X 10^2	CPM X 10^2	*% of Initial ^{14}C	CPM X 10^2	% of Initial ^{14}C	CPM X 10^2	% of Initial ^{14}C
CO_2		7.5		14.6		20.0	
Counts lost to media		28.0		18.3		15.6	
Soluble Neutral & Basic Amino Acids	255.0	108.2	42.5	52.8	20.4	35.0	13.8
Acidic Amino Acids	143.0	71.7	50.0	72.6	50.8	54.2	37.7
Sugars	4.1	4.1	100.0	3.0	73.0	4.1	100.0
Organic Acids	45.7	57.0	125.0	44.0	96.5	53.0	116.0
Insoluble Neutral & Basic Amino Acids	114.4	130.4	114.0	166.4	146.0	183.6	161.0
Acidic Amino Acids	22.3	25.2	113.0	23.6	106.0	31.5	142.0
Neutral Fraction	3.4	3.0	88.0	3.4	100.0	4.5	133.0
Total	587.9	435.1		398.7		401.5	

* % of Initial ^{14}C $\frac{^{14}C \text{ presently in fraction}}{^{14}C \text{ in fraction immediately following pulse}} \times 100$

Table 2

Utilization of ^{14}C-Proline by Excised Corn Roots

Proline-U-^{14}C was present continuously over periods of 10, 20, 30, and 60 minutes in a corn salts media. Forty excised roots were used per sample.

Minutes roots were in tracer media	10	20	30	60
Fraction	CPM X 10^2	CPM X 10^2	CPM X 10^2	CPM X 10^2
Soluble Neutral and basic A.A.	518.0	543.0	837.0	2392.0
Acidic A.A.	41.8	46.7	75.8	211.0
Sugars	2.0	2.2	3.9	9.8
Organic Acids	13.0	13.5	18.0	45.9
Insoluble Neutral and basic A.A.	44.0	69.1	146.0	503.0
Acidic A.A.	4.2	7.5	8.4	18.0
Neutral fraction	0.9	1.4	1.3	2.8
Total	623.9	683.4	1090.4	3182.5

*Note that CO_2 was not measured in this experiment.

Table 3

How Preincubation Time Affects ^{14}C-Acetate Incorporation

A. Preincubation: Effect on ^{14}C-Acetate Metabolism

Time	0	1		3		6 hr.	
	CPM X 10^3	CPM X 10^3	*% time 0	CPM X 10^3	% time 0	CPM X 10^3	% time 0
Total	641	789	125	1,103	174	1,390	217
Soluble							
A.A.	246	449	183	689	281	652	266
N & B	18.7	12.2	652	39.3	210	49.7	266
Acidic A.A.	223	170	76.3	309	139	474	213
Glu	229	129	56.4	211	92.2	347	152
Asp	6	7.8	130	4.3	73.4	12.3	202
Amides	126	179	139	299	238	178	142
GlN	84.5	175	148	205	243	123	146
AsN	1.3	2.6	200	4.85	373	9.8	755
Org A & S	126	144	114	164	130	244	194
Total							
Insoluble							
A.A.	180	316	175	345	192	350	194
N & B	17.9	26.3	147	41.5	232	38.0	213
Acidic	105	187	178	231	220	240	229
Glu	47.9	108	226	124	259	153	320
Asp	7.5	10.0	214	24	320	39.15	522
Neut. Fr.	310	218	70	155	50	136.0	43.7

$$* \% \text{ time } 0 = \frac{^{14}C \text{ content with preincubation}}{^{14}C \text{ content without preincubation}} \times 100$$

Table 3

How Preincubation Time Affects ^{14}C-Acetate Incorporation

B. Into Amino Acids of Corn Roots

Values for individual amino acids determined using chromatography and strip counter (see Methods).

Time of Preincubation (Hours)	0	1		3		6	
	CPM X 10^3	CPM X 10^3	% time 0	CPM X 10^3	% time 0	CPM X 10^3	% time 0
Soluble							
Total N & B	18.70	12.21	55.4	39.3	100.5	49.73	112
Ala	4.40	3.40	65.5	12.87	160.0	12.40	119
Thr	3.26	1.65	42.3	2.93	49.0	4.44	63.5
Pro	.63	.53	72.0	1.15	100.0	2.24	150
γAB	3.37	1.33	30.0	5.74	83.4	14.05	157
Val	1.13	.96	72.3	1.15	55.5	2.69	100
Leu	1.46	.89	37.5	1.61	60.4	2.24	116
Slow	4.02	3.43	71.7	9.40	128.0	11.10	64.6
Insoluble							
Total N & B	17.92	26.34	130	41.50	201	38.01	108
Ala	0.69	1.38	181	2.16	278	2.40	181
Thr	0.94	1.92	156	5.95	550	4.68	255
Pro	0.13	0.64	234	8.95	5920	9.56	372
Val	0.89	1.59	194	1.19	239	1.11	136
Leu	0.94	9.80	112	9.30	86	7.35	40
Slow	4.83	8.84	183	13.94	288	13.37	276
Soluble and Insoluble							
Total	36.66	38.50	106	80.82	221	87.71	240
Ala	5.09	4.78	94	15.03	295	14.80	291
Thr	4.20	3.57	85	8.88	212	9.12	218
Pro	0.76	1.17	154	10.10	1330	11.80	1550
γAB	3.37	1.33	44	5.74	170	14.05	418
Val	2.02	2.55	126	2.34	115	3.80	188
Leu	10.40	10.69	102	10.91	105	9.59	95
Slow	8.85	12.27	138	23.34	263	24.47	277
Total A.A. & Amides	426	765	180	1448	340	1741	410

Table 4

5 mm roots were excised using sterile technique and preincubated for times of 1, 3 or 6 hours in corn salts media, with or without sucrose. Roots were then transferred to fresh media of similar composition which contained ^{14}C-acetate. (Roots were also added to media containing sucrose without preincubation.) Incubation time was 2 hours for all treatments.

A. Effect of Sucrose in Acetate Metabolism

Time of Preincubation Hours	0	1	1	3	3	6	6
1% Sucrose	+	-	+	-	+	-	+
Fraction	CPM X 1,000						
Total Soluble	641.2	983.2	789.2	1,047.0	1,103.0	999.2	1,391.0
Amino Acids	246.0	373.9	448.9	504.3	688.8	524.8	651.9
Neut & Basic A.A.	18.7	12.2	12.2	23.0	39.3	22.1	49.7
Acidic A.A.	222.6	195.3	170.1	256.0	308.1	409.1	473.6
Glu	229.6	174.3	129.4	189.0	211.2	344.0	347.3
Asp	6.0	4.0	7.8	8.0	4.3	14.8	12.3
Amides	126.4	191.3	179.1	202.6	299.0	172.9	178.0
GlN	84.5	130.8	125.0	177.6	204.8	88.6	122.6
AsN	1.25	1.3	2.6	8.47	4.85	11.6	9.8
Org.A. & Sug	126.0	230.0	144.0	176.4	164.4	205.0	244.0
Total Insoluble	180.0	318.0	316.0	306.0	345.0	260.0	350.0
Neut & Basic A.A.	17.9	27.9	26.3	27.1	41.5	20.6	38.0
Acidic A.A.	104.6	223.8	187.1	220.6	231.1	164.4	239.6
Glu	47.9	102.8	108.0	146.8	124.0	112.6	153.2
Asp	7.5	12.4	16.0	25.2	24.1	28.3	39.1
Neutral Fraction	310.0	268.0	218.0	248.0	155.0	189.0	135.0

Table 4

B. Amino Acids

from ^{14}C-acetate with and without Sucrose. Preincubation periods 0, 1, 3, 6 hours.

Time of Preincubation (Hours)	0	1	1	3	3	6	6
1% Sucrose	+	-	+	-	+	-	+
Soluble				CPM X 1000			
Ala	4.40	3.72	3.40	8.77	12.87	6.45	12.40
Pro	0.63	.30	.53	2.04	1.15	2.87	2.24
γAB	3.37	1.10	1.65	2.55	5.74	2.87	14.05
Leu	1.46	1.17	.89	1.65	1.61	.54	2.24
Val	1.13	.85	.96	2.67	1.15	1.17	2.79
Other	4.02	3.18	3.43	3.30	9.40	6.45	11.10
Thr	3.26	1.81	1.65	2.04	2.93	1.79	4.44
Total	18.7	12.13	12.2	23.02	39.30	22.14	49.70
Insoluble							
Ala	.69	1.51	1.38	2.93	2.16	2.92	2.40
Pro	.13	0.70	.64	2.30	8.95	6.43	9.50
Leu	8.94	11.60	9.80	8.10	9.30	3.23	7.35
Val	.89	1.51	1.59	1.94	1.19	1.91	1.11
Other	2.83	10.02	8.84	9.35	13.94	2.92	13.37
Thr	.94	2.32	1.92	2.30	5.95	1.27	4.68
Total	17.90	27.66	26.30	26.92	41.50	20.63	38.00
Total Neut & Basic A.A.							
Ala	5.09	5.23	4.78	11.70	15.03	9.37	14.80
Pro	.76	1.00	1.11	4.34	10.10	4.30	11.80
γAB	3.37	1.10	1.33	2.55	5.74	2.87	14.05
Leu	10.40	12.80	10.64	9.75	10.91	3.77	9.59
Val	2.02	2.36	2.55	4.61	2.34	3.08	3.80
Other	8.85	13.20	12.27	12.65	23.34	9.37	24.47
Thr	4.20	4.12	3.57	4.34	8.88	3.06	9.12
Total	36.6	39.82	38.5	49.94	80.8	40.82	87.7

Table 5

5 mm corn roots were preincubated for 3 hours in corn salts media containing 1% sucrose and varying concentrations of proline. Roots were then transferred to similar media containing ^{14}C-acetate for 2 hours. Following this roots were removed from media, rinsed briefly and crushed in alcohol. Amino acid radioactivity was determined by descending chromatography in BAW followed by scintillation counting of areas containing amino acids (see Methods).

A. Effects of Proline on ^{14}C-Acetate Metabolism

Concentration of Proline (M)	0	2×10^{-4}		4×10^{-4}		6×10^{-4}		8×10^{-4}		10×10^{-4}	
Total Sol.	3,271	3,438	105	3,290	101	3,413	99.4	3,481	106	3,490	107
Amino Acids	2,114	2,200	104	2,010	95	2,456	115	2,285	106	2,335	110
Neutral and Basic A.A.	82	78	95	81	94	82	99.5	84	102	74	90
Acidic A.A.	1,054	1,112	105	1,120	106	1,102	105	1,100	104	1,230	106
Glu	792									991	125
Asp	127									132	104
Amides	720	727	101	740	103	817	113	853	118	798	111
AsN	88									87	99
GlN	616									673	109
Organic Acids and Sugars	846	939	111	954	113	985	116	980	115	947	112
Total Ins.	419	420	100	426	101	442	105	420	96	445	106
Total A.A.	333	364	110	370	111	381	114	334	100	372	112
N & B	109	113	104	115	105	116	106	97.8	90	118	108
Acidic A.A.	224	234	104	237	107	282	126	256	115	253	113
Glu	129									165	129
Asp	78									78	100
Neut. Fr.	30	47	155	512	165	49.3	162	48	156	48	156

Table 5

B. Effects of Proline on Production of ^{14}C Neutral and Basic A.A. from ^{14}C-Acetate

Concentration of Proline (M)	0	2×10^{-4}		4×10^{-4}		6×10^{-4}		8×10^{-4}		10×10^{-4}	
		CPM	% time 0	CPM	% time 0	CPM	% time 0	CPM	% time 0	CPM	% time 0
Soluble											
Total N & B	82.4	78.0	95	81.0	98.4	82.0	100	83.6	102	74.2	90
Ala	25.4	24.4	85	24.1	95	20.4	80.5	25.8	102	24.4	96
Thr	9.1	8.4	90	8.5	93	8.9	98	8.0	88	7.2	72
Pro	4.9	3.0	61	2.3	47	3.0	61.2	3.7	75.5	2.0	40.8
γAB	24.0	23.2	97	27.2	114	26.5	111	27.9	116	20.8	87.7
Val	4.6	4.7	102	4.6	100	5.1	111	4.1	89	4.1	89
Leu	2.9	2.6	90	3.3	114	2.3	79	2.6	90	2.3	80
Slow	10.0	12.0	120	11.1	111	11.5	115	11.6	116	12.2	122
Insoluble											
Total N & B	109.0	113.0	104	115	106	116	107	97	89	118	108
Ala	5.3	5.6	106	6.7	127	6.2	117	5.4	102	6.8	129
Thr	12.0	11.5	96	10.5	87.5	13.8	113	11.1	92.5	13.7	114
Pro	16.2	15.7	103	10.6	65.4	8.0	49.3	4.8	29.7	5.3	32.6
Val	4.3	5.2	121	5.3	123	6.1	142	4.2	97.6	6.1	142
Leu	32.7	34.4	105	39.1	120	38	116	31.6	96.7	38.0	117
Slow	38.2	38.8	102	39.6	104	44	116	39.0	103	48.5	126
Total											
Sol & Ins	191	191	100	196	102	198	104	180	94	192	100
Ala	30.7	30.0	98	31.2	102	26.6	88	31.2	102	31.2	102
Thr	21.1	19.9	94	19.0	90	22.5	106	19.1	90.5	20.9	99
Pro	21.1	19.7	93.5	12.9	61	11.0	52	8.5	44	7.3	35
γAB	24.0	23.0	96.0	27.2	114	26.5	111	27.9	116	20.8	87
Val	8.9	9.9	111	9.9	111	11.2	126	8.3	93	10.2	115
Leu	35.6	37.0	104	42.4	119	40.3	114	34.2	96	40.3	113
Slow	48.2	50.8	106	50.7	105	55.5	115	50.6	105	60.7	126

Table 6

Competition Experiment: Effect of Arginine, glutamic acid, leucine, arginine and proline on the amino acids synthesized from ^{14}C-acetate.

Excised roots were placed in corn salts media containing 1% sucrose and a competitor - glutamic acid, leucine, arginine or proline in the concentrations 10^{-3}, 10^{-4}, 5 X 10^{-3} and 10^{-4} respectively. After a 3 hour preincubation the roots were transferred to a similar media containing ^{14}C-acetate for 2 hours. Fractionation was carried out as described in Methods. All radioactivity was measured using the scintillation counter.

Concentration (M)		Prol. 10^{-4}	Arg. 5 X 10^{-3}	Glut. A. 10^{-3}	Leu 10^{-4}
Soluble					
Lys	3.1	3.5	2.6	2.1	3.7
Arg	.9	1.3	.7	1.1	2.6
Gly, Ser	1.1	1.0	.9	.9	1.8
Thr	6.5	5.3	8.4	6.9	6.7
Ala	18.9	20.4	21.2	23.6	25.8
Pro	2.5	2.6	4.0	3.3	3.0
γAB	18.8	23.6	21.4	27.4	24.3
Val, Meth	2.6	2.3	2.3	3.0	1.4
Isol, Leu	2.2	2.2	2.0	1.7	.36
Acidic A.A.	853	1006	1071	954	1199
Insoluble					
Lys	10.2	8.6	11.5	8.7	11.1
Arg	11.9	12.0	1.9	9.7	18.7
Ser, Gly	1.2	1.5	.8	1.5	1.5
Thr	7.1	8.0	7.2	7.1	10.1
Ala	2.8	4.1	3.7	3.4	3.9
Pro	15.2	16.2	20.0	17.2	24.6
Met, Val	3.5	3.2	3.4	2.8	2.3
Leu, Iso	29.7	30.4	32.3	26.9	3.9
Acidic A. A.	301.7	338.2	207.7	236.3	279.8