STUDIES ON THE AMINO ACID METABOLISM OF HIGHER PLANTS

Thesis by

Bruce Joseph Rogers

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ABSTRACT

It has been known for some time that the protein level of detached leaves decreases as a result of the excision. The possibility that detached leaves are unable to form certain amino acids has been advanced as a cause for the decrease in the protein level. This hypothesis was tested in the present work. The incorporation with time of carbon-14 from C^{14}-carboxyl-labeled acetate and C^{14}-uniformly-labeled sucrose into the free and protein-bound amino acids of excised organs of red kidney bean (*Phaseolus vulgaris*) was studied. Of sixteen amino acids investigated, all were found to have incorporated carbon atoms from both acetate and sucrose in the excised leaves, stems and roots. On the basis of this work, it is felt that the decrease in protein level in detached leaves is not due to an inability of leaves to synthesize amino acids.

The incorporation of radioactive amino acids (produced *in vivo*) into leaf, stem and root protein of red kidney bean was studied. It was found that radioactive amino acids are incorporated into root protein much more rapidly than into leaf or stem protein.

The enzymatic decarboxylation and oxidation of a number of amino acids was studied in a water extract prepared from acorn squash. Great variation in the relative rates of reaction for the amino acids was demonstrated for both the decarboxylative and oxidative reactions.

The partial N-terminal amino acid sequence of pancreatic trypsin inhibitor was found to be arginyl-phenylalanine. It is suggested that the system used would be applicable for the determination of the sequence of small peptides such as those discovered in the work with red kidney bean.

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PART I

Localization of Amino Acid Synthesis in Plants

Introduction

The problem under consideration may be stated as follows: Are amino acids synthesized in all parts of a higher plant (leaves, stems, roots), or is there a localization of synthesis? Are some amino acids made in some organs and not in others? Although there has been no direct evidence that the synthesis of any amino acids is localized in this way, the possibility has been suggested in several indirect ways. Such evidence has come from studies concerned with the protein loss which occurs in leaves after they are detached from the plant.

The fact that abnormal protein loss occurs in the leaf after excision has been well established. The first inference that the protein in detached leaves is hydrolyzed was based on the large increase in asparagine which occurs when the leaves of young oat plants are kept in water in the dark for several days (1). More direct evidence came from the work of Chibnall (2) and Mothes (3) on bean and pea leaves. These two workers showed that an increase in non-protein nitrogen occurs concurrently with the decrease in protein nitrogen in the excised leaf.

At this point it might be worthwhile to consider a

pertinent facet of the problem--whether protein loss is caused by a decreased rate of protein synthesis, an increased rate of protein hydrolysis, or both. Vickery, et al. (4) measured the replacement of nitrogen in various tissues and compounds of intact tobacco plants with N^{15}-ammonia. They found that the rate at which nitrogen is incorporated into plant protein is consistent with the view that a steady state situation exists in which protein hydrolysis is normally balanced by protein synthesis. Similar experiments with sunflower plants supported this view (5).

Chibnall (6) investigated the uptake of nitrogen-15 by detached leaves of runner bean. After an incubation period of 96 hours the leaf protein contained an excess of the heavy isotope. The excess nitrogen-15 content of lysine from a portion of the protein was determined and found to be equally distributed between the two amino groups. Chibnall concludes from this that despite the fact that the protein level dropped during the incubation period, protein synthesis must have continued. Racusen and Aronoff (7) used soybean leaves fed C-14 carbon dioxide and found that with increasing time after excision of the leaf, rate of radioactivity incorporation into the protein decreased. They interpret this as an indication of a decrease in rate of protein synthesis after excision rather than an increase in rate of hydrolysis. A critical evaluation of their supporting data indicates a certain amount of incompatibility with this interpretation. An alternate con-

clusion from their data is that carbon-14 is incorporated into protein in the detached leaf, without reference to the relative rates of synthesis and hydrolysis. At the present time, it would seem that the gross cause of protein loss in detached leaves, whether a decrease in rate of synthesis, an increase in rate of hydrolysis, or both, is not known.

Regulation of the protein content of detached leaves has been studied by many workers. Yemm (8) found that the decrease in protein nitrogen in detached barley leaves is accompanied by successive increases and decreases of amino nitrogen, amide nitrogen, and finally of ammonia nitrogen. Determinations of the carbohydrate level and rate of respiration of the leaves showed that the carbohydrate decrease accounts for only a rapidly decreasing fraction of the carbon dioxide released after 24 hours. Thereafter, an increasing amount of carbon dioxide must come from non-carbohydrate sources--very possibly from the carbon skeletons of amino acids. More recent data collected by Vickery, _et al_. (9) also show a decrease in protein in detached tobacco leaves both in the light and in the dark. The rate of protein loss was only slightly less in the light than in the dark. Leaves in the light produce carbohydrate photosynthetically and this may have had some sparing action on the use of amino acid skeletons for respiration. Similar investigations on grasses have been carried out by Wood, _et al_. (10,11,12) with much the same results. A portion of this work was car-

ried a step further, however. Leaves were detached and allowed to lose protein in an anaerobic (nitrogen) atmosphere. Protein loss was minimized and amino acids, rather than amides and ammonia, accumulated. Leaves incubated in air showed large increases in amide nitrogen as well as amino nitrogen. Thus, in air, oxidative de-amination must take place, with the result that the concentration of amino acids is decreased. Not only is the over-all concentration decreased, but the concentration of certain amino acids must be disproportionately decreased, as will be seen later.

It is difficult to reconcile the above data with those of Phillis and Mason (13). These workers found that leaf discs (from nitrogen-deficient cotton plants) when floated on a medium high in nitrate are able to synthesize protein as rapidly as leaves left on nitrate-fed plants. High soluble nitrogen content was correlated with high protein level. On the basis of their data they suggest that the protein-amino acid ratio controls the rate of protein hydrolysis. This is exactly contrary to the result found in work cited previously on tobacco, barley, beans and peas in which the amino acid nitrogen level rises as the protein level decreases.

The work of several researchers, led by Mothes in 1931, has been along a different line. Mothes (14) found a correlation between water content and protein loss. Water deficiency in detached leaves slowed protein hydrolysis--probably as a result of a greater accumulation of hydrolytic products.

Interestingly enough, Chibnall (15) has found precisely the opposite. Leaves kept under high humidity for six days lost only about one-half the amount of protein lost by a control series kept under lower humidity.

Paech (16), on the basis of his own work and that of others, concludes that a generalized mass action relation controls the protein content of detached leaves, and the concentration of monosaccharides and soluble nitrogenous material determines whether protein loss or synthesis will occur. Chibnall (17), like Paech, concludes that the protein level must depend on the level of carbon and nitrogen sources. He suggests that the complete series of alpha-ketonic acids rather than the monosaccharides are of direct importance.

Wood and Cruickshank (18) determined the concentrations of several amino acids in the detached leaves of Kikuyu grass (_Pennisetum clandestinum_, Hockst.) and Algerian oat (_Avena sterilis_, L.) over a period of 192 hours. The concentration of each amino acid at a specified time was compared with a calculated concentration based upon the concentration of the amino acid in a normal leaf plus the amount of the amino acid liberated by the hydrolysis of protein during the specified time interval. The difference between the determined concentration and the calculated concentration gives a measure of the rate of utilization (that is, the rate of disappearance) of the amino acid. These workers were able to show that cystine disappears most rapidly, while glutamine,

arginine, tyrosine and tryptophan, in the order given, disappear more slowly. They conclude that "...there exists a steady state relationship between protein and amino acids based on the law of mass action, but complicated by the preferential oxidation of some amino acids at a rate more rapid than that of others." Similar work with rye grass has borne out the findings of Wood and Cruickshank (19).

Chibnall has suggested that if a deranged protein synthesis is responsible for the drop in the protein level of detached leaves, then possibly normal protein synthesis is brought about by some "influence" of the root system--perhaps a hormone (17). More recently Chibnall (15) has pursued this thought further, and has found that if detached leaves of runner bean are treated so that roots are formed, the decrease in protein is delayed some 17 days as compared to control leaves. This experiment points directly to the roots as the source for the factor or factors which regulate the protein level in leaves.

To sum up--leaves detached from a plant lose protein more rapidly than normal leaves. This may be due to an increase in the rate of hydrolysis of the protein, a decrease in the rate of synthesis of protein, or both. The separation of the leaf from the plant must be of utmost importance, so one would seek to explain the protein loss on the basis of disruption of the supply of some necessary factor which comes from the roots. A logical factor would consist of one or

more critical amino acids--critical in that the leaf cannot synthesize them in the quantity needed for normal protein synthesis. The following work has been designed to test this hypothesis.

Materials and Methods

There are two obvious methods which may be used to test for the localization of amino acid synthesis in plants: (1) isolation of the various enzyme systems, and (2) addition of labeled compounds to the separated organs of the plant and subsequent measurement of the radioactivity in the isolated amino acids. A third, more indirect method, would be to feed the separated organs a mixture of amino acids and record the protein level over a period of time. The first method is time-consuming. The third method could possibly answer the question but it might not since it is dependent on the rate of entry of all the amino acids. The second method was selected on the basis of rapidity, decisiveness, and the fact that it would provide other information, such as data on the incorporation of labeled amino acids into the protein of the different organs over a period of time.

Plant

The plant selected for this study was red kidney bean (*Phaseolus vulgaris*). It was chosen because it is easy to grow and produces ample amounts of tissue and protein.

The plants were grown in a medium of 50% vermiculite and 50% gravel for a period of five to six weeks in the Earhart Plant Research Laboratory. They were watered twice

a day with a standard nutrient solution. Natural light conditions were used, at a day temperature of 23° C and a night temperature of 17° C. At the time of harvest the plants were 30-45 centimeters high.

Treatment

The plants were separated into the three components--leaves, stems and roots--at the time of harvest. Fifteen to twenty gram aliquots of each tissue were placed immediately in solutions of either uniformly-labeled C^{14}-sucrose or of carboxyl-labeled C^{14}-acetate.

A 0.5% solution of sucrose was used, this being made up of uniformly-labeled C^{14}-sucrose (specific activity 0.17 millicurie per millimole) diluted with C^{12}-sucrose. The measured initial activity of the solution was approximately 67,000 counts per minute per milliliter, in a total volume of 400 milliliters. The final measured activity was about 30,000 counts per minute per milliliter.

The acetate solution was made up to 1% sodium and potassium acetate with C^{14}-carboxyl-labeled acetate (specific activity 1 millicurie per millimole) diluted with C^{12}-acetate. The measured initial activity was approximately 115,000 counts per minute per milliliter in a total volume of 500 milliliters. The estimated final activity was about 70,000 counts per minute per milliliter.

The plant parts were floated in the solutions at a level

just below the surface. The tissues were incubated at room temperature at low light intensities which ranged from total darkness to less than ten foot-candles. Aliquots of leaves, stems, and roots were placed in the appropriate solution for a specific time, removed, and another set placed in the solution for the next time interval. Upon removal the parts were rinsed in water twice and then quick-frozen in a mixture of dry ice and acetone, after which they were stored at -20° C.

Extraction and Separation of Free Amino Acids

The frozen material was homogenized in a Waring blendor in water and the brei filtered through Whatman #1 filter paper. A sufficient amount of trichloroacetic acid was added to the filtrate to make a 2-3% solution in order to precipitate the protein. The precipitate was separated from the supernatant by centrifugation and stored at -20° C for later examination.

The supernatant, essentially a water solution of salts, sugars and amino acids, was reduced in volume *in vacuo* at 50° to 60° C, shaken with ethyl ether to remove as much TCA as possible, and placed on an ion-exchange column (2 x 28 cm.) composed of Dowex-50 in the acid cycle. (20). The resin was washed with 100 milliliters of water to remove the sugars and organic acids, and then with progressively more concentrated solutions of hydrochloric acid in the sequence 1.5 N (100 milliliters), 2.5 N (100 milliliters) and 4 N (300 milli-

liters). The separation of the amino acids by this means was not complete but resulted in separation into groups of amino acids. This was presumably due to the high salt content of the water extract put on the column. The eluates of each group were combined, evaporated to dryness, taken up in water and separated by paper chromatography.

For the paper chromatography, solutions of knowns were made up according to Levy and Chung (21)--an "A" solution which contains 200 micromoles each of lysine, aspartic acid, glycine, threonine, proline, valine, tryptophan, phenylalanine and leucine, and a "B" solution which contains 200 micromoles each of cystine, histidine, arginine, serine, glutamic acid, alanine, tyrosine, methionine, and isoleucine. One spot of each of these two solutions was placed approximately in the center of a sheet of Whatman #1 filter paper ($22\frac{1}{2}$ by $18\frac{1}{4}$ inches) and to either side of these two spots the unknown mixture was streaked along the base line. The large sheets were run, six at a time, in a Chromatocab (Research Equipment Corp., Oakland, Calif.). The solvent used at this step of the process was butanol-acetic acid-water (4:1:5), with the water phase used for equilibration and the butanol phase for separation. Equilibration took place in 4-6 hours and the time required for the solvent to reach the bottom of the sheets was about 18 hours.

When the chromatographs were dry, four vertical streaks were made on the sheets with a small paint brush dipped in a

solution of 0.25% ninhydrin in acetone. Two of the streaks covered the known amino acids, and one streak was made to either side of the center of the sheets to indicate the presence of the amino acids from the unknowns.

It was found that the solvent system used can separate a mixture of amino acids into groups rather more easily than into individual amino acids under the conditions which prevailed in this work. The groups are as follows:

Group 1	Cystine, Peptide I
" 2	Lysine, Arginine, Histidine
" 3	Aspartic Acid, Glycine, Serine
" 4	Glutamic Acid, Threonine
" 5	Alanine, Proline
" 6	Tyrosine, alpha-Amino-butyric Acid
" 7	Valine, Methionine
" 8	Phenylalanine, Leucine and Isoleucine
" 9	Peptide II

Tryptophan was not found in the unknown solutions--most probably it was degraded on the column and during the evaporation process.

Horizontal strips containing each group as defined by the known amino acids and the ninhydrin streaks were marked, cut out and the amino acids eluted. For all groups but No. 6 elution was with water. For No. 6, elution was accomplished with a mixture of acetic acid and water at pH2-3.

The groups were rechromatographed to obtain the individual amino acids, using sheets of Whatman #1 filter paper eleven inches square and ascending chromatography. The solvents used are as described by Berry, et al. (22):

Group		Solvent	Amino Acid	R_f
1		65 ml. pyridine 35 ml. water	Cystine Peptide I	0.50 0.60
2	(A)	65 ml. pyridine 35 ml. water	Histidine Lysine, Arginine	0.50 0.35
	(B)	80 ml. Isobutyric Acid 20 ml. water	Lysine Arginine	0.20 0.30
3	(A)	95 ml. 95% ethanol 5 ml. conc. NH_4OH	Aspartic Acid Glycine, Serine	0.05 0.35
	(B)	65 ml. pyridine 35 ml. water	Glycine Serine	0.50 0.60
4		95% methanol	Glutamic Acid Threonine	0.40 0.50
5		80 ml. Isobutyric Acid 20 ml. water	Alanine Proline	0.40 0.55
6		95% methanol	Tyrosine α-NH_2-butyric acid	0.50 0.60
7		95 ml. ethanol 5 ml.glac.acetic acid	Valine Methionine	0.70 0.25
8		95% methanol	Phenylalanine Leucine,Isoleucine	0.50 0.60
9		Butanol-acetic acid-water (4:1:5)	Peptide II	0.90

It should be noted here that buffered solvents as described by McFarren (23) were not found satisfactory for separation of the crude water extract. Of several solvent systems tried, those listed gave the best results.

Quanitative Determinations

Location and elution of the separated amino acids was accomplished as before. The amount of water used for elution was minimized in order to keep the concentration of each amino acid as high as possible.

Determination of the radioactivity of each amino acid was carried out by depositing one-half milliliter of each eluate on a Tygon-coated planchet, drying under a heat lamp, and counting. Counting was done with either a thin-walled G-M tube or a flow counter as dictated by the activity level.

The absolute amount of each amino acid was determined on one-half milliliter portions of the eluate as described by Moore and Stein (24,25). The intensity of the amino acid-ninhydrin color complex was determined with a Beckman Model B spectrophotometer. Relative intensities were adjusted to leucine as 100. Specific activities were calculated as counts per minute per millimole of amino acid.

Protein-Bound Amino Acids

The water-extractable protein from the various tissues was hydrolyzed by treatment with 6 N HCl at 105° C for 20 hours. The amino acids were separated and the respective radioactivities measured by the same procedures as given above.

Results

The results of the radioactivity measurements are plotted on the following graphs. Data for the stems do not add any significant information and so were not included. It will be noticed that there is a certain amount of scatter in the position of some of the points. Undoubtedly this is a result of the fact that the allowable error had to be balanced against the large number of samples which were measured for radioactivity and the generally low activity of the samples (from 0.5 counts per minute upward). Sufficient counts were made to insure a probable error of $\pm$ 8% for the free amino acid data. Probable error is considered sufficient for most counting problems (26) and indicates that 50% of the time the counting error will be $\pm$ 8% or less. The equation used is

$$\text{Percentage error} = \frac{100\ k}{(N)^{\frac{1}{2}}}$$

where k is the probability constant and N represents the net number of counts (27). The standard error, calculated by the same equation for the same number of counts is $\pm$ 11%.

It is felt that the rather large error allowed in counting the radioactivity of the free amino acids is compensated for by the presence of three closely spaced points--those for 20 minutes, one hour and three hours. In order to establish

that biosynthesis of an amino acid takes place rapidly in a particular organ, these three points were generally considered to be most significant. Certain samples which vary widely from the general trend were counted twice in order to increase their accuracy. In a few cases one or two samples are missing and the curve must rely then on two or three samples.

The radioactivity of some of the amino acids was not measured because insufficient amounts were isolated. This is true for alpha-aminobutyric acid, histidine, proline and tyrosine for the sucrose experiments and for these same amino acids, with the exception of alpha-aminobutyric acid, for the acetate experiments. However, the radioactivity of histidine, proline and tyrosine, isolated from the protein hydrolysates was determined.

The radioactivity of the protein-bound amino acids was measured to a standard error of approximately $\pm$ 5%. The amounts recovered and hence the actual activities counted in every case were greater than for the free amino acids. More counts could be taken during the allotted time and therefore the accuracy is greater.

Tentative identification, based on paper chromatography, was made of the components of the two peptides which were discovered. Peptide I consists of lysine, arginine, glycine, and glutamic acid in estimated relative proportions of 3:1:1:1. This peptide is present in leaves, stems and roots in fairly large quantities and measurements of the incorporation of

carbon-14 were obtained. Peptide II consists of leucine, valine, alanine, glutamic acid, and glycine in estimated relative proportions of 3:1:1:1:1. This peptide is present in small amounts in the leaves and stems and was not discernible in the roots. Measurement of the incorporated carbon-14 was not attempted due to the small quantities available. A more thorough, quantitative examination of these two peptides is planned.

Figures I-IV. Acetate-C^{14}. These figures show the rise in radioactivity with time of the free amino acids of excised red kidney bean leaves and roots during incubation in a solution containing acetate-C^{14}.

Figures V-VIII. Sucrose-C^{14}. These figures show the rise in radioactivity with time of the free amino acids of excised red kidney bean leaves and roots during incubation in a solution containing sucrose-C^{14}.

Identification of curves and points:

Roots--designated by "R", solid line, point o.

Leaves--designated by "L", dashed line, point x.

Where points coincide, the point is shown as ●.

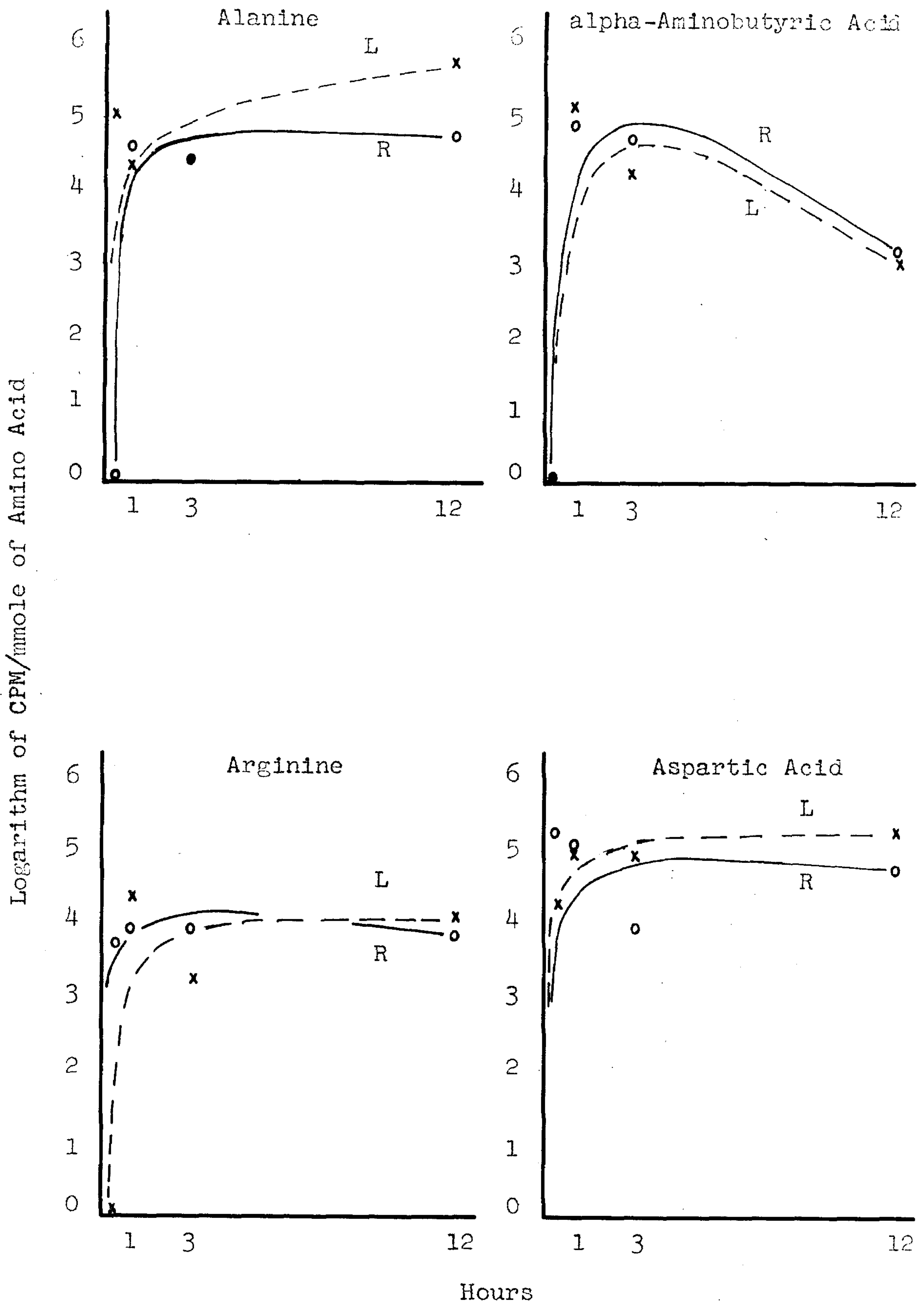


Figure I

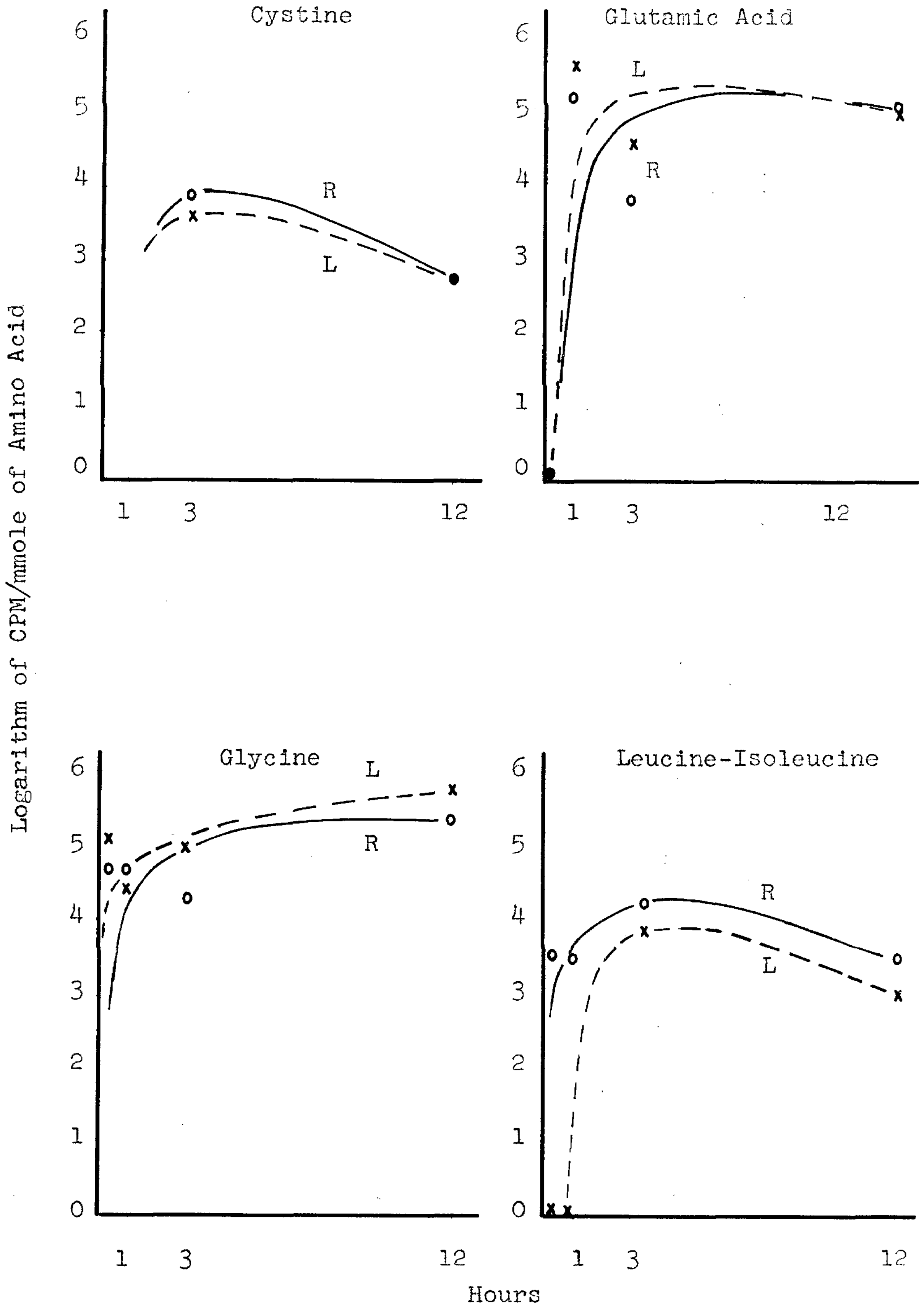


Figure II

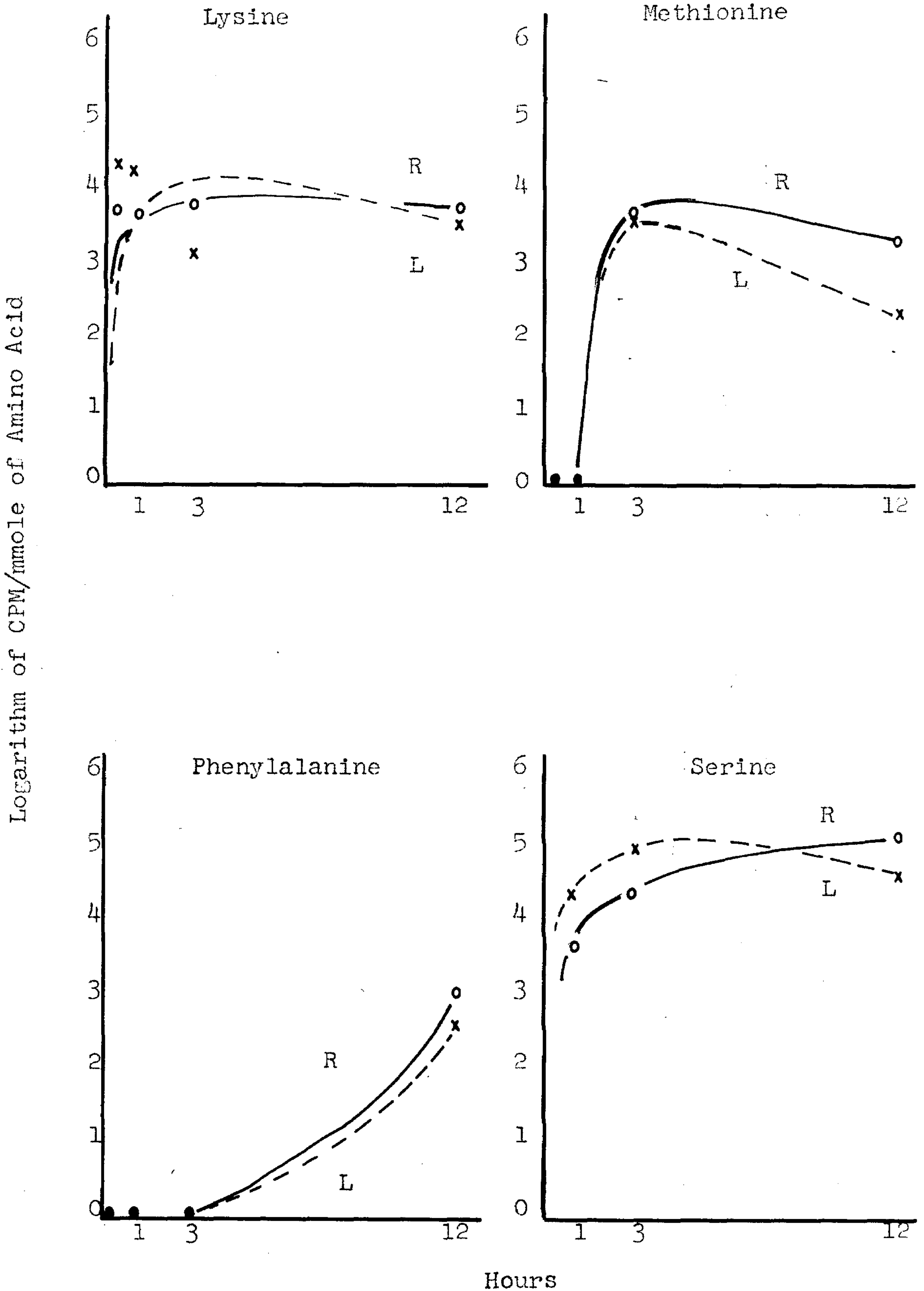


Figure III

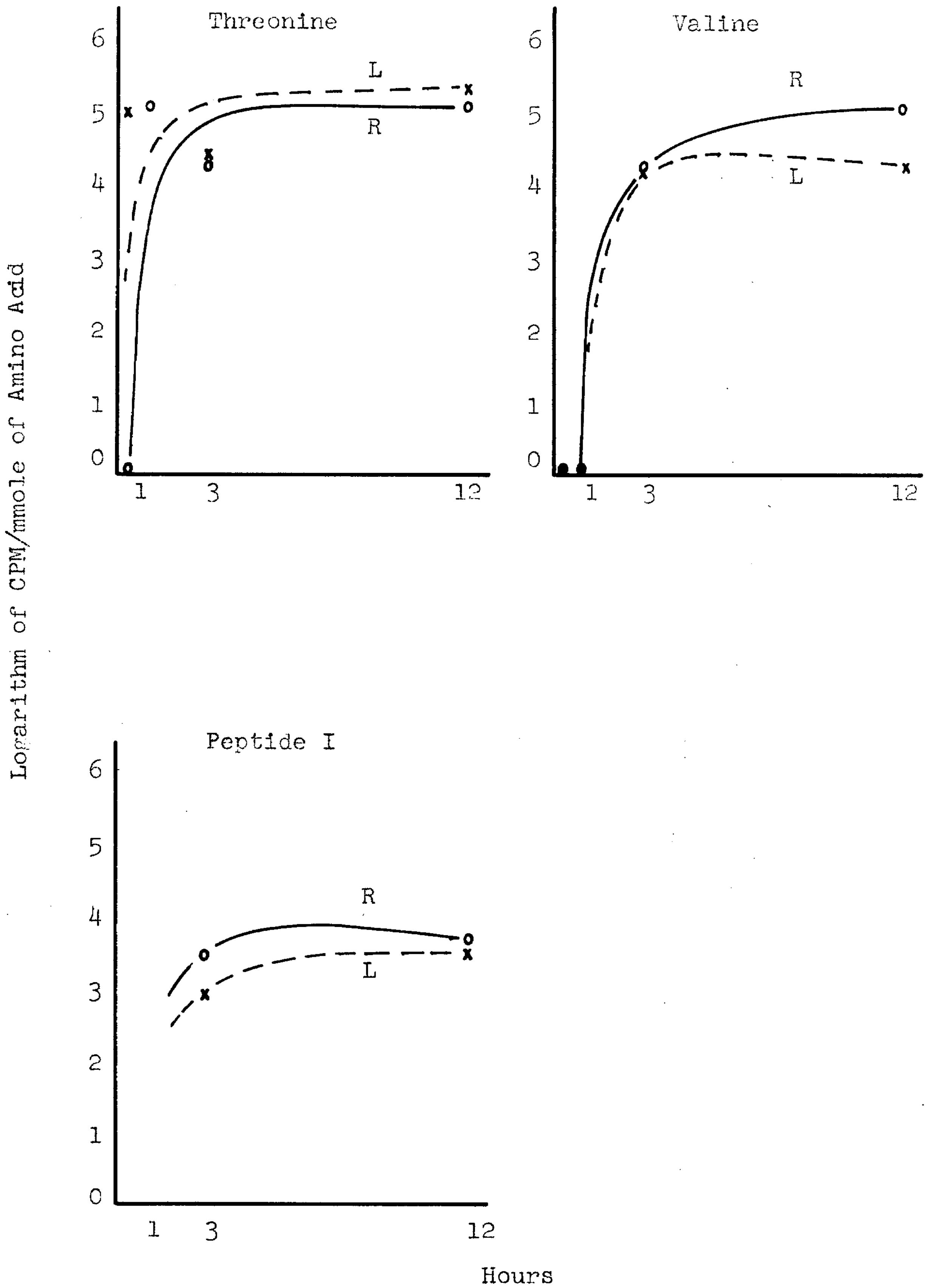
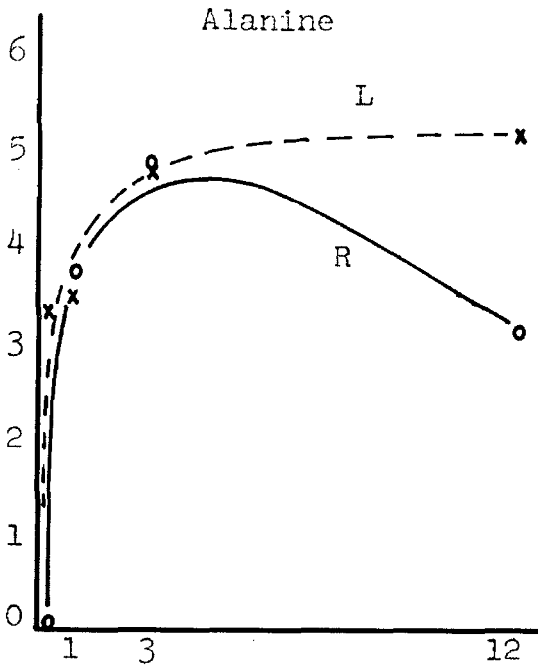
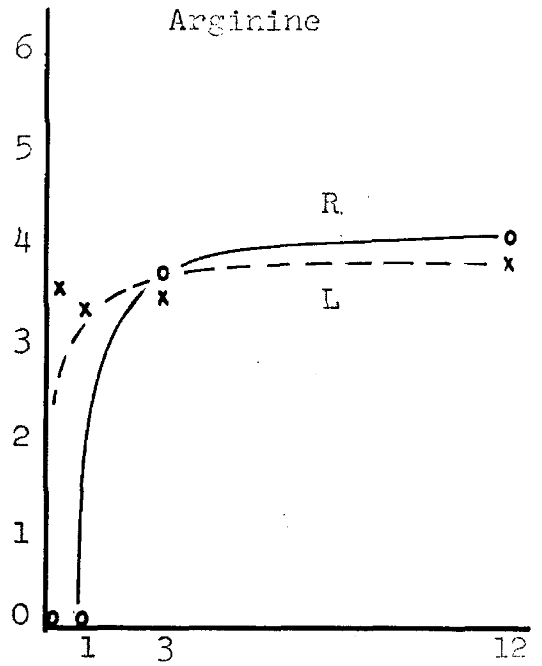


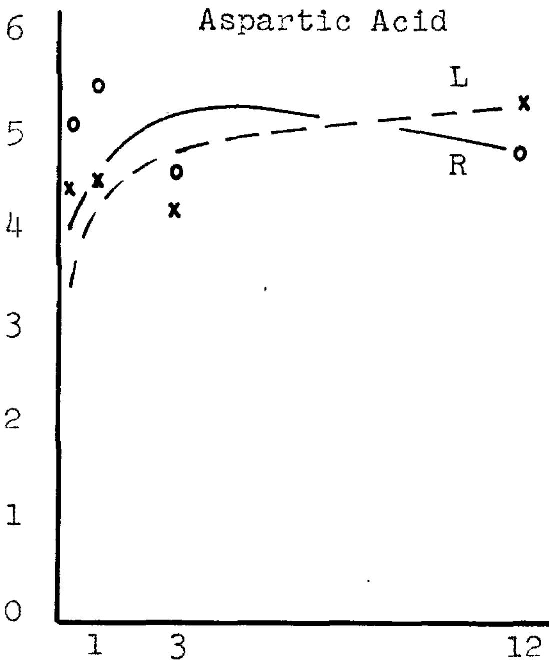
Figure IV

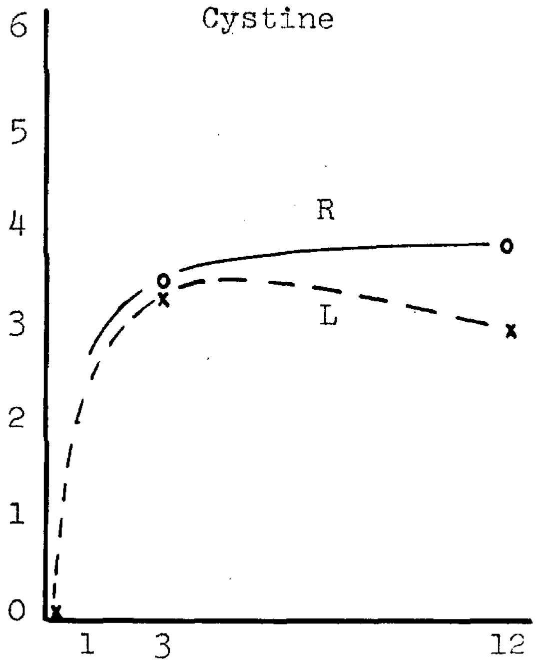
SUCROSE-C^{14}

Logarithm of CPM/mmole of Amino Acid









Hours

Figure V

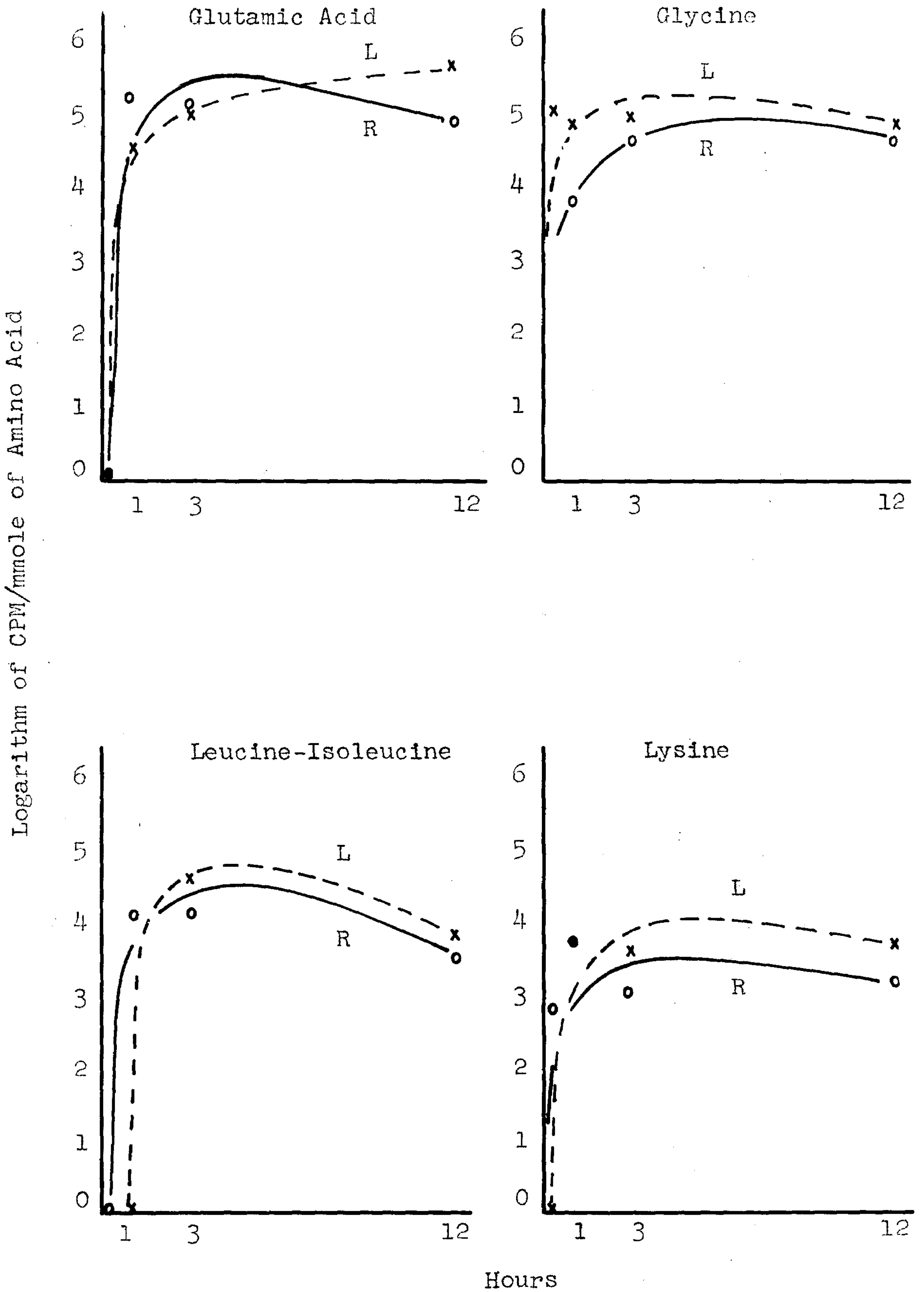


Figure VI

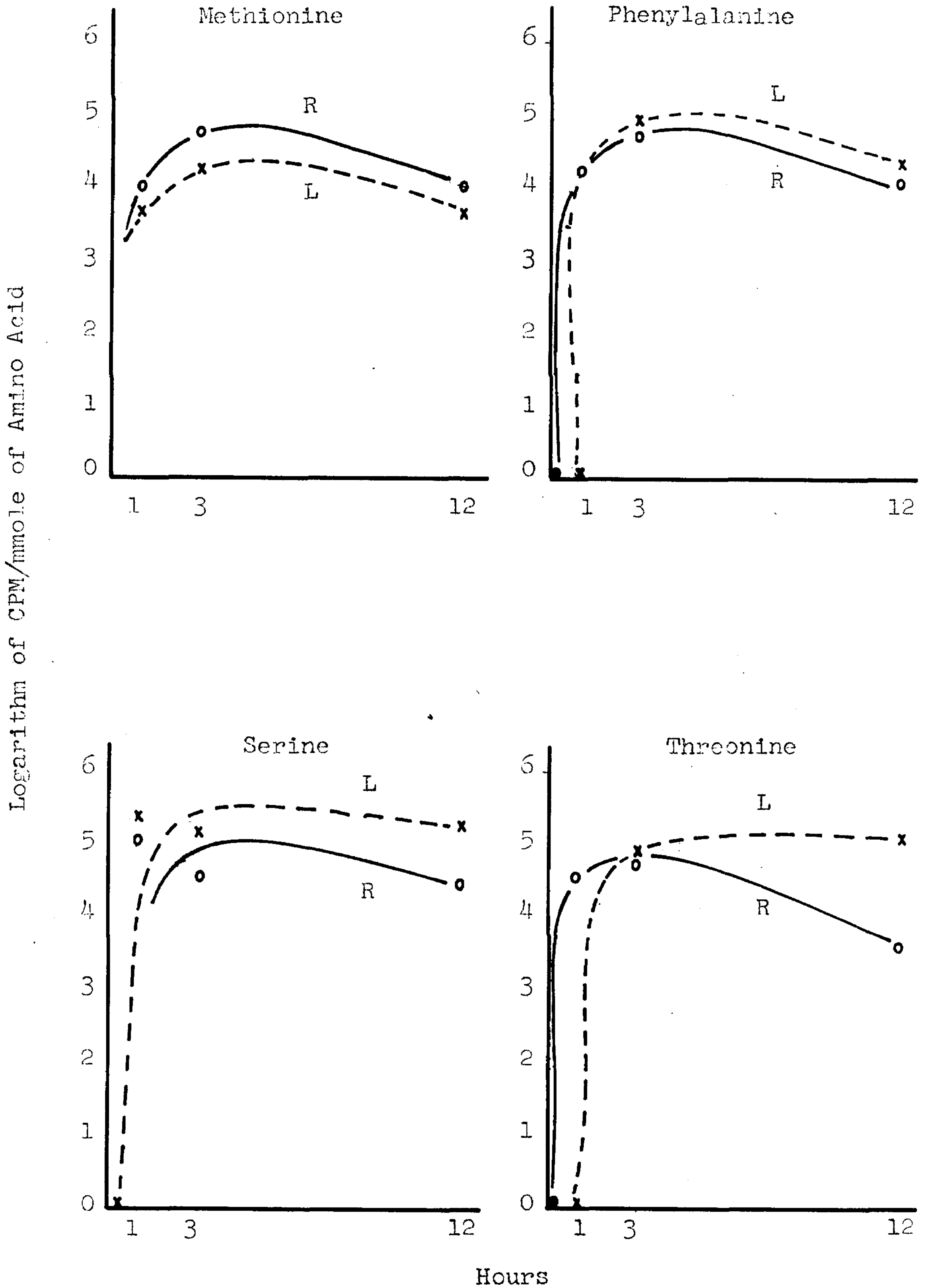


Figure VII

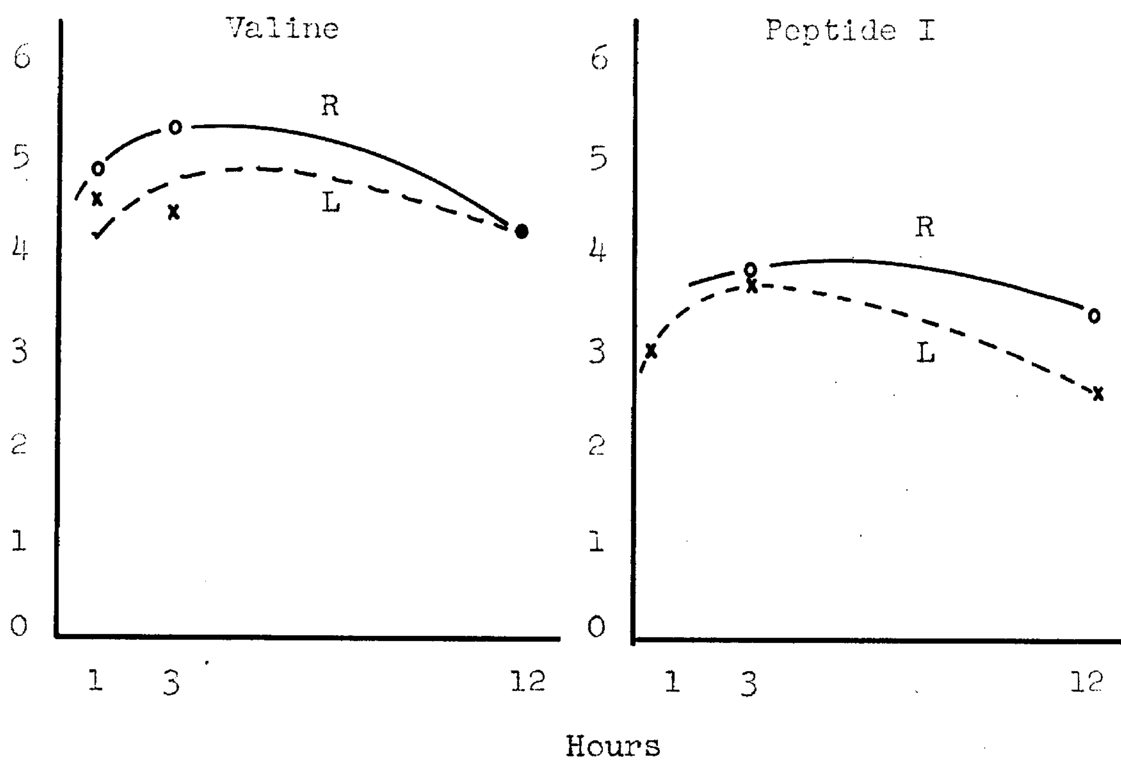


Figure VIII

Table I. Radioactivity of Protein-Bound Amino Acids

Distribution of Radioactivity in Amino Acids of Red Kidney Bean Protein after Twelve Hours Incubation of Excised Organs with Acetate-1-C^{14} and Sucrose-C^{14}
(Counts per minute per millimole of Amino Acid)

Amino Acid	Acetate-1-C^{14}						Sucrose-C^{14}					
	Leaves		Stems		Roots		Leaves		Stems		Roots	
	Intact	$-CO_2$	Intact	$-CO_2$	Intact	$-CO_2$	Intact	$-CO_2$	Intact	$-CO_2$	Intact	$-CO_2$
Alanine	7,800	--	10,300	--	207,500	20	169,000	30	151,700	55	149,400	35
Arginine	18,000	--	50,000	--	24,300	--	24,400	--	58,300	--	50,000	--
Aspartic Acid	30,900	50	42,700	40	--	--	42,600	16	137,100	40	18,200	100
Cystine	4,100	--	1,300	--	700	--	3,400	--	5,100	--	11,200	--
Glutamic Acid	72,100	50	42,200	50	80,000	60	52,900	25	242,000	--	204,000	--
Glycine	148,200	--	28,300	--	79,000	--	12,000	--	50,000	--	116,700	--
Histidine	28,300	--	23,200	--	54,500	--	67,600	--	64,500	--	90,000	--
Leucine*	2,400	100	24,000	30	18,500	14	53,900	25	17,500	40	137,700	60
Lysine	23,000	--	44,000	--	78,200	--	6,200	--	104,000	--	--	--
Methionine	32,600	--	2,000	--	21,500	--	49,200	--	5,400	--	29,200	--
Phenylalanine	9,100	100	32,100	75	19,000	70	46,600	75	21,700	80	90,000	85
Proline	53,000	10	21,400	50	170,000	30	61,700	10	48,600	40	--	--
Serine	3,700	--	40,700	--	139,500	--	26,200	--	284,600	--	--	--
Threonine	7,600	--	39,400	--	237,000	--	59,100	--	140,000	--	262,500	--
Tryptophan**	16,800	--	12,000	--	119,400	--	136,000	--	95,800	--	34,100	--
Tyrosine	12,600	100	22,700	100	139,400	40	20,000	100	114,000	80	70,000	70
Valine	6,600	--	18,000	--	80,000	--	13,300	--	92,100	--	280,000	--

The heading "Intact" refers to the amino acids before degradation. The heading "$-CO_2$" refers to the % of radioactivity remaining in the amino acids after decarboxylation by treatment with ninhydrin.

* Contains leucine and isoleucine

** It is doubtful that tryptophan would remain intact throughout the hydrolytic procedure. However, a small amount of color was noted at the level of tryptophan upon development with ninhydrin after the first chromatographic separation. The strip was eluted, re-chromatographed with a different solvent, eluted and the quantity and radioactivity measured in the same manner as for the other amino acids. The data are recorded as obtained.

Discussion

1. Results as shown in Figures I - VIII and Table I

The preceding data show that carbon-14 from either labeled acetate or labeled sucrose is incorporated into the carbon skeletons of all of the free leaf and root amino acids (and the peptide) on which measurements were made. The amino acids isolated from hydrolyzed protein of the leaves, stems and roots have been shown to be labeled just as are the free amino acids.

The free amino acids may be arranged into groups on the basis of time to the first appearance of radioactivity:

Acetate-C^{14}		Sucrose-C^{14}	
Leaves	Roots	Leaves	Roots
1. Zero time to twenty minutes			
Alanine Aspartic Acid Glycine Lysine Threonine	Arginine Aspartic Acid Glycine Leucine-Isoleucine Lysine	Alanine Arginine Aspartic Acid Glycine	Aspartic Acid Lysine
2. Twenty minutes to one hour			
alpha-Amino-butyric Acid Arginine Glutamic Acid	Alanine alpha-Amino-butyric Acid Glutamic Acid Threonine	Cystine Glutamic Acid Lysine Serine	Alanine Glutamic Acid Leucine-Isoleucine Phenylalanine

	Acetate-C^{14}		Sucrose-C^{14}	
	Leaves	Roots	Leaves	Roots
3. One hour to three hours				
	Leucine-Isoleucine Methionine Valine	Methionine Valine	Leucine-Isoleucine Phenylalanine Threonine	Arginine
4. Three hours to twelve hours				
	Phenylalanine	Phenylalanine		
5. Indeterminate because of missing points				
	Cystine Serine	Cystine Serine	Methionine Valine	Cystine Glycine Methionine Serine Valine

Aspartic acid is apparently the only amino acid which becomes radioactive in the first twenty minutes without regard to organ or carbon-14 source. It is possible that glycine should also be placed in this category. The remaining amino acids are distributed in what seems to be almost a random pattern. There is a slight suggestion that branched-chain and aromatic amino acids incorporate carbon-14 somewhat more rapidly in roots than in leaves. In general, there is a tendency for those amino acids which may be regarded as being closely related to either pyruvic acid or Krebs cycle acids to become radioactive at a somewhat greater rate than aromatic, branched-chain or sulfur amino acids. It is not prudent to state dogmatically, on the basis of the available data, that a certain amino acid is synthesized at one rate

while a second amino acid is produced at a second rate because only the specific activities of the amino acids were measured, and these vary with the amounts of endogenous amino acids. Another factor which must be considered germane is the location of radioactivity in each amino acid. This will be discussed later.

Phenylalanine appears to be anomalous in that it shows a peculiar labeling pattern. After treatment with sucrose-C^{14}, phenylalanine becomes radioactive at a rate normal in relation to the other amino acids. When acetate-C^{14} is supplied to the tissues, however, this amino acid becomes labeled at a very low rate. The fact that acetate is not very effective as a carbon source for this amino acid while sucrose carbon is incorporated more rapidly suggests that glycolytic intermediates are important as precursors. Such a suggestion has been made for tyrosine synthesis by Thomas, *et al*. (28) and by Shigeura, *et al*. (29). Davis has reported that phenylalanine is formed in *E. coli*. from some structure related to glucose (30). Known intermediates include, in order, dehydroquinic acid, dehydroshikimic acid, shikimic acid, prephenic acid and phenylpyruvic acid. Both quinic and shikimic acids have been found in plants (31).

The radioactivities of protein-bound amino acids for tissues incubated twelve hours are shown in Table I. They support the data shown in Figures I to VIII. The generally low activities of amino acids isolated from leaf protein are

obvious for both the acetate and the sucrose experiments. This is in accord with studies on the specific activities of the isolated protein fraction as shown in Part II. Certain differences in the amount of carbon-14 residing in the carboxyl carbon are apparent. For the most part those amino acids with relatively high specific activities show the greatest reduction in radioactivity upon treatment with ninhydrin. There seems to be a rapid turnover of carboxyl carbon, regardless of tissue or carbon source. It is unfortunate that rates of carboxyl carbon exchange as compared with rates of true carbon skeleton formation are not known. The data for ninhydrin-degradation of the amino acids reaffirm the position taken earlier, to the effect that a definite arrangement of the amino acids by rate of carbon-14 incorporation into the carbon skeletons is not possible on the basis of the present work.

2. Individual Amino Acids

A comparison of the results presented in this thesis with results obtained with other organisms will be made in the following section.

Alanine

In the present study, carbon-14 from both C^{14}-labeled sucrose and acetate has been shown to be incorporated rapidly into alanine found in the leaves, stems and roots of red kidney bean. The biosynthesis of alanine by amination of pyruvic acid is well established (32). Further evidence comes from the fact that pyruvic acid is readily formed from alanine (33,34,35). The principal pathway of pyruvate formation is glycolytic. Since the glycolytic pathway has been shown to be operative in plants (31), it may be assumed that carbon-14 in a carbohydrate fed to a plant would be found in alanine isolated from the plant. This has been shown to be true in leaf discs of *Canna indica* fed C^{14}-labeled sucrose, glucose or fructose in the dark (36). Similar results have been obtained with animal tissues (37) and bacteria (38). Some incorporation of carbon-14 from labeled acetate has also been shown to occur in animal tissues (37,39) and yeast (40). A possible pathway is the conversion of acetate to acetyl-CoA and subsequent carboxylation to pyruvate (32).

Alpha-aminobutyric Acid

Data are now available which show a high rate of incorporation of carbon-14 from labeled acetate into the alpha-aminobutyric acid of the organs of red kidney bean which is similar to the data for threonine. It has been

suggested that this amino acid is formed by conversion of threonine to alpha-ketobutyrate, followed by amination of the keto acid (41). The formation of the keto acid has been demonstrated in *Neurospora crassa* (42). Amination of the keto acid by transamination has been shown in animal tissues (43). This relationship to threonine would of course result in a pattern of radioactivity similar to that for threonine.

Arginine

In red kidney bean arginine is readily formed from sucrose or acetate carbon as shown above. Arginine synthesis has been shown in animals (44) and in *Neurospora* (45) to be closely connected with the ornithine cycle. In liver preparations the direct synthesis is from citrulline by way of an intermediate-argininosuccinic acid (46,47,48). Citrulline is formed from ornithine which is closely related to glutamic acid (49,50). Hence the pathway of carbon-14 from carbohydrate or acetate to arginine is fairly clear. Carbon-14 from methyl- or carboxyl-labeled acetate appears in the ornithine moiety of arginine in *E. coli* (51,52). Similar results have been reported in yeast (40). In mouse brain, although glutamic acid was labeled by C^{14}-acetate, no carbon-14 was found in the arginine after 24 hours incubation (37). Incorporation of carbon-14 from labeled glucose and sucrose has been shown in animal tissues (37,53,54), and bacteria (38).

Aspartic Acid

In the present work aspartic acid is labeled to a high degree by both radioactive acetate and sucrose. Aspartic acid may be synthesized from oxalacetic acid by amination (32) or from fumaric acid by the action of aspartase (55). Since aspartic acid synthesis is so closely connected with Krebs cycle intermediates, it is apparent that carbohydrates should furnish the carbon atoms. This has been shown to be true in animal tissues (37,53,54), bacteria (38) and plants (36). Acetate also furnishes carbon atoms to aspartic acid, although to a lesser extent (37,52).

Cystine

The radioactivity of cystine isolated from the organs of red kidney bean is rather low when labeled acetate is supplied. When labeled sucrose is added to the organs the radioactivity of cystine increases fairly rapidly in the roots but slowly in the leaves. This latter pattern is similar to that for serine . In animals the biosynthesis of cysteine (from which cystine is derived by oxidation) has been related to serine (32). Proof has been obtained that methionine donates the sulphur for cysteine (56). It has been shown that carbon-14 from glycine-2-C^{14} is found in cysteine of rats (57). Since nitrogen-15 from N^{15}-serine has been found in the protein cystine of rats (58), it is

suggested that glycine is converted to serine and the serine carbon skeleton is used in cysteine synthesis (32). In more recent work with mouse brain it has been shown that carbon-14 from glucose-C^{14} and C^{14}-formate appears in protein cystine, but carbon-14 from labeled acetate does not (37). Corresponding data for serine and glycine in mouse brain tend to confirm the place of these two amino acids in the synthesis of cystine in animals. Interestingly enough, carbon-14 from C^{14}-glucose was not found in the protein-serine of one day-old mouse liver (54). Studies with E. coli show a definite relation of serine to cysteine synthesis (38).

Glutamic Acid

Glutamic acid has been shown in the present work to incorporate carbon at a high rate from both acetate and sucrose in the roots, stems and leaves of red kidney bean. The relation of glutamic acid to alpha-ketoglutaric acid is obvious. Since the keto acid is a member of the Krebs cycle, carbon from carbohydrate or from acetate should find its way into the carbon skeleton of glutamic acid. This has been demonstrated in animal tissue (37,39,53,54) yeast (40,59), bacteria (38,51) and plants (36).

Glycine

It has been demonstrated in the present work that carbon atoms from sucrose and acetate enter glycine at about the same rate in all parts of red kidney bean. Observations by various workers have indicated that in the rat the beta-carbon of serine (60,61,62) and the alpha-carbon of pyruvic acid (63) are precursors of the alpha-carbon of glycine. The carboxyl-carbon of pyruvic acid appears in the carboxyl group of glycine (63). Thus glycine synthesis is linked with carbohydrate metabolism in the rat. Arnstein and Neuberger (64) have reported that in the rat carbon-14 from carboxyl-labeled acetate appears in the carboxyl group of glycine. According to Weinhouse, acetate is not converted to glycine in the intact rat (65). He has suggested that serine is the precursor of glycine, and that equilibrium exists between glycine, glyoxalate and glycolate. Further evidence along these lines is based on work with mice. Substantial amounts of carbon-14 from labeled glucose and sucrose have been found in the protein-glycine of mice fed these compounds (37). In yeast fed methyl-labeled acetate little carbon-14 was found in the glycine (40). Work with *E. coli* has indicated that serine is a direct precursor of glycine, but that threonine is also involved to a lesser extent (38).

Histidine

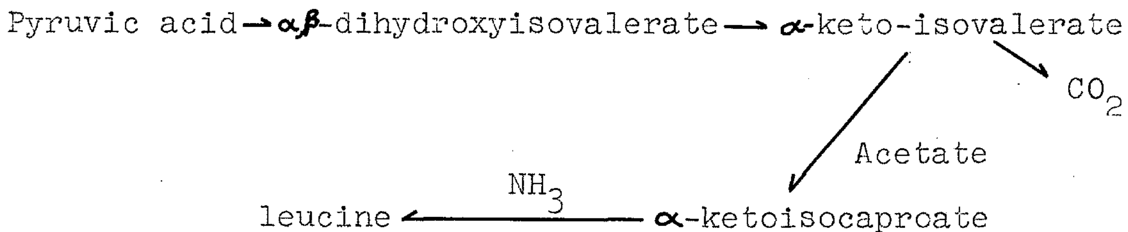
In red kidney bean histidine synthesis evidently involves the carbon atoms of either sucrose or acetate. The possible pathways involved are uncertain. There is some evidence, cited by Tabor (66), that a pentose is involved in histidine synthesis. The degradation of sucrose could provide such a pentose. The utilization of acetate carbon perhaps is through condensation of a two-carbon unit with a triose to form a labeled pentose. The work of Ames indicates that in _Neurospora_ the precursors of histidine may be the phosphate esters of imidazole-glycerol, imidazole-acetol, and histidinol (67). Most animals require either the amino acid or the keto analog in the diet (32). Levy and Coon (68) have demonstrated in yeast the incorporation of formate carbon into the amidine carbon of the imidazole ring. Further work by these two researchers indicates that carbon-14 from methyl-labeled acetate is also found only in the amidine carbon (69). In the same report it was shown that a high level of radioactivity is found in the histidine of yeast grown in the presence of uniformly-labeled glucose. In this case all the carbon atoms in histidine were labeled. When methyl-labeled acetate was added the radioactivity was relatively very high in glutamate and low in histidine and the authors suggest that this argues against glutamate as a direct precursor of histidine, although glutamate has been demonstrated as a degradation product of histidine (32). Addition of

uniformly-labeled C^{14}-sucrose to the diet of mice resulted in no radioactivity in the histidine isolated from protein (53). Incubation of one day-old mouse brain with labeled glucose yielded much less radioactivity in isolated histidine than did the tissue incubated with carboxyl-labeled acetate (37). Similar experiments with one day-old mice showed essentially no incorporation of carbon-14 from labeled glucose into the histidine isolated from the liver and intestinal protein (54).

Leucine

Since leucine and isoleucine were not separated, it is impossible to compare the data from the present work with those from other organisms. At most it may be said that in the roots incorporation of acetate carbon into the leucine-isoleucine fraction compares quite favorably with that from sucrose. In the leaves, incorporation of carbon-14 from either sucrose or acetate into the leucine-isoleucine fraction is slow. Work with *E. coli* has suggested that carbon-14 incorporation into isoleucine, regardless of the source of carbon-14 is approximately equal to the incorporation into threonine (52). Therefore, in order to obtain a value for the radioactivity of isoleucine, the value found for threonine was subtracted from that found for the leucine-isoleucine fraction. This amino acid, when catabolized, produces ketone compounds. The assumption is sometimes made that synthesis is a reversal

of degradation. There is, however, no concrete evidence for a role of ketone bodies in leucine synthesis. It is known that the alpha-keto acid corresponding to leucine as well as the alpha-hydroxy acid will replace the amino acid in cultures of microorganisms if vitamin B_6 is present (70). When yeast is supplied with C^{14}-methyl-labeled:C^{13}-carboxyl-labeled acetate, both carbon-14 and carbon-13 in excess amounts are found in significant amounts in leucine isolated from the protein (40). Radioactive leucine was isolated from one day-old mouse brain incubated with labeled glucose, while under the same conditions with carboxyl-labeled acetate substituted for labeled glucose the leucine was without radioactivity (37). In the same experiment, one day-old mouse liver incubated with labeled glucose contained no radioactive leucine. Work with E. coli (38) suggests the following sequence of steps in the synthesis of leucine:



From this it may be seen that both carbohydrate and acetate could supply carbon atoms for leucine synthesis.

Lysine

In both leaves and roots of red kidney bean carbon atoms from sucrose and acetate may be used in the formation of lysine, although the utilization of sucrose carbon proceeds rather slowly in the leaves. In animals, this amino acid must apparently be supplied entirely through the diet as lysine (32). Although it is known that lysine is metabolized in animals to alpha-aminoadipic acid, the process has not been shown to be reversible (71,72). At least two mutant strains of *Neurospora crassa* can use alpha-aminoadipic acid in place of lysine (73). Work with yeast indicates that acetate, rather than carbohydrate, supplies the carbon atoms utilized in lysine synthesis (40,74). In many bacteria lysine is formed by decarboxylation of diaminopimelic acid (75,76). Aspartic acid has been shown to furnish carbon atoms in the production of diaminopimelic acid (51). More recent work points to the incorporation of small but significant amounts of carbon-14 from labeled glucose into the protein-lysine of one day-old mouse brain. A check on the position of the label in this lysine indicated that it was all in carboxyl carbon (37). When labeled acetate replaced the glucose no radioactivity was found in lysine. The lysine of *E. coli* appears to be synthesized in part from aspartic acid (38). Also *E. coli* grown in methyl-labeled or carboxyl-labeled acetate produces labeled lysine. Ravel,

et al. have published a report in which they suggest that aspartic acid is intimately involved in the synthesis of lysine in Lactobacillus spp. (77).

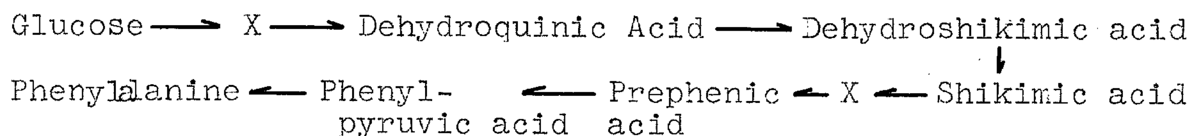
Methionine

Sucrose carbon appears quite soon in the methionine of leaves and roots. The appearance of acetate carbon is slow. It is possible that sucrose metabolism is more capable than acetate metabolism of active methyl group production, and that this is the limiting step. Most probably methionine is synthesized by the methylation of homocysteine. Homocysteine is in turn derived from cystathionine which is formed from the condensation of cysteine with a four-carbon compound--possibly homoserine. The reverse reaction has been shown to occur in rats (32). The actual synthesis of methionine by this pathway is indicated by experiments with Neurospora crassa mutants (78). In one day-old mouse brain after incubation with labeled glucose the protein-methionine had about twice the specific activity of cystine and rather more than did serine. Incubation with labeled acetate yielded non-radioactive methionine (37). The methionine of one day-old mouse liver after incubation with C^{14}-glucose contained small amounts of radioactivity while cystine was without radioactivity (54). Serine, however, was well labeled. Work with E. coli has indicated that methionine is related to aspartic acid by way of homoserine

(38), a compound produced in yeast by reduction of aspartyl phosphate (79).

Phenylalanine

It was found in the present study that phenylalanine synthesis utilizes carbon atoms from sucrose in the roots and leaves, but that acetate carbon appears at a very low rate. This is explainable, as discussed previously, on the basis of glycolytic intermediates. Studies on the biosynthesis of this amino acid have been largely confined to microorganisms. Gilvarg and Block (40,80) have shown that in yeast glucose supplies the carbon atoms for phenylalanine and acetate is not involved. Work with E. coli has suggested that phenylalanine is produced from the keto acid, phenylpyruvic acid (38). Davis has been able to identify most of the intermediates for phenylalanine synthesis in E. coli (30). His scheme is as follows:



One day-old mouse brain incubated with labeled glucose was found to contain radioactive phenylalanine and tyrosine (37). Further work with C^{14}-formate showed an incorporation of radioactivity into the phenylalanine-tyrosine fraction almost equal to that from the glucose. This apparent synthesis of an essential amino acid is partially negated

by other studies in which it was shown that half of the carbon-14 incorporated into brain protein-phenylalanine and -tyrosine is located in the carboxyl carbon (81). In vivo studies with labeled sucrose showed negligible amounts of radioactivity in the phenylalanine isolated from the total protein of mice (53).

Proline

As may be seen from the protein-amino acid data, carbon 14 in proline is provided by both labeled sucrose and labeled acetate. Carbon-14 from labeled glutamic acid fed to rats has been found in the proline of the protein (82). Further work has shown that glutamic acid semialdehyde and pyrroline carboxylic acid are intermediates in the bio-synthesis of proline (83). The relation of glutamic acid with proline has been indicated by studies on microorganisms (50,84,85). More recent experiments with E. coli indicate a direct pathway from glutamic acid to proline (38,51,52). Both carbon-14 and carbon-13 have been found in the protein proline of yeast fed doubly-labeled acetate (40). After incubation of one day-old mouse brain with labeled glucose it was found, contrary to the work on rats, that although the glutamate was highly labeled there was no radioactivity found in proline. When labeled acetate replaced the glucose a slight amount of radioactivity in the proline and a large amount in the glutamate was recorded (37). Addition of C^{14}-

sucrose to the diet of mice led to the incorporation of a significant amount of radioactivity in the proline of the total body protein after three days as would be expected (53). The failure of glucose carbon, as cited in the previous studies, to be incorporated into the proline of brain protein is not understandable.

Serine

In red kidney bean carbon atoms from both sucrose and acetate are rapidly incorporated into the serine of leaves and roots. The synthesis of serine from glycine plus a 1-carbon unit is well established in animals, yeast and microorganisms (32,62). Pyruvic acid has also been implicated in the metabolism of serine (86) and although the reaction has not been shown to be reversible it is entirely possible that here is a direct connection of carbohydrate with serine synthesis. Acetate supplies carbon atoms for the production of serine in yeast (40). In one day-old mouse brain little carbon-14 from labeled acetate is incorporated into serine (37), while large amounts of carbon-14 from labeled glucose (37,54) or sucrose (53) were found in the serine of mouse brain, liver and total body protein.

Threonine

Addition of either labeled acetate or labeled sucrose to the tissues of red kidney bean results in the formation of radioactive threonine. Data from work with Neurospora crassa mutants indicate that threonine may be synthesized by conversion of either homoserine or alpha-aminobutyric acid (32). Some evidence from rat liver preparations suggest that alpha-aminobutyric acid is a product of the catabolism of threonine rather than a precursor (41). Recent work indicates this same trend in Neurospora (87). In E. coli the pathway of synthesis seems to run from aspartic acid to homoserine and then to threonine (38,51,88). In agreement with this is work with Lactobacillus spp. (77). Aspartic acid in this case acts as a link with Krebs cycle intermediates. It has been shown that both acetate and glucose supply carbon atoms for the synthesis of threonine in E. coli (38,52). As would be expected from the fact that threonine is an essential amino acid for animals, when labeled acetate or carbohydrate is fed to mice or incubated with their tissues, essentially no carbon-14 is found in threonine isolated from the protein (38,53,54).

Tyrosine

That carbon atoms from acetate and sucrose are incorporated into the tyrosine of red kidney bean is evident (Table I). The utilization of sucrose carbon is understandable in terms of work with yeast. The part which acetate plays is most easily explained by a reversal of glycolysis. In animals the conversion of phenylalanine to tyrosine has been well established. However, work with microorganisms and molds suggests strongly that the conversion is indirect and that both are synthesized from a common precursor (32). Gilvarg and Bloch have shown that in yeast phenylalanine and tyrosine are synthesized from glucose in some manner as yet not fully elucidated and that acetate contributes little directly to the synthesis (40,80). Thomas, et al. (28) have confirmed this in yeast and suggest that the citric acid cycle does not participate directly in the formation of tyrosine from pyruvate, acetate or glucose. They base this suggestion on the fact that the pattern of labeling in tyrosine makes necessary certain intermediates which are not equilibrated with symmetrical intermediates from glycolysis or the Krebs cycle. Davis has some evidence which suggests that prephenic acid is a precursor of tyrosine in E. coli (30). In vivo studies with one day-old mouse brain indicated that a certain amount of carbon in the carboxyl group is furnished by glucose (37,54).

Valine

Sucrose and acetate suffice in red kidney bean as carbon sources for valine. Tatum and Adelberg (89) have shown in a mutant strain of *Neurospora crassa* that the valine skeleton may be synthesized from acetate carbon. The results of several studies on *Neurospora* and *E. coli* suggest certain interrelationships between valine, isoleucine and threonine (32). A contrary result was obtained by Moldave, *et al.* (37) who found that one day-old mouse brain incubated with labeled acetate incorporated no carbon-14 into valine (isolated from the brain protein). Incubation with labeled glucose resulted in formation of a small amount of C^{14}-labeled valine. The incorporation into the brain protein-valine of carbon atoms from glucose confirms previous work by Winzler, *et al.* (54) who also measured incorporation of carbon-14 into mouse liver protein-valine and found no radioactivity after incubation with labeled glucose. It is known that yeast is able to utilize acetate as a carbon source for valine to a certain extent (40). Abelson and coworkers have suggested, on the basis of experiments with *E. coli*, that the pathways of synthesis of valine and of isoleucine are somewhat different in that valine is synthesized by a pathway which commences with pyruvic acid whereas synthesis of isoleucine starts with aspartic acid (38,51).

Peptide I

Of the two peptides, only peptide I was present in sufficient quantity to be measured for radioactivity. It quickly became radioactive, which suggests that it is a peptide formed *de novo* from free amino acids rather than a fragment from some protein.

3. Localization of amino acid synthesis

The question posed in the first paragraph of the introduction has largely been answered. Data concerning the synthesis of sixteen amino acids have been accumulated and it has been shown that they are all formed *de novo* in the three general parts--the leaves, stems and roots--of red kidney bean plants. Furthermore, the amino acids formed are present in the free state, in small peptides and in protein. The amino acids studied do not include such uncommon amino acids as hydroxylysine or other as yet undiscovered variations of the "normal" amino acids. That such amino acids may be of importance to the plant cannot be ruled out as yet. On the whole, however, the answer to the question posed in the introduction must be that all of the amino acids found in plant proteins are formed in the three main parts of the plant. There is no localized synthesis of amino acids such as might have been suggested by work with other kinds of detached leaves.

With this fact now at hand, the evidence for abnormal protein loss in detached leaves has to be re-evaluated and an answer sought in other directions. Recently some unpublished information pertinent to the subject was unearthed. Detached tobacco leaves placed under constant high light intensities (2000 foot candles or more) and in optimal temperatures and nutrient solution were found to show an increase in protein content over a period of several weeks (90). Vickery, et al. (9) performed similar experiments with detached tobacco leaves. In their work, the leaves were placed either in a darkroom or in a greenhouse supplied with artifical light at night. The base of each leaf was submerged in nutrient solution. The protein nitrogen level, over a period of days, was found to decrease in both light and dark, but the decrease in light was about two-thirds that in the dark. Pearsall and Billimoria have reported that discs removed from the actively growing region of daffodil leaves showed an increase in protein nitrogen under light intensities of the order of 1000 meter-candles, and a loss in protein nitrogen in the dark (91). With this in mind, the work of Chibnall and others (17) was reviewed more critically. Chibnall states that his work was carried out in "strong diffused daylight". It must be assumed then, that the work was done in a laboratory rather than in a greenhouse, and most probably other investigators of this problem, as cited by Chibnall, also carried out their work in laboratories. This, of course, is the most convenient

place for such research. More recent work by Chibnall (15) on runner bean leaves was also, with one exception, done in "strong diffused daylight". The exception was an experiment in which the leaves were placed in a greenhouse where it may be assumed that they were under direct sunlight at least part of the day. The leaves in the greenhouse lost little more than half the amount of protein lost by leaves in the low light condition.

High light intensity may be, then, essential to protein synthesis in the leaves of plants. It is reasonable to wonder what purpose might be served by such light intensities. Photosynthetic products may be necessary, although Vickery and coworkers found that a glucose solution in place of an inorganic nutrient solution had no effect on protein loss in the dark and actually increased protein loss in light. A possible need for light is suggested by the work of Vishniac and Ochoa (92,93). It was shown that the photochemical reduction of diphosphopyridine nucleotide by chloroplasts can be coupled to the synthesis of adenosine triphosphate (ATP). This may be an important mechanism in leaves. Roots do not need light energy for ATP synthesis since they may use for this purpose a more than adequate supply of carbohydrate. The role which ATP might play in decreasing protein loss must be considered. Webster has reported that a greatly enhanced incorporation of amino acids into plant mitochondria exists from addition of ATP

to an in vitro system (94). Similar results have been found for the mitochondria and microsomes of rat liver (95). Data presented in a later chapter of this thesis (Part II) actually show that incorporation of amino acids into leaf protein proceeds at a low rate as compared to the rate found in roots. Amino acids isolated from leaf protein have been shown to possess lower specific activities than those from root protein, despite the fact that the specific activities of the free amino acids in leaves and roots are roughly comparable in many cases. The implication here, of course, is that incorporation from complete amino acid mixtures is tantamount to protein synthesis.

It is realized that the preceding thoughts are speculative in nature and several assumptions have been made which may prove to be unwarranted. However, the suggestions outlined are certainly amenable to experimentation.

The idea that transmissible substances formed in the roots may govern protein synthesis in leaves is not entirely disproven. Among the laboratory experiments reported by Chibnall are two in which runner bean leaves were induced to form roots by application of indoleacetic acid. These leaves lost protein to an extent intermediate between the amount lost by the laboratory root-less leaves (low light) and the root-less leaves placed in the greenhouse (high light). Such root-produced regulatory substances, if they do exist, would not appear at the present time to be amino acids.

SUMMARY

1. Carboxyl-labeled C^{14}-acetate and uniformly-labeled C^{14}-sucrose have been supplied to the detached organs of red kidney bean plants. The amino acids and protein have been isolated and the incorporated radioactivity measured.

2. It is shown that the amino acids studied are all synthesized, at least partially and probably wholly, in each of the organs tested--leaves, stems and roots, and that carbon atoms necessary for synthesis may be provided by either sucrose or acetate.

3. The data suggest that those amino acids closely related to either pyruvic acid or Krebs cycle acids become radioactive (are synthesized) at greater rates than aromatic, branched-chain or sulfur amino acids. The data also suggest that branched-chain and aromatic amino acids are synthesized more rapidly in the roots than in the leaves.

4. The labeling pattern for phenylalanine is unusual and indicates glycolytic intermediates as precursors to the synthesis of this amino acid.

5. A study was made of amino acids isolated from protein for each of the three organs. Amino acids isolated from stem and root protein had, in general, higher specific activities than amino acids isolated from leaf protein.

It was found that a large fraction of the radioactivity of certain amino acids resides in the carboxyl carbon, indicating a rapid turnover of this group.

6. A tentative determination has been made of the composition of two small peptides. The rate of incorporation of radioactivity has been measured for one of them and found to be comparable to rates of incorporation found for the free amino acids. It is felt that this indicates de novo synthesis of the peptide.
7. The present data are compared with similar data gained by work with other organisms.
8. The bearing which the collected data have on the loss of protein in detached leaves is discussed.

PART II

Incorporation of Labeled Amino Acids into Plant Protein

Introduction

In Part I of this thesis, it was shown that the cellular amino acids of bean plants become labeled with carbon-14 if the tissue is supplied with an appropriate carbon-14-labeled precursor. One would expect that these labeled amino acids might become incorporated into the protein of the leaves, stems, or roots. This might be by incorporation into existing protein or as a result of de novo synthesis of protein.

Little study has been devoted to the incorporation of labeled amino acids into higher plant protein. The first work was that of Arreguin, Bonner, and Wood (96) in which carboxyl-labeled C^{14}-acetate was fed to guayule plants for 48 hours. The root and stem protein was extracted, hydrolyzed, and the activity in the separated amino acids measured. The bulk of the carbon-14 in the hydrolysate was found in leucine, glutamate, and aspartate.

Boroughs and Bonner (97) fed C^{14}-carboxyl-labeled leucine and glycine to corn and oat coleoptiles over an incubation period of six hours. The solutions used were buffered and contained 3% sucrose in addition to the labeled amino acid. The protein was isolated, hydrolyzed, and the resultant amino acids decarboxylated. The radioactivity of the carbon

dioxide from the decarboxylation was measured. Carbon-14 from both amino acids was incorporated into the protein of the coleoptiles. Whether or not some of the carbon-14 given as glycine or leucine appeared in other amino acids was not determined. It was impossible, as usual, to ascertain the method of incorporation--whether into pre-existing protein, or into new protein synthesized during the incubation period.

More recent work has been carried out by Webster (94) on the incorporation of C^{14}-carboxyl-labeled glycine, glutamate, and aspartate into bean hypocotyl mitochondria. Incorporation of the carbon-14 took place in a minimal solution but the rate of incorporation was doubled by addition of adenylic acid and Krebs cycle intermediates. This increase in rate was inhibited by respiratory poisons. Carbon-14 from glutamate or aspartate was incorporated at about twice the rate of carbon-14 from glycine.

Webster has been able to show the appearance of carbon-14 from C^{14}-labeled glutamate in the protein of different plants (98). He has also shown that in bean hypocotyl sections the particulate fraction which sediments between 10,000 and 70,000 x g incorporates labeled glutamate more rapidly than any other fraction, while the nuclear fraction incorporates this substrate most slowly.

Racusen and Aronoff (7) measured the incorporation of

carbon-14 from $C^{14}O_2$ into the protein of soybean leaves. They found a decrease in the rate of incorporation with time after excision of the leaves. Leaves in the light and carrying out photosynthesis incorporated more carbon-14 into the protein than did leaves kept in the dark.

Materials and Methods

The water-soluble protein contained in the homogenates of the various plant parts was precipitated and stored as indicated in Part I. The protein was further purified by two washings in 3% trichloroacetic acid (TCA). It was then dissolved in 20% potassium hydroxide, and re-precipitated by the addition of TCA. The effectiveness of this treatment in removing adsorbed radioactivity was tested on three samples by again dissolving the protein and re-precipitating. There was no significant change in the specific activity of any of the three samples.

The purified protein was spread evenly on aluminum planchets and the incorporated radioactivity was measured with a thin-window G-M tube by standard techniques. Measurements were made on protein samples from the leaves, stems and roots for the 20 minute, one hour, three hour and twelve hour intervals for both the sucrose and acetate experiments. Correction was made for self-absorption according to the recommendations of Calvin, *et al*. (27).

Results

The data on the incorporation of carbon-14 into protein of the varied tissues are presented in two graphs; Figure I for the sucrose experiments and Figure II for the acetate experiments. The rate of incorporation of carbon-14 from a total mixture of labeled amino acids into the protein of intact organs of higher plants has been demonstrated for the first time. The greater amount of radioactivity in the root protein is of interest. This difference cannot be explained on the basis of greater labeling of the free amino acids of the roots. Thus, an increased rate of incorporation into the root protein is suggested.

The protein isolated from stems and leaves contained some chloroplasts since the protein from these two sources was green, while the root protein fraction contained no chloroplasts, of course. The effect of inclusion of chloroplastic protein (by weight) into the calculations for specific activities of the protein samples is impossible to determine from the present data. It is known that the rate of decrease of chloroplastic protein is roughly proportional to the rate of decrease of cytoplasmic protein (10,99). Whether the proportionality in decomposition may be construed as an indication that rates of incorporation are also similar in both the cytoplasmic and chloroplastic fractions or not is debatable.

Figure I.

The incorporation, with time, of carbon-14 from sucrose-C^{14} into leaf, stem, and root protein of red kidney bean.

Figure II.

The incorporation, with time, of carbon-14 from acetate-C^{14} into leaf, stem and root protein of red kidney bean.

Identification of curves:

Roots--designated by "R."

Leaves--designated by "L."

Stems--designated by "S."

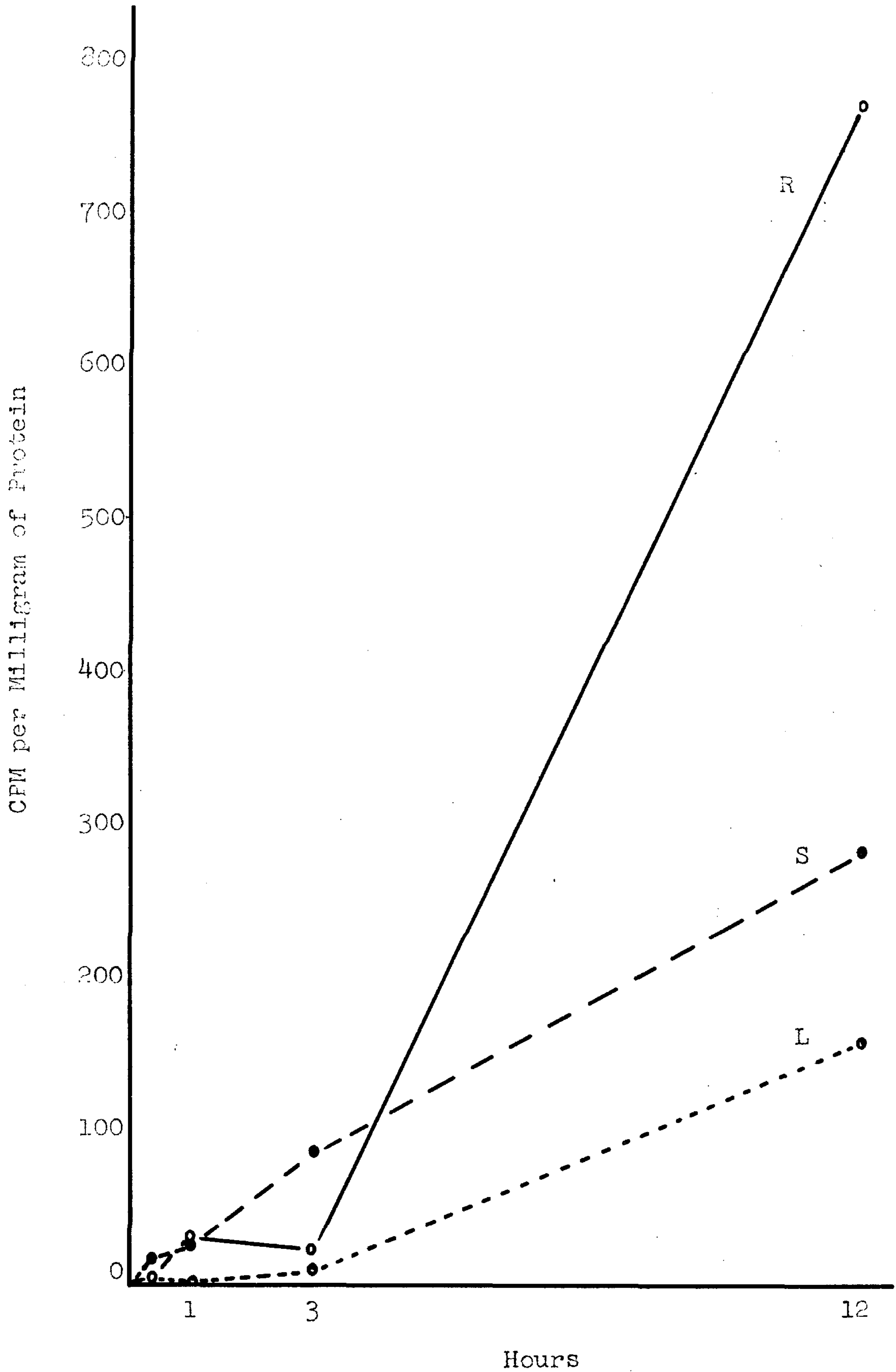


Figure I

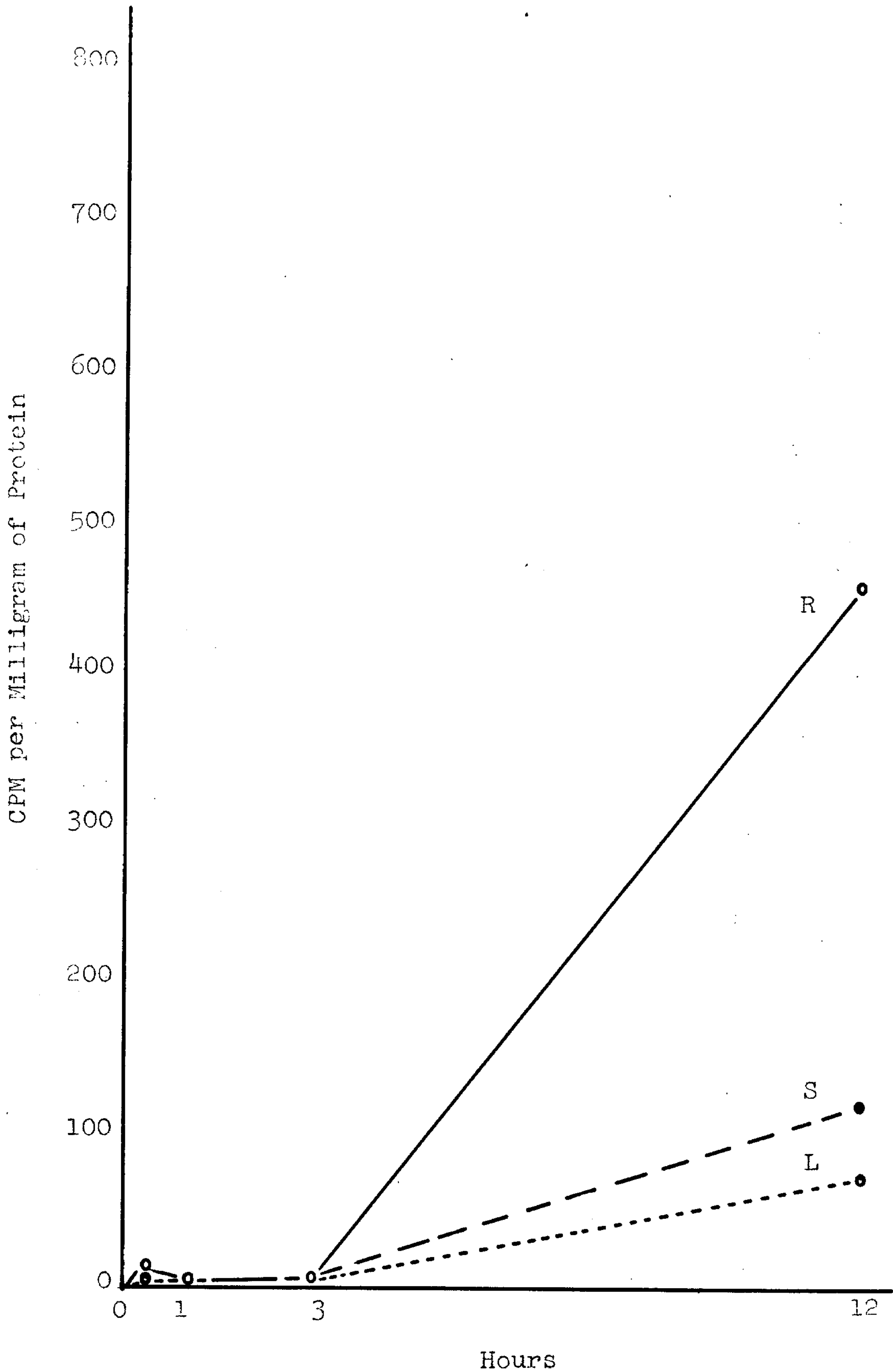


Figure II

Discussion

The relative distribution of specific activities at 12 hours for both the acetate and sucrose experiments are approximately what one might predict from the results of experiments of Vickery, et al. (4) with nitrogen-15. The pertinent data from intact tobacco plants gathered by these workers are as follows:

	Protein N meq.	Atom % Excess of N^{15} Found in Protein after 72 Hours
Leaves	29.55	0.099
Stalk	3.77	0.184
Roots	5.32	0.222

Comparable data from the present experiments for carbon-14 incorporation from acetate over a 12-hour period and for the water-extractable protein are:

	Relative Amounts of Water-Extractable Protein	Specific Activity of Protein CPM/mg.
Leaves	2	71
Stems	2	120
Roots	1	456

Both sets of data show a higher rate of incorporation into the root protein than into either leaf or stem protein.

It may be noticed that the nitrogen-15 data show an amount of incorporation into root protein double that for the leaf protein. The carbon-14 data show six times as high

a specific activity for the root protein as for the leaf protein. Since the nitrogen-15 data are based upon intact plants and the carbon-14 data on excised plant parts, it is attractive to speculate that excision may have had little effect on incorporation into root protein, but has greatly lowered the rate of incorporation into leaf protein. Before this notion can be entertained however, consideration must be given to (1) the metabolism of kidney bean and tobacco which may differ sufficiently to account for the difference in the rates of incorporation, (2) the fact that rates of nitrogen-15 and carbon-14 incorporation may or may not be comparable, although Borsook (100) suggests that the two are comparable in animals, (3) the periods of application--12 hours for kidney bean versus 72 hours for tobacco, and (4) the method of application--to the leaves directly in the case of the carbon-14 and by means of translocation from the roots in the case of the nitrogen-15. An experiment might be done which would do much to clarify the situation. One could feed C^{14}-labeled substrates (or N^{15}-labeled) to both intact plants and the excised parts and examine the relative rates of incorporation.

The rate of initial carbon-14 incorporation into protein is more rapid for the sucrose experiment than for the acetate experiment. A possible explanation of this is that certain of the amino acids are labeled more rapidly by sucrose-C^{14} than by acetate-C^{14}. At least one such amino acid--glutamic

acid--has been shown by Racusen and Aronoff (7) to be incorporated into the protein of soybean leaves much more rapidly than any other amino acid. They conclude that in soybean leaf protein there is present either a selectivity in protein turnover, or a net synthesis of glutamate-rich protein. MacVicar and Burris found that N^{15}-fed (as ammonium sulfate) tomato plants incorporated much more nitrogen-15 into protein glutamic acid than was incorporated on the average by the other protein amino acids (101). Webster (94) has shown that both glutamate and aspartate are incorporated at a greater rate into bean hypocotyl mitochondria than is glycine. Borsook (100) has cited much evidence of differential rates of incorporation of amino acids into animal proteins.

The lag period in the rise in specific activity of protein is most probably correlated with the rate of synthesis of amino acids from the respective carbon sources. A certain amount of time is needed before sufficient carbon-14 has accumulated in the free amino acid pool to be incorporated into the protein to a measurable extent. Differentiation in the rate of incorporation as between roots, stems and leaves is not greatly noticeable until after three hours. This may be due to the fact that the specific activities of the various protein samples are so low that any differences are hidden, or it may be that the effects of excision are not felt strongly until some time beyond the third hour after excision.

Some evidence against this latter supposition comes from work by Yemm (8). His determinations showed a steady drop in the insoluble nitrogen level of excised barley with no hint of a lag period.

Summary

1. The rate of incorporation of carbon-14 from sucrose-C^{14} and acetate-C^{14} into leaf, stem and root protein of red kidney bean has been investigated for the first time.
2. It has been shown that the rate of incorporation of C^{14}-labeled amino acids varies with the organ under consideration. The level of radioactivity rises much more rapidly in root protein than in either leaf or stem protein.
3. This work, accomplished with excised organs, has been compared with the incorporation of nitrogen-15 into leaf, stem and root protein of intact tobacco plants.

PART III

Metabolic Pathways of Amino Acids in Plants

Introduction

Little attention has as yet been paid to the oxidation and decarboxylation of amino acids by higher plant tissues.

The oxidation of L-amino acids in animals was first well defined by Krebs (102). Preparations of liver and kidney of a variety of animals were found to have the ability to oxidatively deaminate alanine. Similar activities were found in microorganisms (103), molds (104-106), and snake venoms (107,108). The L-amino acid oxidase of snake venom has been shown to be a flavo-protein which contains flavin-adenine-dinucleotide (108). Cell-free enzyme preparations from rat liver and kidney were found by Blanchard, *et al*. (109) to act on all L-amino acids except serine, threonine, arginine, ornithine, lysine, aspartic acid, glutamic acid, and glycine. Such oxidative activity, with the exception of glycine oxidase (110), has not been found in any other mammal.

The oxidative deamination of glutamic acid by a higher plant was first studied by Thunberg (111) and by Andersson (112). The enzyme responsible--glutamic dehydrogenase--has

been identified in a variety of plants (31,113,114). Diphosphopyridine-nucleotide is necessary for the reaction (115). This enzyme is also found in animals (115,116), bacteria (117), and yeast (116,118).

The L-amino acid oxidases as found in animals, microorganisms, and Neurospora have not yet been obtained from higher plants. A possible exception may be tryptophan oxidase, which is associated with a peroxide-mediated oxidation in pea-seedling tissue (119).

Decarboxylation of amino acids has been investigated extensively in microorganisms, to a lesser extent in animals, and in higher plants only glutamic acid has been found to be decarboxylated (120-123). Pyridoxal phosphate has been shown to be a co-factor for decarboxylases which have been purified to any extent (124,125).

Glutamic acid decarboxylase has apparently been found wherever sought in higher plants. Okunuki first reported this enzyme as occurring in a variety of plants (126). Later Schales, et al. (127-129) reported extensively on the properties of the enzyme. This group has also been able to prepare a clear extract containing the enzymatic activity. Okunuki had previously failed to bring the enzyme into solution and had suggested that it might be attached to the cell wall (126). Morrison (130) also has found the activity to be associated with the insoluble portion of a homogenate pre-

pared from rhubarb leaves. Schales, et al. found that when the active extract is dialyzed it loses much of its activity, which can however be restored by addition of pyridoxal or even increased above that of the original extract by addition of pyridoxal phosphate. The pH optimum was found to be in the range of 5.3 to 5.9 for a carrot preparation. The manometry was carried out under nitrogen. A number of other amino acids do not act as substrates for the enzyme, among them D-glutamic acid, aspartic acid, lysine, arginine, leucine, glycine, cystine, tyrosine and histidine.

The product of glutamic acid decarboxylation was first identified in radish plants as gamma-aminobutyric acid by Hasse and Schumacher (131). Beevers later confirmed this in an extract from barley roots (132).

Materials and Methods

Decarboxylation

The enzyme preparation used was extracted from a homogenate of acorn squash obtained from a local market. Squash was used because of the ease of preparation and the fact that Schales, et al. (127) have shown that an active glutamic acid decarboxylase is found in squash. Only the ovary wall of the squash was used. Approximately 20 grams of squash tissues were ground in a Waring blendor with 100 milliliters of M/15 phosphate buffer, pH 5.8. It was found that when the homogenate was squeezed through several thicknesses of muslin, cell wall debris was adequately removed. The extract was used without further purification and was stable for as long as two weeks at -20° C.

Decarboxylation was measured manometrically by standard techniques (133). A total of five milliliters of reaction mixture was used in each reaction vessel. This was made up of four milliliters of squash extract containing 20 milligrams of protein and one milliliter of a solution containing 33 micromoles of the desired amino acid. In the case of tyrosine the concentration of substrate was somewhat less--a saturated solution was used. The experiments were carried out at 35° C. Both aerobic and anaerobic (under nitrogen) conditions were used. Results are expressed as microliters

of carbon dioxide produced per hour. Appropriate controls on carbon dioxide evolution from endogenous substrates were run simultaneously. All readings less than 20% above the readings of the controls were assumed to be not significant.

Oxidation

The enzyme preparation used for the study of amino acid oxidation was the same as for the decarboxylation studies, but was prepared at twice the concentration, and contained ten milligrams of protein per milliliter of squash extract. A total of 3.7 milliliters of reaction mixture was placed in each reaction vessel. This was composed of two milliliters of squash extract, 0.5 milliliter of an amino acid solution containing 33 micromoles of an amino acid, one milliliter of phosphate buffer of pH 7, and 0.2 milliliter of 10% potassium hydroxide contained in the center well. The temperature was maintained at 35° C. Results are expressed as microliters of oxygen absorbed per hour. Appropriate controls on oxygen absorption by endogenous substrates were run simultaneously. All readings less than 20% below the readings of the controls were assumed to be not significant.

Results

Data on the rates of oxidation and decarboxylation for a variety of substrates are contained in Table I. The catabolism of a large number of amino acids and two amides was investigated. Wherever possible the L isomers were used. In addition, non-nitrogenous compounds related to certain of the amino acids were used as substrates. In conjunction with other studies carried out in this laboratory, some data are available for the production of carbon dioxide by the enzymatic degradation of urea. Since the system was buffered at pH 5.8, the ammonia produced remained in solution and only the rate of carbon dioxide evolution was measured.

Further work was carried out to determine the cellular location of the glutamic decarboxylase. The squash extract, strained through muslin, was fractionated by centrifugation into three portions: material sedimented at 10,000 x g, probably mitochondrial (134), material sedimented at 100,000 x g which may be called the microsomal fraction (135,136), and the supernatant from this last separation. The carbon dioxide evolved was measured in the same manner as before. Four milliliters of extract containing a total of 40 milligrams of protein were fractionated. The reaction mixture consisted of 0.5 milliliter of a solution which contained 33 micromoles of glutamic acid, one milliliter of phosphate buffer of pH 5.8, and one milliliter of a water solution of

one of the fractions or a combination of the fractions. The protein content of the fractions was not determined. The data are shown in Table II.

Table I

Catabolism of Exogenous Substrates by an Extract Prepared from Squash

Substrate	Decarboxylation		Oxidation
	µl of CO_2 evolved per hour per 20 milligrams of squash protein		µl of O_2 absorbed per hour per 20 milligrams of squash protein
	Air	Nitrogen	Air
alpha-L-Alanine	41	0	12
beta-Alanine	0	0	0
alpha-Aminobutyric Acid	0	0	10
gamma-Amino-butyric Acid	0	0	7
L-Arginine	46	0	10
L-Asparagine	38	0	10
L-Aspartic Acid	81	0	3
Canavanine	0	-	4
DL-Citrulline	0	0	4
L-Cysteic Acid	0	0	10
L-Cysteine	80	0	50
L-DOPA	0	0	0
L-Glutamic Acid	392	303	7
L-Glutamine	130	0	14
Glycine	0	0	0
L-Histidine	0	0	0
Hydroxy-L-Proline	71	0	3
L-Isoleucine	42	0	7
L-Leucine	0	0	0
L-Lysine	45	0	6
L-Methionine	0	0	0
L-Ornithine	105	0	9
L-Phenylalanine	0	0	6
L-Proline	31	-	6
L-Serine	0	0	0
L-Threonine	0	0	0
L-Tryptophan	0	0	0
L-Tyrosine	0	0	0
DL-Valine	0	0	0
alpha-Ketobutyric Acid	76	0	10
alpha-Ketoglutaric Acid	0	0	10
Glyoxylic Acid	0	0	0
Glutaric Acid	0	0	-
Pyruvic Acid	111	0	0
Urea	66	-	0

Table II

Intracellular Location of Glutamic Decarboxylase in Squash

Fraction	Decarboxylation µl of CO_2 evolved per hour
Mitochondrial	5,5
Microsomal	5,4
Supernatant	210,213
Supernatant plus microsomal	225
Supernatant plus microsomal plus mitochondrial	228

Discussion

1. Decarboxylation

The amino acids undergoing oxidative decarboxylation may be divided into four groups as follows:

No Significant Decarboxylation	31-46 ul/hr.	71-130 ul/hr.	400 ul/hr.
beta-Alanine	alpha-Alanine	Aspartic Acid	Glutamic Acid
alpha-Aminobutyric Acid	Arginine	Cysteine	
gamma-Aminobutyric Acid	Asparagine	Glutamine	
Canavanine	Isoleucine	Hydroxyproline	
Cysteic Acid	Lysine	Ornithine	
Citrulline	Proline		
Glycine			
Histidine			
Leucine			
Methionine			
Phenylalanine			
Serine			
Threonine			
Tryptophan			
Tyrosine			
Valine			

Glutamic acid was the only amino acid which yielded significant amounts of carbon dioxide under anaerobic conditions.

A comparison of the foregoing list with data on decarboxylation of amino acids compiled by Schales in 1951 (120) may be made. Microorganisms have been shown to decarboxylate anaerobically arginine, histidine, lysine, ornithine, tyrosine, glutamic acid, aspartic acid, and possibly tryptophan, leucine and serine. No one type of organism, under the condi-

tions used, could decarboxylate all of these. For instance, of 151 strains of E. coli studied by Gale (122), 70-95% were able to decarboxylate arginine, lysine, ornithine and glutamic acid. Only 14 could decarboxylate histidine and only six could act on tyrosine. This work was done at low pH values, with washed suspensions of bacteria.

Slices or extracts of mammalian tissues decarboxylate the following amino acids at neutral or slightly alkaline pH: histidine, tyrosine, dihydroxyphenylalanine, tryptophan, cysteic acid, hydroxyphenylserine, phenylalanine and glutamic acid (120,121). In higher plants glutamic acid decarboxylation has been observed most widely (120). The reported occurrence of both histamine and hydroxytyramine (137) in plants makes it appear possible that histidine and dihydroxyphenylalanine decarboxylases also occur in plant tissues. Recently putrescine, the decarboxylation product of ornithine, has been found in barley leaves under a condition of potassium deficiency (138).

In the present work most of those amino acids which did not show significant carbon dioxide evolution will not be considered further. Each of the amino acids which did show significant decarboxylation will be discussed briefly.

alpha-Alanine

The keto-acid analog of this amino acid, pyruvic acid, was decarboxylated at four times the rate for the amino acid.

Neither was decarboxylated under nitrogen. An alpha-alanine oxidase was shown to be present, whereas pyruvic acid was not oxidized. Presumably in the squash, alpha-alanine is oxidatively deaminated and oxidatively decarboxylated.

Arginine

Both Gale (122) and Blaschko (121) state that amine formation from arginine in bacteria and animals occurs as well under nitrogen as in air. Under the conditions of the present work, arginine was only decarboxylated aerobically. There are two possible mechanisms which would fit the data. The enzyme arginase may function as in animal tissues and in Neurospora. This would result in the production of urea (139,140). If urease is present, then carbon dioxide would be evolved. The degradation of urea is shown to occur in squash. Or, as has been found in some invertebrates (141, 142), arginine may be oxidized to delta-guanidino-alpha-ketovaleric acid and then decarboxylated to produce gamma-guanidobutyric acid. This oxidation product has also been produced by a preparation of Crotalus spp. venom (143).

Asparagine and Aspartic Acid

Both of these compounds were decarboxylated only under aerobic conditions. Presumably oxidative deamination is followed by oxidative decarboxylation. A non-oxidative deamination is possible through the action of aspartase. The end-

products were not studied. Oxalacetic acid would be formed by deamination and deamidation. This compound, under the conditions used, breaks down spontaneously. If deamidation takes place slowly, malonamide could be formed.

Cysteine and Cysteic Acid.

Two mechanisms are at hand to explain the decarboxylation. Oxidation of the cysteine to cysteic acid and subsequent decarboxylation of the latter as happens in rat liver (144) seems to be ruled out since cysteic acid was not decarboxylated. More probably, through the action of a cysteine desulfhydrase, pyruvic acid was formed and oxidatively decarboxylated. This type of reaction has been shown to occur in animal tissues, yeast, and bacteria (145-148).

Glutamic Acid

The decarboxylation of glutamic acid differs from that of all the other amino acids studied in that no oxidative step needs to precede the decarboxylation. Many workers have shown this (120) and have also shown the product of the decarboxylation to be gamma-aminobutyric acid (131,132), which does not undergo further decarboxylation in the preparation used here. The fact that alpha-ketoglutaric acid is not decarboxylated indicates a requirement for the alpha-amino group if decarboxylation is to take place.

Glutamine

It is difficult to suggest a plausible reason for the lack of anaerobic decarboxylation of this compound. Certainly deamidation does not require oxygen, but obviously glutamine was not converted to glutamic acid. Glutamine does serve as a substrate for oxygen uptake. This may be due to an oxidative deamination. The alpha-ketoglutaramide formed may subsequently have been decarboxylated to produce the amide of succinic acid.

Hydroxyproline and Proline

Both of these amino acids served as substrates for oxygen uptake. Proline was probably converted to glutamic semialdehyde and hydroxyproline to gamma-hydroxyglutamic semialdehyde (83,149). Decarboxylation of the semialdehyde is a possibility since no decarboxylation took place anaerobically.

Isoleucine

Presumably this amino acid was oxidized to the keto-acid (alpha-keto-beta-methylvaleric acid) and oxidatively decarboxylated to yield alpha-methyl-butyric acid (32). It is interesting to note that leucine was not oxidized and was not decarboxylated.

Lysine

Like arginine, lysine was apparently not decarboxylated to form the amine since anaerobically no decarboxylation was observed. Very possibly the lysine was converted to pipecolic acid which may have then been converted to alpha-aminoadipic acid. This may well have been deaminated and decarboxylated to form glutaric acid (150-152). Glutaric acid was not decarboxylated by the preparation used, although oxidation of glutaric acid to alpha-ketoglutarate has been suggested by <u>in vivo</u> studies with rats (153). It may be that CoA is necessary for enzymatic degradation of glutaric acid.

Ornithine

In bacteria this amino acid is decarboxylated to the amine (154). In squash it is apparently oxidized to the keto-acid analog (alpha-keto-delta-aminovaleric acid) and this material is then decarboxylated to the amine of butyric acid. It is not probable that the oxidation of both amino groups to form alpha-keto-glutarate takes place since this compound is not decarboxylated by the preparation used.

Neither tyrosine nor DOPA was decarboxylated to a significant extent. This may well have been due to the relative insolubility of these two amino acids at pH 5.8. It is noteworthy that while alpha-ketobutyric acid was decarboxylated, alpha-aminobutyric acid was not, although the latter caused

as much oxygen uptake in the oxidation experiments as did the former. Neither glycine nor glyoxylic acid was decarboxylated, nor did either cause absorption of oxygen. This suggests that glycine may not be degraded as such but that it may be metabolized as a two-carbon part of some other compound, such as serine.

Glutamic acid decarboxylase is evidently not attached to a particle but is a soluble enzyme in squash. These data concur with the results of Schales, *et al*. (127).

2. Oxidation

As for the section on decarboxylation, the amino acids may be divided into four groups according to their rate of oxidation:

No Oxygen Taken Up	2-4 µl/hr.	6-14 µl/hr.	50 µl/hr.
DOPA	Aspartic Acid	alpha-Alanine	Cysteine
Glycine	Hydroxyproline	Arginine	
Histidine	Tryptophan	Asparagine	
Leucine	Citrulline	Glutamic Acid	
Methionine	Canavanine	Glutamine	
Serine		Isoleucine	
Threonine		Lysine	
Tyrosine		Ornithine	
Valine		Phenylalanine	
beta-Alanine		Proline	
		Cysteic Acid	
		gamma-Aminobutyric Acid	
		alpha-Aminobutyric Acid	

These results may be compared with those compiled by Fruton and Simmonds (32) of the L-amino acid oxidase activities of preparations of rat kidney, *Proteus vulgaris*, *Neurospora* and

cobra venom. *Histidine, leucine, methionine and tyrosine,* which are generally oxidized by the four organisms mentioned above, were not found to be oxidized by the squash preparation.

The oxidative processes for most of these amino acids have been metnioned already in the section on decarboxylation and therefore only the more interesting cases will be discussed further. All five organisms failed to oxidize glycine. Preparative methods may be at fault. Alternatively, the action of glycine oxidase may be extremely slow. Of the five sources of enzyme, only the Neurospora preparation oxidizes serine and threonine, and those at a rather low rate. The deamination of these two amino acids in animal tissues, yeast and bacteria has been shown to be non-oxidative in character and catalyzed by a dehydrase (32,155,156). Glutamic acid is apparently deaminated through the action of the ubiquitous L-glutamic dehydrogenase (120). In the Neurospora and the squash preparations aspartic acid caused a little uptake of oxygen. Aspartate was not oxidized by the other three organisms. The major pathways of aspartic acid deamination are through transamination and the action of aspartase (157-159).

Oxidation of cysteine to cystine and/or to cysteic acid could account for the large amount of oxygen uptake recorded with cysteine as the substrate.

Tryptophan oxidation could be expected to produce either

the corresponding keto acid, indole-3-pyruvic acid, or as mentioned previously, a peroxide-mediated oxidation to 3-hydroxykynurenine could have taken place (119).

Summary

In summary, these points may be reiterated:

1. Many amino acids have been found to be decarboxylated by a water extract from squash. The relative amounts of decarboxylation vary considerably.
2. Glutamic acid shows by far the greatest amount of decarboxylation, and unlike any of the other amino acids studied, is decarboxylated under anaerobic as well as aerobic conditions.
3. Contrary to the reported results from bacterial suspensions and animal tissues, arginine, lysine and ornithine are oxidatively decarboxylated in squash.
4. In the squash preparation glutamine is apparently not converted to glutamic acid before decarboxylation, but is more probably decarboxylated as alpha-ketoglutaramide.
5. Evidence has been presented that glutamic acid decarboxylase in the squash is a soluble enzyme.
6. The oxidation of many amino acids has been shown to be mediated by the squash preparation. The particular amino acids oxidized and the relative amounts of oxidation among

the amino acids are at variance with published results from at least four other tissue preparations; namely, rat kidney, *Proteus vulgaris*, *Neurospora*, and cobra venom.

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APPENDIX A

Studies on the Possibilities for Carbon-14 Contamination of the Isolated Free Amino Acids

The possibility that the free amino acids isolated by the procedures outlined in Section I might be contaminated by carbon-14 of C^{14}-sucrose, of C^{14}-acetate, or of compounds formed *in vivo* from these substances has not been rigorously ruled out. A series of tests were therefore made to gain some idea as to the extent of any such contamination and as to which of the amino acids, if any, might not be fully separated from possible contaminants.

Sucrose

Although the plant organs, after immersion in the C^{14}-sucrose solution, were rinsed twice, it is to be expected that considerable free labeled sucrose is still contained inside the cells of the tissue. In addition, other labeled compounds, such as glucose, fructose and organic acids are undoubtedly synthesized by the tissue. The soluble fraction of the homogenized plant tissue of course contains these contaminants, together with the free amino acids. In an attempt to eliminate as much of this non-amino acid material as possible, aliquots of the whole water extract were placed

on the Dowex-50 column and then eluted by 100 milliters of water, followed by successive portions of acid. A comparison of the radioactivity of the water eluate with that of the combined acid eluates is shown in Table I.

The greater part of the radioactivity resides in the water eluate. This activity is in part due to the C^{14}-sucrose present in the tissue plus that adsorbed on the surface and not removed by the rinsing procedure. A further part is due to other sugars and organic acids. A rough determination was made of the amount of sucrose which remained on the column. An aliquot of sucrose solution was passed through the column in the same manner as for the water extract. Estimation by the amount of carmelization during concentration of the eluates plus semi-quantitative tests by use of a resorcinol-trichloroacetic acid spray (1) indicated that over 95% of the sucrose was eluted by the first 100 milliliters of liquid to pass through the column. Elution of the remainder was proportional to the amount of liquid washed through the column. Other sugars and organic acids would be expected to act in a similar manner.

It is probable, as shown above, that some C^{14}-sucrose is eluted together with the amino acids. Therefore, the position of sucrose on the paper chromatograms used for further separation of the amino acids was determined. It was found that when butanol-acetic acid-water was used as a solvent the R_f value of sucrose was approximately 0.1, which

Table I

Approximate Total Radioactivity of Various Eluates Collected from Dowex-50 Columns in The Acid Phase

	Radioactivity, counts per minute	
Tissue	Water Eluate	Total Acid Eluate
Stems		
20 minutes	7500	860
1 hour	10530	960
3 hours	-	1540
12 hours	7200	8280
Leaves		
20 minutes	29280	440
1 hour	54930	4840
3 hours	77610	9140
12 hours	193560	20000
Roots		
20 minutes	10650	-
1 hour	8100	-
3 hours	4500	-
12 hours	18800	-

coincides with that of the cystine-peptide fraction. Since a 65% pyridine solution was used to separate the two latter compounds, sucrose was also chromatographed with this solvent. It was found that sucrose in this solvent has an R_f value of 0.95, which places it far above both cystine and the peptide.

The location of other sugars and organic acids was not determined. Published R_f values for the solvent butanol-acetic acid-water indicate that glucose falls near sucrose and that fructose has an R_f value which places it on a level with alanine (2).

Acetate

Since acetate must be expected to be present in the water extract of acetate-treated tissue, the possibility of acetate contamination of the amino acids was also investigated. It was found that substantial amounts of the radioactivity of acetate-treated tissue behave as do the non-amino acid activities of sucrose-treated tissue. In a further experiment a solution of C^{14}-acetate containing 27,000 counts per minute was placed on a Dowex-50 column and eluted by the same system used for purification of the water extract of free amino acids. These data are recorded in Table II. The total activity recovered is only a small portion of that placed on the column. Since the solutions were concentrated *in vacuo* at 50° C before the activity was measured, it seems probable that much of the acetate was

Table II

Total Radioactivity of Acetate Eluates Collected

From a Dowex-50 Column in the Acid Phase

Eluant	Volume of Eluant Milliliters	Total Radioactivity Eluted counts per minute
Water	100	570
1.5 N HCl	100	30
2.5 N HCl	100	42
4.0 N HCl	300	138
20% Acetic Acid	300	30

volatilized and lost. Another source of loss could be expected from adsorption of acetate on the resin. That this happens is indicated by the fact that radioactivity was recovered by elution with 20% acetic acid.

The behavior of acetate during paper chromatography was further determined. A solution of C^{14}-acetate was applied at the base of a large sheet of Whatman #1 paper in a manner similar to that used to separate the amino acids. Butanol-acetic acid-water was the solvent system used. After development, horizontal strips were cut from the paper at the level characteristic of each amino acid group, the strip eluted with water and the eluate counted (Table III). The acetate radioactivity is distributed in a pattern roughly proportional to the width of the horizontal paper strips. Thus, as might be expected, acetate is distributed uniformly on the chromatogram developed with a solvent containing acetic acid.

The distribution of C^{14}-acetate was now determined for the second amino acid isolation procedure in which small sheets of Whatman #1 paper were used. C^{14}-acetate was applied to such sheets and these then were developed with the solvents used to separate the various amino acids. The radioactivity at the position of each amino acid was determined (Table IV). It is shown that acetate is fairly uniformly distributed, with the exception of the amount which appears at the level characteristic for proline.

Table III

Distribution of Acetate Upon Paper Chromatograph after Development with Butanol:acetic acid:water

Amino Acid-Equivalent Location of Radioactivity	Total Radioactivity Eluted counts per minute
Point of application	10
Cystine-Peptide	10
Lysine-Arginine-Histidine	12
Glycine-Aspartic Acid-Serine	22
Glutamic Acid-Threonine	8
Alanine-Proline	7
Tyrosine-alpha-Aminobutyric Acid	5
Methionine-Valine	6
Leucine-Phenylalanine	14
To top of sheet	100

Table IV

Distribution of Acetate Upon Paper Chromatographs After Development with Various Solvents

Amino Acid-Equivalent Location of Radioactivity	Solvent System	Total Radioactivity Eluted: counts per minute
Alanine	Phenol	3
alpha-Aminobutyric Acid	Methanol	2
Arginine	Pyridine	1
Aspartic Acid	Ethanol-Ammonia	4
Cystine	Pyridine	1
Glutamic Acid	Methanol	1
Glycine	Ethanol-Ammonia	1
Histidine	Pyridine	1
Leucine	Methanol	9
Lysine	Pyridine	1
Methionine	Ethanol-Acetic Acid	2
Phenylalanine	Methanol	2
Proline	Phenol	13
Serine	Ethanol-Ammonia	1
Threonine	Methanol	2
Tyrosine	Methanol	1
Valine	Ethanol-Acetic Acid	4
Peptide I	Pyridine	1

It is evident that the possibility for contamination of the isolated amino acids exists in both the sucrose and the acetate experiments, although the opportunity for contamination by sucrose, *per se*, is very small. The very fact that acetate is distributed so uniformly throughout the isolation procedures argues against significant contamination by this compound since certain of the amino acids did not show even low amounts of radioactivity at the shorter time intervals. The conversion products of both sucrose and acetate are an indeterminate factor and the only valid argument against contamination from this source is that radioactivity appeared in the free amino acids in patterns which would be expected on the basis of work done with other organisms and also on the basis of the appearance of radioactivity in amino acids isolated from the protein hydrolysates.

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APPENDIX B

The Partial Amino Acid Sequence of a Protein

During the last few years more and more has been done to determine the amino acid sequence of peptides and proteins. The amino acid sequences of several naturally occurring peptides have been elucidated (1), and many more are being studied, particularly those which have physiological activity. These studies have made it possible to synthesize some naturally occurring peptides. In the case of one peptide--glutathione--it has been possible to purify the enzyme system which catalyzes the natural synthesis (2,3,4,5). For only one protein, insulin, has the amino acid sequence been followed through to completion (6,7). Partial sequences for many other proteins have, however, been established. Where similar proteins from different organisms have been studied, differences in the terminal series of amino acids have been found, and thus some light has been shed on the basis for species differences.

Studies on amino acid sequences have been made possible by the advent of a great number of new techniques or new applications of old methods. Much of the work up to 1952 has been adequately reviewed by Khorana (8). The first work on amino acid sequence started as investigations of the N-

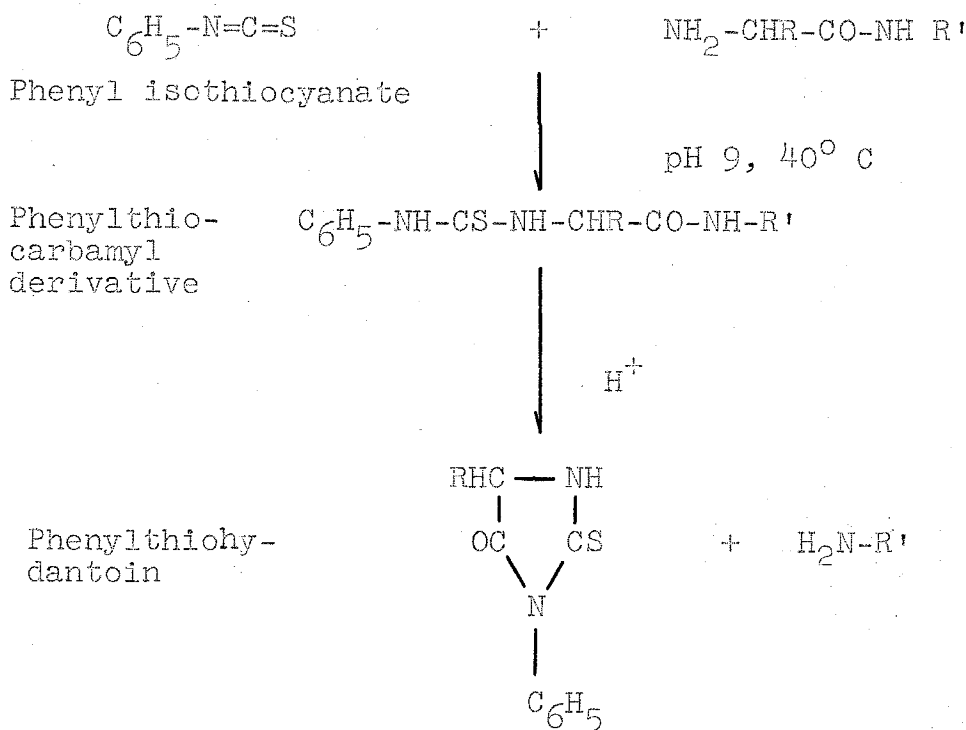
terminal and C-terminal amino acids since these are equipped with a "handle" by which they may be grasped, and after hydrolysis, identified. In the case of the N-terminal groups, most approaches involved the acylation of the free amino group. Reagents such as naphthalene-β-sulphonyl chloride (9) and chloro-2:4-dinitrobenzene (10) were used. Sanger successfully developed fluoro-2:4-dinitrobenzene as a reagent for this work (11). Other compounds which have been used are p-iodo(I^{131})phenyl-sulphonyl chloride (12), phenyl isocyanate (13,14) and phenyl isothiocyanate (15). Conversion of a peptide to the thiocarbamate derivative by reaction with CS_2, followed by acid hydrolysis to form the 2-thio-5-thiazolidone has been proposed by Levy (16). Kenner and Khorana (17) devised a reaction of the sodium salts of peptides at room temperature with alkyl alkoxydithioformates and this was successfully applied to simple synthetic peptides. A rather interesting method is that introduced by Reith and Waldron (18). They synthesized a deep orange isothiocyanate which they call 4-dimethyl-amino-3:5-dinitrophenyl isothiocyanate, or DDPT. When this compound reacts with amino acids or with peptides it forms an orange thiocarbamyl derivative (DDP-thioureidoamino acid) in excellent yields. The appropriate hydrolytic treatment produces the substituted 2-thiohydantoin.

The C-terminal amino acids have been reacted with ammonium thiocyanate to form the substituted 2-thiohydantoin (19,20,21),

with di-4-tolyl-carbodi-imide to form an acylurea (22), and with metal hydrides to form the amino alcohol (23,24,25). They have also been oxidized in methanol to an amino-methoxy residue which can be hydrolyzed to the aldehyde (26,27). In addition, several workers have used carboxypeptidase to investigate not only the terminal amino acids, but also the amino acid sequence from the C-terminal end (28,29,30,31,32, 33).

The most successful study of a protein sequence to date has been that of Sanger, who used a combination of reaction with FDNB and systematic partial hydrolysis. This technique worked very well as applied to insulin (6,7).

In 1950 Edman suggested the use of phenyl isothiocyanate as a reactant for the amino group of small peptides (34). He demonstrated this method on synthetic di- and tri-peptides. The essential characteristic here as opposed to the FDNB method is the fact that the peptide is not destroyed by the mild hydrolytic conditions employed to obtain the terminal amino acid. The method could thus conceivably be used to obtain the amino acids consecutively in their proper sequence in a protein. The steps as outlined by Edman are as follows:



Edman then hydrolyzed the phenylthiohydantoin in barium hydroxide and recovered the amino acid.

Other workers since then have modified the original technique. Dahlerup-Petersen, et al. (35) found that milder ring closure conditions are needed. Ottesen and Wollenberger applied the method to peptides released in the conversion of ovalbumin to plakalbumin (36). They modified the reaction mixture and also used a means of measuring the yield of thiohydantoin that was suggested by the Fraenkel-Conrats (37).

Christensen attempted to apply the reaction to insulin five successive times and was able to recover the first five amino acids in a sequence which agreed with that determined by Sanger and his group. By the third time, however, several other amino acids appeared out of turn (38).

Recently Landmann, et al. (39) used the Edman method to determine the partial sequence of lyzozyme. The sequence of the first five amino acids was found to be the same as that found or predicted by Schroeder with the FDNB method (40). Instead of hydrolyzing the phenylthiohydantoin as previous workers had done, Landmann simply separated the derivatives by chromatography in appropriate solvents.

Fox has used the phenyl isothiocyanate method in his studies on the sequence of amino acids in ACTH (41). Fraenkel-Conrat and Singer investigated the N-terminal groups of tobacco mosaic virus and measured the number of end groups (42). The latest use of the method is that of Thompson (43) who established the first two amino acids of human and bovine serum albumin and of carboxypeptidase. Several different cleavage reagents were tried, with essentially similar results in all cases. Thompson found that cleavage was good with the material used, but that recovery of the phenylthiohydantoins was poor. Fraenkel-Conrat (44) has developed a technique which removes one obstacle from the use of the Edman method. The phenylthiocarbamyl-proteins are fairly insoluble in the acid media needed for formation of the phenylthiohydantoins. He applied the protein to a strip of filter paper and carried out the reaction on the paper. Harris used this development successfully in an examination of the amino acid sequence of ACTH (45).

The sequence studies from the C-terminal end have been rather few. Turner and Schmerzler (21) feel that the reaction with ammonium thiocyanate can be used on a small scale for the successive degradation of peptides and proteins, but their results do not appear promising. Gladner and Neurath have found that the use of carboxypeptidase on the diisopropyl phosphate derivative of chymotrypsin gives somewhat equivocal results (32). Harris on the other hand has been able to work out a partial sequence for ACTH using carboxypeptidase. He obtained the successive liberation of three amino acids. The reaction failed on the fourth, which was shown by pepsin digestion of the protein to be proline, an amino acid resistant to the action of carboxypeptidase (33).

Materials and Methods

Since the phenylthiohydantoin method of Edman works so well with small peptides, it appeared to be sensible to try it on a small protein. The protein selected was five times recrystallized bovine pancreatic trypsin inhibitor (PTI), prepared according to the method of Kunitz and Northrup (46) (Worthington Biochemical Sales Co., Freehold, New Jersey). The reasons for selection of PTI are two-fold: (1) this protein has an approximate molecular weight of 9000 as found by osmotic pressure measurements (47) and (2) it was suggested (48) that since PTI is one of several proteins isolated from pancreas in a fairly pure form, it would be of interest to compare the amino acid sequence of PTI with that of other isolated pancreatic proteins, such as insulin.

The PTI was used as received, in the damp cake form of the five times recrystallized product. In a comparison of trypsin inhibitors Laskowski, et al. (49) prepared PTI according to the method of Kunitz and Northrup, recrystallized the product four times and found it to be electrophoretically homogeneous.

Green and Work (47) have already established by use of FDNB that the first amino acid on the N-terminal end is arginine, and have made an amino acid analysis by paper chromatography (Table I).

It seemed advisable, before the study itself commenced,

Table I

Amino Acid Analysis of Pancreatic Trypsin Inhibitor (47)

Amino Acid	% by Weight	Molecules per Molecule of Inhibitor
Aspartic Acid	8	6
Glutamic Acid	8	6
Cystine	2.5	1
Threonine	4.5	4
Serine	3.5	3
Glycine	7.5	10
Alanine	14.5	18
Valine	2.5	2
Proline	5.5	5
Leucines	6	5
Phenylalanine	8	5
Tyrosine	9	5
Tryptophan	0	0
Methionine	1.5	1
Lysine	7.3	5
Arginine	15	9
Histidine	0	0

to try the proposed method on some small peptide of well-established sequence. Glutathione was chosen, although it was realized that the phenylthiohydantoin would be formed by reaction with the free carboxyl group and not the gamma-glutamyl group involved in the peptide linkage. The Dahlerup-Petersen, et al. (35) modification of Edman's technique was followed. Under this plan, 0.1 milliliter of phenyl isothiocyanate was added to six milligrams of glutathione in a solution of 2 milliliters of water:diozane (1:1). This mixture was stirred constantly at 40° C, pH 8. In 45 minutes the reaction was complete as judged by the uptake of 1 N NaOH needed to keep the pH constant. The excess phenyl isothiocyanate was extracted by repeated washings with cyclohexane followed by benzene. The residue was then evaporated to dryness. To the residue, phenylthiocarbamyl-peptide, were added 1 milliliter of 0.05 M aqueous citrate buffer at pH 4.6 and 1 milliliter of benzene. This mixture was shaken at 50° C for 36 hours--the object being to cleave the phenylthiocarbamyl-peptide and simultaneously extract the phenylthiohydantoin from the aqueous solution into the benzene. The benzene extract was then dried in vacuo, taken up in 0.25 N $Ba(OH)_2$ and heated at 140° C for 48 hours. The barium was precipitated as $BaCO_3$, the liquid evaporated to a small volume, and chromatographed. Treatment with ninhydrin revealed only one reactive spot, that corresponding to glutamic acid.

It seems most likely that the cleavage of the gamma-glutamyl peptide bond was not due to formation of the phenylthiohydantoin, but actually was a result of the instability of the gamma-glutamyl bond to acid hydrolysis.

The remaining di-peptide--cysteinylglycine--was treated in a similar fashion, but no cysteine could be recovered. Evidently the action of hot alkali on cysteine is so severe as to decompose it beyond identification. The glycine was not looked for in the remaining solution.

Despite the unpromising results of the preliminary trial, the method was applied to the protein. A total of 153 milligrams of PTI were added to a mixture of 2 milliliters of phenyl isothiocyanate, 2 milliliters of water and 2 milliliters of dioxane. The procedure which produced glutamic acid from glutathione was used, i.e., cleavage in citrate buffer. After hydrolysis with $Ba(OH)_2$ and subsequent chromatography, no amino acid could be detected.

At this point, a publication on the chromatography of phenylthiohydantoins appeared in which the author mentions the fact (which had been overlooked) that serine, threonine, cystine, arginine, asparagine and glutamine are decomposed by hot alkaline hydrolysis (50). As arginine was expected to be the N-terminal amino acid, such decomposition may explain the absence of a recoverable amino acid after hydrolysis.

In order to confirm the position of arginine, resort was made to the technique of Sanger (11). The DNP-PTI derivative was formed. The reaction mixture used was 10 milligrams of PTI in 1 milliliter of 1% trimethylamine plus 0.1 milliliter of FDNB in 2 milliliters of ethyl alcohol. The product was hydrolyzed in 6 N HCl at 105° C for ten hours. The hydrolyzate was extracted with ethyl ether and both the ether extract and the aqueous residue (in which the arginine might be expected) were chromatographed on paper using butanol-acetic acid-water (4:1:5) as suggested by Kent (51). The control was DNP-arginine made as recommended by Porter and Sanger (52). The R_f of a spot from the aqueous extract coincided with that of the control DNP-arginine. A second run was made with a mixture of the DNP-arginine and the DNP-amino acid from the protein, and only one spot appeared on the paper. This was taken as confirmation of arginine as the N-terminal amino acid of PTI.

The residue left from the first phenyl isothiocyanate treatment of PTI was treated again. After hydrolysis and chromatography, four ninhydrin-positive spots were found. These were tentatively identified as serine, threonine, phenylalanine and methionine.

A third and fourth treatment yielded nothing from the alkaline hydrolysis. It was thought that a superior method might be to chromatograph the phenylthiohydantoins as such and exclude the alkaline hydrolysis. Therefore the phenyl-

thiohydantoin derivatives of several of the amino acids were made according to the method of Edman (15). The melting points checked with those given by Edman. The derivatives were applied to paper in an acetone solution. The chromatographic techniques of Sjöquist (50) and of Landmann (39) were used.

The fifth time the protein was treated by the Landmann modification. The procedure used is shown in Figure I. Determination of the amino acid sequence of a second portion of PTI was started. The Landmann modification, as outlined, was used from the beginning. Five reactions were run and the thiohydantoins recovered and chromatographed. Four different solvents have been found to give satisfactory results--two suggested by Landmann, et al. (39) and the A and B solvents of Sjöquist (50). Landmann and co-workers have developed a spray which gives characteristic colors with the thiohydantoins. The color agent--Grote's solution--is based on sodium nitroprusside and reacts with the sulfur of the thiohydantoins. The resultant color is dependent on the particular amino acid involved. Sjöquist was able to adapt an iodine-azide color agent which is specific for divalent sulfur. This agent is ten to twenty times as sensitive as Grote's solution, but is non-specific as to the amino acid involved. The color agents and the solvents, of course, may be used interchangeably. An addition can be made to Sjöquist's table of R_f values. The thiohydantoin of serine was prepared (53) and found to have an R_f value of 0.30 in Solvent A and 0.10 in Solvent B.

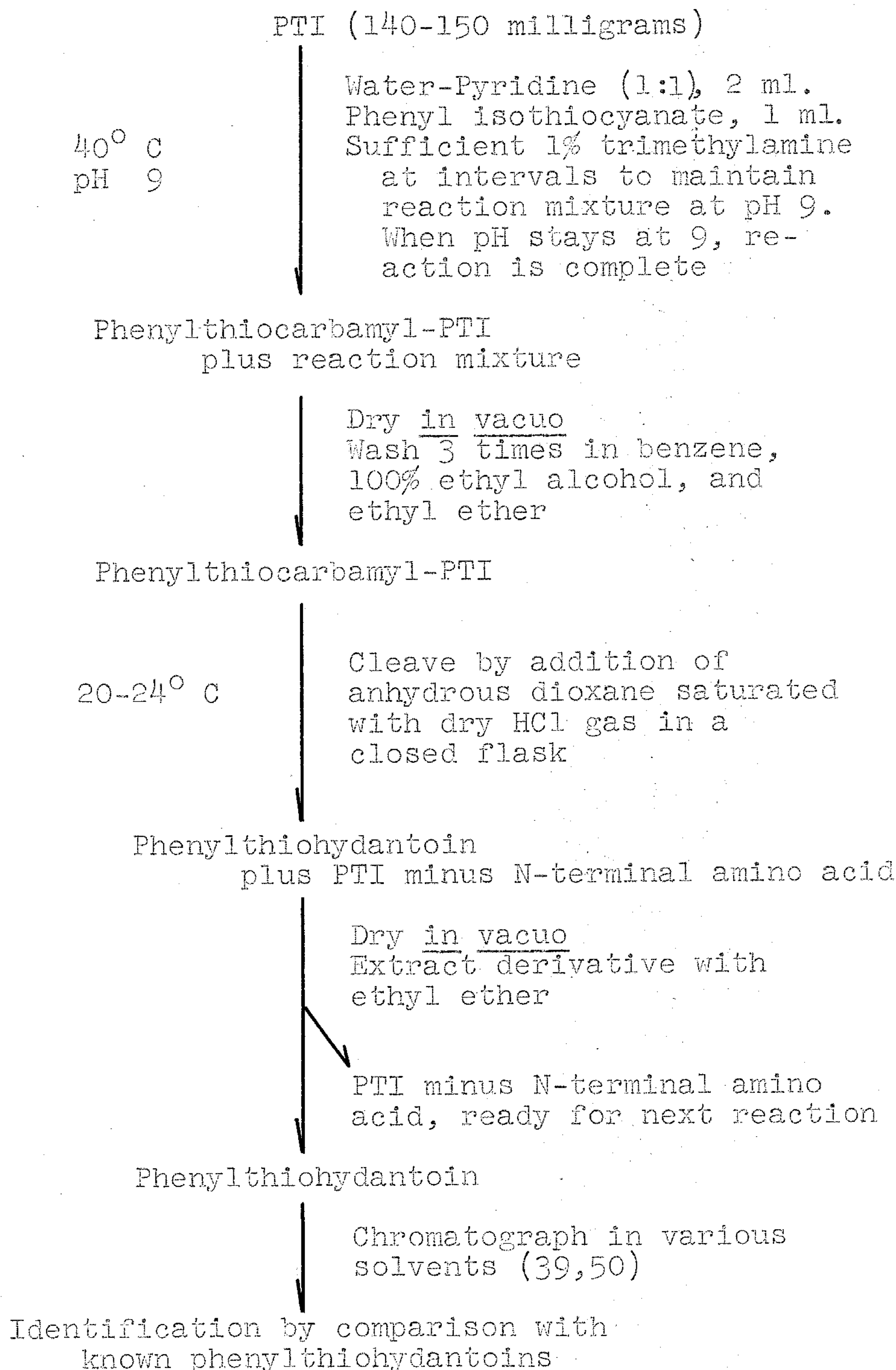


Fig. I. Procedure used for successive cleavage of amino acids from PTI.

Results and Discussion

The results obtained by successive treatment of two aliquots of PTI with phenyl isothiocyanate are shown in Table II. It is obvious that after the second amino acid the results are ambiguous. The data are of the same type as those found by Christensen in his study on insulin (38). Only the first two amino acids--arginine and phenylalanine--are well established. For the other reactions it was impossible to determine absolutely the proper amino acid by chromatography of the thiohydantoin and there was insufficient material for a crosscheck by hydrolysis of the thiohydantoin to produce the amino acid. In certain cases the evidence for a particular amino acid was stronger than for the others, which are enclosed in parentheses. The indeterminate nature of the results would seem to be a result of three causes: (1) incomplete reaction, (2) incomplete extraction, and (3) partial hydrolysis of the protein. The first two causes might be reduced fairly simply by longer reaction times and more complete extraction procedures. However, to reduce the amount of undesirable hydrolysis the reaction time should be reduced to a minimum--a move in opposition to the desire for a more complete reaction.

A procedure was evolved during the course of this work which should give satisfactory results with small peptides and with the first few amino acids of proteins:

Table II

Results of the Sequential Degradation of Pancreatic Trypsin Inhibitor by Successive Treatments with Phenyl Isothiocyanate

Reaction	PTI Aliquot I	PTI Aliquot II
1	Arginine	Arginine
2	Phenylalanine (Serine, Threonine, Methionine)	Phenylalanine
3	Threonine (Phenylalanine)	Phenylalanine (Methionine)
4	---------- (Serine)	Methionine
5	(Phenylalanine, Methionine, Alanine)	(Phenylalanine, Methionine, Alanine)

1. React 200-400 milligrams of the peptide (or protein) in three milliliters of water, three milliliters of pyridine, and 1.5 milliliters of phenyl isothiocyanate for 45 minutes at 35° C, pH 8.
2. Dry _in vacuo_ and wash thoroughly with benzene, 100% ethyl alcohol and ethyl ether, in that order.
3. Carry out the cleavage with anhydrous dioxane saturated with dry hydrogen chloride gas in a closed flask at room temperature for two hours.
4. Again dry _in vacuo_ and extract the thiohydantoin by repeated extraction with ethyl ether.
5. Divide the ether solution into two aliquots. Hydrolyze one portion with barium hydroxide and identify any amino acids produced. Identify the thiohydantoin(s) in the second portion by chromatography, as outlined in the section on materials and methods.

The procedure outlined above is quite similar to that described in Figure I. The major differences are the larger amounts of material used, the slightly gentler conditions, and the expanded identification system.

The evidence which has accumulated suggests that the Edman reaction affords satisfactory results for sequential determinations of small peptides and for partial sequences of proteins. The method would seem to be superior to the

FDNB method as used by Sanger in that the number of necessary peptide fractions might be sharply reduced. The amino acid sequences of peptides produced by the first partial hydrolysis of a protein could be determined without resort to further hydrolysis.

Summary

1. The phenylthiohydantoin, or Edman, method for determination of amino acid sequences has been applied to a small protein-pancreatic trypsin inhibitor.
2. The sequence of the first two amino acids from the free amino-group end has been established as arginyl-phenylalanine.
3. A procedure is suggested which should give good results for the determination of sequences in peptides as those isolated from red kidney bean and described in another part of this thesis.

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APPENDIX C

Some Effects of Light and Temperature on the Germination of Seed of *Eschscholtzia californica*

Introduction

Since the time of Ingenhousz in 1788 investigators have studied the effects of light and temperature on seed germination. For almost one hundred years the seeds studied were those of common crop plants, such as oat, wheat, bean and pea--seeds which show very little response to light. In 1860 Caspary demonstrated a definite effect of light on the germination of the seeds of an aquatic plant, but his work went unnoticed. A few years later other workers found that light is necessary for the germination of mistletoe seeds, but not until 1881 were definitive experiments, on the effect of light on grass seed, carried out (1).

The effects of light on seed germination are of two kinds; (1) the germination of some seeds is enhanced by light, and (2) the germination of some seeds is inhibited by light. The first class consists of, for example, seeds of various species of mistletoe, strangling fig, African violet, South American pampas grass, bluegrass, fireweed, lettuce and *Oenothera*. Several theories have been advanced to account for the favorable action of light on seed germination. Two

of the more valid of these, as listed by Crocker (1,2) are:

1. Light may act by enhancing diurnal temperature fluctuations. Evidence: the germination of bluegrass seed is greatly aided by intermittent temperature fluctuations, even in the dark. However, germination of some seeds is enhanced by one dosage of light (tobacco).
2. Light inhibits the production of an impermeable layer in the seed coat, either through promotion of the rate of germination or by some direct mechanism. Evidence: a number of workers have found that the seed coat is involved in the sensitivity of many different kinds of seeds. Data are available, though, which indicate that the seed coat is not an important factor in the light sensitivity of several seeds.

Seeds of the second class, germination of which is inhibited by light, include _Phacelia_, _Nigella_, and many plants of the lily family. To this class belong also the seeds of _Eschscholtzia californica_, the California poppy. A theory has been advanced by Axentieff to explain the action of light on this class of seeds as well as for seeds which germinate in the light. In certain cases in which the seed coat is involved in light sensitivity it was found that the seeds responded to increased oxygen tension as well as to light. Germination of seeds of this type is enhanced by increased oxygen tension and completely inhibited by low oxygen tensions, regardless of the light regime. On this basis Axentieff

concluded that light affects oxidation processes within the seed, either favorably or unfavorably, while the seed coat regulates the amount of oxygen available to the protoplasm. Thus, if a seed coat is relatively impermeable to gas exchange, and light interferes with the oxidative processes, then germination is hindered. If light aids the oxidation processes, the effect of the seed coat is counteracted (1). A simpler explanation might be that the low oxygen tensions used were physiologically too low for germination under any conditions, and high oxygen tensions merely increased the rate of germination to the point where the inhibitory effects of light were not important.

In some seeds the effect of light is correlated with temperature. For instance, while *Amaranthus caudatus* seeds are inhibited by light between 5 and 20° C, no effect is apparent between 25 and 30° C and between 35 and 40° C light enhances the germination (1). The germination in darkness of lettuce seed (var. Grand Rapids) is increased by a red light treatment between 20 and 30° C and is not affected by red light at 18 or 32° C (3). Treatment with far-red radiation (7300 Å) inhibits the germination of lettuce seed (4,5).

Materials and Methods

The seeds of *Eschscholtzia californica* used in this work were obtained from the Ferry-Morse Seed Company. They were treated with methyl bromide for two hours prior to introduction into the Earhart Plant Research Laboratory where the work was carried out. The seed was stored at room temperature and 50% relative humidity. The viability of the seed was checked. It was found that approximately 80% of the seed were filled and apparently capable of germination. The following data are not corrected for this percent of viability.

For most of the germination tests a standard set of conditions were employed. The seeds were germinated in petri dishes on a layer of water-soaked absorbent cotton, with approximately one hundred seeds per dish. Two dishes of seeds were run for each point on each curve. These conditions were varied for certain specific experiments. Tests for germination of seeds with the seed coats removed were carried out with 10 to 15 seeds per dish. The seed coats were removed by first imbedding the seeds in a water-starch paste which when dry was sufficiently firm to allow removal of the seed coats with the sharpened edge of a flattened dissecting needle. To test for the adsorption of possible inhibitors by charcoal, a circular piece of filter paper was floated in a petri dish on a mixture of charcoal and water. The seeds were placed on the filter paper.

Light in all cases was supplied by fluorescent tubes plus incandescent bulbs. Light intensities, except for certain experiments, were of the order of 800 foot-candles at the level of the seeds. The sets of seeds kept in the light were exposed in a constant temperature room while those in the dark were kept in the same room in a large light-proof cabinet served by a blower fan. Temperatures were controlled within $\pm$ 2° C and all experiments except those shown in Figure I were run at 24° C.

Results and Discussion

During the course of the germination tests a curious phenomenon was noticed. The dry seeds are gray. When they are wet, they become dark in appearance. The surface of seeds placed in the dark became wet in a matter of hours, while seeds placed in the light became wet only after several days. Furthermore, the percent of totally wet seeds was estimated to be roughly proportional to the light intensity. It was thought that this behavior might be related to germination so tests were made to study it. One set of seeds was covered by a sheet of tissue paper which absorbed water from the cotton and kept the seeds under it quite damp. Another set had a sheet of tissue paper placed over the outside of the petri dish so as to equalize the light intensity which reached the two sets of seeds. The decrease in light intensity was slight since the tissue paper is almost transparent when

wet. No difference in the amount of germination was found between the two sets of seeds. It was also noted at the same time that seeds which were dry on top (all, of course, were wet at the point of contact with the cotton) were quite capable of germination. An explanation on purely physical grounds was sought. The water content of the cotton was approximately the same for both dishes. One difference which developed was that water condensed on the inner surface of the cover of dishes in the light. This indicates a cooler surface under the lights, or rather a surface cool in relation to the cotton and water in the petri dish. The probable explanation is that heat radiation from the lights slightly increased the temperature of the entire dish, but since the outside of the petri dish was cooled by air, a temperature differential resulted. Since the seeds on the cotton are dark enough to function as black bodies, it may be that their surface temperature became high enough to evaporate any water which tended to cover the seed surface. Under such conditions convection currents may be set up inside the petri dishes such that once an equilibrium is established, humid air will rise along the warm cotton layer and strike the cool surface of the dish. The air will become cooler and water will be lost to the surface, whereupon the relatively dry air will flow back across the seeds and remove more water. The cycle is complete, of course, when water returns to the bottom of the dish by gravity flow. Either expla-

nation would be more tenable than a vitalistic theory which must explain the creep of water up the outside of the seed coats in the dark and the lack of such movement in the light.

It may be seen in Figure I that the inhibition of seed germination by light occurs throughout a wide range of temperatures. As the temperatures rise however, the differential between light and dark germination decreases. This perhaps may be viewed most simply as an increase, with temperature, in the rate of the germination processes. It was observed that once a seed began to germinate, light no longer had an effect. An alternate explanation might be that a thermolabile inhibitor present in the seed breaks down at higher temperatures.

Figure II shows the effect of light intensity on germination at 24° C. Figure III points out the fact that the first 50 foot-candles of light exert an inordinate effect on germination. The next 750 foot-candles are only able to reduce the germination to about one-half the extent of the lower light intensity. One could envisage a light-mediated process which is saturated at rather low light intensities. It would be appropriate to determine the effect of variations on the light regimes tested. The effect of high intensity light flashes given to seed in the dark might be compared with low intensity light (to the order of ten foot-candles or less) given over long periods of time.

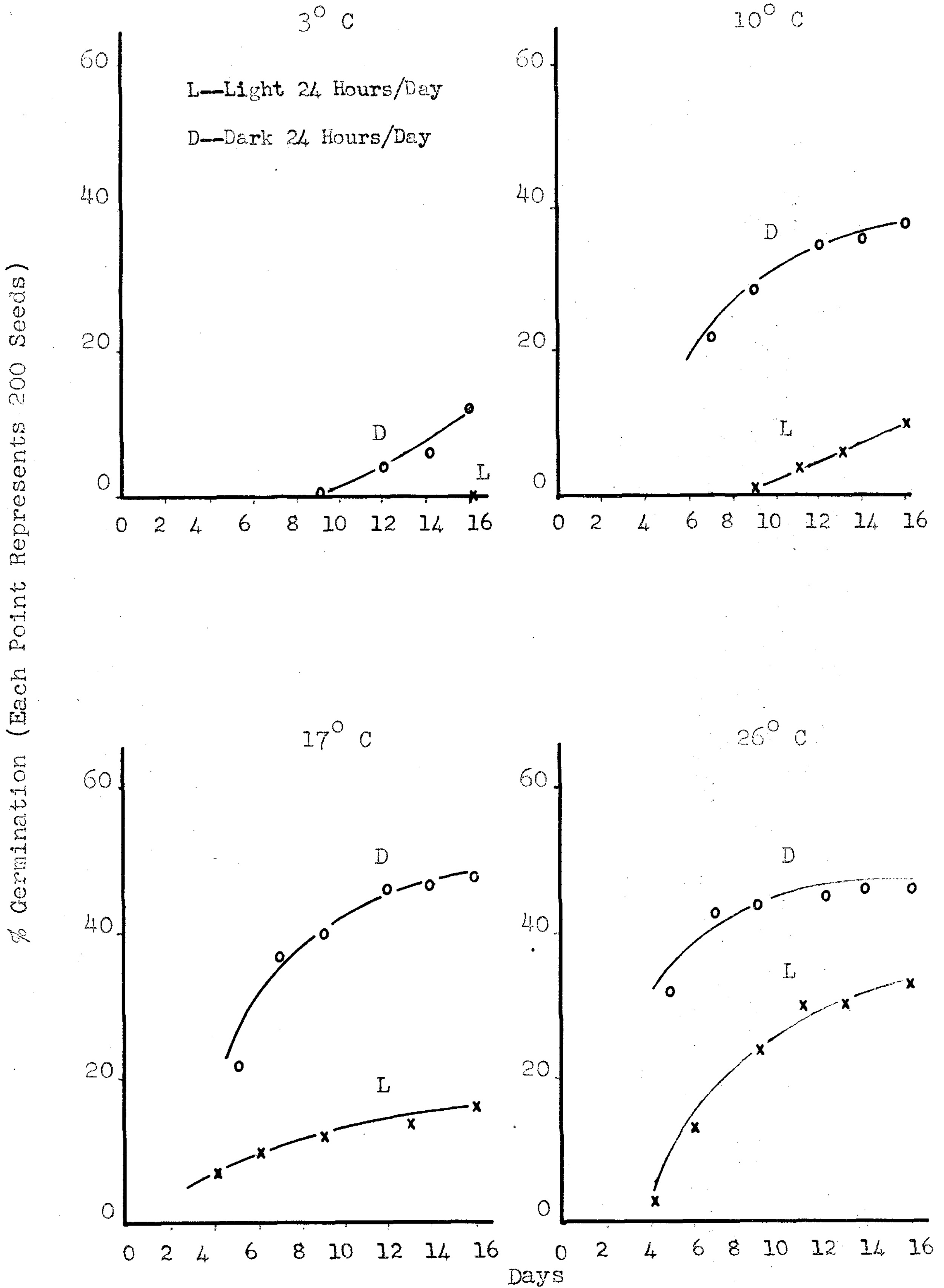
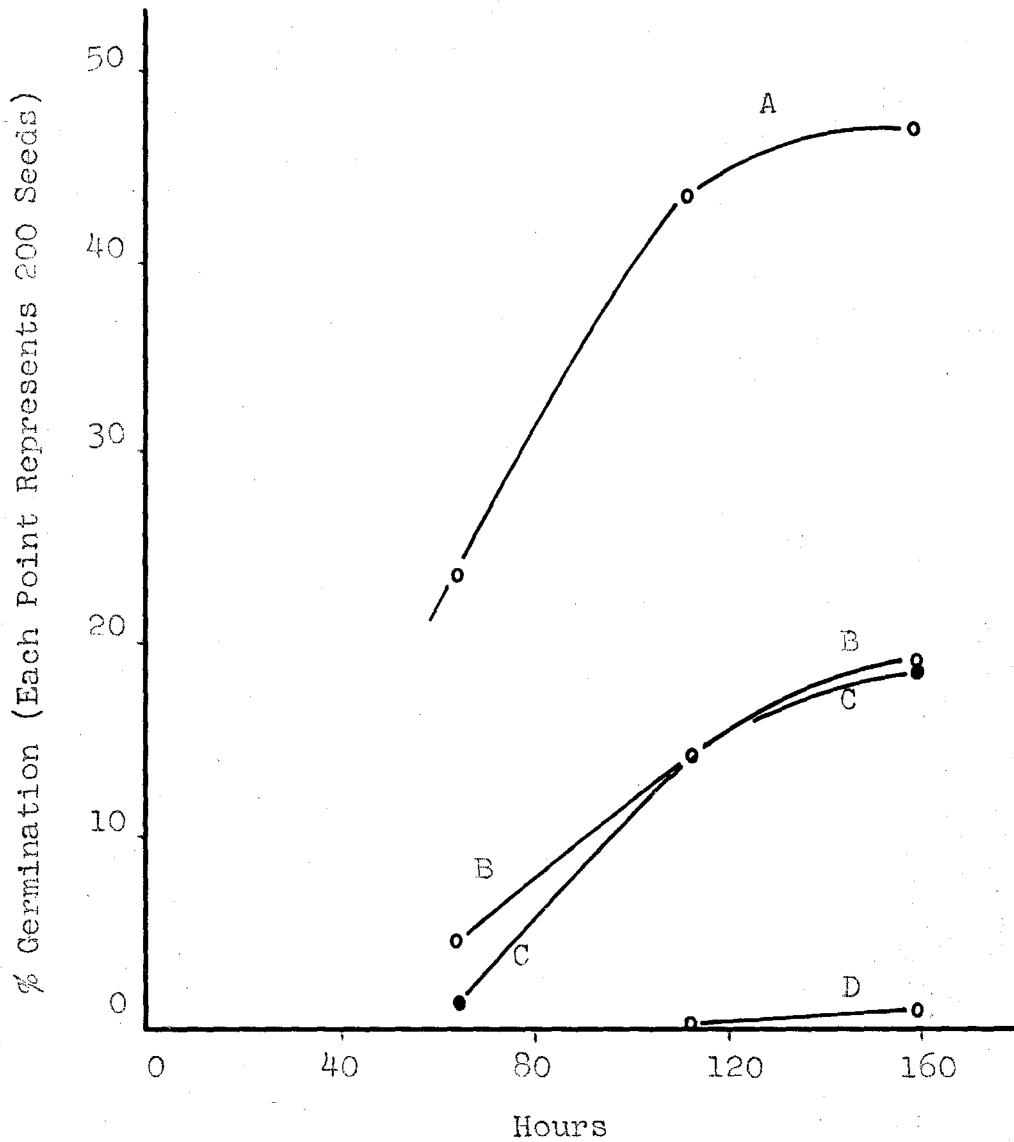


Figure I. Effect of Temperature on Germination.



A Kept in darkness

B 16 hours, 50 F-C, 8 hours darkness

C 16 hours, 300 F-C, 8 hours darkness

D 16 hours, 800 F-C, 8 hours darkness

Figure II. Effect of Light Intensity on Germination.

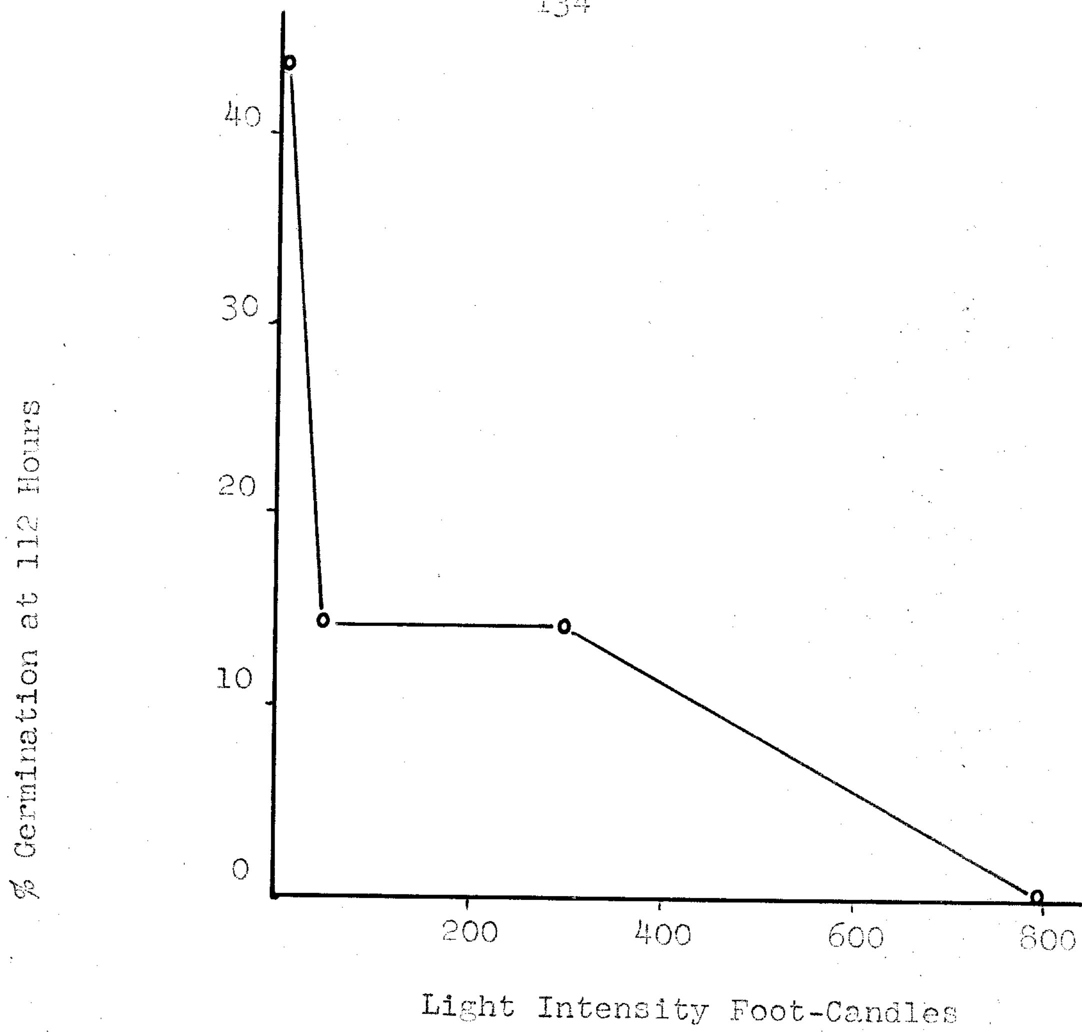
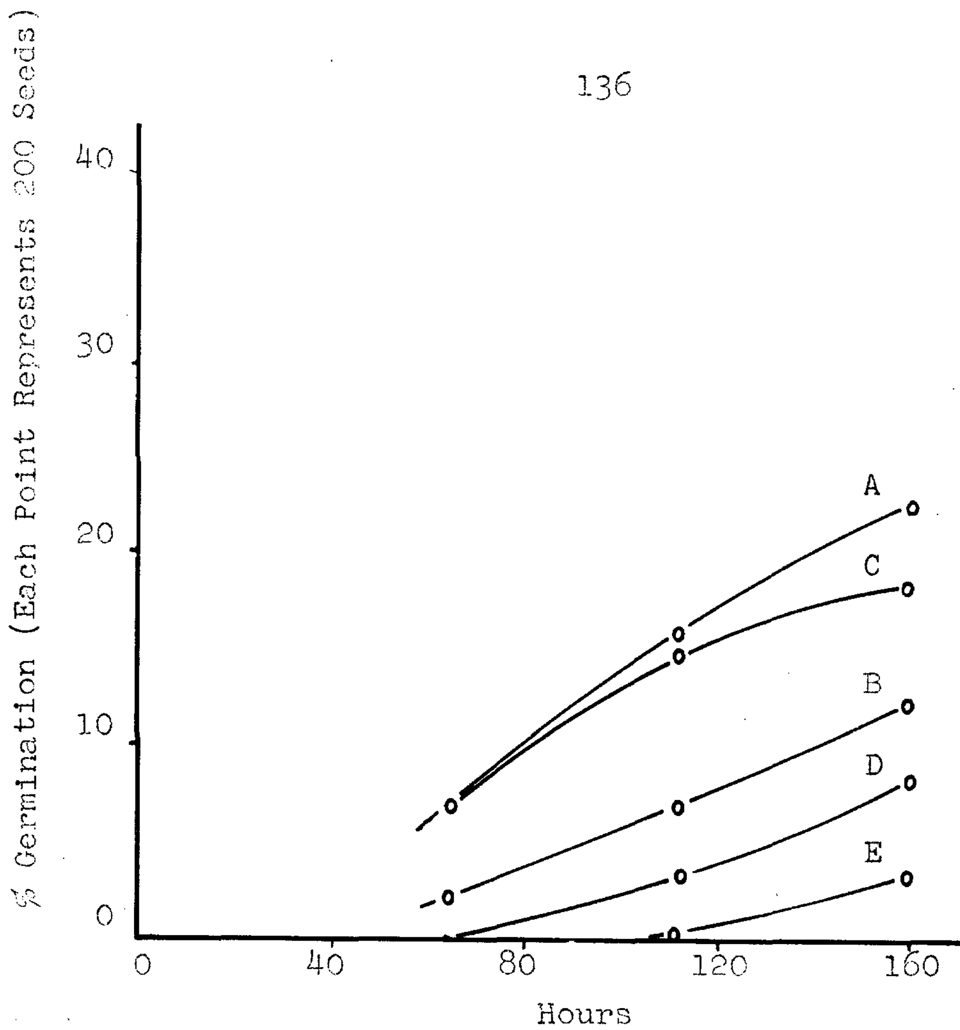


Figure III. Data taken from Figure II, 112 Hours, and Plotted Against Light Intensity.

The effect of light at different times during the first 24 hours of germination is shown in Figure IV. The most plausible explanation for this pattern of light effects is that during the entry of water into the seeds the sensitivity is low, as denoted by curve C. Once the seeds are well soaked even a short period of light is inhibitory, as indicated by curve B. This explanation is strengthened by the results shown in curves D and E. Mes also found that exposure to light during the first 16 hours of soaking produces maximum inhibition (6).

It is known from the work by Mes that exposure of seeds, under germinable conditions, to light for any length of time effectively decreases future germination upon subsequent transfer of the seeds to darkness. This is amply borne out by the data of Figure V. If no inhibition of future germination had occurred, then it would be expected that the germination of set B at 96 hours should have equaled that of set A at 72 hours, and obviously such is not the case. This phenomenon has been reported by several workers, as listed by Crocker (1). It has also been found to occur with seeds which need light for germination. After some time in a dark germinator these seeds will not germinate in light. This has been traced in lettuce seed (which requires light for germination) to competition between red light (6600 Å) and darkness or far-red light (7300 Å) for some photochemical reaction (3,4,5,7). The red portion of the spectrum was found



A 24 hours darkness

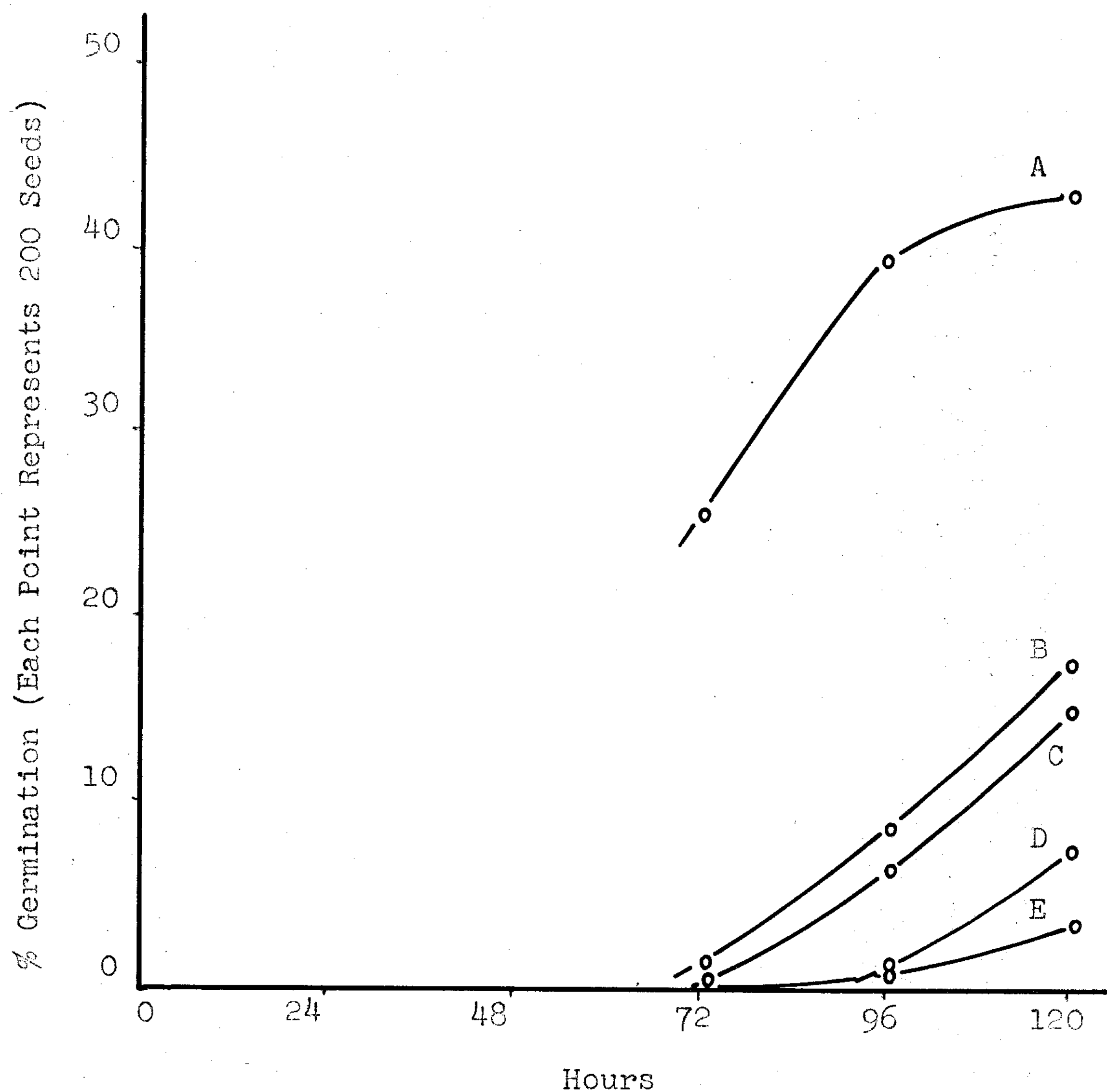
B 12 hours darkness, 4 hours light, 8 hours darkness

C 4 hours light, 20 hours darkness

D 8 hours light, 16 hours darkness

E 16 hours light, 8 hours darkness

Figure IV. Effect of Light During First 24 Hours (Kept in Darkness after First 24 Hours).



A Kept in darkness

B In first 24 hours received 16 hours light, after which kept in darkness

C In first 48 hours received 32 hours light, after which kept in darkness

D In first 72 hours received 48 hours light, after which kept in darkness

E 16 hours of light per 24 hours for entire period

Figure V. Stability of Light Effect.

to enhance and darkness to decrease the germination of lettuce seed. Application of a short period of far-red light was shown to act as a more successful inhibitor than darkness. The effects were demonstrated to be completely reversible--if a red light flash was followed by a flash of far-red shortly afterward, then germination was decreased. If the flash of far-red light was followed by a flash of red light, then germination was enhanced.

Mes was able to demonstrate that light of wavelengths of 6500 Å and longer are mainly responsible for the inhibitory effect of light on poppy seed germination. It is conceivable that the effect of light quality on poppy seed may be just the reverse of that for lettuce. Mes also found that poppy seed placed on activated charcoal would germinate in the light. This was not confirmed by the present work. A difference in the conditions employed may be involved.

Removal of the seed coats enhances germination, as seen in Table I. The way in which the seed coat affects the light response is difficult to imagine. One might postulate a light-mediated reaction which occurs in, or on, the seed coat. A similar occurrence for lettuce seed has been reported by Evenari and Neumann (7). When lettuce seed is de-coated, germination in darkness is equal to that of intact seeds in light and far-red light has no effect on the germination of coat-less seeds. Mes also found increased germination of poppy seed upon seed coat removal in both light and in dark-

Table I

Germination of Seeds With Seed Coat Removed
(24 C)

	Light 800 foot-candles 16 hours per day	Dark 24 hours per day
Total Seeds Prepared	22	28
Number of Seeds Germinated in Six Days	7	15
Percent Germination	32	54
Percent Germination of Seeds with Intact Coats	1	47

ness. Further work by Mes demonstrated that the germination of intact poppy seeds is increased under increased oxygen tension. If seeds placed in the dark were held in a nitrogen atmosphere for the first 16 hours, germination was inhibited for some time after a return to normal conditions. The evidence certainly suggests an intimate connection of the seed coat with light and with the oxygen supply.

A complex inhibitory system could be readily imagined. One would incorporate into this system a seed coat resistant to the passage of gases, an inhibitor formed by a thermolabile mechanism upon imbibition of water or a thermolabile inhibitor formed during seed maturation, and a light-mediated inhibition which may or may not be a part of the thermolabile inhibitory system. Such a system is for the most part speculative--the purpose of this speculation is to point out the need for more pertinent data.

Summary

1. It has been shown that inhibition of the germination of seed of *Eschscholtzia californica* by light decreases with an increase in temperature.
2. The inhibitory effect may be brought about by relatively low light intensities.
3. The increase in sensitivity of the seed to inhibition during the first 24 hours is apparently correlated with the time needed for water absorption by the seed.
4. The effect of light is fairly stable in that exposure to light in the first 24 hours affects future dark germination.
5. The seed coat is in some manner intimately involved in the phenomenon of light sensitivity.

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