STUDIES ON AMINO ACID ACTIVATION AND PROTEIN SYNTHESIS

Thesis by

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ABSTRACT

This thesis concerns the overall subject of protein synthesis in plants. Several different experimental approaches to the problem are described.

The initial studies concern the incorporation of amino acids into tissue sections and homogenates. Tissue sections show a constant rate of incorporation of labeled amino acids into protein for periods of time up to two hours. The rate of incorporation then decreases with time. Conversely, tissue homogenates show ever increasing rates of incorporation of labeled amino acids into protein. This incorporation is indicative of incorporation of labeled amino acids into bacteria within the homogenate rather than incorporation into plant protein. Sterile homogenate preparations do not show the kinetics of incorporation shown by other homogenate preparations. The incorporation of labeled amino acids into acid insoluble material obtained from sterile tissue homogenates is a function of the washing procedure used in the assay.

Amino acid activation has been studied by a hydroxylamine trapping reaction and by pyrophosphate exchange. Plant tissues contain amino acid activating enzymes which may be detected by either method of assay. The pyrophosphate exchange assay is however more sensitive. The hydroxamate assay suffers from some added complications common to plant systems.

The specific nature of amino acid activation in plant preparations has been studied with enzymes isolated from spinach leaves. The activation reaction requires ATP and amino acids, the products being pyrophosphate and probably a mixed anhydride between the 5' phosphate of AMP and the carboxyl group of the amino acid. The enzymes occur in all plant tissues tested and appear to be a part of the soluble fraction of cells. Purification procedures have been developed which make it possible to remove the free amino acids from the preparations. The purified preparation is capable of activating all of the naturally occurring (in protein) amino acids except serine. The possible activation of serine is not excluded. Preliminary evidence is presented which indicates that each amino acid is activated by its own specific amino acid activating enzyme.

The possibility of linking the process of amino acid activation to protein synthesis is considered. Crude particulate containing preparations of spinach exhibit an ATP and RNA dependent incorporation of leucine. Attempts to obtain an amino acid dependent exchange of AMP into ATP with spinach preparations were unsuccessful. Even so it has been possible to demonstrate an ATP dependent RNAase sensitive incorporation of labeled leucine into acid and alcohol washed material.

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I. INTRODUCTION

The synthesis of protein by living things is a complex multi-step process which is as yet not fully understood. The complexity of the process can be sensed by consideration of some of the fundamental principles involved in the synthesis of proteins. First, from a thermodynamic viewpoint, the synthesis of protein from free amino acids themselves is unlikely. Secondly, there are thousands of different kinds of proteins. These differences between proteins appear to involve differences in sequence of the constituent amino acids. Therefore, there is some organizer which directs the sequence of amino acids and which determines the ultimate protein formed. Thirdly, proteins appear to exist in nature in distinct structural forms. The formation of the specific structural forms must be complex as can be seen by the number of possibilities available in a large polyfunctional polymer such as protein. Fourth, the very complexity of the spatial arrangements of cells complicates matters further. The correct structural forms of protein must be located in the correct location within a cell in order to be functional. This may require that particular kinds of protein be made at particular and different sites. All of these complications make the full understanding of the field of protein synthesis a complex and as yet an unattained goal. We do however know some facts which are basic and fundamental to the process of protein synthesis. These are summarized in the following discussion.

A. Thermodynamic Considerations in Peptide Bond Formation

It is known that the formation of peptide bonds by mass action from free amino acids at the generally assumed physiological concentrations and pH is an endergonic process. The reaction of D,L-alanine with glycine to form D,L-alanylglycine illustrates this point. The ΔF of formation of the peptide at 37.5°C is 4130 calories/mole (1). This results in only 8×10^{-4}% synthesis of the peptide, starting with .01 M amino acids. It is unlikely that such an endergonic reaction is involved in the synthesis of proteins. It is more likely that some additional factor is added to increase the probability of peptide or polypeptide synthesis.

There are several ways in which the reactants can be changed to increase synthesis of peptides by mass action. Firstly, increasing the concentration of the reactants favors increased synthesis of the peptide. For example, if one starts with .1 M zwitterionic reactants in the above synthesis of D,L-alanylglycine one obtains 1.6×10^{-2}% synthesis (1). This is in excess of the 8×10^{-4}% synthesis obtained with .01 M reactants. Even so, it is unlikely that this modification alone is of any great importance for peptide synthesis in the cell since the extent of the syntheses obtained are still low and the concentrations necessary to obtain this small increase in synthesis are above normal physiological concentrations.

Secondly, removal of the other ionic groups from the vicinity of the ionic groups involved in the synthesis of

the peptide bond increases the extent of synthesis of the peptide. This may be achieved by blocking the terminal groups of the different amino acids with amine or carboxyl blocking agents or by displacement of the charges not involved in bond formation by increasing the size of the reactants to peptides or polypeptides. In this case, the resulting ΔF of formation favors synthesis to a slightly greater extent than is the case for the formation of the same bond between the same two amino acids as zwitter ions. The overall reaction, however, still favors hydrolysis.

Thirdly, if the peptide formed can be removed from the system in some manner, the synthesis of the peptide will be expected to proceed due to the mass action principle. Use is made of this phenomena in the well known plastein formation (2). Here a concentrated solution of large peptides is incubated with a catalyst, a proteolytic enzyme. The proteolytic enzyme would be expected to complete the hydrolysis of large peptides, but during the hydrolysis transfer reactions occur. These are in part aided by the great separation between the terminal charges of large peptides and the resulting lower ΔF of formation of new peptide bonds. The new and often larger high molecular weight peptides or cyclic peptides formed are often insoluble and therefore remove themselves from the system. This phenomenon is unique to large peptides in high concentration. Plastein formation for example requires peptides larger than tripeptides (3).

Therefore, the phenomenon has no physiological role in the conversion of free amino acids to protein.

Use has been made of these three principles, high concentration of reactants, charge separation, and product removal in attempts to implicate proteases in protein synthesis (4,5,6). In general the few reactions measured which do favor synthesis of peptides over hydrolysis involve unnatural substrates. The insolubility of the unnatural product peptides aids the synthesis observed. The final equilibrium of proteolytic transfer reactions involving natural substrates and peptide bonds or abnormal peptides such as glutamyl peptides (7) is in general towards hydrolysis of the peptides to free amino acids.

Fourthly, the reactants can be altered to favor peptide synthesis. It is possible to couple peptide synthesis with an energy yielding reaction such as hydrolysis of a high energy bond. In these cases the energy of the high energy bond is consumed in driving the reaction towards synthesis of the peptide bond. This has been found to be the case in the synthesis of many simple peptides and amides such as hippuric acid (8), glutathione in animals (9), yeast (10), and higher plants (11,12), glutamine (13,14,15), asparagine (16), and pantothenate (17).

The mechanism by which the energy of hydrolysis of the phosphate anhydrides (ATP) is coupled to peptide synthesis in these reactions is different. Nevertheless, they all

make use of the energy of hydrolysis of the phosphate anhydrides to enhance the synthesis of the peptide bond. This type of coupling of an energy yielding reaction to peptide bond synthesis also appears to be involved in the case of protein synthesis.

B. Energy Considerations in Protein Synthesis

Both in vivo and in vitro experiments indicate that ATP is necessary for the incorporation of amino acids into proteins. For example, treatments known to inhibit ATP synthesis inhibit the conversion of amino acids into proteins. Dinitrophenol inhibits protein synthesis in excised silk glands of the silkworm (Bombyx mori) (18) and amino acid incorporation in rat liver slices (19) or homogenates (20), rabbit reticulocytes (21), and Ehrlich Ascites cells of mice (22). The rate of amino acid incorporation in the dinitrophenol uncoupled system then follows the rate of ATP synthesis by glycolysis (22). Azide inhibits amino acid incorporation in rat liver homogenates (23), and rabbit reticulocytes (21) and inhibits anaerobic adaptive enzyme formation in yeast by blocking ATP synthesis in the glycolytic pathway (24). Cyanide inhibits amino acid incorporation by rat liver homogenates (23). Anaerobiosis inhibits amino acid incorporation in rat liver homogenates (23) and rabbit reticulocytes (21). The removal of ATP by extraction from a thymus nuclei fraction lowers the extent of amino acid incorporation into the protein of this

system (25). The addition of ATP or of an ultimate ATP source enhances amino acid incorporation in many tissues. For example, addition of ATP and magnesium ions increases the incorporation of amino acids into a crude rat liver homogenate (26). An ATP generating system in the form of added mitochondria or glycolytic intermediates is necessary for amino acid incorporation in several systems (20, 27, 28, 29). Krebs cycle intermediates enhance incorporation of amino acids in thymus nuclei (30) and the synthesis of protein in excised silk glands (18). The role of ATP as the energy source of protein peptide bond formation is further indicated in chloroplast suspensions which show a marked enhancement of amino acid incorporation in the presence of light (31). Photosynthetic phosphorylation in chloroplasts requires only water and light to produce ATP from ADP and Pi.

It therefore appears that the energy released on hydrolysis of the high energy phosphates in ATP is coupled to the endergonic synthesis of peptide bonds in proteins. This makes the synthesis of peptide bonds in protein analogous in principle, to the observed synthesis of many peptides.

C. Role of Nucleic Acids in Protein Synthesis

Another and equally important problem in the synthesis of proteins is the problem of protein specificity. There are many different proteins. These differences in proteins

appear to arise from differences in size and amino acid sequence. Because of the uniformity of specific proteins within species it is necessary to envision some controlling mechanism which determines the sequence of the amino acid and the eventual size of individual proteins. This organizer has often been called a template for want of a better name.

Substantial evidence has been accumulated which implicates nucleic acids in protein synthesis. The implication of ribonucleic acid (RNA) is fairly straightforward, but the evidence implicating desoxyribonucleic acid (DNA) is not as clear cut. The evidence concerning nucleic acids falls into three catagories. First the removal of nucleic acids by use of specific hydrolytic enzymes or other methods is known to have marked effects upon the ability of the preparation to incorporate amino acids and to form adaptive enzymes. For example treatment with RNAase inhibits amino acid incorporation into protein in lysed cells of Micrococcus lysodeikticus (32,33), sonicated Staphylococcus aureus preparations (34), onion root tips (35), and rat liver particulate preparations (27,36). RNAase treatment inhibits adaptive enzyme formation in at least one case of bacterial transformation (37). Salt extraction of sonicated S. aureus preparations, which removes nucleic acids, reduces the ability of these preparations both to incorporate amino acids (38) and to produce adaptive enzymes (39). The role of specific removal of DNA is not as clear cut, for varied

results have been obtained. For example, DNAase treatment of lysed Micrococcus lysodeikticus cells enhances amino acid incorporation (32) while DNAase treatment inhibits amino acid incorporation in calf thymus nuclei preparations (30). Inhibition of DNA synthesis in bacteria with mustard gas does not inhibit the ability of these cells to produce adaptive enzymes (40). Care must be used in interpretation of the results of DNAase treatment for they may reflect indirect effects due to changes in sub-cellular organization rather than direct effects upon protein synthesis. The inhibition of amino acid incorporation in calf thymus nuclei by DNAase is due to interruption of the energy source for peptide bond formation rather than interference with a template role (41).

A second way in which nucleic acids are implicated in protein synthesis is the direct requirement for nucleic acids or nucleic acid components by tissues deficient in these forms in order to incorporate amino acids or for adaptive enzyme formation. The addition of either RNA or DNA or pyrimidines and purines to the salt extracted preparations of S. aureus mentioned above restores the ability of the preparation to adapt and incorporate amino acids (38,39). Bacterial mutants which require pyrimidines or purines for growth also need these compounds for enzyme induction (40). In more refined systems, the evidence indicates that RNA is more closely implicated in protein synthesis than is DNA.

Tissues which lack DNA still actively incorporate amino acids into protein. For example, enucleated cells incorporate amino acids for long periods of time after removal of the nucleus (42). Mammalian reticulocytes, which contain no detectable DNA, readily incorporate amino acids into protein (21).

The third way that nucleic acids are implicated with protein synthesis is obtained from in vivo and in vitro observations of incorporation into particulate fractions. Fractions rich in RNA become labeled first upon the addition of labeled amino acids to the system (20,27,43,44). RNA is therefore apparently directly involved in protein synthesis over short periods of time.

The nature of the role of nucleic acids as the organizer or template is not clear. The above evidence indicates that RNA plays a more direct role in the process of protein synthesis than does DNA. The exact nature of the role of nucleic acid must await further investigation.

D. A Proposed Mechanism for Protein Synthesis

The state of our knowledge at the outset of the research presented in this thesis has been outlined above. Since then great strides have been made towards formulation of a hypothesis that utilizes all of the above principles. In this mechanism it is assumed that the energy needed for synthesis of the peptide bonds of proteins is donated to free

amino acids by ATP by the process of amino acid activation. The activated amino acid is then assumed to conserve this energy through a series of acyl transfer reactions involving template materials. The activation energy is finally consumed in the synthesis of the peptide bonds of protein. The proposed mechanism includes the following steps.

1. Amino Acid Activation

The initial step in the synthesis of proteins is assumed to be an activation of the carboxyl group of the free amino acid similar to the mechanism of acetate activation first observed by Berg (45). The reaction results in the formation of an enzyme-AMP-amino acid complex in which the 5' phosphate of AMP is linked to the carboxyl of the amino acid as a mixed anhydride (46). Equation 1 illustrates the proposed mechanism.

$$\text{Enz} + \text{ATP} + \text{AA} \rightleftharpoons \text{Enz-AMP-AA} + \text{PP} \qquad (1)$$

Similar reaction mechanisms have been found for the activation of fatty acids (47), sulfate (48,49), and pantoate (50). After the initial observation by Hoagland in rat liver (51), of such a mechanism involving amino acids, similar systems specific for amino acids have been obtained from 15 different microorganisms (52), beef pancreas (53), hog pancreas (54), guinea pig liver (55) and yeast (56). The enzymes as isolated are specific to the L isomers of the amino acids. Purification studies have been undertaken in a few cases (53,

56, 57), resulting in the isolation of enzymes largely specific to the activation of one amino acid. To this date no tissue has been reported that has activating ability towards all the amino acids naturally occurring in proteins. The presence of amino acid activating enzymes is required for amino acid incorporation in both crude (27,29), and purified (28,55,58) systems.

2. Transfer of the Activated Amino Acid

The activated amino acid at the state of an acyl 5' adenylate is now capable of non endergonic transfers to many possible acceptors (59). If these are acyl transfers, the activation energy initially donated by the hydrolysis of ATP will be conserved. The transferred amino acid will still be capable of forming a peptide bond at any transfer level. The initial acceptor of the activated amino acid may be some form of soluble RNA. This has been determined by the finding of a non particulate RNAase sensitive acceptor of the activated amino acid. This can be measured by an amino acid dependent exchange of AMP into ATP (60) or amino acid incorporation into acid insoluble material (58). In one case, a soluble, extracted and purified RNA has been added back to a purified amino acid activating enzyme (55). This RNA is active in accepting a labeled amino acid.

Support of the hypothesis that the soluble RNA plays a role in protein synthesis is found in the work of Hoagland et al. (58). These workers are able to obtain from

rat liver a preparation that contains a labeled amino acid bonded to a soluble RNA. This preparation can transfer the labeled amino acid to a microsomal preparation in the presence of guanosine triphosphate (GTP). Therefore, it is possible to carry out the stepwise, enzymatic transfer of a free amino acid into what is assumed to be microsomal protein. These steps may be summarized as follows:

Activation:

$$\text{Enz} + \text{ATP} + \text{AA} \rightleftharpoons \text{Enz-AMP-AA} + \text{PP}$$

Transfer to soluble RNA (S-RNA)

$$\text{Enz-AMP-AA} + \text{S-RNA} \rightleftharpoons \text{S-RNA-AA} + \text{Enz} + \text{AMP}$$

Transfer to particulate material

$$\text{S-RNA-AA} + \text{Particle} \overset{\text{GTP}}{\rightleftharpoons} \text{Particle-AA} + \text{S-RNA}$$

The exact nature of the last reaction and any possible further reactions must await further study.

This thesis is concerned primarily with the first or activation step in the above scheme. This thesis will also shed further light upon the plausibility of the above sequence of reactions.

II. MATERIALS AND METHODS

A. Abbreviations and Chemicals

In this thesis reference is made to many materials in the form of abbreviations. The abbreviations commonly used and their meanings are listed below:

tris-HCl	Tris-(hydroxymethyl)-aminomethane titrated with HCl
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
DNA	Desoxyribonucleic acid
GTP	Guanosine triphosphate
UTP	Uridine triphosphate
CTP	Cytidine triphosphate
XTP	Nucleoside triphosphate
UDPG	Uridine diphosphoglucose
AA	Amino acid
AANHOH	Amino acid acyl hydroxamate
TCA	Trichloroacetic acid
Pi	Ortho phosphate
PP	Pyrophosphate
Enz	Enzyme
RNA	Ribonucleic acid
RNAase	Hydrolytic enzyme specific to RNA
DNAase	Hydrolytic enzyme specific to DNA
S-RNA	Soluble RNA
X	Unknown acceptor of transferred material
pH 5_I	Enzyme precipitated at pH 5.0-5.2 once and resuspended at 7.5
pH 5_{II}	Enzyme precipitated at pH 5.0-5.2 twice and resuspended at 7.5
PGA	3-Phosphoglyceric acid

The other abbreviations used are the internationally used chemical symbols.

The source of most of the chemicals used is listed with the individual experiments. The amino acids used in the various assays were either from Nutritional Biochemical

Corp. Cleveland, Ohio, or from the California Foundation for Biochemical Research, 3408 Fowler Street, Los Angeles 63, California. All other chemicals used were reagent grade materials.

B. Source of Materials

All of the plant tissues used in these experiments were either grown at the California Institute of Technology or purchased in the local markets. The preparation of the various plant materials for assay is as follows.

1. Intact Tissue Sections - Bean Hypocotyls

Kentucky Wonder Beans (Phaseolus vulgaris) were dusted with "Semesan" seed disinfectant and then grown in vermiculite at 25°C in the dark for five days. The etiolated hypocotyls were collected and the cotyledons removed. The hypocotyls were then either washed with the commercial detergent "Dreft" (.1%) for two minutes and rinsed with water or washed directly. Half inch sections were cut from the washed hypocotyls and assayed as in Figure 3.

2. Tissue Homogenates

a. Soluble Enzymes from Tobacco. Young leaves and stems of tobacco plants (Nicotiana tabacum) grown in the Earhart Laboratory were ground in a mortar at 2°C (1 gram of material/.5 ml of .4 M sucrose, .03 M potassium phosphate at pH 7.5). The resulting homogenate was filtered through

cheesecloth and then centrifuged at 105,000 g in a Spinco Model L ultracentrifuge (Spinco Div., Beckman Instrument Co., Belmont, Calif.) for 90 minutes in the cold. The supernatant obtained was used in the experiments involving soluble enzymes from tobacco.

b. Spinach. All spinach homogenate preparations were prepared from bunch spinach obtained from Jurgensen's market in Pasadena, California. The spinach was washed to remove adhering dirt. The washed spinach was then ground in an Omni Mixer (Ivan Sorvall Inc., serial number 228) at 2°C. One hundred grams of spinach were ground in 100 ml of .01 M or .001 M tris-HCl (as designated in the experiment) at pH 7.5* for 1 minute. The resulting homogenate was filtered through cheesecloth until more than 100 ml of solution were obtained. The pH was then raised to pH 6.8-7.2 with 1 M KOH in the cold. Small aliquots were removed for pH determination at 25°C and then discarded.

One hundred ml of this solution was then ground with yet another 100 grams of spinach for 1 minute and treated as the previous batch until 100 ml of solution at pH 6.8-7.2 were obtained. One hundred ml of this solution were then further concentrated by grinding with a third 100 gram batch of spinach and treating as before. The final concen-

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*The pH of tris-HCl buffer varies with temperature so all values reported in this paper shall refer to the pH of tris-HCl at 25°C.

trated solution was centrifuged to obtain fractions as follows:

1. Cellular debris and chloroplasts were removed by 5,000 g for 10 minutes.
2. Broken chloroplasts and mitochondria removed by 42,000 g for 15 minutes.
3. Microsomes were sedimented between 42,000 and 105,000 g for 60 min.
4. Soluble enzymes included enzymes not sedimentable at 105,000 g.

The pH of the soluble enzymes was then adjusted to the desired pH with 1.0 N HCl or 1.0 N KOH.

c. Pea Roots Grown in Vermiculite. Peas were washed in .1% detergent before and after imbibition of water and then planted in moist vermiculite. After 96 hours at 25°C in the dark, the peas and accompanying roots were removed and washed with water. The 500 g-40,000 g fraction was prepared from the excised roots as described by Webster and Johnson (61).

d. Sterile Pea Roots. One hundred grams of dry peas (Pisum sativum, var. Alaska) were stirred in 200 ml of a solution of commercial detergent "Tide" (.1%), for 15 seconds and then washed 4 times with distilled water. All further operations were carried out under aseptic conditions in a tissue culture transfer room. The rinsed peas were stirred with 200 ml of 70% ethanol for 5 seconds. The

alcohol was decanted completely by holding a petri dish over the lip of the inverted beaker containing the peas. The peas were then immersed in 1% NaOCl solution for ten minutes. The hypochlorite solution was then decanted completely. The treated seeds were next poured into a sterile 11-1/2" by 7" by 2" enamel tray with a layer of cheesecloth upon the bottom. Three hundred ml of sterile distilled water were then added. The trays were covered with a glass plate and stored at 25°C for 5 days in the dark. At the end of this time the solution around the germinated pea seeds was completely bacteria free as determined by appropriate plating.

The roots were removed (using sterilized gloves) and washed with distilled water. They were next ground briefly in a Waring Blendor at 2°C in .05 M potassium phosphate, .3 M sucrose, .005 M $MgCl_2$ at pH 7.5 (1 gram of roots/1 ml of solution). The slurry was filtered through cheesecloth and spun at 1,000 g for 10 minutes. The 1000 g supernatant was spun at 50,000 g for 30 minutes. The resulting precipitate was resuspended in .05 M potassium phosphate, .3 M sucrose at pH 7.5 (1/10 of original volume). .5 ml aliquots of this solution were assayed as in Figure 5.

e. Pea Epicotyl. Washed pea epicotyls were obtained as described above. All further operations prior to assay were carried out in the range 0-5°C. The epicotyls were ground in a mortar in .4 M sucrose (.5 ml of solution/gram of epicotyls). The pulpy material was removed by filtration

through cheesecloth. The following fractions were obtained by sequential centrifugation in the cold.

Nuclear fraction, 15 minutes at 5,000 g

Mitochondrial fraction, 12 minutes at 42,000 g

Microsomal fraction, 90 minutes at 105,000 g

Supernatant fraction, material not sedimented at 105,000 g

The sedimented fractions were resuspended in .1 M tris-HCl, .3 M sucrose at pH 7.5 as follows:

Nuclear fraction resuspended in 1/10 of original homogenate volume

Mitochondrial fraction resuspended in 1/20 of original homogenate volume

Microsomal fraction resuspended in 1/20 of original homogenate volume

.5 ml samples of these solutions were then assayed in the pyrophosphate exchange assay as described in Table 4.

3. Acetone Powder Preparation

In the preparation of acetone powders of plant tissues, large volumes of cold (-25°C) acetone per amount of plant tissue were always used (e.g. 10 ml acetone/gram tissue). Acetone powders of 8 different tissues were prepared. The preparation of an acetone powder preparation of spinach is described below as an example. Other tissues were treated in approximately the same manner.

a. Spinach. Fresh spinach purchased in the local markets was used for the preparation of acetone powder. In general spinach sold in dampened bunches appeared fresher and yielded enzyme preparations of slightly higher activity than preparations obtained from cellophane packaged spinach such as is sold in large commercial operations.

Two hundred and fifty grams of washed spinach were ground in a Waring Blendor in 600 ml of cold (-25°C) acetone for one minute. The resultant slurry was vacuum filtered on a Büchner funnel. The residue was washed with 50 ml of cold acetone and then removed from the Whatman #1 filter with a spatula. The crude powder was ground in 500 ml of cold acetone in the Waring Blendor for one minute and filtered and washed as above. This refined powder was removed from the filter paper and stored in 300 ml of cold acetone until another batch of refined powder could be prepared in a like manner. The two 300 ml slurries were then combined and ground for 1 minute in the Waring Blendor. After filtration and washing of the powder as above, the powder was ground for a final minute in 400 ml of a 3:1 mixture of cold (-25°C) diethyl ether : acetone. The powder obtained by vacuum filtration of the above material was dried in a vacuum desiccator for 15 minutes and then screened through cheese-cloth at room temperature to assure complete drying. The powder was stored at -25°C.

b. Avena Coleoptile. The top 1.5 inches of coleoptiles and enclosed primary leaves of oats seedlings (variety Siegeshafer) grown in the manner of McRae and Bonner (62) were used for the acetone powder preparations.

c. Corn Seedlings. The tops of young 4 inch high corn seedlings grown by Dr. E. G. Anderson were excised and used for the preparation of acetone powder.

d. Pea Roots. Sterile pea roots grown as described earlier were used for the preparation of acetone powder.

e. Pea Tops. The excised tops of flowering pea plants grown in Earhart Laboratory were used for this acetone powder preparation.

f. Tobacco Leaves. The young leaves from 18-24 inch high tobacco plants grown in Earhart Laboratory were used for these acetone powder preparations.

g. Asparagus. The tips of asparagus shoots obtained in local markets were used for the preparation of asparagus acetone powder preparations.

h. Winter Rye. The leaves of adult winter rye plants grown in the Earhart Laboratory were removed with scissors and cut into small pieces. This preparation was converted to an acetone powder.

4. Extraction of Acetone Powders

Enzyme solutions were prepared from the acetone powders by essentially similar procedures. A typical extraction of enzymes from a powder is described. All extractions were carried out in this manner unless otherwise stated.

A weighed amount of powder was removed from storage at -25°C and mixed quickly with the desired buffer (10 ml of buffer/1 gram of powder) in a vessel cooled in an ice bucket. The slurry was allowed to sit from 5 to 15 minutes in the cold before centrifugation in a refrigerated Spinco Model L Ultracentrifuge. Centrifugation involved 5 minutes at 3,000 g to partially sediment the slurry (solution not decanted) followed by 25 minutes at 40,000 g to compact the undissolved material. The supernatant was decanted and clarified by centrifugation in a clinical centrifuge and then used for assay. The amount of solution returned varied with the hydroscopic nature of the powder used, but in general 7 ml of supernatant solution were obtained from 10 ml of powder slurry. The pH of the supernatant was then adjusted to the desired value with HCl or KOH. Unless otherwise indicated the enzyme was titrated to pH 7.5 with dilute KOH.

5. Concentration of the Enzymes with $(NH_4)_2SO_4$

Concentration of the enzymes from spinach acetone powder extracts was achieved by the addition of 100% saturated (at 2°C) $(NH_4)_2SO_4$ solution titrated to pH 7.5 with NH_4OH) until the desired concentration of salt was achieved. The

precipitated solution was allowed to sit for 30 minutes at 2°C before the precipitate was removed by centrifugation at 8,000 g for 10 minutes in a Servall Superspeed Angle Centrifuge, type SS-1 (Ivan Sorvall, Inc. P.O. Box 230, Pearl Street, Norwalk, Conn.). The supernatant solution was decanted and the precipitate was redissolved in the desired volume of the buffer specified in the experiment.

6. Charcoal Treatment of Enzymes

It was necessary to treat certain of the enzyme preparations with acid washed charcoal to remove various harmful components from the enzyme solutions. A concentrated slurry of Norite A charcoal treated with .1 N HCl and subsequently washed by three vacuum filtrations followed by 15 decantations of added wash water was used in the enzyme treatments. The final pH of the charcoal brei was 5.0. In general 4-12 mg of charcoal were added in the cold per ml of enzyme solution. The charcoal was removed by high speed centrifugation in a refrigerated Spinco Model L Ultracentrifuge. Certain enzyme solutions required a second treatment with charcoal to remove all of the harmful compounds.

C. Assay Procedures

1. Hydroxamate Assay

The experiments involving hydroxamate formation were all assayed by the method of Schweet (63). The enzyme preparations used in the experiments reported here were pre-

pared from homogenates of pea epicotyls or plant acetone powder as described above. The final concentrations of reactants used in the total three milliliter incubation mixtures were 30 μ moles of $MgCl_2$, 3,150 μ moles of $NH_2OH \cdot KCl$, 10 μ moles of tris-HCl (excluding that added with the enzyme) and where indicated 30 μ moles of ATP-K salt, and 5 μ moles of each of 15 L amino acids (alanine, glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, lysine monohydrochloride, arginine monohydrochloride, phenylalanine, tryptophan, tyrosine and histidine). The pH of the reaction mixture was pH 7.0-7.3. These concentrations were obtained by adding 1.4 ml of enzyme in .1 M tris-HCl at pH 7.2 to .7 ml of 4.5 M $NH_2OH \cdot KCl$ at pH 7.0-7.3, .1 ml of .3 M $MgCl_2$ in .1 M tris-HCl at pH 7.2, and where indicated, .3 ml of .1 M ATP-K salt, and .5 ml of a .01 M mixture of the 15 L amino acids. When ATP or the amino acid mixture was omitted from the incubation mixture, the missing solution was replaced by an equal volume of water.

The above mixture was incubated for 2 or 3 hours in a Dubnoff Metabolic Shaker (American Instrument Company, Silver Spring, Maryland) at 37°C. The reaction was stopped by the addition of 1.4 ml of 100% trichloroacetic acid at pH .9 and cooled in an ice bath. After a minute of standing in the cold, .6 ml of 2.0 M $FeCl_3$ was added and the resulting colored solutions were filtered through Whatman number 1 filter paper. After 15 minutes the optical density at 520 mμ was determined by use of a Beckman Model DU Spectrophoto-

meter (Beckman Instruments Company, South Pasadena, California). The optical densities observed in the total 5 ml were converted to μ moles of amino acid hydroxamate by use of Schweet's value: 1 μ mole of amino acid hydroxamate per ml yields an optical density of .77 at 520 mμ.

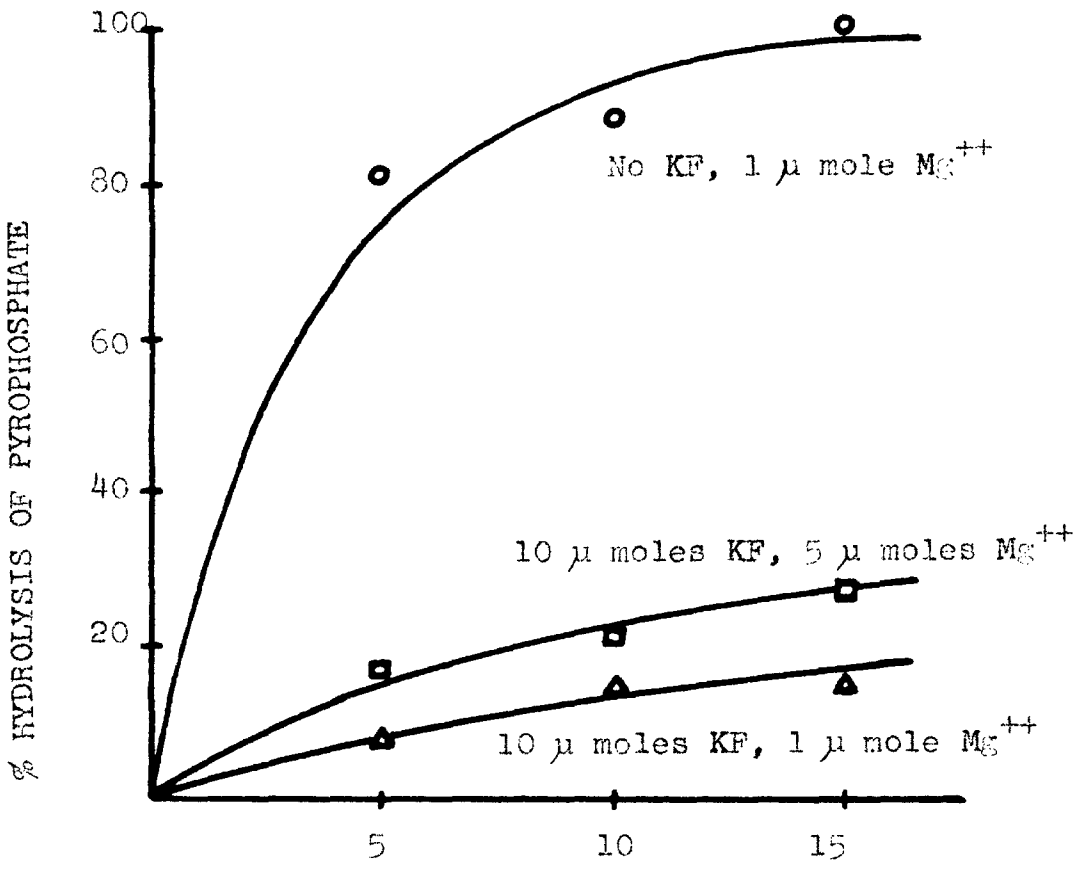
2. Pyrophosphate Exchange Studies

a. Pyrophosphatase Inhibition. In order to carry out an exchange reaction involving pyrophosphate, it is necessary to preserve the integrity of the pyrophosphate during the incubation period. In general, plant systems are endowed with active pyrophosphatases. Therefore, it was necessary to run all of the pyrophosphate exchange studies in the presence of a high concentration of fluoride ion in order to inhibit pyrophosphatase activity. Figure 1 illustrates the inhibition of the pyrophosphatase of a spinach acetone powder extract by fluoride ion.

As can be seen, the presence of fluoride ion exerts a marked effect upon the rate of pyrophosphate cleavage. Accordingly, all pyrophosphate exchange assays were run in the presence of 10 μ moles of potassium fluoride.

b. Synthesis of Labeled Pyrophosphate. P^{32} labeled pyrophosphate was synthesized by pyrolysis of P^{32} ortho phosphate obtained from Oak Ridge. From .2 to 2 millicuries of P^{32} pyrophosphate were pyrolyzed during a pyrophosphate preparation. The P^{32} phosphoric acid and accompany-

Figure 1. Inhibition of Pyrophosphatase Activity of Spinach Acetone Powder Extract by Fluoride Ion



The enzyme was prepared by 10:1 extraction of spinach acetone powder with .1 M tris-HCl at pH 7.5. The final pH of the enzyme was 7.2. .5 ml of the enzyme was added to .5 ml of solution containing 50 μ moles of tris-HCl pH 7.5, 2 μ moles of each of 15 L amino acids, 5 μ moles of potassium pyrophosphate at pH 7.5 and as indicated, 10 μ moles of KF and either 1 or 5 μ moles of $MgCl_2$. Incubation was at 37°C. The reaction was stopped by the addition of .5 ml of warm 95% ethanol. The precipitated protein was removed by centrifugation. The supernatant was assayed for ortho phosphate and pyrophosphate by the method of Flynn, Jones and Lipmann (64). The points indicated represent single determinations calculated from both pyrophosphate present and ortho phosphate released. The experiment is repeatable with qualitatively similar results.

ing hydrochloric acid were neutralized with KOH. Two hundred to 300 μ moles of K_2HPO_4 were then added to the labeled material to bring the pH to that of a solution of K_2HPO_4. The solution was then dried by gentle warming in the vessel in which pyrolysis was to take place. Pyrolysis was carried out in either a Wood's metal bath at 340°C for from 6 to 10 hours, or in a platinum crucible heated to red heat for 4-5 minutes in a flame. Both methods yielded from 95 to 100% conversion of ortho phosphate to pyrophosphate as measured by the method of Flynn, Jones, and Lipmann (64). The resulting $K_4P_2O_7$ was titrated to pH 7.5 with HCl and mixed with sufficient carrier potassium pyrophosphate at pH 7.5 to make a .05 M solution. This solution was further diluted with .05 M carrier potassium pyrophosphate at pH 7.5 to obtain from 50,000 to 200,000 counts/min/5 μ moles of PP^{32}. Five μ moles of this material was incubated with the 1 ml reaction mixture.

c. Pyrophosphate Exchange Procedure. All of the pyrophosphate exchange reactions were conducted in 1 ml mixtures in 12 ml clinical centrifuge tubes. The reaction mixtures all contained .5 ml of enzyme prepared as indicated and .5 ml of reaction substrates etc. The incubation mixtures contained, in addition to the enzymes, 5 μ moles of $MgCl_2$, 50 μ moles of tris-HCl at the desired pH, 10 μ moles of KF, 5 μ moles of potassium P^{32} pyrophosphate at the desired pH and other substrates as indicated in each experiment.

The potassium salt of ATP (Pabst Laboratories, Milwaukee, Wisconsin) was used unless specified otherwise. In general each incubation mixture contained 10 μ moles of ATP titrated to the appropriate pH. These concentrations were obtained by adding the following amounts of solutions (pH determined by pH desired in the experiment)

.1 ml of .1 M ATP-K salt at pH 6.5-9.5

.1 ml of .1 M KF

.1 ml of .05 M potassium P^{32} pyrophosphate ph 6.5-9.5 and where indicated

.1 ml of either a .02 M solution of each of 15 L amino acids (the same ones as in the hydroxamate assay) or a .02 M solution of the various separate amino acids.

When further ingredients were desired the ATP and KF were combined into one solution. One-tenth of a milliliter of .1 M KF, .1 M ATP-K salt, pH 6.5-9.5, were then added allowing further additions not to exceed the .5 ml of final substrates.

Incubations were always carried out in a Dubnoff Metabolic Shaker at 37°C. The reactions were stopped by the addition in the cold (0°C) of 1 ml of 12% TCA. One ml of 6% TCA was then added to the precipitated protein to allow for full dispersion of the coagulated protein. The steps in the procedure from this point forward follow the method of Crane and Lipmann (65) with several modifications. The denatured protein was centrifuged down in a clinical centrifuge (International Clinical Centrifuge, International Equipment Co., Boston, Mass.). The supernatant was decanted

onto 300 mg of charcoal (Norite A) in a 12 ml centrifuge tube. The precipitated protein was washed once with 1 ml of water, and the wash was added to the charcoal slurry. The charcoal solution was stirred with a stirring rod to assure thorough mixing. The rod was then removed with the addition of a few drops of 95% ethanol to break the surface tension of the slurry and allow full wetting of the charcoal scum on the surface. The solution was centrifuged and the supernatant discarded. The sedimented charcoal was then washed by resuspension and centrifugation with 4 consecutive 2 ml washes of water. Each time the precipitate was mixed with a stirring rod. The rod was removed with the aid of a few drops of alcohol.

The final washed precipitate was resuspended in 1 or 2 ml of 1.0 M HCl. The two terminal phosphates of the ATP bound to the charcoal were then hydrolyzed by heating the acidified charcoal solution at 100°C for 10 minutes. The hydrolyzed charcoal solution was cooled by the addition of 2 ml of water and stirred. The stirring rod was removed with the addition of a few drops of ethanol and the charcoal sedimented by centrifugation. The supernatant which now contains the two terminal phosphates hydrolyzed from the ATP was decanted into a calibrated centrifuge tube. The hydrolyzed charcoal was washed once with 4 ml of water as before and the combined wash and hydrolysis supernatant was raised to 10 ml final volume. One-half of a milliliter of each solution was plated out on 3/4 inch diameter copper planchet and counted on a

micro mil thin window gas flow counter (Nuclear Instrument and Chemical Corp., Chicago, Ill.).

Inorganic phosphate determinations were made on samples of the hydrolyzed phosphates from the ATP using the method of Allen (66) or Flynn, Jones & Lipmann (64) as indicated in each experiment.

d. Calculation of the Extent of Exchange of PP and Pi into ATP. The counting of a given volume (.5 ml) of the eluate from the hydrolyzed charcoal bound ATP yields a value for the number of counts contained in any given volume. The phosphorus determination further allows one to calculate the amount of phosphorus in any given sample. Therefore, it is possible to compare these two values for a volume of sample and calculate the specific activity of the hydrolyzed phosphorus (counts/min/μ moles Pi). This value is only of qualitative significance. It cannot be used for comparison of experiments with different specific activities of the initial substrates. Neither does the specific activity yield quantitative information about the extent of exchange of pyrophosphate or of ortho phosphate into ATP. It is necessary to calculate the actual extent of exchange catalyzed by the enzyme in order to obtain quantitative information about the sample assayed. This is achieved by first calculating the apparent exchange of pyrophosphate or ortho phosphate into ATP followed by conversion of this value to the actual exchange.

The apparent exchange or percent exchange is calculated in the following manner. The value obtained for counts/min/ μ mole Pi is first multiplied by 20 to obtain the counts/ min/10 μ moles ATP. This is possible because the acid hydrolysis only releases the two terminal phosphates of ATP and, in theory, these are the only two phosphates which contain P^{32} phosphorus. It is now necessary to compare this extent of exchange with that for complete equilibration or complete exchange of pyrophosphate or ortho phosphate into ATP.

In most of the pyrophosphate exchange experiments reported in this paper the initial concentrations of ATP and of PP^{32} are 10 μ moles/ml and 5 μ moles/ml respectively. Therefore, at complete equilibration of the free labeled pyrophosphate with the pyrophosphate of ATP, one would expect two-thirds of the labeled pyrophosphate to reside within the ATP. In terms of counts:

$$\text{counts/min in ATP at 100\% exchange} = \text{2/3 X counts/min initially in pyrophosphate}$$

Therefore, the percent of exchange in any given sample is:

$$\frac{\text{counts/min/10 } \mu \text{ moles ATP}}{\text{2/3 X counts/min in initial PP}} \text{ X 100 = \% exchange}$$

In Table 7 only 5 μ moles of nucleotide triphosphate is used per ml. In this case the argument above indicates that:

$$\frac{\text{counts/min/5 } \mu \text{ moles XTP}}{1/2 \text{ X counts/min in initial PP}} \text{ X } 100 = \% \text{ exchange}$$

In Table 5 the exchange of ortho phosphate into ATP is measured. Ten μ moles of ATP/ml and 5 μ moles of Pi^{32}/ml are used. It is assumed that exchange of Pi^{32} only occurs into the terminal phosphate of ATP. The possible transfer of the terminal phosphate of ATP into the intermediate phosphate of ATP by the action of adenylic kinase is ignored. Since only the terminal phosphates of the 10 μ moles of ATP are involved in exchange with the 5 μ moles of Pi^{32}, complete equilibration of the counts originally in the Pi^{32} would result in 2/3 of the labeled Pi^{32} residing in the ATP. Therefore:

$$\text{2/3 X counts/min initially in Pi} = \text{counts/min in ATP at 100\% exchange}$$

The specific activity (counts/min/μ moles Pi) of the hydrolyzed phosphates of ATP is made up of two kinds of phosphate. One half of the released phosphate has been exchanging with the free Pi^{32}. The other half is assumed not to be involved in exchange with free Pi^{32}. The terminal phosphates of ATP involved in the exchange have been diluted by an equal amount of inert phosphorus. Therefore, the specific activity of the 10 μ moles of terminal phosphate of ATP is as follows:

$$\text{counts/min/}\mu\text{ moles Pi} \times \frac{}{2 \text{ X } 10} = \text{counts/min/10 } \mu \text{ moles of terminal phosphorus}$$

The calculated percentage of exchange is then:

$$\frac{\text{counts/min/10 } \mu \text{ moles of term. Pi in 10 } \mu\text{moles ATP}}{2/3 \text{ X counts/min initially in Pi}} \text{ X } 100 =$$

% exchange

The values obtained in the form of percent exchange are useful in a qualitative sense in the comparison of experiments in which different initial specific activities of substrates are used. They are not however quantitatively impeccable. As the apparent exchange of labeled substrate into ATP increases, the chance that some of the labeled substrate in the ATP will be exchanged back out of the ATP into the free substrate pool also increases. It is necessary to correct for this coincidence. The coincidence correction utilized in these calculations takes the form of (see appendix):

$$\mu \text{ moles exchanged} = -2.303 \frac{(A)(B)}{(A)+(B)} \log_{10} (1 - \text{fraction exchange})$$

where

(A) = number of μ moles of labeled substrate

(B) = number of μ moles substrate exchangeable in nucleotide triphosphate

fraction exchanged = percent exchanged/100

In most of the experiments reported, 5 μ moles of PP^{32} and 10 μ moles of ATP are used. In this case the above equation assumes the following form:

$$\mu \text{ moles PP exchanged} = -2.303 \frac{5 \times 10}{5 + 10} \log_{10} (1 - \text{fraction exchanged})$$

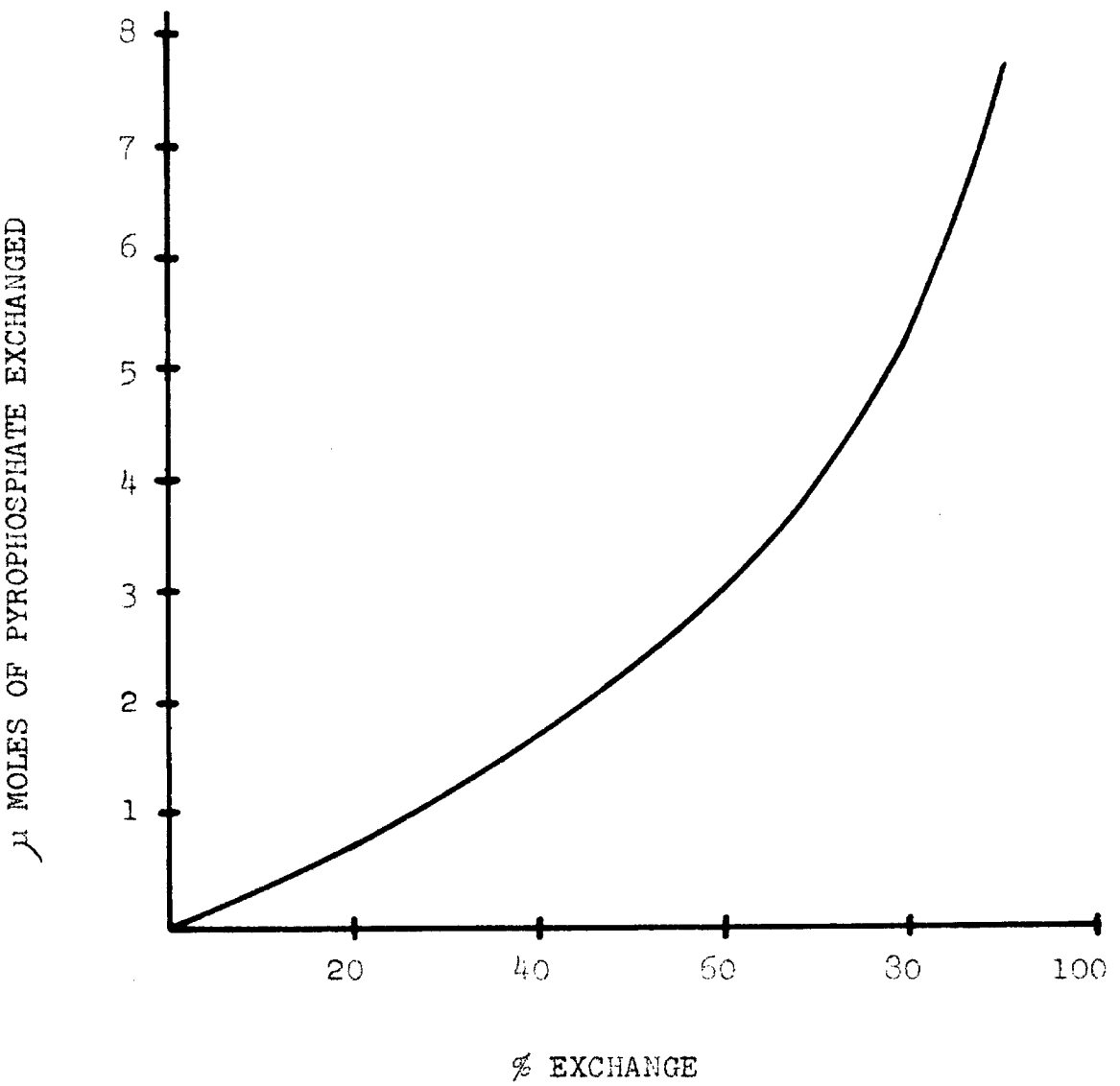
which generates the relationship between the actual number of μ moles of pyrophosphate exchanged and percent or apparent exchange shown in Figure 2.

e. Proof that Hydrolyzed Counts Originate from ATP. In the procedure outlined above, the activity of the ATP is determined by acid hydrolysis of the washed charcoal and subsequent analysis of the hydrolysate. It is necessary to demonstrate that all of the counts released by hydrolysis originate from ATP and not from some other form of phosphate. This can be approached in three ways.

Firstly, no counts are released by hydrolysis of the washed charcoal from preparations incubated in the presence of 5 μ moles of P^{32} pyrophosphate and in the absence of nucleotide triphosphate. Therefore, the incorporation of pyrophosphate is nucleotide triphosphate dependent. Pyrophosphate, by itself, is not incorporated into any hydrolyzable component of the enzyme solution alone.

Secondly, elution of count containing washed charcoal with 50% aqueous pyridine or 1 M NH_4OH, 47.5% ethanol removes most of the counts from the charcoal. When the eluate is

Figure 2. Relationship of % Exchange and Actual Exchange



chromotogramed by the method of Cohn and Carter (67) in isoamyl alcohol, 5% Na_2HPO_4, all of the counts as determined by radio autography are in chromatographically pure ATP. No other radioactive materials are found.

Thirdly, it is possible to count the ATP obtained by charcoal elution and subsequent chromatography. Since the extinction coefficient of ATP is known, it is possible to obtain the concentration of the chromatographically pure eluted ATP. The specific activity (counts/min/μ mole of ATP) of the eluted ATP is therefore known. The specific activity obtained in this way is 92% of the specific activity of ATP as calculated from acid hydrolysis. This is within the limits of experimental error considering all of the steps involved in this assay.

Consideration of the three lines of evidence discussed above leaves little doubt that the counts released from washed charcoal by hydrolysis originate from ATP.

3. Adenosine 5' Monophosphate Exchange

All of the exchange reactions involving exchange of C^{14}-AMP into ATP were performed in the manner of Holley (60). One half milliliter of enzyme solution prepared as indicated in each experiment was incubated with .5 ml containing 5 μ moles of $MgCl_2$, 50 μ moles of tris-HCl, 10 μ moles of ATP-K salt, 5 μ moles of potassium pyrophosphate, 10 μ moles of KF, .5 μ moles of AMP-8-C^{14} (Schwartz Laboratories Inc., 230 Washington St., Mount Vernon, N.Y. - .7 μ curies/mg

diluted with cold AMP (Pabst) until it contained approximately 9,000 counts) and where indicated, 2 μ moles of each of 15 amino acids (same as those used in the hydroxamate assay). The substrates were all at pH 7.5. These concentrations were obtained by combining .1 ml of .05 M $MgCl_2$, .5 M tris-HCl at pH 7.5 with .1 ml of .1 M ATP-K salt pH 7.5, .1 ml of .1 M KF, .05 M potassium pyrophosphate at pH 7.5, .05 ml of .01 M AMP-8-C^{14} at approximately pH 7.5, and where indicated .1 ml of a .02 M solution of each of 15 amino acids. The final volume was made up to .5 ml before the addition of the enzyme solution. When enzymes were treated with RNAase, 1 ml of enzyme was mixed with .05 ml of a solution of RNAase (Armour Laboratories, 200 gamma/ml) and preincubated at room temperature for 5 minutes before incubation. Incubation of the enzyme was at 37°C. The reaction was stopped by the addition of 1 ml of 12% TCA in the cold. The reactions were then treated as described by Holley (60). The resulting slurry of the barium salt of ATP in water was plated out on tared copper planchets containing a disc of lens tissue cemented to the planchets with tygon. The results are expressed in terms of counts/min/mg Ba salt of ATP.

4. Incorporation Studies Involving C^{14} Amino Acids

The various methods of incorporation are too varied to discuss as a group and are therefore discussed in connection with each experiment. The sources of the labeled amino

acids are listed in the experiments. When dilutions in the specific activity of the amino acids were performed, L isomers of commercially obtainable amino acids were used.

5. Protein Determinations

Protein determinations were performed in one of two ways. Where indicated, the biuret method of Cornell et al. (68) was used. In the others, protein concentration was determined by TCA precipitation, digestion in H_2SO_4, and use of the colorometric Nessler assay. The protein was precipitated by the addition of two volumes of 12% TCA. After storage at 2°C for 24 hours the precipitate was sedimented in a clinical centrifuge and washed twice with 2 ml washes of 12% TCA. The precipitate was then treated with 2 ml of 12% TCA at 90°C for 10 minutes. The precipitate which remained after cooling and addition of 2 additional ml of 12% TCA was washed once more with 2 ml of 12% TCA. The precipitated material was digested in .5 ml of concentrated sulphuric acid at 230°C for at least six hours. The dark brown solution was clarified by the addition of a drop or two of 30% H_2O_2 and an additional hour of heating. The digested material was then diluted out to 10 ml. Three-tenths to 1 ml of this solution was combined with an equal volume of 2 N NaOH (added just before Nesslers reagent), .1 ml of 2% gum ghatti and made up to 10 ml. Five ml of Nesslers reagent prepared by the method of Bock and Benedict (69) were then added and the

optical density of the sample was determined. Comparison with known $(NH_4)_2SO_4$ solutions yielded the concentration of nitrogen in digested sample. Protein was determined by assuming that proteins contain 16% nitrogen.

III. RESULTS

A. Studies on the Incorporation of Amino Acid into Protein

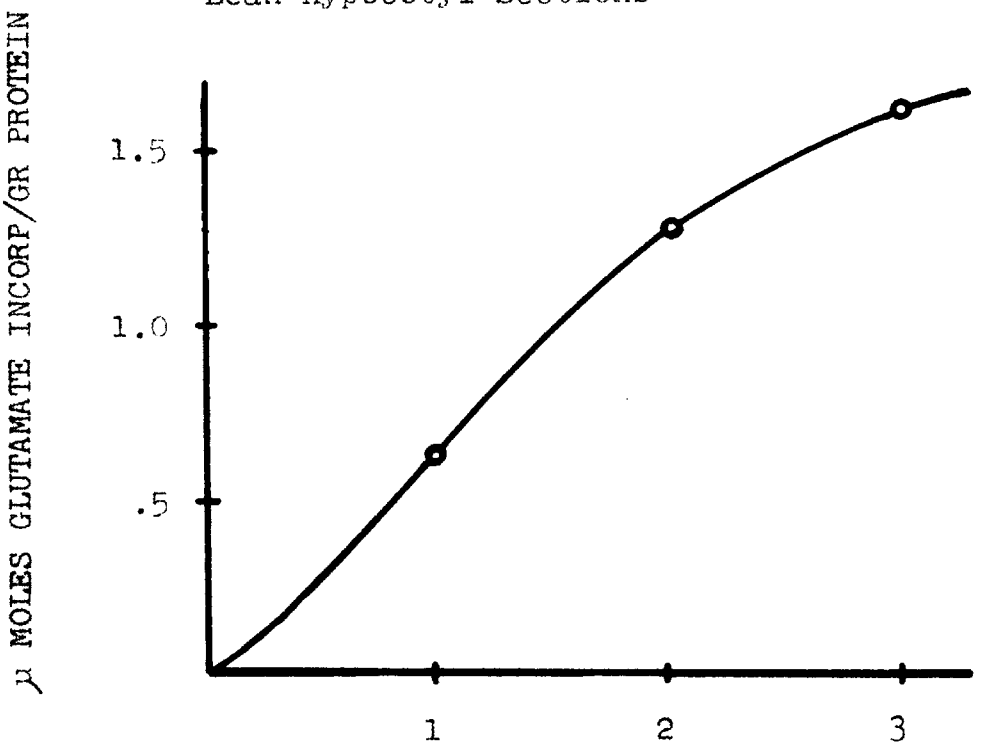
1. Tissue Section Studies

It is possible to demonstrate an incorporation of D,L-glutamate-2-C^{14} into TCA precipitable material obtained from plant tissue sections. Detergent washed 1/2 inch sections of 5 day old etiolated red kidney bean hypocotyls show this phenomenon when incubated with .009 M C^{14}-labeled glutamate, .045 M potassium phosphate at pH 6.5. Figure 3 shows such an incorporation in bean hypocotyl sections. The results are similar to those reported by Webster (70).

2. Tissue Homogenate Studies

The result of the experiment of Figure 3 indicates that it should be possible to show an incorporation of a radioactive amino acid into TCA precipitable material in tissue homogenate preparations. One might expect two major difficulties with this approach. First, the disruption of the natural organization of the tissue would be expected to result in a drop in the ability of the tissue to incorporate amino acids. Secondly, there is danger of bacterial contamination in homogenates. Homogenates, by their nature, no longer possess the resistance to infection common to the internal tissues of most plants. Instead, all of the forms of contamination which are present on the outside of intact tissue, are free to multiply in the tissue homogenate and

Figure 3. Incorporation of Glutamate into Protein by Bean Hypocotyl Sections



5 day old etiolated bean hypocotyl (cotyledons removed) were washed in .1% detergent ("Dreft") for 2 minutes, and then rinsed three times in distilled water. The hypocotyls were cut into 1/2 inch sections. 15 of these sections were incubated with 8 ml of .009 M D, L-glutamate-2-C^{14} (Tracerlab Inc., Waltham, Mass., 2370 counts/min/μ mole), .045 M potassium phosphate at a final pH of 6.5. After incubation at 25°C the sections were removed and washed three times with water. The sections were then pulverized with 1.5 ml of water in a glass homogenizer and the insoluble material removed by centrifugation at 500 g for one minute. The insoluble material was washed once with .5 ml of water. The combined washings and supernatant from the 500 g centrifugation was precipitated by the addition of 4 ml of 12% TCA. The resulting precipitate was washed with 2 ml of 12% TCA three times. The precipitates were then plated out and counted. Protein determination was by the biuret method of Gornell et al. (68) on the solution prior to addition of TCA.

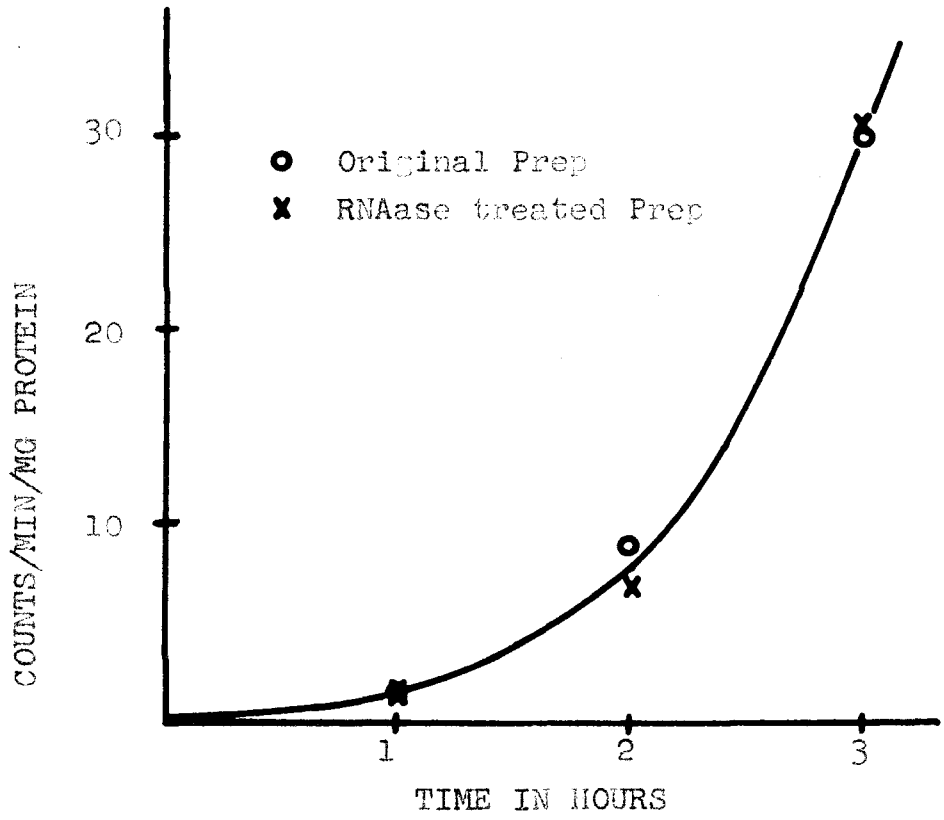
to use not only the contents of the cells but the added substrates as well. With these considerations in mind, one can however effectively study the incorporation of labeled amino acids into plant tissue homogenates.

It is possible to obtain from detergent washed and vermiculite germinated pea roots, a preparation of the 500 g-40,000 g fraction in .45 M sucrose .05 M potassium phosphate at pH 7.5 prepared in the manner of Webster & Johnson (61). When .5 ml of this preparation was incubated in the manner of Webster and Johnson (61) with .5 ml of substrates containing 10 μ moles of D,L-glutamate-2-C^{14} (23,648 counts/min), 10 μ moles of potassium phosphate at pH 7.5, and .1 mg of each of 17 amino acids, the incorporation of the glutamate into the TCA precipitable material follows the time course shown in Figure 4.

As can be seen, the incorporation follows a lag period. RNAase does not appreciably affect the incorporation. These observations tend to indicate incorporation of the labeled amino acid into bacteria rather than into the pea preparation.

In contrast, it is possible to grow pea roots under absolutely sterile conditions. When such roots are homogenized in .05 M potassium phosphate, .3 M sucrose, .005 M $MgCl_2$ in the cold, and the 1,000 g-50,000 g fraction is precipitated by centrifugation and resuspended in .05 M potassium phosphate, .3 M sucrose at pH 7.5 (one tenth the

Figure 4. Incorporation of D,L-Glutamate-2-C^{14} into a Pea Root Homogenate Preparation

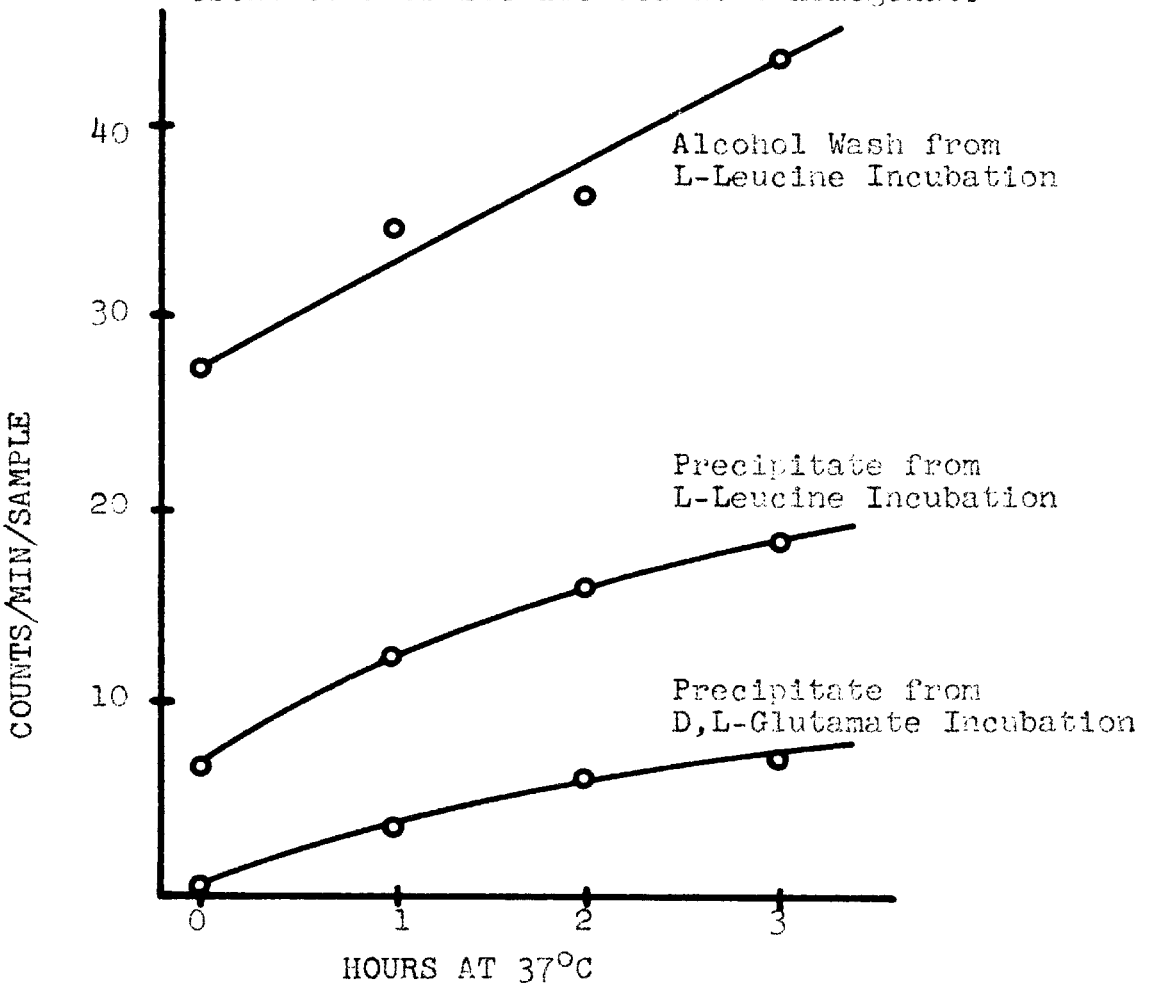


Peas were washed in .1% detergent before and after imbibition of water and then planted in moist vermiculite. After 96 hours at 25°C in the dark the peas and accompanying roots were removed and washed with water. The 500 - 40,000 g fraction was prepared from the roots and assayed as described by Webster and Johnson (61) using 10 μ moles of D,L-glutamate containing 23,700 counts/min (Tracerlab Inc., Waltham, Mass.). The RNAase treated sample was preincubated with 100 gamma of RNAase (Armour Labs, Chicago) for 30 minutes at 37°C before assay. The precipitate obtained upon addition of 2 ml of 12% TCA was washed with 2 ml of 12% TCA three times. The final precipitate was resuspended in water, plated out, and counted. Results are expressed in terms of counts/min/mg protein as determined by the biuret method (68).

original volume) a preparation is obtained which upon incubation with labeled amino acids, does not show the kinetics of the above homogenate incorporation. On the other hand, it shows what appears to be two types of incorporation. In this experiment the precipitate obtained upon addition of 2 ml of 12% TCA to the 1 ml reaction mix was washed in a more complete manner. After 3 washes with 2 ml volumes of 12% TCA, the precipitates were washed once with 12% TCA at 90°C for 5 minutes. The precipitate was then washed twice more with 2 ml volumes of 12% TCA and finally washed with 1 ml of 95% ethanol. The precipitate was then suspended in water, plated out, and counted. It was noted that the alcohol wash turned cloudy when 12% TCA was added; therefore, the 1 ml alcohol washes from the leucine incubation were plated out and counted also. Figure 5 shows the results of this experiment.

The results of these two experiments indicate the extensive difficulties encountered in the study of amino acid incorporation in crude homogenates. Once sterile preparations are obtained the exact nature of the incorporation is hard to interpret. A more easily defined approach to the study of protein synthesis is the study of individual steps involved in the mechanism.

Figure 5. Incorporation of Amino Acids into Fractions Obtained from Sterile Pea Root Homogenate



All of the enzyme preparations were carried out under sterile conditions in the cold. The homogenate was homogenized as described above using 1 gm of sterile roots/ml of solution. The resulting slurry was filtered through cheese cloth. After removal of the 1,000 g fraction (10 min at 1,000 g) the 1,000 - 50,000 g fraction was obtained by centrifugation for 30 minutes and resuspension in .5 M potassium phosphate, .3 M sucrose at pH 7.5 (1/10 original volume). .5 ml of the enzyme was incubated at 37°C with .5 ml of solution at pH 7.2 containing .6% yeast extract, 15 μ moles of $MgCl_2$, 1 μ mole of GTP, CTP, & UTP (Pabst Laboratories, Milwaukee, Wis., Na salts), 15 μ moles of ATP-K salt, .1 mg of each of 19 amino acids (L-aspartate, L-asparagine, L-glutamine, L-glutamate, L-alanine, L-proline, glycine, L-tyrosine, L-phenylalanine, L-tryptophan, L-leucine, L-valine, L-methionine, L-cysteine, L-serine, L-threonine, L-arginine·HCl, L-lysine·HCl and L-histidine), and 10 μ moles of either L-leucine-C^{14} (8,950 counts/min/μ mole, Nuclear of Chicago, Chicago, Ill.) or 10 μ moles of D,L-glutamate-2-C^{14} (17,400 counts/min/μ mole, Tracerlab Inc., Waltham, Mass.).

B. Studies on Amino Acid Activation in Plant Tissues

The further work reported in this thesis concerns studies upon enzyme mechanisms which appear to be involved in protein synthesis.

As has already been pointed out, it is necessary to have an energy donating system in the formation of peptide bonds. This energy donation is assumed to involve the formation of a new bond between a previously free amino acid and another compound. This new bond is formed at the expense of some preexisting bond. This process has been termed amino acid activation.

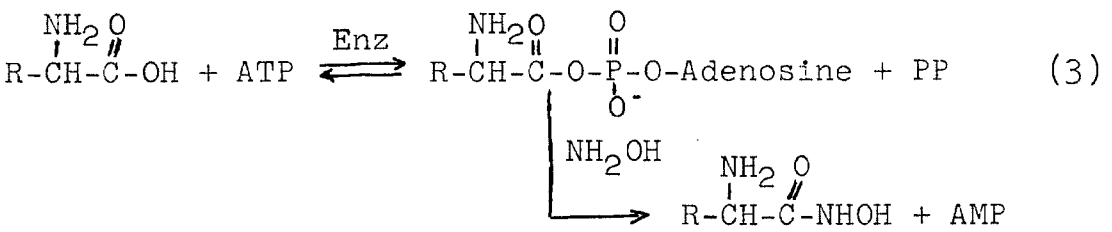
1. Amino Acid Activation as Studied by Hydroxamate Formation

One of the possible ways of detecting the presence of amino acid activating enzymes in plant tissues is the hydroxylamine trapping reaction originally employed on an enzymatic system by Lipmann (71). It is assumed that any carboxyl activated compound, preexisting or enzymatically formed, will react non enzymatically with hydroxylamine to yield the corresponding hydroxamate as shown in equation 2:

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}X + NH_2OH \rightleftharpoons R\text{-}\overset{O}{\overset{\|}{C}}\text{-}NHOH + HX \qquad (2)$$

The hydroxamate present can then be assayed colorimetrically by making use of the fact that these compounds yield intense color in the presence of ferric iron in acid solution.

Enzymatic activation of an amino acid in the presence of hydroxylamine, using ATP as an energy source, should result in the formation of the corresponding amino acid hydroxamate as shown in equation 3:



(3)

An amino acid and ATP dependent hydroxamate formation can therefore be taken to indicate amino acid activation, and Hoagland (51) has used this method of assay. The sensitive and quantitative amino acid hydroxamate assay of Schweet (63) was used in the present experiments.

The use of homogenates of intact plants in the hydroxamate assay is beset with difficulties as is shown by experiments with pea epicotyl homogenates preparations. Firstly, cellular particles are not cleanly precipitated by the trichloroacetic acid used in the assay procedure. Only the soluble supernatant fraction (70,000 g soluble) can therefore be assayed colorimetrically. Secondly, compounds within the juices of plants yield high non enzymatic base colors as shown by the high level of color formed in boiled enzyme preparations. This color is due to non enzymatic cleavage by hydroxylamine of preexisting activated complexes

in the plant juice and to the complexing of ferric iron with the common phenolic compounds of plants. Thirdly, free amino acids present in the intact cells of the plant persist in the homogenates making the detection of any amino acid dependent reactions difficult. Fourthly, there is a visible reduction of the ferric iron added to the system. This makes the assay useless. Accordingly, attempts must be made to remove the endogenous color yielding materials and other harmful chemical compounds from the activating enzymes.

Total precipitation of the enzymes in the soluble supernatant fraction of peas by $(NH_4)_2SO_4$ and resuspension of the precipitate is ineffective in removing substrates for the non enzymatic color formation. As an alternative, the removal of the small molecular weight components from the proteins of plant cells by preparation of acetone powders is an effective way to gain the purification necessary to render the hydroxamate assay useful. Acetone powder preparations of spinach yield a slight indication of an amino acid and ATP dependent hydroxamate formation, but still yield a high endogenous base color. The color forming material can be removed by dialysis of the enzyme, but the dialyzed material is enzymatically inactive. However, further success may be achieved with acetone powder extracts which have been subjected to $(NH_4)_2SO_4$ precipitation. While this degree of purification of the enzymes from spinach still

leaves an appreciable endogenous base color, it is none the less possible to detect an amino acid and ATP dependent hydroxamate formation as shown in Table 1.

The enzymatic activity at this stage is stable to freezing and thawing.

Even with this degree of purification, the large amounts of color obtained in the boiled enzyme preparations render the assay only qualitatively useful. Dialysis of the enzyme in .1 M tris-HCl, pH 7.5, at 2°C for a period of time long enough to lower the endogenous color formation results in loss of enzymatic activity.

As an alternative, the quick removal of the harmful materials from the enzyme by the use of charcoal is more effective. The enzyme, extracted from acetone powder, is treated with sufficient acid washed charcoal to remove the materials which result in color formation in the presence of ferric iron. It is then subjected to an $(NH_4)_2SO_4$ precipitation followed by resuspension. The resulting preparation carries out a clear amino acid and ATP dependent hydroxamate formation. Table 2 shows the results obtained with such a preparation.

In order to ascertain whether the phenomenon of amino acid activation as studied by hydroxamate formation is common to plants, acetone powder preparations of oat coleoptiles, corn seedlings, pea root, pea tops, and tobacco leaves were prepared. These powders were extracted with

Table 1. Hydroxamate Formation in $(NH_4)_2 SO_4$ Precipitated Enzyme from Spinach Acetone Powder

	OD 520	μ moles of hydroxamate/ml	μ moles of hydroxamate/ml minus .735
Boiled enzyme	.555	.735	-
Enzyme alone	.680	.884	.149
Enzyme & ATP	.720	.938	.203
Enzyme & AA	.750	.978	.243
Enzyme & ATP & AA	.840	1.090	.355

Enzyme prepared by 10:1 extraction of spinach acetone powder with .1 M tris-HCl at pH 7.5 followed by precipitation from 80% saturated $(NH_4)_2 SO_4$ and resuspension of the enzyme in 30% of the original volume in .1 M tris-HCl pH 7.5. Assay was that of Schweet (63) using 1.4 ml of enzyme and 10 μ moles of ATP-K salt, 10 μ moles of $MgCl_2$ and 1.67 μ moles of each of 15 amino acids/ml of reaction mix all at pH 7.2. Incubation at 37°C for 2 hours was followed by hydroxamate determination. The above readings are on 5 ml samples after TCA and $FeCl_3$ has been added.

Table 2. Hydroxamate Formation in Charcoal Treated and $(NH_4)_2 SO_4$ Precipitated Spinach Acetone Powder Extracts

	OD 520	μ moles hydroxamate/ml
Enzyme alone	.085	.110
Enzyme & ATP	.151	.196
Enzyme & AA	.128	.166
Enzyme & AA & ATP	.277	.360

Enzyme preparation and assay as in Table 1 except that the enzyme was treated with 8.7 mg of acid washed charcoal/ml of acetone powder extract. The charcoal was removed by centrifugation before $(NH_4)_2 SO_4$ precipitation of the enzyme.

.1 M tris-HCl, pH 7.5. The resulting enzyme solutions were all found to become deeply colored upon the addition of ferric iron, even before the addition of hydroxylamine.

If these varied preparations are treated with sufficient acid washed charcoal to remove the materials responsible for the portion of the base level color due to complexing of ferric iron, enzymes adequately purified for assay may be obtained. All of the enzymes contain large amounts of free amino acids. Therefore, only the differences between minus ATP and plus ATP values can be considered as relevant to amino acid activation. Table 3 shows the results of an assay conducted in the usual manner.

The large amounts of color obtained with three of the preparations even in the absence of ATP may be due to non enzymatic cleavage by hydroxylamine of peptides liberated by proteolysis, to the trapping of enzyme bond amino acids as they are liberated by proteolysis, or to the reaction of hydroxylamine with esters or amides in the preparation.

The data presented above demonstrate the existence of amino acid activating enzymes in various plant tissues. Even so, the difficulties experienced with the hydroxylamine assay for amino acid activation in plant tissues are many. There are present in the preparations various compounds which form color complexes with ferric iron even in the absence of hydroxylamine. There appear to be other enzymatic reactions which yield hydroxamates. The existence of non enzymatic

Table 3. Hydroxamate Color Formation in Charcoal Treated Acetone Powder Extracts of Plants

	OD 520 on 5 ml samples	
	+AA, -ATP	+AA, +ATP
Avena coleoptile	1.36	1.56
Corn seedlings	.20	.27
Pea roots	1.43	1.54
Pea tops	.92	.96
Tobacco leaves	.56	.59

Enzymes prepared in .1 M tris-HCl pH 7.5 and treated with charcoal as in the section on methods. Enzymes were incubated for 3 hours at 37°C before assay for hydroxamate formation. Otherwise as in Table 1.

cleavage reactions of preexisting activated materials with hydroxylamine is not precluded; and lastly, the optical densities obtained with crude amino acid activating preparations are very low. The following calculation will further point out the need for a more sensitive assay.

If we assume protein is synthesized in a growing plant at a rate of 1% per hour, then 10 μ grams of protein are formed/mg protein/hour. Let us further assume that activation is necessary for protein synthesis. Ten μ grams of total amino acids must then be activated/mg protein/hour. If we assume that the molecular weight of each amino acid is 150, then:

$$\frac{10\ \mu\ \text{grams}}{150\ \text{grams/mole}} = .066\ \mu\ \text{moles of total amino acids activated/mg protein/hr}$$

or during any two hour period such as used in the hydroxamate assays on crude enzyme preparations one could expect

$$2 \times .066 \text{ or } .132\ \mu\ \text{moles of amino acid activated/mg protein/2 hours}$$

The preparation from spinach acetone powder in Table 2, charcoal treated and concentrated with $(NH_4)_2SO_4$, contained 18 mg of protein/1.4 ml of enzyme. The enzyme was incubated with substrates for 2 hours. This preparation when diluted to 5 ml showed upon colorimetric assay, at the most the formation of .36 μ moles AANHOH/ml or

$$\frac{5 \text{ ml} \times .36 \mu \text{ moles hydroxamate/ml assay}}{18 \text{ mg protein/5 ml assayed}} = .1 \mu \text{ moles hydroxamate/mg protein/2 hours}$$

in the total 5 ml of assay solution.

Therefore the spinach acetone powder preparation, even though it yields such low optical densities when examined with colorimetric assay of Schweet (63), is operating at

$$\frac{.1 \mu \text{ moles hydroxamate/mg protein/2 hours (observed)} \times 100}{.132 \mu \text{ moles hydroxamate/mg protein/2 hours (calculated)}} =$$

77% of calculated maximum efficiency. With crude unpurified preparations the sensitive hydroxamate assay of Schweet is therefore not sensitive enough to detect small levels of amino acid activation, and is certainly not sensitive enough to detect the activation of individual amino acids. The use of a more sensitive assay is therefore necessary.

2. Amino Acid Activation as Studied by Pyrophosphate Exchange

a. Introduction. Another method for the detection of the presence of amino acid activating enzymes involving ATP is provided by the enzymatic, amino acid dependent, exchange of pyrophosphate into ATP. If one examines the mechanism of activation presented in equation 3, one can see that an equilibrium should exist between the activated and non activated amino acid. It is only the removal of the activated amino acid by hydroxamate formation that drives the overall reaction far to the right. In the absence of

hydroxylamine it should be possible to incubate ATP, amino acids, and radioactive pyrophosphate with the appropriate enzyme system, and, upon the reisolation of the ATP, to measure the exchange of radioactive pyrophosphate into ATP. The extent to which such exchange is catalyzed by amino acids would then be a direct measure of the amount of amino acid activating enzymes present. The reaction can be stopped at any given time and the percent exchange and actual exchange of pyrophosphate into ATP can be calculated as discussed in the section on experimental methods.

This method provides a far more sensitive assay than that which uses hydroxamate formation. If one uses pyrophosphate with a high specific activity, it is still possible to detect small amounts or differences in radioactivity in the isolated ATP. Secondarily, it has subsequently been reported by Demoss & Novelli (52) in slightly purified bacterial amino acid activating preparations, by Davie et al.(53) with a purified tryptophan activating enzyme, and by Schweet et al. (54) with a purified tyrosine activating enzyme that the specific activity of the respective enzyme preparations for pyrophosphate exchange exceeds that of the same enzymes for hydroxamate formation. This implies that either the hydroxylamine in the reaction mixture inhibits the amino acid activation or that the hydroxylamine is not totally efficient in trapping out activated amino acid. Accordingly, in the following experiments use is made of the pyrophosphate procedure discussed in the section on methods.

b. Initial Studies. With preparations of the soluble enzymes from spinach homogenates or acetone powder extracts of spinach acetone powders as a source of enzymes, it is possible to demonstrate a definite enzymatic amino acid enhanced exchange of pyrophosphate into ATP. Two μ moles of each of 15 different L amino acids/ml of incubation material were provided to obtain the enhancement of exchange in the experiments considered in Table 4.

The values obtained in the experiments of Table 4 are larger than those obtained in the hydroxamate assay, further justifying the use of the pyrophosphate exchange assay. The high values of exchange obtained in the minus amino acid samples are of some concern. The enzyme prepared from acetone powder contains, prior to incubation, 12.75 u moles of free amino acid per half ml. according to the colorimetric procedure of Yemm and Cocking (72). This indicates the possible source of the exchange in the samples without added amino acids. One would expect qualitatively a larger amount of free amino acids in the fresh tissue homogenate than in the acetone powder extract, and the exchange values are in accord with this expectation.

The acetone powder preparation offers a more efficient source of material than does the homogenate for the study of pyrophosphate exchange, because of its higher specific activity, the ease of storage of large amounts of acetone powder, and the relatively lower activity in the absence of

Table 4. Specific Activities of Soluble Enzymes and Acetone Powder Extracts of Spinach

	μ moles PP exchanged/10 min./mg protein	
	+ Amino Acids	- Amino Acids
Soluble enzymes of homogenates in .01 M tris-HCl pH 7.5	1.29	.97
Acetone powder extracted with .1 M tris-HCl pH 7.5	1.45	.82

Soluble enzymes of homogenate and acetone powder extract are prepared as in the section on methods. The calculations are as described earlier. The incubation system contained .5 ml of the above enzymes and 10 μ moles of ATP-K salt, 5 μ moles of radioactive potassium pyrophosphate, 10 μ moles of KF, 5 μ moles of $MgCl_2$, 2 μ moles of each of 15 amino acids where indicated, and 50 μ moles of tris-HCl buffer all at pH 7.5 and contained in .5 ml. Incubation was for 10' at 37°C. Reactions were stopped with TCA in cold. ATP isolated and exchange calculated as described in the section on methods. Protein determination by TCA precipitation followed by hot TCA treatment, digestion, and Nesslers assay. Phosphate determined by the method of Allen (66). The zero time controls showed less than .01 μ moles of PP exchanged/mg protein.

added amino acids. Accordingly, acetone powder preparations are used in the following experiments.

c. Studies on Mechanism. It will now be shown that the mechanism of amino acid activation in the present system is in accordance with the formulation of Equation 4.

$$ATP + AA \overset{Enz}{\rightleftharpoons} AA\text{-}AMP + PP \qquad (4)$$

(1). Role of Pyrophosphate. The radioactivity measured in the ATP cannot be due to incorporation of phosphate in any other form than pyrophosphate. Ortho phosphate, released by a pyrophosphatase, is excluded. This has been shown by three different approaches. Figure 1 shows that the level of pyrophosphatase activity in the crude enzyme in the presence of 10 μ moles of potassium fluoride per ml is unlikely to produce sufficient ortho phosphate to account for the incorporation. Secondly, as shown in Table 5, there is no amino acid enhanced exchange of ortho phosphate into ATP. Thirdly, Table 6 shows that it is possible to prepare the enzymes in an ortho phosphate buffer without reduction in specific activity of the enzyme as compared to that of enzyme prepared in tris-HCl buffer at the same pH. If the exchange proceeded by way of an ortho phosphate intermediate, one would expect a lower specific activity for the enzyme prepared in ortho

Table 5. Exchange of Pyrophosphate and Ortho Phosphate into ATP in 10 min. at 37°C

	μ moles of material actually exchanged	
	+ AA	- AA
5 μ moles of PP^{32}/ml	2.00	1.27
5 μ moles of Pi^{32}/ml	.32	.31

Enzyme prepared by 10:1 extraction of spinach acetone powder, with .1 M tris-HCl pH 7.5. Otherwise the system is as in Table 4 except that the phosphate determination of Flynn et al. (64) was used. Calculations of % exchange for both Pi and PP as in the section on methods.

Table 6. Activities of 10:1 Spinach Acetone Powder Extracts in .1 M Buffers at pH 7.5

	counts/min/μ mole Pi/mg protein	
	0 min. at 37°C	10 min. at 37°C
Enzyme in .1 M tris-HCl	10	3081
Enzyme in .1 M potassium phosphate	10	3098

Assay procedure and determination as in Table 4 except the enzyme in phosphate buffer was incubated in the presence of 50 μ moles of potassium phosphate buffer/ml in place of 50 μ moles of tris-HCl/ml.

phosphate buffer due to the dilution of the specific activity of the Pi^{32} by the buffer. These lines of evidence leave no doubt that the exchange of labeled pyrophosphate into ATP is in fact due to pyrophosphate exchange.

(2). Role of ATP. In the absence of added amino acids, radioactive pyrophosphate is incorporated to a detectable extent into GTP, UTP, and CTP as well as into ATP. Only ATP however yields an amino acid enhanced exchange of pyrophosphate into nucleotide triphosphate as is shown in Table 7.

The relatively high levels of exchange in preparations containing UTP are probably due to the well known uridyl transferase which catalyzes the reaction of UTP and glucose-1-phosphate and causes the formation of uridine diphosphoglucose (73) as illustrated in equation 5:

$$\text{UTP} + \text{Glucose-1-phosphate} \rightleftharpoons \text{UDPG} + \text{PP} \qquad (5)$$

It is possible to test for the presence of the uridyl transferase in the preparation. In a system limiting in glucose-1-phosphate, as would be expected with an acetone powder extract, the addition of a small amount of glucose-1-phosphate (K-salt, Nutritional Biochemical Corp., Cleveland, Ohio) should increase the exchange of pyrophosphate into UTP. Table 7 shows that this is in fact the case. Therefore, the uridyl transferase is present in the preparation

Table 7. Exchange of Pyrophosphate into Various Nucleotide 5' Triphosphates

	μ moles of PP exchanged/10 min. at 37°C	
	+AA	-AA
5 μ moles of ATP	2.55	.62
5 μ moles of GTP	.17	.18
5 μ moles of CTP	.20	.15
5 μ moles of UTP	.79	.79
5 μ moles of UTP & 2 μ moles of Glucose -1-phosphate	--	3.08

Enzyme prepared by 10:1 extraction of spinach acetone powder with .1 M tris-HCl pH 7.5. Otherwise as in Table 4, except that only 5 μ moles of the sodium salts of the nucleotide 5' triphosphates were used.

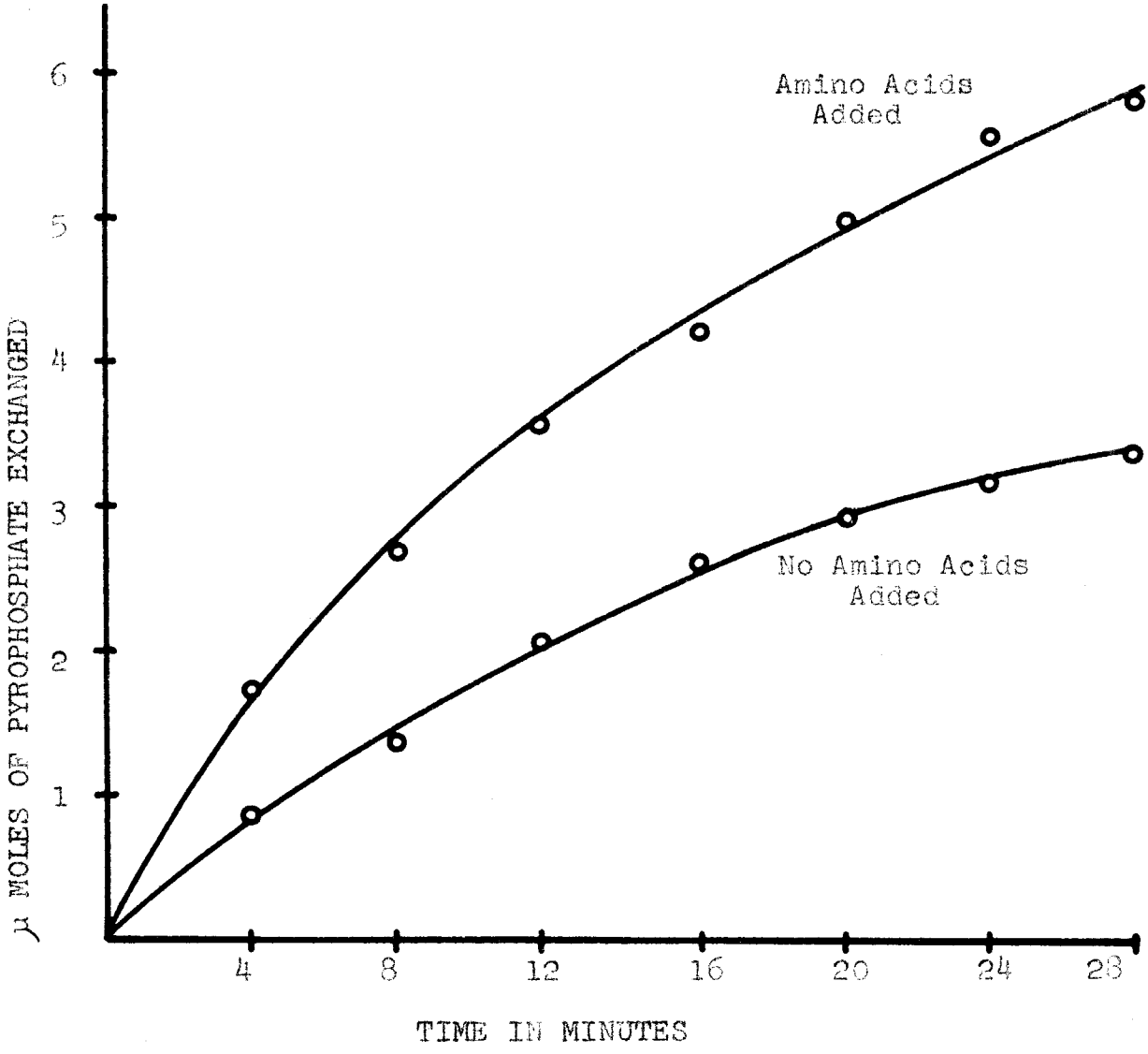
and it is no doubt responsible for the high level of non amino acid dependent exchange exhibited by the preparation in the presence of UTP.

It has already been shown that all of the counts released by acid hydrolysis of a preparation, incubated in the presence of ATP, originate from counts incorporated in the ATP. The evidence, taken together, shows that there is little doubt but that the enzymes involved in amino acid activation are specific for ATP.

(3). Role of Amino Acids. The time course of the pyrophosphate exchange reaction as determined, using as an enzyme a preparation made from spinach acetone powder (.1 M tris-HCl, pH 7.5), yields the progress curves shown in Figure 6. It is of interest to note that the rate of pyrophosphate exchange under these conditions is not linear. The progress curves for exchange in the absence of added amino acids as well as that for exchange in the presence of added amino acids exhibit a decrease in rate with time.

There is definite enhancement of exchange rate due to the addition of amino acids. It is also necessary to consider the nature of the exchange which takes place in the absence of added amino acids. As mentioned earlier, this preparation, made in the cold, contains 12.75 μ moles of free amino acid per half ml of enzyme. Incubation of the enzyme at 37°C for 30 minutes, with concomitant proteolysis,

Figure 6. Pyrophosphate Exchange Exhibited by an Acetone Powder Extract of Spinach



Enzyme prepared in .1 M tris-HCl pH 7.5 and assayed as in Table 4. There is no major loss of ATP during the incubation (8 μ moles of ATP returned from time 0 sample and 7.5 μ moles of ATP returned from 28 minute sample).

increases the level of free amino acids to 13.4 μ moles of free amino acids per half ml of enzyme. It seems probable therefore, that activity in the absence of added amino acids is due to an amino acid dependent exchange of pyrophosphate into ATP. It is possible, by treating the enzyme with charcoal, to lower the extent of pyrophosphate exchange in the absence of added amino acids without decreasing the level of exchange in the presence of added amino acids. This is illustrated by the data of Table 8.

The charcoal treatment would be expected to remove a portion of the aromatic amino acids such as tryptophane and tyrosine from the enzyme solution. The fact that charcoal treatment decreases exchange in the absence of added amino acids further supports the hypothesis that such exchange is in fact due to free amino acids present in the preparation.

Further support of the above hypothesis has been achieved by complete removal of the free amino acids from the preparation. Dialysis of the charcoal treated preparation of Table 8 for 16 hours at 2°C yields a preparation that has only an amino acid dependent exchange as is shown in Figure 7.

It is evident that all of the exchange of this system is dependent upon added amino acids.

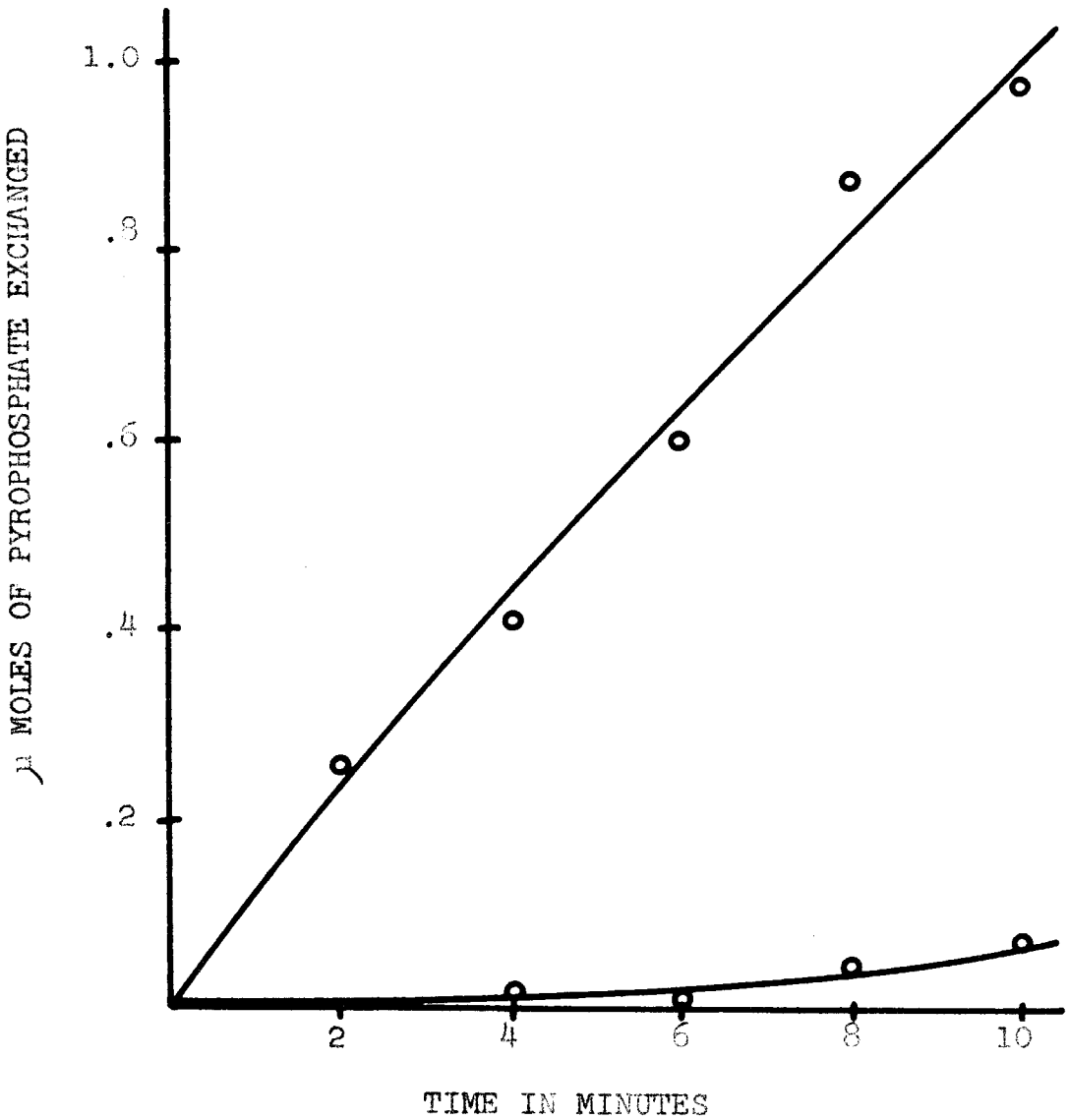
The level of exchange activity is lowered by dialysis of the preparation. The drop in activity is due to inability

Table 8. Effect of Charcoal Treatment upon Spinach Acetone Powder Extracts

	μ moles PP exchanged/10 min. at 37°C	
	+AA	-AA
Enzyme in .1 M tris-HCl pH 7.5	2.09	1.24
Charcoal treated enzyme in .1 M tris-HCl pH 7.5	2.09	.94

Assay and reagents as in Table 5. Charcoal treated enzyme was treated with 5.2 mg of acid washed charcoal/ml of enzyme. The charcoal was removed by centrifugation before incubation of the enzyme with substrates.

Figure 7. Rates of Exchange in Dialysed Spinach Acetone Powder Extracts

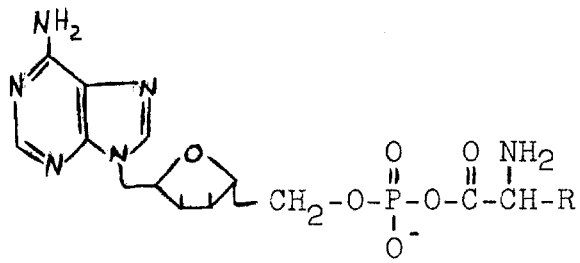


The enzyme was extracted from spinach acetone powder and treated with charcoal as in Table 8. The charcoal treated enzyme was dialysed for 16 hours against .1 M tris-HCl pH 7.2 in the cold. Assay as in Table 4.

of the amino acid activating enzymes to withstand dialysis. It is not due to denaturation of enzymes involved in an amino acid independent exchange. The hypothesis that there is a separate dialysis sensitive enzyme activity responsible for exchange in the absence of added amino acids can be eliminated by study of the enzyme preparation after dialysis for various periods of time. The activity of the dialyzed enzyme drops quickly as assayed in the absence of added amino acids. This drop is due to removal of free amino acids. At the same time the activity of the dialyzed enzyme assayed with added amino acids gradually decreases due to the slow inactivation of the amino acid activating enzymes (Figure 11). If there were a separate dialysis sensitive activity responsible for the exchange in the absence of added amino acids, then the activity expressed upon assay in the presence of amino acids should decrease, upon dialysis, at a rate equal to or faster than the decrease in activity in the absence of amino acids. This is not the case. Therefore, we again see that the exchange is actually due to the presence of free amino acids in the preparation. Consideration of all of the above lines of evidence leaves no other alternative for explanation of exchange in the absence of added amino acids.

(4). Role of Amino Acid - Adenosine 5' Monophosphate. No direct investigations have been made of the exact nature of the activated amino acid. Even so, it is possible to

conclude that the activation of the amino acid is through the carboxyl group. This follows from the formation of acyl hydroxamates in the hydroxylamine trapping experiments. Since pyrophosphate is the material released in the cleavage of ATP, it is logical to conclude that AMP is somehow involved with the activated amino acid. The evidence therefore suggests that the activated amino acid may be held in the form of a mixed anhydride of the amino acid and AMP as indicated below:



This is in accord with the findings of Demoss et al. (46) who were able to convert leucyl-AMP into ATP and leucine in the presence of pyrophosphate and an enzyme from E. coli. It is not possible to conclude whether the amino acid-AMP remains bound to the enzyme as originally suggested by Hoagland et al. (74).

It has now been shown that the enzymes isolated from spinach acetone powders carry out activation of amino acids by the mechanism proposed in equation 4:

$$AA + ATP \overset{Enz}{\rightleftharpoons} AA\text{-}AMP + PP \qquad (4)$$

Still yet to be answered are questions concerning amino acid specificity, properties of the enzymes, occurrence of the enzymes within various plants, location of the enzymes within plant cells, and implication of the enzymes in the mechanism of protein synthesis.

d. The Occurrence of Pyrophosphate Exchange in Various Plant Tissues. The phenomenon of an amino acid dependent exchange of pyrophosphate into ATP exists in other plant tissues besides spinach as can be seen in Table 9. Amino acid activating enzymes have been found in every plant in which they have been sought. The data of Table 9 plus that of Table 3 using the hydroxylamine assay, suggest that amino acid activation is ubiquitous to plant tissues. This is in full accord with the concept that amino acid activation is necessary for the synthesis of proteins in plants.

e. Properties of Spinach Acetone Powder Extracts. In order to investigate matters concerned with amino acid activation, it is first necessary to know some of the properties of the enzymes involved. This section covers several of the properties of the enzymes obtained from spinach acetone powder. Spinach acetone powder preparations are used in this and succeeding sections unless otherwise stated because of the high yield of protein from the acetone powder, the ease with which material could be

Table 9. Specific Activities of Enzymes from Various Plant Sources Toward Pyrophosphate Exchange

	μ moles PP exchanged/mg protein/10 min	
	+AA	-AA
Pea epicotyl homogenate in approx. .3 M sucrose pH 7.2	.91	.60
Extract of acetone powder of Avena coleoptiles in .1 M tris-HCl pH 7.3	1.62	1.13
Extract of acetone powder of asparagus tips in .1 M tris-HCl pH 7.0	.64	.61
Extract of acetone powder of winter rye grass in .1 M tris-HCl pH 7.2	1.14	.65
Extract of acetone powder of tobacco leaves in .1 M tris-HCl pH 7.0	.44	.12
Extract of acetone powder of spinach leaves in .1 M tris-HCl pH 7.5	1.45	.82

Avena coleoptile powder extracted 12.5:1 and rye powder extracted 16.7:1 with .1 M tris-HCl pH 7.5. Other powder enzymes prepared by 10:1 extraction of powders with .1 M tris-HCl pH 7.5. Pea epicotyl homogenate prepared by grinding 6 day old etiolated pea epicotyles in .4 M sucrose (1 gram of epicotyles/.5 ml of solution) in a mortar at 0°C followed by filtration through cheese cloth. Assay as in Table 4.

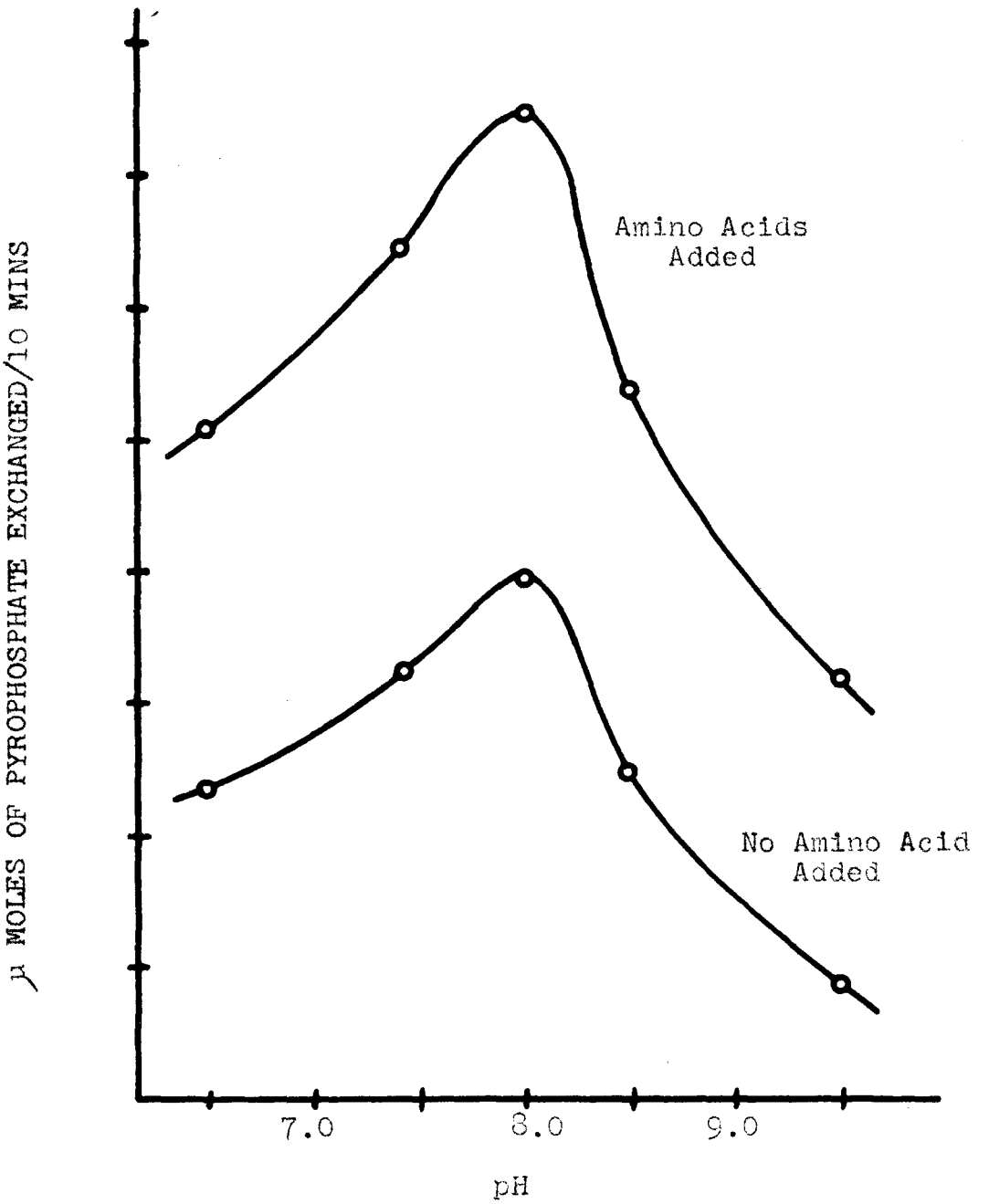
obtained from the local markets, and the high specific activities of the preparations.

(1). Optimal pH. The enzymes prepared in .1 M tris-HCl buffer exhibit the pH curve shown in Figure 8. The pH optimum is at pH 8. The experiments reported in this thesis were principally done at pH 7.5, a pH near the optimum for the preparation.

(2). Nature of Buffer Used in Powder Extraction. There is a large variation both in specific activity and in protein concentration of enzyme preparations obtained by extraction of spinach acetone powder with buffers of different concentration but at the same pH. Table 10 shows how equal amounts of acetone powder yield enzymes of different specific activities when extracted with equal volumes of different buffers. As can be seen, it is possible to obtain preparations of higher specific activity by extracting the powder with buffers of lower ionic strength. On the other hand, the lower the molarity of buffer used, the smaller the amount of protein extracted from the powder, as is shown in Figure 9.

In terms of total enzymatic activity extracted, preparations made in high and low salt are nearly equal as is shown by the calculation below:

Figure 8. Relationship of pH to Amino Acid Activation in Spinach Acetone Powder Extracts



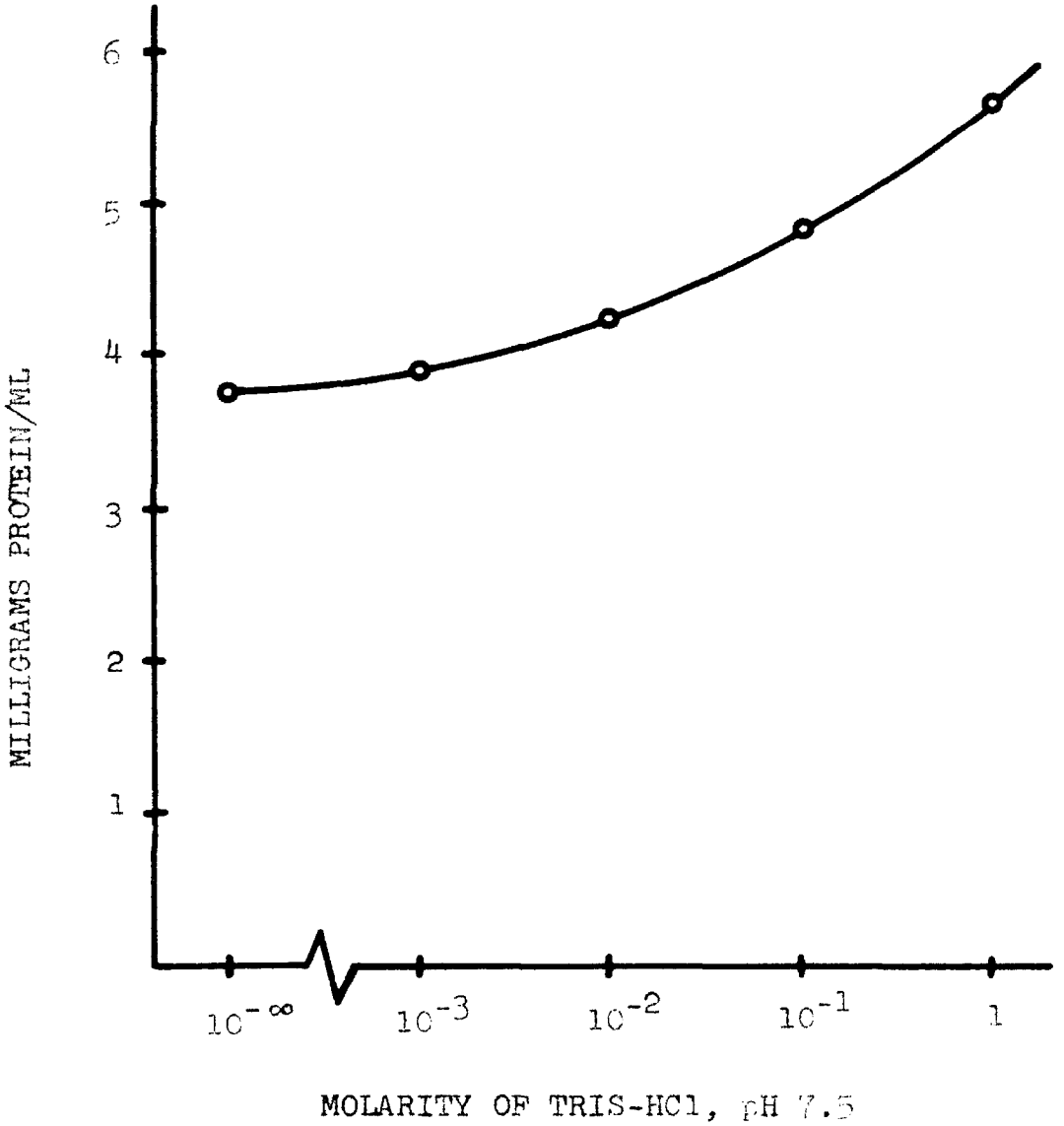
Assay as in Table 4. All reagents and the enzyme titrated to the correct pH before assay.

Table 10. Specific Activities of Powder Extracts Prepared by 10:1 Extraction of Spinach Acetone Powder with Different Buffers at pH 7.5

	μ moles PP exchanged/mg protein/10 min	
Molarity	tris-HCl buffer pH 7.5	K-PO4 buffer pH 7.5
.1 M	1.45	1.45
.01 M	--	1.51
.001 M	1.96	--
.1 M & .1 M KCl	.77	--
Water	1.54	

All enzymes prepared by 10:1 extraction of powder with given buffer. The pH of the resulting solution was raised to pH 7.5 with KOH before assay. Assay as in Table 4 with each preparation containing 2 μ moles of each of 15 amino acids/ml. The values expressed represent averages of two or more separate experiments.

Figure 9. Amounts of Protein Extracted from Spinach Acetone Powder



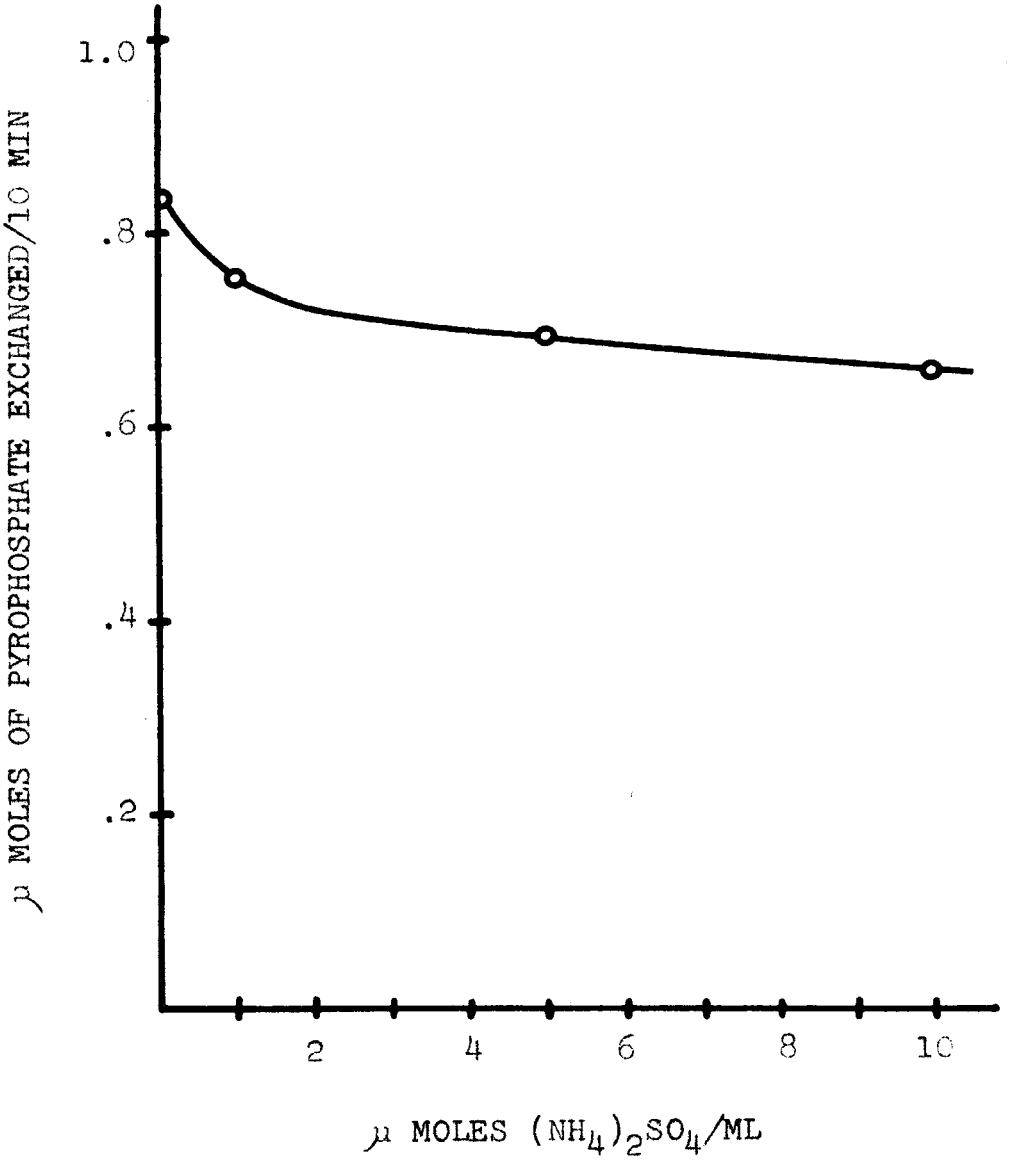
Protein determined by Nessler's assay as in section on methods.

	μ moles PP exch/mg protein/10 min	X	mg protein/.5 ml	=	total μ moles PP exch/10 min
.001 M Tris-HCl	1.96	X	1.97	=	3.86
.1 M Tris-HCl	1.45	X	2.43	=	3.52

The fact that total enzymatic activity does not increase in the higher ionic strength buffer even though higher protein concentrations are extracted can be the result of one or more effects. Firstly, it is possible that .001 M tris-HCl extracts all of the amino acid activating activity from the powder. The higher salt concentrations would then extract, in addition, other proteins from the powder, which are either inactive or are involved in competing or inhibitory reactions. Secondly, higher salt concentrations may inhibit the amino acid activating enzymes. Thirdly, some substrate, rather than the enzyme, may be the limiting component in the system. The result of extraction of the powder with .1 M tris-HCl, .1 M KCl at pH 7.5 (shown in Table 10) favors the second of these possibilities.

(3). Effect of Ammonium Sulphate upon Pyrophosphate Exchange. Because of the use of ammonium sulphate fractionation at a later point in this thesis, it is necessary to ascertain the effect of residual $(NH_4)_2SO_4$ upon the amino acid activating enzymes. Figure 10 shows that increasing the concentration of $(NH_4)_2SO_4$ present in a

Figure 10. Effect of $(NH_4)_2SO_4$ Concentration upon Pyrophosphate Exchange in the Presence of Added Amino Acids



Enzyme prepared as in Figure 7 and frozen until used. Assayed as in Table 4. $(NH_4)_2SO_4$ titrated to pH 7.0 before addition to enzyme.

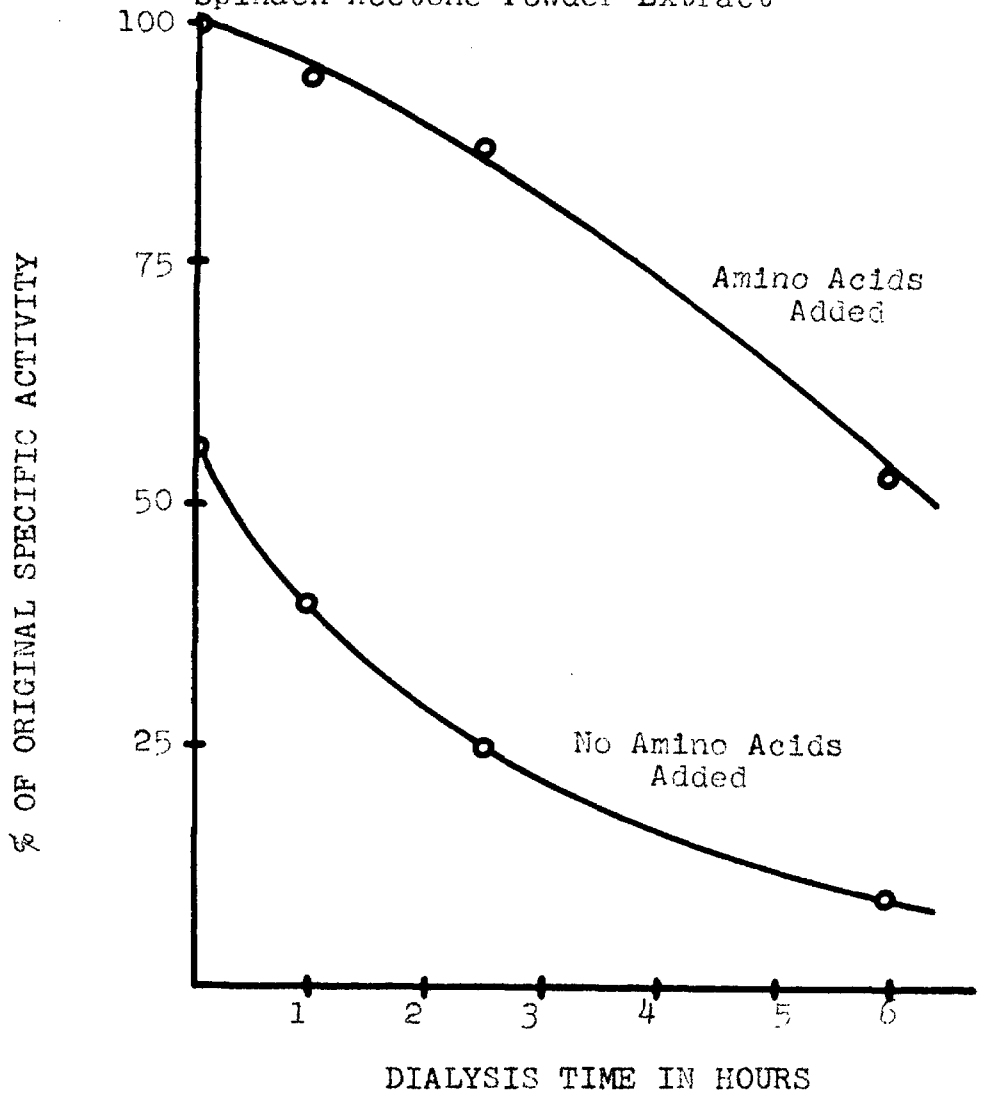
dialyzed acetone powder extract markedly decreases the exchange of pyrophosphate into ATP in the presence of added amino acids. Therefore, dialysis of ammonium sulphate fractionated protein appears to be necessary.

(4). Stability to Dialysis. The enzyme preparations in .1 M tris-HCl are not very stable to dialysis in .1 M tris-HCl. A study of the extent of enzyme degradation after a given period of dialysis time in .1 M tris-HCl shows that the pH range 7.2-7.5 yields the least degradation of the enzymes. When an enzyme preparation in .1 M tris-HCl at pH 7.5 is dialyzed in the same solvent, it is still actively degraded as is shown in Figure 11.

The degradation of activity in enzymes prepared in 001 M tris-HCl pH 7.5 and dialyzed against the same is not as pronounced, but is still apparent. This property makes the detection of the activities towards individual amino acids difficult. It is not possible to dialyze away the free amino acids without considerable loss in activity of the enzyme. The loss in activity upon dialysis could be due to any one of many possibilities including harm caused by non-protein materials in the preparation, removal by dialysis of a cofactor, proteolysis, etc.

f. Site of the Amino Acid Activating Enzymes in Cells. It is necessary to know the site of the amino acid activating enzymes in cells in order to understand fully their

Figure 11. Effect of Dialysis Time Upon Activity of Spinach Acetone Powder Extract



Enzyme prepared by 10:1 extraction of spinach acetone powder with .1 M tris-HCl at pH 7.5. The preparation was dialyzed at 2°C in .5 inch diameter tubing against .1 M tris-HCl at pH 7.5. Assayed as in Table 4. The specific activities (μ moles pyrophosphate exchanged/mg protein/ 10 minutes at 37°C) are converted to % of the original specific activities.

role in protein synthesis. Determination of the extent of pyrophosphate exchange in various cellular fractions obtained from etiolated pea epicotyls shows that the soluble supernatant fractions (105,000 g soluble) contain the highest specific activity and total activity towards pyrophosphate exchange. Table 11 shows these results.

It appears that the amino acid activating enzymes are among the soluble enzymes of cells. Even so, the possibility still exists that the activating enzymes are particle-bound and that homogenization of the tissue releases the enzymes to solution. This hypothesis is partially discredited by the fact that the large number of enzymes already in the soluble fraction would dilute out the activating enzymes released from any particles to such an extent that it is unlikely that the specific activity of the supernatant would be the highest of all the cellular tissues. The concept that activating enzymes are a natural component of the soluble fraction of cells is in agreement with Hoagland et al. (73).

It can be seen from Table 11 that the fractions containing large quantities of nucleic acids (nuclear and microsomal) contain the next highest specific and total activities of amino acid activating enzymes. This suggests that these particulate fractions may coprecipitate the amino acid activating enzymes. The high activity in

Table 11. Specific and Total Activities of Various Cellular Fractions of Pea Epicotyles in Pyrophosphate Exchange

Fraction	μ moles PP exchanged/mg protein	Total μ moles PP exchanged
Nuclear (0-5,000 g) + AA	.52	19.7
Nuclear (0-5,000 g) - AA	.40	15.2
Mitochondrial (5,000-42,000 g) + AA	.44	7.2
Mitochondrial (5,000-42,000 g) - AA	.36	5.9
Microsomal (42,000-105,000 g) + AA	.71	13.4
Microsomal (42,000-105,000 g) - AA	.44	8.3
Soluble Supernatant (105,000 g) + AA	.94	88.2
Soluble Supernatant (105,000 g) - AA	.77	72.7

The particulate fractions resuspended in .1 M tris-HCl .3 M sucrose pH 7.5 are obtained from a homogenate of etiolated pea epicotyles prepared in approximately .3 M sucrose as described in the section on methods. The fractions are assayed as in Table 4. The values of μ moles of PP exchanged/mg protein obtained from the .5 ml of enzymes assayed are multiplied by the total milligrams of protein in the fraction (mg protein/ml X number of milliliters in fraction) to obtain the total exchange values.

these fractions would then be merely an artifact of preparation. Experiments with the microsomal fraction suggest that this is in fact the case. When the specific activity of an original microsomal fraction is compared with that of a purified (ultracentrifugally purer) preparation of microsomes (prepared as by Ts'o et al. (75)), it is observed that the purified preparation is lower in specific activity (by 61%) towards pyrophosphate exchange in the presence of amino acids than is the original preparation. Table 12 shows this to be the case.

This evidence further supports the concept that amino acid activating enzymes are in fact a part of the soluble component of cells. In light of this evidence and the evidence of other workers in other tissues, it seems likely that the majority of the amino acid activating activity of plants is not associated with any particulate fraction of the cell.

g. Studies on the Purification of Spinach Acetone Powder Extracts. Two properties of the acetone powder extracts obtained from spinach are outstanding and should be considered in all purification attempts. First, the extracts contain high levels of free amino acids. It is necessary to remove these free amino acids in order to study the activation of the individual amino acids. Secondly, the extracts contain a high concentration of nucleic acid material which tends to influence fractionation procedures and secondarily to interfere with the

Table 12. Comparison of Specific Activities of Original and DC-2 Pea Epicotyl Microsomes

	μ moles of PP exchanged/mg protein/10 min
Original microsomes + AA	2.38
Original microsomes - AA	.95
DC-2 microsomes + AA	.92
DC-2 microsomes - AA	.40

$$1 - \left(\frac{.92}{2.38} \times 100\right) = 61\%$$

Etiolated pea epicotyles were ground in sucrose solution (.5 ml of .4 M sucrose/1 gram of epicotyles) in the cold and the fraction sedimentable in 90 minutes between 40,000 g and 105,000 g was obtained. This was resuspended in water in approximately 25% of the original volume and stirred for one hour at 2°C. This material was considered the original microsomes. The DC-2 microsomes were prepared as described by Ts'o et al. (75). The assay procedure is that used in Table 4. The specific activities obtained are larger than those reported in Table 11, but it should be noted that the preparations are in distilled water rather than in .1 M tris-HCl buffer at pH 7.5.

study of any RNA catalyzed reactions. Therefore, fractionation attempts should be aimed at removing these two types of interfering materials with the least loss of activity towards the total number of amino acids activated.

The free amino acids in the enzyme solutions appear to originate from amino acids bound to the powder. The release of amino acids by proteolysis is small. Enzymes prepared in the cold in .1 M tris-HCl at pH 7.2 contain 12.75 μ moles of free amino acid/.5 ml. Incubation of such a preparation for 30 minutes at 37°C raises this level of free amino acids only slightly, to 13.4 μ moles/.5 ml. Therefore, excessively active proteolysis of the preparation does not appear to be the source of the free amino acids.

The nucleic acid material observed would be expected in any acetone powder preparation. The present preparations give $\frac{280\ m\mu}{260\ m\mu}$ ratios in the range of .70 - .75 indicating high nucleic acid content.

In order to remove these two materials it is necessary to investigate several fractionation procedures. Isoelectric precipitation (or coprecipitation) of the active enzymes by lowering the pH to pH 5.1 and resuspension of the precipitate at pH 7.5 would be one way to remove the free amino acids from the solution. Possibly a purification of the enzymes could be obtained at the same time. Such a method has been used successfully by Hoagland et al. (74). Precipitation of spinach acetone powder extracts in

.1 M tris-HCl or .1 M K-PO_4 in the cold by lowering the pH to 5.1 followed by resuspension at pH 7.5 is very harmful to the preparations. The specific activity in the presence of added amino acids drops to less than 25% of value of the original enzymes. Raising the pH of enzyme preparations and resuspension of the resulting precipitates at pH 7.5 is equally harmful to the activity. Therefore some other means of fractionation must be used to remove the free amino acids and nucleic acid from the system.

Ammonium sulphate fractionation is not effective as a means of purification of the enzymes at this stage. There is some degree of purification of the enzymes in an ammonium sulphate fractionation but this degree of purification is lost by the degradation of the enzymes during the dialysis necessary to remove the $(NH_4)_2SO_4$. Therefore, a preparation which has been $(NH_4)_2SO_4$ fractionated and then dialyzed has a lower activity than the original preparation. Table 13 shows this to be the case.

Although this experiment does not yield any positive purification, it does yield some information about the activation of individual amino acids. It is to be noted that the activity towards individual amino acids is generally spread out over a wide range of fractions and yet some general trends are evident. There is no L-cysteine activation by the 0-40% fraction, but this

Table 13. Ammonium Sulphate Fractionation at pH 7.5 of Spinach Acetone Powder Extract

Enzyme Type	μ moles PP exchanged/mg protein/10 min at 37°C				
	15AAs	L-Leucine	L-Isoleucine	L-Cysteine	-AAs
Orig. unfractionated enzyme	.93	-	-	-	.418
Unfractionated enzyme dialyzed	.32	.128	.167	.121	.042
0 - 40% saturated	.14	.096	.087	.060	.060
40 - 50% saturated	.42	.154	.206	.097	.071
50 - 60% saturated	.48	.144	.224	.130	.085
60 - 70% saturated	.19	-	-	-	.047

All operations described below were carried out at 0°C. Spinach acetone powder was extracted 10:1 with .1 M tris-HCl at pH 7.5. The resulting supernatant was treated with 4.5 mg of acid washed charcoal/ml of enzyme. The charcoal was removed by centrifugation. A sample of this enzyme was then assayed to determine the activity of the original unfractionated enzyme. A second sample of unfractionated enzyme was stored at 0°C during the fractionation and then dialyzed with the fractionated preparations. All fractionations were carried out by the addition of 100% $(NH_4)_2 SO_4$ solution titrated to pH 7.5 with NH_4OH. The fractions were obtained by successive additions of salt solution to the same original material until all of the fractions were obtained. The precipitates were removed from each fraction by centrifugation at 10,000 g for 5 minutes. All fractions were resuspended in .1 M tris-HCl pH 7.2 (0-40%, 50-60%, and 60-70% in 1/2 of original volume, 40-50% in 5/8 of original volume). Dialysis was for 6 and 1/2 hours at 5°C against .1 M tris-HCl at pH 7.2. Assay procedure is that of Table 4.

activity increases progressively in the 40-50% and 50-60% fractions. Conversely, L-leucine and L-isoleucine activation occurs generally in all fractions, but the extent of the activation of the two amino acids relative to each other varies. These observations indicate that separate enzymes are involved in the activation of each amino acid.

As can be seen, none of the above methods remove the free amino acids from the enzymes and offer a purification. Absorption of protamine sulphate treated enzymes upon calcium phosphate gel followed by washing of the gel with water to remove free amino acids and subsequent elution of the washed gel with potassium phosphate buffer at pH 7.5 does however yield purified, amino acid free, enzymes. Both the amount of protein released and the specific activity of the eluted protein vary as a function of concentration of the buffer used in the elution. Table 14 shows that elution with .2 M potassium phosphate releases protein of the highest specific activity from the gel.

Elution with .2 M potassium phosphate buffer appears to optimally balance the harmful effects of high buffer strength with the requirement for sufficient buffer to remove absorbed proteins from the gel. A preparation prepared in this manner no longer has the low $\frac{280\ m\mu}{260\ m\mu}$ ratio characteristic of the original material, but has instead a $\frac{280\ m\mu}{260\ m\mu}$ ratio of 1. This is equal to approximately 3%

Table 14. Concentration and Specific Activity of the Proteins Eluted from $Ca_3(PO_4)_2$ Gel as a Function of Strength of Buffer

	mg protein /.5 ml	μ moles PP exchanged /mg protein/10 min	
		+AA	-AA
Original enzyme in .001 M tris-HCl	1.97	1.96	.76
Gel eluted with 1.0 M $K\text{-}PO_4$ 7.5	.93	.72	--
Gel eluted with .5 M $K\text{-}PO_4$ 7.5	.92	1.70	.11
Gel eluted with .2 M $K\text{-}PO_4$ 7.5	.77	2.53	.14
Gel eluted with .1 M $K\text{-}PO_4$ 7.5	.52	1.44	.12

Spinach acetone powder was extracted 10:1 with .001 M tris-HCl pH 7.5. The supernatant obtained after removal of the non soluble material was titrated to pH 7.0 with KOH. Protamine sulphate (.05 ml of a 1.5% protamine sulphate Nutritional Biochemical Corp., Cleveland, Ohio solution at pH 6.7/ml of enzyme) was added and the flocculent material was removed by centrifugation at 10,000 g for 10 min. This supernatant was raised to pH 7.5 and 1 ml of a $Ca_3(PO_4)_2$ gel slurry (34 mg gel/ml) prepared in the method of Singer & Kearney (76) was added to each 4 ml of enzyme. The gel was collected by centrifugation in a clinical centrifuge and each ml of original gel was washed once with .5 ml of water. The gels were eluted with 4 ml of the above buffers/3 ml of original gel and the eluates were assayed as in Table 4.

nucleic acid.

It is now possible to study the activation of the individual amino acids. Enzymes prepared by the washed gel elution method have very low levels of endogenous exchange due to free amino acids. This base level exchange can be lowered still more by washing of the protein bound to the gel with two .5 ml washes of water/ml of gel instead of one. This is accompanied by only a slight drop in activity of activating enzymes.

h. Amino Acid Specificity. Heretofore we have considered the properties of the amino acid activating enzymes towards a mixture of 15 L amino acids. It is now necessary to consider which amino acids are responsible for the enhancements in exchange caused by this amino acid mixture.

Homogenates of intact spinach dialyzed for six hours in .001 M tris-HCl at pH 7.5 show an enhanced pyrophosphate exchange in the presence of any of 9 individual L amino acids (L-Leucine, L-Isoleucine, L-valine, L-cysteine, L-tyrosine, L methionine, L-tryptophan, L-histidine, and glycine). The enhancement is reflected over a fixed base level of exchange presumedly due to free amino acids in the solution.

Dialyzed acetone powder preparations from spinach show activity toward these same 9 amino acids as well as toward 3 others (L-alanine, L-arginine free base, and

L-aspartic acid). The activities toward each amino acid relative to the activities towards other amino acids of the two preparations, intact homogenate supernatants and acetone extracts, are roughly equal. Therefore, no major loss in activity toward any individual amino acid occurs in the preparation of acetone powders. Here again the enhanced exchange is measured above a base level of exchange due to free amino acids still present within the preparation.

As we have seen earlier, it is possible, by protamine sulfate and calcium phosphate treatment of an acetone powder extract, to obtain a purified preparation that exhibits very low pyrophosphate exchange in the absence of added amino acids. Such a preparation may be tested for enhancement of pyrophosphate exchange as catalyzed by individual amino acids. It is possible to demonstrate activity of the preparation towards an even larger spectrum of amino acids. Table 15 shows the activities of the three preparations mentioned toward individual amino acids.

As can be seen, spinach preparations prepared in various manners have the ability to activate all of the amino acids tested except serine and hydroxyproline. It is possible that the small level of free amino acids in the gel eluate still contains sufficient serine to saturate the amount of serine activating enzyme present, thereby making the detection of activity towards serine impossible at this

Table 15. μ Moles of Pyrophosphate Exchanged by Various Preparations in 10 minutes at 37°C

Substrate		Dialyzed intact spinach homogenate in .001 M tris-HCl pH 7.5	Charcoal treated and dialyzed acetone powder extract	$Ca_3(PO_4)_2$ gel eluate of protamine sulphate treated powder extract
No amino acids added (Blank)		.352	.187	.0407
L-Leucine		1.02	.59	.276
L-Isoleucine		1.00	.63	.344
L-Valine		.57	.35	.206
L-Cysteine		.48	.42	.219
L-Tyrosine		.48	.33	.116
L-Methionine		.43	.30	.109
L-Tryptophan		.42	.23	.061
L-Histidine	pH 7.0	.40	.25	.044
Glycine		.38	.31	.067
L-Alanine		.36	.25	.047
L-Arginine	pH 7.0	.36*	.23	.042*
L-Aspartic Acid	pH 7.0	-	.27	-
L-Asparagine		-	.25	-
L-Proline		.34	.21	.051
L-Hydroxyproline		-	-	.042
L-Lysine	pH 7.0	.37*	.21*	.047*
L-Threonine		.37	.22	.045
L-Serine		.33	.20	.040
L-Phenylalanine		.35	.20	.044
L-Glutamic Acid	pH 7.0	-	.20	-
L-Glutamine		-	.18	-
α-Ketoisocaproic		-	-	.143
α-Ketoisocaproic & L-Leucine		-	-	.302
15 L Amino Acids		1.45	1.20	.840
Difference from blank for significance at 1% level		.039	.062	.0028

All of the enzyme preparations mentioned in the following discussion were carried out at 0°-2°C.

The homogenate supernatant of intact spinach was prepared in .01 M tris-HCl pH 7.5 as described in the sections on methods. The resulting supernatant enzyme was titrated to pH 7.5 with KOH and dialyzed for 6 hours against .001 M tris-HCl pH 7.5. One-half ml aliquots of this enzyme were used for assay.

The acetone powder extract was prepared by 10:1 extraction of

spinach acetone powder with .1M tris-HCl at pH 7.5. The supernatant obtained upon centrifugation was treated with 4.5 mg of acid washed charcoal/ml of enzyme. The supernatant obtained after charcoal removal was dialyzed for 6 hours against .1 M tris-HCl at pH 7.2. One-half ml aliquots of this dialyzed enzyme were used for assay.

The gel eluate preparation was prepared in the following manner. Spinach acetone powder was extracted 10:1 with .001 M tris-HCl pH 7.5. The supernatant obtained upon centrifugation was raised to pH 7.0 with 1.0 M KOH. Five-hundredths of a milliliter of a 1.5% solution of protamine sulphate at pH 6.7 was added to each ml of the above supernatants, and allowed to combine with the solution for 5 minutes. The flocculent material was removed by centrifugation at 10,000 g for 10 minutes. The pH of the solution was then raised to pH 7.5. One ml of a $Ca_3(PO_4)_2$ gel solution (34 mg $Ca_3(PO_4)_2$/ml) prepared by the method of Singer and Kearney (76) was added to each 4 ml of the enzyme solution. The gel was centrifuged down with a clinical centrifuge. The gel precipitate was washed twice by resuspension in water (.5 ml of water/1 ml of original gel) in order to remove free amino acids. The gel was then eluted with .2 M potassium phosphate at pH 7.5 (4 ml of buffer/3 ml of original gel solution). One-half ml aliquots of this gel eluate were used in the assay.

The assay procedure used was that of Table 4 except that 2 μ moles of each of the substrates as indicated were used (.1 ml of a .02 M solution). The samples incubated without added amino acids received an equivalent volume of water.

A t-test was used to calculate significance of deviation in pyrophosphate exchange between the blanks and their respective amino acid treatments. Four replicates were used to calculate the blank values and two replicates were used for each amino acid treatment.

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* Indicates monohydrochloride used.

stage.

It is not possible to state whether the activity expressed towards asparagine and aspartic acid is amino acid activation. The activity expressed in the presence of aspartic acid or asparagine could be due to an orthophosphate exchange into ATP such as that proposed by Webster & Varner (16) in asparagine synthesis. The aspartic acid dependent exchange also could represent a combination of the action of β-aspartokinase and L-aspartic β-semialdehyde dehydrogenase as outlined by Black & Wright (77). The enzyme solution in combination with 10 μ moles of potassium fluoride still contains sufficient pyrophosphatase activity to release the amount of ortho phosphate needed to satisfy the above enzyme systems.

The activity toward L-cysteine is not due to sulfhydryl activation of amino acid activating enzymes and resultant higher base level exchange. It can be seen in Table 16 that other sulfhydryl compounds do not increase the exchange in the absence of added amino acids. Furthermore, it can be seen that sulfhydryl inhibitors have no effect upon the reactions. Therefore, sulfhydryl groups are not involved in the reaction mechanism.

The activity towards leucine, isoleucine, and valine can not be due to degradation of these amino acids to acetate followed by acetate activation with accompanying pyrophosphate exchange similar to that of Berg (45). This

Table 16. Sulfhydryl Studies on Dialyzed Spinach Acetone Powder Extracts

Substrate	μ moles pyrophosphate exchanged/10 min at 37°C
L-Cysteine	.21
Glutathione	.14
2-mercaptoethanol	.13
no amino acids added	.13
15 amino acids	.70
15 amino acids .1 μ mole iodoacetate	.70
no amino acids .1 μ mole iodoacetate	.13

The enzyme was prepared by 10:1 extraction of spinach acetone powder with .1 M tris-HCl pH 7.5. The resulting enzyme solution was dialyzed against .1 M tris-HCl pH 7.2 for 7 and 1/2 hours at 2°C and then frozen until assayed. One half ml aliquots were assayed as in Table 4. Two μ moles of each of the substrates were added (2 μ moles of each of 15 amino acids in the 15 amino acid mix).

is proved by the observation that dialyzed preparations capable of activating leucine, isoleucine, and valine show no enhanced pyrophosphate exchange when incubated with sufficient acetate or acetate and CoA (Pabst Laboratories, Milwaukee, Wisconsin) to saturate any acetate activating system.

The activity of the activating enzymes is specific for the L isomers (where tested) of the individual amino acids. The D isomers are inactive in enhancing exchange and do not inhibit the exchange catalyzed by the corresponding L isomers. Table 17 illustrates this point.

It is interesting to consider whether the activation of each amino acid is carried out by an enzyme specific for that one L amino acid or whether the activating enzymes have a wider specificity and activate more than one amino acid. It has already been shown that some degree of separation of specific activating activities can be obtained by $(NH_4)_2SO_4$ fractionation. Davie et al. have isolated a purified enzyme largely specific to the activation of L-tryptophan (53). Schweet has isolated a pure enzyme specific to the activation of L-tyrosine (57). These observations suggest that there may be individual enzymes specific for the activation of each L amino acid.

In contrast to this theory, it can be seen in Table 15 that addition of the activities expressed towards 15 of the individual L amino acids yields a much larger value of

Table 17. The Effect of the D isomers of Amino Acids upon Pyrophosphate Exchange in Dialyzed Spinach Acetone Powder Extracts

Substrate	μ moles of pyrophosphate exchanged/10 min at 37°C
no amino acids added	.10
L-Leucine	.29
L-Leucine + D-Leucine	.29
D-Leucine	.10
L-Isoleucine	.27
L-Isoleucine + D-Isoleucine	.27
D-Isoleucine	.11

Enzyme prepared by 10:1 extraction of spinach acetone powder with .1 M tris-HCl at pH 7.5. The enzyme was prepared in the manner of Table 16. One μ mole of each of the substrates listed was added to the reaction mixture. Assay as in Table 4.

exchange than can be obtained from the same 15 amino acids assayed as a group. Such an observation has also been made by Demoss & Novelli (52). There are at least two ways to account for this apparent discrepancy.

1. The enzymes involved are specific for more than one amino acid.
2. The activation of the individual amino acid by individually specific enzymes is inhibited by the presence of other amino acids.

The complete answer as to which hypothesis is correct must await the isolation and assay of pure activating enzymes, but the data presented in Table 13 support the second hypothesis.

C. Attempts to Link Amino Acid Activation with Protein Synthesis

1. Amino Acid Dependent Exchange of AMP into ATP

In the previous section it has been established that plant tissues are well endowed with the ability to activate amino acids. Now it is necessary to consider the linking of this energy donating system to protein synthesis.

The hypothesis of Holley (60) states that amino acid activation is followed by the transfer of the activated amino acid to some RNAase sensitive acceptor (X) releasing the AMP involved in the initial activation of the amino acid as shown in equations 1 and 6.

$$\text{Enz} + \text{ATP} + \text{AA} \rightleftharpoons \text{Enz-AMP-AA} + \text{PP} \qquad (1)$$

$$\text{Enz-AMP-AA} + \underbrace{\text{X} \rightleftharpoons}_{\text{RNAase sensitive}} \text{Enz} + \text{AMP} + \text{X-AA} \qquad (6)$$

If this is in fact the mechanism and if the preparation under study contains the acceptor (X), it should then be possible to demonstrate an amino acid dependent exchange of AMP into ATP. Such an exchange must be measured above the non amino acid dependent exchange of AMP into ATP catalyzed by the enzyme adenylic kinase as follows:

$$\text{ATP} + \text{AMP} \rightleftharpoons 2\ \text{ADP} \qquad (7)$$

Initial preparations of tissues usually have such a high level of non amino acid dependent exchange that it is necessary to fractionate away from adenylic kinase activity in order to detect any small level of amino acid dependent exchange that might be present. Holley precipitated the preparation at pH 5.1 and resuspended at a higher pH to fractionate away the adenylic kinase.

Extracts of spinach and tobacco acetone powders also have a very active adenylic kinase, thus making the detection of an amino acid dependent AMP exchange difficult. Precipitation of the spinach extract at pH 5.1 and resuspension fail to yield any amino acid dependent exchange

of AMP into ATP. This is to be expected for it has already been shown that precipitation of spinach acetone powder preparations at pH 5 inactivates the enzymes involved in amino acid activation.

Intact tissue homogenates offer a better source of material for study of amino acid dependent exchange of AMP into ATP. They can be precipitated at pH 5.0 and resuspended without total loss of amino acid activating activity. Also, the chance of destruction or loss of the RNAase sensitive acceptor by acetone powder formation is avoided. Homogenates of spinach, tobacco, or pea epicotyls show no amino acid dependent exchange of AMP into ATP. Preparations made by precipitation of the enzymes from the soluble supernatant of spinach at pH 5 and resuspension at pH 7.5 once or twice (designated pH 5_I and pH 5_{II}) also do not exhibit any amino acid dependent AMP exchange.

The homogenates and even the pH 5 precipitated and resuspended enzymes contain large amounts of free amino acids. The possibility still exists that an amino acid dependent exchange does take place, but that the system is saturated with amino acid. If this were the case, then RNAase treatment of the preparation should inactivate the amino acid dependent exchange of AMP into ATP by removing the RNAase sensitive acceptor. Preincubation of homogenate or of pH 5_I preparations from spinach with 10 gamma of RNAase/ml of enzyme for 5 minutes at 25°C does not affect the AMP

exchange exhibited by these preparations. Table 18 summarizes these data.

2. Coupling of Spinach Amino Acid Activating Enzymes to Various Tissues

Experiments in which it was attempted to couple amino acid activating enzymes obtained from spinach to particulate preparations from various plant sources have been in general unsuccessful. Spinach acetone powder extracts do not cause incorporation of labeled leucine into the proteins contained in Hevea latex. Soluble enzyme preparations do not enhance the incorporation of leucine into pea microsomal preparations (75) washed in the manner of Hoagland et al. (58) even when an ATP regenerating system and a mixture of nucleotide triphosphates are included in the incubation mixture. Table 19 illustrates this point.

It is quite probable that some further essential factor is missing or that the amino acid activating enzymes are species specific. In either case, it would be impossible to link spinach amino acid activating enzymes to other systems.

3. Activity of Spinach Preparations towards L-Leucine-C^{14}

Another way to implicate the amino acid activating enzymes is to study systems obtained from only one tissue, spinach. In this case it has been possible to obtain in-

Table 18. Exchange of AMP into ATP in Spinach Homogenate Preparations

	Minutes at 37°C	Counts/min/ mg Ba-ATP	Counts/min/mg Ba-ATP/mg Protein
homogenate + AA	0	7	1
homogenate + AA	5	128	24
homogenate + AA + RNAase	5	125	23
homogenate (only)	5	132	24
$pH5_I$ + AA	0	5	1
$pH5_I$ + AA	5	153	21
$pH5_I$ + AA + RNAase	5	158	22
$pH5_I$ (only)	5	156	22

Original homogenate prepared as in the section on methods in .01 M tris-HCl. The $pH5_I$ enzyme was prepared by lowering the pH of the homogenate to pH 5.1 in the cold with .5 M HCl and sedimenting the precipitate at 10,000 g for 10 minutes. Resuspension of the precipitate in .01 M tris-HCl pH 7.5 in a homogenizer in 30% of the original volume was followed by titration to pH 7.5 and clarification of the solution by centrifugation at 15,000 g for 10 minutes. The resultant supernatant is designated as $pH5_I$ enzyme. Assay is that of Holley (60).

Table 19. Incorporation of L-Leucine-C^{14} into Pea Microsomes

	Counts/min/mg of microsomes		
	0 min	20 min	40 min
Pea microsomes alone	6.1	15.1	15.2
Pea microsomes + spinach activating enzyme	4.4	10.7	13.2

Spinach activating enzymes were prepared in .001 M tris-HCl at pH 7.5 as in the section on methods, final pH 7.0. Pea microsomes were prepared in the manner of Ts'o et al. (75). The reaction mixture of .44 ml at pH 7.5 contained 5 μ moles of ATP-K salt, 5 μ moles of KF, 5 μ moles of $MgCl_2$, 2 μ moles of creatine phosphate and .012 mg of creatine kinase isolated from rabbit muscle (creatine kinase added as .02 ml of .05 M cysteine free base, 5×10^{-4} glycine buffer pH 9.0), .0912 μ moles of L-Leucine-C^{14} (970,000 counts/min) and .25 μ moles of GTP, CTP and UTP as Na salts. To this was added either .2 ml of a 1% solution of microsomes or .2 ml of microsomes plus .45 ml of spinach activating enzyme. Incubation was at 37°C. The reaction was stopped by the addition of 1 ml of 6% TCA in the cold. The resulting precipitates were washed in the manner of Hoagland et al. (58) or 3 times in the cold with 2 ml washes of .2 M perchloric acid, followed by one wash with 2 ml of ethanol : .2 M perchloric acid 5:1 at room temperature and one wash at 50°C for 2 minutes in 2 ml of ethanol : ether 3:1. The washed precipitates were resuspended in water and plated out on tared planchets. The measured counts were divided by the weights of microsomes (average weights in the case of added amino acid activating enzymes) to obtain the final results.

corporation of a labeled amino acid into a bound form. When one incubates the 5,000 g supernatant prepared from spinach leaves in .001 M tris-HCl (the best buffer strength for the amino acid activation system) at a final pH of 7.0 with L-Leucine-C^{14}, one can obtain a consistently repeatable, energy dependent, and RNAase sensitive incorporation. Figure 12 shows this incorporation in a preparation of the 5,000 g supernatant washed in the rigorous manner of Hoagland et al. (58).

The finding of such an energy dependent, RNAase sensitive incorporation in a crude system known to contain an abundant supply of activating enzymes raises the question of the exact mechanisms of the incorporation. The RNAase sensitivity implicates some RNA factor such as the soluble RNA (S-RNA) proposed by Hoagland et al. (58). This would then justify formulation of the following series of reactions:

$$\text{ENZ} + \text{AA} + \text{ATP} \rightleftharpoons \text{ENZ-AMP-AA} + \text{PP}$$

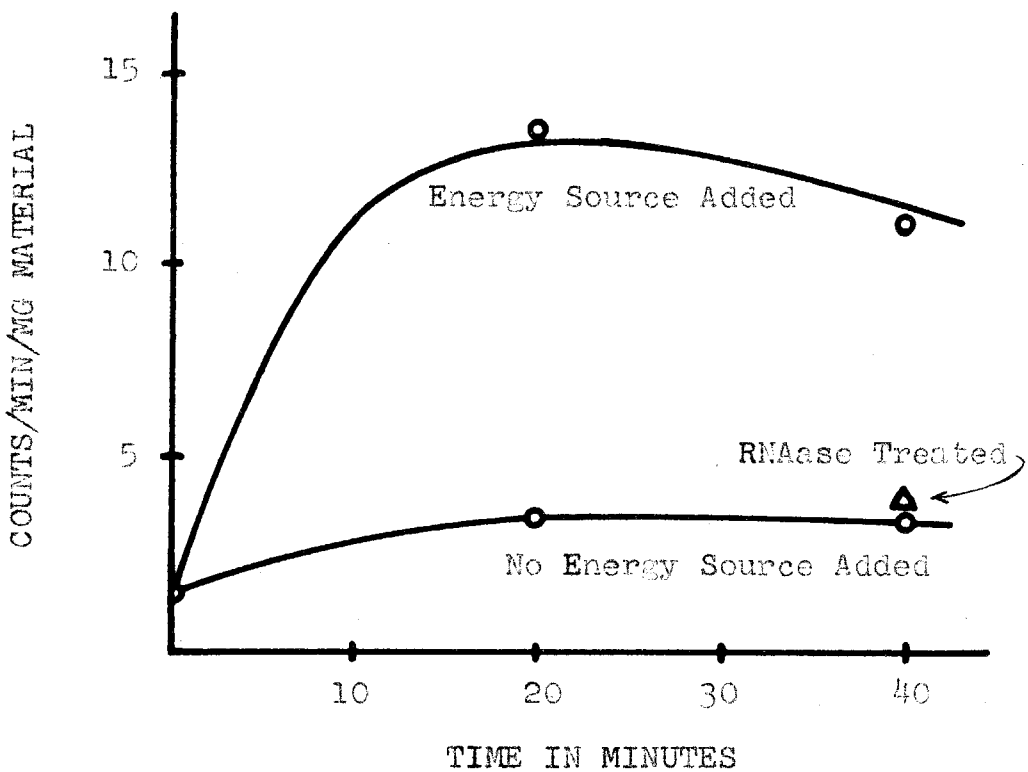
$$\text{ENZ-AMP-AA} + \text{S-RNA} \rightleftharpoons \text{ENZ} + \text{AMP} + \text{S-RNA-AA}$$

$$\text{S-RNA-AA} + \text{particulate system} \rightleftharpoons \text{particulate-AA} + \text{S-RNA}$$

The exact nature of the third equation is extremely vague.

Even so, if the scheme does represent reality, it should be possible to determine the presence of amino acid bound to the S-RNA as described in the second equation. This is in fact the case.

Figure 12. Incorporation of L-Leucine-2-C^{14} into 5,000 g Supernatant of Spinach Homogenate



The 5,000 g supernatant at final pH 7.0 in .001 M tris-HCl was prepared as in the sections on methods. The reaction mixture, assay and washing procedure are identical with Table 19. The measured counts were divided by the weights of protein obtained to yield the final results.

Two approaches to this problem have been tried. In the first, labeled amino acid and appropriate substrates are fed to an enzyme system obtained from acetone powder of spinach. The system lacks any form of particulate material; therefore the third reaction is excluded. The enzyme had previously been treated with charcoal to remove harmful pigments, and then dialyzed to remove the free unlabeled amino acids. This preparation still contains a high concentration of nucleic acid as shown by its $\frac{280\ m\mu}{260\ m\mu}$ ratio of .72. The reaction is stopped by lowering the pH to pH 4 in the cold. The precipitated material is then washed three times with a 47.5% ethanol 1% perchloric acid solution and plated out. The results are summarized in Table 20.

These results are qualitatively interesting although their exact quantitative significance is questionable. One hundred fifty-three counts of leucine represents 3×10^{-6} μ moles of leucine incorporated into some bound form in the preparation. It is possible that this value in part represents that leucine retained in the activated form, leucyl-AMP. It may on the other hand represent transfer of leucine to some other acceptor such as S-RNA.

A preparation from intact tissue should not have suffered the harsh treatment involved in acetone powder formation. Such preparations might therefore offer a better way to get at the fate of the activated amino acid. It

Table 20. Results of Incubation of C^{14} Labeled Leucine with Dialyzed Spinach Acetone Powder Preparation

	time at 37°C	counts/min in precipitate
Enzyme + ATP	0	40
Enzyme + ATP	10 min	153
Enzyme - ATP	10 min	27
Inert Protein Solution	10 min	50

Enzyme prepared by 10:1 extraction of powder with .1 M tris-HCl pH 7.5, followed by charcoal treatment (4.5 mg charcoal/ml enzyme, charcoal removed by centrifugation) and dialysis for 7.5 hours against .1 M tris-HCl pH 7.2. The enzyme was frozen at -80°C and stored at -25°C before use. Incubation solution contained 10 μ moles of ATP-K salt where indicated, 10 μ moles of KF, 50 μ moles of tris-HCl, 5 μ moles of $MgCl_2$, .25 μ moles of L leucine C^{14} ($\sim$ 1,300,000 counts/min) all in .4 ml at pH 7.5. To this was added .7 ml of enzyme in .1 M tris-HCl at pH 7.2. Incubation was stopped by addition of .1 ml of 6% TCA (resulting pH 4.1-4.0). The resulting precipitate was washed 3 times with .5 ml washes of 47.5% ethanol 1% perchloric acid in the cold. The inert protein control used was a mixture of ovalbumin and protamine sulfate at pH 7.2 (7 mg/ml). This experiment has only been performed once and the results reflect single samples rather than duplicates.

should be possible to prepare from homogenates, particle free supernatants, and to demonstrate the transfer of activated amino acids to an acceptor such as the soluble RNA of Hoagland et al. (58).

This possibility is tested in the following manner. Labeled leucine is incubated with the soluble enzymes of a spinach homogenate in the presence or absence of ATP, an ATP regenerating system, and catalytic amounts of the other nucleotide triphosphates. An accompanying incubation of an RNAase treated enzyme with the appropriate energy sources is also run. All reactions are stopped by the addition of an equal volume of 6% TCA in the cold and then washed in the drastic (including room temperature and 50°C) manner of Hoagland et al. (58). Even under these conditions, it is possible to show an ATP dependent, RNAase sensitive incorporation of leucine into some bound form as shown in Table 21.

The exact quantitative significance of these data is not clear due to the fluctuation in duplicates. The qualitative result is nonetheless quite clear. There can be therefore little doubt that it is possible to obtain incorporation into some soluble, RNAase sensitive factor obtained from spinach homogenates. This incorporation is energy dependent indicating that the abundant amino acid activating enzymes are involved. This serves

Table 21. Incorporation of L-Leucine-C^{14} into Protein Intermediates by Soluble Enzymes of Spinach.

	t at 37°C	counts/min/mg material
Supernatant + ATP + ~Pi source	0	5.4
Supernatant + ATP + ~Pi source	15	18.9
RNAase treated supernatant + ATP + ~Pi source	15	4.2
Supernatant only	15	3.9

The supernatant enzyme was prepared from a homogenate of intact spinach in .001 M tris-HCl as described in the section on methods, final pH 6.8. The reaction mixture contained 5 μ moles of KF, 5 μ moles of $MgCl_2$, 5 μ moles of tris-HCl, 1.5 μ moles of Cysteine free base, .0912 μ moles of L-Leucine-C^{14}(970,000 counts/min) and where indicated 5 μ moles of ATP-K salt, .5 μ moles of GTP, CTP, and UTP (Na salts), 2 μ moles of creatine phosphate (Na salt), and .012 mg of creatine kinase isolated from rabbit muscle in a final volume of .44 ml at pH 7.5. To this was added .65 ml of soluble enzyme. The RNAase treated enzyme had previously been incubated for 5 minutes at 25°C with 100 gamma of RNAase (Armour Labs., Chicago). Incubation was at 37°C. The reaction was stopped by the addition of 1 ml of 6% TCA in the cold. The resulting precipitate was washed in the manner of Hoagland et al. (58) as described in Table 19. The counts obtained are divided by the weights of the material in order to obtain the results presented.

as preliminary verification in a plant system of the scheme for amino acid incorporation proposed by Hoagland et al. (58) in which the activated amino acid is transferred to soluble RNA and thence on into protein.

IV. DISCUSSION

This thesis has presented evidence for the presence of amino acid activating enzymes in plant tissues. The possible implication of these enzymes in a system that incorporates amino acids into proteins has also been discussed. It is now possible to consider some of the quantitative aspects of the information presented.

Firstly, the decrease in the observed rate of PP exchange with time presented in Figure 6 can be interpreted in light of other evidence presented in this thesis. This decrease in rate cannot reflect removal of ATP since there is no significant loss of ATP after 30 minutes of incubation. On the other hand, there is extensive destruction of the pyrophosphate present by the active pyrophosphatase (Figure 1). Therefore, it is possible that the drop in the rate of exchange during incubation reflects, in fact, the gradual removal of pyrophosphate from the system. Pyrophosphate is required to drive the exchange reaction, and removal of the radioactive pyrophosphate prior to attainment of full equilibrium will decrease the rate of exchange.

Secondly, study of the rates of amino acid activation obtained yields information about the equilibrium of the amino acid activation reaction. In order to derive this information, it is necessary to make three assumptions,

all reasonable. First, one must assume that the maximum rate of *in vitro* exchange obtained is not in excess of the *in vivo* rate. Second, that the rate of exchange of pyrophosphate represents an equal rate of amino acid activation. Third, that an average molecular weight of an amino acid is 150. With these assumptions it is possible to make the following calculation.

The maximum rate of PP exchange obtained from spinach is

1.96 μ moles PP exchanged/mg protein/10 minutes

which equals

282 μ moles PP exchanged/mg protein/day

If we assume that an amino acid has a molecular weight of 150 and that PP exchange represents amino acid activation, then

$$150 \frac{\text{grams}}{\text{mole}} \times 282\ \mu \text{ moles} = .0423 \text{ grams AA activated/mg protein/day}$$

$$= 42.3 \text{ mg of AA activated/mg protein/day}$$

This value is clearly greater than that which would be needed to account for *in vivo* protein synthesis. No variety of spinach known exhibits a 42-fold increase in protein content within a day. The rate of amino acid activation

is clearly in excess of that of protein synthesis. There must therefore be some step in the mechanism of protein synthesis that limits the overall synthesis of proteins. This can be either a rate limiting reaction or an unfavorable equilibrium.

It is possible that the equilibrium of the activation reaction is far to the left or so:

$$\text{Enz} + \text{ATP} + \text{AA} \longleftrightarrow \text{Enz-AMP-AA} + \text{PP}$$

This may be checked in three ways. Firstly, this hypothesis in another form states that the free energy of an acyl phosphate bond is greater than that of a phosphate anhydride. This is verified by study of the analogous phosphoglycerokinase reaction (78).

$$\text{1,3-diphosphoglyceric acid} + \text{ADP} \xrightleftharpoons{\text{Enz}} \text{ATP} + \text{PGA}$$

$$K = 3 \times 10^{3}, \text{ therefore } \Delta F = -4.9 \text{ Kcal/mole}$$

in which the acyl phosphate is known to be in a higher energy state than the phosphate anhydride of ATP.

A second check of this hypothesis is to be found in the very high levels of hydroxylamine needed in the hydroxylamine trapping reaction. Hydroxamate formation experiments are carried out in 1 M $NH_2OH \cdot KCl$. This high concentration of hydroxylamine is necessary to trap the small amounts of activated amino acid present even in the presence of 10 μ moles of ATP and excess amino acid.

A third proof of the hypothesis is to be found in an amino acid incorporation experiment in a system obtained from the soluble fraction of guinea pig liver (55). It is possible to fractionate this system into two fractions. The first fraction activates leucine but does not incorporate leucine into soluble RNA. The second fraction obtained by heat treatment is largely nucleic acid and does not activate or incorporate leucine. When fractions one and two are combined, they together possess the ability to incorporate leucine into the soluble RNA of the second fraction. Therefore this system provides a separable acceptor for the activated amino acid.

When an amount of the leucine activating fraction sufficient to activate 1.20 μ moles of leucine/10 mins (measured by pyrophosphate exchange) is incubated with a fixed amount of the S-RNA fraction, only .89 mμ moles of leucine are incorporated in 10 minutes. When lower amounts of the activating fraction are used, the level of leucine incorporation drops proportionally. Therefore, even with what appears to be an excess of activating activity as measured by pyrophosphate exchange, the rate limiting step in the incorporation is the amino acid activation reaction. This again indicates that the equilibrium of the activation equation is in favor of the free amino acid. The pyrophosphate exchange which measures the rate of formation of the activated amino acid, does not give a true impression

of the equilibrium of the reaction. The pyrophosphate exchange reaction is overly efficient. Each activation is recorded because the activated amino acid is driven back to the free state by the 5 μ moles of pyrophosphate in the mixture.

This hypothesis that the activation equilibrium is far to the left would also account in part for the excessive specific activity of amino acid activating preparations for pyrophosphate exchange compared to that of the same enzymes for hydroxamate formation (52,53,54). Either of two theories or a combination of them can account for this result. Firstly, it has already been shown that the pyrophosphate exchange assay is overly efficient because every activation is recorded. If the hydroxylamine used in the hydroxamate assay does not trap out each activated amino acid formed, before the nature of the equilibrium removes it, some specific activations would go unrecorded. This would cause the specific activity as measured by the hydroxamate assay to be lower than that obtained from the pyrophosphate exchange assay. Secondly, hydroxylamine trapping reactions are carried out in high salt and hydroxylamine concentrations. It is quite likely that the high concentration of hydroxylamine operates effectively in trapping out most of the activated amino acid. On the other hand the high salt or hydroxylamine concentration could tend to lower the rate of activation by affecting

the enzymes. The pyrophosphate exchange assay does not have such high concentrations of material and would not be inhibited in this manner. Therefore, a higher specific activity would be obtained from the pyrophosphate exchange assay.

Preliminary evidence presented in this thesis indicates that there are specific activating enzymes for each amino acid, but that their activities towards specific amino acids are affected by the presence of other amino acids. Thus the total specific activity obtained when the amino acids are assayed individually is greater than when they are assayed as a group. Further support of this hypothesis is supplied by the work of Nisman (79) with a lysed E. coli preparation. Nisman has also found inequality between the total activity expressed towards individual amino acids assayed separately and total activity expressed by the same preparation towards the same amino acids as an equimolar group. When the relative proportions of the individual amino acids in the mixture are adjusted to resemble that present in E. coli, then the inequality between the summed rate and combined rate is reduced. This again indicates that the specific activation of one amino acid is influenced by the presence of another amino acid. Further purification of the lysed E. coli system shows this, in fact, to be the case. Amino acids, not activated by particular enzyme fractions, can inhibit the activation of the

amino acids normally activated by these fractions. These findings then support the hypothesis that activation of amino acids is carried out by specific enzymes, and that the rates of activation of individual amino acids are influenced by the presence of other amino acids.

If the activation of each amino acid requires a specific enzyme and activation is necessary for synthesis, then it follows that it should be possible to detect activating enzymes for each amino acid that participates in protein synthesis. The systems so far isolated have been limited in the number of activities expressed towards amino acids (74,80). In contrast, the system obtained from spinach preparations is active towards a wide spectrum of amino acids. Only serine, among the amino acids assayable, is lacking in activity. The possible activation of the serine is not eliminated. The level of serine activating activity could easily fall below the detectable level of assay in this preparation. The argument that amino acid activation is not involved in protein synthesis because activity towards all the amino acids is lacking is eliminated now that a tissue preparation has been found containing activity towards a wide spectrum of amino acids. This observation plus the finding of amino acid activating enzymes in all the tissues investigated serves to further implicate the amino acid activating enzymes with protein synthesis.

The incorporation of labeled amino acids into soluble RNAase sensitive material reported in this thesis resembles that reported by Hoagland et al. (58) and by Schweet (55). The exact nature of the incorporation is not known at present, but it is possible to hypothesize as to the nature of the linkages. The amino acid is presumedly linked through its carboxyl group since this is the group where activation has taken place. The acceptor is apparently RNA as indicated by the RNAase sensitivity of the incorporation, and Schweet's (55) finding that RNA obtained from his preparation by phenol extraction is active as an acceptor. Preliminary findings indicate that the soluble RNA possesses specificity towards individual amino acids (55). There appear to be four possible sites for linkage of carboxyl groups to RNA. These are:

1. The doubly esterified phosphates
2. The singly esterified terminal phosphates
3. The 2' or 3' hydroxyl groups of the ribose units
4. The function groups of the bases

The possibility of linkage through several of these sites can be reduced or eliminated on various grounds. Linkage to the doubly esterified phosphates of RNA is largely excluded due to the known lability of tertiary phosphates. The singly esterified terminal phosphates are also partially eliminated as a final site of the activated amino acids. This can be done on both theoretical and chemical

grounds. Terminal groups would not be as susceptible to specificity determination by the adjacent bases due to their location. Also, nucleotide 5' acyl amino acids rapidly convert themselves spontaneously to nucleotide 2' amino acid esters in aqueous solution (81). There would be no terminal 3' phosphate in non degraded, polynucleotide phosphorylase synthesized RNA. Due to the structure of RNA, only a terminal ribose would have an unesterified 3' hydroxyl. This leaves only the 2' hydroxyl groups of the ribose and the functional groups of the bases as logical sites on RNA for the reception of the activated amino acid.

On strictly theoretical grounds the 2' hydroxyl groups appear to be the most logical site for the activated amino acid. The functional groups of the bases should form two types of groups, amides and esters, with carboxyl activated amino acids. It is more logical to assume that only one general type of bond is formed by activated amino acids. Then the specificity would be determined by the bases adjoining the bond forming site rather than allowing the nature of the bond to influence the specificity. Secondarily, one must ask the question, why is RNA the acceptor rather than say DNA. This could be specifically because RNA has a 2' hydroxyl group.

It is necessary to also consider the indirect chemical evidence relating to the possible site of the amino acid.

The washing procedures used to isolate the S-RNA-AA complex involve weak acid washes performed at 0°C. This procedure implies a bonding of the amino acid to the soluble RNA through a more unstable bond than the ester linkage proposed above, such as a terminal acyl phosphate anhydride. It is not known whether the extreme caution used in the washing procedures is, in fact, necessary. The final answer as to what is the nature of the bond between the activated amino acid and the soluble RNA must await further investigation.

It has not been possible in this thesis to obtain a successful amino acid dependent exchange of AMP into ATP. On the other hand the same soluble preparations show a RNAase sensitive energy dependent incorporation of amino acids. This can be construed in part as evidence backing the 2' hydroxyls or sites of equal energy as the site of the activated amino acid on soluble RNA. An ester linkage of the amino acid to soluble RNA through a 2' hydroxyl group would be at a lower energy state than that of the acyl phosphate of the activated amino acid. Amino acids bond as esters would therefore be less likely to carry out an amino acid dependent exchange of AMP into ATP in the manner described by Holley (13). Only a tissue with both an active activation of amino acid and an active transfer of amino acids to soluble RNA would be expected to catalyze such a transfer if the 2' hydroxyl of the ribose of the

soluble RNA were the acceptor of the activated amino acid. Hoagland (58) has already shown that the rat liver preparations used by Holley have these activities in large amounts. In contrast, the spinach preparations presented in this thesis do not catalyze extensive exchange of the activated amino acid to RNA. The lack of activity in the exchange of AMP is therefore not unreasonable if the 2' hydroxyl is the final site of activated amino acid on RNA.

These data are therefore in accord with the concept that protein synthesis does in fact proceed by a series of sequential reactions. The initial activation of the individual amino acids is followed by a transfer of the activated amino acids to a soluble RNA. The nature of the fate of the activated amino acids beyond this point is vague, but appears to involve particulate matter (58), apparently the microsomes.

APPENDIX

Derivation of the Expression Equating % Exchange with Actual Exchange

A. Given symbols and facts

1. Let:

(A) = number of μ moles initially labeled compound

(B) = number of μ moles of ATP

α = number of labeled μ moles in pool A or activity

β = number of labeled μ moles in pool B or activity

then it follows that:

$$\alpha_o = \alpha + \beta \qquad \text{or} \quad \beta = \alpha_o - \alpha$$

where α_o = activity of pool A at time 0

2. Pools A & B exchange at a given rate R or:

$$A \overset{R}{\rightleftharpoons} B$$

3. Then the rate times the duration of exchange will equal the actual or total exchange

B. We want an expression for $\frac{d\alpha}{dt}$ that involves both the decrease in α due to removal of label and the increase in α due to return of label from pool B so:

1. $$\frac{d\alpha}{dt} = -R\frac{\alpha}{(A)} + R\frac{\beta}{(B)}$$

where:

$$\frac{\alpha}{(A)} = \text{fraction hot in A,}$$

$$\frac{\beta}{(B)} = \text{fraction hot in B}$$

2. Substituting $\beta = \alpha_o - \alpha$ yields:

$$\frac{d\alpha}{dt} = -R\frac{\alpha}{(A)} + R\left(\frac{\alpha_o}{(B)} - \frac{\alpha}{(B)}\right)$$

$$= -R\alpha\left(\frac{1}{(A)} + \frac{1}{(B)}\right) + R\frac{\alpha_o}{(B)}$$

3. If we now define two constants

$$x = R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)$$

and

$$y = \frac{\alpha_o}{(B)} R$$

4. Then the expression of section 2 reduces to the form

$$\frac{d\alpha}{dt} = -x\alpha + y$$

5. Which upon integration yields

$$\alpha = Ce^{-xt} + \frac{y}{x}$$

where C is an arbitrary constant which cannot be solved from the differential equation alone.

6. C can be solved by considering the initial conditions at time O.

when $t = 0$ $\alpha = \alpha_o$

therefore substituting into the equation of 5

$$\alpha_o = Ce^{-x \cdot 0} + \frac{y}{x}$$

but $e^0 = 1$, therefore:

$$C = \alpha_o - \frac{y}{x}$$

or upon substitution of the values for y and x

$$C = \alpha_o - \frac{\frac{\alpha_o}{(B)} R}{R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)}$$

$$= \alpha_o - \frac{\alpha_o}{(B)\left(\frac{1}{(A)} + \frac{1}{(B)}\right)}$$

$$= \alpha_o - \frac{\alpha_o(A)}{(A) + (B)}$$

7. Substituting the values of x and y and C into the equation of 5 yields:

$$\alpha = \left(\alpha_o - \frac{\alpha_o(A)}{(A)+(B)}\right) e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right) t} + \frac{\frac{\alpha_o}{(B)} R}{R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)}$$

$$= \left(\alpha_o - \frac{\alpha_o(A)}{(A)+(B)}\right) e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right) t} + \frac{\alpha_o(A)}{(A)+(B)}$$

8. The quantity measured in an experiment is the percent or fraction of exchange into pool B, $\frac{\beta}{\beta_\infty}$ where β_∞ = the activity of pool B at 100% exchange or at time infinity. Therefore it is necessary to obtain an expression involving only the measurable quantities % exchange, (A), (B), t, etc.

Therefore we proceed by substituting $\beta = \alpha_o - \alpha$ into the equation of 7 so:

$$\beta = \alpha_o - \left[\left(\alpha_o \frac{\alpha_o(A)}{(A) + (B)}\right) e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)t} + \frac{\alpha_o(A)}{(A)+(B)}\right]$$

then factoring out an α_o

$$\beta = \alpha_o \left[1 - e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)t} + \frac{(A)}{(A)+(B)} e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)t} - \frac{(A)}{(A)+(B)} \right]$$

followed by factoring the part of the equation in brackets:

$$\beta = \alpha_o \left[1 - \frac{(A)}{(A)+(B)} \right] \left[1 - e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)t} \right]$$

and simplifying the first two terms on the right yields:

$$\beta = \frac{\alpha_o (B)}{(A)+(B)} \left[1 - e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right) t} \right]$$

9. Now consider the state of the final equation of 8 at time infinity. Substituting $t = \infty$ into this equation from section 8 yields

$$\beta = \frac{\alpha_o (B)}{(A)+(B)} \left[1 - e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)\infty} \right]$$

but since $e^{-\infty} = 0$, the above equation reduces to:

$$\beta_\infty = \frac{\alpha_o (B)}{(A)+(B)}$$

where β_∞ equals the activity in pool B at infinite time.

10. It is now possible to obtain an expression involving only the measurable quantities and the rate. This is achieved by substituting equation 9 into the equation of section 8 so:

$$\beta = \beta_\infty \left[1 - e^{-R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)t} \right]$$

or upon the substitution of $x = R\left(\frac{1}{(A)} + \frac{1}{(B)}\right)$

$$\frac{\beta}{\beta_\infty} = 1 - e^{-xt} = \text{fraction exchange}$$

or

$$e^{-xt} = (1 - \text{fraction exchange})$$

which when converted to natural logs reduces to the form:

$$\ln (1 - \text{fraction exchange}) = - xt$$

or substituting the value for x:

$$\ln (1 - \text{fraction exchange}) = -R \left(\frac{1}{(A)} + \frac{1}{(B)}\right) t$$

11. When the equation of 10 is rearranged it takes the form:

$$Rt = \frac{-\ln\ (1 - \text{fraction exchange})}{\frac{1}{(A)} + \frac{1}{(B)}}$$

But the total or actual exchange is equal to the rate times the time, therefore

$$\text{total exchange} = \frac{-\ln\ (1 - \text{fraction exchange})}{\frac{1}{(A)} + \frac{1}{(B)}}$$

which when converted to log to the base 10 and rearranged equals:

$$\text{total exchange} = -2.303\ \frac{(A)(B)}{(A)+(B)}\ \log_{10}\left(\begin{array}{l}1 - \text{frac-}\\ \text{tion}\\ \text{exchange}\end{array}\right)$$

REFERENCES

1. Borsook, H., In Greenberg, D. M., Chemical Pathways of Metabolism Vol. 2, pp. 173-222, Academic Press, New York (1954)
2. Borsook, H., Physiol. Revs. 30: 206-219 (1950)
3. Virtanen, A. I. and Kerkkonen, H. K., Nature 161: 888-889 (1948)
4. Bergman, M. and Fruton, J. S., J. Biol. Chem. 124: 321-329 (1938)
5. Fruton, J. S., Johnston, R. B., and Fried, M. J., J. Biol. Chem. 190: 39-53 (1957)
6. Dowmont, Y. P., and Fruton, J. S., J. Biol. Chem. 197: 271-283 (1952)
7. Hanes, C. S., Hird, F. J. R., and Ischerwood, F. A., Biochem. Jour. 51: 25-35 (1952)
8. Chantrenne, H., J. Biol. Chem. 189: 227-233 (1951)
9. Block, K. and Anker, H. S., J. Biol. Chem. 169: 765-766 (1947)
10. Snoke, J. E. and Block, K., J. Biol. Chem. 213: 825-835 (1955)
11. Webster, G. C. and Varner, J. E., Archives of Biochem. & Biophy. 52: 22-32 (1954)
12. Webster, G. C. and Varner, J. E., Archives of Biochem. & Biophy. 55: 95-103 (1955)
13. Speck, J. F., J. Biol. Chem. 179: 1405-1426 (1949)
14. Elliott, W. H., J. Biol. Chem. 201: 661-672 (1953)

15. Webster, G. C. and Varner, J. E., J. Amer. Chem. Soc. 76: 633 (1954)

16. Webster, G. C. and Varner, J. E., J. Biol. Chem. 215: 91-99 (1955)

17. Maas, W. K., J. Biol. Chem. 198: 23-32 (1952)

18. Shimura, K., Koide, F., Itabashi, H., and Hironobu, F., J. of Biochem. (Japan) 42: 285-294 (1955)

19. Frantz, I. D., Zamecnik, P. C., Reese, J. W., and Stephenson, M. C., J. Biol. Chem. 174: 773-774 (1948)

20. Siekevitz, P., J. Biol. Chem. 195: 549-565 (1952)

21. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., J. Biol. Chem. 196: 669-694 (1952)

22. Rabinovitz, M., Olson, M. E., and Greenberg, D. M., J. Biol. Chem. 213: 1-9 (1955)

23. Winnick, T., Friedberg, F., and Greenberg, D. M., J. Biol. Chem. 175: 117-126 (1948)

24. Spiegelman, S., Cold Spring Harbor Symposia Quant. Biol. 11: 256-274 (1946)

25. Osawa, S., Allfrey, V. G., and Mirsky, A. E., J. Gen. Physiol. 40: 491-513 (1957)

26. Peterson, E. A., Winnick, T., and Greenberg, D. M., J. Amer. Chem. Soc. 73: 503-504 (1951)

27. Zamecnik, P. C., and Keller, E. B., J. Biol. Chem. 209: 337-354 (1954)

28. Littlefield, J. W. and Keller, E. B., J. Biol. Chem. 224: 13-30 (1957)

29. Keller, E. B. and Zamecnik, P. C., J. Biol. Chem. 221: 45-59 (1956)

30. Allfrey, V. G., Proc. Nat. Acad. Sci. 40: 881-885 (1954)

31. Stephenson, M. L., Thimann, K. U., and Zamecnik, P. C., Archives of Biochem. & Biophy. 65: 194-209 (1956)

32. Lester, R. L., J. Amer. Chem. Soc. 75: 5448-5449 (1953)

33. Beljanski, M., Biochim. et Biophy. Acta 15: 425-431 (1954)

34. Gale, E. F. and Folkes, J. P., Biochem. Jour. 55:XI (1953)

35. Brachet, J., Nature 174: 876-877 (1954)

36. Allfrey, V., Daly, M. M., and Mirsky, A. E., J. Gen. Physiol. 37: 157-175 (1953)

37. Minagawa, T., Biochem. et Biophy. Acta 16: 539-552 (1955)

38. Gale, E. F. and Folkes, J. P., Biochem. Jour. 59: 661-675 (1955)

39. Gale, E. F. and Folkes, J. P., Biochem. Jour. 59: 675-684 (1955)

40. Pardee, A. B., Proc. Nat. Acad. Sci. 40: 263-270 (1954)

41. Allfrey, V. G. and Mirsky, A. E., Proc. Nat. Acad. Sci. 43: 589-598 (1957)

42. Brachet, J. and Chantrenne, H., Nature 168: 950 (1951)

43. Holloway, B. W. and Ribley, S. H., J. Biol. Chem. 196: 695-701 (1952)

44. Littlefield, J. W., Keller, E. B., Gross, J. and Zamecnik, P. C., J. Biol. Chem. 217: 111-123 (1955)

45. Berg, P., J. Amer. Chem. Soc. 77: 3163-3164 (1955)

46. Demoss, J. A., Genuth, S. M., and Novelli, G. D., Proc. Nat. Acad. Sci. 42: 325-332 (1956)

47. Peng, C. H. L., Biochim. et Biophy. Acta 22: 42-48 (1956)

48. Hilz, H. and Lipmann, F., Proc. Nat. Acad. Sci. 41: 880-890 (1955)

49. De Meio, R. H. and Wizerkanink, M., Biochim. et Biophy. Acta 20: 428-430 (1956)

50. Maas, W. K., Federation Proc. 15: 305-306 (1956)

51. Hoagland, M. B., Biochim. et Biophy. Acta 16: 288-289 (1955)

52. Demoss, J. A. and Novelli, G. D., Biochim. et Biophy. Acta 22: 49-61 (1956)

53. Davie, E. W., Koningsberger, V. V., and Lipmann, F., Archives of Biochem. & Biophy. 65: 21-38 (1956)

54. Schweet, R. S., Holley, R. W., and Allen, E. H., Archives of Biochem. & Biophy. 71: 311-325 (1957)

55. Schweet, R. S., Bovard, F. C., Fleshman, K., and Allen, E., J. Amer. Chem. Soc. (in press)

56. Berg, P., J. Biol. Chem. 222: 1025-1034 (1956)

57. Schweet, R. S., J. Biol. Chem. (in press)

58. Hoagland, M. B., Zamecnik, P. C., and Stephanson, M. L., Biochim. et Biophy. Acta 24: 215-216 (1957)

59. Jencks, W. P., Biochem. et Biophy. Acta 24: 227-228 (1957)

60. Holley, R. W., J. Amer. Chem. Soc. 79: 658-662 (1957)

61. Webster, G. C. and Johnson, M. P., J. Biol. Chem. 217: 641-649 (1955)

62. McRae, D. H. and Bonner, J., Physiol. Plantarum 6: 485-510 (1953)

63. Schweet, R. S., Biochim. et Biophy. Acta 18: 566 (1955)

64. Flynn, R. M., Jones, M. E., and Lipmann, F., J. Biol. Chem., 211: 791-796 (1954)

65. Crane, R. K. and Lipmann, F., J. Biol. Chem. 201: 235-243 (1953)

66. Allen, R. J. L., Biochem. Jour. 34: 858-865 (1940)

67. Cohn, W. E. and Carter, C. E., J. Amer. Chem. Soc. 72: 4273-4275 (1950)

68. Gornell, A. G., Bardawill, C. J., and David, M. M., J. Biol. Chem. 177: 751-766 (1949)

69. Bock and Benedict Formula, In Hawk, P. B., Oser, B. L., Summerson, W. H., Practical Physiological Chemistry 12th Ed., p. 1230, Blakiston Co., Philadelphia, Pa.

70. Webster, G. C., Plant Physiol. 29: 382-385 (1954)

71. Lipmann, F. and Tuttle, L., J. Biol. Chem. 159: 21-28 (1945)

72. Yemm, E. W. and Cocking, E. C., The Analyst 80: 209-213 (1955)

73. Kalckar, H. M., Science 125: 105-108 (1957)

74. Hoagland, M. B., Keller, E. B., and Zamecnik, P. C., J. Biol. Chem. 218: 345-358 (1956)

75. Ts'o, P. O. P., Bonner, J., and Vinograd, J., J. of Biophy. & Biochem. Cytology 2: 451-466 (1956)

76. Singer, T. P. and Kearney, E. B., Archives of Biochem. & Biophy. 29: 190-209 (1950)

77. Black, S. and Wright, N. G., Amino Acid Metabolism, pp. 591-600, Johns Hopkins Press, Baltimore, Maryland (1955)

78. Bücher, T., Naturwissenschaften 30: 756-764 (1942)

79. Nisman, B., Seminar, California Institute of Technology, October 1957.

80. Demoss, J. A. and Novelli, E. D., Biochim. et Biophy. Acta 18: 592-593 (1955).

81. Raacke, D., unpublished data.