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ABSTRACT

The development of an ATPase activity in the course of the germination of pea seeds was studied in extracts from the cotyledons and from the shoot-root axis (S-R). The specific activity (S.A.) in the S-R extracts was much higher than in those from the cotyledons, but there was little activity in either extract until the radicle had broken through the seed coat. Differential centrifugation of the extracts revealed that the ribonucleoprotein (RNP) fraction had the highest S.A. This fraction under appropriate conditions of extraction could be divided into a gelatinous pellet with a greatly reduced activity, and a looser "fluffy" overlay containing most of the activity.

Attempts to repeat the results of Webster (30) and Raacke (32) met with failure. Neither an incorporation of labelled amino acid nor a net synthesis of protein could be demonstrated in vitro despite various modifications in technique. Examination of the reaction mixtures showed that the added labelled amino acid remained intact, but that the added nucleotides were rapidly degraded.

In a series of experiments arising out of the in vitro studies, pea seeds were germinated in the presence of phenylalanine uniformly labelled with C^{14}. The label was almost completely absorbed even when considerably diluted with unlabelled phenylalanine. Some of the added activity was incorporated into proteins in both the cotyledons and the shoot-root axis, but much of it was lost as unidenti-

fied volatile products of metabolism. Dilution of the label with the unlabelled analogues, p-fluorophenylalanine or B-thienylalanine, had little effect on the distribution of the activity, although growth of the seedlings was inhibited.

RESUME

On rapporte les résultats d'une étude du developpement d'une activité ATPase dans la graine de pois en germination. L'activité spécifique de l'enzyme est beaucoup plus élevée dans les extraits provenant de l'axe tige-racine que dans les extraits des cotylédons. Cependant l'activité enzymatique ne fait son apparition dans ces extraits qu'après la percée du tégument par le radicule.

Les granules de nucleoprotéines (RNP) obtenus par centrifugation différentielle possèdent l'activité spécifique la plus elevée. En variant les conditions d'extraction ces granules peuvent être séparés en un culot gélatineux surmonté d'une masse floconneuse. Cette dernière contient la plus grande partie de l'activité enzymatique.

Nous avons essayé de repétér les travaux de Webster (30) et de Raacke (32). Ces tentatives ont abouti à un échec complet. In vitro nous n'avons pas pu obtenir d'incorporation d'acides aminés marqués au carbone 14 ni d'augmentation de la quantité de protéines. Au cours de ces essais l'acide aminé radioactif a pu être récupéré inchangé, mais nous avons constaté que les nucléotides étaient rapidement hydrolysés.

Dans une autre série d'expériences, des graines de pois ont germé en présence de phénylalanine marquée au carbone 14. L'acide aminé radioactif est rapidement absorbé dans les graines

même s'il est dilué avec de la phénylalanine non-radioactive. Une partie de l'acide aminé radioactif est incorporé dans les protéines des cotylédons et de l'axe tige-racine et une partie est transformée en métabolites volatils que nous n'avons pas pu identifier. La présence d'analogues non-radioactifs, tels que la p-fluorophénylalanine et la B-thienylalanine ne change pas la distribution de la radioactivité mais retarde la croissance des graines.

Abbreviations used in the text.

a.a. - amino acids, ala-alanine, ade-adenosine, ATP, ADP, AMP- adenosine tri-, di-, mono- phosphate, ATPase - adenosine triphosphatase (used to denote the enzyme activity displaying an ability to split free phosphate from ATP, ADP, and GTP.)

B.S.A. - bovine serum albumin.

c/m - counts per minute, cots - cotyledons, CTP, CMP - cytidine tri- and mono- phosphate.

DL - DL phenylalanine (unlabelled), DNP-aa - dinitrophenol-amino acids.

E - molar extinction coefficient, E.M. - extraction medium (in which the seedlings were ground), E.U. - enzyme unit.

F - p-fluorophenylalanine, FDNB - fluorodinitrobenzene, fr.wt. - fresh weight.

germ. - germination (a seed was considered to have germinated if the radicle had punctured the seed coat.) G F - radioactive counting by gas-flow, GSH - glutathione, GTP, GDP - guanosine tri- and di-phosphate.

L S - radioactive counting by liquid scintillation.

M.W. - molecular weight, mμ - millimicrons (measure of wave

length), µg - microgram.

O.D. - optical density.

PEP - phosphenolpyruvic acid, PGA - phosphoglyceric acid, phe - phenylalanine, Pi - inorganic phosphate, PPi - inorganic pyrophosphate, POPOP - p-bis(2-(5-phenyloxazolyl)benzene), PPO - 2,5-diphenyloxazolyl.

rf - ratio of the distance travelled on a chromatogram by a compound over that travelled by the solvent. R.M. - reaction mixture, RNA - ribonucleic acid, RNase - ribonuclease, RNP - ribonucleoprotein (used as abbreviation for the precipitate of 100,000xg centrifugation).

S.A. - specific activity (enzyme activity per mg. protein), S.R. - shoot-root axis, sup - supernatant, sRNA - soluble ribonucleic acid,

TE - B-thienylalanine, Tris - Tris (hydroxymethyl)aminomethane.
UV- ultraviolet

PART ONE

INTRODUCTION

The pea seedling has been widely used as experimental material in plant physiological and biochemical studies because of its large size and the ease with which it can be cultured. Owing to the ample food storage in the cotyledons, it can be grown for considerable periods of time in darkness, thereby eliminating the complications introduced by photosynthesis and chlorophyll synthesis. It is especially suited to the study of protein metabolism since a large part of the storage material consists of proteins which have been given considerable study (1). The processes through which these reserve proteins in the cotyledons are mobilized to provide the structural material for the developing shoot-root axis have not been intensively studied, however, and much still remains to be learned.

1. The fate of the storage proteins in germination.

It would be expected that the process of hydrolysis of the reserve proteins would predominate in the course of germination, and Danielsson (2) found this to be so. The content of reserve proteins decreased slowly at first, then more rapidly as the shoot-root axis developed. Lawrence et al (3) reported essentially similar results in their study of nitrogen mobilization in pea seedlings. The distribution of the various nitrogen fractions was followed, e.g. protein-N, alcohol soluble-N etc. The results indicated that the reserve proteins broke down in the

cotyledons to soluble intermediates which increased in concentration at first, and then were transported to the shoot-root axis where synthesis of new protein occurred. Goksøyr et al (4), however, found that non-protein-N was used first before the reserve proteins were broken down, but this observation might have resulted from the use of different growing conditions. A detailed examination of the free amino acids (5) showed that the amount and proportions of the individual amino acids varied considerably with age in the different parts of the developing seedling. The results indicated that an extensive conversion and new synthesis of amino acids occurred along with the simple release of amino acids in the course of hydrolysis.

Obviously many complex reactions are coordinated in the course of the mobilization of protein reserves, and the factors controlling them are not well understood. The difficulty in determining the precise point at which a protein becomes a peptide, and therefore non-protein-N, adds a further complication.

2. The development of enzyme activities.

The development of enzyme activities in the course of germination of pea seeds as a measure of protein mobilization has been studied by numerous authors. In the anaerobic phase, i.e. before the radicle punctures the seed coat, a considerable increase in alcohol dehydrogenase activity, which decreases abruptly by about the third day, has been shown to occur (4). Stafford (6) sampled germinating seeds at zero, three, and six days, and found that amylase, phosphorylase,

and oxidase activities increased. Holden and Pirie (7), and Barker and Douglas (8) demonstrated an increase in ribonuclease and phosphatase activities. Glutathione and cystine reductase activities were shown to increase by Russian workers (9). Young and Varner (10) reported an increase in phosphatase and amylase to occur in cotyledons.

The above list is not intended to be exhaustive, but merely to serve as an example of the variety of enzymic activities that develops in the course of germination. Except for the last mentioned, however, these studies do not attempt to discriminate between activation of proenzymes and de novo synthesis.

3. The de novo synthesis of a phosphatase activity.

Young and Varner (10) have presented evidence that the phosphatase activity observed to increase in the course of germination did so as a result of protein synthesis. The influence of the shoot-root axis on this synthetic activity of the cotyledons was considerable and has been the object of further study (11). The occurrence of protein synthesis in a storage organ is interesting enough in itself, but the type of enzyme synthesized is of even greater interest. Even in crude preparations, the activity showed a high specificity for ATP and ADP and hence could be termed an adenosine polyphosphatase, or apyrase. It appeared in the shoot-root axis as well, especially in the regions of meristematic activity.

4. Possible functions of the newly synthesized phosphatase.

The presence of an ATPase activity in pea seedlings was

first mentioned by Elliott (12), who described the precipitation by protamine of an ATPase in the course of purification of glutamine synthetase. It was also mentioned by Varner and Webster (13) before being studied more fully by Young and Varner (10), and Young et al (11). A similar activity is of widespread occurrence in plant preparations in general. In extracts from potato tubers it has been the object of several recent studies (14-16). It has also been studied in extracts from wheat germ (17), cabbage leaves (18), and in a cell wall preparation from corn coleoptiles (19).

In a system such as a germinating seed which would presumably require all available energy for synthetic activity, the question of the function of an ATPase is a legitimate one. It is possible, of course, that it is only an artifact of preparation. Racker (20) has suggested that non-specific phosphatase activities in cell-free preparations may be a result of group transfer reactions that have developed into simple hydrolases in the course of preparation of the extracts. Moustafa and Lyttleton (21) have suggested that the activity in their extracts from wheat germ is derived from broken mitochondria and obtained a reduced activity when the plant material was extracted in sucrose. Assuming that this activity is not an artifact, other functions for it can be suggested. Meyerhof (22) has demonstrated the role of ATPase in providing ADP for glycolysis in yeast cells. An ATPase activity has been reported in bacterial membranes that apparently controls the permeability of the membrane to substrate (23). Sol-gel transformations have been re-

ported to be mediated by an ATPase activity in Amoeba proteus (24) and slime molds (25), a function that could conceivably affect protoplasmic streaming in higher organisms. ATPase activity is involved in the active transport of ions across a membrane in animal tissues (26). The possibility that there is a similar function in plant tissues is suggested by nutrition studies in which the manipulation of magnesium and calcium levels affected the level of ATPase in sunflower shoots (27). ATPase activity has long been associated with embryonic development (28), and has recently been demonstrated in the mitotic apparatus of sea urchin eggs (29). The occurrence of an ATPase activity in the ribosomes (30, 31) suggests a possible role in protein synthesis.

Thus several possible functions for the activity studied by Young and Varner (10), especially for the initial ATP to ADP step, can be suggested, but what its role or roles in the living pea seedling might be still remains an unanswered question..

5. The study of protein synthesis in cell-free in vitro systems.

Protein synthesis in the pea seedling has also been studied by means of cell-free in vitro systems. The work of Webster (30) and of Raacke (32, 33) are especially notable in this respect, both having obtained a measurable net increase in protein. Despite the success of their experiments, many questions as to the steps involved in protein synthesis remain unanswered. The problem has been further complicated by reports of inability to repeat the results already published (34, 35). Other than those already mentioned, studies in this

field with plant systems have been limited in number, but studies with animal and bacterial preparations have been more numerous, and have produced less conflicting results.

The gradual elucidation of the intricate series of steps involved in protein synthesis is the subject of several recent reviews (36-40). Reading of these reviews leads one to the conclusion that the first steps in the formation of the polypeptide chain from free amino acids have been clearly defined and can be duplicated in cell-free systems. The later stages, involving the release of the newly synthesized protein and the assumption of secondary and tertiary structure, are not fully understood.

The development and refinement of the cell-free in vitro systems is largely a result of improvements in differential centrifugation. The cells of the experimental material, whether animal, plant, or bacterial, are broken by an appropriate method in an extracting medium of variable complexity. The resultant homogenate is centrifuged at a relatively low speed, ca 40,000xg, to remove the heavier material such as unbroken cells, nuclei, mitochondria etc. The supernatant is then decanted and centrifuged at a relatively high speed, ca 100,000xg, to separate the microsome fraction from the supernatant, or soluble fraction. The microsome fraction consists of the ribonucleoprotein particles or ribosomes which may or may not be associated with a phospholipid membrane, depending on the source material. (41).

The first two steps of protein synthesis have been demon-

strated to occur in the soluble fraction alone. These are:

1) activation of the free amino acids by the reaction, enzyme + aa + ATP $\longrightarrow$ enzyme-aa-AMP + PPi.

2) formation of the soluble RNA-amino acid complex by the reaction, enzyme-aa-AMP + sRNA $\longrightarrow$ enzyme + AMP + sRNA-aa.

The first step is usually demonstrated as an exchange of P^{32} between ATP and labelled pyrophosphate, or by formation of hydroxamates which can be detected chemically or chromatographically. The same enzyme seems to catalyse both steps, and each amino acid has an enzyme specific for it. The soluble or transfer RNA also seems to be in a form specific for each amino acid, although the isolation of the specific forms has been complicated by the similarity in physical and chemical properties of the different types. In practice, a more or less crude preparation of sRNA is employed, the association of label with the RNA precipitate being taken as evidence of complex formation with radioactive amino acids.

The subsequent steps of protein synthesis require the presence of the microsome fraction, or more specifically the RNP particles. These are:

3) transfer of amino acid from sRNA to the RNA of the ribosomes,

4) formation of the polypeptide chain,

5) release of the newly-formed polypeptide.

Step 3 is catalyzed by a transfer enzyme, requiring GTP as an essential cofactor, present in the soluble fraction. The ribosomes contain a high

proportion of RNA (40-60%), (42), but recent discoveries point to the intervention of a third type of RNA, termed messenger RNA, in this step. The messenger RNA is visualized as being synthesized directly on the DNA template and therefore contains the information stored in the genes. This concept has allowed an experimental approach to be made to the perplexing problem of how the transfer of information occurs. Such experimentation has already been done independently in the laboratories of Nirenberg (43), and Ochoa (44). Using synthetic polynucleotides of known composition as messenger RNA in cell-free bacterial systems, and assuming that the position of each amino acid on the RNA template is determined by a trio of adjacent nucleotides, they have arrived at a probable code for each amino acid (45).

A further development from this discovery has been the concept of multiple ribosomes, termed variously polysome (46) or ergosome (47). This concept has arisen from the application of simple arithmetic. Since the ribosome is known to be at most only 230 Å in length, while the messenger RNA must be at least 1500 Å in order to accomodate a polypeptide such as hemoglobin, it follows that several ribosomes must combine to provide the foundation for the messenger.

Step 4, formation of the polypeptide chain, is assumed to occur automatically on the ribosome as a result of the positioning of the activated amino acids on the template, and is accompanied by the release of the sRNA. The involvement of lipids in this step has been postulated (48), with the formation of an intermediate phospholipoprotein.

Steps 3 and 4 have been demonstrated in vitro only through the use of labelled amino acids, since the yield of polypeptide has been too low in most cases to be detected in a chemical reaction. The results reported by Webster (30) and Raacke (32, 33) are notable exceptions. The former measured a net synthesis of 0.4 mg. per mg. of RNP protein, while the latter found an increase in protein reaction in a much simpler system of 0.3 mg. per mg. of original protein. In some studies, an increase in enzyme activity has been taken as a measure of protein synthesis (30, 49, 50), although this method is open to the possibility that the enzymes were already preformed and were merely released from the ribosomes.

In most studies, a distinction between bound and released protein has not been sought; the increase in the activity of the total protein precipitate after removal of the RNA has been taken as evidence of protein synthesis. In some studies, however, (30, 51, 52), the ribosomes have been separated from the soluble fraction before precipitation, and the activity in the soluble protein fraction measured separately as an indication of the release of newly-formed polypeptide. With the exception of the results of Webster (30), the amount of labelled soluble protein found in this way has been very small. Lett and Takahashi (34) could not repeat Webster's results, and Webster himself has since reported (35) that only certain "active" batches of peas are suitable.

One of the reasons for the low yield of protein synthesis is thought to be that the newly-formed polypeptides are not released

from the ribosomes, and therefore synthesis stops when the binding sites are loaded. The process of release has been studied in vitro with pre-labelled RNP (53-55), and there seems to be a specific enzyme involved, with a high concentration of potassium ion being essential.

The assumption of secondary and tertiary structure of the proteins is presumed to follow automatically from the order of the amino acids, and recent studies with ribonuclease (56) support this concept. The intrusion of an enzyme even in this step, however, is suggested by the results of the continued study of ribonuclease reactivation (57).

As Zamecnik (58) has outlined, many questions remain unanswered in the field of protein synthesis. While the scheme reviewed above has gained wide acceptance, there has been a growing body of evidence that synthesis does not occur only by the steps indicated, but may also occur by other routes. Hendler (59) has pointed out that the rate of protein synthesis in vitro is only 1/100-1/1000th that occurring in vivo. Whether this discrepancy is a result of poor technique, or of the loss of another system is not yet decided. The nucleus (60, 61,) mitochondria (62, 63), and chloroplasts (64) are capable of considerable protein synthesis, and while this may occur on ribosomes located in these structures, this point is not firmly established. The polypeptide synthetase studied in detail by Beljanski (65) omits amino acid activation in the synthesis of peptides. The peptidyl-nucleo-

tides isolated from yeast (66) and of widespread occurrence in mammalian tissues (67) appear to be products of protein catabolism, but may also play a role in synthesis. Studies on protein synthesis in the silkworm (68) indicate the involvement of a lipid fraction associated with the cellular debris. A preliminary report recently published (69) describes a soluble amino acid incorporation system from E. coli that does not require ribosomes.

6. The use of labelled amino acid for the study of protein synthesis in vivo.

The development of in vitro systems for the study of protein synthesis has contributed a great deal in the past decade, but in vivo studies still provide valuable information. With animals, a simple injection into the blood stream is usually adequate. The rapid growth of bacterial cultures makes the provision of the label in the nutrient medium the obvious choice. The situation with plants, however, is more difficult, although advances in tissue culture have been exploited by Steward and his co-workers (70). Here atypical growth may lead to conclusions that are not universally applicable, e.g. the synthesis of a collagen-like protein containing a high concentration of hydroxyproline (71). In most studies, isolated plant parts have been incubated with the labelled amino acid, and the incorporation of label into the protein fraction followed. Thus Ts'o and Sato (72) used pea epicotyl tips, Lyndon and Steward (73) slices of tuber, Stephenson et al (64) tobacco leaf discs, Webster (74) tissue sections from a variety

of plants, and Young et al (11) slices of pea cotyledons. The uptake of label under these conditions, even with several hours exposure, is usually in the neighbourhood of only 1%. An unusual study with whole plants is that of Virtaanen and Miettinen (75) who fed pea plants grown under aseptic conditions C^{14} amino acids through the roots. Almost all the activity was removed from the nutrient solution within 12 hours. The rapid uptake of liquid by an imbibing seed would seem to be an ideal way of injecting labelled test material into living cells, but it does not seem to have been widely used. Swaramarkrishnan and Sarma (76) germinated seeds of Phaseolus radiatus in the presence of labelled glutamic acid or glucose in a study of asparagine synthesis, but this approach is more commonly used in the study of germination inhibitors.

7. The aims of the present work.

In the experimental part of this thesis, the problem of protein synthesis in the germinating pea seedling has been approached from three interrelated directions:

1) A study of an adenosine polyphosphatase (ATPase) activity appearing in pea seedlings in the course of germination, and the effect of the extraction medium on its activity in extracts from 3-4 day old shoot-root axes.

2) A study of protein synthesis using the RNP fraction isolated from shoot-root axes. This was begun as an attempt to repeat the promising results already published by Webster (30) and Raacke (32), with the intention of proceeding towards a study of some of the still obscure as-

pects of protein synthesis.

3) A preliminary study of the uptake and utilization of a labelled amino acid imbibed in the course of germination.

PART TWO

EXPERIMENTAL

A. METHODS

1. Experimental material.

a. Germination of the seeds. Alaska pea seeds, obtained locally and from Howick Seed Co., P.Q., were given a preliminary two minute surface sterilization in 5-10% Javex, a commercial preparation of sodium hypochlorite, followed by a thorough rinsing. Damaged seeds were then picked out and the remainder were placed in Petri plates, supplied with tap water sufficient in amount for complete imbibition (20-25 ml. per 100 seeds), and left in an incubator in the dark at 27°C. After about 5 hours, when the seeds were fully swollen, they were transferred to moist vermiculite to grow in darkness in the incubator for the required length of time.

b. Preparation of extracts. This was done using the methods described by Young and Varner (10), Webster (30), and Raacke (32). In general, the seedlings were washed two or three times in tap water to remove the vermiculite from the roots, and separated into cotyledons and shoot-root axes, the seed coats being discarded. The seedling material was then rinsed two or three times in distilled water and blotted dry for fresh weight determination. The tissues were ground by hand with a chilled glass mortar and pestle, usually without sand, in an extraction medium varied to suit the experimental purpose and described in the

appropriate place in the results. The homogenate so obtained was then strained with hand pressing to remove as much of the liquid as possible through a double layer of cheesecloth, and transferred to centrifuge tubes. This crude extract was then centrifuged either in the high speed head of the International refrigerated centrifuge, Model PR2, at top speed (25,000xg) for one hour, or in two stages in a Spinco ultracentrifuge, #40 head, first at 40,000xg for 10-20 minutes to remove cellular debris, nuclei, and mitochondria, and then at 100,000xg for 1-2 hours. In the former case, the supernatant containing soluble proteins and the bulk of the microsomal fraction was reserved and is subsequently termed the 25,000xg supernatant. In the latter case, the soluble proteins were separated into the supernatant (100,000xg supernatant) from the microsomes in the precipitate (RNP) and both fractions were kept. The RNP was suspended in a small volume of water or 0.5M sucrose with the aid of an homogenizer. All operations were carried out as close to 0°C as possible. Any major variation in the technique used is detailed in the appropriate section.

c. Fractionation of extracts. A few unsuccessful attempts were made to isolate a fraction of greater specific activity in regard to ATPase using the Karler-Misco electrochromatography apparatus, ammonium sulfate, and acid precipitation. Preparations of the "pH 5 enzyme" fraction from the 100,000xg supernatant were made for use in protein synthesis reaction mixtures following the method used by Lund (49).

d. Administration of C^{14}-amino acids in vivo. The process of im-

bibition was used as a means of feeding labelled amino acid to the seeds. Eighty seeds were germinated in 15 or 20 ml. of water containing 2 μM of the amino acid. Details of individual experiments are given in the results.

e. Preparation of the seeds for extraction of protein etc. The seeds were removed after the desired lapse of time to a beaker of water for washing. They were transferred to a second beaker of water, drained, and then either frozen or dropped into 5% TCA. The water washings were combined and evaporated to dryness for counting. The washed seeds were either ground whole or first separated into cotyledons - seed coat and shoot-root axis. Further treatment was as described in the preparation for counting procedures.

f. Trapping of respiratory CO_2. The carbon dioxide given off by the seeds during germination was collected in only one experiment and by a rather inefficient method. The Petri plates containing the seeds were placed in dessicators over 50% KOH. The dessicator stopcocks were left open to admit air from which the CO_2 had been removed. At the sampling period, the KOH solutions were treated with $BaCl_2$ to precipitate the trapped CO_2 as $BaCO_3$ which was then collected by centrifugation, dried, and counted.

2. Analytical methods.

a. Protein determination.

Colorimetric method. In every experiment, the protein content of the extracts was estimated by the widely used phenol method of Lowrey

et al (77), the amount of the reagents being adjusted to give a final volume of 6.5 ml. Standard curves were prepared, using solutions of egg albumin in 0.1N NaOH and bovine serum albumin (B.S.A.) in water. The normality of the NaOH-sodium carbonate reagent was reduced accordingly when the sample was dissolved in NaOH. The optical density (O.D.) of the reaction tube was measured at 750 mμ in a Bausch and Lomb spectrophotometer. With this instrument, it is difficult to read the O.D. accurately when it is in excess of 0.7. Therefore the % transmittance (T) was taken at higher values and transformed into O.D. readings by means of the formula: O.D. = -log T.

The total protein reaction of the extracts or reaction mixtures (R.M.) was determined, i.e. a suitably diluted aliquot was tested without previous precipitation with trichloroacetic acid (TCA) or, as in the case of R.M., without separation of the TCA precipitate. As well, many of the extracts and R.M. were first separated into 10% TCA-soluble and TCA-insoluble fractions. The precipitate was dissolved in N NaOH, and diluted to a final 0.1N NaOH for analysis. Small precipitates obtained from samples of R.M. were dissolved directly into the sodium carbonate reagent. The TCA supernatant was tested without neutralization.

Turbidometric method. (78)

In the preparation of a standard curve, a solution of B. S.A. (25 mg./ml.) was used in the following procedure: 7 ml. of 3% TCA were mixed thoroughly with 0.2 ml. of solution containing 1-10 mg.

of protein. Exactly 30 seconds after the addition of TCA, the O.D. was measured at 540 mμ.

Optical density method. The O.D. of an appropriately diluted protein sample was measured at two wave-lengths, 260 mμ and 280 mμ. The equation, 1.45 x $O.D._{280}$ minus 0.74 x $O.D._{260}$ (79) was then applied to obtain an estimation of the mg./ml. of protein in the diluted sample.

b. Phosphate determination. Phosphate was determined as the inorganic ion (Pi) using the method of King (80) which is simple, rapid, and sensitive and relies on the formation of a blue phosphomolybdate complex. A calibration curve was prepared using K_2HPO_4 as standard and taking readings at 660 mμ. In some cases, total and acid-soluble phosphate were also determined as Pi after appropriate treatment of the samples witn acid.

c. Pyruvate determination. The method of Kachmar and Boyer (81) was used, and a standard curve was prepared with sodium pyruvate. The O.D. of the resultant hydrazone was measured at 440 mμ.

d. Amino acid determination. A ninhydrin method modified from that of Moore and Stein (82) was used. The modification, which consisted mainly of an increase in the ninhydrin (1.5x) and a reduction of the hydrintantin (0.6x), made the test more sensitive. DL-alanine was used as a standard in the preparation of the calibration curve, and the O.D. was measured at 570 mμ.

e. Ammonia determination. Ammonia was estimated by a modified

Conway microdiffusion method (83).

f. RNA determination. The concentration of the RNA preparations made for inclusion in protein synthesis reaction mixtures was estimated from the O.D. at 260 mμ. The value of 200 was used for the molar extinction coefficient (E) as suggested by Kirby (84).

g. Paper chromatography. The standard methods of descending paper chromatography were used (85) with Whatman #1 or #3MM paper. TCA was removed from samples only when 0.05 ml. or more was to be evaporated on one spot.

Separation of nucleotides. Pabst solvent I (86) was used to separate the nucleotide components of ATPase and protein synthesis reaction mixtures (R.M.), using samples taken before or after precipitation of proteins with TCA. After the spotted samples had dried, the papers were equilibrated for at least one hour before the addition of the solvent.

Separation of amino acids. The solvent system, butanol-acetic acid-water (4:1:5) was used to separate the components of protein hydrolysates and protein synthesis R.M. This is a two phase system, the upper layer being used in the solvent trough, and the lower in the chromatograph jar. For a better separation of hydrolysates, a double run was used, i.e., the papers were left until the solvent front was at the end of the paper, then removed, dried, and placed back in the solvent for a second run.

Elution of nucleotides. The nucleotide spots were eluted from the paper by cutting out a rectangle large enough to include all the UV-ab-

sorbing area and fitting it into a small test tube. It was covered with 0.02M phosphate buffer, pH 7, and heated in a water bath at 100°C for one hour. The O.D. of the eluted sample was read at several wavelengths in the Beckman DU spectrophotometer to obtain a UV absorption spectrum which served both for identification of the spot and to estimate the amount present through use of the formula: concentration (moles/l.) = O.D. ÷ E (molar extinction coeff.). In cases where two nucleotides occupied the same spot, as with ATP and GDP, the amount of each one was estimated through the use of the equations given by Loring (87).

Detection of compounds on paper chromatograms. Nucleotides or other UV-absorbing compounds were located by scanning with a Mineralight UV lamp, Model R-51. Amino acids were detected by means of a ninhydrin spray (0.1%, w/v. in ethanol). Phosphates were located by the Hanes-Isherwood spray (1% ammonium molybdate, 3% perchloric acid, and 0.4% HCl). After spraying, the papers were air-dried and exposed to UV light for a few minutes. Acids and bases were shown as yellow or blue spots, respectively, by means of dipping the paper in a solution of bromphenol blue (0.04% in ethanol and made a reddish color with N NaOH). C^{14}-labelled compounds were detected by exposure of the chromatograms to Kodak No-Screen X-ray film for periods of one week to two months, depending on the amount of activity. The film was then developed by a standard photographic procedure.

h. Radioactive counting. Two systems, both from Nuclear-Chicago,

were used for counting: 1) a gas-flow (G.F.) counting system with scaler (Model 182), detector (Model D-47 with micro-mil window), and low background automatic sample changer (Model C110A); and 2) the automatic liquid scintillation (L.S.) system, Model 703 with 8000 series instruments.

Gas-flow counting. The number of counts taken on each sample was varied according to the accuracy desired. With active samples, or those representative of a large fraction, 2560 counts were taken. With samples of low activity, duplicate or triplicate counts at a lower level, usually 80 or 160, were taken and an average value computed. The appropriate corrections for self-absorption in solid samples were made; e.g. for a 100 mg. sample in a planchet 2.4 cm. in diameter, the actual count was divided by the factor 0.161 to obtain a corrected count. This correction was based on the assumption that the surface of the sample was perfectly smooth, which in practice it seldom was, but no further correction was made for any error introduced in this manner.

Liquid scintillation counting. The samples counted were either solid samples or liquid samples spotted on paper discs. Undiluted toluene with the dissolved scintillation compounds was therefore used. This procedure eliminated quenching in the counting mixture but introduced errors from self-absorption in the sample itself, and was mainly used because of the convenient manner in which samples could be counted in both systems with a minimum of additional preparation. Duplicate or triplicate 10 minute or 30 minute counts were taken on each sample at

two levels.

In some cases, it was desirable or necessary to convert readings obtained in the gas-flow to those to be expected in the scintillation counter or vice versa. This was done simply by counting some samples in both instruments and assuming that the relationship so obtained would hold for other similar samples.

A correction curve for solid samples was prepared by counting various weights of the same sample in toluene.

i. N-terminal analysis of proteins. A dinitrophenylation method (91) was used with samples of radioactive precipitates for the isolation of the N-terminal amino acids of proteins as dinitrophenol (DNP) derivatives. Each sample was suspended in 5% sodium bicarbonate using a glass homogenizer to obtain a uniform suspension. Fluorodinitrobenzene (FDNB), dissolved in a minimum of ethanol, was added to the bicarbonate suspension in the ratio of 0.1 ml. FDNB/100 mg. sample. The reaction was allowed to proceed in a shaking water bath at room temperature for two hours. The resultant heavy yellow precipitate was then removed by centrifugation, washed 1x with water, the water washings being added to the supernatant; 2x with ethanol, and finally 1x with ether. The supernatant was washed 2x with 2 volumes of ether, then made acidic with 6N HCl. The resultant precipitate was removed by centrifugation and washed as above. The supernatant was washed 2x with 2 volumes of ethyl acetate and evaporated to dryness. The following fractions, their probable composition indicated in brackets, were thus obtained for count-

ing: 1) ethanol washings of the precipitates (excess FDNB), 2) combined ether washings (excess FDNB), 3) ethyl acetate washings (free DNP-amino acids and DNP-peptides), 4) final washed supernatant, 5) bicarbonate-insoluble precipitate (DNP-proteins), and 6) HCl-insoluble precipitate (DNP-proteins). This latter fraction was always very small.

The precipitates, usually only those of fraction 5, were hydrolysed in 6N HCl by heating at 100°C overnight in a sealed tube. The insoluble black residue (humus) was removed by filtration (fraction 5a). The filtrate was evaporated to dryness, and the residue washed with ether to remove DNP-amino acids released from the N-terminal position by hydrolysis (fraction 5b). The washed residue then represented the internal unreacted amino acids (fraction 5c).

j. Isolation of aromatic compounds. The charcoal adsorption method of Partridge (92) as well as the method of Udenfriend and Cooper (93) were used in attempts to isolate radioactive phenylalanine and tyrosine from protein hydrolysates. In the latter method, 5 ml. of ethanol were added to 5 ml. of solution containing the aromatic substance, followed by 80 ml. of acetone. Salts and amino acids are reported to be precipitated and removed by filtration, leaving the major part of the aromatic amino acids in solution.

3. Preparation of RNA for use in protein synthesis R.M.

a. Preparation of soluble RNA. Two methods of preparing sRNA were used, that of Kirby (84) and that of Rosenbaum and Brown (88). Both

methods were developed for extraction of sRNA from rat liver, and not from pea seedlings. They utilize the same principle, i.e. extraction of the RNA into the water layer of a phenol-water mixture, and its subsequent precipitation with ethanol. The former method starts with the 100,000xg supernatant which presumably contains only the sRNA, methyl cellosolve being used to separate polysaccharide impurities from the final product. The latter method uses frozen tissue as starting material, and a buffered phenol-NaCl extraction medium. In the course of grinding, presumably all the RNA in the tissue is removed into the buffer-NaCl layer. High M.W. RNA is subsequently removed by precipitation when the concentration of NaCl is increased to 1 N. Both methods were used essentially as described except that in the latter, a final step of resuspending the "sRNA" fraction in a minimum of water was added in order to remove the bulk of the relatively insoluble material, presumably starch.

Extracts prepared in sucrose were used in the Kirby method and frozen seedling material in the Rosenbaum and Brown method. In both methods, samples were removed at various stages for measurement of UV absorption.

b. Preparation of high M.W. RNA from the ribosomes. The method of Nirenberg et al (43) was used for a single preparation of this type, the product being used as messenger RNA in a few protein synthesis R.M. (e.g. Feb. 23, Table 17).

Associated with the preparation of RNA were a number

of minor techniques: 1) phenol was distilled by the method of Draper (94), 2) potassium iodide was used in a test for peroxides in ether (95), and 3) bound amino acids were removed from sRNA using the dilute KOH method of Zamecnik (96) in order to obtain an estimate of their quantity.

4. Preparation of samples for radioactive counting.

a. The TCA precipitate from protein synthesis R.M. This precipitate was usually that from a 0.5 ml. sample. It was collected by centrifugation, dissolved in 1 ml. of 0.2N NaOH containing an excess of unlabelled amino acid to ensure the complete removal of unbound labelled amino acid, and reprecipitated with TCA. The precipitate was again collected and washed with 5% TCA, suspended in ethanol, and transferred to a glass homogenizer with 2 volumes of ether. It was ground to a fine powder, filtered onto 2.5 cm. filter paper discs (#1 Whatman) using suction and a separable glass funnel with sintered disc, washed several times with ether, and left to dry over concentrated H_2SO_4. For GF counting, the entire paper with the packed powder was placed on a copper planchet.

In some experiments, smaller samples (0.05-0.1 ml.) of the R.M. were spotted directly onto 2.5 cm. discs of Whatman #3MM filter paper which were then dried briefly under a hair dryer, dropped into cold 10% TCA to precipitate the proteins into the paper, and prepared for counting after the method of Mans and Novelli (90).

b. Phenol and TCA-insoluble fractions from the entire seedling. Two

methods of extracting seedlings from the in vivo studies were used: a) the phenol method used for sRNA and b) grinding of the seeds in 5% TCA after the method of Haber (89). In both cases, preparation of the powder was the same. The insoluble fractions were collected by centrifugation, washed once with phenol or TCA as the case might be, then with ethanol, followed by exhaustive extraction with ether in a glass mortar until a fine white powder had been collected on filter paper in a Buchner funnel. Samples of 100 mg. were weighed into stainless steel planchets for GF counting, or into bottles for LS counting.

c. Liquid samples. Samples expected to be of low activity, as for instance most washings, were evaporated to dryness in 2.4 cm. planchets and counted as a dry residue with due correction being made for weight. In one experiment (Expt. 4, H_2O wash, Tables 25 and 26), the samples were completely absorbed into paper discs for LS counting. Small aliquots (0.01-0.02 ml.) of samples of high activity were placed in 5-7 symmetrically distributed spots on 3 cm. steel planchets and evaporated to dryness for GF counting. For LS counting, a larger aliquot (0.05-0.1 ml.) was absorbed onto a 2.5 cm. disc.

5. Preparation of stock solutions.

Adenosine triphosphate (ATP) was obtained from Mann Chemical Co. as the disodium salt (M.W. 605). It was dissolved in water and neutralized in an ice bath with N NaOH. The potassium salt was prepared in a few instances by using Dowex-x8 H^+ ion exchange resin to remove the Na^+, then neutralizing with N KOH. The final concentration was

approximately 40 μM/ml.

Adenosine diphosphate (ADP) was obtained from Mann Chemical Co. as the monosodium salt (M.W. 510). A stock solution of about 40 μM/ml. was prepared as above.

Adenosine monophosphate (AMP) was obtained as muscle adenylic acid from Nutritional Biochemicals Co. Stock solutions of about 20 μM/ml. were prepared as above.

Guanosine triphosphate (GTP) was obtained from Mann Chemical Co. as the disodium salt (M.W. 603) and was neutral when dissolved in water.

In most cases, the concentration of the stock solutions was checked by measuring the optical density of a suitably diluted aliquot in the Beckman spectrophotometer, and using the value for the molar extinction coefficient (E) suggested in the Pabst circular (86). A test for free phosphate and occasionally total phosphate was also made, along with paper chromatography.

Tris (Tris-(hydroxymethyl)aminomethane) was obtained from Fisher Scientific Co. (M.W. 121.1). A molar stock solution was made, and from this 0.1M or 0.2M buffer solutions were prepared after titration of the Tris with N HCl to the desired pH.

Glutathione (GSH), (M.W. 307.33) was obtained from Nutritional Biochemicals Co. It was acid in solution, and was neutralized with either N NaOH or with the stock Tris solution.

Amino acid mixture. The mixture suggested by Webster was pre-

pared as follows: 3 mg. of each of the L-amino acids and amides: arginine. HCl, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, histidine. HCl, isoleucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine, and 6 mg. of each of the DL-amino acids alanine and serine, were dissolved in 10 ml. of water.

Radioactive amino acids. Three labelled amino acids were used in the course of this work, and all were obtained from Atomic Energy of Canada. These were:

DL-alanine-1-C^{14}, (M.W. 89.1)- 200,000 c/m/mg. (GFcount).

DL-phenylalanine-2-C^{14}, (M.W. 165)- 42,677 c/m/mg. (GF count).

L-phenylalanine, uniformly labelled (M.W. 165) 260,000 c/m/mg. (GF count).

Stock solutions containing 2 μM/ml. were prepared and stored in the freezer.

Mixture for scintillation counting. Since solid samples or paper discs were always used, pure toluene containing 0.4% of 2,5-diphenyloxazole (PPO) and 0.01% of p-bis(2-(5-phenyloxazolyl)benzene)(POPOP) was used.

Phosphoglyceric acid (PGA) was obtained as the barium salt (M.W. 357.4) from California Biochemicals. It was put into solution with the aid of Dowex-50 H^+ resin, the resin being removed by filtration, and rinsed with water. The combined filtrates were neutralized with N NaOH.

Phosphoenolpyruvic acid (PEP) was obtained as the barium-silver salt (M.W. 446.3) from California Biochemicals. The free acid was prepared following the procedure of Kachmar and Boyer (81) and neutralized with NaOH.

The total and free phosphate concentrations of the final stock solutions of these two compounds were analysed to determine the actual concentration of PGA and PEP.

Pyruvic kinase was obtained from California Biochemicals as a solution containing 2 mg. of protein per ml., with a stated activity of 44 E. U./mg.

6. Test of pyruvic kinase activity.

The method of Kachmar and Boyer (81) was used to test the activity of the kinase on its arrival. To test its ability to compete with the ADPase activity of the RNP, two additional R.M. were included: ADP, and 0.5 mg. RNP protein; and ADP, PEP, kinase, and RNP. Phosphate and pyruvate determinations were made on 0.3 ml. samples withdrawn at zero, 5, and 15 minutes to 0.3 ml. of 10% TCA.

7. Measurement of ATPase activity.

In experiments with the 25,000xg supernatant, the reaction mixture(R.M.) was modelled after that used by Young and Varner (10). Each ml. of R.M. contained 33.3 μM of Tris-HCl, pH 7.5, 3-4 μM of Na-ATP, and 1 μM of $MgCl_2$. The extract, 0.05-0.10 ml., was added last after the mixture had equilibrated in a shaking water bath at 37°C for a few minutes. Duplicate samples, 0.1-0.2 ml., were removed im-

mediately after mixing, (zero time), and again after 15 minutes, to an equal volume of cold 10% TCA. When it was made necessary by a large amount of protein precipitate, the samples were centrifuged before phosphate determination. Control flasks without ATP were run simultaneously.

In tests with the 100,000xg sup and RNP the procedure was modelled after that of Webster (30), the Tris-HCl content being increased to 50 μM and the $MgCl_2$ being replaced by $MgSO_4$. The protein content was reduced to less than 0.5 mg. and as a result: a) control flasks could be omitted because the reaction contributed by such small amounts was negligible, and b) no centrifugation was necessary because the amount of precipitate was so small as not to interfere in the phosphate analysis. Although duplicate samples at zero and 15 minutes were still sometimes used, the practice of removing several single samples at several time intervals was more frequently followed in order to obtain a time-course curve of the reaction.

In nearly every experiment, the left-over R.M. was frozen for chromatography, and in some experiments time samples were also removed for this purpose.

The same reaction mixtures and procedures were used in the tests for hydrolytic activity with other phosphorylated compounds, the amount of extract and the timing of the samples being varied to suit the expected rate of the reaction.

8. Measurement of protein synthesis in a cell-free in vitro system.

The reaction mixtures described by Webster (30) and Raacke (32) were tested at first, but these were later modified as detailed in Tables 11 - 17. The various solutions were pipetted into 25 ml. Erlenmeyer flasks usually in the order given in these tables and left to equilibrate in a shaking water bath at 37°C for a few minutes. All extract fractions, whether supernatant, RNP, etc., were added after equilibration, the RNP always being the last one added. Samples were removed immediately after mixing (zero time) and at 1/2 hour intervals thereafter. Depending on the experiment, the samples taken were one or more of several types:

a) duplicate or triplicate samples removed to an equal volume of cold 10% TCA for total protein determination. These were always taken, the size varying with the expected concentration of protein. One or more were sometimes separated by centrifugation into TCA-soluble and TCA-insoluble fractions before analysis.

b) a single 0.1 ml. sample removed to a separate Erlenmeyer flask containing 0.9 ml. of an ATPase reaction mixture (30) from which zero and 15 minute samples were removed for phosphate analysis.

c) a 0.5 ml. sample removed to 0.5 ml. of cold 10% TCA, the precipitate of which was later prepared for radioactive counting. Various analyses were usually performed on the TCA-soluble fraction of this sample.

d) a 0.05 ml. sample removed either to cold TCA or directly to the freezer for later chromatography.

e) a 0.05 or 0.1 ml. sample absorbed directly onto a 2.5 cm. disc of filter paper and dropped into cold 5% TCA for later preparation for LS counting.

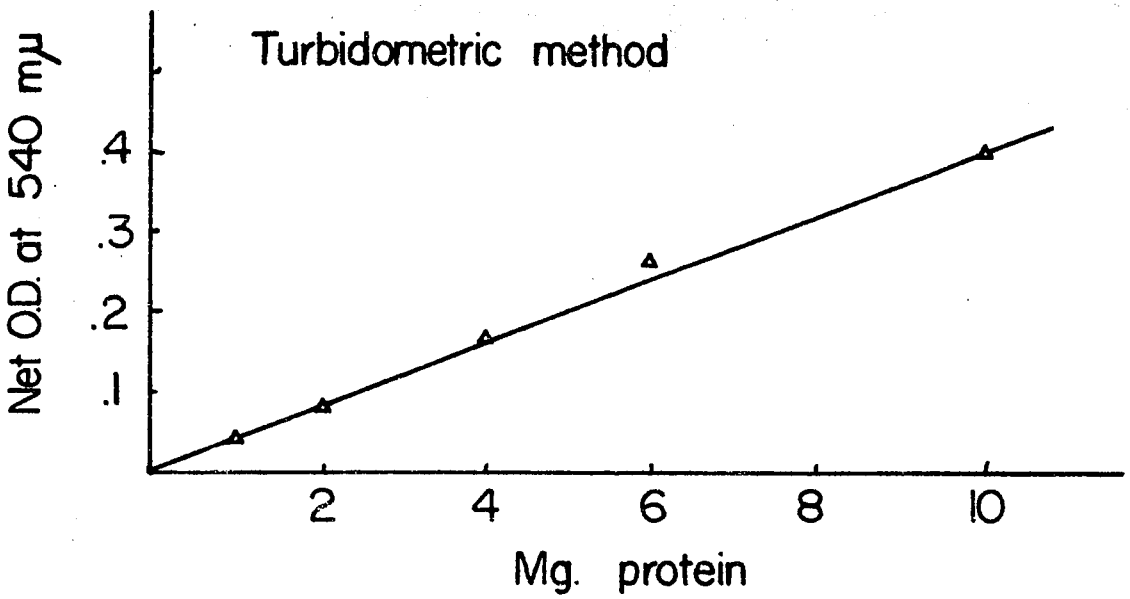
B. RESULTS AND DISCUSSION

1. Analytical methods.

a. Protein determination.

Colorimetric method. The calibration curves obtained with the two standards are presented in Fig. 1. It is to be noted that, as Lowrey et al (77) had observed, a straight line reaction was not obtained with increasing concentration. The effect of increasing amounts of extract on the protein reaction was also tested. It was found that an unprecipitated extract yielded a straight line relationship between amount and O.D., but that a TCA precipitate redissolved in NaOH gave a curved relationship similar to that in Fig. 1. For this reason, samples were diluted to give an O.D. reading of 0.3 or lower where a straight line reaction obtained in all cases, and a conversion factor of 330 was used (i.e. net O.D. x 330= μg protein/ml. of diluted sample). On occasion, the samples from protein synthesis reactions exceeded this value, and the O.D. readings were then converted by reading from Curve B in Fig. 1. This is why in some of the protein synthesis results, the total protein/ml. R.M. is less than that of the added RNP (e.g. Tables 14, 16, and 20). Since the purpose of these tests was to detect a relative increase within each R.M., it was not considered necessary to correct this anomaly.

In the experimental results, protein content is given as total protein since it was the only determination made in every experiment. It was higher than the value given by the TCA-insoluble fraction



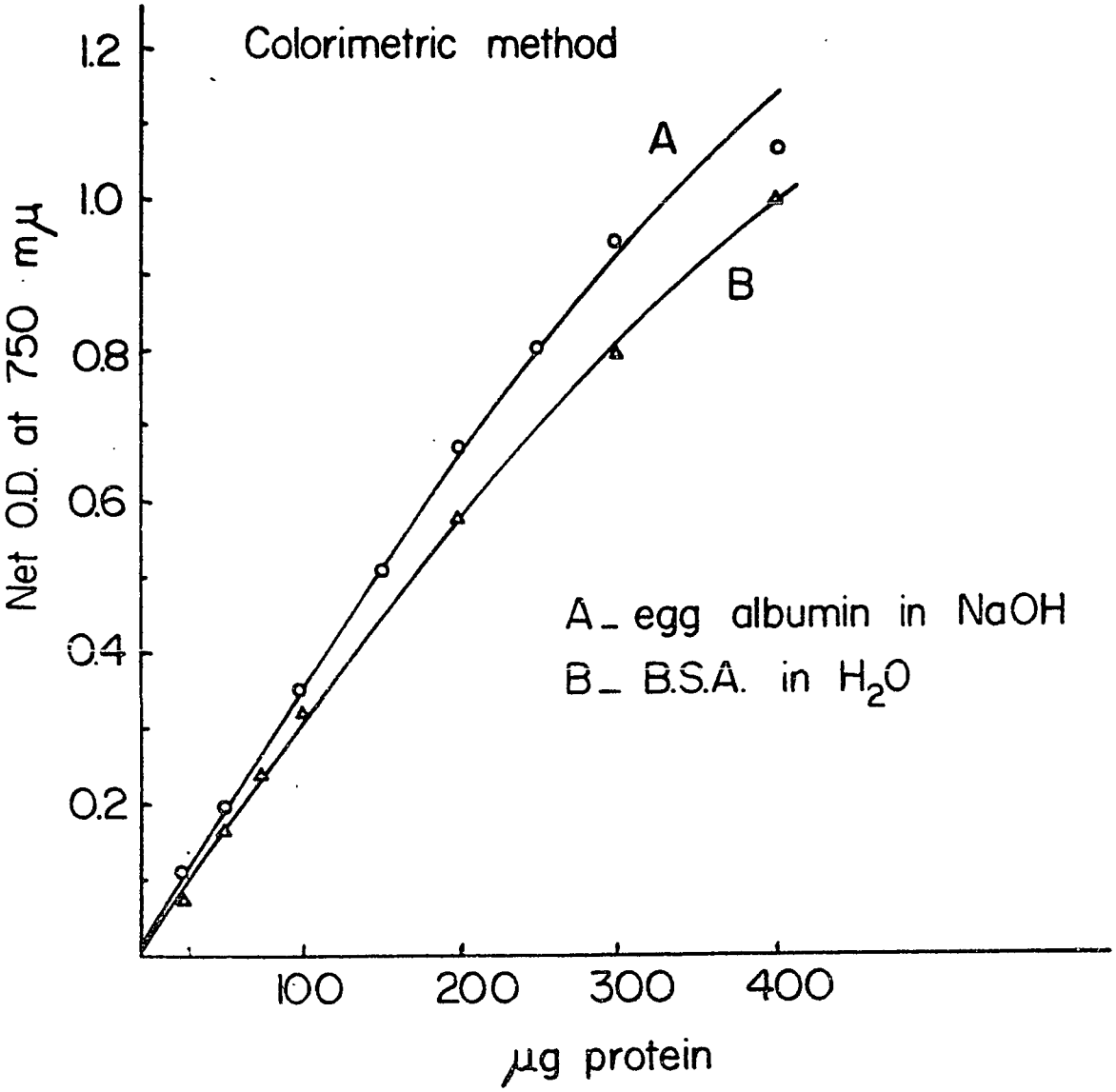


Fig. 1 Calibration curve for protein

alone. The relationships between these two values which usually prevailed in the various extracts were as follows:

Extract	% TCA ppte.prot./Total prot.
25,000xg sup (cots)	ca 90
" " (S-R)	" 60
100,000xg " "	50-60
RNP "	70-80

Turbidometric method. The calibration curve shown in Fig. 1 is a straight line. This method was mainly useful in obtaining preliminary estimates of concentration.

Optical density method. With the extracts used in the course of this work, the O.D. method yielded an estimate about two times higher than that of the Lowrey method.

b. Phosphate determination. The straight-line calibration curve obtained is shown in Fig. 2. Occasional anomalous behaviour was encountered which was probably a result of interfering reactions in the experimental mixtures employed. It was important to take the readings as close to the stated 5 minutes interval as possible, especially with samples of reaction mixtures from which the protein had not been removed. In these samples, a slow hydrolysis of indigenous phosphate-containing complexes, presumably RNA, occurred.

It was noticed that the extracts themselves contained an amount of inorganic phosphate which increased with the age of the seedling material. In the 25,000xg supernatant from cotyledons, there was an amount of Pi equivalent of 28 μM/100 seeds after one day, which had

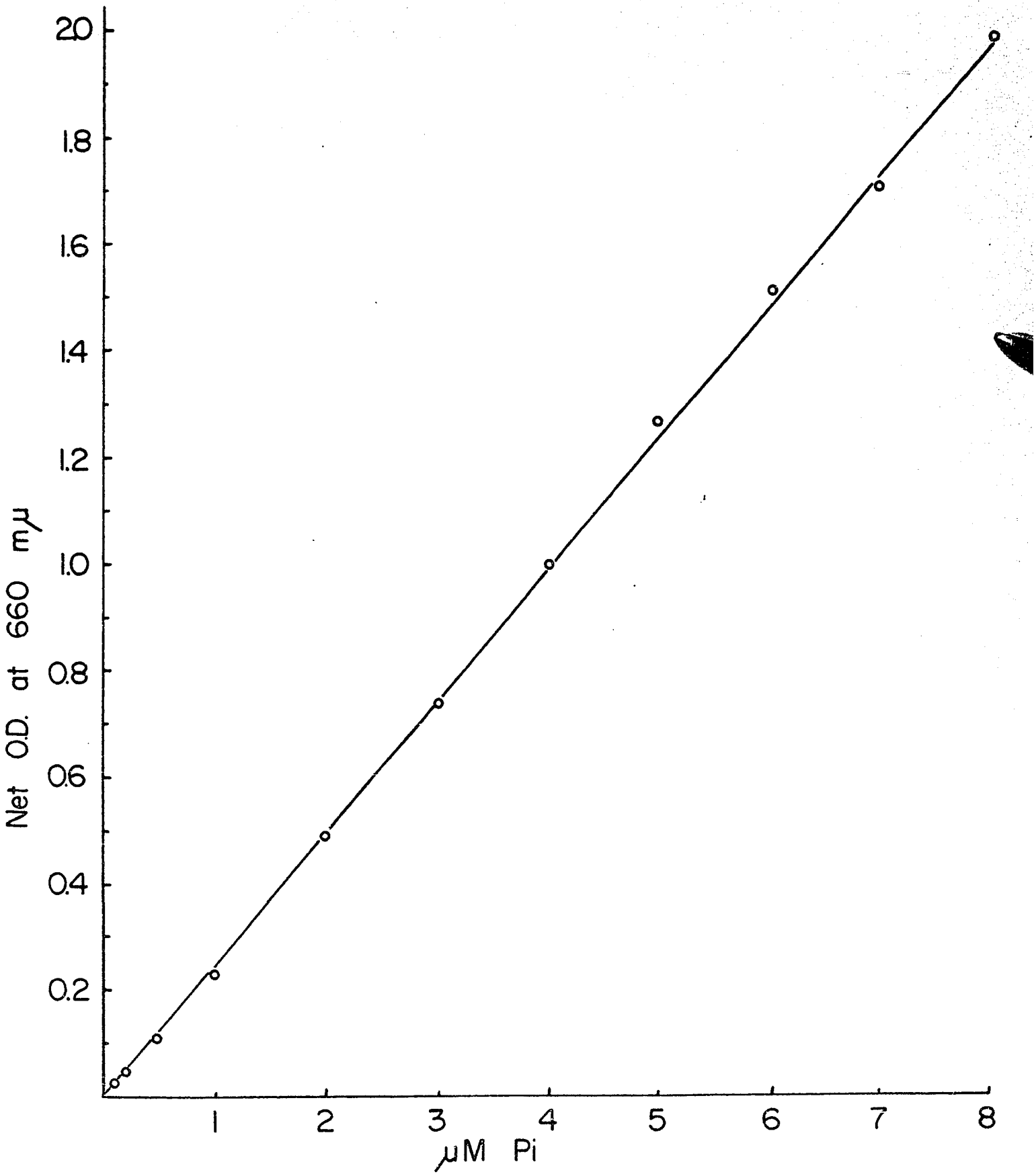


Fig. 2 Calibration curve for phosphate

increased to 68 μM by 10 days. In the S-R extracts, these values were 6 μM at one day, and 54 μM by 10 days.

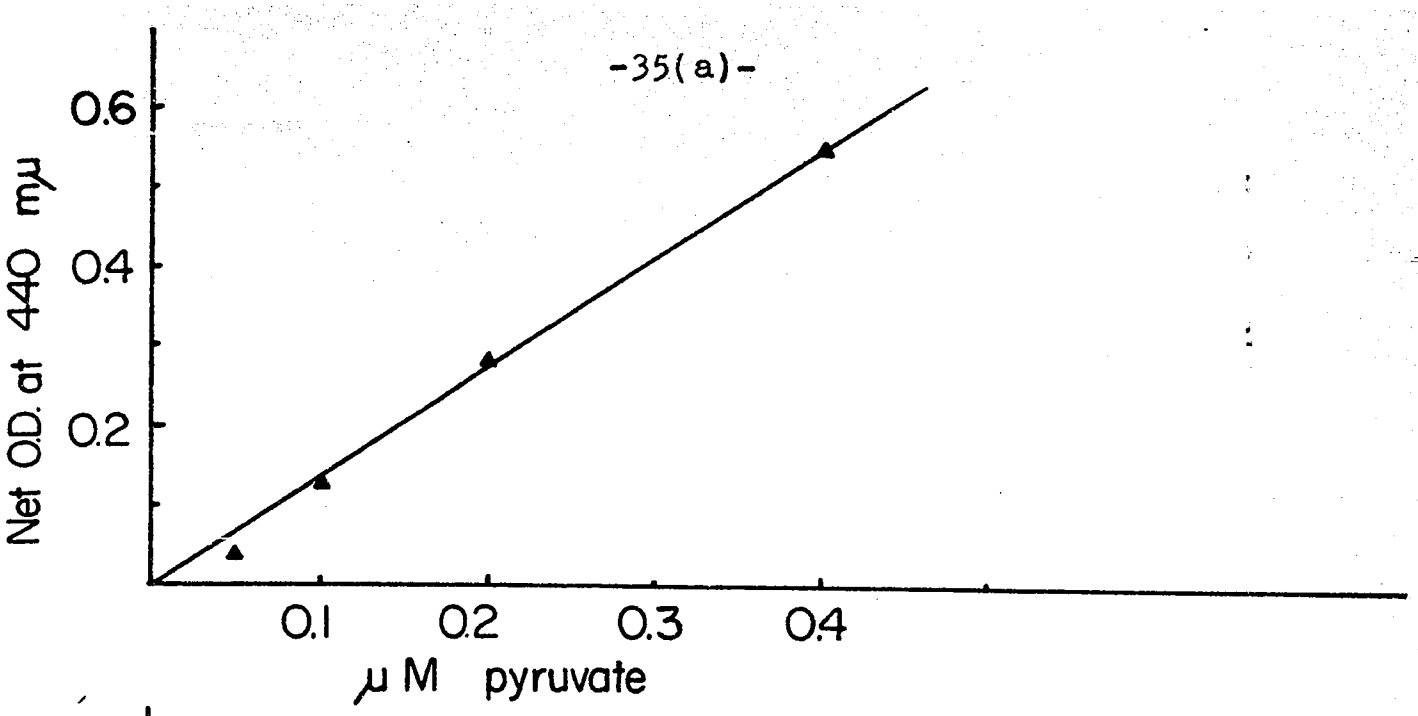
c. Pyruvate determination. The calibration curve obtained is presented in Fig. 3 and shows a straight-line reaction. For amounts of pyruvate higher than 0.04 μM, however, the concentration of hydrazine was not enough to give a proportional reaction.

d. Amino acid determination. The calibration curve presented in Fig. 3 shows that a straight-line reaction was obtained over the range of concentrations used.

e. Paper chromatography

Separation of nucleotides with the Pabst solvent. After a 16 hour run, the solvent front had travelled about 40 cm. from the origin. With the unwashed papers used in these studies, a secondary front about 10-15 cm. behind the solvent front showed up under the UV lamp. On occasion, the solvent ran much faster than expected and in such cases, this front did not appear. This phenomenon was attributed to the use of an old bottle of concentrated ammonium hydroxide, and was corrected by the addition of a ml. or so of the ammonium directly to the chromatograph jar. Magasanik et al (97) have made a study of this solvent and the importance of pH in its operation. The rf values obtained were higher than those given in the Pabst circular (86) and were the following: ATP- .22 (.20 in Pabst circular), ADP- .34 (.30), AMP- .51 (.45), GTP- streak from origin (.05), GDP- .21 (.09).

Separation of amino acids with the butanol solvent. The rf values of



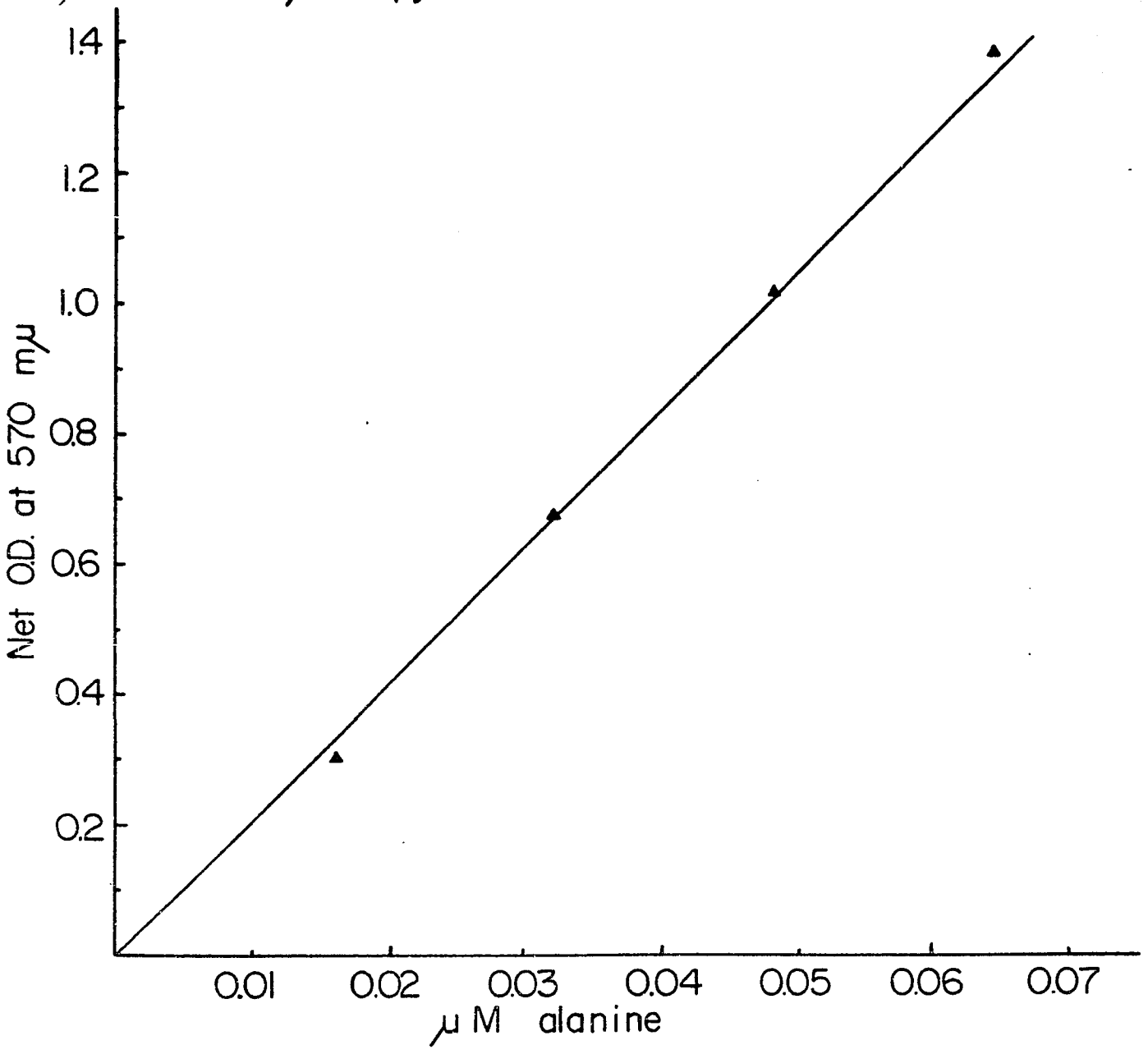


Fig. 3 Calibration curves

the amino acids used in these studies, alanine (ala), phenylalanine (phe), p-fluorophenylalanine (F), and B-thienylalanine (TE), were not computed per se because of the double run technique used. Instead, their positions in relation to phe = 100 were calculated and were found to be: ala- 43, F- 108, TE- 87.

f. Radioactive counting.

The gas flow (GF) counter with its low 2 c/m background was excellent for samples of low activity. The background of the liquid scintillation (LS) counter was relatively high, ca 30 c/m. and fluctuated from 25-35 c/m. These fluctuations made it unsatisfactory for samples of low activity despite its greater sensitivity.

Representative results of comparative LS and GF counting are presented in Table 1 and show that the GF count of paper discs was only 16-18% that of the LS count. When the sample was spotted on a planchet, a procedure that was calculated to yield an efficiency of 5%, the GF count increased to 52-53% that of the LS. Mans et al (90) reported that the disc method was ca 55% efficient, but extrapolation from these results would indicate that an efficiency of only ca 10% was obtained in these tests.

The GF count of 100 mg. solid samples was only 10% of that obtained in the LS, but increased to 60-63% when the correction factor was applied. If the corrected GF count represents an efficiency of 5%, and the LS method yields an efficiency of 10%, then the GF count should have been only 50% that of the LS count. This discrepancy is

Table 1

Interconversion of liquid scintillation (LS) counting and gas-flow (GF) counting.

Liquid samples

*Sample #	net count per min. 1 LS (paper)	2 GF (paper)	3 GF (planchet)	%2/1	%3/1	%2/3
phenylalanine	22,150	3,725	-	17	-	-
"	9,100	1,480	-	16	-	-
"	-	3,022	9,638	-	(52)	31
TCA sup 6	34	6	18	18	53	33
(Expt. 4) 9	44	7	22	16	52	32

Solid samples

Sample #	mg.	net count per min. 1 LS	2 GF	3 GF (corrected)	%2/1	%3/1
TCA ppte 16	100	1,013	99	615	10	63
(Expt. 4) 17	"	612	61	379	10	62
18	"	904	91	566	10	63
20	"	1,290	125	776	10	60

* 0.05 ml. sample on paper, 0.02 ml. sample on planchet; counts corrected in the latter for equal volumes.

then an indication of a loss of about 15% of the LS count owing to absorption in the sample. This loss is indicated graphically by the curve in Fig. 4, which is a plot of the weight of sample against the LS count. In practice, a standard amount, 100 mg., of the preparations from entire seeds or cotyledons was counted. The error from absorption was presumed to be the same for each and was not corrected for in Table 26.

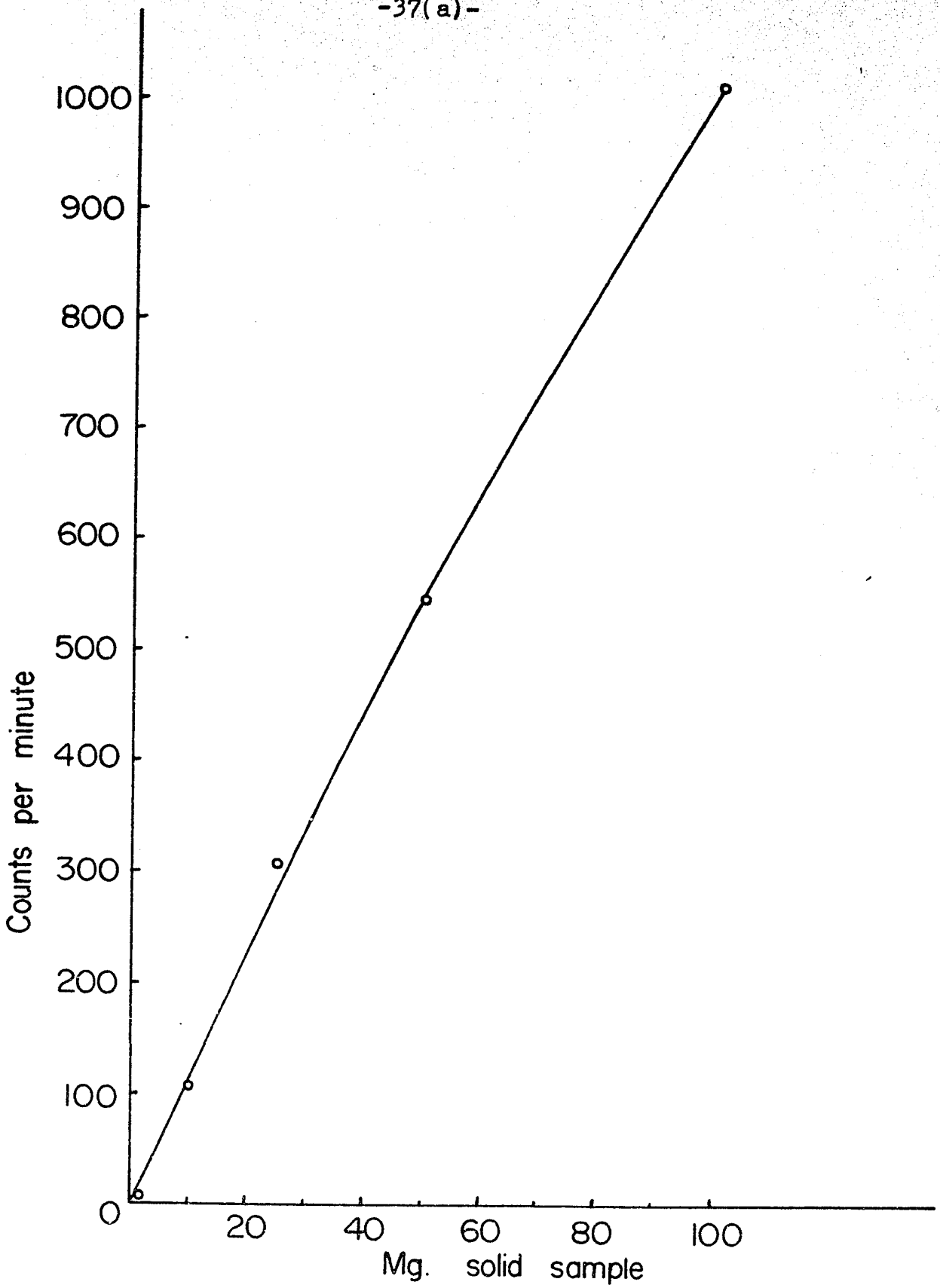


Fig. 4 Calibration curve for radioactive counting of solid samples

2. Preparation of RNA

a. Preparation of sRNA. Representative results obtained with the two methods used are summarized in Table 2 (5 out of 12 preparations by the Kirby Method (84)), and Table 3 (5 out of 8 preparations by the Rosenbaum and Brown method (88)). From these results, it is considered that the latter method is superior both in terms of yield and apparent purity. In the former, cellosolve was not effective in removing polysaccharide impurities from the sRNA fraction of pea seedlings. The method, probably because of the greater number of manipulations, did not give consistent results from one preparation to another.

The $O.D._{260}/O.D._{280}$ ratios were slightly lower than those reported by Rosenbaum and Brown ($O.D._{260}/O.D._{280} = 2.13$) with both methods, but were more so with the Kirby method.

The amino acids attached to sRNA were measured on the samples of the last 2 columns of Table 3, and, expressed as alanine equivalents, were found to be about 0.07 μM/mg. in the S-R sRNA and 0.06 μM in the cot sRNA.

b. Preparation of high M.W. RNA from the RNP. Only one preparation of this type was made, using the RNP fraction prepared in the usual way from 3-4 day shoot-root axes stored in the freezer for one week as starting material. The final white powder obtained was equivalent to a yield of 1.23 mg/100 seeds (O.D. measurement indicated 4.3 mg.), and gave an $O.D._{260}/O.D._{280}$ ratio of 1.67 in solution.

3. Study of ATPase activity.

The study of the ATPase activity of the extracts

Table 2

Representative results of sRNA preparation from the 100,000xg supernatant of pea seedling extracts.

Date	Jan. 28	Mar. 7	Mar. 15	May 19	Jan. 9
Source of extract	S-R	S-R	S-R	S-R	cot
mg. sRNA/100 seeds by dry wt.	-	3.5	1.0	4.4	39.5
* by O.D. determ'n	1.8	0.01	0.7	0.5	11.0
** % "purity"	-	0.4	71.0	11.0	27.5
$O.D._{260}/O.D._{280}$	1.52	1.85	1.85	1.79	-

*Calculated from $O.D._{260}$, E assumed to be 200.
**mg. sRNA by O.D./ mg. by dry wt. x 100.

Table 3

Representative results of sRNA preparation from frozen seedlings.

Date	May 26	May 31	Oct. 26	Jan. 15	Jan. 15
Source	S-R	S-R	S-R	S-R	cot
Age (days)	3	3	3	3	3
mg. RNA/100 seeds * "Total" RNA (O.D.)	9.9	14.4	10.9	10.9	50.0
* High M.W. RNA (O.D.)	0.6	2.6	0.8	5.8	4.1
$O.D_{260}/O.D_{280}$	1.64	1.92	2.04	2.00	2.04
*sRNA (O.D.)	4.4	3.2	1.8	4.9	22.2
dry wt.	12.1	9.7	6.6	10.7	98.2
** % "purity"	36.4	33.0	27.3	46.2	22.6
$O.D._{260}/O.D._{280}$	1.92	2.00	2.08	1.96	2.08

*Calculated from $O.D._{260}$, E assumed to be 200.
**mg. sRNA by O.D./ mg. by dry wt. x 100.

can be divided into two major parts: 1) experiments using the 25,000xg supernatant and 2) experiments using the two fractions obtained after 100,000xg centrifugation of the above supernatant.

a. Studies with the 25,000xg supernatant.

Effect of age of the seedling on the ATPase activity of the extract.

Young and Varner (10) and Young et al (11) reported that there was an increase in the specific activity (S.A.) of ATPase in the cotyledons of germinating pea seeds up to a maximum at about 4 days, followed by a decline. In this study the ATPase activity in extracts from both the cotyledons and the shoot-root axis was followed for periods of up to 8 days. The results of 2 separate determinations are presented in Table 4 and Figure 5, from which it can be seen that the specific activity in both extracts did indeed increase in the early days of germination. However, the decline in the S.A. of the cotyledon extracts reported by Young and Varner (10) was not observed to occur, (their results are indicated by the dotted line in Fig. 5A), but the S.A. showed a tendency to continue rising at least as far as the 8th day. The S.A. of the shoot-root extracts increased rapidly to a maximum by the 2nd or 3rd day and thereafter showed a slow decline.

The differences between the two series apparent in Figure 5 exist primarily in the cotyledon extracts and can be accounted for by the differences in extraction method. In the May series, 20 ml. of Tris buffer were used per 10 seeds in extracting the cotyledons, the method used by Young and Varner (10), and an amount approximately 10

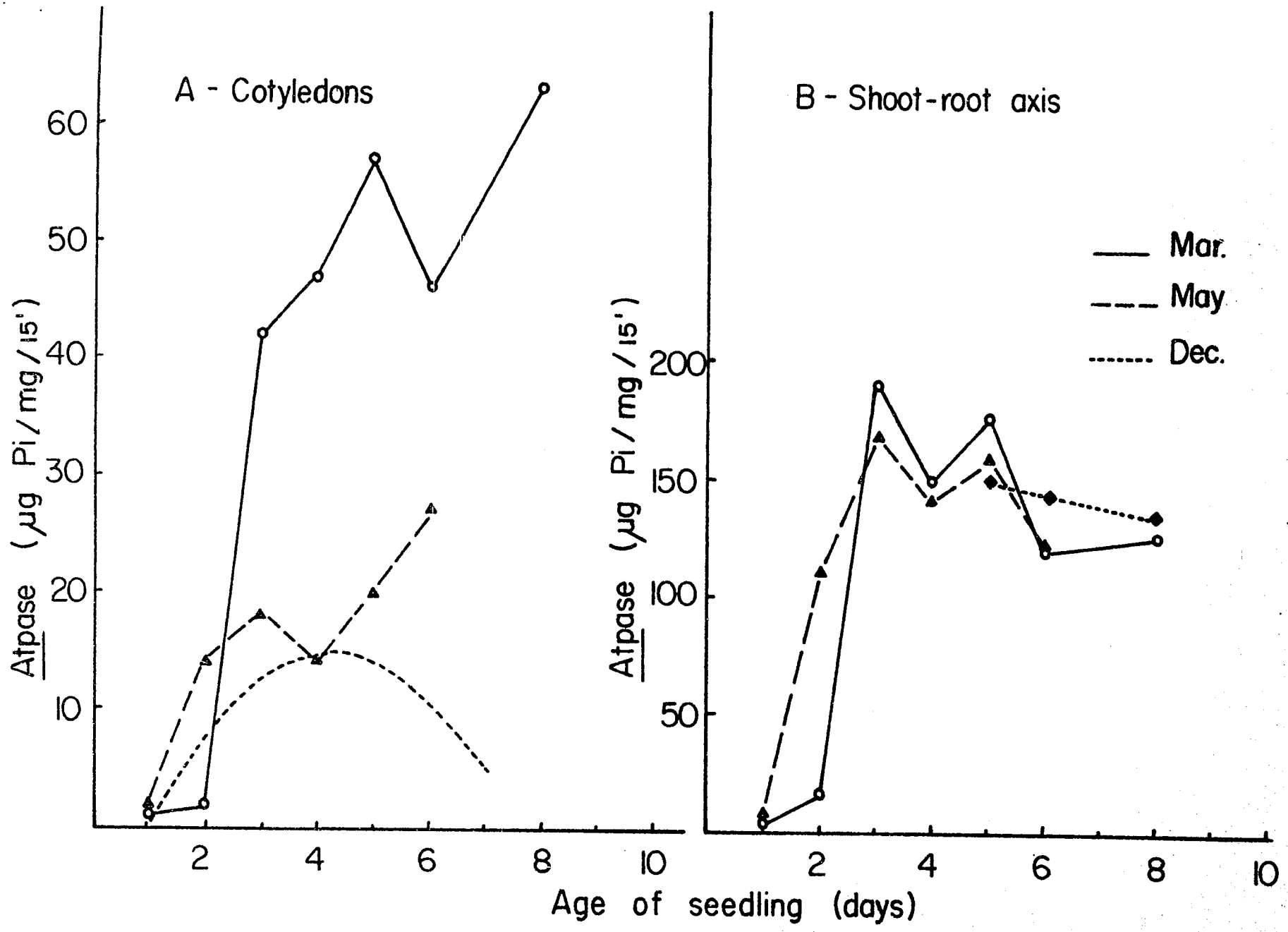


Fig. 5 The effect of the age of the seedling on atpase activity of the extracts

Table 4

Effect of the age of the seedling on the ATPase activity of the 25,000xg supernatant.

Date	Age, Days	Shoot-root axis					Cotyledons		
		Fr.Wt. (g.) 1)	Av. Length (cm.) Root	Shoot	Prot. (mg.) 1)	ATPase 2)	Fr.Wt. (g.) 1)	Prot. (mg.) 1)	ATPase 2)
Mar/61 3)	1	0.8	-	-	15	2	41.0	1917	1
	2	1.3	-	-	52	16	37.0	1093	2
	3	7.1	3.7	0.3	43	191	28.2	1092	42
	4	12.9	5.6	1.0	96	150	30.2	725	47
	5	20.6	8.1	2.7	128	176	27.3	739	57
	6	25.0	10.2	3.5	181	121	27.2	1000	46
	8	20.1	10.2	3.7	134	127	28.8	777	63
May/61 4)	1	1.5	0.5	-	57	7	33.3	1634	2
	2	4.2	2.4	0.2	112	112	32.0	1862	14
	3	10.0	4.4	0.7	133	168	27.6	1660	18
	4	13.4	6.5	1.2	140	143	29.7	1612	14
	5	13.4	8.4	2.1	176	160	32.4	1707	20
	6	33.8	12.1	6.0	336	122	28.9	1294	27
Dec/61 5)	5	17.2	5.9	1.8	208	150			
	6	22.4	8.7	2.7	286	144			
	8	19.8	9.8	2.3	232	135			

1) Fresh weight and mg. protein are calculated on a 100 seed basis.

2) ATPase expressed as µg Pi released/mg. protein in R.M./15 min.

3) March seedlings ground in 0.5 volumes of 0.1M Tris, 0.05 ml. extract/ml. R.M.

4) May seedlings ground in 2 volumes of 0.1M Tris, 0.1 ml. S-R, 0.3 ml. cot extracts/ml. R.M.

5) Dec. seedlings ground in 1 volume of 0.5M sucrose, 0.04 ml. extract/ml. R.M.

times more than was used in the March series. (Two volumes of Tris buffer per g. fresh weight were used in extracting the shoot-root axis, or 4 times more than was used in the March series.) As a result, there was a greater extraction of proteins from the cotyledons, presumably not enzymic in nature, which would therefore reduce the specific activity in the crude extract.

The reason for not obtaining an exact duplication of the results reported by Young and Varner (10) may be merely the different methods used in determining protein content. The O.D. method used by Young and Varner (10) which gives a higher estimate than the Lowrey method used in this investigation, would reduce the apparent S.A. If, in addition, it did not reveal a drop in protein content as the Lowrey method tended to do, then a further reduction in S.A. would be apparent. The O.D. of the extracts was not measured in these tests, but O.D. measurements made on the RNP fraction from cotyledons, indicated that no drop in protein occurred with age, while the Lowrey method showed that one did.

It can be seen from Table 4 that the protein extracted from the seedlings tended to increase with age in the shoot-root axis, as would be expected, and slowly to decline in the cotyledons. However, this determination was subject to considerable variation, being dependent on the thoroughness of the grinding which was done by hand.

A further incidental observation to be made from Table 4 is that the fresh weights and shoot-root lengths were not too consis-

tently related to the apparent age of the seedlings. These are average values taken from material that showed considerable variability in individual rates of development. Also, the age group in each series was not selected daily from a large lot of seeds all started at the same time. Instead, the reverse was done, i.e., small lots were started on succeeding days, and all were harvested on the day of the experiment. In this way, two variabilities were introduced that would account for the above observation. These were: 1) minor variations in handling the seed from day to day, and 2) inherent variability between samples introduced in the chance selection of a small number of seeds (ca 50). This then raises the question of what constitutes the true age of a seedling, the chronological age dated from the beginning of germination, or the physiological age, as determined by the stage of development, and which is affected not only by environment, but by inherent factors as well.

In the light of this argument, ATPase activity in the extracts can be correlated with the length of the root used as a measure of the age of the seedlings. In two experiments, summarized in Figure 6A seedlings of the same chronological age were divided into groups on the basis of the root length. In the first experiment, seedlings nominally 2 days old were divided into 4 lots, ranging from those with the radicle just broken through the testa to those whose roots were approximately 2 cm. in length. In the second experiment, the seedlings were nominally 1 day old, and were divided into 2 lots: those

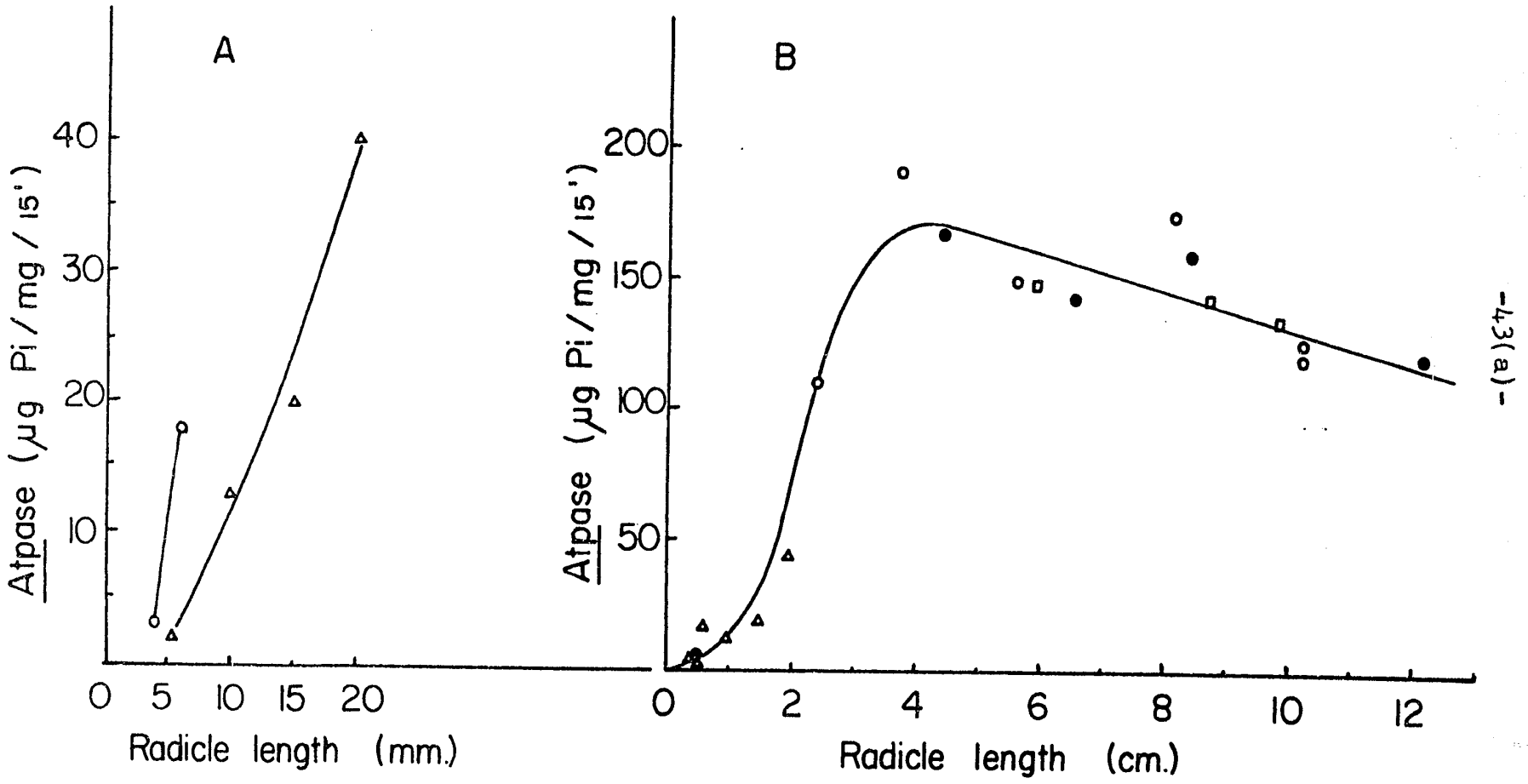


Fig. 6 Correlation between length and atpase activity of extracts from the shoot-root axis

whose radicles had just broken through the testa, and those with the radicle already protruding a few mm. Both tests showed that the ATPase activity could not be demonstrated in the shoot-root axis except at a very low level at the stage of germination at which the seed had just begun aerobic respiration (testa broken), but could be demonstrated at increasingly higher levels as the root length increased.

The correlation between root length and S.A. of the extracts can be observed in Fig. 6B, which is a collation of the data from Table 4 and Fig. 6A. From Fig. 6B, it can be seen that the S.A. of the ATPase increased rapidly in the shoot-root axis to a maximum when the root was 3-4 cm. long, and then slowly declined as the root elongated.

The specificity of the enzymic activity for ATP. Young and Varner (10), had already determined the highly specific character of their cotyledon extracts towards ATP and ADP. In these experiments, substrates other than ATP were not studied to any great extent (Table 5). Paper chromatography of R.M. containing either cotyldeon or S-R extracts showed that ATP was hydrolyzed to AMP with the intermediate formation of ADP, and so presumably ADP would also serve as substrate. Inorganic pyrophosphate and phosphoglyceric acid were also tested in one or two experiments, but these substances were hydrolysed much more slowly than ATP; the rate for pyrophosphate being 1/14, and that for PGA being 1/4 that of the rate for ATP.

Raacke (31) has reported that GTP, uridine triphosphate

Table 5

Hydrolysis of ATP, and other substrates by extracts from 3-5 day old shoot-root axes.

Extract	Substrate added					
	ATP	ADP	AMP	GTP	PGA	PPi
	(µg Pi/mg. prot./15 min.)					
25,000xg supernatant	137	-	-	-	-	10
	132	-	-	-	34	-
100,000xg " (PO_4)*	105	397	-	-	-	-
" " (Tris)*	109	256	-	-	-	-
" " (sucrose)*	66	194	0	-	-	-
RNP (PO_4)*	290	882	4	-	-	-
" (sucrose)*	235	1013	-	715	-	-
" "	225	402	0	386	6	-
" (Tris)*	421	725	-	-	-	-

* (PO_4) etc. refers to the E.M. in which the extracts were prepared.

(UTP), and cytidine triphosphate(CTP) as well as ATP are hydrolysed by extracts from pea seedlings. The conditions of incubation of the R.M. were different (pH 8.1, 28°C), and the rates of hydrolysis reported were lower than those of this study (25 µg Pi/mg./15 min. for ATP, increasing with each nucleotide to a maximum for CTP of 78 µg.Pi/mg./15 min.).

Factors during extraction affecting the ATPase activity of the extract. The effect of the volume of the E.M. on the activity of cotyledon extracts has already been noted. In addition, there were many minor variations in preparing the extracts whose affect on ATPase

activity was undetermined. These included the degree of hand grinding, small differences in temperature or in length and speed of centrifugation and the inherent variability of the experimental material itself. However, the composition of the E.M. was subject to control, and the effect of varying this was studied to a limited extent.

The medium used by Young and Varner (10), 0.1M Tris buffer, pH ca 7.5, was used for most extractions, and only occasionally was a sucrose-containing medium used. Moustafa and Lyttleton (21) claimed that the ATPase observed in their extracts from wheat germ was reduced when sucrose was used during extraction. This they attributed to the mitochondria being kept intact. In this investigation, the effect of the presence or absence of sucrose was not deliberately studied, but there was no indication of any marked effect of sucrose on the ATPase activity of extracts from the shoot-root axis of pea seedlings. For example, in Table 4, the S.A. of the March and May extracts prepared in Tris was similar to the S.A. of the December extracts prepared in sucrose. Perhaps the concentration of sucrose used, 0.5M, was not adequate to prevent the breakage of at least some of the mitochondria.

It seems reasonable to assume that the pH of the E.M. would affect the enzyme extracted in a manner quite different from the effect of pH on the already extracted enzyme, which Young and Varner (10) had shown to have a pH optimum in a broad range around 7.0. Sucrose alone, or sucrose adjusted to varying pH with Tris buffer

was used as E.M. in the two experiments reported in Table 6. Shoot-root axes were used, one lot being fresh, and one lot being frozen prior to extraction. In both experiments, the pH had a marked effect

Table 6

The effect of the pH of the extraction medium on ATPase activity in extracts from fresh and frozen shoot-root axes.

Tissue	Age (days)	pH *E.M.	pH extract	** Prot. (mg.)	*** ATPase	pH R.M.
Fresh S-R	3	6.61	6.44	68	486	6.85
		7.45	6.90	83	358	6.87
		7.88	7.65	96	410	6.92
		8.39	8.16	107	325	7.06
Frozen S-R	3	6.30	6.31	190	37	6.65
		7.41	6.66	196	45	6.62
		7.68	7.25	198	62	6.80
		8.26	7.75	230	73	6.82

* E.M. = 2 volumes of 0.5M sucrose, or 0.5M sucrose-0.1M Tris. HCl.

** mg. protein extracted from 100 seeds.

*** ATPase expressed as μg Pi/mg. prot./15 min., (0.05 ml. extract /ml. R.M.).

on the appearance of the extracts. These were normally a green color, but they became increasingly yellow in color as the pH was increased. From Table 6, it can be seen that the effect on ATPase activity of varying the pH was not as marked as the effect of freezing. Freezing prior to extraction reduced the S.A. of the ATPase to about

1/10th of that usually obtained, but approximately doubled the amount of protein extracted. There appeared to be some effect of pH also, although more experimentation would be needed to establish the significance of the relatively small differences. The amount of protein extracted tended to increase with pH. The ATPase activity tended to be higher in the sucrose preparation and to show a maximum at pH 7.6 with the sucrose-Tris preparations from fresh tissues, but to increase with pH in extracts from frozen tissues. In all cases, as shown in Table 6, ATPase activity was measured at a pH close to 7.0.

Freezing of the tissues sometimes occurred when grinding was done in a too-cold mortar, and the extracts prepared from such tissues invariably showed an ATPase activity much below normal. The effect of freezing after extraction, however, as occasionally occurred during centrifugation, seemed to be negligible as far as ATPase activity was concerned. Hence it would appear that freezing of the tissues altered the cellular makeup in such a way that the ATPase activity was not extracted in an active form, perhaps because the active site was bound to a cellular fraction. It is tempting to draw an analogy with the preparation from muscle described by Molnar and Lorand (98). In their case, a microsomal fraction that served as a phosphoryl group acceptor exhibited an ATPase activity when treated with a detergent to remove the phosphoryl group acceptor activity. If the analogy is valid, then it would seem that the ATPase activity observed *in vitro* is not the true role of the enzyme

in vivo, and would be in this sense an artifact of extraction.

Fractionation of the extracts. Preliminary attempts to fractionate the extracts by electrochromatography, ammonium sulfate fractionation, or acid precipitation met with no success. Differential centrifugation was the only method tested that was effective in producing a fraction with an increased S.A. With shoot-root extracts, the RNP fraction from 100,000xg centrifugation showed an activity 2-3 times that of the 25,000xg supernatant from which it was obtained.

b. Studies with the fractions from 100,000xg centrifugation.

Effect of the age of the seedling on the distribution of activity between RNP and supernatant. Young and Varner (10) studied the distribution of ATPase activity in fractions obtained by differential centrifugation of cotyledon extracts. They found that the activity in the mitochondria and cell debris fractions remained at a low level, while that of the microsome and supernatant fractions increased with age of the seedling to a maximum. The rate of increase in the microsomes exceeded that of the supernatant. In this investigation, extracts were prepared from the cotyledons and shoot-root axes of seedlings of varying ages, and separated into RNP and supernatant fractions as usual. The ATPase activity of each fraction was determined and the results are presented in Table 7. From this table, it can be seen the S.A. of the ATPase of the RNP fraction from the shoot-root axis was as much as 10 times that of the supernatant. This activity tended to decrease with time, while the total amount of protein in the fraction increased.

Table 7

The effect of the age of the seedling on the ATPase activity of the 100,00xg fractions.

	Age (days)	*Fr. Wt. (g)	*Prot.(mg.) RNP	*Prot.(mg.) sup	**ATPase RNP	**ATPase sup	RNP $\frac{O.D._{260}}{O.D._{280}}$	RNP $\frac{O.D._{260}}{0.1\ mg.prot.}$
*** S-R	1	1.3	6	-	9	-	1.59	3.42
	2	4.6	11	102	450	44	-	-
	3	10.2	18	166	399	52	-	-
	4	11.3	16	-	300	-	1.50	0.95
	6	24.4	29	405	226	54	-	-
	8	46.8	31	-	182	-	1.48	0.82
***cots	1	35.5	93	-	3	-	1.51	0.54
	2	34.1	113	2040	17	3	-	-
	3	32.8	103	1934	8	6	-	-
	4	35.4	65	-	8	-	1.56	0.66
	8	35.2	61	-	3	-	1.57	0.73

*** cot and 2, 3 and 6 day S-R extracts prepared in 2 volumes of 0.5M sucrose, 1, 4 and 8 day S-R ground in 1 volume of 0.5M sucrose, then diluted with 10, 0.5, and 1 volume of sucrose respectively.

* Fr. wt. and mg. protein per 100 seeds.

** µg Pi/mg. prot./15 minutes.

The S.A. in the supernatant on the other hand, tended to remain at a constant low level, while the protein content increased.

Also Table 7 shows that the S.A. of the RNP fraction from the cotyledons was very low by contrast with that from the shoot-root axis, and after a slight initial increase, showed a decline with age. Only 2 samples of the supernatant, those from the 2 and 3 day extracts, were tested for ATPase activity, but the results indicate that its activity was even lower than that of the RNP.

Effect of age on appearance of the extracts. The variations in the ATPase activity of the RNP and supernatant fractions with age can be correlated with variations in the appearance of the extracts. Because this correlation is considered to be of significance in an interpretation of the intracellular location of the enzymic activity, it is gone into in considerable detail, both here and in a subsequent description of the effect of the extracting medium on activity.

1) Extracts from the shoot-root axis. Low speed centrifugation in general yielded a white precipitate with a yellow-brown overlay, presumably the mitochondria and nuclei, regardless of the age of the seedlings. High speed centrifugation of the supernatant so obtained yielded an RNP fraction from 1 day old seedlings that consisted only of a small, translucent, brownish pellet. In preparations from 2 and 3 day old seedlings, a considerable "fluff" overlay this pellet, but did not separate readily from it when the supernatant was decanted. In extracts from 4 day and older seedlings, the "fluff" tended to pour off

with the supernatant, but was retained with the RNP for the purpose of the ATPase test.

2) Extracts from the cotyledons. Low speed centrifugation removed a large amount of white material with a loose overlay which was a clear green color in preparations from 1 day old seedlings, but which gradually faded to a yellow-green color as the seedling material grew older. After high speed centrifugation, the RNP from 1 day old seedlings was found to consist of a small amount of gelatinous brown precipitate attached to the tube, and a considerable green "fluff". The supernatant was very turbid, and yielded a further greenish precipitate on standing. With preparations from 2 day old seedlings, the "fluff" had paled in color, but was still considerable in amount. With preparations from 3 and 4 day old seedlings, only the gelatinous deposit was present with a small amount of overlay. A slight turbidity remained in the final supernatant. By the time the seedlings were 8 days old, only a small patch of translucent material was obtained in the RNP fraction.

These changes can be compared with the microscopic changes occurring in the pea seed cotyledon during germination as described by Varner and Schlidovsky (99). The "fluff" may be formed from the considerable endoplasmic reticulum they noted in the cells after 1 to 3 days germination. Chloroplast lamellae were also present, as well as starch granules and large protein bodies which lost their identity in the course of germination. By the 7th day of germination, the cells of the cotyledon consisted only of a few large vacuoles and a thin

sheet of cytoplasm at the periphery.

The appearance of the "fluff" in the extracts from 2 day old shoot-root axes coincided with a marked increase in the ATPase activity of the RNP fraction. In extracts from cotyledons, the gradual disappearance of the "fluff" layer from the RNP fraction of 2 day and older seedlings can be correlated with the decline in its ATPase activity. Presumably this "fluff" corresponds to the "light microsomes" described by Loening (100), a fraction which he found to increase in root tips with age. In his study, the root tip was separated into sections corresponding to 1) the area of meristematic activity, 2) the area of elongation, and 3) the area of differentiation. No "light microsomes" were obtained in the first area, but they appeared in the second, and were considerable in the third. Electron microscope studies of the "light" and "heavy" fractions revealed that the latter contained a high proportion of ribosomes, and the former many vesicles. RNA/protein ratios were greater than 1 in the latter, and less than 1 in the former.

Although RNA determinations per se were not made on any of the preparations from S-R axes and cotyledons, the $O.D._{260}$ and $O.D._{280}$ of suitably diluted aliquots were measured on many of them. From these data, an approximate RNA/protein ratio can be calculated in two ways: 1) the $O.D._{260}/O.D._{280}$ ratio, and 2) the $O.D._{260}$/mg. protein ratio. In both cases, the ratio would be higher in an RNP fraction containing a high concentration of ribosomes. These

values are given in Table 7, and indicate decreased ratios with the shoot-root preparations, as would be expected from the increased amount of "fluff". In cotyledon preparations, on the other hand, the ratios increased, as would be expected from the decreased amount of "fluff".

Effect of the extraction medium on the ATPase. This study was carried on in conjunction with attempts to demonstrate an in vitro synthesis of protein. The ATPase activity of most of the preparations of RNP used in these tests, and that of the supernatant as well, was routinely measured. These preparations were made from 3-4 day old S-R axes in a variety of E.M., the compositions of which are indicated in Table 8, but given in more detail in Tables 12-17. The average results obtained with each type of E.M. are presented in Table 8 together with the number of preparations on which the average was based and a calculation of the standard error. The differences in pH which occurred were ignored since they were slight within each group.

1) Extraction media not containing GSH. From Table 8, it would appear that RNP prepared in sucrose-phosphate buffer (E.M. Type B) had a higher ATPase than other preparations. RNP prepared in sucrose-Tris buffer (E.M. Types C and D) had the second highest ATPase and sucrose preparations (E.M. Type A) had the lowest activity. In all these preparations, the activity of the RNP exceeded that of the supernatant. The ATPase of the supernatant tended to be higher in the sucrose-phosphate and sucrose preparations than in the Tris prepara-

Table 8

The effect of the extraction medium on the ATPase activity of the 100,000xg fractions.

E.M. without GSH

Type	A	B	C	D
*Comp. of E.M				
sucrose	x	x	x	x
phosphate	-	x	-	-
Tris	-	-	x	x
Mg++	-	-	-	x
Cl-	-	-	x	x
# preparations	4	5	6	5
**Prot.-RNP	14±3***	16±2	6±1	11±3
-sup	119±24	122±9	124±28	147±20
****ATPase-RNP	318±28	689±79	468±37	457±70
-sup	148±95	141±16	30±15	88±6

E.M. with GSH

Type	E	F	G	H	I	J
*Comp. of E.M.						
sucrose	x	-	x	-	-	x
Tris	x	x	x	x	x	x
Mg++ (low)	-	-	-	-	x	x
" (high)	x	x	-	-	-	-
GSH	x	x	x	x	x	x
K+	-	-	-	-	x	-
Cl-	x	x	x	x	x	x
# preparations	2	1	1	1	1	1
**Prot.-RNP	7±1	7	7	8	4	8
-sup	98±33	128	102	103	63	63
****ATPase-RNP	115±20	67	338	328	223	276
-sup	232±30	250	49	63	27	94

*given for individual preparations in Tables 12-17. Sucrose = 400-500 μM; phosphate, Tris, and high Mg++ = 100 μM; low MG++ = 1-55 μM; GSH = 6 μM; K+ = 50 μM/ml. E.M.

**Mg. protein extracted from 100 seeds.

***Standard error.

****μg Pi/mg. protein/15 minutes.

tions. The amount of protein extracted into the two fractions was about the same in all the media, with the exception of the RNP from Type C preparations in which it was somewhat lower, and the supernatant in Type D preparations in which it was a little higher.

In appearance, sucrose preparations were invariably in two fractions after 100,000xg centrifugation; 1) a tightly packed 2-layered pellet of an opaque brownish color, and 2) a clear, pale-colored supernatant. When the supernatant was decanted, and the tube inverted to drain, the 2-layered RNP fraction remained more or less intact. Tris preparations were very similar to sucrose preparations, except for the change in color of the supernatant to a more yellow cast at the higher pH values. Phosphate preparations, however, were different. The RNP fraction seemed much bulkier, and was not well packed so that when drained, it tended to separate into a tightly packed gelatinous pellet that adhered to the tube, and a loose, brownish "fluff". In most cases, the "fluff" was retained with the pellet by not inverting the tube to drain it. With one such preparation, however, the "fluff" was allowed to drain into the supernatant, and two effects of this action were observed: 1) the S.A. of the supernatant was higher than usual (515 μM Pi/mg.), and 2) the protein content of the RNP was lower than usual (4 mg.).

2) Extraction media containing GSH. A series of extracts were prepared which contained GSH (E.M. Types E-J, Table 8). In most of these, (E.M. Types G-J), the ATPase activity of the RNP exceeded

that of the supernatant and tended to be similar to that of a sucrose preparation, (E.M. Type A), while the ATPase of the supernatant approximated that of sucrose-Tris preparations (E.M. Types C and D). In appearance, the extracts were generally similar to those prepared in sucrose with the RNP showing a separation into two layers. In the type H preparation, however, the appearance of the RNP was more like that of a sucrose-phosphate preparation.

Two media (E.M. Types E and F) yielded extracts in which the ATPase of the supernatant exceeded that of the RNP, and that of the other supernatant preparations as well, while that of the RNP was the lowest ever obtained from fresh (as opposed to frozen) tissues. Both these E.M. contained a concentration of magnesium 10 times higher than intended, and differed from each other only in the absence of sucrose in E.M. Type F. The RNP consisted only of a more or less homogenous, gelatinous, small pellet. Apparently most of the "fluff" had not been precipitated, but was dispersed in the supernatant.

Although the activity of the fractions obtained in the E.M. Type G was not unusual, the appearance of the extract was. Three distinct fractions were apparent after centrifugation: the supernatant, the "fluff", and the pellet. The ATPase of the "fluff" was determined separately, and is not included in Table 8. It was higher (425 μM/mg.) than that of the RNP (338 μM/mg.).

From these limited results, it is not possible to draw any conclusions as to the effect of GSH <u>per se</u>. However, it is

apparent that manipulation of the E.M. with respect to GSH and Mg++ content produced marked changes in the appearance of the RNP fraction, so that the whole spectrum from a small homogeneous pellet, to a clear separation into pellet and "fluff" was obtained, with corresponding changes in the ATPase activity.

O.D. measurements were made on many of these preparations, but no consistent correlation with the S.A. of the ATPase or the appearance of the RNP was observed.

The specificity of the enzymic activity of the RNP and supernatant fractions for ATP. Only GTP, ATP, ADP, AMP, and PGA were tested as substrates and the results are given in Table 5. From this table, it is apparent that the rate of hydrolysis of ADP exceeded that of ATP. This rate was so rapid, in fact, that some of the values in the table are an underestimation of the true rate owing to failure to take a sample early enough. With either ATP or ADP as substrate, AMP accumulated as the product, as was shown by paper chromatography. The hydrolysis of AMP, if it occurred at all, was so slow that phosphate determinations were not a reliable indication of its occurrence. Adenosine appeared to be the product since a UV-absorbing spot appeared in the position it would be expected to occupy on paper chromatograms. The eluted spot showed a maximum absorption at 260 mμ, but the amounts were too small for further identification. Whether or not AMP would be hydrolysed did not seem to be predictable. Adenosine tended to appear on chromatograms of R.M. containing: 1) RNP from older

shoot-root axes, 2) RNP that had been frozen, and 3) RNP at a 10 times higher concentration than usual. Attempts to impose deliberately one or more of these conditions were not always successful, especially those attempts to demonstrate the effect of age. Further experimentation to clarify this puzzling situation is indicated. No deamination of the adenine nucleotides apparently occurred since no ammonia could be detected in the reaction mixtures. GTP was rapidly hydrolysed to GDP, but no further hydrolysis seemed to occur. The hydrolysis of PGA occurred at a slow rate.

Raacke (31) found that a nucleotidase active towards ATP, GTP, UTP, and CTP appeared in both the supernatant and ribosome fractions of pea seedling extracts. Her studies with inhibitors, etc. indicated that several enzymes were involved, those of the supernatant being different from those of the ribosomes.

The effect of combining two substrates was tested in one experiment, using a 5-day S-R RNP preparation, with the results shown in Table 9. These results indicate, both by phosphate determination and by the amount of ATP on the chromatogram, that the addition of AMP, PGA or GTP reduced the extent of ATP hydrolysis. No significance can be attached at the present time to the calculations of percentage recovery etc. also included in this table since there is no measure of experimental error.

The effect of storage on the ATPase activity of the RNP. Some of the RNP preparations were stored in an ice bath for periods of up to two months. Their ATPase activity was measured again on one or

Table 9

The effect of combining ATP with AMP, PGA, or GTP on the hydrolysis of each substrate.

Nucleotide added	ATP	AMP	ATP AMP	PGA	ATP PGA	GTP	ATP GTP
µM added/ml. R.M.	2.58	3.58	6.16	4.0	2.58	2.66	5.24
" Pi/ml. in 30'	5.65	0.0	4.98	0.16	5.32	3.53	6.87
Calculated µM Pi	4.48	0	3.96		4.98	2.06	6.72
% calc./actual	79		64		94	58	98
* " eluted/ml. R.M.							
GTP	-	-	-	-	-	0.05	0.04
ATP	0.05	-	0.67	-	0.47	-	1.87
GDP	-	-	-	-	-	2.06	2.11
ADP	0.01	-	0.88	-	0.12	-	0.55
AMP	2.24	-	5.12	-	2.43	0.02	2.03
Total µM recovered	2.30	-	6.67	-	3.02	2.13	6.60
% recovery	89	-	108	-	117	80	126

*paper chromatography in Pabst solvent I (86) of 0.025 ml. of R.M.

Table 10

The effect of storage at 4°C on the ATPase activity of the RNP fraction.

Days in storage	1	2	4	5	6	9	11	18	25	26	39	53
% orig. activity												
suc. RNP (7)*	80	78	79	69	63	65	-	60	-	29	27	14
PO_4 " (2)	-	-	64	-	-	-	57		60			
Tris (3)	47	-	49	-	-	-	-	35				

*the numbers in brackets refer to the number of extracts on which these percentages are based.

more occasions. This was not done in a systematic or deliberate manner, but judging from the results in Table 10, it would seem that a gradual loss of activity occurred with the passage of time. This loss was most rapid in the RNP that had been prepared in a sucrose-Tris buffer (E.M. Type C). In the few extracts in which it was tested, ADPase and GTPase activities also declined with time, but retained the same relative rates.

4. The study of protein synthesis in the pea seedling using a cell-free in vitro system.

a. Using the 25,000xg supernatant. In this study, the initial attempts to demonstrate an in vitro synthesis were performed with the 25,000xg supernatant, a preparation assumed to contain the ribonucleoprotein fraction but not the mitochondria. In three experiments, extracts were prepared from the shoot-root axis of 3 day old seedlings and were added to reaction mixtures modelled after that of Webster (30). Total protein was determined in all three experiments, and in addition, a test for an increase in ATPase activity was made in two experiments. The results are presented in Table 11. From this table, it is apparent that a net increase in the protein reaction occurred only in one control flask (Nov. 10), a decrease of protein reaction being the more usual case. An increase in ATPase activity occurred in the first experiment, but was unaccompanied by any increase in protein reaction. This did not seem to be a fruitful approach, and the preparation of more refined systems was undertaken.

Table 11

The results of attempts to demonstrate an in vitro synthesis of protein using the 25,000xg supernatant.

Date		Nov. 3 1960	Nov. 10 1960		Dec. 9 1960	
Age (days)		3	3		3	
Extraction medium						
*µM sucrose		-	-		800	
" Tris-HCl		100	100		-	
pH E.M.		7.5	7.5		-	
ml./g.fr.wt.		0.5	0.5		0.5	
Reaction mixture		(1)	(1)	(2)	(1)	(2)
*µM Tris-HCl		50	50	51	35	35
" $MnCl_2$		0.03	0.30	0.31	0.30	0.30
" KCl		0.01	0.10	0.10	0.10	0.10
" ATP		0.10	4.10	4.10	0.10	0.10
" PGA		-	-	-	95.3**	95.3**
*mg. a.a.		0.66	-	0.66	-	0.66
ml. extract		0.1	0.1	0.09	0.36	0.36
mg. prot./ml. (TCA ppte)	0'	1.28	1.29	1.16	3.51	3.60
	20'	-	-	1.12	-	-
	30'	1.23	-	-	3.53	3.52
	40'	-	1.34	1.12	-	-
	60'	1.26	-	-	3.26	3.35
ATPase (µM Pi/ml./15')	0'	4.61	-	-	7.52	8.38
	15'	5.64	-	-	5.46	6.93
	30'	5.82	-	-	4.76	6.16

*concentration expressed as µM or mg./ml. E.M. or R.M.; amino acid mixture used by Webster (30).

**concentration of PGA 10 times higher than intended.

b. Using the RNP fraction. Webster (30) has published the results of experiments in which a net synthesis of protein and enzymic activity in cell-free systems from pea seedlings were obtained. He used 0.5M sucrose as the extraction medium (E.M.) in the preparation of the RNP fraction from 3 day old shoot-root axes. Ten attempts to duplicate these experiments were made and representative results are presented in Table 12. The reaction mixture (R.M.) given in the table was that used by Webster (30), with the exception that labelled amino acids were omitted. Evidence of protein synthesis was looked for in the total protein reaction and in a test for ATPase. The RNP and supernatant in the R.M. were not separated before analysis as Webster had done. Only 2 tests (Jan. 14, March. 10, Table 12) gave a clear increase in protein reaction. ATPase activity also showed an increase in a few cases (e.g. Jan. 14, Mar. 10; Table 12), but this did not always coincide with an increase in protein reaction. The Jan. 14 attempt, the first one made, was remarkable in that it showed a net synthesis of 1.5 mg./mg. RNP protein, an amount well in excess of the 0.4 mg./mg. RNP reported by Webster (30). This preparation was the first one tested and had been made from the last of an old batch of seeds. Perhaps this was an instance of the "active" seeds described by Webster (35) in an effort to explain his own difficulty in repeating his results. On the other hand, it may have been a result of some experimental error. An estimate of experimental error can be made from the duplicate Mar. 10 R.M. of Table 12 which differed only in the sRNA content. The Jan. 12, 1962,

Table 12

Representative results of attempts to demonstrate an in vitro synthesis of protein, using RNP prepared in sucrose.

Date	Jan. 14 1961	Mar. 10 1961		Mar. 17 1961	Apr. 20 1961	May 19 1961	Jan. 12 1962
Age (days)	3	3		3	3	3	2
E. M.							
*µM sucrose	500	500		500	500	500	500
" Tris	-	-		-	-	20	-
" NaOH	-	-		-	**	-	-
pH E. M.	-	-		-	-	9.00	-
ml./g. fr. wt.	2	2		2	2	2	2
pH extract	-	-		6.10	7.3	7.40	6.50
R. M.	(1)	(1)	(2)	(1)	(1)	(1)	(1)
*µM Tris	50.0	50.0	50.0	50.0	50.0	50.0	50.0
" ATP	0.1	0.1	0.1	0.1	0.1	0.1	1.35
" GTP	0.3	0.3	0.3	0.3	0.3	0.3	4.46
" $MnCl_2$	0.3	0.3	0.3	0.3	0.3	0.3	5.7
" KCl	0.1	0.1	0.1	0.1	0.1	0.1	0.96
" PGA	10.0	10.0	10.0	9.1	13.5	10.0	-
" PEP	-	-	-	-	-	-	9.07

Table 12 continued

Table 12 - continued

*mg. a.a.		0.66	0.66	0.66	0.66	0.66	0.66	0.63
* " sRNA(S-R)		0.36	0.3	-	0.001	0.213	0.33	0.93
" " (cot)		-	-	0.3	-	-	-	-
" kinase		-	-	-	-	-	-	0.03
" RNP(S-R)		0.46	2.50	2.50	1.44	0.37	0.26	-
" " (cot)		-	-	-	-	-	-	1.01
Tot. prot. (mg./ml)	0'	1.07	3.04	3.13	1.43	0.74	0.86	1.20
	30'	1.78	3.38	3.20	1.44	0.75	0.83	1.24
	60'	-	-	-	-	-	-	1.25
***ATPase	0'	0.0	1.11	0.79	2.42	0.26	0.34	-
	30'	0.34	1.11	0.89	1.99	0.39	0.34	-
***ATPase of RNP		229	137	98	521	218	406	17

* concentration expressed as µM or mg./ml. E.M. or R.M.; amino acid mixture used by Webster (30); sRNA prepared by the method of Kirby (84).

** extract titrated with an undertermined amount of NaOH.

*** ATPase expressed as µM Pi/ml R.M./1t min., and for the RNP as µg Pi/mg. prot./15 min.

attempt in this table was the only one in which the RNP from cotyledons was used.

Webster (30) stated that his preparations were inactive if the pH fell below 7, and that ions commonly used as buffers in the extraction media were inhibitory to synthetic activity if present in any but a very low concentration. In these experiments, the pH of the extracts was below 7 when prepared in sucrose. Four attempts to alter the pH by titrating the extracts with NaOH or Tris were unsuccessful in producing active preparations (e.g., Apr. 20, May 19; Table 12), although the Apr. 20 preparation did show a slight increase in enzymic activity.

Table 13 is a summary of results with Webster-type preparations done at a later date, in which the effect of growing the seeds in the presence of GSH was tested. In preparing the RNP fraction for the protein synthesis test, the extracts from the seedlings grown in 25, 50, and 500 μM GSH were mixed in approximately equal proportions in order to provide sufficient material. Part of the RNP fraction prepared on Aug. 24 (Table 13) was washed with 12 ml. of 0.5M sucrose, and recentrifuged at 100,000xg. Incorporation was not improved with these washed ribosomes, however, and the results are not included in Table 13. A further modification,introduced in both tests, was the use of the paper disc method of counting (90). The R.M. used was similar to that of Webster (35) and differed mainly in that a much higher concentration of magnesium ion was added. The method proposed by Webster (35) of adding aliquots of the supernatant at 5, 10, and 15 minutes was also

Table 13

Results of attempts to demonstrate an in vitro synthesis of protein, using RNP prepared in sucrose from seedlings germinated in the presence of glutathione.

Date	July 24, 1962			August 24, 1962		
Age (days)	3	3		3	3	3
µM GSH/80 seeds	0	mixture		0	mix.	200
Extraction medium						
*µM sucrose	500	500		500	500	500
ml./g.fr.wt.	2	2		2	2	2
pH extract	6.40	6.43		6.30	6.40	6.50
Reaction mixture	(1)	(1)	(2)**	(1)	(1)	(1)
*µM Tris-HCl	50.0	50.0	50.0	5.0	5.0	5.0
" ATP	0.22	0.22	0.22	0.22	0.22	0.22
" $MgCl_2$	-	-	-	6.0	6.0	6.0
" $MgSO_4$	1.0	1.0	1.0	-	-	-
" PEP	0.16	0.16	0.16	0.16	0.16	0.16
" C^{14}-phe	2.0	2.0	2.0	2.0	2.0	2.0
*mg. kinase	0.02	0.02	0.02	0.12	0.12	0.12
" sup	1.63	2.92	2.92	-	-	-
" RNP	0.62	1.65	1.65	1.80	1.51	1.65
Tot. prot. 0'	2.46	4.74	4.70			
(mg./ml.) 30'	2.40	4.32	5.76			
60'	2.46	4.44	5.48			
***c/m/						
mg.RNP 0'	276	73	52	26	29	48
30'	129	81	47	23	65	31
60'	161	67	112			
ATPase of RNP						
(µg.Pi/mg./15')	43****	44****		210	225	159

* concentration as µM or mg./ml. E.M. or R.M.

** 2.92 mg. supernatant protein added at 5 and 15 minutes (35).

*** counted by paper disc method (90).

**** tissue froze in mortar during grinding.

tested (July 24, R.M. 2, Table 13). As far as incorporation of activity was concerned, this modification had only slight success.

When the Webster system, as described in his early paper (30) was found to be unsatisfactory, it was abandoned in favor of the Raacke system (32). This investigator had also demonstrated protein synthesis in pea seedling extracts, but with a quite different and much simpler system than that of Webster. Three to 7 day old shoot-root axes were ground with phsophate buffer to keep the pH of the extract over 7, and the RNP fraction was incubated in the presence of the buffer, magnesium, labelled amino acid, and sometimes ATP as well. Only total protein and incorporation of label into the TCA precipitate were determined as measures of protein synthesis.

Fourteen preparations were made to test the Raacke system and representative results are given in Table 14. As with the Webster-type preparations, labelled amino acids were not used in the first tests, but even when they were, no significant incorporation into the TCA precipitate was observed, (e.g. Nov. 14, Table 14). In the Nov. 14 and similar experiments, a high concentration of phenylalanine (5μM/ml. R.M.) resulted in a higher than usual binding of activity after 30 minutes incubation. This did not show a continuing increase with time and was not necessarily associated with an increase in protein reaction. Raacke (33) had made a similar observation with washed ribosomes and concluded that the incorporation of labelled phenylalanine was not a reliable measure of protein synthesis since it occurred in the

Table 14

Representative results of attempts to demonstrate an in vitro synthesis of protein using RNP prepared in a phosphate buffer with sucrose.

Date	June 23 1961		June 27 1961		Nov. 14 1961		Nov. 24 1961	
Age (days)	4		5		4		4	
E.M. (Type B)								
*µM $(K)PO_4$	100		100		100		100	
" sucrose	450		450		450		450	
pH of E.M.	7.75		7.60		7.75		7.90	
ml./g. fr. wt.	1		1		0.81		1	
pH of extract	**25xg - 7.25 100xg - 7.18		**R. -6.98 S. -7.10		7.27		7.20	
R.M.	(1)	(3)	(5)	(R)	(S)	(1)	(2)	(1)
*µM$(K)PO_4$	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
" ATP	-	0.1	0.1	0.1	0.1	1.05	1.05	1.2
" GTP	-	0.3	0.3	-	-	-	-	-
" $MgCl_2$	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
" PGA	-	10.0	10.0	-	-	-	-	-
" C^{14}-ala	-	-	-	-	-	-	1.0	-
" " -phe	-	-	-	-	-	5.0	-	1.0
" GSH	-	10.0	10.0	-	-	-	-	-
*mg. a.a.	-	0.66	0.66	-	-	-	-	-
* " sRNA	-	0.3	0.3	-	-	-	-	-
" RNP	1.42	1.42	-	1.51	1.51	3.18	3.18	2.52
** " 25,000 xg RNP	-	-	1.76	-	-	-	-	-

Table 14 continued

Table 14 continued

Tot. prot.	0'	1.50	2.55	3.34	1.22	1.07	3.32	3.46	2.17
(mg./ml.)	30'	1.47	2.52	3.31	1.25	1.10	3.46	3.46	2.34
	60'	-	-	-	-	-	3.46	3.44	-
	75'	1.27	2.37	3.14	-	-			-
	90'				-	-			2.28
	120'				1.31	1.17			
c/m/mg.RNP	0'						6.7	1.6	1.9
	30'						17.2	1.8	-
	60'						15.8	3.3	-
	90'								3.3
ATPase of RNP (µg Pi/mg.prot.)							850		565

* concentration expressed as µM or mg./ml. E.M. or R.M.; amino acid mixture as used by Webster (30); sRNA prepared by the method of Rosenbaum and Brown (88).

** homogenate divided in two parts; 1 part being centrifuged at 25,000xg, 1 part at 100,000xg, for 1 hour.

*** shoot-root axes separated into roots and shoots before grinding.

absence of a net synthesis in the manner described above.

The 25,000xg RNP (i.e. the pellet obtained on 25,000xg centrifugation of the supernatant from a preliminary low speed centrifugation of the crude extract) was included in the June 23 experiment (Table 14) and in 3 others not included in the table as a test of this fraction which Webster (101) had found to be more active in the incorporation of activity than the RNP sedimenting at 100,000xg. These preparations did not behave any differently in this investigation than those prepared by high speed centrifugation.

In one experiment (June 27, Table 14), the roots and shoots were separated for RNP preparation. The RNP from both parts yielded a slight but probably insignificant increase in protein. In most experiments, Raacke's simple R.M. and a more complex R.M. of the Webster-type were tested on each RNP preparation. Various other additions to the R.M., e.g. 10μM of GSH, aliquots of the 100,000xg supernatant, or a "mitochondrial" fraction, were all without effect in inducing a net protein synthesis. From these negative results, it was concluded that the Raacke system also had failed. Raacke herself (102) has since reported difficulties in obtaining consistent results.

With the failure of the Webster and Raacke systems, attention was turned to systems used by other workers. Various modifications of the methods used in the study of protein synthesis in preparations from bacteria, animal, or other plants were tested. Tris-HCl-sucrose was used as extraction medium in three experiments

Table 15

Results of attempts to demonstrate an in vitro synthesis of protein using RNP prepared in Tris buffer with sucrose.

Date		Mar. 21 1961	Mar. 27 1961		Sept. 12 1961	
Age		3	3	1	*7	3
E.M. (Type C)						
**µM Tris-HCl		200	96		60	
" sucrose		450	480		450	
pH of E.M.		7.50	-		7.47	
ml./g. fr.wt.		2	2	4	4	4
pH of extract		7.60	6.83	7.30	7.05	6.77
R.M.						
**µM Tris-HCl		50.0	50.0	58.7	58.7	58.7
" ATP		0.1	0.1	0.68	0.68	0.68
" GTP		0.3	0.3	0.32	0.32	0.32
" $MgSO_4$		-	-	10.5	10.5	10.5
" $MnCl_2$		0.3	0.3	-	-	-
" KCl		0.1	0.1	-	-	-
" PGA		10.0	10.0	10.5	10.5	10.5
" C^{14}-ala		-	-	1.05	1.05	1.05
**mg. a.a.		0.66	0.66	0.7	0.7	0.7
** " sRNA		0.001	0.001	0.044	0.044	0.044
" RNP		0.72	1.01	0.53	0.54	0.51
Total protein	0'	1.00	1.12	0.87	0.89	0.83
(mg./ml. R.M.)	30'	0.97	1.01	0.91	0.89	0.83
	60'	-	-	0.88	0.86	0.83
c/m/mg. RNP	0'			4.6	4.8	4.8
	60'			6.0	6.5	6.2
ATPase	0'	1.74	2.11			
(µM Pi/ml./15')	30'	1.73	1.56			
ATPase of RNP (µg Pi/mg./15')		748	651	52	228	603

Table 15 continued.

*extract prepared from apical tip of shoot only.

**concentration expressed as μM or mg./ml. of E.M. or R.M.; amino acid mixture as used by Webster (30); sRNA prepared by the method of Rosenbaum and Brown (88).

reported in Table 12. From this table, it can be seen that none of the tests showed a net protein synthesis. A test for change in ATPase activity showed a decline after 30 minutes (Mar. 21 and 27 R.M., Table 15). The net incorporation of activity, 2-3 c/m/mg. RNP protein in the Sept. 12 test was insignficant. In this test, the RNP had been prepared from 1 day old and 3 day old shoot-root axes, and from the apical tips of 7 day old etiolated shoots, to see if the age of the seedling would have any effect.

Rabson et al (103) have reported a small success in obtaining incorporation of labelled amino acid with corn seedling extracts prepared in E.M. containing magnesium ions. Their system, or modifications of it, was tested and representative results are presented in Table 16. Six preparations, the E.M. of which contained varying amounts of Mg++ were tested, but only one (Oct. 26, R.M. 1, Table 16) showed an apparent net increase in protein, and then only in the first 30 minutes. The incorporation of label was practically negligible, although the July 5 preparation (Table 16) showed a net increase of almost 30 c/m/mg. RNP protein after 1 hour. This incorporation was assumed to be associated only with RNA since there was

Table 16

Representative results of attempts to demonstrate an in vitro synthesis of protein using RNP prepared in Tris buffer with sucrose and magnesium ion.

Date	Oct. 16 1961	Oct. 26 1961		Nov. 24 1961		July 5 1962
Age (days)	3	3		4		3
E. M. (Type D)						
*µM Tris-HCl	100	100		100	100	
" sucrose	450	425		450	450	
" $MgCl_2$	1	55		10	10	
pH of E. M.	7.75	7.65		8.00	7.77	
ml./g. fr. wt.	2	2		1	1	
pH of extract	7.37	7.27		7.45	7.25	
R. M.	(1)	(1)	(2)	(1)	(1)	(2)**
*µM Tris-HCl	47.7	52.7	50.0	50.0	50.0	50.0
" ATP	0.84	1.11	1.05	1.2	1.04	1.04
" GTP	0.60	3.04	2.89	3.0	1.0	1.0
" $MgCl_2$	9.5	10.5	-	10.0	10.0	10.0
" $MnCl_2$	-	-	0.3	-	-	-
" KCl	0.2	-	0.1	-	1.0	1.0
" PGA	9.5	5.3	10.0	10.0	-	-
" PEP	-	-	-	-	5.0	5.0
" C^{14}-ala	0.17	0.19	-	-	-	-
" C^{14}-phe	-	-	-	1.0	1.0	1.0
*mg. a.a.	-	-	0.66	0.66	0.66	0.66
*" sRNA	-	-	0.97	0.97	0.34	0.34
" kinase	-	-	-	-	0.03	0.03
" pH 5 enz.	-	-	-	-	0.52	0.52
" RNP	0.81	2.80	0.53	2.87	2.84	1.56**
Tot. prot. 0'	0.78	2.34	1.17	2.66		
(mg./ml. R. M.) 30'	0.73	2.39	1.14	2.62		
60'	0.66	2.14	1.12	-		
90'	-	-	-	2.58		
c/m/mg. RNP 0'	1.2	1.7	-	1.4	12.2***	0.7***
30'	2.0	1.3	-	-	16.1	20.7
60'	3.0	1.2	-	-	13.7	30.0
90'	-			1.9	-	-
ATPase of RNP (µg./mg./15 min.)	252	676		421	63****	91

* concentration expressed as µM or mg./ml. of E. M. or R. M.; amino acid mixture as used by Webster (30); sRNA prepared by the method of Rosenbaum and Brown (88).

** RNP washed and recentrifuged at 100,000xg.

*** determined by the paper disc method (90).

**** tissue frozen before grinding.

no activity after hydrolysis of the samples for 30 minutes at 90°C in 5% TCA.

Table 17 is a selection of the results obtained in 12 tests made with extracts prepared in the presence of GSH. GSH has been shown to protect protein sulfhydryl groups from oxidation in extracts from rat liver (104), and it is assumed that it would do the same for pea seedling extracts. The role of GSH in protein synthesis also has been demonstrated at the stage of the transfer of amino acids from sRNA to the ribosomes (105). For these reasons, it was decided to test the effect of the presence of GSH in the E.M. As can be seen from Table 17, 3 of the preparations showed a net increase in protein reaction after one hour (Feb. 16, 23, and June 29), but incorporation was insignificant.

From the results presented in Tables 11-17, it is apparent that little or no success was obtained either in amino acid incorporation or in net protein synthesis. What positive results were obtained could not be repeated at will. A discussion of the probable reasons for this lack of success follows in section c.

c. An examination of various aspects of the attempts to demonstrate an in vitro synthesis of protein.

The pH of the extract and factors affecting it. As is apparent in Tables 12 and 13, the pH of the extracts prepared without buffer was in the range of 6.0-6.5 reported by Tsuo and Sato (73) and not over 7.0 as reported by Webster (30). A further indication of the mildly acid

Table 17

Representative results of attempts to demonstrate an in vitro synthesis of protein using RNP prepared in Tris buffer with glutathione

Date	Feb.12 1962	Feb.16 1962		Feb.23 1962	Jun.29 1962	July 16 1962
Age (days)	3	3		3	3	3
E.M.(Type --	(F)	(G)	(H)	(I)	(E)	(J)
*µM Tris-HCl	100	100	100	100	100	100
" sucrose	-	450	-	-	450	450
" $MgCl_2$	100	-	-	10	100	10
" GSH	6	10	6	6	6	6
" KCl	-	-	-	50	-	-
pH of E.M.	8.0	8.0	7.98	8.03	7.80	
ml./g.fr.wt.	1	0.5	1	1	1	1
pH of extract	7.51	7.06	7.66	7.63	7.27	7.50
R.M.						
*µM Tris-HCl	50.0	50.0	50.0	50.0	50.0	50.0
" ATP	1.0	0.1	0.13	0.13	0.1**	1.1
" GTP	2.5	5.0	0.5	0.5	1.0	2.5
" $MgCl_2$	10.0	0.3	6.7	6.7	3.0	10.0
" KCl	1.0	1.0	1.0	1.0	1.0	1.0
" PEP	4.74	5.9	5.88	5.88	5.9	5.0
" C^{14}-ala	2.0	-	0.67	0.67	-	-
" C^{14}-phe	-	1.0	0.47	0.47	1.0	1.4
*mg. a.a.	0.66	-	-	-	-	-
* " sRNA	0.31	1.67	3.35	3.35	2.35	-
* " RNA	-	-	0.36	0.36	-	-
" kinase	0.02	0.02	0.02	0.02	0.03	0.02
" pH5 enzyme	-	-	0.05	0.05	-	-
" sup	-	0.15	-	-	-	-
" RNP	1.54	1.62	2.62	1.49	3.0	2.48
Tot.prot. 0'	1.93	2.08	3.00	1.85	3.40	2.72
(mg./ml.) 30'	1.86	2.16	3.34	1.69	3.54	2.48
60'	1.89	2.32	3.20	1.68	3.64	2.48
c/m/mg.RNP 0'	0.4	2.1	1.4	0.9	6.7***	6.5***
30'	0.3	-	-	-	3.3	6.8
60'	0.4	6.3	4.4	5.8	0.0	10.1
ATPase of RNP (µg./mg/prot./15')	57	338	328	223	135	276

* concentration expressed as µM or mg./ml. of E.M. or R.M.; amino acid mixture as used by Webster (30); sRNA prepared by the method of Rosenbaum and Brown (88); RNA = that prepared from the ribosomes.

** K-ATP rather than Na-ATP

*** sample counted on paper disc. (90).

nature of the extracts was the fact that the pH of a buffered extract was always lower than that of the buffer (Tables 14-17). On the hypothesis that it might lead to the formation of an extract active in protein synthesis, some tests were made to see if the peas could be induced to produce an extract above pH 7 without the addition of buffer. In these tests, the pH measurements were made only on the crude homogenate obtained before centrifugation (the pH tended to drop slightly with fractionation). Since it was noted that the pH of the extracts prepared in water was about the same as that prepared in sucrose, the tests were in some cases further simplified by using water as the extraction medium.

1) The source of the seed. Neither Webster nor Raacke could supply samples of their seeds when requested to do so, but Alaska pea seeds were obtained from 8 seed companies as indicated below:

Seed company	location	source of seed	pH of extract
Alberta Nurseries	Alberta	not given	6.05
W. Atlee Burpee Co.	Pennsylvania	Idaho	6.00
Dominion Seed House	Ontario	not given	6.20
Howick Seeds Ltd.	Quebec	Alberta	6.05
Geo. Keith & Sons, Ltd.	Ontario	England	6.05
Lindenberg Bros.	Manitoba	not given	6.45
W.H. Perron Cie.	Quebec	B.C.	6.15
Tobe's	Ontario	Alberta	6.45

Samples of the seeds were germinated and grown in the dark for 3 days, when the shoot-root axes were excised and ground in sucrose for pH determinations. As can be seen above, the pH was low in them all.

2) Seed variety. Seven different varieties of garden pea were ob-

tained from Ritchie Feed and Seed Co., Ottawa. These were: Alaska, American Wonder, Laxton's Progress, Little Marvel, Stratagem, Telephone, and Thomas Laxton. Forty seeds of each variety were tested in a water extract after 3 days germination, but the pH in all 7 was practically the same, ranging from a minimum of 6.23 to a maximum of 6.35.

3) The age of the seedling, or the seed itself. As the age of the seedling increased, the pH of the extract tended to decrease. For example, one day old S-R yielded an extract of pH 6.5, and 3 day old S-R an extract of pH 6.2 This drop in pH could be correlated with the lengthening of the axis. In 3 day old S-R, the pH of the extract from the tips was 6.5, while that from the shafts was only 6.0.

To test the effect of the age of the seeds themselves, seeds fresh from the combine were obtained through Howick Seed Co. The pH of the extracts prepared from them was only 6.3 This value tended to become lower with the passage of time, whether the seeds were stored in a laboratory cupboard or in a dry atmosphere over $CaCl_2$.

4) Conditions of germination. Preliminary attempts to alter the environment during germination by growing the seeds in light, or removing the seed coat prior to germination, had no effect on the pH of the extract. The imbibition of 0.05M KCl had a slight effect (the pH increased from 6.36-6.52) but NaCl, $MgCl_2$, and $MgSO_4$ had none. All the salts tended to inhibit germination. The effect of the imbibition during germination of GSH was tested in three experiments. The

pH of the extracts tended to increase slightly with increasing concentration of GSH (e.g. Table 13). No attempt was made to measure either the amount of GSH absorbed or its oxidation-reduction state.

Webster (35) has apparently come to the conclusion that the previous history of the seed is the determining factor in obtaining "active" preparations (i.e. active in an in vitro protein synthesis R.M.), and presumably also extracts with a high pH, although he does not discuss this point. Support for this conclusion can be derived from the work of Highkin (106) who found that the environment of the parent plant had marked effects on the progeny, quite apart from those of a strictly genetic character.

The ATP generating system. In the early studies (up to the end of 1961), PGA alone was used as an ATP-generating system. Webster (30) had stated that the pea seedling preparation contained its own kinase activity, and this statement was taken at its face value. However, if this were the case with the preparations used here, pyruvic acid should have appeared in the TCA supernatant as a result of the series of reactions:

$$\text{3-PGA} \longrightarrow \text{2-PGA} \longrightarrow \text{PEP} \underset{\text{ADP}}{\overset{\text{kinase}}{\rightleftharpoons}} \text{pyruvic acid.} \quad \text{ATP}$$

A test for pyruvate was not made in the Webster-type preparations (Table 12) since samples had been taken only for total protein and ATPase determinations. When radioactive amino acids were included in the reaction mixtures, a source of surplus material - the TCA super-

natant of the sample prepared for counting - became available. Pyruvate determinations were made on 3 such preparations with the results shown in Table 18. The values for pyruvate given in this table are extremely small when one considers that about 10 μM of PGA were added. They do not indicate whether the pyruvate arose as the result of kinase activity, or from direct dephosphorylation of the PGA. Since it was shown that a small amount of inorganic phosphate was released from

Table 18

The determination of pyruvate in protein synthesis reaction mixtures.

Date		net μM pyruvate/ml. R.M.				ATPase of RNP
R.M. with PGA		0 min.	30 min.	60 min	net	μg. Pi/mg./min
Oct. 16	1	.032	.045	.094	.062	252
	2	.032	.045	.072	.040	
Oct. 19	3	.076	.113	.234	.158	519
Oct. 26	3	.075	.151	.234	.159	676
R.M. with PEP						
Feb. 12	1	0.74	3.23	4.16	3.42	67
	2	1.24	4.11	4.79	3.55	95
Feb. 16	1	2.18	-	3.54	1.36	338
Feb. 23	1	0.83	-	1.36	0.53	328
	2	0.66	-	1.30	0.64	223

PGA in an ATPase R.M. (Table 5), the latter possibility is the more likely one, especially in view of the extremely active ATPase which would remove the ADP essential to the kinase reaction.

For the later experiments, PEP and its kinase were obtained and tested for activity. The results of this test are presented

in Table 19, and show that while the enzyme was active, it was not as active as stated. Forty-four μM/mg./min. of pyruvate should have been formed but in the R.M. used, only 15.6 μM/mg./min., or 35%

Table 19

Results of the test of kinase activity.

R.M.	Time elapsed	net μM Pi/ml.	net μM pyruvate/ml.	E.U.*
PEP, ADP, kinase	5'	0.08	0.27	18.0
	15'	0.25	0.70	15.6
ADP, RNP	5'	1.59	0.12	
	15'	1.38	0.09	
				(net)
PEP, ADP, kin., RNP	5'	1.71	0.17	3.3
	15'	2.29	0.27	4.0

*E.U. - enzyme units, one E.U. - 1μM pyruvate released/mg. enzyme protein/min.

of this rate was realized. In the presence of the RNP, the production of pyruvate was greatly reduced. In view of the active ADPase activity, the pyruvate may have been a result of direct dephosphorylation of the PEP. Nevertheless, PEP and its kinase were included in subsequent R.M. as indicated in Tables 12, 13, 16, and 17. Again a few tests for pyruvate were made with the results given in Table 18. These results, in terms of pyruvate formation, were much improved over those with PGA alone. The fact that the ATPase of the Feb. 12 preparation was unusually low was no doubt a determining factor in the

almost complete conversion of the PEP to pyruvate. These preparations, however, did not yield a net protein synthesis, or even incorporation of label (e.g. Table 17). This is taken as an indication that an active production of pyruvate, presumably through the regeneration of ATP, was not the only factor lacking in the R.M.

Paper chromatography of the R.M. This was done with three types of samples: 1) the last remnants of the R.M. after all samples were removed, 2) the TCA supernatant left from the samples for counting, and 3) time samples taken especially for this purpose. The aims were twofold: 1) to determine the fate of the added nucleotides, and 2) by radioautography to determine the fate of the added C^{14}-amino acid.

1) The fate of the added nucleotides. As to this first aim, the probable appearance of nucleotides or their breakdown products from the RNP or RNA itself would complicate matters. Lett and Takahashi (34) found that more than half of the RNA originally present in the RNP was TCA-soluble after one hour incubation. Also the low concentration of nucleotides usually added made it necessary to apply a relatively large amount of the R.M. (up to 0.2 ml.) in order to approach an optimum concentration for chromatography. This would have the effect of increasing any addition products from the RNP. Also, except in the test reported in Table 20, no control samples without added nucleotides were prepared.

Table 20 shows the results of testing various com-

Table 20

A study of the fate of various ingredients of the protein synthesis R.M.

Date		Sept. 20, 1961							
Age (days)		5							
E.M.									
*µM sucrose		500							
ml./g.fr.wt.		2							
pH of extract		6.24							
R.M.		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
*µM Tris-HCl		50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
" ATP		-	0.32	-	-	0.32	0.32	-	0.32
" GTP		-	-	0.27	-	0.27	-	0.27	0.27
" $MnCl_2$		3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
" KCl		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
" PGA		-	-	-	3.0	-	3.0	3.0	3.0
*mg. RNP		2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
Tot. prot. (mg./ml.)	0'	1.84	1.80	1.82	1.80	1.90	1.74	1.75	1.80
	20'	-	1.62	1.80	1.74	1.61	1.64	1.61	1.61

Table 20 continued

Table 20 continued

**Chromatog. Sample time	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20
GTP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PGA	-	-	-	-	-	-	x	x	-	-	x	x	x	x	x	x
ATP, GDP	-	-	-	-	x	-	-	-	x	x	-	-	x	-	x	x
ADP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMP	-	x	x	x	-	x	-	x	x	x	x	x	-	x	x	x
Ade	-	-	-	x	-	-	-	-	-	x	-	x	-	-	-	x

* concentrations expressed as μM or mg./ml. E.M. or R.M.

** 0.02 ml of R.M. run in Pabst I solvent; position of UV-absorbing spots indicated by x.

ponents of the protein synthesis R.M. separately. As can be seen, an apparent loss of protein occurredin every case. Each R.M. was chromatographed in Pabst solvent with the position of the UV-absorbing spots indicated in the diagram below the table. The following was observed to occur, and presumably would occur with other RNP preparations also: a) a spot corresponding to AMP appeared after 20 minutes from the RNP alone (R.M. 1); b) ATP was already hydrolyzed to AMP by the time the zero time sample was taken (R.M.2) which was within 30 seconds of the addition of the RNP, and appeared to an undetermined extent as adenosine by 20 minutes in every R.M. to which it had been added (R.M. 2, 5, 6, 8); and c) GTP appeared only as GDP in the zero time sample, and had disappeared completely by 20 minutes (R.M.3,7), but seemed to be spared to some extent by the presence of ATP (R.M. 5), or PGA and ATP (R.M. 8).

These results were more or less observed in the R.M. from other tests as well, although the adenosine spot did not always appear, and there were sometimes unidentified, fast-running, UV-absorbing spots.

With the butanol solvent, the nucleotides all remained at or near the origin, and yet in the chromatograms of the R.M. tested, many fast-running UV-absorbing spots were observed. No attempt to identify these spots was made.

It would therefore appear that considerable breakdown of the added nucleotides, and presumably also the added RNP

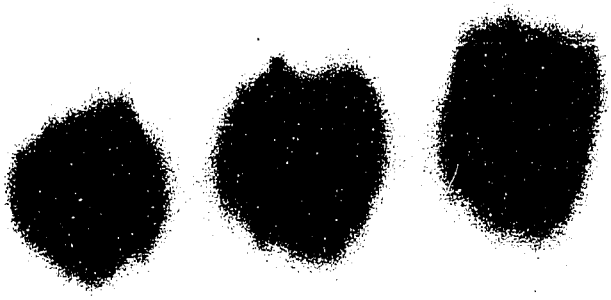
and RNA occurred in the reaction mixtures. The effect on the ability of the RNP to incorporate label or synthesize protein was no doubt deleterious. These results also provide further indication that PGA or PEP and its kinase were not effective in regenerating ATP under the conditions of the protein synthesis R.M.

2) The fate of the amino acids. The answer to this second question as obtained from chromatograms of the TCA supernatants run in Pabst solvent was clear and unequivocal; the amino acids ran in the expected positions on the paper (Fig. 7) and therefore had emerged from the reaction unchanged. Fig. 7A is taken from a radioautograph (6 weeks exposure) of 0.02 ml. of a reaction mixture containing C^{14}-alanine (Oct. 16, Table 16). Fig. 7B is taken from a radioautograph (2 months exposure) of 0.05 ml. of a reaction mixture containing C^{14}-phenylalanine (June 29, Table 17). No other radioactive spots were visible even when as much as 0.2 ml. of the R.M. had been spotted and with exposures of up to 3 months. This observation eliminated the possibility that TCA-soluble peptides had been formed as a result of a polypeptide synthetase activity such as was studied by Beljanski (66) in bacterial preparations. When samples of the unprecipitated R.M. were spotted, a small amount of activity was usually visible at the origin after 2 months exposure. Presumably this represented activity that would be removed in the washing procedure to which these samples had not been subjected. In one case in which a reaction mixture containing a washed RNP fraction (July 5, R.M. 2, Table 16) had been spotted,

Figure 7.

Representative radioautographs of chromatograms of the TCA supernatants of protein synthesis R.M. run in Pabst solvent.

A.



ala R.M. 0' R.M. 60'

B.

phe R.M. 0' R.M. 60'

A. R.M. containing C^{14}- alanine (0.02 ml. Oct.16, Table 16), rf ala, 0.46.

B. R.M. containing C^{14}-phenylalanine (0.05 ml. June 29, Table 17), rf phe, 0.81.

this activity seemed to increase with time. After hydrolysis of the RNA (90), however, further exposure to the film for 3 months showed no trace of activity. It is concluded that this had been activity associated with the RNA as a result of amino acid activation.

In the butanol solvent, the results were not as clear since considerable streaking occurred at the concentrations spotted, especially with those R.M. containing phosphate buffer, phenylalanine, or a high concentration of ATP.

Incorporation of label into the TCA precipitate. No more than a barely detectable incorporation of activity into the TCA precipitate was ever observed in these experiments. Webster (35) also found that "inactive" preparations from pea seedlings did not even incorporate label, let alone synthesize protein. Assuming that incorporation and protein synthesis occur by the steps outlined in the introduction, the following reasons for the failure to observe significant incorporation with pea seedling preparations can be suggested:

1) Activation of the labelled amino acid may not have occurred because: a) the activating enzymes were not present in the preparation. (In these experiments, only alanine and phenylalanine were used as the source of label and the absence of the activating enzymes for these two amino acids alone would be adequate to prevent incorporation. An attempt to measure amino acid activation in the 25,000xg extract was made using the hydroxamate method (107), but it was found extremely difficult to control the pH at 0.6 and the results are inconclusive.)

b) the sRNA either added or indigenous in the preparation may have been already loaded with the unlabelled amino acids. (The amount of amino acids bound on the sRNA was measured on two preparations and estimated to be 0.07 μM alanine equivalents per mg. RNA. If the M.W. of the sRNA is taken as approximately 50,000, then each μM of RNA had 3.5 μM of alanine associated with it. It would appear then that the sRNA may well have been already loaded. This situation requires the additional condition that no unloading of the sRNA occurred in the course of the experiment as a result of the transfer of label to the ribosomes. A shortage of binding sites on the ribosomes themselves has been shown to be a limiting factor in this transfer in preparations from mouse liver (108), and the inability of the ribosomes to release newly synthesized protein in vitro would accentuate this problem.)

c) conversely, the sRNA may have lost the critical CMP and AMP end groups and have been incapable of accepting amino acids.

d) a high concentration of AMP, which is an end product, may have inhibited activation. (Hele (109) found with rat liver preparations that AMP depressed an amino acid dependent pyrophosphate exchange. A high concentration of AMP is reported to be present in pea seeds,(110) and might therefore be present in the undialyzed RNP fractions used in these experiments. The appearance of a spot running in the AMP position after incubation of an RNP fraction for 20' has already been noted (Table 20). In addition, the ATPase activity of the RNP would result in an increased concentration of AMP in the R.M.).

2) Activation and incorporation of the labelled amino acid may have occurred, but the activity was lost in the washing procedure. Any activity bound only to the RNA in the precipitate would be lost as a result of redissolving it in NaOH, a procedure which is known to hydrolyse RNA (111). Any label bound in this way would be discarded with the NaOH-TCA supernatant. In one experiment, however, (Feb. 16, Table 17), the TCA precipitate was not redissolved in NaOH, but was washed only with TCA, ethanol, and ether. The net count obtained with this preparation was slightly better than usual (4 c/m/mg. RNP), but was hardly a significant figure. The experiments in which paper discs were used were also examples of the omission of the NaOH treatment, but these preparations showed very little if any net incorporation (July 24 and Aug. 24, Table 13; July 5, Table 16; June 29 and July 16, Table 17). A further loss of activity could possibly occur in the ethanol-ether washing step. TCA precipitation increases the solubility in non-polar solvents of serum albumin (112), and since the ethanol-ether washings were never saved, their effect if any in solubilizing these preparations is unknown.

Protein determinations as a measure of synthesis. Lett and Takahashi (34), who also attempted without success to repeat the results of Webster (30), found that an increase in soluble proteins (i.e. those left after 100,000xg centrifugation of the R.M. did indeed occur as Webster had stated. This increase could be accounted for by an equal

loss from the particulate fraction. Some of these soluble proteins were lost after incubation at 37°C, presumably as a result of protease activity.

In the tests reported here, the soluble proteins were not separated, nor was proteolytic activity tested for directly, but the decrease in total protein reaction which occurred in the majority of the experiments is regarded as an indication of its presence. Such activity would create confusion, especially in those R.M. in which no label was used. How could one detect a small increase or synthesis of protein in the presence of a concomitant decrease or proteolysis of protein? In view of the experimental error of the determination, (triplicate samples varied by as much as ± 10%), any small net increases of protein reaction that did occur would not be significant evidence of protein synthesis in the absence of supporting evidence. In these experiments, supporting evidence such as amino acid incorporation was lacking.

5.*The utilization of phenylalanine in vivo by the germinating pea seed.

Pea seeds were germinated in the presence of phenylalanine uniformly labelled with C^{14} with the intention of isolating sRNA from them. It was hoped that in the process of activation and incorporation of the labelled amino acid into protein some of the labelled amino acid would be isolated in the activated form, attached to sRNA. This labelled sRNA it was proposed to include in an in vitro protein synthesis R.M.

*This portion of the thesis was presented at the 4th scientific meeting of the Canadian Society of Plant Physiologists, Winnipeg, June, 1963.

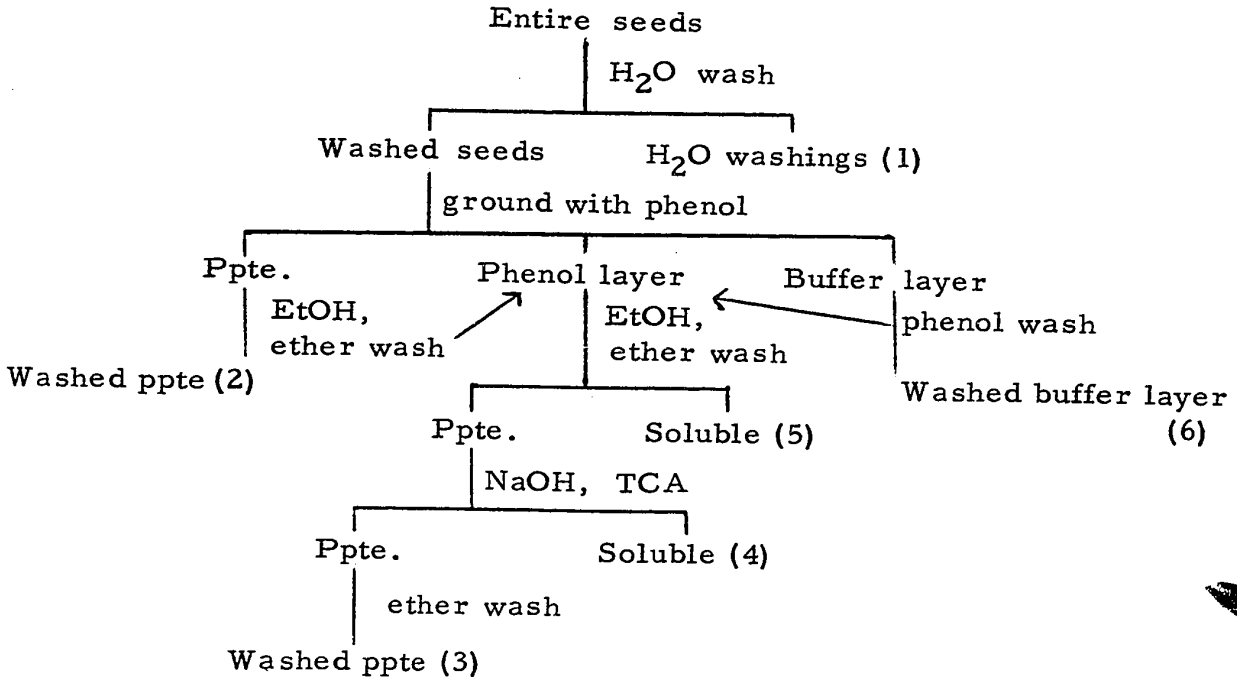
as Webster (113) had done as a means of by-passing the amino acid activation step. Very little activity was found in the RNA fraction, however, but its distribution in the phenol soluble and insoluble fractions indicated that the amino acid had already been incorporated into the proteins of the seedling. This observation seemed worthy of further investigation and the in vitro studies were temporarily put aside in order to pursue the question of the utilization of the labelled amino acid in vivo. Four experiments were done and are outlined in detail below. Only brief comments are made on the results of the individual experiments, since these are discussed more fully in a consideration of the results as a whole.

a. Experiment 1. Eighty seeds were left to germinate in the presence of 2 μM of uniformly labelled phenylalanine in 15 ml. of water for 66 hours. The seedlings were then removed, washed, weighed, and stored on dry ice. The frozen seeds, weighing 8.6 g./20 seeds including the seed coats, were ground for 5 minutes at high speed in the Omnimix in a phenol-salt-phosphate buffer mixture used for sRNA extraction (88). The resultant slurry was centrifuged and fractionated as indicated in Fig. 8 to yield six fractions. The washed buffer layer (fraction 6) was washed in the usual steps for sRNA preparation. The results of counting given in Table 21 show that there was very little activity in the buffer layer which contained the sRNA. The bulk of the activity, 42%, was in the three phenol-soluble fractions (fractions 3, 4, and 5), while 33% was in the washed precipitate (fraction 2).

Figure 8

Fractionation procedures used with pea seeds germinated for 66 hours in the presence of a C^{14}-amino acid.

Experiment 1



Experiment 2

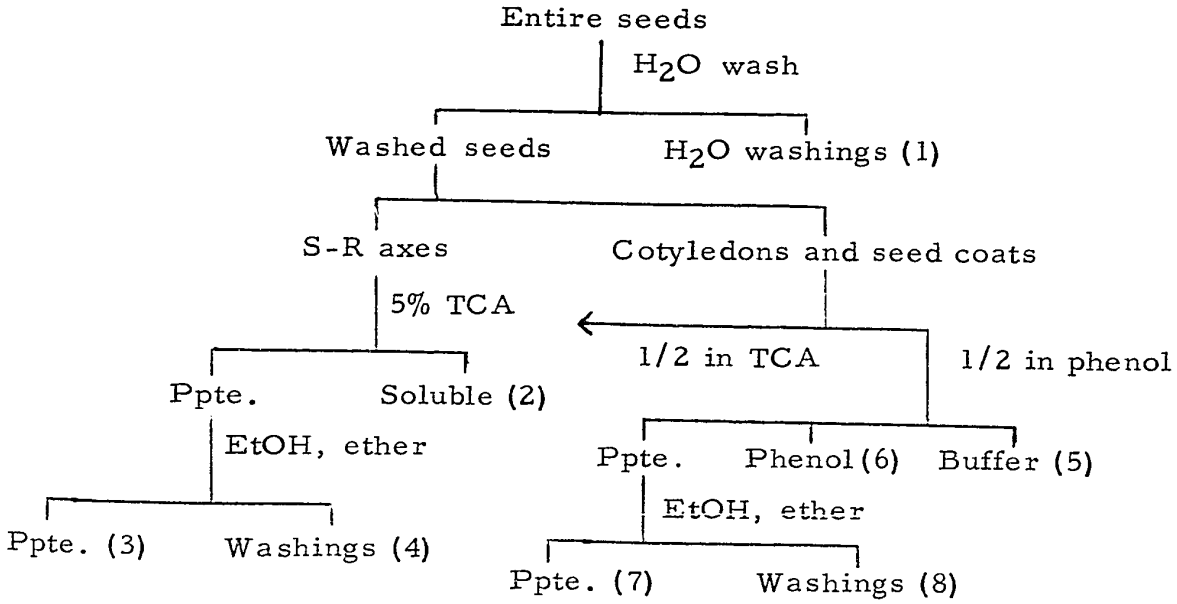


Table 21

The distribution of activity in the various fractions of seeds germinated 66 hours in the presence of C^{14}-phenylalanine.

Fraction	Wt. (g)	Total activity (c/m)	(%)
1) H_2O wash	-	484	2
2) Phenol insoluble ppte.	2.11	7,320	33
3) Phenol soluble	0.40	5,346	24
4) Phenol soluble	-	1,686	8
5) Phenol soluble	-	2,108	10
6) Buffer layer	-	213	1
Total activity recovered		17,157	78
Total activity added		22,000	100

From these results, it would appear that the amino acid had already been incorporated into protein, and a sample of the precipitate of highest specific activity, fraction 3, was tested for N-terminal activity (91). The results of this analysis are presented in Table 29, and show that the bulk of the activity was in the internal-amino acid fraction, while very little appeared in the N-terminal fraction or the free amino acid fraction. This was taken as evidence that the amino acid had been incorporated into protein.

b. Experiment 2. This experiment was essentially a duplication of the first, but with two groups of seeds, one given phenylalanine as before, and the other alanine-1-C^{14}. The seeds were placed in Petri plates in a dessicator over a layer of 50% KOH to trap CO_2, and allowed

access to CO_2-free air. After 66 hours germination, they were harvested and washed. The shoot-root axes were dissected out, frozen, and ground separately in a mortar with 5% TCA. The cotyledons and seed coats were separated into two groups, frozen, and ground with either phenol-buffer as in Experiment 1, or with 5% TCA. The fractionation procedure used was as indicated in Fig. 8, and the results of counting are presented in Table 22. From this table, it is apparent that very little of the alanine label was recovered, even in the CO_2. The distribution of activity in the phenol fraction of the phenylalanine group was similar to that in the first experiment, except that the unwashed buffer layer had 10 x more activity. In the TCA fractions, on the other hand, the bulk of the activity, 68%, was in the precipitate. A smaller amount, 10% in all, appeared in the shoot-root axes.

Seedling development was not uniform in either group. At the time of harvest, only 45% of the seeds had germinated with roots up to 5 cm. in length. The fresh weights of the cotyledons and seed coats was 7.69 g./20 seeds in the alanine group and 7.80 g. in the phenylalanine group. The shoot-root axes weighed 0.45 g. and 0.44 g. in the alanine and phenylalanine groups respectively.

c. Experiment 3. In this experiment, the seedlings were sampled at four time intervals, 5, 20.5, 28, and 49 hours, after the initiation of germination. In view of the uneven development of the seeds, as already noted in Expt. 2, the samples were selected to include as uniform a representation as possible of the varying stages present. Thus

Table 22

The distribution of activity in the various fractions of seeds germinated 66 hours in the presence of C^{14}-phenylalanine or alanine.

Fraction	Alanine-fed			Phenylalanine-fed		
	Wt. (g)	Total activity c/m	%	Wt. (g)	Total activity c/m	%
$BaCO_3$	0.03	1.036	3.4	0.01	2	<0.1
1) H_2O washings	-	19	<0.1	-	162	0.7
Cots and testa						
2) TCA sol.	-	285	0.9	-	1,695	7.4
3) " ppte.	3.32	331	1.1	3.27	15,742	68.5
4) washings	0.28	76	0.3	0.25	473	2.1
5) Buffer layer	-	-	-	-	2,405	10.4
6) Phenol "	-	-	-	-	7,616	33.1
7) " ppte.	-	-	-	2.32	6,176	26.8
8) Washings	-	-	-	0.04	224	1.0
S-R axes						
2) TCA sol.	-	88	0.3	-	861	3.7
3) " ppte.	0.03	17	<0.1	0.03	1,242	5.4
4) Washings	0.01	6	<0.1	0.02	235	1.0
Activity recovered		1,858	6.1	(TCA)⟶	20,412	88.6
				(Phenol)⟶	18,923	82.2
Activity added		30,256	100.0		23,025	100.0

at 5 hours, 18 fully imbibed seeds, and 2 not yet fully imbibed, were selected. At this time also, approximately one ml. of the solution still remained in the Petri plate, and two samples (0.1 ml.) were taken for counting. At 20 hours, 19 fully imbibed seeds and one with

Table 23

The distribution of activity in the TCA fractions at various intervals in the course of germination. (Expt. 3).

Time (hrs.)	H_2O wash			TCA fractions									% Tot. c.
				Sol.		Ppte.			Wash				
	Wt. (mg.)	c/m	%	c/m	%	Wt. (g.)	c/m	%	Wt. (mg.)	c/m	%		
5	3	500	2	12,100	56	3.24	990	5	256	1,210	6		68
20.5	4	39	<1	5,950	28	3.26	7,420	34	322	515	2		64
28	4	26	<1	7,040	32	3.05	10,280	48	108	712	3		83
49	11	38	<1	3,280	15	3.15	10,280	48	201	1,016	5		68

the radicle broken through the seed coat (5% germination) were selected. At 28 hours, 15 not germinated, and 5 germinated (25% germination) were selected. At 49 hours, 14 not germinated, and 6 germinated (30% germination) were left for the final sample. With each sample, the entire seeds were ground in 5% TCA and the slurries fractionated as shown in Fig. 8 (S-R axes). The results of counting are presented in Table 23.

From this Table, it is apparent that the activity associated with the TCA precipitate gradually increased, while that associated with the supernatant decreased. Again the activity in the water wash, and in the ethanol-ether wash was only a small fraction of the total activity.

d. Experiment 4. This experiment was similar to the third in that four time samples were taken. The seeds, however, were germinated in 20 ml. of test solution rather than in 15 as previously, and the number of plates was expanded to include additional tests with phenylalanine analogues:

Group #	Germinated in:
0	water only
1	phenylalanine U-C^{14} (2 μM)
2	" and 80 μM DL-phenylalanine (DL)
3	" " " " p-fluorophenylalanine (F)
4	" " 400 μM DL-phenylalanine (DL)
5	" " " " B-thienylalanine (TE)

The concentrations of the analogues were selected on the basis of those used by Rabinowitz et al (114). The samples were washed with water, and stored at 4°C in 5% TCA for later crushing in a mortar.

A summary of the comparative growth and other parameters of the 6 groups is presented in Table 24, from which it can be seen that there were no seeds germinated in the first 2 sampling periods (9 and 17 hours). All but 2-4 ml. of the medium had been absorbed in the course of imbibition by 17 hours. At that time the surplus was removed and saved for counting. As can be seen from the table, the samples with 400 μM of DL or TE were 10 times more active than the control or 80 μM-treated samples. By 29 hours, over half the seeds in Groups zero, 1, and 2 had germinated, but less than half in Groups 3, 4, and 5. The analogues F and TE and 400 μM of DL delayed, but did not inhibit germination, since at 42 hours, when the third sample was removed, most of the seeds had germinated and in the first two groups the roots were up to 2 cm. in length. All the remaining seeds were removed in the final sample at 124 hours. The shoot-root axes were dissected out and ground separately in the last 2 samples. The higher concentrations of phenylalanine and the analogues had a deleterious effect on growth which, as can be seen from the table, was reflected in decreased shoot and root length, and fresh weight. The roots of the B-thienylalanine-treated seedlings were a decided yellow-brown color. As well, there was some brownish discoloration in the F and 400 μM DL seedlings. The fractionation procedure was the same as

Table 24

The effect of added DL-phenylalanine (DL) or its analogues β-thienylalanine (TE) and p-fluorophenylalanine (F) on the absorption of U-C^{14}-L-phenylalanine by pea seedlings, and the effect on germination and growth of the shoot-root axis.

Germ. time, hrs.	Effect on	Water cont. #0	μM added to 2 μM U-C^{14}-L-phe				
			#1	#2	#3	#4	#5
			cont.	80DL	80F	400DL	400TE
17	ml. left	2.0	3.1	3.1	2.5	3.7	2.9
17	c. left	-	496	496	400	5258	4348
	" on seed	-	320	372	268	1026	2608
17	% c. left	-	0.4	0.4	0.3	3.7	2.9
17	% germ.	0	0	0	0	0	0
29	% germ.	70	57	70	38	35	35
42	% germ.	83	87	90	87	-	85
124	% germ.	100	95	95	95	100	100
124	Root, cm.	3.0	3.2	2.6	1.6	2.5	1.7
	Shoot, cm.	1.6	1.5	1.4	0.8	1.1	0.6
124	Fr.Wt, g.	2.2	2.0	1.9	1.1	1.6	0.9

that used in Expt. 3, and the results of counting are presented in Tables 25, 26, and 27. In this experiment, counting was done by liquid scintillation as well as by gas-flow, although all samples were not counted by both methods. The results in Table 25 are those for the first group only, and show essentially the same picture as was obtained in Expt. 3, i.e. a gradual increase in activity in the TCA precipitate, and a decrease in the supernatant. The results in Table 26 are those determined

Table 25

The distribution of activity in the TCA fractions at various intervals in the course of germination (Expt. 4).

Time (hrs.)	H_2O wash		TCA fractions								% Tot. c.
			Sol.		Ppte.			Wash			
	c/m*	%	c/m	%	Wt. g.	c/m	%	Wt. (mg.)	c/m	%	
9	62	<1	19,363	64	3.45	4,270	14	29	938	3	85
17	46	<1	11,580	38	3.11	6,560	22	86	1,470	5	65
42	18	<1	7,978	26	3.01	11,849	39	118	1,408	5	70
124	145	<1	5,426	18	2.25	16,595	55	222	1,048	4	70

*sample evaporated on paper discs, count corrected from LS count.

Table 26

The distribution of activity in the TCA fractions at various time intervals in the course of germination: the effect of added DL-phenylalanine and its analogues (Expt. 4).

Fraction	Hours of germination							
	9		17		42		124	
	*c/m	%	*c/m	%	*c/m	%	*c/m	%
Grp. 1								
H_2O wash	109	<1	80	<1	31	<1	254	<1
TCA sol.	26,800	51	21,420	41	10,686	20	3,605	7
TCA ppte.	6,900	13	12,260	23	20,240	38	25,233	48
**Wash	1,675	3	2,624	5	2,530	5	1,827	4
Recovery	35,484	67	36,384	69	33,487	63	30,694	58
Grp. 2 (DL)								
H_2O wash	288	<1	93	<1	56	<1	353	<1
TCA sol.	40,200	76	40,800	77	22,800	43	10,230	19
TCA ppte.	3,280	6	2,623	5	11,528	22	20,768	39
**Wash	2,104	4	2,446	5	1,883	4	2,505	5
Recovery	45,872	87	45,962	87	36,267	68	33,567	63
Grp. 3 (F)								
H_2O wash	294	<1	67	<1	58	<1	488	<1
TCA sol.	27,520	49	28,160	50	11,235	20	5,880	10
TCA ppte.	4,115	7	7,935	14	21,688	39	27,178	48
**Wash	1,942	4	3,932	7	1,384	2	3,316	6
Recovery	33,871	61	40,094	72	34,365	61	35,622	64
Grp. 4 (DL)								
H_2O wash	1,905	4	255	<1	38	<1	300	<1
TCA sol.	33,150	68	35,192	72	29,380	60	10,860	22
TCA ppte	1,824	4	2,303	5	6,435	13	11,863	24
**Wash	1,942	4	3,022	6	2,131	4	1,359	3
Recovery	38,821	80	40,772	84	37,984	78	24,221	50
Grp. 5 (TE)								
H_2O wash	2,002	3	652	1	78	<1	426	<1
TCA sol.	45,780	76	36,960	61	21,352	37	7,276	12
TCA ppte.	6,500	11	13,607	22	33,010	55	36,060	59
**Wash	2,610	4	2,533	4	1,943	3	2,356	4
Recovery	56,892	94	53,752	88	56,383	93	45,874	76

*counts per minute as determined in the LS counter.

**c/m corrected from GF count.

Table 27

The distribution of activity in the TCA fractions of the shoot-root axes at various time intervals in the course of germination in the presence of C^{14} phenylalanine, DL-phenylalanine, and its analogues.

Fractions	Hours of germination					
	42			124		
	Wt. (mg.)	*c/m	%	Wt. (mg.)	*c/m	%
Grp. 1						
TCA sol.	-	936	1.8	-	2285	4.3
TCA ppte.	27.3	1430	2.7	79.3	3853	7.3
Wash	30.2	60	0.1	58.3	446	0.8
Grp. 2 (DL)						
TCA sol.	-	3600	6.8	-	6450	12.2
TCA ppte.	31.0	1767	3.3	92.4	5043	9.5
Wash	36.1	55	0.1	43.6	740	1.4
Grp. 3 (F)						
TCA sol.	-	75	0.1	-	3780	6.8
TCA ppte.	28.5	1509	2.7	74.6	2345	4.2
Wash	31.6	46	0.1	45.9	448	0.8
Grp. 4 (DL)						
TCA sol.	-	740	1.5	-	1890	3.9
TCA ppte.	28.5	682	1.4	87.1	2549	5.2
Wash	20.1	39	0.1	37.5	317	0.7
Grp. 5 (TE)						
TCA sol.	-	292	0.5	-	756	1.2
TCA ppte.	25.9	1852	3.0	54.5	2397	3.9
Wash	13.4	29	<0.1	35.7	754	1.2

* counts per minute as determined in the LS counter.

in the LS counter for all 5 groups, and show a generally similar distribution of activity to that already remarked on for Grp. 1. In all groups, the shoot-root axes acquired a small part of the label (Table 27), but the amount varied from group to group.

e. Discussion of the combined results of Experiments 2, 3, and 4.

Recovery of total count. In these experiments, a sample of the initial feeding mixture was reserved for counting in order to obtain a more exact estimate of the activity supplied to the seeds. As can be seen from Tables 21, 22, 23, 25, and 26, in no experiment was 100% of the activity initially supplied recovered in the various samples. It could be postulated that this loss was in the form of untrapped CO_2, but the results of Expt. 2 would indicate that such loss was slight, and certainly not enough to account for the total.

If an extrapolation can be made from the group grown on alanine, then the following calculation can be made to estimate the loss of activity from phenylalanine as CO_2: only the carboxyl carbon of alanine was labelled, and only 9.6% of the counts were recovered. Assuming that all this activity was lost as CO_2 in the series of reactions: alanine $\longrightarrow$ pyruvic $\longrightarrow$ acetaldehyde and CO_2

$(CH_3.CHNH_2.C^{14}OOH \longrightarrow CH_3CO.C^{14}OOH \longrightarrow CH_3.CHO$ and $C^{14}O_2)$,

then only 3.8% of the CO_2 (1036 counts) was trapped by the method used. Since there were only 10 counts in the CO_2 fraction of the phenylalanine-fed seeds, which was presumably only 3.8% of the total given off, then

it can be calculated that 263 counts in all were given off as CO_2. Such a calculation leaves several thousand counts unaccounted for.

The addition of cold phenylalanine and its analogues tended to yield an increased recovery, especially in the case of TE. With this group of seeds, 88% of the added activity was recovered as compared to only 64% in the control.

A further observation to be made is that the activity recovered from each sample group was not the same, but tended to be lower in the later samples than in the early ones. In Expt. 3, however, the 28 hour sample showed a much higher recovery than those both preceding and following it. Whether or not this was a result of experimental error cannot be definitely stated. The work of Cossins (115) might indicate that it was not entirely a result of error, but could be the result of a shift in metabolic pattern that occurs when the seed coat is split by the emerging radicle. (In this experiment, 25% of the seeds had germinated by 28 hours.) Working with pea seeds, he demonstrated that the ethanol given off in the initial anaerobic stage of germination was absorbed and utilized by the seed when respiration became aerobic following rupture of the seed coat. He has demonstrated with 2-C^{14}-ethanol and slices of cotyledons from germinated seeds that this utilization of ethanol is rapid, the label appearing in keto acids, Krebs cycle acids, and amino acids after only 30 minutes incubation. Perhaps there is an analagous occurrence in the case of phenylalanine breakdown products, i.e. a volatile compound is formed under anaerobic conditions which is converted to a more

stable compound under aerobic conditions. Therefore fluctuations in the recovery of added activity could conceivably be a result of fluctuations in the metabolic pattern of the seedling material.

Activity not absorbed. The water washings of the seedlings combined with the samples taken from the feeding mixtures are a measure of the activity not absorbed. A composite of the results obtained from the four experiments is presented in Table 28, in which the volume of feeding mixture left over, and its activity, are given as percentage of that

Table 28

Activity not absorbed as determined from the water washings and unabsorbed germination medium.

Expt. #	3	4	4	3	2	4
Germ. time (hrs)	5	17	42	49	66	124
% vol. left	7.0	15.5	-	-	-	-
% total c.	3.2	0.2	-	-	-	-
% tot. c. in wash	2.5	0.2	0.1	0.2*	0.7*	0.5*

*these samples include the rinse of the Petri plate as well as the rinse of the seeds.

originally added to each 20 seeds. It would appear from this table that the labelled amino acids moved into the seeds more rapidly than did the water, since in Expt. 3 only 3.2% of the activity remained in the 7% of solution left at 5 hours. In Expt. 4, 15.5% of the water was left at 17 hours, but only 0.2% of the activity. An important difference

between these two experiments was that 15 ml. of solution were added in Expt. 3, not quite as much as the seeds could absorb, but 20 ml. were added in Expt. 4, an amount slightly in excess of that needed. Nevertheless, it would appear that the phenylalanine was taken into the seeds by an active process, rather than being passively carried in in the course of imbibition.

A second point to note from Table 28 is that a small amount of activity remained adsorbed on the seed coat for at least 42 hours, and possibly longer. The starred figures in this table are those taken from the final sample in each experiment, which included a rinse of the Petri plate as well. Hence they represent residual activity adsorbed on the glass as well as that adsorbed on the seed coat.

Judging from the water wash of the seeds, it appears as though the addition of 80 and 400 μM of phenylalanine decreased the rate of absorption (Table 26) as might be expected, and so also did the analogues. From the activity washed from the 9 hour samples (Table 26) it would appear that almost 3 times more activity was unabsorbed in the presence of 80 μM of either DL or F (288c and 294c vs 109), and almost 20 times more (ca 2000c) in the presence of 400 μM of DL or TE. By 17 hours, the activity in both the water wash and the unabsorbed solution of the 80 μM-treated groups was similar to that of the control (ca 500c-600c in all, (Table 24)). In the 400 μM groups, the total activity, (water wash and unabsorbed solution), was slightly higher in the DL group (5558c vs 4774c). This would indicate, as might be ex-

pected, that TE did not compete with the labelled phenylalanine for absorption to the same extent as did the DL. The capacity to absorb added phenylalanine was obviously considerable. Presumably 42 µM were taken up by the third group, assuming no distinction to be made between the D and L forms. In the fifth group, all but 13 µM of the 402 µM total was absorbed. This latter figure was arrived at in the following manner: ca 200,000c representative of 402 µM were added to 80 seeds. Only 5258c were unabsorbed and 255x4 were on the seed coats at 17 hours, a total of 6268c, representative of 13 µM.

The analogues were also absorbed into the seed, as indicated by the gradual disappearance of their easily identifiable spots from the chromatograms of the water wash samples.

A further observation to be made from the water wash itself is that the movement of material did not occur only into the seed. Substances were also excreted from the seed, as indicated by: 1) the increase in weight of the evaporated water wash (Expt. 3, Table 23), 2) the coloration of the germination medium after a few hours of imbibition, and 3) the apparent increase and variety of ninhydrin-reacting material which was apparent following chromatography of this fraction in the butanol solvent. The liberation of organic substances, particularly amino acids and peptides, from germinating seeds is a commonly observed phenomenon (116), although one that has not been very much studied.

The chromatograms were exposed to X-ray film for

6 weeks before spraying with ninhydrin, but except for the early samples from the groups given 80 μM or 400 μM of DL, F, or TE, no activity appeared in the expected position of phenylalanine. This might have been a result of the low activity present, but in some samples, a small activity was visible in a position ahead of that of phenylalanine, and/or at the origin.

Activity in the TCA supernatant and precipitate fractions. The results of adding 2 μM of C^{14}-phenylalanine in Expts. 2-4 is summarized in a graph (Figure 9) and show an initial rise in activity in the supernatant followed by a decline, and a steady increase in the activity of the precipitate. It may be of significance that the hump that occurred at 30-50 hours coincided with the time at which the radicle normally breaks through the testa. This hump recalls to mind the increased recovery of activity in the 28 hour sample of Expt. 3.

The effects of adding cold phenylalanine or its analogues are shown in Figure 10. The result of adding excess phenylalanine (curves 1, 2, and 3) is what one would expect: dilution of the labelled molecules with the unlabelled so that there was a slower rate of decline in the supernatant, and a decrease in the apparent incorporation into the precipitate. The curves obtained, however, do not diverge in the proportionate manner that might be expected. The activity in the supernatant was almost the same as that of the group given 400 μM of phenylalanine, while that of the TCA precipitate of the last sample from the 80 μM DL had almost reached the activity of group 1. The analogue,

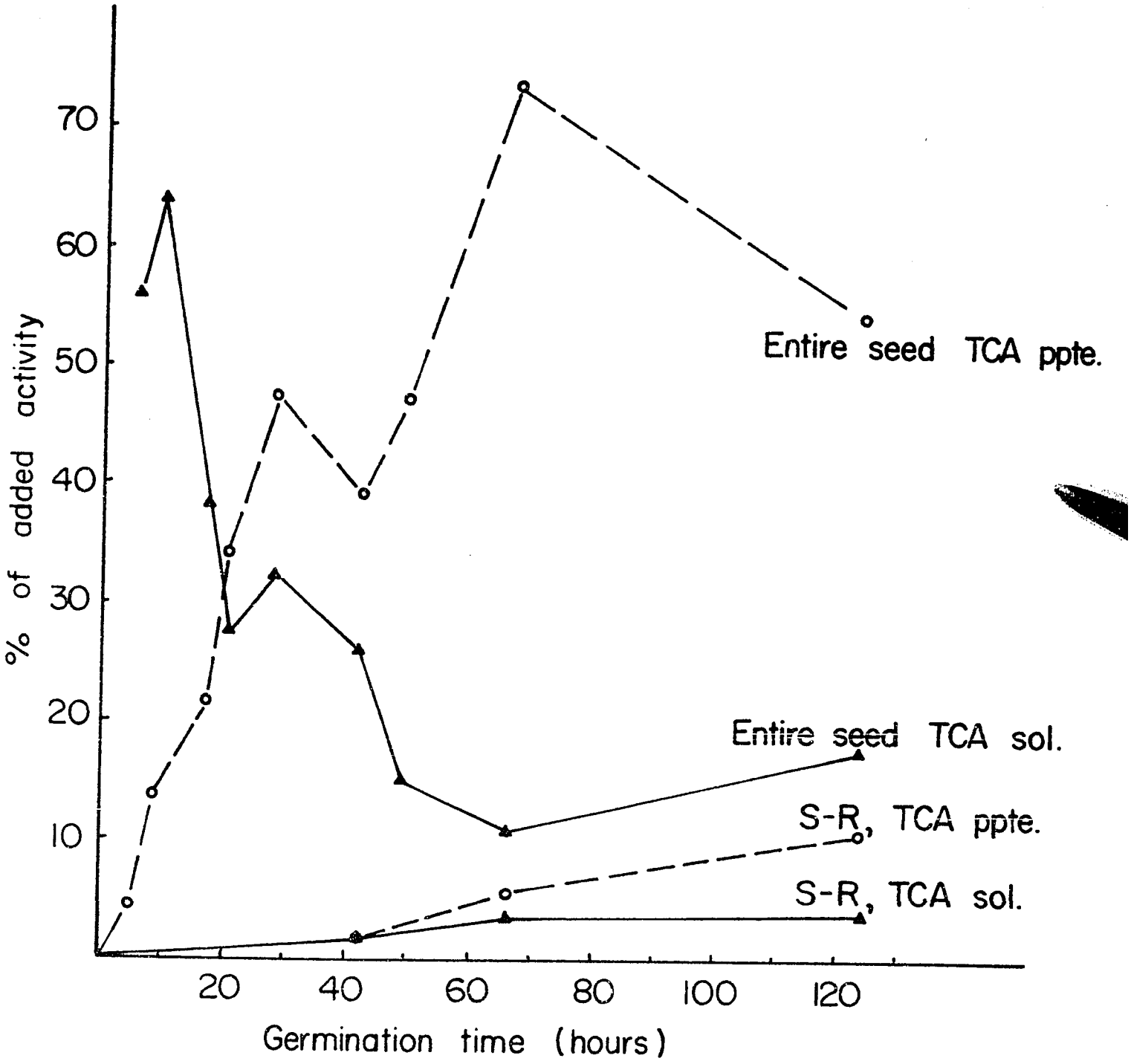
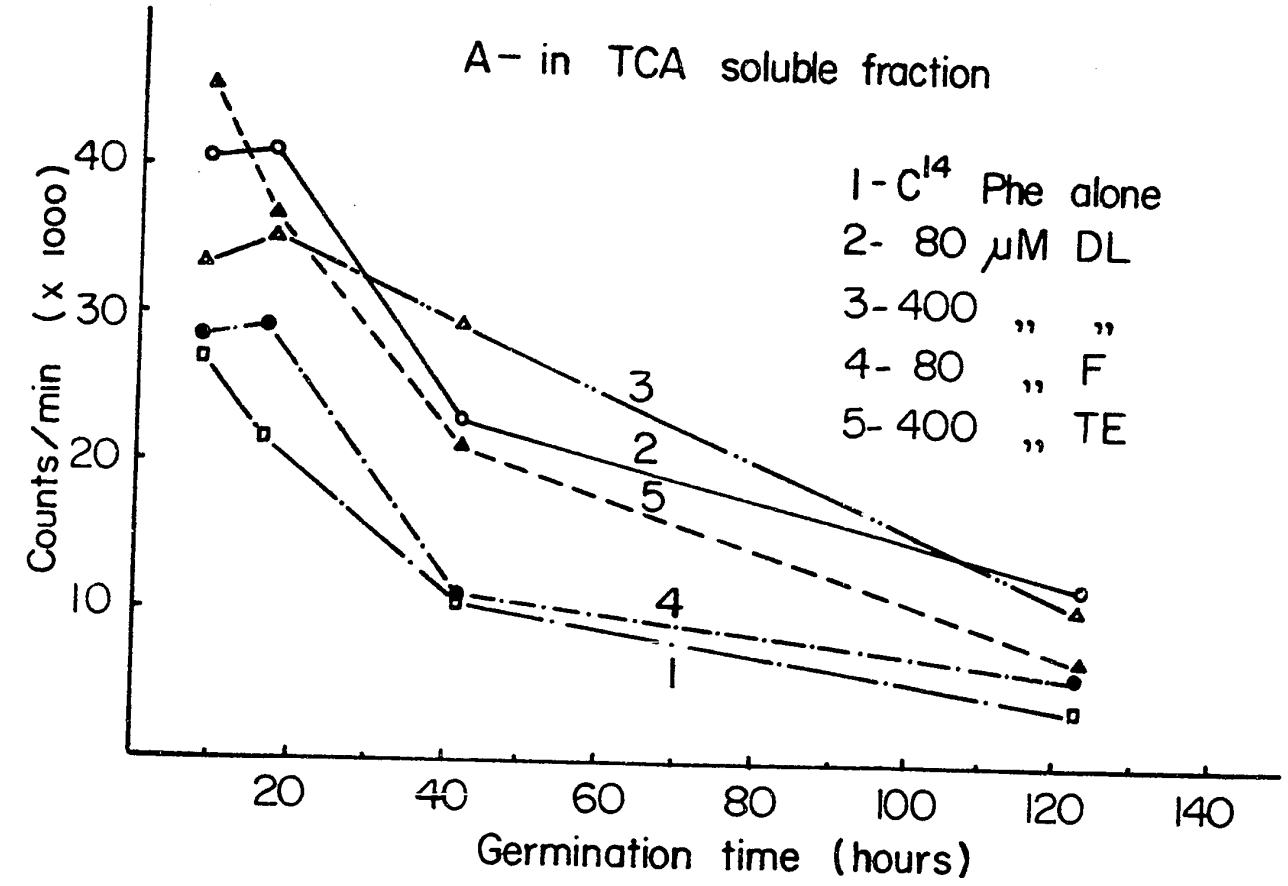


Fig. 9 Distribution of activity between the TCA soluble and TCA precipitate fractions



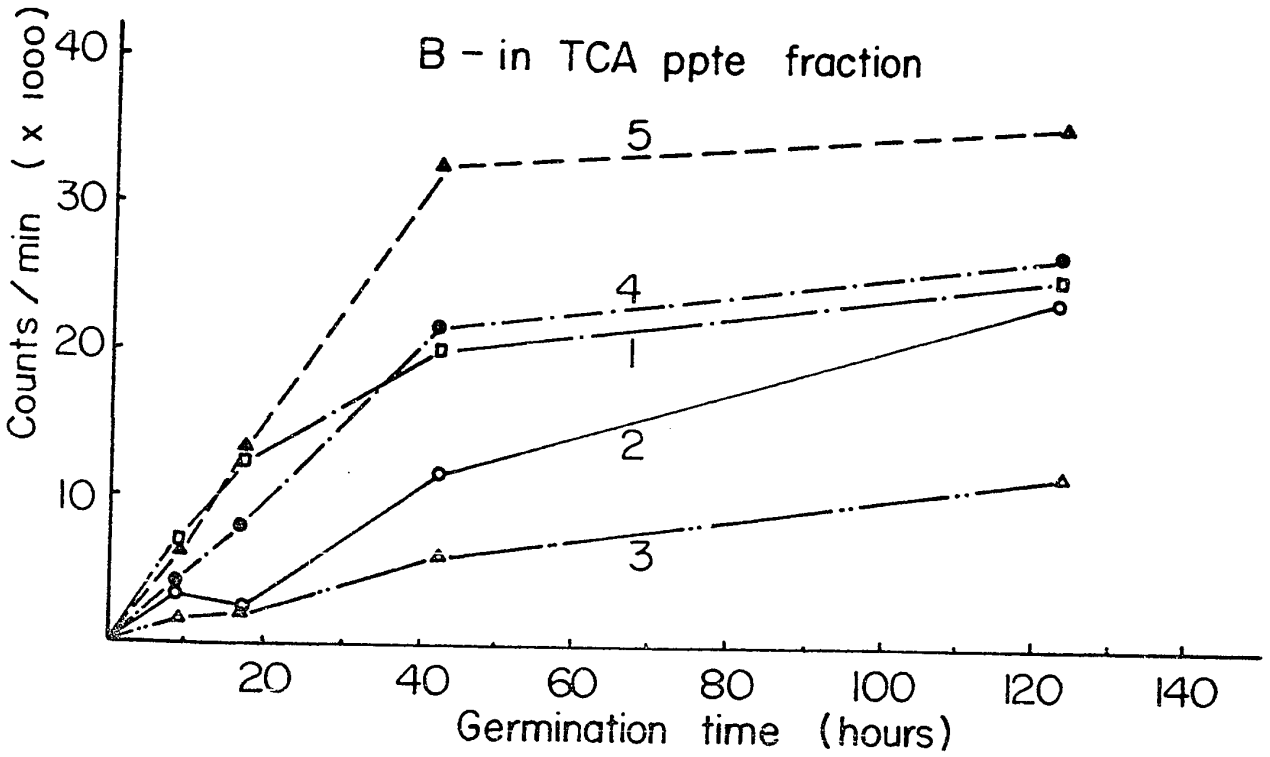


Fig. 10 Effect of adding unlabelled phenyl_alanine or its analogues on the distribution of activity

p-fluorophenylalanine, curve 4, seemed to have very little effect except for a slight initial delay in the decline of activity in the supernatant, and its increase in the precipitate. B-thienylalanine, curve 5, on the other hand, resulted in a higher level of activity in both the supernatant and the precipitate, in correlation with the greater recovery of activity in this group.

In the S-R, Table 27, the proportion of activity in the TCA soluble and precipitate varied from group to group, but was higher in the precipitate in all except Group 2 (42, 124 hours), Group 3 (124 hours) and Group 4 (42 hours).

The form of the activity in the TCA fractions.

1) TCA supernatant. Presumably the activity in this fraction would consist of unincorporated phenylalanine or its metabolic products. The TCA supernatants from Expts. 2 and 4 were first evaporated on a boiling water bath to reduce their volume, then washed with ether to remove TCA. The ether washings were discarded. Three volumes of ethanol were added to the residue, and the resultant precipitate was separated by centrifugation. Very little activity, 3% or less, was found in this precipitate, and at most 35% in the ethanol-soluble fraction. The TCA supernatants from Expt. 3 were extracted first with ether, then reduced in volume by evaporation before the residue was treated with 3 volumes of ethanol as above. Again, 1% or less of the activity was found in the ethanol-insoluble fraction, but as much as 71% was recovered in the ethanol-soluble fraction, while 20% or less

was found in the ether-soluble fraction.

Since the activity in the ethanol fractions from Expts. 2 and 4 was considerably less on a percentage basis than in those of Expt. 3, it is concluded that some volatile component was driven off in the course of the preliminary evaporation. Here is a possible cause of the loss of total activity already noted. The TCA supernatant fraction was counted only after a sample, usually 0.02 ml., had been evaporated to dryness in a stream of air, or occasionally on a slightly warmed hot plate. These drying procedures would result in the loss of volatile fragments of phenylalanine.

A small part of the ethanol-soluble fractions of Expt. 3 was chromatographed in the butanol solvent. Gas-flow counting of a strip cut from the 20 hour sample indicated that the activity streaked slightly from the origin, but mainly appeared in the position occupied by tyrosine. Extraction of the remainder of this sample with acetone-ethanol (93) showed that only 33% of the activity was recovered in the aromatic fraction. A small part of the ethanol fraction from Expt. 4 was also chromatographed, and the papers radioautographed. Activity in the first 3 samples appeared mainly in the position ahead of that occupied by phenylalanine, with a lesser activity in the area occupied by tyrosine.

It must be concluded that the phenylalanine did not long remain as such in the free form, but was broken to smaller fragments, although some was hydroxylated to tyrosine.

2) TCA precipitate. The activity in this fraction was presumably that attached to, or bound into, a larger molecule that was not soluble in 5% TCA. In these crude preparations, this fraction would include starch, cell wall material, nucleic acids, and proteins. As has been seen from Figure 7, the activity associated with the fraction gradually increased. To determine whether the activity was associated with protein or not, the following tests were made:

a) N-terminal analysis. A sample of the phenol-soluble fraction from Expt. 1 had already been tested by this method, and the results indicated that a large part of the activity was associated with the internal amino acid fraction. The TCA precipitates from the 42 and 124 hour samples of Group 1, Expt. 4, were also tested, both those from the cotyledons and those from the shoot-root axes. The results are presented in Table 29, and again showed that the bulk of activity was in the internal amino acid fraction. A considerable activity was lost in the "humus", and very little or none at all appeared in the N-terminal or free amino acid fractions. A loss of activity in the course of FDNB treatment was usual, and tended to be higher in the samples from older seedlings.

b) Solubility in NaOH. Stephenson et al (64) made the comment that TCA precipitates from plant materials were especially susceptible to contamination with substances adsorbed from the cell sap. In Expt. 4, the 100 mg. samples from the cotyledons or entire seeds that had been used in gas-flow counting were suspended in 1 ml. of N NaOH

Table 29

Results of N-terminal analysis of precipitate fractions from Expt. 1 and Expt. 4.

Fraction	Expt. 1	Expt. 4			
		cots		S-R	
	c/m	42 hr. c/m	124 hrs. c/m	42 hrs. c/m	124 hrs. c/m
1. Ethanol wash	13	1	0	3	6
2. Ether wash	39	0	0	0	0
3. EtAc. sol.	4	2	2	0	0
4. Residue	6	0	0	0	1
5. Bicarb. ppte.	-	251	347	308	543
6. HCl ppte.	-	5	7	2	2
5a. Humus	-	22	110	75	244
b. N-term. a.a.	9	0	4	2	4
c. Intern. a.a.	1000	207	258	288	295
Activity added	1000	449	804	424	1115
Activity recovered	1071	287	381	370	550
% recovery	107	64	47	87	49

for 24 hours. The material still insoluble at this time was removed by centrifugation, and washed once with water. One ml. of 100% TCA was added to the supernatant, and the resulting precipitate collected by centrifugation and put without washing into a planchet to dry. After drying, the precipitates were washed with ether to make a smooth preparation for counting. The results showed that considerable activity (25-63%) was not recovered in either the NaOH-soluble or insoluble precipitates. It is concluded that this activity was merely adsorbed onto the TCA precipitate. The TCA-NaOH supernatants from

these tests were discarded but additional tests showed: a) that activity was lost to the same extent even when the original precipitate was suspended in NaOH for only 15 minutes, and b) that only a small fraction (ca 10%) of the released activity could be recovered after evaporation of the supernatant on a water bath. The remainder was presumably in the form of a volatile compound or compounds.

Some preliminary attempts to isolate and identify the form of this released activity have been made, but the small amounts present make this a difficult problem. Attempts to determine the amount of phenylalanine or tyrosine actually incorporated have been hampered by the low specific activity of most of the fractions. Their contamination with carbohydrates made acid hydrolysis and the isolation of aromatic amino acids by charcoal adsorption (92) from the hydrolysates unsatisfactory because of the formation of "humus". Nevertheless, a small fraction of relatively high S.A. was obtained in the course of the NaOH solubilization discussed above. Chromatography and radioautography of this fraction after hydrolysis showed that the activity was present mainly in the position of tyrosine, and possibly in that of phenylalanine also.

Activity in the ethanol-ether wash of the TCA precipitate. The TCA precipitates when first collected had a slight greenish cast. After exhaustive extraction with ether following a single ethanol wash, the green color was concentrated in the ethanol-ether washings. These washings, as is evident from Tables 22, 23, 25 and 26, contained from

2-7% of the added activity. Activity associated with this fraction could be of several types: 1) ethanol or ether-soluble products of phenylalanine metabolism that had been adsorbed on the precipitate, 2) ethanol or ether-soluble protein or polypeptides into which phenylalanine had been incorporated, and 3) lipid components to which activity was attached. These washings were not subjected to controlled conditions so that the significance, if any, of the variations from one time sample to another cannot be assessed. In Expts. 2 and 3, the washes were merely evaporated to a tarry deposit and counted as such. The samples from Expt. 4 were separated into parts. The ether-soluble pigments, etc. were largely removed and concentrated onto paper discs for LS counting, while the ether-insoluble fraction was weighed and counted separately. The activity associated with the former is not included in Tables 25 and 26. It was relatively small, being in the neighbourhood of only 100 c/m.

PART THREE

SUMMARY AND CONCLUSIONS

1. ATPase activity

A study of the ATPase found in the cotyledons of pea seedlings had already been made by Young and Varner (10), and Young et al (11). These studies were repeated to a limited extent in the experiments presented here, but greater emphasis was given to the study of a similar activity appearing in extracts from the developing shoot-root axis.

It was found that the ATPase activity of the extracts increased rapidly from a very low level to a maximum within two days after the seed coat had been broken by the emerging radicle. As the seedling aged, however, the specific activity of the ATPase declined slowly although the amount of protein extracted tended to increase. When the extracts were separated by high speed centrifugation into precipitate (RNP) and supernatant fractions, the fraction of highest specific activity was found to be the RNP. Studies of variation in distribution of the activity resulting from manipulation of the extraction medium indicated that the ATPase was apparently associated primarily with the loosely packed "fluffy" layer of the RNP fraction.

The possibility that the ATPase studied in the extracts was an artifact of preparation and not the true role played in the living cell was suggested by the observation that tissues frozen

either prior to or during extraction yielded an extract of greatly reduced activity. An analogy with the phosphoryl-group-acceptor activity of muscle microsomes (98) has been proposed, i.e. as a result of freezing, the active sites were bound by some group that would ordinarily be removed in the course of extraction if the tissues had not been frozen. The fact that more protein was extracted from frozen tissue would make less likely the alternative possibility that the ATPase was only partially extracted.

ADPase and GTPase, both of greater activity than the ATPase, were also associated with the extracts. The available evidence supports the hypothesis that these activities and the ATPase were catalyzed by the same enzyme, but more experimentation is needed.

It is tempting to speculate that the ATPase activity associated with the "fluff" is involved in the transport of materials across the cell membranes. This would be an important function in a rapidly developing tissue dependent for its nutrition on the food stored in the cotyledons. Such a function would be necessary in the cotyledons themselves to aid in the transfer of sugars, amino acids, etc. out of the storage cells.

It is likely, however, that apart from the above mentioned activity, other activities are included under the general ATPase label. The fact that some ATPase activity always remained associated with the RNP even after more or less complete removal

of the "fluff", or after washing and recentrifugation, would support this view. Also the activity of frozen tissue extracts was only reduced not completely removed. A possible function for an ATPase associated with the ribosomes themselves has been suggested by Warner (46), i.e. that it acts as the coupler in the formation of multiple ribosomes. The energy required in the transfer of amino acids to the ribosomes and the release of the finished polypeptide would also call for enzyme activities of this type. Raacke (31) has found four distinct nucleotidases to be present in the ribosome and also finds the supernatant activity to be only partially inhibited by fluoride.

In view of the complex and contentious nature of an in vitro ATPase activity and the unknown relation to the role it plays in vivo, these preparations and the results obtained therefrom point the way to many interesting experiments. Such studies could include the enzymological problem of sorting out the various phosphatase activities in a pure form, and the physiological problem of their function, and factors affecting their development in vivo.

2. Attempts to demonstrate protein synthesis in a cell-free in vitro system.

The ribosome is universally regarded as the locus of protein synthesis and various methods of preparing this fraction from pea seedlings for in vitro tests were tried. Webster (30) with sucrose preparations, and Raacke (32) with sucrose-phosphate pre-

parations had reported considerable amounts of *in vitro* synthesis with the pea seedling system. Numerous attempts to repeat their experiments were unsuccessful. Various other extraction media were tested, also with negative results. It would appear that the very active ATPase associated with the ribosome fraction quickly hydrolysed the added ATP to AMP, and GTP to GDP, thereby preventing their participation in the amino acid ativation and transfer reactions. The breakdown of the nucleotides was verified by paper chromatography of the reaction mixtures.

Although there was an apparent increase in net protein reaction in some experiments there was little or no supporting evidence to indicate that a net protein synthesis had indeed occurred. Radioautography of the reaction mixtures indicated that the labelled amino acid emerged from the reaction unchanged, with no indication that any significant activation or binding to soluble RNA had occurred. Presumably the activating enzymes were not present, or the essential terminal groups of the sRNA were not available. If any polypeptide synthetase activity, as distinct from amino acid activation, were present, it occurred in amounts too small to be detected by the methods used.

Failure with the pea system has been reported by other workers (34), as well as by Webster (35) and Raacke (102) themselves, and raises the puzzling question as to why the original results cannot be repeated. Perhaps the isolated ribosomes do not function

in most cases because they do not have the proper structural relationship with a phospholipoprotein membrane, and the ATPase activity is a symptom of this lack.

3. The utilization of labelled amino acid in vivo.

In a series of 4 experiments, pea seeds were germinated in the presence of one of two labelled amino acids. The distribution of the label between phenol-soluble and insoluble fractions and TCA-soluble and TCA-insoluble fractions was determined. Alanine was used in one experiment in the carboxyl-labelled, DL form. Very little activity was recovered, most of it apparently being lost as CO_2. Phenylalanine was applied in a uniformly-labelled L form, and was used in all 4 experiments. Total recovery of added activity was not obtained with this amino acid either, but the loss did not seem to occur to any great extent as CO_2.

The seeds rapidly and almost completely imbibed the added label (2 μM per 80 seeds), although a small amount seemed to remain on the seed coat from which it was removable by washing. Fractions prepared with phenol after 66 hours showed an almost equal distribution of label between the phenol layer and the precipitate. Very little activity appeared in the water layer. TCA preparations were made from samples taken at different intervals, and a time-course curve of incorporation was obtained. Activity in the TCA supernatant was initially high, as might be expected, but dropped quickly, while the converse occurred with the TCA precipitate. A

small amount of activity (10% in 66 hours) appeared in the S-R. This picture of incorporation, however, was not so simple as it appeared.

From examination of the TCA fractions obtained, it became obvious that the phenylalanine had not remained unchanged. When the TCA supernatant was fractionated, a large part of the activity was not recoverable, but was lost as unidentified volatile compounds. Approximately 40% of the activity associated with the TCA precipitate was also lost as a volatile fraction when it was suspended in NaOH and reprecipitated. Hydrolysis of the "protein" fraction indicated that labelled tyrosine was present as well as labelled phenylalanine. N-terminal analysis indicated that the activity was in an internally-bound position, and that a part of the added amino acid had been incorporated into protein, although the percentage was not determined.

In the final experiment, the effect of adding additional unlabelled phenylalanine (DL), or the analogues, p-fluorophenylalanine (F), or B-thienylalanine (TE), was tested. The DL had the expected effect of reducing the apparent incorporation but F had little or no effect, while TE increased it. Since F inhibits protein synthesis at the concentrations used (10), these results would suggest that most of the activity was not incorporated into the protein fraction through synthesis, but by some other means, e.g. transpeptidation. All the additions had a deleterious effect on seedling growth.

The results of these four experiments raise a number of intriguing questions, which can be answered only by further experi-

mentation. For example:

1) Did the administered amino acid actually enter the cells of the cotyledons and mix with the indigenous pool, or was it broken down in large part exterior to this pool? That this latter possibility occurred is indicated by the fate of alanine, only 6% of whose activity was recovered. An idea of the extent of dilution of the indigenous amino acids can be obtained from the study by Lawrence and Grant (5). They found alanine to be present at a concentration of 22 μM per 80 seeds before germination, and to increase to 157 μM after 5 days germination. Phenylalanine and tyrosine were present in smaller amounts, namely 6 and 7 μM respectively, before germination and 57 and 13 μM respectively after 5 days.

2) By which route or routes did the breakdown of phenylalanine occur, and did this breakdown function in the seed as a protective mechanism? A search of the literature indicates that there are a number of possible pathways of phenylalanine metabolism, many of which have been demonstrated in plant tissues:

a) It can be converted to tyrosine by an hydroxylase activity (117), a reaction that seems to have occurred in these preparations.

b) Transaminase activity has been identified in pea seed extracts that would convert phenylalanine to phenylpyruvic acid (118).

c) Decarboxylation, for which there is indirect evidence in higher plants (118) would lead to the formation of phenylethylamine. Kenton and Mann (119) have shown that amine oxidase activity increased in

the course of germination of pea seedlings, phenylethylamine being oxidized to phenylacetaldehyde. This latter compound has been shown to be further metabolized to benzaldehyde and formic acid by an enzyme from pea roots (120). Phenylacetaldehyde could conceivably arise as well by decarboxylation of the phenylpyruvic formed by transaminase activity.

d) The possibility of microbial activity cannot be overlooked. The seeds were surface sterilized before germination but this would not be very effective against spore-forming organisms. A test for seed-borne diseases was negative (courtesy of Dr. V. Wallen, Plant Research Inst.) and no further precautions were taken to exclude or control microbial activity. No test for the presence of bacterial contaminants was made, but there were no visible mold contaminants. It is possible that bacteria might also have metabolized the phenylalanine, possibly by the route: phenylalanine $\longrightarrow$ tyrosine $\longrightarrow$ phenol, pyruvic acid, and ammonia (117).

These routes should be adequate to account for the presence of volatile components in the TCA preparations. However, other reactions by which the aromatic ring would be broken are not ruled out, and the likelihood that the activity was dispersed into a wide range of compounds is high.

4. Concluding remarks.

The interesting studies by Sutcliffe (121) and Jacoby and Sutcliffe (122) suggest a means by which the three apparently di-

vergent studies pursued in this thesis could be coordinated. These workers used washed slices of storage tissue, but the results they obtained are applicable to the developing shoot-root axis. Both tissues are dependent on external sources for the absorption of ions and amino acids, processes which Sutcliffe and Jacoby concluded are related to protein synthesis, since both are inhibited by chloramphenicol. The ATPase activity studied *in vitro* may be the type of protein synthesized in the course of absorption, an enzyme involved in transport and itself dependent on *de novo* synthesis for continued activity. That portion of the labelled amino acid imbibed by a germinating seed and thereafter remaining intact would serve as a marker of the transfer of amino acids from the cotyledons to the shoot-root axis. It would be interesting to determine if this label would appear in the fraction which showed the highest ATPase activity, *i.e.* the RNP "fluff" fraction.

The final conclusion, and the note on which this dissertation is ended, is that the experiments performed are of importance not so much for the questions answered but for the questions raised. Many avenues for further research into the intricacies of protein metabolism in the germinating seed are thereby indicated.

REFERENCES

1. Danielsson, C.E. Ann. Rev. Plant Physiol. 7, 215 (1956).

2. Danielsson, C.E. Acta Chem. Scand. 5, 541 (1951).

3. Lawrence, J.M., Day, K.M., and Stephenson, J.E. Plant Physiol. 34, 668 (1959).

4. Goksøyr, J., Boeri, E., and Bonnichsen, R.K. Acta Chem. Scand. 7, 657 (1953).

5. Lawrence, J.M. and Grant, D.R. Plant Physiol. 36 suppl., xviii (1961).

6. Stafford, H.A. Bull. Torrey Bot. Club 79, 351 (1952).

7. Holden, J. and Pirie, N.W. Biochem. J. 60, 39 (1955).

8. Barker, G.R. and Douglas, T. Nature 188, 943 (1960).

9. Zveva, E.S. and Proskuriakov, N.I. Biochimia 25, 897 (1960).

10. Young, J.L. and Varner, J.E. Arch. Biochem. Biophys. 84, 71 (1959).

11. Young, J.L., Huang, R.C., Vanecko, S., Marks, J.D., and Varner, J.E. Plant Physiol. 35, 288 (1960).

12. Elliott, W.H. J. Biol. Chem. 201, 661 (1953).

13. Varner, J.E. and Webster, G.C. Plant Physiol. 30, 393 (1955).

14. Liebecq, C. Arch. Biochem. Biophys. 97, 609 (1962).

15. Molnar, J. and Lorand, L. Arch. Biochem. Biophys. 93, 353 (1961).

16. Traverso-Cori, A. and Cori, O. Biochim. Biophys. Acta 57, 158 (1962).

17. Joyce, B.K. and Grisolia, S. J. Biol. Chem. 235, 2278 (1960).

18. Mazelis, M. Plant Physiol. 34, 153 (1959).

19. Kivilaan, A., Beaman, T.C., and Bandurski, R.S. Plant Physiol. 36, 605 (1961).

20. Racker, E. Physiol. Rev. 35, 1 (1955).

21. Moustafa, E. and Lyttleton, J. W. Biochim. Biophys. Acta 68, 45 (1963).

22. Meyerhof, O. J. Biol. Chem. 157, 105 (1945).

23. Abrams, A., McNamara, P., and Johnson, F. B. J. Biol. Chem. 235, 3659 (1960).

24. Sells, B.H., Six, N., and Brachet, J. Expt. Cell Res. 22, 246 (1961).

25. Ts'o, P.O.P. J. Gen. Physiol. 39, 325 (1956).

26. Skou, J.C. Biochim. Biophys. Acta 58, 314 (1962).

27. Grover, R. Absts., West. Sect. Am. Soc. Plant Physiol., June (1956).

28. Kalchar, H.M. in The Enzymes Vol. I I, p.151 (Boyer, P.D. and Lardy, H., Eds.) Academic Press, New York (1959).

29. Mazia, D., Chaffee, R.R., and Iverson, R.M. Proc. Nat. Acad. Sci. U.S. 47, 788 (1961).

30. Webster, G.C. Arch. Biochem. Biophys. 85, 159 (1959).

31. Raacke, I.D. Fed. Proc. 22, 348 (1963).

32. Raacke, I.D. Biochim. Biophys. Acta 34, 1 (1959).

33. Raacke, I.D. Biochim. Biophys. Acta 51, 73 (1961).

34. Lett, J.T. and Takahashi, W.N. Arch. Biochem. Biophys. 96, 569 (1962).

35. Webster, G., Whitman, S.L. and Heintz, R.L. Expt. Cell Res. 26, 595 (1962).

36. Bonner, J. Fortschritte de Chem. org. Naturstoffe 16, 139 (1958).

37. Harris, R.J.C. (Ed.) Protein Biosynthesis, Academic Press, New York, (1961).

38. Hoagland, M.B. in The Nucleic Acids Vol. IV, p. 349 (Chargoff, E. and Davidson, J.N. Eds.), Academic Press, New York (1958).

39. Simpson, M. Ann Rev. Biochem. 31, 333 (1962).

40. Zamecnik, P.C. Harvey Lectures 54, 256 (1960).

41. Birbeck, M.S.C. and Mercer, E.H. Nature 189, 558 (1961).

42. Ts'o, P.O.P. Ann. Rev. Plant Physiol. 13, 45 (1962).

43. Nirenberg, M.W. and Matthaei, J.H. Proc. Nat. Acad. Sci. U.S. 47, 1588 (1961).

44. Lengyel, P., Speyer, J.F., Basilio, C. and Ochoa, S. Proc. Nat. Acad. Sci. U.S. 48, 282, 1962).

45. Eck, R.V. Science 146, 477 (1963).

46. Warner, J.R., Knopf, P.M., and Rich, A. Proc. Nat. Acad. Sci. U.S. 49, 122 (1963).

47. Wettstein, F.O., Staehelin, T., and Noll, H. Nature 197, 430 (1963).

48. Hunter, G.D. and Godson, G.N. Nature 189, 140 (1961).

49. Lund, H.A. Biochim. Biophys. Acta 33, 347 (1959).

50. Straub, F.B., Ullmann, A., and Venetianer, P. Biochim. Biophys. Acta 43, 152 (1960).

51. Korner, A. Biochem. J. 83, 69 (1962).

52. Lingrel, J.B. and Webster, G. Biochem. Biophys. Res. Comm. 5, 57 (1961).

53. Hultin, T. Biochim. Biophys. Acta 61, 916 (1963).

54. Lamborg, M.R. Biochim. Biophys. Acta 55, 719 (1962).

55. Lingrel, J.B. and Webster, G. Biochim. Biophys. Acta 61, 942 (1962).

56. Anfinsen, C.B. and Haber, E. J. Biol. Chem. 236, 1361, (1961).

57. Goldberger, R.F., Epstein, C.J. and Anfinsen, C.B. J. Biol. Chem. 238, 628 (1963).

58. Zamecnik, P.C. Biochem. J. 85, 257 (1962).

59. Hendler, R.W. Nature 193, 821 (1962).

60. Allfrey, V.G. Ann. N.Y. Acad. Sci. 88, 722 (1960).

61. Birnstiel, M.L. Biochim. Biophys. Acta 55, 728 (1962).

62. Das, H.K. and Roy, S.C. Biochim. Biophys. Acta 54, 447 (1961).

63. Truman, D.E.S. and Korner, A. Biochem. J. 83, 588 (1962).

64. Stephenson, M.L., Thimann, K.V., and Zamecnik, P.C. Arch. Biochem. Biophys. 65, 194 (1956).

65. Beljanski, M., Beljanski, M., and Lovigny, T. Biochim. Biophys. Acta 56, 559 (1962).

66. Davies, J.W., Harris, G., and Neal, G.E. Biochim. Biophys. Acta 51, 95 (1961).

67. Szafrajski, P. and Bagdasariam, M. Nature 190, 719 (1961).

68. Miura, Y., Ito, H., Tanaka, S., Momose, K., Sunaga, K., and Moriyama, A. J. Biochem. (Tokyo) 50, 458 (1961).

69. Kaji, A., Kaji, H., and Novelli, G.D. Biochem. Biophys. Res. Comm. 10, 406 (1963).

70. Steward, F.C., Bidwell, R.G.S., and Yemm, E.W. Nature 178, 734 (1956).

71. Pollard, J.K. and Steward, F.C. J. Expt. Bot. 10, 17 (1959).

72. Ts'o, P.O.P. and Sato, C. J. Biophys. Biochem. Cytol. 5, 59 (1958).

73. Lyndon, R.F. and Steward, F.C. J. Expt. Bot. 14, 42 (1963).

74. Webster, G.C. Plant Physiol. 29, 382 (1954).

75. Virtanen, A.I. and Miettinen, J.K. Soc. Expt. Biol. Symp. 13, 218 (1959).

76. Swaramarkrishnan, V.M. and Sarma, P.S. Biochim. Biophys. Acta 14, 579 (1954).

77. Lowrey, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. J. Biol. Chem. 193, 265 (1951).

78. Hoagland, M.B., Keller, E.B., and Zamecnik, P.C. J. Biol. Chem. 218, 345 (1956).

79. Warburg, O., and Christian, W. Biochem. Z. 303, 40 (1939).

80. King, E.J. Biochem. J. 26, 292 (1932).

81. Kachmar, J.F. and Boyer, P.D. J. Biol. Chem. 200, 669 (1953).

82. Moore, S. and Stein, W.H. J. Biol. Chem. 211, 907 (1954).

83. Hochster, R.M. and Nozzolillo, C.G. Can. J. Microbiol. 7, 389 (1961).

84. Kirby, K.S. Biochem. J. 64, 405 (1956)

85. Block, R.J., Durrum, E.L., Zweig, G. A manual of paper chromatography and paper electrophoresis. Academic Press, New York, (1958).

86. Circular OR-15. Pabst Laboratories, Milwaukee, Wis., U.S.A. (1959).

87. Loring, H.S. in The Nucleic Acids Vol. I, p. 191 (Chargoff, E. and Davidson, J.N. Eds.) Academic Press, New York, (1958).

88. Rosenbaum, M. and Brown, R.A. Anal. Biochem. 2, 15 (1961).

89. Haber, A.H. Amer. J. Bot. 48, 431 (1961).

90. Mans, R.J. and Novelli, G.D. Arch. Biochem. Biophys. 94, 48 (1961).

91. Fraenkel-Conrat, H., Harris, J.I., and Levy, A.L. in Methods of Biochemical Analysis, Vol. II, p.359, Academic Press, New York, (1955).

92. Partridge, S.M. Biochem. J. 44, 521 (1949).

93. Udenfriend, S. and Cooper, S.R. J. Biol. Chem. 203, 953 (1953).

94. Draper, O.J. and Pollard, A.L. Science 109, 448 (1949).

95. Weissburger, A. (Ed.) Technique of organic chemistry, Vol. VII, Interscience Publishers Inc., New York (1955).

96. Zamecnik, P.C. and Stephenson, M.L. Ann. N.Y. Acad. Sci. 88, 708 (1960).

97. Magasanik, B., Vischer, E., Doniger, R., Elson, D., Chargaff, E. J. Biol. Chem. 186, 37 (1950).

98. Molnar, J. and Lorand, L. Arch. Biochem. Biophys. 98, 356 (1962).

99. Varner, J.E. and Schlidovsky, G. Plant Physiol. 38, 139 (1963).

100. Loening, U.E. Biochem. J. 81, 254 (1961).

101. Webster, G.C. and Johnson, M.P. J. Biol. Chem. 217, 641 (1955).

102. Raacke, I.D. personal correspondence (1963).

103. Rabson, R. and Novelli, G.D. Proc. Nat. Acad. Sci. U.S. 46, 484 (1960).

104. Jocelyn, P.C. Biochem. J. 85, 480 (1962).

105. Hülsmann, W.C. and Lipmann, F. Biochim. Biophys. Acta 43, 123 (1960).

106. Highkin, H.R. Amer. J. Bot. 45, 626 (1958).

107. Raacke, I.D. and Bové, J. Separatum Experientia 16, 195 (1960).

108. Robinson, C.L. and Novelli, G.D. Arch. Biochem. Biophys. 96, 456 (1962).

109. Hele, P. Biochim. Biophys. Acta 42, 546 (1960).

110. Brown, E.G. Biochem. J. 85, 633 (1962).

111. Fleck, A. and Munro, H.N. Biochim. Biophys. Acta 55, 571 (1962).

112. Grimbleby, F.H. and Ntailianas, H.A. Nature 189, 839 (1961).

113. Webster, G.C. Arch. Biochem. Biophys. 89, 53 (1960).

114. Rabinovitz, M., Olson, M.E., and Greenberg, D.M. J. Biol. Chem. 210, 837 (1954)

115. Cossins, E.A. and Turner, E.R. J. Expt. Bot. 14, 290 (1963).

116. Börner, H. Bot. Rev. 26, 393 (1960).

117. Meister, A. Biochemistry of the amino acids, 485pp., Academic Press, New York (1956).

118. Street, H.E. in Encyclopedia of Plant Physiology, Vol. VIII, p. 674, (Ruhland, W. Ed.) Springer-Verlag, Berlin, (1958).

119. Kenton, R.H. and Mann, P.J.G. Biochem. J. 50, 364 (1952).

120. Kenton, R.H. Biochem. J. 55, 350 (1953)

121. Sutcliffe, J.F. Nature 188, 294 (1960).

122. Jacoby, B. and Sutcliffe, J.F. J. Expt. Bot, 13, 335 (1962).