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THE UNIVERSITY OF ALBERTA

SOME ENZYMES AND INTERMEDIATES INVOLVED IN THE BIOSYNTHESIS OF L-ARGININE IN *PISUM SATIVUM* L.

by

PETER DOUGLAS SHARGOOL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled SOME ENZYMES AND INTERMEDIATES INVOLVED IN THE BIOSYNTHESIS OF L-ARGININE IN *PISUM SATIVUM* L. submitted by Peter Douglas Shargool in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The pathway of L-arginine biosynthesis was examined in germinating pea seeds using ^{14}C labelled precursors and by extraction of enzymes involved in the pathway.

When DL-ornithine-^{14}C was fed to excised root tips of 3 day old pea seedlings, labelled citrulline and arginine were formed together with aspartate, glutamate and alanine. In pulse-feeding experiments, the root tips rapidly metabolized the supplied ornithine-5-^{14}C to yield citrulline and arginine. When L-citrulline-^{14}C was fed to imbibing pea seeds, more than 50% of the label was incorporated into both free and protein bound arginine.

In order to ascertain the possible intermediary role of argininosuccinate in the conversion of citrulline to arginine, enzymes catalyzing the synthesis and cleavage of this compound in pea cotyledons were examined. Argininosuccinate lyase was isolated from pea root tips and cotyledons, and partially purified using isoelectric precipitation, calcium phosphate gel fractionation, and negative adsorption on C.M. cellulose. Enzyme activity was assayed spectrophotometrically by continuous measurement of fumarate formation from argininosuccinate. The reverse reaction was studied by a chromatographic technique. The general properties of this enzyme were studied, including reaction equilibria, the effects of sulphydryl group inhibitors and stability. The Michaelis-Menten constants for argininosuccinate and arginine were determined at pH 7.9. Under the

experimental conditions employed, the reaction appeared to favour the cleavage of argininosuccinate.

Argininosuccinate synthetase was also demonstrated in enzyme extracts prepared from cotyledons. The enzyme was partially purified and found to have an absolute requirement for citrulline,aspartate, ATP, and magnesium. The general properties of this enzyme were examined and compared with those of the corresponding synthetases from bovine liver and *Neurospora crassa*. Attempts to separate argininosuccinate lyase activity from the synthetase activity were unsuccessful. It is suggested that the two enzyme activities might be associated with a single protein complex.

It was concluded that the biosynthesis of arginine in pea seeds occurs via the partial reaction of the ornithine cycle. The significance of this synthesis during the early stages of germination is discussed.

ACKNOWLEDGEMENTS

I am very happy to acknowledge the pleasant stay that was spent in the department of Botany, whilst working on this programme. I would particularly like to thank Dr. E.A. Cossins for his help and guidance throughout the course of these studies, and during the preparation of this thesis.

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LIST OF ABBREVIATIONS

ATP : Adenosine triphosphate

ADP : Adenosine diphosphate

NADP : Nicotinamide adenine dinucleotide phosphate

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced).

CPM : Counts per minute

Tris: Tris (hydroxymethyl) aminomethane

EC. Enzyme Commission *

* The names of enzymes used in this thesis are the systematic and trivial names recommended by the International Commission on Enzymes.

INTRODUCTION

The first evidence that the ornithine cycle outlined by Krebs and Henseleit in 1932, existed in plants, was the isolation of the constituent amino acids of the cycle from plant tissues. Wada in 1930 discovered the existence of citrulline in the juice of the watermelon (*Citrullus vulgaris* Schrad) fruit. Kasting and Delwiche (1957) showed that ornithine and arginine as well as citrulline, were present in watermelon seedlings. The amounts of these compounds varied between 0.2 and 0.9 μmoles/g fresh weight. Kasting and Delwiche also examined wheat and barley plants and noted that citrulline was present in much smaller amounts, up to 0.03 μmoles/g fresh weight. Bollard (1957) discovered that citrulline made the major contribution to the total nitrogen of the xylem sap in certain trees. These included the alder (*Alnus glutinosa* L. Gaertn), the southern beech (*Nothofagus fusca* Oerst), the chinese persimmon (*Diospyros kaki* Lf.) and the cypress (*Chamaecyparis lawsoniana* Parl).

Concomitant with these discoveries have been feeding experiments employing radioactive isotopes. These experiments have been designed to label the amino acids of the cycle in different plant species. Bone (1959) studied the synthesis of some of the intermediates of the cycle in mung bean (*Phaseolus aureus* Roxb) mitochondria. Using incubation mixtures containing magnesium, $NaH^{14}CO_3$, ammonium chloride, ATP, sucrose and mitochondria, Bone was able to demonstrate the synthesis of

labelled carbamyl phosphate which was isolated as the barium salt. When N-acetyl glutamate was added to the reaction mixture, it was found to have no effect on the rate of synthesis. Thus the carbamyl phosphate must have been synthesized by the action of carbamyl phosphate synthetase (ATP: carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5). When ornithine was added to the above reaction mixture, citrulline synthesis occurred presumably by the action of ornithine transcarbamylase (carbamyl phosphate:L-ornithine carbamyltransferase, EC 2.1.3.3).

Similar evidence for the presence of the ornithine cycle in trees was obtained by Barnes and Naylor (1959). They investigated the products of $NaH^{14}CO_3$ metabolism in sand pine (*Pinus clausa* [Chapm.]Vasey) and pond pine (*Pinus serotina* Michx). When pine root cultures were allowed to take up the labelled solution for 24 hours in darkness, it was found that in addition to typical dark fixation products such as malic acid, ^{14}C was also present in citrulline and arginine. When $NaH^{14}CO_3$ was presented together with ornithine to isolated pond pine roots, then as much as 20% of the total ^{14}C was incorporated into citrulline, more than in any other compound. Similar results were obtained when using root callus tissue from pond and sand pine. When ornithine-2-^{14}C was fed to pond and sand pine roots, and callus tissue, it was freely converted into citrulline-^{14}C, and arginine-^{14}C, as much as 41% being found as citrulline in pond pine callus tissue. When the tissue was pretreated with $10^{-2}M$ ornithine for 6 to 10

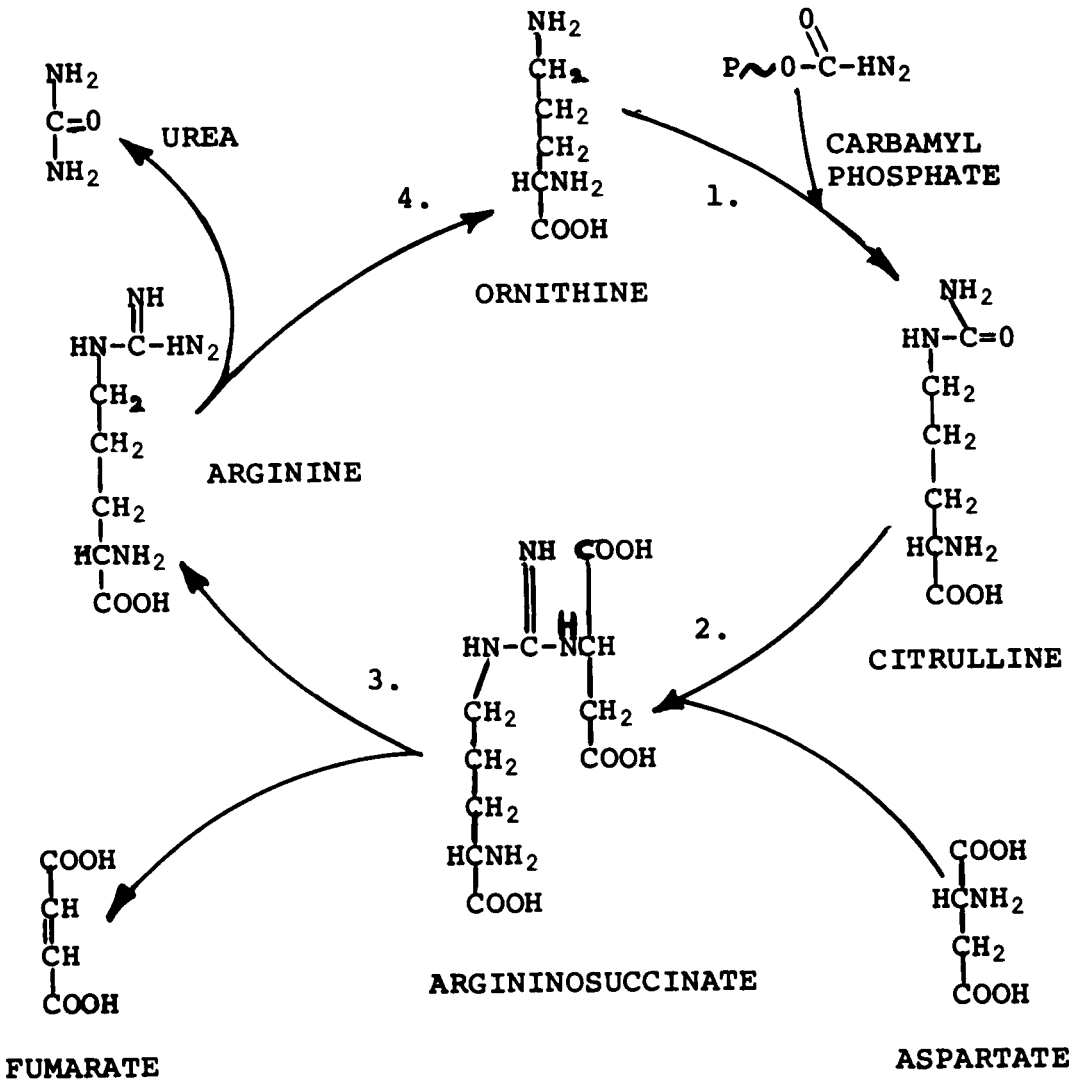
days, the amount of ^{14}C found in citrulline and arginine was increased, as much as 50% of the radioactivity being present as citrulline and 16% as arginine in pond pine callus tissue. Other workers such as Racusen and Aronoff (1954) have also reported the fixation of $^{14}CO_2$ into the ornithine cycle intermediates of plants. In these experiments, the isolated arginine was essentially labelled in the carbon atom of the guanidino group.

From a consideration of the data presented above, it appears that there is strong evidence for the existence of the ornithine cycle in plants (fig. 1). One of the major differences between the ornithine cycle of plants, and that present in animal tissues appears to be the absence of urea production in plants. Methods used in the past to demonstrate urea in plant tissues were criticized by Tracey (1955). Tracey pointed out that most of these methods would have brought about the decomposition of labile ureides present, such as allantoin and allantoic acid, to yield urea. In view of the uncertainty regarding the presence of urea in plant tissues, it follows that the physiological significance of arginase and urease in plants is also uncertain.

Recent work by Durzan (1968 a, b, and c) suggests that arginine present in some conifer tissues may be degraded via compounds other than the established ornithine cycle intermediates. He also suggests that the synthesis of arginine may not be due to the operation of the ornithine

FIGURE 1

The Ornithine cycle, and the enzymes catalyzing its partial reactions.



1. Ornithine transcarbamylase.
2. Argininosuccinate synthetase.
3. Argininosuccinate lyase.
4. Arginase.

cycle. Durzan fed L-arginine-U-^{14}C, to apices of the leader shoots of white spruce (*Picea glauca* [Moench]Voss) saplings. He found that arginine and certain other monoguanidino compounds accounted for 41-57% of the activity extracted in 70% ethanol. The monoguanidino compounds which were identified included α-keto-δ-guanidino valeric acids, and γ-guanidinobutyric acid. Durzan postulated that γ-guanidinobutyric acid is formed from α-keto-δ-guanidino valeric acid by the action of a transaminase or L-aminoacid oxidase. At least seven other monoguanidino compounds were found, but were not identified.

Dougall and Fulton (1967) recently obtained data which suggest the possible existence of a pathway involving acetylated intermediates of the ornithine cycle. Suspension cultures of Paul's Scarlet Rose (Rosa sp.) tissue, were shown to contain α-N-acetyl-L-ornithine:2 ketoglutarate aminotransferase (EC 2.6.1.11) activity. The enzyme was highly specific for α-ketoglutarate and α-N-acetyl-L-ornithine. In isotope competition experiments, Dougall found that α-N-acetyl-L-ornithine reduced the amount of arginine-^{14}C synthesized from glucose-U-^{14}C by 70%, compared with control experiments. Using L-ornithine and glucose-U-^{14}C, he found that the amount of arginine-^{14}C produced was lowered by 80%. L-citrulline reduced the yield of arginine-^{14}C from glucose-U-^{14}C by 90%. From these results it would appear that the pathway utilizing α-N-acetyl-L-ornithine for arginine biosynthesis is not as active as the pathway which

utilizes L-ornithine.

With a few notable exceptions, little work has been carried out to purify and study the enzymes catalysing the partial reactions of the ornithine cycle of plants. This is in marked contrast to the extensive studies that have been made with animal tissues (Ratner, 1956). O'Neal and Naylor (1968) published a preliminary report on the properties of a carbamyl phosphate synthetase isolated from 10-15 day old pea seedlings (*Pisum sativum* L). They partially purified the enzyme 10-15 times and found that 2-mercaptoethanol, L-glutamine, or L-ornithine had to be present to stabilize enzyme activity. The partially purified enzyme required L-glutamine, two molecules of ATP, and bicarbonate ion, for the synthesis of carbamyl phosphate. Lineweaver-Burk plots showed that the enzyme was inhibited competitively with respect to ATP, by UMP, UDP, and AMP. ADP was found to inhibit non-competitively with respect to ATP. O'Neal and Naylor calculated the inhibition constants (Ki values) for UMP, ADP, AMP, and ADP. They also noted that hyperbolic kinetics were obtained for UMP inhibition, indicating a possible allosteric transition in the enzyme molecule.

Kleczkowski (1965) showed that L-ornithine transcarbamylase activity could be demonstrated in acetone extracted powders of 10-14 day old pea plants. Kleczkowski showed that these preparations required L-glutamine and magnesium for maximum activity, which occurred at pH 7.4 to

7.6, N-acetylglutamate was without effect in the assay. These studies were extended by Kleczkowski and Cohen (1964) who prepared a very highly purified preparation of L-ornithine transcarbamylase from 5-10 day old pea seedlings. This preparation was found to be highly specific for L-ornithine; D-ornithine producing only 8% of the activity displayed by the L-form. The enzyme was inhibited by sodium flouride, p-hydroxymercuribenzoate, and chloroacetophenone.

Fotyma, Kleczkowski and Reifer (1961) demonstrated the presence of argininosuccinate synthetase (L-citrulline, L-aspartate ligase (AMP) , EC 6.3.4.5), in wheat seedlings. The demonstration relied on the estimation of ammonia produced from incubation mixtures containing L-citrulline, L-aspartate, magnesium, ATP, arginase, urease, and crude enzyme extracts. The enzyme extracts were produced by homogenising the seedlings in 0.2 M phosphate buffer, pH 7.2. Synthetase activity could not be demonstrated for pea seedling extracts produced in a similar manner, until the extracts had been passed through G-50 Sephadex. Grabarek, Reifer and Kleczkowski (1965) postulated that the lack of activity of the pea seedling extract was due to the presence of an inhibitor which was removed by the Sephadex treatment.

Argininosuccinate lyase (L-argininosuccinate arginine lyase, EC 4.3.2.1) has been demonstrated by several workers in crude plant extracts. It was first partially purified from pea seeds by Davison and Elliot (1952). The

enzyme was extracted by stirring pea meal with water, and subsequently partially purified by isoelectric precipitation. Davison and Elliot demonstrated the ability of their preparations to synthesize argininosuccinate when incubated with high concentrations of arginine and fumarate. They did not, however, examine the reverse reaction. Rosenthal and Naylor (1966) published a short communication on the argininosuccinate lyase of jackbean meal. The enzyme was reported to be cold labile, and unstable even to short periods of dialysis. Acetone powders of 20 day old pea seedlings, imbibed pea seeds, and of jackbean meal have also been shown to contain argininosuccinate lyase activity (Walker and Myers, 1953 , Buraczewski, Kleczkowski and Reifer, 1960).

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) activity has been detected in ether-extracted powders produced from the embryos of various gymnosperm species, by Guitton (1957). The species examined included *Pinus pinaster* Sol, and *Abies pectinata* Lamk, D.C. Guitton noted that particularly high levels of arginase were present in the endosperm of these species. He also noted that the arginase levels rose rapidly after the embryo had fully imbibed water. These facts led him to assume that the enzyme had importance in the nitrogen metabolism of these tissues. Muszyńska and Reifer (1968) performed detailed studies of an arginase preparation isolated from plants of the bitter lupin (*Lupinus angustifolius* L). The preparation was obtained from

acetone dried lupin plants, and was purified 75 times using ammonium sulphate fractionation, and filtration through Sephadex G-100. The Michaelis constant for arginine was determined at pH values ranging from 8.75 to 10.40, in the presence of various metal ions. The authors noted that the Km values for the plant enzyme obtained in the presence of manganous ions were similar to the values obtained for arginase preparations isolated from various animal sources.

A number of studies have been carried out which draw attention to the importance of arginine as a nitrogen storage compound in plants. Oland (1959) made a detailed study of the principal nitrogenous storage compounds in apple trees. The trees were divided into groups, some received nitrogen during an experimental period but were later starved of nitrogen. Oland discovered that in the nitrogen rich trees the soluble nitrogen which could be extracted by treating the tissue samples with sodium chloride/sodium phosphate buffer for 24 hours at 4°C, was quantitatively the most important reserve of the stem. Of the compounds contained in these extracts, arginine had the characteristics of the most important nitrogen storage compound present. Thus in the spring, arginine amounted to at least 70% of the soluble fraction. In trees that became depleted of arginine by growth on a nitrogen poor medium, the level of this amino acid fell until only a trace remained. This behaviour was not noted for other amino acids, the amide fraction for example remained relatively constant in all

plant parts analysed. Durzan (1967) obtained similar results when he studied the effect on aminoacid levels of growing white spruce (*Picea glauca* [Moench]Vose) and jack pine (*Pinus banksiana* Lamb), in sand cultures irrigated with various nutrient solutions. The nutrient solutions used were as follows: low sodium, nitrate or ammonia as the nitrogen source, low nitrogen, low phosphorous, and low potassium. He found that the trend in all of the treatments given was for arginine to be the dominant amino acid. Thus he noted that in white spruce stems and roots, in five out of the seven treatments, approximately 90% of the total soluble nitrogen could be accounted for as arginine. He also noted that the arginine content was reduced when the spruce and pine were grown in a nitrogen deficient medium. In the case of spruce leaves, the arginine content was reduced to 2% of the total soluble nitrogen. These data can be interpreted as showing that arginine is an important nitrogen storage compound in these tissues.

The importance of the ornithine cycle as a possible pathway for the utilization of stored arginine was suggested by the studies of Boulter and Barber (1963). These workers examined the levels of different amino acids in germinated and ungerminated seeds of the broad bean (*Vicia faba* L.) growing without an external supply of nitrogen. They found that in the ungerminated beans, arginine was by far the most prominent amino acid, accounting for 25% of the total amino acid nitrogen. After 14 days growth, however, less than 6%

of this amount remained but the levels of all other amino acids had increased. Boulter and Barber also found that argininosuccinate, citrulline, and ornithine did not accumulate in appreciable quantities until the fourteenth day of germination. They were thus led to postulate that arginine nitrogen was being utilized by a reversal of the ornithine cycle via argininosuccinate, citrulline and finally ornithine. This would mean that one nitrogen atom of the guanidino group would appear as aspartate. The other nitrogen atom would ultimately appear as ammonia, if it were not removed from citrulline in a transamination reaction. Their suggestions were supported by data from feeding experiments when arginine-U-^{14}C was supplied to bean cotyledon slices. Labelling was observed in argininosuccinate and proline after 5 mins, and in glutamate after 30 mins. Ornithine, citrulline, and aspartate were not labelled until after 24 hrs incubation.

More recent research on the ornithine cycle has tended to concentrate on possible control mechanisms. For example, Bernlohr (1966) discovered the existence of two forms of ornithine transcarbamylase in *Bacillus licheniformis*. One form which was presumed to have a biosynthetic function was repressed by arginine. The other form was induced by arginine but repressed by glucose, and was synthesized towards the end of the growth cycle. A similar situation was found to exist in a strain of *Pseudomonas fluorescens* (IRC-204), by Ramos, Pierard and Wiame,(1966 a & b). They also discovered

the existence of two ornithine transcarbamylases. One appeared to be anabolic in nature and was repressed by arginine, the other enzyme appeared to be catabolic in nature and was induced by arginine. While the anabolic enzyme appeared to be unable to catalyse the break down of citrulline, the catabolic enzyme appeared to have a limited ability to catalyse the synthesis of citrulline. The enzymes had different pH optima. The catabolic enzyme had an optimum pH of 6.7, which is similar to that for the catabolic enzyme arginine deiminase (L-arginine iminohydrolase, EC 3.5.3.6). The pH optimum for the anabolic enzyme was 8.5. Nazario (1967) reported studies on the control of ornithine transcarbamylase levels in a mutant strain of *Neurospora crassa* (arg - 10). This strain had no detectable argininosuccinate lyase, and thus accumulated argininosuccinate when grown on an arginine containing medium. Nazario found that the ornithine transcarbamylase level was progressively repressed by increasing levels of arginine. If, however, the argininosuccinate level in the medium was indirectly increased by the addition of citrulline, then the ornithine transcarbamylase appeared to be derepressed. The mechanism of derepression was found to be via the competitive inhibition of arginyl transfer RNA synthetase by argininosuccinate. Arginyl-t-RNA formation is necessary to achieve repression of the arginine biosynthetic enzymes (Eidlic and Neidhardt, 1965 a and b, Bock, Faiman and Neidhardt, 1966). When arginyl-t-RNA synthetase is inhibited then the arginine biosynthetic enzymes

are derepressed.

Eliasson and Strecker (1966) have reported experiments on the control of arginase levels in tissue cultures of Changs liver cells. These workers found that arginase appeared to be repressed by proline or Δ'-pyrroline-5-carboxylic acid. They postulated that an initial increase in the level of ornithine transcarbamylase in the liver cells would lead to a decrease in the level of proline formed. This would occur because of competition between ornithine transcarbamylase, and ornithine-δ-transaminase for the available ornithine, and would lead to a derepression of arginase synthesis. Since argininosuccinate lyase and argininosuccinate synthetase have been shown to be repressed by arginine (Schimke, 1964), the increase in arginase activity would be expected to remove the repressor of the arginine synthesizing enzymes.

The studies outlined above, have helped provide a much clearer picture of the ornithine cycle in a variety of animal and microbial species. In order that our understanding of the ornithine cycle in plants may also be enlarged, the following basic studies should be carried out.

1. Purer preparations of argininosuccinate lyase which has already been examined in the form of crude extracts, should be produced. These preparations may then be examined in detail with regard to the characteristic properties such as the Michaelis constants for different substrates. This in turn should enable us to say if the enzyme has more

affinity for arginine or for argininosuccinate, and thus the degree of reversibility of the cycle in plants should become more apparent.

2. Argininosuccinate synthetase has not yet been isolated from a higher plant source. This should be attempted and the enzyme examined to determine if its properties resemble those of the enzyme prepared from animal tissues (Ratner, 1949 and 1967), and that from *Neurospora crassa* (Wampler and Fairley, 1967). Such studies as these would clearly assist in revealing the importance of the ornithine cycle in the nitrogen metabolism of higher plants. They would also pave the way for more sophisticated studies upon the mechanisms involved in controlling the cycle and the level of its intermediates in plants.

MATERIALS AND METHODS

General Chemicals

Argininosuccinic acid (barium salt), 2-phosphoenol pyruvic acid (potassium salt), fumarate hydratase and L-citrulline were obtained from the California Corporation for Biochemical Research, Los Angeles, California, U.S.A. Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) and adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3), were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Inorganic pyrophosphatase (Pyrophosphate phosphohydrolase, EC 3.6.1.1) and arginase were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. L-aspartic acid, and ATP (disodium salt) were obtained from Nutritional Biochemicals, Cleveland, Ohio. Sephadex gels were obtained from Pharmacia Canada Ltd., Montreal.

Labelled Compounds

L-arginine-5-^{14}C (2.8 μc/μ mole), DL-ornithine-5-^{14}C (10.0 μc/μ mole) and L-citrulline-carbamyl-^{14}C (0.95μc/μ mole), were purchased from New England Nuclear Co., Boston, Massachusetts, U.S.A. L-citrulline-carbamyl-^{14}C (29.2 μc/μ mole), and aspartate-U-^{14}C (8.7 μc/μ mole) were purchased from the Radiochemical Centre, Amersham, England. The purity of these compounds is shown in Table 1A.

Ion Exchange Resins

Two types of ion-exchange resin were obtained from the California Corporation for Biochemical Research, Los Angeles, California, U.S.A.

TABLE 1 A

The purity of ^{14}C labelled isotopes used in feeding and other experiments.

Isotope	Purity (%)	Chromatographic System used.
DL-ornithine-5-^{14}C	98	Butan-1-ol: acetic acid: water (5:1:4, by volume)
DL-arginine-5-^{14}C	98	as above
L-citrulline-carbamyl-^{14}C	97	Electrophoresis in borate succinate Buffer (pH 7.0) as described in the materials and methods section (P. 24).
Aspartate-U-^{14}C	97	Butan-1-ol: water: acetic acid (37:25:0, by volume) and Phenol: water (80:20, by volume)

1. Cation exchange resin - Dowex 50W-X8, (hydrogen form) 200-400 mesh.
2. Anion exchange resin - Dowex 1-X10, (chloride form) 200-400 mesh.

Preparation of Ion Exchange Resins

Before use the ion exchange resins were made into a slurry with distilled water. The slurry was then packed into 1 cm diameter chromatography columns to a depth of 6 cm. The cation exchange resin was now washed with distilled water until the pH of the effluent was 5.5. The anion exchange resin which was purchased in the chloride form, was converted to the acetate form before use (Canvin and Beevers, 1961). This was accomplished by extensive washing of the resin with 1 M sodium acetate solution until the effluent from the column gave a negative chloride test with silver nitrate. The resin was then washed with 0.1 N acetic acid (8 ml/ml of resin bed), and finally with distilled water until the effluent had a pH value of 5.5.

Plant Materials

Feeding experiments and enzyme studies were conducted on the cotyledonary tissues, root tips and leaves of the pea plant (*Pisum sativum* L. var Homesteader). To provide material for the extraction of enzymes, dry seeds were washed thoroughly in distilled water and then soaked in distilled water for 24 hr at room temperature. The fully imbibed seeds were removed and used for experimental purposes. When required in a germinated condition the fully

imbibed seeds were planted in moist vermiculite at 28°C in darkness for periods of up to 5 days. Under these conditions the seeds germinated rapidly and uniformly. The approximate stage of germination and seedling development over this period being shown in Plate 1. Cotyledons were obtained after removal of the testas and embryos.

Feeding Experiments

1. Feeding of DL-ornithine-^{14}C to root tips

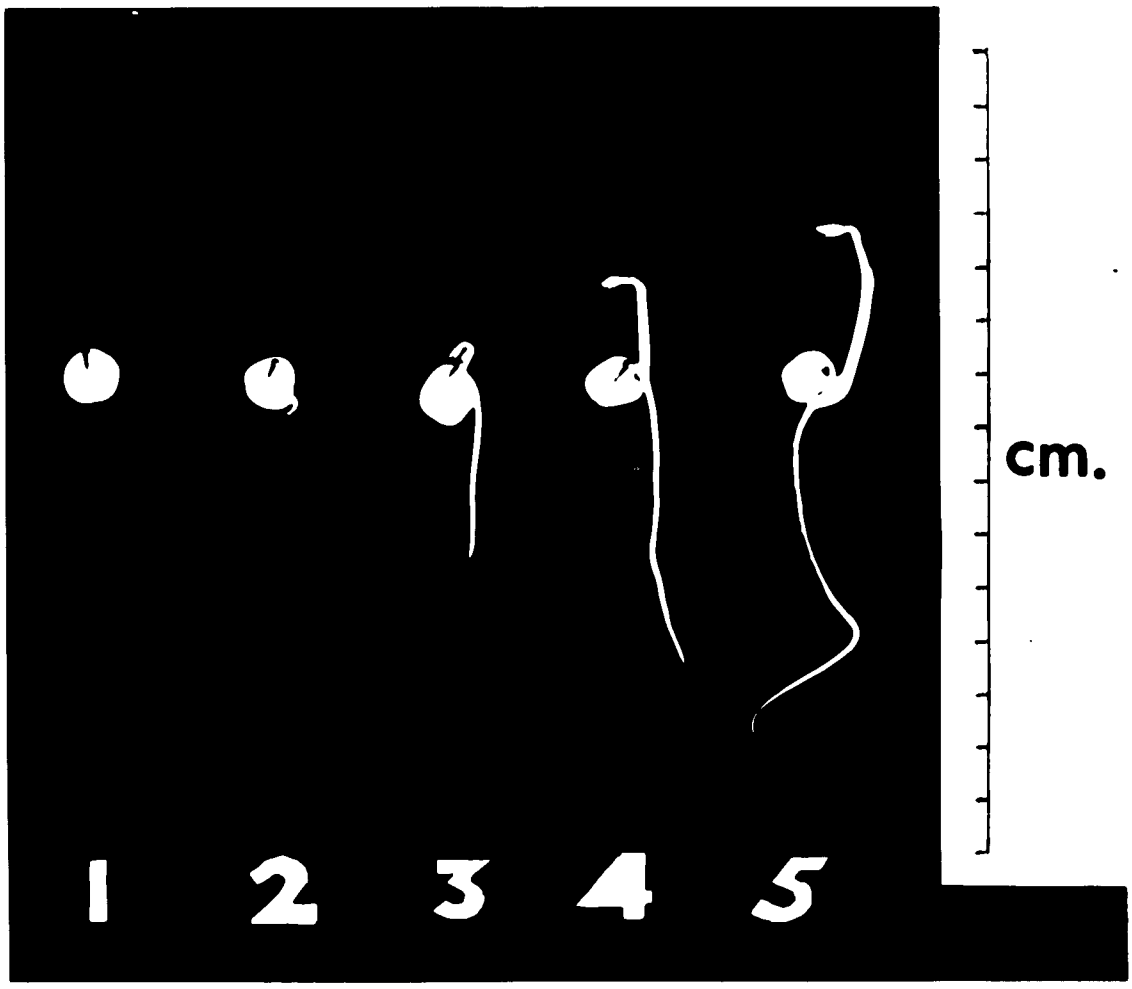
1 gm samples of root tips, 1 cm long and from three day old peas, were incubated at 30°C in Erlenmeyer flasks of 60 ml capacity, provided with a centre well and capped with a rubber serum vial cap. In addition to the plant material, each flask contained 2 ml of 0.1 M potassium phosphate buffer (pH 5.5) and DL-ornithine-5-^{14}C (4.0 µc, 0.4 µmole). The centre well of each flask contained 0.5 ml of 20% (w/v) potassium hydroxide solution to absorb carbon dioxide evolved during the experiment. At the end of the experimental period, the potassium hydroxide was removed from the centre well, and the absorbed carbon dioxide converted to barium carbonate, by the addition of 5% (w/v) barium chloride solution. The root tips were washed quickly in distilled water to remove excess isotope. They were then killed by boiling for 5 min in 50 ml of 80% (v/v) ethanol. The roots were next homogenized in the 80% ethanol, using a glass Broeck tissue homogenizer. The homogenate was then centrifuged at 12,000 x g for 15 min at 4°C in a Sorvall refrigerated centrifuge (Ivan Sorvall

PLATE 1

Stages of germination of imbibed pea seeds.

Pea seeds which had been allowed to imbibe water for 1 day, were planted in moist vermiculite, and incubated in darkness at 28° for the length of time indicated(Days).


1 2 3 4 5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Inc., Norwalk, Connecticut, U.S.A.). The sedimented material was then washed with 50 ml of 50% (v/v) ethanol, followed by 80 ml of distilled water, and the centrifugation repeated. The supernatants from these steps were combined, and dried *in vacuo* at 40°C using a flash evaporator (Buchler Instruments, Fortlee, New Jersey, U.S.A.).

Lipids were extracted from the dried material by washing with three consecutive 10 ml aliquots of anhydrous diethyl ether. The ether washings were combined, and then extracted in a separatory funnel with three consecutive 10 ml aliquots of distilled water. The combined aqueous layers from the latter extraction were then used to dissolve the ether insoluble material remaining in the evaporating flask. The solution obtained was now subjected to ion exchange chromatography accor ding to the method of Canvin and Beevers (1961). This resulted in separation of the extracts into four fractions; a) organic acids, b) sugars, c) neutral and basic amino acids, and d) acidic amino acids.

2. Pulse feeding of DL-ornithine-^{14}C to pea root tips

Two hundred root tips were excised from 3 day old pea seedlings and placed in 30 ml of 0.1 M potassium phosphate buffer (pH 5.6), at 4°C in a 250 ml beaker. DL-ornithine-5-^{14}C (10 µc, 1.0 µmole, in 0.1 ml of the phosphate buffer) was added and the tissue incubated at 30°C ın a Dubnoff shaker for 3 hr. At the end of this time, the root tips were quickly separated from the labelled medium by suction, using a Buchner funnel. They were then quickly washed in 0.1% (w/v) ornithine

solution and finally in distilled water. Thirty root tips were taken at this stage for a zero time sample, these were killed and the amino acids extracted by treatment with boiling 80% (v/v) ethanol. The remaining root tips were quickly transferred in 50 ml of 0.1 M potassium phosphate buffer (pH 5.6) contained in a large glass funnel (350 ml vol) having a sintered glass plate fused in its base. Moist CO_2-free air was now forced through the sintered glass disk and thus the root tips were gently aerated and kept in suspension. Samples of 30 root tips were collected at 30 min intervals, over a period of 3 hr. Carbon dioxide was collected by passing the air stream from the roots through 2N potassium hydroxide followed by precipitation with barium chloride. A fresh sample of potassium hydroxide was used every 30 min. The root tips were killed as described above and the amino acids extracted in 80% (v/v) ethanol followed by 50% (v/v) ethanol and water as already described for the previous experiments.

3. Feeding of L-citrulline-^{14}C to pea cotyledons

In order to feed L-citrulline-carbamyl-^{14}C to pea cotyledons, samples of 15 dry peas were first surface sterilized with Captan compound (Orthoagricultural Chemicals, Vancouver). The seeds were then washed in sterile distilled water, and placed in a small petri dish containing 2 ml of sterile distilled water and L-citrulline-^{14}C (1 µc, 0.035 µmole). After approximately 60 min, a further 2 ml of sterile water were added. One lot of peas was sampled after six hours,

5 ml of water being added to the other samples which were incubated for a further 18 hr. At the end of the experimental period, the testas and embryos were removed, the cotyledons rinsed with sterile distilled water and blotted dry on filter paper. They were then ground in a chilled mortar and pestle with 50 ml of cold (4°C) 80% (v/v) ethanol, containing 2% (v/v) of concentrated ammonia (Bieleski and Turner, 1967). They were finally completely homogenized in a glass Broeck tissue homogenizer, the homogenate was then spun at 12,000 x g in the Sorvall centrifuge at 4°C. The insoluble residue was washed with 50 ml of 50% (v/v) alcohol containing 2% (v/v) concentrated ammonia and the suspension again spun at 12,000 x g in the Sorvall centrifuge. The supernatant fractions were combined, reduced to dryness using a flash evaporator and then extracted with ether as previously described. The ether insoluble material was dissolved in water, and then fractionated by means of ion exchange resin into amino acids and organic acids (Canvin and Beevers, 1961).

4. Feeding of DL-Arginine-^{14}C to pea cotyledon slices

The testas and embryos were excised from 1 day old peas and the cotyledons sliced as thinly as possible with a scalpel blade. The slices were placed immediately into a beaker of ice cold 0.1 M potassium phosphate buffer (pH 5.5), and washed thoroughly. The slices were finally blotted dry with filter paper, and duplicate samples of 1 g were placed in a Warburg flask. Two millilitres of 0.1 M potassium phosphate buffer (pH 5.5), were added to the main compartment,

and 0.4 ml of 20 (v/v), potassium hydroxide added to the centre well for the collection of CO_2. The flasks were fitted with ground glass stoppers which were greased lightly with silicone grease, and left to equilibrate for 10 min at 30°C in a Dubnoff shaker. At the end of this time, DL-arginine-5-^{14}C (5 µc, 1.88 µmole, in 0.1 ml of the phosphate buffer), was added to each flask, and the flasks then incubated at 30°C for a further 3 hr. At the end of this time, the cotyledon slices were removed and washed with 0.1 M phosphate buffer (pH 5.5), and the amino acids extracted in ammoniacal alcohol as previously described. The potassium hydroxide was also removed and absorbed carbonate precipitated with barium chloride.

5. Feeding of DL-Arginine-^{14}C to pea leaf disks

Pea plants were grown at 25°C in a growth chamber under 14 hr light per day for 14 days by which time they had acquired approximately 6 leaves. Four samples of 30 leaf disks (5 mm diameter) were then prepared using a sharp cork borer. The disks were placed in Warburg flasks and 2 ml of 0.1 M phosphate buffer (pH 5.5), added to the main compartment. Two of the flasks were covered with aluminium foil to exclude light. The flasks were then sealed with rubber serum bottle caps. The flasks were placed on a glass plate held approximately 6 inches from a battery of 6 GE 75 watt photoflood lights. A water filter was interposed between the experimental flasks and the lights to minimize heat transfer to the samples. Under these conditions the flasks received a light intensity

of 2,500 foot candles. The flasks were then allowed to equilibrate for 10 min and DL-arginine-5-^{14}C (5 μc, 1.88 μmoles, in 0.1 ml of the phosphate buffer), added to each. The flasks were then illuminated for a total period of 3 hr, during which time the temperature on the glass plate did not rise above 27°C. At the end of this time the experiments were terminated by washing the disks in a cold 0.1% (w/v) arginine solution followed by killing and extraction of the amino acids in alcohol as previously described.

Hydrolysis of the insoluble residue

The insoluble residue obtained after extraction of plant tissues with ethanol and water was routinely hydrolyzed with hydrochloric acid. The hydrolysis was accomplished by heating the residue in 10 ml of 6N hydrochloric acid for 18 hr at 100°C (Greenberg and Rothstein, 1957). At the end of this time the hydrolyzate was filtered through a glass fibre disk, and the filtrate taken to dryness at 40°C using a flash evaporator. Any remaining traces of hydrochloric acid were removed by blowing a stream of air into the flask. The sample was then dissolved in 10 ml of distilled water, neutralized by the addition of ammonium hydroxide solution, and passed through a column of Dowex resin in the hydrogen form as previously described.

Thin layer chromatography of amino acid mixtures

Amino acids resulting from the hydrolysis of the insoluble residues and present in the alcohol soluble fraction

were separated by two dimensional thin-layer chromatography. The supporting medium was prepared from a mixture of 12 g Avicel (microcrystalline cellulose) plus 5 g silica gel H (E. Merckag, Germany). This mixture was homogenized in 110 ml of distilled water for 1 min, using a Virtis homogenizer. It was then spread 0.33 mm thick on 20 x 20 cm glass plates, using a Desager thin-layer spreader (Canadian Laboratory Supplies, Edmonton). The plates were dried at 80-100°C for 6 hr, and afterwards stored in a dessicator. The solvent system used for the first dimension was propan-1-ol:water: propyl acetate:acetic acid:pyridine (120:60:20:4:1 by volume). Two successive runs of 4 hr duration were carried out in this solvent. The solvent system for the second dimension was phenol:water (91:9 v/v), the duration of this run being 6 hr. One dimensional chromatography in butan-1-ol:acetic acid: water (5:1:4 by volume), and phenol water was also carried out using silica gel thin layers.

Thin layer electrophoresis of ornithine cycle amino acids

Enzyme incubation mixtures containing arginine, citrulline, argininosuccinic acid, and ornithine, were separated by electrophoresis at pH 7.0 in 0.05 M sodium borate/ 0.05 M succinic acid. Between 5 and 20 µl of deproteinized sample were applied to sheets of silica gel G thin layer (Eastman Kodak, Rochester, N.Y., U.S.A.) and subjected to electrophoresis in a Gelman deluxe electrophoresis tank (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.) using a potential difference of 500 volts for a period of 80 min. The buffer was cooled to 4° initially and the lid of the tank

covered with a sheet of styrofoam to reduce condensation inside the tank. The degree of separation of the ornithine cycle amino acids obtained using this method is shown in Plate 2. Under the conditions given above, ornithine, arginine, and citrulline migrated towards the cathode, the distances moved relative to the origin being 5.8 cm, 4 cm, and 1.5 cm respectively. Argininosuccinic acid moved towards the anode, for a distance of 1.3 cm relative to the origin.

Detection of Amino Acids

After separation the amino acids were detected by spraying the thin layer plates with 0.3% (w/v) ninhydrin in butan-1-ol containing 3% acetic acid (v/v). After incubating at 80°C for 30 min, the colour obtained was rendered permanent by spraying with 5% (w/v) nickel sulphate solution. Labelled amino acids were detected on the thin layer plates by autoradiography. Samples for autoradiography were placed in contact with Kodak NO-Screen X-ray film in a light tight cassette, and exposed for periods of up to one week. The exposed films were subsequently developed using G.E. Supermix X-ray developer and fixer (General Electric X-ray Co., Edmonton).

Counting of Radioactive Samples

In early feeding experiments samples obtained from the ion exchange columns were assayed for ^{14}C by plating duplicate aliquots (0.1 ml) on to stainless steel planchets and counting in a Nuclear Chicago gas-flow counter (model number C110B). The counting efficiency was approximately

PLATE 2

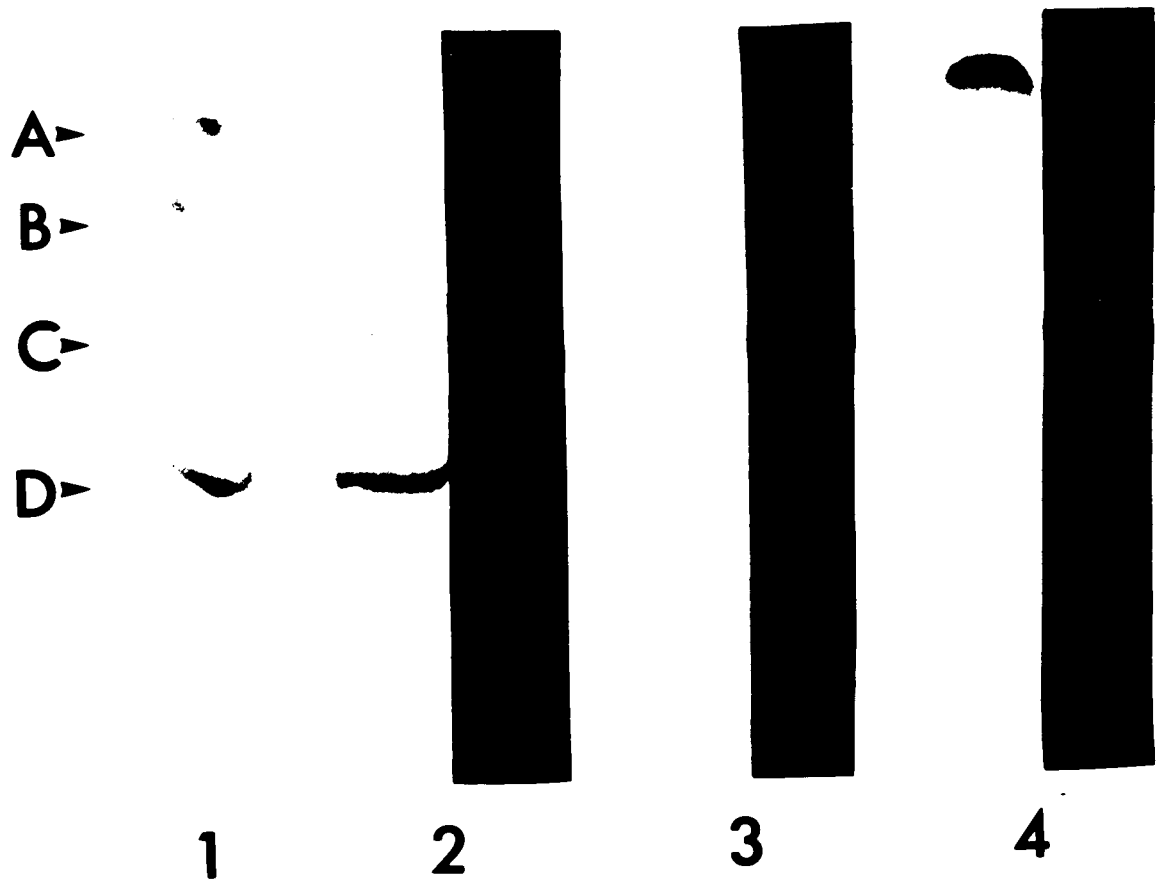
Separation of ornithine cycle intermediates by electrophoresis in borate succinate buffer (pH 7.0).

1. Mixture of standard amino acids: -
   - A: - ornithine
   - B: - arginine
   - C: - citrulline
   - D: - argininosuccinate
2. Deproteinized incubation mixture , in which there has been approximately 50% conversion of citrulline to arginine, over the reaction period of 1 hour. Argininosuccinate was added to this aliquot prior to electrophoresis to act as a reference compound.

3 & 4. Aliquots of the same incubation mixture but with arginine added to 3, and ornithine added to 4.

The autoradiograms of separations 2, 3 and 4 are included beside the corresponding thin layer strips for comparison.

A
B
C
D


A
B
C
D
1
2
3
4

20%. In all other experiments involving ^{14}C-labelled compounds, radioactivity was assayed in a liquid scintillation counter (Nuclear Chicago Corp., Unilux II model). Labelled areas of thin layer chromatograms prepared on glass plates were carefully scraped and the thin layer material sucked into scintillation vials. The vials were filled to the neck with Cabosil (finely divided silica purchased from Henry Cabot Co., U.S.A.) and 15 ml of a toluene fluor added. The fluor contained 1,4-bis[2-(5-phenyloxazolyl)] (POPOP) 0.05 g; and 2,5-diphenyloxazole (PPO) 5.0 g; in a litre of toluene. The vials were shaken vigorously for a few seconds to mix the contents, and were then counted under predetermined balance point conditions. For electropherograms prepared on the plastic backed silica gel thin layers, the areas corresponding to radioactive amino acids were cut out and counted directly in 15 ml of the toluene fluor. The efficiency of this method using a range of dilutions prepared from a sample of arginine-C^{14} was found to be 65%. Experiments were performed to compare the activity of equal amounts of isotope placed on pieces of thin layer, which varied in size. It was found that the activity obtained did not vary with the area of thin layer over which the samples were spread.

The cabosil method was investigated with regard to the possibility of variations in the efficiency of counting individual samples, when varying amounts of thin layer material were present, or when very high count rates were

being recorded. It was found that varying amounts of thin layer material (silica gel G) did not lead to varying counts when using a constant amount of isotope. When the channels ratio method was used to check the possible quenching of samples, with varying levels of radioactivity (4,400 - 176,000 c.p.m.) it was found that a constant channels ratio was obtained. This indicated that the degree of quenching obtained was constant from sample to sample. Using a series of dilutions prepared from a sample of arginine-C^{14}, the efficiency of the method was found to be 62%. Routinely, four counts of ten minutes duration each were obtained for each sample. The four counts were averaged and corrected for background.

Extraction of Argininosuccinate lyase from pea cotyledons

The testas and radicles were removed from germinating pea seeds at the required stage of germination, to give 200 g of cotyledons. These were homogenized with 600 ml of 0.0025 M potassium phosphate buffer (pH 7.9) in a Waring Blendor at 4°C. The blendor was operated at half speed for 40 sec, followed by full speed for a further 40 sec. The homogenate was stirred with a magnetic stirrer for 15 min at 4°C and then passed through fine muslin to remove cellular debris. After this step, the homogenate was centrifuged at 14,600 x g for 20 min in a Sorvall refrigerated centrifuge operated at 4°C by immersing in a bath of crushed ice and the pH adjusted to 5.1 with the addition of cold (4°C) 1 M acetic acid. Protein precipitated by this treatment was removed by

a further centrifugation at 14,600 x g for 20 min at 4°C. The supernatant from this step was again cooled to 4°C and the pH lowered to 4.2 by further additions of 1 M acetic acid. Protein precipitated by this treatment contained appreciable levels of argininosuccinate lyase activity, and was collected by centrifugation at 20,000 x g for 20 min at 4°C. This protein was then washed by stirring in 100 ml of 0.01 M potassium acetate buffer (pH 4.2) and collected by centrifugation. The protein pellet was then suspended in 20-30 ml of 0.1 M potassium phosphate buffer (pH 7.9) and dialyzed against this buffer for 18 hr at 4°C. Protein not in solution after dialysis was removed by centrifugation, and discarded.

Partial Purification of Argininosuccinate lyase

1. Treatment with Calcium phosphate gel

The enzyme from the isoelectric precipitation step was now treated with calcium phosphate gel. The gel was prepared as described by Keilin and Hartree (1938). Prior to gel treatment, the enzyme extract was dialyzed against 0.02 M potassium phosphate buffer (pH 7.9) for 18 hr at 4°C. The gel was first washed with 0.02 M potassium phosphate buffer (pH 7.9) and then mixed with the enzyme. Enzyme protein was readily absorbed by the gel when the protein/gel (w/w) ratio was 0.024. After standing at 4°C for 30 min the mixture was centrifuged at 15,000 x g at 4°C in the Sorvall Centrifuge. Enzyme activity was now eluted from the gel by successive washings with 0.1 M and 0.15 M phosphate

buffer at pH 7.9. In each case the gel-buffer mixture was allowed to stand for 30 min at 4°C, with intermittent stirring. The mixtures were then centrifuged to recover supernatants containing enzyme activity.

2. Treatment with carboxymethyl (C.M.) cellulose

The C.M. cellulose was washed three times with distilled water, and allowed to settle between each wash and the fine particles removed by suction. After washing with 0.5 M potassium phosphate buffer (pH 6.15) for 2 hr, the cellulose was sucked dry on a sintered glass funnel and suspended in 0.005 M potassium phosphate buffer, (pH 6.15). It was now packed into a jacketed glass column to give a bed of 3 x 25 cm. This was equilibrated with 0.005 M phosphate buffer, (pH 6.15) by washing with 10-15 litres of this buffer until the pH was 6.15. Prior to column chromatography the gel-treated enzyme was dialyzed against the above buffer for 18 hr at 2°C. Approximately 170 mg of protein was then applied to the column, this was followed by elution with 0.005 M potassium phosphate buffer (pH 6.15). Elution was carried out at a rate of 1.7 ml/min with fractions of 5 ml being collected at 4°C using a Buchler fraction collector. Argininosuccinate lyase activity was located in fractions 17-23.

Assay of enzyme activity

Argininosuccinate lyase activity was assayed spectrophotometrically using argininosuccinic acid from which the barium had been removed by the addition of potassium

sulphate (Havir, *et al* 1965). The production of fumarate from argininosuccinate was followed continuously by measuring changes in absorbance at 240 mμ. The assay was carried out in quartz cuvettes with a 1 cm light path. The reaction system contained the following in a total volume of 3 ml; argininosuccinate, 1 μmole; 0.01 - 0.02 units of enzyme activity; and potassium phosphate buffer (pH 7.9) 260 μmoles. The reference cuvette contained all of these components with the exception of argininosuccinate which was replaced by distilled water. The cuvettes were preincubated at 37°C for 20 min followed by initiation of the reaction by addition of argininosuccinate. Changes in absorbance at 240 mμ were measured in a Beckman double beam spectrophotometer (model DB-G, Beckman Instruments, Fullerton, California) equipped with a 10-inch potentiometric recorder. The cuvette compartment was maintained at 37°C throughout the reaction. Initial reaction velocities were calculated from the tangent drawn to the time course curve. The amounts of fumarate produced were calculated using the millimolar extinction coefficient for fumarate as 2.44 (Alberty, Massey and Frieden, 1954). This figure was checked using a standard solution of fumaric acid. One unit of enzyme activity is defined as the amount of enzyme catalyzing the production of 1 μmole of fumaric acid per min under the defined experimental conditions.

Identification of reaction products and reversibility

Fumarate was identified as a reaction product by its

extinction at 240 mμ and by demonstrating ability of this production to act as a substrate for purified fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2). Arginine and argininosuccinate were both identified by comparing their mobilities on paper chromatograms with those of the authentic compounds. Arginine had an R_f of 0.87 and argininosuccinate of 0.22. Descending paper chromatograms (Whatman No. 1) were developed for 8 hr in phenol-water (91:9 v/v) containing 0.4% (w/v) 8-hydroxyquinoline. An atmosphere of NH_3 was provided during development by placing 100 ml of 0.02 N NH_4OH solution in the bottom of the chromatographic chamber. Arginine and argininosuccinic acid were detected on the chromatograms by spraying with the ninhydrin reagent described previously.

In studies of the reversibility of the reaction, the production or disappearance of arginine and argininosuccinate was followed using the chromatographic method described above. In these experiments the reaction system contained in a total volume of 0.5 ml:enzyme,0.033 units; arginine and fumarate or argininosuccinate 1 or 10 μmoles, and 20 μmoles of phosphate buffer (pH 7.9). Incubations were carried out at 37°C for periods up to 1 hr and then terminated by addition of 0.5 ml of absolute ethanol at -20°C. The precipitated protein was removed by centrifugation, washed three times with 1 ml of distilled water and the combined supernatants dried in a stream of cold air at 20°C. The residue from this evaporation was then dissolved in 0.5 ml of distilled water and aliquots of 5-50 μl were applied to paper chromatograms. The chromatograms were developed as described above, dried at room tempera-

ture in a fume hood and after spraying with the ninhydrin reagent were stored for 48 hr in darkness at room temperature. The ninhydrin positive areas were then cut from the chromatograms together with suitable paper blanks taken from the same level of the chromatograms as the samples. These were then eluted with 5 ml of 50% (v/v) aqueous ethanol for 45 min, during which time the tubes were shaken occasionally. After centrifuging at 20,000 x g the extinction of the supernatants was measured at 570 mμ in a Beckman DB-G spectrophotometer. Standard curves were constructed for each assay using known quantities of arginine and argininosuccinate which had been subjected to the chromatographic procedure.

Extraction of Argininosuccinate synthetase from pea cotyledons.

Samples of the cotyledons (200 g) from 1 day old peas were homogenized with 600 ml of 0.01 M potassium phosphate buffer (pH 8.0) in a Waring Blendor at 4°C. The blendor was operated at half speed for 30 sec followed by full speed for a further 30 sec. The homogenate was stirred at 4°C with a magnetic stirrer for 20 min, and then passed through fine muslin to remove cellular debris. The homogenate was now centrifuged at 14,600 x g for 20 min, in a Sorvall centrifuge operated at 4°C. The supernatant was cooled to approximately 0°C in an ice bath, and stirred with a magnetic stirrer. Solid ammonium sulphate was added to 30% of saturation and the suspension obtained stirred for 30 min in the cold. At the end of this time, the suspension was centrifuged at 14,600 x g for 20 min at 4°C.

The supernatant from this step was again cooled to approximately 0°C, and more solid ammonium sulphate added to give 50% of saturation. After stirring for 30 min the suspension was centrifuged at 14,600 x g for 20 min at 4°C. This precipitated protein was dissolved in 0.1 M Tris acetate buffer (pH 8.0) at 4°C, to give 25-30 ml of enzyme solution. This was now divided in half and each half desalted by passage through a column of Sephadex G-50, measuring 35 x 1.5 cm. The column had previously been equilibrated with 0.1 M Tris acetate buffer (pH 8.0) at 4°C. Enzyme protein was eluted with the same buffer, and collected in 5 ml fractions at 4°C, using an L.K.B. ultrorac fraction collector (Fisher Scientific Co., Edmonton).

Partial purification of argininosuccinate synthetase with Sephadex G-200

A column of Sephadex G-200 measuring 3.5 x 50 cm, was equilibrated with 0.1 M potassium phosphate buffer (pH 8.0) at 2°C. Protein precipated by ammonium sulphate treatment (30-50% of saturation) was desalted by passage through a column of Sephadex G-50 that had been equilibrated with 0.1 M potassium phosphate buffer (pH 8.0). Enzyme protein (870 mg) from the Sephadex G-50 column was now applied to the column of Sephadex G-200. Elution was carried out with this buffer at the rate of 30 ml per hr, with fractions of 5 ml being collected at 2°C. Argininosuccinate synthetase activity was located in fractions 20-30.

Assay of enzyme activity

Argininosuccinate synthetase was assayed at 30°C using L-citrulline-carbamyl-^{14}C. The reaction system contained the following, in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C,2.0 µc (0.07 µmole), L-citrulline 2.0 µmoles, L-aspartic acid, 2 µmoles, ATP, 3 µmoles, magnesium 5 µmoles, 100 µmoles of Tris-acetate buffer (pH 8.0) and 0.3 ml of enzyme equivalent to form $8 \times 10^{-4} - 6 \times 10^{-3}$ units of enzyme activity. One unit of enzyme activity is defined as the amount of enzyme catalyzing the production of 1 µmole of arginine per min under the defined experimental conditions. The mixtures were incubated at 30°C for 1 hr, and the reaction then terminated by the addition of 1 ml of cold (-20°C) absolute ethanol. The precipitated protein was removed by centrifugation and the supernatant reduced to 1 ml by evaporation in a stream of cold air.

Routinely aliquots (5 µl) of the deproteinized reaction mixtures were now applied to 1 inch wide strips of plastic backed silica gel G thin layer. The sample was applied as a band exactly half way along the length of the strip, and the amino acids now separated using electrophoresis in borate-succinate buffer as previously described, and shown in Plate 2. After electrophoresis, the strips were dried in warm air and then subjected to autoradiography for 3 days, as previously described. At the end of this time, areas of the thin layer strips which corresponded to labelled arginine, citrulline, and argininosuccinate

respectively, were cut out and the radioactivities determined. Control experiments were always included in which the enzyme was omitted and buffer substituted in its place. These samples were then subjected to the incubation,deproteinization and filming procedures. Low levels of radioactivity found to correspond to arginine or argininosuccinate were estimated and subtracted from the values obtained for these compounds in the test samples containing enzyme.

Identification of reaction products

Arginine was identified by its electrophoretic and chromatographic mobilities, and also by its ability to act as a substrate for purified arginase. The arginase was dissolved in 0.05 M manganese-malate buffer (pH 7.4), to give a final concentration of 1 mg per ml (Greenberg, 1955). This solution was then incubated for 3-4 hr at 37°C. An aliquot (0.5 ml) was then added to 0.5 ml of a deproteinized incubation mixture, (pH 9.5), suspected to contain labelled arginine. This solution was now incubated for 1 hr at 37°C. The reaction was then terminated by addition of absolute ethanol and the denatured protein removed by centrifugation. The volume of the supernatant was reduced to 1 ml, and 10 µl aliquots taken for analysis. These aliquots were applied to silica gel G thin layer sheet, and chromatography carried out in butan-1-ol; acetic acid; water; (5:1:4 by volume), for 3 hr. At the end of this time the chromatograms were removed, and allowed to dry overnight

at room temperature in a fume hood. The urea-^{14}C produced was located by autoradiography for a period of 5 days, and its radioactivity then determined as previously described. This product was identified by co-chromatography with authentic urea, and was located on chromatograms using Erhlich's reagent (3% p-dimethyl-aminobenzaldehyde in 95% (v/v) ethanol). After spraying with Erhlich's reagent, colour development was achieved by heating at 80°C for 15 min and subsequent exposure to HCl vapour. The value for urea in this solvent system was 0.56. Ornithine, citrulline, arginine and argininosuccinate, were not separated under these conditions, running as a common area with an R_f value of 0.19.

Reaction mixtures were prepared as already described for the assay of synthetase activity, with the inclusion of 2 μc of L-aspartate-U-^{14}C (0.23 μm). In these experiments the added citrulline was not labelled. The reaction was terminated and deproteinized with absolute ethanol as described before. After concentration to 1 ml, aliquots of 20 μl were then applied to thin layer sheets of silica gel G, together with authentic malic and fumaric acids. These were then chromatographed in an ether-formic acid mixture (7:2, v/v), for a period of 1 hr. At the end of this time, the chromatogram was left to dry in a fume hood for 16 hr, and then subjected to autoradiography. The

areas of thin layer corresponding to labelled malate, fumarate and aspartate were cut out, and the radioactivity determined in the scintillation counter. Malate-^{14}C and fumarate-^{14}C were identified by co-chromatography with the authentic compounds. These acids were located on the chromatograms by spraying with aniline xylose reagent (Block, Durrum & Zweig, 1955). Under these conditions malate, fumarate and aspartate were well separated having R_f values of 0.96, 0.66 and 0.44 respectively.

Estimation of protein content of enzyme preparations.

The protein content of enzyme preparations was estimated colorimetrically as described by Lowry, Rosebrough, Farr and Randall (1951). Crystalline egg albumin was used as a reference standard.

RESULTS - Part I

Isotope feeding experiments.

In preliminary experiments, various pea tissues were examined for their ability to utilize arginine, citrulline, and ornithine, under different experimental conditions. Labelled amino acids were supplied in micromolar quantities and after periods of incubation the tissues were extracted and examined for labelled products.

1. Feeding DL-ornithine-5-^{14}C to pea root tips.

This experiment was carried out by incubating 1 g quantities of root tips from 3 day old pea seedlings, with DL-ornithine-^{14}C, in 0.1 M potassium phosphate buffer (pH 5.5). The material was contained in Warburg flasks which were incubated at 30°C, for periods of time ranging from 1.5 to 12 hr. Table 1 shows that a considerable amount of the ornithine-^{14}C that entered the soluble fraction of the root tips, was metabolized to yield citrulline and arginine. The radioactivity in the arginine of the soluble fraction reached a maximum value of 18%, in the 5.5 hr experiment. The arginine-^{14}C content of the protein fraction was also clearly increasing slowly with the time of incubation. The highest amount of radioactivity found in citrulline, was 18% from the 1.5 hr experiment. In all subsequent experiments, the amount of ^{14}C present in citrulline was seen to fall below this figure. Large amounts of ^{14}C entered aspartate, glutamate, and carbon dioxide. The introduction of ^{14}C from the 5 position of ornithine into glutamate and eventually into the Krebs cycle, presumably occurred as a result of the action of ornithine δ-transaminase (Fig. 2).

TABLE 1

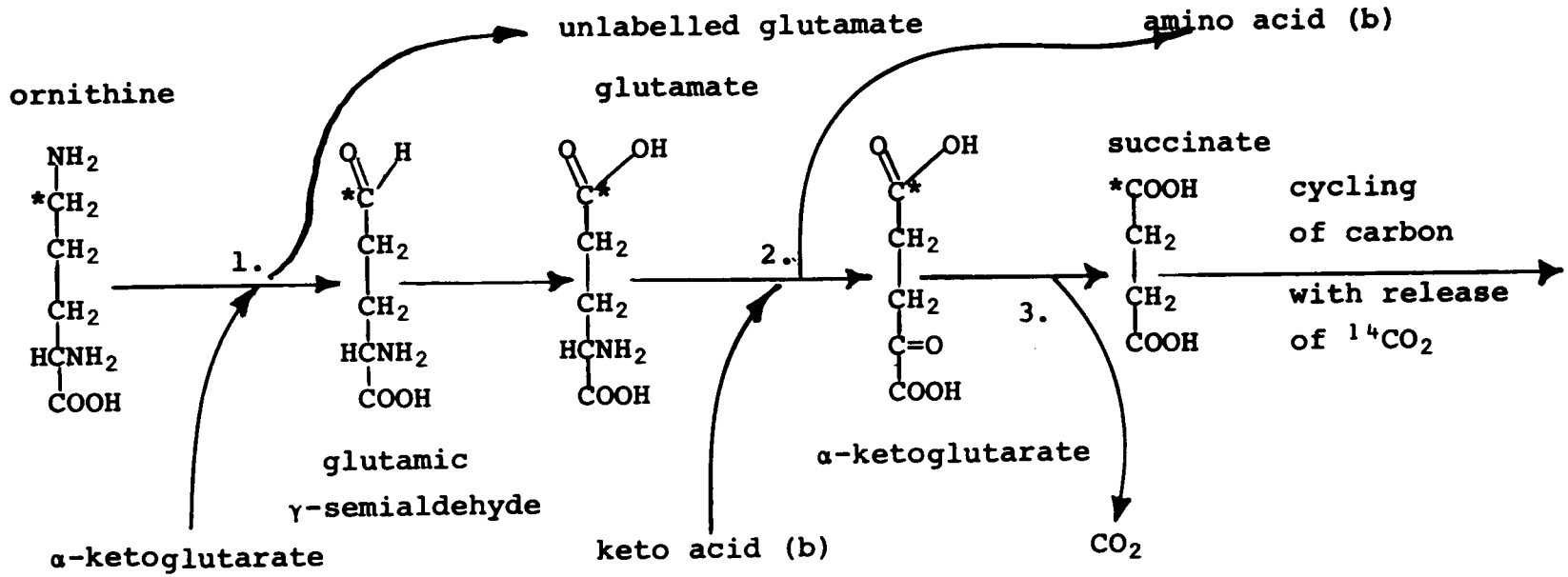
Radioactivity in the soluble and insoluble fractions isolated from pea root tips supplied with DL-ornithine-5-^{14}C.*

Incubation Time (hr)	% of recovered ^{14}C				
	1.5	3	5.5	8	12
Fraction					
Lipids	8.5	9.0	3.2	2.7	3.0
Organic acids	3.5	3.8	1.8	2.1	1.4
Sugars	2.1	2.7	1.5	2.4	2.3
Free amino acids					
ornithine	37.8	23.5	38.5	36.2	42.0
arginine	12.4	10.5	18.0	15.5	17.5
citrulline	18.1	12.5	6.3	6.5	9.8
alanine	nil	nil	10.7	6.5	nil
aspartic + glutamic	10.2	8.2	8.0	6.2	3.1
Protein amino acids					
arginine	nil	0.8	3.3	3.4	4.0
alanine	1.3	4.4	0.7	1.2	1.3
aspartic + glutamic	3.0	3.3	0.8	0.6	0.5
Carbon dioxide	3.2	21.3	7.2	16.7	15.1
Total ^{14}C recovered from tissues (cpm)	218,100	282,300	774,000	842,300	824,400

* Samples of pea root tips (1g fresh weight) were incubated with 4.0 µc (0.4 µmoles) of DL-ornithine in 2 ml 0.1 M potassium phosphate buffer (pH 5.5) at 30°C, for the time periods indicated.

FIGURE 2

Partial reactions involved in the production of labelled glutamate and CO_2 from Ornithine-5-^{14}C.



1. Ornithine-δ-transaminase.
2. An amino acid transaminase.
3. α-ketoglutarate decarboxylase.
* position of labelled carbon atom.

On introduction of ^{14}C into the Krebs cycle as shown in Fig. 2, labelled CO_2 would be quickly released. No label was detected in glutamine or asparagine during this experiment.

2. Pulse feeding of DL-ornithine-5-^{14}C to pea root tips.

Root tips from 3 day old peas were allowed to metabolize labelled ornithine for 3 hr at 30°C. After this they were transferred to unlabelled medium, for a further 3 hr period. The levels of the various ^{14}C labelled amino acids were determined in samples of the root tips taken every 30 min. In this way it was hoped to establish which of the amino acids labelled during the 3 hr feeding period, were subject to the highest rate of metabolic turnover, under the experimental conditions. The data given in Table 2, and Fig. 3 showed that the level of labelled ornithine dropped during the course of the post-pulse period to a value almost half that recorded at the beginning of this period. The level of labelled arginine appeared to drop to almost half the zero time value, by 1.5 hr, but then it appeared to have reached a steady level. Citrulline levels appeared to fluctuate, and after an initial steady decline, finally reached a value almost equivalent to the zero time figure. Labelling in alanine was not detected after 30 min. Radioactivity in glutamate and aspartate also fell steadily, this fact may have been coupled with the steady evolution of $^{14}CO_2$ from the root tips (Table 2). The levels of ^{14}C in the other labelled fractions did not fluctuate appreciably during the post-pulse period. In the

FIGURE 3

Activity in the major components of the soluble fractions isolated from root tips pulse-fed with DL-ornithine-^{14}C.

Root tips from 3 day old peas were incubated with 10 μc of DL-ornithine-5-^{14}C for 3 hr. They were then transferred to unlabelled medium as shown by the zero time point on the abscissa. Samples of the root tips were taken for analysis at zero time, and at 30 min intervals, for a total time period of 3 hr.

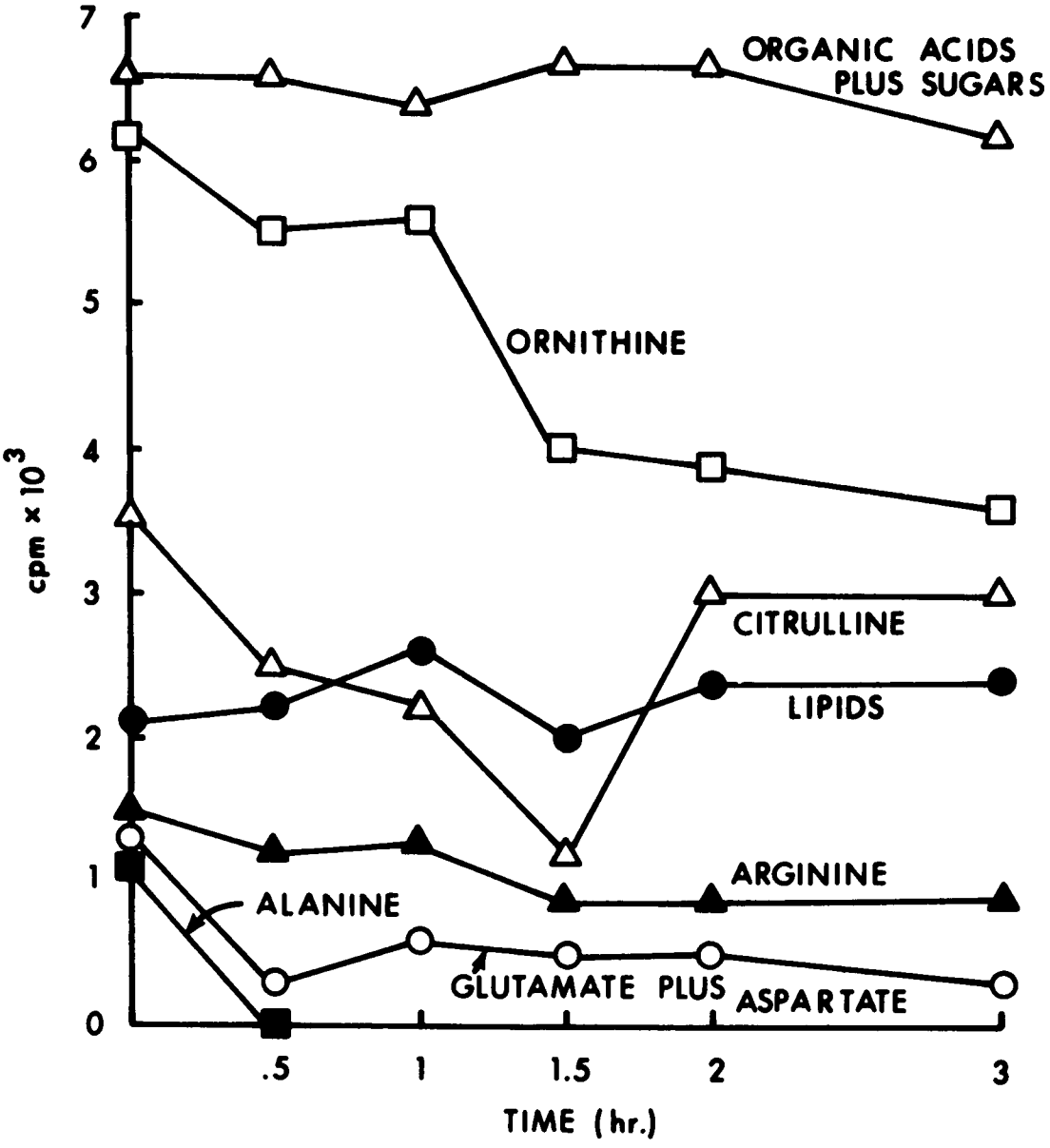

7
6
5
4
3
2
1
0
cpm x 10^3
ORGANIC ACIDS
PLUS SUGARS
ORNITHINE
CITRULLINE
LIPIDS
ARGININE
ALANINE
GLUTAMATE PLUS ASPARTATE
.5
1
1.5
2
3
TIME (hr.)

TABLE 2

Radioactivity in the soluble and insoluble fractions isolated from pea root tips pulse-fed with DL-ornithine-5-^{14}C. *

	Radioactivity in fractions (cpm)					
Incubation time (hr)	0	0.5	1.0	1.5	2	3
Fraction						
Lipids	2,100	2,200	2,600	2,000	2,400	2,400
Organic acid + sugars	6,600	6,600	6,400	6,700	6,700	6,200
Free amino acids						
ornithine	6,200	5,500	5,600	4,000	3,900	3,600
arginine	1,500	1,200	1,300	900	900	900
alanine	1,100	nil	nil	nil	nil	nil
citrulline	3,500	2,500	1,200	3,100	3,000	2,800
aspartic + glutamic acids	1,300	300	600	500	500	300
Protein amino acids						
arginine	600	800	700	600	600	200
alanine	200	300	400	200	300	200
Carbon dioxide	nil	400	600	700	700	800
Total ^{14}C recovered from tissues	23,100	19,800	19,400	18,700	19,000	17,400

* Experimental conditions were the same as described for Figure 3.

Protein amino acids (Fig. 4) the amounts of arginine-^{14}C dropped significantly, while alanine-^{14}C remained virtually constant.

The loss of ^{14}C from the labelled ornithine pool could be partially explained by its entry into other compounds, as a result of metabolic turnover. Thus the increasing levels of ^{14}C in citrulline, and the maintainence of arginine-^{14}C levels after 1 hr, could be accounted for by label obtained from ornithine-^{14}C. Some labelled ornithine must also have been used for the continual formation of labelled glutamate, aspartate, organic acids, and CO_2. However, if the total ^{14}C recovered from each experiment is noted, it can be seen that the values slowly decreased over the 3 hr period. Therefore, labelled compounds must presumably have been lost to the external medium.

3. Feeding of L-citrulline-carbamyl-^{14}C to whole peas.

In these experiments labelled citrulline was supplied to germinating pea seeds *in vivo* in order to assess the importance of this amino acid in arginine biosynthesis. Carbamyl labelled citrulline was chosen from these experiments as diversion of ^{14}C into ornithine and the Krebs' cycle intermediates would be minimal. Although the possibility of bacterial contamination cannot be entirely rulled out, it is clear that the supplied citrulline was extensively converted into arginine (Table 4). When L-citrulline-^{14}C was fed for 6 hr and 24 hr, 4.1% and 55% of the recovered radioactivity was incorporated into arginine-^{14}C respectively. Apart

FIGURE 4

Labelled components of the protein fraction isolated from root tips pulse-fed with DL-ornithine-^{14}C.

Experimental conditions were as described in Figure 3.

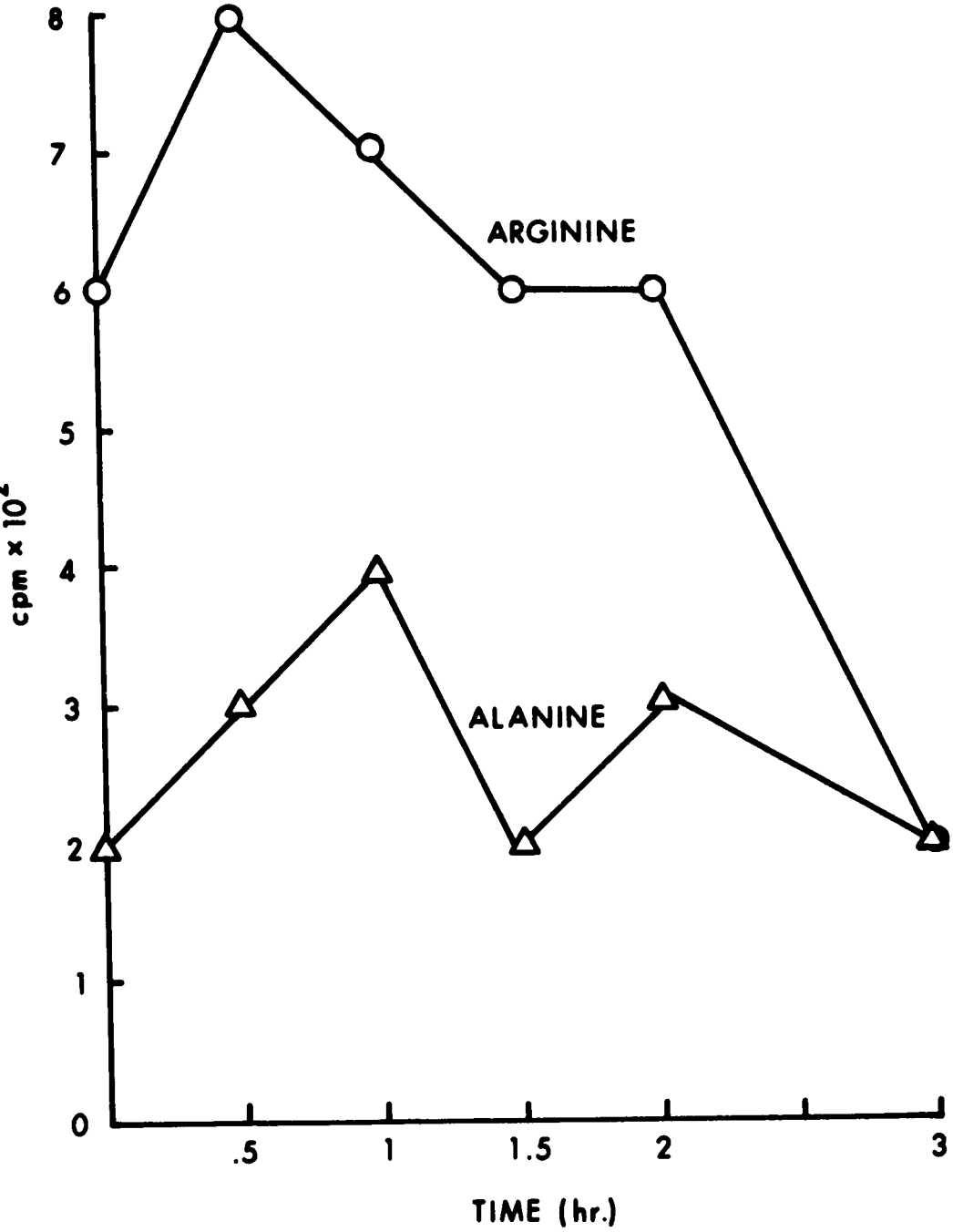

8
7
6
5
4
3
2
1
0
cpm x 10^2
ARGININE
ALANINE
.5
1
1.5
2
3
TIME (hr.)

TABLE 3

Radioactivity in the soluble and insoluble fractions isolated from pea cotyledons supplied with L-citrulline-carbamyl-^{14}C.*

	% of recovered ^{14}C	
Incubation Time (hr)	6	24
Fractions		
Organic acids + Sugars	2.9	1.7
Free amino acids		
arginine	4.1	54.9
citrulline	92.9	31.3
Protein arginine	0.1	12.1
Total ^{14}C incorporated (cpm)	63,854	94,450

* Surface sterilized pea seeds were allowed to imbibe 1 µc (0.035 µmole) of L-citrulline in a total of 9 ml of sterile distilled water at 24°C, for the time periods indicated.

from citrulline-^{14}C, no other labelled amino acid was detected. When the insoluble protein from the 24 hr experiment was hydrolyzed, a single radioactive amino acid was obtained. This was subsequently shown to be arginine-^{14}C.

4. Feeding of DL-arginine-5-^{14}C to pea cotyledon slices.

Before use, the slices were washed thoroughly in distilled water, and then incubated together with the DL-arginine-^{14}C in 0.1 M potassium phosphate buffer (pH 5.5). The material was contained in Warburg flasks, and incubated at 30°C for 3 hr. Duplicate experiments were performed and at the end of 3 hr, the free amino acids and the protein amino acids extracted. In both cases arginine-^{14}C was the only labelled amino acid present (Table 4). Arginine-^{14}C was also shown to be the only amino acid present in the protein hydrolyzate (Table 4).

The results of the L-citrulline-^{14}C and DL-arginine-^{14}C feeding experiments indicate that 1 day old pea cotyledons are active in arginine biosynthesis. However, there does not appear to be any appreciable regeneration of ornithine from the arginine produced.

5. Feeding of DL-arginine-5-^{14}C to pea leaf disks.

Pea leaf disks were collected as described in the Materials and Methods section, and incubated with DL-arginine-^{14}C in 0.1 M potassium phosphate buffer (pH 5.5). Four experiments were carried out, 2 experiments were for a 1 hr period, one being carried out in the light, the other in

TABLE 4

Radioactivity in the soluble and insoluble fractions isolated from cotyledon slices fed with DL-arginine-5-^{14}C. *

	% of Recovered ^{14}C	
	Expt. A.	Expt. B.
Arginine in soluble fraction	97.8	97.1
Arginine in protein fraction	2.2	2.9
Total ^{14}C incorporated (cpm)	679,000	697,000

* Slices (1g fresh weight) of 1 day old pea cotyledons, were incubated with 5 µc (1.88 µmoles) of DL-arginine-5-^{14}C in 2 ml of 0.1 M. potassium phosphate buffer, (pH 5.5) for 3 hr at 30°C.

TABLE 5

Radioactivity in the soluble and insoluble fractions isolated from pea leaf disks supplied with DL-arginine-5-^{14}C. *

Incubation time	% of recovered ^{14}C			
	1 hr		3 hr	
	light	dark	light	dark
Fraction				
Lipids	12.1	11.0	9.7	9.9
Organic acids + sugars	22.7	25.1	25.7	19.6
Free amino acids				
arginine	42.6	48.1	37.8	51.5
glutamine	7.2	1.9	8.6	4.0
proline	0.7	1.1	1.0	1.8
glutamic + aspartic acids	13.0	11.7	15.0	11.9
Protein amino acids				
arginine	1.5	1.1	2.1	0.9
glutamic + aspartic acids	0.2	nil	0.1	0.4
Total ^{14}C recovered from tissues	65,000	63,400	75,300	76,200

* Samples of 30 pea leaf disks (5mm diameter) were supplied with 5 µc (1.88 µmoles) of DL-arginine-5-^{14}C in 2 ml of 0.1 M potassium phosphate buffer (pH 5.5) for the time periods indicated.

darkness. The other two experiments were for a 3 hr period, one being carried out in the light, and the other in darkness. The results of these experiments are shown in Table 5. It is obvious that arginine-^{14}C was metabolized by the disks to some extent, particularly when they were illuminated. The results obtained in this experiment are therefore similar to those obtained by Naylor (1959) using walnut leaves. In the two experiments that were exposed to light, more arginine-^{14}C was utilized. This greater utilization was apparently related to the biosynthesis of glutamate, aspartate and glutamine. Naylor (1959) explained similar results by postulating a conversion of arginine to ornithine which was then rapidly metabolized to glutamic γ-semialdehyde, and then to glutamate.

RESULTS - Part II

Studies of enzymes involved in the biosynthesis of arginine.

The isotope feeding experiments clearly indicated that synthesis of arginine from ornithine and citrulline occurred in various tissues of the pea plant. In further experiments the biochemical nature of these interconversions was examined using tissue extracts which were fractionated to obtain partial purification of the enzyme activity present. In such experiments emphasis was placed on the enzymatic synthesis of arginine from citrulline via argininosuccinate. The enzymes studied were therefore argininosuccinate synthetase and argininosuccinate lyase. Pea cotyledons were chosen as a source of these enzymes for the following reasons. Feeding experiments (Results, Part I) and analytical studies (Lawrence and Grant, 1963, Larson and Beevers, 1965) have demonstrated that arginine is readily synthesized from citrulline, and that the levels of arginine in cotyledons are appreciable during the early stages of germination. Furthermore, these tissues are readily available in quantities necessary for the extraction of enzymes.

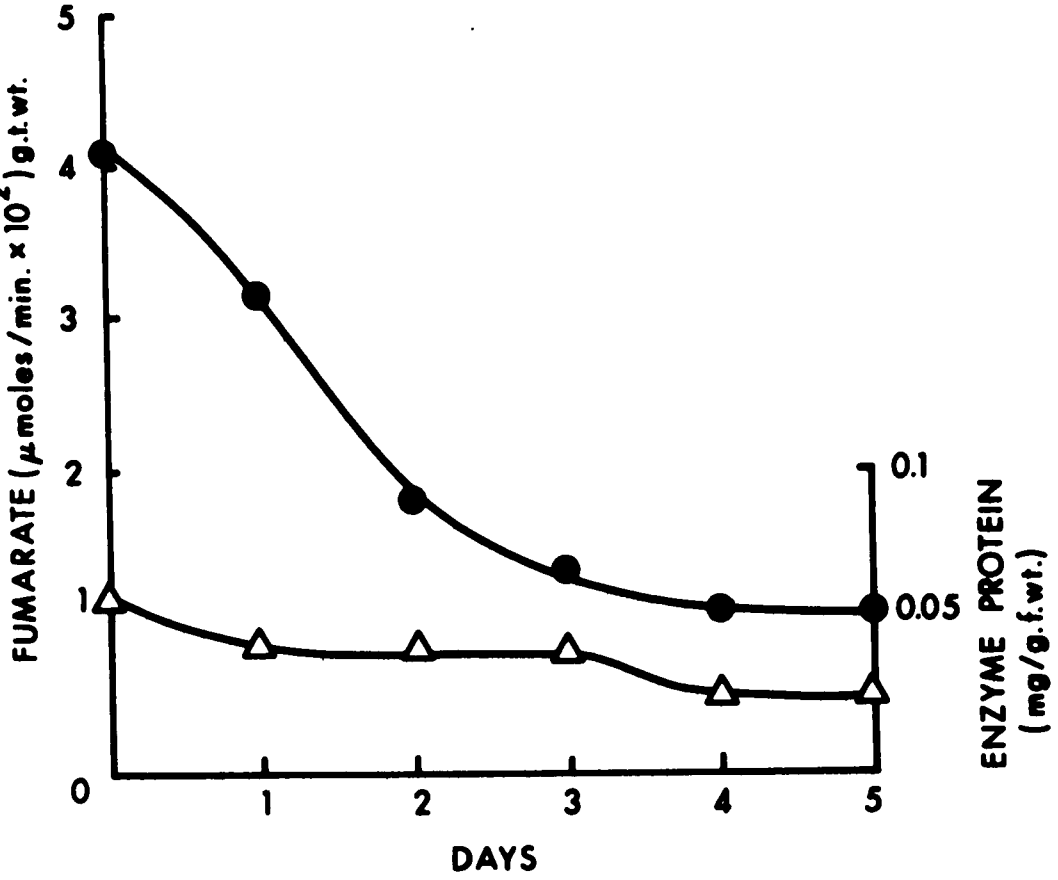
1. Studies of argininosuccinate lyase.

Extracts of 0-5 day old cotyledons were prepared and fractionated as far as the isoelectric stage described in the Materials and Methods section. Lyase activity of these extracts was then assayed, the results are shown in Fig. 5. It can be clearly seen that dry peas and cotyledons from 1 day old peas possessed a greater enzyme activity than cotyledons from peas at a later stage of germination. Since the

FIGURE 5

Changes in argininosuccinate lyase activity and protein content, during the germination of pea seeds.

Enzyme activity in protein obtained by isoelectric precipitation was assayed spectrophotometrically as described in the Materials and Methods section.


FUMARATE (μmoles/min. × 10²) g.f.wt.
5
4
3
2
1
0
1
2
3
4
5
DAYS
0.1
0.05
ENZYME PROTEIN
(mg/g.f.wt.)

protein content of the tissue extracts is also falling with the age of the cotyledons, specific activity and total activity remain roughly comparable. In all subsequent work, 1 day old cotyledons were used as a source of the enzyme.

Attempts to purify argininosuccinate lyase.

Several methods of purification were used in an endeavour to increase the purity of enzyme preparations. Ammonium sulphate fractionation of the crude homogenate was unsuccessful. All enzyme activity being lost. Havir *et al*, (1965) found that the partially purified mammalian enzyme was sensitive to ammonium sulphate. Thus 0.26 M ammonium sulphate produced a 46% inhibition of activity in their experiments. These workers noted that the inhibition was partially reversed by dialysis of the enzyme, and also that the degree of inhibition was greater with purer enzyme preparations. It may well be that the enzyme obtained from pea cotyledons is even more sensitive to ammonium sulphate than the mammalian enzyme. A procedure employing isoelectric precipitation, calcium phosphate gel treatment and chromatography on C.M. cellulose was found suitable for partially purifying the enzyme as outlined in Table 6. From the table it can be seen that the lyase was purified approximately 28 times. Recoveries of approximately 37% of the initial activity were obtained.

Stability of the partially purified enzyme.

Ability to catalyze the cleavage of argininosuccinate was retained after storage in 0.1 M potassium phosphate buffer

TABLE 6

Partial purification of argininosuccinate lyase.

Stages of Purification	Total volume (*ml*)	Activity (*units/ml*) x 10^3	Total units x 10^3	Protein (*ug/ml*)	Specific activity (*units/mg protein*) x 10^3	Recovery (%)
pH 5.1 supernatent	475	9.5	4,341	8.5	1.1	100
isoelectric precipitate	15	261.8	3,927	24	10.9	90
$Ca_3(PO_4)_2$ gel treated enzyme	20	131.0	2,620	9.4	13.9	60
C.M. cellulose treated enzyme	15	107.0	1,605	3.4	31.5	37

(pH 7.9) at -20°C, for periods up to 1 year. The enzyme was also stable to dialysis against all the buffers used in these studies (see Materials and Methods). However, dialysis against 0.1 M Tris-KCl buffer (pH 7.9) for periods up to 48 hr at 0°C, resulted in complete inactivation of the enzyme. This inactivation could be partially reversed by incubation in 0.1 M potassium phosphate buffer (pH 7.9), for 20 min at 37°C. It was found that approximately 10-20% of the initial activity could be restored in this manner. This behaviour is similar to that observed by Havir *et al*, (1965), working with the argininosuccinate lyase from bovine liver. These workers found that the inactivation caused by Tris buffer at 0°C was due to separation of the enzyme into inactive subunits. Thus after 48 hr at 0°C, 81% of the enzyme activity was lost. This loss could be partially reversed, however, by treatment of the enzyme with phosphate buffer, or by incubating the enzyme mixture for at least 6 min at 38°C. Although the possibility of thermal reactivation of the plant enzyme was not directly investigated, all samples were routinely pre-incubated at 37°C for 20 min before measuring initial reaction velocities.

Factors affecting the initial velocity of argininosuccinate cleavage.

The initial reaction velocity was found to be linear for periods of up to 4 min, depending upon the amount of the enzyme preparation taken. It was not affected by the addition of purified arginase plus manganese ions. This suggested that

the initial reaction velocities were not affected by the concomitant accumulations of arginine.

A linear relationship was found to exist between initial reaction velocity and enzyme concentration when less than 2 mg of protein was used in the reaction system (Fig. 6). The effect of pH on the initial velocity of argininosuccinate cleavage is shown in Fig. 7. The pH values plotted in Fig. 7, were measured before and after the reaction had taken place, and were found to stay constant. It can be seen that the enzyme exhibits maximal activity at pH 7.9. This value is similar to the figure of 7.5 obtained for the mammalian enzyme by Havir *et al*, (1965).

The effect of substrate concentration upon initial reaction velocity was investigated by incubating a suitable range of argininosuccinate concentrations with aliquots of purified enzyme containing 1.2 mg of protein. It was found that 1 μmole of argininosuccinate was sufficient to saturate 1.2 mg of enzyme protein. When these results were plotted according to the method of Lineweaver and Burk (1934), as shown in Fig. 8, a linear relationship was found. The Michaelis constant (Km) was calculated from the intercept on the abscissa to be 2×10^{-4}M. From the slope term, the Km was calculated to be 2×10^{-4}M. The Km of the enzyme isolated from mammalian sources was found to be 1.5×10^{-3}M (Havir *et al*, 1965). Thus the apparent affinity for argininosuccinate of the pea cotyledon enzyme is approximately 8 times that

FIGURE 6

The relationship between argininosuccinate lyase activity, and protein concentration.

Reaction mixtures contained the following in a total volume of 3 ml:argininosuccinate, 1 μmole; potassium phosphate buffer (pH 7.9), 260 μmoles; and varying amounts of enzyme activity as indicated. Both the assay and the reference cuvette were incubated at 37°C for 20 min before initiating the reaction by addition of the argininosuccinate.

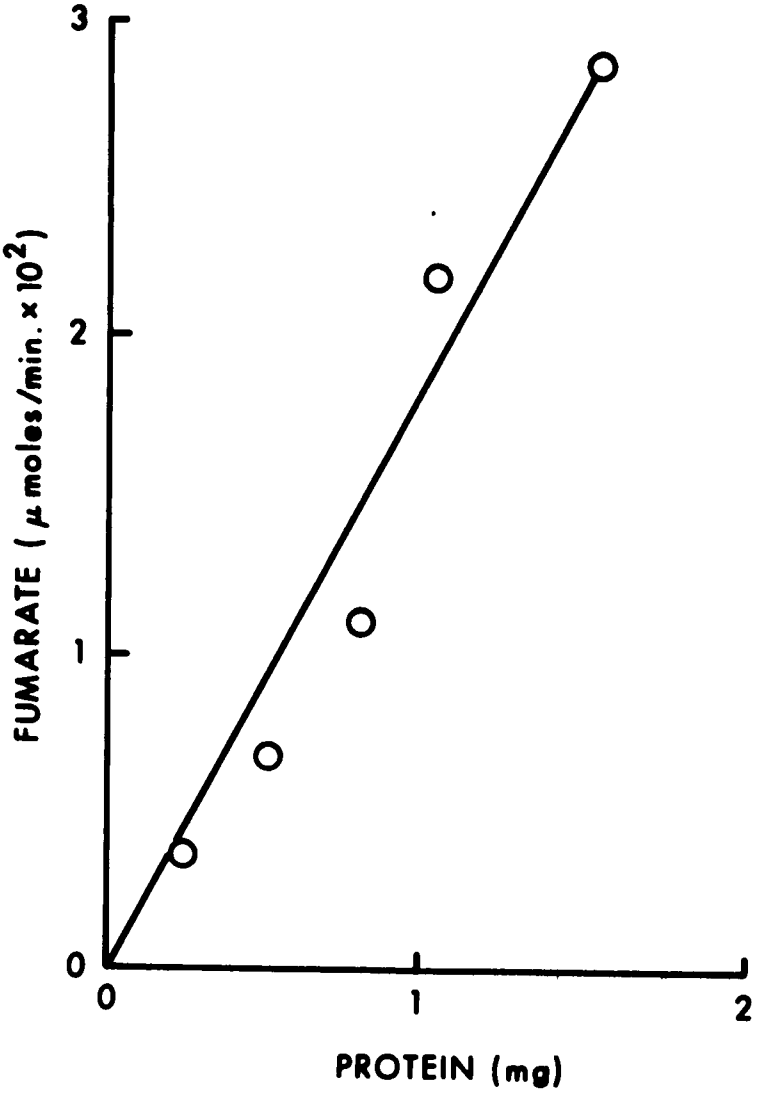

3
2
1
0
0
1
2
FUMARATE (μ moles/min. x 10²)
PROTEIN (mg)

FIGURE 7

The relationship between argininosuccinate lyase activity and pH.

Reaction mixtures contained the following in a total volume of 3 ml: argininosuccinate 1 μmole; potassium phosphate buffer, 260 μmoles; and 0.01 - 0.02 units of enzyme activity. Both the assay and the reference cuvette were incubated at 37°C for 20 min before initiating the reaction by addition of the argininosuccinate.

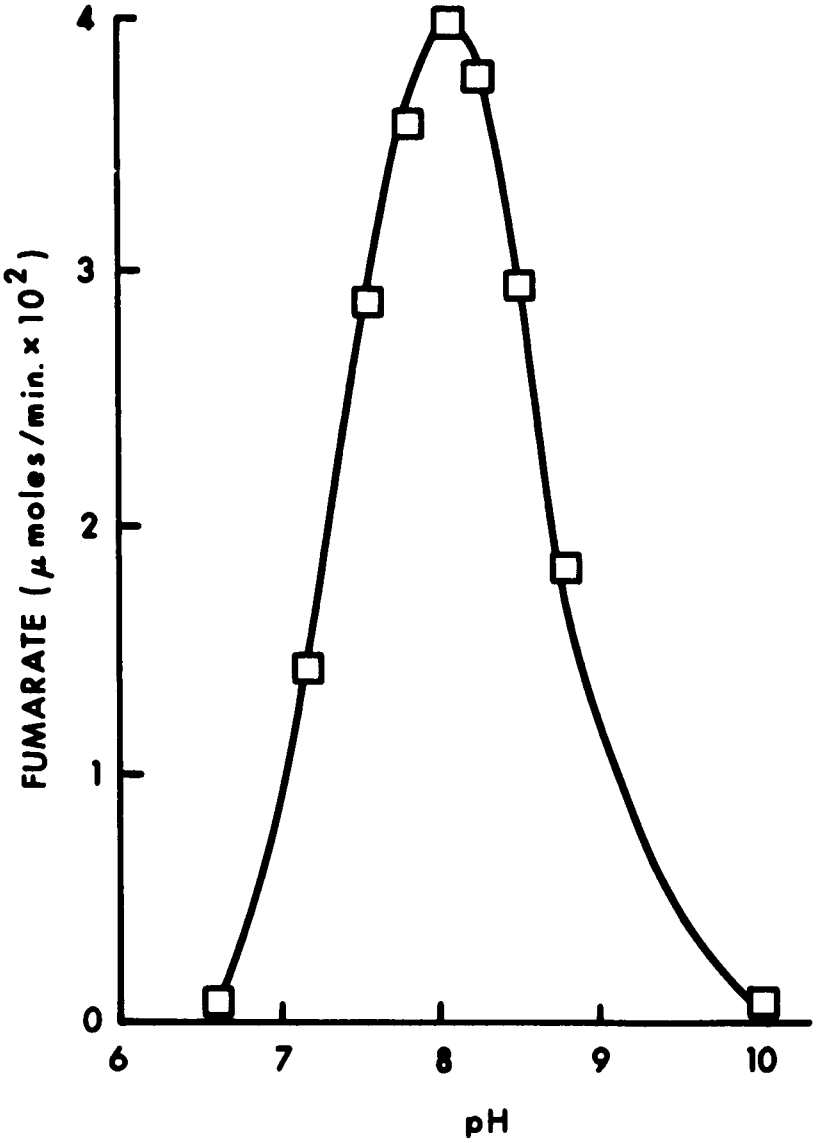
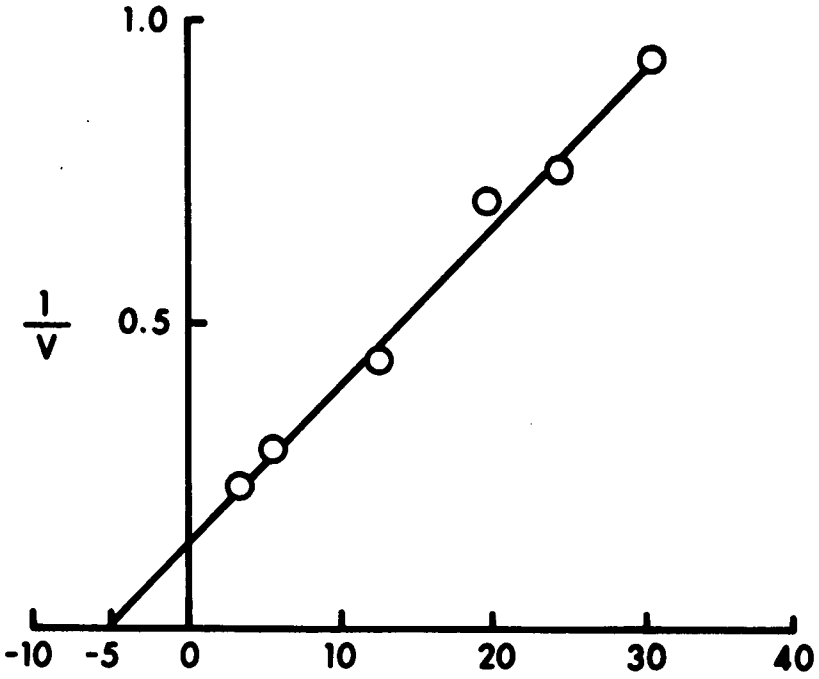

FUMARATE (μ moles/min. × 10²)
4
3
2
1
0
6
7
8
9
10
pH

FIGURE 8

Double reciprocal plot of reaction velocity versus argininosuccinate concentration.

The reaction mixtures contained the following in a total volume of 3 ml: 0.01 - 0.02 units of enzyme activity; potassium phosphate buffer (pH 7.9), 260 μmoles; and varying amounts of argininosuccinate as indicated. Both the assay and the reference cuvette were incubated at 37°C for 20 min before initiating the reaction by addition of the argininosuccinate.


1.0
0.5
1/V
-10
-5
0
10
20
30
40
1/[ARGININOSUCCINATE] mM

displayed by the mammalian enzyme.

Reaction products and reversibility.

The formation of fumaric acid from argininosuccinate was followed spectrophotometrically as shown in Fig. 9. The increase in absorbance at 240 mμ was in fact due to the production of fumarate, as this increase was readily reversed on addition of purified fumarase. This formation of fumaric acid was accompanied by the formation of arginine, as shown by the chromatographic technique. In all cases the ninhydrin-positive compound produced after the enzyme was incubated with argininosuccinate, displayed chromatographic mobility that was identical with that shown by authentic arginine. Similarly the production of argininosuccinate from arginine and fumarate was also confirmed by the chromatographic method.

In order to determine the degree of reversibility of the reaction, aliquots of the purified lyase were incubated with argininosuccinate for periods up to 1 hr at 37°C. The incubation mixtures were then analyzed using the chromatographic procedure described in the Materials and Methods section. The data in Table 7 are derived from duplicate analyses in which approximately 1 μmole of argininosuccinate, or arginine and fumarate/per 0.5 ml of reaction mixture were used. The results obtained are also shown in Figs 10 and 11. Equilibrium was established after 30 min at 37°C, when approximately 80% of the argininosuccinate and 20% of the arginine had been interconverted. The data shows that although the

FIGURE 9

The production of fumarate from argininosuccinate.

Enzyme activity was assayed spectrophotometrically for 3 min at 37°C, then purified fumarate hydratase was added to the reaction and reference cuvettes, as indicated by the arrow.

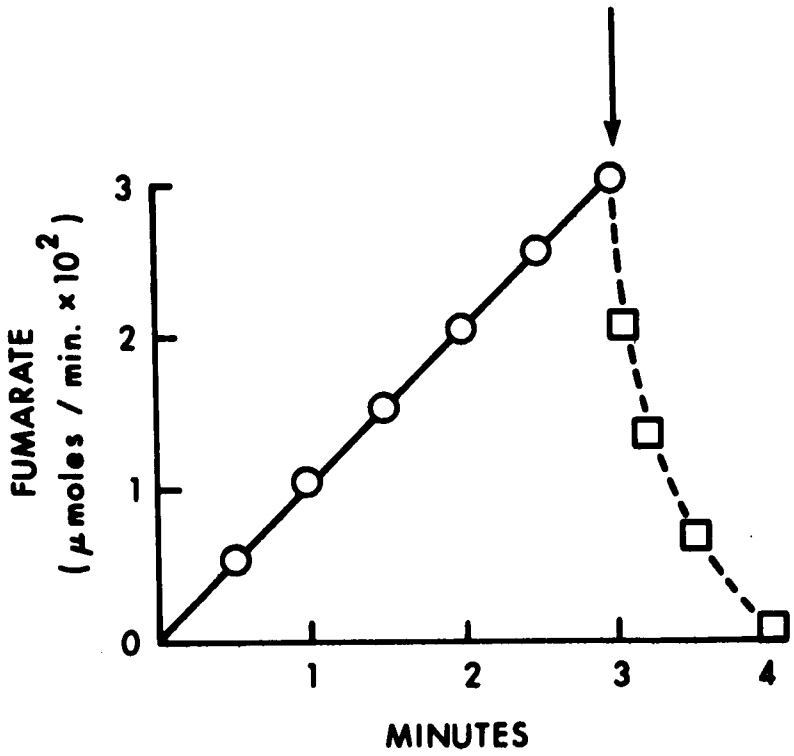

FUMARATE
(μmoles / min. x 10²)
3
2
1
0
1
2
3
4
MINUTES

TABLE 7

Interconversion of arginine and argininosuccinate at low substrate concentrations.*

Reaction	*arginine + fumarate → argininosuccinate*	
Incubation time (min)	arginine	argininosuccinate
0	0.90	0
10	0.78	0.15
20	0.68	0.17
30	0.67	0.20
40	0.70	0.20
60	0.70	0.20
Reaction	*argininosuccinate → arginine + fumarate*	
Incubation time (min)	arginine	argininosuccinate
0	0	0.89
10	0.47	0.38
20	0.59	0.30
30	0.67	0.23
40	0.69	0.21
60	0.69	0.21

* Expressed as μmoles substrate/0.5 ml reaction system.

FIGURE 10

The interconversion of argininosuccinate and arginine in the presence of argininosuccinate lyase.

Reaction mixtures contained the following in a total volume of 0.5 ml: 0.033 units of enzyme; potassium phosphate buffer (pH 7.9), 20 μmoles; and 1 μmole of argininosuccinate. Incubation of the mixtures was carried out at 37°C for periods of time up to 60 min as indicated. The reaction was terminated in each case by the addition of cold (-20°C) ethanol.

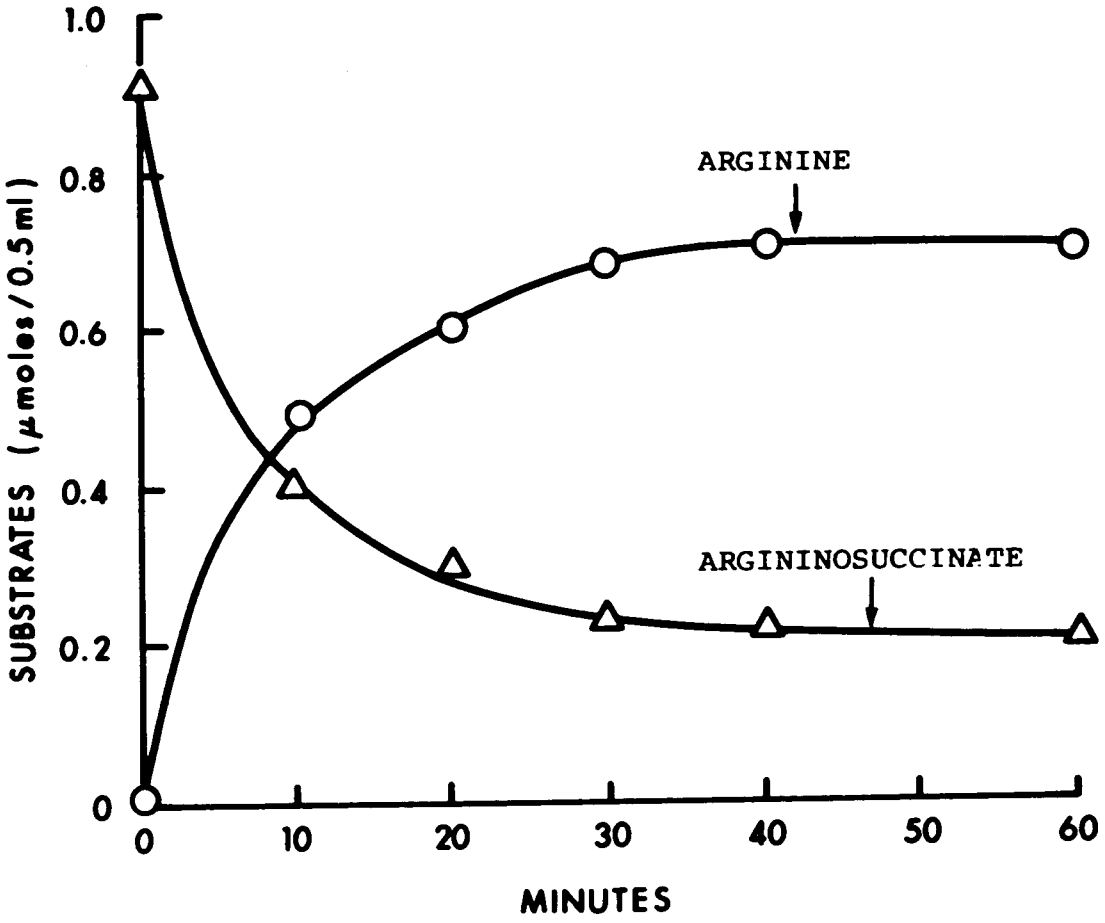
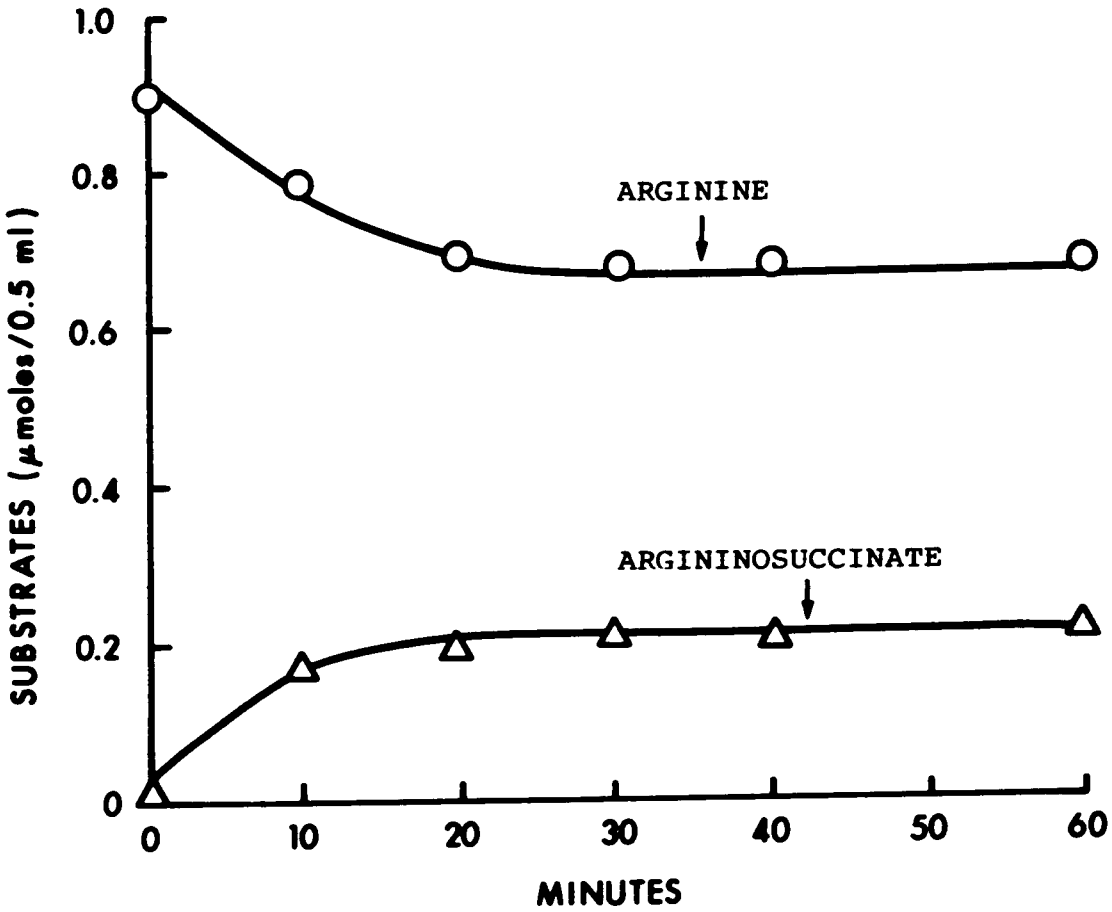

1.0
0.8
0.6
0.4
0.2
0
SUBSTRATES (μmoles/0.5ml)
ARGININE
ARGININOSUCCINATE
0
10
20
30
40
50
60
MINUTES

FIGURE 11

The interconversion of arginine and argininosuccinate in the presence of argininosuccinate lyase.

Reaction mixtures contained the following in a total volume of 0.5 ml: 0.033 units of enzyme; potassium phosphate buffer (pH 7.9), 20 μmoles; and 1 μmole each of arginine and fumarate. Incubation of the mixtures was carried out at 37°C for periods of time up to 60 min as indicated. The reaction was terminated in each case by the addition of cold (-20°C) absolute ethanol.


1.0
0.8
0.6
0.4
0.2
0
SUBSTRATES (μmoles/0.5 ml)
ARGININE
ARGININOSUCCINATE
0
10
20
30
40
50
60
MINUTES

cleavage of argininosuccinate is freely reversible, the formation of arginine is favoured when low concentrations of the substrates are supplied. In a second set of experiments, a 10 fold higher concentration of the substrates was used. The results obtained for these experiments are shown in Table 8. It is apparent that equilibrium was only attained after 60 min of incubation and that approximately equal quantities of arginine and argininosuccinate were present. From the results obtained in the first set of experiments where low substrate concentrations were used, values for the equilibrium constant (Keq) can be calculated.

For the forward direction (cleavage of argininosuccinate)

$$Keq\ (forward) = \frac{[arginine]\ [fumarate]}{[argininosuccinate]}$$

$$Keq = 4.5 \times 10^{-3}M$$

A similar expression for Keq (reverse) yields a value of $4.9 \times 10^{-3}M$.

In these calculations it was assumed that the quantities of fumarate formed would be equivalent to those of arginine.

The free energy change ΔG°, can be calculated utilizing the expression.

$$\Delta G^{\circ} = Rt\ \ln Keq$$

Where R is the gas constant measured in calories/mole/degree, and T is the temperature measured in degrees absolute.

TABLE 8

Interconversion of arginine and argininosuccinate at high substrate concentrations.*

Reaction	*arginine + fumarate → argininosuccinate*	
Incubation time (min)	arginine	argininosuccinate
0	10.2	0
10	9.5	0.3
20	8.8	1.2
30	8.5	1.2
40	7.6	2.4
60	5.1	4.6
Reaction	*argininosuccinate → arginine + fumarate*	
Incubation time (min)	arginine	argininosuccinate
0	0	10.0
15	0.6	8.8
30	3.7	5.7
60	4.9	4.6
90	4.9	4.3
120	4.9	4.6

* Expressed as μmoles substrate/0.5 ml reaction system.

Thus $\Delta G^{\circ} = -2.303 \times 1.987 \times 310 \log 4.5 \times 10^{-3}$

$= -2.303 \times 1.987 \times 310 \ (-2.3468).$

$= +3,329$ calories.

For the mammalian enzyme Ratner *et al*(1953) obtained values of 11.4×10^{-3}M for Keq, and +2,778 calories for ΔG°. Both values of ΔG° are in accord with the ready reversibility observed for the cleavage and synthesis of argininosuccinate.

The Michaelis constant of the reverse reaction.

To determine the Km of argininosuccinate lyase for arginine, a range of substrate concentrations were incubated together with aliquots of enzyme at 37°C for 10 min, and the reaction then terminated. The products were subsequently analysed by the chromatographic and colorimetric techniques previously described. The results obtained for duplicate analyses plotted according to Lineweaver and Burk (1934), are shown in Fig.12. From the intercept on the abscissa Km = 6.7×10^{-3}M. From the slope term Km = 6.8×10^{-3}M. For the mammalian enzyme, Ratner et al (1953), obtained a value of 1.5×10^{-2}M, as the Km for arginine. Thus it can be calculated that the enzyme isolated from pea cotyledons has an apparent affinity for arginine approximately 2 fold higher than the enzyme isolated from animal sources.

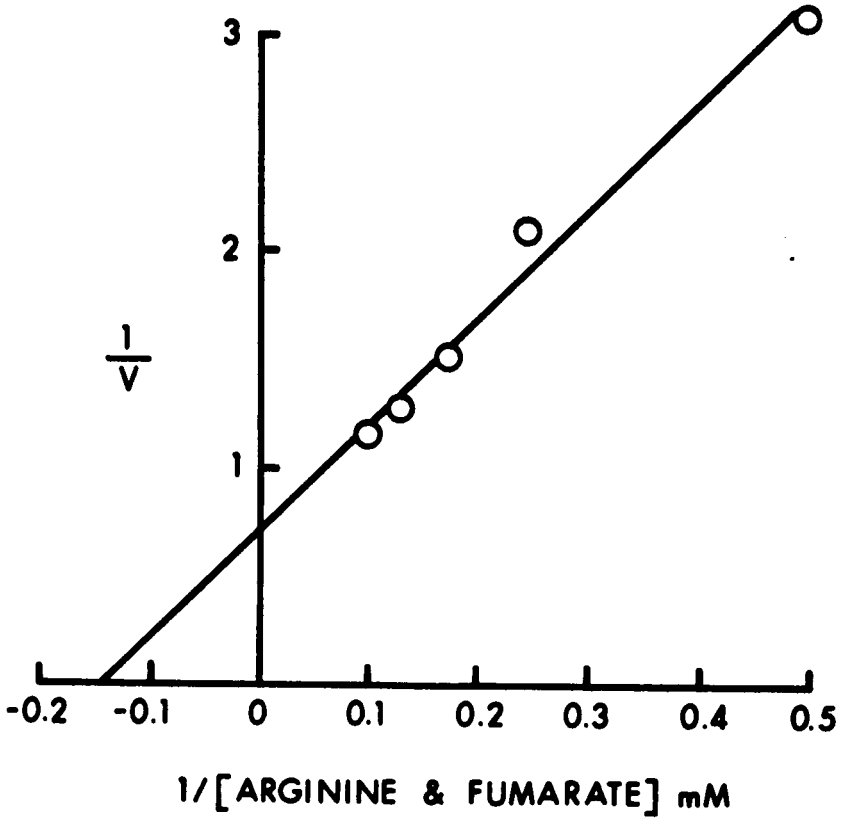
The effect of Sulphhydryl group reagents.

Preliminary studies of the effect of sulphhydryl-group reagents on enzyme activity were carried out using the spectrophotometric assay. The reagents used were p-chloromercuribenzoate (PCMB),iodoacetic acid, and iodoaceta-

FIGURE 12

Double reciprocal plot of reaction velocity versus arginine concentration.

The reaction mixtures contained the following in a total volume of 0.5 ml; 0.033 units of enzyme, potassium phosphate buffer (pH 7.9), 20 μmoles; and varying amounts of arginine and fumarate as indicated. Incubation of the mixtures was carried out at 37°C for 10 min, and the reaction terminated by the addition of 0.5 ml of absolute ethanol at -20°C.


3
2
1
1/V
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/[ARGININE & FUMARATE] mM

mide. All of these reagents appeared to inhibit enzyme activity with varying efficiencies, depending upon the concentration used. Thus 10^{-4}M PCMB, or 10^{-3}M iodoacetate appeared to completely inhibit enzyme activity. It was discovered however, that these levels of inhibitor were able to reduce the extinction of fumarate to zero. Thus the spectrophotometric assay could not be used for this type of study.

The effect of these inhibitors on enzyme activity was therefore investigated using paper chromatography. It was found that 10^{-4}M PCMB inhibited enzyme activity by 50-60% over controls containing no inhibitor. When iodoacetate and iodoacetamide were used (10^{-3}M), it was found impossible to obtain quantitative recoveries of arginine and argininosuccinate from the reaction mixtures. It was thought that in these cases, side reactions were occurring, since a third ninhydrin positive spot appeared on the chromatograms having an R_f of 0.077. From the results obtained using PCMB however, it would appear that sulphhydryl group inhibitors do have a marked effect on the activity of argininosuccinate lyase isolated from pea cotyledons. This result is markedly different from data obtained with the enzyme isolated from mammalian sources (Havir *et al*, 1965). Here a wide variety of sulphhydryl group inhibitors were examined including PCMB. However, no inhibition of enzyme activity was observed.

2. Studies of argininosuccinate synthetase.

The isotope feeding experiments previously discussed in Part I of these results indicated that large amounts of arginine-^{14}C were readily synthesized from citrulline-^{14}C in the cotyledons of germinating pea seeds. For this synthesis to take place, argininosuccinate synthetase must be present in these tissues. Also studies of argininosuccinate lyase suggested that the high level of arginine present in ungerminated and 1 day old pea seedlings might be correlated with the activity of arginine, synthesizing enzymes (Part II, Results; Shargool and Cossins, 1968). In view of these findings, it was decided to investigate 1 day old pea cotyledons as a source of argininosuccinate synthetase.

Extraction and partial purification of argininosuccinate synthetase.

Preliminary attempts to extract the enzyme from pea cotyledons were made using acetone treated cotyledon powders. Enzyme activity was extracted from the powders by homogenization in 0.01 M potassium phosphate buffer (pH 8.0). After filtering through cheese cloth, and centrifuging at 14,600 x g, the ability of the extracts to synthesize argininosuccinate was investigated using ^{14}C labelled citrulline. It was found that labelled arginine together with a small amount of labelled argininosuccinate were produced when aliquots of the crude extracts were incubated with L-citrulline-carbamyl-^{14}C, ATP, L-aspartate, and magnesium. The

presence of arginine in the reaction system suggested that argininosuccinate lyase was also present in these extracts. This fact was verified by using the following criteria. Firstly, the production of arginine from argininosuccinate when this amino acid was incubated with aliquots of the enzyme extracts. Secondly, the production of labelled fumarate and malate when aspartate-U-^{14}C was substituted for citrulline-^{14}C in the incubation mixtures. Attempts to resolve the lyase from synthetase were however unsuccessful, even when the enzyme protein was subjected to treatment with Sephadex G-200 as described below. For this reason synthetase activity was routinely assayed by measuring the production of arginine and argininosuccinate from the supplied citrulline. Extracts having this ability were fractionated for all subsequent studies as summarized in Table 9. Samples of the cotyledons were homogenized directly in phosphate buffer, and the homogenate was fractionated with ammonium sulphate. After desalting with Sephadex G-50, the enzyme protein was further fractionated with Sephadex G-200. Enzyme activity was purified approximately 36 times by this procedure (Table 9). It is of interest to note from Table 9, that there was a marked increase in enzyme activity after treatment with Sephadex G-50. The most obvious explanation for this increase in activity might be the removal of ammonium sulphate which is known to inhibit the lyase.

TABLE 9

Partial purification of argininosuccinate synthetase.

Stage of Purification	Total vol *(ml)*	Activity *(units/ml)* x 10^3	Total units x 10^3	Protein *(mg/ml)*	Specific activity *(units/mg protein)* x 10^4	Recovery (%)
Crude enzyme*	520	2.5	1,300	16	1.6	100
30-50% $(NH_4)_2SO_4$ fraction	23	7.7	177	75	1.0	13.6
Sephadex G-50 treated enzyme	23	18.2	419	58	3.1	32.2
Sephadex G-200 treated enzyme	23	4.6	106	0.8	57.5	8.2

* in 0.1 M potassium phosphate buffer (pH 8.0).

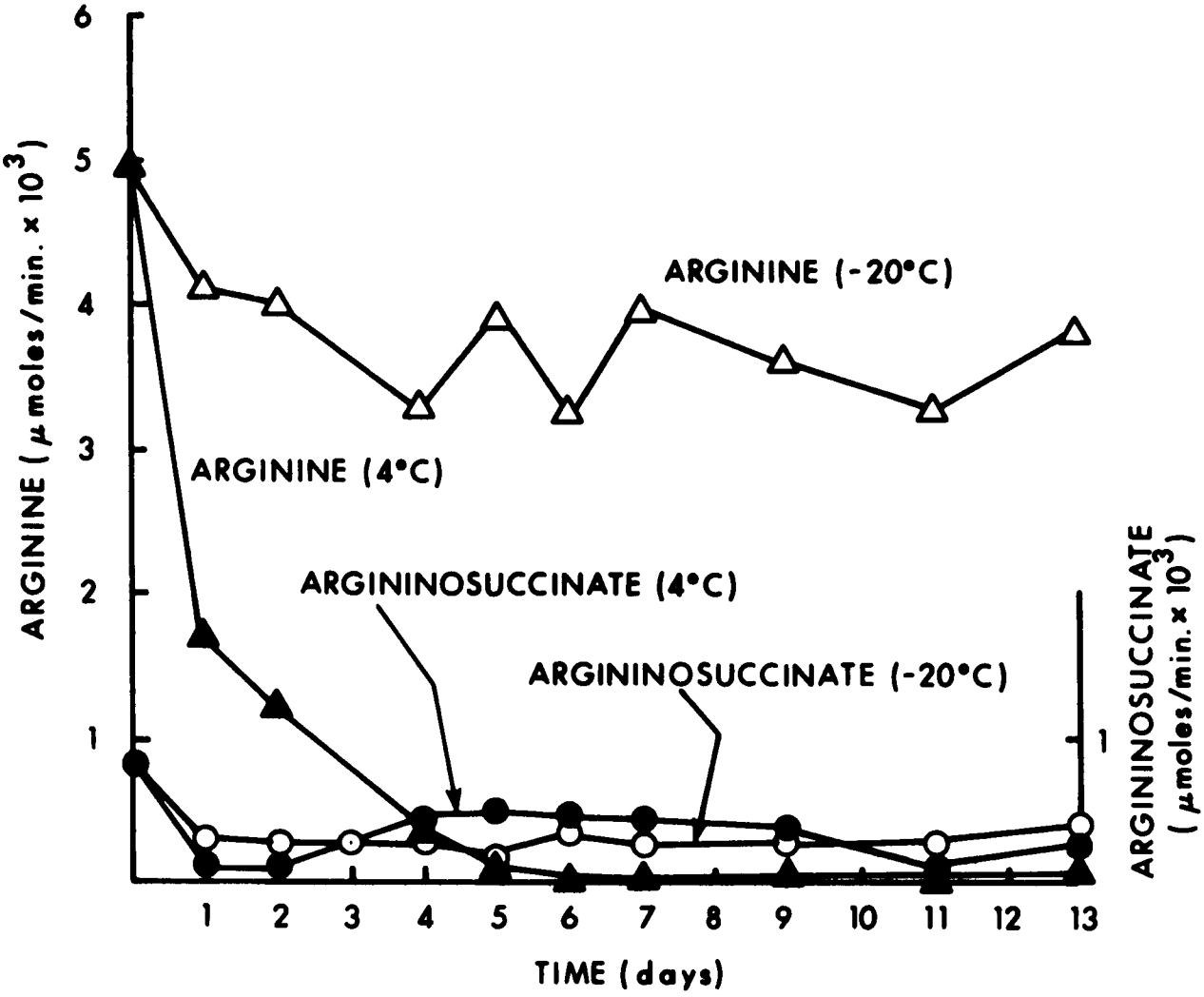
Stability of the partially purified extracts.

The effects of storage at -20°C and 4°C in 0.1 M Tris acetate buffer, (pH 8.0) is illustrated in Fig. 13. It can be seen that at -20°C the arginine synthesizing ability is lowered, whilst it is reduced to zero after 6 days storage at 4°C. It has already been noted in the discussion of the lyase that it was extremely unstable when stored in Tris buffer at 4°C. Thus it might be assumed that the loss of ability to synthesize arginine was due to an inactivation of the lyase component. This explanation also appeared likely as the small amount of argininosuccinate produced in the reaction was not appreciably affected by these treatments (Fig. 13). In order to test this possibility, an active preparation of the lyase was added to the incubation mixture, containing a sample of the synthetase enzyme which had been stored at 4°C in Tris buffer for 8 days. The ability to synthesize arginine still remained low. It was also found that preparations of the lyase obtained by the techniques previously described, possessed synthetase activity. This activity was only evident in freshly prepared samples and disappeared completely on storage in 0.1 M potassium phosphate buffer (pH 7.9) at -20°C. These samples were still active, however, in the cleavage of argininosuccinate. Thus it may well be that the synthetase is protected by Tris buffer.

FIGURE 13

The effects of storage at -20°C and 4°C on the production of arginine by Sephadex G-50 treated extracts.

Reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2 µc, (0.07 µmole); carrier L-citrulline 2 µmoles; L-aspartate, 2 µmoles; ATP, 3 µmoles; magnesium sulphate, 5 µmoles; Tris-acetate buffer (pH 8.0), 100 µmoles; and approximately 6 x 10^{-3}units of enzyme. After incubation at 30°C for 60 min, the reaction was terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.


ARGININE (μmoles/min. x 10³)
ARGININOSUCCINATE (μmoles/min. x 10³)
TIME (days)
ARGININE (-20°C)
ARGININE (4°C)
ARGININOSUCCINATE (4°C)
ARGININOSUCCINATE (-20°C)
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
12
13

Factors affecting the synthesis of arginine from citrulline.

The reactions leading to the production of arginine-^{14}C from citrulline-^{14}C, were shown to be completely dependent upon the presence of enzyme, aspartate, ATP, and magnesium (Table 10). The omission of inorganic pyrophosphatase resulted in a slight decrease in the yield of arginine (Table 10). The relationship between between arginine production and the levels of citrulline and aspartate present in the reaction mixtures is shown in Figs 14 and 15. In obtaining the data for these curves, the concentration of aspartate was kept constant at 5 µmoles/ml, citrulline at 2 µmoles/ml. The concentration of ATP was kept constant at 3 µmoles/ml, and the magnesium concentration was held constant at 5 µmoles/ml. It can be seen that the enzyme became saturated with citrulline at a concentration of 2 µmoles/ml. Aspartate became saturating at a concentration of approximately 1 µmole per ml. At concentrations above 1.5 - 2 µmoles, aspartate was inhibitory. The influence of ATP concentration upon arginine formation is shown in Fig. 16. Maximal arginine synthesis occurred at an ATP concentration of 3 µmoles per ml. ATP concentrations above this value were found to be inhibitory. The addition of an ATP regenerating system which included phosphoenol pyruvate, pyruvate kinase, and adenylate kinase, in conjunction with inorganic pyrophosphatase did not increase arginine production under these conditions.

Arginine formation was markedly affected by pH as shown in Fig. 17. An optimum pH range was found to exist

TABLE 10

Requirements for synthesis of arginine from citrulline by cotyledon extracts.*

Omission from reaction mixture	Arginine-^{14}C formed (% of complete system)
none	100
aspartate	0
ATP	0
magnesium	0
enzyme	0
inorganic pyrophosphatase	85-90

* after treatment with Sephadex G-50.

FIGURE 14

The effect of citrulline concentration on arginine production by Sephadex G-50 treated extracts.

The reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2 µc (0.07 µmole); L-aspartate, 5 µmoles; ATP, 3 µmoles; magnesium sulphate, 5 µmoles; Tris-acetate buffer (pH 8.0), 100 µmoles; and approximately 6 x 10^{-3} units of enzyme. The amounts of carrier citrulline present were varied as indicated. After incubation at 30°C for 60 min, the reaction was terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.

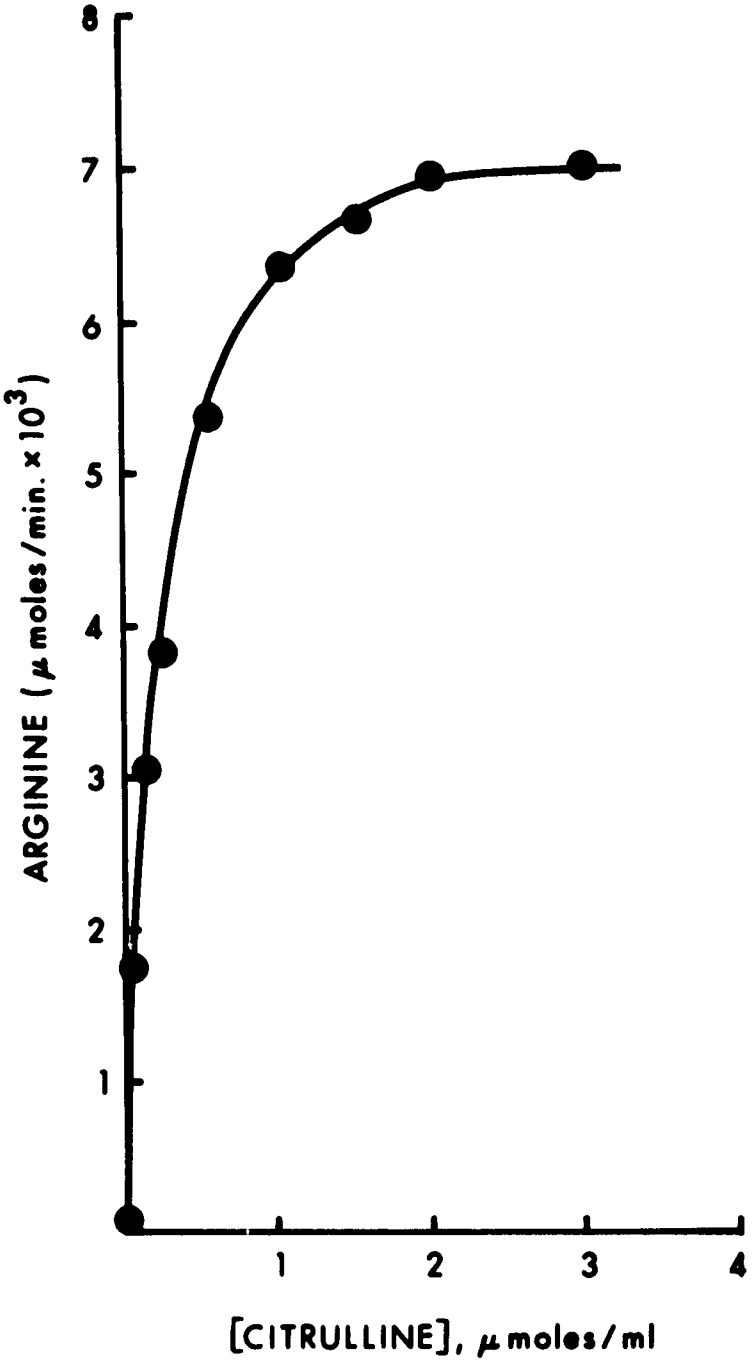

8
7
6
5
4
3
2
1
ARGININE (μ moles/min. × 10³)
1
2
3
4
[CITRULLINE], μ moles/ml

FIGURE 15

The effect of aspartate concentration on arginine production by Sephadex G-50 treated extracts.

Reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2.0 µc (0.07 µmole); carrier L-citrulline, 2 µmoles; ATP, 3 µmoles; magnesium sulphate, 5 µmoles; Tris-acetate buffer (pH 8.0), 100 µmoles; and approximately 6 x 10^{-3} units of enzyme. The amounts of L-aspartate present were varied as indicated. After incubating at 30°C for 60 min, the reaction was terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.

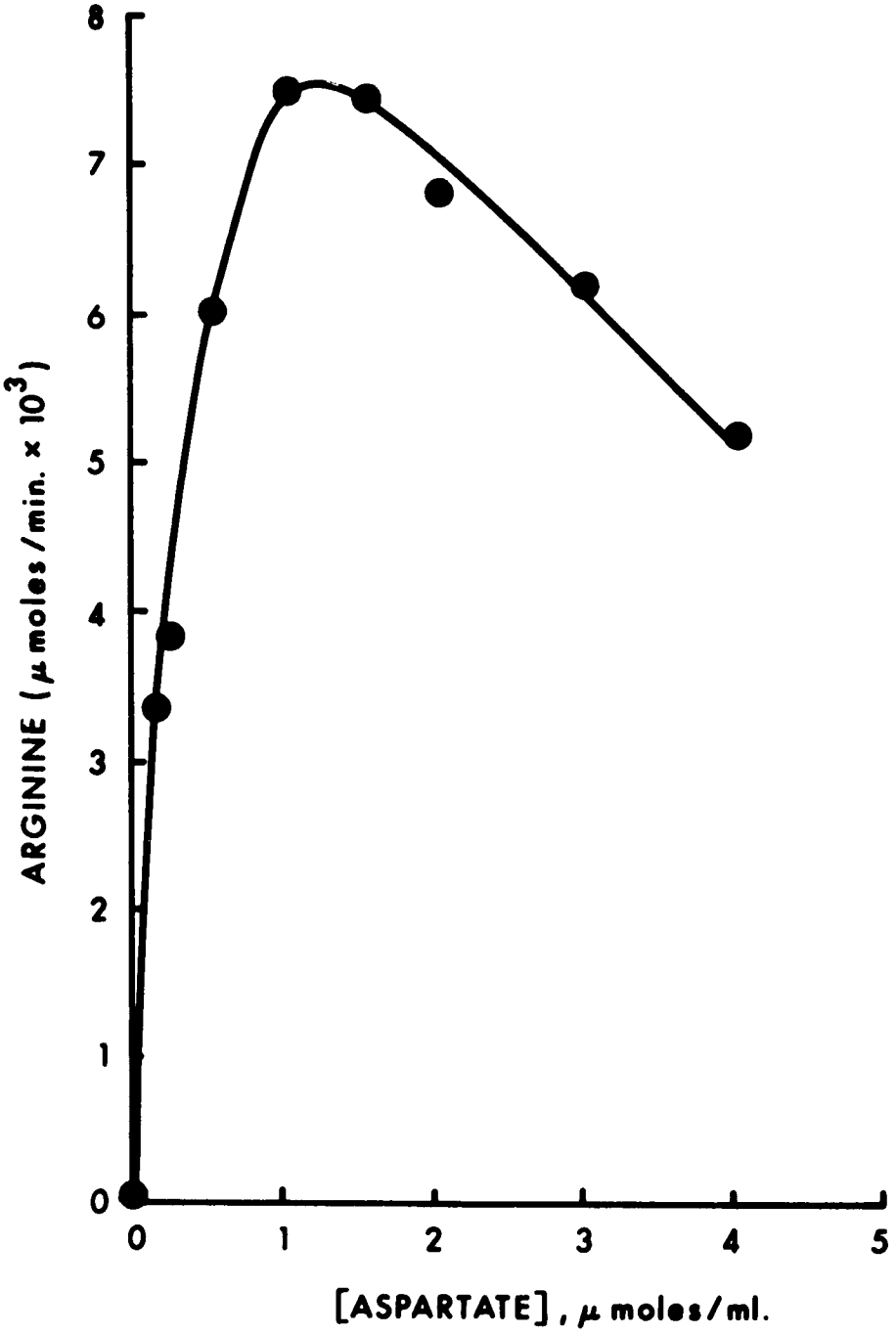

8
7
6
5
4
3
2
1
0
ARGININE (μ moles/min. × 10³)
0
1
2
3
4
5
[ASPARTATE], μ moles/ml.

FIGURE 16

The effect of ATP concentration on arginine production by Sephadex G-50 treated extracts.

Reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2.0 µc (0.07 µmole); carrier L-citrulline, 2 µmoles; L-aspartate, 5 µmoles; magnesium sulphate, 5 µmoles; Tris-acetate buffer (pH 8.0), 100 µmoles; and approximately 6 x 10^{-3} units of enzyme. The ATP concentration was varied as indicated. After incubating at 30°C for 60 min, the reaction was terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.

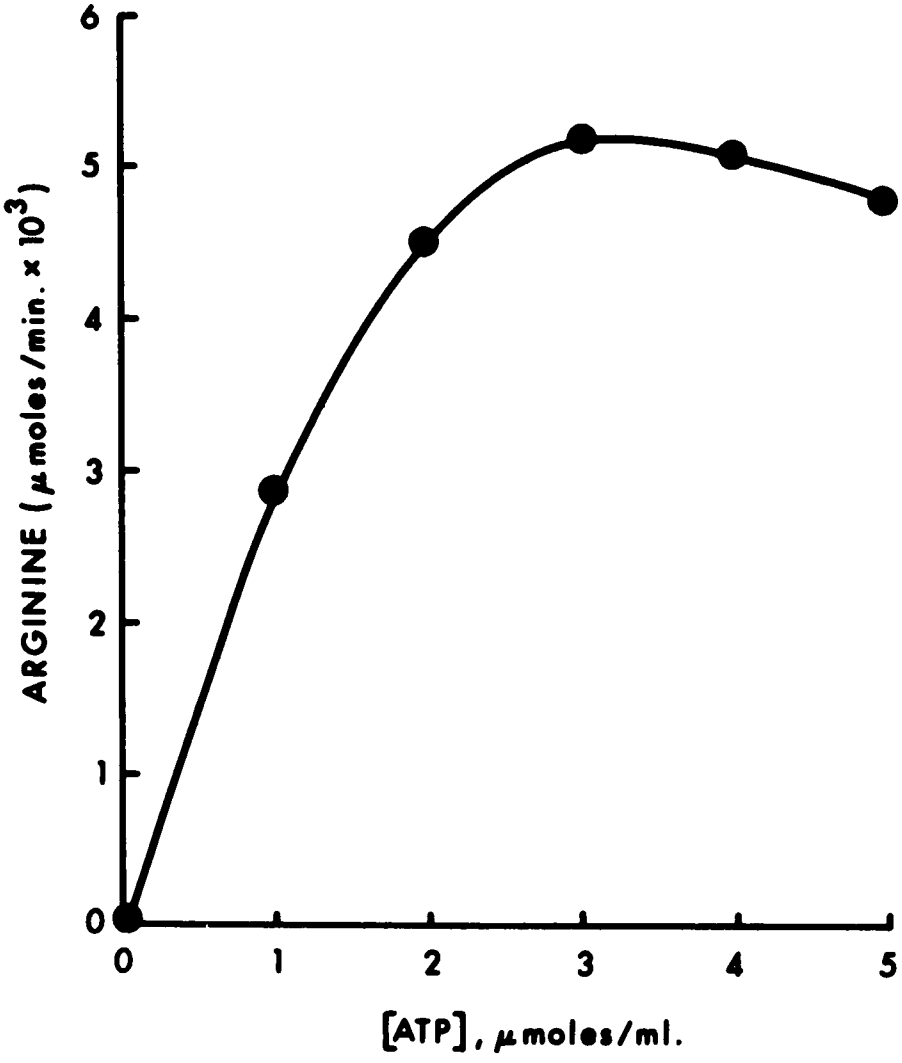
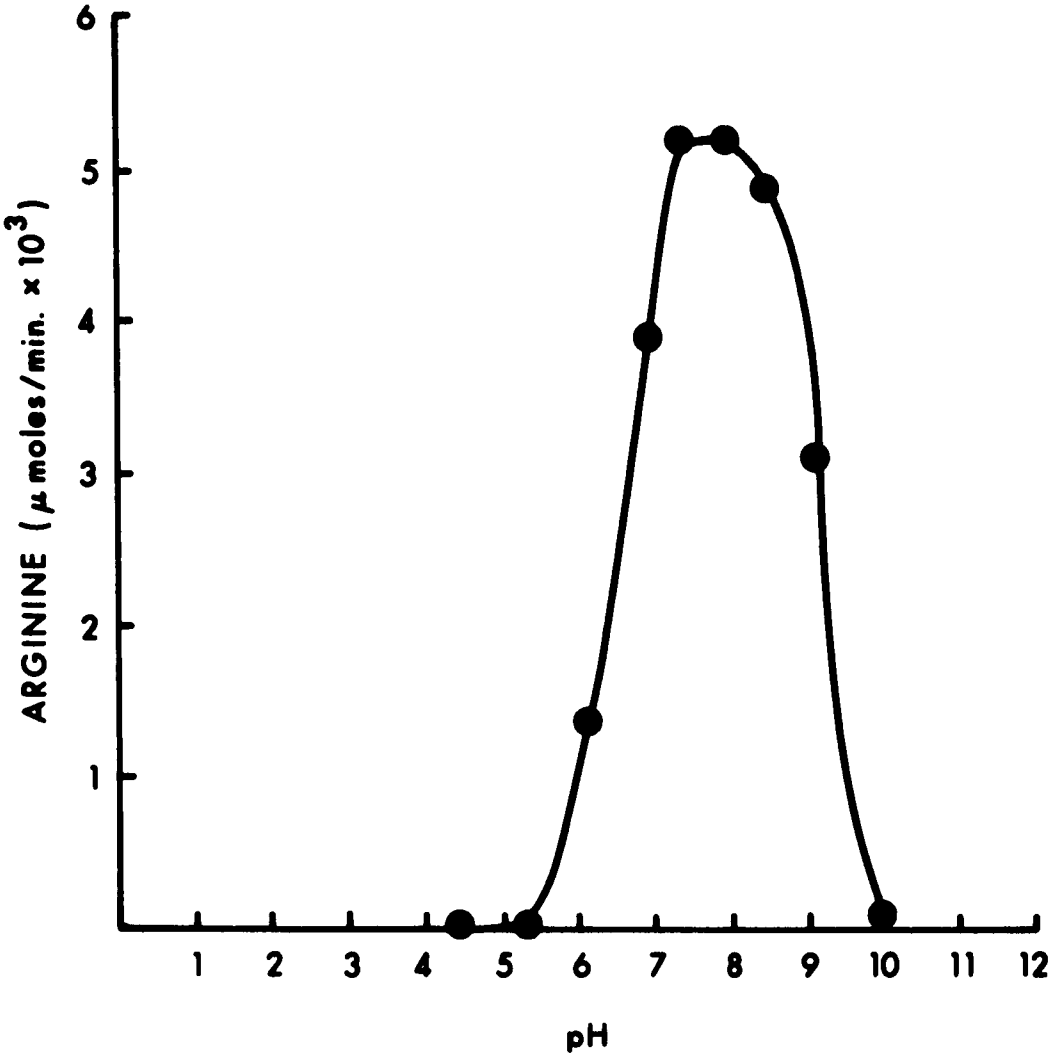

ARGININE (μmoles/min. × 10³)
6
5
4
3
2
1
0
0
1
2
3
4
5
[ATP], μmoles/ml.

FIGURE 17

The effect of pH on arginine production from citrulline.

Reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2 µc (0.07 µmole); carrier L-citrulline, 2 µmoles; L-aspartate, 2 µmoles; ATP, 3 µmoles; magnesium sulphate, 5 µmoles; Tris-acetate buffer, (100 µmoles); and approximately 6 x 10^{-3} units of Sephadex G-50 treated enzyme. After incubating at 30°C for 60 min, the reaction was terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.


ARGININE (μ moles/min. x 10³)
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
12
pH

between 7.4 and 8.0. The pH of the incubation mixtures was measured before and after the reaction was carried out, and found not to vary. Using a sample of enzyme which had been purified by treatment with Sephadex G-200, a linear relationship was found to exist between product formation and protein concentration, when less than 0.3 mg of protein were used in the reaction system (Fig. 18). In early studies of the synthetase enzyme isolated from ox-liver, Ra Ratner and Pappas (1949), used an assay system employing argininosuccinate lyase plus arginase, and estimated the urea formed as a final product. Using this system these authors found that saturation with aspartate occurred at a concentration of 4 μmoles/ml of reaction mixture, and with citrulline saturation was achieved at 3 μmoles/ml reaction mixture. ATP concentration was found by Ratner and Petrack (1951), to be optimal at approximately 4 μmoles/ml. Above this concentration it was inhibitory. The optimum pH of the overall reaction was found to be 7.5. Wampler and Fairley (1967), did not state the saturation values for citrulline and aspartate in work with the *Neurospora* enzyme. They did however, note that ATP was inhibitory in concentrations over 1×10^{-3}M and that the optimum pH was about 7.8.

The effects of arginine and argininosuccinate upon product formation were investigated as shown in Fig. 19. It was obvious that the presence of arginine (Fig. 19) had a marked effect on the amounts of arginine-^{14}C

FIGURE 18

The relation between arginine synthesis and protein concentration.

Reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2.0 µc (0.07 µmole); carrier L-citrulline, 2 µmoles; L-aspartate, 2 µmoles; ATP, 3 µmoles; magnesium sulphate, 5 µmoles and Tris-acetate buffer (pH 8.0), 100 µmoles. The amounts of Sephadex G-200 treated enzyme present were varied as indicated. After incubation at 30°C for 60 mins, the reaction was terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.

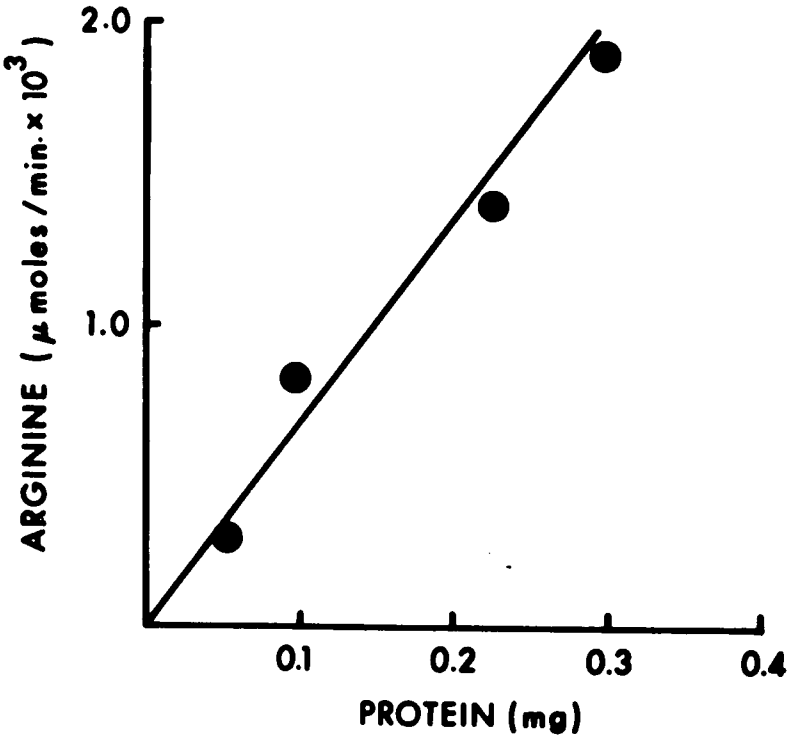
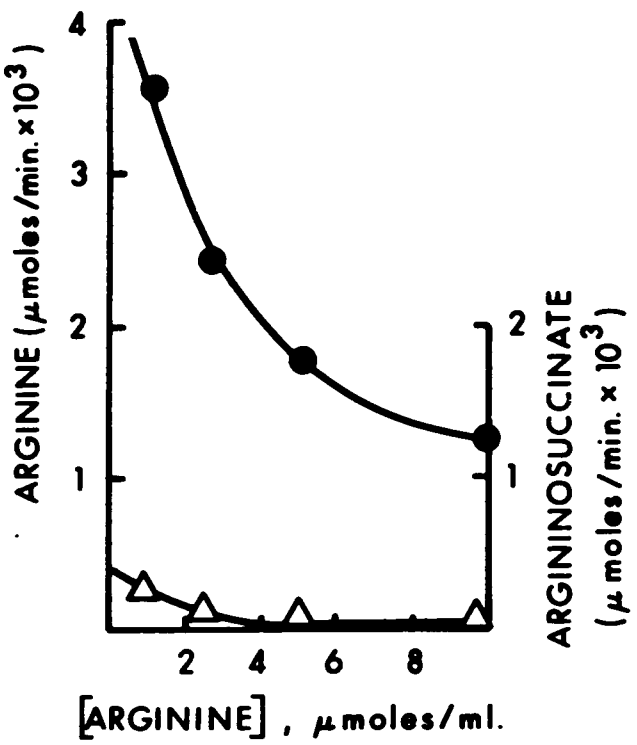
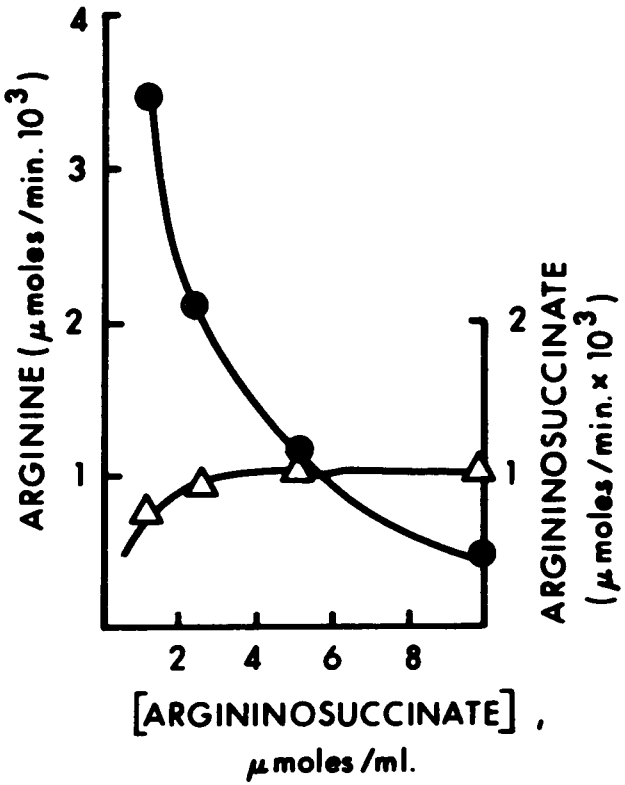

2.0
1.0
ARGININE (μ moles / min. × 10³)
0.1
0.2
0.3
0.4
PROTEIN (mg)

FIGURE 19

The synthesis of arginine and argininosuccinate from citrulline.

Reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2.0 µc (0.07 µmole); carrier L-citrulline, 2 µmoles; L-aspartate, 2 µmoles; ATP, 3 µmoles; magnesium sulphate, 5 µmoles; Tris-acetate buffer (pH 8.0), 100 µmoles; and approximately 6×10^{-3} units of Sephadex G-50 treated enzyme. Varying amounts of arginine or argininosuccinate were added as indicated. After incubating at 30°C for 60 min, the reaction was terminated by the addition of cold (-20°C) absolute ethanol.


ARGININE (μmoles/min.×10³)
ARGININOSUCCINATE (μmoles/min.×10³)
[ARGININE], μmoles/ml.


ARGININE (μmoles/min.10³)
ARGININOSUCCINATE (μmoles/min.×10³)
[ARGININOSUCCINATE], μmoles/ml.

and argininosuccinate-^{14}C that were formed. Wampler and Fairley (1967), found that the synthetase from *Neurospora* was inhibited by arginine. The inhibition being competitive with respect to citrulline concentration. This situation may also prevail with the enzyme isolated from pea cotyledons. A further explanation could be that the increased arginine concentration slowed down the lytic activity of the argininosuccinate lyase (Part II, Results; Shargool and Cossins, 1968). If this were the case however, a build up of argininosuccinate-^{14}C should have occurred. The fact that this did not happen suggests that arginine might be inhibiting the production of argininosuccinate by the synthetase enzyme. When argininosuccinate (Fig. 19) was added to the reaction mixtures, the levels of argininosuccinate-^{14}C were increased slightly. The amount of arginine-^{14}C produced rapidly declined however. This is presumably due to the presence of a pool of unlabelled substrate for the lyase.

When the production of arginine was investigated as a function of time (Fig. 20), product formation was found to be linear for approximately 20 min.

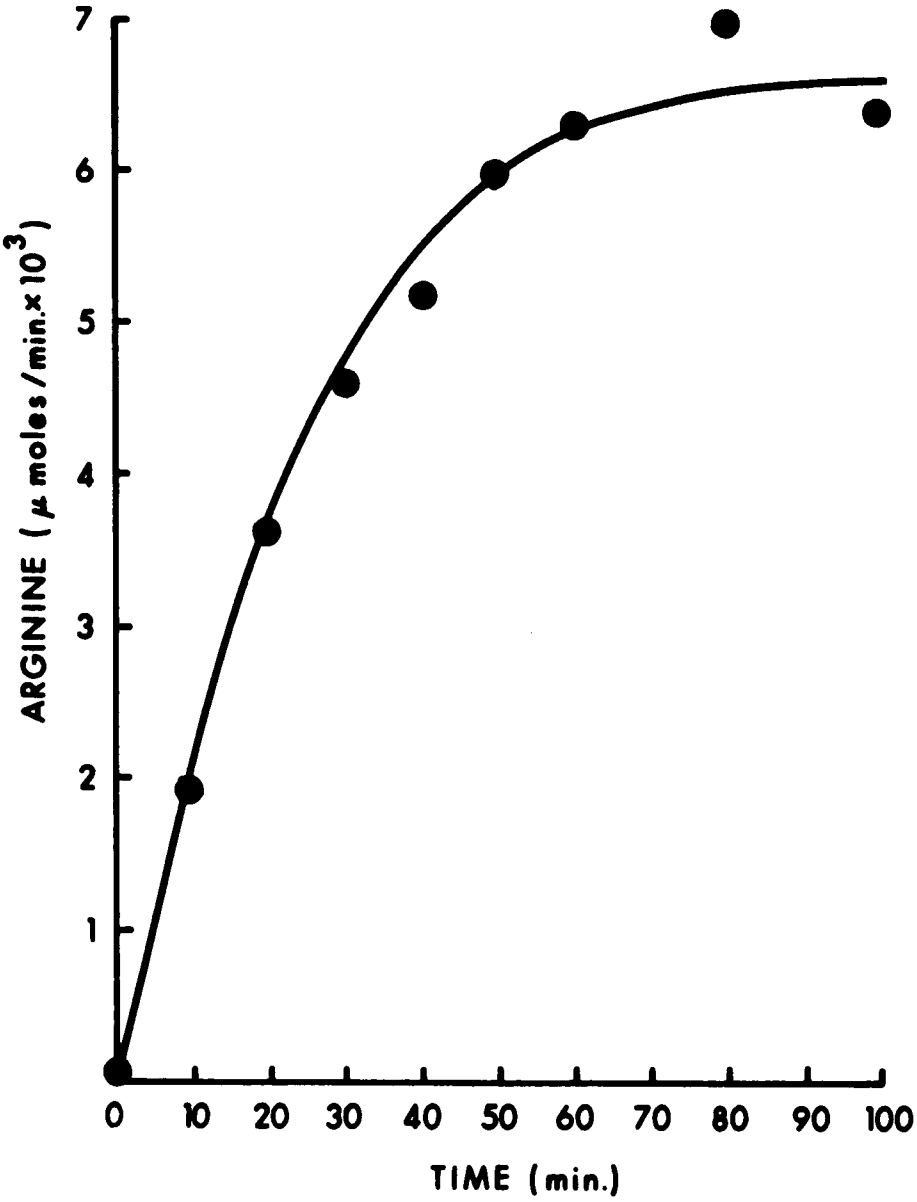
Michaelis constants for citrulline and aspartate.

Reaction mixtures were prepared in which either aspartate was present at saturating concentration, and citrulline was varied, or citrulline was present at saturating concentration and aspartate was varied. In both cases, ATP and magnesium were present at optimal concentrations.

FIGURE 20

The amount of arginine produced by enzyme substrate mixtures in relation to time.

Reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2 μc (0.07 μmole); carrier L-citrulline, 2 μmoles; L-aspartate, 2 μmoles; ATP, 3 μmoles; magnesium sulphate, 5 μmoles; Tris-acetate buffer (pH 8.0), 100 μmoles; and 6 x 10^{-3} units of Sephadex G-50 treated enzyme. Incubation was carried out at 30°C as indicated, and finally terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.


7
6
5
4
3
2
1
ARGININE (μ moles /min.× 10³)
0
10
20
30
40
50
60
70
80
90
100
TIME (min.)

Incubation was carried out for 15 min at 30°C, after which time the reaction was terminated and the products analysed as previously described. The data obtained were plotted according to the method of Lineweaver and Burk (1934), and are shown in Figs 21 and 22. From the plot obtained for citrulline (Fig. 21), the Michaelis constant (Km) is 2.8×10^{-4}M from the slope term and 2.7×10^{-4}M from the intercept on the abscissa. From the plot obtained for aspartate (Fig. 22) the Km was calculated to be 3.5×10^{-4}M from the slope term, and 3.3×10^{-4}M from the intercept on the abscissa. It appears therefore, that the apparent affinities for aspartate and citrulline are approximately equal in this reaction system. Rochovansky and Ratner (1967), obtained values of 4.6×10^{-5}M and 3.5×10^{-5}M for the Km values of citrulline and aspartate respectively, using the synthetase isolated from ox-liver. Similar values were obtained by Rochovansky and Ratner (1967), for the synthetase isolated from hog kidney. Wampler and Fairley (1967), obtained Km values of 6.8×10^{-3}M and 1.07×10^{-3}M for citrulline and aspartate respectively in work on synthetase from *Neurospora*.

FIGURE 21

Double reciprocal plot of arginine synthesis versus aspartate concentration.

The reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2 μc (0.07 μmole); carrier L-citrulline, 2 μmoles; ATP, 3 μmoles; magnesium sulphate, 5 μmoles; Tris-acetate buffer (pH 8.0), 100 μmoles; and approximately 6 x 10^{-3} units of Sephadex G-50 enzyme. The quantities of carrier aspartate present were varied as indicated. After incubation at 30°C for 15 min, the reaction was terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.

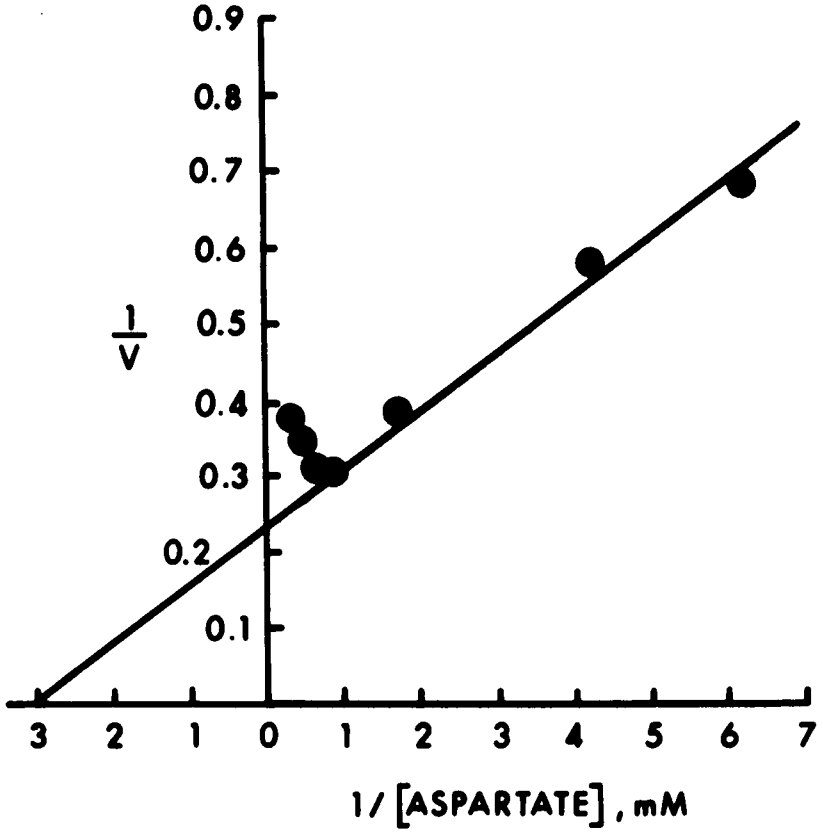
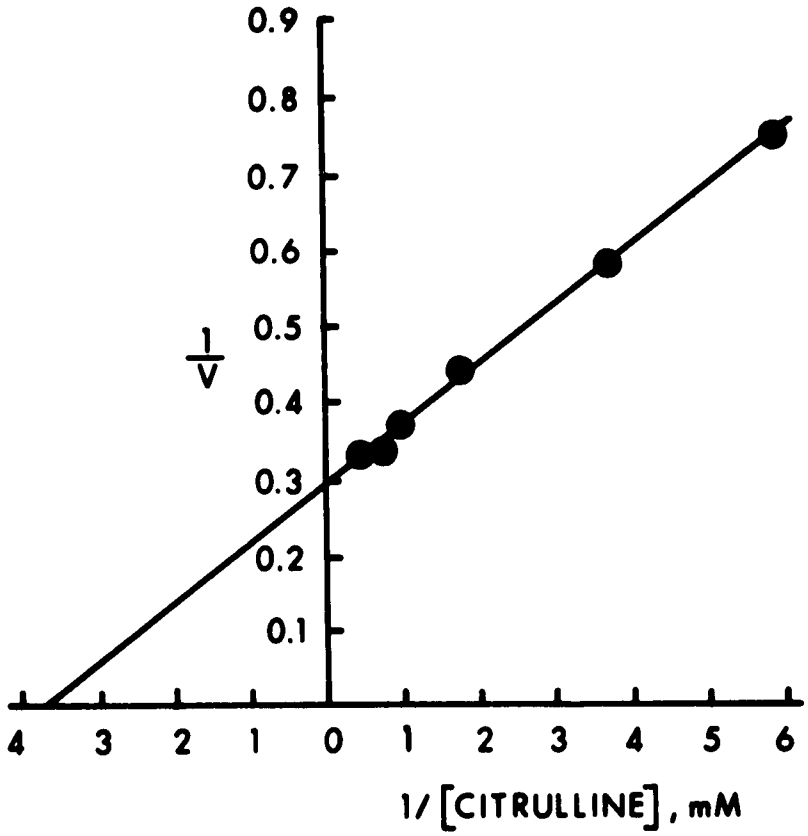

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/V
3 2 1 0 1 2 3 4 5 6 7
1/[ASPARTATE], mM

FIGURE 22

Double reciprocal plot of arginine synthesis versus citrulline concentration.

The reaction mixtures contained the following in a total volume of 1 ml: L-citrulline-carbamyl-^{14}C, 2 µmoles, aspartate 2 µmoles, ATP, 3 µmoles; magnesium sulphate, 5 µmoles; Tris-acetate buffer (pH 8.0), 100 µmoles; and approximately 6 x 10^{-3} units of Sephadex G-50 treated enzyme. The quantities of carrier citrulline present were varied as indicated. After incubating at 30°C for 15 min, the reaction was terminated by the addition of 1 ml of cold (-20°C) absolute ethanol.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/V
4
3
2
1
0
1
2
3
4
5
6
1/[CITRULLINE], mM

DISCUSSION

While the significance of certain observations has been discussed in the appropriate section, it is proposed to consider their implications in more detail in this discussion under the following sub-headings.

The sources of arginine in germinating peas.

Labelled arginine was formed in experiments where either ornithine-^{14}C was fed to pea root tips (Table 1 and 2, Figs. 3 and 4), or citrulline-^{14}C was fed to whole germinating peas (Table 3). This data, together with the extraction and partial purification of argininosuccinate synthetase and argininosuccinate lyase from pea root tips and cotyledons, provides evidence that the biosynthesis of arginine in the germinating pea proceeds via the partial reactions of the ornithine cycle. The initial reactions of the cycle involve the condensation of a molecule of carbamyl phosphate with a molecule of ornithine (Fig. 1). The synthesis of carbamyl phosphate has been studied in a number of organisms including peas (Wilkoszewska, Kleczkowski, and Reifer, 1961; Kleczkowski 1965; O'Neal and Naylor, 1968).

The studies of Bone (1959), indicated that in mung bean ornithine was synthesized via a similar pathway to that occurring in *Neurospora crassa* (Vogel and Bonner, 1964). This involves a reversal of the sequence of reactions shown in Fig. 2 (page 41), resulting in synthesis of ornithine from glutamate. When Bone fed acetate-1-^{14}C to mung bean mitochondria, he obtained the synthesis of labelled

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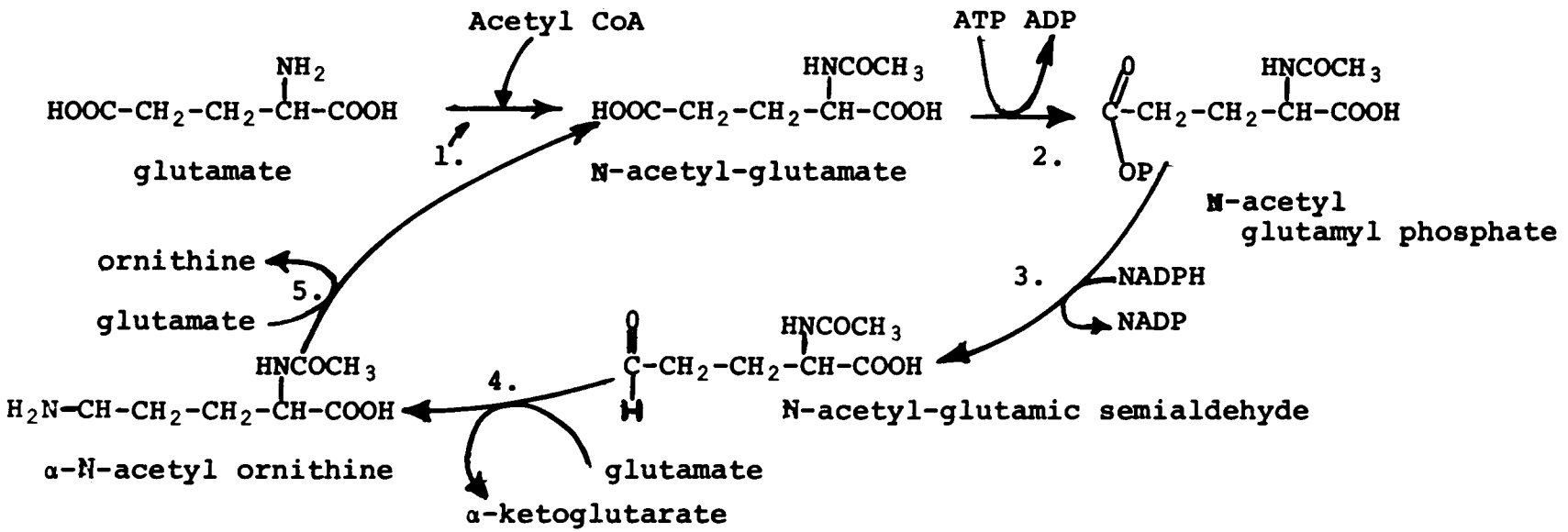
glutamate together with labelling in ornithine. Thus it appeared that ornithine could be synthesized in these tissues from glutamic acid produced from the Krebs cycle reactions. Bone (1959) was able to demonstrate the conversion of ornithine to glutamic γ-semialdehyde by mitochondrial preparations when incubated with ornithine and α-ketoglutarate. Thus indicating that the mitochondria contained ornithine δ-transaminase activity. However, attempts by the writer to demonstrate the presence of this enzyme in extracts of pea root tips, using the colorimetric method of Strecker (1965), and also using α-ketoglutarate -^{14}C, were unsuccessful in spite of the significant conversion of ornithine-^{14}C to glutamate-^{14}C in the feeding experiments carried out with this tissue (Table 1). Although preliminary attempts to detect ornithine δ-transaminase activity were not successful, it is possible that in peas, ornithine may be synthesized by reactions involving acetylated derivatives (Fig. 23). There is considerable experimental evidence for the occurrence of such a reaction sequence in fungi (DeDeken, 1962; Middlehoven, 1963; and Vogel and Vogel, 1963), algae and photosynthetic bacteria (Hoare and Hoare, 1966), and other bacteria (Meister, 1965). In such microorganisms as *Neurospora crassa* (Vogel and Vogel, 1963), the pathway outlined in Fig. 23 is now considered to be the major pathway of ornithine synthesis. Furthermore, the presence of an α-N-acetyl-ornithine aminotransferase in tissue cultures of rose cells (Dougall and Fulton, 1967), may indicate that such a pathway is operative

FIGURE 23

Synthesis of ornithine from glutamate via acetylated intermediates.



1= N-acetyl glutamate synthetase.

2= N-acetyl glutamate phosphokinase.

3= N-acetyl glutamyl γ-phosphate NADPH reductase.

4= α-N-acetyl ornithine δ-transaminase.

5= N-acetyl glutamate ornithine transacetylase.

in higher plants. Although the present work has not included studies with acetylated derivatives, it is clear that such investigations are needed before the origin of ornithine in peas is fully understood.

Apart from the synthesis of arginine which is evident in the tissues studied, some arginine is likely to be provided by the hydrolysis of protein reserves. The main reserve proteins of pea cotyledons are the globulins, vicillin and legumin, which contain 11.5 and 13% arginine respectively (Danielsson and Lis, 1952). These reserve proteins are located in distinct subcellular entities called protein bodies (Varner and Schidlovsky, 1963). Electron microscopy indicated that the disappearance of these protein bodies was correlated with growth and development of the pea embryo. Hydrolysis of the proteins presumably occurs under the influence of proteases which are known to be present in germinated peas (Young and Varner, 1959).

Role of argininosuccinate lyase during the germination of pea seeds.

From the results of the isotope feeding experiments discussed earlier, it is obvious that the biosynthesis of arginine was most active in the cotyledons of pea seeds, during the earliest stages of germination. This result is in accord with data obtained by Lawrence and Grant (1963), who found arginine to be the most prominent amino acid in the cotyledons of 1 day old peas. These workers observed that the level of arginine in cotyledons fell steadily after 1 day,

whilst the levels of all other amino acids rose.

During the present studies it was found that the level of argininosuccinate lyase in cotyledons apparently fluctuated in a manner similar to that of the arginine levels found by Lawrence and Grant (1963). Thus it is highly probable that the high initial levels of argininosuccinate lyase in the cotyledons during the early stages of germination were related to an active synthesis of arginine.

Both the cleavage and the synthesis of argininosuccinate by the lyase preparations were investigated during the present studies. It was found that the apparent substrate affinity for argininosuccinate (Km = 2×10^{-4}M) was appreciably greater than that for arginine (Km = 6.7×10^{-3}M). Although the standard free energy change indicated that the reaction was readily reversible, the Km values imply that cleavage of argininosuccinate is favoured *in vitro*. Also studies of the reaction equilibrium (Figs. 11 and 12), indicated that at low substrate concentrations (2 µmoles/ml) arginine formation was favoured. At higher concentrations of arginine and fumarate (20 µmoles/ml), the equilibrium mixture contained approximately equal amounts of arginine and argininosuccinate (Table 8). Therefore, if the arginine pool related to the lyase reaction, is maintained at low levels *in vivo* there would be a continuous production of arginine from argininosuccinate. It appears that argininosuccinate is indeed rapidly converted to arginine *in vivo* as micromolar quantities of citrulline-^{14}C were readily converted to arginine without accumulation of ^{14}C in

argininosuccinate (Table 3, Page 47).

Data from the feeding experiments and the enzyme studies therefore support the contention that argininosuccinate lyase has a major role in the synthesis of arginine during germination of pea seeds.

The importance of argininosuccinate synthetase in arginine biosynthesis.

In animal tissues it is clear that argininosuccinate synthetase is a key enzyme in the overall conversion of citrulline to arginine (Ratner, 1956). The catalytic properties of this enzyme from bovine liver and hog kidney have been thoroughly examined by Ratner and Pappas (1949), and Rochovansky and Ratner (1967). The absolute requirements for ATP, magnesium and aspartate reported by these workers are identical to the requirements for arginine synthesis by pea cotyledon extracts (Table 10). It is therefore concluded that pea cotyledons contain this enzyme and furthermore the high levels of activity argue for an involvement in arginine biosynthesis *in vivo*.

In addition to direct involvement in the pathway of arginine synthesis, the levels of the synthetase can be affected in many tissues by concentrations of arginine (e.g. Schimke, 1964). Arginine was also found to be a competitive inhibitor of the reaction in *Neurospora crassa* (Wampler and Fairley, 1967). It is interesting to note in this connection that preliminary studies on the effect of arginine concentrations (Fig. 19), indicated that this amino acid

might give a similar inhibition of the plant enzyme. Further work might in fact show that arginine biosynthesis in higher plants is partially controlled by this amino acid through its inhibitory effects on the argininosuccinate synthetase reaction.

Possible relationships between argininosuccinate lyase and argininosuccinate synthetase.

Despite the use of different isolation procedures enzyme extracts obtained from the cotyledons contained both argininosuccinate lyase and synthetase activities (Page 71). The presence of both enzyme activities in identical fractions from C.M. cellulose and Sephadex G-200 columns can be interpreted as indicating that both enzymes are similar in overall charge and molecular size. Alternatively these distinct catalytic properties may be associated with a single protein complex. The overall conversion of citrulline to arginine by such a complex would not necessarily involve the release of argininosuccinate. It is of interest to note that during the utilization of citrulline-C^{14} by the enzyme extracts, argininosuccinate-C^{14} accounted for no more than 3% of the total ^{14}C in the reaction system. This figure could not be increased by raising the concentrations of ATP, aspartate or citrulline. The reaction however, proceeds via argininosuccinate ^{14}C, since added arginine caused the accumulation of ^{14}C in this compound during the experiment summarized in Fig. 19. Furthermore, additions of argininosuccinate drastically reduced the incorporation of citrulline-

C^{14} into arginine (Fig. 19).

Work on these two enzymes from mammalian sources (Ratner and Pappas, 1949) has clearly established that they are distinct proteins. For example, these workers were able to separate the enzymes by ammonium sulphate fractionation. Further, evidence for the existence of two distinct enzymes in mammalian tissues was derived from work by Petrack and Ratner, 1958, who concluded that both enzymes act independently. Thus removal of argininosuccinate by added lyase did not stimulate the production of this compound by the synthetase.

Although the existence in pea cotyledons of a protein having both argininosuccinate synthetase and lyase activities cannot be entirely ruled out, more detailed examination of the protein extracts is required to elucidate this point. This would conceivably include detailed electrophoretic and sedimentation studies.

The significance of arginine biosynthesis in germinating pea seeds.

The results and discussion, have pointed out that an active pathway exists in pea cotyledons for the biosynthesis of arginine. In considering the significance of this synthesis reference to other studies with this tissue is important. For example, it is clear that several enzymes are synthesized in pea cotyledons during germination (Young and Varner, 1958 , Brown and Wray, 1968). In such protein synthesis, arginine would presumably play an import-

ant role. In agreement with this suggestion, it will be recalled that considerable amounts of citrulline-^{14}C were incorporated into protein arginine in the experiments with intact germinating peas (Table 3, Page 47). Arginine is also an important constituent of structural proteins and these will assume importance as seedling development proceeds.

Thus arginine synthesized in the cotyledons may be utilized for protein synthesis within this organ. In addition to this, considerable amounts of arginine will be translocated into the seedling and utilized there during subsequent growth and development.

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