ABSTRACT

Ph.D. Terry M. Rosenbaum Agricultural Chemistry

STUDIES ON COLUMN ELECTROPHORESIS OF PROTEINS AND ITS APPLICATION TO THE FRACTIONATION OF THE WATER SOLUBLE PROTEINS OF THE FIELD PEA (Pisum sativum L.)

An improved apparatus for powder column electrophoresis was designed and constructed for the preparative fractionation of proteins. Two methods were developed for concentrating the starting zone prior to electrophoretic fractionation of the sample. The water-soluble pea proteins were separated by the electrophoretic method and by subsequent gel filtration into seven fractions. The fractions were analyzed by disc electrophoresis and by disc isoelectric focusing and their molecular weights and isoelectric points were estimated. The sequence in which the pea proteins migrated under the conditions of powder column electrophoresis was different from that in which they migrated in acrylamide gel electrophoresis.

It was demonstrated, by high resolution autoradiography, that the calcium in cooked peas is associated mainly with the cell membrane and the cytoplasmic matrix.
Suggested short title -

STUDIES ON THE COLUMN ELECTROPHORESIS OF PEA PROTEINS

ROSENBAUM
STUDIES ON COLUMN ELECTROPHORESIS OF PROTEINS AND ITS APPLICATION TO THE FRACTIONATION OF THE WATER SOLUBLE PROTEINS OF THE FIELD PEA (Pisum sativum L.)

by

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A. GENERAL INTRODUCTION

Dried peas constitute an important source of dietary protein for the people of many Asian countries. Dried peas are also widely used by food processors and consumers in America, for the preparation of soups. Peas can be stored in the dried state for long periods of time, without deterioration. The high nutritional value and good keeping quality of dried peas makes them an ideal staple food stuff.

The modern consumer is usually conditioned to accept only foods that are easy to prepare and hence dried peas are not used extensively, directly by the consumer. Dried peas are usually soaked in cold water for about 24 hours and are then cooked until they are tender. Often, peas do not all soften to the same degree after they are cooked. This is troublesome to the housewife and unacceptable to the food processor who aims at the production of products of uniform quality. The lack of uniformity in the cooking quality of peas was observed as early as 300 B.C. (Hart, 1916). The peas that are produced commercially today still lack uniformity in cooking characteristics. Many reports have been written on the subject of pea texture but there is still very little known about the factors which affect the cooking
quality of peas.

The present study has been undertaken to further our knowledge of factors which influence the cooking properties of dried peas. A large part of this project involved the design and construction of a column electrophoresis apparatus for the preparative fractionation of the water soluble proteins of dried peas. Previous workers (Bourdillon, 1951; Kyoko et al., 1967; Zimmermann et al., 1967) have presented evidence which suggests that this protein fraction may be linked with the problem of cooking quality.
B. LITERATURE REVIEW

B.1. Isoelectric Focusing

Isoelectric focusing is a technique that is used to fractionate ampholytes according to their isoelectric points. When amphoteric molecules are placed in a pH gradient and are subjected to an electric field, they migrate to the region at which the pH corresponds to their isoelectric point.

Ampholytes or amphoteric molecules are species which have incorporated into their structures both acidic and basic groups. The term applies to many biological materials such as amino acids, peptides, proteins as well as many other substances which possess an isoelectric point. The isoelectric point of an ampholyte is the pH value at which the net charge on the molecule is zero. An ampholyte will have a net negative charge on the alkaline side of its isoelectric point and a net positive charge on the acid side of its isoelectric point.

The principle involved in isoelectric focusing is by no means new. Ikeda and Suzuki (1912) used it to isolate glutamic acid from an hydrolyzate of plant proteins. They designed a rectangular electrolysis apparatus with electrodes mounted at both ends; the space in between was divided by
two membranes, into three compartments. A potential difference was maintained between the electrodes and this caused the acidic amino acids to migrate to the anode and the basic amino acids to migrate to the cathode. The central compartment contained the neutral amino acids. The membrane across each compartment prevented remixing of the separated species.

Foster and Schmidt (1923) incorporated the ideas of Ikeda and Suzuki into their own design of an electrolytic cell. Their apparatus consisted of a wooden box divided into three sections by gelatin coated linen membranes. The solution of hydrolyzed amino acids which was to be separated, was placed in the central compartment. A potential difference (110 volts DC; 1.5 amps) was applied to the electrodes which caused the basic amino acids to accumulate at the cathode. They found that histidine could be collected in the central compartment if the reaction inside the compartment was maintained at pH 7.5. Foster and Schmidt (1926) used this device to isolate dicarboxylic amino acids which accumulated in the anode compartment. Williams and Waterman (1929) used a similar apparatus to separate acidic, basic and amphoteric materials. They introduced the name electrodialysis to describe their technique. The principle of the method was essentially the same as that of the method described by
Ikeda and Suzuki (1912). Williams and Waterman (1929) discussed some of the theoretical aspects of the technique. They stated, "If the substance sought is an ampholyte it may be concluded that the pH of that portion of the solution which contains the substance in maximum concentration approximates the isoelectric point of the substance."

Williams (1935) used the technique to fractionate a liver extract and a sample of deproteinized milk. The apparatus which was used in these experiments consisted of a series of cups connected in series by siphons. With this apparatus, he was able to avoid the use of membranes to separate the chambers. He claimed that membranes gave rise to endosmosis and advocated the use of high potentials and very dilute solutions to achieve good separation of the components. Vigneaud et al. (1938) used a modification of the apparatus described by Williams (1935) for the fractionation of a posterior pituitary extract. These workers noted that there was a pH gradient in the compartment between the cathode and anode, as a result of the applied potential.

Tiselius (1941) referred to the focusing of ampholytes in a pH gradient as "stationary electrolysis". He claimed that in such a system, ampholytes concentrate at their isoelectric points until a steady state is reached. At the steady state there is an equilibrium between the electric
migration and diffusion of the ampholyte at its isoelectric pH.

Kolin (1954, 1955) pointed out the necessity of using an electrolyte of high conductivity in order to get a more uniform field strength across the gradient. He also advocated the use of a density gradient to prevent mixing caused by convection currents. Kolin prepared a solution which had a pH gradient, by use of two buffer solutions of different pH values. He incorporated sucrose into the system to increase the density and hence reduce convection currents. Hoch and Barr (1955) applied Kolin's method to paper electrophoresis for the separation of human serum and plasma. Phosphate buffers were used in the anode compartment (pH 4.3) and in the cathode compartment (pH 7.4). The sample was applied to the centre of the strip where the two buffers converged. The applied electric field caused a pH gradient to form. Under these conditions the proteins in the sample arranged themselves in order of their isoelectric points. It was noted that proteins which had isoelectric points that were close together, did not separate well.

Kauman (1957) discussed from a theoretical point of view, the behavior of amphoteric electrolytes under the combined action of a pH gradient and an electrical potential.
He stated that a pH gradient will form in a solution between the anode and cathode compartments if a potential difference is applied to the electrodes. An ampholyte that is placed in this solution will migrate to the region where the pH of the gradient corresponds to the isoelectric point of the ampholyte. Under these conditions a stationary state will be reached in which the concentration gradient of ampholyte may be represented by a bell-shaped curve with the maximum concentration at the isoelectric point. He reported that the width of the band of the ampholyte will depend on the magnitude of the pH gradient.

Svensson (1961) presented an excellent review and a theoretical account of the behavior of ampholytes in a pH gradient under the conditions of an applied electric field. He used the term artificial pH gradient as one which is stabilized by buffers and a natural pH gradient as one which is not stabilized by buffers, but is formed automatically when a current is passed through the solution. He reported that the artificial pH gradients created by the use of buffers of different pH values, have the advantage in that conductance and electric field strength can be chosen and maintained at constant values during the experiment. He pointed out however, that artificial pH gradients will eventually
be converted to natural pH gradients by the passage of current through the solution. For example, a system, in which sodium phosphate buffers are used to create an artificial pH gradient, will eventually reach equilibrium under an applied electric field. At this point the anode compartment will contain phosphoric acid and the cathode compartment will contain sodium hydroxide. The pH gradient which forms between the anode and cathode compartments under these conditions is referred to as a natural pH gradient. If a system of this kind contains an ampholyte, the ampholyte will concentrate somewhere between the anode and cathode compartments in a region where the pH corresponds to the isoelectric point of the ampholyte. At equilibrium the concentration gradient of the ampholyte may be represented by a bell-shaped curve. At this point the electric current will be low provided that the pH gradient can be stabilized against convection currents. There will always be a slight current flow at the steady state, because of the diffusion of ampholyte away from the point of maximum concentration. The migration of the ampholyte due to diffusion, is compensated by the electric migration of the ampholyte toward its isoelectric point. The author pointed out that some current will also flow due to the mass flow of hydrogen ions towards
the cathode. Svensson (1961) mentioned that simple ampholytes should be useful as "carrier ampholytes" in the separation of proteins by this technique which he called isoelectric fractionation. He claimed that "carrier ampholytes" could theoretically be used to obtain pH gradients of any desired range. He noted however, that it was unfortunate that suitable "carrier ampholytes" were not readily available.

Svensson (1962a) stated that for good separation of proteins in a natural pH gradient, low molecular weight ampholytes are needed which possess good conductance at pH values close to their isoelectric point. He found that good ampholytes are isoelectric between two closely spaced pK values. Svensson reported that amino acids and peptides with pK values close to their isoelectric points, should make good carrier ampholytes. Histidine and peptides containing histidine, were reported to fulfill these requirements.

Vesterberg and Svensson (1966) prepared some carrier ampholytes by hydrolysis of hemoglobin. They used hemoglobin because of its high content of histidine. These peptide ampholytes were used to separate two components whose isoelectric points differed by 0.1 pH units. The separation was carried out in a simple column (Svensson, 1962b) in
which the desired pH gradient was stabilized in a density gradient prepared by use of sucrose. The authors found however, that peptide ampholytes were not ideal for protein separations because they both contained peptide bonds and hence gave a positive biuret test. Peptide ampholytes also absorbed light at 280 \SI{}{\mu \text{m}}. This made detection of protein zones very difficult. Vesterberg and Svensson (1966) made reference to the discovery by Vesterberg of a procedure for the synthesis of a system of carrier ampholytes that did not contain peptide bonds and did not absorb light at 280 \SI{}{\mu \text{m}}. Earland and Ramsden (1969) later described these synthetic ampholytes as a mixture of a large number of aliphatic amino-carboxylic acids of molecular weight between 300 and 600. They showed that upon hydrolysis the ampholytes yielded products which resembled glutamic acid, aspartic acid and arginine. They cautioned workers to remove the ampholytes from protein preparations before they were analyzed for amino acid content.

The procedure for the synthesis of the carrier ampholytes was patented recently by the Swedish Company, LKB-Produkter AB (1970). The synthesis involved the polymerization of a carboxylic acid containing at least one double bond, with a low molecular weight compound containing at
least two amino or imino groups. They gave as an example the polymerization of acrylic acid with triethylenetetramine.

Once a supply of good carrier ampholytes became available many workers used the synthetic ampholytes in columns such as the one designed by Svensson (1962b) for the preparative separation of proteins (Vesterberg and Svensson, 1966; Vesterberg, 1967; Quast and Vesterberg, 1968; Jonsson and Pettersson, 1968; Jonsson et al., 1969; Susor et al., 1969). The pH gradient which formed in the column was stabilized by a density gradient prepared by use of sucrose. Earland and Ramsden (1968) reported that the sensitivity of the method was such that traces of protein impurities from the sucrose could interfere with the protein zones of the sample under investigation. These workers recommended the use of a mixture of ethylene glycol, glycerol and water (50:25:25) to form the density gradient instead of sucrose.

Several workers (Fawcett, 1968; Leaback and Rutter, 1968; Wrigley, 1968; Catsimpoolas, 1969) have recently used acrylamide gels to stabilize the pH gradient that is produced by isoelectric focusing. The technique provides a rapid and simple method for the fractionation of microgram quantities of proteins.

Radola (1969) applied the technique of isoelectric
focusing to thin layer chromatography. In these experiments the plates were coated with a slurry of Sephadex gel mixed with ampholytes. The Sephadex stabilized the pH gradient.

Several rapid methods for staining the focused protein zones in acrylamide gels have been developed. Awdeh (1969) used Bromophenol Blue dye and Chrombach et al. (1967) and Carrel et al. (1969) used Coomassie Brilliant Blue dye. These two stains are useful because they eliminate the need to remove the ampholytes from the gel before staining.

Wrigley (1968) and Catsimpoolas (1969) used Amidoschwarz 10 B and removed the ampholytes from the gel before the staining.

B.2. Column Electrophoresis

B.2.1. Development of Column Electrophoresis

Early studies (Foster and Schmidt, 1923; Foster and Schmidt, 1926; Williams and Waterman, 1929) demonstrated that "electrolysis" and "electrodialysis" might be useful techniques for the separation of substances with greatly different mobilities.

Consden et al. (1946) maintained that the electrophoretic separation of components with similar mobilities
would require some type of density gradient to stabilize the system against convection currents. These workers were able to separate a mixture of glutamic acid and aspartic acid by electrophoresis in silica gel.

Coolidge (1939) designed a column electrophoresis apparatus for the preparative fractionation of serum albumin and globulin. The device consisted of a straight, water-jacketed glass tube with electrode chambers at each end. Glass powder was packed into the tube to reduce convection currents. The porous nature of the glass powder permitted the elution of the protein zones after electrophoresis.

Brewer et al. (1946) tested various materials including sand, glass wool, cotton and glass beads to see whether or not they would be useful for the prevention of convection currents during electrophoresis. Butler and Stephen (1947) used asbestos fiber in their electrophoresis column. The asbestos column was divided into one-inch sections, each separated by filter paper barriers. The sections were removed from the apparatus after electrophoresis and were eluted to recover the sample.

Haglund and Tiselius (1950) designed a water cooled apparatus similar to the one designed by Coolidge (1939). These authors used glass powder as the stabilizing material.
The sample which was layered at the top of the column, was first fractionated electrophoretically and then the separated zones were eluted from the column.

Flodin and Porath (1954) recommended the use of starch for column electrophoresis. They claimed that starch has a low absorption affinity for most high molecular weight proteins. Starch was also recommended because it was found to give a low electroosmotic flow. Flodin and Kupke (1956) later found that cellulose treated with acid-ethanol was even more useful in column electrophoresis than was starch. The apparatus used by these workers consisted of a straight tube fitted with electrodes at each end of the tube. The separation of serum proteins by use of the apparatus was carried out in a two-step process. The proteins were first separated electrophoretically and then the separated zones were eluted from the column as they are in column chromatography.

Porath (1956) performed a series of experiments to determine the optimum condition for zone electrophoresis on vertical columns. He indicated that elution of the zones from the column after electrophoresis, was the main cause of zone spreading. He found that zone spreading is increased if the support material is not uniformly packed on the column. High flow rates during elution were also found to
contribute to zone spreading.

Porath (1956) described a method for the concentration of the starting zone in column electrophoresis. He showed that the thickness of the starting zone could be reduced if the conductivity of the sample zone was made lower than that of the surrounding buffer. This was accomplished by dilution of the buffer which contained the sample. Experiments in which the concentration of the starting zone was varied, indicated that the diffusion of the zones during electrophoresis was independent of the concentration of material in the starting zone. He warned that the concentration of material in the starting zone should be regulated so that conductivity of the starting zone does not exceed that of the surrounding buffer. If this were to happen, the front of the zone would spread due to the higher potential in front of the zone.

A major advance in the field of column electrophoresis came with the introduction of an apparatus with an arrangement for the continuous removal of the zones during electrophoresis (Porath et al., 1958). With this apparatus the separated zones were removed from the bottom of the column by a stream of buffer which flowed in the direction which was opposite to that of the electrophoretic migration. This
device was used by Gelotte et al. (1962) for the separation of human serum proteins. These workers used Sephadex G-25 as the anticonvection material.

Hjertén (1963) separated human serum into five fractions by electrophoresis on an agarose column. He claimed that the large pore size of the agarose suspension enabled the zones to be eluted from the column after electrophoresis with very little mixing of the zones. He indicated that the electrophoretic migration velocities of the proteins on agarose suspensions were the same as they were in free solution.

Hochstrasser et al. (1963) developed an electrophoresis apparatus in which the column was cooled by running water. The protein fractions were recovered from the column by means of a "Serpentine type" elution chamber located at the top and at the bottom of the column.

Porath (1964a, 1964b) designed a column which used a more efficient cooling system than the one designed by Hochstrasser et al. (1963). The protein zones were eluted from the bottom of the column by a stream of buffer which flowed in a direction opposite to that of the electrophoretic migration.

Ornstein (1964) and Davis (1964) introduced a method to improve the resolving power of acrylamide gel electrophoresis.
The method involved the concentration of the starting zone prior to electrophoresis by the use of a discontinuous buffer system.

Jovin et al. (1964) used the method introduced by Ornstein and Davis for the preparative separation of proteins. These authors used an annular column of acrylamide gel, the inner and outer surfaces of which were cooled to maintain a uniform temperature across the column. A discontinuous buffer system which was similar to that suggested by Ornstein and Davis was used to concentrate the starting zone prior to the electrophoretic separation of the sample. The electrophoretic fractions were recovered as they emerged from the column, in a stream of buffer which was introduced at the bottom of the column. The resolution of protein with this device was found to be better than that which could be achieved by electrophoresis on cellulose columns. The capacity of the acrylamide column (Jovin et al., 1964) however, was lower than that of the cellulose column introduced by Porath (1964a, 1964b).

Hollmen and Kulonen (1966) described an apparatus which was similar to that described by Hochstrasser et al. (1963), in which starch gel was used as the supporting medium.
Bergrahm and Harlestam (1968) described an apparatus which featured an efficient elution chamber for the removal of the fractions from the bottom of the column during electrophoresis. Buffer was forced through a ring of porous plastic located at the base of the column. The buffer left the elution chamber through an opening at the bottom of the chamber carrying with it the emerging zones.

B.2.2. Concentration of the Starting Zone

Haglund and Tiselius (1950) described a technique in which sharp zone boundaries could be obtained in column electrophoresis. The method involved the formation of a concentration gradient of buffer by continuous dilution of buffer in the apparatus. The sample under the influence of an electric field was made to migrate from a region of low buffer concentration to one of higher buffer concentration. Regions in the more concentrated buffer had a higher conductivity and were subjected to a lower potential, than were those regions of lower buffer concentration. As a result, the front of the protein zone was always exposed to a lower potential gradient than was the back of the zone. The front of the zone which was subjected to a low potential gradient, moved more slowly than did the back of the zone which was
subjected to a higher potential gradient. This caused the
zone to concentrate.

This technique was modified by Porath (1956) for the
concentration of the starting zone in column electrophoresis.
Porath found that the starting zone could be concentrated if
the sample was dissolved in more dilute buffer than was used
in the remainder of the column. Under an applied electric
field a region in the diluted buffer had a lower conductivity
and was subjected to a higher potential gradient than that
of the more concentrated buffer in front of the sample.
Under these conditions the sample became more concentrated
as it migrated from the dilute buffer into the more concen-
trated buffer.

Hjertén (1959) used the same principle to concentrate
the starting zone in paper electrophoresis. He placed a
dialysis bag containing concentrated buffer in contact with
the paper in the region where the sample was applied. The
conductivity in the region of the bag was higher than that
in the rest of the paper, and therefore the potential grad-
ient was correspondingly lower. The proteins were concen-
trated when they migrated into the region of lower potential
gradient.

Smithies (1955) noted that the starting zone was
automatically concentrated in starch gel zone electrophoresis. He claimed that the protein sample, which was introduced into a slot in the starch gel, concentrated when the proteins entered the gel. Sharpening of the zone occurred because the proteins had a greater mobility in the slot than they did in the gel.

Davis and Ornstein (1959) introduced a method to concentrate and stack the proteins in a sample prior to their electrophoretic separation. The technique was based on a principle that was described by Kohlrausch (1897) and was used by Kendall (1928) for the separation of isotopes. The method which was discussed in detail by Ornstein (1964) and Davis (1964) involved the use of a discontinuous buffer system. These authors referred to the technique as "disc electrophoresis" partly because a discontinuous buffer system was used and partly because the concentrated proteins appeared as thin discs in the starting zone. The buffer zones were arranged in such a manner that the protein sample was bordered in front by a zone of fast moving chloride ions and in back by a zone of slow moving glycinate ions. Under an applied electric field the sample anions were concentrated to form very thin discs, one stacked on top of the other in order of decreasing mobility, with the last disc followed
immediately by glycinate ions. The concentration of sample in each of the discs was controlled by the "Kohlrausch Regulating Principle" (Ornstein, 1964) given below:

\[
\frac{[A]}{[B]} = \frac{(X_b^-)(C_a^-)}{(X_a^-)(C_b^-)} = \frac{(\mu_a^-)(Z_b^-)}{(\mu_b^-)(Z_a^-)} \frac{(\mu_b^-) - (\mu_{r+})}{(\mu_a^-) - (\mu_{r+})}
\]

where subscripts \( a^- \), \( b^- \) refer to the anions of substances A and B respectively, subscript \( r^+ \) refers to the buffer cation which is common to both solutions. \( Z \) is the electric charge on the ion, \( C \) is the concentration of the ion and \( \mu \) is the mobility of the ion. \( X \) is the fraction of dissociation and can be calculated from the Henderson-Hasselbach equation given below:

\[
\text{pH} = \text{pK}_a - \log_{10} \left[ \frac{1}{X} - 1 \right]
\]

The authors (Ornstein, 1964; Davis, 1964) observed that all the discs in the stacked sample zone migrated at the same velocity in an increasing potential gradient. This increase in the potential gradient caused the trailing disc of ions which had the lowest effective mobility (mobility \( \times \) degree of dissociation) to migrate at the same velocity as the leading disc which had the highest effective mobility. The potential gradient formed automatically in order that the same current would be maintained throughout the column. The stacked discs did not separate from each other until the
sample zone had migrated out of a region of low pH and into a region of higher pH. At the higher pH value the effective mobility of glycine increased sufficiently so that the glycinate ions were able to overtake the sample zone. When this occurred the stacked discs (sample) were able to separate according to their electrophoretic mobility.

Reisfeld et al. (1962) modified this technique so that a sample of positively charged proteins could be stacked and concentrated prior to separation by zone electrophoresis. During the stacking phase, the sample was bordered in front by a zone of fast moving potassium ions and in back by a zone of slow moving $\beta$-alanine cations. The effective mobility of the trailing $\beta$-alanine cations increased when the cations passed from a region of high pH into a region of low pH. This allowed the $\beta$-alanine cations to "overrun the stacked sample zone so that the stacked zone could separate electrophoretically.

Racussen and Foote (1968) described a method in which a mixture of positively and negatively charged proteins could be concentrated and stacked prior to electrophoresis. The method was essentially a combination of the techniques used by Ornstein (1964) and Reisfeld et al. (1962).
C. EXPERIMENTAL

C.1. Materials and Methods

C.1.1. Preparation of Buffers for Column Electrophoresis

C.1.1.a. Tris-Borate Buffer pH 8.35

Tris (hydroxymethyl) aminomethane (30 gm, Fisher Scientific Co., N.J.) and boric acid (15.5 gm) were dissolved in distilled water and the volume adjusted to 10 liters. Sodium azide (0.5 gm, Fisher Scientific Co., N.J.) was added as a preservative. The reaction of the solution was pH 8.35.

C.1.1.b. Tris-Chloride Buffer pH 7.5

Tris (hydroxymethyl) aminomethane (3.0 gm) was dissolved in distilled water (700 ml) and the reaction of the solution was adjusted to pH 7.5 with HCl (0.1N; approx. 200 ml). The volume of the solution was adjusted to 1 liter with distilled water. Sodium azide (0.05 gm) was added as a preservative.

C.1.1.c. Tris-Chloride Buffer pH 8.15

Tris (hydroxymethyl) aminomethane (3.0 gm) was dissolved in distilled water (700 ml) and the reaction of the solution was adjusted to pH 8.15 with HCl (0.1N; approx.
130 ml). The volume of the solution was adjusted to 1 liter with distilled water. Sodium azide (0.05 gm) was added as a preservative.

C.1.1.d. Tris-Chloride Buffer pH 8.3

Tris (hydroxymethyl) aminomethane (3.0 gm) was dissolved in distilled water (700 ml) and the reaction of the solution was adjusted to pH 8.3 with HCl (0.1N; approx. 105 ml). The volume of the solution was adjusted to 1 liter with distilled water. Sodium azide (0.05 gm) was added as a preservative.

C.1.1.e. Tris-Chloride Buffer pH 8.9

Tris (hydroxymethyl) aminomethane (3.0 gm) was dissolved in distilled water (700 ml) and the reaction of the solution was adjusted to pH 8.9 with HCl (0.1N; approx. 39 ml). The volume of the solution was adjusted to 1 liter with distilled water. Sodium azide (0.05 gm) was added as a preservative.

C.1.1.f. Tris-Citrate Buffer pH 8.65

Tris (hydroxymethyl) aminomethane (30 gm) and citric acid (3.45 gm) were dissolved in distilled water and the volume adjusted to 10 liters. Sodium azide (0.5 gm) was added as a preservative. The reaction of the solution was pH 8.65.

C.1.1.g. Tris-Glycine Buffer pH 8.05

Tris (hydroxymethyl) aminomethane (30 gm) and glycine
(644 gm) were dissolved in distilled water and the volume of the resultant solution was adjusted to 10 liters. Sodium azide (0.5 gm) was added to the solution as a preservative. The reaction of the solution was pH 8.05.

C.1.1.h. **Tris-Glycine Buffer pH 8.15**

Tris (hydroxymethyl) aminomethane (30 gm) and glycine (390 gm) were dissolved in distilled water and the volume of the resultant solution was adjusted to 10 liters. Sodium azide (0.5 gm) was added as a preservative. The reaction of the solution was pH 8.15.

C.1.1.i. **Tris-Glycine Buffer pH 8.3**

Tris (hydroxymethyl) aminomethane (30 gm) and glycine (144 gm) were dissolved in distilled water and the volume adjusted to 10 liters. Sodium azide (0.5 gm) was added as a preservative. The reaction of the solution was pH 8.3.

C.1.2. **Preparation of Dye Mixture**

Equal volumes of the following indicator solutions were mixed together to form the Dye mixture:

Bromocresol Purple (0.1 gm) was dissolved in a solution of sodium hydroxide (0.1N, 18.5 ml) and the volume was adjusted to 250 ml with distilled water.

Bromophenol Blue (0.1 gm) was dissolved in a solution
of sodium hydroxide (0.01N, 14.9 ml) and the volume was adjusted to 250 ml with distilled water.

Phenol Red (0.1 gm) was dissolved in a solution of sodium hydroxide (0.01N, 28.2 ml) and the volume was adjusted to 250 ml with distilled water.

Methyl Red (0.02 gm) was dissolved in 60 ml of ethanol and the volume was adjusted to 100 ml with distilled water.

Thymol Blue (0.1 gm) was dissolved in a solution of sodium hydroxide (0.01N, 21.5 ml) and the volume was adjusted to 250 ml with distilled water.

C.1.3. Preparation of Water-Soluble Pea Protein

C.1.3.a. Tris-NaCl Buffer (pH 8.0) for Extraction of Pea Protein

Tris (hydroxymethyl) aminomethane (24.23 gm) was dissolved in distilled water (3 liters) and the reaction of the solution was adjusted with HCl (1.0N; approx. 100 ml) to pH 8.0. Sodium chloride (46.76 gm) and sodium azide (0.5 gm) were added and the volume of the solution was adjusted to 4 liters with distilled water.

C.1.3.b. Isolation of Water-Soluble Pea Protein

Dried peas (Sterling variety) were ground with the aid of a hammer mill to pass through a sieve (U.S. Std. No. 18).
The ground peas (300 gm) were then extracted (occasional stirring) with tris-NaCl buffer (pH 8.0; 4 liters) for 24 hours at 5°C. The slurry was then passed through linen cloth to remove most of the insoluble solids and the filtrate was centrifuged (10,000 rpm; No. 872 angle head, International Centrifuge) for 30 minutes. The clear supernatant was dialyzed against tap water (15°C) for 24 hours and against seven changes of distilled water (5°C) for 60 hours. The precipitate was recovered by centrifugation (30 minutes, 10,000 rpm; No. 872 angle head, International Centrifuge) and the centrifugate which contained the water soluble fraction was lyophilized and the resultant product was stored (-15°C) in tightly stoppered bottles.

C.1.4. Analytical Disc Electrophoresis

Analytical disc electrophoresis was carried out by the method described by Davis (1964). A disc electrophoresis apparatus similar to the one described by Davis (1964) was constructed. A drawing of the apparatus which was constructed is presented in Figure I. At the completion of electrophoresis the gels were removed from the tubes and were stained for one hour in a 1% solution of Buffalo Black NBR (Allied Chemical, Morristown, N.J.) in acetic acid (7%
Disc Electrophoresis Apparatus
aqueous solution). The gels were then placed in a destaining rack (Figure II) and the rack was placed in an aqueous solution of acetic acid (7%) between two carbon electrodes. A potential difference of 60 volts (0.5 amps) was applied between the electrodes to remove the background stain from the gels. The acetic acid solution was changed every 30 minutes until the gels were destained (2 hours). The gels were stored in acetic acid (7% aqueous solution) until they were photographed.

C.1.5. Disc Isoelectric Focusing

Disc isoelectric focusing was carried out by a modification of the methods described by Fawcett (1968), Wrigley (1968) and Catsimpoolas (1969).

Stock Solutions A and B were prepared in advance and could be stored (50°C) up to six months in dark brown bottles without apparent deterioration. Solution C was prepared daily. The three solutions were prepared as follows:

Stock Solution A - Acrylamide (15 gm) and N,N'-methylenebisacrylamide (0.6 gm, Eastman Kodak Company, Rochester, N.Y.) were dissolved in distilled water and the volume of the resultant solution was adjusted to 100 ml with distilled water.
Figure II
Destaining Rack
Stock Solution B - N,N,N',N'-tetramethylethylenediamine (0.20 ml, Eastman Kodak Company, Rochester, N.Y.) and riboflavin (0.003 gm) were dissolved in distilled water and the volume of the resultant solution was adjusted to 100 ml with distilled water.

Solution C - Ampholine (1.8 ml, 40% solution, LKB Produkter AB, Sweden) was dissolved in distilled water (10.2 ml).

The protein sample (0.5–1 mg) was placed in a test tube and was dissolved in stock Solution A (1.0 ml/test tube). Stock Solution B (1.0 ml/test tube) and Solution C (1.0 ml/test tube) were then added and the contents of the test tube were mixed. Glass tubes (5 mm ID, 14 cm long) were placed in a polymerization rack (Figure III) and each tube was filled to within 1 cm from the top with acrylamide solution. A miniscus was allowed to form at the top of the acrylamide gel and this was used as a reference mark to distinguish one end of the gel from the other. The acrylamide solution in the tubes was photopolymerized by exposure to fluorescent light for 45 minutes. The tubes were then removed from the polymerization rack and placed in an apparatus identical to the one used for disc electrophoresis (Figure I). The end of the gel tube with the miniscus was arbitrarily placed in such a way that it made contact with the solution.
Figure III
Polymerization Rack
in the upper buffer reservoir. The upper buffer reservoir (cathode) was filled with an aqueous solution of ethylenediamine (5%) and the lower buffer reservoir (anode) was filled with an aqueous solution of phosphoric acid (5%). A potential of 200 volts (constant voltage) was applied for the first hour. It was then increased to 450 volts and maintained at this level until the current had decreased to 0.5 ma per tube and had remained at this level for at least two hours.

The gels were removed from the tubes by rimming with a hypodermic needle under a stream of water as described by Davis (1964). The gels were stained (Awdeh, 1969) for one hour with a Bromophenol Blue solution [0.2% Bromophenol Blue in a mixture of ethanol, water and glacial acetic acid (50: 45:5)]. The gels were destained overnight in several changes of a mixture containing ethanol, water and glacial acetic acid (30:65:5) and were then photographed. When the pH gradient in the gel was to be determined the gel was not stained, but was cut up into 1 cm segments. Each segment was allowed to stand overnight in 10 ml of distilled water and the pH of the extract was measured. The reaction of the extract obtained from each segment gave the approximate pH in that particular segment of gel. The pH value which corresponded
to the position of a stained protein band was used as a measure-
ment of the approximate isoelectric point of that protein.

C.1.6. Molecular Weight Estimation
by Gel Filtration

Molecular weights were estimated by a modification of
the methods described by Andrews (1964), Laurent and Killan-
der (1964), Andrews (1965) and Granath and Kvist (1967).

Bio-Gel P-200 (100-200 mesh, Cal Biochem, California)
or Sephadex G-75 (40-120 μ meter pore size, Pharmacia Fine
Chemicals, Sweden) was suspended in potassium phosphate
buffer (0.05M, pH 6.95) which contained 0.1M NaCl and 0.02%
sodium azide, and this mixture was allowed to stand for two to
three days. The fine particles were removed by decantation
and the remainder of the suspension was placed in a glass
tube (2.8 cm I.D.). The height of the gel column was approx-
imately 42 cm. A piece of nylon screen (40 micron pore size,
Pharmacia Fine Chemicals) was placed on the top of the column.
Buffer was allowed to pass (15 ml/hr) through the column for
24 hours to ensure that the column was packed uniformly. At
the beginning of an experiment the level of buffer was allowed
to drop to the surface of the column and an aliquot (1 ml) of
buffer which contained the sample (5-10 mg) was placed on
top of the column. The volume of buffer required to elute
the sample from the column was taken as the elution volume. The absorbancy (280 m\(\mu\)) of the effluent from the column was monitored with the aid of a Uviscan III Continuous Ultraviolet Scanner (Buchler Instruments, N.J.) and a Recti-ritter recorder (Texas Instrument, Texas). \(K_a\) values for each of the samples were calculated from their elution volumes using the following equation (Laurent and Killander, 1964): 

\[
K_a = \frac{V_e - V_o}{V_t - V_o}
\]

where

\(V_e = \) elution volume of sample

\(V_o = \) void volume of column (\(V_e\) for Blue Dextran)

\(V_t = \) volume occupied by column

The void volume of the column was the elution volume of a sample (5 mg) of Blue Dextran (Pharmacia Fine Chemicals). The column was calibrated periodically with a mixture (5 mg each) of protein standards (glucagon, mol. wt. 3,500; ribonuclease, mol. wt. 13,700; chymotrypsinogen, mol. wt. 25,000, ovalbumin, mol. wt. 45,000; aldolase, mol. wt. 158,000).

Figure IV shows the calibration curves which were obtained using Sephadex G-75 and Bio-Gel P-200 columns.

C.2. Column Electrophoresis Apparatus

C.2.1. Introduction

Zone electrophoresis on gel supports have been used
Calibration Curves for Molecular Weight Estimation
successfully for the small scale or analytical fractionation of protein mixtures (Smithies, 1955; Ornstein, 1964; Davis, 1964). Various attempts have been made to apply the technique of zone electrophoresis to the separation of proteins on a preparative scale (Hochstrasser et al., 1963; Porath, 1964a, 1964b; Jovin et al., 1964; Bergraham and Harlestam, 1968). Preparative separations unlike analytical separations, required that the fractions be recovered. It is important in a preparative method that relatively large amounts of material can be fractionated and recovered.

The capacity of an apparatus for column electrophoresis depends on a number of factors including the cross sectional area of the column which in turn is limited by the efficiency of the cooling system. Hochstrasser et al. (1963) and Jovin et al. (1964) used an annular column which was cooled on the inside and outside and hence excess heating and temperature gradients were reduced. The capacity of the electrophoresis apparatus also depends on the type of anticonvection material which is used to make the column. Porath (1964a) claimed that with a cellulose column he was able to separate in one run 10 grams of material. Jovin et al. (1964) fractionated approximately 40 mg of hemoglobin on a column of polyacrylamide. The authors indicated that resolution was superior with the polyacrylamide gel column than
with the cellulose column. The better resolution with acryl-
amide as compared to that with cellulose columns was attri-
buted in part to the narrow starting zone that was obtained
with polyacrylamide. Smithies (1955) noted that the start-
ing zone was concentrated when the sample migrated from the
solution into the starch gel. Ornstein (1964) noted that
the starting zone was concentrated when the sample migrated
from an acrylamide gel with a large pore size into one with
a small pore size. Ornstein (1964) and Davis (1964) used a
discontinuous buffer system to concentrate the starting zone
and to stack the protein components in order of decreasing
mobility. They showed that a concentrated and stacked sam-
ple could be fractionated more efficiently than one which
was not stacked and concentrated at the outset.

An effective column electrophoresis apparatus should
have an efficient elution chamber to recover the separated
components. A simple and efficient elution system was de-
signed by Jovin et al. (1964) for washing the zones from the
bottom of an acrylamide column. They used a stream of buffer
which entered at the circumference of the column, to wash
the zones up the outlet tube which was located at the centre
of the annular column. Bergraham and Harlestam (1968) used
a similar type of arrangement in their apparatus in which
a simple (non-annular) column of powdered cellulose was used.

The aim of the present study was to design and construct an apparatus for the electrophoretic fractionation of proteins on a preparative scale.

C.2.2. Description of Apparatus

Figure V shows a diagram of the apparatus which was constructed. The tube (A; 24" x 2" OD; acrylic plastic) which supports the column is cooled by a water jacket (B; 3" ID) and an inner cooling tube (C; 3/4" OD). The bottom of the inner tube (C) is attached to a perforated disc (D; 1 7/8" dia.; 1/4" thick) and this disc has a fine (pore size 40 μ) nylon screen (Pharmacia Fine Chemicals, Uppsala, Sweden) secured to its upper surface by means of O-rings (Q, R). The holes (1 mm dia.; 0.5 mm apart) through the perforated disc are located in the area between the inner cooling tube and a point 2 mm from the outer circumference. The perforated disc sits on a plastic ring (E) and this has stretched over its upper surface a semi-permeable membrane held in place by means of an O-ring (S). The bottom surface of the perforated disc next to the membrane was recessed 0.25 mm to within 2 mm of the periphery. This forms a very narrow elution chamber. An annular well (T; 1.5 mm
Figure V
Column Electrophoresis Apparatus
deep, 1 mm wide) was cut into the bottom surface of the perforated disc (D) 1.5 mm from the periphery. This well is connected by means of holes, to a narrow space between the wall of tube A and the side of the disc. This space, which is contained by the two O-rings R and S, forms a buffer compartment for the elution buffer. Plastic tubing (P) inserted through the wall of tube A in the region between O-rings R and S, supplies buffer to this compartment. The elution buffer flows into the annular well (T) and then sweeps across the bottom of the disc (D) and exits through the tube (N; 1/16" ID) at the centre of the disc. The top end of tube N is connected to a piece of Tygon tubing (1/16" ID). The other end of the Tygon tubing is placed one foot below the level of the disc (D) in order to maintain a slight negative pressure inside the elution chamber.

A plastic ring (F) which has a concave upper surface is fitted into the bottom of tube A. Tube G is attached to this plastic ring. A hole which is made in the two plastic end-plates (H,I) enable tubes C and G to pass through the plates. The plates (H,I) are grooved to fit the ends of tubes A and B and are clamped together by four brass bolts. O-rings are used to make leak-proof seals between the end-plates and the tubes.
The apparatus was mounted over a reservoir containing three liters of buffer. An electrical current was supplied by electrode J at the top of the column and electrode K which was wrapped around tube G and dipped into the buffer reservoir. The sample applicator (O) consisted of a nylon tube (1/8" OD) which could be raised or lowered through a hole in the top plate (H). One end of this applicator was shaped in the form of a ring which had holes drilled around its circumference. It was possible by using this device to distribute the sample evenly on the surface of the column.

C.2.3. Preparation of Column

The apparatus was inverted and the lower section (L) of tube A was filled completely with buffer through tube G. A piece of filter paper was held over the end of this tube and the apparatus was placed over the buffer reservoir with tube G dipping into the buffer. Care was taken so that no air entered the lower section (L) of tube A. A portion (40-50 ml) of buffer was placed in tube A through entry port M. Some of this buffer was drawn through tubes N and P after which the tubes were closed by means of pinch clamps.

A tight fitting sleeve made from a piece of rubber tubing was placed around the sample applicator (O) so that an air-tight seal was made between it and the top plate (H).
Similarly an O-ring around the centre hole in plastic plate H formed a seal between the plate and the cooling tube (C).

The end of the sample applicator (0) was closed and a vacuum was applied to entry port M to remove air bubbles that might have been trapped in the perforated disc (D). A layer of sand (1/4 inch) was then spread over the nylon screen which was attached to the disc (D). The exit tube was then opened and a slurry of the supporting gel (Sephadex, or Bio-Gel, or cellulose powder) in buffer was poured into tube A through entry port M and was allowed to settle to form a column of approximately 11 inches. Three or four void volumes of buffer, introduced through the sample applicator tube (0), was allowed to run through the column to ensure that the column was thoroughly packed. The flow rate of buffer through the column was regulated by either raising or lowering the buffer reservoir which was attached to the sample applicator tube (0). Tap water (15°C) was passed through the cooling chambers at a rate of two liters per minute.

C.2.4. Operation of Column Electrophoresis Apparatus

After the column had been washed with buffer, excess buffer at the top of the column was removed by lowering the
sample applicator (O) to the surface of the gel and by the subsequent use of suction. Last traces of buffer were drained through the exit tube (N). The sample which was dissolved in buffer was then placed on top of the column by means of the applicator and this was allowed to move into the gel by opening the exit tube (N). The exit tube (N) was then closed and the upper portion of tube A was filled with buffer so that electrode J made contact with the buffer. A potential (400-600 volts) was maintained until the Bromophenol Blue tracking dye which was added to the sample had migrated to within one inch of the elution chamber. At this time the buffer in the upper reservoir was replaced with fresh buffer. A piece of rubber tubing was attached to the sample applicator (O) and the other end of the tubing was placed in a beaker of buffer. The level of buffer in the beaker was adjusted so that it was even with the perforated disc (D). Suction was applied to the upper buffer reservoir through entry port M, in order to replace the air in the sample applicator with buffer from the beaker. Entry port M was then closed and hence the pressure in the upper reservoir was able to equilibrate with the atmosphere only through the sample applicator tube (O). Pressure in the upper reservoir from gas which was produced at electrode J
was automatically released as a result of this arrangement.

With exit tube N open, the flow of buffer through the column was regulated by raising or lowering the level of buffer in the beaker. The flow rate of buffer down through the column was maintained at 10 ml/hour throughout the elution period. The elution buffer was pumped through tube P at a rate of 80 ml/hour to wash the zones as they emerged from the bottom of the column. Gas that accumulated in the upper buffer reservoir was removed periodically during the experiment through the application of suction to the entry port (M).

C.3. Preliminary Experiments on Column Electrophoresis

C.3.1. Effect of Column Support

A series of experiments was performed in which a tris-glycine buffer (pH 8.3) was used in conjunction with a number of different supporting media (powdered cellulose, Munktell's cellulose, Grycksbo Pappersbruk AB, Sweden; Sephadex G-100, Sephadex G-200, Bio-Gel P-150, Bio-Gel P-200, Bio-Gel P-300) for the fractionation of water soluble pea protein. The column (11 inch) was packed in the usual manner and a sample (75 mg) of protein and two drops of Bromophenol Blue
dye were dissolved in tris-glycine buffer (7.5 ml; pH 8.3) and placed on the column. The current (35 ma, 550 volts) was applied and the elution was started when the blue indicator dye was one inch from the elution chamber. Figure VI shows typical elution profiles which were obtained with the different supporting media. It will be noted that the greatest number of peaks was obtained with Bio-Gel P-300, the gel with the largest pore size.

C.3.2. Cooling Capacity of the Column

Experiments were carried out to test the cooling efficiency of the column and to examine the electrophoretic separation of the protein sample under different conditions of column temperature. The temperature of the column was governed by the temperature of the cooling water. The objective was to find the lowest column temperature which would give the best protein separation. High temperatures were to be avoided because of the risk of protein denaturation and of bacterial contamination.

An electrophoresis column (11 inch) was prepared using Bio-Gel P-300 and tris-glycine buffer (pH 8.3). A sample of water soluble pea protein (75 mg in 7.5 ml tris-glycine buffer, pH 8.3, containing two drops of Bromophenol
Figure VI
Elution Profiles (Bio-Gel P-300, Bio-Gel P-200, Bio-Gel P-150, Sephadex G-200, Sephadex G-100, Powdered Cellulose) of Water Soluble Pea Protein by Column Electrophoresis
Blue dye solution) was placed on the column and the potential difference between the electrodes was maintained at 550 volts (approx. 35 ma). In four separate trials the temperature of the cooling water was 5°C, 10°C, 15°C and 20°C. It was observed that the protein separation was impaired with the two lowest cooling temperatures. The best separations were achieved when the cooling water was maintained at 15°C or 20°C. There was no apparent difference in the electrophoretic separation when temperatures of 15°C and 20°C were employed. It is possible that the lower cooling temperatures reduced the pore size of the Bio-Gel beads and this altered in an adverse manner the filtering properties of the column.

The next experiments were performed to determine the maximum electrical output that could be used without producing a temperature gradient in the column. In an electrophoretic separation, long running times are accompanied by excessive diffusion of the zones which reduces the efficiency of the separation. High potential gradients decrease the running time. The magnitude of the potential gradient is limited, however, by the cooling efficiency of the apparatus.

An electrophoresis column (Bio-Gel P-300, 11 inch) was prepared using a continuous tris-glycine buffer (pH 8.3) system. A sample of dye (10 drops dye mixture in 7.5 ml
tris-glycine buffer, pH 8.3) was used in these experiments since it was desired to observe the zones during the separation. The temperature of the cooling water was maintained at 15°C in each of the experiments. The following potentials and corresponding currents were used: 500 volts (29 ma), 600 volts (35 ma), 800 volts (47 ma) and 1,000 volts (58 ma). It was observed that: (a) the rate of migration of the dyes increased as the potential gradient increased and (b) a potential gradient larger than 600 volts (35 ma) caused the dye zones to become dish shaped as they approached the bottom of the column. It is possible that at the higher voltages the part of the dye zone which was farther away from the cooling surface moved slightly faster than did that which was nearer the cooling surfaces. This suggested that a temperature gradient across the column was created when the power output was greater than 21 watts (600 volts x 35 ma = 21 watts).

C.4. Concentration of the Starting Zone

C.4.1. Introduction

The purpose of this study was to devise satisfactory conditions for the concentration of the starting zone prior to electrophoresis on powder columns. Various kinds and
combinations of buffers were used in this study. In these experiments the term continuous buffer system refers to a system in which one continuous buffer phase extends from the top buffer reservoir through the column, to the bottom buffer reservoir. The term discontinuous buffer system refers to one in which the buffer in the column or in one section of the column is replaced by a different buffer. The buffer in this section of the column is referred to as the discontinuous buffer, while the buffer in the remaining parts of the column and in the buffer reservoirs, is referred to as the continuous buffer.

C.4.2. Control Experiment

An experiment was performed to demonstrate the degree to which large samples of material could be separated by column electrophoresis, without first concentrating the starting zone. The results from this experiment provided a comparison for studies in which different methods were used to concentrate the starting zone.

A Bio-Gel P-300 column (11 inch) was prepared using a continuous buffer system (tris-glycine, pH 8.3). A sample of dye mixture (20 drops in 30 ml of tris-glycine buffer, pH 8.3) was placed on the column. Photographs (Figure VII)
Photographs Showing the Electrophoretic Separation of the Dye Mixture Without First Concentrating the Starting Zone

A:– At start, current off

B:– Current on (35 ma, 550 volts) 20 min.

C:– Current on (35 ma, 550 volts) 40 min.

D:– Current on (35 ma, 550 volts) 4 hours
were taken before the current was turned on and after 20 minutes, 40 minutes and 3 hours of electrophoresis (35 ma, 550 volts). It will be noted that only four of the five dyes had separated after three hours and that the dye zones were broad and not very straight.

C.4.3. Method No. 1 (Ornstein's Method for Disc Electrophoresis)

Ornstein (1964) and Davis (1964) used a combination of buffers to concentrate and stack the sample starting zone in disc electrophoresis. These buffers were used in such a manner that the stacked starting zone began to separate only after the sample had migrated from the low pH buffer of the large pore gel into the higher pH buffer of the small pore gel. Ornstein (1964) found that the sample zone was stacked when the glycinate ions from the upper buffer reservoir were made to trail the sample zone. The proteins remained stacked and were unable to separate from each other until the trailing glycinate ions were made to overtake the stacked sample zone. The effective mobility (mobility x degree of dissociation) of the glycinate ions was regulated by adjustment of the pH of the tris-glycine buffer. At pH 8.3 the effective mobility of glycine was -0.5 mobility units (1 mobility unit=10^{-5} volts/cm^2/sec). At pH 9.5 the effective
mobility of glycine was -7.5 mobility units. Ornstein found that at pH 9.5 the effective mobility of glycine was greater than that of the stacked protein zones. At this pH value the glycinate ions over-ran the sample zone and this allowed the stacked proteins to separate from each other. An interesting feature of Ornstein's buffer system is that the pH of the tris-glycine buffer is regulated by the pH of the tris-chloride buffer ahead of it. The author found that the reaction of the tris-glycine buffer was pH 9.5 when the reaction of the tris-chloride buffer ahead of it was pH 8.9.

In the following experiment a Bio-Gel P-300 column (11 inch) was prepared using a tris-chloride buffer (pH 8.9). Tris-glycine buffer (pH 8.3) was placed in the lower buffer reservoir. A sample of water soluble pea protein [75 mg, in 7.5 ml tris-chloride buffer (pH 8.9) containing 2 drops of dye mixture] was placed on the column. Tris-glycine buffer (pH 8.3) was then added to the upper buffer reservoir. The current was maintained at 38 ma (constant current, approx. 500 volts) throughout the experiment. It was observed that the dye zone in the sample was concentrated to a very thin band (approx. 1/16 inch thick) and it remained concentrated as it moved down the column. The sample was eluted from the bottom of the column when the dye zone had
migrated to within one inch of the elution chamber. The elution profile of the sample is given in Figure VIII. The dye zone was eluted from the column at the same time as the first protein peak. This indicated that this protein zone was probably stacked together with the dye mixture. The sharpness of the first peak showed that this particular protein had been concentrated in a similar manner to the tracking dye. It was assumed that the sample of protein would concentrate in this fashion if the mobility of the protein was greater than that of glycinate ions. The protein sample would then be trapped between the leading chloride zone and the trailing glycinate zone.

Ornstein (1964) who used a similar buffer system in disc electrophoresis, found that the proteins were slowed down sufficiently by the acrylamide column to allow the glycinate ions from the upper buffer reservoir to over-take the entire sample zone. As a result the stacked zone was able to separate into its different components. In the present experiment the nature of the Bio-Gel column was such that the mobility of the proteins were not sufficiently low to enable the glycinate ions to overtake the entire protein zone. The width of the second peak (Figure VIII) which was much greater than that of the first peak, indicated that
Elution Profile (Column Electrophoresis, Bio-Gel P-300) of Water Soluble Pea Protein by Use of Buffer System Devised for Disc Electrophoresis
it was not concentrated as was the first peak. This suggested that the mobility of the proteins in the second peak was less than that of the glycinate ions. Under these conditions the proteins in the second peak were able to separate from the rest of the proteins which were stacked and concentrated in peak No. 1.

The results of this experiment indicate that some of the components in the protein sample migrate too fast through the Bio-Gel column to allow the glycinate ions to over-take the entire sample zone. It was concluded that the discontinuous buffer system used by Ornstein (1964) and Davis (1964) in disc electrophoresis was not satisfactory for column electrophoresis under the present set of conditions.

C.4.4. Method No. 2 (Dilution of Buffer in the Starting Zone)

An attempt was made to use the method described by Porath (1956) to concentrate the starting zone. A column (Bio-Gel P-300, 11 inch) was prepared using a continuous buffer system (tris-glycine, pH 8.3) and then 30 ml of diluted buffer (5 ml tris-glycine buffer, pH 8.3 + 25 ml water) was placed on top of the column followed by another 30 ml of the same diluted buffer containing 20 drops of the dye mixture. The undiluted buffer (tris-glycine, pH 8.3) was
placed in the upper buffer reservoir and the current was
turned on and maintained at 25 ma (300 volts) for one hour,
after which time it was maintained at 35 ma (550 volts).

Figure IX shows a schematic representation of the
dye zone when it was first placed on the column (Figure IX,A)
and when it was at the point of maximum concentration (Fig-
ure IX,B). It will be noted that only the front edge of
the dye zone became concentrated. The trailing edge of the
zone remained diffuse at the point of maximum concentration.

C.4.5. Method No. 3 (Distilled Water
in Upper Buffer Reservoir)

During one of the preliminary experiments in which
dyes were being separated in a continuous tris-citrate
buffer system (pH 8.65), it was observed that the dye zone
became concentrated several hours after the current was
turned on. The high pH in the upper buffer reservoir after
the zone had concentrated, suggested that the concentrating
effect was related to the fact that the buffer in the upper
reservoir was depleted of citrate ions. This caused a
boundary to move through the column with a void of citrate
ions behind the boundary. The potential gradient behind
the boundary should be higher than that in front because of
the low conductivity in this region caused by the void of
Concentration of the Starting Zone by the Method Devised by Porath (Method No. 2)

A) Starting zone before current turned on

B) Starting zone at point of maximum concentration
CATHODE

A

B

DIL. BUFFER

CONC. BUFFER

SAMPLE
citrate ions. It seemed reasonable to assume that the dye zone had concentrated when this boundary had moved through it. An attempt was made to duplicate this concentrating effect by replacement of the tris-citrate buffer in the upper buffer reservoir, with distilled water at the beginning of the electrophoretic run.

A column (Bio-Gel P-300, 11 inch) was prepared using tris-citrate buffer (pH 8.65). The same buffer was placed in the lower buffer reservoir. The sample of dye (20 drops of dye mixture in 30 ml of tris-citrate buffer, pH 8.65) was placed on the column and distilled water was added to the upper buffer reservoir. The current was turned on at 11 ma (500 volts). It was observed that the trailing edge of the colored dye zone started to move faster than did the leading edge. After two hours the zone had concentrated to a very thin band (approx. 1/16 inch thick) and had moved a distance of one inch down the column. The distilled water in the upper buffer reservoir was then replaced by tris-citrate buffer (pH 8.65) and this caused the current to increase to 42 ma (500 volts). Approximately one minute after the tris-citrate buffer had been added to the upper buffer reservoir the concentrated dye zone began to separate into four separate zones. Figure X shows photographs that were
Figure X
Concentration of the Starting Zone by Use of Tris-Citrate Buffer (Method No. 3)

A) Dye zone 1 hour after current turned on (11 ma, 500 volts). Distilled water in upper buffer reservoir

B) Dye zone 2 hours after current turned on (11 ma, 500 volts). Distilled water in upper reservoir

C) Dye zone 1 minute after tris-citrate buffer added to upper reservoir. Current at 42 ma (500 volts)

D) Dye zone 7 hours after tris-citrate buffer added to upper reservoir. Current at 42 ma (500 volts)
taken during the electrophoretic run.

This experiment indicated that the starting zone could be concentrated if distilled water was substituted for the tris-citrate buffer (pH 8.65) in the upper buffer reservoir at the start of the experiment. Further experiments showed that the same technique could be used with other buffer systems such as tris-chloride buffer (pH 8.3). It was noted that this technique could not be used to concentrate the starting zone in a tris-glycine buffer (pH 8.3) or in a tris-borate buffer (pH 8.35). This suggested that the technique could be used only if the negatively charged buffer ions had an effective mobility (mobility x degree of dissociation) that was greater than that of the negatively charged sample ions. Table I gives the effective mobilities of some buffer anions at definite pH values (Longsworth, 1959; Ornstein, 1964). Above pH 8.0 the effective mobility of most proteins is between -0.5 and -7.5 mobility units (Ornstein, 1964). It should be pointed out that only buffer anions whose effective mobility is greater than -7.5 mobility units can be used with this technique.

In two separate experiments, samples (75 mg) of water soluble pea protein were fractionated (Bio-Gel P-300, 11 inch column) using (a) tris-citrate buffer (pH 8.65) and
(b) tris-chloride buffer (pH 8.3). In both experiments the starting zone was concentrated by the method just described. The elution profile (Figure XI) of the protein samples indicated that these buffer systems did not fractionate the sample on the Bio-Gel P-300 column as well as did the tris-glycine buffer (Figure VI).

Table I. Effective Mobilities of Buffer Ions

<table>
<thead>
<tr>
<th>Buffer Ion</th>
<th>Mobility (Mobility x Units)¹</th>
<th>Degree of dissociation</th>
<th>Effective Mobility (Mobility Units)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borate (pH 8.35)</td>
<td>-11.5</td>
<td>0.145</td>
<td>-1.7</td>
</tr>
<tr>
<td>Citrate (pH 8.65)</td>
<td>-12²</td>
<td>0.99</td>
<td>-12 (approx)</td>
</tr>
<tr>
<td>Chloride</td>
<td>-37</td>
<td>1</td>
<td>-37</td>
</tr>
<tr>
<td>Glycinate (pH 8.3)</td>
<td>-15.0</td>
<td>0.034</td>
<td>-0.5</td>
</tr>
<tr>
<td>Glycinate (pH 9.5)</td>
<td>-15.0</td>
<td>0.5</td>
<td>-7.5</td>
</tr>
<tr>
<td>Tris (pH 8.3)</td>
<td>+ 9.0</td>
<td>0.625</td>
<td>+ 5.6</td>
</tr>
</tbody>
</table>

¹Mobility Unit = 10⁻⁵cm²/volt/sec.

²Estimated value

C.4.6. Method No. 4 (Discontinuous Buffer System)

Experiments were performed to check the possibility
Elution Profile (Column Electrophoresis, Bio-Gel P-300) of Water Soluble Pea Protein

a) Tris-citrate buffer (pH 8.65)

b) Tris-chloride buffer (pH 8.3)
of using a discontinuous buffer system for the concentration of the starting zone. It will be recalled that Ornstein (1964) used a buffer system of this kind to concentrate the starting zone in disc electrophoresis.

A column (Bio-Gel P-300, 11 inch) was prepared using tris-glycine buffer (pH 8.3). Tris-glycine buffer (pH 8.3) was placed in the lower buffer reservoir. Before the sample of dye mixture was placed on the column, tris-chloride buffer (pH 8.3, 30 ml) was placed on top of the column and allowed to flow into the column. A dye solution (20 drops dye mixture in 30 ml of tris-chloride buffer, pH 8.3) was allowed to flow into the column behind the zone of tris-chloride buffer. The tris-chloride buffer formed a zone one and one-half inches thick at the top of the column. The dye sample occupied the top three-quarter inch of this zone. Tris-glycine buffer (pH 8.3) was then placed in the upper buffer reservoir and the current was turned on (25 ma, approx. 400 volts). It was observed that the trailing edge of the dye zone moved faster than the front of the zone and this led to the concentration of the dye zone. At the end of the first hour the sample had concentrated to a very thin straight band (approx. 1/16 inch thick) and had moved down the column approximately one inch. At this point the current was
increased to 32 ma (550 volts). The thin dye zone moved down the column a distance of about one-half inch before it began to separate into its five components. Two minutes after the zones began to separate the five dyes appeared to be stacked on top of the other. The distance between the colored dye zones increased as the bands moved down the column. The colored bands also diffused slightly as they migrated, and each had a thickness of about one-quarter inch when it reached the bottom of the column. The leading phenol red dye migrated down the column at the rate of approximately one and one-quarter inches per hour. Photographs were taken of the column during the electrophoretic run and are presented in Figure XII. The diagrams shown in Figure XIII represent the conditions which are assumed to exist in the column at different stages of the experiment and which led to the concentration of the dye zone and the electrophoretic separation of the dye components.

This method for the concentration of the starting zone and the one used by Ornstein (1964) for the concentration of the sample zone in disc electrophoresis are both based on the "Kohlrausch Regulating Principle" (Kohlrausch, 1897). They differ however, in the method used to introduce buffer anions into the concentrated starting zone. It was
Figure XII
Concentration of the Starting Zone by Use of a Discontinuous Buffer System (Method No. 4)

A) Dye zone at start

B) Dye zone 15 minutes after current turned on (25 ma, 400 volts)

C) Dye zone one hour after current turned on (25 ma, 400 volts)

D) Dye zone four hours after start. Current on at 32 ma (550 volts)
Diagram Showing the Conditions Which Exist in the Column When Starting Zone is Concentrated by a Discontinuous Buffer System (Method No. 4)

A) The tris-glycine buffer is in the lower section of the column and the discontinuous tris-chloride buffer is in the upper section above the tris-glycine buffer. The upper portion of the tris-chloride buffer contains the sample.

B) When the current was turned on, the chloride ions which have a high mobility (-37 mobility units; Ornstein, 1964), began to move past the sample ions and overtake the glycinate ions in the lower portion of the column. The high conductance of the tris-chloride buffer compared to that of the tris-glycine buffer, resulted in a low potential gradient in the region occupied by the tris-chloride buffer. The portion of the sample which was in the tris-chloride buffer was, therefore, in a low potential gradient and hence migrated slowly down the column. The high potential gradient that was created behind the chloride zone caused the sample to concentrate as the chloride zone moved through the sample. The trailing edge of the sample zone formed a sharp boundary because of the high potential gradient behind the sample zone.

C) After the chloride zone had moved through the sample zone, the sample zone became concentrated. The sample was trapped between the fast moving chloride zone ahead of it, and the slow moving glycinate zone behind it.

D) The sample zone has moved into the tris-glycine buffer that was placed in the lower portion of the column. Once the sample entered the tris-glycine buffer the dye components of the sample separated by electrophoresis.

E) The chloride zone has migrated ahead of the sample and the different species in the sample are able to separate according to their electrophoretic mobility.
essential to introduce buffer anions into the concentrated sample zone to enable the anionic species of the sample to separate electrophoretically. In Ornstein's system, buffer anions were introduced into the sample zone when the trailing zone of glycinate ions was made to over-take the sample zone. The effective mobility of the glycine increased from -0.5 to -7.5 mobility units when the reaction of the tris-glycine buffer was increased from pH 8.3 to pH 9.5. Ornstein found that the mobility of the glycinate ions must be increased to -7.5 mobility units in order for them to over-take the fastest moving proteins. As a result the electrophoretic separation of the sample took place in a tris-glycine buffer at pH 9.5.

In the present method, the concentrated starting zone was forced to over-take the zone of tris-glycine buffer ahead of it in the column. This enabled the glycinate ions to enter the region that was occupied by the sample so that the sample could separate electrophoretically. There was no need, therefore, to change the effective mobility of the glycinate ions as was necessary in Ornstein's method.

It will be recalled (Figure XII) that the dye mixture was concentrated effectively when a discontinuous tris-chloride buffer (pH 8.3) was used in combination with a tris-glycine buffer (pH 8.3). According to the "Kohlrausch
Regulating Principle the pH of the tris-glycine buffer which is behind the dye zone, should be higher than that of the tris-chloride buffer which is ahead of the dye zone. If the reaction of the tris-glycine buffer is higher than pH 8.3, then some of the slower moving sample anions may not be concentrated. To check this point experiments were conducted to determine if the pH of the leading tris-chloride buffer zone affected the pH of the trailing tris-glycine buffer. In these experiments a discontinuous tris-chloride buffer was used in combination with a tris-glycine buffer (pH 8.3). The reaction of the discontinuous tris-chloride buffer was pH 7.6, 8.15 and 8.3 in these trial runs. The dye mixture was applied to the column as described previously, and the current was maintained at 25 ma (400 volts) only until the starting zone had concentrated. The buffer was then decanted from the upper buffer reservoir and samples of the Bio-Gel slurry were removed from the column at positions that were approximately one inch above and one inch below the concentrated dye zone. The samples were taken by inserting a glass tube into the column and withdrawing the slurry with the aid of suction. Table II gives the pH values of the samples that were taken when the three different tris-chloride buffers were used. It will be noted that the
Table II. The Influence of the Tris-Chloride Buffer on the Reaction (pH) of the Tris-Glycine Buffer

<table>
<thead>
<tr>
<th>Reaction of Tris-Chloride Buffer (pH)</th>
<th>Reaction of Buffer in Column (pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Inch Behind</td>
</tr>
<tr>
<td></td>
<td>Concentrated</td>
</tr>
<tr>
<td></td>
<td>Starting Zone</td>
</tr>
<tr>
<td>8.3</td>
<td>8.3</td>
</tr>
<tr>
<td>8.15</td>
<td>8.3</td>
</tr>
<tr>
<td>7.6</td>
<td>8.3</td>
</tr>
</tbody>
</table>

reaction of the tris-glycine buffer behind the concentrated sample was not changed appreciably by the discontinuous tris-chloride buffer which was ahead of the tris-glycine buffer. The tris-chloride buffer did however, lower the pH of the buffer in front of the concentrated sample zone. The low pH of the tris-glycine-chloride buffer in front of the sample zone was expected since the addition of chloride ions to a tris-glycine buffer should cause the pH of that buffer to decrease. It was observed that at the lower pH values (tris-chloride buffer) the dye zone moved a shorter distance before it was concentrated than it did at the higher pH values. This indicated that the velocity at which the dyes moved was lower at the lower pH values than it was at the higher pH values. It was suspected that the conductivity
of the tris-chloride buffer increased as the pH of the buffer decreased. The low velocity of the dye zone in the low pH tris-chloride buffer could be attributed, therefore, to the low potential gradient through this zone of buffer.

Experiments were performed to check whether or not the sample zone could be concentrated more efficiently if the reaction of the tris-glycine buffer was slightly lower than pH 8.3. The two pH values (tris-glycine buffer) that were checked were pH 8.15 and pH 8.05. Tris-chloride buffer (pH 8.3) was used as the discontinuous buffer. One can assume that the starting zone should be concentrated more efficiently if the effective mobility of the glycinate ion was decreased to a lower value than it was at pH 8.3. The results of these experiments showed that the starting zone was not concentrated when tris-glycine buffer, having a reaction of pH 8.15 or pH 8.05, was used. These results appeared to be inconsistent with the theory that was presented earlier concerning the application of the "Kohlrausch Regulating Principle" (Ornstein, 1964) to concentrate the starting zone. It is desirable, therefore, to examine the buffer system more closely to see if some explanation may be offered for this phenomenon.

The dissociation constant \((K_b)\) for tris is \(1.202 \times 10^{-6}\) (Merck Index, 1968). This corresponds to a pK\(_a\) value
of 8.08. When the pH of a tris buffer decreases to its pKa value, the tris molecules pick up a proton and become ionized. The increase in the degree of ionization of the tris molecules becomes greater as the pKa of the molecule is approached. It follows, therefore, that the degree of ionization of tris is much greater at pH 8.08 than it is at pH 8.3. In contrast, the change in the degree of ionization of glycine is very small for the same pH change (pKa glycine is 9.8). As the reaction of a tris-glycine buffer decreases to pH 8.05, the effective mobility of the tris ion increases greatly while the effective mobility of the glycinate ion decreases only slightly. As a result, there is a net increase in the conductivity of the solution. Because of the high conductivity of the tris ion at pH 8.05, it follows that this ion should carry most of the current in an applied potential gradient, with only a small portion of the current being carried by the glycinate ions. When the starting zone is concentrated and stacked according to the "Kohlrausch Regulating Principle" the anions in the sample are arranged in order of decreasing mobility. Since the current must be uniform throughout this zone, the region with the slower moving anions is subjected to a higher potential gradient than is the region which contains the faster moving anions. Because of the high mobility
of the tris ion, any increase in the potential gradient will greatly increase the velocity of the tris ion. As a result, when the effective mobility of the tris ion is high, a small change in the potential gradient is needed to regulate the current. It seems reasonable to assume that the boundaries between the stacked zones (sample) would increase in sharpness as the increase in the potential gradient becomes greater. If the increase in the potential gradient is small, then the boundaries between the stacked zones should be poorly defined. The starting zone probably failed to concentrate when the reaction of the tris-glycine buffer was pH 8.05 or pH 8.15 because of the high conductivity of the tris ion. This high conductivity would lead to a small increase in the potential gradient towards the back of the starting zone and hence to poorly defined stacked zones. It was interesting to note that the dye zone concentrated well if the reaction of the tris-glycine buffer was pH 8.3 or higher. At the higher pH values, the tris ion has a much lower conductivity and therefore the increase in the potential gradient towards the back of the sample zone would probably be much greater than it was at the lower pH values.

In another experiment, a tris-borate buffer (pH 8.35) was used instead of the tris-glycine buffer, in a
discontinuous buffer system with tris-chloride (pH 8.3) as the discontinuous buffer. It was observed that the dye zone concentrated and its components separated electrophoretically in a similar manner as when the tris-glycine buffer was used. It should be noted, however, that the effective mobility of borate ions (pKa 9.14) at pH 8.35 should be greater than that of glycinate ions (pKa 9.8) at the same pH value. It would be suspected, therefore, that the borate buffer would not be as effective in the concentration of a low mobility protein sample as would the glycine buffer.

C.5. Preparative Fractionation of Water Soluble Pea Protein by Column Electrophoresis

A column (Bio-Gel P-300, 11 inch) was prepared in the usual manner using tris-glycine buffer (pH 8.3). Tris-chloride buffer (pH 8.15; 30 ml) was placed on the top of the column followed by the protein sample (800 mg plus 3 drops Bromophenol Blue in 25 ml tris-chloride buffer; pH 8.15). This gave the discontinuous buffer system that was necessary to concentrate the sample zone. The current was maintained at 25 ma (approx. 400 volts) until the blue dye had concentrated to form a thin (approx. 1/16 in) zone. The current was then increased to 35 ma (approx. 550 volts) for
the remainder of the electrophoretic run (approx. 16 hours). When the blue tracking dye had migrated to within one inch of the bottom of the column, the elution procedure was commenced (section C.2.4.). The elution buffer (tris-glycine; pH 8.3) was pumped into tube P (Figure V) at the rate of 80 ml per hour and this removed the proteins from the bottom of the column. Buffer from the upper buffer reservoir was allowed to flow through the column at the rate of 10 ml per hour during the elution period. The optical density (280 m\(\mu\)) of the eluate was measured by means of a Uviscan III (Buchler Instruments, N.J.). The temperature of the cooling water of the column was maintained at 15\(^\circ\)C throughout the entire experiment. At the end of the experiment, tris-glycine buffer (500 ml; pH 8.3) was flushed through the column so that the column could be reused to fractionate another sample of protein. It was observed that after five runs had been made using the same column, the time required to flush the column with tris-glycine buffer had increased from the normal 12-hour period to almost 24 hours. When this happened the column was repacked with a new Bio-Gel slurry. It was noted that the elution patterns which were obtained on the newly packed columns were similar to those obtained on columns that had been used five times. Figure XIV shows a
typical elution profile that was obtained with the water soluble pea protein preparation. The eluate from the column that corresponded to each of the peaks was pooled and the resultant fractions ($F_1$, $F_2$, $F_3$, $F_4$, $F_5$, $F_6$) were dialyzed against seven changes of distilled water ($5^\circ$C) for a total of 60 hours and then the fractions were lyophilized. The lyophilized material of each of the fractions was weighed and then nitrogen analyses (Kjeldahl) were performed on each of these fractions. The results of this experiment are presented in Table III.

Table III

Nitrogen Content of the Protein Fractions Separated by Column Electrophoresis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percentage of Total Material Recovered*</th>
<th>Nitrogen Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_1$</td>
<td>0.5</td>
<td>trace</td>
</tr>
<tr>
<td>$F_2$</td>
<td>0.5</td>
<td>trace</td>
</tr>
<tr>
<td>$F_3$</td>
<td>3</td>
<td>16.2</td>
</tr>
<tr>
<td>$F_4$</td>
<td>20</td>
<td>16.5</td>
</tr>
<tr>
<td>$F_5$</td>
<td>35</td>
<td>16.4</td>
</tr>
<tr>
<td>$F_6$</td>
<td>41</td>
<td>15.9</td>
</tr>
</tbody>
</table>

*Quantity of protein recovered = approx. 400 mg
Figure XIV
Elution Profile (Bio-Gel P-300) of Water Soluble Pea Protein in Which Sample was First Concentrated (Method No. 4) and then Fractionated by Column Electrophoresis
The first two fractions (F₁, F₂) gave positive Schiff's tests and negative biuret tests. They contained pentose (Bial's orcinol test), but did not give an absorption maxima at 260 μm which is characteristic of nucleic acids. The fractions (F₁, F₂) each contained 6.5% total sugars (Fairbairn, 1953), a trace of uronic acids (Galambos, 1967) and no amino sugars (Cessi and Piliego, 1960). These tests indicated that the material in these fractions was neither protein, nucleic acid nor pectin. No further attempts were made to identify these two fractions.

Fractions F₁, F₂, F₃, F₄, F₅, F₆ were analyzed by disc electrophoresis and by disc isoelectric focusing. The results of the analyses are presented in Figure XV, Figure XVI and Figure XVII and are summarized in Table IV.

Fractions F₁ and F₂ were stained for carbohydrate (Zacharius and Zell, 1969) since they did not contain protein. Fractions F₁, F₂, and F₃ each contained a component which had the same electrophoretic mobility (disc electrophoresis). It was also observed that a mixture of fractions F₁, F₂, and F₃ was not separated by disc electrophoresis, but migrated at the same rate as the tracking dye. Fraction F₃ gave one major and two minor components when subjected to disc isoelectric focusing. The isoelectric points of these
Figure XV

Disc Electrophoresis of Fractions Obtained by Column Electrophoresis (Figure XIV)

1) Fraction F3
2) Fraction F4
3) Fraction F5
4) Fraction F6
Figure XVI

Disc Isoelectric Focusing of Fraction F3
Figure XVII

Disc Isoelectric Focusing of Fractions Obtained by Column Electrophoresis (Figure XIV)

1) Fraction F_4  
2) Fraction F_5  
3) Fraction F_6
Table IV

Summary of the Results of Disc Electrophoresis and Disc Isoelectric Focusing Analyses of the Protein Fractions Separated by Column Electrophoresis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Components by Disc Electrophoresis</th>
<th>Components by Disc Isoelectric focusing</th>
<th>Estimated Range of Isoelectric points (pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F_1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F_2</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F_3</td>
<td>1</td>
<td>3</td>
<td>4.80 - 5.00</td>
</tr>
<tr>
<td>F_4</td>
<td>13</td>
<td>18</td>
<td>5.20 - 6.60</td>
</tr>
<tr>
<td>F_5</td>
<td>12</td>
<td>22</td>
<td>5.20 - 6.75</td>
</tr>
<tr>
<td>F_6</td>
<td>7</td>
<td>17</td>
<td>5.40 - 6.60</td>
</tr>
</tbody>
</table>

proteins were estimated (disc isoelectric focusing; ampholyte range pH 3-6) to be between pH 4.8 and pH 5.0. Fraction F_4 gave (disc electrophoresis) one major and one minor component that were fast moving and 11 minor components that were slow moving. Fraction F_5 contained (disc electrophoresis) trace amounts of two fast moving zones that had the same mobility as the two fast moving components of Fraction F_4, in addition to 10 slower moving components. Fractions F_4 and F_5 were combined and the resultant mixture was
analyzed by disc electrophoresis. The results of the analysis appear in Figure XVIII. It will be noted that the mixture contains 21 different electrophoretic components. This suggested that at least four components in fraction $F_4$ were the same as four components in fraction $F_5$. Fraction $F_6$ contained (disc electrophoresis) three major components that were fast moving and four poorly defined components that were slow moving. Fraction $F_6$ was the slowest moving fraction on the electrophoretic column. However, the mobilities of its components (disc electrophoresis) were greater than many of the components of fraction $F_5$. This would be expected if the components of fraction $F_6$ have lower molecular weights than those of fraction $F_5$. Other workers (Andrews, 1964; Laurent and Killander, 1964; Male, 1967; Granath and Kvist, 1967; Bly, 1970) have shown that proteins of high molecular weights are eluted from a column (gel filtration) before proteins of lower molecular weight.

C.6. Estimation of Molecular Weight of Pea Proteins by Gel Filtration

Protein fractions $F_3$, $F_4$, $F_5$, $F_6$, which were obtained by column electrophoresis, were subjected to gel filtration using either Sephadex G-75 or Bio-Gel P-200. The molecular weights of the protein components were estimated by the
Figure XVIII

Disc Electrophoresis of Mixture of Fractions F₄ and F₅
method described previously (section C.1.5.). Figure XIX shows a typical elution profile of fraction F₃ on a Sephadex G-75 column. Figure XX shows typical elution profiles of fractions F₄, F₅, and F₆ on a Bio-Gel P-200 column. The results of these experiments are summarized in Table V. The elution profile of fraction F₃ on the Sephadex G-75 column indicates that only one component was present and this had a molecular weight of 7,500 (average of two determinations). The elution profiles of fractions F₄ and F₅ on a Bio-Gel P-200 column were similar in that each had one distinct peak. The first traces of protein from both samples were eluted from the column in a volume which corresponded to the void volume of the column. The protein concentration in the eluate decreased slowly from this point to form a long "tailing" curve. This indicated that some of the proteins in the sample had a molecular weight which was above 200,000. It should be pointed out that this is the exclusion limit of the gel. The tailing of the curve suggested that there might be some intermolecular linkages between the proteins in this fraction. Besemer and Clauss (1968) studied the soluble proteins of pea seedlings by use of disc electrophoresis. They found that reproducible separations could be obtained only when the extraction media contained
Elution Profile (Gel Filtration, Sephadex G-75) of Fraction F₃
FRACTION  F - 3

VOID VOLUME  =  53.7 ml

ELUTION VOLUME (ML)

OD
Elution Profiles (Gel Filtration, Bio-Gel P-200) of Fractions F₄, F₅ and F₆
FRACTION F - 4

FRACTION F - 5

FRACTION F - 6

void volume: 60.3 ml

ELUTION VOLUME (ML)
Table V
Molecular Weights of Protein Fractions Separated by Column Electrophoresis

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Type or Gel</th>
<th>Estimated Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>F₃</td>
<td>Sephadex G-75</td>
<td>7,000</td>
</tr>
<tr>
<td>F₄</td>
<td>Bio-Gel P-200</td>
<td>&gt;200,000</td>
</tr>
<tr>
<td>F₅</td>
<td>Bio-Gel P-200</td>
<td>&gt;200,000</td>
</tr>
<tr>
<td>F₆</td>
<td>Bio-Gel P-200</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td>154,000</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>84,000</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>52,000</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>31,000</td>
</tr>
</tbody>
</table>

mercaptoethanol and polyvinylpyrrolidine or polyethylene glycol. They claimed that the mercaptoethanol split the protein complexes which existed in the sample.

The elution profile (Figure XX) of fraction F₆ obtained with a Bio-Gel P-200 column indicates that the fraction contained four components (A, B, C, D). The molecular weight of each of the components were estimated by gel filtration (Bio-Gel P-200) and the results are presented in Table V. The effluent from each peak was pooled and dialyzed against seven changes of distilled water (5°C) for 60
hours and was then lyophilized. Samples from each of the fractions (A, B, C, D) were analyzed by disc electrophoresis and disc isoelectric focusing (ampholyte range pH 5-8). Results of these analyses are presented in Figure XXI and Figure XXII and are summarized in Table VI. The results showed that as the molecular weight of the proteins increased, the electrophoretic mobility (disc electrophoresis) decreased. It will be recalled that all these proteins migrated at the same rate when subjected to electrophoresis on the Bio-Gel P-300 column. It seems reasonable to assume that the proteins of low molecular weight were held back by the Bio-Gel P-300 column more than were the proteins of higher molecular weight. This was compensated for by the higher free electrophoretic mobility of the smaller proteins so that all these proteins appeared to migrate at the same rate on the electrophoretic column. This also indicated that relative rates of migration of the proteins in column electrophoresis was not the same as those of the identical proteins in disc electrophoresis. The results obtained by disc isoelectric focusing indicated (Table VI) that the average isoelectric point of the proteins decreased as their molecular weight decreased. The decrease in isoelectric point that accompanied the decrease in molecular weight of the
Figure XXI

Disc Electrophoresis of Sub-Fractions (Gel Filtration, Bio-Gel P-200) of Fraction F₆

1) Sub-Fraction A
2) Sub-Fraction B
3) Sub-Fraction C
4) Sub-Fraction D
5) Fraction F₆
Figure XXII

Disc Isoelectric Focusing of Sub-Fractions (Gel Filtration, Bio-Gel P-200) of Fraction $F_6$

1) Sub-Fraction A  2) Sub-Fraction B
3) Sub-Fraction C  4) Sub-Fraction D
5) Fraction $F_6$
## Table VI

Properties of Proteins in Fraction $F_6$

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Molecular Weight (Average for 2 Determinations)</th>
<th>Relative Rate of Migration (Disc Electrophoresis)</th>
<th>No. Components (Disc Electrophoresis)</th>
<th>No. Components (Disc Isoelectric Focusing)</th>
<th>Estimated Range of Isoelectric Points (pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>158,000</td>
<td>4 (slowest)</td>
<td>1 (major)</td>
<td>6 (poorly defined)</td>
<td>6.0 - 6.75</td>
</tr>
<tr>
<td>B</td>
<td>91,000</td>
<td>3</td>
<td>2 (major)</td>
<td>7 (major)</td>
<td>6.0 - 6.45</td>
</tr>
<tr>
<td>C</td>
<td>48,000</td>
<td>2</td>
<td>2 (major)</td>
<td>3 (major)</td>
<td>5.4 - 5.6</td>
</tr>
<tr>
<td>D</td>
<td>34,000</td>
<td>1 (fastest)</td>
<td>2 (major)</td>
<td>3 (major)</td>
<td>5.1 - 5.4</td>
</tr>
</tbody>
</table>

*Note: D indicates disease.*
proteins might account for the corresponding increase in the mobility of these proteins as observed by disc electrophoresis.

C.7. General Discussion

The purpose of this study was to develop a preparative method for the fractionation of water soluble pea proteins. Column electrophoresis was chosen because it can be easily adapted to the fractionation of proteins on a preparative scale and because it can separate proteins into groups according to molecular weight and electrophoretic mobility. Powder columns were used instead of solid acrylamide or starch gel columns because of the greater capacity of powder columns as compared to solid gel columns (Porath, 1964a, 1964b). In addition, powder columns are better suited to the fractionation of materials of high molecular weight than are acrylamide or starch gel columns. This is because high molecular weight materials may not always be able to penetrate the pores of a solid gel column. On the other hand, high molecular weight materials will always pass through powder columns. It has also been shown (Hochstrasser et al., 1964) that powder columns may be reused many times thus avoiding the necessity of repacking
the column. It is well known that acrylamide columns in disc electrophoresis may be used only once.

The apparatus for column electrophoresis described in this thesis is unique in that it can support powder columns and is equipped with both an efficient cooling system and an efficient elution chamber. The elution chamber of the apparatus is similar in principle to the one described by Jovin et al. (1964) and by Bergrahm and Harlestan (1968). The elution buffer enters around the periphery of the chamber and washes the bottom of the column as it flows through the outlet at the centre of the column. A slight negative pressure inside the chamber holds the membrane which forms the bottom of the chamber, up against the top of the chamber (plastic disc D, Figure V) and hence an extremely narrow slit is formed through which the elution buffer flows.

Various types of powder columns were tested to determine which one was best suited to the fractionation of water soluble pea proteins. The results indicated that a Bio-Gel P-300 column was better suited to the fractionation of the proteins than were columns made from gels with lower exclusion limits. It was observed that the separation of proteins on a Bio-Gel P-300 column was adversely affected when the temperature of the cooling water dropped below 15°C. It was suspected that the colder temperatures caused
the gel beads to contract and this decreased the internal pore size of the particles which interfered with the filtering properties of the column.

Attempts were made to improve the efficiency of the column by the use of thin starting zones. Ornstein (1964) indicated that the fractionation of a protein sample could be improved if the starting zone is first concentrated and stacked. He claimed that samples which are stacked in a narrow starting zone could be fractionated on shorter columns and in much less time than ones which are not first stacked and concentrated.

The discontinuous buffer system introduced by Ornstein (1964) and Davis (1964) to concentrate the starting zone and to separate its components by disc electrophoresis was found to be ineffective when applied to a powdered Bio-Gel P-300 column. Experiments showed that the starting zone was concentrated using this buffer system, but the subsequent electrophoretic separation was not satisfactory. It was concluded that the components of the sample did not separate because they were trapped between the zone of fast moving chloride ions ahead of them, and the zone of slow moving glycinate ions which were behind them. Ornstein had designed this buffer system so that the trailing zone of
glycinate ions would overtake the concentrated protein zone once the reaction of the tris-glycine buffer rose to pH 9.5. Only after the glycinate ions had overtaken the starting zone could the components in the sample be separated. This did not happen, however, with this buffer system when a Bio-Gel P-300 column was used, because the mobilities of the proteins were probably greater than -7.5 mobility units which is the effective mobility of glycine at pH 9.5. As a result the glycinate ions were unable to overtake the protein zone and therefore the proteins remained stacked and concentrated as they migrated down the column. This indicated that the buffer system which is used in disc electrophoresis could not be applied effectively to powder columns.

Porath (1956) developed a method to reduce the thickness of the starting zone on powder columns. His technique involved the reduction of the conductivity of the buffer within the starting zone. Experiments showed that this technique led to the concentration of the leading edge of the starting zone only and not to the trailing edge of the starting zone.

An attempt was made to concentrate the starting zone by replacement of the buffer in the upper reservoir by distilled water. The starting zone was concentrated when the
buffer in the rest of the apparatus was either tris-citrate (pH 8.65) or tris-chloride (pH 8.3), but not when it was tris-glycine (pH 8.3) or tris-borate (pH 8.35). It was concluded that the starting zone could be concentrated in this manner only if the buffer anion had a mobility that was greater than that of the sample anions. As a result when the current was turned on the buffer anions in the column moved ahead of the sample. A sharp boundary was formed with buffer anions on one side of this boundary and a void of buffer anions on the other. A very high potential gradient was created behind this boundary because of the low conductivity in the region. As this boundary moved through the sample the high potential gradient behind the moving boundary caused the starting zone to concentrate and stack. Once the zone was concentrated, the distilled water in the upper reservoir was replaced with buffer. This provided anions which migrated through the stacked zone and allowed the components of the sample to separate. The above method to concentrate the starting zone was used with both tris-citrate buffer (pH 8.65) and tris-chloride buffer (pH 8.3) to fractionate a sample of water soluble pea protein by column electrophoresis (Bio-Gel P-300, 11 inch). The results (Figure XI) indicated that the separation was not as good
as that obtained with tris-glycine buffer (pH 8.3) in which the starting zone was not first concentrated (Figure VI, Bio-Gel P-300).

A discontinuous buffer system was developed to concentrate and stack a sample of negatively charged ions prior to electrophoresis on powder columns. The buffer system differed from that used by Ornstein (1964) and Davis (1964) in disc electrophoresis although both systems utilized the "Kohlrausch Regulating Principle" to concentrate the starting zone. In the present system the effective mobility of the glycinate ions was less than that of the sample anions. Under an applied electric field the sample was trapped between the zone of fast moving chloride ions ahead of it and the zone of slow moving glycinate ions behind it. Because of the low mobility of the trailing glycinate zone, a higher potential gradient was produced in this region so that the current here would be the same as that in the chloride zone ahead of it. This increase in the potential gradient behind the chloride boundary caused the starting zone to concentrate and stack according to the "Kohlrausch Regulating Principle". It was shown that the starting zone concentrated best when the mobility of the buffer cation (tris-) was kept at a low value. This was done by ensuring
that the pH of the buffer was well above the pKa (8.08) of tris. On the other hand the pH of the buffer had to be low enough so that the mobility of the glycinate ions would be as low as possible. The glycinate ions must have a mobility which is less than that of the sample, to enable the sample to concentrate. After the starting zone had concentrated, it migrated into the zone of glycinate ions ahead of it in the column. Once the glycinate ions had entered the stacked sample zone, the various components in the sample were able to separate according to their electrophoretic mobility. It should be pointed out that in disc electrophoresis the stacked starting zone began to separate only after the trailing glycinate zone was made to overtake the stacked sample. This was done by increasing the pH of the tris-glycine buffer until the effective mobility of the glycinate ions was higher than that of the sample ions. In the present system there was no need to change the pH of the buffer after the starting zone was concentrated. As a result the sample was concentrated and stacked at the same pH value as that used to separate the stacked sample. In disc electrophoresis the sample is stacked and concentrated at pH 8.3, but the electrophoretic separation is carried out at pH 9.5.
The discontinuous buffer system, which was devised in the present study, provided for the first time, a simple and effective method to concentrate, stack and then to fractionate proteins on a powder column. Because the "Kohlrausch Regulating Principle" was used to concentrate the sample, the various components in the concentrated sample were stacked one on top of the other in order of their electrophoretic mobility. As a result the sample was actually fractionated once the zone had been concentrated.

The subsequent electrophoretic separation only increased the space between the stacked zones. This made possible the use of very short columns and running times to achieve good separations. It was observed in preliminary experiments with the dye mixture, that no improvement in the separation of the dyes was produced when the column length was increased beyond 11 inches. Any increase in the distance between the dye zones was offset by the greater diffusion of the zones as a result of the longer running times. It is worth noting that Porath (1964a, 1964b) used 68 inch columns when he fractionated proteins by column electrophoresis and that the separations required from 60 to 140 hours.

The discontinuous buffer system that was developed in the present work was used to fractionate, by column
electrophoresis, (Bio-Gel P-300 column) water soluble pea protein on a preparative scale. The elution profile (Figure XIV) showed that the sample had been separated into six fractions. Fractions F₁ and F₂ were not protein and contained 6.5% sugars. It was interesting to note that the electrophoretic mobilities (column electrophoresis) of fractions F₁ and F₂ were greater than that of fraction F₃. However, the fractions (F₁, F₂, F₃) migrated at the same speed when subjected to disc electrophoresis. This showed that the present system of column electrophoresis was more effective in the fractionation of fast moving components than was disc electrophoresis. Theoretically, disc electrophoresis, as described by Ornstein (1964), can only fractionate material whose effective mobility is less than -7.5 mobility units which is the effective mobility of glycine at pH 9.5. The results of the analyses by disc electrophoresis indicated that all of the components of F₁, F₂ and F₃ had effective mobilities that were greater than -7.5 mobility units. Disc electrophoretic analysis (Figure XV) indicated that fraction F₃ was homogeneous; however, analyses by disc isoelectric focusing (Figure XVI) showed that it contained three components. Disc electrophoretic analyses suggested (Figure XV, Figure XVIII) that many of the components of F₄ were different from those of F₅. Gel filtration
studies showed that some of the proteins in fractions $F_4$ and $F_5$ (Figure XX) had molecular weights that were greater than 200,000. The results also suggested that the proteins in these fractions may be joined together by intramolecular bonding. Intramolecular bonding in pea proteins was also suggested in a previous study by Besemer and Clauss (1968).

It was observed (Figure XV) that the major components of $F_6$ had a higher electrophoretic mobility (disc electrophoresis) than many of the components of $F_5$. This gave the first indication that the relative rates of migration of the proteins in column electrophoresis (Bio-Gel P-300 column) were different from those that were obtained with gel electrophoresis (solid acrylamide column). It will be recalled that with polyacrylamide or starch gel columns, for example, the materials of high molecular weight are retained longer than are those of low molecular weight. The results of the present study showed that the electrophoretic mobility of many of the proteins in the Bio-Gel column was opposite to that which would be expected in free solution. These results disagree with the findings of Hjerten (1963) who claimed that the electrophoretic mobilities of proteins on a powdered agarose column were the same as they were in free solution.
Fraction F6 (Figure XIV) was separated by gel filtration (Bio-Gel P-200) into four sub-fractions (A, B, C, D). It was noted (Table VI) that the fraction with the highest molecular weight had the lowest free mobility and the fraction with the lowest molecular weight had the highest free mobility. Both of these fractions, however, migrated at the same speed under conditions of column electrophoresis. These fractions would migrate in this fashion in powder column electrophoresis if their mobilities were governed both by gel filtration effect and by their electrophoretic mobilities. Gel filtration studies have shown that materials of low molecular weight enter the pores of the gel beads and are retained by the column longer than are materials of high molecular weight which cannot enter the pores of the beads (Laurant and Killander, 1964; Andrews, 1965; Fawcett and Morris, 1966; Granath and Kvist, 1967; Bly, 1970). It is not difficult to see, therefore, why the proteins of high molecular weight and of low electrophoretic mobility, and the proteins of low molecular weight and high electrophoretic mobility were eluted from the electrophoresis column at the same time.

The water soluble proteins from peas were separated into nine fractions by use of column electrophoresis and of gel filtration. Varner and Schidelovsky (1963) separated
water soluble pea protein into six fractions by ion exchange chromatography. Montgomery et al. (1968) separated an aqueous extract of dried peas into two fractions by gel filtration (Sephadex G-100).

Steward et al. (1965) examined by disc electrophoresis a protein extract which they obtained from germinated pea cotyledons and detected seven electrophoretic components. Polter (1967) detected (disc electrophoresis) 30 protein components in an extract of the roots of pea seedlings. He noted that the number of electrophoretic components varied with the method used to extract the proteins from the plant tissue. He observed that protein extracts that had been dialyzed against tris-glycine buffer or distilled water gave fewer numbers of bands.

Flinn and Pate (1968) used disc electrophoresis to study the albumin fraction of pea cotyledons which were at different stages of maturation. They detected (disc electrophoresis) 20 protein components in the albumin fraction of mature cotyledons. They noted that the electrophoretic pattern of the proteins varied as the pea cotyledon matured.

The present study has led to an effective method for the fractionation of water soluble pea proteins on a preparative scale. Analyses by disc electrophoresis and by disc
isolectric focusing indicated that the nine fractions contained a total of 35 and 60 components, respectively.
D. SUMMARY

1. A column electrophoresis apparatus for the fractionation of proteins on a preparative scale was designed and constructed. It was built for use especially with powder columns. The very small volume of the elution chamber permitted the efficient isolation of the protein fractions as they emerged from the column.

2. Different kinds of anticonvection media (Bio-Gel P-300, Bio-Gel P-200, Bio-Gel P-150, Sephadex G-200, Sephadex G-100, powdered Cellulose) were tested for their suitability for the fractionation of water soluble pea (Pisum sativum) proteins. The results indicated that Bio-Gel P-300 was superior to the others that were tested.

3. Preliminary experiments indicated that the filtering properties of Bio-Gel P-300 were adversely affected when the temperature of the cooling water was below 15°C. It was observed that when the heat energy released in the column exceeded 21 watts, the sample zones became dish-shaped as they moved down the column. This was to be avoided as dish-shaped bands could not be separated efficiently in the
elution chamber.

4. Two methods were devised to concentrate the starting zone prior to electrophoresis. The first method involved the use of a buffer in which the effective mobility of buffer anions was greater than -7.5 mobility units. Experiments showed that tris-citrate (pH 8.65) and tris-chloride (pH 8.3) buffers could be used to concentrate the starting zone and to separate the electrophoretic components of a dye mixture. The second method involved the use of a discontinuous buffer system. It was observed that the starting zone was concentrated most effectively when the conductivity of the cations and anions of the continuous buffer were as low as possible. The conductivity of a buffer is low when the pH of the buffer is below the pKa of the anions and above the pKa of the cations. It was advantageous, therefore, to choose a buffer in which the pKa values of the anions and cations were far apart. Experiments showed that a system comprising a continuous tris-glycine (pH 8.3) and a discontinuous tris-chloride buffer (pH 8.15) was effective for the concentration of the starting zone and for the electrophoretic separation of water soluble pea proteins.
5. Samples (800 mg) of water soluble pea proteins were separated by column electrophoresis (Bio-Gel P-300; 11 inch column) into six fractions (F_1, F_2, F_3, F_4, F_5, F_6). The fractions were analyzed chemically and by disc electrophoresis and by disc isoelectric focusing. The following observations were made: (a) F_1 and F_2 were non-proteinaceous (biurette) and contained approximately 6.5% sugar (Fairbairn, 1953). (b) F_3 comprised one disc electrophoretic component and three disc isoelectric (focusing) components. The isoelectric points of F_3 were estimated between pH 4.8 and pH 5.0. (c) F_4 comprised thirteen disc electrophoretic components and eighteen disc isoelectric (focusing) components. (d) F_5 comprised twelve disc electrophoretic components and twenty-two disc isoelectric (focusing) components. (e) F_6 comprised seven disc electrophoretic components and seventeen disc isoelectric (focusing) components.

6. The molecular weights of fractions F_3, F_4, F_5 and F_6 were estimated by gel filtration. The following results were obtained: (a) F_3 had a molecular weight of 7,500. (b) F_4 and F_5 contained components, many of which had molecular weights above 200,000. (c) F_6 was further fractionated by gel filtration to yield sub-fractions A, B, C and D. The molecular weights of these sub-fractions were 158,000, 91,000, 48,000 and 34,000.
respectively.

7. The electrophoretic mobilities (disc electrophoresis) of sub-fractions A, B, C and D were inversely proportional to their molecular weights.

8. The relative rate of electrophoretic migration of the pea proteins in column electrophoresis (Bio-Gel P-300) was not the same as it was in acrylamide gel electrophoresis.
E. CLAIMS TO ORIGINAL RESEARCH

(Note:- Claims to original research based on the studies in the Appendix are presented on page 157)

1. The design and construction of an improved column electrophoresis apparatus for the preparative separation of proteins. The apparatus is claimed to be superior to others of its kind in respect of ease of operation, efficiency of the elution chamber and ability to support an annular-shaped powder column.

2. The development of a novel method whereby the starting zone in powder column electrophoresis is concentrated and stacked by use of a continuous buffer system in which the anion of the buffer has an effective mobility greater than that of the sample anions. By this method it was possible to concentrate the starting zone when a tris-citrate buffer (pH 8.65) or a tris-chloride buffer (pH 8.3) was used for the electrophoretic separation.

3. The development of a novel technique whereby the starting zone in powder column electrophoresis is concentrated and stacked by use of a discontinuous buffer system. This was the first time that a discontinuous buffer system has been used to concentrate and stack a starting zone on a
powder column. This discontinuous buffer system permitted for the first time, the concentration and subsequent electrophoretic fractionation of samples which contained components of high electrophoretic mobility.

4. The first successful attempt to use short powder columns (11 inch) for the preparative fractionation of proteins by column electrophoresis.

5. The first fractionation of water soluble pea proteins by column electrophoresis on powder columns. Six fractions were isolated of which only the four slower moving fractions contained protein. The fastest moving of the protein fractions (F₃) was found to contain one component by disc electrophoresis. This was the first isolation of this protein from peas.

6. The molecular weight of fraction F₃ was 7,500 as estimated by gel filtration.

7. The isoelectric point of fraction F₃, as determined by disc isoelectric focusing, lay between pH 4.8 and pH 5.0.

8. The slowest moving of the four protein fractions (F₆) yielded four sub-fractions (A, B, C, D) when subjected to gel filtration. These four sub-fractions were isolated for
the first time and their molecular weights and isoelectric points were estimated by gel filtration and disc isoelectric focusing respectively.

9. The first demonstration that the sequence in which proteins migrate in powder column electrophoresis (Bio-Gel P-300) may not be the same as that in which they migrate in acrylamide gel electrophoresis.
LITERATURE CITED

Estimation of molecular weights of proteins by Sephadex gel-filtration.

The gel-filtration behavior of proteins related to their molecular weights over a wide range.

Staining method for proteins after isoelectric focusing in polyacrylamide gel.
Science Tools, 16: 42.

Bergraham, B. and Harleston, R. 1968.
Polyacrylamide gel electrophoresis with continuous elution in the Uniphor 7900 column electrophoresis system.

Besemer, J. and Clauss, H. 1968.
Disc electrophoresis of soluble plant proteins.

Gel permeation chromatography.
Science, 168: 527.

Bourdillon, J. 1951.
A crystalline bean seed protein in combination with phytic acid.

The concentration of $^{39}K$ and $^{41}K$ by balanced ion migration in a counterflowing electrolyte.
Science, 104: 156.
An apparatus for preparative electrophoresis.

Gel-electrofocusing in combination with immunodiffusion.
J. Chromatog., 45: 483.

Catsimpoolas, N. 1969.
Micro isoelectric focusing in polyacrylamide gel columns.

The determination of amino sugars in the presence of
amino acids and glucose.

A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis.

Consden, R., Gordon, A.W., Martin, A.J.P. 1946.
Ionophoresis in silica jelly.

Coolidge, T.B. 1939.
A simple cataphoresis apparatus.

Davis, B.J., and Ornstein, L. 1959.
A new high resolution electrophoresis method.

Davis, B.J. 1964.
Disc electrophoresis. II. Method and application to human serum proteins.
Annal. of the N.Y. Acad. of Sci., 121: 404.
Artefacts arising from the use of sucrose solutions in electrofocusing techniques.  
J. Chromatog., 35: 575.

Hydrolytic decomposition products of some carrier ampholytes used in electrofocusing.  
J. Chromatog., 41: 259.

Fairbairn, N.J. 1953.  
A modified anthrone reagent.  
Chem. Ind., 72: 86.

Molecular seive chromatography of proteins on granulated polyacrylamide gels.  

Fawcett, J.S. 1968.  
Isoelectric fractionation of proteins on polyacrylamide gels.  
FEBS. Letters, 1: 81.

Flinn, A.M., and Pate, J.S. 1968.  
Biochemical and physiological changes during maturation of fruit of the field pea (Pisum arvense L.).  

Zone electrophoresis in starch columns.  

Zone electrophoresis on cellulose columns.  

Foster, G.L., and Schmidt, C.L. 1923.  
The separation of the hexone bases from certain protein hydrolysates by electrophoresis.  

Foster, G.L., and Schmidt, C.L. 1926.  
The separation of the dicarboxylic amino acids from certain protein hydrolysates by electrical transport.  
The reaction of carbazole with carbohydrates. II.  
Effect of borate and sulfamate on the ultraviolet  
absorption of sugars.  
Anal. Biochem., 19: 133.

Fractionation of human plasma proteins by gel filtration and zone electrophoresis or ion-exchange chromatography.  

Molecular weight distribution analysis by gel chromatography on sephadex.  
J. Chromatog., 28: 69.

Zone electrophoresis in a glass powder column.  

Hart, Sir Arthur. 1916.  

Hjertén, S. 1959.  
Zone-sharpening in paper electrophoresis - A method allowing application of dilute protein solutions.  

Zone electrophoresis in columns of agarose suspensions.  
J. Chromatography, 12: 510.

Hoch, H., and Barr, G.H. 1955.  
Paper electrophoresis with superimposed pH gradient.  

An improved column for preparative electrophoresis.  
Separation of the components in collagen by preparative starch-gel electrophoresis.


Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. VI.
Isoelectric spectrum of bovine carbonic anhydrase B.

Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. VIII.
Continuous recording of pH and light absorbance of column effluent after isoelectric focusing.

An apparatus for preparative temperature regulated polyacrylamide gel electrophoresis.

Kauman, W.G. 1957.
The electrophoretic separation of ampholytes in a medium of nonuniform pH.

Kendall, J. 1928.
Separation by the ionic migration method.
Science, 67: 163.

Kohlrausch, F. 1897.
Uber concentrations-verscheibungen durch electrolyse im innern von losungen und losungsgemischen.
Cited in Ornstein, 1964.

Kolin, A. 1954.
Separation and concentration of protein in a pH field combined with an electric field.
Electrophoretic "line spectra".  

Protein-calcium-phytic acid relationships in soybean.  
Part I. Effects of calcium and phosphorus on solubility characteristics of soybean meal proteins.  

A theory of gel filtration and its experimental verification.  
J. Chromatog., 14: 317.

A new technique for the electrophoresis of proteins.  
Biochem. J., 108: 19P.

Ampholyte supports.  
French Patent No. 1, 554,045.  

Longsworth, L.G. 1959.  

Molecular sieves and their use in separations of biological materials.  
Lab. Practice, 16: 863.

Merck Index. 1968.  
Published by Merck and Co., Inc., Rahway, N.J., U.S.A.  
Eighth edition.

Purification and substrate and inhibitor specificities of carboxylesterases of the pea (Pisum sativum L.).  
Ornstein, L. 1964.
Disc electrophoresis. 1. Background and theory.

Separation of soluble plasma proteins of the pea root
by acrylamide disc electrophoresis.

Porath, J. 1956.
Methodological studies of zone-electrophoresis in
vertical columns. 1. Fractionation in cellulose
powder columns of substances of low molecular weight
exemplified by amino acids and related compounds.

Migration-elution of zones in an electrophoresis col-
umn.

Some recent developments in column electrophoresis
in granular media.

Porath, J. 1964a.
Column electrophoresis on a large scale.

Porath, J. 1964b.
Some recent developments in preparative electrophor-
esis and gel filtration.

Quast, R., and Vesterberg, O. 1968.
Isoelectric focusing and separation of hemoglobins
of Maxine glutinosa L. in a natural pH gradient.

Doubly discontinuous electrophoresis on sucrose grad-
ients for the analysis of plant peroxides.
Radola, B.J. 1969.
Thin-layer isoelectric focusing of proteins.

Disc electrophoresis of basic proteins and peptides
on polyacrylamide gels.

Smithies, O. 1955.
Zone electrophoresis in starch gels. Group variations
in the serum proteins of normal human adults.

Acrylamide gel electrophoresis of soluble plant pro-
teins: A study on pea seedlings in relation to devel-
opment.

Heterogeneity of presumably homogeneous protein pre-
parations.

Isoelectric fractionation, analysis and characteri-
ation of ampholytes in natural pH gradients. I.
The differential equation of solute concentrations at
a steady state and its solution for simple cases.

Isoelectric fractionation, analysis and characteri-
ation of ampholytes in natural pH gradients. II.
Buffering capacity and conductance of isoionic ampho-
lytes.

Isoelectric fractionation, analysis and characteri-
ation of ampholytes in natural pH gradients. III.
Description of apparatus for electrolysis in columns
stabilized by a density gradient and direct determi-
nation of isoelectric points.
Tiselius, A. 1941.
Stationary electrolysis of ampholyte solutions.

Intracellular distribution of proteins in pea cotyledons.
Plant Physiol., 38: 139.

Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. IV.
Further studies on the resolving power in connection with separation of myoglobins.

Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. V.
Separation of myoglobins and studies of their electrochemical differences.

Electrophoresis of posterior pituitary gland preparation.
J. Biol. Chem., 123: 45.

Williams, R.J., and Waterman, R.E. 1929.
Electrodialysis as a means of characterizing ampholytes.

Williams, R.J. 1935.
Fractional electrical transport as a tool in biochemical research.

Wrigley, C.W. 1968.
Analytical fractionation of plant and animal proteins by gel electrofocusing.

Glycoprotein staining following electrophoresis on acrylamide gels.

The distribution of protein-lysine and methionine, and antitryptic activity in the cotyledon of some leguminous seeds.
I. GENERAL INTRODUCTION

Mattson (1946) and Mattson et al. (1951) claimed that peas which are blanched before they are soaked cook better than those that are not blanched. These workers suggested that blanching increased the permeability of the seed coat and enabled the peas to soften faster when they were cooked. They also suggested that the blanching treatment inactivated phytase, an enzyme which attacks phytic acid. They claimed that the presence of phytic acid in peas improved their cooking quality. It followed, therefore, that blanched peas which contained little or no phytase should have good cooking quality.

Rosenbaum et al. (1966) studied the cooking characteristics of two groups of peas. One of the group (Avion) was judged by the supplier to have good cooking quality and the other group (3CW) to have poor cooking quality. These workers found that the Avion peas took less time to soften than did the 3CW peas when they were cooked in distilled water with their seed coats intact. They observed, however, that the two groups of peas took the same time to soften when they were cooked in distilled water, with their seed coats removed. If the two groups of peas (seed coats...
removed) were cooked in water which contained calcium ions, the 3CW peas again took longer to cook than did the Avion peas.

Rosenbaum and Baker (1969) studied the calcium distribution in peas that were soaked or cooked in water which contained $^{45}\text{Ca}$. They found that the peripheral region of the pea contained a higher concentration of $^{45}\text{Ca}$ than did the interior of the pea and that the peripheral region cooked less readily.

Mattson (1946) claimed that a pea is considered cooked when the cellular cement dissolves and allows the individual cells of the pea to separate. He suggested that calcium ions in the pea form cross linkages between the pectic materials in the middle lamella and this decreases the solubility of the cellular cement.

The present investigation was undertaken to determine (a) the effect of blanching and (b) the role which the seed coat plays in the distribution of calcium in the pea. High resolution autoradiography was used to locate $^{45}\text{Ca}$ inside the cells of pea tissue.
II. LITERATURE REVIEW

II.1. Cooking Quality of Peas

II.1.1. Measurement of Cooking Quality

Organoleptic methods have been developed for the determination of the quality of cooked peas (Sayre et al., 1931). The subjective nature of the taste panel has led to the development of more objective methods for the assessment of pea texture. A number of mechanical instruments have been used to measure the texture of peas. Bonney et al. (1931) and Sayre et al. (1931) used a device which crushed the peas. Martin (1937a, 1937b) introduced the "tenderometer", a device which simulated the action of the jaw.

Christel (1938) used a texturemeter to measure pea texture. The sample of peas to be tested was placed in a cylindrical cup placed directly below a cylinder fitted with 25 steel rods (3/16" dia.). The force required for the rods to penetrate the peas was used as a measure of texture.

Mattson (1946) constructed an apparatus that measured the time required to cook peas. The device consisted of 100 hollow tubes positioned directly above 100 conical holes.
drilled into a brass plate. One soaked pea was placed in each of the crater-like holes in the brass plate. A weighted plunger was inserted into each of the hollow tubes so that it rested on the surface of the soaked pea. The apparatus was then placed over a tray of boiling water and the peas were cooked in an atmosphere of steam. As the pea cooked, the weighted plunger penetrated the pea. Cooking curves were made by plotting the percent of peas that were cooked as a function of time.

Rosenbaum et al. (1966) described a device which was used to measure the cooking quality of one half of a single pea. A half pea was placed on a stationary brass plate which was fitted with brass nails (1 mm dia.) arranged 1.5 mm apart. A plunger was lowered into position so that it rested on the convex surface of the pea. A weight-arm was adjusted so that it rested on the plunger. The assembly was immersed in a beaker of water (25°C) and the temperature of the water was raised to the boiling point. As the pea softened, the plunger forced the pea into the prongs and the movement of the plunger was used as a measure of the cookability of the pea. The apparatus was later modified (Rosenbaum, 1966) by the attachment of a pen to the weight arm. As the pea softened the weight arm moved downward and the
pen traced out a curve on a kymograph. The slope of the curve at any point was taken as a measure of the cookability of a particular section of the pea cotyledon.

Some indirect methods have also been used to measure cooking quality of peas. The Brine Test gives a measure of the specific gravity of peas and this has been shown to be related to pea texture (Smith and Kramer, 1946). Bourne (1967) used size and density measurements as a means to distinguish good cooking peas from poor cooking peas.

Walls and Kemp (1940) suggested that there was a relationship between alcohol insoluble solids of peas and tenderometer (Martin, 1937a) readings.

II.1.2. Theories Related to Cooking Quality

II.1.2a. Hydration Properties of Peas

Adam and Siddappa (1935) noted that peas normally absorb about 75% to 80% of their own weight of water when they are soaked for 18 hours. The peas absorb additional water when they are cooked, and as a result they continue to swell until eventually the cotyledon ruptures.

The cooking quality of dried peas is closely associated with the capacity of the pea to absorb water. Adam and Siddappa (1935) and Snyder (1936) have shown that peas
which do not absorb water when soaked, remain hard when they are cooked. These findings have been confirmed by Mattson et al. (1951), Crean and Haisman (1963a) and Bhatia et al. (1967). Several workers have suggested that the seed coat may inhibit water absorption (Adam and Siddappa, 1955; Snyder, 1936; Mattson et al., 1951; Hamad and Powers, 1965; Rockland and Mitzler, 1967; Larson, 1968). Denny (1917), Harrington (1949), Mattson et al. (1951) and Hamad and Powers (1965) have indicated that a layer of cutin on the surface of the seed coat is partly responsible for the slow hydration of peas. These workers have shown that the permeability of the pea may be increased by the removal of the surface layer of cutin by scratching. This treatment, which is commonly known as scarification, usually leads to an improvement in the cooking quality. Harrington (1949) suggested that a low moisture content may reduce the permeability of the colloidal cutin layer on the seed coat. He recommended the storage of peas under conditions of high humidity in order to improve their permeability and cooking quality. This assumption was substantiated by the findings of Snyder (1936), Crean and Haisman (1963a) and Morris (1963) who found that peas with a high moisture content cooked faster than did those with a low moisture content.
Other workers, however, have found that peas that were stored under conditions of high humidity did not cook as well as those stored under conditions of low humidity. (Muneta, 1964; Wood, 1964; Burr et al., 1968).

Mattson (1946) and Mattson et al. (1951) found that blanching may improve the cooking quality of peas. They suggested that this treatment destroyed the layer of cutin on the seed coat and therefore increased the permeability of the pea.

Adam and Siddappa (1935) found that peas which were cooked or soaked in water containing calcium ions, swelled less than did peas cooked or soaked in distilled water. Snyder (1936) reported similar results and suggested that any treatment which altered the amount of water absorbed by peas would also alter their cookability. She noted that peas which were cooked in solutions containing monovalent cations and pectic solvents such as ammonium citrate, gained more weight and cooked more easily than did peas which were cooked in distilled water. Rosenbaum (1966) confirmed Snyder's findings, that calcium ions in the cooking water reduced the weight gained and cooking quality of peas. He also demonstrated that good cooking peas lost more solids when they were soaked (25°C) in water containing calcium
ions than did poor cooking peas.

Manery (1966) showed that calcium ions increased the rigidity of cytoplasmic surfaces and reduced membrane permeability. He suggested that calcium ions dehydrate membranes at cell surfaces. Garrard and Humphreys (1967) found that calcium ions reduced the leakage of sucrose from corn scutellum slices. They suggested that calcium ions regulate the permeability of cell membranes. Ciric and Jovanovic (1969) also indicated that calcium and magnesium ions in the cooking water penetrate the peas and combine with pectin. They claimed that this reduces the diffusion of soluble substances out of the pea. Jones and Lunt (1967) prepared a comprehensive review on the possible role of calcium in membranes, chromosomes and cell walls.

II.1.2b. Cellular Cement

Sosnin (1927) studied the problem of cooking quality in peas with special reference to the importance of the pectin in the middle lamella which binds the cells together. Mattson (1946) suggested that when peas are cooked the pectin in the middle lamella dissolves and allows the individual cells to separate. He suggested that the cellular cement consists of long chains of pectin which are crosslinked by divalent cations such as calcium and magnesium ions. He
claimed that the solubility of the pectin in the middle lamella depends on the number of calcium cross-linkages between the parallel pectin strands. The greater the calcium ion concentration the more cross-linkages and the less soluble is the pectin. He also suggested that any material which ties up calcium ions or removes them from the pectin molecules, also increases the solubility of the cellular cement and therefore improves the cooking quality of peas. Earlier, studies by Sayre *et al.* (1931), Adam and Siddappa (1935) and Snyder (1936) showed that peas of poor cooking quality contained more calcium than those of good cooking quality. These results agreed with Mattson's (1946) findings and gave some support to his hypothesis concerning the effect of calcium ions on the solubility of pectin in the middle lamella.

Adam and Siddappa (1935) and Bjälffe (1944) found a positive correlation between the phosphorus content of peas and cooking quality. Mattson's (1946) results were in agreement with these findings and he suggested that most of the phosphorus in peas was in the form of phytic acid (myo-inositol hexa phosphoric acid). He pointed out that phytic acid has a very high affinity for calcium and therefore suggested that phytic acid competes with pectic for the available calcium ions in the pea. Peas with a high phytic acid
content should therefore contain soluble pectin and should cook more easily than peas with a low phytic acid content.

Mattson's (1946) hypothesis, which stated that cooking quality is directly proportional to phytic acid content and inversely proportional to calcium ion content of peas, has been tested by several workers. Mattson et al. (1951), Fowler (1957), Smithies (1960), Gfeller and Halstead (1967) and Muller (1967) obtained results which supported Mattson's hypothesis. However, Morris and Seifert (1961) and Halstead and Gfeller (1964) were unable to find any relationship between the phytic acid content of peas and the cooking quality of peas. Rosenbaum et al. (1966) found that within a given sample of poor cooking peas there was no significant correlation (5% level) between phytic acid and cooking quality if the peas were cooked (seed coat removed) in water containing calcium ions. There was a correlation (2% level) between phytic acid and cooking quality within this same sample of peas if the peas were cooked (seed coats removed) in distilled water. The authors demonstrated that peas of poor cooking quality are more sensitive to calcium ions in the cooking water than are peas of good cooking quality. There was, however, no significant difference (5% level) between the average phytic acid content of
poor cooking and good cooking peas.

Rosenbaum and Baker (1969) later reported that the peripheral region of the pea took longer to cook than did the interior region of the pea. They also found that the peripheral region contained about 25% more phytic acid than did the interior of the pea. These findings do not support the hypothesis (Mattson) that high phytic acid is associated with good cooking quality.

Albersheim (1959) and Albersheim et al. (1960) studied the mechanism by which pectin is hydrolyzed in neutral solution. They claimed that pectin in the middle lamella is converted to shorter, more soluble units, when heated in neutral solution and that the rapid breakdown of pectin is dependent on the presence of methylester groups on the pectin molecule. The authors suggested that the mechanism of hydrolysis in neutral solution is similar to the mechanism of alkaline degradation of pectin (Garrick, 1958; Neukom and Deuel, 1958). Albersheim et al. (1960) pointed out that the presence of sodium salts in the cooking water may provide the buffering conditions necessary for the rapid hydrolysis of the pectin in the middle lamella. He disagreed with Mattson's (1946) suggestion that sodium ions in the cooking water increased the solubility of pectin through
the removal of calcium cross-linkages between adjacent pectin molecules. Albersheim (1965) in a review on cell wall polysaccharides, indicated that the nomenclature which is commonly used for pectin substances, is misleading. He stated that cell wall polysaccharides are normally divided into groups according to their solubilities but these groups were named according to their chemical structure. Pectin is defined as a polymer of galacturonic acid and hemicellulose is defined as a polymer of pentosans. The author pointed out that the solubility of each of these fractions depends on the susceptibility of the material to hydrolysis or degradation. Each fraction was extracted only after sufficient covalent bonds had been broken. The more easily hydrolyzed material was extracted first and was referred to as pectin while the material which was more resistant to hydrolysis was called hemicellulose. Albersheim pointed out that the soluble material which was known as pectin may not be pure polygalacturonic acid. In some instances the fraction which was believed to be pectin was found to contain only a small percentage of galacturonic acid and should more appropriately be called hemicellulose. Bonner (1936), Olsen et al. (1939), Jones (1951) and Northcote (1963) have also demonstrated that pectic materials which
could be extracted from various plant tissues contained co-
valently bound hemicellulose.

Bonner (1950), Powrie et al. (1960) and Albersheim
and Killias (1963) noted the presence of pectic materials
in the cell walls and middle lamella of plant tissues.
Esau (1953) stated that it is usually very difficult to
distinguish between the cell wall and middle lamella of
plant tissue because of the similarity in the composition
of the two structures.

Reeve (1947) made a histological examination of peas
that were at different stages of maturation. He found that
the middle lamella which is mainly pectic in nature, becomes
"encrusted" with pentosans or hemicellulose as the pea
matures.

Recently, evidence has been presented that suggested
that proteins which are high in hydroxyproline may be in-
volved in cellular adhesion (Ginsburg, 1958, 1961; Lamport,
1967). King and Bayley (1965) found that hydroxyproline
was present only in the cell wall fraction and not in the
cell contents of pea stems.

Cleland and Karlsnes (1967) suggested that hydroxy-
proline containing proteins of the end wall of Alaska pea
epicotyls are involved in cell elongation. Bhatia et al.
(1967) found that pulses which had been treated with the proteolytic enzyme papain had a higher cooking quality and were not affected by the hardness of the cooking water as were untreated pulses.

II.1.2c. Phytic Acid and Pectin Metabolism

Many workers have found relationships between the phytic acid content and cooking quality of peas (Mattson, 1946; Mattson et al., 1951; Smithies, 1960; Fowler, 1957; Rosenbaum et al., 1966; Gfeller and Halstead, 1967; Muller, 1967). Other studies have shown that the nature of the cellular cement may have a direct bearing on the texture of peas (Mattson, 1946; Dudkin et al., 1968).

It is generally accepted that as growth proceeds in plant tissues the polysaccharides of the cell wall undergo a change in their chemical composition. Several workers have shown that the synthesis and degradation of pectin and hemicellulose is controlled or regulated in part by the growth hormone indole acetic acid (Ordin et al., 1955; Albersheim, 1963; Ray and Baker, 1965; Cleland, 1967; Katz and Ordin, 1967; Wada et al., 1968).

Although there has been no direct evidence to suggest a relationship between phytic acid and pectin synthesis in peas it is interesting to note that myo-inositol is a
precursor of both pectin and phytic acid in many plant tissues.

Excellent reviews on the metabolic pathway for the synthesis of pectin have been written by Wilson (1964), Loewus (1967) and Hassid (1967).

Loewus and Kelly (1962) demonstrated that labeled glucose is converted through a cyclization reaction to myo-inositol in parsley leaves. Chen and Charalampous (1965) showed that an enzyme system from yeast catalyzes the cyclization of glucose-6-phosphate to inositol-1-phosphate.

Dietz and Albersheim (1965) found an enzyme system in mung bean (*Phaseolus aureus*) which selectively phosphorylates myo-inositol. English *et al.* (1966) found that myo-inositol kinase from mung bean seeds converts myo-inositol to myo-inositol-1-phosphate. They suggested that myo-inositol-1-phosphate was an intermediate in phytic acid biosynthesis and not in cell wall synthesis. Roberts and Loewus (1968) showed that *Wolffiella floridans*, an aquatic angiosperm, converts labeled myo-inositol to phytic acid and that very little of the activity goes into the polysaccharides of the cell wall. Loewus *et al.* (1958), however, noted that in the ripening strawberry myo-inositol is converted to pectin and hemicellulose. Loewus *et al.* (1962) studied the
metabolism of myo-inositol and found that it was converted primarily to D-glucuronic acid in strawberry fruit and in the leaves of parsley. Loewus and Kelly (1963) found that myo-inositol-2-C is cleaved between carbon atoms 1 and 6 and is converted to galacturonate-5-C in strawberries. Roberts et al. (1968) incubated excised root tips from young Zea mays plants in a solution of myo-inositol-2-C. Their results indicated that 50% of the activity was incorporated into the ethanol insoluble residues of the cell wall. Hydrolysis of the ethanol insoluble residue yielded labeled D-glucuronic acid, D-xylose and L-arabinose. They also noted that phytic acid may be broken down to myo-inositol which is then converted to cell wall polysaccharides. An excellent review on the metabolism of inositol in higher plants has been written by Loewus (1969).
III. EXPERIMENTAL

III.1. Materials and Methods

III.1.1. Selection of Non-Radioactive Peas

Dried peas (Sterling variety) were selected from individual plants that were grown in a field near Portage la Prairie, Manitoba in 1966. The peas that were obtained from the same pod were stored together.

III.1.2. Growing Peas Containing $^{45}$Ca

Peas (Sterling variety) were grown in a greenhouse and were subjected to 16 hours of artificial light per day. The plants were injected with radioactive calcium (0.1 ml/blossom; 15.6 $\mu$ curries) at a point in the internode which was just below the node carrying the blossom. The injection was made five days after full flowering. The peas were harvested after an additional period of 40-50 days and were stored for one month at room temperature before they were used.

III.1.3. Preparation of $^{45}$Ca Stock Solution

A solution (0.48 ml) containing radioactive calcium
(2M curies $^{45}$Ca in 0.48 ml; 1.3N HCl; Atomic Energy of Canada, Ottawa) was added to a sodium hydroxide solution (12.30 ml; 0.050N). The resulting stock solution contained 156M curies/ml $^{45}$Ca. The hydrogen ion concentration of this solution was approximately 0.0007 eq/liter. This represents a pH of 3.15.

III.1.4. Preparation of Pea Section for X-ray Autoradiography

Pea cotyledons that were soaked or cooked and which contained $^{45}$Ca were sectioned (0.5 mm thick) by means of a scalpel. The cotyledon was sliced in a plane that was perpendicular to the flat surface of the cotyledon. Slicing was begun at a point farthest away from the micropyle. Each section was mounted on a glass slide which was then wrapped in Saran wrap (Dow Chemical Company, Midland, Michigan). The slide was placed in a cassette and was covered with a sheet of X-ray film (Kodak Medical X-ray Blue brand). The film was exposed for seven days and was then developed.

III.1.5. Preparation of Cooked Peas for High Resolution Autoradiography

Peas (Sterling variety) were selected at random and their seed coats were removed. The cotyledons were cooked
(100°C) for 30 minutes in a solution of calcium chloride (100 ppm calcium) which contained 156 μ curies of $^{45}$Ca per liter of solution. The cotyledons were then soaked (25°C) for an additional hour in a non-radioactive solution which contained calcium chloride (100 ppm calcium). The cotyledons were rinsed in distilled water and sectioned (10 μm thick) on a freezing microtome. The sections were rinsed in three changes of distilled water to remove the calcium which was not tightly bound to the tissue. The rinsed sections were placed on glass slides that were coated with a thin film of Meyer's albumin. The slides were dried on a warming plate (38°C) and stored in a slide box at room temperature.

III.1.6. Preparation of Soaked Peas for High Resolution Autoradiography

The peas used in this study were obtained from plants that had been injected with $^{45}$Ca (section III.1.2.). The radioactive peas were harvested 23 days after full flowering and were then dehydrated at room temperature in the following series of solutions:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment (hrs)</th>
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<tbody>
<tr>
<td>Water</td>
<td>50%</td>
</tr>
<tr>
<td>Ethanol</td>
<td>40%</td>
</tr>
<tr>
<td>Tert butyl alcohol</td>
<td>10%</td>
</tr>
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</table>
The dehydrated peas in solution (f) were mixed with tissuemat (56°C mp, Fisher Scientific) and the mixture was maintained at 56°C for four hours. The liquid was replaced six times every four hours with melted tissuemat. The embedded peas were then placed in wax blocks and were sectioned (10 μm) by means of a rotary microtome. The sections were mounted on slides and then dipped in xylene to remove the wax.

III.1.7. Application of Photographic Emulsion

The coating technique used for high resolution autoradiography was the same as that described by Kopriwa and
Leblond (1962).

The slides bearing the unstained sections (10 μm thick) were first dipped in a mixture of ethanol and ether (50:50 v/v) which contained 1% celloidin (Tissue embedding solution, No. M 4700, Randolph Products Co., Carlstadt, N.J.). Subsequent steps in the procedure were carried out in a dark room. A wratten safelight No. 2 (Eastman Kodak) was placed three feet away from the working area.

Gelled NTB2 emulsion (Eastman Kodak Co., Rochester, N.Y.) was placed in a water bath (42°C) one hour before the slides were to be coated. The slides were then dipped into the melted emulsion for one to two seconds. The back surface of the slides were wiped clean with a piece of paper tissue and the slides were rested in a vertical position for one hour (safelight off). The slides were then placed (emulsion side down) in a black plastic slide box which contained a small package of drying agent (silica gel). The slide box was sealed with black plastic tape and placed inside a black metal tin. The tin was stored in a refrigerator (5°C) during the exposure period. The exposure period for the peas that were cooked in a solution of 45Ca was 10 days and for the peas that were grown in the greenhouse (plants injected with 45Ca) was 27 days.
III.1.8. Developing Process for High
Resolution Autoradiography

The method used to process the autoradiograms was that outlined by Kopriwa (1967). After the slides had been exposed, they were placed horizontally (emulsion side up) in a developing rack. The developing rack was made from a slide box (3½ x 3") in which the front and back panels had been cut out. The slide rack which contained the slides was placed in developer solution (18°C) for six minutes. The developer solution was made by dissolving 4.5 gm amidol developing agent (May & Baker Canada Ltd.) in 200 ml of stock D-170 developer solution which was first diluted to 1,000 ml with distilled water. Developer solution (stock D-170) was made by dissolving anhydrous sodium sulfite (125 gm) and potassium bromide (5 gm) in distilled water. The volume of this solution was then made to one liter with distilled water.

After the slides had been developed for six minutes in the developer solution the rack was removed and placed in distilled water (18°C) for 30 seconds. The slide rack was then placed in a sodium thiosulfate solution (24%, 18°C) for three minutes. The slide rack was then rinsed in cold running tap water for 10 minutes. The slides were dehydrated
for two minutes in 95% alcohol and for two minutes in each of two changes of absolute alcohol. The slides were then removed from the developing rack and were placed in a mixture of cedarwood oil and absolute alcohol (1:1 v/v) for one hour. The slides were transferred to a mixture of Canada-balsam and xylene (1:1 v/v) for at least one hour. The slides were mounted with a cover glass and dried in a horizontal position in an oven at 37°C. After the preparation had dried, the excess Canada-balsam was removed from the slides with xylene. The slides were examined by means of a microscope and were photographed.

III.2. Results and Discussion

III.2.1. Effect of Blanching on the Distribution of Calcium in Peas

Peas from the same pod were divided into two groups. One group of peas was blanched (100°C) for 5 minutes in distilled water and then soaked (25°C) for 6 hours in a solution of calcium chloride (100 ppm calcium) which contained 156 μ curies of 45Ca per liter of solution. The other group of peas was first soaked (25°C) for 6 hours in a solution of calcium chloride (100 ppm calcium) which contained 156 μ curies of 45Ca per liter of solution and then cooked.
(100°C) for 5 minutes in distilled water. The peas were sectioned by hand and X-ray autoradiograms were prepared. The results shown in Figure 1 indicated that the peas which were not blanched before soaking accumulated most of the 45Ca at the periphery of the pea. The peas which were blanched first contained a double line of 45Ca near the periphery of the pea cotyledon. These results show that blanching affects the distribution of calcium in the pea. The cause of the double band of calcium in the blanched pea is unknown. The autoradiogram shown in Figure 1 indicated that 45Ca has diffused farther into the blanched pea than in the unblanched pea. The blanching process may have increased the permeability of the pea to calcium ions and thus the 45Ca was able to diffuse farther into the blanched pea during soaking than into the unblanched peas.

It is also possible that blanching may have increased the capacity of certain regions of the pea to bind calcium ions. This may account for the double band of 45Ca in the blanched pea cotyledon. If this were true then it would be reasonable to assume that a heat treatment following a soaking period would also produce a similar distribution of calcium in the pea cotyledon. The results show clearly, however, that the double band of 45Ca is present only in the peas that
were heat treated before soaking (Figure 1B) and not in the ones that were heat treated after soaking (Figure 1A).

It is also possible that the heat treatment may have reduced the pea tissue's capacity to bind calcium ions only in the region between the two bands of $^{45}$Ca (Figure 1B). It is also possible that the $^{45}$Ca may have moved through this region which has a low affinity for calcium (high permeability) while the $^{45}$Ca may never have reached this region in the unblanched peas because of the lower permeability of these peas.

III.2.2. Effect of Seed Coat on the Distribution of $^{45}$Ca in Peas

The peas which were used in this experiment were obtained from the plants that had been injected with $^{45}$Ca. Four peas of approximately equal size were selected from a single pod. The seed coats were removed from two of the peas and the other two peas were left intact. All four peas were then soaked ($25^\circ$C) for 6 hours in calcium chloride solution (100 ppm calcium). Two peas (one with seed coat removed and one intact pea) were then cooked ($100^\circ$C) in this solution for 30 minutes. All four peas were then sectioned by hand and X-ray autoradiograms were prepared (section III. 1.4.). Pictures of typical X-ray autoradiograms are
presented in Figure 2 and represents the results of four separate trials. It can be seen that the pea which is soaked with its seed coat removed (Figure 2A), contains less $^{45}\text{Ca}$ at the periphery of the cotyledon than does the one (Figure 2C) which is soaked with its seed coat intact. The low concentration of $^{45}\text{Ca}$ near the periphery of the cotyledon (Figure 2A) may result from a diffusion of calcium out of the cotyledon during soaking. It may also result from the migration of calcium toward the centre of the cotyledon, caused by the movement of water into the pea. The high concentration of $^{45}\text{Ca}$ at the periphery of the other cotyledon (Figure 2C) may result from the transfer of calcium to the cotyledon from the seed coat. The seed coat may also prevent calcium from diffusing out of the cotyledon during soaking. The low concentration of calcium at the centre of this pea (Figure 2C) as compared to that of the other pea (Figure 2A) suggests that the migration of calcium toward the centre of the cotyledon during soaking may be reduced by the seed coat.

A comparison of Figure 2A with Figure 2B and a comparison of Figure 2C with Figure 2D indicate that calcium diffuses away from the centre of the soaked cotyledon when they are cooked. Most of the $^{45}\text{Ca}$ in the cooked peas is in a thin band at the periphery of the cotyledon.
Figure 2

Autoradiogram of Cooked and Soaked Peas from Plants Injected with $^{45}\text{Ca}$

A) Peas (seed coat removed) soaked (25°C) for 6 hours.

B) Peas (seed coat removed) soaked (25°C) for 6 hours and then cooked (100°C) for 30 minutes.

C) Peas (seed coat intact) soaked (25°C) for 6 hours.

D) Pea (seed coat intact) soaked (25°C) for 6 hours and then cooked (100°C) for 30 minutes.
The results of this experiment also indicate that the cotyledons which were soaked and cooked with their seed coats intact (Figure 2D) contain more $^{45}$Ca than do those cooked without their seed coats (Figure 2B). It can be concluded, therefore, that the amount of calcium in the cotyledon of a pea is influenced by its seed coat.

III.2.3. High Resolution Autoradiography of Peas Injected with $^{45}$Ca

Green peas were harvested (23 days after flowering) from plants that were injected with $^{45}$Ca. One pea from each pod was sectioned (0.5 mm thick) with the aid of a scalpel and an X-ray autoradiogram (exposure 7 days) was made of the pea slices (section III.1.4.). The X-ray autoradiogram (Figure 3) of the green pea shows that most of the calcium is concentrated in the seed coat. There is also a higher concentration of calcium at the periphery of the cotyledon than at the centre of the cotyledon. The other peas in the pod were dehydrated, imbedded in wax and sectioned by the procedure described previously (section III.1.6.). The sections were coated with photographic emulsion (section III.1.7.) and stored in the dark ($5^\circ$C) for 27 days. The slides were developed (section III.1.8.) and were then photographed on a microscope. Figure 4 shows a typical
Figure 3
Autoradiogram of Green Pea from Plant Injected with $^{45}\text{Ca}$

Figure 4
High Resolution Autoradiogram of Green Pea from Plant Injected with $^{45}\text{Ca}$
high resolution autoradiograph of the pea sections. It was observed that most of the $^{45}$Ca in the cotyledon is concentrated in the middle lamella. Crean and Haisman (1964) found that the calcium in rehydrated pea cotyledons was associated mainly with the cytoplasmic membrane.

III.2.4. High Resolution Autoradiography of Peas Cooked in a Solution Containing $^{45}$Ca

Field grown dried peas were selected at random and cooked in a solution containing $^{45}$Ca (156 µ curries/liter solution). The cooked peas were sectioned on a freezing microtome and mounted on slides. The slides were then coated with photographic emulsion (section III.1.7.) and stored in the dark (5°C) for 10 days. The slides were developed (section III.1.8.) and were then photographed under a microscope. A photograph of a typical autoradiogram of a cooked pea is presented in Figure 5. The results indicate that most of the $^{45}$Ca in the cell had accumulated at the cell membrane and in the cytoplasmic matrix around the starch granules. Very little $^{45}$Ca was found in the middle lamella and in the cell wall. These results were in agreement with those of Crean and Haisman (1964) who found that there was very little calcium in the cell wall or middle
Figure 5

High Resolution Autoradiogram of Pea Cooked in Solution Containing $^{45}\text{Ca}$
lamella as compared to the content of calcium in the cells of cooked peas. Crean and Haisman (1964) used a histological staining technique to detect the calcium in the pea tissue.

The high concentration of calcium in the cytoplasmic matrix of cooked peas may result from the binding of calcium by protein in the cytoplasm or cell membrane. This may effect the permeability of the pea tissue and therefore the cooking quality of the peas. A study of the protein of peas may therefore provide information that will lead to a better understanding of the changes that take place when dried peas are cooked.
IV. SUMMARY

1. It is well known that dried peas which are blanched before they are soaked, cook faster than those that are not blanched. Earlier work has suggested that the distribution of calcium in dried peas may influence the cooking quality of the peas. In the present study blanched and unblanched peas were soaked in water containing $^{45}$Ca and the location of the $^{45}$Ca in the peas was ascertained by X-ray autoradiography. It was shown that the $^{45}$Ca in blanched peas was distributed in two bands which were located near the periphery of the cotyledon. The $^{45}$Ca in unblanched peas was distributed in only one band which was located at the periphery.

2. It is well known that the seed coat influences the cooking properties of dried peas. An experiment was performed to determine whether the seed coat affects the distribution of calcium in soaked and cooked pea cotyledons. Pea plants were grown in a greenhouse and were injected with $^{45}$Ca. Radioactive dried peas were harvested from these plants and were soaked and cooked in distilled water with and without their seed coats. The $^{45}$Ca in these peas was then located by use of X-ray autoradiography. The
results indicated that peas which were soaked and cooked without their seed coats contained less calcium than those which were soaked and cooked with their seed coats intact.

3. Peas which were obtained from plants that were injected with $^{45}$Ca were harvested while they were still green. These peas were subjected to high resolution autoradiography to determine the location of calcium in the pea cotyledon at the cellular level. The results showed that the calcium was located mainly in the middle lamella.

4. High resolution autoradiography was used to locate the calcium in peas that were cooked in water containing $^{45}$Ca. The results indicated that the calcium was located mainly in the cell membrane and cytoplasmic matrix.
V. CLAIMS TO ORIGINAL RESEARCH

(Note:- Claims to original research based on the studies in the main body of the thesis are presented on page 110).

1. The first demonstration that the distribution of calcium in blanched dried peas is different from the distribution in unblanched dried peas; and that the blanching increased the permeability of the pea cotyledon to calcium.

2. The first demonstration that the seed coat either supplies calcium to the pea cotyledon or that it prevents the loss of calcium by the pea cotyledon during soaking and cooking.

3. The first use of high resolution autoradiography to demonstrate that the calcium in the tissue of the cotyledon of a fresh green pea is located in the middle lamella.

4. The first use of high resolution autoradiography to demonstrate that the calcium in a cooked pea is associated mainly with the cell membrane and the cytoplasmic matrix. Very little calcium was found in the cell wall and middle lamella.
VI. LITERATURE CITED

Adam, W.B. and Siddappa, G.S. 1935.
The composition and texture of dried peas.

Physical methods of measuring quality of canned peas.
Food Tech., 12: 96.

Albersheim, P. 1959.
Instability of pectin in neutral solutions.

Splitting of pectin chain molecules in neutral solution.

Albersheim, P. 1963.
Hormonal control of myo-inositol incorporation into
pectin.
J.B.C., 238: 1608.

Histochemical localization at the electron micro­
scope level.

Albersheim, P. 1965.
Biogenesis of the cell wall. In: "Plant Biochemistry."
York, pp 298.

Bhatia, B.S., Ramanathan, L.A., Prasad, M.S., and Vyayaragh­
Use of papian in the preparation of quick cooking
dehydrated pulses and beans.
Food Technol., 21: 105.

Bjälftve, G. 1944.
Arternas kokbarket och fosfathalt.
Lantmannen, 28: 44.
Cited in Mattson, S. 1946.

158


Christel, W.F. 1938. Texturemeter, a new device for measuring the texture of peas. Canning Trade, 60: 34.


A note on the slow rehydration of some dried peas. 

Interaction between phytic acid and divalent cations during the cooking of dried peas.  

The cytological distribution of calcium in raw and cooked seed peas.  

Denny, F.E. 1917.  
Permeability of certain membranes to water.  
Botanical Gazette, 63: 468.

The enzymic phosphorylation of myo-inositol.  

Composition and hydrolysis kinetics of hemicellulose and cellulose of pea and bean seeds.  

Myo-inositol kinase. Partial purification of product.  
Science, 151: 198.

Esau, K. 1953.  
"Plant Anatomy."  
John Wiley and Sons, New York, N.Y.

Fowler, H.D. 1957.  
The changes in orthophosphate in relation to phytin formation in maturing peas.  

The effect of divalent cations on the leakage of sucrose from corn scutellum slices.  
Phytochem., 6: 1085.
Garrick, P. 1958.  
Alkaline degradation of pectin.  
Chem. and Ind., p. 1430.

Selection for cooking quality in field peas.  

Evidence for a protein component in the middle lamella of plant tissue: a possible site for indolacetic acid action.  

Evidence for a protein gel structure cross-linked by metal cations in the intercellular cement of plant tissue.  

Halstead, R.L. and Gfeller, F. 1964.  
The cooking quality of field peas.  

Imbibition and pectic content of canned dry-line beans.  
Food Technology, 12: 216.

Harrington, J.F. 1949.  
Hard seed in beans and other legumes.  
Seed World, 64: 42.

Transformation of sugars in plants.  

Jones, J.K.N. 1951.  
The chemical composition and properties of pectins.  
Chem. and Ind., 430.

The function of calcium in plants.  
Metabolic turnover in cell wall constituents of *Avena sativa* L. coleoptile sections.

A preliminary analysis of the proteins of the primary walls of some plant cells.

Improvements in the coating technique of radioautography.
*J. Hist. and Cytol.*, 10: 269.

Kopriwa, B.M. 1967.
Personal communication.

Hydroxyproline-0-glycosidic linkage of the plant cell wall glycoprotein extension.

The effect soaking pea seeds with or without seed-coats has on seedling growth.

The conversion of Cl4-labeled sugars to L-ascorbic acid in ripening strawberries. IV. A comparative study of D-galacturonic acid and L-ascorbic acid formation.
*J.B.C.*, 232: 533.

Conversion of glucose to inositol in parsley leaves.

Metabolism of myo-inositol in plants: conversion to pectin, hemicellulose D-xylose and sugar acids.

Inositol metabolism in plants. I. Labeling patterns in cell wall polysaccharides from detached plants given myo-inositol-2-t or -2-C14.


Muller, F. M. 1967.
Cooking quality of pulses.

Muneta, P. 1964.
The cooking time of dried beans after extended storage.
Food Tech., 12: 130.

Alkaline degradation of pectin.
Chem. and Ind., p. 683.

The biology and chemistry of the cell walls of higher plants, algae, and fungi.

Pectin studies, relation of combining weight to other properties of commercial pectin.

Influence of auxin on cell-wall metabolism.

Chemical, anatomical and histochemical studies on the navy bean seed.

The effect of auxin on synthesis of oat coleoptile cell wall constituents.

Relation of histological characteristics to texture in seed coats of peas.
Food Research, 12: 10.

Inositol metabolisms in plants. V. Conversion of myo-inositol to uronic acid and pentose units of acidic polysaccharides in root-tips of Zea Mays.
Inositol metabolism in plants. VI. Conversion of myo-inositol to phytic acid in **Wolffiella floridana**.

Quick-cooking lima and other dry beans.
Fd. Technol., 21: 26A.

Chemical composition in relation to the cooking quality of field peas (**Pisum sativum** L.).
M.Sc. Thesis, McGill University, Montreal, Quebec.

Constitution of leguminous seeds. VI. The cookability of field peas (**Pisum sativum** L.).

Constitution of leguminous seeds. VII. Ease of cooking field peas (**Pisum sativum** L.) in relation to phytic acid content and calcium diffusion.

Factors affecting the quality of commercial canning peas.

The brine test for maturity of canned peas.
The Canning Trade, p. 7.

Smithies, R.H. 1960.
"Texture in Foods." Effect of chemical constitution of peas.
S.C.I. Monograph No. 7, p. 119.

Snyder, E.B. 1936.
Some factors affecting the cooking quality of peas and Great Northern Type of dry beans.
Sosnin, A.V. 1927.
Die Untersuchung der Kochfähigkeit der Erbsensorten.
Cited in Mattson, S. 1946.

An evaluation of objective methods for determining
the maturity of canning peas.

Cell elongation and metabolic turnover of the cell
wall as affected by auxin and cell wall degrading
enzymes.

Walls, E.P. and Kemp, W.B. 1940.
Relationship between tenderometer readings and alcohol-
insoluble solids in Alaska peas.

Wilson, R. 1964.
The growth of plant cell walls.

Quality in Blue Peas.