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PRELIMINARY STUDIES ON THE ELECTROPHORETIC
PROPERTIES OF PLANT PROTEINS

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PROPERTIES OF PLANT PROTEINS

L. R. Wetter

Department of Plant Science

A THESIS

submitted to the University of Alberta
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PRELIMINARY STUDIES ON THE ELECTROPHORETIC PROPERTIES OF PLANT PROTEINS

L. R. Wetter

INTRODUCTION

For the past six or seven years fundamental research on protein properties has been interrupted by the war. During this period research was directed into more practical channels, e.g. dried blood plasma. With research returning to a peace-time basis, more time will be spent on fundamental research regarding the basic properties of proteins. Probably only after more is known of these properties can proteins be fully utilized in industry.

In the summer of 1944 the Departments of Physics, Chemistry, and Plant Science, undertook to study some of the fundamental properties of the plant proteins. This investigation was made possible by a grant from the University Committee on Agricultural Research Grants. As a result of this, the Department of Plant Science purchased an electrophoresis apparatus to aid in these studies.

Electrophoretic studies are designed to give information on the electrostatic properties of the protein molecule. These properties arise from the electric charges carried on

the protein molecule. When the protein is in solution, the charges vary with variations in the free groups subject to ionization. If all the protein molecules in a solution have the same charge, they will behave the same in the electrophoresis apparatus--i.e. only one boundary will be present. If the solution contains a mixture of proteins, the components will tend to be separated under the influence of a direct current.

Since, in this method of study, the protein molecules actually move it can be used to separate the electrostatically homogeneous components. This separation has been carried out in a special type of cell (40). These pure components can be used for further physical studies, but the studies are limited by the small sample obtained. The pure components can also be used for the determination of the amino-acid content of the component. There are other possibilities for study, one being that electrophoretic measurements may aid as a guide in fractionating the protein mixtures into their component parts by chemical methods.

The method has been widely used in the study of animal proteins, especially blood serum and plasma. Animal proteins lend themselves well to this method of study because they are already in solution. In plant seeds, one of the major problems is to get the protein into solution in its native state. This probably is the chief reason that the electrophoresis method has not been used to any great extent on plant proteins. There are other problems to which Vickery

(43) draws attention.

The first problem studied was the peptization of a variety of plant proteins. An investigation was made to see if most of the protein could be peptized in neutral salts. Then a preliminary study was carried out to see whether these extractions would lend themselves to electrophoretic studies.

This thesis is divided into two sections: the first deals with the extraction work; the second with the electrophoretic properties of these extractions.

PART I

DISPERSION STUDIES OF SOME OF THE PLANT PROTEINS

LITERATURE REVIEW

During the 18th and 19th centuries various workers--e.g. Kessel-Meyer, Einhof, Boussingault, Liebig, Ritthausen, and others (28)--studied some of the plant proteins. Osborne contributed more to the study of plant proteins than any other previous worker. He found that water extracted very little protein from cereal seeds (28) but relatively large amounts were extracted from legume seeds. In neutral salt solutions the amount of protein peptized was less for cereal seeds than for oil seeds. Alkaline extractions usually disperse a greater percentage of the protein than either water or neutral salt solutions. Osborne noted that certain proteins were soluble in 70 to 80% alcohol solutions.

Cereals

Investigations of wheat proteins have been more extensive than those of the other cereal proteins. This is no doubt a result of the great economic importance of wheat.

Wheat

Staker and Gortner (37) found that wheat proteins were peptized to a lesser degree than were oat proteins. Four different solutions were used: 0.5 N KF, KCl, KBr, and KI. The amount of protein peptized in these solutions gave a definite lyotropic series, i.e. $KF < KCl < KBr < KI$. The results indicate that the amount of the total protein peptized depends on the extracting solution used. The total nitrogen peptized varied from 25% of the total for KF to 54% for KI. A "globulin-albumin" ratio was calculated but this ratio varied considerably depending on the solvent and concentration used. Hoffman and Gortner (11) arrived at the same conclusion. They found that different solvents extracted different amounts of protein from the same meal. These extractions evidently include various proteins, including the water-soluble fractions as well as part of the alcohol-soluble fraction. The protein composition varies as the concentration of the same salt is varied.

Smith et al (35), using Brill winter wheat, noted that there was an increase in the amount of protein peptized as the NaCl concentration increased. This increase was evident up to a concentration of 0.10 N, after which there was a levelling off. Smith and Circle (34) showed that the amount of protein dispersed depended on the pH of the extracting solvent. Minimum dispersion occurred between the pH's 3.5 and

6.4 (23.1 to 20.2% respectively). Maximum dispersion occurred at pH 1.7 (51%) and pH 11.0 (97.3%).

Gortner et al (8) raised an interesting question as to what a globulin actually is, since different salt solutions peptize different amounts of protein. They conclude that this variability is not due to constitutional differences in the protein but rather to differences in the colloidal properties.

Oats and Barley

Osborne (28) records having isolated a globulin known as avenalin from oats, and a prolamine known as hordein from barley. Staker and Gortner (37) carried out a preliminary investigation on both oats and barley to determine what percentage of the proteins could be peptized. They found that the proteins in oats peptized to a greater degree than did those in barley. This was particularly true when solutions of KBr and KI were used as extracting agents. The protein peptized ranged from 26% to 55% for oats and from 25% to 34% for barley.

Stamberg and McBain (38) found that the percentage of protein dispersed increased for different solvents in the following manner: $KF < KCl < KBr < KI$. This is in agreement with Staker and Gortner (37). Smith et al (35) observed that increasing concentrations of NaCl solutions did not increase to any marked degree the percentage of the protein peptized.

Legumes

Both Osborne (28) and Staker (37) observed that the proteins of legumes are more easily peptized than those of the cereals.

Peas

Staker and Gortner (37) dispersed Andes peas in neutral salt solutions and found that the dispersion power of the solutions followed a lyotropic series. This lyotropic series had one change in the position of the anions ($KCl < KF < KBr < KI$). The percentage protein dispersed was considerably higher for peas than for the cereals. If the meal was previously treated with ether, the percentage protein extracted increased. Staker and Gortner point out that this increase is a result of the removal of a layer of fat-like substances which prevent the penetration of the peptizing solvent.

Smith and Circle (34) obtained maximum peptization of protein in Alaska peas at pH 2.0 and 10.0, the extraction being 83% and 98% respectively. Minimum peptization occurred at pH 3.9. They suggest that the minimum peptization occurs close to the isoelectric point of most of the protein present in the meal.

Brown (3), in using 1% solution of sodium sulphate, sodium salicylate, and sodium chloride, found that the pep-

tization of the pea proteins was depressed. If solutions of 2% or over were used, the extractions were considerably higher. Variety had no effect on the amount of protein dispersed.

Soybeans

Smith et al (35) noticed that soybean meal behaved differently from any other meal studied. A water extraction disperses more protein than neutral salt solutions. Nagel (25) suggests that this can be explained by the presence of lecithin. Lecithin is an effective dispersing agent, and being in intimate contact with--or in actual combination with--the protein may account for the completeness of the dispersion in water.

The same investigators found that very weak salt solutions depressed the amount of protein peptized. This depression was greater but over a narrower range for divalent cations than for univalent cations.

Smith and Circle (34) obtained a minimum peptization of protein at pH 4.2; this is very close to the isoelectric point of soybean protein. A combination of acids and neutral salts at varying pH's gave results similar to those obtained for neutral salts alone.

Alfalfa and Sweet Clover Leaf Meal

Very little material is available concerning these

meals. Osborne and Wakeman (29), comparing fresh and dry spinach leaves, obtained approximately the same protein yields for extractions in ether, alcohol, water, and alkaline solutions. Thus they concluded that there was very little change in the constituents of the protein when the cells were dried.

Chibnall and Nolan (4) carried out investigations on the proteins of the fresh alfalfa leaf. They used an ether-water extracting solution. The vacuole yielded 0.56% of the leaf protein, the cytoplasm 8.61%. Great difficulty was encountered in trying to extract all the leaf protein.

Tottingham et al (42) studied the effect of desiccation of the proteins of sugar beet leaves. Drying leaves at 40°C. without aeration lowered the amount of protein that could be dispersed by 45%. They did not succeed in finding a temperature or type of aeration that did not reduce the "solubility" of the proteins. Their conclusion was that proteins in dried leaves are more difficult to disperse than those in fresh leaves.

Other Sources

Linseed Oil Meal

No literature was found that dealt with linseed oil meal but flax seed has undergone some investigation. Staker and Gortner (37) found that from 54% to 64% of the total

protein was peptized, depending on the extracting solution used.

Smith et al (35) observed that an increase in the concentration of NaCl from 0 to 0.2 N gave an increase in the amount of protein peptized. The increase was from 65% to 81%, but any additional increase in concentration did not increase the percentage of the protein dispersed.

O'Hara and Saunders (27) used different normalities of NaCl, varying from 0.125 N to saturated solutions. Water dispersed 24% of the protein, while 2 N NaCl dispersed 65%. From 2 N NaCl the percent protein peptized dropped continually until, for a saturated solution, only 45% had been peptized.

MATERIAL

The materials used in these extraction studies were oats, barley, wheat, linseed oil meal, sweet clover meal, alfalfa leaf meal, alfalfa meal, and soybeans. The oats, barley, and wheat were Victory, O.A.C.21, and Reward, respectively. The soybeans were obtained from Brooks, variety unknown. The linseed oil meal was a commercial sample. The alfalfa and sweet clover were collected at the University of Alberta. The latter were air-dried.

The samples were all ground in the Wiley mill, then ball-milled for six hours. The samples were stored in sample jars and total nitrogen and moistures were determined, the

latter at various periods of time.

The meals were all dispersed in an 8% sodium salicylate solution unless otherwise stated.

TABLE I

Total nitrogen* and moistures of the various meals

Meal	Nitrogen per gram dry weight, mgm.	Moistures, %
Wheat	33.45	8.69
Oats	22.48	10.56
Barley	24.76	10.86
Linseed oil	65.00	8.58
Sweet clover	52.68	8.75
Alfalfa leaf	42.22	7.33
Alfalfa	35.66	8.28
Soybean	73.37	6.15

* "Nitrogen" refers to the protein plus all other nitrogenous compounds.

METHODS

A known weight of meal was placed in a 200 cc. centrifuge bottle, to which was added a known volume of extracting solution. The meal was mixed to a smooth paste to ensure thorough mixing. The mixture was then shaken intermittently, i.e. 2 minutes shaking, 5 minutes no shaking, for one-half hour on a mechanical shaker. Then the mixture was centrifuged for 10 minutes at 2000 r.p.m. The supernatant liquid was removed and an aliquot taken for a nitrogen determination.

The Kjeldahl method was employed to determine the nitrogen present, using mercuric oxide as catalyst. The percentage nitrogen dispersed was based on the total amount present in the dry weight of the meal.

EXPERIMENTAL RESULTS

The Effect of Varying the Amount of Solvent

Eight meals were used to study the effect that the amount of solvent has on the percent protein extracted. Large volumes of solvent were avoided by varying the amount of meal used.

The results in Figure 1 indicate that the solvent-meal ratio has very little effect on the amount of protein extracted. Nagel et al (24) showed that the solvent-meal ratio was not critical when working with soybeans. The present study shows that this is true for all meals except linseed oil meal. This meal gave a significant decrease in the percentage protein dispersed as the amount of meal was increased.

Barley protein is difficult to disperse in 8% sodium salicylate as compared with protein from wheat and oats. Staker and Gortner (37) noted the same relation for the three cereals when using the potassium halides as extracting solutions.

Figure 1

The peptization of plant proteins in 8% sodium salicylate using different solvent-meal ratios (40 cc. of solvent)

Alfalfa meal (including the stalks) disperses a greater percentage of the total nitrogen than the alfalfa leaf meal. Both meals were harvested at the same time and received the same treatment. Alfalfa leaf meal contains only the leaves; alfalfa meal contains the entire plant. The difference in extraction results from the fact that the nitrogen in the stalk is more soluble than that in the leaf. When the percentage nitrogen extracted is based on total nitrogen present, the increase in the percentage is because of this difference in solubility. Table II shows that the amount of nitrogen peptized is not significantly different but the percent peptized is.

TABLE II

Comparison of the amounts of nitrogen extracted from alfalfa meal and leaf meal

Weight of meal, gm.	<u>Alfalfa meal</u>		<u>Leaf meal</u>	
	<u>Nitrogen extracted</u>		<u>Nitrogen extracted</u>	
	mgm.	%	mgm.	%
0.5	26.4	73.7	25.6	60.2
1.0	25.9	71.7	26.0	57.8
2.0	25.9	71.7	24.9	53.6
2.5	24.9	70.3	24.5	53.1
3.0	24.4	70.4	24.6	55.3

In order to determine whether or not the first extraction was representative of the total extraction, a calculation

was made taking into account the volume of original solvent and the volume retained by the meal. Table III gives these results.

TABLE III
Comparison of nitrogen extractions, %

Meal	Total represented in 1st extraction	Total extracted
Linseed oil meal	63.3	64.5
Oats	70.9	69.9
Alfalfa meal	71.7	72.7
Sweet clover leaf meal	54.9	55.6
Alfalfa leaf meal	53.6	58.9
Wheat	66.1	69.4
Soybeans	69.7	78.9
Barley	49.2	57.7

The results for the first four meals in the table indicate that the first extraction gives a representative sample of the total nitrogen in the meal. The four remaining meals indicate that subsequent extractions remove additional fractions of protein. This is especially true of the barley and soybean meals.

The Effect of Removing the Fats

Brown (3) found that the removal of fats or fat-like substances from the meal increased the amount of protein

dispersed. This increase was slight, however, and can hardly be considered significant. Nagel et al (25) reported that the increase in the amount of protein dispersed is considerable if soybean meal is first extracted with ether.

Three meals--linseed oil meal, soybeans, and oats--were treated with ether as described by Brown (3). Table IV summarizes these results.

TABLE IV

A comparison of nitrogen extracted in 8% sodium salicylate, using "fat-free" meals and "natural" meals

Weight of meal	Linseed oil meal		Soybeans		Oats	
	Fat-free	Natural	Fat-free	Natural	Fat-free	Natural
0.5 gm.	77.8	73.0	78.8	78.5	82.5	71.6
1.0 "	76.0	75.4	74.2	78.2	81.9	72.8
2.0 "	66.8	64.5	71.5	78.9	70.1	67.0
2.5 "	57.8	60.2	77.8	67.4	74.6	66.5
Average	69.6	68.3	75.6	75.8	77.3	69.5

The amount of protein extracted in soybeans does not appear to increase when the meal was previously treated with ether. This is not in agreement with what Nagel et al (25) found. It is hard to explain why there is this discrepancy in results. Nagel's extractions, however, were carried out in water; the above were carried out in 8% sodium salicylate. It

is known that salt extractions tended to depress the dispersion of soybean proteins. Another consideration is that sodium salicylate may penetrate the fat layer more easily than water and thus ether treatment would not increase the dispersion of the proteins. This latter statement, however, does not explain why the removal of fats increases the dispersion of the oat proteins.

A considerably higher proportion of the protein of oats was extracted after the fats and oils had been removed. The removal of fat-like substances is desirable when the extraction is to be used for electrophoresis analysis. This treatment tends to remove some of the pigmented compounds which interfere in an electrophoresis determination.

The Effect of Varying Concentrations of Sodium Salicylate

Spencer and McCalla (36), working with gluten, found that as the concentration of sodium salicylate was increased the total nitrogen dispersed increased. It was therefore decided to use varying concentrations of sodium salicylate to see if there was a concentration at which a maximum dispersion could be obtained. Six levels of sodium salicylate were used. The meals chosen for this investigation were barley, oats, wheat, linseed oil meal ("fat-free" and

"natural") and alfalfa meal.

Figure 2 gives a graphical representation of the results. The cereals all follow the same general trend: the amount of protein peptized increases up to 0.500 to 0.625 M sodium salicylate, after which there is a general levelling off. The other two meals did not behave in a similar manner. Approximately the same amount of protein was extracted from these meals regardless of the concentration of sodium salicylate used.

It is not surprising that the cereals all have a similar type of curve. Spencer and McCalla (36) found that gluten acted in much the same manner as the wheat in the present experiment. The similarity of the curves obtained for wheat, oats, and barley would suggest that the proteins involved in these extractions must be of similar nature. The extraction curves indicate that the proteins of linseed oil meal and alfalfa meal are different from those of the cereals.

The hydrogen ion concentration was measured by using a glass electrode, measurements being taken on the solution before and after the extraction. The results in Figure 3 indicate that all the meals except oats behave in a similar manner, i.e. increasing the salt concentration has no effect on the pH of the first extraction. Oats is the only meal that behaves differently. Here the substances causing the change in pH are less potent in concentrated solutions than in dilute solutions.

Figure 2

The peptization of plant proteins in
varying concentrations of
sodium salicylate

Figure 3

The pH's of the first extraction in varying concentrations of sodium salicylate

Figure 4 indicates that each successive extraction differs in its pH. All the meals, except linseed oil meal, behaved as barley, i.e. the pH of each successive extraction approaches that of the initial pH of the solvent. Linseed oil meal, however, became more basic for each successive extraction or the pH recedes from the initial pH. This is probably the result of more basic substances' being extracted in each successive extraction.

The Use of the Waring Blendor

It was found that the Waring Blendor worked very successfully in extracting sugars from green barley plants. This led to a preliminary study of the use of the Waring Blendor for the dispersion of proteins. Harris and Johnson (9) used the Waring Blendor to disperse gluten samples in K_2SO_4 . They found that it worked very satisfactorily and caused very little denaturation in spite of the foaming.

The ratio of meal and solvent was the same as used previously. These mixtures were thoroughly blended. Table V gives the results obtained for various periods of blending. It is evident that the use of the Waring Blendor reduces the peptization of the protein. This decrease in peptization may be the result of either excessive heat or excessive foaming. When the Blendor was operated continuously for over 10 minutes the temperature of the solution rose to $58^{\circ}C$.

Figure 4

The pH's of the three successive extractions
of barley and linseed oil meal in
varying concentrations of
sodium salicylate

TABLE V
Effect of blending time on dispersion
of nitrogen in oats and
barley

Length of treatment* (minutes)	Total N dispersed, %	
	Oats	Barley
0	69.0	57.7
5	63.3	40.7
10	45.9	38.4
15	42.9	34.4
20	42.4	30.5

* 0 - ordinary extraction method, total of 3 extractions.

5 - 5-minute period of continuous blending.

There is no doubt that long periods of blending are deleterious. Table VI shows the results for wheat, oats, and barley, when the mixture was blended for 2 minutes every 10 minutes for a period of 30 minutes, and followed by two ordinary extractions.

TABLE VI
Comparison of the percent nitrogen dispersed
by mechanical shaking and by using
the Waring Blender

Meal	Total nitrogen dispersed, %	
	Shaking	Blending
Oats	69.0	82.4
Wheat	69.4	88.7
Barley	53.8	56.2

Oats and wheat show a significant increase in the amount of protein dispersed, while barley shows very little improvement.

This method gives superior dispersions in some cases, but there are some serious disadvantages as listed by Brown (3). The chief disadvantage is that it also puts other materials--chiefly starch--into a colloidal suspension. This material may seriously hamper the carrying out of subsequent analysis, particularly electrophoretic measurements.

The Preparation of a Salt-Soluble Protein

Fraction from Peas

The Department of Chemistry is attempting to determine the molecular weight of some of the plant proteins. Because of the work done by Brown (3) on peas, the proteins of this legume are of especial interest. The proteins of the pea are largely water-soluble and salt-soluble proteins. Therefore, an attempt was made to separate a salt-soluble protein fraction that would be pure enough for these molecular weight determinations.

The method used was a modification of one used by Krishnan and Krishnaswamy (13) for the extraction of globulin from water melon seeds. They extracted the meal with water, followed by two extractions of two hours each with warm 10% NaCl. The extracts were combined and filtered through paper

pulp. The extraction was diluted 15 times with distilled water at 40°C. This was stored over night in an ice chest. Then the precipitate was centrifuged and redispersed in 10% NaCl. The dilution was repeated and stored in an ice chest. The precipitate was separated once more by centrifuging, washed free of chloride ions, dehydrated with acetone, and dried at room temperature.

Modifications of the above method were necessary as later results will show. Table VII indicates that there is very little difference caused by using different dilutions.

TABLE VII

Effect of dilutions on the precipitation of the salt-soluble protein fraction of peas

Dilution	Salt-soluble fraction, %*
1:5	36.4
1:10	36.8
1:15	35.7
1:20	34.9

* Based on total extraction

It was decided to use the 1:10 dilution not because of the higher results, but because the dilution was not too great and thus it was not necessary to handle such large volumes.

Later yields were always much lower than had been

anticipated and this led to an investigation of the effect of pH. The pH was adjusted to five different levels and Table VIII shows the results.

TABLE VIII

Effect of pH on the precipitation of the salt-soluble protein fraction of peas

pH of 1st dilution	Salt-soluble fraction,%**
6.30*	15.9
5.75	18.6
5.50	19.5
5.25	20.4
5.00	14.3
4.75	15.0

* No pH adjustment was made

** Based on total extraction.

The pH was adjusted with 0.1 N acetic acid. Optimum precipitation was obtained in the neighborhood of pH 5.25. The pH had to be adjusted only after the first dilution; subsequent dilutions had no effect on the pH.

This material appears to be complex in nature, as it precipitates as the temperature is lowered. At 0°C. almost all of the protein has precipitated.

This material is essentially all protein as a nitrogen determination gave a yield of 98.2% protein on a dry-weight basis, using conventional conversion figures. This protein fraction is not necessarily homogeneous. On the contrary,

thermal precipitation indicates that this protein is a mixture of proteins.

PART II

ELECTROPHORETIC STUDIES OF SOME PLANT PROTEINS

LITERATURE REVIEW

It is only recently that the phenomenon of electrophoresis has been employed for protein studies. It had, however, been observed and used by Picton and Linder (31) in 1892 with colored dispersed systems. These earlier experiments made use of differently colored solutions so that the movement of the solutions could be observed visually. It is only since the moving-boundary method has been developed and improved by Tiselius (40) that the electrophoresis technique has been applied to the study of protein mixtures. Previous to this the microelectrophoresis apparatus was used. Its chief value is in the determination of the isoelectric point of pure proteins.

Briefly, the microelectrophoresis cell consists of a small glass cell in which the protein molecules are adsorbed on quartz particles. These particles are then subjected to an electric current and movement of the particles is observed by means of a microscope (1).

The micro method has the following advantages (2):

1. During the period of observation the environment of the particle does not change.

2. Electric mobilities of particles may be compared by watching them simultaneously.

3. Measurements of electrical mobility may be observed in fairly concentrated salt solutions as well as in weak solutions.

4. Single determinations require only a few minutes.

Recently, however, interest has shifted to the moving-boundary method as developed by Tiselius (40). This is probably because of its advantages over the micro method. Longsworth (2) summarizes these advantages as follows:

1. It is applicable to a variety of high molecular weight substances in solution in both the native and denatured forms.

2. When applied to such mixtures the following information is obtained:

a. The number of electrically separable components in the mixture

b. The mobility of each component

c. The degree of electrical homogeneity of each mixture

d. The concentration of each component.

3. It is possible to separate into a pure state the components of the mixture.

Animal Proteins

The moving-boundary method has been used almost entirely in the study of certain of the animal proteins, especially blood serum and plasma proteins. Tiselius (40) diluted and dialyzed blood serum against a large volume of buffer; when this buffered material was subjected to a direct current distinct differences in the mobilities were observed. Serum albumin had the greatest mobility followed by three globulins, α , $\beta + \gamma$. Longsworth et al (20) confirmed Tiselius' findings; in addition they noted that blood plasma had still another component designated as the fibrinogen fraction. Hock and Morris (10) found that human blood serum albumin was made up of two components and not one. This result was obtained by passing the current through the solution for 22 hours. Previous to this, it was thought that the albumin component was electrophoretically homogeneous. Neither Tiselius (40) nor Longsworth (20) observed these components probably because they worked over shorter periods of time.

Longsworth (17) found that he obtained two globulin fractions, but that their difference in mobility depended upon the type of buffer used. A sodium diethylbarbiturate buffer at pH 8.6 produced a previously unrecognized component. However, when a phosphate buffer at pH 7.7 was

used, this component had the same mobility as that of the albumin component. In addition, in this buffer, the fibrinogen component was hardly distinguishable, while it was very pronounced in a sodium diethylbarbiturate buffer.

In passing, it is of interest to note that patterns for normal blood remain so constant that patterns of pathological bloods can be made and compared with those of normal blood patterns (20). This technique may serve as an auxiliary diagnostic method.

Another interesting application of electrophoresis analysis has been in the study of the egg-white proteins. This complex protein mixture is usually classified as ovalbumin, conalbumin, ovomucoid, mucin, and globulins. Longsworth et al (19), using the Tiselius apparatus found two egg albumins, A₁ and A₂; ovomucoid, O; conalbumin, C; and three globulins, G₁, G₂, and G₃. They (19) crystallized the egg albumin with ammonium sulphate but the first precipitation did not separate the albumin entirely from the globulins. Three precipitations were required to obtain a pure albumin fraction which was made up of conalbumin. Longsworth et al (19) determined the relative concentrations of the proteins from the electrophoretic pattern. Their results are in general agreement with those of Sorensen, who used chemical methods.

Only two of the animal protein mixtures have been dealt with here, but many other proteins have been studied. Some of these are: fibrous proteins (wool), muscle proteins,

enzymes and hormones, antigens, and antibodies (these may not be strictly proteins but they can be studied electrophoretically).

Plant Proteins

Up to the present plant proteins have received very little attention as far as the moving-boundary technique is concerned. This is so probably because these proteins are much more difficult to work with than the naturally occurring protein fluids of the animal body. Vickery (43) states that many plant proteins are insoluble at the low temperature and ionic strengths used in electrophoretic analysis. To overcome this handicap the proteins must be buffered at a high pH. Another factor to be considered is the difficulty encountered in peptizing all the protein present in plants.

The work that has been done on various plant proteins is reviewed in order.

Cereal Proteins

Most of the literature reviewed dealt with wheat. Kemp (12), using the microelectrophoresis cell determined the isoelectric point of glutenin to be at pH 4.90 at "infinite dilution". As the concentration of the salt or buffer increased the isoelectric point of glutenin shifted to the acid side. The isoelectric point of gliadin was at pH 6.60 but, when the

gliadin was stored in 70% alcohol for four days the isoelectric point had dropped to pH 5.92. This seemed to indicate that denaturation had taken place. Kemp further observed that when an extraction was made of the flour there was evidence of more than two protein constituents.

Martin (21), using a streaming-potential method, observed that the isoelectric point of proteins extracted from gluten with varying concentrations of ethanol varied. When the concentration was 8.60% ethanol the isoelectric point was at pH 7.59, while at 68.69% ethanol the isoelectric point was at pH 5.34. This indicates that different proteins or fractions were extracted with each different solvent.

The most detailed report on cereal proteins recorded in the literature to date is that of Schwert et al (32) and their work deals with gliadin. When the gliadin was dispersed in an acetate buffer (pH 3.8 $\pm$ 0.1) of varying ionic strength different patterns were obtained. The patterns indicated that there was evidence of complex formation between the components present.

These workers used four different preparations of gliadin. Small differences in the patterns were observed but they were relatively small in magnitude. These differences, however, indicate that every preparation of gliadin is likely to have small differences in composition or ratio of components.

Standard conditions of pH, ionic strength, and temperature demonstrated the following facts:

1. If protein concentration and field strength were held constant, at the end of equal intervals of time identical patterns were obtained.

2. The same conditions as (1) gave constant mobilities for every recognizable peak independent of time of electrophoresis.

3. If protein concentration was held constant, the mobility of every recognizable peak was independent of field strength.

4. If field strength was held constant and protein concentration varied, patterns were somewhat similar but mobilities of the peaks varied.

There was evidence of a fast fraction and a slow fraction; these were separated electrophoretically by a special cell. When patterns were made of these fractions there was evidence that these components were still mixtures but a certain amount of separation had been obtained. The isoelectric point was determined by using a microelectrophoresis cell. The isoelectric point of the fast fraction and the original gliadin were the same, i.e. at pH 7.0, while that of the slow fraction was at pH 5.0.

Schwert et al (32) conclude that gliadin is not an electrophoretically homogeneous material. The components do not migrate independently in solution under any conditions which they studied.

Chlorophyll-Protein Complex

Neish (26) observed that a suspension of chloroplast granules isolated from clover leaves is electrophoretically negative in distilled H₂O (pH 6.5) and positive in 0.2 N HCl. Fishman and Moyer (6) found that the green particles which made up the suspension are isoelectric at pH 4.7 in M/50 acetate buffer at 25°C. These particles migrated uniformly regardless of size of particles. When the centrifuge removed these microscopic particles and quartz particles were placed in the solution, the isoelectric point was still at pH 4.7.

Fishman and Moyer (6) also found that these chlorophyll complexes denatured rather easily. When the solution was subjected to a pH of 3.1 (0.04 N acetic acid) then returned to a more basic solution, it was observed that the isoelectric point had shifted to pH 5.0. This denaturation is quite rapid: it was found to be complete in 5 to 10 minutes.

Plant Viruses

Eriksson-Quensel and Svedberg (5) reported that the isoelectric point of chemically isolated tobacco mosaic virus was pH 3.49. Other investigators agree fairly closely with this figure. This isolate behaved as if it would be electrophoretically homogeneous.

Frampton and Takahashi (7), in comparing the proteins extracted from healthy tobacco plants with those from diseased

1919
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plants noticed an additional component which they designated as the tobacco mosaic virus. They found that proteins from healthy young leaves gave the same pattern as healthy leaves three months old. After the plants had been inoculated with tobacco mosaic virus, the additional component appeared only after the plant showed symptoms of the disease. They concluded, although with no definite experimental evidence, that the virus develops without interfering with or changing the composition of those components found in the healthy plant.

Lauffer and Ross (14), in using the ultracentrifuge, concluded that alfalfa mosaic virus appeared to be homogeneous. Electrophoresis analysis was employed in an attempt to substantiate this conclusion. Alfalfa mosaic virus appeared to be electrophoretically homogeneous. There was some indication, however, that this virus was made up of a mixture of proteins of almost the same electrophoretic mobility. This became evident after prolonged electrophoresis, but much of this spreading may have been the result of diffusion.

McFarlane and Kekwick (23) found that bushy stunt virus behaves as if it were homogeneous and has an isoelectric point of pH 4.11.

Thus it appears that virus proteins are typical because of their homogeneity as concluded from electrophoretic and ultracentrifuge studies.

APPARATUS

The electrophoresis apparatus employed for the studies that follow is similar to the one designed by Tiselius (40) and used by Longworth (15, 16) at the Rockefeller Institute, New York. Longworth has added a number of improvements to the original apparatus (15, 16, 17). Various methods can be used to observe the movement of the boundary, and this apparatus makes use of either the schlieren method or the Svensson-Philpot method of observation.

The apparatus consists of four distinct parts: the light source, the thermostated water bath, the camera, and the control panel. A detailed description of the optical system appears in one of Longworth's papers (15).

A diagram of the complete unit as set up in the Plant Science laboratory is shown in Figure 5. The optical bench is made up of four steel beams (B) mounted on four concrete pillars (A). The concrete pillars are set on four inches of cork matting, which in turn rests on a concrete pad supported by concrete shafts sunk approximately five feet into the ground. The concrete pillars are completely isolated from the floor. The apparatus made up of the light source (L), the water bath, and the camera (C) is mounted on the steel beams.

The light source consists of a 100-watt mercury arc which supplies light for a slit which is placed immediately in

Figure 5

A diagram of the optical bench including
the low-temperature bath

front of it. The light from this slit passes through the electrophoresis cell and then the slit is focused on the schlieren diaphragm (S), immediately in front of the camera objective (O). The light from the slit provides the illumination for the observation of the boundary in the cell.

The bath supports the electrophoresis cell, which contains the protein solution under investigation. The bath is constructed so that the water inside can be kept at a constant temperature of 4°C. or lower (40). The low temperature is maintained by a refrigeration unit which is thermostatically controlled by a mercury thermo-regulator. The schlieren lens (D) is mounted in the wall of the bath nearest the light source, while an ordinary double glass window is mounted in the opposite wall. The double windows help to prevent the condensation of moisture on the glass. Longworth (15) describes how this condensation may be prevented.

The camera (C) supports a schlieren diaphragm which is synchronized with the plate holder (G). Both are driven by means of a small electric motor. The camera objective is provided with a series of aperture stops (E) which are used when the scanning method of observation is employed. If the Svensson-Philpot method of observation is used, then a sector-type shutter driven by a small synchronous motor is employed.

The current supplied to the cell is measured and controlled from the control panel. An electric timer which

measures the length of an analysis is mounted on the control panel.

Operation of the Apparatus

The cell used is the type designed by Tiselius and employed by Longworth et al (19). The cell must be filled in a definite manner and details for filling it can be found elsewhere (17, 19). After the cell has been filled it is then attached to the electrode vessels, the latter having been partially filled with buffer. The assembly is then placed in the water bath and allowed to come to thermal equilibrium.

Before the boundaries are formed, the silver-silver chloride electrodes are inserted. The electrode vessel which takes a ground glass stopper is sealed tightly. This step facilitates the use of the "compensator". 1 N KCl is introduced around the electrodes (17). The initial boundary can now be formed as described by Longworth (17). The initial boundary is brought out from behind the opaque horizontal plates of the cell by using the "compensator". The initial boundary is now photographed. Then the potential is applied in the appropriate direction by adjustment at the control panel.

Difficulties in Operating the Apparatus

The apparatus was received by the Plant Science laboratory supposedly ready for immediate operation. When it was assembled many parts of the apparatus did not function properly.

The water bath and thermostat caused the most trouble. The stirrer for the water bath would not operate until brass bushings had been fitted into the boxing. The stirrer itself eventually had to be discarded as it was too heavy and not properly balanced. This stirrer was replaced by a glass one, which operated very smoothly. The mercury thermo-regulator would not operate until several necessary adjustments were made.

The camera objective lens was found not to have the focal length that had been specified. This was remedied by changing the position of the camera so unit magnification of the cell could be observed at the plate holder.

Other minor adjustments had to be made. A few of these were: the support for the cell and electrode vessels had to be aligned; the mercury relay switch had to be adjusted; the slit at the light source had to be ground parallel.

Runs had to be made in the evening as the power in this laboratory during the day dropped to 90 to 95 volts. This voltage was insufficient to keep the lamp operating.

METHODS

The meal was extracted with a suitable buffer of a known ionic strength and pH. This extraction was usually carried out for at least two hours and only one was used. The solvent or meal ratio was changed to 5 grams of dry meal to 50 cc. of extracting solution. This was found necessary if the extraction was to contain enough protein to make a satisfactory run (0.5% or better).

The extracted meal was then centrifuged in a Servall angle centrifuge. The supernatant liquid was removed and filtered if necessary to remove macroscopic material. Nitrogen content of this extracted material was determined.

A known volume of the extracted material was dialyzed at 2°C. against the buffer solution used for extracting the protein. At first the buffer was changed every day and then the electrophoresis run was made against fresh buffer. According to Longworth et al (20) better results are obtained if the buffer is not changed and a large enough volume is used so that it can be placed in the electrode vessels later on. Dialysis took place through collodion tubes which were made in the laboratory. Cellophane would have been more satisfactory but at the time it could not be obtained.

It was assumed that equilibrium was established after dialysis had continued for three days. After this length of

time the pH was approximately the same on both sides of the membrane. Actually, this test is not accurate enough as it is not only important to have the same pH but the specific conductivity must be the same on both sides of the membrane. Specific conductivity could not be determined for the early runs because this equipment was lacking. New equipment will be available soon.

The material from the collodion tube was centrifuged in the angle centrifuge at a temperature of 2°C. The supernatant liquid was then removed from the precipitate by decantation. Nitrogen was determined on an aliquot part of the supernatant liquid and on the precipitate. This supernatant liquid was used for the subsequent electrophoresis run.

Nitrogen was determined either as mentioned in Part I or by the micro-Kjeldahl method. In the latter method the ammonia was caught in a 2% boric acid solution and titrated with 0.01 N HCl. The indicator used was a mixture of methylene blue and methyl red (39). The percentage protein was calculated on a weight basis.

EXPERIMENTAL RESULTS

Efficiency of the Apparatus

One run was made on the proteins of egg-white to see if Longsworth's work could be duplicated (19). The protein solution was prepared by diluting egg-white eight times with a sodium phosphate buffer, ionic strength 0.1 and pH 7.86 (19). This material was dialyzed against the buffer for two days and then a run was made. Figure 6 shows the patterns obtained after 212 minutes, using a current of 6.72 ma. In the figures the initial boundary is represented by the vertical line on the tail of the arrow used to indicate the direction of migration.

These patterns are approximately similar to those obtained by Longsworth (19), when working with the same buffer at a pH 8.00. Longsworth's patterns are a bit more detailed but this is probably because the run was carried out for a longer period of time.

The patterns in Figure 6 certainly illustrate that the two patterns are not identical. Longsworth (18) states that the rising boundary (boundary is ascending into buffer) is usually sharper than the descending boundary (boundary is descending into protein). Perlmann and Kaufman (30) maintain that this difference in patterns can be partly overcome by

Figure 6

Electrophoresis patterns for egg white at pH
7.86 in sodium phosphate buffer, ionic
strength 0.1. Protein concentration
1.14%. Time 212 minutes;
current 6.72 ma.

using more concentrated solutions.

Extraction Results of Pea Proteins

Peas used for these experiments were a sample of Early Blue grown at the University of Alberta.

Table IX gives the results obtained for various extraction agents used. The reason these extractions were carried out in different buffer solutions was that the protein solution must be buffered in the same buffer solution as is placed in the electrode vessels. If this is not done, differences in pH, and especially ionic strength, will cause disturbances and movement of the boundary which are not the result of the charge carried by the protein molecule. Uneven ionic strengths will also cause diffusion of ions at the boundary, thus causing excessive spreading of the boundary.

Table IX seems to indicate that very few of the buffer solutions extract as much protein as do ordinary salt extractions. Brown (3) extracted 90% of the protein when using neutral salt solutions. He found that 1% solutions, approximately an ionic strength of 0.1 to 0.2, tended to depress the dispersion of pea protein. This in part may account for the low dispersions in some cases. Part of this variability in the dispersion of the protein is also due to the pH of the extracting solution (34). The tendency is that, as the pH

TABLE IX

Effect of dialysis on the extraction of protein
from peas by various solvents

Extraction solvent		Extraction		Protein solution after dialysis			Ppt(after dialysis)	
	μ	pH	%	pH	% N of tot.ext.	% N of tot.meal	% N of tot.ext.	% N of tot.meal
NaAc - HAC	0.1	5.38	31.6		24.6	7.8	7.4	2.3
3% NaCl dialysed								
against above	0.1	5.38	66.1		19.1	12.6	54.1	35.8
Na ₂ HPO ₄ - NaH ₂ PO ₄	0.1	7.95	69.6		57.2	39.8	11.9	8.3
" "	0.1	8.08	85.4		76.6	65.4	9.0	7.7
K ₂ HPO ₄ - KH ₂ PO ₄	0.3	6.06	45.7	5.73	38.0	17.4	15.4	7.0
" "	0.3	7.03	71.7	6.90	73.9	53.0	1.6	1.1
Na Sal - H Sal	0.1	5.42	67.5	6.22	27.6	18.4		
Na Sal + Na phosphate	0.3	7.39	72.4	7.05	72.8	52.7		
Na Sal + Na phosphate	0.42	6.95	71.5		82.4	58.9		
NaCl + Na phosphate	0.42	7.63	72.0	6.32	81.6	58.8	10.6	7.6
Borate	0.1	9.00	78.1		64.8	50.6		

* Protein calculated as N x 6.25

becomes more alkaline the extraction increases. This increase appears to be evident in spite of the different salts used for the extraction agent.

The type of buffer solution used as the extracting agent has the greatest effect on the amount of protein dispersed. Comparing the sodium acetate buffer extraction with the sodium salicylate buffer extract indicates this clearly. Both solutions have the same pH and the same ionic strength, but the sodium salicylate buffer solution disperses more than twice as much protein. The phosphate buffers appear to be as satisfactory dispersing agents as the sodium salicylate buffer or the salicylate-phosphate buffers.

The second column under the heading "Protein solution after dialysis" represents the fraction of the total protein in the meal which can be used for electrophoresis runs. The best that was obtained was 65% which is probably somewhat high. The average of several of the best is approximately 50% and this was obtained when phosphate buffers or sodium salicylate buffered with phosphate was used as an extraction agent.

During dialysis some of the protein precipitates out. This precipitate becomes much more pronounced if the buffer is changed every day, but very little precipitation is evident if the buffer solution is not changed. The last column in Table IX indicates that the loss, as a result of this precipitation, is not very great.

Electrophoresis Results for Pea Proteins

The pattern for a 0.62% pea protein solution in a sodium phosphate buffer is shown in Figure 7. This was the first run attempted and the results were very encouraging. In the cathode compartment (descending) there are indications that the pea protein is made up of more than one component. After the current had been applied for 75 minutes, there was evidence of two and perhaps three distinct components. These tend to spread out into a number of components which are not distinct. The pattern of the anode compartment (ascending) is supposed to give a mirror image of the one in the cathode compartment. This, however rarely occurs, especially in this sample of pea protein. Longworth and MacInnes (18) state that usually the boundary that moves into the buffer is sharper. In Figure 7 the opposite is true: i.e. the boundary moving into the protein solution is sharper. The smaller irregularities at the base of the pattern are not protein components but simply irregularities in the wall of the cell.

When the pea protein was buffered against a borax-boric acid buffer patterns similar to those of a phosphate buffer were obtained. Figure 8 (85 minutes) gives a pattern which is very similar to that of Figure 7 (75 minutes). The boundaries in Figure 8 appear to move more quickly than in Figure 7. This is to be expected as the pH was more alkaline

Figure 7

Electrophoresis patterns for pea protein at pH 8.08 in sodium phosphate buffer, ionic strength 0.1. Protein concentration 0.62%. Time as indicated; current 11.03 ma.

Figure 8

Electrophoresis patterns for pea protein at pH 9.00 in borax-boric acid buffer, ionic strength 0.1. Protein concentration 0.68%. Time as indicated; current 6.02 ma.

and therefore the protein molecule would be more negatively charged. In the borax-boric acid buffer the protein appears to be made up of more components than in the case of the phosphate buffer. Figure 8 (37 minutes) indicates that at this point the two components are beginning to separate. This separation is quite evident later on.

When a sodium salicylate buffer is used the patterns obtained are quite different (Figure 9). These photographs were taken 415 minutes after the current was applied. The boundaries travel very slowly when the above time is compared with those in Figures 7 and 8. The pH difference is not great enough to account for this greatly reduced mobility. This difference is likely the result of the type of buffer used and the concentration at which it is used.

Perlmann and Kaufman (30) found that high ionic strength buffers (0.4 and higher) caused a marked difference in the apparent distribution of the components. Part of this variation is undoubtedly due to the ionic strength of the solution. The velocities of the components differ greatly, depending on the buffer used and the ionic strength. The components in the sodium salicylate buffer travelled very much more slowly than in the case of the phosphate buffers or the borax buffer. Tiselius and Svensson (41) showed that observed mobilities are always less than the ideal values, and that this difference becomes greater if the ionic strength is increased. This reduction in mobility can be attributed to

Figure 9

Electrophoresis patterns for pea protein at pH 6.95 in 6 % sodium salicylate buffered with sodium phosphate, ionic strength 0.42. Protein concentration 0.71%. Time 415 minutes; current 14.81 ma.

the reduction of the electrokinetic potential caused by the double layer around the particle. This in part accounts for the reduction in the mobilities of the component parts when a solution of a high ionic strength is used.

It has been suggested that sodium salicylate might form some loose chemical bonding on the surface of the protein molecule (22). Thus some of the charges on the surface of the protein molecule may be covered and this may reduce the number of charges as well as the mobility of the protein component or components.

To test this hypothesis, a sodium chloride buffer was used with the same ionic strength as the sodium salicylate buffer. The electrophoretic pattern for sodium chloride is shown in Figure 10. The velocity for the sodium chloride buffer is considerably less than for the sodium salicylate buffer, in spite of the fact that the pH for the latter was somewhat lower.

The results indicate that loose chemical bonding does not entirely explain the low mobility for the sodium salicylate buffer. This decrease in mobilities is much greater for the sodium chloride buffer than for the sodium salicylate buffer. No doubt much of this decrease is the result of the high ionic strength, but this is not the complete answer. Actual measurement of the mobilities would likely furnish a more complete answer.

The patterns are somewhat similar but the similarity

Figure 10

Electrophoresis patterns for pea protein at pH
7.63 in 0.37 N sodium chloride buffered
with sodium phosphate, ionic strength
0.42. Protein concentration 0.74%.
Time 360 minutes; current
19.59 ma.

is not nearly as close as what one might expect considering that the ionic strength is the same. Part of this difference, however, is due to the difference in extraction when two different salts are used for extraction agents. Diffusion is greater in the case of the sodium chloride buffer than in that of a sodium salicylate buffer. The symmetry between descending and ascending boundaries is greater than in the case of weak buffer solutions.

The spreading of the boundary is very noticeable as is shown in Figures 7 and 8. This very likely is due to a combination of diffusion, heat convection (18) and electrophoretic heterogeneity.

Diffusion takes place at the same time that electrophoresis occurs, but the effect of diffusion will be slow and, as these runs were short, this effect was small. Some of this spreading is no doubt the result of heat convection caused by using a potential gradient that was too high. The heterogeneity of the protein solution perhaps accounts for much of this spreading. At first the peaks were very distinct, but in a short time they had spread out and were hardly distinguishable. If the original peak was made up of a number of components that differed but slightly in mobility, they would tend to separate into components of relatively small concentrations, thus causing the spreading of the boundary. This problem is one that can well be further investigated.

Extraction Results of Some Other Plant Proteins

The extraction and dialysis results for the four meals--barley, oats, soybeans, and wheat--are given in Table X. These results are by no means complete since, because of various technical difficulties, certain analyses could not be made. The most apparent point is the difficulty that is encountered in trying to extract representative samples of protein. The results for oats are very incomplete but it is evident that a phosphate buffer of ionic strength of 0.1 removes a very small proportion of the total protein. The amount that precipitated out during dialysis is large. This is partly the result of changing the buffer every twenty-four hours.

The results with soybean meal are encouraging as a protein solution that accounted for nearly 50% of the total protein present in the meal was obtained. Further extraction and analyses should add to the information we have.

Wheat proteins behaved much as might be expected: i.e. they are very difficult to extract. The phosphate buffers accounted for only about 12% of the total protein present. The amount that precipitated while the solution was dialyzed was very small. The sodium salicylate-phosphate buffer proved to be quite successful as an extracting agent. This buffer may be of some value but further investigations are necessary

TABLE X

Effect of dialysis on the extraction of protein
from plant meals by various solvents

Extraction solvent		Extraction		Protein solution after dialysis			Ppt(after dialysis)	
Buffer	μ	pH	%	% N of tot. ext.	% N of tot.meal	% prot.*	pH	% N of tot.ext. tot.meal
<u>Barley</u>								
Na Sal + phosphate	0.42	7.84	14.6	6.70	66.8	9.8	0.38	7.52 35.3 5.2
<u>Oats</u>								
Na ₂ HPO ₄ - NaH ₂ PO ₄	0.1	8.02	24.0	7.53	43.0	9.2	0.16	7.90 30.1 7.2
" "	0.1	8.05	21.4	7.27	34.2		0.25	7.81 20.1 4.3
" "	0.1	8.13			80.6	44.2	0.39	25.6
Na Sal + phosphate	0.42	7.84	54.8	6.95			1.40	7.49 13.2 7.2
<u>Soybeans</u>								
Na ₂ HPO ₄ - NaH ₂ PO ₄	0.1	8.10	61.2		79.6	48.7	1.51	7.87 9.3 5.7
<u>Wheat</u>								
Na Sal + phosphate	0.42	7.50	55.4	7.34	87.1	48.2	1.04	7.50 0.0
Na ₂ HPO ₄ - NaH ₂ PO ₄	0.1	7.90	17.2	7.60	63.2	10.9	0.29	8.16 20.9 3.6
" "	0.1	7.98	19.4	7.18	61.6	12.0	0.28	7.78 26.9 5.2
" "	0.1	9.45	20.5	7.96	67.7	13.9	0.35	8.92 16.2 3.3

* Protein calculated as N x 6.25

as it may alter the electric charge on the protein molecules. This was mentioned previously and, in the case of peas, does not seem to be true, but an entirely different picture may be presented if similar work were done on the cereals. In any case, the use of sodium salicylate appears to alter the electrophoretic patterns. Part of this alteration undoubtedly is the result of a greater amount of protein being dispersed.

Electrophoresis Results for Other Proteins

Oat Protein

Patterns for the descending boundary are very similar as is shown in Figures 11 and 12. This is both interesting and encouraging as these protein solutions were prepared at different times from the same meal. It clearly indicates that patterns can be fairly well reproduced if the same method of extraction is used. Longsworth et al (20) found that this was possible in the case of normal blood serum and plasma.

To begin with, just one component was evident, but as the current continued to pass through the solution there was evidence of more than one. The spreading of the boundary was very pronounced in oat protein and perhaps greater than in the case of pea proteins. This spreading is no doubt partially the result of the heterogeneity of the protein.

Figure 11

Electrophoresis patterns for oat protein at pH 8.02 in sodium phosphate buffer, ionic strength 0.1. Protein concentration 0.16%. Time as indicated; current 17.85 ma.

Figure 12

Electrophoresis patterns for oat protein at pH 8.05 in sodium phosphate buffer, ionic strength 0.1. Protein concentration 0.25%. Time as indicated; current 6.70 ma.

Oat protein differed quite markedly from pea protein in that it showed very little evidence of boundaries appearing in the ascending boundary. Perlmann and Kaufman (30) state that the increase of salt concentration of the buffer increases the similarity between the ascending and descending patterns. The weak salt concentration of the buffer in part may explain why the two patterns were so unlike. Also, the low protein concentration likely contributed to this asymmetry.

Figure 13 indicates what happens when a more concentrated buffer is used. The ascending boundaries produce a pattern that is almost a mirror image of the descending boundaries. Two very pronounced components are evident, but there is no indication of any other components' appearing after the run had continued for 615 minutes.

The boundary that appears at the vicinity of the initial boundary is undoubtedly due to the " δ " and " ϵ " boundaries (18). These boundaries are not true protein components but rather arise from a gradient of concentration left behind during electrophoresis. These boundaries are usually more noticeable in the descending side but may occur in both sides.

What appears to be a boundary in the descending boundaries (Figures 11 and 12) is not likely a boundary due to a protein component. This boundary remained stationary throughout the experiment and is very likely the result of a fatty layer. Oats contain some fatty substances and these

Figure 13

Electrophoresis patterns for oat protein at pH
7.49 in 6% sodium salicylate buffered with
sodium phosphate, ionic strength 0.42.

Protein concentration 1.00%.

Time 615 minutes;

current 16.22 ma.

probably formed a boundary when the initial boundary was formed and did not move because of their unionized character. The " δ " and " ϵ " boundaries can hardly explain this phenomenon completely.

Soybean Protein

The pattern for this protein solution indicates that it is comparatively homogeneous when compared with the other proteins studied. The predominant peak remains fairly constant throughout the entire run, as is shown in Figure 14. Only in the last two photographs is there any indication that there may be other components that are electrophoretically different. Another point of interest is the similarity between the patterns in the cathode and in the anode compartments.

The use of different buffers will undoubtedly reveal more interesting information: i.e. whether the component is as homogeneous as it appears to be in the phosphate buffer.

Wheat Protein

These proteins are not only difficult to extract, but most buffers give very poor patterns (Figure 15). Both phosphate and phosphate-NaOH buffers give somewhat similar patterns. The patterns are so poor that it is very doubtful

Figure 14

Electrophoresis patterns for soybean protein at
pH 8.10 in sodium phosphate buffer, ionic
strength 0.1. Protein concentration 1.00%
Time as indicated; current 10.91 ma.

1.

2.

Figure 15

Electrophoresis patterns for wheat protein.

1. Sodium phosphate buffer at pH 7.90, ionic strength 0.1. Protein concentration 0.29%. Time as indicated; current 11.04 ma.
2. Sodium phosphate-sodium hydroxide buffer at pH 9.45, ionic strength 0.1. Protein concentration 0.35%. Time as indicated; current 6.74 ma.

that any definite conclusions can be reached. Both treatments give evidence of two components being present early in the run, but one of these is apparently lost as the run is continued. The descending boundaries give the clearest pattern: in the ascending boundaries there are indications of different components but these disappear very rapidly. In the latter case it is difficult to tell whether these are actual components or irregularities in the cell itself.

The patterns shown in Figure 16, however, are very different and show definite possibilities. The meal was extracted with a 6% sodium salicylate-phosphate buffer and the protein concentration was 1.04%. These patterns were obtained near the end of the run, and they illustrate how a peak which appears homogeneous at an early stage in the run later may separate into more components. These patterns indicate that the protein in wheat consists of a number of components which are electrostatically different. The smaller peaks are very likely the result of components which will vary with the buffer used, pH, ionic strength, etc. When an acetate buffer was used, Schwert et al (32) found that the electrophoresis patterns for gliadin broke up into a number of small components. This indicates that this protein is electrophoretically heterogeneous. McCalla (22), in a personal communication with Putnam, found that the patterns obtained when gliadin was buffered against an acetate buffer

Figure 16

Electrophoresis patterns for wheat protein at pH 7.50 in 6% sodium salicylate buffered with sodium phosphate, ionic strength 0.42. Protein concentration 1.04%. Time as indicated; current 26.94 ma.

were made up of a number of components. Only one distinct component was obtained for gliadin when 8% sodium salicylate buffered with veronal was used. There was evidence, however, that when these protein solutions were subjected to a direct current for 860 minutes the main component broke up into several components. It would appear from this that the pattern for any one protein will vary, depending on the buffer used.

Barley Proteins

Only one result was obtained for barley because these proteins are very difficult to extract. Where oats and wheat give extractions which represent 44% and 48% respectively of all the proteins present, in the case of barley only 10% is represented. Up to the present barley proteins have defied all ordinary extraction methods.

Figure 17 shows the patterns obtained when a 6% sodium salicylate-phosphate buffer was used. There are at least two components with a suggestion of a third having a greater mobility than the two more prominent components. Some of the spreading here is the result of diffusion, but it should be noted that this pattern is not nearly so sharp as that for oats (Figure 13). The reason may be that oats contains two components which have two definite mobilities but, in the case of barley, the faster component spreads out as the result of a number of components the mobilities of which

Figure 17

Electrophoresis patterns for barley protein at pH 7.52, in 6% sodium salicylate buffered with sodium phosphate, ionic strength 0.42. Protein concentration 0.38%. Time 600 minutes; current 16.16 ma.

vary very little from one another.

DISCUSSION

Although the results to date are not so accurate as one would like and cannot be used for the calculation of mobilities or concentration of components, there are, the writer believes, distinct possibilities in this type of study. The patterns that have been obtained are in some cases poor, but in others there are indications that further study is warranted.

The work to date has more or less involved trying to develop a technique of extraction that will yield material suitable for electrophoretic work. There is very little doubt that the more concentrated buffer solutions are more satisfactory than the weaker ones. More concentrated solutions give higher yields of protein extracted and a better electrophoretic pattern. There are drawbacks to this increase in concentration which are very serious and perhaps, for this reason, high concentrations will have to be abandoned. If solutions are too concentrated there is a variation in the apparent distribution of the components (30). This would lead to errors in the calculation of the distribution of various components. A second drawback is one pointed out by Tiselius

and Svensson (41) that the actual mobility of a protein molecule is only approached in solutions of "infinite dilution". Thus, it appears that some sort of compromise will have to be made between degree of extraction and accuracy of results.

Increasing salt concentration retards the movement of the boundary. This is evident from the electrophoretic patterns of pea protein. If Figures 8 and 9 are compared, it appears that salt concentration decreases the mobility of a component. This decrease in mobility can also be attributed to variations in the pH of the protein solutions. The higher pH would have a greater mobility if it is assumed that the isoelectric point for these proteins is on the acid side of neutrality. Nothing definite can be stated, however, until further and more precise work has been done.

It has been suggested by Vickery (43) that electrophoresis runs must be made at high pH's if any satisfactory results are to be expected. Work done here at the laboratory indicates that when extractions are carried out at a pH of above 9.00, the extraction becomes highly pigmented. This material is very unsatisfactory as it cuts out the light and usually no photographs can be obtained. This is what happened in one run made on wheat (Figure 15).

Present results do not permit or justify any definite conclusions, but it is the opinion of the writer that

electrophoresis has distinct possibilities of adding much to our knowledge of the properties of plant proteins.

SUMMARY

1. The solvent-meal ratio is not an important factor in peptizing nitrogen in eight meals. Linseed oil meal is an exception.
2. The removal of fats by an ether extraction did not increase the nitrogen peptized in subsequent extractions, except in the case of oats. Ether extractions remove some of the pigment compounds that interfere in the electrophoresis analysis.
3. Increasing concentrations of sodium salicylate gave increasing dispersion of nitrogen in wheat, oats, and barley, but had no effect on alfalfa meal and linseed oil meal.
4. The Waring Blender increases the percentage nitrogen dispersed, but its use is limited because it also disperses contaminants, e.g. starch.
5. A salt-soluble protein fraction was prepared from peas. This fraction was 98% protein.
6. Different buffers were used to extract the protein

from plant materials suitable for electrophoretic analysis. The most promising buffer is sodium salicylate buffered with a phosphate buffer.

7. The ionic strength of the extracting buffers must be relatively high (0.2 to 0.4). This undoubtedly has an effect on the mobilities of the components.

8. Electrophoretic patterns were obtained for peas, soybeans, wheat, oats, and barley. The best patterns were obtained in 6% sodium salicylate buffered with 0.05 μ $K_2HPO_4 - KH_2PO_4$. Preliminary studies indicate that the plant proteins are electrostatically heterogeneous in all cases.

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