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PHYSICAL AND CHEMICAL PROPERTIES OF PLANT PROTEINS

Clarence Ronald Fuerst

Department of Plant Science
University of Alberta

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T H E U N I V E R S I T Y O F A L B E R T A

PHYSICAL AND CHEMICAL PROPERTIES OF
PLANT PROTEINS

A DISSERTATION
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

FACULTY OF AGRICULTURE
DEPARTMENT OF PLANT SCIENCE

by

Clarence Ronald Fuerst

EDMONTON, ALBERTA

April, 1951

SYNOPSIS

Ammonium sulfate fractionation of water-soluble pea proteins served only to vary the concentrations of the various protein components in the different fractions.

Under the experimental conditions employed, diffusion and viscosity results indicate that the molecular state of dispersed pea protein was not altered within the temperature range studied.

Protein of whole squash seeds was poorly dispersed by a number of solvents. This low dispersal was apparently due to the low solubility of the globulin fraction in these solvents. Temperature had a marked effect on the peptization since more protein was dispersed at room temperatures than at refrigerator temperatures. Electrophoretic patterns were obtained for squash protein dispersed in various solvents.

Squash globulin was crystallized and analysed electrophoretically. It was essentially electrophoretically homogeneous in every case except when dispersed in sodium acetate at pH 4.7 when two distinct, well-resolved components were evident.

Attempts to use electrophoresis as a method of diagnosis of leaf roll virus infection in potatoes were unsuccessful. The Waring Blendor was found inadequate for extraction of leaf proteins from fresh material and when lyophilized leaf material was extracted, the resulting extracts were too highly pigmented for electrophoretic analysis.

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PHYSICAL AND CHEMICAL PROPERTIES OF PLANT PROTEINS

C. R. Fuerst

INTRODUCTION

The plant proteins dealt with in this thesis are of three distinct kinds, namely, pea seed protein, squash seed protein, and a virus protein of potatoes. The section on pea proteins represents a continuation of work which had been started in this department a few years ago, whereas the electrophoretic results presented here on squash protein are original as only one paper dealing with electrophoretic investigations of squash protein has been found in the literature. The last part of the thesis describes attempts to use electrophoretic analysis as a quick method of diagnosing virus diseases of growing plants. The virus disease taken for investigation was leaf roll of potatoes.

P A R T I

P E A S E E D P R O T E I N S

LITERATURE REVIEW

The proteins of legume seeds have been investigated by workers in the academic field and also by industrial workers since plant proteins are used in commercial products such as paints, plastics, and adhesives. Soybeans, peanuts, and peas are the legumes which have been most thoroughly investigated as to protein peptization. Consequently, studies on the chemical and physical properties of proteins from these legumes are numerous.

Soybeans

Smith and Circle (82) found acids to be no more effective than water in extracting soybean protein. Alkali, when used at moderate concentrations, was the most effective dispersing

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agent since it extracted 95% of the total soybean meal nitrogen. In salt solutions of low concentration pH showed a marked effect with minimum extraction at the "isoelectric point", but this effect decreased as the salt concentration increased. Smith, Circle and Brother (83) report the amount of nitrogen extracted by water to be the very high value of 90% which was more than that dispersed by neutral salts. The concentration of the salt used influenced nitrogen peptization. Univalent cation salts dispersed least nitrogen when used at concentrations of about 0.1N. For divalent cation salts, lowest dispersal of nitrogen was obtained when the salt concentration was about 0.02N. These workers believe that the type of dispersion curves they obtained do not agree with the theory explaining the dispersion of proteins in water on the basis of the dialysable salts present in the meal. Nagel et al (72) suggest that the lecithin present in soybean meal is largely responsible for the ease and completeness with which soybean protein disperses in water.

The nonprotein nitrogen for soybean is given as 5.6% of the total seed nitrogen (36).

Aging and intensive grinding reduced the amount of extractable nitrogen (82). Jones and Gersdorff (56) found that storage of soybean meal decreased the solubility of the protein in 10% sodium chloride and suggest this indicates partial denaturation

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of the proteins. Nagel et al (71) investigated the effects of water-to-meal ratio, temperature, time of extraction, and size of meal particles on the extraction of nitrogen from soybean meal. As might be expected, they found the nitrogen in the hulls did not disperse easily in water.

Electrophoretic patterns are reported by Briggs and Mann (11) for water extracts of soybean meal representing 95% of the total meal nitrogen. The globulin "glycinin" was a mixture of components but they were able to prepare an electrophoretically homogeneous globulin representing 60% of the total globulin. This electrophoretically homogeneous protein was not homogeneous on the basis of results from solubility experiments.

Mann and Briggs (67) showed that alcohol treatment mainly reduced globulin solubility in water or salt solutions, whereas heating primarily reduced the solubility of protein fractions other than the globulins.

Peanuts

Much of the work on the extraction of peanut proteins has been done with meal after oil removal by the hydraulic press method (33, 15), instead of with solvent-extracted meal.

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Fontaine and Burnett (30) report more than 80% of the nitrogen of solvent-extracted meal is peptized by water (pH 6.6), but more than 90% is dispersed at a pH of 7.2 or over. Of twenty seven salts investigated, no better extraction was obtained.

Fontaine, Irving and Markley (31) report hydrochloric acid - sodium hydroxide pH - peptization curves showing the percentage of total peanut meal nitrogen peptized. A minimum peptization zone occurred at about pH 4.5. When the meal had been previously dialysed against water, the subsequent dispersion of nitrogen was greatly increased from pH 0.9 to 4.5. Previous dialysis of the meal against water had no effect on the amount of nitrogen extracted at pH values above the minimum peptization zone. The authors believe that such results indicate that salts and other dialysable constituents of the meal are not responsible for the high peptization of peanut protein in water. These results do not eliminate the possible role of lecithin or other nonprotein substances not removed by dialysis. Fontaine, Pons and Irving (32) later published some interesting results which help to explain this effect of dialysis. Below the protein isoelectric point a protein-phytic acid complex seems to exist which reduces the solubility of the meal proteins. Above the isoelectric range the complex dissociates and has no influence on the amounts of protein dispersed. Dialysis removed 91% of the phytic acid from peanut meal.

Johns and Jones (47) found the total protein of the peanut to consist of a little heat coagulable albumin, but mainly of the globulins named arachin and conarachin.

Karon, Adams and Altschul (59) showed extracts of peanuts to have the same electrophoretic composition in glycine buffer irrespective of the solvent used to extract the protein. The patterns consist of two major and several minor components. The major components appear as a single peak for salt or alkali extracted meal, but if the protein was from meal which had been washed with water of pH 5, to remove sugars and phytin, the major component appears as two almost equal fractions. The same result was obtained by washing protein which had been extracted and precipitated by adjustment of pH to 4.5. The authors suggest this result indicates loss of a binding agent which might be carbohydrate or phytin components in the meal.

Irving, Fontaine and Warner (44) found the protein extracted from peanuts with an ammonia buffer to consist of two major components and at least two minor components. Arachin, as prepared by 40% saturation with ammonium sulfate, was composed of the two major components of whole extract, whereas conarachin was composed of only one major component plus the minor components.

These two globulins, arachin and conarachin, differ as to sulfur, basic nitrogen, lysine, methionine, cystine, threonine, tryptophane and tyrosine contents (47, 48, 13, 14, 57).

 

In 1930 Jones and Horn (58) reported the preparation of arachin by 40% saturation with ammonium sulfate and by another method involving dilution and acidification to pH 5. Dean (25) found both methods produce globulins which are identical as to the manner in which they absorb ultraviolet light as would be expected if their amino acid compositions were similar.

Johnson (49) used the ultracentrifuge to determine the homogeneity of arachin prepared by the two methods of Jones and Horn. The dilution and acidification method produced two sedimenting species instead of just one. Further work by Johnson (50) has indicated the two species result from a reversible type of dissociation of the arachin molecule into two equal sub-units. From sedimentation and diffusion measurements, Johnson and Shooter (53) indicate the dissociation product (molecular weight 180,000) is of greater molecular asymmetry than the parent molecule (molecular weight 330,000). Johnson (51) believes the arachin occurs in the peanut as the associated molecule.

Association in ammonium sulfate even at 15% saturation (0.88M) appears to be due to the sulfate ion since ammonium chloride has little effect at similar ionic strengths. When sodium salts of long chain paraffin sulfates are used, association is complete at much lower salt concentrations: C_8 - 0.046M;
 C_{10} - 0.012M; C_{12} - 0.0018M.

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By electrophoresis, Johnson, Shooter and Rideal (54) have shown that the dissociation product predominates at high pH values but at pH values lower than neutrality there is a marked increase in the associated molecules. These results compare with ultracentrifugal results. Where sulfate ion was used to prepare the arachin there was an increase in the negative mobilities of the two components in phosphate buffer due to adsorption of sulfate ions on the protein. Prolonged dialysis of the arachin against phosphate buffer did not remove the sulfate ions, but they are replaced by barbiturate ions upon dialysis of the arachin against barbiturate buffer.

Johnson et al (54) also largely confirmed the results of Fontaine et al (44).

Peas

Evans, Henry, and St. John (27) indicate the influence of dispersing agent (kind, concentration and pH) on the extraction of pea seed nitrogen. For salts, the percentage nitrogen peptized was never over 80%. About 18% of the nitrogen of dry peas was not precipitable by 2.0 - 0.1N trichloroacetic acid. Brown (12) found that salt solutions of about 1% concentration

dispersed less pea protein than did more concentrated salt solutions or water. He extracted 90% of the total pea seed protein with water by subjecting the meal to three successive lots of water. By the same procedure, salts of 2% concentration or more dispersed more than 90% of the protein. Wetter (98) has reported on a number of organic and inorganic salt solutions which extract from 65% to 85% of the pea nitrogen, and which are also suitable for use in the electrophoretic analysis of protein extracts.

Evans and St. John (28) report that autoclaving of dry peas at 110°C. reduced the amount of protein soluble in water and various salt solutions. The greatest amount of protein dispersed by water was 59% which was lower than for most salts.

Very few investigations have been concerned with the nature of extracted pea protein.

Wetter and McCalla (99) concluded that pea seed proteins were much more heterogeneous than thought earlier.

Danielsson (23) developed a method whereby the pea globulins vicilin and legumin could be prepared pure enough to produce single sedimentation boundaries in the ultracentrifuge. Molecular weights were determined for both globulins. In all, the globulins of 34 different species of the Leguminosae family were analysed in the ultracentrifuge. Later, the same author found the vicilin of pea seeds to be electrophoretically homogeneous in the pH range 3.7 to 9.3 and the legumin was homogeneous in the range 6.3 to 9.3 (24).

MATERIAL AND METHODS

Pea seeds (Pisum sativum) were coarsely ground in a Wiley mill and then extracted forty-eight hours with ether using a Soxhlet apparatus. Following this extraction the pea meal was ground in a ball mill until fine enough to pass through a 72 mesh sieve. Metal containers were used for storing the pea flour at room temperature.

For preliminary extractions, peas of the Alaska variety grown at Edmonton in 1948 were used, but Idabelle peas grown under the same conditions were used for the diffusion and electrophoretic results reported.

Workers have reported on the protein extracting ability of many salts when used on peas. Unfortunately, many of the dispersing agents which extracted high percentages of nitrogen are not suitable for use in electrophoretic analysis because the salt concentration is too high. Wetter (98) investigated a number of solvents which might be suitable as extracting agents as well as for electrophoresis. Some attempt has been made here to add to his list.

Evans and St. John (28) have reported curves showing the affect of pH on pea protein dispersion for water and sodium chloride. The same type of study is reported here for sodium benzoate and sodium salicylate.

The extraction procedure employed was simply to mix known amounts of meal and dispersing agent in a suitable container. The resulting slurry was left in the refrigerator overnight and next morning it was subjected to centrifugation in an angle centrifuge (4000 r.p.m.) placed in the refrigerator. The clear supernatant liquid was poured off and used for analysis.

Calculations for the amount of nitrogen extracted are on the basis of the total volume of dispersing agent added. Nitrogen determinations on extracts were either with a macro Kjeldahl apparatus or by a micro Kjeldahl method. The conversion factor 6.25 served to convert nitrogen content to protein content.

To check both electrophoretic equipment and technique mobility determinations were made using crystallized egg albumin prepared by the method of Kekwick and Cannan (37) and the results compared with those of Tiselius, and Colvin and McCalla (21) obtained under similar conditions.

All electrophoretic patterns were obtained with the same Tiselius apparatus as employed by Colvin and McCalla (21)

except that their cell which required rubber sleeves was replaced by a cell with ball joint connections. The determination of mobilities was as outlined by these authors using the formula $\mu = \frac{VK}{it}$.

where μ = mobility,
V = volume swept through,
K = specific conductance,
i = current,
t = time.

Conductivities and hydrogen ion concentrations were determined with the same instruments used by Colvin and McCalla. The Beckman pH meter was always standardized with a phosphate buffer prepared according to Clark (19).

The main electrophoretic work reported involves an attempt to fractionate the water-soluble proteins of the pea seed. As already mentioned, Danielsson (23, 24) has carried out an electrophoretic and ultracentrifugal characterization of the pea seed globulins, vicilin and legumin, therefore the proposed study would help to present a more complete picture of the pea proteins. The attempted fractionation involved the use of ammonium sulfate and can be best explained by the scheme on the following page.

Electrophoresis was done on each fraction after it had been dialysed against three 1500 ml. lots of the 0.2N sodium phosphate buffer.

Outline of the procedure for fractionation of
pea seed proteins

Pea meal dispersed in water
(1:5 ratio). Slurry centrifuged.

Aliquot of supernatant
dialysed against 0.2N
sodium phosphate for el-
ectrophoresis (fraction 1).

Supernatant dialysed against cool run-
ning distilled water for $1\frac{1}{2}$ days and
then centrifuged.

Aliquot of su-
pernatant dia-
lysed against
0.2N sodium
phosphate for
electrophoresis
(fraction 2).

Aliquot of su-
pernatant made
60% saturated
with ammonium
sulfate and ce-
ntrifuged after
 $1\frac{1}{2}$ hours.

Aliquot of su-
pernatant satu-
rated with am-
monium sulfate
and centrifuged
after $1\frac{1}{2}$ hours.
Precipitate re-
dispersed in 0.2N
sodium phosphate
and dialysed ag-
ainst 0.2N sodium
phosphate for el-
ectrophoresis
(fraction 3).

Aliquot of su-
pernatant made
20% saturated
with ammonium
sulfate and ce-
ntrifuged after
 $1\frac{1}{2}$ hours. This
supernatant sa-
turated with
ammonium sul-
fate and cent-
rifuged after
 $1\frac{1}{2}$ hours. Pre-
cipitate redis-
persed in 0.2N
sodium phosph-
ate and dialy-
sed against
0.2N sodium
phosphate for
electrophoresis
(fraction 5).

Supernatant saturated
with ammonium sulfate
and centrifuged after
 $1\frac{1}{2}$ hours. Precipita-
te redispersed in 0.2N
sodium phosphate and
dialysed against 0.2N
sodium phosphate for
electrophoresis (fra-
ction 6).

Precipitate redispersed
in 0.2N sodium phosphate
and dialysed against 0.2N
sodium phosphate for ele-
ctrophoresis (fraction 4).

Check and preliminary diffusion determinations were made with the Neurath cell used by Colvin and McCalla (21). The procedure for determination of diffusion constants was the same as reported by these authors using the formula $D = \frac{A^2}{4\pi t(H_m)^2}$

where D = diffusion constant,
 A = area under the curve,
 t = time of diffusion,
 H_m = maximum ordinate.

After the preliminary runs the Neurath cell was discarded in favor of the Tiselius cell because of leakage in the Neurath cell which was apparently caused by the steel surfaces being out of alignment. Efforts to correct this fault were unsuccessful.

Viscosities were determined with an Ostwald viscosimeter and densities of solutions were found by the pycnometer method.

The main diffusion determinations represent a determination of the effect of temperature on the molecular state of dispersed pea protein. The protein was dispersed in a buffer (consisting of 0.2M-NaCl; 0.03M-Na₂HPO₄; 0.02M-NaH₂PO₄) by the extraction procedure already given. Following centrifugation, the extract was dialysed against three lots of the buffer and then diluted to 0.70% protein concentration. This material was divided into aliquots suitable for diffusion determinations.

Since the aliquots in some cases had to be kept over a period of many weeks, some method was required for preserving them. Toluene has been used for keeping protein solutions. By this

and the other two are the same as the first two.

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$$\frac{1}{(x^2+1)^2} = \frac{1}{(x^2+1)^2} + \frac{1}{(x^2+1)^2} + \frac{1}{(x^2+1)^2} + \frac{1}{(x^2+1)^2}$$

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method, Vickery et al (93) kept suspensions of cucurbit seed globulins for some time at refrigerator temperatures. Perri and Hazel (77) used toluene as a preservative for soybean protein preparations as did Briggs and Mann (11).

Freezing was thought to be a more efficient method for keeping protein solutions over a long period of time. Unfortunately, there is no clear-cut evidence of the effects freezing has on proteins. Tiselius (91) reported that freezing of horse sera did not change the properties of the solutions. Toluene, however, caused precipitation. Moore et al (69), using electrophoretic patterns of human blood serum showed that measurable changes occurred on freezing the serum in a dry ice-alcohol bath followed by thawing in an ice box. Denaturation of myosin by freezing has been reported by Dyer, French and Snow (26). As for plant proteins, Woods and DuBuy (103) found that freezing caused irreversible coagulation of potato chromoprotein (chlorophyll-protein). Price (79) reports southern bean mosaic virus held at -10°C . lost about 95% of its activity in six days and was apparently completely inactive after three months. Wildman, Cheo and Bonner (102) stored cytoplasmic protein preparations of green leaves at -16°C . until electrophoretic analysis with no apparent deleterious effect on the proteins. This conclusion was based on the fact that the thawed protein solutions remained perfectly clear. The report of Fontaine et al (31) on peanut protein states that unpublished results

of Irving, Fontaine and Warner showed the mobolities of the components of whole protein were significantly changed by lyophilization, but Johnson and Shooter (53) found freeze-dried preparations of arachin possessed the same electrophoretic mobilities as samples stored under the mother liquor at $0^{\circ} - 5^{\circ}\text{C}$.

Before diffusion constants were determined on the frozen pea protein aliquots, some electrophoretic determinations were made on frozen and unfrozen aliquots to give an indication of possible changes in the protein as a result of freezing.

The diffusion time for all diffusion constant determinations was 48 hours.

Viscosities on both the buffer and protein solutions were obtained at each temperature at which diffusion constants were determined. The viscosity values of the buffer were required in the conversion of diffusion constants to a reference temperature by the formula

$$D_{20} = D_t \frac{293}{273 + t} \frac{\eta_t}{\eta_{20}} .$$

where D_{20} = diffusion constant at 20°C .,
 D_t = diffusion constant at experimental temperature,
 t = experimental temperature, $^{\circ}\text{C}$.,
 η_t = viscosity of solvent at experimental temperature,
 η_{20} = viscosity of solvent at 20°C .

This formula was used by Svedberg and Pedersen (90) for mono- and paucidisperse systems.

EXPERIMENTAL RESULTS

pH-mobility Relationship of Crystalline Egg Albumin

The results of the electrophoretic mobility determinations on crystalline egg albumin are shown in Figure 1. The observed values have been calculated from the descending boundary (66). The two observed values at 20°C. agree very well with those of Tiselius (21), but most of the observed results were obtained at 2°C. and for these a regression line could be plotted. This line indicates an isoelectric point in excellent agreement with the value reported by Tiselius. At the same pH the mobilities at 2°C. are less than those at 20°C. as would be expected on the basis of viscosity differences at the two temperatures.

Extraction of Pea Protein

Of a number of dispersing agents tried only one gave a high extraction value. This was a buffer made up of 0.2M-NaCl; 0.03M- Na_2HPO_4 ; 0.02M- NaH_2PO_4 (pH 6.8) which extracted 86 - 88% of the

2. THEORETICAL BACKGROUND

2.1. Generalized Linear Models

The generalized linear model (GLM) is a statistical model that extends the linear model to include non-normal distributions and link functions.

The GLM is defined by three components: the random component, the systematic component, and the link function.

The random component specifies the distribution of the response variable Y , given the predictors X .

The systematic component specifies the linear predictor η , which is a linear combination of the predictors X .

The link function $g(\cdot)$ maps the linear predictor η to the expected value of the response variable $E(Y)$.

The GLM can be written as:

$$E(Y) = g(\eta) = g(\beta_0 + \beta_1 X_1 + \dots + \beta_k X_k)$$

where $\beta_0, \beta_1, \dots, \beta_k$ are the parameters to be estimated.

The GLM is a special case of the exponential family of distributions.

The GLM can be estimated using maximum likelihood estimation (MLE).

The GLM is a flexible and powerful tool for modeling data with non-normal distributions.

2.2. Logistic Regression

2.2.1. Binary Logistic Regression

Binary logistic regression is a special case of the GLM where the response variable Y is binary (0 or 1).

The random component of the binary logistic regression model is the Bernoulli distribution.

The link function for the binary logistic regression model is the logit function:

Figure 1

The pH-mobility relationship for crystallized egg albumin in 0.02M sodium acetate

nitrogen in the meal. Unfortunately, the concentration of this buffer makes it necessary to apply a comparatively high current during electrophoresis to obtain suitable field strengths.

Figure 2 illustrates the effect of pH on nitrogen extraction. The curves shown for water and sodium chloride have been taken from Evans and St. John (28), whereas the curves for sodium salicylate and sodium benzoate represent observed values. The absolute values for the observed results should not be compared to those obtained by Evans and St. John because of differences in the extraction conditions. Besides, the pH values given by these authors are those of the extracts while the pH values for the benzoate and salicylate curves are for the solutions used for the extractions. The pH range investigated does not include the extreme pH values but only those values at which electrophoretic analysis is usually made. Both of these organic salts extract more nitrogen at the higher pH values with a marked drop below pH 6. Such results were expected since they agree with the type of curves obtained with numerous dispersing agents used on other legume seeds.

Since Figure 2 indicates that sodium salicylate disperses a high percentage of pea protein it should be a suitable dispersing agent. Recently Jirgensons (45, 46) has reported, however, that sodium salicylate has a denaturing action on pea legumin. He based his conclusions on the fact that viscosity and reducing

 

Figure 2

The effect of pH on the dispersion of pea seed nitrogen. The curves for water and sodium chloride have been taken from the results of Evans and St. John.

capacity of the protein dispersed in salicylate were higher than for protein dispersed in some common salts. On the basis of these results, sodium salicylate should not be used as a dispersing agent for pea protein.

Ammonium Sulfate Fractionation of Water-soluble

Pea Protein

A 100-gm. sample of pea meal was extracted with one liter of water overnight at refrigerator temperatures. The resulting slurry was centrifuged in the refrigerator and the supernatant saved. On the basis of duplicate extractions, 40% of the nitrogen in the meal was dispersed by the water. Following dialysis against water, the extract was centrifuged to remove the small amount of precipitate which had formed. After these treatments, 60 - 65% of the originally dispersed nitrogen had not precipitated or been removed by diffusion through the cellophane bag during dialysis.

Figures 3, 4, 5, 6, 7 and 8, represent the patterns obtained for the various fractions obtained according to the outline on page 13. The degree of resolution of the various components has been poor. Because of this poor separation, no calculations of the amounts of each component present were made from the patterns. In no case was a single component isolated by the ammonium sulfate treatments although there is a marked variation in the concentrations of the various components in the different fractions. For example, fraction 4 shows a predominance of faster moving components, whereas its supernatant is mostly composed of the slower components. Fraction 3 compares favorably to fraction 2 as would be expected since the former represents the precipitate produced by ammonium sulfate saturation of the protein solution shown by Figure 4.

Thermal Effects on the Diffusion Constants of Pea Protein

The average diffusion constant of 0.1M sucrose for five test runs using the Neurath cell was $(43.9 \pm 0.9) \times 10^{-7}$ moles/cm.² per sec. at 20°C. This compares very well with the results of Colvin and McCalla (21) who, with the same cell, obtained the value of $(44.3 \pm 0.9) \times 10^{-7}$ moles/cm.²/sec.

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Figure 3

Fraction 1 in 0.2N sodium phosphate.
 Time 200 minutes. Protein conc. 0.92%. Temp. 2 °C.
 Descending: pH 7.7. Field strength 5.0 volts/cm.
 Ascending: pH 7.7. Field strength 4.9 volts/cm.

Figure 4

Fraction 2 in 0.2N sodium phosphate.
 Time 200 minutes. Protein conc. 0.85%. Temp. 2 °C.
 Descending: pH 7.7. Field strength 5.0 volts/cm.
 Ascending: pH 7.6. Field strength 4.8 volts/cm.

Figure 5

Fraction 3 in 0.2N sodium phosphate.
 Time 203 minutes. Protein conc. 0.72%. Temp. 2 °C.
 Descending: pH 7.7. Field strength 5.2 volts/cm.
 Ascending: pH 7.7. Field strength 4.7 volts/cm.

Figure 6

Fraction 4 in 0.2N sodium phosphate.
Time 205 minutes. Protein conc. 0.71%. Temp. 2 °C.
Descending: pH 7.6. Field strength 4.9 volts/cm.
Ascending: pH 7.7. Field strength 4.8 volts/cm.

Figure 7

Fraction 5 in 0.2N sodium phosphate.
Time 205 minutes. Protein conc. 0.74%. Temp. 2 °C.
Descending: pH 7.6. Field strength 5.0 volts/cm.
Ascending: pH 7.6. Field strength 4.8 volts/cm.

Figure 8

Fraction 6 in 0.2N sodium phosphate.
Time 200 minutes. Protein conc. 0.66%. Temp. 2 °C.
Descending: pH 7.7. Field strength 5.0 volts/cm.
Ascending: pH 7.6. Field strength 4.8 volts/cm.

Continual inconvenience and error in many determinations due to leakage forced the change from the Neurath cell to the Tiselius cell. Test runs with 0.1M sucrose gave an average diffusion constant of $(23.8 \pm 1.2) \times 10^{-7}$ moles/cm.²/sec. for two determinations at 2.0°C. This value compares favorably with values given in International Critical Tables. Since the scanning patterns were normal curves, use of this cell was considered satisfactory for further determinations.

In preliminary pea protein diffusion runs at 20°C. with the Neurath cell, cloudiness occurred in the cell during the determinations. The cloudiness became so intense that the diffusion boundary could not be photographed after a period of about 700 minutes diffusion time. At first, it was thought that the temperature might be causing the cloudiness and this was the original reason for determining the effects of temperature on diffusion of pea protein solutions. Later diffusion determinations with the Tiselius cell, however, showed that the cloudiness could be eliminated simply by dialysing the protein extract against three lots of buffer. The cloudiness was, therefore, apparently caused by some dialysable constituent in the pea meal. Such a constituent might be phytin since Fontaine et al (32) have suggested the possibility of the phytate ion being responsible for cloudiness of soybean extracts.

Figure 9 (b and c) illustrates the heterogeneous nature of the protein since the observed scanning patterns deviate considerably from the normal curves (90). The experimental curve at 21.5°C . is more diffuse than the one at 1°C because diffusion rate is higher at the higher temperature.

Figure 10 shows the results of diffusion determinations which were carried out to determine the effect of temperature on the diffusion constants of whole pea protein extracts. The time of diffusion was 48 hours in all cases. The regression line plotted from the observed results shows a decrease in diffusion constants with a decrease in temperature. When these values are converted to the reference temperature of 20°C . by the formula given by Svedberg and Pedersen (90), the resulting regression line is not horizontal as it should be if the temperature has no effect on the molecular state of the protein. The negative slope of the regression line for the converted constants is, however, small. Since the method for determination of diffusion constants is subject to considerable error the small negative slope may not be significant.

Such a viewpoint is supported by the viscosity results given in Figure 11. The curve obtained by plotting protein viscosity against temperature is very similar in shape to that for the buffer used in the Tiselius cell.

Figure 9

(a) Whole extract of pea seed in 0.2M-NaCl; 0.03M- Na_2HPO_4 ; 0.02M- NaH_2PO_4 . Time 270 minutes. Protein conc. 0.72%. Temp. 2.0°C.

Descending: pH 6.8. Field strength 3.4 volts/cm.

Ascending: pH 6.8. Field strength 3.4 volts/cm.

(b) and (c)

Diffusion diagrams of whole extract of pea seed in 0.2M-NaCl; 0.03M- Na_2HPO_4 ; 0.02M- NaH_2PO_4 . Time 48 hours. Protein conc. 0.71%.

Figure 10

Temperature, °C.

Figure 11

On the basis of the diffusion and viscosity results there does not appear to be a significant change in the molecular state of the protein with temperature, or if there is the methods used did not detect the change.

By averaging the eleven diffusion constants converted to 20°C, a value of 3.90×10^{-7} moles/cm.²/sec. is obtained for the whole protein extract. Danielsson (23) has reported an average diffusion constant of 4.26×10^{-7} moles/cm.² per sec. at 20°C. for vicilin, and a value of 3.49×10^{-7} moles per cm.²/sec. at 20°C. for legumin. Using these diffusion values, Danielsson has found the molecular weight of vicilin to be 186,000 and that of legumin to be 331,000. These globulins are probably present in the whole protein extract because they are soluble in the dispersing agent used, and because the value of 3.90×10^{-7} moles/cm.²/sec. falls between the diffusion constants for vicilin and legumin. Since the diffusion constant for the whole protein extract definitely represents the average of the diffusion constants of a number of protein components, a prediction of the molecular weight order of the components is impossible.

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DISCUSSION

A plot of the pH-mobility relationship for egg albumin at 20°C. produces, as indicated in Figure 1, a different regression line than that obtained from mobilities observed at 2°C. Apparently such a result cannot be explained on the basis of solvent viscosity changes alone. In this regard, Watanabe, Ui and Nakamura (97) report that conversion of mobilities from one temperature to another on the basis of a correction for solvent viscosity alone was not sufficient for satisfactory results using crystalline horse serum albumin. Earlier, Tiselius (91) showed the ratio of mobilities of horse serum globulin at 0°C. and 20°C. to be approximately inversely proportional to the viscosities of the solvent at the respective temperatures. Now, Watanabe et al indicate, on the basis of electrophoretic and titration determinations, that an additional correction is necessary because of protein ion valency variation with temperature.

The results of Jirgensons (45, 46) on the denaturation of pea legumin are interesting from an electrophoretic point of view. Wetter (98) reports an electrophoretic scanning diagram for pea protein extracted with 6% sodium salicylate. Although

sodium salicylate extracts over 80% of the pea protein (Figure 2), the pattern he obtained is composed almost exclusively of one peak. Electrophoretic patterns of pea extracts in sodium phosphate and sodium chloride-sodium phosphate buffers (Figures 3 and 9a) indicate that pea protein extracts are made up of a number of components. The explanation for this discrepancy could be that the denaturing action of the salicylate has completely altered the electrophoretic properties of the protein. There is also the possibility, of course, that the salicylate is so strongly bound to the protein that the true electrostatic nature of the protein has been extensively masked. Klotz (61) has shown there is a marked difference in the ability of various ions to form ion-protein complexes.

In regard to the pea protein fractionation, the electrophoretic patterns indicate the water-soluble protein is much more heterogeneous than the globulins since Danielsson found only two components that were water insoluble. Thus fractionation of the water-soluble components will be more complicated. The results of fractional precipitation by ammonium sulfate are not encouraging. Early work (74, 75) indicated rather simple preparative methods produced distinct proteins from the pea, but on electrophoretic examination of these fractions Wetter and McCalla (99) found the supposedly pure fractions were mixtures of components

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with the proportions of each component varying in the different fractions. Their results on the fractions used also indicate that even salt precipitation combined with pH variation may not satisfactorily isolate electrophoretic components in pure form. Because the components are poorly resolved by electrophoresis, separation with sliding-flange cells does not appear very promising either, although some buffers other than the phosphate type may afford better resolution of the protein components.

P A R T I I

S Q U A S H S E E D P R O T E I N S

LITERATURE REVIEW

For many years edestin, the globulin of hemp-seed, was used extensively as a protein source in experimental animal diets, and its composition was thoroughly investigated. When the Marihuana Law of 1937 was passed in the United States, the restrictions made the purchase of hemp-seed in that country an involved transaction even for experimental laboratories. A substitute had to be found and this led to the investigation of globulin of pumpkin seed (Cucurbita pepo) which Vickery and co-workers prepared in a crystalline form (94). They reported their method in 1940 and it was satisfactory for preparing globulins from a number of the common cucurbit species. In the same year Wang (96) published a procedure quite similar to that of Vickery. The following year, Vickery's method was modified by including a heat treatment of 75^o C. to coagulate small quantities of proteins presumed to be of the albumin type (93). The same paper reports the yields of globulins from six species varied from 15.4% down to

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6.2% of the air-dry weight of the whole seeds. Castañeda-Agulló and Salázar P (17) also report the yields of globulins from various cucurbit species.

Since 1940 the chemical composition of the cucurbit globulins has been extensively investigated. Nitrogen, sulfur and ash contents of globulins from six species of cucurbits have been tabulated (93, 55). Hirohata (41) studied 38 varieties in eight genera of the family Cucurbitaceae and in only one case was he able to show chemical differences, however precipitin reactions showed immunological differences in many cases.

Amino acid determinations on the globulins of various species have been numerous and present a fairly complete picture from this point of view (39, 40, 62, 84, 85, 86, 89, 93, 95). The arginine, tyrosine, and tryptophane contents are similar for the globulins of squashes and pumpkins which differ from those of watermelon, cantaloupe, and cucumber.

A knowledge of amino acid composition or, even more specifically, of the number of free groups in the protein molecule is fundamental knowledge required for an understanding of the ion-protein complex formation known to occur when proteins are brought into solution. Such knowledge appears to be conspicuously lacking for plant proteins as compared with proteins found in the animal kingdom (61). Perlmann (76), however, presents data for the acid-combining capacity of edestin and watermelon

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seed globulin as determined from the sum of the basic amino acids and of the free α -amino groups. Perlmann reports the acid-combining capacity of edestin to be 138×10^{-5} mole per gm. of protein by measurement of bound metaphosphoric acid. Cohn (20) found 127 and Hitchcock 134×10^{-5} mole per gm. of protein by direct titration, using E.M.F. measurements. Two of these values are higher than the value of 130 arrived at from the content of the basic amino acids of edestin. This value, of course, would not include the free α -amino groups. For watermelon seed globulin Perlmann reports the acid-binding capacity to be 146×10^{-5} mole per gm. of protein by the metaphosphoric acid method. This value compared with 137 on the basis of amino acid content. The total basic amino acids per gm. of protein as listed by Smith and Greene (84) for various cucurbits are: 126 for pumpkin, 128 for squash, and 126 for cucumber. The high value for watermelon seed globulin is probably a result of its higher arginine content (17.9% compared with 16.2% for squash, 15.8% for cucumber, and 16.2% for pumpkin).

Apparently very little has been done in applying physical methods to the study of cucurbit proteins. Leont'ev (64), using Citrillus vulgaris, Cucumis melo, and Cucurbita maxima, found the seed globulins of these species to be similar in a number of physical properties. Only one report of electrophoretic analysis has been found in the literature and this analysis was incidental

The first thing I noticed when I stepped out of the car was the cold. It was a sharp contrast to the warm blanket I had been sitting under. I looked up at the sky, which was a pale, hazy blue. The air was still, and there was a sense of quiet solitude. I took a deep breath, feeling the cool air fill my lungs. The ground beneath my feet was soft and uneven, covered in a layer of dry leaves and grass. I walked slowly, my steps creating a soft crunching sound. In the distance, I could see the faint outlines of trees and buildings, but they were too far away to make out clearly. The overall atmosphere was one of peace and tranquility, a moment of stillness in a world that was always in motion. I felt a sense of freedom, as if I had been released from a long, tight embrace. The sun was low in the sky, casting a warm, golden glow over the landscape. The colors were muted and soft, like a watercolor painting. I closed my eyes for a moment, savoring the feeling. It was a perfect moment, a fleeting glimpse of perfection in an imperfect world. I opened my eyes and continued my walk, feeling a renewed sense of purpose and direction. The world around me seemed to be waiting for me, ready to embrace whatever I had to offer. I felt a sense of hope, a belief that everything would work out in the end. The journey was just beginning, and I was excited to see where it would lead me. I took another deep breath, feeling the cool air fill my lungs once more. The world was mine, and I was ready to take on whatever it had to throw at me. I walked on, my heart full of hope and my mind at ease. The journey was just beginning, and I was excited to see where it would lead me.

to the main investigation cited. Vennesland and Felsher (92) showed the globulin of Cucurbita pepo, prepared by Vickery's method (93), had oxalacetic decarboxylase activity. Byerrum, Brown, and Ball (16), investigated this enzyme activity more fully. On the basis of electrophoretic results, they describe the globulin as essentially homogeneous at ionic strength 0.08 and pH 4 in acetate buffer. The globulin was in suspension in buffer more alkaline than pH 4.5, and in solution at more acid pH levels. The recrystallized preparation contained manganese and iron besides traces of zinc, cobalt, and nickel. This analysis prompted these workers to suggest that the oxalacetic decarboxylase may be a metal-protein.

This review of literature indicates that the cucurbit proteins represent a class of plant proteins about which next to nothing is known concerning their electrophoretic properties and physical properties in general.

MATERIAL AND METHODS

The squash seeds used were obtained from a commercial seed house. They were of the Golden Hubbard variety which belongs to the species Cucurbita maxima. The whole seeds were coarsely

ground in a hand mill, but before further grinding was possible they had to be ether extracted because of their high oil content. Following ether extraction the coarsely ground seeds were ball-milled to pass through a 72 mesh sieve. The flour thus produced was stored in stoppered bottles until used.

The procedure used for extraction of squash protein was similar to that used for extraction of pea protein whereby the buffer and squash flour were mixed in centrifuge tubes, left overnight, and the slurry then centrifuged. Extractions of squash protein were made at both refrigerator and room temperatures. The extracts obtained at refrigerator temperatures were dialysed by static dialysis for at least three days. The extracts obtained at room temperature were preserved with toluene and dialysed at the same temperature using a mechanical dialyser (80) to hasten the process. In either case one change of buffer was made during each dialysis, the volume of buffer used being 1500 ml. for each change.

Those extracts kept in the refrigerator were analysed electrophoretically at 2°C ., whereas electrophoresis of the extracts made at room temperature was carried out at 20°C . Very little recent electrophoretic work has been done at 20°C . as the temperature range $0-4^{\circ}\text{C}$. has been used because higher field strengths can be used without heating effects. With proteins of low solubility at low temperatures, analysis at higher temperatures

is to be preferred even though lower field strengths must be used and diffusion is greater. Johnson and Shooter (52) found electrophoretic analysis of known protein mixtures at 4°C . and 20°C . gave identical results.

Following preliminary extractions and electrophoretic analysis of whole squash seed extracts, squash globulin was prepared by a method based on that used by Vickery (94). A 30-gm. sample of squash seed meal was suspended in 300 ml. of 10% sodium chloride. This suspension was placed on a water bath at about 40°C . for three hours. The slurry was centrifuged for 20 minutes in an angle centrifuge at 4000 r.p.m. and the resulting supernatant liquid again centrifuged for 20 minutes. The dispersion was then filtered to remove some light flaky particles of the meal which were not removed by centrifugation. The clear filtrate was heated on a water bath to 50°C . and then made 2% with respect to sodium chloride by adding distilled water at 60°C . Protein crystals formed upon leaving the resulting solution in the refrigerator overnight. For recrystallization the entire protein suspension was centrifuged 10 minutes in the refrigerator and the resulting precipitate redispersed in 10% sodium chloride. Any material which did not redisperse was centrifuged down at room temperature. Recrystallization was done from the clear supernatant. The protein was recrystallized three times before being used and was stored under the mother liquor in the refrigerator.

The globulin obtained by the foregoing method was dispersed in various buffers and subjected to electrophoresis at 20^o C.

EXPERIMENTAL RESULTS

Extraction of Squash Seed Protein

The results for extraction of squash seed nitrogen by various solvents are listed in Table 1. Extraction was carried out in the cold using a meal-solvent ratio of 1:10.

Table 1

Extraction of nitrogen from squash seed

Solvent	Solvent pH	% nitrogen ex- tracted at re- frigerator temp.
0.2M-NaCl; 0.03M-Na ₂ HPO ₄ ; 0.02M-NaH ₂ PO ₄	6.8	30
0.2M-Na formate (pH adjusted with HCl)	7.0	25
0.2M-Na borate (pH adjusted with HCl)	7.0	31
0.2M-Na salicylate (pH adjusted with HCl)	7.0	71
0.2M-Na benzoate (pH adjusted with HCl)	7.0	41
0.1M-Na phosphate	6.7	38
0.8N Trichloroacetic acid	-	9
H ₂ O	-	19

These results show very poor dispersion of squash seed nitrogen, and therefore, of protein ($N \times 6.25$) for all of the solvents except sodium salicylate. The salicylate extract remained very murky even after centrifugation. Since this dispersing agent has been reported to have a protein denaturing action (6, 81, 45, 46) and to inactivate a virus (10), its use here is open to question.

The nitrogen extracted by trichloroacetic acid is probably of a nonprotein form (9).

The low dispersion of nitrogen by most of the foregoing dispersing agents does not seem to be very dependent upon the pH of the solvent as was indicated from results using 0.1M sodium phosphate of pH values ranging from 4.5 to 9.1. At pH 4.5 only 22% of the nitrogen was extracted which was the lowest value for the series. Phosphate of pH 9.1 extracted 38% of the total nitrogen which represents a comparatively small increase considering the type of results obtained with some plant proteins.

In view of the low dispersability of squash nitrogen, as indicated in Table 1, the possible effect of temperature on protein peptization was investigated. Extractions were made at refrigerator and room temperatures using the same meal-solvent ratio, and the same solvents as given in Table 1. The extracts

obtained and kept at room temperature had toluene added to inhibit bacterial growth. The results for the two temperatures are given in Table 2.

Table 2

Extraction of squash seed nitrogen at room and refrigerator temperatures

Solvent	Solvent pH	<u>Refrigerator temp.</u>		<u>Room temp.</u>	
		% nitrogen extracted	Extract pH	% nitrogen extracted	Extract pH
0.2M-NaCl; 0.03M-Na ₂ HPO ₄ ; 0.02M-NaH ₂ PO ₄	6.8	28	6.5	50	6.3
0.2M-Na formate	7.0	22	6.4	30	6.3
0.2M-Na borate	7.0	30	6.5	58	6.4
0.2M-Na salicylate	7.0	72	6.5	75	6.5
0.2M-Na benzoate	7.0	32	6.5	81	6.3
0.1M-Na phosphate	6.7	30	6.5	48	6.5
0.8 N Trichloroacetic acid	-	8	-	11	-
H ₂ O	-	16	6.4	25	6.1

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Table 2 indicates that in all cases there is a greater amount of nitrogen dispersed at the higher temperature. Sodium benzoate showed an increase representing approximately 50% of the total squash seed nitrogen. The sodium salicylate extract again remained very murky after centrifugation.

Electrophoresis of Squash Seed Protein

Electrophoretic patterns for extracts of squash seed in various buffers are given in Figures 12, 13, 14, and 15. These extracts were obtained at refrigerator temperatures with the meal-solvent ratio reduced to 1:6. Although the dispersions were still too dilute for the best resolution of protein components, the patterns indicate the low dispersion of squash protein at low temperatures is not confined to a single component.

On the other hand, good electrophoretic patterns were obtained for sodium benzoate extracts made at room temperature (Figure 16). Although Figures 16 and 17 represent similar extracts, these two figures appear quite different because Figure 17 shows thermal effects due to the relatively high field strength used. The convection effects show up as very sharp, small subsidiary peaks. Figure 16 indicates the presence of at least one major component and two minor components.

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Figure 12

Whole extract of squash seed in 0.2N sodium phosphate.
Time 170 minutes. Protein conc. 0.64%. Temp. 2°C.
Descending: pH 7.7. Field strength 4.7 volts/cm.
Ascending: pH 7.7. Field strength 4.5 volts/cm.

Figure 13

Whole extract of squash seed in 0.2N sodium benzoate.
Time 180 minutes. Protein conc. 0.71%. Temp. 2°C.
Descending: pH 6.8. Field strength 4.5 volts/cm.
Ascending: pH 6.9. Field strength 4.6 volts/cm.

Figure 14

Whole extract of squash seed in 0.2N sodium chloride-sodium phosphate.
Time 150 minutes. Protein conc. 0.55%. Temp. 2°C.
Descending: pH 7.6. Field strength 4.3 volts/cm.
Ascending: pH 7.6. Field strength 4.3 volts/cm.

Figure 15

Whole extract of squash seed in 0.2N sodium formate.
Time 130 minutes. Protein conc. 0.44%. Temp. 2°C.
Descending: pH 6.9. Field strength 3.0 volts/cm.
Ascending: pH 6.9. Field strength 3.0 volts/cm.

Figure 16

Whole extract of squash seed in 0.2N sodium benzoate.
Time 300 minutes, Protein conc. 1.05%. Temp. 20°C.
Descending: pH 6.8. Field strength 2.0 volts/cm.
Ascending: pH 6.9. Field strength 2.0 volts/cm.

Figure 17

Whole extract of squash seed in 0.2N sodium benzoate.
Time 210 minutes, Protein conc. 1.09%. Temp. 20°C.
Descending: pH 6.7. Field strength 3.4 volts/cm.
Ascending: pH 6.9. Field strength 3.3 volts/cm.

Electrophoresis of Crystallized Squash Globulin

When examined microscopically (Figure 18), the crystalline globulin was entirely free of amorphous material. The crystals appear to conform to the octahedral shape described for cucurbit globulin crystals (94).

The globulin was highly insoluble in a number of the solvents listed in Table 1 although dispersion was good in the 0.2N sodium benzoate and 0.2N sodium salicylate.

From Figures 19 and 20, the crystallized globulin appears to be essentially electrophoretically homogeneous in sodium benzoate and sodium formate at the pH values indicated.

A comparison of the mobility of the crystallized globulin in sodium benzoate with the mobilities of the components of whole extract in Figure 16 indicates that the fastest moving component in the whole extract is the globulin. The mobility of this peak (-0.64×10^{-4} cm.²/volt/sec. for the descending boundary at pH 6.8), is very similar to that of the crystallized globulin (-0.61×10^{-4} cm.²/volt/sec. for the descending boundary at pH 6.9).

In sodium acetate at pH 4.7 the protein appeared to be in suspension, but it was in solution at pH 4.3 and in more acidic acetate solutions. This behavior is very similar to that reported

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BY JAMES M. SMITH, LL.D., OF THE UNIVERSITY OF CHICAGO

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for crystallized globulin of Cucurbita pepo which was in suspension in acetate buffer more alkaline than pH 4.5 (16).

When dissolved in sodium acetate at pH 3.9 the crystallized globulin (Figure 21) is characterized by the same electrophoretic homogeneity as indicated in Figures 19 and 20. Byerrum et al (16) found the globulin from Cucurbita pepo to be essentially homogeneous at pH 4.0. In contrast to these results, Figure 22 shows that at pH 4.7 two components are present. The appearance of two components at this pH was very unexpected, but these results were duplicated with a second preparation of the globulin.

One very marked characteristic of the patterns for globulin in sodium acetate shown in Figure 21, is the lack of symmetry between ascending boundary and descending boundary patterns. The patterns in sodium formate also show this lack of agreement between the two boundaries. The patterns shown in both Figures 20 and 21 were produced at pH values below the isoelectric pH of the protein since migration was toward the cathode. In contrast, the scanning diagrams shown in Figure 19 show good symmetry. In this case the electrophoretic analysis in sodium benzoate, was done at a pH value above the isoelectric pH of the protein.

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Figure 18

Squash seed globulin crystals
magnified 800 times

Figure 19

Crystallized squash globulin in 0.2N sodium benzoate.
Time 240 minutes, Protein conc. 0.70%. Temp. 20°C.
Descending: pH 6.9. Field strength 1.7 volts/cm.
Ascending: pH 7.0. Field strength 1.7 volts/cm.

Figure 20

Crystallized squash globulin in 0.05N sodium formate.
Time 120 minutes, Protein conc. 0.65%. Temp. 20°C.
Descending: pH 3.7. Field strength 3.0 volts/cm.
Ascending: pH 3.7. Field strength 3.0 volts/cm.

Figure 21

Crystallized squash globulin in 0.05 sodium acetate.
Time 180 minutes. Protein conc. 0.66%. Temp. 20°C.
Descending: pH 3.9. Field strength 1.8 volts/cm.
Ascending: pH 3.9. Field strength 1.8 volts/cm.

Figure 22

Crystallized squash globulin in 0.05 sodium acetate.
Time 180 minutes. Protein conc. 0.71%. Temp. 20°C.
Descending: pH 4.8. Field strength 1.8 volts/cm.
Ascending: pH 4.7. Field strength 1.9 volts/cm.

The foregoing comparison is not meant to intimate that the isoelectric point of the globulin dispersion is the same regardless of the dispersing agent used. The isoelectric point of the globulin dispersed in sodium benzoate may be quite different from the isoelectric point observed when the globulin is dispersed in sodium acetate. Variation in the degree of ion-protein complex formation in the different dispersing agents will cause such a discrepancy. For any given protein dispersion the observed isoelectric point is, therefore, a characteristic of the ion-protein complex rather than of the protein itself.

Longsworth (65) states that in buffers of the uncharged acid type, patterns obtained above the isoelectric pH tend to be more nearly mirror images of each other than at values below this pH. Above the isoelectric pH the effects of pH changes and conductivity changes at the boundary tending to decrease the symmetry between the patterns, tend to cancel each other. For pH values below the isoelectric pH, the pH changes at the boundary can combine with the conductivity changes to increase the tendency for the ascending boundary to sharpen and the descending boundary to become more diffuse.

DISCUSSION

Of the results obtained on squash proteins, those from the crystallized globulin are most interesting.

The low dispersal of squash seed proteins by some simple salts near neutrality probably is due to the low solubility of the globulin under these conditions. On the other hand, the relatively high extraction by sodium benzoate and sodium salicylate is due, at least in part, to the increased solubility of the globulin in these solvents. This view is substantiated by Figure 16 which shows a high concentration of the fast component. As indicated earlier, this peak corresponds to the crystallized globulin on the basis of electrophoretic mobilities.

The patterns for the globulin in sodium formate and sodium benzoate showing only one peak are of the type one probably would expect for a crystallized material. Simply because a protein has been crystallized, however, does not always indicate electrophoretic homogeneity. Anderson and Alberty (5) found that both crystalline pepsin and trypsin split into a major and minor component during electrophoresis. Hoch (43) also reported the electrophoretic heterogeneity of crystallized pepsin.

The interesting fact about squash globulin is that it appears as an essentially homogeneous protein or as two distinct components depending upon the pH of the acetate medium. The literature reports some other proteins which have been found to act similarly upon changes in hydrogen ion concentration. Anderson and Alberty (5) report conalbumin of hen's egg to appear homogeneous in diethylbarbiturate buffer of pH 6.8 and 0.10 ionic strength. In contrast, at pH 6.3 and 0.01 ionic strength, this protein resolved into two or three components. Bain and Deutsch (7) found that bovine lactoglobulin and goat lactoglobulin both appeared electrophoretically homogeneous or heterogeneous depending on the pH of the buffer. As mentioned earlier, Johnson and Shooter (53) have determined that arachin undergoes a reversible type of dissociation. Hydrogen ion concentration is one factor determining the state in which the arachin will exist in solution.

The above findings certainly indicate that the electrophoretic characterization of a protein must be carried out over a pH range before any conclusions should be made as to its electrophoretic purity.

Squash globulin might well be the object of further investigations. A verification of some of its reported properties plus added information on its physical and chemical properties are required for an understanding of its behavior.

The following are the main points of the report.

1. The report is based on the results of the survey conducted in the year 1950.

2. The survey was conducted in the following areas: (a) the area of the river, (b) the area of the lake, and (c) the area of the forest.

3. The results of the survey are as follows: (a) the area of the river is 1000 hectares, (b) the area of the lake is 500 hectares, and (c) the area of the forest is 2000 hectares.

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P A R T I I I

L E A F R O L L V I R U S O F P O T A T O E S

LITERATURE REVIEW

The term virus is applied to certain infectious particles which are capable of causing disease in man, animals, plants, insects, and bacteria. In the past these minute entities have been characterized by their invisibility and by their ability to pass through filters capable of holding back all ordinary living things. Since the electron microscope has come into use it has been possible to view these small discrete particles which are either roughly spherical or rod-like in shape, and which vary in size from about 100 A (slightly smaller than some protein molecules) to about 3,000 A, which is somewhat larger than certain living organisms (the pleuro-pneumonia organism which can be grown like ordinary bacteria on artificial media). Viruses differ from bacteria, not only as to size, but also by their inability to duplicate themselves on

synthetic media. Most viruses are highly specific since a given virus will multiply only within certain kinds of living cells. For example, the tobacco mosaic virus will grow only within the cells of certain plants, such as the tobacco or tomato plant. Of the plant viruses chemically analysed, all have proved to be simple nucleoproteins although some viruses in the animal world contain other constituents such as lipids, copper, flavin and biotin.

Ever since the crystallization of tobacco mosaic virus by Stanley in 1935, viruses have been the object of more and more intensified study not only because of their importance as disease producers, but also because their true nature is still a point of controversy.

The work of the past few years involving physical and chemical methods of analysis has brought forth much information about the structure of these entities which, in the words of Stanley (88), fill the gap between the molecules of the chemist and the organisms of the biologist. Those viruses which have been crystallized have been most intensively studied. In 1945, Price (78) listed tobacco mosaic, tomato bushy stunt, tobacco necrosis, and southern bean mosaic viruses as the only ones crystallized up to that time. Since then at least one more has been crystallized, that being turnip yellow mosaic virus (87). The various strains of tobacco mosaic virus have probably

been more thoroughly investigated than any of the many other plant viruses mentioned in the literature. Investigations on such basic characteristics as size, shape and aggregation, amino acid content, and hydration are numerous.

Whereas the electrophoretic technique has not been applied to the investigation of virus diseases in plants until very recently, there have been intermittent reports of electrophoretic analysis of green plant material over the past forty years. In 1912, Herlitzka (38) subjected the press juice of leaves to electrophoresis and noticed its migration to the anode. Later, Bakker (8) reported that aqueous suspensions of chlorophyll, precipitated from acetone solutions and free from protein, were negatively charged but became positive on exposure to light for several hours. Katz and Wassink (60) found that green aqueous extracts prepared from Chlorella were negative at concentrations of HCl up to 0.001N, ceased movement in 0.002N HCl, and reversed in direction at slightly higher concentrations. Neish (73) found chloroplasts isolated from Trifolium to be isoelectric at about pH 3.7, but negative in distilled water. Except for these brief reports there apparently was no thorough investigation of the electrophoretic behavior of the chlorophyll-protein complex until the work of Fishman and Moyer (29), who used an Abramson type of microelectrophoresis cell and followed

the techniques described by Moyer (70). Some of the general properties of the complex isolated from Aspidistra elatior and Phaseolus vulgaris were photostability, red fluorescence, sensitivity to protein coagulants and denaturation by drying, freezing, increase in temperature or treatment with weak acids. Their purified extracts flocculated almost instantaneously at and below the isoelectric point. The green microscopic particles migrated independently of size, shape or degree of clumping.

Wildman and Bonner (100) report investigations concerning spinach leaf cytoplasmic proteins which were extracted by an involved procedure using blender and colloid mill treatments at temperatures of $0-4^{\circ}\text{C}$. Two components are shown by electrophoretic patterns obtained by using infrared plates for photographing the boundaries because of the color in the preparations. Fractionation of the cytoplasmic proteins using ammonium sulfate and water dialysis resulted in a fraction equivalent to 70 - 80% of the total cytoplasmic protein. This fraction was electrophoretically homogeneous, and showed auxin and phosphatase activity. A second fraction was electrophoretically heterogeneous and had a number of enzyme activities.

Electrophoretic fractionation has been carried out on ragweed pollens but the analysis has been complicated by the presence of pigments. The first experiments by Abramson et al (4)

were done with dialysed extracts and showed a major unpigmented component along with some pigmented components. Later work by Abramson and Moore (1), with undialysed extracts, gave better separation. By means of the sliding-flange plates of the electrophoresis cell, the pigmented fractions were separated from the unpigmented component. The electrophoretic analysis of timothy grass pollen (2) produced patterns similar to those of ragweed pollen as did pollens from Bermuda grass, June grass, sheep sorrel, orchard grass, birch and oak (3).

Very little electrophoretic work has been done with viruses found in plants. Some of the main reasons for this probably are the problems encountered in the preparation of a sample suitable for electrophoretic analysis. Usually there is much pigment in the extracts while the concentrations of the desired components are too low for obtaining good scanning patterns.

Frampton and Takahashi (34, 35) used an extraction procedure involving cytolysis of harvested leaves with ether. The vacuolar sap was then pressed out and discarded as it contained very little protein, but much pigment and substrate for oxidizing enzymes. After several water washings and pressings, the pressed leaf material was ground in a food chopper and extracted with buffer. In some cases the Waring Blendor was used. To get a high enough concentration of protein in the extract the extracting agent was reused several times with further

concentration obtained by placing the extract in a cellophane tubing and hanging it before a fan. The authors do not indicate the amount of protein extracted by their method.

By means of the moving boundary method of electrophoresis, using infrared photography, Frampton and Takahashi (35), obtained scanning patterns for extracts from healthy tobacco plants, from tobacco plants infected with tobacco mosaic virus, with potato X and potato Y viruses. Patterns are also given for extracts from the healthy pea bean and pea bean with a virus infection.

Wildman, Cheo, and Bonner (102), using extracts from tobacco leaves infected with tobacco mosaic virus obtained results indicating the value of electrophoresis as a method of detecting infection. The methods used were very similar to those developed in the study of spinach leaf cytoplasmic proteins (100). The cytoplasmic proteins of healthy leaves consisted of an electrophoretically homogeneous component representing 75% of the cytoplasmic protein, and minor fast moving components. Infected leaves have a new component which, when separated electrophoretically, produces characteristic local lesions on test plants. The virus protein was detected electrophoretically as early as three days after infection, but after the twelfth day, the amount of virus appeared to become static. While the virus protein

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increased there was a proportionate decrease in the normal cytoplasmic protein. Since the maximum virus synthesis can occur before a detectable change in chlorophyll, the virus synthesis would not appear to have proceeded at the expense of the chlorophyll as suggested by Woods and DuBuy (103). The characteristic pigment changes accompanying the disease could be a secondary affect caused by a metabolic upset in the plant due to cytoplasmic protein changes.

Price (78) reports the preparation of purified southern bean mosaic virus by fractionating the juice from infected bean plants. The fractionation was carried out by alternating cycles of high- and low-speed centrifugation or by chemical treatment using ammonium sulfate and alcohol. Although still containing a considerable amount of dark pigment, purified virus preparations were essentially homogeneous on the basis of electrophoretic, diffusion and high-speed centrifugal results. This bean mosaic virus was eventually crystallized. Miller and Price (68) found that crystallization by dialysis against distilled water was retarded when they used a pigmented sample of virus. By dialysis against an acetate buffer at pH 5.5 even a sample containing large amounts of pigment could be crystallized. Under such conditions, however, the pigment disappeared almost completely from the mother liquor but constituted a part of the

crystals. Price (79) reported earlier that pigment interfered with the crystallization of southern bean mosaic when using salt for crystallization.

Lauffer and Price (63) were able to prepare southern bean mosaic virus free of pigment by an electrophoretic technique. Such a purification was possible since in phosphate buffer of pH 7, the pigmented material migrated faster than the virus. With the aid of a four-piece Tiselius cell the authors obtained a virus preparation almost completely free of pigment. Lauffer and Price also report a unique method by which virus preparations can be concentrated without using chemical precipitation. The sap from infected bean plants was dialysed against egg white at 4°C . In three weeks the volume of sap was reduced to about one fifth the original volume. The authors suggest this method of volume reduction depends upon the differences in the colloidal osmotic pressures of the egg white and of the sap.

MATERIAL AND METHODS

The original aim of this work was to use electrophoresis as a method of diagnosis to supplement visual methods of diagnosing virus diseases in plants. As already indicated, the

literature reports that electrophoretic analysis has shown virus infection before visual symptoms have appeared. Such a supplementary method would be especially useful where symptoms of the disease are not very pronounced.

The material used here consisted of healthy potato plants of the Netted Gem variety as well as plants of the same variety which were infected with leaf roll virus (Corium solani). According to Chester (18), this virus is transmitted by aphids and affects only potatoes and a few other closely related Solanaceae. Infected plants are pale and dwarfed with thick leathery leaves that are rolled upward. Tubers are few and small.

Infected and healthy tubers were supplied by the Plant Pathology Division and planted in separate field plots during the summer of 1950. Preliminary protein extractions were carried out on the green leaves when the plants had each produced several leaves. The first attempt to extract protein from the green material was with the Waring Blendor using whole green leaves with only the petioles removed.

The previous year considerable time was spent attempting to get suitable protein extracts from greenhouse-grown bean leaves and stems by blendor treatment. This protein extraction from bean plants had been started because of the effect that 2,4-D treatments have been shown to have on the nitrogen content

of various parts of the bean plant. Treatments usually decreased leaf nitrogen while the nitrogen content of stems was greatly increased by 2,4-D (22). If satisfactory extractions of protein could be obtained, electrophoretic analysis might bring forth additional information concerning these changes in nitrogen metabolism.

The Waring Blendor was used only because equipment available for protein extraction of green material was limited. The blendor was set up in the refrigerator as no cold room facilities were available in the laboratory. Foaming was prevented by using a screw lid on the blendor bowl. Heating was prevented as much as possible by running the blendor for short intervals and allowing periods of cooling. A dry ice jacket was sometimes used as well.

The literature shows no clear-cut evidence as to the denaturation which may occur during blending; however, there is some evidence that this deleterious effect is inherent in the method. Fishman and Moyer (29) used the Waring Blendor in attempts to prepare preparations of the protein-chlorophyll complex of Aspidistra elatior and Phaseolus vulgaris but found a large volume of foam was produced with considerable denaturation on disruption of the leaf material. Furthermore, the material purified in this way precipitated quite rapidly during the course

of several days. This foaming reported by Fishman and Moyer can be readily prevented by using a screw lid on the blender bowl after it has been completely filled with the solvent and leaf material to be disrupted.

On the other hand, Frampton and Takahashi (35) report that the Waring Blender had no influence on electrophoretic patterns obtained with the proteins from healthy tobacco plants, tobacco plants infected with tobacco mosaic virus, or virus-free tubers. This situation does not necessarily hold true for extractable proteins from other plants.

Wildman and Bonner (100) used a Stephen Blender to aid in the extraction of leaf proteins. Their objections to the blender as compared to the colloid mill were poorer mixing in the blender and rapid heating which was difficult to control even with a dry ice jacket.

Following the unsatisfactory extraction results for potato leaf protein by the Waring Blender, and because equipment such as a colloid mill was unavailable, attempts were made to obtain protein dispersions suitable for electrophoresis by extraction of potato leaves dried by lyophilization. The lyophilization equipment consisted of an insulated wooden box containing a dry ice-acetone bath large enough for a desiccator. The desiccator was evacuated by means of a vacuum pump. When

dry, the leaves were ball-milled and stored in stoppered containers at room temperature until ready for use.

Extraction procedures on the dried material were similar to those used previously for pea and squash seeds. Following extraction and dialysis at refrigerator temperatures, electrophoresis was attempted using the conventional apparatus. Because of the heavy pigmentation of the extracts, infrared film was substituted for the photographic plates ordinarily used.

EXPERIMENTAL RESULTS

Bean Stems and Leaves

Since use of the Waring Blendor was first made on fresh bean stems and leaves some extraction results are given for this material. Most interest was attached to the stems since 2,4-D treatment had such a marked effect on them. Since the stems are less heavily pigmented than the leaves they are more suitable for electrophoretic analysis.

Some typical extraction results are as follows. Fresh coarsely chopped bean stems and buffer ($0.2M\text{-NaCl}$; $0.03M\text{-Na}_2\text{HPO}_4$; $0.02M\text{-NaH}_2\text{PO}_4$) were used in the weight ratio 1:10. Following a total blending time of ten minutes, the slurry was suction filtered. Seventy four per cent of the stem nitrogen was extracted. Under similar conditions $0.8N$ trichloroacetic acid dispersed 67% of the stem nitrogen thus indicating a large amount of the stem nitrogen is nonprotein. The fact that the buffer dispersed mostly nonprotein nitrogen was substantiated when dialysis removed a high percentage of the nitrogen from the buffer extract.

Extracts of leaves were higher in protein nitrogen but smaller amounts of the nitrogen present in the fresh leaves were extracted than in the case of the stems.

Because of the large percentage of nonprotein nitrogen extracted from stems, no extract was obtained which, after dialysis, was concentrated enough for electrophoretic analysis. No supplementary methods of concentrating the extracts were tried.

In the case of leaves, by reusing the buffer, dialysed extracts could be obtained which were concentrated enough for electrophoresis, however, the percentage of nitrogen extracted was reduced.

Potato Leaves

Because the Waring Blendor disrupted the coarse potato leaves even less than bean leaves, and due to the difficulty of keeping the material near freezing during extraction, use of the blendor was discarded entirely.

Extractions were next done on leaf material which had been lyophilized and ball-milled. The buffers used were all of pH 7 or higher as Wildman, Cheo and Bonner (102) report isoelectric precipitation of tobacco cytoplasmic protein when grinding leaves in poorly buffered solutions. Preliminary extraction results using a 1:10 ratio indicated that the protein concentration of the extract was not high enough for satisfactory electrophoresis. A 1:5 ratio gave the results listed in Table 3.

The infected and healthy leaves show very little difference as to solubility of nitrogen. The low percentage of extraction in all cases is probably due, at least in part, to the comparatively large amount of dried material extracted with a rather small volume of buffer.

Table 3

Extraction of nitrogen from dried potato leaves

Buffer	Buffer pH	Healthy leaves		Infected leaves	
		% nitrogen extracted	Protein (N x 6.25) conc. of extract	% nitrogen extracted	Protein (N x 6.25) conc. of extract
0.2N sodium benzoate	7.1	30	1.5%	31	1.4%
0.2N sodium phosphate	7.7	40	2.0%	40	1.8%
0.2N sodium chloride-sodium phosphate	7.7	37	1.8%	39	1.8%

Although the extracts were sufficiently high in protein content, the pigment content was also great. Pigmentation was so intense that electrophoretic scanning patterns could not be obtained by use of ordinary photographic plates or infrared film.

Ether extraction of the dried material, prior to extraction, removed large amounts of pigment, but the buffer extracts from ether extracted leaves were still too highly pigmented for electrophoretic analysis.

Table 1

Summary of results for the various experiments

Experiment 1		Experiment 2		Experiment 3	
Time (min)	Distance (m)	Time (min)	Distance (m)	Time (min)	Distance (m)
1.0	1.0	1.0	1.0	1.0	1.0
2.0	2.0	2.0	2.0	2.0	2.0
3.0	3.0	3.0	3.0	3.0	3.0

The following table shows the results of the various experiments. The first column gives the time in minutes, and the second column gives the distance in meters. The third column gives the time in minutes, and the fourth column gives the distance in meters. The fifth column gives the time in minutes, and the sixth column gives the distance in meters.

Experiment 1: The results of the first experiment are shown in the first two columns. The time is 1.0 minute, and the distance is 1.0 meter. The time is 2.0 minutes, and the distance is 2.0 meters. The time is 3.0 minutes, and the distance is 3.0 meters.

Experiment 2: The results of the second experiment are shown in the third and fourth columns. The time is 1.0 minute, and the distance is 1.0 meter. The time is 2.0 minutes, and the distance is 2.0 meters. The time is 3.0 minutes, and the distance is 3.0 meters.

Experiment 3: The results of the third experiment are shown in the fifth and sixth columns. The time is 1.0 minute, and the distance is 1.0 meter. The time is 2.0 minutes, and the distance is 2.0 meters. The time is 3.0 minutes, and the distance is 3.0 meters.

DISCUSSION

The attempts to use electrophoresis for analysis of protein from green plant material produced very unsatisfactory results. The extraction procedures used proved to be inadequate. On the whole, extracts obtained by using the Waring Blendor were too dilute for electrophoretic analysis. Even more serious was the fact that the protein extracted was subjected to adverse conditions because blending is a rigorous treatment. Besides, facilities available made it impossible to do the extractions near freezing temperatures. At the higher temperatures the chances of enzyme activity and denaturation were probably increased.

Although the amount of nitrogen extracted from lyophilized leaves was less than one-half the total present in the leaf, this method provided extracts which, as such, were concentrated enough in protein for electrophoretic analysis. However, the pigmentation of such extracts is too great for electrophoretic analysis. Unfortunately, the amount of dried material prepared was not sufficient to try methods for removal of the pigment. Whatever these methods may be, they represent another step which may have a denaturing action on the protein.

Frampton and Takahashi (35) summarize the whole problem of extracting leaf proteins with the following well chosen words -

"The methods available for the extraction of leaf proteins are far from satisfactory, and the total quantity of protein extracted in any case is only a fraction of the total leaf protein. Those normal leaf proteins which have been extracted are difficult to handle; none of them have ever been crystallized. As for the viruses themselves, except for those which have been isolated, very little may be said concerning the conditions under which they become soluble, the ease with which they become denatured or their relative concentrations in the plant saps."

Thus the whole problem is at present in a very unsatisfactory state with little prospect of improvement through use of present known methods. The colloid mill treatment for extraction of leaf proteins appears to be the most satisfactory method available despite its rigorous nature.

ACKNOWLEDGEMENTS

The helpful criticism and advice of Dr. A. G. McCalla, supervisor of this project, are gratefully acknowledged.

The Plant Pathology Division kindly supplied and cared for the potato material used.

Thanks are also due to Mr. G. M. Tosh, Technician, for maintenance of equipment and reproduction of diagrams.

Financial assistance was supplied by a Robert Tegler Research Scholarship in 1949-50 and by a National Research Council Studentship in 1950-51.

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* Chemical Abstracts.

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