A STUDY OF THE SUNFLOWER SEED PROTEIN

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ABSTRACT

Practically all of the protein of oil-free sunflower seed meal is extracted by a glycine-sodium hydroxide buffer of pH 9. Electrophoresis, filter paper electrophoresis and solubility tests show that it is a single substance with an isoëlectric point of pH 9.0 - 9.1. The amounts of eighteen amino acids --the essential amino acids and alanine, aspartic acid, cystine, glutamic acid, glycine, proline, serine, and tyrosine--in the protein were determined--these make up approximately eighty-three percent of the total protein.

INTRODUCTION

INTRODUCTION

Proteins are necessary constituents of all living matter and are required in the food of all animals. Their nutritive value is determined by the amino acid contents, particularily of the "essential" ones. The life processes that take place in living cells require that many proteins exist in the same cell and it is unlikely that a protein could be directly extracted in the pure state from such a complex mixture without great difficulty. With few exceptions, an extract of animal or vegetable substance is a complex mixture of proteins in colloidal suspension. Various methods have been employed in attempts to separate these mixtures, the classical one being based upon the solubilities of proteins in various solvents.

Until recently the formation of homogeneous crystals, whose melting point remains unchanged after repeated recrystallization, was considered conclusive evidence of a pure substance. However, recent studies on the physical properties of proteins have indicated that many crystalline precipitates are not homogeneous. Now it is believed that two or more proteins may form a single crystal lattice and that crystallizability alone may not be taken as a criterion of the purity of a protein.

Another test of protein purity which has been used is

solubility in one solvent. This is constant at saturation and is not altered by any excess of a solid homogeneous substance. However this method cannot be applied to the solubility of proteins in pure water since the solubility of some of them is dependent on traces of electrolytes and of hydrogen or hydroxyl ions. Usually a concentrated salt solution is employed as a solvent, one in which all of the protein--or, at least all of the protein of major interest--is soluble.

One of the best methods of protein fractionation is electrophoresis, based on the principle that protein molecules carry electric charges, which vary with the hydrogen ion concentration of the medium in which they are suspended. When an electric current is passed through such a suspension, the particles move in a direction and at a speed regulated by the charge on the particle. Thus a protein with a positive charge would move toward the negative electrode and the one with the negative charge would move toward the positive electrode. Thus it would be possible to separate two similar substances easily and even more complex mixtures in the same way.

Paper electrophoresis is a modified form of the above procedure in which electrophoresis is carried out in a filter paper strip. This technique was employed in our studies. The advantage over the conventional electrophoresis method lies in the cheapness of

the equipment. Heretofore, this process has been mainly used as a diagnostic aid for diseases which cause abnormal blood sera but it also may be applied to any protein solution.

The investigation reported herein was prompted by the increased importance of the sunflower crop in Manitoba. Osborne and Campbell, in 1897, studied the sunflower seed protein using salt extraction methods and concluded that it consists of a single globulin. It was decided to test this conclusion using the newer methods of constant solubility and electrophoresis.

It was also decided to determine the amino acid composition of the protein or proteins if there should prove to be more than one. Several analyses have been reported over the years, but newer chemical and microbiological methods are available which were not known to the earlier workers. Also, since the first data were reported, new amino acids have been isolated, and, of course, only recent analytical data are available for them. Since sunflower meal is an important source of protein in animal and poultry feeding, most analyses have been for the "essential" amino acids. It was desired to extend this study to indicate as many as possible. Actually our data are for eighteen.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Sunflowers have been grown for many years. Their seeds yield an edible oil of high quality and the oil-free meal remaining is used as feed. Like other oil seed meals it makes a satisfactory feed because of its comparatively high content of protein.

This protein has been the subject of several scientific investigations, though it has not been studied as much as many others. In 1897, Osborne and Campbell (31) concluded that the principal protein of the sunflower seed is a single globulin, identical with the edestin of other oil bearing seeds. They extracted ten samples of fat-free meal with ten percent sodium chloride solution and separated the protein from the extract by dialysis, dilution, or saturation with sodium chloride and subjected it to elementary analysis. In each case, the composition was the same as that for edestin. Their preparations were colored with helianthotannic (chlorogenic) acid which could be removed in part by preliminary extraction with ethyl alcohol. Earlier than this Ritthausen (34) in 1880 and Vines (44) in 1883 reported results of elementary analysis of protein separated from sunflower seed by extraction and precipitation methods. In each case fewer samples were tested than were done by Osborne and Campbell. In 1948 Smith and Johnson (37) studied the isolation of the protein by the method of alkali extraction and

acid precipitation.

The usefulness of sunflower seed protein in feeds depends on the extent to which it provides indispensable or essential amino acids to the animals which consume it. Mitchell, Hamilton, and Beadles (29) reported that the biological value of the protein was much lower than that of properly heated soybean meal protein. Since the protein content of sunflower seed meal was high, the "net protein value" was higher than that of both soybean oil meal and coconut oil meal. Grau and Almquist (23) noted that if sunflower seed oil protein supplied all of the dietary protein for chicks, a sufficient amount of lysine was present. However, McGinnis, Hsu and Carver (28) reported that when sunflower seed oil meal was combined with cereal proteins which are notably deficient in lysine, the combination did not supply enough lysine to meet the requirements of the chicks.

In the light of modern knowledge the conclusions of Osborne and Campbell (31) should be reviewed anew. It is conceivable that a mixture of globulins may be separated by salt extraction and dialysis, and that the composition of these may be so alike that different mixtures of them would show no elemental differences on analysis. More modern methods of determining whether a protein is a single substance or a mixture are based on electrophoretic and solubility behaviour, and it would seem wise to apply these to sunflower seed protein preparations. This was done in the

investigation reported in this thesis.

ELECTROPHORESIS

Electrophoresis may be defined as the migration of a charged particle suspended in a liquid due to an imposed electromotive force. The direction of migration indicates the sign of the particle (colloid micelle), the micelle migrating through the liquid towards the pole of opposite sign. If a mixture of the micelles of different protein—the micelles of each having a charge different in size or kind, or both, from those of the others —is subjected to electrophoresis, the mobilities will be different for the various proteins and they may be separated, and the separate fractions recognized in this way.

One of the three general variations of the electrophoresis method is the micro method, which uses a microscope and visual measurement of microscopically visible particles. The macro, or moving boundary, method employs the detection and following of the displacement of a boundary between a colloidal suspension and its dispersion medium. In the filter paper method, the protein solution is placed on a strip of filter paper, soaked in a buffer solution and subjected to an electric potential along its length. After a suitable period of time, the position of the protein fractions is determined by a dyeing treatment.

There are many reports on the electrophoresis of amino acids,

peptides and proteins in capillary systems. Consdon, Gordon, and Martin (14) describe electrophoresis of amino acids and peptides in silica gel. Similarly, Gordon, Keil and Sebasta (20) experimented with the electrophoresis of proteins using a gel. Butler and Stephen (12) employed a polystyrene tube which was filled with alternate layers of filter paper and asbestos. A combined separation of amino acids by electrophoresis and paper chromatography was carried out by Haugaard (24). Using a chamber specified by Wieland (45) for electrophoresis of amino acid and peptides, Turba and Enenkel (39) set forth a micro method for electrophoretic separation of serum proteins. This method of separation specified that the proteins be dyed with azocarmine in aqueous methanol containing acetic acid. Further work along these lines was done by Grassmann and Hannig (27) who described no technical details of their process. A method similar to that of Turba and Enenkel (39) for separating serum proteins on filter paper was reported by Biserte (3) and Elseneich and Eder (17). The method of electrophoretic fractionation used in the present investigation employs filter paper strips and is based on a modification of the method of Cremer and Tiselius (13). To avoid troubles due to heating and vaporizations, they enclosed the filter paper strip between two sheets of glass and surrounded it by a coolant (monochlorobenzene). The dyeing process involved the saturation of the strip in a solution containing one percent bromphenol blue in ninety-six percent

alcohol saturated with mercuric chloride. A rather complicated system of glass traps was used in the electrode assembly. Goa (19), a pupil of Tiselius, described a method for the quantitative determination of serum proteins by paper electrophoresis. In his method, the paper strips were in contact with glass on one side and saturated air in the other. The electrode assembly used was similar to that of Cremer and Tiselius (13). The modifications used in this investigation were in the electrode assembly and dyeing procedure--azocarmine was used instead of bromphenol blue.

A complicating factor may be introduced when absolute mobilities are to be determined. This is the mobility due to electroosmosis, which is the rate of motion of a liquid relative to a solid under the influence of an external electrical field applied tangentially to the interface. In the micro method a position of field of view in relation to the cell wall can be found, whereby the electroosmotic effect is cancelled out. On the other hand, in the macro method the cell is of such large dimensions that the effect is negligible. Since filter paper is composed of many capillaries, the electroosmotic effect cannot be eliminated directly. However, if the isoelectric point of one protein fraction is known, the mobility of the protein due to electroosmosis can be measured and corrections made accordingly.

SOLUBILITY

Solubility offers another method for determing the purity of proteins. For a pure chemical compound, the solubility is independent of the amount of solid present. This is also true of proteins. Sörensen (38) demonstrated that egg albumin at fixed temperature, pressure, salt concentration and pH behaves very nearly like a single component with a solubility almost independent of the amount of the solid phase in concentrated ammonium sulfate solution. Northrop et al (30) reported that some proteins may be obtained in sufficient purity to fulfill rigorously the requirements for a pure single component.

To measure protein solubility, varying amounts of protein are placed in a series of test tubes. These are filled completely with solvent and stoppered to prevent surface denaturation of the protein by air. The tubes are slowly rotated and the contents stirred by small glass beads placed in the tubes. The tubes are kept at constant temperature and pressure and rotated until equilibrium it attained. The solutions are then filtered and the amount of protein in the filtrate determined. The amount of protein in solution is plotted against the total amount of protein present. Figure 1 shows diagrammatically the results that may be obtained: (A) represents the solubility curve of a single pure protein, (B) that of a mixture of two proteins, and (C) that of a solid solution of two or more protein components.

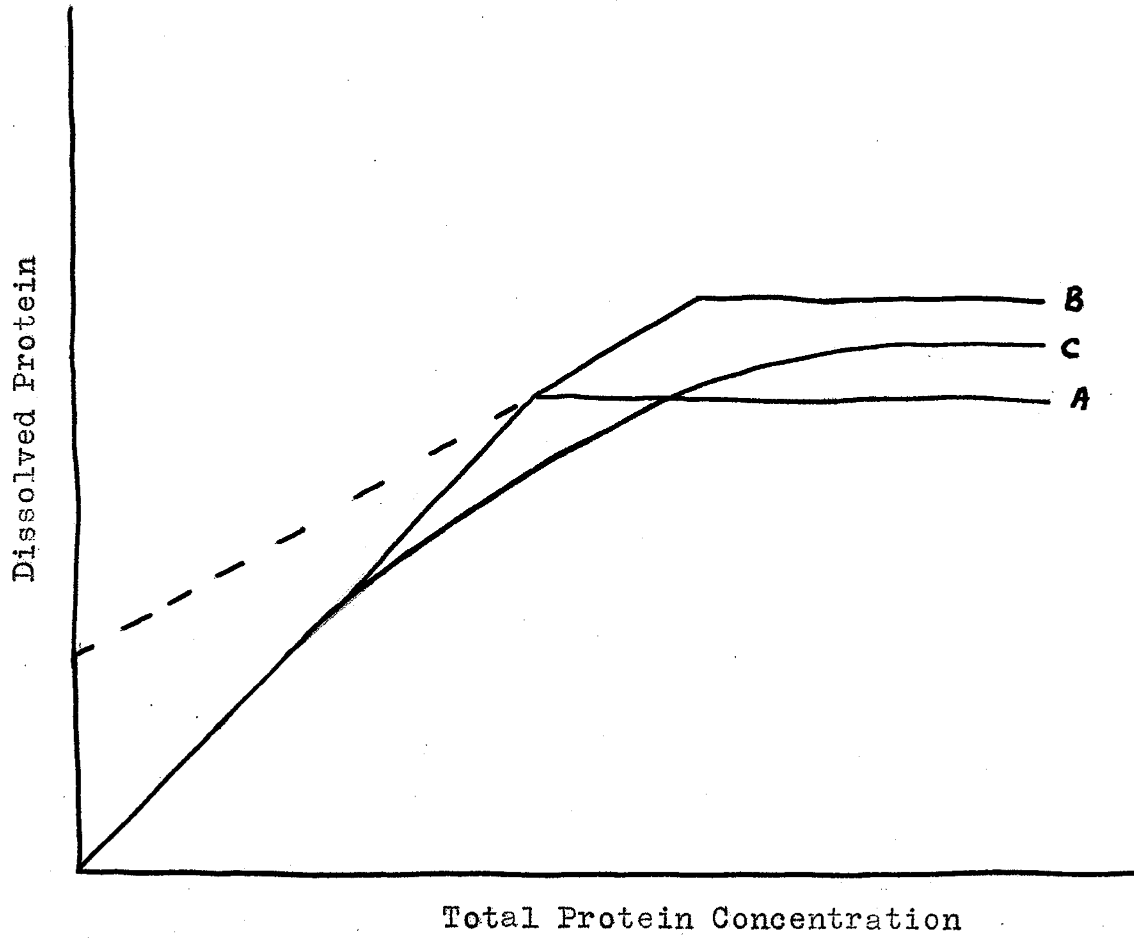

Figure 1.

Diagrammatic Solubility Curves (Bull) (11).

For a pure chemical compound, the solubility is independent of the amount of solid phase present. No solid phase remains undissolved until the break in the line is reached at which point no more of the added solid dissolves.

AMINO ACID COMPOSITION OF THE SUNFLOWER SEED PROTEIN

All protein molecules are known to consist primarily of amino acids linked together through the amino and carboxyl groups or through other reactive groups present in the amino acids concerned. Investigations in which hydrolytic methods were used provided the real clue to the structure of the proteins. These amino acids have been identified over a long period of time and new amino acids have been found in comparatively recent times. It follows that any analysis reported for sunflower seed or other protein must be judged in the light of the time it is reported. Some amino acids may not be reported because they were not known at the time the analysis was made--some may be reported inaccurately because an unknown acid is included with the one named.

The first indication that an amino acid was a primary decomposition product of proteins came from the work in 1810 of Braconnot (9), who performed an acid hydrolysis of meat and isolated and identified glycine. Earlier, in 1810, Wollaston had prepared cystine from urinary calculi, Proust, in 1818, found leucine was a unit of the protein molecule (21).

No further amino acids were discovered until 1846 when Liebig (21) isolated a crystalline substance from casein which had been hydrolysed by strong alkali. The compound was later identified as tyrosine. By hydrolyzing silk with sulfuric acid Cramer discovered serine. Kuhne (21) identified tyrosine and leucine as primary decomposition products of protein. Aspartic acid and glutamic acid, isolated from vegetable proteins by acid hydrolysis by Ritthausen (34), were added to the list. Schützenberger and Bourgeois isolated alanine from silk by barium hydroxide hydrolysis. Schulze and Barbieu isolated phenylalanine from the sap of germinated seed of Lupinus luteus, while in 1889, Drechsel identified lysine as a constituent of proteins (21).

From 1890-1900, three additional amino acids--arginine, histidine and iodogorgoic acid were reported (21). Within the next ten years, the discovery of hydroxytryptophane and the seven commonly occuring amino acids--tryptophane, proline, hydroxyproline, isoleucine, valine, cystine, and cysteine was announced (21).

In the following decade, six new members--norleucine, α-aminobutyric acid, dopa, 3,5-dibromotyrosine, citrulline and thyroxine were added to the list of known amino acids. Since 1920 additional amino acids, including methionine and threonine (21), were isolated from biological sources, and it is probable that other amino acids still remain to be discovered.

METHODS FOR AMINO ACID DETERMINATION

A quantitative determination of eighteen amino acids of the sunflower seed protein was undertaken to obtain a complete picture of its nutritive value. It was felt that the use of more modern methods involving newer reagents and techniques would be an appreciable contribution to the knowledge in this field. To accomplish this, the protein must be hydrolyzed into its constituent amino acids. Hydrolysis may be done by any of several different methods but not all are satisfactory. Strong mineral acids may be used but these destroy the tryptophane with the formation of humin. Alkalies give rapid and complete hydrolysis. However this procedure has not proved satisfactory, since racemization is believed to occur as well as deaminization of amino acids. It is used for special cases, as in the analysis for tryptophane which is destroyed by acid hydrolysis. Enzymes may also be used for hydrolysis but the process is slow and an equilibrium mixture may result.

The procedures for the quantitative study of the mixture obtained by the hydrolysis of proteins may be divided into two general groups: (a) methods for the identification of groups of constituents and (b) methods of isolation and quantitative estimation of individual amino acids.

The earliest group method was that of Hausmann (25) who proposed the characterization of a protein by distributing the

nitrogen content of its hydrolytic decomposition products into three groups : (a) ammonia nitrogen which was a measure of dicarboxy amino acids, (b) basic nitogen from the diamino acids, and (c) nonbasic nitrogen given by monoaminomonocarboxy acids and monoaminodicarboxy acids. Osborne (32) later modified the Hausmann procedure by the addition of a fourth fraction, humin nitrogen, which was considered a quantitative measure of the tryptophane content of a protein.

The Hausmann method with Osborne's modification does not indicate the presence or absence of any individual amino acid, with the possible exception of tryptophane. Many proteins have somewhat similar contents of basic and nonbasic amino acids; such proteins are more or less indistinguishable from each other by the Hausmann technique.

Van Slyke (41, 42, 43) determined amino nitrogen and the ratio between total nitrogen and free amino nitrogen in certain of the amino acids. Employing the Hausmann procedure also, he estimated quantitatively certain of the amino acids in proteins. His method required a relatively small amount of protein and permitted a more or less quantitative determination of arginine, histidine and lysine. However it does not give the composition of the group of monoaminomonocarboxylic and monoaminodicarboxylic acids. Also, "arginine nitrogen", "histidine nitrogen", and

"lysine nitrogen" so obtained do not reflect necessarily the true arginine, histidine, and lysine contents of the material since any compound which would decompose under the action of strong alkali to yield ammonia would be included in the calculations. The balance of the nonamino nitrogen, such as histidine nitrogen and the purines and pyrimidines and other nitrogenous substances, would distribute itself, if present, among the arginine, histidine and lysine fractions.

Methods for the isolation and identification of individual amino acids are also available. Fischer (18) based his ester method on the distillation of ethyl esters in vacuo without appreciable decomposition. The ester fractions are hydrolysed by boiling with water and the individual amino acids are separated by a process of fractional crystallization. The procedure is not completely satisfactory. Osborne and Jones (33) listed the following sources of error: (a) incomplete hydrolysis, (b) loss of amino acids due to humin formation, (c) incomplete esterification, (d) decomposition of the esters by hydrolysis prior to distillation, (e) unavoidable loss in separating the amino acids by fractional crystallization and (f) conversion of the amino acid esters into diketopiperazines.

Dakin (15, 16) employed a method of extraction of amino acids with n-butyl alcohol after hydrolysis. Those amino acids which are not readily obtainable in the crystalline form from aqueous solution will crystallize when extracted from the aqueous

solution with n-butyl alcohol.

In 1930, the Brazier-Schryver (10) copper-zinc method was introduced. This involves the hydrolysis of the protein and the conversion of the amino acids into their copper salts. Those of leucine, phenylalanine and aspartic acid remain as an insoluble residue in water. The copper salt of aspartic acid is changed to barium aspartate and those of phenylalanine and leucine are converted to zinc salts. From the barium and zinc salts, the free amino acids can be obtained. The copper salts which were soluble in water are separated from each other on the basis of their solubility in absolute methyl alcohol. However, the procedure must be adapted somewhat for each protein studied and the analysis of each new protein through the series of metallic salts must be regarded as a research problem.

Boyd (7, 8) noted that amino acids may be changed to uramino acids with potassium cyanate and then to hydantoins with hydrochloric acid. The advantages of the procedure are due to the characteristic melting point and crystallographic properties of the uramino and hydantoin derivatives since the amino acids from which they are derived may be characterized by the physical properties of the derivatives. The amino acids may be regenerated by hydrolysis of the derivatives but usually racemization occurs.

Kossel and Kutscher (27) suggested a method whereby

arginine, histidine and lysine could be extracted in pure form and determined quantitatively. These amino acids could be readily prepared but results are somewhat lower than for the analyses with Van Slyke's procedure.

Separation of amino acids from a mixture has also been attempted with electrodialysis, ion exchange, adsorption and partition.

Many chemical methods have been proposed and these have been compiled by Block and Bolling (5). Most of the determinations in this investigation were made by these methods which involved the development of color by appropriate reagents and comparison with the color given by a known amount of the pure amino acid.

Microbiological procedures have recently been employed for amino acid determinations, including those in the sunflower seed protein. Appropriate organisms are allowed to grow in a nutrient medium deficient in the amino acid in question. A sample of the substance under investigation for the amino acid is used as a source of that particular amino acid. The amount is determined by measuring the response of the organism in growth or in acid production and comparing it with those obtained with known amounts of the amino acid.

A comparatively new method is that of chromatographic

analysis which involves the use of a mixture of substances dissolved in a suitable solvent, slowly drawn through a tube containing the adsorbent and washed with the same or another solvent. Thus the various constituents of the mixture, according to the ease or difficulty with which they are adsorbed, are concentrated in various zones, the most readily adsorbed constituents in the upper zones and the less readily adsorbed in zones in the order of decreasing affinity for the adsorbent. If each constituent is a different color, measurement is comparatively easy. However, if they are colorless, a reagent must be employed which imparts a different color to each adsorbed layer. A modification of this type of analysis is filter paper chromatography.

AMINO ACID COMPOSITION OF THE SUNFLOWER SEED PROTEIN

The earliest experiments on amino acid analysis of sunflower seed protein were performed by Abderhalden and Reinbold (1) in 1905, using Fischer's ester method and results for eleven amino acids were given. Jones, Gersdoff and Moeller (26) in 1924 determined tryptophane and cystine colorimetrically while Blagoveschenskiĭ and Schubert (4) in 1934 gave results for five amino acids of the sunflower seed protein by the same method.

In 1945 Block and Bolling (6) reported results for twelve amino acids, nine of which were analysed colorimetrically and three--leucine, valine and isoleucine--microbiologically. The

next year, research workers at the University of Rochester, N. Y. (40), determined ten amino acids of the sunflower seed protein microbiologically. Slinger et al (36) in 1949, using microbiological methods, obtained data for lysine and methionine. All of these results are shown on the following pages.

	Abderhalden and Reinbold	Jones, Gersdoff, and Moeller	Blagoveschenskii and Schubert
Year Reported	1905	1924	1934
Arginine			9.1*
Histidine			14.29
Isoleucine			
Leucine	12.9		
Lysine			1.8
Methionine			0.8
Phenyl-alanine	4.0		
Threonine			
Tryptophane		2.54	
Valine	0.6		
Tyrosine	2.0		
Cystine	≁	1.56	
Proline	2.8		5.26
Glycine	2.5		
Alanine	4.5		
Serine	0.2		
Aspartic acid	3.2		
Glutamic acid	13.0		

*--Expressed as percent of total protein

	Block and Bolling	University of Rochester, N. Y.	Slinger et al
Year reported	1945	1946	1949
Arginine	8.2*	11.30	
Histidine	1.7	2.96	
Isoleucine	5.2	5.10	
Leucine	6.2	6.80	
Lysine	3.8	3.02	2.7
Methionine	3.4	3.35	1.3
Phenyl-alanine	5.7	4.96	
Threonine	4.0	3.41	
Tryptophane	1.3	2.38	
Valine	5.2	5.60	
Tyrosine	2.6		
Cystine	1.4		

*-Expressed as per cent of total protein

In this investigation both microbiological and colorimetric procedures were employed in analyses for eighteen amino acids.

EXPERIMENTAL

EXPERIMENTAL

Selection of Samples

Hulled sunflower seeds and sunflower seed meal were provided by Co-Operative Vegetable Oils Limited of Altona, Manitoba, from the 1951 and 1952 crops. These are referred to subsequently as 1951 Altona hulled seeds, 1951 Altona meal, 1952 Altona hulled seeds and 1952 Altona meal, respectively. Also, sunflower seeds from the 1952 crop were obtained from the Department of Soils of The University of Manitoba. This sample is designated as 1952 University hulled seeds. All the hulled samples were extracted with cold ether to remove the oil; meals were prepared from them by grinding in a Wiley mill. None of the samples used were from a designated variety of sunflowers--all were commercial mixtures.

Extraction of the Protein

All of the meals were analysed for total protein by the Kjeldahl method, using a factor of 6.25. The amounts of protein extracted by distilled water, 10 per cent sodium chloride, 1 N sodium hydroxide and--for one meal--70 per cent alcohol were determined by shaking 2.50 gram portions of meal with 100 mls. of solvent for 24 hours and determining the amount of protein in an aliquot of the filtrate. The results are shown in Table I. These tests were performed to give some indication of the kinds of proteins present as shown by classical solubility methods.

TABLE I

Extraction of Protein Using Various Solvents

	Water Soluble Protein %	10% NaCl Soluble Protein %	95% Alcohol Soluble Protein %	1 N NaOH Soluble Protein %	Total Protein N x 6.25
1951 Altona Meal	7.5	13.0	4.5	100.0	44.86
1951 Altona Hulled Seeds	14.4	28.6	---	100.0	36.44
1952 Altona Meal	10.2	15.7	---	100.0	43.86
1952 Altona Hulled Seeds	17.4	32.5	---	100.0	28.41
1952 University Hulled Seeds	14.8	26.9	---	100.0	30.52

FILTER PAPER ELECTROPHORESIS

Selection of Buffer

The extract of protein which is subjected to electrophoresis must be adequately buffered. The buffer determines the charge on the particles and this in turn regulates the mobility of the protein fractions. Obviously the buffer must have a pH which will not cause any chemical change of the protein and it must be one which will keep all of the protein in solution. The following fifteen buffer mixtures were tested:

1. KCl-HCl
2. Phthalate-HCl
3. Phthalate-NaOH
4. Potassium dihydrogen phosphate-NaOH
5. Boric acid-KCl-NaOH
6. Glycine-HCl
7. Glycine-NaOH
8. Sodium monohydrogen phosphate-potassium dihydrogen phosphate
9. Sodium borate-HCl
10. Sodium borate-NaOH
11. Sodium citrate-HCl
12. Sodium citrate-NaOH
13. Michaelis barbital
14. Acetic acid-sodium acetate
15. Disodium monohydrogen phosphate-citric acid

Of these only two, glycine-NaOH and sodium monohydrogen phosphate-potassium dihydrogen phosphate were satisfactory. Both were used in subsequent tests.

Filter Paper Selection

Four types of filter paper were tested, Whatman #1 and #100 and Schleicher and Schüll #595 and #598. The last type was found to be most satisfactory since it is pre-washed, whereas both Whatman papers and the Schleicher and Schüll #595 required a pretreatment in distilled water. The #598 paper was of such a thickness that more protein could be deposited on it. The size of the paper strips was 2 ½ " by 24", the electrical length being determined actually by the length of strip between the buffer dishes.

Design of Apparatus

The apparatus used for electrophoresis is shown in Figures 2 and 3. Previous investigations in this laboratory (2) showed that the Goa method, Figure 2, is unsatisfactory for the dry winter climate because of evaporation from the surface of the filter paper. However, the method of Cremer and Tiselius does not have this difficulty since the filter paper is immersed in the coolant, and it was selected for use in this study.

The Cremer type apparatus was tested using human blood serum in a barbital buffer at pH 8.4. The electrophoretic pattern for blood proteins is well known and served as a check

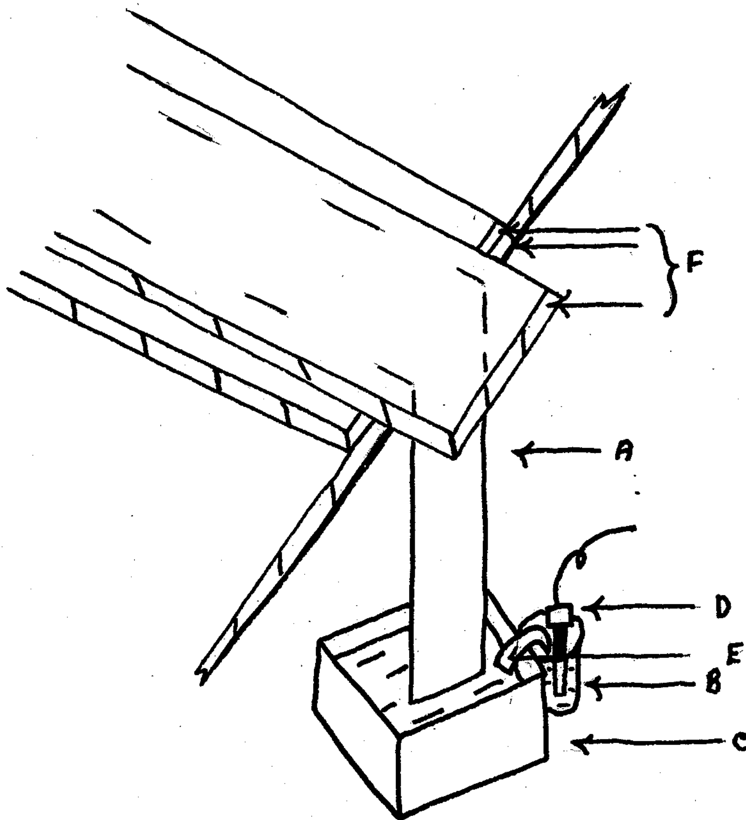

Figure 2.

The Goa Apparatus.

(A) filter paper strip, (B) beaker, (C) microscope slide dish, (D) electrode, (E) filter paper wick, (F) glass plates.

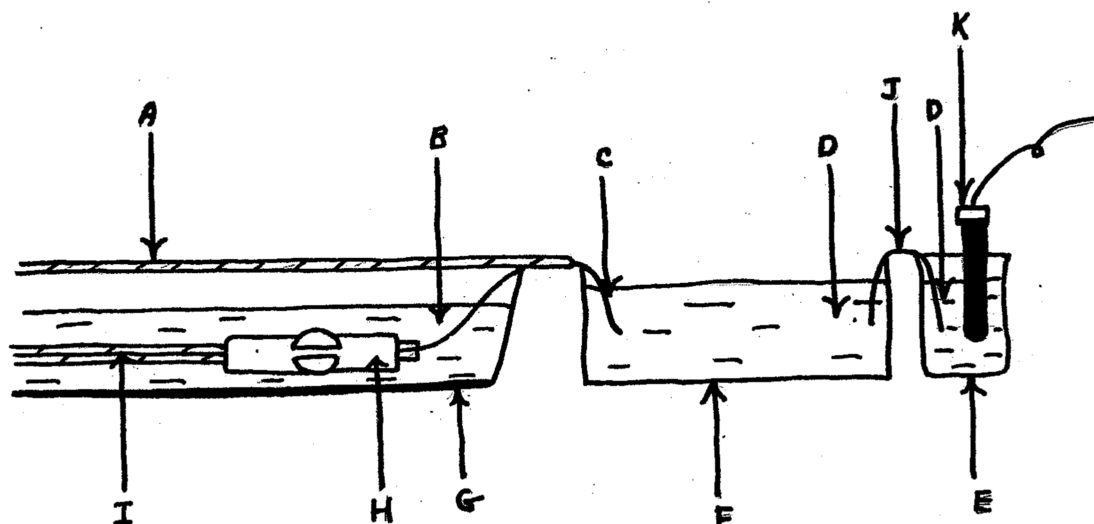

Figure 3.

The Cremer and Tiselius Apparatus.

(A) glass cover, (B) chlorobenzene, (C) filter paper strip, (D) buffer solution, (E) beaker, (F) microscope slide dish, (G) Pyrex utility dish, (H) "Bulldog" clip, (I) glass cover plates, (J) filter paper wick, (K) carbon electrode.

for the accuracy of the method. The results obtained were in agreement with those reported in the literature (19).

A view of the apparatus is shown in Figure 4 and a diagram of it in Figure 3. Carbon rods were used as electrodes and these dip into the buffer solution. To avoid interference of impurities which tend to separate from the carbon rods, the positive electrode is provided with an extra beaker and filter paper wick. The coolant dishes, Pyrex utility #231, are covered with a sheet of glass to prevent evaporation. The coolant used was reagent monochlorobenzene but technical grade is satisfactory. The buffer dishes into which the filter paper strips are dipped are microscope slide dishes. The glass sheets between which the filter paper strips are clamped are single diamond glass 3" by 7½". Four "Bulldog" clamps 2½" long are used to clamp the glass and filter paper together.

Electrical Supply.

A Regulated Power Supply, model 25, manufactured by Lambda Electronics Corporation, Corona, New York, U. S. A., capable of delivering 100 milliamperes at controlled voltages, was employed. The potential used was 140 volts on the electrodes. Using Schleicher and Schüll #598, paper, 2 milliamperes were drawn by each strip at pH 9.0. In theory fifty tests could be made at one time. However, only twelve tests were made in this investigation.

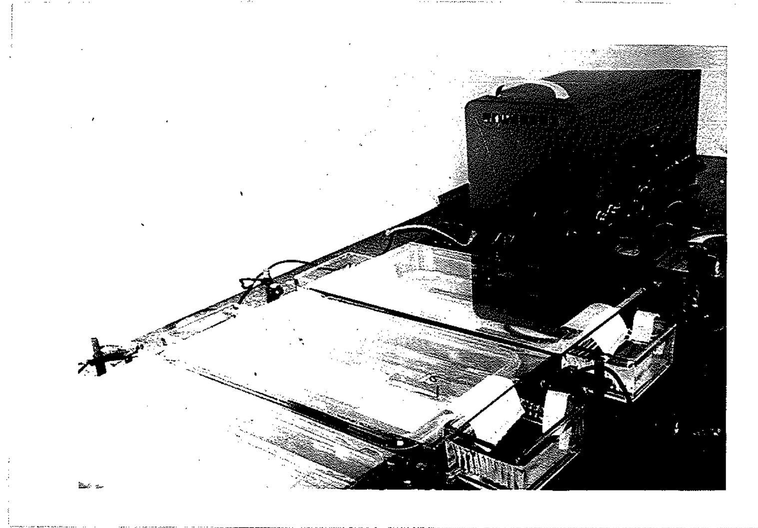

Figure 4.

View of Apparatus Showing Detail.

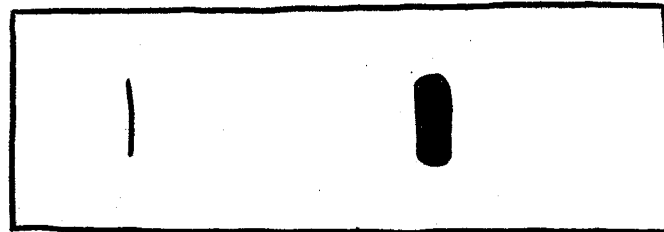

Figure 5.

Dyed Filter Paper Strip

after Electrophoresis.

Procedure of Electrophoresis

Using an Agla micrometer syringe, 0.1 ml. of the protein extract was applied in layers to the filter paper strip in a line one inch long perpendicular to the flow of current. Twelve strips were treated at the same time and were dried after each application. The dried strips were dipped into the appropriate buffer solution, laid on the glass plates, blotted, and covered with another glass plate. These were clamped together with four "Bulldog" clamps and placed in a coolant dish with the ends dipping into the buffer dishes. A potential of 140 volts was applied for twenty-two hours after which the strips were removed for processing.

Dyeing Procedure

The dye method employed was similar to that of Turba and Enenkel (39). The dye was prepared by adding glacial acetic acid to a mixture of 50 per cent ethyl alcohol and water to make the final concentration of acetic acid ten per cent. This solution was saturated with B. D. H. Azocarmine B and filtered. After fractionation, the filter paper strips were immersed in this solution for ten minutes, then washed in a ten per cent acetic acid bath for twenty minutes.

Electrophoresis of the Sunflower Seed Protein

The electrophoresis first attempted made use of 1 N NaOH as solvent. Buffers ranging from pH 1.2 to pH 13.1 were used. However, only a glycine-sodium hydroxide buffer and a disodium

phosphate-monopotassium phosphate buffer allowed movement of the protein. No separation was observed although the voltage and time were varied. It was believed that possibly the protein had been denatured by the high alkali content and the electrophoresis was repeated using the two buffer solutions both as solvent and buffer. Again, no separation of the sunflower seed protein resulted. Since this process was followed for the 1951 and 1952 meal samples prepared at the oil extraction plant, it was believed that possibly the heat involved in the oil extraction had denatured the protein, making it impossible to fractionate it. The work was repeated using whole sunflower seeds from which the oil was extracted by cold ether. However, similar results were obtained. A typical example of a dyed paper electrophoresis strip is shown in Figure 5. These results suggested that either the sunflower seed protein was composed of only one fraction, or that all the fractions were so similar that separation by filter paper electrophoresis was impossible. As a further step in this investigation, the same protein extracts were tested by the moving boundary electrophoresis method by Mr. P. A. Adie of the Defence Research Laboratory, Ralston, Alberta. He obtained similar results. Photographs of these are shown in Figures 6 and 7.

The approximate isoelectric point is the pH at which no movement of the protein from the point of its deposition resulted. This was determined to be 9.0-9.1. However, it does not take

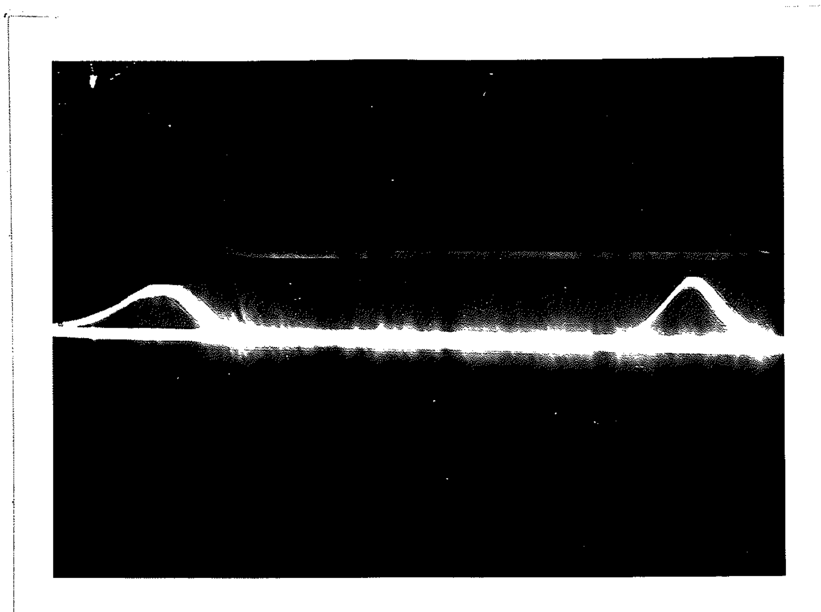

Figure 6.

Starting Positions of the Protein Boundaries.

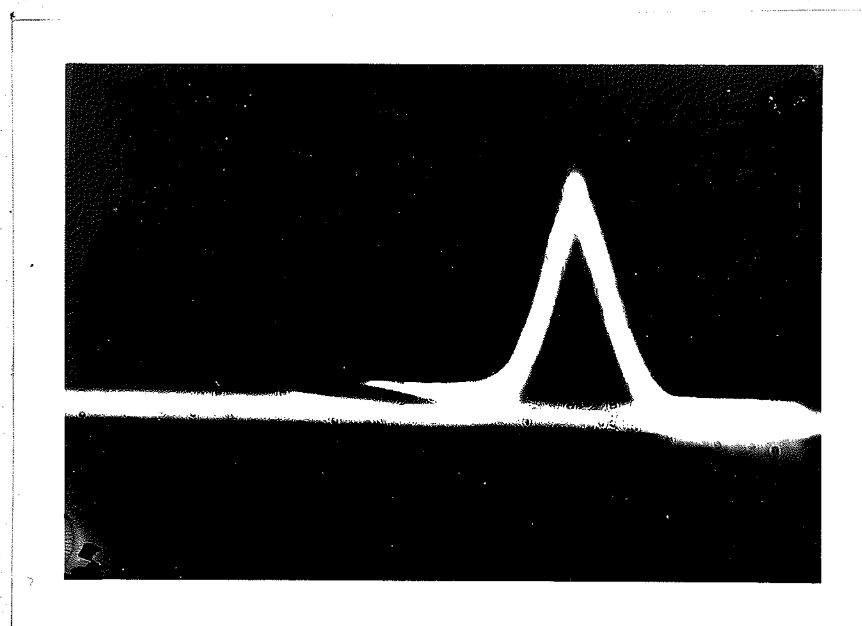

Figure 7.

Ascending Boundary at End
of Electrophoresis Test.

into account the electro-osmotic effect due to the many capillaries in the filter paper. Mr. Adie determined the isoelectric point to be near 8.98. In the moving boundary method which he used, the electro-osmotic effect is negligible.

THE SOLUBILITY TEST

As a further check, the solubility test for protein purity was performed. For this, the 1951 meal sample and a sodium borate-NaOH buffer mixture pH 9.1 were employed. This buffer solution can extract all of the protein in the sunflower seed meal. Varied quantities of meal were placed in a series of stoppered glass tubes, to which glass beads and the solvent were added. These were slowly shaken for twelve hours at room temperature in order that the solution could reach an equilibrium. The solutions were filtered and the percentage protein in each tube determined. The total protein used was plotted against total dissolved protein. These data are shown in Table II. The type of curve obtained, Figure 8, is the same as that given for a single pure substance and substantiates the earlier findings that the sunflower seed protein consists of only one fraction.

Samples of the pure protein were obtained by dialysing samples of borate buffer extractions against distilled water, and the solubility test again performed. The results as shown in Table III and Figure 9. again indicated only one fraction.

AMINO ACID DETERMINATIONS

The methods of the amino acid determinations and the results so found are listed in Table IV. The aminoacids selected were the ten essential ones and glycine--which is essential for poultry--and others for which suitable methods--either chemical or microbiological--were available.

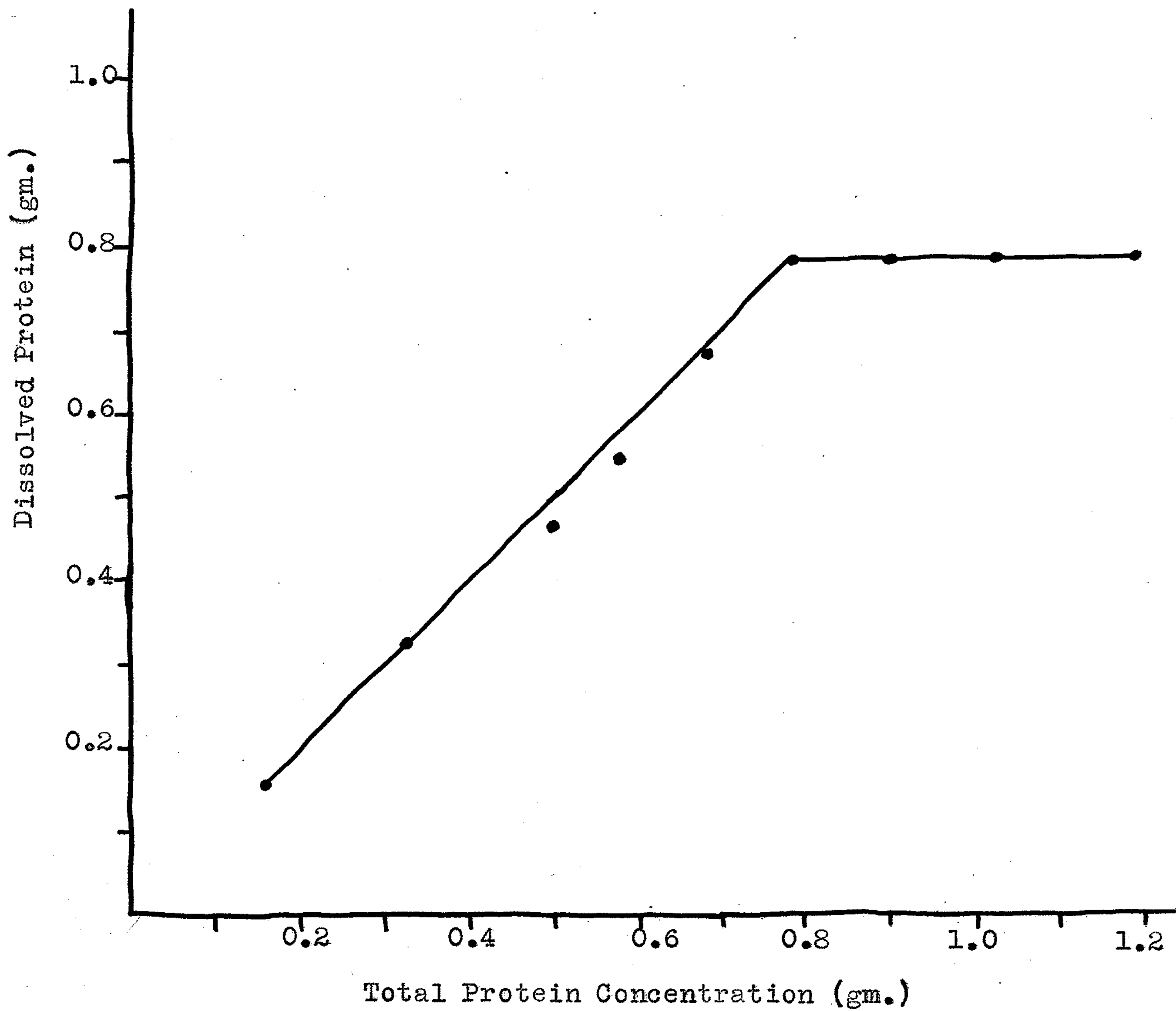

Figure 8.

Total Protein Concentration Plotted against Dissolved Protein using Sunflower Seed Meal.

TABLE II

Data for Solubility Test

Using Sunflower Seed Meal

Total Protein Concentration (gm.)	Dissolved Protein (gm.)
0.15	0.15
0.33	0.33
0.50	0.46
0.58	0.56
0.68	0.67
0.78	0.78
0.90	0.78
1.03	0.78
1.19	0.78

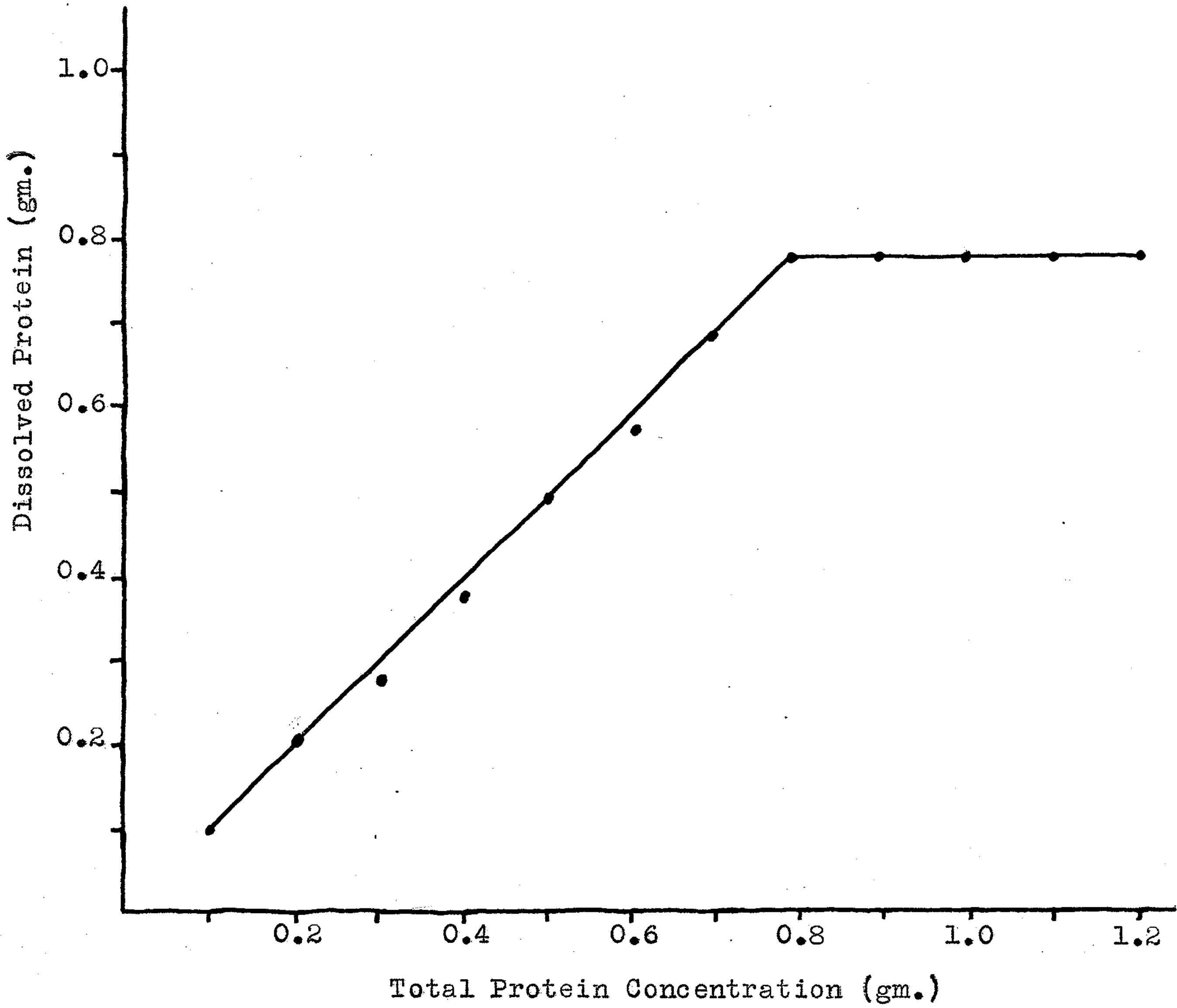

Figure 9.

Total Protein Concentration Plotted against Dissolved Protein Using Pure Sunflower Seed Protein.

TABLE III

Data for Solubility Test

Using Pure Sunflower Seed Protein

Total Protein Concentration (gm.)	Dissolved Protein (gm.)
0.10	0.10
0.20	0.20
0.30	0.28
0.40	0.38
0.50	0.50
0.60	0.59
0.70	0.70
0.78	0.78
0.90	0.78
1.00	0.78
1.10	0.78
1.20	0.78

TABLE IV

Determination of Eighteen Amino Acids of the Sunflower Seed Protein by the Method of Block and Bolling (5).

Amino Acid	1951 Altona Meal %	1951 Hulled Altona %	1952 Altona Meal %	1952 Hulled Altona %	1952 Hulled Manitoba %	Average %
Phenylalanine	5.58	5.54	5.56	5.58	5.54	5.56
Tyrosine	2.81	2.16	2.58	2.70	2.62	2.57
Tryptophane	1.42	1.01	1.19	1.21	1.29	1.22
Cystine	1.95	1.80	1.88	1.91	1.92	1.89
Methionine	3.70	3.47	3.64	3.66	3.64	3.62
Proline	5.61	5.28	5.09	5.60	5.19	5.35
Glycine	3.90	3.48	3.66	3.80	3.69	3.70
Arginine	9.08	8.96	9.00	9.04	9.02	9.02
Alanine	5.33	5.20	5.30	5.25	5.28	5.27

TABLE IV (continued)

Amino Acid	1951 Altona Meal %	1951 Hulled Altona %	1952 Altona Meal %	1952 Hulled Altona %	1952 Hulled Manitoba %	Average %
Histidine	1.80	1.77	1.87	1.82	1.81	1.81
Glutamic Acid	14.45	14.08	14.83	14.01	14.36	14.35
Isoleucine	5.41	5.26	5.38	5.30	5.39	5.35
Leucine	6.74	6.70	6.68	6.75	6.66	6.71
Valine	5.38	5.20	5.33	5.39	5.34	5.33
Lysine	3.30	3.22	3.37	3.43	3.35	3.33
Threonine	4.25	4.08	4.15	4.28	4.21	4.19
Aspartic Acid	4.46	4.02	4.26	4.55	4.33	4.35
Serine	0.32	0.19	0.25	0.30	0.25	0.26

DISCUSSION

DISCUSSION

Filter paper electrophoresis has been used successfully to separate blood sera (19) and other protein mixtures (2) into their constituent fractions. In the case of the blood sera, for example, six fractions have been identified, of which five are globulins and one is an albumin. Here, a filter paper strip shows six distinct colored portions after electrophoresis and dyeing. When the distance travelled by the various fractions is plotted against extinction, a curve with six peaks results. In our investigation, the dyed filter paper strip shows only one colored portion, as shown in Figure 5, after electrophoresis, and thus indicates only one protein fraction in the extract. When the macro method was used, the beginning ascending and descending boundaries before electrophoresis and the final ascending boundary after electrophoresis were photographed. These are reproduced in Figures 6 and 7. Only one peak appears in the curve. This confirms that there is only one protein fraction in the extract.

Solubility tests are also used as a test for protein purity. As shown in Figure 1, when the dissolved protein is plotted against total protein present, line A denotes the graph for a pure substance and line B that for a mixture of substances. Results for the sunflower seed protein as recorded in Figures 8 and 9 show a curve of the type given by a pure substance.

From this it may be concluded to be a single chemical substance.

An alternate suggestion may be that the sunflower seed protein is composed of fractions so similar to one another in electrophoretic properties and solubility that it would be almost impossible to separate them by these methods. However, it does not seem likely that all fractions--if they exist--would be identical in such varied properties. One is justified in concluding that the protein of the sunflower seed which is extracted by glycine-sodium hydroxide and sodium borate-sodium hydroxide buffers --and this includes all of the protein in the sunflower seed--is a single chemical entity.

In Table IV appear the results of analyses of the sunflower seed protein for eighteen different amino acids. For each amino acid are shown individual results for the five samples and the average for all five. The analyses were made with the meal but were calculated and are reported as per cent of the protein in the meal.

There is some variation in the amounts of any one amino acid in different samples. The range is from 0.04 per cent for phenylalanine to 0.77 per cent for glutamic acid. The amount of variations is less than 0.25 per cent for two-thirds of the amino acids. Each figure is the average for four or five replicate samples and these checked within 0.20 per cent. Therefore we conclude that the variations encountered are not due to

experimental error but are normal variations in the amino acid content of the protein concerned.

On pages 20 and 21 are shown the results of analyses reported in the literature. On comparison, our results showed close agreement with those of Block and Bolling (6) for phenylalanine, tyrosine, tryptophane, histidine, cystine and threonine but differed from those of the other workers. Their results for phenylalanine, tyrosine, cystine and threonine are somewhat lower than ours, while those for tryptophane are approximately twice as high and vary from one and one-half to seven times as high for histidine. The data for methionine, isoleucine, valine, lysine and leucine agree with those of Block and Bolling (6) and workers at the University of Rochester, N. Y. (40) but not with those of earlier investigators whose results for isoleucine and lysine are slightly lower, the amount of methionine one-quarter to one-third, the figure for valine one-ninth and the result for leucine approximately twice that found by us. The amounts of proline and arginine are very similar to those given by Blagoveschenskiĭ and Schubert (4) but differ from the results of other workers. Our result is approximately fifty per cent higher than the other one reported in the literature while the amount of arginine found by these other investigators are from 0.8 per cent less to 2.3 per cent more than we found. The result for serine agrees closely with that of Abderhalden and Reinbold (1) which is the only other figure reported for this amino acid. Results for the others--

aspartic acid, glutamic acid, alanine and glycine--have been reported only in 1905 by Abderhalden and Reinbold (1). The amounts as determined in our investigations are all somewhat higher than those found in this earlier study. This may be due to more accurate methods of analysis or to differences in the varieties of the sunflower or both.

The results for the essential amino acids and glycine may be compared with beef muscle protein, which is considered a typical complete protein from the nutritional point of view. The sunflower seed protein is found to be slightly richer in phenylalanine, methionine, and valine and approximately two per cent higher in arginine. However, it is slightly lower in tryptophane, approximately one percent less in glycine, histidine, isoleucine, leucine and threonine and almost five per cent lower in lysine than beef muscle protein. This deficiency in lysine is probably its most serious nutritional deficiency. In this characteristic it resembles most other plant protein.

SUMMARY

SUMMARY

1. Extracts of sunflower seed meal from three different lots of seeds were subjected to filter paper electrophoresis. No separation of the protein into fractions occurred.

2. No separation of the protein of the extract into fractions was obtained when the macro method was employed.

3. Solubility tests on the sunflower seed meal and on the pure sunflower seed protein also indicate there is only one protein fraction.

4. The sunflower seed meal was analysed for eighteen amino acids. The results show the protein has the following composition: alanine 5.27 per cent, arginine 9.02 per cent, aspartic acid 4.35 per cent, cystine 1.89 per cent, glutamic acid 14.35 per cent, glycine 3.70 per cent, histidine 1.81 per cent, isoleucine 5.35 per cent, leucine 6.71 per cent, lysine 3.33 per cent, methionine 3.62 per cent, phenylalanine 5.56 per cent, proline 5.35 per cent, serine 0.26 per cent, threonine 4.19 per cent, tryptophane 1.22 per cent, tyrosine 2.57 per cent and valine 5.33 per cent.

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