SOME PHYSICAL PROPERTIES OF PROTEINS

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

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This is to certify that the undersigned have read and recommended to the Committee on Graduate Studies for acceptance, a thesis submitted by W. B. McConnell, B.Sc. entitled

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GENERAL INTRODUCTION

Proteins belong to a group of the most complex of chemical substances. They are characterized by the fact that upon hydrolysis they yield a mixture of as many as twenty-six α amino acids, and are known to have molecular weights ranging from 16,000 and upwards.

The structural pattern of protein molecules is not known in detail. Certain general features of their structure can be regarded as established and much is known about the relative amounts of the different amino acids contained in the molecule. The greatest problems in the structural chemistry of proteins are, however, still unsolved.

Proteins are highly specialized molecules that can be formed only by living cells. The general elements in the fundamental pattern of structure are capable of wide variation in order to adapt them to their biological function. They vary greatly in size, shape and chemical composition.

The proteins elaborated by plants are up to the present not so well known as those from animals. This is

probably due to the fact that many animal proteins are readily obtained in a relatively pure state and are more distinctive in properties and function. The medical profession has naturally been instrumental in much of the fundamental investigation related to animal proteins.

The study of the physical chemistry of hemoglobin is an excellent example of the relation between the chemistry of a protein and its specialized function in the living organism. In the chemistry of plant proteins there is a very large field which as yet is largely untouched. As plant proteins become better known we may confidently expect to increase our understanding of their functions and special adaptations. Investigations along this line will lead ultimately to the discovery of underlying relations fundamental both to the science of chemistry and to botany.

The measurement of osmotic pressure has been used as a means of estimating the molecular weights of polymers and has been successfully adapted to use with protein solutions.

It is with this approach to the measurement of the molecular weight of proteins that the work presented herein is largely concerned.

FUNDAMENTAL THEORY

When a solution is separated from the pure solvent by a membrane which is perfectly permeable to the solvent but will allow no passage of the solute, the solvent will tend to pass into the solution. A membrane as described above is termed a perfect semi-permeable membrane. The theoretical development following is dependent upon the use of such a membrane.

Consider two bags composed of semi-permeable material: (Fig. 1). The bags, one containing a solution and the other containing the solvent, are suspended in a closed chamber.

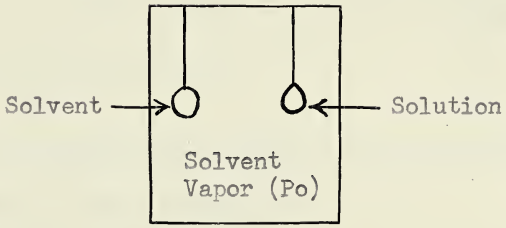


Figure 1.

The pure solvent will tend to vaporize until the pressure of solvent vapor in the chamber is identical with the vapor pressure of pure solvent at the temperature of the apparatus. The vapor pressure of the solution, however, is

lower than that of the solvent, so that a point will be reached at which the solvent vapor will tend to condense into the solution. There will effectively be a distillation of solvent from pure solvent to solution. As this process continues there will be a hydrostatic pressure built up inside the bag containing the solution due to the additional liquid which has entered. A time will be reached when the increased pressure on the solution has raised its vapor pressure to the same magnitude as that of the solvent. There will no longer be any net transfer of material and the system will be in equilibrium.

By considering the thermodynamics of this equilibrium system, it is possible to derive a relation between osmotic pressure and molecular weight.

The work required to transfer one gram molecular weight of the solvent to the solution is

$$P_h \bar{V}_1$$

where:

P_h = hydrostatic pressure inside the sac.

$\bar{V}_1$ = partial molar volume of solvent.

If we consider the transfer to be carried out reversibly at constant temperature and pressure, the work done is equal to the free energy change. During this change we have changed

one mole of solvent from a vapor pressure of P_o to a vapor pressure of P_1. The free energy of this process is

$$RT \ln \frac{P_o}{P}$$

where

P_o = Vapor pressure of solvent

P = Vapor pressure of solution

Equating the two expressions for free energy we have

$$\Delta F = P_h \bar{V}_1 = RT \ln \frac{P_o}{P} \qquad (1)$$

In order to eliminate the term $\frac{P_o}{P}$ from equations (1) we may use Raoult's law. This law states that for dilute solutions

$$\frac{P_o - P}{P_o} = \frac{n_2}{n_1 + n_2}$$

where

n_1 = moles of solvent present

n_2 = moles of solute present

Raoult's equation may be rearranged to give

$$\frac{P}{P_o} = \frac{n_1}{n_1 + n_2} \qquad (2)$$

Substitution in equation (1) gives

$$P_h \bar{V}_1 = -RT \ln \frac{n_1}{n_1 + n_2}$$

and

$$P_h \bar{V}_1 = - RT \ln \left(1 - \frac{n_2}{n_1 + n_2}\right)$$

For dilute solutions n_2 is very small compared to n_1 and so the natural logarithm is very nearly equal to $-\frac{n_2}{n_1+n_2}$

Therefore

$$P_h\bar{V}_1 = RT\ \frac{n_2}{n_1+n_2} \qquad (3)$$

Osmotic pressure is defined as the additional pressure which must be applied to the solution in order to prevent a transfer of material across the membrane[16]. P_h is identical with osmotic pressure (P_{op}) and equation (3) can be written in the form

$$P_{op} = \frac{RT}{\bar{V}_1} \cdot \frac{n_2}{n_1+n_2} \qquad (4)$$

$\bar{V}_1$ is very nearly equal to $\frac{M_1}{\rho_1}$

where M_1 = Molecular weight of solvent

ρ_1 = Density of solvent

For dilute solutions n_2 is very small compared to n_1 and (n_1+n_2) is very nearly equal to n_1.

Equation (4) may now be rearranged to give

$$P_{op} = \rho_1 \frac{RT}{M_1} \cdot \frac{n_2}{n_1} \qquad (5)$$

$M_1 n_1$ = total weight of solvent present (w_1)

n_2 = number of moles of solute dissolved in w_1 grams of solvent

If we let w_1 be equal to 100 grams solvent and C be the mass solute dissolved in 100 grams solvent then:

$$n_2 = \frac{C}{M_2}$$

where

M_2 = the molecular weight of solute

By substituting for M_1 n_1 and for N_2 equation (5) becomes

$$P_{op} = \frac{\rho_1 RTC}{100M_2}$$

If osmotic pressure is expressed in centimeters of water R has the value

$$R = 22{,}414 \times 76 \times 13.597$$

$$= 8.48 \times 10^4 \text{ c.c. cm. } H_2O \text{ per degree}$$

where

22,414 is molecular volume at 76.0 cm. of mercury pressure and 13.597 is density of mercury at 0°C. Using the value 0.9970 as density of water at 25°, equation (6) may be written as a working equation for the special case where temperature is 25°C.

$$P_{op} = \frac{0.9970 \times 8.48 \times 10^4 \times 278}{100} \cdot \frac{C}{M_2}$$

$$P_{op} = 2.525 \times 10^5 \cdot \frac{C}{M_2} \qquad (7)$$

or

$$M_2 = \frac{2.525 \times 10^5 \times C}{P_{op}} \qquad (8)$$

Equation (8) is a simple expression linking osmotic

pressure and molecular weight. Concentration may be readily determined and since the osmotic pressure of materials such as proteins is of a convenient magnitude for laboratory measurement, this method has been frequently employed for estimating molecular weight.

Donnan Equilibrium

A factor which must be considered in an attempt to use osmotic pressure measurements to estimate molecular weight is that of a possible uneven distribution of buffer salts due to Donnan equilibrium.

Bull[6] gives a theoretical discussion of this effect in relation to the study of osmotic pressure of proteins and gives the following equation.

$$\frac{[NaCl]_o}{[NaCl]_i} = 1 + \frac{C_1}{C_2} \qquad (9)$$

where

$[NaCl]_o$ = equilibrium salt concentration outside

$[NaCl]_i$ = equilibrium salt concentration in protein solution

C_1 = equivalent concentration protein ions

C_2 = initial concentration of salt outside

Examination of equation (9) reveals that if the

concentration of charged protein particles is finite, there will be a greater concentration of salt outside than there is inside. It is also evident that as the equivalent concentration of salt becomes large compared to that of the protein, the right hand side of the equation approaches unity and the relative salt concentration gradient tends to disappear.

If the protein is at its isoelectric point, it carries no net charge and does not give rise to an uneven salt distribution across the membrane with its consequent disturbing influence on osmotic pressure. It is therefore, common practice to make osmotic pressure measurements on proteins at their isoelectric points, and even in such cases[5] electrolyte is usually added to depress any possible Donnan equilibrium effects.

Wagner[39] has pointed out that osmotic pressure data obtained at the isoelectric point is often poor even in the presence of excess salts. He suggests that this is due to the fact that at this point the conditions of minimum solubility and maximum aggregation occur. He recommends that the hydrogen ion activity of the solution be buffered at a point about one pH unit from the isoelectric point of the protein and that a sufficient concentration of buffer be provided to reduce the osmotic pressure contribution due to diffusible ion to a small

value which, if significant, can be applied as a calculated correction.

SEMI PERMEABLE MEMBRANE

The successful measurement of osmotic pressure depends largely upon the performance of the membrane used in the determination. The ideal membrane, aside from the obviously necessary condition of semi-permeability, should have as high a rate of solvent transference as possible. The material should be such that it can be obtained or produced with a uniform and reproducible fine structure.

The actual cause of semi-permeability is even at present somewhat obscure and a number of theories have been advanced regarding it. The earliest suggestion regarding the origin of semi-permeability was made by M. Traube in 1867, who thought such membranes acted as sieves, retaining the larger molecules but allowing the passage of the smaller ones. It is doubtful whether the sieve theory accounts completely for the behavior of these membranes. It has been stated for example, that membranes with pores many times larger in diameter than solute molecules are able to prevent the passage of the latter.

A number of experiments have lead to the suggestion

that semipermeability occurs when the solvent is soluble in the membrane material but the solute is not[17]. A simple experiment, which illustrates this point of view is to layer chloroform, water, and ether in a cylinder in the order given. The ether being soluble in water to an appreciable extent is able to pass through the water and down into the layer of chloroform. The chloroform owing to its insolubility in water is unable to reach the ether layer. The water is thus acting as a semipermeable membrane. A similar effect (Imitation of osmosis) occurs when an animal membrane well soaked in water is used to separate ether and benzene.

It is not easy to understand how the above theory could be used to explain the passage of water through collodion as is the case with the osmotic pressure determinations being presented herein. Collodion and water appear to be highly insoluble in each other. In recent years the tendency has been to regard semipermeability as an absorption phenomenon. According to this view a membrane is supposed to be permeable to the molecules it absorbs positively but impermeable to those which are negatively absorbed.[37]

This view, nevertheless, regards the pore size to be of considerable importance as the channels must be small

enough so that the materials therein will be under complete control of surface forces.

There is probably no single theory which is capable of explaining the behavior of all types of membranes.

Bull[6] elects to divide artificial semipermeable membranes into two classes

(1) Sieve membranes, such as cellophane and collodion.

(2) Homogeneous membranes such as would occur when an oil phase separates two water phases.

Pore Diameter.

The term pore diameter is frequently employed when describing membranes. This term can be very misleading because it is quite likely that one is dealing with holes and cracks that have a great variation as to size, shape, and pathway through the material. In spite of this the "Average Pore Diameter" is determined. These measurements serve to characterize membranes and are useful in making qualitative estimates of their performance. It would be dangerous to interpret such results as absolute physical measurements.

A calculation of average pore diameter involves

Poiseuille's law of flow through circular capillaries. The method requires the use of some rather questionable assumptions.[6] Erbe[6] has developed a method for measuring the pore size distribution in a membrane, although the method also involves many uncertainties, all studies on this distribution show the membranes to have a fairly wide range of pore diameters. The distribution follows that of a probability curve.

Electrical Properties of Sieve Membranes

Michaelis[29] observed that collodion membranes, which had been well dried so as to reduce them to very low porosity showed a differential permeability for cations and anions. The cations were able to penetrate at a greater rate than the anions. When potassium chloride solutions of different concentrations were placed on two sides of the membrane, a potential difference was observed across the membrane. With the more dense membranes at 25°C this potential approached the theoretical limit of 59 millivolts for a concentration gradient of 1 to 10 across the membrane. These membrane potentials were maintained for several months. Sollner and Abrams[35] reinvestigated this problem and concluded that this differential permeability to ions shown by dense membranes is due to acid groups in the

collodion. They found it impossible to develop such potentials with the purer grades of collodion on the market. If, however, the collodion was oxidized with sodium hypobromite (NaBrO), it acquired the electrical properties described. Sollner argued that the electrical behavior of such membranes must be due to the presence of carboxyl groups in the collodion which would attract the cations and allow them to pass through. The negatively charged anions would of course be repelled and could not pass the membrane.

Differential ion permeability leads to what is known as anomalous osmosis. It has been found that under proper conditions water will flow through the membrane from a concentrated to a dilute solution. After a time, however, the forces causing this flow decrease and eventually concentration becomes identical on both sides. Anomalous osmosis is a kinetic rather than an equilibrium condition. The conditions giving rise to these effects are briefly outlined.

Negative Anomalous Osmosis.

The term negative anomalous osmosis is used to describe the motion of water from the concentrated to the dilute solution. It will occur when the ion which has a charge of the same sign as the membrane has the greater mobility through the

membrane. In order for this condition to arise, the pores of the membrane must be quite large and an electrolyte must be used which gives ions with considerable difference in mobility.

A good example of this phenomenon is presented by the flow of lithium chloride through a magnesium silicate membrane. The chloride ion has a much greater mobility than the lithium ion and in spite of its negative charge passes through the negative membrane at the greater rate. The dilute side of the membrane thus becomes negative with respect to the concentrated side. Water molecules being positively charged in the negative pores are drawn from the more concentrated to the dilute solution by electrosmosis.

Positive Anomalous Osmosis.

This term describes an accelerated flow of water from the dilute solution to the concentrated solution. It occurs when the membrane pores are very small. In such a situation only ions with opposite charges to the membrane would be expected to penetrate it freely. The other ions would tend to be repelled and the more concentrated solution would acquire a charge of the same sign as the membrane. The water in the membrane would consequently have a charge opposite to the more concentrated solution and would pass into it with increased velocity.

The osmotic pressure measurements on egg albumin to be presented herein were carried out in a solution of sodium acetate-acetic acid buffer. Collodion membranes were used. It would be of interest to consider the probable osmotic effects which would arise out of this situation.

Consider the collodion membrane to be negatively charged due to the presence of an undetermined number of acid groups ($R-\overset{O}{\overset{\|}{C}}-O^-$).

If the membrane pores are very large the ion with the greatest mobility may be expected to penetrate the membrane at the greatest rate.

The mobilities for the ions involved at a temperature of 25°C are:[40]

Na^+ - 50.9 ohms^{-1}

H^+ - 350 "

$C_2H_3O_2$ - 40.8 "

The positive ions have the greater mobility. They would penetrate the membrane faster and the conditions for positive anomalous osmosis, as described above, would be met.

If the pore size is small only positive anomalous osmosis can occur.

In both cases then, one may expect the system to show an initial rapid transfer of water from the dilute to the more concentrated buffer solution. The magnitude of this effect would depend upon the relative concentration of buffer salts in the inside and the outside solutions.

Summarizing, one can say that anomalous osmosis arises out of the electrical properties of the membrane and is a kinetic phenomenon. It is undoubtedly of more importance in considering the time in which equilibrium is reached and probably has no effect upon the actual condition of equilibrium.

Membrane Materials.

The most common materials used for osmotic pressure measurements are collodion and cellophane. Although other substances have been suggested[28, 18] the above are preferred due to the fact that they are easily obtained and can be prepared with a wide range of porosity.

McBain and Stuewer[27] discuss the properties of cellophane. They point out that commercial sheet cellulose as now manufactured is much less porous than formerly. They describe a method of swelling the membrane in 63% zinc chloride solution in order to make it freely permeable to all electrolytes. Flory[13] describes cellophane membranes which were found

satisfactory for osmotic pressure measurements on solutions of the polyisobutanes.

The membrane material used in practically all osmotic pressure measurements to be reported here was collodion. Collodion is a solution of nitro-cellulose in an ether-alcohol mixture. It is very convenient for osmotic pressure work because it can be readily formed into a membrane of the desired shape and size.

The permeability of collodion membranes may be varied in a number of ways. A few suggestions for controlling porosity are listed below:

(1) The higher the concentration of alcohol in the solvent the greater the permeability. The usual ratio is 25 volumes of alcohol and 75 volumes of ether.

(2) Elford[11] finds that in general the addition of good solvents for nitrocellulose decreases membrane porosity and addition of non-solvents increases the porosity. Amyl alcohol for example decreases porosity while acetone increases it.

(3) Collodion membrane porosity can be varied by soaking it in different concentrations of alcohol for twenty-four hours and then washing with water.

(4) The extent to which the collodion is dried before

washing in water is also significant in relation to the porosity of the membrane. The longer the period of drying the smaller the permeability.

These indications of the possible means of varying collodion together with the fact that commercially prepared solutions seem to vary considerably in their inherent properties explains why there are so many different variations in the recommended procedure for membrane manufacture. Bull[5] used Merck U.S.P. collodions and cast the membranes on the inside of a small test tube. These were gently rotated until the material had set in a uniform film and were then dried under vacuum for fifteen minutes. Each bag had two coats of collodion so applied. Adair[1] says a good membrane should be rigid and be capable of standing two atmospheres pressure unsupported. He prepared his membranes by putting from five to seven coats of collodion on the outside of a glass mould. Each coat was dried by rotating the mould before a gas fire and the finished membrane was removed by forcing water under pressure through a small hole in the bottom of the tube. Adair emphasizes the importance of the operator's technique and comments that after several years of practice the proportion of failures was below 10%. Marrack and Hewitt[25] when making osmotic pressure determinations on haemoglobin prepared collodion sacs using 8%

solution of collodion (British Drug House) in a mixture of alcohol, ether, and glacial acetic acid in the ratio of 5:5:1.

Some workers have described methods of preparing membranes of any desired porosity. Such methods involve a systematic changing of one or more of the controlling factors mentioned above. The work of Elford[11] and Pierce[34] can be referred to in this connection. Other authors giving information on the preparation of membranes are Montanna and Jilk[30], Ferry[12] and Flory[13].

METHODS FOR MEASURING OSMOTIC PRESSURES

Osmometers may be divided into two classes:

(1) The Static Elevation Cell

(2) The Dynamic Equilibrium Cell

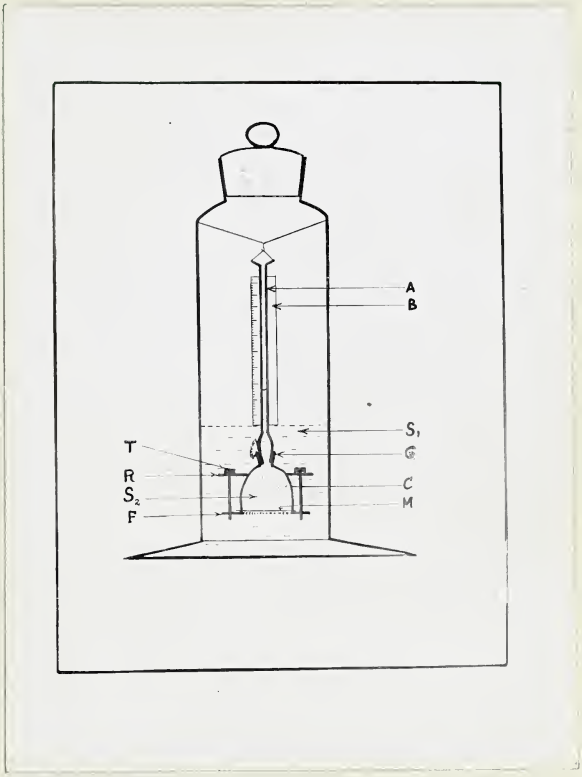
In the static elevation osmometer the osmotic pressure of the solution is balanced by the flow of solvent into the solution. This type of cell has the advantage of being relatively compact, inexpensive to construct, and simple to assemble and operate. The chief disadvantage is the length of time required for the attainment of equilibrium.

The operation of a static elevation osmometer is illustrated by the modified Schultz osmometer shown in

Figure 2. The membrane is fastened across the mouth of the glass chamber C by covering it with the perforated plate F which is then clamped in position by means of the metal ring R and the bolts T. The chamber C is filled with solution S_2 and the tube A fastened into the ground glass joint G in such a manner as to exclude all air. This assembly is suspended in a cylinder so that the lower portion is immersed in the solvent S_1. As the solvent passes into the solution, the liquid level in the tube A rises. When the pressure due to the column of liquid in the tube balances the osmotic pressure the level remains constant and can be read from scale B. The height of this column corrected for any capillary forces acting in the tube A is a direct measure of osmotic pressure.

Bourdillon[4] has described an osmometer better suited to the measurement of low osmotic pressures, especially aqueous solutions for which it was intended. Its design and operation are such as to avoid the necessity for any correction for density and capillarity of the solution. A very significant feature of this instrument is the small sample of solution required to carry out a determination (0.2 ml.)

Other workers who have used the static elevation cell are Adair[1], Marrack and Hewitt[25,26] and Gee[15].



MODIFIED SCHULTZ OSMOMETER

Figure 2

The dynamic equilibrium cell is designed so that the osmotic pressure can be counterbalanced by an external applied pressure of known magnitude. The external pressure required to prevent flow of solvent into or out of the solution is equivalent to the osmotic pressure of the solution. This method is advantageous where a rapid determination of the pressure is essential. Unfortunately this cell is more elaborate in design and requires considerable experience to fill and operate successfully. The time saving features would not be so pronounced in the measurement of buffered protein solutions which require buffer distribution to reach equilibrium before the osmotic pressure registered can be attributed to protein alone.

The dynamic equilibrium cell has been used by Flory[13] and Montanna and Jilk[30]. One of the best cells of this type was designed by Fouss and Mead[14].

A rather unique method for measurement of osmotic pressure is the porous disc method, first used by Townsend in 1928[38]. This apparatus employs the air-solution interface as the semi-permeable membrane.

OSMOTIC PRESSURE OF PROTEINS

Osmotic pressure measurements have been used primarily for estimating the weight of large molecules. The application of the technique to protein solutions introduces some special factors which will be discussed here together with a brief consideration of the results of some of the workers in this field.

(1) The concentration of a protein solution is often determined by a residue weight method. The protein in solution usually has associated with it considerable water of hydration (egg albumin estimated at 36%). The implications of this fact are:

1. The molecular weight obtained is that of the dry protein molecule rather than its effective molecular weight in solution.

2. During the determination of concentration the water of hydration is lost along with the water effective as solvent. Unless the extent of hydration is known a small error is introduced in concentration, expressed as weight of protein in unit weight of solvent.

(2) Proteins are amphoteric. In solutions acid to their

isoelectric point they behave as positive ions and in solutions basic to their isoelectric point they act as negative ions. Protein molecules can exist as neutral particles only if the solution has a particular pH. The significance of the charge on the protein molecule has been referred to in the section on Donnan Equilibrium.

(3) The experimenter working with proteins is quite restricted in his choice of solvents. With the exception of the prolamins and gliadins they are almost universally insoluble in organic solvents. A few dissolve in pure water but many require a special concentration of salt, acids or alkali.

Discussion of Literature.

Bull[5] in 1940 undertook to use osmotic pressure measurements to eliminate some of the uncertainty regarding the molecular weight of egg albumin. He presents a table showing the various values for the molecular weight that has been reported, and describes a new and simple apparatus for making the measurements. A modification of Bull's apparatus and procedure was used in carrying out the work presented herein and it will be described in some detail in a subsequent section.

Bull obtained a value of 45,160 for the molecular

weight of egg albumin. His results showed a standard deviation from the mean of 180.

In 1946, Bull and Currie[7] used a similar method for the estimation of the molecular weight of β - lactoglobulin. They report a value of 35,050.

Adair[1,2,3] presented a critical study of the direct measurement of osmotic pressure of haemoglobin. He points out that haemoglobin may be regarded as a colloidal system and is consequently somewhat unstable. The experimenter is in the difficult position of having to choose between two evils. If an extended period of time is allowed for the system to reach equilibrium there is a possible chemical change in the material under consideration and a consequent erroneous conclusion. On the other hand if approximate equilibrium is accepted at a shorter period of time, the results can never have the finality which alone would justify so difficult a method.

Adair gives a survey of the possible sources of error in osmotic pressure measurements and suggests the following criteria for true equilibrium.

(1) Steadiness. Adair made his measurements at 0°C and required that levels remain constant for long periods of time (several weeks). Rejection of runs that did not measure

up to this standard eliminated errors due to change of the chemical constitution of the solution or to passage of protein through the membrane.

(2) Capacity for Repeating Measurements. The levels of the osmometer liquids were reset at different places and it was required that they return to the same point of equilibrium. This practice will eliminate errors due to simple inertia.

(3) Agreement of Results when Technique was Varied. It was considered that the results should be independent of reasonable changes in procedure and technique.

Marrack and Hewitt carried out osmotic pressure studies on the serum proteins[25] and on egg albumin[26]. In the case of the work on egg albumin, they were interested in determining the effect of various salts on the linearity of the osmotic pressure concentration curve. They showed that concentrations varying from 0.1 to 1.1% of sodium chloride, sodium acetate or ammonium sulphate produce no deviation from the ordinary straight line but that deviations did occur with salt concentrations either greater or less than this. They observed that this effect was more pronounced with ammonium sulphate and conclude that it has a special effect upon the activity of egg albumin. Marrock and Hewitt report a value of

43,000 for the molecular weight of egg albumin.

Burk and Greenberg[8] carried out osmotic pressure measurements, using 6.66 molar urea as solvent. They present the following reasons for the choice of medium.

(1) Few proteins are soluble in water or dilute salts thereby cutting down on comparative studies. Urea solutions, however, have a powerful solvent action on many proteins regardless of class.

(2) The use of acid or alkali may cause chemical reaction and mask effects (e.g. aggregation) which might have been useful.

(3) The use of a new solvent might bring out results which would ordinarily be obscured.

These workers allowed periods up to two to three months for attainment of equilibrium and also give criteria for judging when true equilibrium is reached.

PLANT PROTEINS

A good deal of the initial work on plant proteins was done by Osborne and his co-workers[31,32,33]. They isolated proteins from a large number of plant materials obtaining many of them in a crystalline form. Octahedral crystals, for example, were obtained from the seeds of hemp, flax, oats and squash.

They were unable to get crystals from the legumes.

Osborne and Harris[33] found that peas contained three protein fractions which they named and described as follows.

1. Legumin. A globulin which is soluble in saline solution. The solutions remain clear when heated to 100°C.
2. Vicilin. A globulin, soluble in more dilute saline. It could be coagulated by heating the solution to 95°C.
3. Legumelin. This is an albumin-like protein, soluble in pure water. It can be completely coagulated by heating to 80°C. It is quite distinct from the other two proteins.

Csonka, Murphy and Jones[22] found the isoelectric points of vicilin and legumin to be 5.2 and 5.35 respectively.

Osborne's method for fractionating pea proteins involves much lengthy dialysis. This is a distinct disadvantage because there are proteolytic enzymes associated with the proteins which may bring about chemical changes in the proteins. G. W. Hodgson modified Osborne's method in such a way that the time required for the separation of legumin, vicilin and legumelin was substantially reduced.

PREPARATION OF MATERIALS

Preparation of Egg Albumin.

Two methods were used for preparing egg albumin solutions. The method of Kekwick and Cannan[21] using Na_2SO_4 was used for six of the nine preparations and the method of Sorenson[19] employing $(NH_4)_2SO_4$ was used for the remainder. Three of the preparations were carried out in cooperation with G. W. Hodgson who was using samples of the protein solutions for film balance work. Hodgson was using a microkjeldahl nitrogen determination to obtain the egg albumin concentration, and consequently, the use of Na_2SO_4 as crystallizing agent rather than $(NH_4)_2SO_4$ which contains nitrogen was preferred. The two methods gave products which could not be distinguished as far as their osmotic behavior was concerned. In the preparation according to Keckwick and Cannan, the whites of one dozen fresh eggs were collected and the membranes were broken up. To this was added an equal volume of Na_2SO_4 solution prepared by dissolving 400 gms. of the anhydrous salt in one litre of warm water. The mixture was stirred gently for about two hours and the precipitate removed by filtration. The pH was then adjusted to 4.7 by the addition of 0.2 N H_2SO_4 and solid Na_2SO_4 was added to the clear solution until a permanent opalescence appeared. The liquid was then decanted from any undissolved salt

and the solution put in a warm place (above 21°C) for one or two days. The crystallized egg albumin was then collected on a filter paper and dissolved in distilled water. The preparation was recrystallized three times by addition of solid Na_2SO_4 to opalescence and proceeding as above. The final solution was dialysed until outside solution gave a negative test for sulphate.

During these preparations it was customary to use a microscope to make sure that the precipitate was crystalline in nature. Under the microscope the egg albumin crystals appeared as a beautiful mass of very fine needles[19].

In order to have satisfactory crystallization it was essential that only strictly fresh eggs be used.

Preparation of Vicilin.

Vicilin is characterized by being soluble in 3% sodium chloride solution but insoluble in water. Its preparation was in general as follows: Pea meal was extracted with a solution of 10% NaCl. This removed a large percentage of the protein from insoluble material. The salt concentration was then reduced to about 3% by electrodialysis. This brought about the precipitation of legumin which is the fraction soluble in 10% NaCl. The material was then centrifuged and the solid material discarded. The supernatent solution containing the vicilin was subjected to further electrodialysis until the salt concentration was

reduced to about 0.5%. This precipitated the vicilin which was collected by centrifuging. The water soluble solution of legumelin was discarded. In order to rid the solution as completely as possible from legumin and legumelin this fractionation process was repeated several times.

Detail of the actual steps carried out in the preparation are shown in a flow sheet (Figure 3).

Preparation of Legumelin

The steps involved in the preparation of legumelin were as follows:

1. A sample of 80 gms. of pea meal was stirred with distilled water for three hours.
2. The suspension was centrifuged and the supernatant liquid filtered. The filtrate was dialysed against water for about 18 hours.
3. This material was centrifuged again. The supernatant saturated with ammonium sulphate and the precipitate of legumelin collected on a filter paper.
4. The precipitate was dissolved in water and the resulting solution dialysed against water for two days. A considerable precipitate was filtered off and discarded.
5. This solution of water soluble legumelin was prepared as

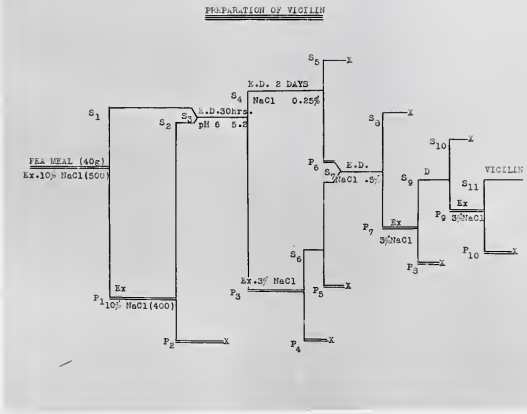

PREPARATION OF VICILIN
PEA MEAL (40g)
Ex.10% NaCl(500)
Ex
10% NaCl(400)
E.D.30hrs.
pH 6 5.2
E.D. 2 DAYS
NaCl 0.25%
Ex.3% NaCl
E.D.
NaCl .5%
Ex
3%NaCl
D
Ex
3%NaCl
VICILIN

Figure 3.

an inside solution for osmotic pressure measurements by dialysing against 0.05 M sodium chloride for twenty four hours.

Preparation of Membranes.

The results of the first ninety osmotic pressure measurements on egg albumin were highly irregular and it was felt that the membranes being used must be faulty. During these runs the procedure for making membranes was varied a good deal in what was essentially a trial and error attempt to obtain a satisfactory membrane. The elements which were varied are listed below.

(1) Membrane material. Materials tried were:

a. Collodion U.S.O. supplied by Baker Chemical Company.

b. Collodion C.P. supplied by Merck and Company Inc.

c. Cellophane sausage casing - supplied by Visking Corporation Union Stock Yards, Chicago.

d. Collodion U.S.P. supplied by British Drug Houses (Can.)

(2) Manner of Drying Collodion. The time of drying was varied from ten minutes to thirty minutes with the pressure ranging from a low value of about 1.5 cm. Hg to 6 cm. Hg. A few attempts were made to dry collodion on the exterior of a glass tube but difficulty was experienced in removing the

membrane. A number of membranes were cast on the interior of a short wide tube in the hope of getting more uniform drying. These, however, were difficult to fix to a glass tube and appeared in no way superior.

(3) Thickness. Membranes of one, two and three coats of collodion were used.

In all of the earlier attempts the variations of osmotic pressures measured were so large that no definite conclusions could be drawn regarding the relative merit of different methods. A few points of interest are, however, listed below.

1. The Merck C.P. Collodion and the cellophane gave membranes which were very slow in attaining equilibrium and were regarded as having too small a pore size.

2. Very few membranes were observed to be permeable to protein molecules. Passage of protein through the membrane could be detected by boiling a sample of the outside solution, or by addition of tannin.

3. The stock bottle of collodion must be kept tightly stoppered at all times to prevent loss of ether by evaporation and a consequent alteration in the porosity of resulting membranes.

4. Considerable trouble was experienced due to a tendency for the drying membrane to peel from the interior of the glass

tube. This was overcome by allowing the collodion to drain from the tube for fifteen seconds so that the coat would not be too thick. It was also found necessary , in this connection, to keep the pressure at a value not lower than four cms. Hg.

5. Two membranes prepared by impregnating an alundum thimble with collodion were unsatisfactory.

The procedure which was finally adopted and which gave satisfactory results is as follows: A thoroughly clean glass sample tube of inside diameter about 0.8 cms. was filled with U.S.P. collodion (British Drug House) and emptied again with a rotating motion. The tube was slowly rotated and allowed to drain at an angle of about 45° for fifteen seconds. The tube was then shaken sharply once, to remove any drops of collodion adhering to the lip of the tube. It was then rotated in a horizontal position until the liquid formed a gel. The tube was then placed in a small flask and the pressure maintained at four cms. Hg. for a period of fifteen minutes. A second coat was applied in the same manner and the bag finally removed from the sample tube with distilled water. The bag was washed for two hours in water before using.

Preparation of Buffer.

Materials: Sodium hydroxide pellets - Merck (reagent)
Glacial acetic acid - Baker's (C.P.)

Osmotic pressure measurements on egg albumin were all done in a sodium acetate-acetic acid buffer. The preparation was carried out by neutrallizing sodium hydroxide with acetic acid. A stock solution of any desired pH was made up to be 0.10 N with respect to sodium acetate; the excess acetic acid required was calculated using the Hasselbach-Henderson equation

$$pH = pKa + \log \frac{[salt]}{[acid]}$$

A Coleman electrometer was used to check the final pH.

To obtain the outside solution for the osmometer, stock solution was diluted with an equal volume of distilled water. The inside solution was obtained by diluting stock solution with an equal volume of dialysed egg albumin solution.

APPARATUS AND PROCEDURE

The osmometer used for all measurements reported herein was a modification of one described by Bull[5]. The first eighty two determinations, many of which were unsatisfactory were carried out with a pyrex glass osmometer having a capillary of bore 0.53 cm. diameter. Subsequent runs were made with a soft glass osmometer which utilized the stem of a broken thermometer as a capillary. A measurement of the mean capillary diameter of one of these thermometer stems gave a value of 0.02 cms.

The object of changing the design was to increase the

sensitivity of the instrument as much less transfer of material was required for the attainment of equilibrium.

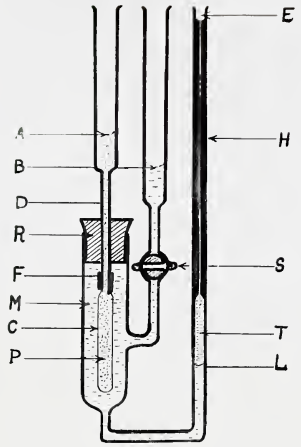
The only essential difference in the two types was the size of capillary bore. Both osmometers were as represented in Figure 4.

Assembly of Apparatus.

The assembly and operation of the osmometer is described by referring to Figure 4.

Attachment of Collodion Sac.

After the rubber stopper R had been put over the tube B a 3/4" length of rubber tubing was moistened with glycerol and slipped over the tube. The rubber tube was then rolled back on itself from the outer end to form a double thickness of rubber and a small rubber band stretched over this. The collodion bag was then placed in position close to the rubber tube and creased to fit tightly around the glass tube. The rubber band was then rolled from its position onto the sac to hold it firmly in place. A very thin film of stopcock grease was rubbed about the exposed interior of the rubber tube and the top of the sac. The rubber tube was then rolled out over the collodion sac but not over the rubber band. Any excess stopcock grease was removed. The interior of the bag was rinsed with the protein



OSMOMETER

Figure 4.

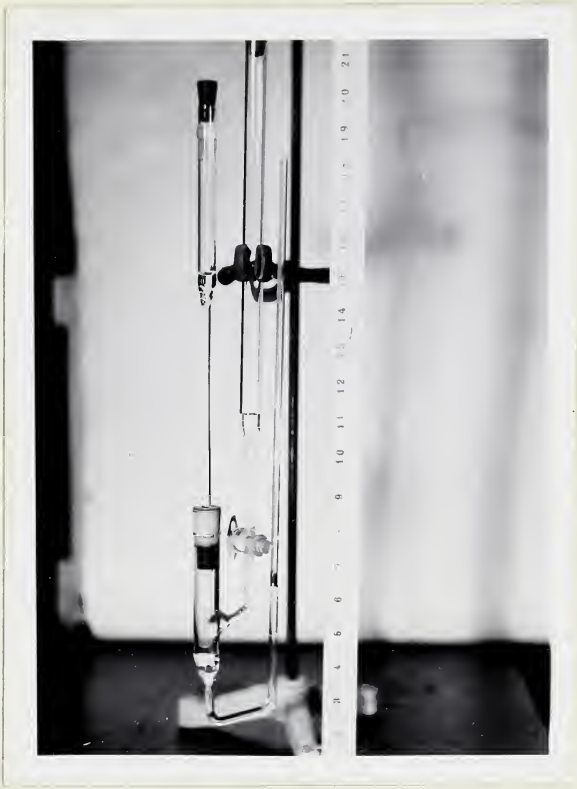


Figure 4b.

solution P and the tube filled to the level A before it was inserted in the osmometer.

This manner of fixing the membrane was simply performed, and after a little experience invariably gave a joint which did not leak.

Order of Assembly.

To assemble the pyrex osmometers, the small cup E was stoppered and stopcock S opened. The osmometer was then filled with buffer solution M and the protein solution put into the apparatus by firmly forcing the rubber stopper R into place. The stopcock S was opened, the stopper removed from E and the buffer solution allowed to flow around to the position L before closing the stopper again. Toluene was added to the cup E, the stopcock opened again, and by means of gentle air pressure the toluene was forced through the capillary to the position T. The air pressure was now removed, and the toluene forced the last traces of air out through the capillary and rose to its natural level in the capillary H. The open ends of the tubes were then stoppered with one holed stoppers to reduce evaporation to a minimum and the apparatus clamped in a thermostated water bath at 25°C.

One side of the bath (Figure 5) was made of plate glass to provide a clear vision of the osmometers through a

cathetometer.

After a period of about fifteen minutes had been allowed for the osmometer to reach the bath temperature, the initial level of the protein at A, the buffer at B and the toluene in the capillary were recorded. The stopcock was then closed and any net transfer of material through the membrane was reflected in a change of the toluene level. Readings of the level of the toluene were taken at intervals until it had reached the point of equilibrium. The stopcock was then opened and the initial readings checked. From these readings the osmotic pressure can be calculated.

The initial pressure across the membrane is equal to the difference of the levels of protein and buffer (A - B). The change of the toluene in the capillary represents a change of this pressure. The osmotic pressure is thus equal to the protein level minus the buffer level plus any adjustment arising out of the change of the toluene level. This may be expressed by the formula:

$$P_{op} = (A - B) + D_T \Delta T$$

where

P_{op} = osmotic pressure in cm. of water at 25°C

A = level of protein solution

B = level of buffer solution

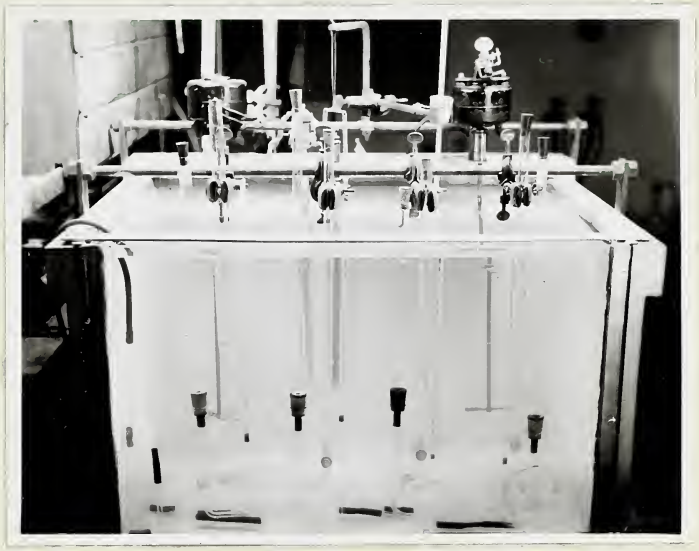


Figure 5.

D_T = density of toluene compared to water 25°C

ΔT = initial level of toluene minus equilibrium level of toluene.

Bull in his work with lactoglobulin[7] continued to adjust the level of protein in the osmometer so that the change in toluene was as small as possible. This small change was then applied as a correction to the osmotic pressure as given by A - B. The practice in the work done here was to adjust the initial levels to approximately the osmotic pressure expected and made no alterations during the run.

It is to be noted that all capillary forces cancel out in this method and can be neglected providing the bore of the capillary is uniform.

DETERMINATION OF PROTEIN CONCENTRATION

All protein concentrations were obtained by residue weight. The problem is somewhat complicated by the fact that the protein solution contains an appreciable amount of dissolved sodium acetate. Ignition of the sample to burn off the proteins proved unsatisfactory due to the fact that sodium acetate is unstable at high temperatures. Tests showed that sodium acetate when ignited suffered a somewhat variable loss in

weight. This loss also was not sufficient to represent a quantitative conversion to sodium oxide.

The method adopted as the most satisfactory is as follows: A sample of about 2 ml. of the protein solution was removed from the osmometer immediately after completion of the run and weighed in a previously dried and weighed crucible. This was dried in an oven at 110°C for a period of four hours or more and again weighed. These weights gave the total weight of sample and the total weight of residue. Previous blank determinations carried out on the stock solution of buffer gave the residue of salt to be expected. This salt residue was then deducted from the total residue to give the total weight of protein present. Protein concentration was expressed as the grams of protein in 100 grams solvent.

This method was regarded as adequate because duplicate determinations were always made and these usually checked to within the accuracy to be expected from the balance used.

A modification of the above procedure which proved satisfactory was to take simultaneous samples of the inside and outside solutions. The difference in the residue per gram from these was regarded as due to the protein.

Samples of egg albumin dried at 110°C in an

ordinary oven showed no change in weight when dried for an additional two hours in a vacuum oven at 105°C.

FACTORS TO BE CONSIDERED IN MEASURING OSMOTIC PRESSURE

Effect of Temperature Variations.

The theoretical osmotic pressure is directly proportional to the absolute temperature and any error in reading the temperature of the bath would be expected to produce an error in the calculated molecular weight. The temperature during any run would be within 0.1°C of the desired 25°C and would ordinarily not fluctuate over a range of more than 0.01°C. This amount of variation could not produce more than a very small error due to the effect mentioned above.

Temperature also has an effect upon the density of the solutions and the isoelectric point of the protein. Here again their magnitude is extremely small for the temperature ranges listed above.

Temperature variation of a very small amount does have a marked effect upon the toluene level in the capillary; especially in the case where thermometer capillaries were used. A rise and fall in the temperature of osmometer solutions will produce a simultaneous rise and fall of the toluene meniscus.

This effect is well illustrated in Figure 6

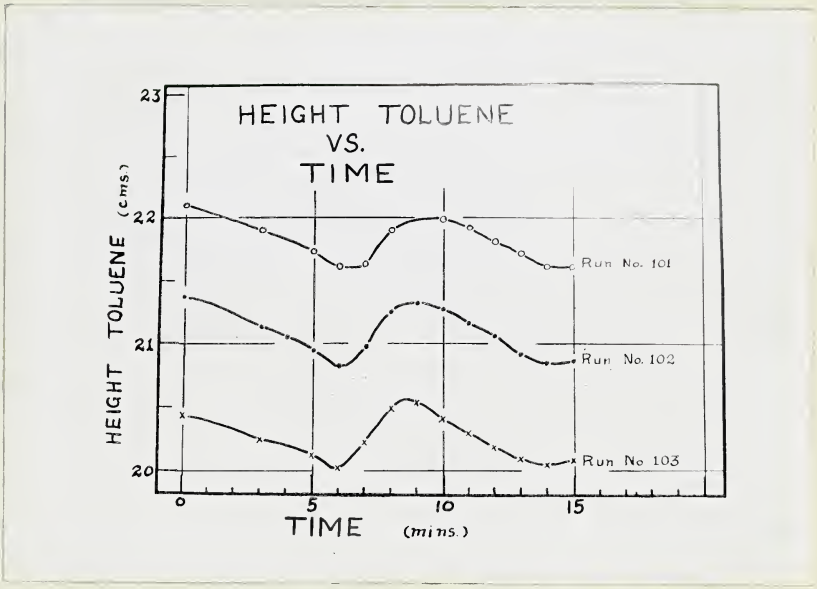

HEIGHT TOLUENE
VS.
TIME
HEIGHT TOLUENE (cms.)
23
22
21
20
Run No. 101
Run No. 102
Run No. 103
0
5
10
15
TIME (mins.)

Figure 6.

Toluene elevations were recorded at regular intervals on runs number 101, 102 and 103. The readings were taken when the runs were near the equilibrium condition. The slow cooling of the bath due to evaporation followed by a more rapid rise in temperature is reflected in the toluene elevations.

In order to calculate the osmotic pressure, it is necessary to have a single value for the elevation of toluene at equilibrium. In order to arrive at what was felt to be the best value for the final toluene level the elevations were read at regular intervals over one or more complete cycles of temperature fluctuations and then averaged. Essentially the same result was obtained by taking the point midway between the maximum and minimum of the wave forms.

This temperature variation is also evident in Figures 7 and 9.

The temperature effects described above were not noticed with the pyrex glass osmometers (see Figure 8). The fact that they had a cross-sectional area of more than six times that of the thermometer bore would account for this.

Time Required to Reach Equilibrium.

Two factors are involved in the time required to reach equilibrium.

(1) The time necessary for the membrane to transfer sufficient material to move the toluene meniscus to its equilibrium position.

(2) The time required for any uneven distribution of buffer salts across the membrane to disappear.

The time required for the first of these factors can be reduced by using a smaller capillary bore, and it was with this object in view that the use of the soft glass osmometer was adopted.

The time needed for salt distribution to be erased depends upon the initial concentration difference, the permeability of the membrane to buffer salts and upon the diffusion rate of the ions in question. In order to reduce this time as much as possible the inside and outside solutions were made as nearly equal in salt concentration as ordinary volumetric measurements would permit. In a number of cases, the protein solution was dialysed against the buffer, before the osmotic pressure measurements were made. Some workers have considered some type of stirring to be essential[24,25].

Equilibrium was judged to exist when the calculated toluene level (see discussion temperature effects) remained constant to within a range of about 0.1 cm. for a period of three hours

or more. A period of 24 hours was usually ample for attaining this state but in a number of cases a longer period was required.

Figure 7 shows the way in which equilibrium is approached when a thermometer bore capillary is used. In runs number 101, 102 and 103, the osmotic pressure which would be calculated at any time from the beginning of the run is plotted against time. The solutions were of approximately the same concentration, so they immediately begin to move together. After a period of from 20 to 30 minutes, the curves flatten out and continue to approach equilibrium at a much slower rate.

Figure 8 is the same type of plot for the behavior of a pyrex osmometer which has the larger capillary. A period of about four hours is required for the curve to level out.

Figure 9 illustrates the behavior of a fine capillary osmometer over a longer period of time. Attention is drawn to the fact that after the first rapid motion there was a reversal of the direction of the motion of the toluene. The approach to equilibrium in this second phase is very much slower. The first rapid motion of the toluene is regarded as a result of the transfer of material through the membrane

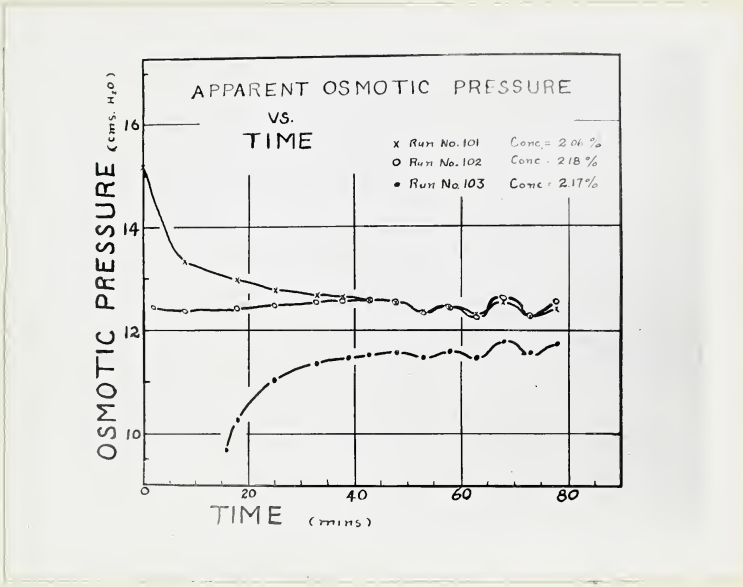

APPARENT OSMOTIC PRESSURE
VS.
TIME
x Run No. 101 Conc. = 2.04 %
o Run No. 102 Conc = 2.18 %
• Run No. 103 Conc = 2.17%
OSMOTIC PRESSURE (cms. H2O)
16
14
12
10
0
20
40
60
80
TIME (mins)

Figure 7.

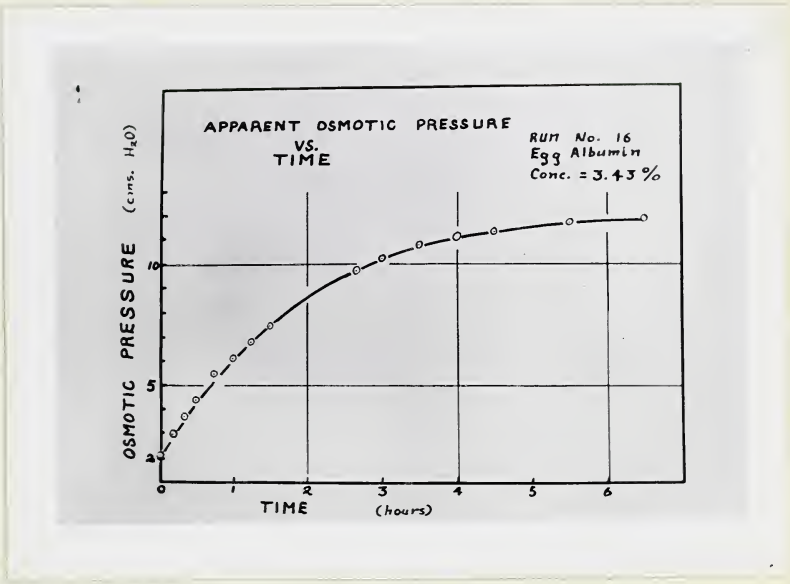

APPARENT OSMOTIC PRESSURE
VS.
TIME
RUN No. 16
Egg Albumin
Conc. = 3.43 %
OSMOTIC PRESSURE (cms. H_2O)
2
5
10
0
1
2
3
4
5
6
TIME (hours)

Figure 8.

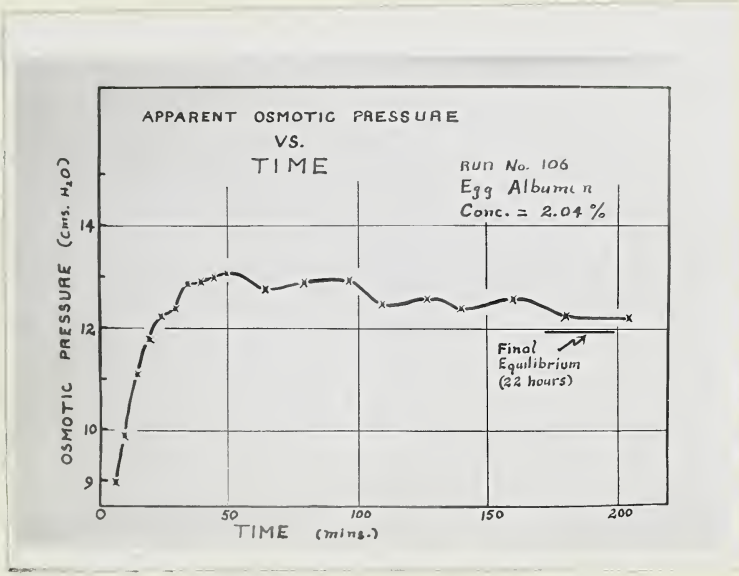

APPARENT OSMOTIC PRESSURE
VS.
TIME
RUN No. 106
Egg Albumen
Conc. = 2.04 %
OSMOTIC PRESSURE (Cms. H2O)
14
12
10
9
0
50
100
150
200
TIME (mins.)
Final
Equilibrium
(22 hours)

Figure 9.

in response to the osmotic pressure as it actually exists at this early stage. As time goes on the buffer salts slowly become evenly distributed and the protein alone contributes to the osmotic pressure. This slow evening up of salt concentration probably explains the gradual downward motion in the later half of the curve of Figure 9.

Sources of Error.

The errors which might occur in making an osmotic pressure measurement are listed and discussed briefly.

Mechanical errors.

(1) Variation in cross section of a Capillary Bore.

For the capillary of a pyrex osmometer the bore uniformity was estimated by measuring the length of a thread of mercury as it moved through the bore. The mercury thread was then removed and weighed, and the results used to calculate the bore diameter at intervals along the capillary.

In order to illustrate the manner in which possible errors were estimated, the data obtained in the study of bore variations is presented in Table I.

Table I.

Bore Variations in Pyrex Capillary

	Elevation of Top of Thread (cms)	Elevation of Bottom of Thread (cms)	Length of Thread (cms)	Diameter of Bore (cms)
1	42.886	41.510	1.356	0.0529
2	41.110	39.760	1.350	0.0534
3	39.651	38.300	1.351	0.0533
4	38.068	36.724	1.344	0.0533
5	36.720	35.370	1.350	0.0534
6	34.354	33.396	1.358	0.0531
7	33.256	31.884	1.372	0.0528
8	31.350	29.958	1.392	0.0524 minimum
9	30.074	28.774	1.300	0.0543 maximum
10	39.302	37.950	1.352	0.0532
			Average diameter	0.05324

The manner in which the error was estimated is shown below:

Calculation of diameter

Weight of mercury recovered = 0.0278 gms.

Density of mercury at 22.5°C = 13.521 gms./ml.

Volume " " = $\frac{0.0278}{13.521}$ = 0.205 ml.

but $D = 2\sqrt{\frac{V}{\pi}\cdot\frac{1}{L}} = \frac{0.0618}{\sqrt{L}}$ cm.

where D = diameter of bore

V = volume of mercury = 0.205 ml.

L = length of mercury

for measurement no. 1

$$D = \frac{0.0618}{\sqrt{1.36}} = 0.0529 \text{ cm.}$$

In order to estimate the probable effect of these variations upon the osmotic pressure, the capillary rise of toluene at 25°C was calculated using the equation

$$h = \frac{4s}{grD}$$

where h = capillary rise of toluene

s = surface tension for toluene at 25°C = 34 dynes/cm.

r = density of toluene at 25°C = 0.862

D = diameter of capillary

g = acceleration due to gravity

Capillary rise for minimum diameter = 3.07 cm.

Capillary rise for maximum diameter = 2.96 cm.

Difference 0.11 cm. toluene

= 0.09 cm. H_2O.

If such variations in capillary rise were to occur during a run it would produce an error of the same magnitude.

For a solution of material with a molecular weight of 40,000 and concentration of 2%, the theoretic osmotic pressure would be 12.6 cms. and an error of 0.09 cm. would be equivalent to error of 0.7%.

(2) Stickiness of Toluene Meniscus.

Any error caused by sticking of the toluene was negligible if the capillary was clean and dry. If dirt or moisture were present errors of serious mature would arise.

(3) Errors Due to Light Refraction Through the Water.

No appreciable error occurred due to this when the following conditions were met:

1. The osmometer was submerged far enough that all readings could be taken below the water surface.

2. The osmometers and the glass wall of the bath were perfectly vertical.

(4) Errors in Making Cathetometer Readings.

The error of any single cathetometer reading is probably considerably less than 0.01 cm. provided that the instrument is properly focused to eliminate all parallax.

It is considered that if the proper precautions are taken the total error in an osmotic pressure measurement due to imperfections of the measuring apparatus will not be greater

than 0.10 cm. H_2O.

Errors Inherent in the Membrane.

This type of error is very hard to evaluate or explain.

Imperfections that may occur are:

1. Large pores or small leads which would allow the passage of protein. If protein passed through the membrane it could be detected in the outside solution by precipitating it with tannin.
2. Slow passage of buffer ions or elasticity of the membrane allowing volume changes. These imperfections would give rise to a very slow approach to equilibrium.
3. Absorption of the protein by the membrane. This was not tested for in the work reported herein, it was assumed that it did not cause appreciable error.

Blank Runs.

When an osmometer is set up containing only buffer salts at the same pH, it should come to equilibrium at such a point that the calculated osmotic pressure is zero. The equilibrium should be independent of the relative initial concentration of the inside and outside solutions. If a method for measuring osmotic pressure does not give this zero

osmotic pressure for a "blank run", it cannot be regarded as satisfactory.

Two such runs were carried out using the apparatus and technique finally adopted as most successful. Osmotic pressure of 0.07 cm. H_2O and 0.02 cm. H_2O were obtained. As this is within the possible experimental error the method was regarded as having met this requirement.

RESULTS

All osmotic pressure measurements were carried out in the manner previously described and the calculations are as indicated in the sample calculation given below:

Sample Calculation.

Run # 119 Egg Albumin Concentration 2% Approx. Feb. 25, 1947.

Osmotic Pressure Measurement.

	Initial	Final
Toluene level (stopcock open)	19.54	19.54
Protein Level	20.12	20.12
Buffer Level	9.69	9.68
(Protein-Buffer)	10.43	10.44

Equilibrium Toluene Level (14 readings, 1 cycle) 20.14

Drop in Toluene Meniscus (19.54 - 20.14) = -0.60

H_2O equivalent of drop in toluene (0.60 x .862) = -0.52

Osmotic pressure = Initial Pressure + Drop toluene

= (10.44 - 0.52) = 9.92 cm. H_2O.

Determination of Concentration.

	(1)	(2)
Wt. empty crucible	10.9914	9.4434
Wt. crucible + solution	12.975	11.437
Sample	1.984	1.994
Wt. after Drying	11.0328	9.4846
Total Residue	0.0414	0.0412
Residue per gram of Sample	0.0208	0.0207
Theoretical Residue Salt	0.0030	0.0030
Wt. Protein/gm. Sample	0.0178	0.0177
Wt. Protein/gm. Solvent	0.0181	0.0180

Average 0.0181

Composition 1.81%

Calculation of Molecular Weight

$$\text{Molecular weight} = 2.525 \times \frac{C}{P_{op}} \times 10^5$$

$$= 2.525 \times \frac{1.81}{9.92} \times 10^5 = 46{,}100$$

Osmotic Pressure Measurements on Egg Albumin.

The object of making osmotic pressure measurements on egg albumin was to test the method and procedure using a fairly well known material. The first results were very erratic as is shown by the distribution given in Table 2.

Table 2.

Distribution of Calculated Molecular Weights of Egg Albumin.

First 90 determinations.

Range of Molecular Weight	No. of Determinations
Under 40,000	3
40,000 - 50,000	25
50,000 - 60,000	12
60,000 - 70,000	13
70,000 - 80,000	5
80,000 - 90,000	3
90,000 - 100,000	1
Over 100,000	9
Discarded runs	19
Total	90

The very high results were nearly all among the first fifteen runs (see appendix II).

Common reasons for discarding a determination were

a leaking membrane, traces of water in the capillary, rise of toluene out of capillary bore and failure to reach a steady equilibrium.

These runs are obviously so variable that in themselves they have little value and no definite conclusion can be drawn from them.

The experience gained in this phase of the work led to the development of a satisfactory method and technique. After the first ninety runs, this acceptable method was used consistently. Table 3 gives the results obtained in using this technique with egg albumin.

Table 3.

Osmotic Pressure on Egg Albumin.

Run No.	Concentration gms/100 gms. Solvent	Osmotic Pressure (cm H_2O)	Molecular weight	Deviation from Mean
91	2.00	11.01	45,900	100
92	2.25	12.81	44,300	1,500
94	2.24	12.77	44,300	1,500
96	2.04	11.33	45,400	400
97	2.04	11.15	46,100	300
101	2.10	11.05	48,000	2,200
102	2.22	12.22	45,800	000
103	2.21	11.90	46,800	1,000
104	2.23	11.95	47,100	1,300
105	2.22	12.61	44,100	1,400
106	2.21	11.99	46,500	700
108	2.99	16.84	44,800	1,000
110	2.96	16.35	45,700	100
111	2.93	16.28	45,400	400
112	2.95	15.99	46,600	800
115	1.82	10.34	44,300	1,500
116	1.25	6.73	46,900	1,100
117	3.45	18.49	47,100	1,300
118	2.61	14.70	44,800	1,000
119	1.81	9.92	46,100	300
123	2.68	14.82	45,600	200
Average Molecular Weight			45,800	
Mean Deviation				860
Average Deviation of the mean				188

Runs # 115, 116, 117, 118, 119 presented in Table 3 were done at a pH of 4.45; the remainder were at the isoelectric point of 4.7. Runs at this slightly lower pH were satisfactory and gave the same results.

Measurements at other pH values are not included in the table. Such runs were few in number and were not consistent. A measurement at a pH of 3.8 for example gave a molecular wieght of 49,300. This was to be expected as the protein solution in the collodion bag and in the stock bottle became cloudy on standing. Marrack and Hewitt[26] also observed this effect below a pH of 4.0.

Discussion.

Although the measurements do not cover a very wide range of concentrations, a plot of osmotic pressure against concentration (Figure 10) gives the straight line characteristic of egg albumin[5,26].

The value of 45,800 although somewhat high is in quite good agreement with the results of Bull[5] who, with the same type of apparatus and procedure, reported the molecular weight of egg albumin as 45,160.

For purposes of comparison, a selection of molecular weights reported for egg albumin are given in Table 4.

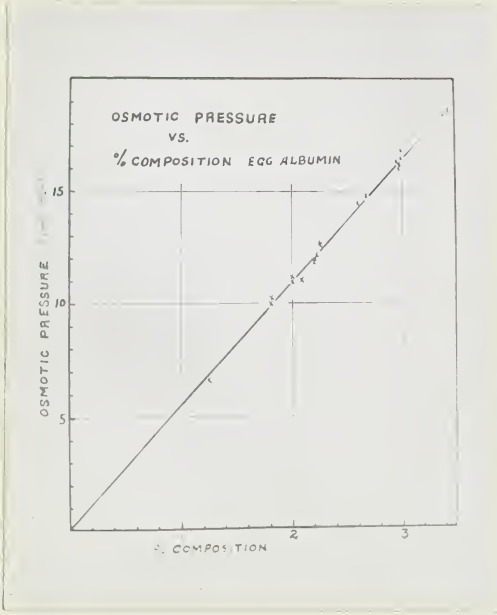

OSMOTIC PRESSURE
VS.
% COMPOSITION EGG ALBUMIN
OSMOTIC PRESSURE
15
10
5
2
3
% COMPOSITION

Figure 10

Table 4.

Values for Molecular Weight of Egg Albumin Reported by Various Workers.[5]

Method	Molecular Weight	Workers
Osmotic Pressure	43,000	Marrack and Hewitt (1929)
Osmotic Pressure	46,000	Taylor Adair and Adair (1932)
Osmotic Pressure	34,000	Sorensen (1917)
Ultracentrifuge (Equilibrium)	34,500	Svedberg and Nichols (1926)
Ultracentrifuge (Sedimentation)	44,000	Svedberg and Pederson(1940)
Chemical Analysis	36,800	Bernhart (1940)
Film Balance	43,200	Hodgson[20] (1947)
Osmotic Pressure	45,160	Bull (1941)
Osmotic Pressure	45,800	Present investigation.

Study of Electrodialysed Egg Albumin.

In 1926, Svedberg and Nichols[36] concluded from work with the ultracentrifuge that egg albumin, even after several recrystallizations, contained two components. They report the molecular weight of the main component as 34,500 and suggested that the second component, present in small amounts, was a

globulin of molecular weight about 170,000. This globulin, they said, was probably held in solution by small amounts of absorbed salts. They reported that electrodialysis to a very low conductivity removed this large molecular weight material. Longworth, Cannan and MacInnes[24] studied egg albumin by means of electrophoresis. They also report the presence of two components even after repeated recrystallization.

These suggestions together with the fact that the literature reports molecular weights for egg albumin ranging from about 35,000 to 46,000 prompted this work on electrodialysed egg albumin.

A solution of egg albumin (about 6.8%) which had previously been dialysed against distilled water until it was apparently sulphate free was electrodialysed with a standard type apparatus using parchment as a membrane. A continuous flow of distilled water, redistilled from barium carbonate through a block tin condenser was passed through the cathode and anode compartments. The protein in the middle compartment was stirred gently.

Colvin measured the specific conductance of the material upon completion of the dialysis and found it to be 1.78×10^{-5} ohms^{-1}.

Osmotic pressure measurements were made upon this material in the same manner as with egg albumin which had not been electrodialysed. Results are given in Table 5.

Table 5.

Osmotic Pressure Measurement on Electrodialysed Egg Albumin

Run No.	Concentration gms/100 gms. solvent	Osmotic Pressure	Molecular Weight
1	3.26	19.62	41,900
2	3.25	19.92	41,200
3	2.35	14.94	39,700
4	2.30	15.00	38,700
	Average Molecular Weight		40,400
	Average Deviation of the Mean		600

R. Colvin carried out an electrophoresis analysis on the electrodialysed egg albumin. This type of analysis makes use of the fact that proteins, not at their isoelectric point will move under the influence of an electric field and thus produce a slow motion of a protein buffer boundary. Different proteins differ in molecular mass and charge and consequently will migrate at different rates. The electrophoresis apparatus is so arranged that the photographic record shows a single

"peak" for a single component and additional peaks for other components that move at a different rate.

Figure 11 is a print obtained from the photographic plate of the electrophoresis run on electrodialysed egg albumin. Unfortunately, no electrophoresis run was made on the material before electrodialysis and it is impossible to be sure what changes the electrodialysis produced in the electrophoretic behavior of this particular preparation. A drawing on the lower unexposed portion of the plate illustrates what would be expected from the material before electrodialysis. It was sketched from work reported by Longsworth[24]. The pH of the electrodialysed egg albumin was 6.7. The sketch illustrates a run done at a pH of 5.3.

The electrophoresis pattern indicates that although a second component is indicated by a hump on the side of the peak (see arrow), it has been much reduced by the electrodialysis.

Discussion.

Although only four measurements for the molecular weight of electrodialysed egg albumin are reported these are all well below any of those reported in Table 3.for the same material before electrodialysis.

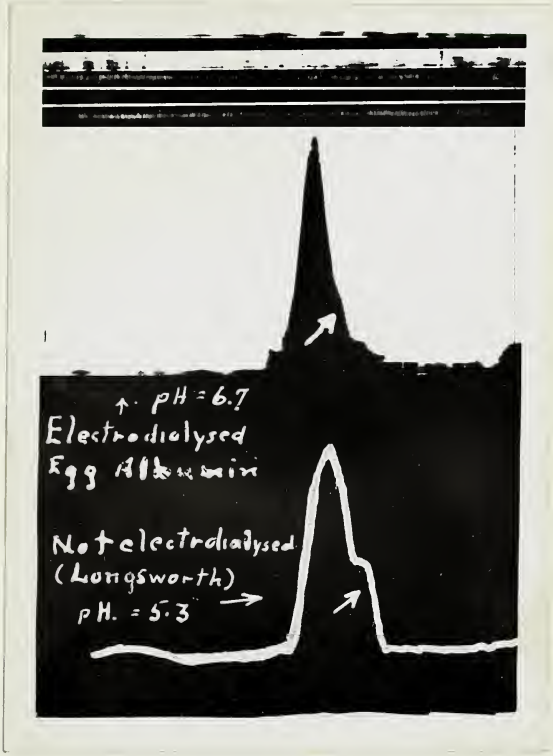

pH = 6.7
Electrodialysed
Egg Albumin
Not electrodialysed
(Longsworth)
pH. = 5.3

Figure 11.

There is a strong indication that this treatment has in some manner reduced the average molecular weight. Although the information is not complete enough to permit the formation of definite conclusions about what has taken place, the following suggestions are offered.

1. The electrodialysis may have partially precipitated some high molecular weight material (e.g. a globulin as suggested by Svedberg), which is not separated by the ordinary recrystallization procedure. The electrophoresis results indicate that at least some of the second material is still present. It is impossible to estimate the molecular weight of the two components without knowledge of the amounts of each present.

A precipitate did form during the dialysis but this could have come from denaturation of the egg albumin with stirring.

2. The high molecular weight material may be a complex built up of the basic egg albumin molecules. It has been suggested that such complex molecules might be formed in a definite proportion to the egg albumin molecules[26]. There is a possibility that such a complex was partially broken down by the electrodialysis and would slowly reform until it was present in what might be termed equilibrium amounts.

3. Electrodialysis is quite a drastic treatment for protein molecules and it is conceivable that it caused some fragmentation of particles of molecular weight 45,800 with a consequent reduction of the average molecular weight.

Osmotic Pressure Measurements on Vicilin.

Osmotic pressure measurements on vicilin at its isoelectric point of 5.2 with a 3% NaCl solution outside gave results as presented in Table 6.

Table 6.

Osmotic Pressure Measurements on Vicilin.

Run No.	Concentration gms/100 gms solvent	Osmotic Pressure (cms. H_2O)	Molecular Weight	Deviations from Mean
1	0.74	1.07	174,000*	----
2 Discard	--	--	--	----
3	0.63	0.68	234,000*	----
4	0.66	0.86	196,000	2,000
5	0.58	0.74	198,000	0,000
6	0.61	0.74	208,000	10,000
7	0.68	0.89	192,000	6,000
8	0.74	1.42	133,000*	
9	0.43	0.93	201,000	3,000
10	0.76	1.10	190,000	8,000
	Average Molecular Weight		198,000	
	Average Deviation of the mean			2,000

* Molecular weights discarded statistically.

Discussion.

The vicilin was obtained in a relatively dilute solution. This coupled with the fact that the material has a high molecular weight made the osmotic pressures to be measured less than one cm. of water. The reliability of a single run is consequently greatly reduced and is reflected in relatively greater deviations from the mean. The results are, however, consistent enough to show that vicilin as prepared for these measurements has a molecular weight in the vicinity of 200,000.

The term molecular weight as applied to a protein fraction such as vicilin is perhaps misleading. The term average particle weight would probably be more explicit. It is probable that many plant proteins exist with quite a wide range of particle weight and that the average weight of these particles varies with varying conditions of preparation, pH, etc. This point of view is supported by the film balance work of Hodgson[20].

He found that when samples of the same preparation of vicilin as used in the above osmotic pressure measurements were spread as a monolayer the molecular weight was 28,400. Hodgson's work indicates that a breakdown of vicilin into

smaller fractions occurs when it is thus spread.

Osmotic Pressure Measurements on Legumelin.

The results of four osmotic pressure determinations on legumelin are presented in Table 7.

Table 7.

Osmotic Pressure Measurements on Legumelin

Run No.	Concentration gms/100 gms. Solvent	Osmotic Pressure After 16 hours	Molecular Weight
1	0.38	6.33	15,200
2	0.38	6.03	15,900
3	0.38	6.06	15,800
4	0.38	6.18	15,500
*		Average molecular weight	15,600
		Average Deviation of the mean	125

* To be regarded as approximate only (see discussion)

Discussion.

The results presented in table 7, although agreeing quite well with each other are not conclusive for the following reasons:

1. Steady Equilibrium was never reached.
2. The concentration at the end of sixteen hours was not available. During the first few hours these runs, all of the same initial concentration, behaved normally and next morning, after a period of sixteen hours, calculations gave osmotic pressures as shown in Table 7. They continued, however, to show a slow but distinct change. Runs numbers one and four moved towards higher osmotic pressures; the other two moved toward lower osmotic pressures. At the end of four days they gave values ranging from 3.85 to 9.00 cms. H_2O, had not yet reached equilibrium, and had partially precipitated.

In order to estimate the molecular weight, the osmotic pressure at the end of sixteen hours was chosen because:

(1) The material had been previously dialysed against buffer and after sixteen hours would ordinarily be very close to equilibrium (see Figure 9).

(2) The chemical change in sixteen hours would be less than at any later time.

(3) The osmometers had by this time approached much the same values from opposite sides of equilibrium.

(4) Subsequent changes were very slow.

The concentration of the protein at the end of the sixteen hour period was, of course, not available. For purpose

of calculation, the concentration of the material at the start of the runs was used. It may be regarded as a reasonable approximation because each collodion sac was rinsed twice with the legumelin solution before it was filled, and no evidences of a precipitate was observed after the sixteen hours.

The irregular behavior of the osmotic pressure over periods of two or more days is difficult to understand. It could be partially explained by assuming that the membranes differed considerably in pore size. It is quite possible that fragmentation of the protein was occurring due to proteolytic enzymes which are known to be present[20]. If these fragments were passing through the different membranes at different rates results similar to those observed would be obtained.

Microkjeldahl nitrogen measurements were obtained on the outside solutions at the end of the four day runs. An increase in nitrogen content was observed in all cases. It represented about 5% of total protein nitrogen in a case where osmotic pressure was rising, and was somewhat more (about 6%) in the case where osmotic pressure was falling. This is the order to be expected in the light of the discussion in the previous paragraph.

The situation is complicated by the fact

that precipitation is occurring at the same time as any breakdown into fragments. Much work is required to obtain a complete picture of the changes occurring in legumelin at room temperature.

Hodgson[20] obtained a molecular weight of 13,500 for the same preparation of legumelin. His measurements were made with a surface balance.

Summary.

A satisfactory technique was developed for measuring the osmotic pressure of protein solutions.

The molecular weight of several proteins were investigated by means of osmotic pressure measurements. The results reported are given as:

1.	Crystallized egg albumin	45,800
2.	Electrodialysed egg albumin	40,400
3.	Vicilin from peas	192,000

Legumelin from peas was found to have a low molecular weight (probably close to 15,600).

The results on egg albumin are in fair agreement with the results of other workers who used a similar method.

Electrodialysis changes the osmotic behavior of egg

albumin solutions.

Legumelin from peas undergoes marked changes in its physical state if retained in dilute salt solution at 25°C.

Suggestions for further work.

The possibilities for further work in this field appear almost limitless. A few suggestions are given below.

1. An investigation into possible ways of speeding up the approach of equilibrium in osmotic pressure measurements should prove useful, especially when unstable materials such as proteins are to be studied. One way of doing this might be to introduce some stirring mechanism to prevent the development of a concentration gradient from the membrane surface to the bulk of the solutions.
2. The work on electrodialysed egg albumin should be extended. The molecular weight of the material could, for example, be measured at regular intervals after the dialysis in an effort to determine whether the large molecular weight component was being re-formed again.
3. Molecular weight determinations on vicilin could be extended in several ways to answer such questions as
   (1) Can the preparation be repeated to give material of the same molecular weight as already observed?
   (2) What effect does the pH have upon the molecular weight

of vicilin?

(3) Is the osmotic pressure concentration curve a straight line?

4. The osmotic pressure measurements on legumelin should be repeated. If the anomalous behavior described is again observed, an investigation into its cause might produce valuable information. Osmotic pressure measurements near 0°C might prove more satisfactory with legumelin as enzyme activity would be much reduced.

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Appendix I.

VISCOSITY MEASUREMENTS

Introductory Remarks.

It is recognized that a general relation exists between the viscosity of a solution and the molecular weight of the solute. The amount by which the viscosity of a solvent is increased with addition of a particular solute depends upon the shape as well as the size of the solute molecule. The interpretation of viscosity measurements is often very difficult and no attempt will be made to discuss the problem in any detail.

The relation between viscosity and molecular weight of material over 10,000 is given by the following equation[39]

$$n = KM^a \qquad (1)$$

where

n = the intrinsic viscosity

M = Molecular weight of solute

K and a are constants characteristic of a given solute solvent system.

The intrinsic viscosity is identical with the ratio $\frac{N_{sp}}{C}$ at infinite dilution.

where N_{sp} = specific viscosity

C = concentration expressed as grams solute in 100 ml. of solution.

N_{sp} can be obtained from the time of flow in an Ostwald type viscometer. It is given by the equation

$$N_{sp} = \frac{t_c - t_o}{t_o} \qquad (2)$$

where t_c = time of flow with solute concentration C

t_o = time of flow of pure solvent

The valves of the constants K and a are however more difficult to determine accurately.

Measurements were made with two Ostwald Viscometers

on Egg Albumin and Vicilin. The chief object was to test the usefulness of this type of viscometer with protein solutions. Results are given in Figure 12.

Discussion.

The results obtained with the two viscometers were in good agreement and the points fall on a fairly smooth line.

The legumelin which was very dilute (0.38%) could not be measured satisfactorily. The increase in viscosity caused by its presence was very small and errors in the method were so large in proportion that no smooth line could be obtained. It was obvious, however, that the slope of the time of flow - concentration curve was very small. The dotted lines in Figure 12 are included to indicate the probable position of the curve.

The method is not regarded as wholly satisfactory for viscosity work on protein solutions. The points show some scattering, and make the exact shape of the curve somewhat doubtful.

Proteins are surface active so they foam very easily. Stable bubbles tend to form in this type of viscometer and unless extreme care is taken, accurate measurements are hard

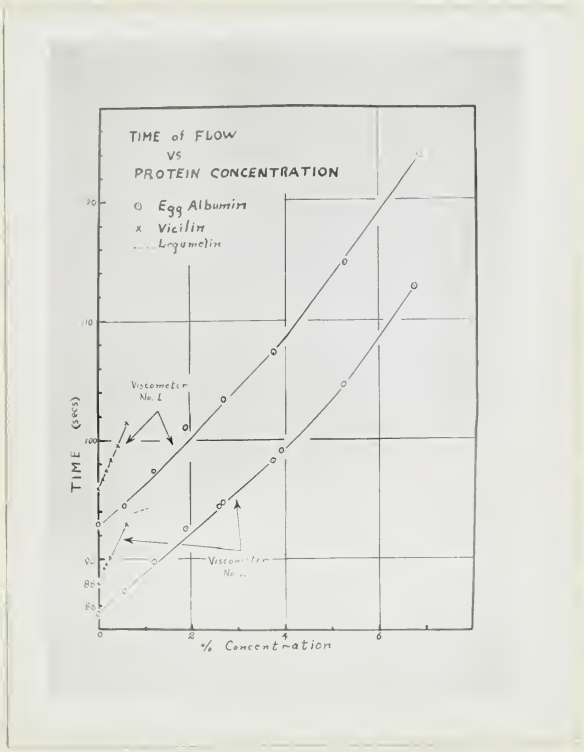
TIME of FLOW
VS
PROTEIN CONCENTRATION
o Egg Albumin
x Vicilin
Legumelin
Viscometer No. 1
TIME (secs)
% Concentration

Figure 12.

to obtain.

There is a qualitative agreement of the slopes of the lines in Figure 12 with the molecular weights of the proteins as obtained by osmotic pressure measurements. The order of increasing slope is also the order of increasing molecular weight.

The values of K and a for these systems are not known with certainty so it was impossible to calculate the molecular weights accurately. An estimation with the information available was made for vicilin.

The equation

$$n = KM^a$$ was used.

n for egg albumin and vicilin was obtained from Figure 12.

The calculation required the following assumptions.

(1) a was taken as 0.67. (The only value for a found in the references[39] with water as solvent).

(2) K for egg albumin was assumed to be the same as K for vicilin. Using the molecular weight of egg albumin as 45,000 and a = 0.67 the K could be calculated.

The value obtained for the molecular weight of

vicilin was 192,000.

It must be made clear that the assumptions used are subject to a great deal of criticism.

In summary, viscosity measurements on plant proteins were encouraging but the Ostwald viscometer was found to have limitations for this type of work.

APPENDIX II

The results of the first ninety runs on egg albumin at the isoelectric point are listed below.

Run No.	Molecular Wt.	Run No.	Molecular Wt.	Run No.	Molecular Wt.
1	*	31	48,880	61	45,600
2	73,500	32	*	62	80,400
3	70,000	33	42,500	62A	60,000
4	65,500	34	48,500	63	79,000
5	79,700	35	49,500	64	66,500
6	126,500	36	48,500	65	61,800
7	59,600	37	48,300	67	60,600
8	99,500	38	52,000	68	*
9	132,000	39	51,500	69	*
10	206,000	40	48,500	70	50,300
11	120,000	41	45,600	71	29,900
12	154,000	42	46,400	72	21,100
13	214,000	43	*	73	48,000
14	150,000	44	*	74	68,100
15	High	45	55,700	75	155,000
16	71,000	46	*	76	*
17	106,500	47	*	77	*
18	*	48	*	78	45,900
19	52,000	49	47,500	79	*
20	*	50	56,000	80	60,000
21	82,500	51	62,000	81	47,000
22	75,500	52	60,000	82	48,700
23	43,200	53	46,100	83	*
24	60,000	54	87,500	84	51,000
25	68,200	55	49,900	85	31,000
26	48,100	56	52,800	86	42,600
27	*	57	44,400	87	40,000
28	*	58	53,300	88	44,000
29	67,700	59	55,000	89	51,300
30	42,200	60	60,000	90	46,200

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