THE PREPARATION OF THE GRADED COLLODION MEMBRANES OF ELFORD AND THEIR USE IN THE STUDY OF FILTERABLE VIRUSES

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(Accepted for publication, April 18, 1934)

During the past 50 years many attempts have been made to develop a method for preparing permeable collodion membranes of graded and uniform pore size. Such membranes would constitute an exceedingly important apparatus in biological investigation and should be especially useful in the study of filterable viruses.

The method of preparation which has been most extensively used is that of Bechhold (1–3), consisting of impregnating filter paper with solutions of nitrocellulose in glacial acetic acid, the permeability being regulated by the concentration of nitrocellulose used. These membranes have the serious defect of considerable variability in the size of pores; Elford (4) estimates that the largest pores in such a membrane may have a radius ten to twenty times greater than the average pore radius.

A number of investigators have attempted to devise a method by which the use of filter paper could be eliminated and strong membranes of graded and uniform porosity be prepared. Bigelow (5), Walpole (6), Bartell and Carpenter (7), Hitchcock (8), Pierce (9), and others have controlled the permeability of membranes by varying the time allowed for evaporation of solvents from an ether-alcohol solution of collodion. Eggerth (10) graded the membrane permeability by varying the alcohol content of the collodion solution. Brown (11, 12) and also Nelson and Morgan (13) treated air-dried collodion membranes with different concentrations of alcohol in water, and so effected variation in permeability. Among non-volatile reagents added to ether-alcohol solutions of collodion for the purpose of altering membrane permeability are ethylene glycol (Pierce (9)), and glycerol (Schoep (14)). Asheshov (15) was the first to utilize volatile reagents, adding varying amounts of acetone to increase, or amyl alcohol to decrease, membrane permeability. In later work he recommends the use of acetic and formic ethers (16), and states that the results obtained by adding amyl alcohol are unreliable.

Elford (4), using the same ingredients as Asheshov, found that acetone and

amyl alcohol are mutually "antagonistic" in their solvent action on nitrocellulose. While either one in conjunction with alcohol and ether is an excellent solvent, the presence of both, combined in certain concentrations, results in coagulation of nitrocellulose. Consequently he prepared a concentrated solution of nitrocellulose in ether and alcohol and to this added certain fixed proportions of amyl alcohol and acetone. When this solution was poured on glass plates and the solvents were allowed to evaporate, the amyl alcohol, being the least volatile, increased in relative concentration and in the presence of acetone initiated an aggregation of the nitrocellulose, while the residual ether and alcohol maintained a spontaneous gelling process. The resulting membranes were highly permeable and had considerable tensile strength. Elford also found that the addition of small amounts of glacial acetic acid to the collodion solution decreased the permeability of the membranes, while the addition of water increased it. Utilizing these findings he devised a very satisfactory method by which finely graded membranes can be prepared, having adequate tensile strength, and ranging in size from 10 mμ to 3 μ in average pore diameter.

A need of membranes suitable for ultrafiltration has been felt in this laboratory for some time. After a review of the various methods recommended for the preparation of graded collodion membranes, Elford's method appeared to be the most practical. Consequently it was selected for study.

EXPERIMENTAL

Preparation of Stock Collodion

A small supply of Necol, the collodion preparation recommended by Elford, was secured from England and was used in early experiments. Considerable difficulty was encountered at the beginning in attempts to prepare membranes similar to those he described. Although his technique was followed as closely as possible, it was found impossible to produce membranes of a similar quality on successive attempts. Occasionally membranes satisfactory in all properties were obtained; however, using the same collodion mixture and duplicating the procedure as closely as possible, membranes of an entirely different quality would result. After some experimentation and a study of all the factors involved, we eventually succeeded in preparing consistently satisfactory membranes from Necol, and thus confirmed Elford's results.

Necol cannot be purchased in this country, and when our original supply became exhausted a new lot was ordered from England. After considerable delay, it was supplied to us from the firm of Du Pont De Nemours, of Parlin, New Jersey, to which our order apparently had been referred. Their product was labelled Collodion X-660-18 and showed the same consistency and appearance as Necol. Membranes made from it were similar in quality to those made from Necol.

Although membranes made from Necol or Collodion X-660-18 were quite satisfactory as far as graded permeability was concerned, their thickness was

excessive, ranging from 0.2 mm. to 0.3 mm. As thinner membranes are desirable Collodion X-660-18 was diluted with one-half its weight of a solvent consisting of 75 per cent anhydrous ether and 25 per cent absolute alcohol by weight. This dilution was made before any acetone or amyl alcohol was added. The results were unsatisfactory since the membranes obtained were very friable and were difficult to remove from the plates. Also, their porosity could not be regulated by the addition of glacial acetic acid.

Inasmuch as the identity and proportions of the solvents used in commercial collodion preparations such as Necol and Collodion X-660-18 are unknown to us, it seemed advantageous to prepare a stock collodion of known chemical constitution which could be diluted, if desired, without danger of disturbing the effective equilibrium between ingredients. After some experimentation a collodion was prepared which was found to give consistently good results. The most convenient form of nitrocellulose was found in Du Pont's parlodion, which is sold in shreds immersed in water. The solvent selected consisted of 75 per cent anhydrous ether and 25 per cent absolute alcohol, by weight. The exact composition of the collodion is as follows:

Parlodion shreds	150 gm.
Absolute alcohol	250 "
Anhydrous ether	750 "
Acetone	1150 "
Amyl alcohol (primary)	575 cc.

The parlodion shreds are washed six times in distilled water, twice in 95 per cent alcohol, and twice in absolute alcohol. They are not dried after washing. In early experiments dried shreds were used. The resulting solutions were quite turbid and, on standing, showed a quantity of precipitate. Membranes made from these solutions were found to be less permeable than if drying had been omitted.

In our early experiments the chemicals used for solvents were subjected to additional purification, as recommended by Elford (4). It was later found that untreated chemicals of the analytical reagent grade gave equally satisfactory results: consequently the additional purification was thereafter omitted.

After the final washing, 250 gm. (316 cc.) of absolute alcohol, freshly prepared by drying 95 per cent alcohol over lime and distilling, is added to the parlodion and the shreds allowed to swell overnight. On the following day 750 gm. (1043 cc.) of anhydrous ether is added and the mixture shaken at intervals until the parlodion is completely dissolved. The solution is then diluted with its own weight of acetone (1453 cc.) and agitated in a mechanical shaker for 2 hours. Primary amyl alcohol is then added at the rate of 10 cc. to each 40 gm. of the mixture (575 cc.) and the solution is again shaken for 2 hours. This stock solution must be allowed to stand 2 to 3 weeks before use. To date seven different solutions have been prepared by this method and all have given uniformly satisfactory results.

It will be noticed that acetone and amyl alcohol are used in the same proportion as recommended by Elford (4).

Recently we obtained a supply of parlodion manufactured by the Mallinckrodt Chemical Works. This is sold in the dry state although the shreds have the same appearance as the Du Pont product. A solution was prepared using this product as the source of nitrocellulose and following the above mentioned procedure, with the exception that the washing of the parlodion was omitted. The resulting collodion was water-clear, and the membranes prepared from it were of excellent quality.

Preparation of Membranes

The apparatus used in the preparation of membranes is similar to that described by Elford and is illustrated in Fig. 1. For routine purposes the membranes are cast in glass cells 40 cm. in diameter. The cell is made from two pieces of plate

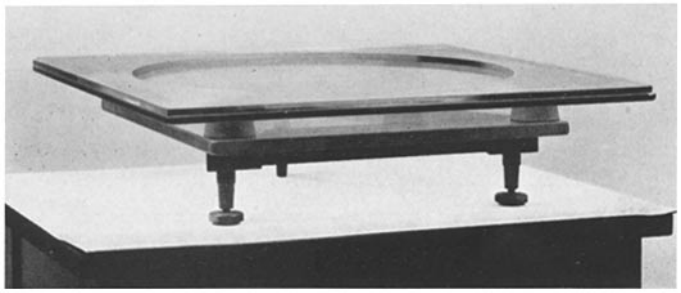

FIG. 1. Cell and stand for preparation of membranes

glass, each 50 cm. square and 7 mm. thick. A circular hole 40 cm. in diameter is cut in the center of one plate and the two plates are cemented together with egg white.

The levelling table for supporting the cell is made of a piece of plate glass 15 inches square and 5/8 inch thick. This is fastened to a heavy metal triangle supported at one corner by a solid leg and at the remaining two corners by adjustable screws. To insulate the cell from the levelling table and to allow a free air circulation between them, four large rubber stoppers are placed at the extreme corners of the levelling table and on them the cell rests. If the cell lies directly on the levelling table, its temperature becomes uneven during the evaporation process, owing to retention of heat by the metal triangle. This in turn results in uneven permeability in the membrane. The cell is carefully levelled by aid of the adjustable screws, making observations with two sensitive spirit levels placed at right angles, thus insuring against unequal distribution of the collodion.

The stock collodion solution is diluted with an equal volume of a diluent con-

sisting of one part by weight of absolute alcohol to nine parts by weight of anhydrous ether. The diluted solution is measured in 200 cc. amounts into a series of 6 ounce bottles having screw caps lined with tin-foil. Membranes prepared from this solution will have an average pore diameter of 0.6 to 0.8μ, depending on the humidity and temperature at which the solvents are allowed to evaporate. If more, or less, permeable membranes are desired, water or glacial acetic acid is added to the solution. The addition of 2 cc. of water to 200 cc. of the diluted collodion gave membranes with an average pore size of 1.0 to 1.2μ. If larger quantities of water are added considerable precipitate is formed and the resulting membranes have but little tensile strength. When membranes of less than 0.5μ average pore size are desired, glacial acetic acid (analytical reagent grade) is added in amounts varying from 0.2 to 3.6 cc. for each 200 cc. of diluted collodion. As indicated above, conditions other than the chemical constitution of the diluent have considerable influence on the membrane permeability; therefore it is impossible to establish a fixed scale of the exact quantities of ingredients necessary to produce a membrane of a given pore size. In our experience, 1 cc. of glacial acetic acid added to 200 cc. of the diluted collodion solution, reduced the average pore size to 200 mμ. The addition of 2 cc. reduced it to 100 mμ, while the addition of 3 cc. reduced it to 15 mμ.

After the addition of the required amount of acetic acid or water, the bottles are placed in a shaking machine and agitated for 2 hours. The collodion solution is then carefully poured into the center of the levelled cell, where it quickly spreads over the entire surface. The contents of one bottle (200 cc.) is used for each 40 cm. cell. The optimum evaporation time was found to be 75 minutes. If this time is prolonged the membranes become less permeable and the surface becomes corrugated. Shorter periods of time yield rather thick membranes of low tensile strength and spongy consistency. The temperatures during the evaporation ranged from 22–24°C., and the relative humidity from 60 to 65 per cent.

The membranes are prepared in a room measuring 12 by 24 feet with a ceiling 11 feet high. The temperature is regulated by opening or closing the steam radiators. The desired humidity is obtained by running hot water from the faucets. The doors and windows must be kept shut as it was found that air movement over the cell during the evaporation time resulted in the formation of a membrane, one part of which was more permeable than the other. During the summer months when the outdoor temperature exceeded 24°C., no satisfactory membranes could be prepared. To assure a fair degree of reproducibility in membrane characteristics both the temperature and humidity must be kept constant. Since the temperature of the collodion solution falls considerably below that of the room during the evaporation process, moisture from the air is condensed into the collodion solution. This absorbed water increases the coagulation of the nitrocellulose, and, therefore, increased atmospheric humidity will result in increased membrane permeability. Likewise, drier air will cause decreased permeability.

After the collodion solution has evaporated for the standard length of time, the

cell containing the membrane is submersed in tap water in a tank measuring 55 by 55 by 15 cm. The membrane separates itself completely from the glass after 5 to 10 minutes immersion. The membranes are then washed for periods of 2 to 3 weeks in distilled water. For this purpose photographic trays measuring 45 by 50 cm. are used. Six membranes, separated from each other by large filter papers, are placed in each tray. The distilled water is changed daily.

When the washing is completed, discs are cut from the membranes by means of a steel punch. The portion of the membrane within 3 cm. from the edge is discarded. Our standard size disc used for ultrafiltration measures 38 mm. in diameter. Consequently one large membrane yields 40 to 45 small discs. These are stored under water in Atlas E-Z seal fruit jars without the addition of preservative. The membranes will keep well in distilled water for a number of months. It was found, however, that on standing some membranes shrink considerably and become less permeable, while others remain unchanged for a period of 6 months or longer. It is therefore advisable to recalibrate membranes which have been stored for a month or more before using them for filtration experiments.

Calibration of Membranes

The average pore sizes of the membranes are calculated by an application of Poiseuille's law, using data obtained by measurement of the thickness of the membrane, the amount of water passing through a measured area in a given time and under a known hydrostatic pressure, and the volume of pores in the membrane as shown by differences between its wet and dry weight. This law was derived to express the rate of flow through one capillary tube of a uniform diameter and was first applied to the calculation of pore diameters by Guérout (17), who tested the size of the pores in bladder, gold beater's skin, and parchment. Since then it has been applied to the determination of the size of pores in collodion membranes by Hitchcock (18), Bjerrum and Manegold (19), Elford (20), and Cox and Hyde (21), among others. The legitimacy of its application to this purpose has been investigated by Bigelow (5) and by Duclaux and Errera (22). Both investigations indicated that the passage of fluids through collodion membranes is controlled by the same laws which determine the passage of liquids through capillary tubes.

Calculation of Average Pore Size

Poiseuille's law governing the passage of water through a capillary tube is stated as

(1) $$v = \frac{\pi p r^4}{8 l \eta} t$$

where V is the volume of water passing in the time t under a pressure p, r the radius of the capillary, l the length of the capillary, and η the coefficient of viscosity of water at the temperature used.

To apply the law of Poiseuille to the measurement of the average pore size of membranes, it becomes necessary to assume that the membrane represents a bundle of capillaries oriented at right angles to the membrane surface and equal in length to the thickness of the membrane. To compute the number of pores the following calculations may be made: If l represents a length of the capillaries (equivalent to the thickness of the membrane), then the volume of each capillary is $\pi r^2 l$. If W^2 represents the weight of the membrane when wet, and W^1 represents the weight of the same membrane when dry, the differences designated as W must represent the volume of pores. Consequently $\frac{W}{l}$ must represent the total cross-section area of all the pores, and since the area is πr^2, $\frac{W}{\pi r^2 l}$ represents the number of capillaries. Now if V is the amount of water passed through the membrane in the time t, then $\frac{V}{\frac{W}{\pi r^2 l}}$ will represent the volume of water passed through one capillary, or the sum of the volumes may be expressed by Poiseuille's law as given in Equation 1. Transposing this formula to solve for r we obtain

(2) $$r^2 = \frac{8 l^2 \eta v}{w p t}$$

or, simplifying,

(3) $$r = 2l \sqrt{\frac{2\eta v}{w p t}}$$

Equation 3 is the one used for determining the average pore size of membranes. r, the radius of the average pore, and l the thickness of the membrane, are expressed in centimeters. Since the measurement of rate of flow of water is done at room temperature, η, the coefficient

of viscosity of water, is taken as 0.00893. V, the volume of water passing through the membrane is expressed in cubic centimeters, and t, the time required for this passage, is expressed in seconds. W, the difference between the wet and dry weight, is expressed in grams and must be corrected to represent an area of 1 sq. cm. The value V must be similarly corrected. p, the average pressure producing the flow, is expressed in dynes, and since 1 cm. water pressure is equivalent to 980.6 dynes, the value becomes 980.6 × average pressure in centimeters of water. The final formula then becomes:

$$(4) \qquad r = 2l \sqrt{\frac{2\left(\begin{matrix}\text{Coefficient of viscosity}\\ \text{of water at } 25^\circ\end{matrix}\right)\left(\dfrac{V}{2\pi\,(\text{radius of disc})^2}\right)}{\left(\begin{matrix}980.6\ (\text{Average pressure in}\\ \text{centimeters of water})\end{matrix}\right) t \left(\dfrac{\text{Wet weight} - \text{dry weight}}{2\pi\,(\text{radius of disc})^2}\right)}}$$

Assigning concrete values, where the rate of flow was measured through an area of membrane 1.575 cm. in diameter and the difference in wet and dry weight was determined for a membrane 3.15 cm. in diameter, the final formula is:

$$(5) \qquad r = \frac{2}{10} \sqrt{\frac{2 \times 0.00893}{\left(\dfrac{1.575}{3.15}\right)^2} \cdot \frac{1}{980.6} \cdot l \sqrt{\frac{v}{wpt}}}$$

Since the values in the first part of this equation are fixed, they can be represented by a constant K, and the working formula simplified to:

$$(6) \qquad r = Kl \sqrt{\frac{v}{wpt}}$$

K, constant for fixed condition of measurements.
l, thickness in millimeters.
v, volume of water passed through membrane in cubic centimeters.
W, difference in weight in grams.
p, average hydrostatic pressure in centimeters of water.
t, time in seconds.

Logarithms are most conveniently used in all computations.

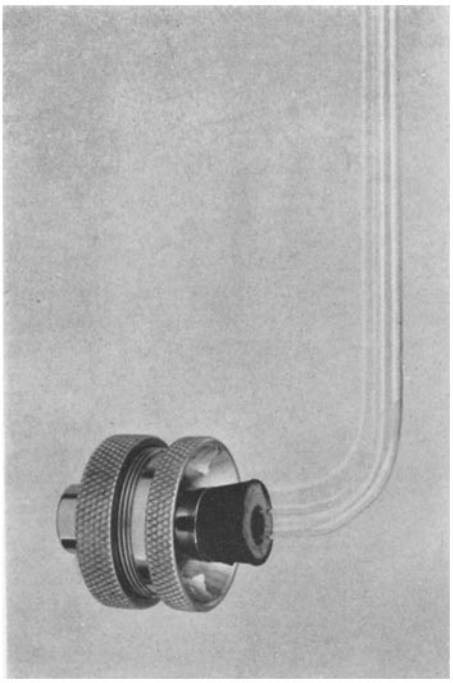

FIG. 2. Instrument used for determining the rate of flow of water

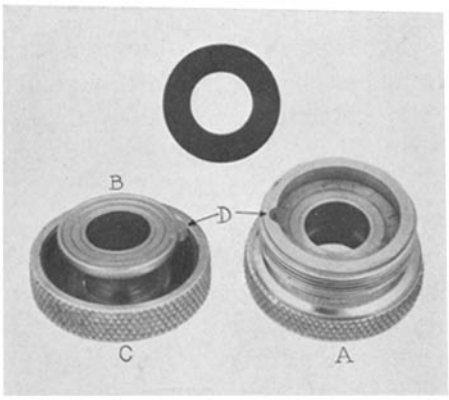

FIG. 3. Instrument used for determining the rate of flow of water, showing details.

Determination of Average Pore Size

To obtain the data for calculations, three determinations must be made on each membrane; the thickness, the rate of flow of water, and the difference between wet and dry weights.

The thickness is measured by use of a micrometer caliper, such as is used industrially in determining the thickness of paper. It is graduated to 0.01 mm. The average of fifteen to twenty measurements is used in calculations.

The apparatus used for determining the rate of flow is indicated in Fig. 2. The glass tube is a Folin microburette as used in blood sugar determinations, with a capacity of 5 cc., graduated to 0.02 cc. but readable to 0.01 cc. The glass stop-cock is cut off and a right angle tube fused on in its place, this tube being of such a length that the distance from the 0.00 graduation to the center of the bend is 100 cm. The details of the apparatus designed to hold the membrane are shown in Fig. 3. The diameter of the orifice through which the water passes is 15.75 mm.; consequently this becomes the diameter of the membrane disc tested and is a value used in computation. To determine the rate of flow, the membrane to be tested is cut to the appropriate size with a steel punch and placed in the depression in *A*. The rubber washer, also with a hole 15.75 mm. in diameter, is placed above it. The flange *B* is then placed over it with the locking device *D* fitted together so as to insure against damage to the membrane by rotation of *B*. The collar *C* is then tightened and the rubber stopper on the burette secured in place. This last operation is done with the holder submerged in water to prevent the formation of air bubbles. The burette is then filled about 1 cm. above the zero mark with water and the level is allowed to fall to 0.00. When that point is reached, a stop-clock is started. After an appropriate time the volume of water passed is noted. For membranes between 40 and 200 mμ, this time is usually 10 minutes. Membranes with a pore diameter of less than 10 mμ may require as long as 18 hours before the passage of sufficient water to give an accurate reading. The average pressure producing the flow is determined by measuring the distance from the zero mark on the burette to the point where the reading was made. Since the zero mark represents a pressure of 100 cm., the average pressure be-

comes (100 minus half the distance of fall). A volume/pressure curve can profitably be constructed by which average pressures can be read directly as a linear function of volume. For determining the rate of flow through membranes with an average pore diameter of more than 200 mμ, a 50 cc. burette is used in the same manner.

The water content of the membrane (equivalent to the pore volume) is determined by weighing the same membrane in the wet and dry states. Discs 3.15 cm. in diameter are blotted with absorbent paper to remove surface water and are rapidly weighed in small, covered, Petri dishes using an analytical balance. They are then dried for 48 hours over sulfuric acid in a vacuum desiccator, and reweighed.

Since sterile membranes are required for work with viruses and since a slight contraction of the membrane takes place during sterilization, all the measurements given above are made on membranes which have been sterilized by steaming for 1 hour in an autoclave at 0 pressure.

Determination of Maximum Pore Size

There is no satisfactory method available by which the size of the largest pores in a membrane can be estimated. Elford (4) recommends a method based on the application of Cantor's formula

$$r = \frac{2\sigma}{p}$$

where r is the pore radius, σ the surface tension air/water, and p the pressure required to force air through the wet membrane, as indicated by the escape of air bubbles from a submersed membrane under a measured air pressure. In our experience, this method was found applicable only to very permeable membranes and even then readings could be obtained accurate only to a pressure of about 5 pounds per sq. in. To force air through a water-filled capillary 50 mμ in diameter several hundred pounds pressure per square inch is required, which is much in excess of the strength of any membrane. To reduce this pressure Bechhold, Schlesinger, and Silbereisen (23) substitute isobutyl alcohol and water mixture for the air/water system. The system (isobutyl alcohol saturated with water/water saturated with isobutyl alcohol) has a surface tension of 1.85 dynes per sq. cm., as

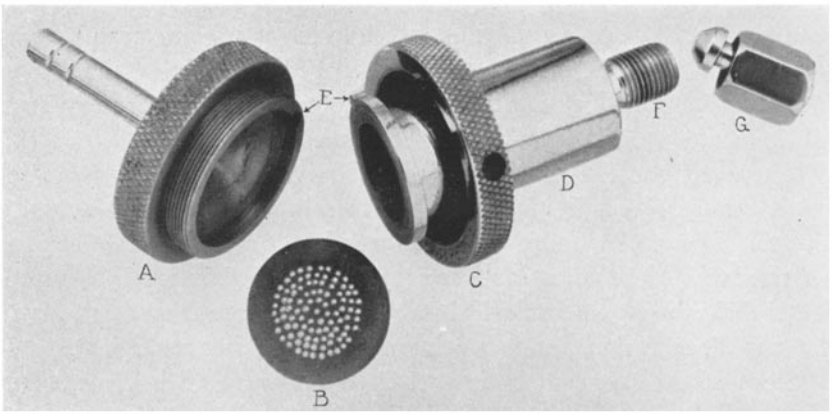

FIG. 4. Metal filter showing details of different parts

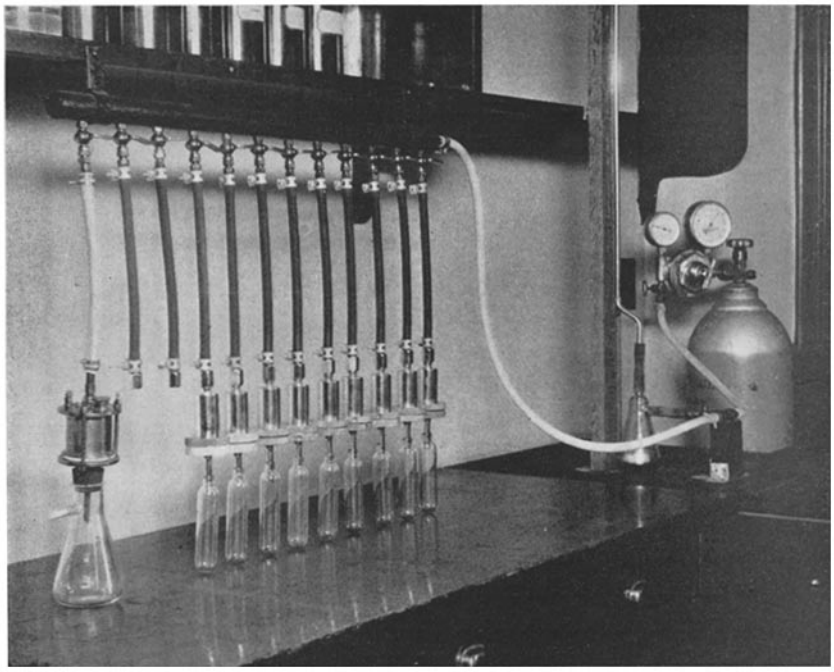

FIG. 5. Arrangement of apparatus used in filtration

contrasted with 73 dynes for the air/water system, and consequently correspondingly lower pressures can be used. However, it was found that collodion membranes exposed to isobutyl alcohol became swollen and softened and consequently the method could not be applied.

The Use of Membranes in Ultrafiltration

The filters used are similar to those described by Elford (20) and are illustrated in Fig. 4. The body of the filter and the collar are made of brass and are chromium plated. The perforated plate, intended to support the membrane, is made of monel metal or stainless steel. A rubber washer with a hole 27 mm. in diameter is prepared from a special rubber sheeting, 1.5 mm. thick, manufactured for use as gaskets in hot water lines. Ordinary laboratory rubber sheeting is unsatisfactory, since it does not stand autoclaving. Discs of hard filter paper (Schleicher and Schüll No. 575) cut to the same size as the membrane discs (38 mm. diameter) are also required. All parts of the filter except the membrane itself are sterilized by autoclaving; the membrane is sterilized by steaming for 1 hour.

In practice the procedure is as follows: The metal plate *B* is placed in the portion of the filter designated as *A*. On this is placed the disc of hard filter paper, which protects the membrane and prevents it from stretching, and also permits filtration throughout the whole area of the membrane. Without the paper, filtration will take place only in the areas directly over the perforations in the plate. The membrane is placed directly over the paper and on this is placed the rubber washer previously described. The barrel of the filter, *D*, is now put in place, with the projection *E* fitting into a corresponding seat in the stem portion, thus preventing rotation. The collar, *C*, is tightened with a wrench, during which process the stem portion, *A*, is secured in a vise. The material to be filtered is introduced through the stem, *F*, which is threaded to take the tapered compression fitting, *G*.

Filtrations are carried out under positive pressure, using an arrangement shown in Fig. 5. The manifold has twelve outlets, each provided with a pet-cock, a length of high pressure rubber tubing, and the compression coupling shown under *G* in Fig. 4. The manifold is connected to a nitrogen tank through a reducing valve, with a mercury manometer between the reducing valve and manifold. For pressures

up to 100 cm. of mercury, the manometer is used. When higher pressures are required, the valve connecting the manometer is closed and the pressure read directly from the reducing valve gauge.

With this arrangement the material to be filtered can be passed through a series of membranes of varying average pore diameters under uniform conditions of pressure.

Properties of Membranes as Shown by Ultrafiltration Experiments

Although there is no satisfactory method by which the sizes of the largest and smallest pores in a membrane can be determined, the sharp, clear-cut end-points observed in our filtration experiments suggest that the pores in any membrane are remarkably uniform in size.

Inasmuch as these membranes are intended primarily for the study of filterable viruses, the virus of yellow fever was employed in an investigation of their filtration properties. As shown by Bauer and Mahaffy (24) and by Dinger (25) this virus becomes progressively inactive when suspended in a protein-free medium, such as distilled water or saline solution. It therefore becomes necessary to utilize diluents containing a considerable amount of protein, and consequently having a relatively high viscosity. Filtration of such solutions through collodion membranes affords an excellent opportunity to study adsorption phenomena and other factors involved in the filtration of viruses.

The virus-containing material used consisted with a few exceptions of animal tissue ground in a mortar and suspended in a diluent which had been previously tested and found to sustain activity for an adequate length of time. This suspension is centrifuged at high speed for 30 minutes and the supernatant decanted off and passed through a Seitz filter. When passage through membranes with an average pore diameter of less than 75 mμ is required, the Seitz filtrate is not suitable for use, since it contains particles large enough to clog the membrane pores. Consequently it is necessary to pass this filtrate through a collodion membrane with an average pore diameter of about 250 mμ before passing it through the less permeable membranes. It was found that a preliminary passage of hormone broth through the Seitz filter pad greatly reduced the adsorption of virus. Attempts to utilize the sand and paper pulp filter described by Barnard and Elford

(29) for preliminary filtration were unsuccessful, since the filter invariably became clogged after the passage of a few cubic centimeters of virus-containing suspensions.

As emphasized by Elford (26), the adsorption capacity of the membrane for proteins is greatest near the isoelectric range of the protein and is diminished as the reaction becomes more alkaline. Since most viruses are undamaged in pH ranges from 7.0 to 9.0, this range is most useful for filtration. The adsorption of protein from the virus-containing material can be to a large extent eliminated by passing a small amount of alkaline (pH 8.0) hormone broth through the membrane prior to the filtration. That the passage of broth facilitates filtration was first demonstrated by Ward and Tang (27), and its use in ultrafiltration is strongly recommended by Elford (26). We have observed, however, that a preliminary passage of broth through membranes alters the filtration end-point. A series of tests were made in which broth was incorporated into a diluent for the yellow fever virus together with some protein such as serum. As will be shown in another communication, when the preliminary passage broth was omitted the end-point for the virus was invariably found to be 55 mμ, a figure in agreement with the results of Findlay and Broom (28). When the virus was suspended in a medium exactly similar, but passed through membranes which had previously been treated with broth, the end-point was reduced to 50 mμ.

To secure information as to the uniformity of pore size in a given membrane and also the range of particle size of the virus, titrations of the virus content were made with a series of membrane filtrates as indicated below. Infected brains were ground up in a mortar, suspended in a suitable diluent, and passed first through a Seitz filter and then through a membrane with an average pore size of 250 mμ. Such filtrates are usually infective when 0.02 cc. is inoculated intracerebrally into mice in a dilution of 1:100,000. It was found that filtrates passing membranes with average pore diameters of 70, 66, and 60 mμ were infective in a dilution of 1:10,000, while 55 mμ filtrates were infective in a dilution of 1:1000 and a 50 mμ filtrate infective in a dilution of 1:100. Filtrates passing membranes with average pore diameters of 45 and 40 mμ contained no demonstrable virus. These results indicate that there is a remarkable uniformity

both in the size of the virus-containing particles and in the pore diameters.

It was observed that adsorption of protein by the membranes was greater when the diluent contained 5 to 10 per cent serum than if it contained 25 to 50 per cent ascitic fluid. Three samples of ascitic fluid were tested for protein content and were found to vary from 0.6 to 5.0 per cent. In a majority of the filtration experiments the protein content of the stock filtrate was determined and was compared with that of the membrane filtrates. Where the protein content of the filtrates was greatly reduced the acidity was frequently excessive, due to loss of buffering material. The use of Esbach tubes and Tsuchiya's reagent was found most convenient for these estimations. When virus suspensions were filtered through membranes having an average pore diameter of less than 75 mμ, some loss of protein always resulted even though broth had previously been passed through the membrane. Membranes made from the identical stock collodion solution under identical conditions may differ markedly in their adsorptive capacity. A few were encountered which held back practically all protein and so had to be discarded. We have not yet determined the factors responsible for such variations.

Obviously, membranes cannot operate in an uncomplicated sieve-like manner. Even though they are as thin as paper, the ratio of the length of the pore to its diameter is enormous. The virus of yellow fever passes through a membrane which has an average pore diameter of 50 mμ and measures 0.15 mm. in thickness. Comparing the pore diameter of 50 mμ to its length of 150,000 mμ, the ratio is 1 to 3000. If we consider the surface and capillary forces which operate in such a long and narrow channel, it becomes apparent that a particle able to transverse this passage must have a diameter considerably smaller than that of the pore diameter. Elford (26) has discussed this matter fully and from the results of quantitative experiments with particles of known size concludes that membranes with average pore diameters ranging from 10 to 100 mμ must have pore diameters of two to three times the diameter of the particle to permit its passage. In membranes having an average pore diameter of less than 10 mμ, or more than 100 mμ, this ratio is decreased.

It appears from Elford's observations, as well as our own, that a rela-

tively large group of viruses has a filtration end-point between 25 and 100 mμ. No observations have as yet been made establishing the pore/particle ratio using proteins of known molecular sizes within these limits, since only one protein substance, hemocyanin of the snail, *Helix pomatia*, (Svedberg and Chirnoaga (30)), with a spherical molecule of suitable size is known. To obtain data applicable to this group of viruses, previous investigators have tested suspensions of metal sols and aniline dyes. Since the colloidal behavior of these substances differs radically from that of proteins, we believe that the results so obtained may not be strictly applicable to biological materials.

Two proteins having molecular sizes smaller than the size of most of the viruses were tested for filtration end-points. Crystalline ovalbumin was prepared by the method of Hopkins and Pinkus (31) and was purified by recrystallization. Oxyhemoglobin was prepared from the blood of sheep and monkeys by washing cells three times in saline, laking them with distilled water, and oxidizing by the passage of air. A mixture for ultrafiltration was made as follows:

5 per cent hemoglobin suspension	25.0 cc.
Hormone broth, pH 8.0	20.0 "
4 per cent crystalline ovalbumin	20.0 "
Phosphate buffer, M/15, pH 8.4	20.0 "
Distilled water	10.0 "
1 per cent chinosol solution	5.0 "

Since filtration through membranes with average pore diameters capable of holding back albumin is extremely slow, the chinosol was added as a preservative, being especially suitable for this purpose since it does not coagulate protein. The mixture was passed through a Seitz filter and then through a membrane of 400 mμ and, finally, one of 85 mμ average pore diameter. This stock filtrate was divided into portions and passed through membranes ranging from 9 mμ to 2.3 mμ average pore diameter under a pressure of two atmospheres. The filtration period was 18 or 19 hours. The results of the experiment are shown in Tables I and II.

The oxyhemoglobins from the sheep and monkey were similar in size, passing through pores of 8 mμ but not passing pores of 7.0 mμ diameter. The albumin in each case passed through pores of 6.2,

but not through 4.2 mμ diameter. The albumin was detected by the precipitin reaction, while hemoglobin was recognized by its color.

Our results obtained on filtration of ovalbumin are in full agreement with those of Elford (26), who also found the end-point to be 6 mμ.

TABLE I

Ultrafiltration Experiment with Oxyhemoglobin from Sheep and Recrystallized Egg Albumin

No. of membrane	Average pore diameter	Amount of filtrate collected	Filtration time	Presence in filtrates	
				Oxyhemoglobin	Albumin
	mμ	*cc.*	*hrs.*		
128	9.0	7.0	18	+++	+++
125	8.0	5.5	18	+++	+++
120	7.0	5.0	18	0	+++
143	6.2	6.5	18	0	++
144	4.6	6.5	18	0	0
145	3.7	6.0	18	0	0
146	3.2	6.0	18	0	0
156	2.3	4.0	18	0	0

TABLE II

Ultrafiltration Experiment with Oxyhemoglobin from Monkey and Recrystallized Egg Albumin

No. of membrane	Average pore diameter	Amount of filtrate collected	Filtration time	Presence in filtrates	
				Oxyhemoglobin	Albumin
	mμ	*cc.*	*hrs.*		
128	9.0	7.5	19	++++	+++
125	8.0	5.5	19	+++	+++
120	7.0	6.0	19	0	+++
143	6.2	7.5	19	0	++
144	4.6	7.0	19	0	0
145	3.7	6.0	19	0	0
146	3.2	4.5	19	0	0
156	2.3	3.5	19	0	0

The results obtained with oxyhemoglobin, however, differ somewhat. Elford used hemoglobin from horse blood, and found the end-point to be 10 mμ. Consequently he assigns a pore/particle ratio of 2/1

for hemoglobin and 1.5/1 for ovalbumin. The size of the oxyhemoglobin molecule has been determined by Northrop and Anson (32), using diffusion measurements, and by Svedberg (33), using ultracentrifugal analysis. The results obtained are almost identical, indicating that the hemoglobin molecule has a dissymmetry ratio of 1.25 and a diameter of about 5 mμ. Svedberg also determined the molecular size of ovalbumin and found it to be spherical with a diameter of 4.34 mμ. Using these values in connection with our filtration results, we find a pore/particle ratio of 1.5/1 for both ovalbumin and oxyhemoglobin.

We have had occasion to carry out filtration experiments with several viruses which have been studied by Elford, and the filtration endpoints are found to be in close agreement. These results will be published in later communications.

SUMMARY

1. The method described by Elford for the preparation of graded collodion membranes suitable for ultrafiltration was found to give excellent results, and his findings are fully confirmed.

2. A formula is given for the preparation of collodion from which satisfactory membranes of graded porosity can be prepared.

3. The technique and apparatus used in the preparation, and standardization of membranes are described in detail.

4. The technique and apparatus required for ultrafiltration experiments are described, and some drawbacks encountered in the experiments are discussed.

5. The results of ultrafiltration experiments show that the pores of the membranes are remarkably uniform in size.

BIBLIOGRAPHY

1. Bechhold, H., *Z. phys. Chem.*, 1907, **60**, 257.
2. Bechhold, H., *Z. phys. Chem.*, 1908, **64**, 328.
3. Bechhold, H., *Biochem. Z.*, Berlin, 1908, **6**, 379.
4. Elford, W. J., *J. Path. and Bact.*, 1931, **34**, 505.
5. Bigelow, S. L., *J. Am. Chem. Soc.*, 1907, **29**, 1675.
6. Walpole, G. S., *Biochem. J.*, London, 1915, **9**, 284.
7. Bartell, F. E., and Carpenter, D. C., *J. Phys. Chem.*, 1923, **27**, 252.
8. Hitchcock, D. I., *J. Gen. Physiol.*, 1925, **8**, 61.

9. Pierce, H. F., *J. Biol. Chem.*, 1927, **75**, 795.
10. Eggerth, A. H., *J. Biol. Chem.*, 1921, **48**, 203.
11. Brown, W., *Biochem. J.*, London, 1915, **9**, 591.
12. Brown, W., *Biochem. J.*, London, 1917, **11**, 40.
13. Nelson, J. M., and Morgan, D. P., *J. Biol. Chem.*, 1923–24, **58**, 305.
14. Schoep, A., *Z. Chem. u. Ind. Kolloide*, 1911, **8**, 80.
15. Asheshov, I.-N., *Compt. rend. Soc. biol.*, 1925, **92**, 362.
16. Asheshov, I.-N., *J. Bact.*, 1933, **25**, 323, 339.
17. Guérout, M., *Compt. rend. Acad.*, 1872, **75**, 1809.
18. Hitchcock, D. I., *J. Gen. Physiol.*, 1926, **9**, 755.
19. Bjerrum, N., and Manegold, E., *Kolloid-Z.*, 1927, **42**, 97.
20. Elford, W. J., *Brit. J. Exp. Path.*, 1929, **10**, 126.
21. Cox, H. R., and Hyde, R. R., *Am. J. Hyg.*, 1932, **14**, 667.
22. Duclaux, J., and Errera, J., *Rev. gên. colloïdes*, 1924, **2**, 130.
23. Bechhold, H., Schlesinger, M., and Silbereisen, K., *Kolloid-Z.*, 1931, **55**, 172.
24. Bauer, J. H., and Mahaffy, A. F., *Am. J. Hyg.*, 1930, **12**, 175.
25. Dinger, J. E., *Zentr. Bakt., 1. Abt., Orig.*, 1931, **121**, 194.
26. Elford, W. J., *Proc. Roy. Soc. London, Series B*, 1933, **112**, 384.
27. Ward, H. K., and Tang, F. F., *J. Exp. Med.*, 1929, **49**, 1.
28. Findlay, G. M., and Broom, J. C., *Brit. J. Exp. Path.*, 1934, **14**, 391.
29. Barnard, J. E., and Elford, W. J., *Proc. Roy. Soc. London, Series B*, 1931, **109**, 360.
30. Svedberg, T., and Chirnoaga, E., *J. Am. Chem. Soc.*, 1928, **50**, 1399.
31. Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898, **23**, 130.
32. Northrop, J. H., and Anson, L. M., *J. Gen. Physiol.*, 1929, **12**, 543.
33. Svedberg, T., *Kolloid-Z.*, 1930, **51**, 10.