MULTI-MEMBRANE ELECTRODECANTATION AND ITS APPLICATION TO ISOLATION AND PURIFICATION OF PROTEINS AND VIRUSES

by

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If a protein solution is subjected to electrodialysis between semipermeable membranes in the absence of stirring the protein will gradually disappear from the top and will become concentrated at the bottom of the container. This phenomenon can be explained by the movements of the protein in the electric field to one or other of the membranes where zones of high protein concentration are formed. The fluid in the immediate vicinity of the membranes will, because of higher density, tend to move downwards. Eventually all the protein from the upper layers will be concentrated in the bottom of the vessel.

Several workers have made use of this phenomenon for the separation and purification of organic as well as inorganic colloids. In an excellent review by SVENSON[1] these are summarized. Recently KIRKWOOD[2] described an apparatus in which fractionation of proteins occurs between two membranes in close proximity. The space between the membranes communicated with the top and bottom compartment. When a mixture of proteins is treated in this apparatus the proteins that migrate in the electrical field are concentrated in the bottom compartment and only the proteins that are at their iso-electric points remain in the top compartment. In the electrodecantation apparatus of GUTFREUND two electrode vessels are separated from a central compartment by two semipermeable membranes. The protein mixture to be electrodecanted is placed in the central compartment. A direct current is passed through the apparatus and the protein component which is at its iso-electric point remains in solution in the general contents of the cell while the components that migrate at the particular pH employed are concentrated in the bottom layers of the separation cell.

The apparatus of GUTFREUND has several disadvantages: 1. The average distance which protein molecules must migrate to reach the membranes is large so that experiments must be extended over long periods; 2. Only relatively small current densities can be employed as any excessive heating which would result from stronger currents causes convection; 3. Temperature cannot be adequately controlled.

THE MULTI-MEMBRANE ELECTRODECANTATION APPARATUS

In the apparatus to be described in this work an attempt has been made to overcome the disadvantages of GUTFREUND's apparatus.

References p. 325.

1. The distance which protein molecules must migrate is reduced to a minimum by the subdivision of the separation cell into many small units. This is accomplished by assembling a large number of cellophane membranes separated from one another by perspex frames about 1 mm in thickness.

2. The introduction of cooling sections through which chilled buffer is circulated makes it possible to use larger current density without fear of heat convection. These cooling sections serve the additional purpose of collecting electrolytic byproducts that diffuse back into the apparatus from the electrode chambers thereby maintaining the pH at an approximately constant value.

Fig. 1 shows the simplest form of the multi-membrane electrodecantation apparatus. It consists of two electrode compartments A_1 and A_2, two buffer cooling compartments B_1 and B_2, and a separation cell C. The compartments are separated from each other

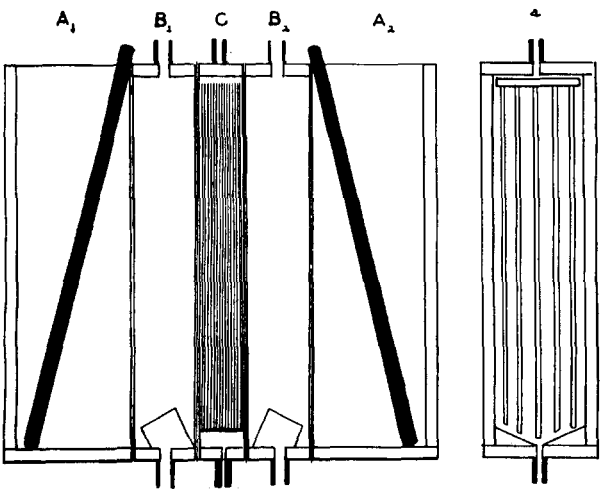


Fig. 1. Simplest multi-membrane electrodecantation apparatus. A_1 and A_2 are electrode compartments, B_1 and B_2 are compartments through which cooled buffer is circulated and C is the separation cell packed with "cellophane" membranes and "perspex" frames.

Fig. 1a. Is the cross section of the separation cell showing a perspex frame.

by thick cellophane membranes clamped between rubber gaskets. The buffer compartments are provided with inlet and outlet tubes for the circulation of cooled buffer. Perspex baffles are inserted to direct the chilled buffer onto the separation cell thereby increasing the effectiveness of the cooling. The apparatus is held together in a clamp which is not shown in the figure. The separation cell is composed of a series of membranes separated from one another by perspex frames of 1 mm thickness (Fig. 1a). In a separation cell of 2 cm width there are usually 18–20 such frames and cellophane membranes. The electrodes consist of 2 sets of four carbon rods. Perspex wedges are inserted into the bottom of the separation cell to assist in the removal of the separated protein fractions.

Prevention of heat convection currents

In the electrodecantation apparatus used in the initial stages of this work heat

References p. 325.

convection currents were a serious source of error. This difficulty was overcome by the establishment of a temperature gradient vertically down the cell.

In the earlier type of apparatus the electrodes were held parallel to one another and the generation of heat in the separation cell was therefore homogeneous throughout the cell. In subsequent models of the apparatus the electrodes were sloped with the top ends closer together than the lower ends so that more current flowed through the shorter distance separating their upper portions and consequently more heat was generated in the upper layers of the separation cell than lower down thereby establishing a temperature gradient. This temperature gradient together with the density gradient set up by the protein separation in the cell reduces the convection currents.

Because of the low heat conductivity of the cellophane membranes and the fluid between them there will be, in addition to the vertical temperature gradient, a horizontal gradient in the separation cell. As the spaces between the membranes are very narrow each of the sections will have a different vertical but negligible horizontal gradient, consequently each space will act as a unit for separating the protein components.

Circulation and cooling of buffer

To remove the generated heat and any electrolytic byproducts that diffuse through the membranes of the electrode compartments the cooling buffer is chilled by circulating through a glass condenser cooled with iced water from a water bath at 0–4° C. The Hilger-Watt electrophoresis bath was found very suitable for this purpose. From the cooling condenser the buffer flows through the cooling sections of the apparatus and then with the aid of a pump or air lift back to the reservoir.

Suitable protein concentrations for electrodecantation

The rate at which protein fractions separate in the separating cell is dependent on the concentration of the protein solution. Thus, if undiluted serum is electrodecanted at pH 6.6 for the separation of γ-globulin, the albumin and other protein fractions which migrate at this pH separate out very slowly. When this serum is diluted with an equal volume of buffer the separation is very much faster. This is in accordance with what has been observed in electrophoresis where the rate of migration of a protein is also dependent on the concentration.

Displacement of non-migrating components by the separating components

The displacement of the stationary components by the migrating components forms the basis of the separation method of KIRKWOOD (*l.c.*). The quantitative recovery of the "stationary" component is not possible in the type of separation cell we have described unless upward displacement of the component being separated takes place. A certain amount of the stationary component would otherwise always be lost in the layer of protein and debris separating in the bottom of the cell. In our experiments the displacement effect was shown by electrodecantation of various mixtures of ovalbumin and haemoglobin at the iso-electric point of haemoglobin. The concentrated ovalbumin which separated in the bottom of the cell was invariably free of all but a trace of haemoglobin whilst the haemoglobin remained in the upper part of the cell.

Avoidance of electrolyte accumulation in the bottom of the separation cell

It has been observed that during electrodecantation there is always an accumulation

References p. 325.

of electrolyte in the bottom of the separation cell. This tends to interfere with effective separation probably because of heat convection currents set up. The formation of this layer of concentrated electrolyte can be accounted for quantitatively as follows when, for instance, phosphate buffer is used. Na_2HPO_4 will dissociate into the following ions: Na^+, $NaHPO_4^-$, HPO_4^{-2}, H^+ and PO_4^{-3}. Of these ions Na^+ and $NaHPO_4^-$ will be present in greatest concentration. The Na^+ and $NaHPO_4^-$ ions will migrate in opposite directions. These ions together with a certain amount of fluid migrate through the pores of the membrane. The direction of migration of the fluid will be in the direction of the migration of the Na^+ ions. The flow of the liquid through the pores of the membrane impedes the passage of the $NaHPO_4^-$ ions in the opposite direction to the electrosmotic flow. $NaHPO_4^-$ ions will accumulate on the membrane surface and consequently a region of higher Na_2HPO_4 concentration is formed on the membrane surface. The fluid in this region will have a higher density than the fluid in the vicinity and will thus tend to sink.

An alternative explanation for the concentration of electrolyte is suggested from the theory of SÖLLNER[3]. If there are charged groups within the pores of the cellophane membrane as a result of dissociation of –COOH groups into COO^- and H^+ ions, the negatively charged $NaHPO_4^-$ ions will be repelled by the COO^- charges but the positively charged sodium ions will be driven through the pores in the opposite direction by the potential gradient between the electrodes assisted by the oppositely charged COO^- ions on the walls of the pores in the membrane. As a result of the retardation of the motion of the anion and the acceleration of the cations, a region of concentrated electrolyte will be formed on the membrane surface. This relatively dense electrolyte solution will sink to the bottom of the separation cell. It is of course possible that the separation of electrolyte depends on a combination of the two factors—electro-osmosis and repulsion by ions of like charge on the pore surfaces.

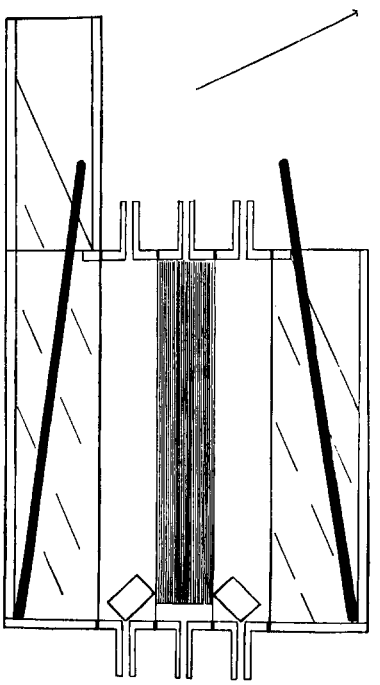


Fig. 2. Multi-membrane electrodecantation apparatus in an inclined position. The arrow indicates the vertical.

An important effect of this separation of the electrolyte in the bottom of the separation cell will be that more current will pass through the lower part of the separation cell than through the upper regions, consequently more heat will be generated in the lower regions than in the top regions of the cell, which in turn will tend to give rise to convection currents. The formation of an electrolyte gradient can be reduced considerably by the addition of a small amount of divalent cation (Mg^{+2}). The divalent cation acts by lowering the ζ potential of the membranes and accordingly reduce the electrosmotic flow.

OBLIQUE MULTI-MEMBRANE APPARATUS

If the apparatus is mounted in such a way that the separation cell is oblique rather than vertical (Fig. 2) the speed of separation is accelerated. This is probably accounted for by the reduced tendency

References p. 325.

of components migrating to the lower one of any pair of membranes to "fall" back into the cell as would be the case in the vertical apparatus.

The oblique cell is particularly suitable for the separation of γ-globulin from serum. It can however be used equally conveniently for the isolation of the other components of serum provided the γ-globulin is first removed. The procedure we have followed is to change the pH of the serum buffer mixture to that of the iso-electric point of α- and β-globulins (pH 5.4) after the γ-globulin has been removed. By passing the electric current through the cell the albumin now separates out leaving the α- and β-globulins in the supernatant. The α- and β-globulins can themselves be separated by electrodecantation at a pH where they have widely different mobilities. The α-globulin which will separate out first is repeatedly electrodecanted at that pH until the desired degree of purity is obtained. The supernatant will contain the β-globulin.

CONTINUOUS FLOW APPARATUS

For the separation of single protein components from others contained in large volumes a continuous flow apparatus was constructed (Fig. 3). This consisted of three separation cells separated from one another by cooling sections through which chilled buffer could be circulated. The separation cells had free space above the membranes and each one was provided with an outlet through which the contents of the lower portion could be removed. The crude mixture of protein in buffer with its pH at the iso-electric point of the component for separation is introduced in the bottom of the first separation section where, being of lesser density than the concentrated material which tends to accumulate in the bottom of the cell, it rises. From the top of the first separation cell the fluid, now partially freed of contaminating protein, is passed into the bottom of the second cell, and from it through the third. The protein solution thus becomes progressively freed of all components except that which has as iso-electric point the pH of the buffer employed. The object of the free space above the membranes in each cell is to provide a region into which purified component in solution can accumulate before passing on to the next cell. During operation the concentrated material in the bottom of each separation section can be removed through the outlets provided.

The efficiency of the apparatus is greatly increased by operating it in an oblique position. The temperature gradient can be maintained in the same way as in the apparatus with the cells in the vertical position, by appropriate sloping of the electrodes.

The purity of the final product is dependent on the number of separation cells in series and also on the rate of introduction of the crude product into the system.

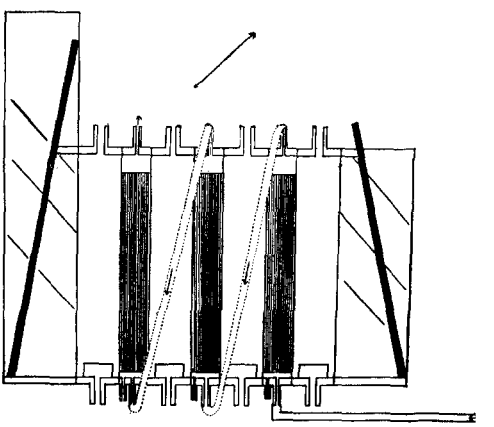


Fig. 3. Multi-membrane electrodecantation apparatus used for the continuous separation of protein fractions. The supernatant from the first separation section is passed into the bottom of the second and the supernatant of this section is passed into the bottom of the third. The supernatant from this section passes into a receiver. The arrow indicates the vertical.

References p. 325.

For the separation of γ-globulin from serum it must be kept in mind that the iso-electric point of pH 6.6 as given in the literature (ABRAMSON, MOYER AND GORIN[4]) is in reality the mean iso-electric point of a number of components present in γ-globulin. If therefore electrodecantation of serum is carried out at pH 6.6 and the rate of the flow is slow, only components with iso-electric points close to pH 6.6 will be present in the effluent from the continuous flow apparatus and all components which have different iso-electric points will have separated out. With faster rates of flow components with a somewhat wider range of iso-electric points will remain in the effluent. The total yield of γ-globulin will thus be dependent on the rate of introduction of the serum.

RESULTS

The various types of apparatus have been used for the purification or concentration of serum proteins, viruses and the enzyme trypsin.

RESULTS OBTAINED ON THE SEPARATION OF γ-GLOBULIN FROM HUMAN SERUM WITH THE DIFFERENT TYPES OF APPARATUS

Vertical separation cell

Human sera were electrodecanted at pH 6.6, the iso-electric point of γ-globulin, in a phosphate buffer-$MgSO_4$ mixture with an average voltage gradient of 5 volts/cm across the electrodes. At the end of each experiment the contents of the separation cell was divided into two fractions, a bottom layer of 100 ml containing most of the protein that migrates at this pH, and a supernatant of 400 ml which contained the γ-globulin. These fractions were subjected to electrophoresis in the apparatus described by POLSON[5]. In a typical experiment it was found that after electrodecantation for 3 to 4 hours 30% of the proteins present in the supernatant were composed of albumin, α- and β-globulins. The yield of γ-globulin in the supernatant fluid appeared to be 100%. After electrodecantation for 7 and 9 hours in two separate experiments there was less than 5% contaminating protein in the supernatant. The yield of γ-globulin was reduced to 70% and 60%, respectively. After 17 hours electrodecantation contaminating proteins were not detectable in the supernatant fluid by electrophoresis but the yield of γ-globulin was only 30% of that present in the original serum.

In these prolonged electrodecantation experiments there was usually a significant decrease in the pH of the circulating buffer. Such a change in the pH of the medium can cause also some of the γ-globulin to migrate thereby reducing the yield of the γ-globulin in the supernatant fluid.

The separation of albumin, α-, β- and γ-globulins from human serum has been achieved with all types of apparatus described. The electrophoresis diagrams of components isolated with the oblique apparatus are shown in Fig. 4. The albumin fraction was electrodecanted twice at pH 5.4 in order to remove traces of α- and β-globulins which contaminated the product of the first separation. The γ-globulin was isolated by electrodecantation at its iso-electric point of approximately pH 6.6. The α-globulin was isolated by repeated electrodecantation at pH 8.0 each time the layer that contained all the α-component was electrodecanted to reduce the amount of β that separated

References p. 325.

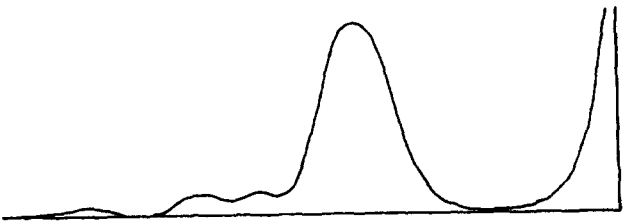
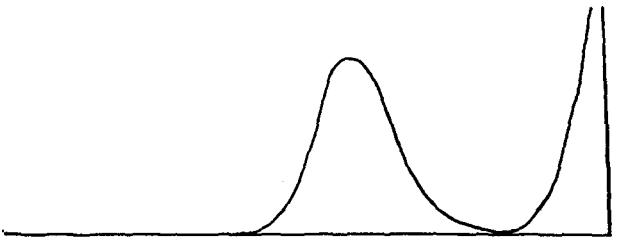


Fig. 4a. Electrophoresis diagrams of human albumin in borate buffer at pH 8.6. Top curve was obtained of albumin after removal of globulins by sodium sulphate precipitation. Bottom curve is of the same material after purification by multi-membrane electrodecantation.



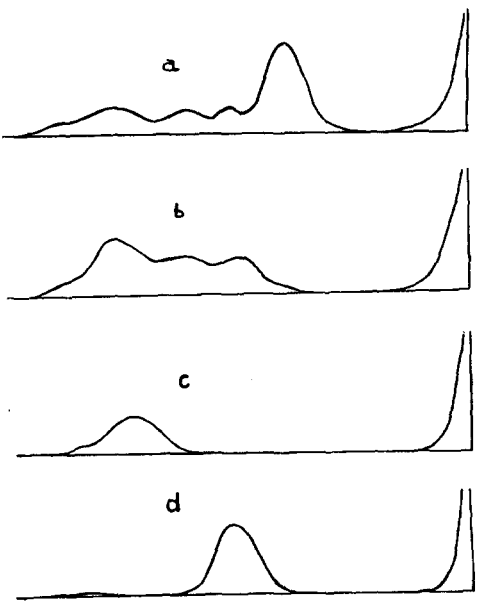


Fig. 4b. Electrophoresis diagrams of serum and globulin fractions.

a. Electrophoresis diagram of original serum.
b. Electrophoresis diagram of globulin obtained by sodium sulphate precipitation.
c. γ-globulin obtained from globulin precipitate by multi-membrane electrodecantation.
d. α-globulin separated from globulin precipitate by M.M.E.D.

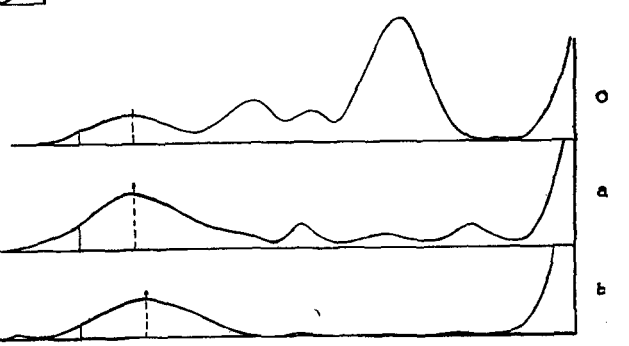


Fig. 5. Electrophoresis diagrams of original serum.

a. γ-globulin separated at flow rate of 0.45 l/h
b. γ-globulin separated at flow rate of 0.22 l/h

together with the α-component. Unfortunately the β-globulin was not electrophoresed in this experiment.

Fig. 5 shows the result of electrophoresis analysis at pH 8.6 of two samples of γ-globulin. The first was separated at the rate of 0.45 l/h and the second at 0.22 l/h in the continuous flow apparatus. It is apparent that the sample separated at a rapid rate was contaminated with appreciable amounts of other serum components. The sample separated with a slower rate of flow is without detectable contaminant. The main component in the first sample has a slightly lower electrophoretic mobility than the only component of the purer second sample.

The concentration of viruses from crude emulsions of virus-infected tissues has presented certain difficulties only some of which are easily overcome.

The concentration of virus protein in emulsion of virus-infected tissues is usually very low, and the amount of virus obtained from such emulsions is usually almost negligible in comparison with the relatively enormous bulk of tissue protein. Concentration by differential centrifugation has the disadvantage that usually only relatively small volumes of emulsion can be dealt with. The method of multi-membrane electrodecantation has proved particularly useful as a preliminary step in concentration by ultracentrifugation. It has been found that concentration of virus succeeds only in the presence of contaminating proteins which migrate in the same direction in the electric field as the virus. It appears that when such proteins are absent the virus moves onto the membrane to which it then remains adherent. It is not unlikely that, when significant amounts of other protein are present, these in the normal downward movement during concentration carry the virus particles with them into the lower part of the separation cell. When however the product of mobility of the virus × voltage gradient is high the virus particles become so firmly adsorbed to the membrane that, even in the presence of large amounts of protein, concentration of virus into the lower layers of thes eparation cell will not occur.

Electrodecantation in 10% serum buffer solutions at pH values between 6 and 8 and using potential gradients of less than 6 volts/cm appeared to give the best results for the concentration of viruses.

In Tables I and II are given the results of experiments on the MEF_1 strain of Poliomyelitis virus in emulsions of infected suckling mouse brains. The results in Table I were obtained in an experiment carried out at pH 8.7 and with a current of 5 volts/cm acting for 2½ hours. In the experiment from which the results recorded in Table II were obtained a lower pH (6.0) was employed and a current of 5 volts/cm applied for 6 hours. It is apparent that at the higher pH adsorption of the virus to the membranes occurred, but with the lower pH and the consequent slower migration rate of the virus effective concentration was obtained.

TABLE I

ELECTRODECANTATION OF MEF_1 POLIOMYELITIS VIRUS FOR 2 HOURS AT pH 8.7

Current 5 volts/cm

Material	*Volume*	LD_{50}
Original	500 ml	2.36
Supernatant fluid	440 ml	0.83
Bottom layer	60 ml	2.00

References p. 325.

TABLE II

ELECTRODECANTATION OF MEF_1 POLIOMYELITIS VIRUS FOR 6 HOURS AT pH 6.0

Current 5 volts/cm

Material	*Volume*	LD_{50}
Original	490 ml	2.34
Supernatant fluid	427 ml	1.61
Bottom layer	63 ml	3.10

An advantage of this method of concentration is that most of the tissue debris present in the emulsion remains adsorbed to the membranes leaving a concentrate which is relatively free of tissue fragments. This factor is of great importance for the further purification of the virus by ultracentrifugation.

PURIFICATION OF TRYPSIN FROM BOVINE PANCREAS

The very high iso-electric point of trypsin, pH 10.8, as determined recently by BIER AND NORD[6], makes it ideally suited to purification by this technique. Bovine pancreas frozen at —10° C, then rapidly thawed was minced and suspended in NaOH-glycine buffer at pH 10.8 with 0.025 *M* Ca acetate. It was allowed to extract for about 1½ hours and the tissue debris separated by centrifugation. The extract was adjusted to a pH of 10.8 with NaOH and then subjected to electrodecantation at this pH for 5 hours in an apparatus sloped at 45° from the vertical. The voltage difference across the electrodes was 80 volts. Finely divided suspended matter rapidly became adherent to the membranes resulting in a rapid clearing of the fluid. After 5 hours a glass outlet tube was attached to the top of the separation cell and 20% glucose was run into the bottom. Successive samples of effluent from the outlet tube was collected into a series of 4 flasks. The total amount of fluid obtained in this way was 350 ml. The proteolytic activities of the contents of each flask was then determined by titration on gelatine paper. It appeared that although there was a fourfold loss of enzyme activity in the effluents as compared to the solution before electrodecantation the activity was constant in all the samples of effluent. It is probable that the enzyme was partly inactivated by the relatively high temperature (7–30° C) to which it was subjected. The pooled effluents from the separation cell were then dialysed against the buffer saturated with $(NH_4)_2SO_4$ and $MgSO_4$. The volume decreased rapidly and a needle-like crystalline precipitate formed. The crystalline deposit was removed by centrifugation, dissolved in a small amount of *M* 15 phosphate buffer at pH 6.0 containing 10% Mg SO_4 and tested for proteolytic activity by titration on gelatine sheets. It was found that the crystalline material contained the full enzymic activity of the combined effluents.

In experiments with crude Difco trypsin a crystalline product was also obtained which contained the full activity of the supernatant from the electrodecantation apparatus.

DISCUSSION

The field of application of multi-membrane electrodecantation to protein systems is very broad and in the present study it was applied to the separation of widely different substances, *i.e.* serum proteins, enzymes and viruses.

References p. 325.

With its application to the separation of individual serum proteins, especially with the continuous flow apparatus, it fulfils an existing need, *i.e.*, a method for the rapid separation of γ-globulin for prophylaxis against virus diseases like measles and poliomyelitis.

The apparatus has proved successful also for the separation of viruses if care is taken to use moderate voltage gradients, buffer pH values not too far removed from the iso-electric points of the viruses, and ensuring the presence of sufficient protein to assist in the transporation of virus particles to the bottom layer in the separation compartment. It may, therefore, assist materially in the purification and concentration of viruses for the preparation of prophylactic vaccines. Furthermore the easy separation of most of the tissue debris from suspensions of virus in organ emulsions makes electrodecantation an invaluable preliminary step in further purification by ultracentrifugation.

Although there was a loss in total activity of the trypsin in the experiments on the purification of the enzyme from bovine pancreas, presumably caused by the relatively high temperature to which it was exposed during the process of isolation, the enzyme was obtained in a crystalline state in a relatively simple and inexpensive manner. It may be that the apparatus will assist in the isolation of other enzymes and of hormones. Even if complete separation of a single component from a complex mixture is not achieved at least considerable purification of the substance results from a single operation with this apparatus.

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SUMMARY

1. An electrodecantation apparatus is described in which the separation cell is subdivided into a large number of sections with the aid of thin "perspex" frames and "cellophane" membranes.

2. Buffer cooling sections are employed through which chilled buffer from a reservoir is circulated.

3. A temperature gradient and consequently a density gradient is established down the separation cell by appropriate sloping of the electrodes. Such a density gradient minimized convection currents in the cell.

4. Separation of proteins is greatly accelerated if the apparatus is held in an inclined position.

5. A continuous flow apparatus is described which enabled the continuous extraction of proteins from large volumes of solutions.

6. Results are given of attempts at concentrating Poliomyelitis virus from mouse brain suspensions, purification of trypsin from bovine pancreas, and the extraction of γ-globulin from human sera using three different types of the apparatus.

RÉSUMÉ

1. Les auteurs décrivent un appareil à électrodécantation dans lequel la cellule à séparation est subdivisée en un grand nombre de sections à l'aide de cadres de "perspex" et de membranes de cellophane.

2. Les sections sont refroidies par la circulation d'un tampon réfrigéré.

References p. 325.

3. Un gradient de température et par conséquent un gradient de densité peuvent être établis du haut en bas de la cellule de séparation en inclinant convenablement les électrodes. Ce gradient diminue les courants de convection dans la cellule.

4. La séparation des protéines est fortement accélérée si l'appareil est maintenu incliné.

5. Un appareil à écoulement continu est décrit. Il permet l'extraction continue de protéines à partir de grands volumes de solution.

6. Les auteurs donnent les résultats d'essais de concentration du virus de la poliomyélite à partir de suspensions de cerveau de souris, de purification de la trypsine du pancréas de boeuf et d'extraction de la γ-globuline du sérum humain, avec différents modèles de l'appareil.

ZUSAMMENFASSUNG

1. Es wurde ein Elektrodekantierapparat beschrieben, in dem die Trennzelle in eine grosse Anzahl von Abteilungen mit Hilfe eines dünnen "Perspex" gerüstes und Cellophanmembranen unterteilt ist.

2. Es wurden pufferkühlende Abteilungen verwendet, durch welche gekühlte Pufferlösung aus einem Resevoir zirkuliert.

3. Durch geeignete Neigung der Elektroden bildet sich in der Trennzelle ein Temperaturgradient und folglich ein Dichtegradient aus. Ein derartiger Dichtegradient setzt Konvektionsströme auf ein Minimum herab.

4. Die Trennung der Proteine wird beschleunigt, wenn die Apparatur in geneigter Stellung gehalten wird.

5. Es wird ein kontinuierlicher Strömungsapparat beschrieben, der die kontinuierliche Extraktion von Proteinen aus einem grossen Lösungsvolumen ermöglicht.

6. Es werden die Ergebnisse von Versuchen Poliomyelitisvirus aus Maushirnsuspensionen zu konzentrieren, zur Reinigung von Trypsin aus Rinderbauchspeicheldrüsen und zur Extraktion von γ-Globulin aus menschlichem Serum unter Benützung von drei verschiedenen Typen der Apparatur angeführt.

REFERENCES

[1] H. SVENSON, *Advances in Protein Chem.*, IV (1948) 251.
[2] J. G. KIRKWOOD, *J. Chem. Phys.*, 9 (1941) 878;
J. R. CANN, J. G. KIRKWOOD, R. A. BROWN AND O. PLESCIA, *J. Am. Chem. Soc.*, 71 (1949) 1603;
J. R. CANN, R. A. BROWN AND J. G. KIRKWOOD, *J. Am. Chem. Soc.*, 71 (1949) 1609, 2687;
J. G. KIRKWOOD, J. R. CANN AND R. A. BROWN, *Biochim. Biophys. Acta*, 5 (1950) 301.
[3] K. SÖLLNER, *J. Phys. Chem.*, 49 (1945) 47, 171.
[4] H. A. ABRAMSON, L. S. MOYER AND M. H. GORIN, *Electrophoresis of Proteins*, Rheinhold Publishing Corp., New York, 1949.
[5] A. POLSON, *Nature*, 170 (1952) 628.
[6] M. BIER AND F. F. NORD, *Arch. Biochem. Biophys.*, 33 (1951) 320.

Union of South Africa patent application 2293/51.
Patented in Union of South Africa No. 13159 — 3rd Sept., 1951.
Union of South Africa Patent of Addition 273/52.
British Patent Pending No. 17673 — 14th July, 1952.
Patents pending overseas.

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