Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Wadström, T. and Vesterberg, O.(1970) 'Continuous Dialysis of Protein Solutions on a Large Scale. II. Dialysis of Extracellular Proteins from *Staphylococcus aureus* Followed by Adsorption on Ion Exchangers and Other Methods', Separation Science and Technology, 5: 2, 91 — 98
To link to this Article: DOI: 10.1080/00372367008057951
URL: http://dx.doi.org/10.1080/00372367008057951

SEPARATION SCIENCE, 5(2), pp. 91–98, April, 1970

Continuous Dialysis of Protein Solutions on a Large Scale. II. Dialysis of Extracellular Proteins from *Staphylococcus aureus* Followed by Adsorption on Ion Exchangers and Other Methods

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Summary

An artificial kidney constructed according to Kiil and equipped with Cuprophane membranes has been used for a continuous dialyzing process of dilute protide solutions. After cultivation of several strains of *Staphylococcus aureus* in submerged culture, the supernatants have been dialyzed in order to remove salt and other low molecular medium constituents. A total recovery of all the enzymes and toxins studied was noticed after dialysis. A flow rate of 30 ml/min for the supernatant and 150 ml/min for the wash water lowered the ionic strength after one passage to less than 1/6 of that of the culture medium. This effective and rapid large-scale procedure has been followed by rotary evaporation, lyophilization, and ion exchange chromatography as the first steps in concentrating and purifying the extracellular proteins. A combination of these methods for concentration and purification of proteins from dilute solution is discussed with special reference to its proposed general applicability.

Complex media are often needed for cultivation of bacteria to obtain high yields of cells and enzymes. The removal of salts and other low molecular medium constituents after cultivation, is mostly desirable, but it is often a difficult task in preparative work.

Inorganic salts and other low molecular weight compounds increase during the concentration procedure when methods such as lyophilization, freezing-out (*1*), and rotary vacuum evaporation (*2*) are used.

A low ionic strength is also necessary for batch- or columnwise adsorption of proteins on ion exchangers. Elution in a small volume with an increased salt concentration and/or with pH adjustment can then give both an essential concentration and a certain purification.

In order to obtain high yields of cells, extracellular enzymes, and toxins from *Staphylococcus aureus,* the use of complex media seems necessary (*3–5a*). These media are based on acid-hydrolyzed or enzyme-digested proteins such as casein, infusions of animal tissues, and dialyzates of yeast extract to meet the demands of amino acids, vitamins, and other growth factors. Trypticase Soy Broth (BBL, Baltimore, Maryland), Brain Heart Infusion (Difco Laboratories, Detroit, Michigan), and the CCY medium (*2, 5a*) are examples of these. Salt and buffer substances are mostly added to defined as well as to complex media. Phosphate is usually used to get a well-buffered medium and might, as well as sodium chloride, be of significance for the release of extracellular proteins (*6, 7*). The ionic strength is often about 0.3, e.g., in media based on acid hydrolyzates of casein.

Cultivation in large volumes is often necessary for the preparation and purification of several bacterial enzymes and toxins.

For the purification of a nuclease from *Staphylococcus aureus,* a 2000-liter culture of strain V8 was grown with a final yield of a few milligrams (*8a, 8b*). There are other examples where biologically active proteins in culture supernatants of staphylococci are present in low quantities. In spite of a comparatively high enzymatic activity, the amount of hyaluronate lyase per liter of culture medium of a good producing strain (strain M18) has been estimated to be in the order of 0.1 mg (O. Vesterberg, to be published). A very low amount of active protein has also been calculated for staphylokinase (K. Vesterberg, to be published) and β-haemolysin (Wadström and Möllby, to be published). Thus the handling of large volumes is often necessary in order to obtain enough material for an extensive purification and characterization. Conventional methods, such as precipitation with ammonium sulfate and ethanol, have been found unsatisfactory for certain proteins. Removal of salt and low molecular compounds on preparative Sephadex gel columns is often difficult because of the high density and viscosity of the concentrated samples.

In an earlier paper the use of an artificial kidney for dialysis was described (*9*). The apparatus then used (AB Gambro) was found to possess a comparatively efficient dialysis capacity like the one constructed according to Kiil (Nycotron A/S, Drammen, Norway) (*10*).

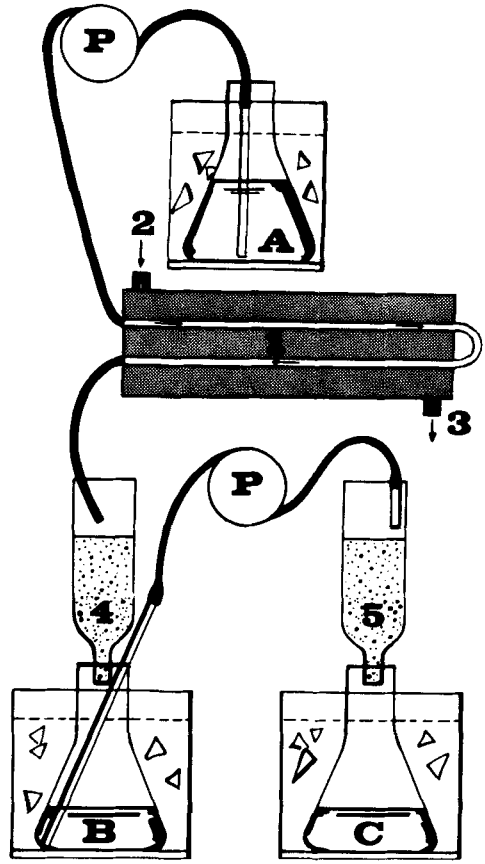

FIG. 1. Arrangement for continuous dialysis of culture supernatants and adsorption of proteins on ion exchangers. A pump (P) supplied the artificial kidney (1; Nycotron) with supernatant (A) (5 liter volumes) at a rate of 30 ml/min. This supernatant passed between membranes of cuprophane (inner compartment), while the washing water was pumped through the outer compartment (2-3) at a rate of 150 ml/min. The dialyzed supernatant ($I < 0.05$) was pumped on a column (4; 6×120 cm), packed with CM-Sephadex C-25 (6×20 cm), and equilibrated in a 0.05 *M* phosphate buffer, pH 6.5. The pH of the effluent was adjusted to about 8.5 with ammonia and was then pumped into a column of similar size (5) but packed with DEAE-Sephadex A-25, equilibrated in 0.05 *M* Tris-HCl, pH 8.3. Elution of the columns was carried out "stepwise" by increasing the concentration of NaCl in a 0.2 *M* phosphate buffer, pH 6.5 (4 and 5). In each step the columns were washed with 1 liter of buffer solution (about 300 ml/hr).

In this investigation most of the experiments have been performed with the latter, mainly because the membranes can be exchanged in a simple way. Dry membranes of Cuprophane (Type 150 PT, 15 g/m^2; Bemberg A.G., Wuppertal, Germany) were put into a 3% acetic acid solution. Two membranes were then carefully placed between each pair of plates and the unit was assembled (Fig. 1). The dialyzing area was 0.64 m^2 with three plates, and the volume of the salt solution compartment was 200–250 ml.

Pumping of the solutions was done with a peristaltic pump (Sigma Co., Middleport, New York) using silicone rubber tubes. A test was made to detect possible leakage of macromolecular substances through defects in the membranes before the dialysis of the culture supernatant Twenty milliliters of a solution containing 2 g of hemoglobin was pumped through the dialyzing compartment. A leakage of about 1% to the other compartment, through which deionized water was pumped, could be detected by the naked eye. When a leakage was detected the membranes were exchanged. This was, however, very seldom the case.

In order to study the permeability of the membranes some dialyzing experiments were made with 400 mg of a preparation of three-times crystallized egg white lysozyme, $M = 14{,}200$ (Sigma Chemical Co., St. Louis, Missouri), dissolved in 20 ml of distilled water. The assay was made as described by Jollés (*11*). Less than 1% enzymatic activity was detected in the wash solution. This degree of permeability is small compared with the loss of small proteins noticed with ordinary dialysis membranes. However, in some of our dialysis experiments a variable loss of lysozyme and staphylococcal DNase activity was observed, probably due to adsorption of negatively charged groups in the membranes. Adsorption of ions and basic proteins, such as calcium (*12*) and egg-white lysozyme (*13*), to dialysis membranes and to glass has been reported. The adsorption is, however, much less pronounced when salt is present in the protein solution or when dialysis is made against a buffer.

A loss of 20–50% of DNase activity was also noticed in a batch procedure after prolonged dialysis in hoses (Visking Co., Chicago, and Unicarbide, New York) with flat widths of 6.35, 15, and 20 mm. This loss has been found to diminish when dialysis is performed in hoses heated in a solution of 1% bicarbonate (*14*) or by using acetylated membranes which shrink the pores (*15*). Thicker membranes, such as "23/32" (Visking), have also been found useful for small proteins (*16*).

However, with these "small pore hoses," dialysis for about 12 hr with several changes of the outer volume was necessary to obtain as effective a desalination as with the continuous process. This prolonged procedure resulted in a considerable loss of some unstable enzymatic activities, e.g., hexosaminidase activity. No significant loss was noticed for most of the biological activities studied after dialysis in the artificial kidney, probably due to the characteristics of the membranes as well as to the short dialyzing time needed.

Several strains of *Staphylococcus aureus* (M18, Wood 46, V8, 524, Foggie, and R1) have been grown in a modified CCY medium (*5a*). Batch and continuous cultivations have been performed under controlled conditions of pH, temperature, aeration, agitation, and chemical foam breaking (*5a, 5b*). The cultures were harvested, cooled to 4°, and centrifuged (8000 *g*, 20 min) in a Sorvall RC-2B centrifuge (GS-3 1 rotor). This high *g* value was important to spin down the whole cells and cell debris which might otherwise rapidly diminish the dialyzing efficiency, probably due to clogging of the pores of the membranes. To prevent further bacterial growth the addition of *n*-butanol (1% v/v) or bensylpenicillin (10 U/ml) was found satisfactory and did not seem to interfere with the further purification.

Volumes of 4–8 liters of culture supernatant were kept in an ice-water bath and subjected to dialysis in an artificial kidney. The flow rates of a culture supernatant and the washing solution of deionized water were varied in some experiments (*9*). In principle, effective desalination was achieved when deionized water was pumped through with a flow rate of about 5 times that of the supernatant. A flow rate of 30 ml/min for the supernatant and 150 ml for the wash water was chosen. With these flow rates one passage of the supernatant is sufficient to lower the salt concentration to less than 0.05 *M* which permits adsorption of the proteins on ion exchangers. A certain gain in time can be achieved if higher flow rates are used, but in order to get a sufficient low salt concentration it is then necessary to recirculate the culture supernatant. An LKB conductolyzer (LKB Produkter, Bromma, Sweden) was used for conductivity determinations and estimations of salt concentration, as earlier described (*9*). According to such measurements,* the salt concentration of a culture supernatant (CCY medium) was 0.3 *M* before dialysis, 0.04 *M* after one passage, and $<0.02\,M$ after two passages. The volume of the dialyzed super-

* The salt concentration was estimated from conductivity determinations using solutions of NaCl of different molarities as standards.

natant then increased about 10%. A second run was of special importance for material to be lyophilized. A volatile buffer, e.g., 0.03 *M* ammonium acetate, pH 6.5, was then usually used instead of deionized water in order to prevent inactivation of unstable proteins. A loss of less than 10% in biological activity was noticed for bacteriolytic activity, hyaluronate lyase, lipase, and phosphatase (strain M18). The same was found for staphylokinase, protease, and nuclease (strain V8) and also for α-haemolysin (Wood 46), β-haemolysin (R1), and δ-haemolysin (Foggie). For the methods used to determine the enzymatic and toxic activities, see Ref. *2*.

After passage of 30 to 50 liters of culture supernatant, the efficiency of the dialysis decreased significantly. The membranes were then exchanged. When only small volumes had been dialyzed, the compartments were filled with a water solution of *n*-butanol 2% v/v and stored between experiments in order to prevent bacterial growth. Microorganisms are known to be able to spoil cellophane membranes (*17*). Merthiolate (0.1 mg/ml) and sodium azide (0.2 mg/ml) have also been tried for this purpose but were found to partially destroy some of the biological activities (e.g., hexosaminidase and lysozyme). If these compounds have been used, the compartments should be carefully rinsed by pumping large volumes of water through them before a protein solution is dialyzed.

ADSORPTION OF PROTEINS ON ION EXCHANGERS

A suggestion for a simple procedure for the concentration and purification of proteins in supernatants from bacterial cultures is presented in Fig. 1.

Thirty liter portions of supernatants (protein content about 0.5 mg/ml) were dialyzed and pumped into a Perspex column packed with CM-Sephadex 6 $\times$ 20 cm (Pharmacia, Uppsala, Sweden) which was equilibrated in 0.03 *M* phosphate buffer, pH 6.5. The amount of CM-Sephadex used could adsorb all the activity of a bacteriolytic hexosaminidase (isoelectric point, pI 9.5) and of a DNase (micrococcal nuclease, pI 10.1) from a culture volume of 40 liters from *S. aureus* strain M18. The flow rate of the effluent was about 30 ml/min. The pH of the effluent was adjusted to 8.3 before it was pumped into a DEAE-Sephadex column of the same dimensions. This column had earlier been equilibrated in 0.03 *M* Tris HCl buffer, pH 8.3.

Acidic nuclease of pI 2–3, 4.5–5.5, and 6.0–6.5 (*18*) and bacteriolytic protein(s) with isoelectric points in the range pH 4–6 (*19*) could be completely adsorbed to the DEAE-Sephadex. The elution of these proteins, which was tried by using solutions of increasing ionic strengths, has, however, given low recoveries until now; but work is in progress to study their elution properties. A nuclease with an isoelectric point (pI) of 10.1, a bacteriolytic hexosaminidase (pI 9.5), and β-hemolysin (pI 9.5) could be eluted from the CM-column by a stepwise procedure of 0.2 *M* phosphate buffer pH 6.5, containing 0.2 and 0.4 *M* of sodium chloride. A concentration of about twenty times and a certain degree of purification were obtained. The yield for these activities was about 80%.

The effluents from the CM- and DEAE-columns were found to contain some activities that adsorbed only to a very low degree to the ion exchangers, e.g., α- (strain Wood 46) and δ- (strain Foggie) hemolysin, and most of the protease (strain V8). Although the activities mentioned did not adsorb to the ion exchangers, a significant purification was obtained since a lot of macromolecular substances, such as proteins and products of autolysis, e.g., nucleic acids, and cell wall components, were adsorbed to the ion exchangers. These partly purified materials were easier to handle when later subjected to a more extensive purification by Sephadex chromatography and the method of isoelectric focusing in a narrow pH range.

In principle, the methods described, which are based on a primary continuous dialysis procedure, would also be applicable to concentration and purification of other dilute protein solutions. After cultivation of other bacteria, such as streptococci, pneumococci, corynebacteria, mycobacteria, and clostridia, the culture supernatants contain biologically-active proteins. Some of these are now of growing interest for the study of virulence factors and for vaccine production, and others are of great interest for basic biological research. Outside the sphere of microbiology there are other fields where dialysis of dilute protide fluids containing a certain salt concentration are of great importance, e.g., for studies of urine- and milk-proteins. Furthermore, a smaller-scale apparatus for dialysis, based on the same principle, would have even greater application for rapid, mild, and efficient dialysis. An improved type of a rapid laboratory dialyser has recently been described by Craig and Chen (*20*). The use of an artificial kidney for simultaneous dialysis and concentration by ultrafiltration is under investigation.

Acknowledgments

We are indebted to Dr. J. Bergström and Miss I. Alstrup, Department of Medicine, St. Erik's Hospital, Stockholm, Sweden, for providing us with an artificial kidney. The cooperation with Dr. S. Arvidson is hereby gratefully acknowledged. The authors are indebted to Professors Berndt Malmgren and Harry Rilbe for their valuable criticism on the manuscript. This work has been supported by grants from The Swedish Medical Research Council, and the Swedish Board for Technical Development, and The Emil and Wera Cornell's Foundation.

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Received by editor September 16, 1969