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NEW APPARATUS FOR ISOELECTRIC FOCUSSING

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CONTENTS

1. INTRODUCTION

The method of isoelectric focussing would be an extremely elegant and useful first step in the separation of proteins. The property being used for the separation is well defined and can readily be determined on small samples using analytical apparatus. This would enable one to get rid of the vast majority of contaminating molecules. So far, however, no apparatus has been available in which large quantities of material can be treated. The analytical methods devised by Svensson[1,2] and Vesterberg and Svensson[3] used a separating medium of carrier ampholytes, consisting of amphoteric buffers with a wide spectrum of isoelectric points, which are conducting when isoelectric. This medium is unfortunately too expensive for use on a large scale, and at pH values widely different from neutrality, affords only a limited time for separation because of its drift towards the electrodes. Therefore, our approach has been to devise a system using only simple buffer solutions. Early work on isoelectric focussing in simple buffers has been reviewed by Kolin[4].

Another condition for large-scale work is efficient removal of heat from the electrolytes. We have chosen to use a series of compartments separated by diaphragms which individually possess isoelectric points, so chosen that the compartments can be maintained at a graded series of pHs. The contents of each compartment are cooled by being pumped through an external electrically insulated heat exchanger. The distance between successive membranes is reduced to the minimum that will allow an adequate flow of electrolyte and thereby as little heat as possible is generated. The extensive early work on compartment apparatus using non isoelectric separators has been reviewed by Svensson[5], who has also suggested the use of amphoteric membranes but not in a situation where they would be isoelectric[1].

With simple buffers it is only possible to maintain stable pHs in such an ap-

paratus with a single pair of electrodes, if one ensures that the transport number of the anions and cations of the buffer is constant throughout the apparatus. This can only be attained by using monovalent ions of each charge, one or both of which may be buffering. Means also have to be provided to maintain the pH of the end compartments at a stable level. This can be done in two ways. (1) Buffer solution can run through the end compartments to waste at a sufficient rate, or (2) the buffer ions can be returned by pumping liquids between the two electrode compartments at the correct rate. The latter method can only be employed if the material to be separated contains no ions which can migrate outside the limit of the pH steps between the end amphoteric membranes. The use of monovalent buffering ions limits the range of pH to about 1.5 units in the case where one ion only is buffering, and to about 3.5 units if both ions are buffers and are chosen with a suitable interval between their pK values. This is not, in fact, a very severe limitation if the aim is to isolate from a mixture a single species with a given isoelectric point. The pH of the buffer can be chosen as optimal for this particular separation and other substances can be discarded, without the necessity of separating them from each other. Of course the purpose of an analytical apparatus is to separate the complete spectrum, but for preparative work the apparatus need only cover a pH range with that of the wanted substance near its centre. By complicating the apparatus and having electrodes for each compartment, it is possible to use multivalent buffer ions with a very much wider buffering range but whose transport number depends on the degree of ionisation.

If the mixture to be purified contains multivalent ions then its rate of addition must be limited to a value which does not cause such a large disturbance of pH that the wanted substance is lost from the apparatus. The pH values should be readjusted before counter-flow is begun.

2. THE ELECTROPHORESIS APPARATUS

Our early work was done with thick agarose gels which were virtually unreinforced. At this time we were attempting to use uncharged membranes and found that available reinforcing materials all carried significant negative charges. It proved impossible to make gaskets which did not injure the membranes and we devised an apparatus consisting of a series of spherical bowls, which rested in spherical gauze separators. A stack of such bowls and separators could be of any desired number. Liquids were introduced and removed from between the membranes by means of flattened polythene tubes. The liquids were syphoned out into the heat exchangers, and pumped back from the heat exchangers into the apparatus.

After we had developed the technique of making amphoteric membranes the charge on the reinforcing fibres could be balanced, and it was possible to use reinforced membranes strong enough to be held satisfactorily using gaskets. We have employed an apparatus constructed of polymethyl methacrylate, using neoprene washers as gaskets, see Fig. 1. A large apparatus built on these principles would be expensive to construct and clumsy to assemble.

For large-scale work the design shown in Fig. 2 is preferable, since only plain rectangular membranes and spacers are required, with strips of rubber as gaskets. It should be very easy to assemble, and disassemble, and plumbing arrangements should be straightforward.

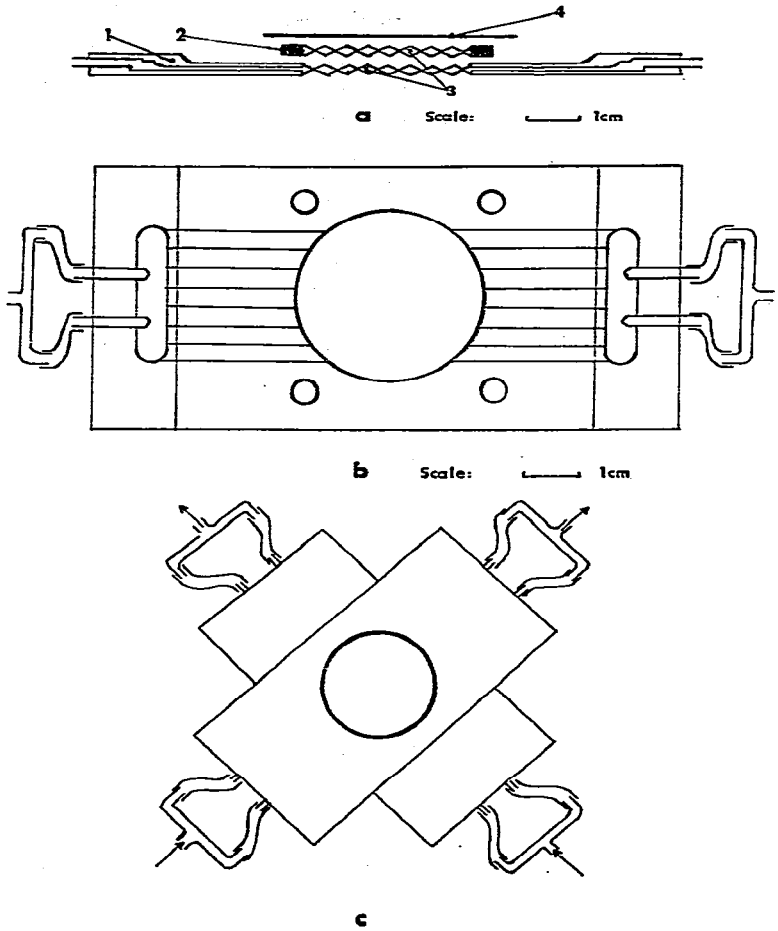


Fig. 1. Apparatus used in small experiments. (a) Exploded section through single compartment with polymethyl methacrylate scale containing flat flow tubes (1), neoprene washer (2), plastic gauze separators (3), isoelectric membrane (4). (b) Plan view of polymethyl methacrylate separator. (c) Method of assembly, but with electrode compartment omitted.

3. THE ELECTRODE COMPARTMENTS

It is essential to avoid unwanted reactions at the electrodes. Most buffers undergo either oxidation at the anode and/or reduction at the cathode. The simplest way of avoiding these is to separate the electrodes from the rest of the apparatus by efficient (dense and with high concentration of charges) ion-exchange membranes. Such membranes have a low electrical resistance and show low electroendosmosis. The anode, which may be immersed in dilute sulphuric acid, is separated from the rest of the apparatus by a cation-exchange membrane containing a high concentration of sulphonic acid residues. The only cation present in the anode compartment is then the hydrogen ion and it carries the whole of the current through the ion-exchange membrane.

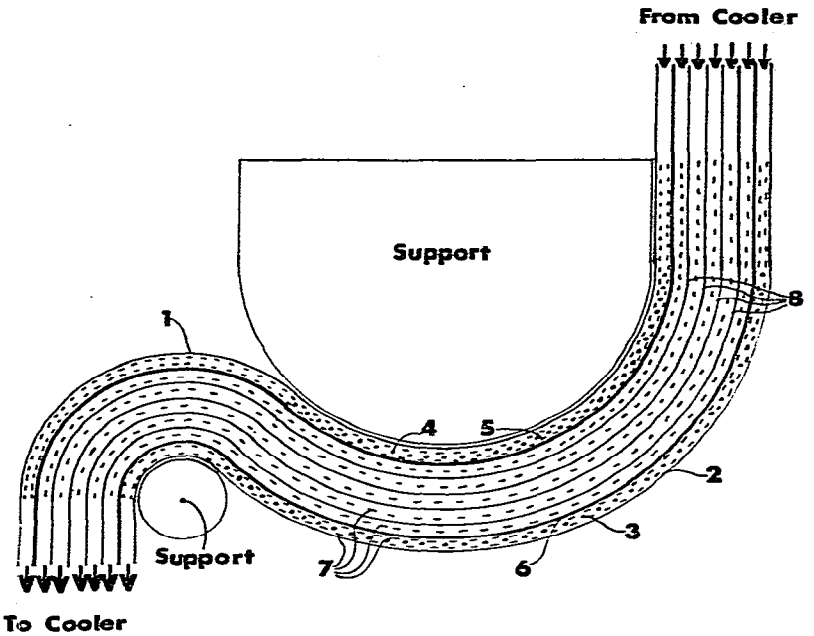


Fig. 2. Section through apparatus suggested for large-scale separations. 1 and 2 = impermeable plastic sheets stretched over formers to hold the sandwich of membranes watertight; 3 = cathode; 4 = anode; 5 = cation-exchange membrane; 6 = anion-exchange membrane; 7 = gauze separators; 8 = isoelectric membranes. The rubber strip gaskets which seal the edges of the membranes and plastic sheets are not visible in this section, but would be seen in an end elevation.

On the side of the membrane away from the anode, the only ions drawn to it are anions which are excluded by the high concentration of negative charges fixed in the membrane.

Similarly, the cathode which may be immersed in sodium hydroxide, is separated from the rest of the apparatus by an anion-exchange membrane containing a high concentration of quaternary amino groups. The only anion, except for any carbonate impurity, is the hydroxyl ion which will carry virtually the whole of the current through the membrane; cations from the non-electrode side of the membrane being excluded by the high density of fixed positive charges. With sulphuric acid as the anolyte a lead electrode which becomes covered with lead peroxide seems entirely satisfactory, and the cathode immersed in sodium hydroxide solution can be made of many metals, mild steel being perfectly satisfactory.

4. PREPARATION OF AMPHOTERIC MEMBRANES

Hardened filter papers (Whatman grade 541) were soaked in hot aqueous agarose solution (4% w/w) and the surplus agarose was removed by pressing the paper between two warm sheets of plate glass. After the impregnated papers had cooled they were soaked for one hour in 2.5 *M* aqueous sodium hydroxide, then after blotting lightly they were suspended overnight in xylene containing 1-chloro-2,3-epoxypropane (1% v/v). The membranes were then soaked for one hour in 2.5 *M* sodium hydroxide

and immersed for exactly 2 h in an alkaline solution freshly prepared by mixing equal volumes of 5.0 *M* sodium hydroxide and 2 *M* chloracetic acid. The membranes were then rinsed in 2.5 *M* sodium hydroxide solution containing diethanolamine (0.06–0.12 *M*). The membranes were then blotted lightly and suspended overnight in xylene containing 1-chloro-2,3-epoxypropane (8%, v/v). This procedure gives membranes with isoelectric points between 4.8 and 5.5, depending on the concentration of diethanolamine used.

The ion-exchange membranes were obtained from Permutit Bobey (Brentwood, Great Britain).

5. THEORY OF AMPHOTERIC MEMBRANES

For satisfactory separation the rate of electroendosmotic flow through the membrane must be low compared with the electrical migration velocity of the wanted substance. It is impracticable to attempt to use uncharged membranes, since absorption of traces of charged compounds always ensues, and causes a large endosmotic flow. To reduce the effect of absorbed substances it is necessary for the membrane to have a relatively high concentration of appropriate charges which will make it isoelectric at a given pH, and which possesses buffering power at this pH. This ensures that absorbed ions will only change the isoelectric point by a small amount.

If such an isoelectric membrane separates two solutions in an electrophoresis apparatus, one of higher pH than the isoelectric point on its cathodic side, and one of lower pH than its isoelectric point on its anodic side, then it automatically reduces the endosmotic flow to a negligible amount. Suppose that there is a flow through the membrane towards the anode, this will cause the membrane to become more alkaline on its cathode side and hence more negatively charged. The solution in the membrane will then carry an excess of positive charges and will be urged by the electric field to move in a cathodic direction. This will cause the flow to reduce until the charges on the anode side of the membrane become more positive. A similar argument applies to flow in the opposite direction. The flow will continue until the electroendosmotic pressure balances the applied difference of pressure. If the pH difference across the membrane is reversed the system is unstable and the electroendosmotic flow will continue in the direction in which it started when the current was first applied.

The membrane may be regarded as a large multivalent molecule, and has the property of all such molecules in spreading the pK values of a given type of dissociating group so that it will buffer over in much wider range than the same group would in free solution. It is possible to adjust the isoelectric point of a membrane by the addition of strong non-buffering ions such as sulphonic acid or quaternary ammonium groups without losing buffering capacity at the isoelectric point. This process is, of course, analogous to that described by Svensson for carrier ampholytes, and as with carrier ampholytes, the membrane will be conducting.

The membranes must obviously be permeable to the substances to be separated and for use with proteins, this limits the choice of material to porous gels such as cross-linked polyacrylamide or agarose, or other gels where the long chains are in contact, leaving free space between them. Such gels are inconveniently weak mechanically, but may be reinforced by stronger material with very much larger pores. Contrary to the difficulties this brings with uncharged membranes, the charge on the

surface of the supports is unimportant if they are filled with buffering gels. The pore size must be large enough not to make a substantial difference to the rate of migration of the substances being separated. If large molecules with high mobility in free solution are very much slowed down by the membrane, then a high-concentration layer will build up against the surface and the charge properties of this layer will come to dominate the properties of the membrane. The farther a substance is from its isoelectric point the larger is its charge and its mobility. The change of charge with pH could be derived from the titration curve of the protein, but cannot in general be predicted.

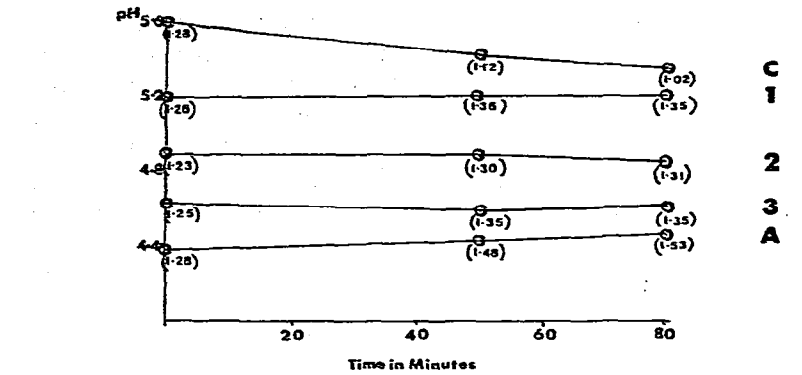
The rate of movement of molecules through the membrane is the algebraic sum of their own mobilities and of any residual electroendosmotic flow, so that the exact pH at which a substance stays within a compartment will be slightly influenced by any small electroendosmotic flow. The constancy of these factors rather than simple diffusion, which seems to be very slow, will limit the resolution of the method. Inevitably, if the difference in isoelectric point between two substances is very small, their separation will be very slow.

As a corollary, the temperature must be maintained constant, since a change will not necessarily have the same effect on the isoelectric points of the two proteins and of the membranes.

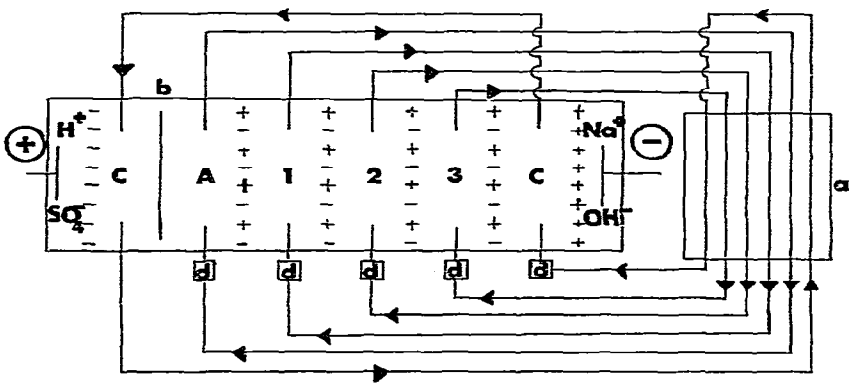
If an ideal uncharged membrane were available, it would be possible to place this between either the anion or the cation-exchange membrane and the rest of the apparatus, and then circulate the liquids between the compartments next to the ion-exchange membranes protecting the anode and cathode so rapidly that the composition would be identical at each end. In this situation where the pH on the anodic side of a membrane is higher than the pH on its cathodic side we have not been able to produce an ideal uncharged membrane. If one were available it would have no effect on the pH or conductivity of the liquids on either side of it. Using a Visking membrane at an acid pH we hoped to minimise the effects of the carboxylic acid groups present in it, in fact, however, slow changes occurred consistent with the membrane having a negative charge. The result of the experiment is shown in Fig. 3, where the membrane is included with a set of amphoteric membranes. This shows the effect of the different transport numbers in the Visking membrane which passes more sodium than amphoteric membranes, but the pH and conductivity changes are confined to the compartments separated by the Visking membrane and the one in circulation with it.

6. MAINTAINING A STABLE SET OF pH STEPS WITH A BUFFER SOLUTION CONSISTING OF MONOVALENT IONS

If the transport numbers of the buffer ions are the same at every membrane, then the requirement for stability is that the concentrations of both positive and negative ions in the end compartments be maintained constant. The simplest way to achieve this is to flow buffer solution of the correct concentration through each compartment at such a rate that its concentration is not sensibly changed during its passage through the apparatus. This will, of course, use a large amount of buffer solution. However, at the beginning of a purification this will serve to wash out of the apparatus charged substances showing no isoelectric point, or whose isoelectric point is outside the pH range employed in the experiment. If the wash buffer effluents from both end



a



b

Fig. 3. The circulation of ions through a compartment separated by a Visking membrane. (a) Variation of pH with time in the individual compartments, the figures in brackets are the conductivities of the solutions in Ω^{-3} cm^{-1}. The starting concentration of sodium ions was 0.02 *M* in each compartment and the current was 1 A. (b) Diagram of the apparatus used. The amphoteric membranes are represented as + − + − +, the anion-exchange membrane as + + + + +, the cation-exchange membrane as − − − − −. a, Coolers and pumps; b, Visking membrane; d, external reservoirs.

compartments are mixed, then the conductivity of the mixture will be higher when added salts are being removed from the apparatus than when they have all disappeared, when the conductivity will fall to the value obtaining before the substance to be separated was introduced. If the buffer solutions in the end compartments are to be kept to a fixed volume and to be recirculated then the condition for stability is simply that cations must be transferred from the most cathodic compartment at the correct rate to the most anodic compartment; at the same time anions must be transferred at the correct rate from the most anodic compartment to the most cathodic compartment. The correct rate for each ion depends on the total current passing through the apparatus and on the proportion of the current carried by the particular ion, *i.e.*, on

its transport number. Some of the ions are converted to the unionised form of each buffer molecule by the H^+ and OH^- ions from the electrodes. These are not, of course, moved by the electric field and can pass through a membrane only by diffusion or electroendosmosis, which is negligible compared to the electrical transport of the ions. They are pumped along with the ions and maintain the appropriate balance.

If the hydroxyl or hydrogen ion concentration is appreciable, then the condition that the transport numbers of the buffer ions remain constant is violated and a stable system cannot be maintained without additional electrodes.

A satisfactory method of maintaining stability is to return the ions by circulation of buffer solution between the end compartments. As has been explained previously, a rapid circulation of liquids is maintained between each compartment, a heat exchanger, and a reservoir. If liquid is circulated from one end reservoir to the other end reservoir and back at the same rate, it is possible to adjust the concentrations of buffer solutions in each reservoir, such that the rate of transfer of ions by the electric field can be exactly balanced by the rate of transfer by circulation. We propose to call this return of ions by circulation "counterflow". The relation between rate of counterflow f (ml/min), the composition of the end compartments and the current I (A) for a buffer consisting of a weak acid HA and a weak base B is:

$$f(A_a^- + HA_a + BH_a^+ + B_a - A_c^- - HA_c - BH_c^+ - B_c) = \frac{I}{F}\left(T_{A^-} - T_{BH^+}\right)$$

where A_a^- is the concentration of A^- in the anodic end compartment, BH_c^+ is the concentration of BH^+ in the most cathodic compartment, and so on. T_{A^-} and T_{BH^+} are the transport numbers of A^- and BH^+, respectively. The pH of the most anodic compartment is then:

$$pH = pK_{HA} + \log A_a^- - \log HA_a$$

$$= pK_B + \log BH_a^+ - \log B_a$$

and of the most cathodic compartment:

$$pH = pK_{HA} + \log A_c^- - \log HA_c$$

$$= pK_B + \log BH_c^+ - \log B_c$$

The simplest way of providing a counterflow with two equal rates of flow is to pump in one direction and allow the return to be over a weir, which is practicable since the electroendosmotic flows are very small.

The effect of this type of circulation is illustrated in Fig. 4. It will be noted that the pH is very much more stable in the inner compartments than in the most cathodic. The reservoir for the most anodic compartment was not easily accessible and no measurements were made of its composition during the experiment. Most of the changes will have been due to temperature rise.

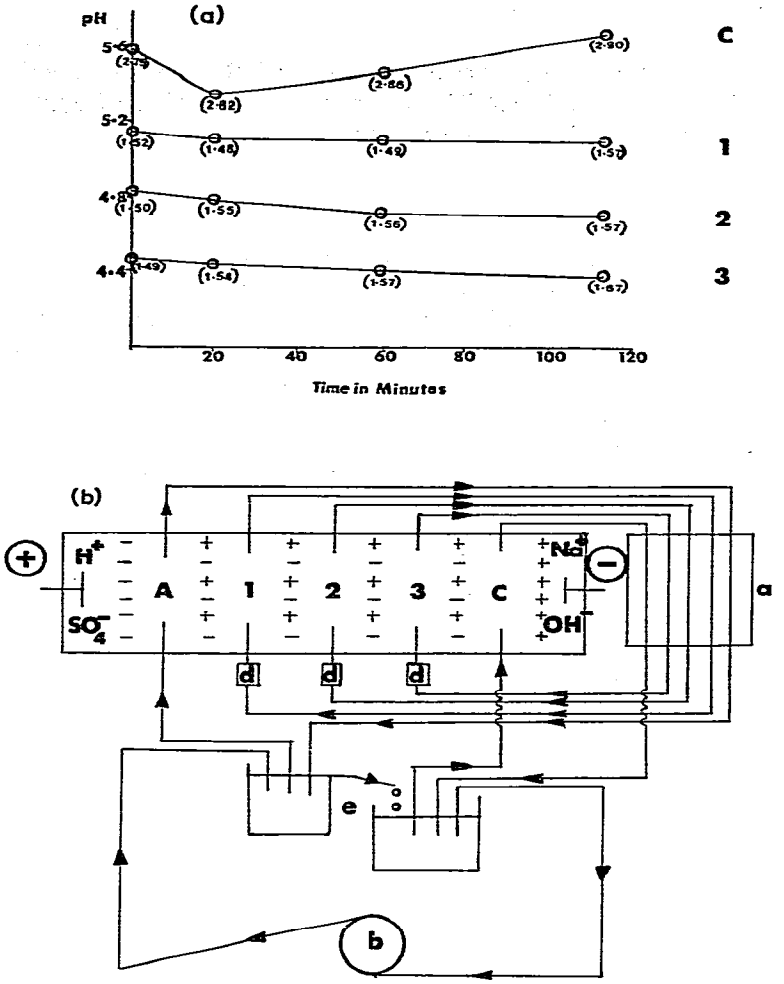


Fig. 4. The use of "counterflow" to recirculate ions. (a) Variation of pH with time in the individual compartments; the figures in brackets are the conductivities of the solutions in Ω^{-3} cm^{-1}. The concentration of sodium ion at the start was 0.02 *M* in compartments A, 1, 2, and 3 and 0.03 *M* in compartment C, the current was 1.0 A and the rate of counterflow was 18.1 ml/min. (b) Diagram of the apparatus. The amphoteric membranes are represented as $+-+-$, the anion-exchange membrane as $++++$, the cation-exchange membrane as $----$. a, pumps and coolers; b, metering pump; d, external reservoirs; e, weir for the counterflow circulation.

7. CONCLUSION

As yet sufficient work has not been done to indicate how good a separation can be achieved in this apparatus, but we feel confident that for preparative purposes it will be adequate. We regard as particularly important the probability that there is virtually no limit to the scale on which such apparatus may be constructed and operated.

8. ACKNOWLEDGEMENTS

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9. SUMMARY

A method is described for isoelectric focussing using a buffer solution containing univalent ions only. Multi-compartment apparatus is described, the compartments being separated by isoelectric amphoteric membranes. The liquid in each compartment is circulated through external heat exchangers. Current is fed into the apparatus through an anode immersed in sulphuric acid and protected by a cation-exchange membrane and by a cathode immersed in sodium hydroxide solution and protected by an anion-exchange membrane. Various means for maintaining a stable system are described, including a metered circulation of electrolytes between the end compartments, to balance the electrical flow of ions.

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