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Preparation of Isoionic Protein by Electrodialysis with Permselective Membranes*

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A water-jacketed apparatus employing permselective membranes for the electrodialysis of water-soluble polymers is described. This apparatus, characterized by its simplicity of construction and operation, is singularly free from leaks because of the incorporation of O-rings in its design. Heat production, type and concentration of salts, electrolysis products, geometry, membrane type and resistance, stirring, etc., are evaluated with respect to the rate and efficiency of the electrodialysis process. Routinely, a 2% solution of albumin in 0.10 M NaCl can be deionized in 4 hours. Two voltage effects, namely, coulombic heat and membrane polarization, are discussed in relation to the denaturation of protein. The values for the isoionic points of some proteins were determined and subjected to a critical analysis.

In spite of the obvious advantage of preparing salt-free protein by electrodialysis this technique has failed to gain wide acceptance for several reasons: electrodialysis with cellophane membranes generally required from 10 to 30 hours for adequate desalting, and the high voltages employed often resulted in denatured product. Frequently the conventional apparatus was prone to leak, resulting in a material loss and the hazard associated with electrical malfunction.

With the advent of commercially available permselective membranes the initial objections are no longer applicable. The instrument described is simple in design and operation, free from leaks, and can readily accommodate volumes ranging from 5 to 35 ml. An analysis is made of the electrodialysis process, with particular reference to the factors which serve to set an upper limit to the current and or voltage which can be applied to this system.

APPARATUS

A water-jacketed electrodialysis unit for use with permselective membranes consisting of two identical glass electrode vessels and a Plexiglass center or sample cell was designed (Fig. 1). Each electrode cell, with a capacity of 15 ml, has shiny platinum electrodes, 28 mm diameter, mounted 6 mm from the membrane surface. In operation, influent distilled water is directed laterally against the membrane and electrode surfaces to prevent the accumulation of electrolysis products and to minimize temperature gradients.

The capacity of the water-jacketed center cell is 35 ml. Its contents can be stirred by either magnetic or mechanical means. For processing smaller volumes, cylindrical glass inserts are placed in this chamber. Burets or electrodes can be introduced through the top ports for *p*H monitoring, while the bottom port serves as the outlet. The membranes used were Nepton-AR-111-A (anion exchange) and Nepton

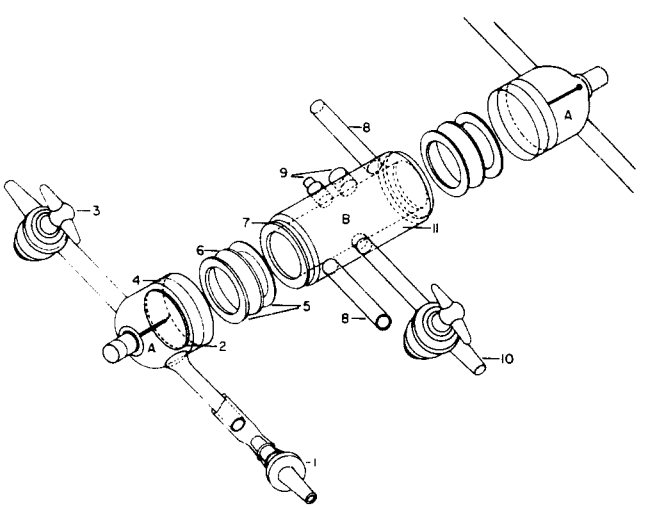

FIG. 1.—An expanded view of the electrodialysis unit. *A*, electrode cell; *B*, center or sample cell; *1*, polyethylene connector for water inlet; *2*, platinum electrode; *3*, needle valve for electrode water outlet; *4*, polyethylene support for membrane; *5*, rubber gaskets; *6*, permselective membrane; *7*, O-ring; *8*, cooling water inlet and outlet; *9*, electrode ports; *10*, sample outlet; *11*, water jacket. The remainder of the unit is identical to the labeled portion.

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CR-6 1(cation exchange).[1] With conservative use the membranes lasted several months or more; however, excessive voltage or heat resulted in their earlier deterioration.

A direct current power supply, continuously variable from 0–500 v and from 0 to 250 ma, was used. The voltage and current readings were such that the calculated resistances were precise to ±3%. The *p*H was measured with a Beckman Model G *p*H meter with fiber-type calomel and glass internal electrodes at 25 ± 1°. The electrodes gave a linear response from *p*H 4.00 to 10.00 at 25° as verified against two sets of Beckman reference buffers. A Beckman Spectrophotometer, Model DU, was used for spectrophotometric analysis.

Method

The unit was assembled by placing a permselective membrane on a rubber gasket seated on the Plexiglass supporting ridge in the electrode compartment. Another washer was placed on the membrane and the sample cell inserted. The other electrode cell was incorporated similarly. Two elastic bands fastened to glass stirrups on the electrode cells maintained positive tension on the unit.[2]

Before electrodialysis, the center cell was filled with water, while distilled water at 4° flowed through the electrode vessels. To test for leaks, the needle valves on the tops of the electrode vessels were shut so that a hydrostatic head of 100 to 200 cm water was applied on the unit. Another check involved shutting the needle valves and opening the bottom ports on the electrode cells to the atmosphere; the absence of dripping was proof of tightness.

During electrodialysis, the flow rate for the coolant was 800 ml/minute, while the electrode-water rate varied from 10–35 ml/minute depending on the wattage. The sample volume must be sufficient to wet 80% of the membrane area or deleterious *p*H effects may occur (see discussion of membrane polarization). To prevent the possibility of protein denaturation, the voltage was initially set at 225 v and reduced to 125 v when the salt content decreased to 0.001 equiv./liter or less.

Effective stirring contributes to the optimum performance of this unit in several ways: by facilitating ion transport from the bulk-solution to the membrane, by reducing protein and salt stratification, and by promoting adequate heat transfer. Even with continuous stirring the contents of sample cell had to be recycled several times to avoid stratification due to electrophoresis-convection (Blank and Valko, 1928; Bier, 1959). When glass inserts were used only intermittent mixing was feasible; therefore, lower voltages and a more frequent recycling of contents were necessary. The results were identical with those obtained by the standard procedure.

The following methodology was used to determine the isoionic point, *pSL*, of a protein. Prior to processing the *p*H was adjusted to a point considerably removed from the *pSL*. Upon completion of electrodialysis, *i.e.*, when the salt content was $\leqq 10^{-5}$ N and a steady-state *p*H obtained, the *p*H was adjusted to the other extreme and the solution reprocessed again. This was repeated for another sample whose concentration differed by at least 50%, but starting from the other *p*H extreme.

[1] These were generously provided by Ionics Inc., Cambridge 42, Mass.

[2] These are not shown in Figure 1.

For proteins insoluble in salt water, electrodialysis was continued to the point of incipient precipitation; then a supporting electrolyte, *e.g.*, NaCl or KCl, was added. This addition was continued until the protein was present in a system of defined composition. Parenthetically, certain water-insoluble proteins remained in solution during electrodialysis, permitting them to be prepared in a salt-free state. In order to redissolve them after freeze-drying, salt solutions were required.

Electrodialysis

Rate Expression.—The rate expression for deionization by electrodialysis, $-dc/dt$, is given by equation (1), where Φ is the electrodialysis coefficient,

$$-\frac{dc}{dt} = \frac{\Phi E}{FRV} \qquad (1)$$

E the applied voltage, F the Faraday constant, R the impedance of the system, and V the volume of solution in the chamber. The equation states and experiments confirm that the rate of desalting varies directly with the applied voltage and inversely with the resistance of the unit. By positioning the electrodes near the membranes an increased effective voltage and a decreased heat production are achieved. The electrodialysis coefficient, Φ, is determined by the membrane type and area, geometry, salts, stirring, temperature, and the polarization of electrodes and membranes.

Membrane.—The membrane type is probably the prime determinant in establishing the efficiency of the unit process (Sollner, 1955). Electrodialysis of 2% gelatin in 0.15 M KCl required 24 hours with cellophane membranes but only 5 hours with permselective membranes (Katz and Maurer, 1957). Another index of membrane performance is the relative electrodialysis rate. This was determined for the following electrolytes at a concentration of 0.01 equiv./liter: sodium chloride, calcium chloride, disodium phosphate, ammonium sulfate, and acetic acid, for which the values were 1, 0.85, 0.8, 0.8, and 0.24, respectively.[3] With the exception of acetic acid (see subsequent discussion) the agreement between these values and the corresponding equivalent conductance in free solution indicates that the possibility of selective or preferential ion transport is negligible (Rosenberg *et al.*, 1957; Harned and Owens, 1950).

The impedance of this unit incorporating permselective membranes and salt is double that observed in the absence of membrane and exhibits a concentration dependence which parallels that of the free solution (see Fig. 2). This resistance increment is explicable in terms of the contribution of the impedances of the membrane and of the medium intervening between the membrane and the electrode. These effects are concentration dependent.

During the initial phase of electrodialysis, when the greatest amount of ion transport occurs, the sample volume decreases by 5–10%. The loss is as water of hydration and because of electro-osmotic flow; the quantity lost varies with the concentration and type of salt present (Rosenberg *et al.*, 1957). This was verified by material balance studies.

To ensure that the *p*H values determined were independent of the membranes used, duplicate experiments were performed with membranes from another

[3] The relative electrodialysis rate is defined as the ratio of the conductance of a given salt relative to that of sodium chloride determined in this unit. The term conductance, used in this context, is the reciprocal of the mean value for resistance calculated from the current-voltage readings taken at four different wattages.

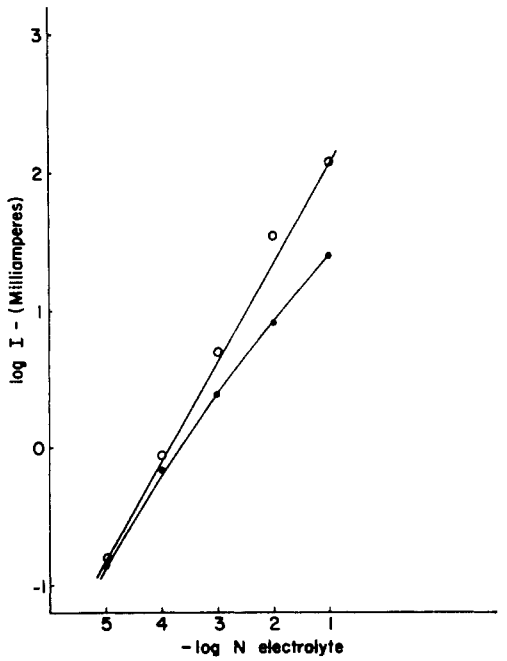

FIG. 2.—Correlation between the electrolyte concentration and current in an electrodialysis cell. O, Ammonium sulfate; ●, acetic acid. Data obtained at 100 v, 25 ± 1°.

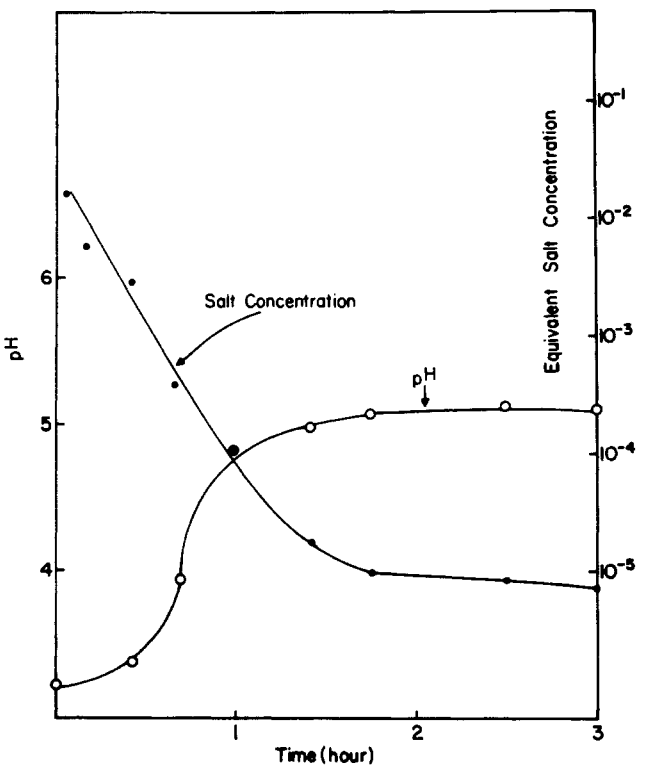

FIG. 3.—The kinetics of desalting a 1.5% bovine serum albumin solution in the electrodialysis cell employing permselective membranes. Initial voltage, 150 v. Right ordinate, the concentration of salts expressed as sodium chloride. Left ordinate, the *p*H of the system.

source.[4] The resulting data were identical, and the processing time agreed within 10% with that for the other membranes.

Quantitative Relationships.—The concentration of dialyzable ions in the cell can be determined from a plot of the logarithm of current, log *I*, *versus* the logarithm of the salt concentration, at a given voltage (Fig. 2). The curves found for strong electrolytes were linear and essentially parallel in the region between 10^{-5} to 10^{-1} N, and their conductivities agreed within 20% in this zone. The concentrations determined by conductivity were in good accord with those found by direct analysis. The presence of protein has no demonstrable effect on the observed conductivity,[5] since there is no protein transport through the membranes, and because the conductivity of isoionic protein is virtually zero (Timasheff *et al.*, 1957; Möller *et al.*, 1961). Weak electrolytes, such as acetic acid, yield a linear relationship only when the concentration is 10^{-3} N or less (Fig. 2). The deviation from linearity at higher concentrations is due to intermolecular association. However, as reference to Figure 2 will reveal, in dilute solutions the voltage-current requirements for both weak and strong electrolytes are comparable.

Desalting Kinetics.—A representative plot for the electrodialysis of a 1.5% solution of bovine serum albumin in 0.05 N NaCl, initial *p*H 3.2, is summarized in Figure 3. The change in conductivity followed first-order kinetics for the initial 95% of this process, in accord with equation (1), while the *p*H rate data, depicted by a sigmoid curve, were more complicated. The *p*H data reveal that the hydrogen ion contribution to the initial ion content is small and its value depends on two factors, namely, its rate of transport and the buffering capacity of protein. The desalting rate is independent of the protein concentration in the range 0–5%. At higher concentrations this rate is reduced because of increased viscosity and electroconvection effects. Routinely, a 2% albumin solution in 0.1 M NaCl can be desalted to 10^{-5} equiv./liter in the course of 4 to 5 hours. The protein recovery was about 95%, as determined spectroscopically at 279 mμ.

To establish the effect of ion-protein interaction on the rate of deionization a large excess of calcium ion was added to bovine serum albumin under conditions which promoted binding (Katz and Klotz, 1953). Since the desalting rate was identical to that in control experiments, apparently protein-metal ion binding does not interfere with electrodialysis as would be expected in view of the rapid reversibility of binding and the marked *p*H dependence of ion binding (Perkins, 1961). The latter factor is the basis for the initial *p*H adjustment to a point removed from the *pSL* prior to electrodialysis. However, the electrodialysis of 2% bovine serum albumin in 0.1 M NaCl and 0.003 M tryptophan initially adjusted to *p*H 11 removed only 30% of the amino acid. This cannot be ascribed to protein binding but rather to the fact that the amino acid is converted to the amphoteric state and therefore does not migrate in an electric field. Maintaining the *p*H at either 3 or 10 keeps the amino acid in a charged form and permits its quantitative removal.[6] Since amphoteric amino acids can exist over a wide range of *p*H and are characterized by a lack of buffering capacity, their presence with isoionic protein has little effect on the *p*H; e.g., the *p*H of 2% isoionic bovine serum albumin in 0.001 N isoionic tryptophan differed from that of isoionic bovine serum albumin by only 0.03 *p*H units.

[4] The AMFION membranes were provided by the the American Machine and Foundry Co., Springdale, Conn.

[5] Providing that uptake of ions by complexing is negligible.

[6] This *p*H adjustment precludes any interference due to tryptophan-albumin binding since this association is maximal at *p*H 9.2 and decreases sharply on either side of this joint (McMenamy and Oncley, 1958).

Current-Voltage Relationships

Coulombic Heat.—The two factors which limit the voltage for the successful electrodialysis of proteins are coulombic heat and the membrane polarization effects. The possibility of protein denaturation by heat led to the analysis of the thermal production and transfer mechanisms operative in this unit. Some data obtained at an ambient temperature of 25 ± 1° and at 8 watts are presented. When the unit was air-cooled and the major heat-sink was the electrode water at 24° circulating at 30 ml/minute, a steady-state temperature of 44° was reached in 18 minutes. Using 22° cooling water in conjunction with electrode water at 4°, at the previous flow rate, resulted in a steady-state temperature of 31°. About 65% of the heat was dissipated by the coolant and the remainder by the electrode water. Since the power input was 5 watts or less, the temperature was invariably under 25°.[7]

Membrane Polarization.—The deleterious effects observed at high voltages often have been attributed to localized temperature gradients; however, more probably these are due to membrane polarization effects. Membrane polarization occurs when an excessive voltage is applied so that the rate of ion transport from the bulk solution to the membrane cannot compensate for the ion removal at the interface and as a result this zone becomes ion depleted. To meet the voltage-current demand, the water adjacent to the membrane hydrolyzes with protons being transferred through the cation-exchange membrane, creating a zone of elevated *p*H. The converse occurs at the other membrane. Rosenberg and Tirrel (1957) noted that the *p*H gradient is a function of the over-voltage, the nature and concentration of the electrolytes, and the rate of exchange or mixing with the solution. An analytical statement for this phenomenon (Cowan, 1960) takes the form of equation (2), where R_s and

$$\frac{E}{I} = (R_s + R_m)_c + \frac{E_d + E_p}{I} = R \qquad (2)$$

R_m are the resistances of the solution and membrane at a given concentration of salt, c; E_d is the decomposition potential at the electrode; and E_p is the membrane polarization voltage, while the other symbols have their usual significance. It follows that when the limiting current is not exceeded, the total resistance, R, should be constant, since the membrane polarization voltage is zero and the other terms are fixed at a given salt concentration. Thus, when E/I is plotted against E, the curve should have a zero slope, as exemplified by 0.005 N NaCl at voltage and power inputs ranging from 0–350v and 0–15 watts (Fig. 4).[8] The slope obtained with 0.0005 N NaCl was zero up to 250v, but at higher voltages this changed to a high positive value, indicative of membrane polarization. Here polarization occurred at less than 1.3 watts, thereby precluding any temperature effects.

The advent of membrane polarization during the electrodialysis of proteins results in turbidity, flocculation, and a product frequently insoluble in aqueous solutions. After membrane polarization the *p*H reaches a steady-state value which often can differ from the isoionic *p*H by as much as 0.3 units. There is evidence that a similar electrolytic action may occur when less than 75% of the membrane surfaces are not wetted.

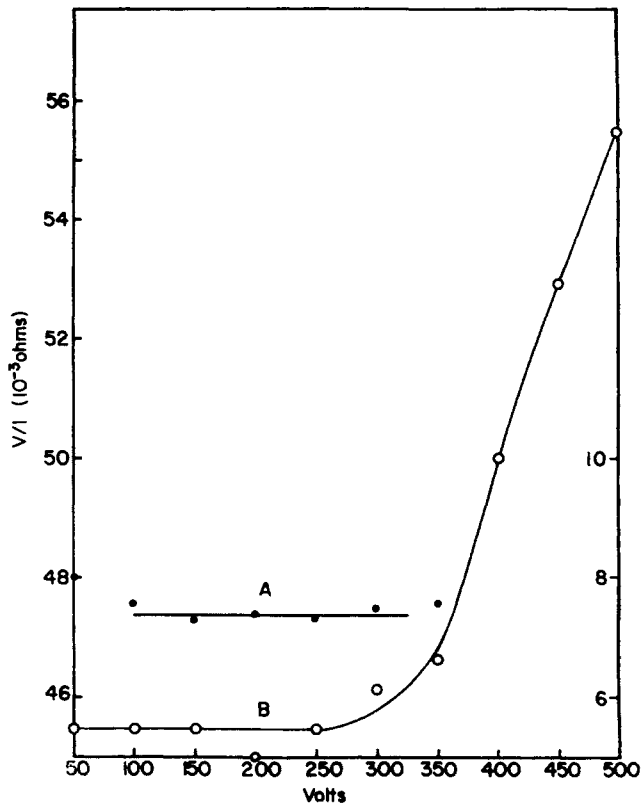

FIG. 4.—The relationship between voltage and membrane polarization as a function of salt concentration in the electrodialysis cell. *A*, 5 × 10⁻³ M sodium chloride. The maximum wattage applied was 12 watts. Corresponding ordinate is to right. *B*, 5 × 10⁻⁴ M sodium chloride, maximum wattage applied was 1.5 watt. The ordinate is to the left.

Isoionic Points

The isoionic points, *pSL*, of some proteins are presented in Table I (Linderstrøm-Lang and Nielsen).[9] A measure of the reproducibility of these data is the standard deviation of 0.018 for the *pSL* of bovine serum albumin as determined in eight experiments conducted over a 6-month period. The *pSL* of 5.10 determined for bovine serum albumin is higher than the other values reported for bovine serum albumin, with the exception of the *pSL* of 5.15 obtained for mercaptalbumin (Scatchard *et al.*, 1957). The difference may be caused by variations in the homogeneity of the sample (Hartley *et al.*, 1960) or the desalting technique employed. Scatchard and Black (1949) found that methanol-extracted human serum albumin had a *pSL* of 4.98 compared to 4.88 for the original material.

Contrary to expectation, bovine γ-globulin, in concentrations up to 2.5%, exhibited no tendency to precipitate during electrodialysis. The *pSL* reported for this protein, 6.87, is that of the salt-free material present in ion-free water. The electrodialysis of bovine hemoglobin resulted in a limited amount of

[7] When cooling water at 14° was used, the steady-state temperature decreased to 18° owing to the increased efficiency of heat transfer.

[8] In this graph, the effective voltage was assumed to be the applied voltage, *i.e.*, no correction was made for *IR* drop between the electrode and the membrane. This explains the deviation from linearity observed in the region of low voltage.

[9] The definition of *pSL* employed is as follows: The isoionic point, *pSL*, is obtained when sufficient isoionic protein is dissolved in pure water to yield a characteristic *p*H value which exhibits a trivial concentration dependence. The protein concentration studied covered the range from 1-3%.

TABLE I

ISOIONIC POINT OF PROTEINS AS OBTAINED BY ELECTRODIALYSIS[a,b]

Type	Purity (Electrophoretic)[c]	*pSL* This Study	*pSL* Others	
Bovine albumin	Crystallized 100%	5.10	4.9	Timasheff *et al.* (1957)
			4.90	Möller *et al.* (1961)
Bovine serum mercaptalbumin			5.15	Scatchard *et al.* (1957)
Ovalbumin	5 × Crystallized >98%	4.62		
Bovine γ-globulin fraction II	2 × Crystallized >98%	6.87		
Bovine hemoglobin[d]	2 × Crystallized	6.85		
Human hemoglobin			7.18	Vodrazka and Cejka (1961)
Trypsin	1 × Crystallized	6.7	6.52	Duke *et al.* (1952)

[a] Solvent: water; temperature 25 ± 1°. [b] All proteins were purchased from Pentex, Inc., Kankakee, Ill. The concentrations used were 1 to 3%. [c] These data were supplied by the manufacturer. [d] Some denaturation occurred during electrodialysis; see Discussion.

denaturation, as evidenced by a slight color change. However, the values obtained at four different processing times agreed to within ±0.02 *p*H units. Apparently the *pSL* of the native and denatured protein were similar and or the extent of denaturation was minimal.

The value reported for trypsin is less reliable for several reasons. The *p*H adjustment to the alkaline side was omitted since autolysis occurs in this region. A *pSL* of 6.7 ± 0.06 was determined from four experiments in which the processing time ranged from 2.5 to 5.0 hours. The sample processed for 5 hours showed evidence of autolysis, since only 95% of the protein was precipitable by trichloroacetic acid. However, this *pSL* is in fair agreement with the value of 6.52 determined by Duke *et al.* (1952) for isoionic trypsin prepared by rapid electrophoresis-convection. Reservations were expressed by this group concerning the validity of their value, since a slight increase in acid-binding capacity was observed which was taken as evidence of autolysis. The considerable difference existing between the *pSL* and the isoelectric point, *pI*, of trypsin of 10.8 reported by Bier and Nord (1951) and a value of 10.5 found for diisopropyl phosphoryl trypsin (Cunningham, 1954), is difficult to reconcile.[10] However, since trypsin's buffering capacity is low between *p*H 6.5 and 10, the complexing of the protein with buffer ions could conceivably result in such a shift. *pI* changes of a similar magnitude for several proteins, dependent on the type and concentration of buffer used, have been documented (Velick, 1949).

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[10] The buffers used for the electrophoretic studies of trypsin contained 0.03 M $CaCl_2$; however, the buffer employed for diisopropyl phosphoryl trypsin did not include calcium or similar bivalent cation. This eliminates the possibility of calcium binding as the major source of this substantial difference.

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