A Method of Preparing Isoelectrically Pure Proteins and Other Ampholytes

Background of the Invention

1. Field of the Invention

The present invention relates to a method of purifying amphoteric molecules, in particular proteins, for both research and commercial use. Proteins purified using this method may be used therapeutically, such as insulin for the control of diabetes; prophylactically, such as for the purification of the protein components of acellular vaccines; or for the preparation of diagnostics, such as for hepatitis B surface antigen. This invention may also be applied to purifying proteins as part of research protocols particularly if the protein is present at low concentrations in the original aqueous solution.

2. Description of Prior Art

Isoelectric focusing is used both to purify and to analyze protein preparations. The earliest mention in the professional literature of an isoelectric focusing technique is in the report of Kolin entitled "Separation and concentration of proteins in a pH field combined with an electric field" in the Journal of Chemical Physics (Vol. 22, pp.1628-1629) in 1954. This publication describes how isoelectric focusing was performed in gradients using solutions with two different pH values. This method of forming the pH gradient was later improved by Svennson, who described how ampholytes could be used to stabilize the pH gradients. This report is given in an article titled "Isoelectric fractionation, analysis and characterization

of ampholytes in natural pH gradients. The differential equation of solute concentrations at a steady state and its solution for simple cases." Acta Chem. Scand. 15: 325-341 (1961). Later, Vesterberg described how to use specially synthesized ampholytes to prepare wide range pH gradients that are particularly useful for analysis of protein mixtures. This is reported in an article titled "Synthesis and isoelectric fractionation of carrier ampholytes." Acta Chem. Scand. 23:2653-2666 (1969).

Isoelectric focusing using synthetic ampholytes to stabilize the pH gradient is one of the most powerful tools for purifying proteins. This is because each protein has a nearly unique isoelectric point, known as its "pI". The isoelectric point is the pH at which the net charge of the protein is zero, i.e. the pH at which the positive and negative charges on the protein are exactly in balance. The electrical charges on proteins and most other soluble amphoteric compounds arise from the ionization of weak acidic and weak basic groups. At high pH values, weak acid groups ionize and contribute a negative charge to the protein. At low or acid pH values, the weak bases take on a hydrogen ion to add a positive charge to the protein. Each protein has different numbers of these ionizable groups and the dissociation constants for these groups vary. If a direct electric current is applied to proteins in a stable pH gradient, each protein moves to the position at which its net charge is zero. Since the pI of each protein species is nearly always different from that of other protein species in an aqueous solution, the different proteins migrate to different positions in the pH gradient. This isoelectric focusing technique not only separates proteins from one another, but

concentrates them as well. This is another reason isoelectric focusing is such a powerful technique.

There are, however, problems associated with the present practice of using synthetic ampholytes to form the pH gradients. First, ampholytes are too costly for the commercial production of many proteins. Second, the ampholytes are difficult to remove from the protein preparations after they have been purified. Both of these problems restrict the use of ampholytes for preparing parenteral products such as protein hormones, cytokines, clotting factors, vaccine components, etc.

Summary of the Invention

The present invention is a method of preparing stable pH gradients, and a method of using the same for isoelectric focusing of amphoteric molecules.

The pH gradient is prepared by adding a first quantity of soluble non-ionizing compound, such as sucrose, to a first quantity of buffer to achieve a saturation concentration of sucrose in the buffer; adding a second quantity of sucrose to a second quantity of buffer to achieve a second concentration of sucrose in buffer; layering the second solution on the saturated solution, and repeating this procedure for as many gradients as desired.

Isoelectric focusing is done by layering a quantity of amphoteric compound, e.g. insulin or other protein, on the pH gradient, and applying a direct electric current across the pH gradient to focus molecules of the insulin at locations within the pH gradient determined by the isoelectric points of the molecules.

Drawing

Figure 1 is a plot of hydrogen ion activities for various hydroxyl containing substrates vs. their molality after addition to 0.10 ionic strength phosphate buffer.

Figure 2 is a plot of the slopes of the lines shown in Figure 1, showing that the shift in a_H is related to the number of hydroxyls on the solute.

Figure 3 is a plot showing the trend of how the initial pH of the buffer affects the degree of pH change when sucrose is added.

Figure 4 is a plot of the slopes of a_H shift upon addition of sucrose to phosphate buffers with different ionic strengths.

Figure 5 is a schematic elevation view of a vessel for the procedure.

Description of the Invention

The present invention relates to a method of isoelectric purification of molecules using polyhydric compounds to stabilize the pH gradient. Polyhydric compounds include all compounds that have a hydroxyl group covalently linked to them. These compounds include alcohols, glycols, glycerols, as well as saccharides and polysaccharides. I have discovered that these compounds affect the acidity of solutions in which they are dissolved. Acidity is defined as the activity of the hydrogen ion (a_H) or pH (= - log a_H) in that solution. An example of how polyhydric compounds affect a_H is shown in the Figure 1 plot of hydrogen ion activities for various hydroxyl containing substrates vs. their molality after addition to

0.10 ionic strength phosphate buffer. It is noted that if the polyhydric compound had two or fewer hydroxyls, the a_H of the aqueous phosphate buffer decreased, i.e. the pH increased with increasing solute concentration. This was seen for ethanol, methanol, and glycol. When there are three hydroxyls, such as on glycerol, the compound did not cause a substantial change in the a_H. If there were four or more hydroxyls, the a_H increased, i.e. the pH decreased upon addition of the compound to the buffer. Further, the greater the number of hydroxyls, the greater the decrease in pH per mole of the compound added. It can also be noted in Figure 1 that the greater the amount of the compound added the greater the change in the activity of the hydrogen ion. In these experiments, sucrose caused the greatest increase in the acidity of the buffers. These results were unexpected and seem to constitute a contradiction to the Hammet equations as presented by R.G. Bates in Determination of pH: Theory and Practice. J. Wiley and Sons, Inc., New York, pp. 196-199 (1964).

In Figure 1, the number of hydroxyls on the compound appears to be related to the increase in the hydrogen ion activity (a_H). When the slopes of the lines in Figure 1 were plotted vs. the number of hydroxyls, a nearly linear relationship was seen. Figure 2 is a plot of the slopes of the lines shown in Figure 1 showing that the shift in a_H is related to the number of hydroxyls on the solute. This seems to predict that even greater shifts in the pH would occur if a compound bearing even higher numbers of hydroxyls were dissolved in the buffer. However, for most purposes, sucrose offers enough capacity to shift the pH for the construction of stable pH gradients to use in isoelectric focusing. The use of sucrose has additional advantages; it

is inexpensive and can be easily removed by dialysis. Further, the dissolved sucrose forms a stable density gradient. Still, it is possible that compounds bearing greater numbers of hydroxyls may be used to construct suitable pH gradients. These will most likely be polysaccharides.

The range of the pH shift caused by the addition of the polyhydric compound is related to the starting pH of the buffer and the buffer's concentration. It was found that the higher the starting pH of the buffer, the greater the decrease in the pH. Thus, the addition of sucrose to buffers in the alkaline range will change the pH by half a pH unit or more, but in the extreme acid range the addition of the same amount of sucrose may increase the pH by less than a tenth of a pH unit. Figure 3 shows the effect of adding sucrose to buffers with different initial pH values. All buffers were prepared at 0.10 ionic strength with the obvious exception of water. This plot shows that the higher the pH the greater the shift in pH upon addition of sucrose. In addition, the initial concentration of the buffer affects the extent to which sucrose can shift its pH. This is shown in Figure 4, which is a plot of the slopes of a_H shift upon addition of sucrose to phosphate buffers with different ionic strengths. This plot shows that the greater the ionic strength of the buffer the greater the shift in pH upon sucrose addition. Both of these effects are important considerations in the construction pH gradients and whether the gradient will be a practical for purifying a particular protein. Because of the shorter ranges of the low pH gradients, one has to be more precise in selecting the buffer pH, and if the pI is very low it may prove impractical. The concentration of the buffer is important

because the focusing requires the passage of a direct electric current through the gradient. If the buffer contains a high concentration of ions, it will take longer to focus the protein. If the buffer concentration is too low, the focusing will be rapid, but there is less buffering capacity and the pH gradient shorter. For this reason an optimal ionic content must be used, e.g., near 0.01 ionic strength.

The previous data pertained to how polyhydric compounds affect the pH of various buffers. The following information is related to how this phenomenon can be used to construct pH gradients and use them for electrophoresing proteins so that they will concentrate in the gradient at their isoelectric point. In most instances, these gradients are limited to less than a pH unit in range, but are suitable for separating of amphoteric molecules such as a protein if its pI is known. There are two kinds of gradients that can be used. They can be either continuous gradients or step gradients. Continuous gradients suitable for this kind of electrophoresis may be prepared using the same kind of mixer used to prepare density gradients and may be purchased from several scientific supply companies. Step gradients can be prepared by simply layering a lower density (concentration) solution of the polyhydric compound, e.g. sucrose, over a higher density solution of the same polyhydric compound in a gravitational field. Both types of gradient rely on the density of the solutions for retaining stability of the pH gradient.

Both types of gradients are also suitable for resolving proteins of different pI. The invention is best suited for the preparation of pure proteins, and not as an analytical tool for determining pI. For this reason it is preferred that the pI of the protein be known before

setting up the gradient. The pI may be found from the published literature, from an experiment using the wide pH range ampholyte isoelectric focusing technique, or from a theoretical calculation based on its amino acid content. Once the pI of the protein is known, a suitable pH gradient can then be constructed for purifying and concentrating the protein. Step gradients are usually adequate for this purpose as long as the pI falls within the pH range defined by the difference between the two concentrations of the dissolved polyhydric compound. In effect, because of the small amount of mixing that occurs as they are prepared, step gradients are actually very steep continuous gradients. The polyhydric compounds that may be used include ethanol, methanol, glycol, glycerol, inositol, dextrose, sucrose, as well as any other saccharides and polysaccharides that do not ionize in the pH range of the gradient. Other possible polyhydric compounds include starches, mannan, cellulose, etc. For most purposes, sucrose has proved suitable for the formation of the gradient.

Buffer selection is also critical, and there are several considerations that need to be addressed for its selection. The first is that has a pKa at or near the pH of the protein one wishes to purify. Buffers are most effective in the pH range near where they ionize, and this will increases the stability of the gradient. A second consideration is the concentration of the buffer. If the buffer is too concentrated, the buffer ions will carry the bulk of the electric current and reduce the mobility of the protein so that the separation process will be very slow. On the other hand, if the buffer is too dilute, the gradient will be shortened as the data in Figure 4

demonstrate. A third consideration is that the buffer should not chemically react with the polyhydric compound in a way that may affect the pH of the gradient. For example, boric acid and molybdic acid react covalently with sucrose resulting in a progressive decrease in the pH that takes several days to reach completion. Suitable buffers include both weak acids and weak bases. These include but are not limited to acetic acid, phosphoric acid, hydroxylamine, ethanolamine, tris[hydroxy-methyl]aminomethane (TRIS) and any other buffer that does not react covalently with the polyhydric compound.

The present invention relates to a method of purifying proteins and other ampholytes by electrophoresing them in pH gradients formed by the addition of polyhydric compounds to aqueous buffer solutions. The selection of the buffer and the polyhydric compound for forming the pH gradient must be adjusted according to the protein or amphoteric molecule that is to be purified and/or concentrated. The best suited buffer is one with a pKa at or near the pI of the protein to be separated. The preferred ionic strength is between .001 and 0.1. The preferred polyhydric compound is sucrose.

The following example shows this method applied to separate one protein from a mixture with other proteins, based on the former protein's pI value.

Example

Figure 5 is a schematic elevation view of a vessel 10 that may be used for the procedure. The vessel 10 is constructed of an electrically non-conductive material, such as a plastic or glass, and includes a bottom, 12; outer sidewalls, 14; and an open top. The interior of the vessel 10 is divided by a first partition, 16, extending upward from the bottom, and a second partition, 18, extending downward from the top, into three chambers; an anode chamber 20, a cathode chamber 24, and a gradient chamber 22 between them. An anode, 26, is disposed near the bottom of the anode chamber 20. A cathode, 28, is disposed near the top of the cathode chamber 24. The partition 16 is lower than the sidewalls 14 and is, therefore, "open" at the top. The partition 18 is open at the bottom. The three chambers thus define a series path between the anode 26 and the cathode 28 that will allow an electric current to pass. The anode 26 and cathode 28 are connected to a D.C. source 30.

To prepare the vessel for use, buffer with a saturation concentration of sucrose is first layered into the bottom of the cathode chamber 24 and gradient chamber 22. This same sucrose-buffer solution is mixed with buffer without sucrose using a gradient mixing device to form a linear gradient layered on top of the sucrose-saturated solution in the gradient chamber 22. Buffer without dissolved sucrose is then added on top of the gradient and into the other chambers to immerse the electrodes. A small amount of sucrose is added to a sample of protein that has previously been dialyzed against the buffer. This sample is then carefully layered on top of the gradient where it meets the sucrose-free buffer. A direct

current is applied across the gradient causing the protein, which has a positive charge in the sucrose-free buffer, to migrate toward the cathode. The protein migrates until it reaches the pH in the gradient that is the same as its pI. The desired purified (or separated or concentrated) product is withdrawn with a syringe or the like.

This kind of vessel was used to separate different isoelectric forms of hemoglobin. The buffer used for preparing the gradient was a 0.01 ionic strength phosphate buffer of pH 7.5. The sucrose-saturated phosphate buffer, which had a pH of 7.1, was layered into the bottom of chambers 24 and 22. The same stock of sucrose-buffer solution was mixed with buffer without sucrose using a gradient mixing device to form a linear gradient layered on top of the sucrose-saturated solution. The length of the final gradient was approximately 7 cm. Buffer, without any dissolved sucrose, was added on top of the gradient and into the other chambers to immerse the electrodes. A small amount of sucrose was added to the sample of protein that had previously been dialyzed against the phosphate buffer. This was then carefully layered on top of the gradient where it met the sucrose free buffer. A direct electric current of 35 amp was applied across the gradient causing the protein, which had a positive charge in the buffer lacking sucrose, to migrate toward the cathode. The protein migrated until it reached the pH in the gradient that was the same as its pI. After an hour, two bands were observed in the gradient portion of the cell. Normal adult hemoglobin can have five different isoelectric forms, and older specimens six forms. Only the bands for A2 (pI=7.4) and methaemoglobin (pI=7.2) fell within the range of the gradient and were resolved. The pH at the

bottom of the gradient was too high to focus the A, A1c, A1a or A1b forms. The bands persisted at the same position in the gradient despite the continued application of the electric current for over three hours. at which time the current was disconnected.

Claims

What is claimed is:

1. A method of preparing stable pH gradients, including the following steps:

a. adding a first quantity of soluble non-ionizing compound to a first quantity of buffer to achieve a saturation concentration of said compound in said buffer;

b. adding a second quantity of said compound to a second quantity of said buffer to achieve a second concentration of said compound in said buffer; and

c. layering the solution from step (b) on the solution from step (a).

2. A method as defined in claim 1, further including the following steps:

d. adding a third quantity of said compound to a third quantity of said buffer to achieve a third concentration of said compound in said buffer; and

e. layering the solution from step (d) on the solution from step (b).

3. A method as defined in claim 1, in which said non-ionizing compound is a polyhydric compound.

4. A method as defined in claim 3, in which said polyhydric compound is sucrose.

5. A method as defined in claim 1, in which said buffer is prepared from a substance selected from the group consisting of (a) phosphoric acid and the salts thereof; (b) pyrophosphoric acid and the salts thereof; (c) acetic acid and the salts thereof; (d) tris[hydroxy-methyl]aminomethane (TRIS) and the acid forms thereof; and (e) hydroxylamine and one of the acid forms thereof.

6. A method of isoelectric focusing of amphoteric molecules, including the following steps:

a. adding a first quantity of soluble non-ionizing compound to a first quantity of buffer to achieve a saturation concentration of said compound in said buffer;

b. adding a second quantity of said compound to a second quantity of said buffer to achieve a second concentration of said compound in said buffer; and

c. layering the solution from step (b) on the solution from step (a);

d. layering a quantity of amphoteric compound on the pH gradient thus formed; and

e. applying a direct electric current across said pH gradient to focus molecules of said amphoteric compound at locations within said pH gradient determined by the isoelectric points of said molecules.

7. A method as defined in claim 6, in which said amphoteric compound is selected from the group consisting of peptides and protein.

8. A method as defined in claim 6, in which said amphoteric compound is protein that can be used as a part of a therapy to treat a disease.

9. A method as defined in claim 6, in which said amphoteric compound is a protein that can be used directly or indirectly to diagnose a disease.

10. A method as defined in claim 6, in which said amphoteric compound is a protein that can be used in a vaccine to prevent disease.

11. A method as defined in claim 6, in which said amphoteric compound is an amphoteric polysaccharide.

12. A method of isoelectric focusing of molecules by electrophoresis of said molecules in pH gradients formed by the addition of polyhydric compounds to aqueous buffer solutions.

13. Electrophoresis apparatus, including:

a vessel having a bottom, sidewalls, and an open top;

a first interior partition extending partially upward from the bottom of said vessel, forming with said sidewalls an anode chamber on one side of said vessel;

a second interior partition extending partially downward from the top of said vessel, forming with said sidewalls a cathode chamber on the other side of said vessel;

said first and second partitions forming a gradient chamber between them;

said anode chamber, said gradient chamber, and said cathode chamber together forming a serpentine series fluid path from the bottom of said anode chamber to the top of said cathode chamber;

14. Electrophoresis apparatus as defined in claim 13, further including an anode electrode electrode disposed at the bottom of said anode chamber;

a cathode electrode disposed at the top of said cathode chamber; and a source of direct current operatively connected to said anode and said cathode.

15. Electrophoresis apparatus, including:

a vessel having a bottom, sidewalls, and an open top;

a first interior partition partially upward from the bottom of said vessel, forming with said sidewalls an anode chamber on one side of said vessel;

a second interior partition extending partially downward from the top of said vessel, forming with said sidewalls a cathode chamber on the other side of said vessel;

said first and second partitions forming a gradient chamber between them to receive: (a) a first quantity of buffer with a saturation concentration of soluble non-ionizing compound; (b) a

second quantity of buffer with a lower concentration of said compound therein; and (c) a quantity of amphoteric compound on the pH gradient formed by (a) and (b);

said anode chamber, said gradient chamber, and said cathode chamber together forming a serpentine series fluid path from the bottom of said anode chamber to the top of said cathode chamber, said anode and cathode chambers adapted to receive quantities of buffer to fill said chambers and complete said fluid path;

an anode electrode disposed at the bottom of said anode chamber;

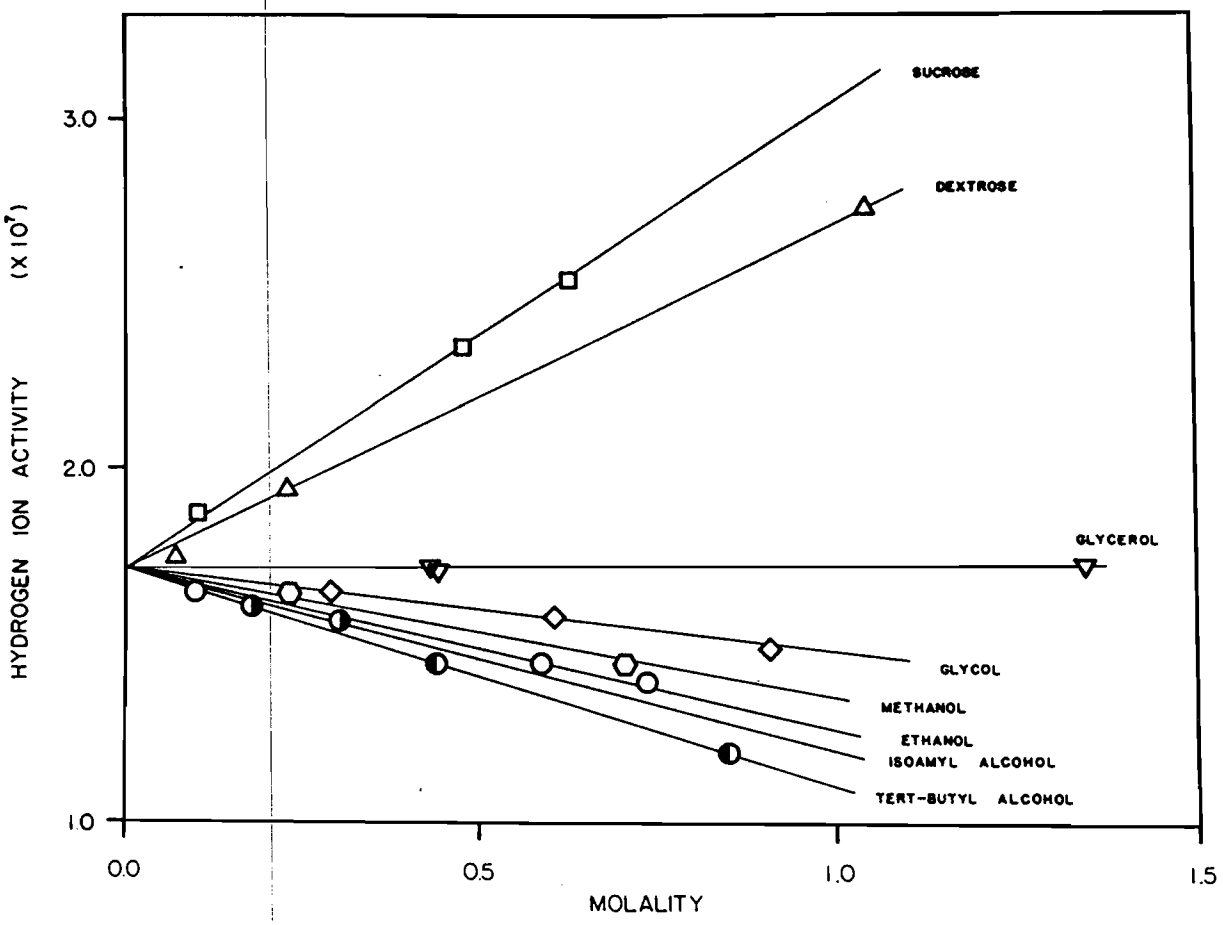
a cathode electrode disposed at the top of said cathode chamber;

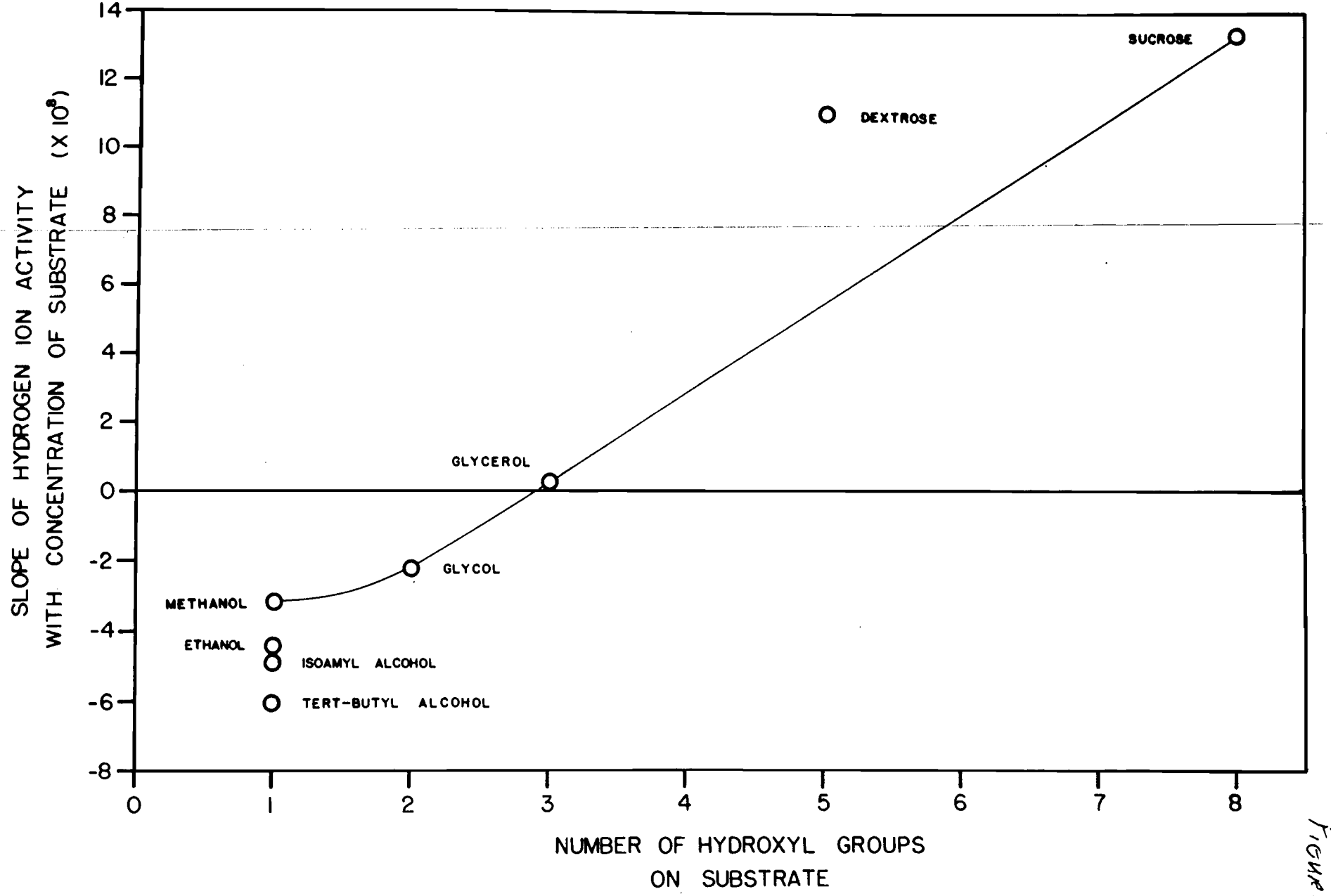
a source of direct electric current operatively connected to said anode and said cathode to cause migration of molecules of said amphoteric compound within said vessel.

Abstract of the Disclosure

A method for preparing stable pH gradients suitable for purifying amphoteric compounds, such as proteins, by isoelectric focusing is disclosed. These pH gradients are formed by dissolving increasing amounts of polyhydric compounds, e.g. sucrose, in aqueous buffer solutions. When a direct electric current is applied to these gradients, the amphoteric compound migrates to the point in the gradient where it has zero net charge. Special synthetic compounds called ampholytes, usually used to prepare pH gradients for isoelectric focusing, are not needed in the method described.

FIGURE 1


SUCROSE
DEXTROSE
GLYCEROL
GLYCOL
METHANOL
ETHANOL
ISOAMYL ALCOHOL
TERT-BUTYL ALCOHOL
HYDROGEN ION ACTIVITY (X 10⁷)
3.0
2.0
1.0
0.0
0.5
1.0
1.5
MOLALITY


SLOPE OF HYDROGEN ION ACTIVITY WITH CONCENTRATION OF SUBSTRATE (X 10⁸)
14
12
10
8
6
4
2
0
-2
-4
-6
-8
0
1
2
3
4
5
6
7
8
NUMBER OF HYDROXYL GROUPS ON SUBSTRATE
SUCROSE
DEXTROSE
GLYCEROL
GLYCOL
METHANOL
ETHANOL
ISOAMYL ALCOHOL
TERT-BUTYL ALCOHOL

FIGURE

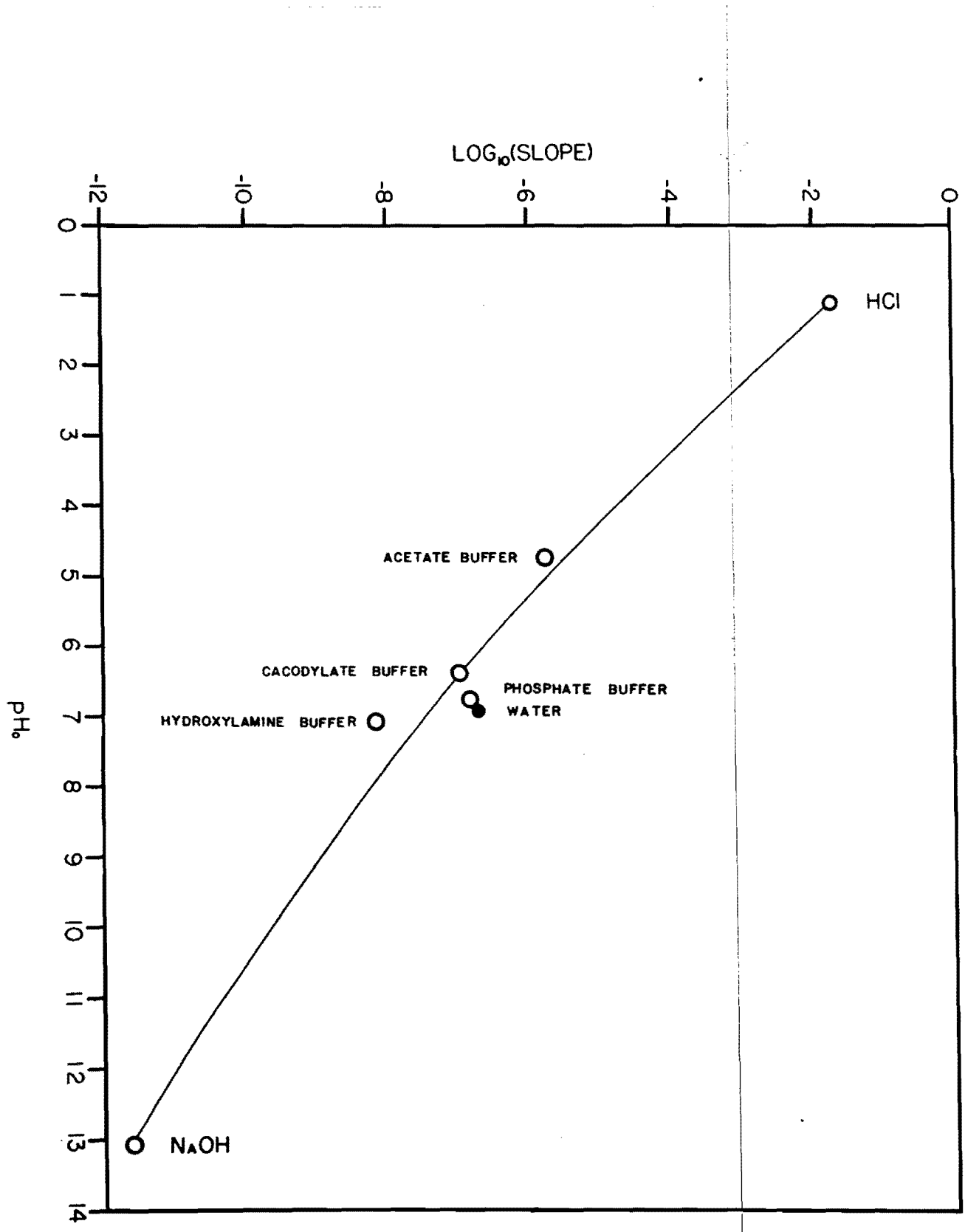

LOG10(SLOPE)
-12
-10
-8
-6
-4
-2
0
pH0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
HCl
ACETATE BUFFER
CACODYLATE BUFFER
PHOSPHATE BUFFER
WATER
HYDROXYLAMINE BUFFER
NaOH

FIGURE 4

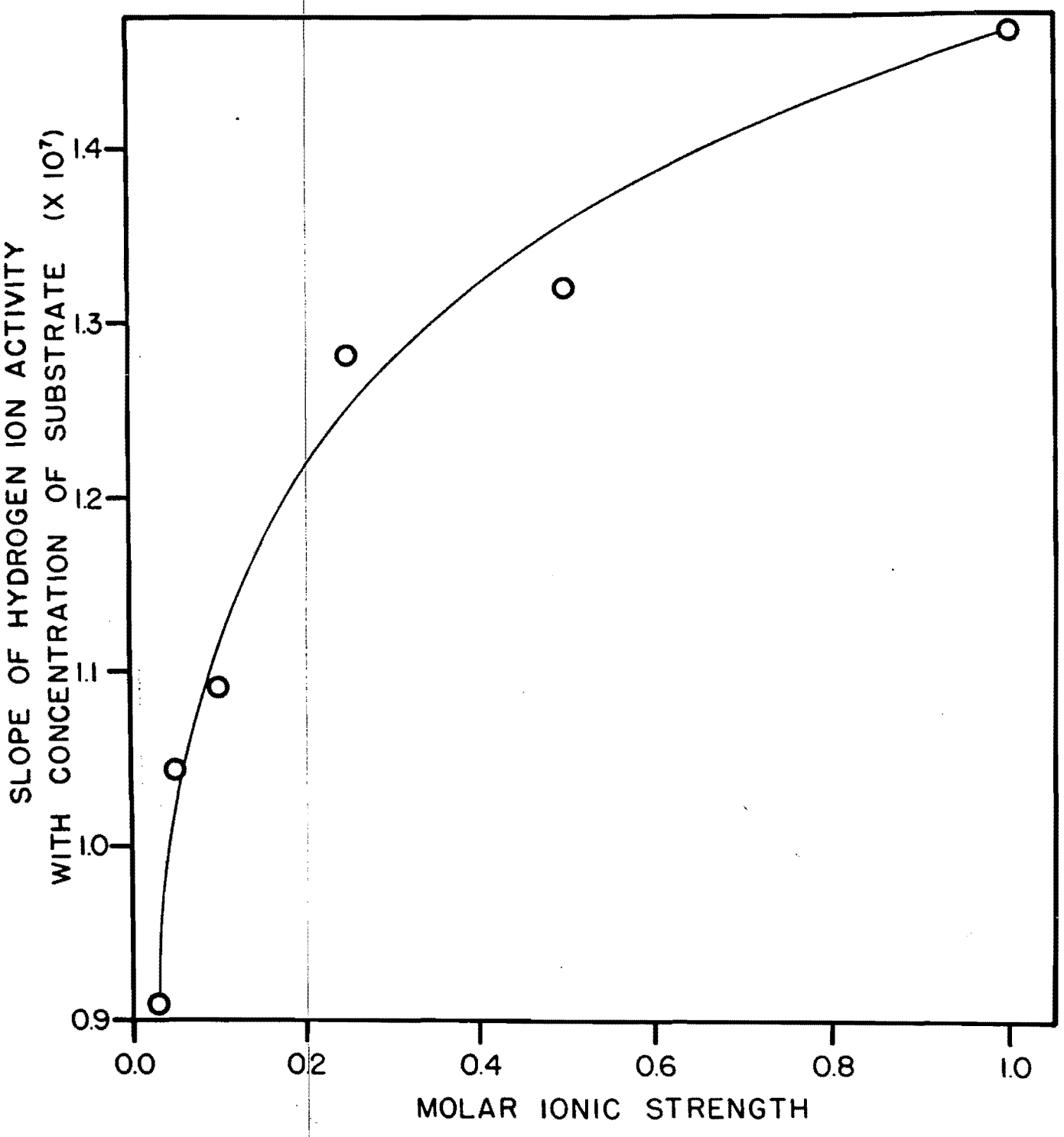
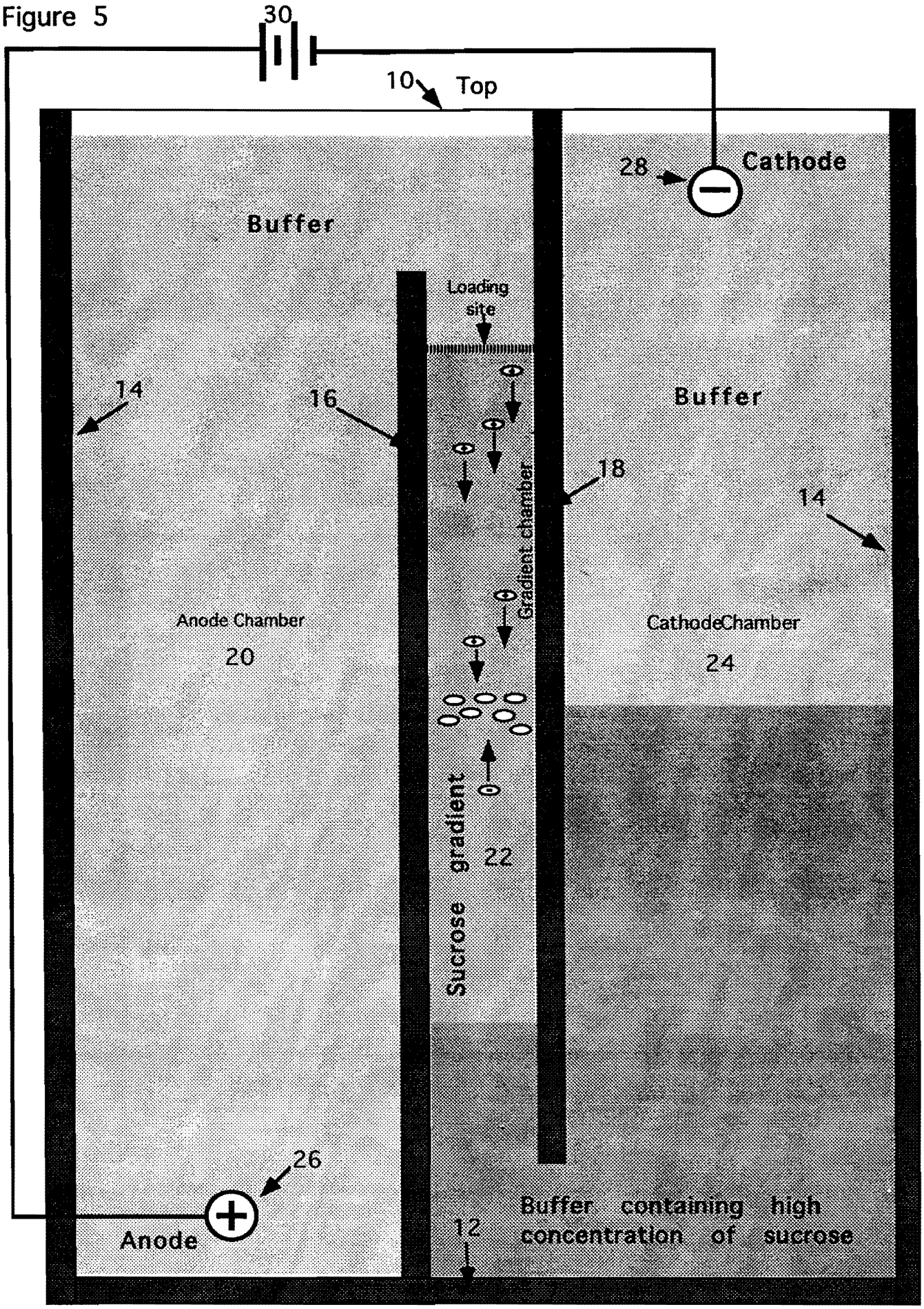

SLOPE OF HYDROGEN ION ACTIVITY WITH CONCENTRATION OF SUBSTRATE (X 10⁷)
1.4
1.3
1.2
1.1
1.0
0.9
0.0
0.2
0.4
0.6
0.8
1.0
MOLAR IONIC STRENGTH

Figure 5


30
10
Top
28
Cathode
Buffer
Loading site
14
16
18
Buffer
Gradient chamber
14
Anode Chamber
20
CathodeChamber
24
Sucrose gradient
22
26
Anode
12
Buffer containing high concentration of sucrose