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Polson et al.

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[54] CHEMICALLY HARDENED GELATIN-SEPARATING MEDIUM FOR CHROMATOGRAPHY AND ION EXCHANGE AND METHOD OF MAKING THE SAME

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[30] **Foreign Application Priority Data**

Dec. 18, 1967 Republic of South Africa.....67/7563

[52] **U.S. Cl.**.....**204/180 G,** 204/299, 210/31 C, 210/198 C, 260/117

[51] **Int. Cl.**.....**B01k 5/00,** B01d 13/02

[58] **Field of Search**.....260/117; 210/31 C, 198 C; 204/180 R, 180 G, 180 S, 299

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Assistant Examiner—A. C. Prescott

Attorney—Connolly and Hutz

[57] **ABSTRACT**

Selected gelatin, in particular the high molecular weight fraction obtained by fractionation with PEG is chemically hardened e.g. with tanning agents and used in a form of beads for exclusion chromatography and/or ion exchange or as a continuous body for electrophoresis. Ion exchange properties may be suppressed or enhanced as required by chemical treatment of the gelatin or by the choice of conditions during separation (pH, salt content). Ion-exchange properties are calibrated by electro-osmosis.

34 Claims, 15 Drawing Figures

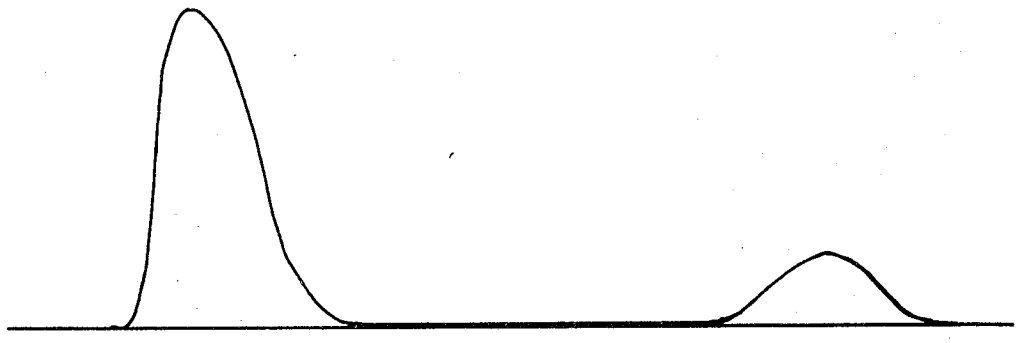


FIG. 1a

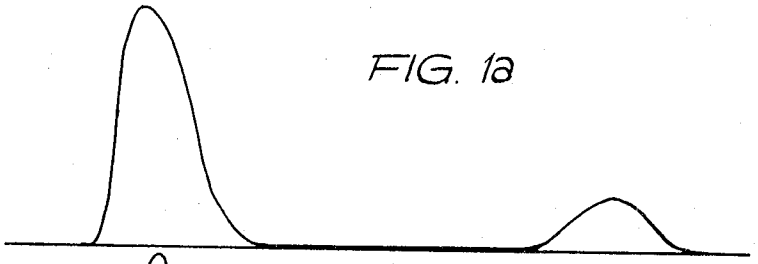


FIG. 1b

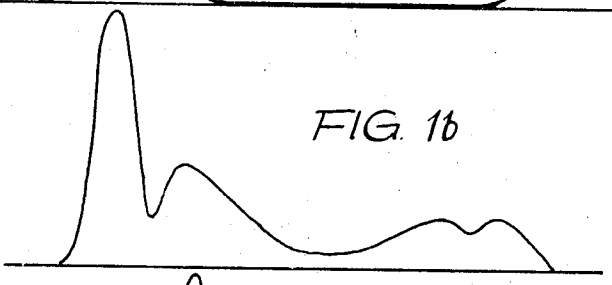


FIG. 2a

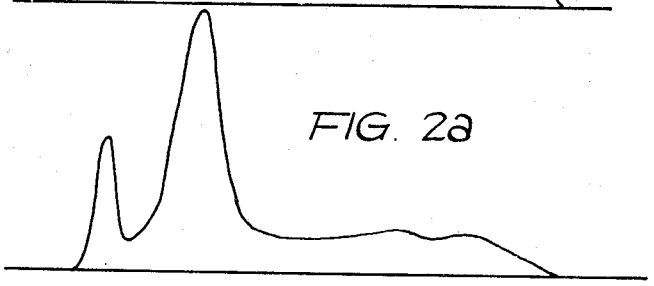


FIG. 2b

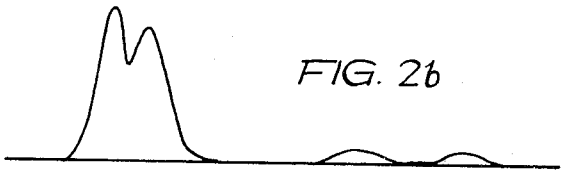


FIG. 3a

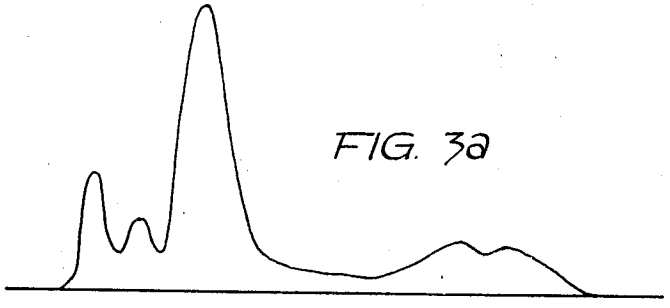


FIG. 3b

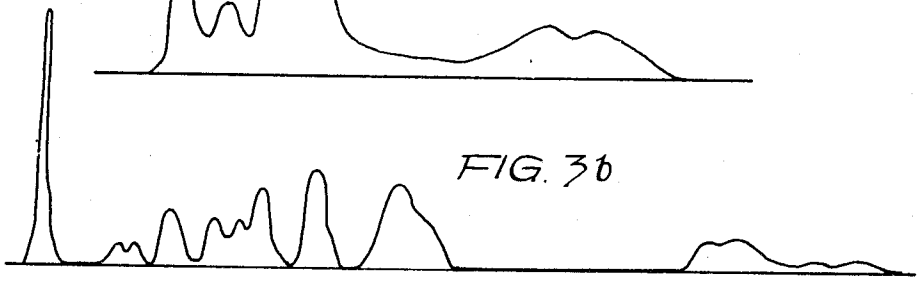


FIG. 4A

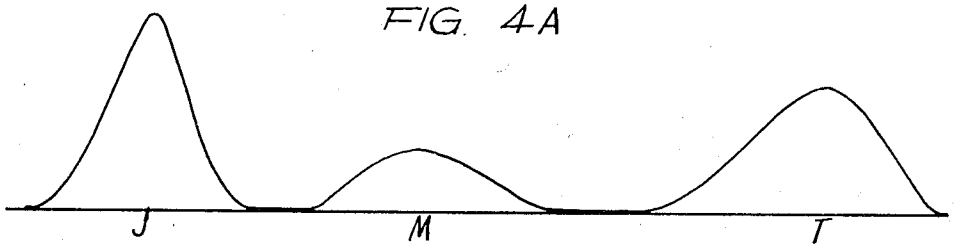


FIG. 4B

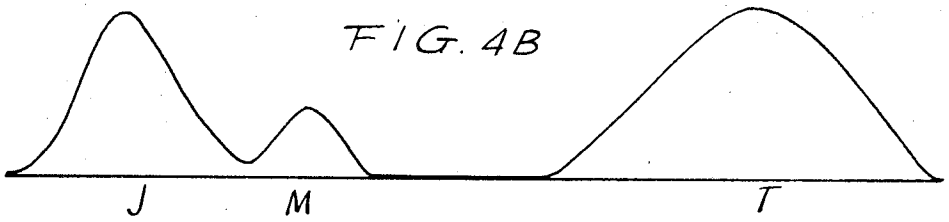


FIG. 4C

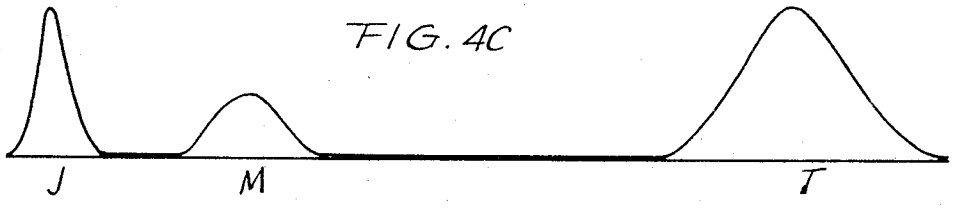
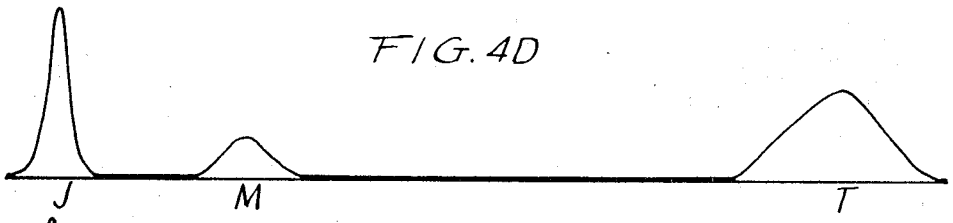


FIG. 4D



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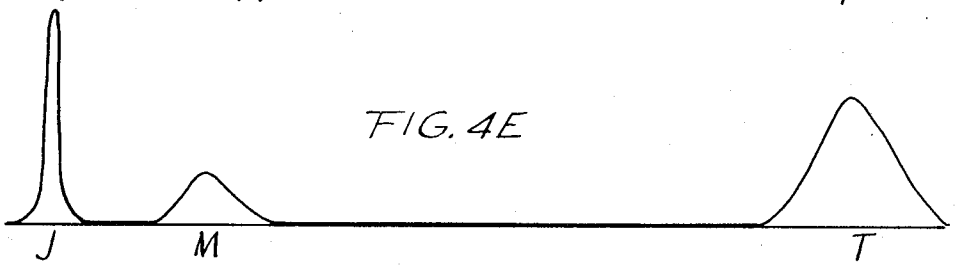


FIG. 4F

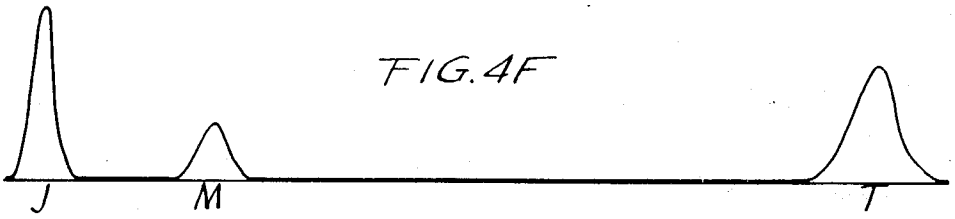


FIG. 5

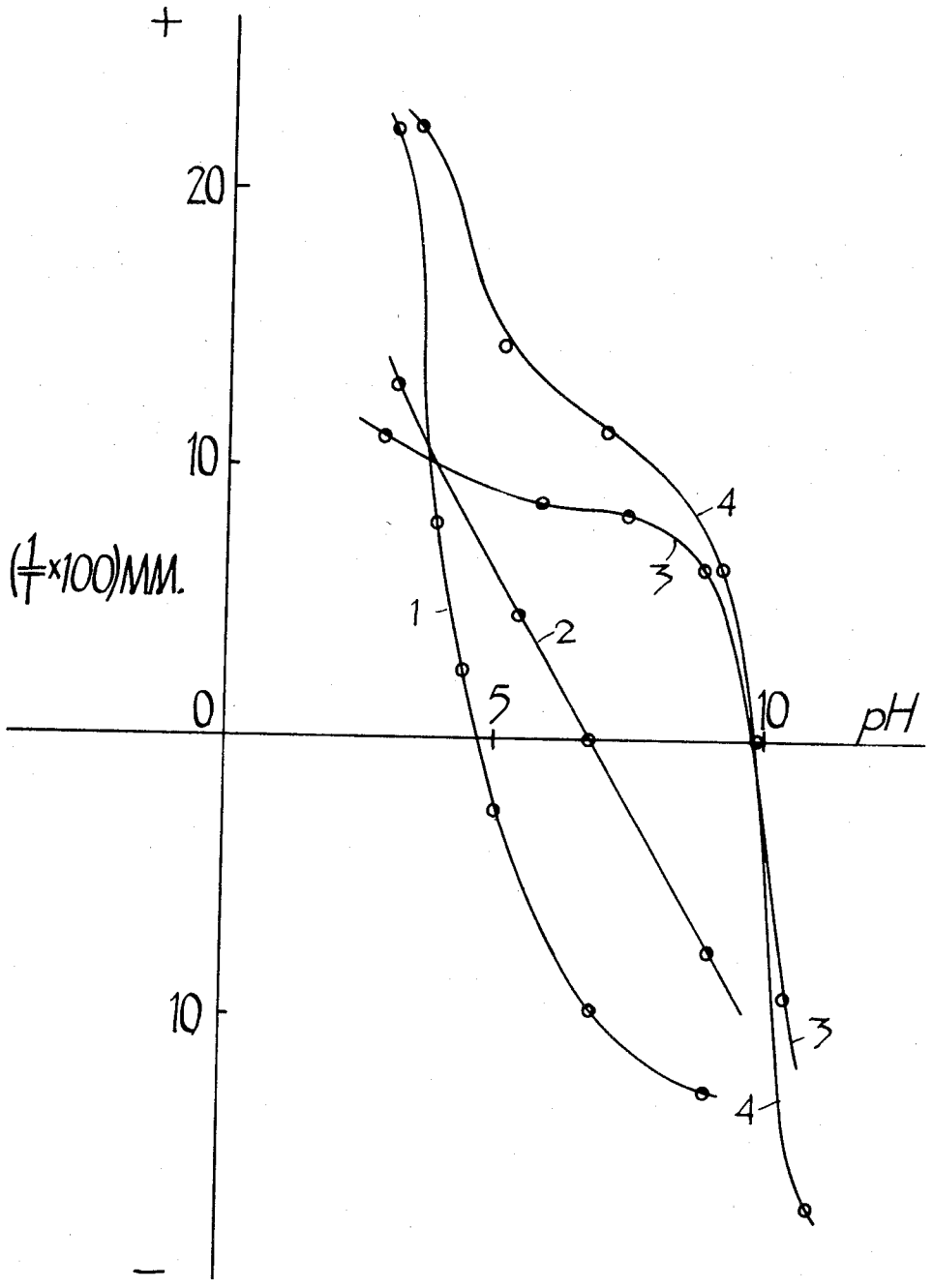


FIG. 6

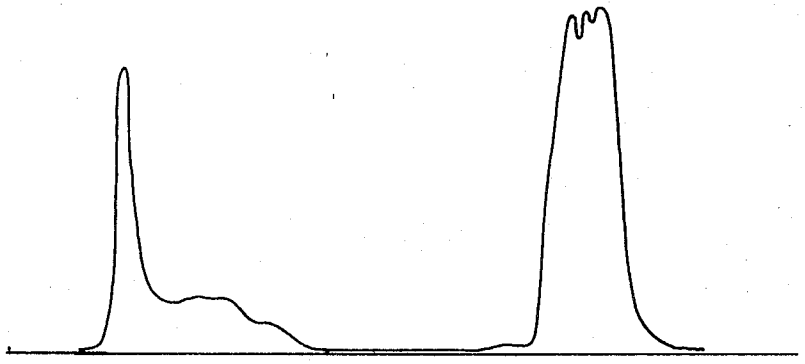
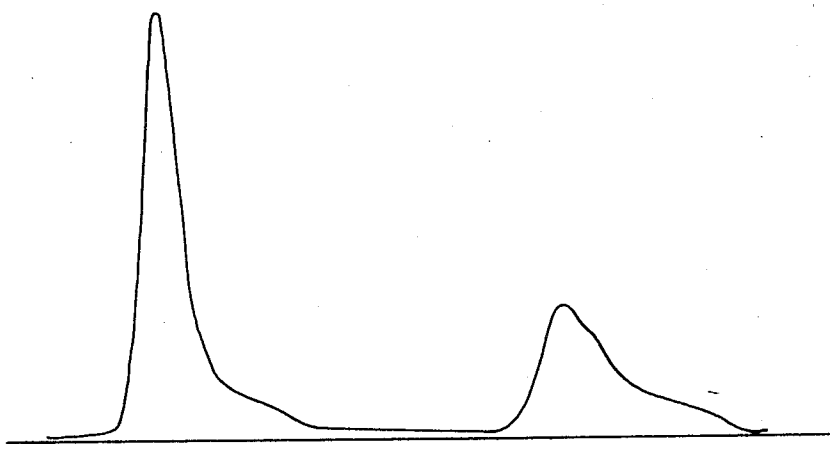


FIG. 7



CHEMICALLY HARDENED GELATIN-SEPARATING MEDIUM FOR CHROMATOGRAPHY AND ION EXCHANGE AND METHOD OF MAKING THE SAME

BACKGROUND OF THE INVENTION

The present invention relates to a medium for separations, more particularly to a novel medium for separations, useful in particular for chromatography, in particular so-called exclusion chromatography or gel filtration by virtue of its ability to act as a molecular sieve, although other properties such as absorptive properties or ion exchange properties may be present as well to a greater or lesser degree which, in accordance with the present invention may be controlled and utilized when desired. The invention also provides for the separating medium to be in the form of cast bodies adapted for electrophoresis. The invention further provides for a process for the production of the separating medium, the use of the separating medium for carrying out separations, and includes in its scope the products of such separations.

A number of media suitable for exclusion chromatography are already known, some of the more important of which in the context of exclusion chromatography are cross-linked dextran of which different preparations may be used for retarding substances in the molecular weight range 300 to 300,000, agar-agar which in the same sense covers the molecular weight range of 10,000 to approximately 4,000,000 and polyacrylates which can retard substances up to a molecular weight of approximately 500,000. There exists a definite need for a larger variety of separating media to enable those skilled in the separating art to select separating media best suited to a particular separating problem. Thus, for example it was found possible with some of the separating media in accordance with the present invention to achieve very superior separations as compared with those possible with previously known media.

SUMMARY OF THE INVENTION

The separating medium in accordance with the invention is of a macromolecular character and comprises, in a form suitable for a separating process a gelatin which has been chemically hardened and is indispersable at temperatures above 7° C., more particularly at least as high as room temperature, preferably at least up to 100° C., and is permeable to the flow therethrough of a chromatographic mobile phase. More particularly, the gelatin is selected with a high molecular weight in the sense of having an intrinsic viscosity of at least 0.9 dl./g., preferably at least 0.95 dl./g. more particular between 0.95 and 1.0 dl./g., say 0.95 dl./g. (deciliters per gram) measured in 4 molar urea, saline, neutral pH, prior to said chemical hardening.

Another measure of suitability is the sedimentation coefficient of the nonhardened gelatin which is preferably in excess of 2.4×10^{-13} cm./sec./dyne (measured in 4M urea saline to avoid gelling or congealing), say at least 2.8×10^{-13} cm./sec.dyne.

In order to be suitable as a separating medium, in particular for the purpose of chromatography the medium should have a high ratio of surface area to volume of gelatin. Preferably said ratio is at least 10 $cm.^{-1}$, for smaller columns at least $\frac{3}{8}\times100$ $cm.^{-1}$ which in the case of beads of the separating medium corresponds to a bead diameter of 100 microns, the preferred bead diameter for chromatography being between 100 and 300 microns, preferably as nearly as possible to 200 microns for preparative columns and smaller, say 25–50 micron for micro-analytical columns, and intermediate between these values for intermediate sizes of columns.

Conceivably however, the separating medium may also be applied as a coating on a substratum, e.g., as a coating of beads of inert material such as glass or any other surface suitable as a support, when the above preferred ratios of surface area to volume of active separating medium apply.

Further details of the separating medium may be deduced from the following description of its method of preparation.

The method of preparation in accordance with the invention comprises transforming a selected gelatin into a form physically adapted as a separating medium for chromatography, permeable to the flow therethrough of a chromatographic mobile phase, including a chemical step of hardening the gelatin and insolubilizing it in the sense of rendering it indispersable in water above a temperature of 7° C. and preferably at least of room temperature or even higher.

Regarding the selection of the gelatin, the above-mentioned intrinsic viscosities apply. The gelatin may be derived from any suitable normal source of gelatin e.g., bone, hides, or collagen, pigskin gelatin (acid processed) giving a very rigid gel even at low gel concentrations, particularly after chromium-tanning and being at present preferred as a source.

To obtain a gelatin of the desired intrinsic viscosity the preferred process includes a separating step by which gelatin fractions having an intrinsic viscosity outside the desired range are separated from a fraction within the desired range. The preferred separating process is carried out by fractionation in aqueous medium with polyethylene glycol (PEG), preferably having a molecular weight above 300, more particularly above 1,000 say of approximately 6,000 to produce a heavy phase in which the high molecular weight gelatin is concentrated and the supernatant phase containing predominantly the lower molecular weight fraction. The separation is for example carried out with PEG employed in concentrations between 5 and 8 percent, by weight based on the total, and the process is described in greater detail in our patent application Ser. No. 627,308 filing date Mar. 31, 1967 now U.S. Pat. No. 3,415,804 which by reference thereto is to be considered as part of the present disclosure. The desired high molecular weight fraction may be washed, for example with water to remove PEG and then with acetone or another suitable solvent to remove water and is then dried e.g., at room temperature.

Instead of PEG which was found to be particularly suitable it is in principle possible to employ other separating agents being capable of fractionating on a basis of molecular weight, for example dextran. Alternative methods such as gel filtration may also be employed.

In order to produce a separating medium of defined properties in respect of its functions as a molecular sieve, the gelatin is dissolved in aqueous medium, more particularly water in a predetermined concentration, e.g., from 1 percent upwards, more particularly in the range of 4 percent to 45 percent depending on the molecular weight of the substances for the separation of which the medium is to be employed. From the aqueous dispersion thus produced it is now possible to produce beads of suitable particle size as a separating medium. This is done, for example by emulsifying with a substantially nonpolar medium, for example a mixture of toluene and carbontetrachloride, and in the presence of a suitable emulsifying agent, stirring, cooling and breaking the emulsion e.g., with methanol or ethanol. The concentration of the emulsifying agent and the rate of stirring influence the particle size of the beads.

A preferred emulsifying agent is a commercial epoxy vegetable oil product, e.g., that produced and marketed by Badische Anilin und Sodafabrik A.G. under the trade name "Emulphor EL." This emulsifying agent is employed in a preferred concentration of between 0.5 and 5 percent W/w more particularly 0.7 to 2 percent based on the aqueous gelatin solution. The preferred ratio of water in the aqueous solution to said nonpolar medium is between 1:1.5 and 1:3 say 1:2.

The preferred ratio of toluene to CCl_4 (when these are employed) is between 5:1 and 2:1.

The chemical hardening of the gelatin may be carried out either before or after the bead formation. In the latter case, the particles are filtered off and then treated, in the former case the chemical hardening agents are added prior to the step of cooling and allowing the beads to set.

In accordance with the preferred method the chemicals employed for the chemical hardening are substantially of the nature of tanning agents known as such in the leather-tanning art. They may be selected in accordance with the particular properties desired of the separating medium. For example, tannic acids or tannins e.g., spray-dried wattle tannin have been employed for the purpose to produce a separating medium having one particular set of separating characteristics, while hardening of the gelatin with formaldehyde (formalin) which reacts with the free NH_2 groups of the gelatin or hardening by chromium tanning with a chromium composition known in the tanning art as "Kromex" or with a combination of the two produced totally different separating characteristics, both of which may be desirable under different circumstances.

At present it is preferred to carry out the hardening with formaldehyde and/or the said "Kromex" (the latter eliminating the free carboxyl groups of the gelatin), interalia because by using these tanning agents it is possible to eliminate partly or wholly either the anionic or the cationic ion-exchange properties of the gelatin or both.

In many cases it is advantageous to harden the gelatin with both formalin and "Kromex," one after the other. Depending on the desired properties, the preferred sequence may be "-Kromex" first, followed by formaldehyde or vice versa.

"Kromex" as commercially available has approximately the following composition:

Mixture of chromic hydroxide, chromide sulphate $Na_2(SO_4)$ and a small quantity of sucrose:

0.915 parts $Cr(OH)_3$+0.542 parts $Cr_2(SO_4)_3$ +1.00 parts $Na_2SO_4\pi$¼0.119 parts $C_6H_{12}O_6$

In principle it is not only possible to eliminate ion-exchange properties but the tanning or hardening agent may even be selected to impart particular ion-exchange properties to the gelatin if such should be desired, or the gelatin may be subjected to chemical reactions prior to or after hardening with the exclusive object of introducing into the gelatin molecule specific ion-exchanging groups. Where ion-exchange properties are desired the use of gel concentrations not less than 25 percent, say from 30 percent upwards is preferred for reasons of adequate ion-exchange capacities.

The ion-exchange properties may in accordance with a preferred feature of the invention be calibrated by electro-osmosis through a membrane of the medium, and the invention includes in its scope the media thus calibrated.

In accordance with the invention there is furthermore provided a fractionating process which comprises introducing a mixture to be fractionated into a separating medium in accordance with the invention, bringing about movement of a component of the mixture through the separating medium and relative to at least one other component of the mixture at least in part by percolation of a liquid through said medium, and recovering at least one of said components separately from the other said component.

Said movement may be brought about by electrophoresis where the substances to be fractionated lend themselves thereto, i.e., to movement under the influence of an applied potential difference.

Important embodiments of the invention provide for said movement to be brought about by percolation of a liquid through a bed of the separating medium. This would include various forms of chromatography whether using only molecular sieve properties of the medium (e.g., by using a gelatin from which all ion-exchange groups have been eliminated or by adjusting with buffers the pH to a value at which ion exchange properties of the medium are suppressed or achieving the same object by carrying out exclusion chromatography under strongly saline conditions) or utilizing ion-exchange properties of the medium.

The separating process in accordance with the invention may be carried out substantially in a manner known per se but employing a separating medium as defined above or as prepared in accordance with the above process.

With beads manufactured in the manner described above, it was possible to achieve substantial insolubility of the gelatin at temperatures above 7° C., more particularly within the normal temperature ranges employed in chromatography and even up to the boiling temperature of water, and furthermore a strengthening of the beads sufficient to prevent clogging of a column under the pressures applied thereto. It is possible to eliminate or increase substantially either or both kinds of ion exchange and in particular it was possible to achieve resolutions vastly superior to resolutions obtained with commercially available molecular sieves by making positive use of this variability. The attainable flow rates were found to be higher than those attainable with many existing molecular sieve preparations.

The ratio of water to gelatine in the gel affects the molecular sieve properties.

BRIEF DESCRIPTION OF THE DRAWING

The drawings are to be read in conjunction with the following examples, FIGS. *1a*, *1b*, *2a*, *2b*, *3a*, *3b* and *4a–4f* chromatograms obtained in accordance with Examples 5 to 8;

FIG. 5 represents a set of calibration diagrams obtained by electro-osmosis on four different samples of chemically hardened gelatin for use as a separating medium in accordance with the invention;

FIGS. 6 and 7 represent chromatograms obtained with ion exchangers in accordance with Examples 10 and 11.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Example 2

a. Preparation of gelatin of high-gel strength

Gelatin (160 g.) was dissolved in 3,800 ml. of distilled water over a boiling water bath with gentle stirring. When all the material had dissolved a solution of polyethylene glycol (240 g. PEG dissolved in 200 ml. of water) was added to the warm solution of gelatin and well mixed. The solution was kept at room temperature for 2–3 hours when it was found that the high gel strength fraction separated out as a viscous oily layer. The upper fraction was completely removed and discarded, and the remaining portion was slowly poured into 4 litres of distilled water at 4° C. which was slowly agitated with a mechanical stirrer. The insoluble gelatin collected round the stirrer and was removed prior to storing it with the water at 4° C. overnight. The following day the ropey product was collected in a large precooled filter funnel containing a cotton wool plug to prevent the gelatin from passing through with the filtrate. The gelatin was washed repeatedly with large volumes of cold distilled water until free of PEG. The swollen strands were squeezed to express water and then dried by immersion in two changes of acetone. Excess acetone was removed by extraction with light petroleum (boiling range 40°–60° C.) and the material was finally dried by passing a warm current of air over it. The temperature of the air should not exceed 50° C. as at higher temperatures of drying a large proportion of the gelatin was found to become insoluble.

The following changes were produced by the fractionation:

i. the gelatin (unfractionated had an intrinsic viscosity in 4 Molar Urea, saline, neutral pH, η=0.76 dl./g. (deciliters/g.)

The same gelatin fractionated with PEG 6,000 had an intrinsic viscosity in 4 M Urea, saline, neutral pH, η=0.95 dl./g. The viscosity measurements were done with an Ubbelohde type viscometer in a thermostatically controlled water bath at 26° C.

ii. Sedimentation coefficient at 0 g./100 ml.

Unfractionated $2.0>10^{-13}$ cm./sec./dyne fractionated $2.8\times10^{-}$B&13 cm./sec./dyne

The measurements of viscosity and sedimentation were done in 4 M Urea saline to avoid gelling (congealing).

iii. The fractionated product was more homogeneous.

b. Preparation of gelatin beads

Beads were prepared as follows:

A known amount of the high molecular weight gelatin was dissolved in 300 ml. of distilled water over a boiling water bath with gentle stirring. The organic liquid phase toluene + carbon-tetrachloride) containing the dissolved stabilizer "Emulphor EL" (Badische Anilin und Soda-Fabrik AG, Ludwigshafen am Rhein, Germany) was preheated to 50° C. and added to the dissolved gelatin. The mechanical stirrer was switched on and after approximately 1 minute the flask containing the suspension was lowered with the stirrer into a beaker containing crushed ice.

Once the temperature had reached ±4° C. approximately 1 liter of ethanol (0° C.) was added to break the emulsion. The beads were allowed to settle at 4° C. for approximately 30 minutes and were then collected on a precooled Buchner filter. They were washed several times with small volumes of cold ethanol until the filtrate gave no turbidity when mixed with water. (Regarded by us as a qualitative test for Emulphor). Untanned beads were kept as close to 0° C. as possible to prevent them redissolving. The concentration of stabilizer, toluene and carbon tetrachloride and the rate of stirring for the preparation of different concentrations of gelatin beads are given in the following table:

Concentration gelatin, g./300 ml. H_2O	Toluene, ml.	CCl_4, ml.	Emulphor EL stabilizer, grams
6	490	110	3.5
12	480	120	5.0
21	450	150	4.5
30	440	160	2.0
45	420	180	2.5
90	420	180	5.0
120	420	180	7.5

The beads of the different formulations differed in their ability to retain substances of different maximum molecular weights.

In the present examples the rate of stirring did not exceed 1,000 revolutions per minute, but this rate could be adjusted according to the size of beads required.

c. Preparation of formalin-tanned beads

Untanned beads prepared as described above were transferred to a beaker containing 1 1. per batch as above of a solution of 20 percent (w/v) formalin and 2 percent (w/v) sodium chloride at pH 9.0 which was then briskly stirred for 2 minutes. The tanning was allowed to proceed for 24 hours after which the beads were washed free of formalin with cold 0.85 percent (w/v) saline, and kept in phosphate-buffered saline pH 7.0 containing sodium azide (1/10,000) as a preservative.

d. Preparation of Kromex-tanned beads.

Untanned beads prepared as described above were transferred to a beaker containing 1 liter of a solution of Kromex 4 percent (w/v) and 2 percent (w/v) sodium chloride at pH 2.8. The suspension was well stirred and tanning was allowed to continue for 4 hours. A harder preparation is produced by tanning for 24 or preferably 48 hours. The beads were collected on a Buchner filter and well washed with 0.85 percent (w/v) saline until quite free of Kromex and kept in phosphate buffered saline pH 7.0 with sodium azide (1/10,000) as a preservative.

e. Preparation of formalin-Kromex-tanned beads

Formalin-tanned beads prepared as described above were tanned with Kromex as previously described.

Example 2

Gelatin beads prepared with a spray gun

A shallow precooled glass trough was partially filled with a cold solution of Kromex-saline pH 2.8 into which was poured twice to three times the volume of ether at 0° C. The trough was surrounded with crushed ice to prevent the temperature from rising above about 40° C. A spray gun having a nozzle with a pore diameter of 0.1 mm. or less was used for spraying the warm solution of gelatin (of various concentrations) previously fractionated as described in Example 1*a*, directly onto the cold surface of the ether, from a height of approximately 30 cm. The gelatin beads passed through the ether and came into contact with the Kromex solution. The ether was removed, and the Kromex bead suspension was kept at 4° C. until the tanning had reached completion. The remaining ether was removed under negative pressure and the beads were collected and preserved as previously described. To obtain spheres of different porosities the gelatin concentration was varied between 4 and 30 percent.

Example 3

Preparation of tanned gelatin granules

Gelatin of known concentration, previously fractionated as described in Example 1*a*, was dissolved in water over a boiling water bath with gentle stirring. The hot solution was allowed to cool to approximately 50° C. and was immediately poured into a precooled enamel dish which was surrounded with crushed ice and water. Once the solution had gelled it was cut into small pieces (about 1-cm. cubes) with a knife and transferred to a beaker containing 1 liter of a solution of Kromex-saline pH 2.8 at 4° C. The tanning was continued at 4° C. for 24 hours after which the entire contents of the beaker were homogenized in a Waring blender until the particles were small enough for use in chromatography. The excess Kromex was removed with numerous washings of saline on a Buchner filter. The granules were suspended in phosphate buffered saline pH 7.0 with sodium azide (1/10,000) as a preservative. Concentrations of gelatin varying between 2 and 40 percent were prepared for exclusion chromatography experiments.

An observation of great interest is that pearls (beads) made by the emulsifying technique in which organic solvents are used are less porous than the granules which are made by the disintegration of the tanned gelatin gel of the same initial concentration. A possible explanation is that the surface of the beads is covered with a shell which has a higher concentration due to loss of water to the organic solvents.

Example 4

The procedure in accordance with Example 1 is modified as follows: e.g.,

The fractionated gelatin is dissolved in water and treated with nonpolar solvent and stabilizer as before. Immediately after starting the stirring the desired tanning agent, e.g., 1.5 grams Kromex and/or vegetable wattle tannin was added and stirring was continued for 10–15 minutes prior to cooling.

After that the procedure was continued as in Example 1*b*.

Example 5

FIGS. 1*a* and 1*b* illustrate the chromatographic partial separation of two haemocyanins, Jasus lalandii (Mol. Wt. 500,000) and Burnupena cincta (Mol. Wt. 6,600,000), confirmed by analytical ultracentrifugation. The separations were carried out with columns of 4 percent gelatin beads prepared in accordance with Examples 2*e* and 1*d* respectively.

The elluting fluid was phosphate buffered saline, pH 6.9 and the experiments were conducted at 21°, the effluent being monitored with an optical recording unit.

Example 6

FIGS. 2*a* and 2*b* illustrate the chromatographic separation of human serum employing 4 percent gelatin beads prepared in accordance with examples 1*d* and 1*e* respectively.

The elluting fluid was phosphate buffered saline, pH 6.9 and the experiments were conducted at 21°, the effluent being monitored with an optical recording unit.

Example 7

It was possible to resolve chromatographically cobra venom to a considerably greater degree using beads prepared from a 30 percent aqueous gelatin solution in accordance with Example 1*e* (FIG. 3*b*) than with the commercial preparation "-Sephadex G–50" (FIG. 3*a*).

The elluting fluid was phosphate buffered saline, pH 6.9 and the experiments were conducted at 21°, the effluent being monitored with an optical recording unit.

It was found that the choice of a pH of 6.9 being considerably on the low side of the isoelectric point of the separating medium (approximately pH 9.7) favored this kind of separation by making good use of the ion-exchange properties of the preparation.

Example 8

FIGS. 4*a*–4*f* illustrate the separation of Jasus lalandii haemocyanin (J), Myoglobin (M) and tryptophan (T) using various beads produced in accordance with Example 1.

The elluting fluid was phosphate buffered saline, pH 6.9 and the experiments were conducted at 21°, the effluent being monitored with an optical recording unit.

Example 9

Calibration of separating medium

Samples of the same gelatin as used for the production of beads are gelled from aqueous dispersion (in this example 20 percent strong) in the form of membranes having a thickness of between about 0.05 and 0.06 cm. The membranes are then tanned as described in the various examples above.

The membranes are subjected to electro-osmosis with 0.05 M glycine as a reference substance, a potential difference of 100 volt, the current being in the range of between 3 and 5.5 milliamperes. The flow rate is measured in terms of $1/T \times 100$ mm. at different pH values, the pH being changed with either 0.05 M HCl or 0.05 M NaOH.

The results are apparent from FIG. 5 in which curve 1 represents that obtained after tanning with formalin only, curve 2 is that obtained with a membrane tanned with Kromex followed by formaldehyde, curve 3 is that obtained with a membrane first tanned with formaldehyde and then with Kromex and curve 4 is that obtained with a membrane tanned with Kromex only.

Example 10

Diethyl amino ethyl tanned gelatin:

Thirty percent chromium-tanned gelatin granules (500 ml. vol.) were washed with distilled water until free of preservative. Excess water was removed from the tanned gelatin granules, which were then transferred to a glass beaker and the pH brought up to 12.5–13.0 with 5N-NaOH. The granules were continuously and vigorously stirred during the addition of the caustic, to prevent localized areas of highly concentrated caustic soda. The pH drops over a period of time and NaOH must be added until it remains constant for at least 30 minutes.

A solution of 2-chlorotriethylamine hydrochloride (35 g.) in 45 ml. of H_2O was added slowly to the slurry of granules which were surrounded with crushed ice and water. The pH of the mixture was controlled by the judicious addition of NaOH and the halogen, so as to maintain the pH at 12.5–13.0. The mixture was heated at 85° C. for 30 min. After cooling 250 ml. of 2 M-NaCl was added and the mixture well stirred.

The suspension of granules was collected on a sintered glass filter (pore No. 3) and the filter cake washed with 0.1 N NaOH. The filter cake was suspended in 1 liter of 0.1 N HCl, excess acid removed on the filter and then resuspended in 0.1 N NaOH and left overnight at room temperature.

The gelatin granules were collected on the filter, washed with water and then with 0.006 M-PO_4 buffer pH 6.9. Once equilibrated against the buffer the granules were packed into a column 2.5×40 cm.

A sample of horse antidog or antibaboon lymphocyte serum was dialysed against the buffer, centrifuged at 10,000 r.m.p. for 10 min. and 1 ml. of the clear SNF applied to the column. The elution diagram is shown in FIG. 6. The first peak (which did not adsorb to the column) was γ-globulin which was identified in the ultracentrifuge (7S). The second peak was that due to albumin and related fractions, and had been adsorbed to the column. The fraction was removed with a NaCl gradient starting with 1 M NaCl in the reservoir vessel.

Example 11

Carboxymethyl tanned gelatin:

30 percent chromium-tanned gelatin (500 ml. vol.) granules were washed with water, excess water removed on the filter and the pH brought to 12.5 with 5 N NaOH with vigorous stirring. A solution of 30 g. of $CH_2Cl.COOH$ in 40 ml. of water was slowly added with the addition of NaOH to maintain the pH at 12.5. The solution was not heated.

Five hundred milliliters of 10 percent Ch_3COOH was slowly added to the suspension which was then diluted to 2 liters with distilled water.

The suspension was collected on a filter and repeatedly washed with 10 percent CH_3COOH and then with distilled water. The filter cake was washed with water until neutral and then equilibrated against 0.006 M KH_2PO_4 pH 5.6.

One millileter of dialysed normal horse serum was applied to a column of CM-gelatin The first peak (FIG. 7) was that due to albumin and related fractions and was not adsorbed. The second peak was that due to γ-globulin and had been adsorbed and was eluted with a NaCl gradient starting with 0.4 M-NaCl in the reservoir.

It is recommended to determine the optimum concentration of caustic soda experimentally for each batch of gelatin.

What we claim is:

1. A separating medium of a macromolecular character for chemical separations by chromatography, comprising a chemically hardened gelatin in a form suitable for a chromatographic separating process and being indispersable in water at temperatures at least above 7° C., and being provided with flow passage means or interleading pores is permeable to the flow therethrough of a chromatographic mobile phase.

2. A separating medium as claimed in claim 1 which is indispersable in water at temperatures at least up to 100° C.

3. A separating medium as claimed in claim 1 wherein the gelatin is selected with a high molecular weight in the sense of having an intrinsic viscosity of at least 0.9 deciliters per gram measured in 4 molar urea, saline, neutral pH prior to said hardening.

4. A separating medium as claimed in claim 1 wherein a gelatin is selected having a sedimentation coefficient in excess of 2.4×10^{-13} cm./sec./dyne, measured in 4 M urea, saline, prior to said hardening.

5. A separating medium as claimed in claim 1, wherein the anionic groups of the gelatin have been at least partly eliminated by said hardening.

6. A separating medium as claimed in claim 1, wherein the cationic groups of the gelatin have been at least partly eliminated by said hardening.

7. A separating medium as claimed in claim 1 wherein said gelatin has been hardened with formaldehyde.

8. A separating medium as claimed in claim 1 wherein said gelatin has been hardened by chromium tanning.

9. A separating medium as claimed in claim 8 wherein said gelatin has in addition been hardened with formaldehyde.

10. A separating medium as claimed in claim 1 wherein ion-exchange groups have been introduced into the gelatin by chemical reaction.

11. A separating medium as claimed in claim 1 wherein the gelatin employed constitutes a fraction of higher molecular weight obtained by a fractionation on the basis of molecular weight.

12. A separating medium as claimed in claim 11 wherein the gelatin has been subjected to fractionation with polyethylene glycol.

13. A separating medium as claimed in claim 1 in a form suitable for chromatography and having a ratio of surface area to volume of at least 10 cm. $^{-1}$.

14. A separating medium as claimed in claim 13 in the form of beads.

15. A separating medium as claimed in claim 14 wherein the bead diameter is from 300 microns downwards.

16. A separating medium as claimed in claim 1 having ion-exchange properties calibrated by electro osmosis.

17. A separating medium as claimed in claim 1 when incorporated in a chromatographic apparatus.

18. A calibrated separating medium of a macromolecular character for chemical separations by chromatography comprising a chemically hardened gelatin medium in a form suitable for a chromatographic separating process, said medium being indispersable in water at temperatures at least above 7° C. and permeable to the flow therethrough of a chromatographic mobile phase and having ion-exchange characteristics which are calibrated by forming a membrane and carrying out electro-osmosis through said membrane at a plurality of pH values.

19. A method of preparing a separating medium which comprises the step of transforming a suitable selected gelatin into a porous form having pores constituting flow passages and physically adapted as a separating medium for chromatography, permeable to the flow therethrough of a chromatographic mobile phase, and a chemical step of hardening the gelatin and insolubilizing it in the sense of rendering it indispersable in water above a temperature of 7° C.

20. A method as claimed in claim 19 wherein said hardening is carried out with a tanning agent.

21. A method as claimed in claim 20 wherein formaldehyde is employed as a tanning agent.

22. A method as claimed in claim 20 wherein a chromium-tanning composition is used as a tanning agent.

23. A method as claimed in claim 19 wherein the gelatin is first gelled and then subjected to hardening.

24. A method as claimed in claim 19 for producing the medium in the form of beads wherein the gelatin is dissolved in aqueous medium, emulsified with a substantially nonpolar medium and in the presence of an emulsifying agent, cooled with stirring and subjected to emulsion breaking.

25. A method as claimed in claim 24 wherein chemical hardening of the gelatin is carried out prior to the cooling.

26. A method as claimed in claim 24 wherein chemical hardening of the gelatin is carried out after the emulsion has been broken.

27. A method as claimed in claim 19 including the step of fractionating the gelatin on a molecular weight basis and recovering a fraction of increased average molecular weight.

28. A method as claimed in claim 19, including the step of adding ion-exchange groups to the gelatin molecules.

29. A fractionating process which comprises introducing a mixture to be fractionated into a separating medium of a macromolecular character for chemical separations by chromatography, said medium being chemically hardened gelatin in a form suitable for a chromatographic-separating process and being indispersable in water at temperatures at least above 7° C., and permeable to the flow therethrough of a chromatographic mobile phase,

bringing about movement of a component of the mixture through the separating medium relative to at least one other component of the mixture at least in part by percolation of a liquid through said medium,

and recovering at least one of said components separately from the other said component.

30. A process as claimed in claim 29 which comprises bringing about said movement by percolation of a liquid through a bed of the separating medium.

31. A process as claimed in claim 30 carried out by way of exclusion chromatography.

32. A process as claimed in claim 29 which comprises adjusting conditions of separating at least in part for ion exchange to take place as at least one factor determining the fractionation.

33. Substances whenever fractionated by a process as claimed in claim 29.

34. A process as claimed in claim 29 further comprising bringing about said movement by electrophoresis.

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