'Tanned' Gelatin Spheres and Granules for Exclusion Chromatography

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1. The preparation of tanned gelatin spheres and granules from high-molecular-weight gelatin is described. This material is comparatively hard, giving high flow rates, is insoluble in water at temperatures between 0° and 100° and is resistant to digestion by trypsin and chymotrypsin. The high-molecular-weight fraction of gelatin was prepared by precipitation with polyethylene glycol, and the spheres and granules prepared from this fraction were hardened and insolubilized by tanning with either formalin or chromium salts or both. 2. The spheres and granules were used successfully for the separation of protein molecules and other proteinaceous materials ranging in molecular weight from 200 to greater than 6 000 000. This gel exclusion material has several properties superior to those of other products used for similar purposes. Further, it was noticed that the porosity of the spheres differed considerably from that of the granules.

Numerous materials are available for exclusion chromatography, but none covers the entire range of molecular size. A certain amount of overlapping exists between agarose and dextran gels, the former having a useful range for fractionation of substances of molecular weights ranging from 100 000 to subcellular particles and the latter being applied usefully over the molecular-weight range 60 to 800 000.

Gelatin has been chemically altered for many purposes, but no reference in the available literature describing gelatin in modified form as a gel exclusion agent has been noted. The present paper describes the preparation and range of tanned gelatin spheres and granules as a new gel exclusion agent. Gelatin was found on treatment with different tanning agents to be insoluble in water at temperatures between 0° and 100°, and was sufficiently hard to ensure satisfactory flow rates even at low concentrations when packed in 2.5 cm. × 50 cm. columns. To achieve this result the material was tanned with chrome salts, spray-dried vegetable wattle tannin or a concentrated solution of formalin.

MATERIALS AND METHODS

When possible chemicals of A.R. quality were used. Formalin (40%, w/v) solution was obtained from the Imperial Chemical Industries Ltd., Cape Town, South Africa. The chrome tanning salt Kromex [composition 0.915 part of $\text{Cr}(\text{OH})_3$, 0.542 part of $\text{Cr}_2(\text{SO}_4)_3$, 1.000 part of Na_2SO_4 and 0.119 part of $\text{C}_6\text{H}_{12}\text{O}_6$ (invert sugar)] was manufactured by Marble Lime and Associated Industries Ltd., Johannesburg, South Africa. The spray-dried wattle

tannin was manufactured by the Natal Tanning Extract Co., Durban, Natal, South Africa.

Buffers. The 66 mm-phosphate-buffered saline, pH 6.9, was prepared as follows. To 200 ml. of 0.13 M- KH_2PO_4 was added 300 ml. of 0.13 M- Na_2HPO_4 and 500 ml. of 0.14 M- NaCl . Sodium azide (0.1 g.) was added as a preservative.

Tanning solutions. The 20% (w/v) formalin-saline, pH 9.0, was prepared as follows. To 1 l. of 40% (w/v) formalin was added an equal volume of 0.14 M- NaCl and the pH was adjusted to 9.0 with N-NaOH.

The 8% (w/v) Kromex-saline, pH 2.8, was prepared as follows. Kromex (160 g.) was dissolved in 2 l. of warm distilled water. After cooling, 17.0 g. of NaCl was dissolved in the Kromex solution, which was kept at 4° until used.

Preparation of high-molecular-weight gelatin. It is advisable to isolate the high-molecular-weight fraction of gelatin, as this material produced spheres and granules that had greater gel strength than unfractionated gelatin of the same concentration. The fractionation was achieved as follows: gelatin (Difco Laboratories, Detroit, Mich., U.S.A.) (160 g.) was dissolved in 3800 ml. of distilled water by gentle stirring over a boiling-water bath. When all the material had dissolved a solution of polyethylene glycol (mol. wt. 6000) (240 g. dissolved in 200 ml. of water) was added to the hot (80°) solution of gelatin and well mixed. The preparation of the fractionated gelatin was similar to that of agarose (Polson, 1963).

The solution was kept at room temperature for 2–3 hr., when it was found that the high-molecular-weight fraction (see below) separated out as a viscous layer. The upper fraction was completely removed and discarded, and the remaining portion was slowly poured into 4 l. of distilled water at 4° that was gently agitated with a mechanical stirrer. The insoluble gelatin collected round the stirrer and was removed before storage in 10 l. of water at 4° overnight. The next day the gelatin strands were collected in a large precooled filter funnel containing a cotton-wool plug

to prevent the gelatin from passing through with the filtrate. The strands were washed repeatedly with large volumes of cold distilled water until free of polyethylene glycol. The material was compressed to remove the excess of water and dehydrated by immersion in several changes of acetone. Excess of acetone was removed by extraction with light petroleum (b.p. 40–60°) and the material was finally dried in a current of warm air. The temperature of the air should not exceed 50°, as at higher temperatures a large proportion of the gelatin was found to become insoluble. Yannas & Tobolsky (1967) have suggested that gelatin becomes covalently cross-linked when its water content falls below a certain level; this is a possible explanation for the insolubility of the above-mentioned product on drying at high temperatures.

Intrinsic viscosity. The intrinsic viscosity, $[\eta]$, of unfractionated gelatin and gelatin fractionated with polyethylene glycol was determined to show that the fractionated product contained longer molecules, so giving a higher gel strength. The samples of gelatin were dissolved in 4.0M-urea in 0.14M-NaCl, pH 7.0, to prevent gelling at room temperature. Viscosity measurements were made with a Ubbelohde-type viscometer in a thermostatically controlled water bath at 26°.

Analytical ultracentrifugation. Sedimentation coefficients were determined at different gelatin concentrations in 4.0M-urea in 0.14M-NaCl at neutral pH. Centrifugation was done in a Spinco model E ultracentrifuge fitted with the An D rotor with the valve-type synthetic-boundary cell. The values obtained were standardized to sedimentation in water at 20°.

Preparation of gelatin spheres. Spheres were prepared according to the method of Hjertén (1964) with modifications. A weighed amount of the high-molecular-weight gelatin was dissolved in 300 ml. of distilled water with gentle stirring over a boiling-water bath. The organic liquid phase (toluene and carbon tetrachloride) containing the dissolved stabilizer Emulphor EL (Badische Anilin und Soda-Fabrik A.G., Ludwigshafen am Rhein, Germany) was preheated to 50° and added to the dissolved gelatin. The mechanical stirrer was switched on and after approx. 1 min. the flask containing the suspension was lowered with the stirrer into a beaker containing crushed ice. Once the temperature had reached 4° approx. 1 l. of cold ethanol (0°) was added to break the emulsion. The spheres were allowed to settle at 4° for approx. 30 min. 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 as a qualitative test for Emulphor). Untanned spheres were kept as close to 0° as possible to prevent them from dissolving.

The concentrations of stabilizer, toluene and carbon tetrachloride for the preparation of different concentrations of gelatin spheres are given in Table 1. The rate of stirring never exceeded 1000 rev./min., but this rate could be adjusted according to the size of the sphere required for gel exclusion, as mentioned by Hjertén (1964).

Preparation of formalin-tanned spheres. The batch of spheres prepared as described above was transferred to a beaker containing approx. 1 l. of formalin-saline, pH 9.0, at 4°. The mixture was briskly stirred for 2 min. and the tanning was allowed to proceed for 24 hr., after which the spheres were washed free of formalin with cold 0.14M-NaCl.

Table 1. *Concentrations of stabilizer, toluene and carbon tetrachloride for the preparation of different concentrations of gelatin spheres*

Concn. of gelatin (g./300 ml. of water)	Toluene (ml.)	CCl ₄ (ml.)	Emulphor EL stabilizer (g.)
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 spheres were kept in phosphate-buffered saline containing NaN_3 as a preservative.

Preparation of Kromex-tanned spheres. A batch of untanned spheres prepared as described above was tanned in approx. 1 l. of Kromex-saline, pH 2.8, at 4° for 24 hr. The spheres were collected on a Buchner filter and washed with large volumes of 0.14M-NaCl. This was achieved by alternately suspending the spheres in a large volume of 0.14M-NaCl and then collecting them on a filter. This was followed by repeated washings on the filter. It is imperative to remove all the free Kromex before suspending the spheres in the phosphate buffer, as failure to do this resulted in the precipitation of the chromium salts and the spheres gave unsatisfactory results. Once free of chromium they were kept in phosphate-buffered saline containing NaN_3 .

Preparation of formalin-Kromex-tanned spheres. Gelatin spheres were double-tanned first with formalin and subsequently with Kromex by the methods described above.

In later experiments it was found possible to tan gelatin spheres during the emulsification process. Once the gelatin had been dissolved and the organic liquids and emulsifier had been added, 1.5 g. of Kromex or spray-dried wattle tannin was added to the emulsion immediately after the stirrer had been started. Mixing was continued while the emulsion was still hot for approx. 10–15 min. before immersion of the flask in crushed ice and water.

Gelatin spheres prepared with a spray gun. A shallow precooled glass trough containing 1 l. of cold Kromex-saline, pH 2.8, and 3 l. of cold ether was continually agitated with a magnetic stirrer. The trough was surrounded with crushed ice and water to keep the temperature as close to 4° as possible. A spray gun (Royal Spray; Electric Systems A.G., West Germany) with a nozzle aperture of diameter not greater than 0.1 mm. was used for spraying a warm solution of gelatin directly on to the surface of the ether from a height of approx. 30 cm. To obtain spheres of different porosity, various concentrations of gelatin between 30% and 4% were sprayed with the gun.

On making contact with the cold ether the spheres gelled immediately and then passed into the layer of cold Kromex-saline, pH 2.8. Tanning was continued for 24 hr. at 4° after the top layer of ether had been poured off. After the tanning the remaining ether was removed under reduced pressure, and the spheres were collected and preserved as described above.

Preparation of tanned gelatin granules. A weighed amount of gelatin was dissolved in distilled water over a boiling-

water bath. The hot solution was cooled to approx. 50° and poured into a precooled enamel dish floating on crushed ice and water. The solidified gelatin was cut into small portions (0.5–1.0 cm. cubes) and transferred to a beaker containing 1 l. of Kromex-saline, pH 2.8, at 4°. The tanning was continued at 4° for 24 hr., after which excess of chromium was removed as described above. The gelatin was then homogenized in a Waring Blendor until the particles were small enough for use as bed material in chromatography. The granules were suspended in phosphate-buffered saline containing NaN₃.

By using the above methods it was possible to prepare gelatin spheres and granules of various concentrations between 2% and 40%, all of these materials exhibiting high flow rates with good resolutions of the proteins.

Packing procedure. Glass columns (50 cm. × 2.5 cm.) with a sintered-glass disk (porosity no. 1) sealed into the bottom of each column were used. The capillary outlet of the column was so constructed as to have a minimum dead volume, i.e. the space between the sintered-glass disk and the capillary outlet was as small as possible. Before the columns were packed a bed of glass Ballotini 1 mm. thick was poured directly on to the sintered-glass disk and the column half-filled with phosphate-buffered saline. The buffer was allowed to drip out of the column slowly and a slurry of gelatin spheres or granules suspended in the same buffer solution was poured into the column and the material allowed to settle without stirring. The procedure was repeated until the height of the packed gelatin in the column had reached 40 cm.

RESULTS

High-molecular-weight gelatin. The results obtained for the measurements of viscosities and the sedimentation coefficients for fractionated and unfractionated gelatin are recorded in Table 2. From these results it is feasible to assume that polyethylene glycol separated a higher-molecular-weight fraction from a mixture of gelatin of different molecular weights. This fraction comprised 60% of the unfractionated material.

Molecular weight of gelatin. The molecular weights of unfractionated and polyethylene glycol-fractionated gelatin were calculated from the equations of Svedberg & Pederson (1940) and Polson (1967), as shown below:

$$\frac{f}{f_0} = 1.19 \times 10^{-15} \frac{M^{\frac{2}{3}} (1 - \bar{v}\rho)}{S_{20,w} \bar{v}^{\frac{2}{3}}} \quad (\text{Svedberg \& Pedersen})$$

$$\frac{f}{f_0} = \frac{1}{0.61} \log \left(\frac{[\eta]}{\bar{v}} \right) \quad (\text{Polson})$$

where f/f_0 is the frictional ratio, $\bar{v}$ is the partial specific volume, assumed equal to 0.738 ml./g., and ρ is the density of medium, which is 1.00 g./ml. The results are recorded in Table 2.

Exclusion chromatography with tanned gelatin. Exclusion-chromatography experiments were per-

Table 2. *Intrinsic viscosities, sedimentation coefficients and average molecular weights of the unfractionated and fractionated gelatin*

Material	$[\eta]$ (dl./g.)	$S_{20,w}$ (s)	Mol. wt.
Unfractionated Difco gelatin	0.76	2.14	89 700
Polyethylene glycol-fractionated Difco gelatin	0.95	2.70	141 000

formed with gelatin spheres and granules tanned as described above. The following substances were used to determine the resolving properties of gelatin spheres: haemocyanin of *Jasus lalandii* (mol. wt. 500 000), sperm-whale myoglobin (mol. wt. 17 800) and DL-tryptophan (mol. wt. 204). *Burnupena cincta* haemocyanin (mol. wt. 660 000) and *J. lalandii* haemocyanin were used to determine the resolving properties of the granules. In all instances the eluting fluid was phosphate-buffered saline, pH 6.9, and the experiments were conducted at 21°, the effluents being monitored with an LKB Produkter Uvicord optical unit.

The results obtained with 30% gelatin spheres tanned with formalin, formalin-Kromex and Kromex respectively gave similar elution diagrams, that depicted in Fig. 1(a) being the same for all three types of tanned material.

The diagrams obtained with 15% and 7% formalin-Kromex-tanned spheres are illustrated in Figs. 1(b) and 1(c). The first peak in all three diagrams was that due to *J. lalandii* haemocyanin, the second peak to myoglobin and the third peak to tryptophan. Chromatography of the same mixture, containing in addition *B. cincta* haemocyanin on 4% formalin-Kromex-tanned spheres, is shown in Fig. 1(d), the first peak being that due to *B. cincta* haemocyanin and the remaining three peaks as described above. A diagram obtained of a gel-exclusion-chromatography experiment on a column packed with 4% tanned spheres on which a mixture of *J. lalandii* and *B. cincta* haemocyanins was applied is represented by Fig. 2(a). The same mixture chromatographed on 4% tanned gelatin granules is shown in Fig. 2(b). The fractions were ultracentrifuged after concentration by pervaporation, and it was confirmed that the first peak (Fig. 2a) was that due to the larger haemocyanin and the second to the smaller of the two proteins. The last two peaks on the same diagram were due to low-molecular-weight breakdown products. The initial large peak (Fig. 2b) contained *B. cincta* haemocyanin, which was confirmed by ultracentrifugation, and the second peak was that due to *J. lalandii* haemocyanin. The small peak was due to breakdown products of the haemocyanins.

Fig. 1. Elution diagrams of a mixture of *J. lalandii* haemocyanin, myoglobin and tryptophan on (a) 30%, (b) 15%, (c) 7% and (d) 4% gelatin spheres (mesh 100-300) tanned with formalin and Kromex. *B. cincta* haemocyanin was added to the above-mentioned protein mixture when it was applied to the 4% gelatin spheres. The buffer was 66mM-phosphate-buffered saline, pH 6.9, the flow rate was 8ml./hr. and the sample was 1 ml. The column dimensions were 2.5 cm. $\times$ 50 cm. The front is indicated by a vertical arrow.

Human serum was chromatographed on 4% tanned spheres and granules respectively, the results of which are shown in Figs. 2(c) and 2(d). The first peak in Fig. 2(c), in which gelatin spheres were used, was due to a mixture of macroglobulin and globulin, and the second peak was due to serum albumin. The two smaller peaks were due to low-molecular-weight proteins. Ultracentrifugation of the effluents obtained from the granules established that the first peak in Fig. 2(d) was that due to macroglobulin and the second peak to unresolved serum globulin and albumin.

Chromatography of *B. cincta* and *J. lalandii* haemocyanins on 2% tanned gelatin granules (Fig. 3) revealed that both the haemocyanins had entered into the pores of the gel.

Venom from the cobra (*Naja nivea*) was chromatographed on 30% gelatin spheres tanned with formalin followed by tanning with Kromex. The elution diagram is shown in Fig. 4(b). As a comparison the venom was chromatographed on Sephadex G-10, G-15, G-25, G-50, G-75 and G-100. The diagram obtained with Sephadex G-50 only is shown in Fig. 4(a). The patterns obtained with the other grades of Sephadex are not included as these showed an inferior resolution. The sedimentation coefficients of the components eluted from the 30% tanned spheres showed that the separation occurred

Fig. 2. Elution diagrams of a mixture of *B. cincta* and *J. lalandii* haemocyanins applied to a column containing (a) 4% spheres and (b) 4% granules both tanned with formalin and Kromex, and elution diagrams of human serum applied to a column containing (c) 4% spheres and (d) 4% granules both tanned with formalin and Kromex. The eluting fluid in all instances was 66mM-phosphate-buffered saline, pH 6.9, the flow rate was 8ml./hr. and the sample volume was 1 ml. The column dimensions were 2.5 cm. $\times$ 50 cm. The front is indicated by a vertical arrow.

Fig. 3. Elution diagram of a mixture of *B. cincta* and *J. lalandii* haemocyanins on 2% gelatin granules tanned with formalin and Kromex. The eluting fluid was 66mM-phosphate-buffered saline, pH 6.9, the flow rate was 8ml./hr. and the sample volume was 1 ml. The column dimensions were 2.5 cm. $\times$ 50 cm. The front is indicated by a vertical arrow.

on a molecular-size basis. Increasing the salt concentration of the eluting buffer to 1M-sodium chloride failed to show any change in the elution diagram, as shown in Fig. 4(b).

The possibility of the large number of peaks (Fig. 4b) being due to breakdown products of gelatin resulting from enzymic action of components in the snake venom was investigated. This did not appear to be the case.

Fig. 4. Elution diagrams of cobra (*Naja nivea*) venom applied to columns containing (a) Sephadex G-50 and (b) 30% gelatin spheres tanned with formalin and Kromex. The eluting fluid was 66mM-phosphate-buffered saline, pH 6.9, the flow rate was 8ml./hr. and the sample volume was 1ml. The column dimensions were 2.5cm. $\times$ 50cm. The front is indicated by a vertical arrow.

Trypsin and α -chymotrypsin failed to digest the tanned spheres, but the effects of other proteolytic enzymes were not investigated. The method for assessing the breakdown of tanned gelatin with trypsin, snake venom and α -chymotrypsin was that described by Schurr & McLaren (1965). A thin slurry of tanned gelatin was prepared on three separate microscope slides and 3 drops of snake venom were added to one slide, 3 drops of trypsin solution (10mg./ml.) to another and 3 drops of chymotrypsin (10mg./ml.) to the final slide. The gelatin spheres and granules were observed under the light-microscope but no digestion of these particles took place, as they retained their shape and size. Schurr & McLaren (1965) observed that their gelatin spheres faded away completely during trypsin digestion. It was noticed, however, that spheres or granules kept in the absence of sodium azide or any other preservative became heavily contaminated with bacteria.

DISCUSSION

Finely divided untanned gelatin, like agar and agarose, may be used in gel exclusion chromatography at low temperatures where the gelatin is insoluble. At higher temperatures, notably above 7°, it is necessary to treat gelatin to render it insoluble. Materials that were found to be satisfactory for the insolubilization of gelatin were the tanning agents used in the leather industry. Certain other substances are capable of rendering gelatin insoluble but they tend to concentrate the molecules by the exclusion of water, thereby changing the initial concentration of the gelatin and decreasing the porosity of the spheres and granules. This, however, was less evident with the leather-tanning agents.

A further advantage of the tanning procedure is

that the gel strength of the reaction product is considerably higher than that of the untreated gelatin of the same concentration.

It was found that when commercially available gelatin was fractionated with polyethylene glycol a fraction was separated that had a higher average molecular weight than that of unfractionated gelatin, as was evident by its higher intrinsic viscosity, sedimentation coefficient and molecular weight. Spheres and granules prepared with the high-molecular-weight fraction of gelatin were found to be more rigid than those prepared with the untreated product.

Tanned gelatin for exclusion chromatography may be prepared in a finely divided state by three methods. These are: (a) the emulsification of solutions of gelatin in organic solvents with the subsequent gelling of the spheres; (b) disintegration of the tanned gelatin in a Waring Blender; (c) dispersion of solutions of gelatin by spraying, in the form of a mist, into cold ether layered on the tanning solution.

The method of tanning is important. If the gelatin is tanned while still in the ungelled state, denser (less porous) particles are formed than those tanned subsequent to solidification. Cross-linking of the gelatin with resorcinol (Braunwald, Gay & Tatoes, 1966) before emulsification also resulted in less-porous spheres than those prepared in the normal manner.

A noteworthy observation is the difference in behaviour of gelatin spheres and granules of identical initial concentration: 4% tanned granules separated macroglobulin from serum globulin and albumin, whereas 4% tanned spheres failed to differentiate between macroglobulin and serum globulin, but separated albumin from these substances. It would therefore be feasible to operate two columns in series, one containing spheres and the other granules, to obtain complete separation of macroglobulin, globulin and albumin from serum. It would be beneficial to concentrate the zones eluted from the initial column before they enter the second. This could be accomplished as outlined by Polson & Russell (1966a,b).

A similar observation to that noted above was that the *B. cincta* and *J. lalandii* haemocyanins chromatographed on 4% tanned spheres and tanned granules behaved differently on these two materials, as can be seen from the positions of the fronts. *B. cincta* haemocyanin was separated from the other protein on both the spheres and granules, but the resolution on spheres was better, suggesting a greater porosity for the granules.

A likely explanation of the difference in behaviour of the two forms of tanned gelatin is that the spheres, while in the ungelled state, lost water to the hot organic phase, resulting in spheres of higher

concentration with the resultant decrease of porosity. This loss of water would not occur on the disintegration of gelatin into granules with a Waring Blendor, and this explains the retention of its porosity.

When spheres of different diameter are prepared, the loss of water from the smallest particles would be expected to be greater than that from spheres with a larger diameter. The result of this is that small spheres would be less porous than the large ones. This is in fact the case with agarose spheres of different diameters (A. Polson, unpublished work).

B. cincta and *J. lalandii* haemocyanins were also chromatographed on 2% tanned gelatin granules and the elution diagram showed that this material was porous enough to allow both haemocyanins to enter the pores. This assumption was confirmed in the ultracentrifuge.

The superior resolution of cobra venom obtained on 30% gelatin spheres compared with that found with Sephadex G-50 may be explained on the assumption that the pores present in the spheres of gelatin are of a more uniform diameter than those present in Sephadex, which might have the effect of producing a better separation.

Tanned gelatin spheres and granules form gel-filtration agents that have been applied successfully to the separation of proteinaceous substances, ranging from amino acids to proteins of molecular weight greater than 6 600 000. As the picornaviruses have molecular weights of about 8×10^6 – 10×10^6

the 2% tanned gelatin granules should be admirably suitable for fractionation of these substances. Further, this material gives high flow rates at low concentrations and minimal zone spreading and is economical to prepare.

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