TANNED GELATIN.

Some Biophysical and Biochemical Properties of a New Gel Exclusion Agent and its Application to the Chromatography of Proteins and Viruses.

by

W. KATZ.

Thesis presented for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Science

UNIVERSITY OF CAPE TOWN.

JUNE 1970.

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SUMMARY.

The principal aim of this study was to develop a new bed material suitable for gel exclusion chromatography, which would embrace all the desirable characteristics of available materials used for this purpose and if possible improve on some of them.

The two most important limitations of existing gel media concern their exclusion limits and rigidity. Agar and agarose have been found satisfactory for the separation of larger molecules including some viruses, but difficulties may arise at low gel concentrations due to the low mechanical strength of the granules. The dextran and polyacrylamide gels have in general a rigidity equal to that of agar and agarose, but cannot be prepared with such large pore sizes and are therefore limited to the separation of smaller molecules.

In order to improve on the available media a gel must be found that is insoluble at room temperature, rigid at all useful concentrations, suitable for the separation of a very wide range of particle sizes and inexpensive to prepare. The properties of tanned gelatin have been investigated and shown to meet these requirements.

Gelatin gels have not been used before as bed material for gel exclusion chromatography. However,

rigid gelatin gels can be prepared, and depending on the concentration, could probably be used for gel exclusion chromatography below 7°, as it is soluble above this temperature. Gelatin was rendered insoluble by "tanning" the gel in much the same manner as is done in the leather industry. This method was investigated because of the close relationship between gelatin and collagen, the main component of hides.

This procedure rendered the gelatin insoluble in water, even at 100° and, with some modifications, very rigid. Biochemical and biophysical studies revealed that the method and sequence of tanning with various agents, and the type of gelatin used, greatly affected the properties of the product. Tanned gelatin may be prepared for use as an exclusion agent or as an ion-exchanger.

Various types of tanned gelatin were applied to the separation of proteins and viruses, and revealed that these gels had wide exclusion limits which resulted in good separation of the particles.

The success of this program has resulted in the development of a new bed material for chromatography.

This material was found to be more rigid than any of the other gels available for this purpose, especially at low concentrations. Also the ease with which they could be prepared, and the relatively cheap cost of producing the material in very large quantities was found to be a decided advantage. Furthermore tanned gelatin was found to be superior in many respects to materials currently available for exclusion chromatography.

CHAPTER ONE

GEL EXCLUSION CHROMATOGRAPHY

1.1. INTRODUCTION.

"The importance of chromatography lies primarily in its use as an analytical tool. It serves as a means for the resolution of mixtures and for the isolation and partial description of the separated substances. Chromatography permits the separation and partial description of unreported substances whose presence is unknown or unsuspected. It is an indispensable exploratory method in all sciences dealing with chemical substances and their reactions. It is among the most selective and the most widely applicable separatory techniques yet devised" (Strain, 1961).

Some of the most significant recent advances in biochemistry may be attributed to, and have to a large extent depended on, new or improved methods of separation. The use of chromatography for this purpose is well known. A feature common to all chromatographic methods is the use of two phases; one stationary and the other mobile. Adsorption, ion-exchange, partition and gel exclusion chromatography are the four main sub-divisions, but only the latter will be dealt with in this study.

During the 1920's McBain (1926) investigating the characteristics of zeolite (crystalline aluminosilicates) adsorption phenomena, developed the idea of "molecular sieves". Thirty years later Synge and Tiselius (1950) observed a "molecular sieving" effect during the separation of proteins and peptides by ion-exchange chromatography. Partridge (1952) noted that his experiments with ion-exchange resins exhibited a similar effect. However the first recorded instance of "molecular sieving" effects on organic compounds was observed by Lathe and Ruthven (1956). Their experiments conducted on starch grains revealed a graded penetration of these particles by solutes varying between 100 and 1000 molecular weight. By swelling the starch grains in thiocyanate, proteins e.g. haemoglobin, were capable of penetrating the particles. Their pioneering experiments with starch could not be developed owing to the limitations imposed by the media which they employed in these experiments.

It was not until the introduction of gel matrices that this sphere of chromatography became established as a means of separating and purifying mixtures of solute molecules. The introduction of particulate dextran gels (Porath and Flodin, 1959), cross-linked with epichlorohydrin, was a milestone in the field of gel chromatography. These gels have been considerably modified since then, and have been used

extensively in the purification and characterization of a wide variety of substances, but owing to their restricted porosity in the macromolecular range their usefulness has been limited to molecules varying in molecular weight from a few hundred to about 600,000.

With the introduction of granulated agar gels (Polson, 1961) the fractionation range of these gels was extended to include viruses and sub-cellular particles. However due to the charged agaropectin molecules associated with the agar certain precautions had to be taken to avoid adsorption of the solute molecules to the gel. This problem was overcome to a large extent by Hjertèn (1962) who devised a method for the isolation of the uncharged fraction of agar, agarose, by precipitation with cetyl pyridinium chloride.

The preparation of granulated polyacrylamide gels for use as bed material for gel exclusion chromatography added yet another member to the growing group of gel matrices available for this technique (Hjertèn and Mosbach, 1962). The fractionation range of these gels is very similar to those of dextran.

Several polymers have been investigated for their exclusion properties (Porath, 1959) and include such substances as polyvinyl alcohol, polysorbitol and cellulose, and cross-linked hyaluronic acid (Laurent, 1964), but the

gels mentioned previously have given far superior results on experimentation. Haller (1965) developed porous glass powder as a non-gel exclusion agent. This material can be prepared to cover the entire fractionation range of proteins from several hundred to several hundred million. Unfortunately due to the high surface charge of glass, adsorption effects must be considered a potential hazard, until the material can be treated in order to neutralise these effects.

It is only a decade since the pioneering experiments of Lathe and Ruthven (1956), but the expansion and development of gel exclusion chromatography has provided one of the most popular tools for the separation and characterization of biological substances.

1.2. THEORY.

Martin (1950) has defined column chromatography as "the uniform percolation of a fluid through a column of a more or less finely divided substance that selectively retards, by whatever means, certain components of the fluid". This definition covers every facet of chromatography, but naturally enough it does not give an indication of the mechanism responsible for the separation of molecules on a molecular size basis.

When a column is packed with gel particles

suspended in a liquid medium (solvent), the solvent may be considered to exist in two states; that which is confined within the gel matrix and that which is located in the interstices between the gel particles. If the solvent is allowed to percolate through the column, while the gel particles remain stationary, the solvent within the particles is prevented from flowing, while the interstitial solvent flows between the granules. Thus the effect of the gel particles is to create two distinct solvent phases, one stationary and one mobile. The principle of gel exclusion chromatography concerns the partition of solute molecules between the stationary and mobile solvent phases. Obviously in order to become part of the stationary phase a solvent would be required to penetrate the molecular framework of the gel; and this penetration would in turn be governed by the size of the molecule and the mean distance between the randomly arranged macromolecular filaments which form the gel particle.

Solutes applied to gel exclusion columns may be divided into three categories: (i) those which are completely excluded from the gel particles and remain in the interstitial spaces (ii) those which are partially included, and (iii) those which are completely included. The volumes at which these molecular species are eluted from a gel exclusion

column provide information as to the stationary and mobile phases. Therefore the volume required to be eluted from a column in order to elute a solute in category (i) would be equivalent to the interstitial volume or void volume V_o. A freely included solute (category iii) would require the elution of a volume equivalent to the total column volume, V_t. Partially included solutes would be eluted at volumes intermediate between these two extremes. The difference between V_t and V_o is the volume of solvent confined within the gel matrix (i.e. the stationary phase) and is designated by the symbol V_i . The distribution coefficient K_d is defined as the fraction of the stationary phase which is accessible to the solute, and is expressed as:

$$K_d = \frac{V_e - V_o}{V_i}$$

where V_e is the elution volume. Therefore it will be seen when $V_e = V_o$ the solute is completely excluded from entering the gel matrix. Similarly from the above equation it will be obvious that when $V_e = V_o$ then $K_d = 0$. If the solute distributes itself equally within and without the gel $K_d = 1$. Consequently solutes capable of entering the gel matrix to different degrees will have K_d values between 0 and 1 . K_d value greater than 1 indicates that

adsorption of the solute to the gel has taken place.

The main theories for the explanation of gel chromatography have been divided into a thermodynamic and a hydrodynamic interpretation by Ogston (1966). The thermodynamic aspect relates to the differential partition of molecules between the interior of the gel matrix and in the buffer surrounding the gel particles. The hydrodynamic aspect is concerned with the restricted diffusion of solute molecules within the gel matrix.

The first theory assumes that the gel is composed of rigid straight chains (Laurent and Killander, 1964) randomly distributed within the gel. During chromatography with this gel a mixture of solute molecules varying in molecular dimensions would be eluted from the column in an order of decreasing molecular weight. The gel, it is assumed, will exclude molecules from entering the matrix if the molecular dimensions of the molecule are greater than that of the pore of the gel. Therefore larger molecules will be excluded to a greater extent than small molecules and will therefore appear in the eluting fluid before the smaller particles. The distribution behaviour, of solutes of varying molecular dimensions in a system, is given by the relationship (Laurent and Killander, 1964):

$$K_{av.} = \frac{V_e - V_o}{V_g + V_i}$$

where $K_{av.}$ is the available volume in the internal gel matrix for the solute molecule and V_g is the volume of the swollen gel.

With regard to the hydrodynamic aspect, Pedersen (1962) demonstrated that large solute molecules migrate faster with respect to small solute molecules in capillaries of diameters approximately 10 times those of the solute molecules. In this concept the gel particles are assumed to have very fine capillaries which would behave in a similar way if a mixture of solutes were chromatographed on this material. If a molecule has greater dimensions than the diameter of the gel capillary pore it will be excluded from entering completely and would appear in the void volume. However molecules of varying molecular dimensions, but all capable of entering the capillary of the gel would appear in the eluate in decreasing order of magnitude, due to the acceleration of large relative to small solute molecules in capillaries.

1.3. COLUMN TECHNIQUE AND FACTORS AFFECTING RESOLUTION.

Glass columns varying in length from 40 to 100 cm.

and having diameters of 1.5 to 4.0 cm. (Andrews,1966) were found to be the most satisfactory. At the base of the column a sintered glass disk (porosity 1) is sealed into the glass walls, and the area immediately below the disk is constructed to have a minimum volume in order to reduce mixing effects in this position when the solutes are eluted from the column. Columns of larger and smaller dimensions than those mentioned above, and utilising other supports than sintered glass have been used with success. Maitland (1966) observed superior resolutions using long in contrast to short columns, and if very long columns are used (100 cm. or more) the gel granules need not be as small in order to give comparable resolutions. Long columns tend to pack with a subsequent reduction in flow rate after continual use. This has been overcome to a certain extent by chromatographing the mixture of solutes through a number of short columns placed in series, or by repeatedly passing the substances through the same column. This technique is known as recycling chromatography and was introduced by Porath and Bennich (1962). This method has been improved upon by Polson and Russell (1966 a,b), who concentrate the eluting zones before re-chromatography on the column. This concentration step has the advantage of sharpening the zones after each cycle through the column.

Flodin (1961) has shown that gel particles of small and uniform diameter give better resolutions than that of larger particles of varying sizes. The resolution is further improved by preparing gels in bead form (Hjertèn, 1964), and the flow rates are considerably enhanced. However good separations depend in the first instance on properly packed columns. The gel when supplied in the dry form (dextran and polyacrylamide) should be swollen in a suitable solvent for at least 24 hr. before packing into the column. Incompletely swollen particles when packed into columns, swell further and cause packing and reduce the flow rate. Once the gel is completely swollen, the thickest possible slurry of the material is then poured or syphoned into the column which is held in a perpendicular position with a clamp. It is advisable to commence pouring the slurry of gel only after the material has been de-aerated. The excess buffer should not be allowed to drain off under a head greater than that to be used in the actual experiment. However, Andrews (1965) has suggested that greater care be taken in packing columns especially with more porous materials. The use of a tap funnel as a reservoir for the gel is used to pack the column. A steady stream of gel particles is run into the column which is filled with the same buffer. If the gel material is too soft to permit reasonable flow rates the technique of reversed flow

(Rothstein, 1965) may be used, which involves introducing the sample for chromatography into the base of the column. The flow of buffer is in the same direction.

Columns should be packed and used at the same temperature (Andrews, 1966 b), as a drop in temperature will cause a decrease in the flow rate, and if the temperature is raised again air bubbles become trapped in the gel. Column irregularities are checked by observing the passage of a coloured substance through the gel material. In these laboratories phenol red has been used with success, but there are a number of other substances e.g. blue dextran, cytochrome C etc. which can be used equally effectively. If the column is irregularly packed it is advisable to remove the gel and start again.

After packing the column the flow of buffer through the column is stopped and the sample for chromatography is carefully pipetted directly onto the surface of the gel after removing the excess buffer. The column is allowed to flow again and once the sample has drained, the walls are washed down with small volumes of buffer and the column refilled with the eluting fluid and the reservoir attached. Samples having high viscosities and or high concentrations can disrupt the flow of the solutes through the column (Flodin, 1961). Furthermore samples of high concentration

containing in addition solutes which are not capable of entering the gel matrix cause the gel to shrink and the effluent volume of the solutes entering the gel matrix are considerably increased (Edmond et al, 1968). The use of a circular piece of filter paper placed over the surface of the gel to avoid disruption of the surface during the application of the sample (Andrews, 1966 a), is not always advisable owing to the adsorption of solutes to the paper.

Choice of a suitable buffer for chromatography depends on the solute as well as the type of gel being used. Buffers having molarities of at least 0.1M in the presence of 0.85% saline are usually satisfactory in inhibiting adsorption effects. The flow rate of the buffer through the column should be between 3 to 10 ml./cm^2 of column cross-sectional area/hr. (Andrews, 1966 a). Slower flow rates will give better resolutions, but rates in excess of those given above give impaired resolutions (Flodin, 1961). However column runs have been accomplished in less than one sec. with only a slight loss in resolution (Curtain and Nayler, 1963).

It is advisable when using column media of an organic nature that preservatives e.g. sodium azide or thiomersalate be added to the buffer in concentrations of at least 0.02% (w/v). Failure to do this may result in

bacterial contamination of the gel with the liberation of carbohydrate from agar, agarose and dextran gels.

1. 4. MOLECULAR WEIGHT ESTIMATIONS.

Granath and Flodin (1961) observed a relationship between the molecular weights of solutes and their behaviour during gel exclusion chromatography on Sephadex gels. Andrews (1962) and Steere and Ackers (1962) noted a similar relationship on agar gels. The method has been developed for column chromatography and for thin layer techniques on plates of suitable dimensions.

In the first method columns are packed with a gel material and after equilibration against the buffer, the column is calibrated by chromatographing mixtures of solutes of known molecular weight and calculating the elution volumes. If the elution volume is plotted as a function of molecular size or molecular weight a non linear curve is obtained. A sample of unknown molecular weight is then passed through the column preferably in the presence of one or two of the standard substances. The elution volume of the unknown substance obtained on the standardized column, can be easily converted into molecular parameters by reference to the standard curve.

To obtain authentic values the standards used for calibration must be well characterised, and their progress

through the column must not be impeded by adsorption, ionic or other similar effects. This method is basically used for the estimation of molecular weights and not for molecular weight determination (Andrews, 1966 b).

A second method for column calibration (Ackers, 1964., Siegel and Monty, 1966) requires the use of one solute of known Stokes radius. The elution volume of this solute obtained by chromatography on the gel is used to determine the pore radius of the gel. Substances of unknown Stokes radii are then chromatographed on the column and their radii or diffusion coefficients calculated from formulae.

A similar technique described by Determann and Michel (1966) also requires only one standard for calibration, the molecular weight being calculated from the formula :

$$\log M = M_o - (6.062 - 5.00d)(V_e/V_o).$$

where V_e is the elution volume of the solute, V_o the void volume, d the wet density of the gel and M_o is a constant obtained in a further experiment with a protein of known molecular weight. It has been mentioned (Andrews, 1966 b) that the possible introduction of errors using one standard only are far greater than in the original method where more than one standard substance is used.

A relationship does exist between the Stokes radius of

molecules in solution and their behaviour during gel exclusion chromatography (Siegel and Monty, 1966., Ackers, 1964., Andrews, 1965). Results obtained with columns calibrated using the Stokes radius of the molecule, should be interpreted with caution. For spherical molecules the Stokes radius is a measure of the dimensions of the particle, but if the particle is asymmetrical the Stokes radius is merely an indication of its translational properties (Andrews, 1966 a) and errors may be introduced in the estimation of the molecular weight. Furthermore the estimation of molecular weights by gel exclusion chromatography requires that the standard and unknown have similar shapes and densities in solution in order to obtain authentic values. Differences in the structural characteristics of molecules may be ascertained if there are wide discrepancies in their molecular weight values. It should be mentioned that the methods of molecular weight determination from the diffusion and sedimentation coefficients of a molecule are more reliable than those estimated by gel exclusion chromatography.

The method has been extensively used for molecular weight estimation of proteins (Andrews, 1965), large protein molecules (Largier and Polson, 1964) and for proteins in the presence of high concentrations of urea or guanidine hydrochloride (Wieland, Duesberg and Determann, 1963). Certain

difficulties have been encountered in molecular weight estimation of carbohydrates (Andrews, 1966 b) due to the fact that they appear to have larger diameters in solution, probably to increased hydration.

Molecular weight estimation of nucleotides (Hohn and Pollman, 1963) has been reported but difficulties due to their filamentous nature and adsorption effects have been observed (Hayes, Hansbury and Mitchell, 1964. Gelotte, 1960).

1.5. GEL EXCLUSION CHROMATOGRAPHY MEDIA.

A number of criteria are in use for evaluating the usefulness of media for gel exclusion chromatography. The medium should exhibit no adsorption properties, it should be insoluble and stable when suspended in buffers of varying composition, it should be sufficiently rigid to allow good flow rates, it should show no tendency towards packing in the column and it should not cause denaturation of the solute molecules. An advantageous feature of a medium would be a narrow range of pore sizes in any preparation, to allow greater selectivity within a particular molecular weight range and hence to separate proteins with similar molecular weights. Such an ideal gel medium unfortunately is still not available but the cross-linked dextran and polyacrylamide gels, and the agar and agarose gels have met these

criteria to a greater or lesser extent.

1.5.1. Cross-linked dextran gels.

The cross-linking of dextran, of bacterial origin, with epichlorohydrin in a non-aqueous medium has been described by Porath and Flodin (1959). The degree of cross-linking can be fairly accurately controlled, thereby producing gels of different degrees of cross-linkage and consequently varying porosity. These white, water insoluble particles of dextran swell in aqueous buffers to form gels which can be used as bed material for gel exclusion chromatography. The most highly cross-linked member of the series is known as Sephadex G-10 and G-200 is the least cross-linked and most porous. The figures refer to 10 times the water regain of the gels (g. H_2O/g. gel.). A new derivative of Sephadex G-25 has recently become available and is the hydroxypropyl ether of dextran which has the property of swelling in both aqueous and polar organic solvents.

Dextran gels are slightly soluble in the normal buffers used for chromatography, liberating carbohydrate after long periods of use. Aromatic and heterocyclic compounds (Gelotte, 1960) are adsorbed to the gel and in the presence of water or weak buffers proteins and other substances containing basic groups are withheld on the column. Substances containing acidic groups however are accelerated

when chromatographed on these gels (Posner, 1963). The adsorption effects have been overcome to a large extent by increasing the ionic strength of the buffer. Andrews (1966 a) observed that small amounts of protein were adsorbed to the gel initially, but after continual use quantitative recoveries of solute were obtained.

1.5.2. Polyacrylamide gels.

Gels which were originally used for electrophoresis by Raymond and Weintraub (1959) have been prepared in granular form by Hjerten and Mosbach (1962), for gel exclusion chromatography. The acrylamide is cross-linked with N,N'-methylene-bisacrylamide and by controlling the conditions of the experiment, gels of different porosity are produced. The acrylamide and the cross-linker are dissolved in water or buffer at pH's above 6 to aid in polymerization. The solution is de-aerated and the catalysts β-dimethylamino-propionitrile and ammonium persulphate are added with gentle stirring to prevent aeration of the solution. Gelation proceeds within a few minutes, and once complete the gel is broken up into particles suitable for bed material. Hjerten (1967) has described a procedure for the preparation of spherical grains of polyacrylamide gel. Bio-Gel P is the trade name for these gels manufactured by Bio-Rad Laboratories, and eleven different porosities are available, Bio-Gel P 2

being the least porous and P-300 the most porous. The material is supplied in dry bead form and the numerical suffix in this case refers to the approximate exclusion limit of the material in terms of solute molecular weight (Bio-Gel P-300 has an approximate exclusion limit of 300,000).

The greater chemical stability and rigidity, and the absence of contaminating carbohydrate provides certain advantages of these gels over the dextran gels.

1.5.3. Agar and Agarose gels.

With the introduction of granulated agar gels (Polson, 1961) it became possible to purify an entirely new range of substances of macromolecular dimensions owing to the greater porosity of these materials. These gels must be used at neutral pH, to reduce the effects of adsorption, but even then they have marked adsorptive and ion-exchange properties (Andrews, 1966 b). As with the Sephadex gels they liberate carbohydrate in trace amounts.

Agar consists of two fractions; a neutral galactose polymer agarose, and the charged sulphated polysaccharide agaropectin (Araki, 1937). It is desirable to remove the charged agaropectin and to use the neutral agarose as bed material for gel exclusion chromatography. There are three principle methods for the preparation of agarose:

(i) The acetylation method described by Araki

(1937), which is tedious and expensive and produces a gel of low gel strength containing residual charges (Russell, Mead and Polson, 1964).

(ii) The precipitation of agaropectin with cetylpyridinium chloride (CPC) (Hjertèn, 1962 a) is based on the selective precipitation of the charged agaropectin with CPC. A hot solution of agar is added to a solution of CPC with stirring and the precipitate of agaropectin is collected by centrifugation. The agarose gel is washed, liquified by heating and added to a hot slurry of Fuller's earth which adsorbs the CPC. The hot solution is centrifuged or filtered to remove the Fuller's earth and any precipitated agaropectin. The agarose gel is washed, liquified and precipitated 3 to 4 times with ethyl alcohol which removes the remaining CPC which is soluble in alcohol. The yield of agarose is approximately 60%.

(iii) Precipitation of agarose with polyethylene glycol (Russell, Mead and Polson, 1964) is based on the effect of linear polymer precipitation. To a hot solution of agar (80°) is added a solution of polyethylene glycol (mol. wt. 6000) at the same temperature. The agarose precipitate is collected and washed with water overnight, and then with acetone before drying in a current of warm air. The whole procedure is repeated twice, which gives an approximate yield of agarose

after the final treatment of 45%.

The purity of agarose prepared by these methods can be ascertained by sulphur determinations or by the measurement of electroendosmosis after electrophoresis of serum in a sample of the gel. CPC prepared agarose when used as an overlay in the plaque technique is toxic to the cells owing to traces of CPC. This is not so with polyethylene glycol precipitated agarose (Philipson, 1967).

Gels of agar or agarose were initially broken up into small particles by mechanical means, but with the introduction of methods for the preparation of bead form gels the method of chromatography has been greatly facilitated. Two methods have been devised for the preparation of agar or agarose spheres (Hjertèn, 1964., Bengtsson and Philipson, 1964).

In the method described by Hjertèn (1964) the agarose is dissolved in hot water and a mixture of organic solvents (carbon tetrachloride and toluene) containing a stabilizer are added to the flask. The mixture is briskly stirred forming a water-oil emulsion of spheres. When the contents of the flask are cooled by immersion in a water-ice mixture the spheres gel, and the organic liquids and stabilizer are removed by washing with ether. The diameter of the spheres is controlled by governing the rate of stirring or by altering the concentration

of the stabilizer. After suspending the spheres in water they are wet sieved in order to obtain spheres of uniform diameter.

The method of Bengtsson and Philipson (1964) resorts to spraying a hot solution of agar (or agarose) into a cold organic liquid. The hot solution of agar is poured into a Seitz filter fitted with a glass nozzle of fine diameter. A hole is made in the asbestos pad of the filter to facilitate passage of the liquified gel. Pressure is applied and the spray of hot agar is directed onto the surface of a trough containing cold ether layered on top of water containing ice. The drops of agar condense on making contact with the cold ether and then sink into the water phase. The spheres are collected and wet sieved.

These gels differ from dextran and polyacrylamide in that the molecules are not chemically cross-linked but are held together, in all probability, by hydrogen bonds. They should therefore not be used at temperatures below 0° or above 40° and the pH should be kept between 4 and 9 (Male, 1966).

The exclusion limits of agarose gels are governed by the concentration of the agarose. At low concentrations the gels are most porous, so that at 2% the approximate fractionation range of molecules is between molecular weights of 500,000 and 150×10^{6}. At higher concentrations notably

10% the porosity is decreased and molecules of molecular weights between 10,000 and 250,000 may be fractionated. These gels are manufactured under the trade name "Sagavac" (Seravac Laboratories) and also under the name of "Sepharose" (Pharmacia Laboratories) and in both cases are supplied in the swollen wet form.

1.5.4. Porous glass particles.

While the capacity of gel columns is dependent on the cross-sectional area of the column the resolving properties are a function of the length of the latter hence greater resolution is generally sought by increasing the column length. Owing to the softness of certain gels however, they tend to compact slowly under their own weight. This has been overcome by packing short columns and arranging them in series, but soft gels even under these conditions tend to resist high flow rates with a consequent increase in working time. Most of the gels are sensitive to changes in temperature and are prone to contamination with bacteria unless used in the presence of preservatives (which is not always desirable) or at low temperatures. Dextran, agar and agarose are slightly soluble in water and in the normal buffers used for chromatography and samples run on columns packed with these gels will be contaminated with traces of carbohydrate. Gels which have become contaminated with bacteria are discarded as their

organic nature does not permit the use of strong oxidizing agents for their rejuvenation. Finally these gels can not be autoclaved and therefore cannot be used in the preparation of vaccines and immune sera.

For these and other reasons the hydrophilic gels described in this study are not ideal media for gel exclusion chromatography. With the introduction of porous glass particles (Haller, 1965) many of the above short comings are eliminated. The preparation of the glass particles involves a controlled heating of bulk glass to develop microheterogenous regions of suitable pore diameter. The glass is crushed and the soluble microphases removed. The material is packed into glass columns under vibration and a suitable buffer added for chromatography.

Good resolution and high flow rates, operation over a wide temperature range, sterilization by autoclaving and no bacterial contamination are some of the advantages of these particles. There is however one major disadvantage and that is the high surface charge of glass which would tend to make adsorption of solutes to the material a hazard especially at low concentrations.

1.6. APPLICATIONS OF GEL EXCLUSION CHROMATOGRAPHY.

The separations effected by gel exclusion chromat-

ography are numerous and diverse. One of the most common techniques is that of desalting solutions of protein on the highly cross-linked dextran or polyacrylamide gels. A macromolecular solute which is completely excluded from entering the gel matrix will be separated completely from a small solute capable of entering the gel pores. This technique can therefore be used as a simple and rapid alternative to dialysis. The use of Sephadex gels for buffer exchange has been described by Flodin (1961). Desalting has been successfully applied in the fluorescent antibody technique (Coons, Leduc and Connolly, 1955) to isolate the unconjugated dye from the fluorescent labelled antibodies on Sephadex G-25. The highly cross-linked dextrans have been used in many routine clinical assays for the removal of low-molecular-weight substances which interfere in the reaction.

An example of this has been reported by Patrick and Thiers, (1963) in the determination of protein in cerebrospinal fluid, and in the inulin clearance test (Davidson, Sackner and Davidson, 1963). The concentration of solutions, containing high-molecular-weight solutes, has been achieved by the addition of dry gel particles (highly cross-linked) of Sephadex or polyacrylamide to the solution. The swollen gel particles which now contain most of the solvent are removed by light centrifugation or are collected by gentle suction

on a filter (Ackers and Steere, 1967). The procedure can be repeated a number of times until the desired concentration is obtained.

Fractionation of mixtures of solutes has been most widely investigated on Sephadex gels. Serum proteins are separated into three main components on Sephadex G-200; macroglobulin, serum globulin and albumin respectively (Gelotte, Flodin and Killander, 1962). These gels have also been successfully used in the preparation and purification of insulin (Humbel, 1963) and gastrin (Tauber and Leonard, 1965).

The fractionation of carbohydrates with dextran gels has not been as successful as with proteins, due to the release of trace amounts of carbohydrate and to the increase in molecular size of these molecules in solution. However oligosaccharides (Edstrom and Heath, 1965) glycopeptides (Montgomery and Wu, 1963) and polysaccharides (Ricketts, 1966) have been studied with these gels.

The separation of nucleotides and oligonucleotides and other small nucleic acid components (Hohn and Pollman, 1963) have been recorded with dextran gels. Soluble ribonucleic acids (Richards and Gratzer, 1964) have also been studied with Sephadex gels but agar or agarose are superior media for the investigation of nucleic acids, viruses, subcellular particles and macromolecules which are excluded from

the dextran and polyacrylamide gels. Killander, Bengtsson and Philipson (1964) have fractionated serum macroglobulin on 3.5% agar, and phage RNA has been separated from ribosomal RNA on agarose (Erikson and Gordon, 1966). Similarly poliovirus was separated from influenza virus on 2.5% agar (Bengtsson, 1966), and Echo 7 virus from Adenovirus type 2 on the same material.

The separation of cellular nucleic acids on 1% or 2% agarose columns give three fractions consisting of DNA, ribosomal RNA and transfer RNA (Philipson, 1967). The same author has drawn attention to the fact that the buffer in these experiments has a profound effect on the elution properties of these nucleic acids. He has suggested that RNA in solutions of low ionic strength is filamentous, but in solutions of high ionic strength or in the presence of divalent metal ions the filaments are altered to more compact coils. It is obvious that a rod will be excluded to a greater extent than a coil or globular molecule and therefore the elution volume of this type of molecule will be greatly influenced by the buffer. This also demonstrates that molecular configuration is primarily responsible for the elution properties of the molecule during gel exclusion chromatography.

The use of porous glass powder as an exclusion

agent has not been used extensively to date. However Haller (1965) has demonstrated the effectiveness of this material by the separation of an artificial mixture of tobacco mosaic virus from tobacco ringspot virus. As mentioned previously the high resolution and flow rates obtained with these materials is a decided advantage.

The use of gel exclusion agents for the study of associations between small molecules and macromolecules has been reported by Hummel and Dreyer, (1962). The associations of proteins with iron (Barber, Dempster and Anderson, 1963) and drugs (Murphy and Pattee, 1964) are only two of the many studies carried out in this field. Nichol and Winzor (1965) have adapted this technique for the study of the kinetics of interaction between molecules, and also for the study of hydrogen exchange between water and macromolecules (Englander, 1963).

Separations based on interactions between solute molecules and the gel matrix have been investigated by Gelotte (1960). This effect is relatively pronounced with agar gels due to the presence of the charged agaropectin molecule. Similar effects have not been reported for the polyacrylamide gels although they may be present. Reversible adsorption on Sephadex is due to the presence of carboxyl groups on the polymer, which cause adsorption of basic and

exclusion of acidic molecules. The separation of three decapeptides on Sephadex G-25 (Mach and Tatum, 1964) is probably due to this effect. The adsorption and ultimate elution with saline of ribonuclease on dextran has been reported by Glazer and Wellner (1962), and the retardation of hydroxyl and borate ions on these gels has been noted by Gelotte (1960). Similarly the exclusion of small acidic molecules, capable of entering the gel matrix under ordinary conditions have been found to be eluted at the void volume of the column in the presence of distilled water (Spitzy, Skrube and Muller, 1961). This factor can however be used to advantage under controlled conditions for the separation of solutes of different electrical charge, but very similar molecular dimensions.

From this brief review it is apparent that the agarose gels and the dextran and polyacrylamide gels complement each other in that together they cover a wide range of molecular weights for gel exclusion chromatography.

This study was initiated as the gels described above had certain disadvantages. A search was consequently made for a material which would cover the entire range of molecular weights encompassed by both agarose and dextran gels, and also for a material which was more stable and economical to produce. As gelatin solutions produce gels at

low temperatures they were investigated with a view to producing gels of different concentration which would meet the criteria outlined above. However due to the solubility of these gels at room temperature a method had to be developed whereby they could be rendered insoluble. Furthermore this treatment would have to increase the rigidity of the gel without affecting the porosity of the material. The preparation from commercially available gelatin of a fraction suitable for development as a new gel exclusion medium, was therefore the initial objective of the study to be reported in this thesis. A fraction thus obtained was "tanned" and prepared in spherical and granular form suitable for gel exclusion chromatography in columns and certain properties of this novel medium are subsequently described. In the final chapters data are presented which were obtained when tanned gelatin was used in the separation of artificial protein mixtures and viruses, and serve as a demonstration of the potential of tanned gelatin in gel exclusion chromatography.

CHAPTER TWO.

FRACTIONATION OF GELATIN WITH POLYETHYLENE GLYCOL AND THE ESTIMATION OF THE MOLECULAR WEIGHT OF THE ISOLATED FRACTIONS.

2,1. INTRODUCTION.

It is common knowledge that solutions of gelatin, at concentrations between 1% and 10%, kept at 4°, set to produce gels which (in comparison with agarose gels of the same concentration) are too soft for use as bed material in column chromatography. During the hydrolysis of collagen for the preparation of gelatin, an inhomogeneous mixture of gelatin molecules is produced which vary considerably in molecular weight due to the different lengths of the polymers. If the hydrolysis is allowed to continue for too long a period the gelatin molecules are reduced to very short chains. It was therefore considered imperative that the longer molecules be isolated since these should have the effect of producing gels of greater rigidity than those produced from unfractionated material.

The fractionation of human serum with polyethylene glycol was successfully achieved by Polson et al (1964). As gelatin is a protein it was decided (Polson and Katz, 1968)

to use polyethylene glycol for the precipitation of the high molecular weight fraction of this material. The molecular weights of these fractions of gelatin were calculated from the sedimentation coefficients and the intrinsic viscosities. This section deals with the preparation of the high-molecular-weight fractions and some of the biophysical properties of these proteins.

2.2. MATERIALS.

2.2.1. Gelatins.

Two different makes of gelatin were used for this study: The acid processed pigskin gelatin was manufactured by Canada Packers Ltd., Gelatine Division, Toronto 4, Canada, and had a Bloom strength of 287 g. Difco gelatin was manufactured by Difco Labs., Detroit, U.S.A. and was of unstipulated Bloom strength. Bloom strength is a measurement used in commerce for the gel strength of gelatin and glue. It is the force required (in grams) to drive a plunger of precise dimensions 14 mm. into the surface of the gel, which has been made up according to a rigorously specified procedure.

2.2.2. Urea (Analar-B.D.H.).

Initially this substance was used without further purification but it was observed that a solution of urea had an "oily" scum on the surface. The urea was therefore

purified as follows: Approximately 500 g. of urea was dissolved in a minimum volume of water by gentle heating over a water bath (40°). Sufficient acetone (Analar) was added to the warm solution until the urea precipitated. The solution was kept at 4° for 24 hr. and the crystals were then collected on a precooled sintered glass filter (porosity 3). The crystals were packed down with a glass stopper, washed several times with cold acetone (4°) and dried in an oven at 40°.

2.2.3. 8. 0M-Urea + 0.14M-NaCl.

This solution was prepared by dissolving 120.2 g. of recrystallized urea in sufficient 0.14M-NaCl to make 250 ml. of solution.

2.3. METHODS.

2.3.1. Preparation of high-molecular-weight gelatin.

Difco gelatin (160 g.) was dissolved in 3,800 ml. of distilled water by gentle stirring over a boiling water bath. When all the material had dissolved a solution of polyethylene glycol (Shell Chemicals., average mol. wt. 6,000) (240 g. dissolved in 200 ml. of water) was added to the hot (80°) solution of gelatin and well mixed. The solution was kept at room temperature for 2-3 hr., after which time the high-molecular-weight fraction had separated out as a

viscous layer. The supernatant layer was completely removed and discarded, and the remaining portion was slowly poured in to 4 litres of distilled water (4°) which was gently agitated with a mechanical stirrer. If the stirring was too vigorous the particles of gelatin were too finely dispersed and difficulties were encountered with subsequent washing for the removal of polyethylene glycol. The insoluble gelatin collected round the stirrer and was removed before storage in 10 litres of water at 4° overnight. The washed gelatin strands were collected in a large precooled filter funnel containing a cotton-wool plug to retain the gelatin. The strands were washed repeatedly with large volumes of cold distilled water to remove the polyethylene glycol. The gelatin 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 final extraction of the gelatin with light petroleum was included since the acetone dried material tended to absorb water from the air and the drying procedure was prolonged because of this. The temperature of the air should not exceed 50°, as at higher drying temperatures a large proportion of the gelatin was found to be insoluble on attempting to redissolve this material in hot water.

High molecular weight acid processed pigskin gelatin was prepared as described above, with minor modifications. After the addition of the polyethylene glycol to the dissolved gelatin, saturated sodium chloride solution in water was added to precipitate the gelatin. The hot solution was well mixed and the fine flocculent precipitate was allowed to settle at room temperature. The supernatant fluid was removed after 1-2 hr. and the precipitate was washed and dried as described above.

2.3.2. Preparation of the gelatin solutions.

An 8%(w/v) solution of unfractionated and polyethylene glycol fractionated gelatin was prepared from each type of gelatin by dissolving the material in 0.14M-NaCl solution in water over a boiling-water bath. The solutions were cooled to 30° and mixed with an equal volume of 8.0M-urea. The solutions were centrifuged at 30,000 r.p.m. for 60 min. to remove any undissolved particles. Two-thirds of the supernatant of each solution was collected and kept at room temperature until used. The concentrations of the gelatin solutions were determined refractometrically (Abbe Refractometer, Hilger & Watts Ltd., London.) using 4.0M-urea + 0.14M-NaCl as standard.

2.3.3. Analytical ultracentrifugation.

Svedberg (1925) who was the first to consider the

theoretical aspects of centrifugation, showed that macromolecules could be made to migrate when subjected to a high gravitational field. The rate of migration was related to the molecular weight and the hydrodynamic properties of the molecules. He expressed this migration, known as the sedimentation coefficient (S), as the rate of movement per unit field of force, expressed as:

$$S = \frac{\frac{dx}{dt}}{\omega^2 x}$$

where ω is the angular velocity (rev./sec. 2π), x the distance from the axis of rotation (cm.) and t the time (sec.). Upon rearrangement we have

$$S\omega^2 dt = \frac{dx}{x}$$

$$S\omega^2 (t_2 - t_1) = \ln x_2 - \ln x_1$$

or

$$S = \frac{\Delta \ln x}{\omega^2 (t_2 - t_1)}$$

$$S = \frac{2.303 \, \Delta \log x}{t \, (2\pi \text{ r.p.s.})^2}$$

As the sedimentation rate of a molecule is small it is convenient to multiply this factor by 10^{13} which gives a convenient figure for most proteins. This unit is termed the

Svedberg (S). Furthermore it is customary to reduce the experimental result to a value equivalent to the rate that the particle would sediment in pure water at 20°. The sedimentation coefficient is then written S_{20w}. The dimensions for the sedimentation coefficient are:

$$S = \frac{\frac{dx}{dt}}{\omega^2 x} = \frac{\frac{l}{t}}{\left(\frac{rev.}{sec.} \cdot 2\pi\right)^2 . l}$$

which simplifies to t (sec.).

During centrifugation the Schlieren diagram is photographed at specific intervals of time. Once the photographic plate is developed the distance from the centre of the peak to the index line is measured in cm. This value is subtracted from the measurement of the distance from the centre of rotation to the index line. This value is 7.3 cm. at low speeds, and 7.33 cm. above 50,000 r.p.m. The logs of the latter measurements are plotted against the time (min.) interval between each photograph. This should give a straight line. Therefore the log value at time t_1 subtracted from that at time t_2 gives $\Delta \log x$.

Sedimentation coefficients of unfractionated and polyethylene glycol fractionated gelatins were determined at different gelatin concentrations in 4.0M-urea + 0.14M-NaCl, in a Spinco model E ultracentrifuge fitted with the An-D rotor

and the plain cell. The values obtained were standardized to sedimentation in water at 20°.

2.3.4. Intrinsic viscosity $[\eta]$.

When a liquid flows through a capillary, as in a viscometer, the layer of liquid at the walls of the capillary is stationary, but those adjacent to this layer move with increasing velocity, especially at the centre where the velocity is maximal. Therefore this laminar or Newtonian flow may be considered to consist of a number of layers moving over each other, all having different velocities. This would create a velocity gradient $\frac{du}{dx}$ through the distance dx. The shearing stress which is a force (F) per unit area, produces an internal resistance to the flow of the faster and slower moving layers of liquid. The coefficient of viscosity (η) is therefore given as the ratio between this force and the velocity gradient

$$\eta = \frac{F}{\frac{du}{dx}}$$

In practice it is found convenient to avoid the calculation of the absolute viscosity by comparing the viscosities of two liquids.

The specific viscosity (η_{sp}) is a measure of the increase in viscosity produced by dissolving for example

a protein in a known solvent. This is given by the formula below and has no dimensions

$$\eta_{sp} = \frac{\eta_{solut.} - \eta_{solv.}}{\eta_{solv.}}$$

The reduced viscosity is obtained by dividing the specific viscosity (η_{sp}) by the concentration (c) which is given as g./100 ml. The dimensions are recorded as dl/g.

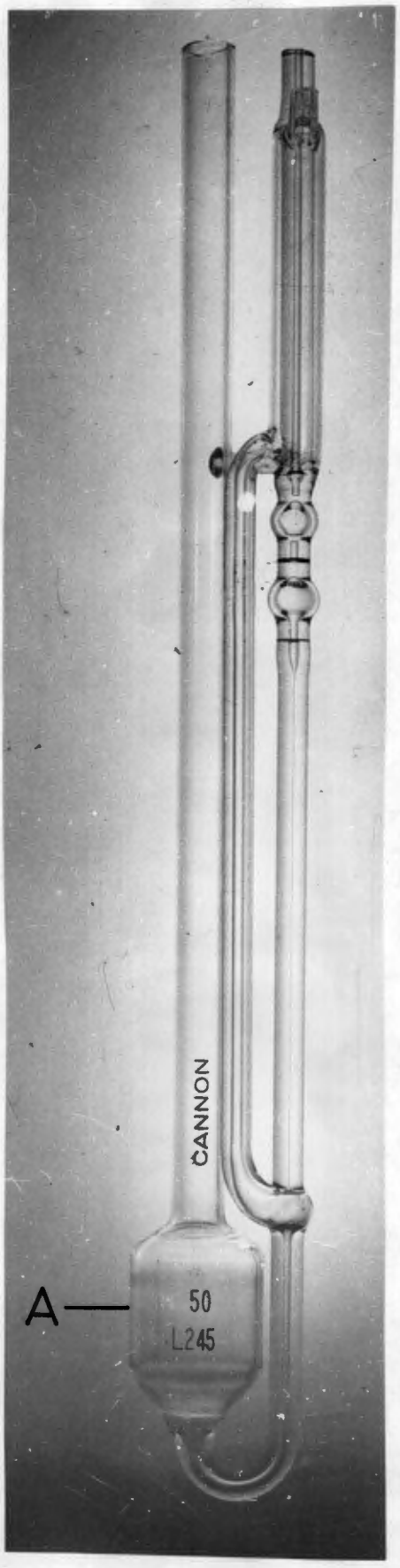
The intrinsic viscosity $[\eta]$, is the reduced viscosity at infinite dilution of the solute $[\eta] = \lim_{c \to 0} \cdot \eta_{sp}/c$. This value is conveniently obtained by plotting η_{sp}/c against c , and extrapolating to zero concentration. The point where the line intersects the ordinate is taken as the intrinsic viscosity $[\eta]$ value.

The intrinsic viscosity of fractionated and unfractionated gelatins was determined with an Ubbelohde-type viscometer (Fig. 1). Cleanliness was of the utmost importance as any particles of dust or dirt in the capillary would affect the flow rate and introduce considerable errors. The viscometer was therefore cleaned with chromic acid followed by numerous rinsings with distilled water. This was followed by several rinsings with Analar acetone and the apparatus was finally dried in an oven at 100°. Filtered air was periodically blown through the apparatus to aid in drying.

The viscosity of the 4.0M-urea-saline solution was

Fig. 1.

Ubbelohde-type Viscometer.


CANNON
A
50
L245

measured as follows: 4 ml. of the urea solution was accurately pipetted into the reservoir (A) of the viscometer. The apparatus was then lowered into a thermostatically heated and controlled water bath at 26°. Once equilibrated (about an hour) the solution was gently drawn up by sucking with a rubber tube until the liquid had completely filled the area between the two index lines. The suction was released and the downward flow of the meniscus in the capillary tube was observed until it just reached the upper index line, when the time was noted on a stop watch. When the meniscus reached the same position on the lower index line the time was again noted. The procedure was repeated three times with the same solution. Sufficient gelatin solution of known concentration was then added accurately to the reservoir and the solutions well mixed by gently sucking the liquid up into the side arm and allowing it to flow out under gravity. The solution was then sucked up into the capillary and allowed to flow out again. This was repeated a number of times to ensure complete mixing. Various concentrations of the same sample of gelatin could therefore be measured by the addition of small known volumes of the concentrated stock gelatin solution. On completion of the experiment, the viscometer was thoroughly washed and the next solution added after re-measuring the urea solution first.

2.3.5. Molecular weight estimations.

Molecular weight estimations from the intrinsic viscosity $[\eta]$ and sedimentation coefficient $S_{20^\circ w}$ values of unfractionated and polyethylene glycol fractionated gelatin were calculated from the equations of Svedberg and Pedersen (1940) and Polson (1967) as shown below:

$$f/f_o = 1.19 \times 10^{-15} \quad \frac{M^{2/3}(1-\bar{\nu}\rho)}{S_{20^\circ w}\,\bar{\nu}^{1/3}}$$

$$f/f_o = \frac{1}{0.61} \log\left(\frac{[\eta]}{\bar{\nu}}\right)$$

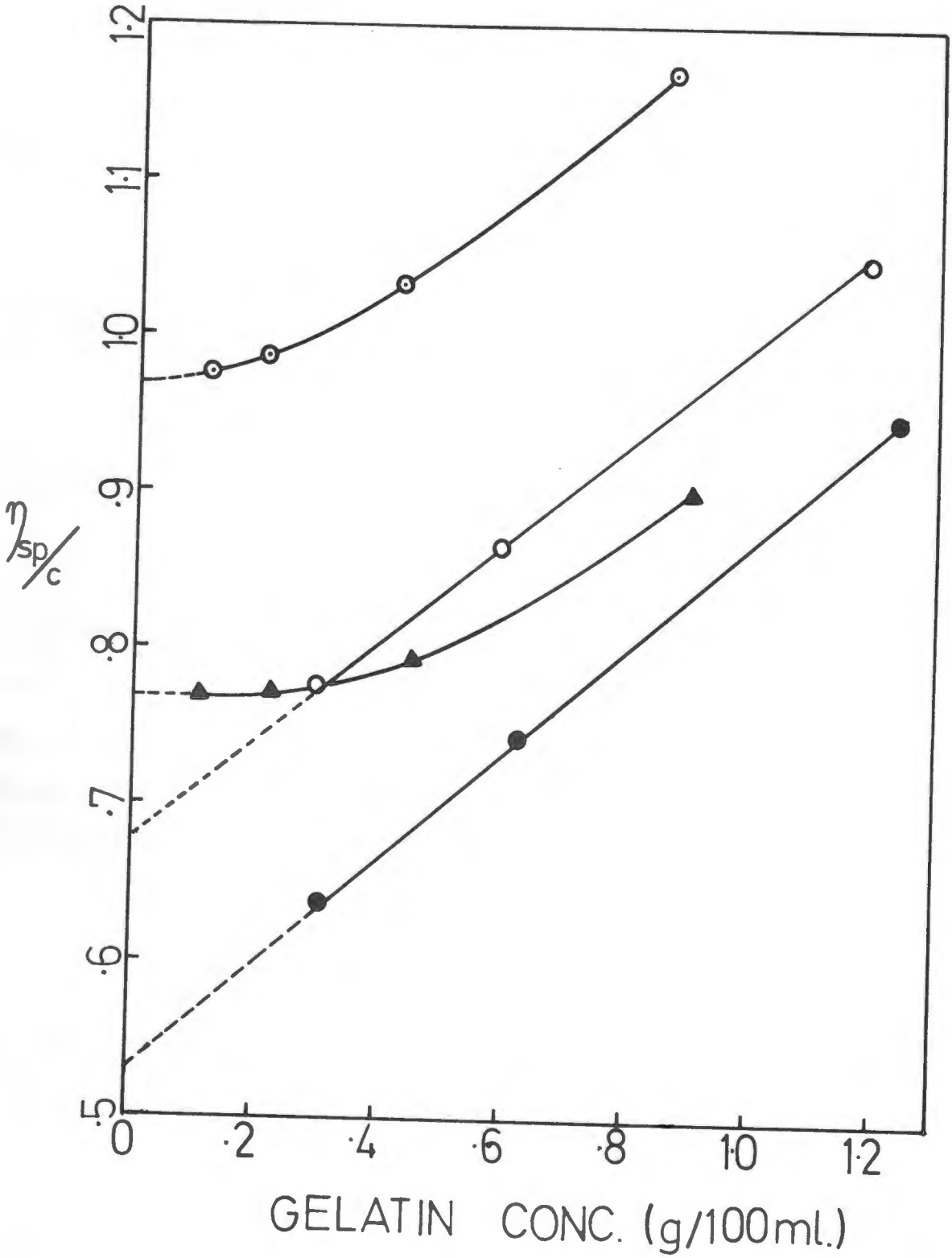
where f/f_o is the frictional ratio, $\bar{\nu}$ is the partial specific volume of gelatin (0.738 ml/g.), ρ is the density of the medium which is 1.00 g./ml and M is the molecular weight.

2.4. RESULTS.

The reduced viscosity and intrinsic viscosity values for unfractionated and polyethylene glycol fractionated Difco and acid processed pigskin gelatins are recorded in Table 1. Both the unfractionated and fractionated Difco gelatins had higher intrinsic viscosity values than those for the pigskin gelatin. A plot of η_{sp}/c against the gelatin concentration (g./100 ml.) for Difco (fractionated and unfractionated)

Fig. 2

The figure is a plot of the reduced viscosity η_{sp}/c against the gelatin concentration (g./100 ml.) for (a) fractionated Difco gelatin (⊙——⊙), (b) unfractionated Difco gelatin (▲——▲), (c) unfractionated pigskin gelatin (○——○) and (d) fractionated pigskin gelatin (●——●). The diluent was 4M-Urea + 0.14M-NaCl


1·2
1·1
1·0
·9
·8
·7
·6
·5
$\eta_{sp/c}$
0
·2
·4
·6
·8
1·0
1·2
GELATIN CONC. (g/100ml.)

and pigskin (fractionated and unfractionated) is illustrated in Fig 2.

Table 1.

The reduced viscosity η_{sp}/c and intrinsic viscosity $[\eta]$ values for unfractionated and polyethylene glycol fractionated Difco and pigskin gelatins: Mean values for five experiments.

Gelatin Concn.	η_{sp}/c	$[\eta]$ (dl./g.)
Difco unfractionated		
0.110%	0.77	
0.225%	0.775	0.77
0.450%	0.815	
0.900%	0.902	
Difco fractionated		
0.105%	0.974	
0.210%	0.982	0.97
0.425%	1.032	
0.850%	1.166	
Pigskin fractionated		
0.311%	0.6386	
0.622%	0.7429	0.53
1.243%	0.9461	
Pigskin unfractionated		
0.294%	0.7628	
0.588%	0.8712	0.68
1.176%	1.041	

The sedimentation coefficients and molecular weights of unfractionated and fractionated Difco and pigskin gelatin are recorded in Table 2.

Table 2.

The sedimentation coefficients and molecular weights of unfractionated and fractionated Difco and pigskin gelatins dissolved in 4.0m-urea + 0.14M-NaCl.

Material	$s_{20^\circ,w}$ (S)	Mol. wt.
Unfractionated Difco	2.14	89,700
Fractionated Difco	2.70	141,000
Unfractionated pigskin	1.98	73,210
Fractionated pigskin	1.76	55,460

From the above table it will be seen that fractionating Difco gelatin had the effect of isolating the high-molecular-weight fraction, but this was not the case with the gelatin derived from pigskin.

2.5. DISCUSSION.

The two types of gelatin used in these investigations reveal a number of interesting facts. Firstly the Difco product was readily fractionated into a high and lov molecular-weight fraction on treatment with polyethylene glycol.

However this effect was not obtained when pigskin gelatin was subjected to the same treatment. In the first instance fractionation of pigskin gelatin produced a substance of lower molecular weight than the original unfractionated material. Furthermore difficulties were encountered in precipitating this material with polyethylene glycol and this was only achieved on the addition of sodium chloride to the solution. Although the fraction of pigskin gelatin which was not precipitated by polyethylene glycol and consequently discarded, could in fact have been one of higher molecular weight, further studies with this material were not pursued as the concentration was low, and satisfactory results were obtained with unfractionated material. Both the intrinsic viscosity and sedimentation coefficient values for the different types of gelatin demonstrated these differences in molecular weight.

Although the lower molecular weight values for polyethylene glycol fractionated pigskin gelatin, may have been a reflection of smaller molecular size in this fraction, another possible explanation for the difference is at hand. Had the gelatin chains, as a result of prior treatments (hydrolysis from collagen) followed by the polyethylene glycol precipitation procedure described above, undergone a change in shape to a more globular configuration, both the

viscosity and sedimentation parameters would have been reduced. This may also have been the reason for the dissimilarity between the slopes of the curves of the two types of gelatin (Fig. 2). It appears that the source and the method of producing these types of gelatin exert an influence on the molecular parameters of these molecules.

Although the fractionated Difco gelatin had a molecular weight nearly double that of the unfractionated pigskin, and nearly three times that of the fractionated pigskin gelatin, both samples were used for further investigations which are described in the forthcoming sections of this study.

CHAPTER THREE.

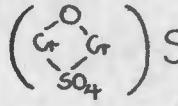
THE PREPARATION OF TANNED GELATIN SPHERES AND GRANULES.

3.1. INTRODUCTION.

Protein molecules, in particular gelatin, have the property of cross-linking with the subsequent binding of similar or different molecules leading to the formation of larger complexes. A single cross-linkage between two large molecules would result in the formation of a molecule having a molecular weight equal to the sum of the molecular weights of the molecules involved. This type of cross-linkage occurs during the tanning of leather, and the resultant product is considerably stabilized and strengthened. Furthermore cross-linking reduces the solubility of the material, increases its resistance to hydrothermal and enzymic effects, reduces its elasticity and in certain instances makes it extremely brittle (Bjorksten, 1951).

As nearly all of our present knowledge regarding tanning is associated with leather, most of the discussion in the present section will relate directly to this material. Leather is tanned by a number of reagents e.g. vegetable tannins, unsaturated oils, aldehydes, basic chromium salts, etc. Of these only vegetable tannin, formaldehyde and

chromium salts were investigated as potential tanning agents for gelatin gels.

The tanning potential of basic chromium salts is well known as they have been found to be the most satisfactory agents to date. Gustavson (1949) showed that the tanning action of basic chromium is a function of the co-ordination properties, the degree of aggregation and ionization of the salt. The basic salt has the sulphate group attached to the chromium atom and is known as a sulphate chromium complex  .

The factors governing tanning are numerous and diverse. Chromium sulphate is superior to either the nitrate or chloride, while the basicity (33%) of the chromium sulphate is also important, since the more basic salts give less satisfactory results. (Gustavson, 1949). The pH is critical, since at values between 3 and 3.5 most of the carboxyl groups are ionized and available for reaction with the chromium. At lower pH values, notably 2, the carboxyl groups are not charged and there is little reaction with the salt. At pH 4-5 all the groups are charged but the basic chromium salts tend to aggregate and collect at the surface of the leather, so preventing penetration of the tanning agent to the interior and consequently the leather remains untanned.

Furthermore Gustavson (1949) found that the uptake

of chromium was reduced by high concentrations of the salt, while at lower concentrations chromium fixation could be further enhanced by the addition of small amounts of Na_2SO_4 to the solution of basic chromium sulphate. The percentage uptake of the salt was also dependent on the duration of tanning and on the temperature, since elevated temperatures increase the uptake of chromium from solution (Merrill and Schroeder, 1929).

Before the introduction of chromium salts as tanning agents, the vegetable tannins had pride of place, and they are still in use for the preparation of different types of leather. The tannins are complex polyphenols which aggregate in acidic solutions, but at optimum pH no aggregation takes place and the reaction between the vegetable tannin and the leather takes place within a few days or hours depending on the degree of tanning required. The reaction appears to involve electrovalent and co-ordinate forces (Gustavson, 1949). As the tannins contain phenolic and many ionic groups on the polymeric structure they react with the collagen forming a multi-point attachment. The initial reaction appears to involve the basic protein groups, followed by co-ordination reactions. Unfortunately, due to the complex nature of the tannins, very little is known of their reaction with collagen.

The aldehydes as a group have a reasonably satisfactory tanning effect. Formaldehyde is the best tanning agent in

the series. The higher aliphatic aldehydes do not tan at all: the aromatic aldehydes do, but the leather is not stabilized after treatment with them. Tanning with formaldehyde was found to be optimal between pH 5 and pH 9 as at lower pH values there is insufficient reaction with the collagen, and at higher pH values the leather swells to such an extent that there is rupturing of the hydrogen bonds and a general disintergration of the structure, so creating unfavourable steric conditions for cross-linking (Gustavson, 1949).

Due to the similarity of gelatin and collagen, it seemed feasible to assume that the former material would also be tanned by one or all of the aforementioned reagents. Gelatin has been chemically altered and used for a variety of purposes but no reference in the available literature describing its use in modified form as a gel exclusion agent has been noted. This section describes the methods of tanning gelatin to render it insoluble and sufficiently rigid for use as bed material in gel exclusion chromatography. A report on this aspect of the study has recently been published (Polson and Katz, 1968).

3.2. MATERIALS.

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 $Cr(OH)_3$, 0.542 part of $Cr_2(SO_4)_3$, 1.000 part of Na_2SO_4 and 0.119 part of $C_6H_{12}O_6$ - 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, South Africa.

3.2.1. Tanning solutions.

The 20% (w/v) formalin-saline, pH 9.0, was prepared as follows: To 2 litres of 40% formalin was added an equal volume of 0.14M-NaCl and the pH adjusted to 9.0 with N-NaOH.

The 8% (w/v) chromium solution, pH 2.8, was prepared as follows: Kromex (160 g.) was dissolved in 2 litres of warm distilled water, and kept at 4° until used.

3.2.2. Buffer.

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

3.2.3. High-molecular-weight gelatin.

This product was prepared from Difco gelatin as described in the previous chapter. (2.4.1.)

3.3. METHODS.

3.3.1. Preparation of gelatin spheres.

Spheres of gelatin were prepared according to the method of Hjertèn (1964), with modifications as described below. A weighed amount of the high-molecular-weight gelatin was dissolved in 300 ml. of distilled water by 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 approximately 1 min. the flask containing the suspension was lowered with the stirrer into a beaker containing crushed ice. Once the temperature had reached 4° about 1 litre of cold ethanol (0°) was added to break the emulsion, a modification of the original technique. The spheres were allowed to settle at 4° for 30 min. and were 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). The temperature of the untanned spheres was maintained as close to 0° as possible to prevent them from melting. The concentrations of stabilizer, toluene and carbon tetrachloride for the preparation of different concentrations of gelatin spheres are given in Table 3. The rate of stirring never exceeded 1000rev./min., but this rate could be adjusted

according to the size of the sphere required for gel exclusion chromatography Hjertén (1964).

Table 3.

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

Conc. of gelatin (g./300 ml. of water).	Volume of Toluene. (ml.)	Volume of CCl_4. (ml.)	Wt. of 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

3.3.2. Preparation of formalin-tanned spheres.

Spheres prepared by the technique described above (3.3.1.) were transferred to a beaker containing approximately 1 litre of formalin-saline, (pH 9.), at 4°. The mixture was briskly stirred for 2 min. and the tanning was allowed to proceed for 24 hr. at 4°, and for a further 24 hr. at room

temperature. The spheres were then washed free of formalin with saline (0.85%) and ultimately resuspended in phosphate buffered saline containing NaN_3 as a preservative.

3.3.3. Preparation of chromium-tanned spheres.

Gelatin spheres prepared by the method described above (3.3.1.) were tanned in approximately 1 litre of chromium solution pH 2.8 at 4° for 24 hr. The spheres and tanning solution were then kept at room temperature for a further 3 days. The spheres were collected on a Buchner filter and washed with large volumes of saline. This was achieved by alternately suspending them in a large volume of saline and then collecting them on the filter. This was followed by repeated washings on the filter. It is imperative to remove all the free chromium before suspending the tanned gelatin spheres in the phosphate buffer, as failure to do this results in the precipitation of the unattached chromium salts in the gel matrix. Once free of unbound chromium the spheres were kept in phosphate buffered saline containing NaN_3.

3.3.4. Preparation of formalin-chromium-tanned spheres.

Gelatin spheres were double tanned first with formalin and subsequently with chromium by the methods described above. (3.3.2. and 3.3.3.)

3.3.5. Spheres tanned during emulsification.

To a solution of dissolved gelatin containing the

organic liquids and emulsifier, 1.5 g. of chromium tanning salt or spray dried wattle tannin powder was added to the emulsion immediately on commencing stirring. Mixing was continued while the emulsion was still hot for approximately 10-15 min. before immersion of the flask in crushed ice and water. The emulsion was broken by the addition of alcohol and the spheres washed with saline until free of uncombined tanning agents. No further tanning was necessary after the organic liquids and stabilizer had been removed.

3.3.6. Resorcinol cross-linked gelatin spheres.

Spheres were cross-linked according to the method of Braunwold, Gay and Tatooles (1966). A known amount of gelatin (12 g./300 ml.) was dissolved in water and 4 g. of resorcinol added while the solution was still hot. Stirring, while hot, was continued for 10 min. after which the organic liquids and stabilizer were added. The spheres were prepared and tanned with chromium as previously described. (3.3.3.)

3.3.7. Gelatin spheres prepared with a spray gun.

A shallow precooled glass trough containing 1 litre of cold chromium tanning solution and 3 litres of cold ether, was surrounded with crushed ice and water to keep the temperature as close to 4° as possible. The chromium solution was continually agitated by means of a magnetic stirrer. 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 onto the surface of the ether from a height of approximately 30 cm. To obtain spheres of varying porosity, concentrations of gelatin between 4% and 30% were sprayed from the gun.

On making contact with the cold ether the spheres gelled immediately and passed into the solution of cold chromium. Tanning was continued for 24 hr. at 4° and for 72 hr. at room temperature after the top layer of ether had been removed. After tanning, the remaining ether was removed under reduced pressure the spheres were collected and kept in phosphate buffer as previously described. (3.3.3.)

3.3.8. Preparation of tanned gelatin granules.

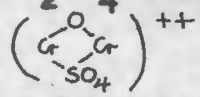
A weighed amount of gelatin was dissolved in distilled water over a boiling-water bath. The hot solution was allowed to cool to 30°, poured into a precooled dish, and kept at 4° until the gelatin had set. The solidified gelatin was cut into small portions (0.5-1.0 cm. cubes) and transferred to a beaker containing 1 litre of either chromium or formalin tanning fluid at 4°. Tanning was continued as previously described. (3.3.2. and 3.3.3.) In certain instances a double strength solution of gelatin was mixed with an equal volume of double strength chromium tanning fluid and allowed to set at 4°. After tanning the gelatin was broken up in a Waring

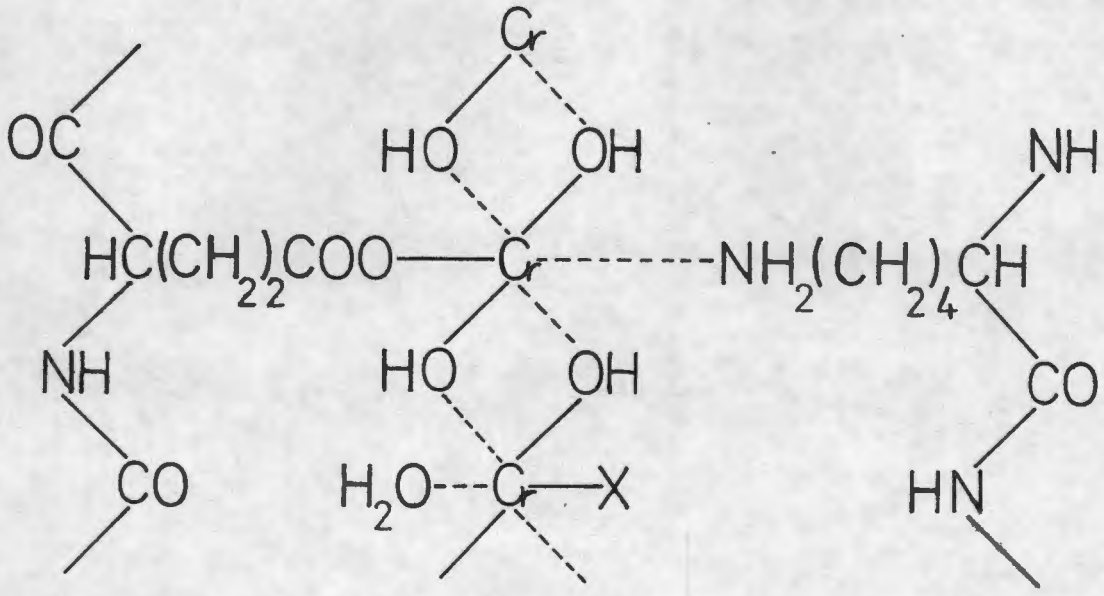
blendor until the particles were small enough for use as bed material in chromatography. The granules were washed free of tanning fluid and suspended in phosphate buffer.

3.4. DISCUSSION.

Finely divided untanned gelatin, like agar and agarose, could probably be used for gel exclusion chromatography at low temperatures where the gelatin is insoluble. At higher temperatures, notably above 7^{o}, it is necessary to treat gelatin to render it insoluble. Materials that were found suitable for rendering gelatin insoluble during this study were the tanning agents used in the leather industry. Certain other substances e.g. resorcinol are capable of bringing about the same effect but they tend to concentrate the molecules by the exclusion of water thereby changing the initial concentration of the gelatin and consequently 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.

In all instances gelatin gels tanned with vegetable tannin, chromium salt or formaldehyde were insoluble in boiling water at 100^{o}. The gel strength of the end product

was found to be a function not only of the type of gelatin, and tanning agent employed, but whether it had been previously fractionated with polyethylene glycol. This phenomenon will be discussed in the forthcoming section in greater detail. Gustavson (1949) has suggested that collagen tanned in the presence of 67% acid chromic sulphate at pH 3.0 reacts initially with the free H_2SO_4 formed, and the positively charged chromium complex  then reacts with the carboxyl group of the leather, the sulphate being compensated by the amino groups. This should result in the formation of a complex, with multipoint attachment of the chromium to the groups of adjacent collagen chains. The possible combination suggested by Gustavson (1949) is as follows:



A certain amount of evidence has been accumulated to support the theory of Gustavson. For instance it has been shown that inactivation of the carboxyl groups of collagen by methylation reduces the chrome fixation by 70% and the leather remains unstable. Furthermore deamination of methylated collagen causes a further reduction in the chromium uptake (Boves, cited by Gustavson, 1949). It is not clear however what other reactions might take place during the deamination of collagen as it has been shown (Ellis, Shuttleworth and Sykes, 1963) that the amino groups do not take part in the reaction. In fact the further apart the amino and carboxyl groups are on the molecule the greater the stability of the chrome tanned leather. It has been shown (Shuttleworth, 1950) that similar reations occur during chrome tannage of gelatin but probably on a far simpler scale as this substance is less complex than leather.

The tanning action of formaldehyde has been fairly well investigated. It appears that formaldehyde tanning inactivates the basic groups of the protein as the isoelectric point of gelatin is displaced to the acid side (Polson, Fawcett and Katz, 1969 a).

Nitschmann and Hadorn (1944) have shown that formaldehyde forms cross-linkages by the formation of methylene bridges ($-CH_2-$) between the $\in$ - amino group of the lysine

residue and the amino group of the peptide link on an adjacent chain.

Fraenkel-Conrat and Olcott (1948) conducting their experiments at room temperature and at pH values between 3 and 7.5, have shown that methylene bridges are formed between amino groups on the one hand and primary amide or quanidyl groups on the other during formaldehyde tanning of gelatin. Partial removal of the quanidyl groups of the arginine residues prior to formaldehyde tanning, did not reduce the stability of leather, suggesting that they are not important for its stabilization. (Gustavson, 1940).

Due to the complex nature of vegetable tannin little is known about its chemical reaction with collagen. However Thomas and Foster (1926) have shown that deamination of collagen (removal of the ϵ -amino groups of the lysine residue) reduces the fixation by vegetable tannin. Caution should be exercised in interpreting these results as insufficient is known about the reaction which occurs during the deamination of collagen. Inactivation of the basic protein groups with pentanenaphthalene tetramethylenedisulphonic acid reduced the uptake of vegetable tannin suggesting their participation in the original reaction (Gustavson, 1949).

Chromium and formalin-tanned gelatin spheres and granules were found to be superior to the vegetable tannin

tanned material, in as much as these materials produced more rigid gels than the latter. The formalin-tanned material had a slight advantage over the other methods of tanning as the absence of colour in the gel allowed the progress of coloured proteins to be observed during gel exclusion chromatography experiments.

A further point of interest was the reduced concentration of stabilizer necessary for the preparation of gelatin spheres in comparison with that required for preparing agarose spheres. Higher concentrations of the stabilizer had the effect of either producing very small spheres or the entire emulsion set as a gel on cooling.

As chromium and formalin-tanned gels appear to have higher gel strengths than vegetable tannin-tanned material, it was thought to be of interest to investigate some of their properties. These are dealt with in the forthcoming section.

CHAPTER FOUR.

PROPERTIES OF TANNED GELATIN GELS.

4.1. INTRODUCTION.

Two important criteria are used in evaluating tanned leather; the hydrothermal stability of leather ascertained by its ability to withstand immersion in boiling water for three minutes, and its resistance to enzymic degradation by trypsin. These criteria have been adopted in this laboratory for assessing the quality of gels of tanned gelatin for use as bed material in exclusion chromatography. Tanned gelatin was found to be insoluble in boiling water, resistant to the action of trypsin and it was sufficiently hard to give good flow rates when packed in columns. It was therefore of interest to investigate some of the other properties of these gels in order to determine the limits of their usefulness as gel exclusion agents.

4.2. EXPERIMENTAL.

4.2.1. Rigidity of tanned gelatin gels.

Gelatin was precipitated with polyethylene glycol to isolate the high-molecular-weight fraction (2.3.1.). It was anticipated that this high-molecular-weight material would give a gel of greater rigidity than a similar sample

of unfractionated gelatin of the same concentration.

Two types of gelatin were investigated: (i) Difco and (ii) acid processed pigskin gelatins. The gelatins were fractionated as described (2.4.1.) and 4% gels were prepared by dissolving the fractionated and unfractionated samples respectively in distilled water over a boiling-water bath. The four samples of gel were all tanned with chromium (3.3.3.) since chrome-tannage gave a more satisfactory end product than the other methods of tanning.

Experiments were conducted as follows: The gels under investigation were packed into identical columns (50 cm. x 2.5 cm.) and equilibrated against approximately one litre of 66mM-phosphate buffered saline, pH 6.9. The reservoir containing the buffer flowing into each column was kept at a constant height during the experiments. The flow rates of each column were noted and they were allowed to flow continuously for a period of five days. The flow rate of each column was noted at the same time each day. The results of these experiments are recorded in Table 4.

Table 4.

The rigidity of 4% fractionated and unfractionated chromium-tanned Difco and pigskin gelatin gels, as determined by flow rate experiments.

Gelatin	Flow rate measurements over a period of days. 1st day.	2nd day.	3rd day.	4th day.	5th day.
Fractionated Difco	++++	+++	++	++	++
Unfractionated Difco	++	+	+	o	o
Fractionated Pigskin	++++	+++	+++	+++	+++
Unfractionated Pigskin	++++	++++	+++	+++	+++

++++ = greater than 20 ml./hr., but less than 30 ml.

+++ = greater than 10 ml./hr., but less than 20 ml.

++ = greater than 5 ml./hr., but less than 10 ml.

+ = less than 5 ml./hr.

o = no flow.

From Table 4 it will be seen that the high-molecular-weight Difco had a faster flow rate than the unfractionated material. Furthermore the tendency towards packing ultimately causing cessation of flow

was far more marked in the unfractionated material.

In testing pigskin gelatins, there appeared to be very little difference between fractionated and unfractionated material, as gels of the same concentration had similar flow rates suggesting comparable degrees of rigidity.

Gels prepared from unfractionated gelatin on comminution in a Waring blendor tended to froth excessively, the froth persisting for a number of hours and in most cases not disappearing completely. Frothing was noted with fractionated gels but did not persist for longer than two or three hours which suggested the liberation of gelatin in the unfractionated samples during comminution.

At higher gel concentrations, notably above 8%, the use of fractionated gelatin for the preparation of more rigid gels was unnecessary as they were sufficiently hard without this treatment, after tanning. Pigskin gelatin, between concentrations of 1% to 40%, always gave gels with good flow rates whether the gelatin was fractionated or unfractionated.

4.2.2. Tanning procedure.

Experiments were conducted to determine the optimum periods for tanning gelatin gels with both formalin and chromium. Although gels of different concentrations

were used in these experiments, the material of 4% concentration was most frequently employed as it gave the most satisfactory indication, by flow rate determinations, of the degree of rigidity after different periods of tanning. The formalin and chromium tanning fluids used were those described previously (3.2.1.). Experiments were conducted as follows: a 4% solution of pigskin gelatin (unfractionated) was prepared and allowed to set at 4^{o}. The gel sheet was cut up into small cubes and the whole divided into two equal portions. One portion of the cubes was transferred to a beaker containing formalin and the other to a beaker containing chromium. Samples were removed from each beaker at 12, 24, 36, 48, 72 and 96 hr. intervals. The samples were thoroughly washed until free of tanning fluid (3.3.3.) and broken up in a Waring blendor until suitable for use as bed material for chromatography. Columns (50 cm. x 2 cm.) were packed respectively with each sample of gel, equilibrated against 66mM-phosphate buffered saline, pH 6.9, and the flow rates determined. The results are recorded in Table 5.

Table 5.

The optimum tanning period of 4% pigskin gelatin with chromium and formalin as determined by flow rate measurements.

Method of tanning.	Tanning time in hours.						
	12	24	36	48	72	96	336
Chromium	o	+	++	+++	++++	++++	++++
Formalin	o	++	++	+++	+++	+++	+++

++++ = greater than 20 ml./hr., but less than 30ml.

+++ = greater than 10ml./hr., but less than 20ml.

++ = greater than 5 ml./hr., but less than 10 ml.

+ = less than 5 ml./hr.

o = the gelatin was soft enough to pass through the sintered disk at the base of the column.

From Table 5 it is apparent that the minimum period of chromium tanning is 24 hr. at 4^{o}, but a more stable and rigid product was obtained by continuing tannage for a further 72 hr. There appeared to be no advantage in tanning for longer periods of time as gels which had been tanned for two weeks or longer had flow rates similar to those tanned for 96 hr. However, the minimum period for optimal gelatin tannage with formalin appeared to be 48 hr.

but with chromium 72 hours gave the best results.

Gelatin gels tanned for less than 24 hr. tended to pack even though they were insoluble in boiling water. In certain instances these gels passed through the sintered disk at the bottom of the chromatographic column because of their softness.

In all instances tanning was commenced at 4°, since the gelatin melted at room temperature, and then continued at room temperature for 72 hr., since Gustavson (1949) noted that the uptake of chrome and the speed of tanning of leather was enhanced at higher temperatures.

The optimum pH for chrome tanning was at 2.8 to 3.0 (Gustavson, 1949) and for formalin tanning at pH 9.0. It is apparent from Table 5 that the flow rate of the formalin tanned material was slightly slower than that of the chrome treated gelatin, indicating a greater rigidity for the latter. The addition of sodium chloride to the tanning fluid in initial experiments to prevent excessive swelling of the gels was found to be unnecessary.

4.2.3. Hardeners.

An attempt was made to increase the rigidity of formalin and chromium-tanned Difco gelatin by immersing these tanned gels in solutions of potassium alum or chrome

potassium alum. The formalin and chromium-tanned gels were prepared as previously detailed (3.3.3.) and granules suitable for use as bed material were prepared by comminution of the material in a Waring blendor. The excess tanning fluids were removed from the gels by repeated washings in saline (0.85% $^{w}/_{v}$) followed by distilled water. An equal portion of each gel was suspended in a saturated solution of potassium alum and chrome potassium alum respectively for 24 hr. at room temperature. A further sample of each type of tanned gelatin was kept in saline as a control. The hardening fluids were ultimately washed out of the gelatin samples with saline (0.85% $^{w}/_{v}$) and each respective gel including the controls were packed into columns and the flow rates determined as previously described (4.2.1.) The results of this experiment are recorded in Table 6.

Table 6.

The effect of hardeners on the rigidity of 4% formalin and chromium-tanned Difco gelatin granules, as determined by flow rate experiments.

Method of tanning.	Experimental.		Control.
	Potassium alum.	Chrome potassium alum.	No hardeners.
Chromium	+++	+++	+++
Formalin	++	++	++

+++ = greater than 10 ml./hr., but less than 20 ml.

++ = greater than 5 ml./hr., but less than 10 ml.

The samples of gelatin suspended in the hardening fluids shrunk during this treatment, but on washing with saline (0.85% $^{w}/_{v}$) returned to their original volumes. From Table 6 it is clear that neither of the treatments hardened these materials, as the controls in each instance continued to give similar results.

4.2.4. High and Low salt concentrations.

The effects of high and low concentrations of salt on chromium-tanned Difco and pigskin gelatins were investigated as follows: 2%, 4%, 6%, 10%, 20%, 30% and 40% tanned gelatins were packed into columns after equilibration against 66mM-phosphate buffered saline, pH 6.9.

The buffer was allowed to flow under a constant head for 48 hr. after which the height of the bed material in each column was noted. Approximately 2 litres of 2M-NaCl in 66mM-phosphate buffer, pH 6.9 was run through each column, the hydrostatic pressure being kept constant and identical with that used during the equilibration process. Once the above amount of buffer had been passed through the column, the height of the gel in the column was again noted and the percent gel shrinkage calculated from the original height of the gel in the column. The results are recorded in Table 7.

Table 7.

Shrinkage of chromium-tanned Difco and pigskin gelatin granules after equilibration against 2M-NaCl.

Gelatin conc.	Fractionated Difco gelatin chromium-tanned.	Unfractionated pigskin gelatin chromium-tanned.
2%	++++	++++
4%	++++	+++
6%	+++	++
10%	+	+
20%	+⟶o	o
30%	o	o
40%	o	o

++++ = greater than 25% shrinkage, but less than 30% of original volume.

+++ = greater than 15% shrinkage, but less than 25% of original volume.

++ = greater than 10% shrinkage, but less than 15% of original volume.

\+ = less than 5% shrinkage of original volume.

o = less than 1% shrinkage of original volume.

In all instances shrinkage was noted, but it was most noticeable in the lower gel concentrations notably 2% and 4%. At concentrations of gelatin between 10% and

20% it was less noticeable and hardly noticeable between 30% and 40%. Furthermore this effect was slightly less marked in the pigskin variety of gelatins.

On completion of these experiments 6mM-phosphate buffer, pH 6.9 was then run through each column. All the gels were found to swell again. At concentrations between 2% and 6% the swelling was so marked that the columns ceased to flow, a condition which became less marked the higher the gel concentration.

4.2.5. Thermal Stability.

The ability of chromium and formalin-tanned Difco and pigskin gelatin granules and spheres to withstand immersion in boiling water was investigated as follows: Chromium and formalin-tanned gelatin spheres and granules of concentrations varying between 2% and 40% were suspended in beakers containing distilled water. The water containing the gel was boiled over an oil bath for 15 min. and then allowed to cool to room temperature. The gelatin was collected on a sintered glass filter (porosity 4) and the filtrate (1 ml.) mixed with 6% perchloric acid (3 ml.). After half an hour the tubes were observed for any signs of turbidity. As no turbidity was produced in any of the tubes it was assumed that under the conditions of the experiment the tanned gelatins were insoluble.

4.2.6. Resistance to trypsin and α-chymotrypsin.

Chromium and formalin-tanned gelatin granules were subjected to enzymic digestion according to the method of Schurr and McLaren (1965) with minor modifications. A solution of trypsin (10mg./ml.) in 66mM-phosphate buffer, pH 8.0, and a solution of α-chymotrypsin (10mg./ml.) in the same buffer and pH were prepared. Onto two microscope slides a slurry of formalin-tanned gelatin, and on two further slides a slurry of chromium-tanned gelatin granules was applied. Three drops of the trypsin solution were added to each of one of the chromium and formalin gelatin slides, and three drops of the chymotrypsin solution was applied to the remaining two slides. Two slides with untanned gelatin granules received respectively three drops of each enzyme solution. The experiments were conducted at 10° since the untanned gelatin does not melt at this temperature. Although this was not the optimum temperature for either enzyme, a digestion period of up to one hour was alloved for each sample. The gelatin particles were observed under the light microscope, but no digestion of the tanned particles took place, as they retained their shape and size. In the untanned samples the particles were noted to fade away

slowly during digestion and the slide contained a clear liquid with no sign of the original particles, after the one hour digestion period. Two slides, one with chromium and the other with formalin-tanned granules, kept under identical conditions, but in the absence of either enzyme, showed on investigation that the gelatin particles retained their shape and size.

A second experiment conducted at 10° on untanned, formalin-tanned and chromium-tanned gelatin slabs gave similar results. A drop of each enzyme solution was applied to all three types of gelatin slab, and the digestion allowed to proceed for an hour. After the digestion period the sheets were washed with cold saline (4°) and the areas previously covered with enzyme solution were examined. The results of both these experiments are recorded in Table 8.

If digestion took place on the slab of gelatin a small hole was produced on the surface of the material, in the area previously covered with the enzyme solution.

Table 8.

The effects of trypsin and α-chymotrypsin on untanned, formalin and chromium-tanned gelatin granules and slabs.

	Gelatin granules.			Gelatin slab.		
	Trypsin	Chymo-trypsin	No enzyme	Trypsin	Chymo-trypsin	No enzyme
Formalin tanned	o	o	o	o	o	o
Chromium tanned	o	o	o	o	o	o
Untanned	+	+	o	+	+	o

o = No digestion.

+ = Digestion.

4.2.7. Effect of diaminoethanetetra-acetic acid, disodium salt (EDTA).

Chromium tanned gelatin granules were suspended in a saturated solution of EDTA with gentle stirring. After three hours the granules were allowed to settle and it was noticed that the supernatant fluid had become coloured (purplish) due to the liberation of chromium, and the granules had become purple in colour (originally green). A similar effect, but to a lesser degree was

noticed when a 0.01M-EDTA solution, pH 7.5 was allowed to percolate through a column containing 2% chromium tanned granules. The liberation of chromium continued visibly until approximately 2 litres of the buffer had passed, when it appeared to stop. If the column flow was stopped for 3 or 4 days and then allowed to flow again a further release of chromium was noticed. Gustavson (1949) observed that chrome tanned collagen was stabilised by immersion in IM-Na_2SO_4 solution for an hour. A similar treatment of chrome tanned gelatin granules for 3 hr. did not inhibit the release of chromium when these granules were suspended in saturated EDTA.

4.2.8. Storage and preservation.

Samples of formalin-tanned and chromium-tanned gelatin kept at room temperature in the absence of preservative became heavily contaminated with bacteria, with the liberation of malodorous fumes.

Initially 0.02% ($^{w}/_{v}$) NaN_3 was added to the tanned gels suspended in 0.85% NaCl solution and kept at room temperature. This concentration of NaN_3 was obviously insufficient as certain of the samples supported the growth of bacteria. The presence of fungi was also often noted in these samples, and it was therefore considered essential

to increase the NaN_3 concentration to 0.2% ($^w/_v$) with the addition of 5 ml. of $CHCl_3$ for each litre of gel. Gels stored at room temperature under these conditions for periods of up to a year were free of fungi and bacteria and continued to give satisfactory results when used as bed material for gel exclusion chromatography.

4.2.9. Removal of chrome tanning fluid.

If the chrome tanning fluid was incompletely removed from the gelatin after tanning, the addition of phosphate buffer to a slurry of gelatin granules caused the precipitation of the uncombined chrome in the gel matrix. This precipitated chrome could be partially removed by washing the gel with 0.5N-HCl, but the effect of this precipitation was to reduce the porosity of the gel. This effect is discussed in greater detail in the forthcoming sections.

4.2.10. Drying.

Chromium-tanned gelatin granules were dried by a number of different methods. The granules were freed of excess water by suction on a Buchner filter and then spread out on a large enamel dish. A current of warm air (50^o) was blown over the granules until they were completely dry. A second batch of granules was suspended in several changes

of absolute ethyl alcohol and a current of cold air blown over the granules until dry. Finally a batch of gelatin granules was suspended in several changes of acetone and finally in petroleum ether (b.p. 40°-60°) to remove the acetone and then dried in a current of cold air.

Each batch of dry granules was independently suspended in 66mM-phosphate buffer, pH 6.9 and allowed to equilibrate overnight. In all instances the water regain was very small and the granules remained hard and brittle even after immersion for three days.

4.3. DISCUSSION.

Tanned gelatin spheres and granules were found to be suitable for gel exclusion chromatography. The greater rigidity of gels prepared from polyethylene glycol precipitated Difco gelatin, could possibly be ascribed to the fact that the polymer-like chain of these proteins, due to their greater length, had many more points on the chain for potential cross-linkages during chrome tannage, thereby producing a more rigid matrix and consequently a harder gel. With acid processed pigskin gelatin this did not appear to be the case. From the intrinsic viscosity and sedimentation coefficient of fractionated and unfractionated pigskin gelatins, it appears that the acid hydrolysis of the

original collagen is not a milder process than the alkaline procedure for the preparation of Difco gelatin. This would not account for the tremendous difference between the rigidity of the pigskin and Difco gelatins as the former had a lower molecular weight than the Difco product. It appears that pigskin gelatin has a greater capacity for cross-linking than the other gelatin, either by the exclusion of water or by some ability which is innate in the molecule for binding with other groups either on the same chain or on adjacent chains.

The "frothing" phenomena observed during comminution of unfractionated gelatin may be due to uncombined lov molecular weight gelatins liberated from these gels during their breakdown to smaller particles. This could occur if the cross-linking of a small molecule was at one or two points on a longer molecule, and during breakdown would easily be disrupted from the parent molecule. At higher concentrations of gelatin, notably above 8%, the number of protein molecules is sufficiently high to have enough points of attachment for cross-linking with the chromium complex, but not enough points for the prevention of the frothing phenomenon, as this was noticed even with 40% tanned gels.

Chromium-tanned gelatin gels are more rigid than gels tanned with vegetable tannin or formalin, and consequently have higher flow rates. This is probably due to the superior cross-linking effect of chromium in contrast with that of formaldehyde. It is not possible to give an explanation for this effect, but the binding of the carboxyl group appears to be a more stable means of reaction than that of formaldehyde where the ϵ-amino group of the lysine residue is involved.

Tanning appeared to be complete after 96 hr. with chromium, but due to the insensitivity of the method used for the measurement of rigidity, complete tanning may need a longer period of time. However for the preparation of chromium tanned gelatin gels this was found to be adequate.

A number of substances have been used for hardening gelatin in the photographic industry. These include carbodiimides (Wilson, 1966), organic acid chlorides (Allan and Carroll, 1955) and the more commonly known compounds such as chrome potassium alum and potassium alum. The chrome and potassium alum hardeners used in this study were incapable of increasing the rigidity of tanned gelatin gels. In most instances the above mentioned hardeners render gelatin insoluble at temperatures below 100°. Chromium is obviously of no use for

photographic work but in this study, where clarity of the gel was not an important criterion, chromium was found to be ideal.

The resistance of tanned gelatin to hydrolysis by either chymotrypsin or trypsin and its hydrothermal stability appears to be a function of the cross-linking effects of formalin and chromium (Bjorksten, 1951). However the chelating agent EDTA was capable of removing the chromium intimately associated with the gelatin. This suggests that the chromium has a greater affinity for EDTA than for the gelatin.

While drying the gelatin after fractionation with polyethylene glycol the temperature had to be rigorously controlled as at temperatures above 50^{o} a large percentage of the gelatin remained insoluble when attempts were made to re-dissolve it in hot water. An explanation for this was given by Yannas and Tobolsky (1967) who suggested that the gelatin molecules became covalently cross-linked when the water content fell below a certain level. With tanned gelatin the material is already insoluble, but it is proposed on the removal of water during drying the already cross-linked molecules are brought closer with a greater opportunity for further

cross-linkage with each other. This would account for the small water regain and would probably cause a reduction in porosity, since the gel matrix would now consist of molecules more closely bound, and the gel pores would be reduced in diameter. This would have the same effect as increasing the concentration of the gelatin in the gel.

Agarose gels are almost completely neutral having a small residual charge, in good preparations, which is due to the presence of carboxyl groups. In assessing the suitability of tanned gelatin gels for exclusion chromatography it was considered advisable to investigate the charge properties of these materials. Experiments were therefore conducted with tanned gels in order to ascertain the charge and polarity of these materials. These studies are described in the following chapter.

CHAPTER FIVE.

THE ISO-ELECTRIC POINT OF TANNED GELATINS.

5.1. INTRODUCTION.

A knowledge of the charge characteristics of tanned gelatin gels is essential if they are to be used as bed material for gel exclusion chromatography. The efficient use of tanned gelatin for this technique depends on a knowledge of the sign, overall charge and the iso-electric point.

Initially it was thought that tanning with formalin would bind the positive (amino) groups, and re-tanning with chromium would then bind the free negative (carboxyl and hydroxyl) groups thus giving a neutral gel similar to agarose. This however was not the case as will be shown in the forthcoming sections.

Because of the difficulties inherent in the measurement of electrophoretic mobility of gels, it was necessary to investigate the electrical characteristics of the differently tanned gelatins by electro-osmosis. The passage of buffer through a gel under the influence of a voltage gradient over a wide pH range provided an indication of the charge and polarity of rigid, porous and insoluble membranes. (Polson, Fawcett and Katz, 1969)

5.2. MATERIALS.

5.2.1. Difco gelatin.

Difco gelatin was fractionated by precipitation with polyethylene glycol as previously described. (2.4.1.) This high-molecular-weight fraction was used in the preparation of all the gelatin membranes.

5.2.2. Tanning solutions.

The formalin and chromium tanning fluids were prepared as previously described. (3.2.1.)

5.2.3. 50 mM-glycine buffer.

This solution was prepared by dissolving 18.75 g. of aminoacetic acid (B.D.H. - Analar) in 5 litres of distilled water. The pH of the buffer was adjusted with either 50mM-NaOH or 50mM-HCl.

5.2.4. Preparation of gelatin membranes.

A 20% ($^{w}/v$) solution of the high-molecular-weight fraction was prepared by dissolving gelatin in water over a boiling-water bath. The solution was allowed to cool to approximately 30° before being poured on to a plane glass surface. Circular gelatin disks 3 cm. in diameter were cut from the gel sheet with a stainless-steel cutter.

5.2.5. Tanning procedure.

Half the gelatin disks were tanned with formalin and half with chromium as previously described. (3.3.2. and

3.3.3.) The membranes were thoroughly washed with normal saline until free of the tanning agents, and half the formalin-tanned disks were re-tanned with chromium and half the chromium-tanned disks re-tanned with formalin. The tanning agents were removed by repeated washings with normal saline and stored in the presence of NaN_3 (0.2%) until used.

5.3. METHODS.

5.3.1. Apparatus.

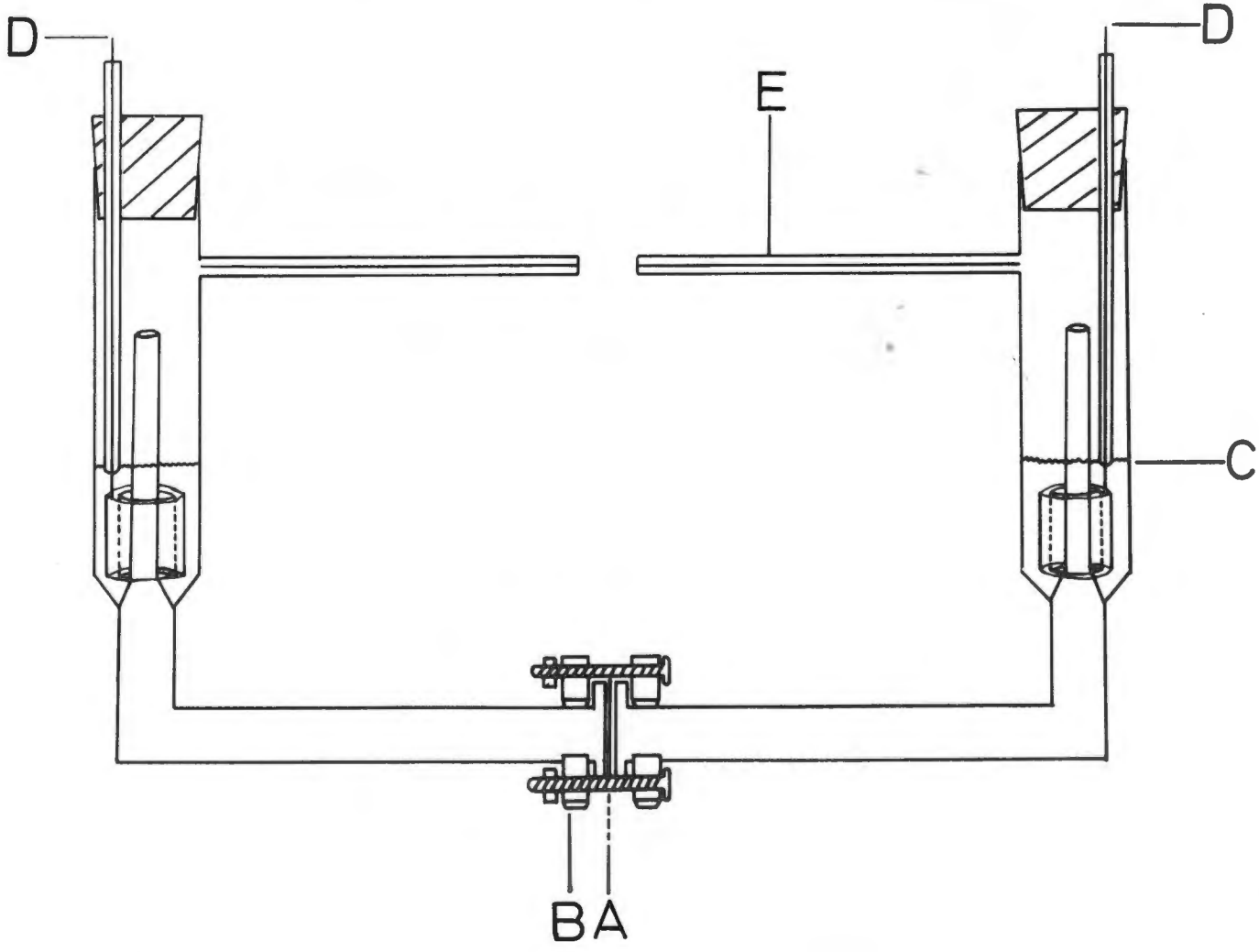
The apparatus for measuring electro-osmosis was that described by Russell (1968) with minor modifications, and is illustrated in Fig. 3. The tanned gelatin membrane (A) was secured in position with Perspex clamps (B). The apparatus was completely filled with glycine buffer of known pH, and the apparatus freed of any adhering air bubbles. The non-polarizing Ag/AgCl electrodes were gently lowered into position and sufficient saturated NaCl solution in water was pipetted into the electrode vessels (C) to cover the electrodes (D), which were secured with a rubber stopper. The entire apparatus up to the level of the capillary side arms (E) was immersed in a water bath, and the water was continually stirred and thermostatically controlled at 22^{o}.

A voltage gradient of IV/cm. was applied across the apparatus. This resulted in a current flow of 3-6 mA

Fig. 3.

Apparatus for the measurement of the iso-electric point of tanned gelatins.

Operational details are given in the text.


D
D
E
C
B
A

depending on the buffer used. The system was alloved to equilibrate for at least 1 hr. before any readings were taken, as the current caused a slight initial rise in temperature. Once equilibrated the time and the positions of the menisci of the buffer in the capillaries were noted. After time had been allowed for migration of the buffer (1 cm.) the time and position were once again noted. The polarity was reversed and the flow rate of the buffer over the same period of time was noted. The entire procedure was repeated with the same membrane in position but in buffer of different pH values. Each of the four differently tanned membranes were treared in a similar manner.

5.4. RESULTS.

The results of the electro-osmosis experiments for (a) chromium-tanned, (b) formalin-chromium-tanned, (c) chromium-formalin-tanned and (d) formalin-tanned membranes are illustrated in Fig. 4, which is a plot of the flow rate of the meniscus over 1 cm. (100 x 1 cm./T) against the pH value. T being the time (min.).

The iso-electric points of the differently tanned membranes are recorded in Table 9.

Fig. 4.

Electro-osmosis experiments with formalin-tanned (⊙), chromium-formalin-tanned (▲), formalin-chromium-tanned, (○) and chromium-tanned (●) gelatin membranes. The curves are plots of the flow rate (100 x 1 cm./T) against the pH value, where T is time (min.).

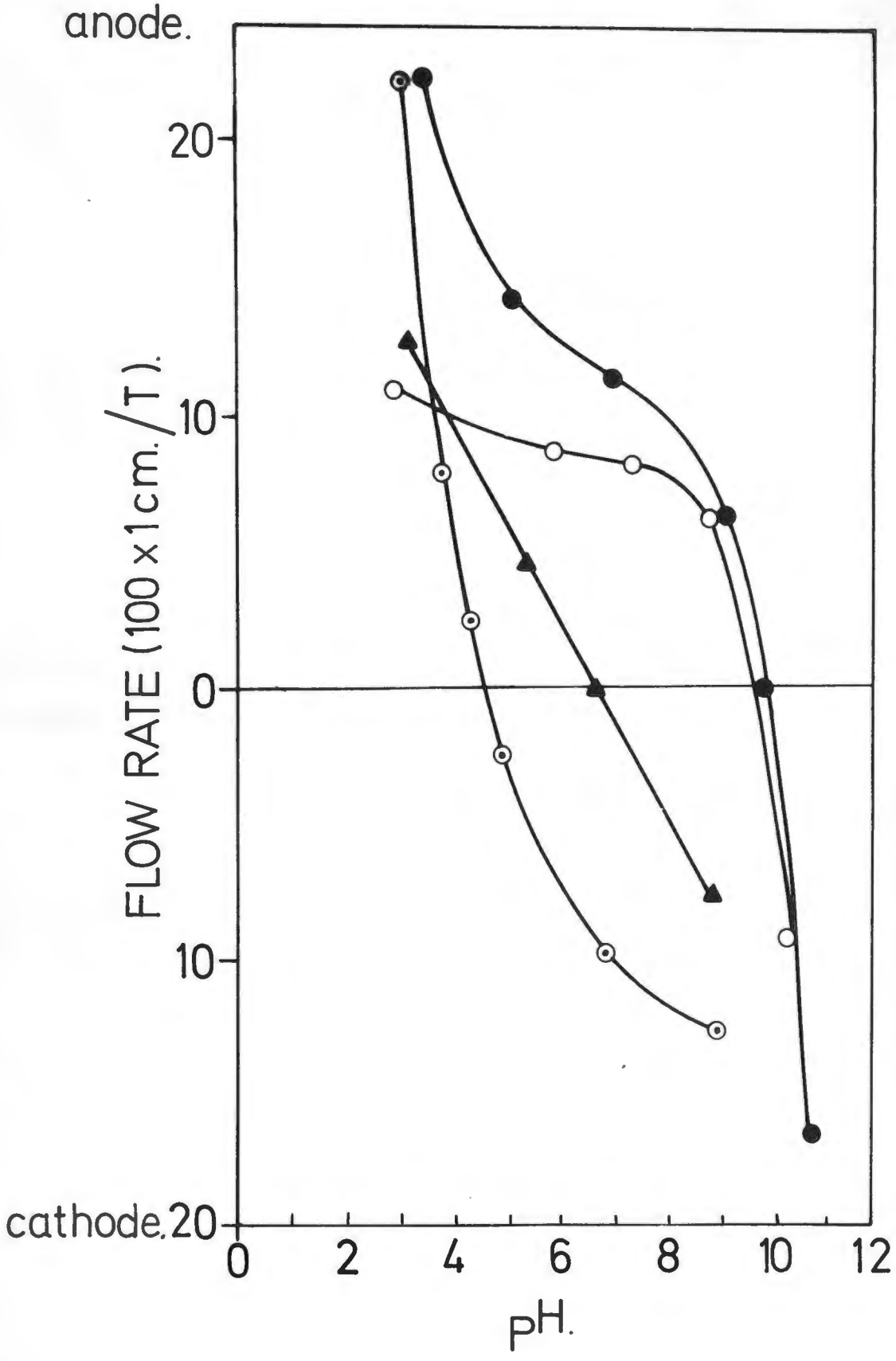

anode.
20
10
0
10
cathode.20
FLOW RATE (100 x 1cm./T).
0
2
4
6
8
10
12
pH.

TABLE 9.

The iso-electric point of tanned gelatins.

Type of Tanning.	Membrane thickness in cm.	Iso-electric point. (pH)
Chromium	0.055	9.9
Formalin-chromium	0.060	9.8
Chromium-formalin	0.047	6.8
Formalin	0.057	4.6

5.5. DISCUSSION.

It has been shown (Fraenkel-Conrat and Olcott, 1948) that the tanning of gelatin by formaldehyde is due to a secondary condensation reaction, in which the formaldehyde forms cross-linking methylene bridges ($-CH_2-$) between ϵ-amino groups of the lysine residue on the one hand and primary amide or quanidyl groups on the other. This would leave the carboxyl, hydroxyl and any other unidentified groups free or unattached, and the formalin-tanned membrane would carry a residual charge due to these groups.

During chrome-tannage of leather the chromic salt, owing to its polynuclear co-ordination complexes forms a bridge of covalent and co-ordinate links between reactive

points on the same or neighbouring chains (Green, 1953). These reactive points have been shown to be the carboxyl, amino and hydroxyl groups, as acetylation of either free amino or free hydroxyl groups of collagen cause a marked decrease in its ability to take up either cationic or anionic chromium complexes from solution. Furthermore, inactivation of the carboxyl groups of collagen by methylation decreases the chromium fixation by 70% and the product remains unstable (Gustavson, 1949). Deamination of methylated collagen decreases the chromium fixation even further. The same reactions occur during the tanning of gelatin with chromium salts as was shown by Shuttleworth (1948).

Tanned gelatin membranes have now been shown to carry a residual charge irrespective of the tanning sequence with either formalin or chromium. It is noteworthy that the iso-electric point of formalin-tanned gelatin (pH 4.6) is raised considerably (pH 9.8) by re-tanning this material with chromium. A possible explanation for this is that the formalin tanning decreases the number of available positively charged groups (ϵ - amino groups of the lysine residue) so making the gel iso-electric at acid pH. On re-tanning this material with chromium the hydroxyl and carboxyl (negatively charged) groups are involved thus producing a gel iso-electric at alkaline pH, due to the residual unreacted

positive groups.

Conversely, the chromium-tanned gelatin would have a residual positive charge due to the amino groups, and is therefore iso-electric at pH 9.9. But on re-tanning this material with formalin the iso-electric point is lowered to 6.8 suggesting that a number of positive groups have taken part in the reaction on re-tanning with formalin, which would be expected. However an explanation must be found to account for the iso-electric point of chromium-formalin-tanned gelatin being two pH units above that of the formalin-tanned membrane. It has been shown by Gustavson (1943) that on re-tanning chrome tanned leather with formaldehyde, the formaldehyde displaces the protein bound acid of chrome-tanned collagen. This, coupled with the fact that a reasonably large percentage of the amino groups take part in the reaction during chrome-tannage (Gustavson, 1949) would possibly leave fewer unreacted groups (ϵ - amino) to react with the formaldehyde on re-tanning, thus the iso-electric point would not drop as low as for the formalin-tanned membrane.

By the judicious selection of buffers of suitable pH value or ionic strength the differently tanned gels could be used for gel electrophoresis and gel exclusion chromatography at their iso-electric points. As these iso-electric points vary from 4.6 to 9.9 a reasonably wide pH range is

available for either of these two techniques.

As tanned gelatin granules appeared to be suitable material for gel exclusion experiments, it was used initially to prove a new theory for gel exclusion chromatography. (See next section) Unfortunately during these experiments a number of new and interesting facts were revealed regarding this material which prohibited its use in this particular investigation. (This will be discussed in greater detail in Chapter 7). Consequently agarose was substituted for tanned gelatin, and the forthcoming section deals with some of these findings.

CHAPTER SIX.

THEORY OF GEL EXCLUSION CHROMATOGRAPHY.

6.1. INTRODUCTION.

A number of terms have been used to describe the separation of substances of varying molecular dimensions on gel materials such as starch, dextran, agar, agarose, polyacrylamide and now tanned gelatin gels. This effect has been variously termed "Gel filtration" (Porath and Flodin, 1959), "restricted diffusion chromatography" (Steere and Ackers, 1962), "molecular sieve chromatography" (Hjertén and Mosbach, 1962) and "exclusion chromatography" (Pedersen, 1962). The wide variety of terms is unfortunately an indication of the lack of knowledge regarding the mechanism of gel chromatography. This criticism is not intended to detract from the attempts made to explain the theories for gel chromatography, but rather to stress the formidable problems associated with an explanation for the mechanisms which are responsible for this phenomenon. (Anderson and Stoddart, 1966).

The passage of a solute through a column packed with a gel is described by the distribution coefficient (K_d) which Gelotte (1960) has defined as that portion of the internal

volume (V_i) of the gel which is accessible to the solute. This has been expressed as follows:

$$K_d = \frac{V_e - V_o}{V_i}$$

where V_e is the elution volume of the solute and V_o is the void volume.

The total volume of the column (V_t) which includes the swollen gel and surrounding buffer is given by

$$V_t = V_g + V_o + V_i$$

where V_g is the volume of the swollen gel. V_i can be calculated from the known dry weight of the gel (a) and the water regain (W_r)

$$V_i = a.W_r$$

or from the wet density of the swollen gel particles (d)

$$V_i = \frac{W_r . d}{W_r + 1}\left(V_t - V_o\right)$$

For large solute molecules unable to enter into the gel matrix $V_e = V_o$ or $K_d = 0$. On the other hand small molecules which are able to enter the gel matrix and therefore capable of distributing themselves equally between gel and surrounding buffer K_d is equal to one. K_d values greater than one indicate that adsorption is also taking place.

The formulation of a theory for gel exclusion is

dependent on the interpretation of the interaction of solute molecules with porous gel materials. Ogston (1966) has distinguished two aspects: (a) thermodynamic and (b) hydrodynamic. In the thermodynamic interpretation Ogston (1966) has suggested that the mobile and stationary phases during gel exclusion chromatography constitute a system in which large solute molecules are excluded from the gel matrix to a greater extent than small solute molecules. Therefore the exclusion mechanism permits the chromatographic separation of solutes according to molecular size the largest molecules being eluted first from the column. Laurent and Killander (1964) have attempted to explain gel chromatography in terms of steric models. They have suggested that dextran gels are composed of straight, rigid polysaccharide chains which are randomly distributed within the gel particles so forming a sieve or matrix with these polymers. When a mixture of solute molecules are chromatographed on this gel those molecules having molecular dimensions greater than the pores of the gel matrix will therefore be excluded from entering, but those having dimensions smaller than the gel pores will enter into the matrix and consequently will be retarded and therefore will be eluted from the column after the larger molecules.

Porath (1963) has assumed that the pores in dextran gels are conical in shape and has derived a theoretical

formula relating K_d to the Stokes radius (a) of a solute molecule. He found that for flexible macromolecules $K_d^{1/3} \propto M^{1/2}$ where M is the molecular weight of the solute. Squire (1964) has enlarged Porath's theory to include "crevices" and "cylinders" which yields

$$\frac{V_e}{V_o} = \left[1 + g(1 - a/r)\right]^3$$

where g is a constant and r is the average radius of the cylinder, cone or crevice.

With regard to the hydrodynamic aspect, Pedersen (1962) has shown that large in comparison with small solute molecules migrate faster in very fine capillaries. The capillary volume is reduced by the presence of a layer of solvent molecules on the surface and therefore the large solute molecules are confined to a smaller volume than the smaller solute molecules. This would result in the larger molecules moving faster in relation to the smaller, and consequently the bigger molecules would be eluted from the column first. The pores in a gel could therefore be interpreted as long capillaries in the gel particles which act as diffusion barriers to the solute molecules in the mobile phase. Ackers has shown (1964) that a process of simple molecular exclusion is in itself not sufficient to explain the process of chromatography on

gels. The probability of large molecules entering the capillary to the same extent as small molecules is unlikely and this could explain the simple exclusion effect. However, molecules which do enter the capillary experience an increase in their hydrodynamic resistance to diffusion and therefore the movement of large relative to small molecules is enhanced and they are eluted first from the column.

It was with the above considerations in mind that a study was undertaken to try and explain the mechanism of gel chromatography approaching the problem from a different aspect. A quantitative theory for gel exclusion chromatography has been devised (Polson and Katz, 1969) and the forthcoming section of this thesis deals with some of its implications.

6.2. THEORETICAL.

If we consider a horizontal section of infinitesimal thickness through a separating zone of a protein component in a gel exclusion column, the osmotic pressure (due to the protein) of the buffer surrounding the gel particles would be given by:

$$\Pi_e = \frac{RTC_e}{M} + RTC_e^2B \qquad (1)$$

where R, T and C_e are the gas constant, absolute temperature and the external concentration of the protein respectively. B is the second virial coefficient and M the molecular weight. B is

related to the mutual exclusion of the protein molecules from a defined space. The details relating to the virial coefficient were those outlined in Tanford (1963).

The osmotic pressure of the protein fraction which diffuses into the gel matrix would be given by:

$$\overline{\Pi}_i = \frac{RTC_i}{M} + RTC_i^2 B + RTC_i G \qquad (2)$$

where C_i is the concentration of protein inside the gel particles. G in equation (2) is a factor related to the interference of the gel filaments with the Brownian motion of the protein molecules which diffused into the gel matrix. This factor is of a similar nature to B in equation (1). The mutual interference or exclusion is between the fraction of concentration C_i and the filaments of the gel. It therefore is a function of $C_i \cdot C_g$ where C_g is the concentration of the gel forming material. As it is not possible at the moment to give a theoretical value for this function the factor pertaining to the gel is expressed as G, and it is assumed that G contains the energy factor RTC as the other members of the equation do.

As there is no transfer of material when the system is at equilibrium

$$\overline{\Pi}_e = \overline{\Pi}_i$$

Therefore equating equations (1) and (2) and dividing throughout by RTC_i we have:

$$\frac{C_e}{MC_i} + \frac{C_e^2 B}{C_i} = \frac{1}{M} + C_i B + G \qquad (3)$$

But $\frac{C_i}{C_e} = K_d$, the distribution coefficient between the gel and the mobile phase. Upon rearranging equation (3) it follows that:

$$G = \frac{1}{M}\left(\frac{1}{K_d} - 1\right) + B\left(\frac{C_e^2 - C_i^2}{C_i}\right)$$

$$= \frac{1}{M}\left(\frac{1}{K_d} - 1\right) + B\left(\frac{1}{K_d} - 1\right) C_t \qquad (4)$$

$$C_t = C_e + C_i$$

which indicates that G may be partly dependent upon the concentration of the protein in the system. At low concentrations, which generally prevail in separating zones, the factor containing B may be disregarded as a first approximation and equation (4) may be written:

$$G = \frac{1}{M}\left(\frac{1}{K_d} - 1\right) \qquad (5)$$

K_d was calculated from the equation $K_d = \frac{V_e - V_o}{V_i}$

6.3. MATERIALS.

Several globular proteins of well established molecular weight were used for these investigations.

6.3.1. Ovalbumin. (Mol. wt. 45,000 Lamm and Polson, 1936)

This was prepared according to the method of Sörenssen and Höyrup (1918). This preparation was freed of bacteria by ultrafiltration and kept at 4° until used. For experimentation 10 ml. was removed aseptically from the stock bottle and dialysed for 24 hr. against 5 litres of 66mM-phosphate buffered saline, pH 6.9. The resulting solution was freed of gross particulate matter by centrifugation at 10,000 r.p.m. for 15 min. and then used immediately.

6.3.2. Bovine plasma albumin. (Mol. wt. 68,000 Svedberg and Pedersen, 1940)

Fraction 5 (Armour Pharmaceutical Co., Eastbourne, England) was used without further purification. A 2% (W/v) solution of bovine plasma albumin in phosphate buffered saline pH 6.9 was prepared, dialysed and centrifuged as described above. (6.3.1.)

6.3.3. Chymotrypsinogen A. (Mol. wt. 25,000, Tristram and Smith, 1963)

This was obtained from Seravac Laboratories, Cape Town, South Africa, and was 6 x crystallized and salt free. A 2% (W/v) solution of chymotrypsinogen A in phosphate buffered saline was prepared dialysed and centrifuged as described above (6.3.1.)

6.3.4. Cytochrome C. (Mol. wt. 13,000 Paleus and Paul, 1963)

Grade I, prepared from horse heart, was obtained from Seravac Laboratories, Cape Town, South Africa. A 2% ($^{w}/v$) solution of cytochrome C in phosphate buffer was prepared and used immediately.

6.3.5. Lactalbumin. (Mol. wt. 16,400 Polson, unpublished data)

This was prepared according to the method of Kekwick (unpublished data) with modifications. To approximately 2 litres of unpasteurized milk was added 5 to 6 drops of rennet and the whole well stirred. After standing at room temperature for 30 min. the casein was removed by centrifuging at 1000 r.p.m. for 15 min. The upper fatty layer and the pellets were discarded, and the cloudy supernatant was then transferred to washed cellulose dialysis bags and dialysed against sufficient distilled water to just cover the bags. Sodium azide was added to a concentration of 0.02% ($^{w}/v$). Dialysis was continued for approximately a week, after which the dialysate was removed and pervaporated (7.3.2.) in order to concentrate the lactalbumin to $\pm$ 3%.

6.3.6. Escherichia coli.

Escherichia coli was grown on Lab-Lemco agar plates at 37^{o} for 24 hr. The bacteria were removed from the surface by scraping with a smooth glass rod after the addition of 3 ml. of normal saline. The suspension of bacteria was centrifuged at 10,000 r.p.m. for 15 min., the supernatant was

discarded and the pellet resuspended in 0.5% ($^{v}/v$) formalin saline to kill the bacteria. This suspension of bacteria was used to determine the void volume of columns packed with agarose gel.

6.3.7. Agarose.

This was prepared according to the method of Russell, Mead and Polson (1964), and agarose spheres according to the method of Hjertén (1964).

6.4. METHODS.

6.4.1. Ultracentrifugation.

The homogeneity of the aforementioned proteins was confirmed by ultracentrifugation in the Spinco model E ultracentrifuge fitted with the An-D rotor and plain cell.

6.4.2. Packing procedure.

Glass columns (34.5 cm. x 2 cm.) with a sintered glass disk (porosity No 1) sealed into the bottom of the column were used. The capillary outlet of the column was constructed to have a minimum dead volume. Before the column was 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 agarose pearls 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 agarose in the column had reached 30 cm.

Due to the inherent difficulties encountered in the determination of the gel volume V_g the partial specific volume of agarose 0.59 (Hickson and Polson, 1968) was multiplied by the dry weight of agarose which was originally packed into the column (W_g). V_i was then calculated from the formula:

$$V_t - (V_e + V_g) = V_i$$

In all instances the eluting buffer was 66mM-phosphate buffered saline, pH 6.9, the experiments were conducted at 22° and the effluents were monitored with a L.K.B. Produkter Uvicord optical unit.

After equilibrating the column with a minimum of 3 litres of the buffer the height of the gel in the column was noted and a further litre of buffer was allowed to percolate through the gel. If the height of the gel remained constant the experiment was commenced. This was done in order to obtain an authentic value for the void volume of the column, as this tended to vary considerably if the gel contracted after all the experiments had been completed.

Approximately 1 ml. of the first protein solution was applied to the column, and the effluent was collected

simultaneously in a preweighed volumetric flask. When the elution peak had reached its mid point on the recording sheet the flask was carefully removed, stoppered and weighed and the volume V_e determined from the density and mass of the solution. The effluent volume was determined in a similar manner for each protein solution in turn. The void volume was then measured. The height of the gel in the column was noted and found to be the same as that at the commencement of the experiment. The total volume V_t of the swollen gel and its surrounding buffer was conveniently determined by filling the glass column with distilled water, run from a pipette to the height previously occupied by the gel in the same column.

The gel was then transferred to a Millipore (Bedford, Mass., U.S.A.) pyrex microanalysis filter holder fitted with a HAWP 025000 filter. The gel was washed with distilled water until free of phosphate and then transferred to a preweighed evaporating dish which was kept at 90° until the particles were completely dry. The dish and contents were cooled over P_2O_5 under vacuum and then weighed and the dry weight of the gel calculated W_g.

6.5. RESULTS.

The partial specific volume of agarose $\bar{\nu}$ was 0.59,

the total volume V_t of the column and gel was 58.5 ml., the void volume was 18.0 ml. and the dry weight of the agarose (W_g) was 4.0468g.

The results of the above experiments are recorded in Table 10.

TABLE 10.

The effluent volumes, distribution coefficients (K_d) and G values of various proteins determined on 10% agarose spheres.

Protein	Mol.wt.	V_e ml.	K_d	$G.10^5$
Cytochrome C	13,000	44.5	0.695	3.37
Lactalbumin	16,400	43.2	0.661	3.13
Chymotrypsinogen A	25,000	38.9	0.548	3.30
Ovalbumin	45,000	33.8	0.415	3.13
Bovine serum albumin	68,000	29.3	0.297	3.48

From Table 10 it is evident that the G value is essentially constant for 10% agarose spheres indicating the validity of equation (5).

6.5.1. Example of a calculation.

This example was calculated from the figures obtained from Cytochrome C.

$$\overline{V} \cdot W_g = V_g$$

$$0.59 \times 4.0468 = V_g$$

$$2.389 = V_g \longrightarrow$$

$$V_t - (V_g + V_o) = V_i$$

$$58.5 - (2.389 + 18.0) = V_i$$

$$38.11 = V_i \longrightarrow$$

$$K_d = \frac{V_e - V_o}{V_i}$$

$$K_d = \frac{44.5 - 18.0}{38.11}$$

$$K_d = 0.695 \longrightarrow$$

$$G_1 = \frac{1}{M}\left(\frac{1}{K_d} - 1\right)$$

$$G_1 = \frac{1}{13{,}000}\left(\frac{1}{0.695} - 1\right)$$

$$G_1 = 3.37 \times 10^{-5} \longrightarrow$$

6.6. DISCUSSION.

The number of proteins used for these investigations is admittedly small, but it appears that other globular proteins with molecular weights between 13,000 and 70,000 would give the same G value on 10% agarose spheres provided that all adsorption effects were eliminated.

Filamentous molecules are known to have smaller K_d values than molecules of similar molecular weight but of globular structure. The present theory of gel chromatography would explain this behaviour as will be shown by the following example. During chromatography of nucleic acids the elution diagrams obtained reveal a "tailing effect" even when the conditions under which the experiments are conducted are optimal. A possible explanation for this tailing effect is that one would expect more interference between the filamentous particles of the gel and the filamentous nucleic acid molecules, therefore retarding their elution from the gel matrix.

As the fibrous molecules of the gel have a restricted vibrational movement it may be expected that they would contribute to the chemical potential of the system, therefore exerting a dynamic effect during exclusion chromatography. This has in fact been established by Freundlich (1926) who

demonstrated that gels exert an osmotic pressure (P) that may be expressed by the equation

$$P = RTC^{\kappa}$$

where R is the gas constant, T the absolute temperature, C the concentration of the gel and κ is a constant > 1.

Edmond, Farquhar, Dunstone and Ogston (1968), have observed that chromatography of bovine serum albumin on Sephadex G-200 in the presence of dextran (non-penetrable solute) increases its elution volume as the dextran concentration is increased. This effect was attributed to the osmotic behaviour of Sephadex beads which were found to shrink appreciably in solutions of dextran (2%-20%), which were not capable of penetrating the matrix of the bead. This shrinkage was a reversible effect. Furthermore equation (2) of this study is compatible with the formulations of the above authors. (equations Nos. 3 and 4)

It is noteworthy that equation (4) indicates a concentration dependency of K_d, which probably offers an explanation for the small increase of K_d with concentration - a phenomenon which has been observed by Winzor and Nichol (1965).

A further point of interest was that experiments conducted with 11% chromium-tanned gelatin granules gave a G value of $3.28 \cdot 10^{-5}$. However it was necessary to increase

the electrolyte content of the elution fluid to between 0.6 and 1 molar to obtain true exclusion free of any adsorption effects. The K_d value of cytochrome C was found to be as high as 3.1 in 0.14-M NaCl but the value decreased to K_d=0.69 when the NaCl concentration was increased to 1M.

It is therefore impossible to prove the validity of equation (5) with tanned gelatin gels, due to the marked adsorption effects exhibited by these gels. Furthermore experiments conducted with tanned gelatin revealed that these ionic effects were reasonably pronounced, even in the presence of buffers of high ionic strength and at the iso-electric point of these gels. These studies were therefore pursued in order to determine the effect they had on gel chromatography. The forthcoming section of this thesis deals with these investigations.

CHAPTER SEVEN.

ADSORPTION-ELUTION AND ION-EXCHANGE CHROMATOGRAPHY ON TANNED GELATIN GRANULES.

7.1. INTRODUCTION.

Philipson (1967) classified the different adsorption media according to their retention characteristics which he divided into dipole interactions, van der Waals dispersion forces and hydrogen bonding between solute and adsorbent. Gel exclusion effects also take place with these media.

Several adsorbents e.g. bentonite, cellulose and Celite (diatomaceous earth) have been used for virus purification, but the reproducibility of the results obtained with these substances is not satisfactory, except in the case of cellulose, which is prepared under standard conditions. Aluminium hydroxide and calcium phosphate gels must also be produced under standard conditions in order to obtain reproducible results.

Polystyrene, one of the first ion-exchange resins, prepared by the copolymerisation of styrene and divinylbenzene, is also used for exclusion chromatography of small

molecules, as the porosity of the resin may be altered within certain limits by controling the degree of cross-linking. By the direct copolymerization of methacrylic acid with divinylbenzene a cation exchanger with carboxyl groups as the reactive molecules is produced (Morris and Morris, 1964). However with the preparation of cellulose derivatives for ion-exchange chromatography (Peterson and Sober, 1956) a new concept was introduced. Due to the hydrophilic nature of cellulose this material was found to be superior to the hydrophobic polystyrene resins for the purification of labile proteins. Peterson and Sober (1956) prepared a number of cellulose derivatives which include diethylaminoethyl (DEAE), carboxymethyl (CM), sulphoethyl (SE), triethylaminoethyl (TEAE), aminoethyl (AE) and ecteola cellulose. These materials are suitable for use with moderately large molecules e.g. globulins and albumins, but not for viruses or other equally very large particles due to secondary reactions between solute and matrix, which cause irreversible adsorption.

Dextran gels have been similarly treated to form ion-exchangers such as DEAE, CM and SE Sephadex. They are not ideal as they shrink considerably during the elution process with increasing salt concentrations. Gelotte (1960)

reported that dextran gels show strong irreversible adsorption of dyes and other aromatic substances (Male, 1966). Wilding (1963) has observed the adsorption of pancreatic amylase, while the exclusion of acidic molecules which appear at the void volume of the column was noted by Gelotte (1960). Both CM agarose (Ghetie and Schell, 1968) and DEAE agarose (Schell, Ghetie and Mihaiescu, 1968) ion-exchangers have been prepared for use as bed materialfor the chromatography of proteins, and in contrast with the dextran derivitives do not shrink appreciably with increasing salt concentration.

The adsorption effects of agar gels are well known and are due to the sulphated agaropectin molecule (Male, 1966). However these effects can be reduced or even eliminated by the judicious choice of buffers of appropriate ionic strength.

It was noted in these laboratories (6.6.) that proteinaceous molecules, during chromatography on chromium-tanned gelatins, were retarded in certain instances depending on the solute or buffer used. It was therefore considered of interest to investigate these properties in an attempt to elucidate the reasons for the retardation.

7.2. MATERIALS.

7.2.1. Gelatin.

Difco gelatin fractionated with polyethylene glycol (2.4.2.) was used in all instances for the preparation of the gel media. Chromium-tanned and chromium-formalin-tanned gelatin granules were prepared as previously described (3.3.2. and 3.3.3.).

7.2.2. Proteins.

The proteins ovalbumin, bovine serum albumin, chymotrypsinogen A and cytochrome C were essentially the same preparations as those described in the previous chapter. (6.3.1., 6.3.2., 6.3.3., 6.3.4.)

7.2.3. Human serum.

This was centrifuged at 20,000 r.p.m. under liquid paraffin to trap lipid molecules. The serum was collected from the bottom of the tube, and kept at 4^{o} until used.

7.2.4. Cobra (Naja nivea) snake venom.

The venom was obtained in the dry state and was redissolved just prior to use, in 66mM-phosphate buffered saline, pH 6.9 to give a 2% ($^{w}/v$) solution.

7.3. METHODS.

Glass columns 34.5 cm. x 2 cm. and 50 cm. x 2 cm. with a sintered glass disk sealed into the base of each of

the columns were used for these investigations.

The determination of the total gel volume (V_t), the void or free volume (V_o), the effluent volume (V_e) and the gel volume (V_g) was calculated as described in the previous chapter (6.5.1.).

7.3.1. Partial specific volume ($\bar{V}$) of tanned gelatin.

The partial specific volume of chromium and chromium-formalin-tanned gelatin granules was determined as follows: A 50 ml. volumetric flask was weighed and then filled to the mark with distilled water and reweighed. Weight of water W_1. Sufficient gel (chromium or chromium-formalin-tanned) equilibrated against distilled water, to occupy approximately 20% of the volume of the flask was then added, freed of adhering air bubbles and the volume readjusted to the mark. Weight of water and gel (W_2). The gel was removed, dried in an oven at 90°, cooled under vacuum over P_2O_5 and then weighed (W_3). The partial specific volume was calculated from the formula:

$$\bar{V} = \frac{W_1}{W_3} - \frac{W_2}{W_3} + 1$$

The G values of the gel media were calculated from the formula (6.2.);

$$G = \frac{1}{M}\left(\frac{1}{K_d} - 1\right)$$

7.3.2. Pervaporation.

The technique of pervaporation was that described by Mead (1962). The sample to be concentrated was treated as follows: An appropriate length of dialysis tubing (Visking Co., U.S.A.) was cut from the roll and immersed in distilled water until soft. The tubing was thoroughly washed with distilled water by tying a knot at one end of the tube and shaking the water up and down for about 30 sec. The water was run out and the procedure repeated. The sample was introduced at the open end and the tubing closed by knotting this end. The washing and soaking procedure described above was always carefully executed as inadequately washed tubing contained impurities (Katz, 1967).

The dialysis sac containing the sample was placed immediately beneath the cooling element of the refrigerator. A small 10 volt direct current electric fan was placed below the sac to accelerate the process of evaporation, by circulating the air in the refrigerator. The tubing was left in the refrigerator for approximately 16 hr. which gave about a four-fold concentration of the sample.

7.3.3. Electrophoresis in gels.

Samples of human serum concentrated by pervaporation were identified by electrophoresis in agarose gels.

A 1% (w/v) solution of agarose was prepared in 0.05M-Veronal buffer, pH 8.6 by heating in a boiling-water bath. The dissolved agarose was poured onto a glass microscope slide and allowed to gel. A small well was cut into the centre of the gel sheet and filled with the sample concentrated by pervaporation. The slide was placed on the water-cooled floor of the apparatus (Paigen, 1956) shown in Fig. 5. Electrical contact between the ends of the gel and the electrode vessels, filled with 0.1M-Veronal buffer, pH 8.6, was made with paper wicks previously soaked in the same buffer. A current producing a voltage drop of 10 V/cm. across the length of the gel sheet was applied for 45 min. The slide was stained with nigrosin black for 10 min. and washed free of uncombined dye with a solution of water, methyl alcohol and acetic acid,
(5 parts H_2O : 5 parts CH_3OH : 1 part CH_3COOH).

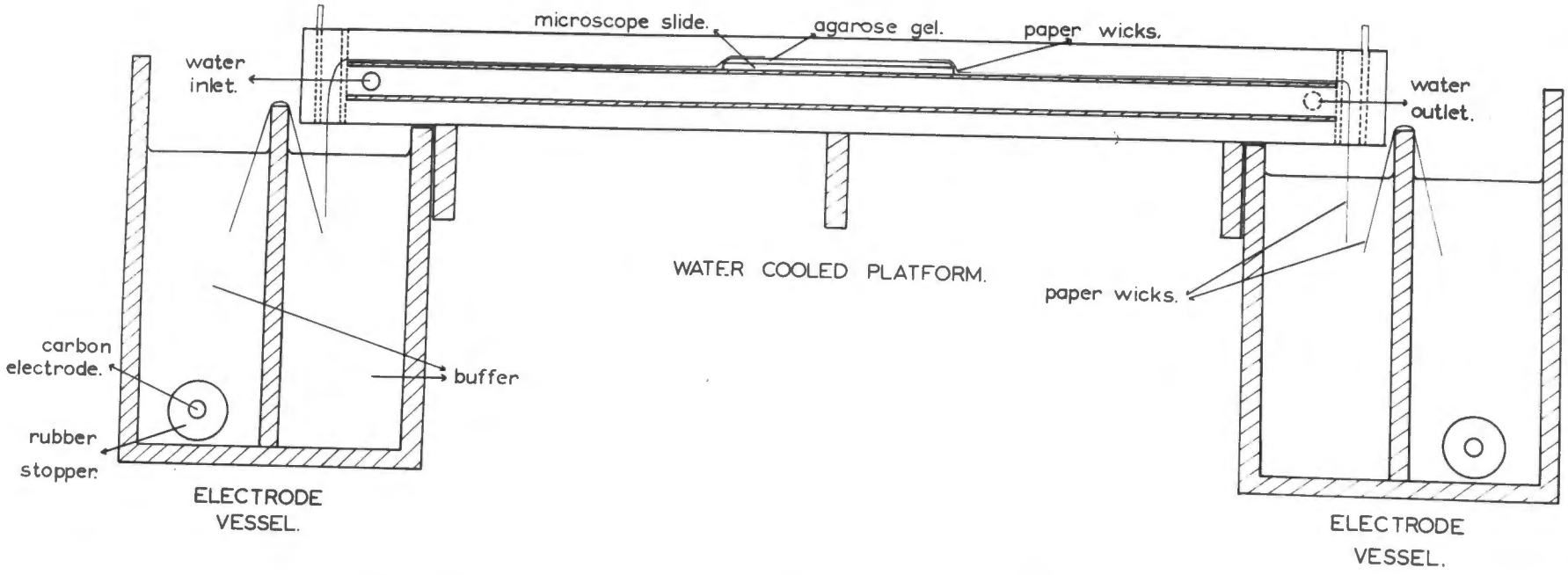
7.4. RESULTS.

7.4.1. Partial specific volume.

The partial specific volume of chromium-tanned gelatin granules and chromium-formalin-tanned gelatin

Fig. 5.

Electrophoresis Apparatus.


microscope slide.
agarose gel.
paper wicks.
water inlet.
water outlet.
WATER COOLED PLATFORM.
paper wicks.
carbon electrode.
buffer
rubber stopper.
ELECTRODE VESSEL.
ELECTRODE VESSEL.

granules are recorded in Table 11. In both instances this was found to be 0.61 c.c./g.

Table 11.

Partial specific volume ($\overline{V}$) of gelatin granules tanned with chromium and chromium-formalin.

Gelatin	W_1 Wt. g.	W_2 Wt. g.	W_3 Wt. g.	Partial specific volume c.c./g.
Chromium tanned	49.8212	50.0680	0.4151	0.61
Chromium-formalin tanned.	49.8481	81.7212	0.7217	0.61

7.4.2. Chromatography.

Chromatography of ovalbumin, chymotrypsinogen A and cytochrome C on 8% chromium-formalin-tanned gelatin granules in the presence of 66mM-phosphate buffer + 0.5M-NaCl, pH 6.9 (iso-electric point of the gel) revealed that both chymotrypsinogen A and cytochrome C had been retarded on the column. This was surprising since the gel was used at its iso-electric point and furthermore in the presence of 0.5M-NaCl. The V_e, K_d and G values are recorded in Table 12.

Table 12.

The effluent volume, distribution coefficient and G values obtained on 8% chromium-formalin-tanned gelatin granules. Eluant fluid: 66mM-phosphate + 0.5M-NaCl, pH 6.9. Column dimensions 34.5 cm. x 2 cm. Void volume = 31.07 ml.

Protein Mol. wt.	V_e ml.	K_d	$G10^5$
Ovalbumin (45,000)	43.02	0.4281	2.976
Chymotrypsinogen A (25,000)	62.37	1.170	+++
Cytochrome C (13,000)	58.99	1.044	+++

+++ Because $K_d > 1$ the G value cannot be calculated.

A similar experiment conducted with the three above mentioned proteins and including bovine plasma albumin (Bpa) on 11% chromium-formalin-tanned gelatin in the presence of 0.14M-NaCl revealed an even greater retardation of some of these proteins. Chromatography of the same proteins on the same gel but in the presence of IM-NaCl revealed that chymotrypsin was retarded to a greater extent than the smaller molecule of cytochrome C. It is difficult to decide whether

the V_e values obtained for the other proteins are authentic as the G values are not at all constant, as one would expect from a gel (e.g. agarose) exhibiting no adsorption or ion-exchange effects. The V_e, K_d, and G values are recorded in Table 13.

Table 13.

The effluent volume, distribution coefficient and G values obtained on 11% chromium-formalin-tanned gelatin granules.

Protein Mol. wt.	0.14M-NaCl buffer			1.0M-NaCl buffer		
	V_e ml.	K_d	$G.10^5$.	V_e ml.	K_d	$G.10^5$.
Bpa (68,000)	40.2	0.3227	3.085	33.4	0.1981	5.954
Ovalbumin (45,000)	45.8	0.4999	2.222	39.0	0.3589	3.969
Chymotrypsin-ogen (25,000)	121	2.878	+++	59.4	0.9445	0.2360
Cytochrome C (13,000)	145.7	3.660	+++	48.7	0.6375	4.377

+++ = Because $K_d > 1$ G values cannot be calculated.

Chromatography of ovalbumin and cytochrome C on 15% chromium-tanned and chromium-formalin-tanned gelatin granules in the presence of 66mM-phosphate buffer, pH 6.9 containing in addition 0.5M-NaCl gave similar results on these two gels. The adsorption effects persisted although to a less marked degree. The V_e, K_d and G values obtained with these materials are recorded in Table 14.

Table 14.

The effluent volume, distribution coefficient and G values obtained on 15% chromium and chromium-formalin-tanned gelatin granules. The eluant was: 66mM-phosphate buffer + 0.5M-NaCl, pH 6.9. Column dimensions 50 cm. x 2 cm.

Protein Mol. wt.	Chromium-tanned gelatin.			Chromium-formalin-tanned gelatin.		
	V_e ml.	K_d	$G.10^5$	V_e ml.	K_d	$G.10^5$
Ovalbumin (45,000)	39.25	0.1949	9.445	38.25	0.1540	12.21
Cytochrome C (13,000)	48.65	0.5543	6.186	47.74	0.5554	6.186

Chromatography of Cobra venom (Naja nivea) on 30% chromium-formalin-tanned gelatin equilibrated against 66mM-phosphate buffered saline, pH 6.9 is illustrated in

Fig. 6.

Elution diagrams of Cobra venom chromatographed on columns containing (a) Sephadex G-50 and (b) 30% formalin-chromium-tanned gelatin spheres. The eluting fluid was 66mM-phosphate buffered saline, pH 6.9, the flow rate was 8 ml./hr. and the sample volume was 1 ml. The column dimensions were 2 cm. x 50 cm. The front is indicated by a vertical arrow.

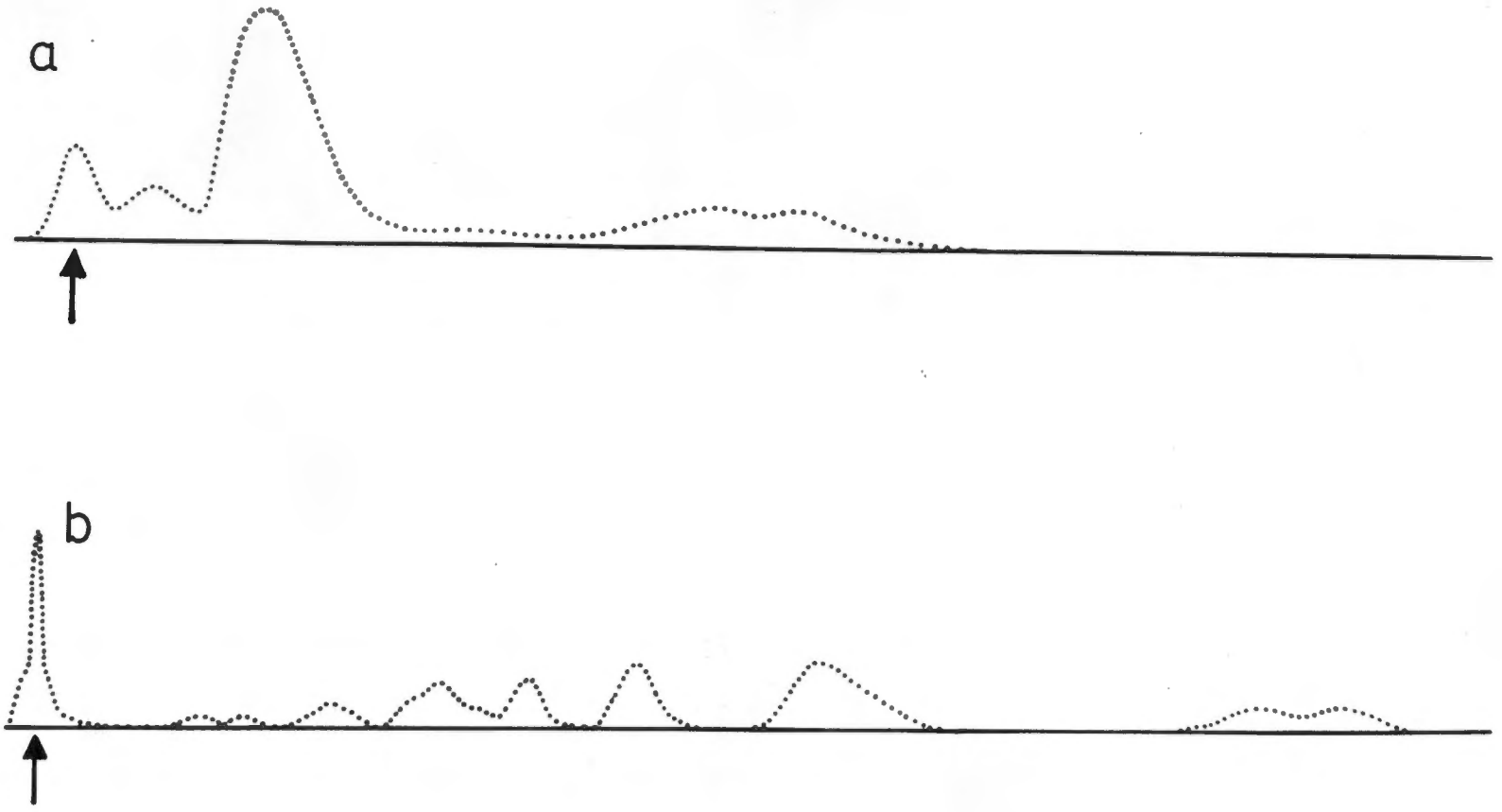

a
b

Fig. 6 (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. 6 (a). The patterns obtained with the other grades of Sephadex are not included as these show less resolution. Increasing the salt concentration of the eluting buffer to 1M-NaCl revealed no change in the elution diagram shown in Fig. 6 (b).

The possibility that the large number of peaks (Fig. 6 b) was due to breakdown products of gelatin resulting from enzymic action of components in the snake venom was investigated according to the method described (4.2.6.). This was found not to be the case.

The ion-exchange properties of 4%, 6%, 10%, 15% and 30% chromium-tanned gelatin granules were investigated with human serum. Chromatography of serum on 4% gelatin in the presence of 0.14M-NaCl is illustrated in Fig. 7 (a). The partially resolved first peak was shown by gel electrophoresis (7.3.3.) to be due to 19S and 7S globulins containing a trace of albumin. After allowing the column to flow overnight no further elution of protein was observed. Sufficient cysteine hydrochloride was added, in order to elute the adsorbed proteins, to the saline to make the concentration

Fig. 7.

Elution diagrams of human serum chromatographed on columns containing (a) 4% chromium-tanned gelatin granules equilibrated against 0.1M-NaCl, (b) 6% chromium-tanned gelatin granules initially equilibrated against phosphate buffer and then washed free of the latter and equilibrated against saline (0.85%) and (c) 10% chromium-tanned gelatin granules equilibrated against 6mM-phosphate buffer, pH 6.9. The flow rate in all instances was 4 ml./hr., the sample volume 3 ml. and the column dimensions were 2 cm. x 50 cm. The front is indicated by a vertical arrow.

c
b
a

0.006M with respect to cysteine. A large peak appeared approximately 90 ml. after applying the new eluting fluid to the column. This peak contained albumin and 7S globulin as shown by gel electrophoresis. The recoveries of protein were not quantitative and it appears that there is an irreversible adsorption to the gel. The buffer was then changed to 0.01M-EDTA pH 7.0, but no further release of protein was observed, although as mentioned previously, chromium is released from the gel.

Chromatography of serum on 6%, 10%, 15% and 30% chromium-tanned gelatin granules also exhibit adsorption effects, but this phenomenon became less marked the higher the concentration of the gelatin. The adsorption on 15% gel was very small, and almost negligible on 30% gelatin.

An experiment conducted on 6% chromium-tanned gelatin which had previously been equilibrated against 66mM-phosphate buffer, pH 6.9 and then washed free of phosphate with saline (0.85%), exhibited no adsorption effects when serum was chromatographed on this material using 0.14M-NaCl as the eluting fluid. The elution diagram is illustrated in Fig. 7(b).

Experiments were conducted with 10% chromium-tanned gelatin which had never been in contact with

phosphate buffer. The columns were equilibrated against 6mM-phosphate buffer, pH 6.9 and human serum chromatographed on both columns. The elution diagram for both columns are illustrated in Fig. 7 (c) as they were identical. The first large unresolved peak in both experiments was due to 19S globulin, and the small second and larger third peaks contained 7S globulin and a trace of albumin. After running both columns overnight no further elution of protein was observed from either column. The buffer of the first column was changed to 66mM-phosphate containing 0.25M-NaCl, pH 6.9, and a fraction containing albumin and a trace of 7S globulin was eluted 90-100 ml. after the buffer change. The buffer of the second column was not altered except that sufficient cysteine was added to bring its concentration to 0.004M, and a peak was eluted at approximately the same volume as for the first column and contained the same proteins as were present in the first column.

Chromium-tanned gelatin granules (10%) which had not been in contact with phosphate buffer were equilibrated against 1M-NaCl, and serum chromatographed on this gel. Only one peak was eluted which contained 19S and 7S globulins. Phosphate buffer,(1M) was substituted for the above buffer, but no further release of protein was observed. However

on addition of cysteine (0.006M) to the buffer the adsorbed proteins were liberated.

Chromium-tanned gelatin granules (10%) which had previously been in contact with both phosphate and cysteine were suspended in 1N-HCl and well mixed. The granules were collected on a sintered glass filter (porosity No. 3) and washed with several changes of 1N-HCl. The gel was then repeatedly washed with distilled water until the pH was nearly neutral, after which it was suspended in several changes of 0.14M-NaCl. The granules were packed into a column and after percolation of a further 2 litres of saline (0.85%) a sample of human serum was applied. No adsorption occurred.

7.5. DISCUSSION.

Experiments conducted with chromium-tanned gelatin revealed that adsorption -elution effects take place even in the presence of high salt concentrations. This adsorption-elution or retardation effect was clearly demonstrated by chromatography of cytochrome C, the elution volume of which could be partially governed by the salt concentration of the buffer. The variation in the G values suggests that all the proteins used in this study were adsorbed to a greater or lesser extent depending on the buffer.

The greater resolution of snake venom on 30% tanned gelatin in comparison with that on Sephadex appears to be due to adsorption-elution effects. Initially it was thought that the pores of gelatin gels were of a more uniform diameter than those of Sephadex, consequently the greater resolution on the former.

This problem was further investigated by chromatography of human serum on gelatin gels. It was assumed that the use of these media at their iso-electric point would eliminate the charge effects and consequently the adsorption, but this was obviously not the case as may be seen from the early experiments (7.4.2.) conducted with these materials. There is very little adsorption to the gel if it is equilibrated against a concentration of at least 66mM-phosphate or 0.004M cysteine hydrochloride. Furthermore as the concentration of the gelatin is increased the adsorption effect is reduced and ultimately disappears completely at high concentrations. On the other hand gels which have not been in contact with phosphate or cysteine adsorb certain amounts of 19S and 7S globulins (50-60%) and nearly all the albumin. The addition of cysteine to the buffer liberates the majority of adsorbed protein but not all of it, but concentrations of phosphate up to 1M do not

release adsorbed protein, possibly because the affinity for the protein adsorbed was greater than that for phosphate. Explanations must therefore be found for (a) adsorption-elution equilibria between gel and solute, (b) adsorption in the presence of 0.006M-phosphate and solutions of NaCl varying in concentration between 0.14M and 1M, (c) the decreasing adsorption with increasing gel concentration and (d) the apparent neutrality of gels equilibrated against phosphate or cysteine hydrochloride even after treatment with 1N-HCl.

In an earlier discussion (3.1.) it was suggested that tanning of gelatin with chromium, in the first instance, involved the carboxyl groups of the protein which reacted with the polynuclear chromium complex (Green, 1953). It appears that the adsorption-elution effect is governed by the salt concentration and by the type of protein molecule involved. If all the chromium atoms have not taken part in the tanning reaction with the available carboxyl groups, or if the chromium, which has a potential valency of six, does not have all these satisfied during the tanning procedure, the chromium complex would adsorb solute molecules during chromatography. Furthermore any other groups which do not react with chromium would probably take part in

secondary reactions.

The retardation effect may be due to a weak reversible bonding between protein solute and chromium complex attached to the gel, which is more marked in dilute buffer and less so in buffers of high salt content. It appears that phosphate and cysteine react irreversibly with chromium as gels equilibrated with buffers containing these salts show no adsorption effects except in the case of phosphate, where retardation of solute molecules still takes place.

The binding of proteins, containing SH groups, by heavy metals such as mercury is well known, and the technique has been extended to gel chromatography (Eldjarn and Jellum, 1963) where the mercurial complex is attached to dextran, and the material used to adsorb SH containing proteins. The proteins are then eluted in order of increasing number of SH groups per molecule.

It appears that a similar effect may be taking place with chromium-tanned gelatin. The chromium would have an affinity for SH proteins, and according to the availability of this group they would be bound to the chromium. The use of thiols (e.g. cysteine) for removing the adsorbed protein is achieved with very dilute solutions

of these salts, although higher concentrations of these solutions do not remove all the protein and it is therefore assumed that secondary reactions must be taking place. This was clearly demonstrated with the use of EDTA which is known to remove the chromium from the gel, but in this instance there was no protein associated with the released chromium.

The decreasing adsorption of protein with increasing gel concentration could be explained in two ways: Firstly as the gel concentration is increased the porosity of the material decreases, and the proteins used in this study would tend to be excluded from the gel matrix to a greater extent as the concentration of the gel increased. It has been shown (Philipson, 1967) that molecules which do not penetrate the matrix of ion-exchangers are adsorbed to a lesser extent than those which do, as the number of charges on the surface of the medium are much reduced in comparison with those in the matrix. Secondly it is thought that as the gelatin concentration is increased there would possibly be more groups available for reaction with the chromium and in all probability the cross-linking effect would reach a saturation point leaving very few or no chromium complexes available for

attachment of the SH proteins.

It has been shown that gels which have been in contact with phosphate or cysteine exhibit no adsorption even after suspension in 1N-HCl, as these complexing agents cannot be removed at low pH.

This phenomenon can however be used to advantage for separating proteins with different numbers of SH groups or other groups capable of reaction with chromium. If the effect is undesirable, equilibration of the gel against cysteine prior to use is recommended when only the gel exclusion properties of the material will operate. Secondly formalin-tanned gels may be used in preference for gel exclusion experiments.

With the above studies in mind, experiments were conducted to investigate the resolving properties of these gels, excluding adsorption effects, using artificial protein mixtures and human serum. The forthcoming section deals with these findings.

CHAPTER EIGHT.

CHROMATOGRAPHY OF ARTIFICIAL PROTEIN MIXTURES ON TANNED GELATIN.

8.1. INTRODUCTION.

The separation and characterization of carbohydrates, amino acids, hormones, lipids, proteins, viruses and a host of other substances has been variously accomplished on columns packed with polyacrylamide, dextran, agar and agarose gels and also on porous glass particles. With the aid of these materials it has been found possible to separate and purify mixtures of particles ranging in molecular weight from several hundred to several hundred million daltons. Because of the wide fractionation range offered by these bed materials and the almost complete absence of denaturation effects, the technique has become one of the most frequently used for the purification of biological substances.

The removal of salts or other low-molecular weight components (desalting) from higher molecular weight particles may be achieved far more rapidly by gel chromatography than by dialysis. Gels of small pore diameter are used in this method, and in certain instances the component being

isolated is normally concentrated by this technique (Flodin, 1961).

Gel exclusion chromatography has been employed for the separation of co-factors from enzymes (Kisliuk, 1960) and for the purification of peptides and other proteins (Bennich, 1961). Similarly the purification of polypeptide hormones (Porath, 1963) and the fractionation of mixtures of proteins or glycoproteins (Carnegie, 1965) has been achieved by this technique.

The purification of oligosaccharides by gel exclusion chromatography was reported by Lee and Ballou (1965) but has not been extensively investigated. Separations of nucleotides and other nucleic acid components on dextran gels was reported by Ingram and Pierce (1962) and by Lipsett (1964). Fractionation of nucleic acids on 1-4% agar or agarose gels has been described by Oberg, Bengtsson and Philipson (1965) and the separation of ribonucleic acid from the intact poliovirus by Oberg and Philipson (1967).

Bengtsson and Philipson (1964) have chromatographed myxoviruses, adenoviruses and picornaviruses on 2.5% agar. The quantitative elution of lipid containing viruses from agar gels requires the presence of a neutral or basic

detergent in the buffer and the ionic strength of the buffer should be above 0.1 molar to minimize adsorption effects (Philipson, 1967).

Fridborg et al (1965) have separated the satellite component from tobacco necrosis virus on 4% agar. With the introduction of porous glass particles for chromatography, Haller (1965) has described the separation of an artificial mixture of tobacco mosaic virus and tobacco ringspot virus.

As proteins are most frequently the object of attempted separations, the efficiency of tanned gelatin gels was consequently tested with artificial mixtures of proteins. Furthermore it was considered of interest to determine the exclusion range of the different concentrations of gelatin gels. These investigations are described in this chapter.

8.2. MATERIALS AND METHODS.

Gelatin spheres and granules were prepared and tanned as previously described (3.3.2. and 3.3.3.). In all instances polyethylene glycol fractionated Difco gelatin was used (2.4.1.). The preparation of cross-linked resorcinol gelatin was essentially the same as that described earlier (3.3.6.).

8.2.1. Analytical ultracentrifugation.

The homogeneity of the proteins used in this study was confirmed by ultracentrifugation in the spinco model E ultracentrifuge fitted with the An-D rotor and the plain cell. The proteins, in the elution peaks after chromatography, were usually identified by determining their sedimentation coefficients.

8.2.2. Jasus lalandii haemocyanin (mol. wt. 500,000 Joubert,1954).

The haemocyanin of the crayfish, Jasus lalandii, was obtained by severing the blood vessel on the ventral surface between the first segment of the abdomen and the carapace. The blood was allowed to clot and then transferred to a muslin bag which was gently squeezed to express the serum, which was centrifuged at 10,000 r.p.m. for 20 min. The serum was freed of bacteria by ultrafiltration and kept at 4° until used.

8.2.3. Sperm whale myoglobin. (mol. wt. 17,800 Crumpton and Polson, 1965).

This was obtained in crystalline form from Seravac Laboratories, Cape Town, South Africa, and was used without further purification.

8.2.4. DL-Tryptophan. (mol. wt. 204, Litt.).

This was a B.D.H. product and was used without

further purification.

8.2.5. Human serum.

This was obtained from samples of clotted human blood. The serum was centrifuged under liquid paraffin at 10,000 r.p.m. for 30 min. to remove lipids and collected by piercing a hole in the bottom of the centrifuge tube.

8.2.6. Burnupena cincta haemocyanin. (mol. wt. 6,600,000 Polson, Ehrenberg and Cramer, 1958).

This was obtained by bleeding freshly collected whelks. The haemocyanin was centrifuged at 12,000 r.p.m. for 15 min. and the supernatant recentrifuged at 30,000 r.p.m. for 90 min. The pellets were resuspended and freed of bacteria by ultrafiltration.

The above mentioned proteins were used to determine the resolving properties of differently tanned gelatin spheres and granules. In all instances the eluting fluid was 66mM-phosphate buffered saline, pH 6.9, the experiments were conducted at 22^{o} and the effluents were monitored with an L.K.B. Produkter Uvicord optical unit. The void volume of each column was determined with Esch. coli as previously described (6.3.7.).

The effluents were collected on elution from the column and concentrated by pervaporation (7.3.2.) and then

Fig. 8.

Elution diagrams of a mixture of J.lalandii haemocyanin, myoglobin and tryptophan on (a) 30%, (b) 15%, (c) 7% and (d) 4% gelatin spheres tanned with formalin and chromium. B.cincta haemocyanin was added to the 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 8 ml./hr. and the sample volume was 1 ml. The front is indicated by a vertical arrow.

a
b
c
d

dialysed against phosphate buffered saline before being subjected to analytical ultracentrifugation.

8.3. RESULTS.

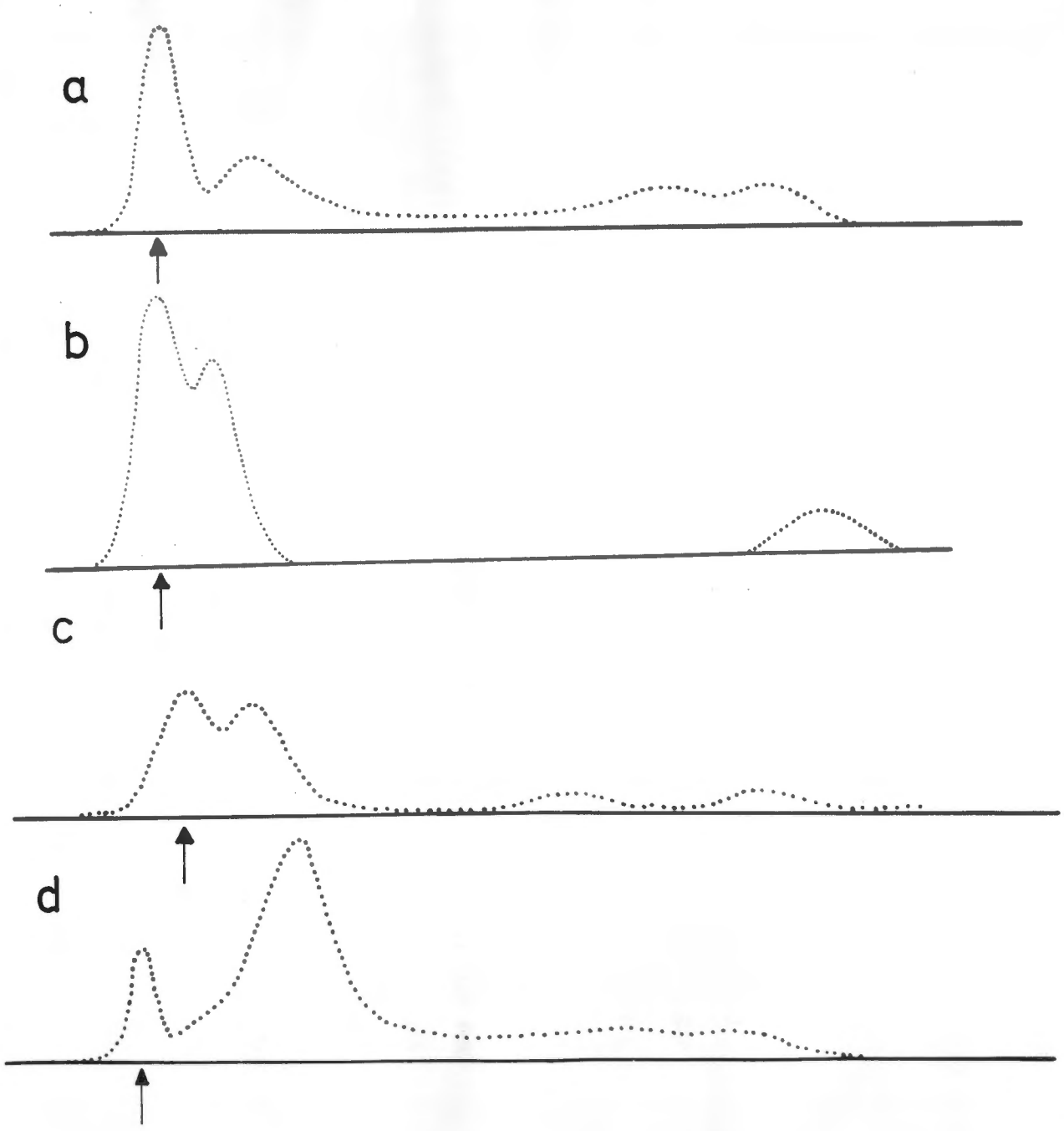
Gelatin spheres (30%) tanned with formalin, formalin-chromium and chromium respectively had similar elution diagrams, that depicted in Fig. 8 (a) being the same for all three types of tanned material.

The elution diagrams obtained on 15% and 7% formalin-chromium tanned spheres after chromatography of a mixture of J.lalandii, myoglobin and tryptophan are illustrated in Fig. 8 (b) and 8 (c). The first peak in all three diagrams consisted of J. lalandii haemocyanin, the second peak myoglobin and the third peak, tryptophan. Chromatography of the same mixture, containing in addition B. cincta haemocyanin, on 4% formalin-chromium tanned spheres is illustrated in Fig. 8 (d), the first peak being that due to B. cincta haemocyanin and the remaining three peaks to J. lalandii, myoglobin and tryptophan respectively.

The elution diagrams of a gel exclusion chromatography experiment conducted on a column packed with 4% tanned granules to which a mixture of J. lalandii and B. cincta haemocyanins was applied is represented by Fig.9(a). The same mixture chromatographed on 4% tanned gelatin spheres

Fig. 9.

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


a
b
c
d

is shown in Fig. 9 (b). The fractions were identified in the ultracentrifuge after concentration by pervaporation and it was confirmed that the first peak (Fig. 9 a) 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 proteins. The initial large peak (Fig. 9b) contained B.cincta and traces of J. lalandii haemocyanin. While the second partially resolved peak contained both components, the smaller (J. lalandii) haemocyanin predominated. The small peak was due to low molecular-weight proteins.

The results obtained when human serum was chromatographed on 4% formalin-chromium-tanned spheres and granules, are illustrated in Figs. 9 (c) and 9 (d) respectively. The first peak in Fig. 9 (c), in which gelatin spheres were used, was due to a mixture of 19S and 7S globulins and the second was due to serum albumin. The two smaller peaks were low molecular-weight proteins. Ultracentrifugation of the effluents obtained from the granules established that the first peak in Fig. 9 (d) was that due to 19S globulin and the second peak to unresolved serum globulin and albumin.

Fig. 10.

Elution diagrams obtained by chromatography of (a) human serum and (b) a mixture of B. cincta and J. lalandii haemocyanins both on 4% resorcinol cross-linked gelatin spheres tanned with formalin and chromium. The buffer was 66mM-phosphate-buffered saline pH 6.9, the flow rate was 8 ml./hr. and the sample volume was 1 ml. The front is indicated by a vertical arrow.

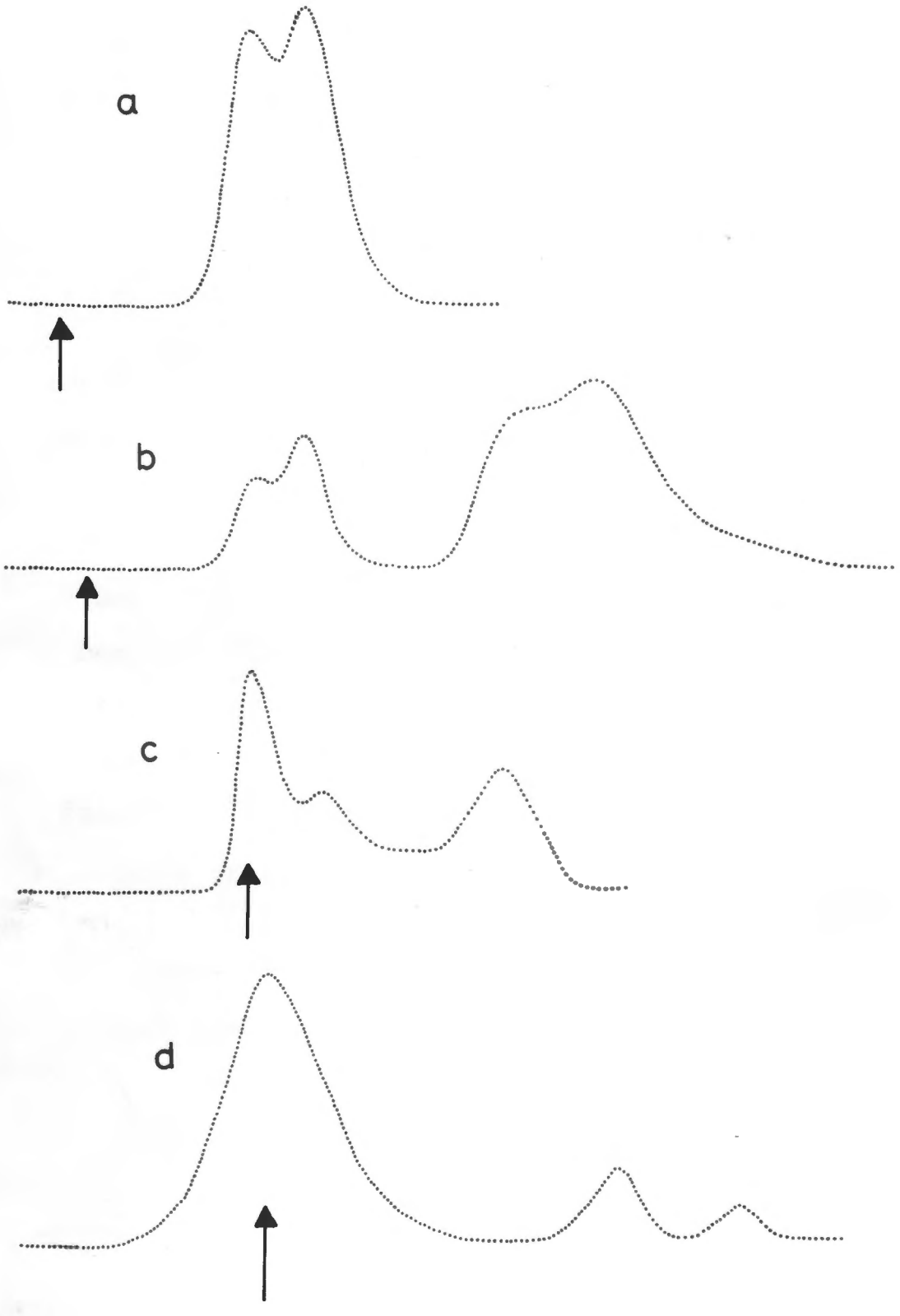
a
b

A sample of human serum chromatographed on 4% resorcinol cross-linked gelatin tanned with formalin and chromium is illustrated in Fig. 10 (a). The first unresolved peak was composed of 19S and 7S globulin and the second was that due to albumin. The two remaining small peaks were due to unidentified small molecules. A mixture of the two haemocyanins chromatographed on the same column is illustrated in Fig. 10 (b). The first peak on the elution diagram was due to both haemocyanins, and the remaining three smaller peaks were low-molecular-weight proteins.

The resolving properties of 4% vegetable tannin tanned gelatin were determined with spheres which had been tanned during the emulsification process before gelling. A 2% ($^{w}/v$) solution of myoglobin in phosphate buffered saline was prepared and applied to the column containing the above mentioned spheres. The elution diagram Fig. 11 (a) revealed one partially resolved peak both components of which were due to myoglobin. On ultracentrifugation of the original mixture only one peak was observed on the Schlieren diagram. A mixture of myoglobin and tryptophan chromatographed on the same column revealed two peaks both partially resolved into a further two peaks (Fig. 11 b).

Fig. 11.

Elution diagrams of (a) a solution of myoglobin alone and (b) a mixture of myoglobin and tryptophan chromatographed in both instances on 4% vegetable tannin tanned gelatin spheres (tanned during the emulsification process), and (c) a mixture of B. cincta, J. lalandii, myoglobin and tryptophan chromatographed on 4% gelatin spheres tanned with vegetable tannin, formalin and chromium after the spheres had gelled, and (d) a mixture of B. cincta and J. lalandii chromatographed on 4% gelatin spheres tanned with chromium and formalin during the emulsification process. The eluting fluid in all instances was 66mM-phosphate-buffered saline, pH 6.9, the sample volume was 1 ml. and the front is indicated by a vertical arrow.


a
b
c
d

The first partially resolved peak was due to myoglobin and the second partially resolved peak to tryptophan. On repacking the column with the same material similar elution diagrams were obtained as with the two previously mentioned substances.

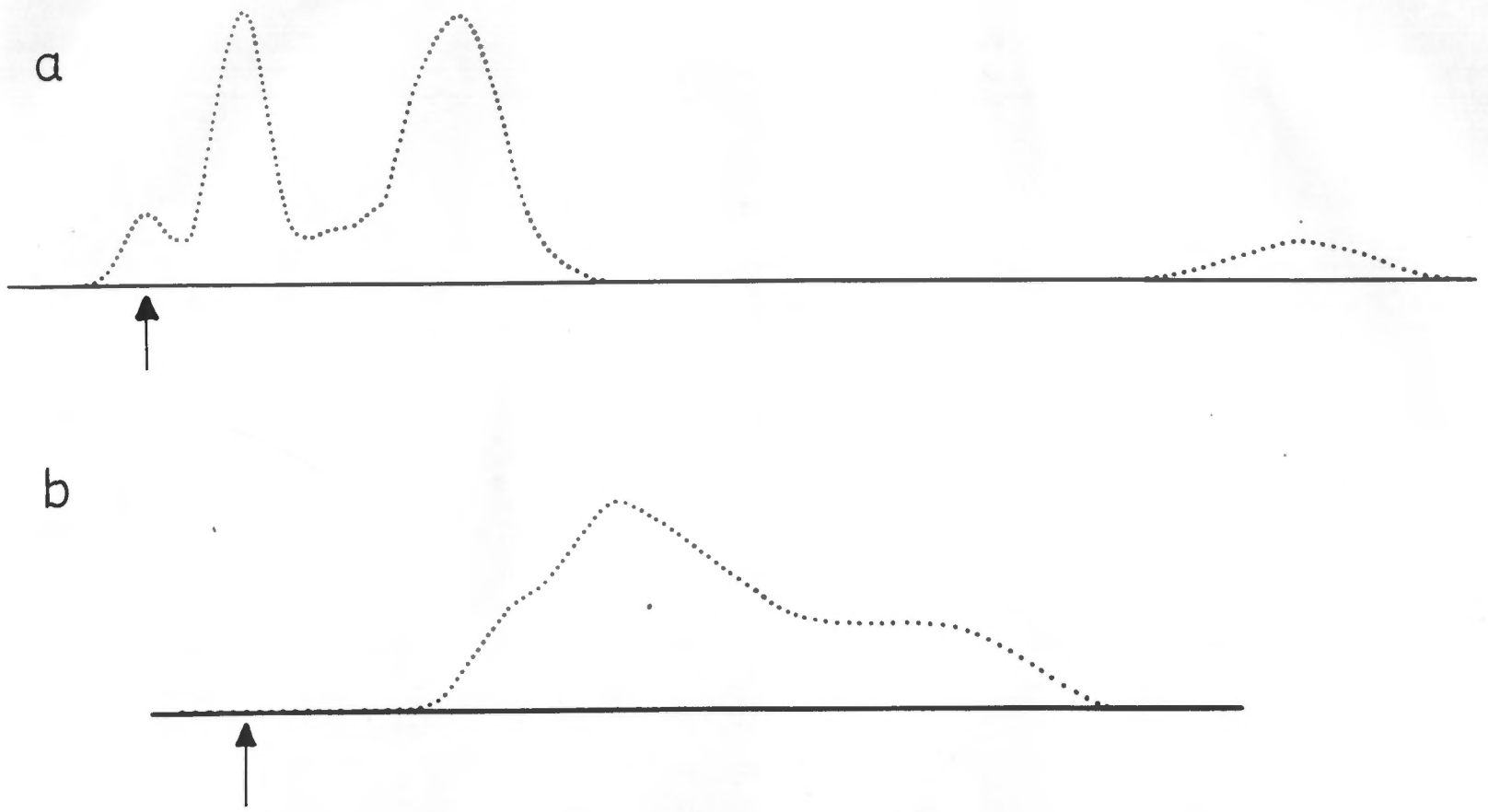
Gelatin spheres (4%) tanned with vegetable tannin, formalin and chromium after the spheres had been gelled and washed free of the organic liquids, were packed into a column and a mixture of B. cincta, J. lalandii, myoglobin and tryptophan chromatographed on the column. The elution diagram is illustrated in Fig. 11 (c). The first peak contained both haemocyanins, the second myoglobin and the third tryptophan. In this case no partial resolution of the myoglobin and tryptophan was observed.

Gelatin spheres (4%) tanned with chromium and formalin during the emulsification process and before the removal of the organic liquids were used to chromatograph a mixture of the two haemocyanins. The first peak on the elution diagram (Fig. 11 d) contained both haemocyanins, while the remaining peaks were low-molecular- weight proteins.

Chromatography of a mixture of Esch. coli, bacteria and the haemocyanins of B. cincta and J. lalandii on 3% chromium-tanned gelatin granules is illustrated in Fig. 12 (a).

Fig. 12.

Elution diagrams of a mixture of (a) *Esch. coli*, *B. cincta* and *J. lalandii* chromatographed on a column containing 3% chromium-tanned gelatin granules, and (b) a mixture of *B. cincta* and *J. lalandii* haemocyanins on 2% formalin-chromium-tanned gelatin granules. The eluting fluid was 66mM-phosphate-buffered saline, pH 6.9, the flow rate was 8 ml./hr., and the sample volume was 1 ml. The position of the front is indicated with a vertical arrow.


a
b

The first peak contained Esch. coli, the second B. cincta, the third J. lalandii and the last was due to low-molecular-weight proteins.

Chromatography of a mixture of the two haemocyanins on 2% formalin-chromium tanned gelatin granules is depicted in Fig. 12 (b). Both haemocyanins were present in the first peak.

8.4. DISCUSSION.

The elution diagrams represented in Figs. 8 (a), (b), (c) and (d) suggest an increasing porosity of the gel material with a decrease in gel concentration. A similar situation is found with agarose gels. The method or sequence of tanning the gelatin did not appear to be important since gels tanned with formalin, formalin-chromium or chromium gave identical elution diagrams when similar mixtures of proteins were chromatographed on them. It is therefore assumed that the porosity of tanned gelatin is not dependant on the sequence of tanning with either formalin or chromium.

A noteworthy observation was the difference in behaviour of gelatin spheres and granules of identical initial concentration. Chromatography of human serum on 4% tanned granules separated 19S globulin from 7S globulin

and albumin, whereas 4% tanned spheres failed to differentiate between 19S globulin and 7S globulin, but separated albumin from these substances. A similar observation was that B. cincta and J. lalandii haemocyanins chromatographed on 4% tanned spheres and granules behaved differently on these two materials, as can be seen from the position of the fronts. B. cincta haemocyanin was separated from the other protein on both the spheres and granules, but the resolution on the spheres was better, suggesting a greater porosity for the granules.

A probable explanation for 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 in porosity. This loss of water would probably take place at the surface of the spheres, forming a coat round the outside which is of a higher concentration than the gelatin at the centre. When spheres of different diameter are formed, the percentage loss of water from the smallest particles should be greater than that from spheres with a larger diameter. The result of this is that small spheres would be less porous than the larger ones. This is in fact the case with agarose spheres

of different diameters (Polson, unpublished work). The loss of water would however not occur on the disintegration of gelatin into granules with a Waring blendor, and this is a possible explanation for the retention of their porosities.

A second source of water loss could be anticipated during the addition of ethanol to break the organic emulsion. It has been observed by Yannas and Tobolsky (1967) that excessive drying of gelatin causes it to cross-link and become insoluble. This additional cross-linking would tend to diminish the pore diameters in the gel matrix with a consequent reduction in its porosity.

The method of tanning was found to be important. Gelatin tanned during the emulsification process was found to be less porous than that tanned after the removal of the organic liquids.

Cross-linking of the gelatin with resorcinol before emulsification also resulted in less porous gels than those prepared in the normal manner.

An interesting observation was that revealed by chromatography on 4% gelatin tanned with vegetable tannin during emulsification. The partial resolution of the myoglobin and tryptophan peaks first appeared to be due to irregularities in the packing of the column. However

after repacking the column with the same material a similar effect was noticed. This could have been due to a second faulty packing of the material or possibly due to ion-exchange effects. If there had been aggregation of the molecules during chromatography this would have been revealed in the Schlieren diagram. The reason for this is not apparent.

Chromatography of the haemocyanins on 3% and 2% chromium tanned gelatin further illustrated the increasing porosity of these gels with decreasing gel concentration.

Tanned gelatin spheres and granules form gel exclusion 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. Furthermore this material gives high flow rates at low concentrations and minimal zone spreading and is economical to prepare. It was therefore of interest to investigate the application of these materials to the purification of spherical and rod-shaped viruses. The forthcoming section deals with these studies.

CHAPTER NINE.

PURIFICATION AND SEPARATION OF VIRUSES ON CHROMIUM TANNED GELATIN GELS.

9.1. INTRODUCTION.

The use of gel exclusion agents for the purification and separation of viruses has not been extensively developed as only the agarose or agar gels are suitable for this type of investigation. Furthermore the volume of the virus sample is limited by the rather small column dimensions which can be used with low concentrations of gel necessary. Methods such as differential centrifugation, density gradient centrifugation (Brakke, 1967), adsorption chromatography (Woods and Robbins, 1961), ion-exchange chromatography (Hodes, Zepp and Ainbender, 1960), counter current distribution (Bengtsson and Philipson, 1964) have all been used with very satisfactory results in virus purification. Other techniques include the use of membranes for separating viruses from unwanted material and include such techniques as dialysis, ultrafiltration and electrodialysis (electrodecantation). Philipson (1967) has described a water-organic solvent phase system for the isolation of viruses, and Polson and Russell (1967) have discussed electrophoresis

as a method of virus purification. The latter method is known to be one of the most effective means of purifying virus samples, but its use is restricted to fairly small volumes of material. Finally the adsorption of viruses to cells and their elution is an effective, simple and rapid means of virus purification (Wasserman, 1967).

The chromatographic separation of the different rod lengths of tobacco mosaic virus has been reported by Steere (1963). He found that by two passages through a 1% agar or agarose column a marked separation of the rods was achieved into four components each differing in molecular size. The infectivity moreover was shown to be associated only with the rods having lengths of 300 mμ.

Haller (1965) reported the separation of an artificial mixture of tobacco mosaic and tobacco ringspot viruses on porous glass powder, which he achieved in a matter of minutes. Bengtsson and Philipson (1964) demonstrated that myxoviruses, adenoviruses and picornaviruses are eluted in order of decreasing molecular size from columns packed with 2.5% agar. All these viruses were retarded on this material indicating that they had entered the gel matrix or had been adsorbed. Furthermore 2-2.5% agar or agarose was shown to be suitable for the separation of most viruses.

With the introduction of tanned gelatin gels it was of interest to investigate their resolving properties with virus samples. Care was taken in interpreting these results as adsorption-elution effects rather than true gel exclusion chromatography may be responsible for the separation of these particles.

9.2. MATERIALS.

9.2.1. Gelatin.

In all instances chromium-tanned gelatin granules were used as bed material. Both Difco and acid processed pigskin gelatins were used after fractionation with polyethylene glycol (2.4.1.). The tanning procedure and the fractionation of the gelatins was carried out as previously described (3.3.3.).

9.2.2. Viruses.

A nonoccluded virus of the Pine Emperor moth (Nudaurelia cytherea capensis), the nuclear polyhedral virus from the lucerne caterpillar (Colias electo) and the rod shaped tobacco mosaic virus (TMV) and Holmes ribgrass virus were used in these studies.

In all instances the experiments were conducted at 22°, the effluents being monitored with an L.K.B. Produkter Uvicord optical unit.

9.3. METHODS.

9.3.1. Virus preparation.

A nonoccluded virus of the Pine Emperor moth (Nudaurelia β virus) was prepared and isolated from infected larvae of the Pine Emperor moth according to the method of Tripconey (1969). The larvae were homogenized in a Waring blendor in the presence of 66mM-phosphate buffer, pH 7.0. The emulsion was centrifuged at 10,000 r.p.m. for 10 min. and the supernatant was recentrifuged at 30,000 r.p.m. for 60 min. The supernatant was discarded and the pellets resuspended in phosphate buffer and kept at 4^{o} until used.

Approximately 4-5 ml. of the virus suspension was chromatographed on columns packed respectively with 2% chromium-tanned Difco gelatin and 1.5% chromium-tanned acid processed pigskin gelatin, both equilibrated with phosphate buffer containing in addition 0.02% ($^{w}/v$) sodium azide.

All the peak fractions obtained from the column during chromatography of the virus were collected and centrifuged at 30,000 r.p.m. for 60 min. in order to sediment the virus or other non-viral particles present in these samples. The supernatant was carefully removed and discarded and the pellets resuspended in a few drops

of phosphate buffer. All the fractions were tested for the presence of virus by the immunodiffusion technique.

9.3.2. Gel precipitin test.

Tests for the detection of viral antigen were performed according to the method of Mead (1962). A 1% (W/v) solution of agarose was prepared in 0.85% (W/v) NaCl solution. The hot solution of agar was poured into a petri dish containing a small quantity of thiomersalate (B.D.H.) to a depth of approximately 1-2 mm. Thiomersalate was not added to the agarose solution while it was being dissolved as it is heat sensitive. After the solution had gelled a hexagonal arrangement of wells was cut in the gel with a cutter producing wells 2 mm. in diameter and with centres 5 mm. apart. The centre well within the hexagonal arrangement of wells was filled with antiserum (see below), the outer wells with concentrated antigen, and the dish transferred to a glass container with a water saturated atmosphere produced by placing wet cotton wool at the bottom of the apparatus. Contact photographs were not made of the lines of precipitation as this technique merely served as a means of identifying virus antigens.

9.3.3. Preparation of antiserum.

Two rabbits were inoculated with purified Nudaurelia β virus samples by the intramuscular route

as follows: 1 ml., 2 ml., 2 ml. and 5 ml. samples of the virus were injected at weekly intervals. After a period of two weeks the rabbits were exsanguinated, the blood was allowed to clot, and the serum collected and treated as previously described (8.2.5.).

9.3.4. Colias electo virus.

Virus preparations from Colias electo were isolated from infected lucerne caterpillars by the method of Bergold (1964). The dead worms were suspended in distilled water and kept at room temperature for 7 days, when the inclusion bodies collected at the bottom of the beaker. The supernatant was carefully removed and discarded and the inclusion bodies resuspended in water. The polyhedra were partially purified by a number of extractions with Arcton 113 (Imperial Chemical Industries, South Africa). To a suspension of the polyhedra was added an equal volume of Arcton and the mixture well shaken. After allowing the solution to stand at room temperature for five minutes the liquids separated into two phases, when the uppermost layer containing the polyhedra was removed and extracted for a second time with an equal volume of fresh Arcton. The aqueous layer was removed and centrifuged at 6,000 r.p.m. for 10 min., the supernatant was discarded and the pellets resuspended in

0.1M-Na_2CO_3 + 0.05M-NaCl. The suspension was kept at room temperature for 1 hr. to hydrolyse the polyhedra and liberate the virus. The virus suspension was centrifuged at 6,000 r.p.m. for 10 min. to remove the gross polyhedral protein, and the supernatant recentrifuged at 20,000 r.p.m. for 60 min. The pellets containing the partially purified virus were resuspended in 2-3 ml. of phosphate buffer and chromatographed on 1.5% chromium-tanned pigskin gelatin. The peak fractions obtained from the above column were collected and the components present were concentrated by centrifugation at 20,000 r.p.m. The pellets were resuspended in a few drops of phosphate buffer, and kept at 4° until needed for further investigation.

9.3.5. Holmes ribgrass virus and Tobacco mosaic virus.

The preparation of Holmes ribgrass virus and tobacco mosaic viruses was that according to the method of von Wechmar (unpublished data), which was a modification of the method of Steere (1965).

Leaves of Nicotiana tobaccum var. sampson infected with Holmes ribgrass virus or TMV were dipped in 0.05M-EDTA buffer, pH 8.5 and macerated in a mechanical mincer. The juice was extracted from the pulp by squeezing through a double layer of cheese cloth. The pH of the juice was

adjusted to 7.5 with 1N-NaOH and centrifuged at 10,000 r.p.m. for 10 min. The supernatant was kept and the pellets discarded. For every 100 ml. of supernatant, 5 g. of activated charcoal and 5 g. of Celite (diatomaceous earth-High Flo Supercel) was added and the whole well mixed. The suspension was filtered on a Buchner filter containing a pad of packed washed Celite approximately 1 cm. thick. The filtrate was concentrated by precipitation of the virus with 4% polyethylene glycol in the presence of 4% NaCl. The virus precipitate was collected and resuspended in 0.01M-EDTA, pH 7.5 or 0.06M phosphate buffer, pH 7.5 to give a concentration of virus of approximately 25 mg./ml. The virus suspension was kept at 4° until used.

9.3.6. Chromatography.

Approximately 2-3 ml. of the ribgrass virus suspension was chromatographed on 1%, 1.5% and 2% chromium-tanned pigskin gelatin equilibrated against 66mM-phosphate-buffered saline, pH 7.5 containing 0.02% sodium azide as preservative. The column dimensions were 110 cm. x 1.5 cm., the flow rate was approximately 3 ml./hr. and samples were collected at intervals along the elution peak.

The partially purified suspension of TMV was chromatographed on 2% chromium-tanned pigskin and Difco gelatins equilibrated against phosphate buffered saline, pH 7.5. The column dimensions were 50 cm. x 2.5 cm., the flow rate was 3 ml./hr. and samples were collected at intervals along the elution diagram.

9.3.7. Electron microscopy.

The virus pellets of Nudaurelia and Colias obtained after centrifugation of the peak fractions from gel exclusion chromatography were suspended in two drops of phosphate buffer. An equal volume of virus suspension and 2% sodium phosphotungstate pH 7.0 were well mixed and applied to formvar-carbon coated grids with a micropipette. Excess solution was removed with the pipette and the samples examined immediately in a Siemens 1 A or Philips 300 electron microscope. This technique was introduced as a second method for detecting the presence of virus and to observe the presence or absence of viral subunits.

The fractions taken at intervals during elution for both ribgrass virus and TMV were not concentrated as there was sufficient virus present for examination in the electron microscope. A pipette having a diameter of at

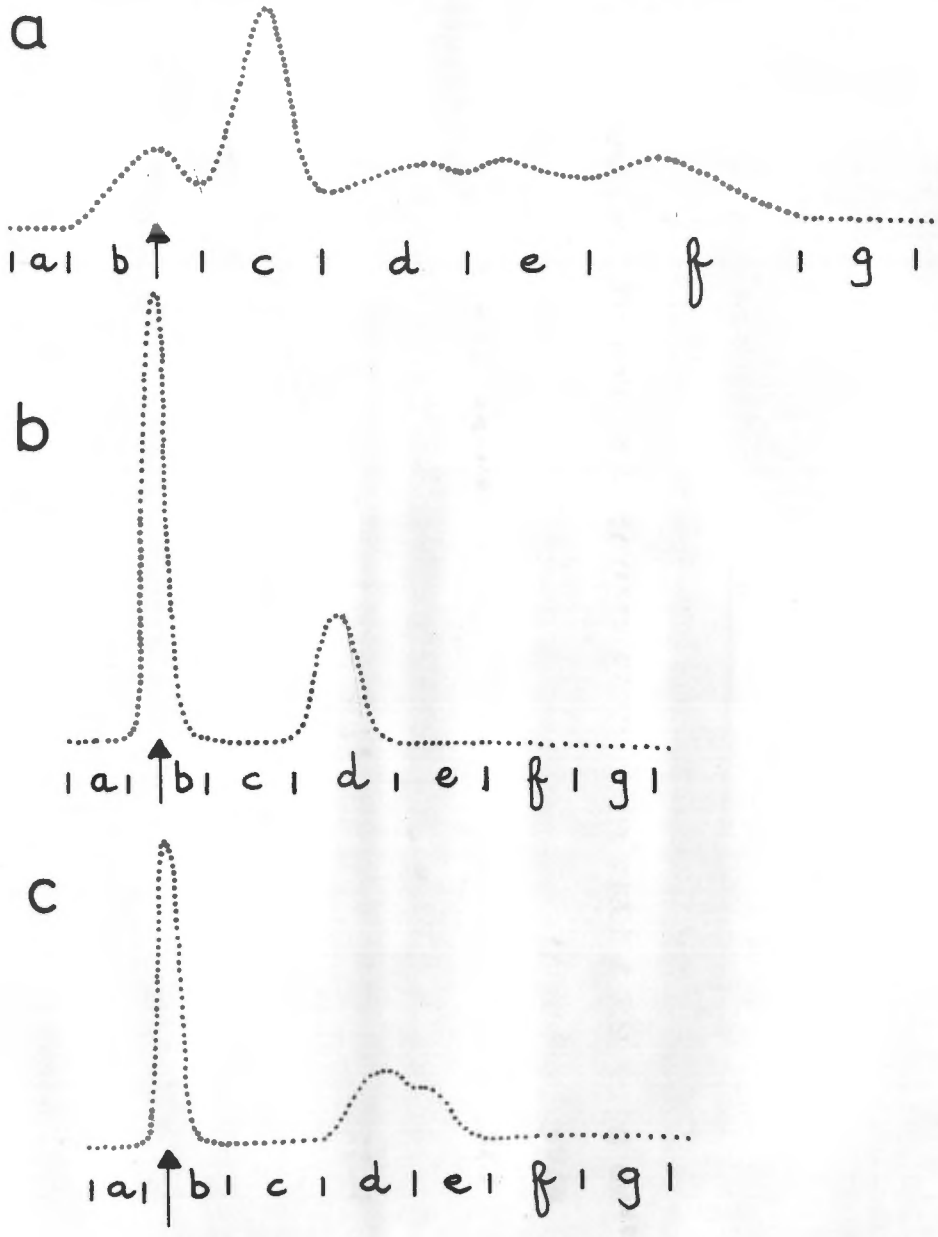
least 2 mm. to prevent breaking the virus rods, was used for mixing samples of virus with an equal volume of 2% sodium phosphotungstate. The grids for electron microscopy were prepared as described above, and then examined immediately. Photographs were made of the electron photograph negative, and all the virus particles were measured. At least 200 measurements were taken of each print. This technique gave an indication of the average size of the virus particles in any one sample. These results have not been recorded as they merely gave an indication of the effectiveness of the column separation.

9.4. RESULTS.

The elution diagrams obtained from chromatography of Nudaurelia β virus revealed that the virus was eluted at the front on 1.5% pigskin gelatin. The first and second peaks obtained from a column packed with 2% Difco gelatin (Fig. 13 a) both contained virus which either entered the gel or was retarded by adsorption effects, as they were eluted after the front. The remaining three peaks were due to low-molecular-weight impurities. The first peak obtained from a column packed with 1.5% pigskin gelatin (Fig. 13 b) was due to virus and the second to low-molecular-weight impurities.

Fig. 13.

Elution diagrams obtained from chromatography of a partially purified suspension of Pseudomonas β virus on (a) 2% chromium-tanned Difco gelatin granules and (b) 1.5% chromium-tanned pigskin gelatin granules. Elution diagram obtained by chromatography of a sample of Colias electo virus on (c) 1.5% chromium-tanned pigskin gelatin granules. The eluting fluid was 66mM-phosphate buffer, pH 6.9, the column dimensions were 2 cm. x 50 cm., the flow rate 8 ml./hr. and the sample volume 3–4 ml. The front is indicated by a vertical arrow.


a
| a | b | c | d | e | f | g |
b
| a | b | c | d | e | f | g |
c
| a | b | c | d | e | f | g |

as was confirmed by immunodiffusion and electron microscopy.

Chromatography of Colias virus suspension on 1.5% pigskin gelatin (Fig. 13 c) gave one large peak at the front and a further partially resolved peak of non viral material. The results of the virus identification by immunodiffusion and electron microscopy are recorded in Table 14.

Table 14.

Identification of fractions obtained from chromatography of Nudaurelia and Colias viruses on tanned gelatin, by the immunodiffusion technique and electron microscopy. Refer to Fig. 13.

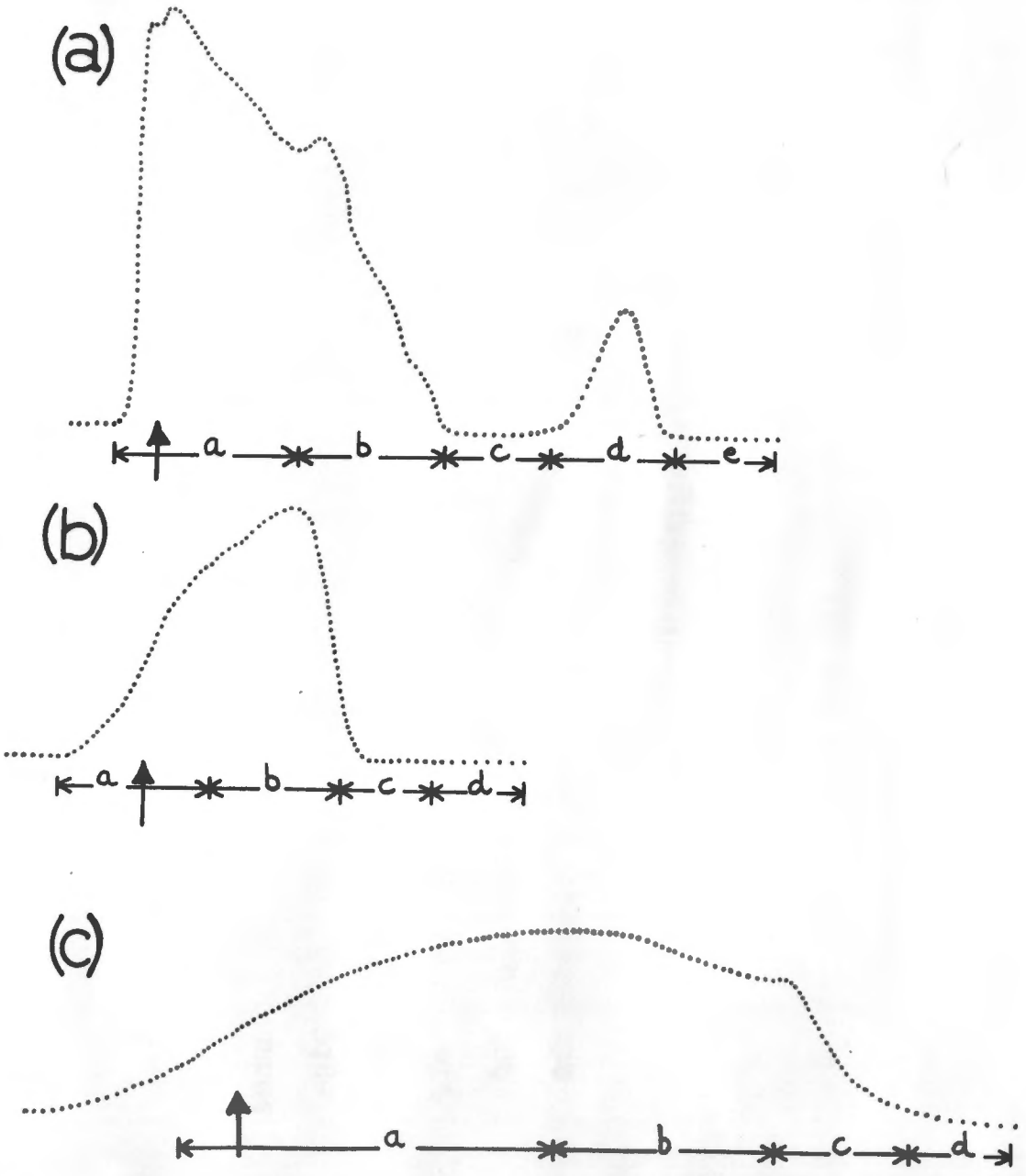
Type of gelatin	Fractions.						
	a	b	c	d	e	f	g
2% Difco (Nudaurelia)	Ag^-	Ag^+	Ag^+	Ag^-	Ag^-	Ag^-	Ag^-
1.5% Pigskin (Nudaurelia)	Ag^-	Ag^+	Ag^-	Ag^-	Ag^-	Ag^-	Ag^-
1.5% Pigskin (Colias)	Ag^-	Ag^+	Ag^-	Ag^-	Ag^-	Ag^-	Ag^-

Ag^- = Viral antigen absent

Ag^+ = Viral antigen present.

Fig. 14.

Elution diagrams of a sample of Holmes ribgrass virus chromatographed on columns containing (a) 2% chromium-tanned pigskin gelatin granules, (b) 1.5% chromium-tanned pigskin gelatin granules and (c) 1% chromium-tanned pigskin gelatin. The eluting fluid in all instances was 66mM-phosphate buffer, pH 6.9, the sample volume 3-4 ml. and the flow rate 3 ml./hr. The column dimensions for the 2% and 1.5% columns were 2 cm. x 50 cm. and for the 1% 1.5 cm. x 120 cm.


(a)
a
b
c
d
e
(b)
a
b
c
d
(c)
a
b
c
d

There was partial penetration of the 2% pigskin gelatin granules by ribgrass virus chromatographed on this material (Fig. 14 a). The largest virus particles (300 mμ) however appeared at the front, but those following had been retarded according to molecular size. The partially resolved first peak (Fig. 14 a) was due entirely to virus and the smaller peak following was due to non viral components confirmed by electron microscopy. There was a gradual decrease in the length of these particles as the elution diagram developed. There were no virus particles present in the smaller peak. These results are recorded in Table 15.

The same virus sample chromatographed on 1.5% chromium-tanned pigskin gelatin granules (Fig. 14 b) revealed a graded penetration of the gel particles except for the largest virus particles (300 mμ) which appeared at the front. Chromatography of ribgrass virus on 1% pigskin gelatin granules (Fig. 14 c) gave an elution diagram with a very much broader base to that shown in Fig. 14 (a and b). This broadening of the elution diagram may have been due to a greater penetration of the particles of the gel matrix. The column length of the experiment conducted with 1% pigskin gelatin was

120 cm. x 1.5 cm. and the flow rate was 3 ml./hr. The results of the two above experiments (Fig. 14 b and c) are recorded in Table 15.

Table 15.

Measurement of Ribgrass virus particles collected after elution from columns packed with tanned gelatin. Refer to Fig. 14.

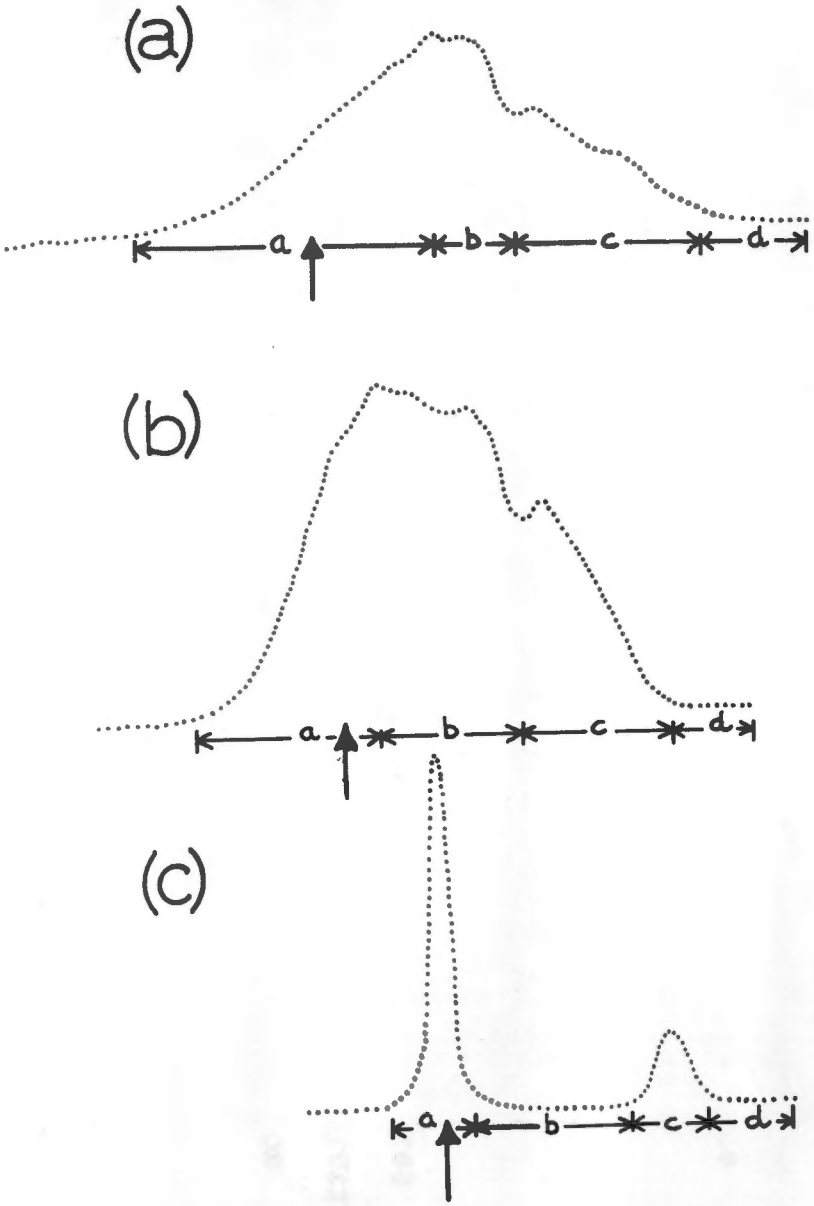
Type of gelatin	Approximate size range of viruses in different fractions.				
	a	b	c	d	e
2% Pigskin.	300-120 mμ.	200-50 mμ.	Ag^-	Ag^-	Ag^-
1.5% Pigskin.	300-200 mμ.	100-20 mμ.	Ag^-	Ag^-	
1.0% Pigskin.	300-250 mμ.	200-100 mμ.	100-20 mμ.	Ag^-	

Ag^- = Virus absent.

Chromatography of a suspension of TMV on 2% pigskin gelatin granules (Fig. 15 a) and 2% Difco gelatin granules (Fig. 15 b) in both cases revealed the partial resolution of a number of viral components all represented by one large peak. Samples taken at intervals along the elution diagrams revealed a separation on a molecular size basis, as was confirmed by measuring samples of virus photographed as previously described. The results of this experiment are recorded in Table 16.

Fig. 15.

Elution diagrams of a sample of TMV chromatographed on columns containing (a) 2% chromium-tanned pigskin gelatin granules, (b) 2% chromium-tanned Difco gelatin granules and (c) 1% chromium-tanned pigskin gelatin granules. The last sample of virus was obtained from the front in (b) and concentrated before application to the 1% material. The eluting fluid in all instances was 66mM-phosphate buffer, pH 6.9, the sample volumes were 3–4 ml. the flow rate was 3ml./hr. and column dimensions 1.5 cm. x 120 cm. The front is indicated by a vertical arrow.


(a)
a
b
c
d
(b)
a
b
c
d
(c)
a
b
c
d

The column dimensions in both experiments were 120 cm. x 1.5 cm. and the flow rate was approximately 3 ml./hr.

A sample of virus which appeared at the front (see Fig. 15 b) was collected and concentrated by pervaporation and rechromatographed on 1% chromium-tanned pigskin gelatin, the elution diagram of which is illustrated in Fig. 15 (c). This fraction appeared to contain particles which were predominantly 300 mμ. in length. The last small peak was due to non viral components. The results of this experiment are recorded in Table 16.

Table 16.

Measurement of TMV virus particles collected after elution from columns packed with tanned gelatin. Refer to Fig. 15.

Type of gelatin.	Approximate size range of viruses in different fractions.			
	a	b	c	d
2% Pigskin	300-200 mμ.	150-100 mμ.	80-20 mμ.	Ag^-
2% Difco	300-250 mμ.	200-120 mμ.	100-60 mμ.	Ag^-
1% Pigskin	300-250 mμ.	Ag^-	Ag^-	Ag^-

Ag^- = No virus present.

DISCUSSION.

Chromium-tanned, fractionated Difco and unfractionated pigskin gelatins, at concentrations between 1% and 3% were found to be suitable as bed material for chromatography of impure virus suspensions.

An interesting fact observed during these experiments was the apparent greater porosity of Difco gelatin in comparison with that of pigskin of the same concentration, both tanned under identical conditions. Nudaurelia β virus which appeared after the front on the Difco gelatin column, appeared at the front on the column packed with pigskin gelatin. Caution must be excercised when interpreting these results. It is tempting to ascribe these results to a greater porosity of the Difco gelatin. However from work presented in an earlier part of this study (6.1.) it would be more feasible to assume that the virus particles investigated could have accessible SH groups in the viral protein coat, and adsorption-elution effects may be taking place, especially on the Difco gelatin. The reason why this should be more marked on the Difco gelatin is not apparent. In comparison with agarose or agar gels, tanned gelatin

appears to be slightly less porous (Bengtsson and Philipson, 1964) as mixtures of viruses have been successfully separated on the former material.

Experiments conducted with ribgrass virus showed that the virus (300 mμ.) appeared at the front in all instances. However there was a graded penetration of the particles into the gel matrix, smaller than 300 mμ. It should be mentioned that the separation of this virus, which is very similar in size to TMV, was not as satisfactory as the separation obtained by Steere (1963) with the same virus on 1% agar. However this fractionation of ribgrass on 1% pigskin gelatin showed the best separation, but not comparable to that on agar.

Similar experiments conducted with TMV showed that this virus also appeared at the front. However a second cycle of chromatography on 1% pigskin gelatin of the virus that appeared at the front on 2% Difco gelatin, gave a superior resolution approaching that observed by Steere (1963).

The use of tanned gelatin gels at low concentration appears to be satisfactory for virus purification. One distinct advantage that this material had over that of any of the other gels at present in use for the separation of biological materials was that tanned gelatin gave higher flow rates in very long columns over a period of a number of weeks of continual use. Whether the separation of viruses was due entirely to molecular exclusion or to some extent to adsorption-elution effects is difficult to assess.

CHAPTER TEN.

CONCLUSIONS.

Since the introduction of starch grains (Lathe and Ruthven, 1956) for use as bed material for gel exclusion chromatography this field of separatory techniques has developed rapidly. A number of substances e.g. dextran, polyacrylamide, agar and agarose gels have been introduced and studied extensively as potential media for gel exclusion chromatography. These materials have proved satisfactory for purifying and separating biological substances, but each gel medium has certain limitations. The same is true for tanned gelatin but as has been shown in the present work this substance has certain distinct advantages over all other bed materials.

Initially it was thought that the longer the molecule responsible for producing the gel matrix, the more rigid would be the gel. These studies have shown that this assumption was only partly correct, and that the bonding or cross-linking between these molecules, in the presence of the tanning agent, was even more important. Fractionation of Difco gelatin with polyethylene glycol produced a gelatin of higher molecular weight than the

unfractionated sample, and a gel of greater rigidity than that produced from the untreated sample of the same gelatin. This does not necessarily imply that the molecular weight is an indication of the length of the gelatin molecules. Pigskin gelatin which had a molecular weight of only half or one third of that of the fractionated Difco gelatin produced a gel of greater rigidity than the latter. It therefore appeared that the method and chemical agent used for tanning was important and that the number of available groups on the gelatin capable of taking part in the tanning reaction was of primary importance. Although the gel strength of pigskin gelatin was always greater than that of Difco, the former was less porous than the latter. This did not appear to be due to the exclusion of water from the pigskin material during tanning, as the gel strength of this material prior to tanning was greater than that of the Difco.

It has been suggested (Ferry, 1948) that the formation of a gelatin gel is due to van der Waals forces between groups on different gelatin molecules as yet unidentified. It is further assumed that these forces, which are situated at loci widely separated from each other, are stabilised by the tanning of gelatin with the substances

previously mentioned. In addition to these forces it has been shown (Gustavson, 1949) that the tanning agents cross-link with a wide variety of chemical groups which would have the effect of further stabilising the temperature dependant bonds present in untanned gelatin. This would account for the thermal stability and enzymic resistance of the tanned gelatin gels. The swelling of these gels in dilute acids and alkali and in water, will also be dependant on the number of cross-links in the gel matrix. As has been shown in this study (4.2.) tanned gels of low concentration swell and shrink to a greater extent than do the higher concentrations. Similarly drying these gels would have the effect of removing water and consequently bringing the molecules into closer proximity with each other, with the formation of further cross-links. This would explain the irreversible swelling of these gels after dehydration.

Both formalin and chromium were found to be more satisfactory tanning agents than vegetable wattle tannins, although all three substances rendered gelatin insoluble after tanning. Chromium however was found to give a tanned gel with the greatest rigidity, due probably to the large number of carboxyl groups present on the gelatin molecule, in contrast with the number of ϵ-amino acid

groups which take part in the reaction during formalin tanning (Fraenkel-Conrat and Olcott, 1948).

Cross-linking of the gelatin with resorcinol (Braunwold et al, 1966) prior to tanning with chromium appears to produce a more rigid gel by the exclusion of water, with a subsequent reduction in the porosity of the material. A similar effect takes place if tanning is carried out during the emulsification process when preparing spheres. However the colour of the end product was usually dull green instead of the usual "sparkling" clear green. This dulling was also observed if phosphate buffer was added to a suspension of chromium tanned spheres or granules which had been incompletely freed of uncombined chromium. The porosity of the two above mentioned gels was also considerably reduced when it had this dull green appearance. The dullness could be removed to a large extent by washing the gel with 0.5N-HCl which appeared to remove the factor responsible for the dulling and with its release the porosity of the material increased. It was more satisfactory to discard the material and prepare a fresh batch.

The method of tanning with chromium or formalin or both did not alter the porosity of gels of identical

concentration. However the iso-electric point of the gelatin was considerably altered by the type of tanning solution and also by the sequence of tanning with chromium and formalin. The iso-electric point was either depressed or elevated according to the method and sequence of tanning, and appeared to be concerned with the reaction of the amino and carboxyl groups of the gelatin with those of formalin or chromium. Gels used at pH's similar to their iso-electric point still revealed that certain molecules were retarded, during chromatography a possible explanation for this is given later in this discussion.

A simple explanation of the mechanism of exclusion chromatography assumes that the bed material acts as a molecular sieve. This assumption presupposes that the gel has fairly well defined pores the diameter of which may be regulated in the case of agarose or agar (Polson, 1961) and tanned gelatin by varying the concentration of the gel; or in the case of dextran and polyacrylamide gels by varying the degree of cross-linking of the gel forming material.

From electron microscopy studies (Hickson and Polson, 1968) of agarose gels it is evident that the

structure of the gel is in reality a matrix of fibres held together in an irregular manner. During chromatography of a mixture of solute molecules of different molecular size it is assumed that these molecules enter into the stationary phase if they have diameters similar to or smaller than those of the gel pore. This retards the progress of smaller relative to larger molecules.

An interesting study concerning gelatin gels has confirmed some of the above assumptions. Wykoff (cited by Bourne, 1963) has described experiments with gelatin gels of concentrations varying between 2% and 4%. After fixing the gels with osmic acid they were examined in the electron microscope, which revealed a network of fibres with pores whose diameter was dependant on the concentration and the type of fixative used. The pores were found to be smaller in gels of higher concentration.

A similar effect has been noticed in experiments conducted during the present study. If gelatin gels were tanned with chromium for 24 hr. only, they were found to be more porous than a gel of identical concentration tanned for 72 hr. This was confirmed by chromatography of identical protein solutions on

columns packed with the respective media. This suggests that the tanning effect of chromium on gelatin tends to reduce the porosity of the gel by literally drawing together the fibres of the gel with the possible exclusion of water - similar to concentrating the gel. It appears therefore in order to obtain gels of sufficient rigidity for use as bed material a sacrifice must be made with regard to the porosity of the material.

Ferry (1948) postulated that the initial formation of a dilute gelatin gel was due to associations between two polypeptide chains, in all probability influenced by van der Waals forces or hydrogen bonding. However as the concentration of the gelatin in the gel is increased there would be further lateral associations between paired chains which would eventually build up, as the concentration of the gel is increased, into localized "bundles" of chains as illustrated by Ferry (1948, Fig. 16).

Ferry (1948, Fig. 4) has furthermore differentiated two types of network in gelatin gels. Firstly the "fine" association, which would tend to be formed with concentrated gels by the association of a macromolecule with many others at a number of points along its length. The second type known as "coarse" association would occur

in dilute gels, where macromolecules would align themselves next to each other for long distances. This would not decrease the supposed pore diameter of the gel to a great extent. It is therefore permissible to assume that the gel has a three dimensional network enclosed within the gel granule or sphere but not composed of pores or capillaries. A mixture of solute molecules of varying molecular dimensions chromatographed on a column of these particles would be retarded by molecular interference as suggested by Polson and Katz (1969). Only those molecules capable of entering the gel matrix would be retarded, due to the interference between themselves and the linear-like molecules of the gel, and would therefore appear in the eluate after the larger molecules, which would not have entered the matrix to any great degree.

The theory of molecular interference would probably account for the "tailing" effect of the elution diagram during chromatography of nucleic acids on various gel media (Polson, unpublished data). The tailing might be due to the belated release of these linear molecules from the gel matrix due to their greater interference with the gel fibres. Philipson (1967) has shown that by increasing the buffer concentration, or by the addition of divalent metal ions, to the buffer the nucleic acid

molecules become more compact and the tailing effect disappears.

An interesting effect exhibited by the chromium-tanned gels was the adsorption-elution and ion-exchange properties of these materials. On investigation it was revealed that substances such as chymotrypsinogen, cytochrome C, ovalbumin etc., were retarded on these gels, and this retardation was found to be a property of the protein, eluting fluid and its concentration. Similarly proteins containing SH groups which are situated on the molecule in a position available for reaction with the chromium complex attached to the gel, are adsorbed and eluted in the presence of weak solutions of thiols. It must be borne in mind that the number of SH groups per protein molecule, and the steric arrangement of these groups will govern their reactivity with the chromium complex. Due to the affinity of mercapto groups for heavy metals this characteristic has been utidized for the isolation of mercaptalbumin, enolase and other substances (Alexander and Block, 1960). The same authors have also made mention of the use of zinc, lead and cupric salts for the precipitation of SH proteins.

From the discussion given in chapter 7 it can be inferred that not only is there a bonding between SH group and chromium but that secondary reactions take place between solute and gel and these appear to be irreversible under normal conditions, but are of a less important nature as only a small percentage of solute is bound in this way. This was probably due in some instances to the low concentration or absence of phosphate, which appears to combine irreversibly with the chromium.

This adsorption effect with heavy metals has a large number of possibilities for the separation of SH proteins. However the use of EDTA as a complexing agent for the elution of these proteins from the gel is to be avoided, as not only the protein but the chromium is released. If the latter complexing agent must be used it is advisable that the gel be washed thoroughly with water after the elution procedure and retanned with chromium for two days at room temperature. Eldjarn and Jellum (1963) have noted in their investigations that cysteine, cysteamine, 2-mercaptoethanol and strong bases (pH greater than 11) elute the SH protein as well as the organomercurial group. However their columns could be rejuvenated by reintroducing the latter group on to the gel matrix.

Chromatography of artificial protein mixtures on tanned gelatin gels revealed good separations of protein components on a molecular size basis as was confirmed by ultracentrifugation. Similarly virus separations were good and were relatively free of contaminating proteins. It is difficult to assess the exact fractionating range of these gels, but it appears in the absence of adsorption effects to lie in the molecular range between a few hundred and at least 1×10^6 daltons, or more realistically for globular proteins ranging in diameter from a few mμ. to at least 100 mμ.

These studies have revealed a number of advantages in using tanned gelatin as bed material for gel exclusion chromatography experiments in preference to those already well established in this field. Agarose gels are practically neutral with regard to charge, and the necessity of using buffers of high ionic strength is obviated. However with both dextran and tanned gelatin gels these effects can be suppressed to a large extent by the judicious selection of buffers.

Tanned gelatin has been found on experimentation to give flow rates, when packed in columns of all dimensions, far superior to either agarose or dextran, and

the compressibility of the former gel is so small in comparison with the others that columns have been used continuously for a number of weeks without any significant decrease in the flow rate. The porosity of agarose gels at low concentrations seems to be greater than that of gelatin at similar concentrations, but not much more so, as the latter has been used successfully for virus purification and isolation. Gelatin has a far wider fractionating range than agarose or dextran individually.

The progress of coloured proteins can be observed during chromatography on agarose or dextran, but not so with chromium tanned gelatin. It has been suggested in an earlier discussion that formalin-tanned gelatin be used for the same purpose, but its use at very low concentrations (1% to 2%) should be avoided due to its lower gel strength in comparison with the chromium-tanned material.

All three gels are prone to bacterial and fungal contamination, and the use of preservatives in all instances is recommended. Both agarose and dextran liberate trace amounts of carbohydrate after long use (Katz, Larsson and Mead, 1968). No liberation of protein

or amino acids has as yet been observed with tanned gelatin which has been effectively preserved with sodium azide or chloroform, a situation similar to that found with polyacrylamide gels. Therefore the use of tanned gelatin gels is recommended for carbohydrate investigations.

Finally the preparation of dextran, agarose and polyacrylamide gels are tedious and expensive to prepare. Gelatin gels on the other hand are economical and easy to prepare, but with the higher concentrations a certain amount of difficulty may be encountered during comminution of the material into particles suitable for bed material due to their high rigidity. Tanned gelatin, due to its low cost of production, is at present being used as bed material in columns (2 metres x 10 cm.) for the preparation of purified anti-lymphocyte serum.

In conclusion it has been shown that tanned gelatin has a number of distinct advantages over the established gel media. The spheres and granules of tanned gelatin form gel exclusion agents which have been successfully applied to the separation and purification of proteins and viruses. The material gives good flow rates at low concentrations with minimal zone spreading of the solute bands.

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ACKNOWLEDGEMENTS.

To Dr. A. Polson, I express my gratitude and indebtedness for his constant encouragement, stimulating advice, guidance and patience.

In addition I should like to thank Professor A. Kipps, Director of the C.S.I.R. and U.C.T. Virus Research Unit, for his interest and advice at all times.

I would like to thank the Council for Scientific and Industrial Research, for the opportunity of conducting this research in their Laboratories, and to Mr. M. Schady, of the South African Developments Corporation, for financial assistance, without which, this work would not have been completed.

To Mr. Hammer, of Olivetti Africa (Pty) Ltd., my sincere thanks for the free loan of a typewriter for the preparation of this manuscript.

I wish to express my appreciation to Mrs. D. Gibbon for the competent typing of this manuscript, and to Mr. L.G. Fowle and Dr. B.W. Russell for much painstaking work with the electron microscope.

APPENDIX.

The author has been associated with the research work and the preparation of manuscripts for the following publications:

KATZ, W. (1967). Rabies Virus Soluble Antigens: A biochemical and biophysical study of the major antigen of Rabies Virus. M.Sc. Thesis. University of Cape Town.

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