SYNTHESIS OF POLYACRYLAMIDE GELS AND IMMUNOSORBENTS FOR FRACTIONATION OF PROTEINS AND ANTIBODIES

by

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TABLE OF CONTENTS

		Page
CHAPTER I	Chromatographic Separation of Proteins	1
CHAPTER II	Theory of Gel Filtration	10
CHAPTER III	Preparation and Evaluation of Polyacrylamide Gels for Gel Filtration	
	Introduction	20
	Experimental	21
	Results	35
	Discussion	69
CHAPTER IV	The Isolation and Purification of Antibodies	76
CHAPTER V	Preparation and Evaluation of Polyacrylamide-BSA Conjugates as Immunosorbents	
	Introduction	86
	Experimental	89
	Results	96
	Discussion	100
SUMMARY		107
CLAIMS TO ORIGINALITY		110
BIBLIOGRAPHY		112

LIST OF TABLES

TABLE		Page
I	Important dates of chromatography	3
II	Composition of 16 acrylamide-methylene bisacrylamide copolymers	23
III	Partition coefficients of various solutes for 7 polyacrylamide gels	41
IV	Theoretical and observed partition coefficients of 7 solutes for gel 5-5	48
V	Theoretical and observed partition coefficients of 7 solutes for gel 5-10	49
VI	Theoretical and observed partition coefficients of 7 solutes for gel 5-15	50
VII	Theoretical and observed partition coefficients of 7 solutes for gel 5-20	51
VIII	Theoretical and observed partition coefficients of 7 solutes for gel 10-15	52
IX	Theoretical and observed partition coefficients of 7 solutes for gel 15-15	53
X	Theoretical and observed partition coefficients of 7 solutes for gel 20-15	54
XI	Composition of 10 acrylamide-acrylic acid-methylene bisacrylamide copolymers	90
XII	Composition of 11 polyacrylamide-BSA immunosorbents	92
XIII	Characteristics of polyacrylamide-BSA immunosorbents	98

LIST OF FIGURES

FIGURE		Page
1	Ideal gel filtration chromatogram	14
2	Typical precipitin curve	29
3	Structural formula of the acrylamide-methylene bisacrylamide copolymers	36
4	Inter- and intra- molecular imidization of adjacent amide groups in polyacrylamide	37
5	Water regain values of polyacrylamide gels	39
6	Fractionation of bovine γ-globulin, hemoglobin, ovomucoid and tryptophane	43
7	Fractionation of anti-BSA antiserum	44
8	Chromatograms of single and multiple component runs of bovine serum albumin and lysozyme	45
9	Chromatogram of single and multiple component runs of bovine serum albumin and ribonuclease	46
10	Plot of K_D vs log M for gel 5-5	55
11	Plot of K_D vs log M for gel 5-10	56
12	Plot of K_D vs log M for gel 5-15	57
13	Plot of K_D vs log M for gel 5-20	58
14	Plot of K_D vs log M for gel 10-15	59
15	Plot of K_D vs log M for gel 15-15	60
16	Plot of K_D vs log M for gel 20-15	61
17	Plot of K_D vs r_s for gel 5-5	62
18	Plot of K_D vs r_s for gel 5-10	63
19	Plot of K_D vs r_s for gel 5-15	64
20	Plot of K_D vs r_s for gel 5-20	65
21	Plot of K_D vs r_s for gel 10-15	66
22	Plot of K_D vs r_s for gel 15-15	67

23	Plot of K_D vs r_s for gel 20-15	68
24	Diagram illustrating the effect of the gradual narrowing of free flow channels on the mode of migration of molecules through a gel filtration column	75
25	Khorana's reaction mechanism for the reaction of carbodiimide with carboxyl and amino groups	88
26	Ultracentrifugal pattern of the purified antibody preparation	101
27	Immunoelectrophoretic patterns of whole rabbit anti-BSA antiserum, the globulin fraction and the purified antibody preparation	102

CHAPTER I

CHROMATOGRAPHIC SEPARATION OF PROTEINS

Introduction

Progress in practically every field of chemical research and technology depends to a large extent on the development and judicious application of appropriate separation methods. This need is particularly eminent in biochemical and pharmaceutical fields, where the materials involved, e.g. animal sera and extracts of natural products, usually contain a multitude of closely related components. Over the past few decades, numerous techniques have been developed for the fractionation and isolation of proteins, such as dialysis, solvent extraction, salt precipitation, multi-membrane electro-decantation, free and convection electrophoresis, ultra-centrifugation and density gradient centrifugation, counter-current distribution and various types of chromatography.

It is beyond the scope of this presentation to give detailed descriptions of all these techniques. It is sufficient to refer the interested reader to an excellent series of reviews in the 1966 May issue of the British Medical Bulletin (1) and to a series of books edited by Alexander and Block (2) and by Neurath (3). Nevertheless, as a general background for this thesis, a brief description will be given of the general principles of the various types of chromatography, and of the more important applications of this technique to the fractionation of proteins. In Chapter II, the theory of gel filtration will be dealt with in greater detail.

Historical Background

Although a number of workers (4) preceded Tswett with methods that might be considered as chromatography, he is generally given the credit for

inventing this method. In 1903, while working with chloroplast pigments, Tswett observed that an adsorbent saturated with one substance was still able to take up and bind another. He also discovered that substitutions might take place and there existed a certain adsorption sequence according to which substances were able to displace one another. Using a calcium carbonate column, Tswett fractionated a petroleum ether solution of pigments and resolved them into various coloured zones. Though aware of the fact that this sequential adsorption phenomenon was not restricted only to coloured substances, he named the method "chromatography" (5).

In spite of the simplicity and power of Tswett's method and of his over fifty publications, the chromatographic technique was not extensively used until 1931 when Kuhn, Lederer and Winterstein achieved a breakthrough by resolving, among other pigments, carrot carotene and egg yolk xanthophyll into their isomeric components (6). An avalanche of reports on the applications of the technique followed. An excellent illustration of this explosive growth of the field is given by Hais and Macek in their bibliographies of paper chromatography covering the years 1943 through 1960, in which twenty thousand references were listed (7,8). Several of the more important dates in the history of chromatography are given in Table I.

TABLE I: IMPORTANT DATES OF CHROMATOGRAPHY

		Ref.
Liquid Phase Column Chromatography		
Adsorption	Tswett, 1903	5
Partition	Martin and Synge, 1941	9
Ion Exchange	Various workers, ~1947	
Gel Filtration	Porath and Flodin, 1959	10
Paper Chromatography		
Partition	Consden, Gordon and Martin, 1944	11
Gas Chromatography		
Adsorption	Hesse and Tschachotin, 1942	12
Partition	James and Martin, 1952	13

Types of Chromatography and Principles

All chromatographic methods depend on the distribution of the materials to be separated between two phases which move with respect to each other. Generally, one phase is fixed and the other is mobile. The fixed phase must be solid or liquid, otherwise it cannot be conveniently immobilized. The mobile phase can be either a liquid or a gas. Thus there are four major kinds of chromatography according to the kinds of fixed and mobile phases used.

All chromatographic methods using a gaseous mobile phase are designated as gas chromatography. In the case of a liquid mobile phase, one may have column or thin-layer (including paper) chromatography depending on the geometry of the supporting medium.

A more realistic, in terms of mechanism, classification of chromatographic methods depends on the kinds of phase equilibria determining the separation process. Thus, if the separation depends on the selective or sequential interaction between the solutes and the surface-active fixed phase (e.g. alumina, activated charcoal), it is called adsorption chromatography. The forces involved here may be Van der Waals, hydrogen bonds, dipole-dipole or ion-dipole interactions ... etc. If it depends on an exchange of ions between the fixed phase and the solutes, it is called ion-exchange chromatography. In this case, both the solute and the fixed phase must contain ionizable groups and there is a chemical equilibrium between the ionic species. Ionic bonds are being formed and broken compared with the surface interactions of adsorption chromatography. The fixed phase of the above two types of chromatography is usually a solid. Finally, in partition chromatography, differences in partition coefficients of the solutes in a pair of usually immiscible liquids are exploited in the separation process. In this case, the supporting medium is usually inert and does not interact with the solutes. The kinds of equilibria involved are comparable to those in fractional distillation and counter-current distribution.

Gel filtration is a particular kind of partition chromatography which has the same liquid in the two phases, but the partition of the solutes between them is controlled by the permeabilities of the solutes into the fixed or gel phase.

It must also be emphasized that often more than one mechanism underlies a given chromatographic separation. For example, due to the huge surface area of the packing material, adsorption always occurs to a lesser or higher extent. In some investigations, a loosely crosslinked ion-exchange column is chosen on purpose, rather than a highly crosslinked one, in order to achieve better separation by virtue of the additional sieving properties

for ions of various sizes. Electrochromatography, more commonly known as zone electrophoresis (on a slab, column or thin layer of some inert material), is a combination of partition chromatography and electrophoresis. In a good number of chromatographic separations using "ion-exchange columns", elution conditions were such that few if any of the "ionizable groups" were ionized; the separation mechanism was purely adsorption or a combination of ion-exchange and adsorption.

Although highly mathematical analyses of the intrinsic mechanisms of chromatography based on theoretical plate and rate concepts have been developed (9,14), chromatography is still considered by a great number of chemists and biochemists purely as a practical tool to be mastered as an art. For further details, the reader is referred to the original references by Martin and Synge (9), Giddings (15,16), Glueckauf (17) and Van Deempter (14) and to books by Feller (18), Heftmann (19), Pecsok (20), and Morris and Morris (21).

Applications of Chromatography to Protein Fractionation

(A) Adsorption Chromatography

The number of adsorbents that have been used for proteins is very large, the more commonly used being calcium phosphate, silica gel, kieselguhr and aluminium hydroxide.

The method has received its greatest impetus through the numerous publications from the laboratory of Tiselius (22), which included experiments on the chromo-proteins (phycoerythrin, phycocyanin, and hemocyanin), on egg albumin and on carbon monoxide hemoglobin with calcium phosphate columns. Similar columns have found application in the fractionation of enzymes (23-26) and of human serum (27). Columns of diatomaceous earth (kieselguhr) have been used for the chromatography of growth hormone from the anterior

pituitary (28). Yeast maltase was purified on passage through aluminium hydroxide (29). The separation of corticotrophin (30) and eledoisin (31) on Amberlite IRC-50 (a carboxylic acid cation exchanger) was, according to Morris (32), mainly due to the high hydrogen bonding capacity of the resin rather than to its normal ion-exchange function. In general, however, separation of proteins by adsorption chromatography does not yield as successful results as by cellulose ion-exchangers or by gel filtration media. The non-specific adsorption and the hydrophobic surfaces of the column packing material often results in non-quantitative recovery even after prolonged elution, and the fractionated proteins often are denatured during the adsorption-desorption process as to lose their biological activities.

Extremely selective adsorbents, exploiting the exquisite specificity of immunological reactions, have been synthesized for the fractionation of antibodies. Various antigens have been insolubilized by coupling to some inert supporting medium, e.g. cellulose, and the resulting immunosorbents have been used for the specific isolation of the corresponding antibodies (33,34). A more detailed description of this phase of work will be given in Chapter IV.

(B) Ion-Exchange Chromatography

Ion-exchangers are usually classified into two kinds, cationic and anionic. In protein fractionation, however, it is more convenient to classify them as hydrophobic or hydrophilic according to the solvation properties of their backbone. The former consists of a whole series of "resins" usually with a polystyrene backbone, e.g. the Dowex and Amberlite resins. The latter belong to the class of derivatives of cellulose and, more recently, crosslinked dextrans and polyacrylamide gels. Hydrophobic ion-exchange resins find few applications in protein fractionation, mainly due to the incompatibility of the solvents for use with biopolymers. The capacity of these resins

for the adsorption of proteins is small, because only the exterior charges are available to the macromolecule. The fact that almost all proteins are bound non-selectively to carboxylic exchangers below pH 5 is not due to the formation of hydrogen bridges between protein molecules and the undissociated acid groups of the exchanger (35), but to the unfolding of the protein molecules under the influence of the hydrophobic matrix of the exchanger. Therefore, only proteins, possessing sufficiently stable secondary and tertiary structures and groups which can adequately interact with the ion-exchanger, can be separated by chromatography on carboxylic resins. Thus, for example, Amberlite XE-64 (a finely divided form of Amberlite IRC-50) has been successfully used as a cation exchanger in the chromatography of relatively basic proteins at pH values that permit the exchanger to be highly ionized (36).

Derivatives of cellulose (37), crosslinked dextrans (38) and polyacrylamide (39) with ionizable functional groups have provided a wide range of affinities for essentially all types of proteins. The porous structure of these hydrophilic ion-exchangers permit ready penetration by large molecules, resulting in very high capacities for the adsorption of proteins. The chemical stability of these ion-exchangers allows the use of an almost unlimited variety of buffer species over a wide range of pH. Carboxymethyl (CM) and diethylaminoethyl (DEAE) celluloses are the most commonly used. They have found extensive applications in the fractionation of enzymes (40-43), serum proteins (44-48), hormones (49-52) ... etc. For experiments at very low pH, phosphorylated (P) cellulose (37) and sulfoethyl (SE) cellulose (53), and for experiments at very high pH guanidinoethyl (GE) cellulose (54) and triethylaminoethyl (TEAE) cellulose (53) may be used. The crosslinked dextrans and polyacrylamide ion-exchangers have shorter histories and their full potentialities remain still to be realized.

(C) Partition Chromatography

The principle of partitioning between two immiscible or partially miscible phases is applicable also to proteins. However, considerable disadvantages are encountered in relation to proteins, due to their (i) low solubility in the organic phase (especially at low temperatures at which most of these experiments are performed), and (ii) denaturation in the organic solvents and at the interfaces of the two liquids. Consequently, examples of separation and purification of proteins by this method are somewhat limited. One of the first applications, however, was the purification of catalase by partition between ethanol and aqueous ammonium sulphate (55). Other examples are fractionation of ribonuclease (56) by the use of the aqueous ammonium sulphate-cellosolve system, of insulin (57), chymotrypsin (58) and γ-globulins (59) with the ethylbutylcellosolve-sodium phosphate system, and of phenylcarbamyl derivatives of insulin (60) with the butanol-trichloroacetic acid system.

If one considers gel filtration as a special type of partition chromatography, the number of applications of this technique to protein fractionation becomes enormous, which have been covered in reviews by Determann (61) and Andrews (62). The elegance of this method can be ascribed to (i) the inertness of the column materials which possess negligible adsorption properties and have practically no denaturating effect on proteins, (ii) mild elution conditions since no extreme salt or hydrogen ion concentrations are required, and (iii) predictable elution volumes since in the absence of other mechanisms, all solutes are eluted in a volume not greater than that of the bed (c.f. theory in Chapter II). Column materials include starch (63), dextran (10), agar or agarose (64-68) and polyacrylamide (69-72) gels. Applications range from "desalting" of protein solutions, i.e. removal of salt and other small molecules (21, 72-83), to fractionation of

peptides (84-95), oligo- and polysaccharides (96-108), nucleotides and nucleic acids (101, 109-118), enzymes and serum proteins (63,66,72, 119-128), viruses (67,129) and even subcellular particles (65). The application of this method to the evaluation of molecular parameters of proteins will be described in Chapter II together with the theory of gel filtration.

(D) Paper and Gas Chromatography

Although paper chromatography has been extensively applied to the fractionation of amino acids and peptides, its use with proteins is rarely known. Together with electrophoresis (electrochromatography), however, it has been frequently used especially for characterization and identification of proteins and peptides. The application of gas chromatography to protein separations is impractical because of the involatility of proteins of high molecular weight.

CHAPTER II

THEORY OF GEL FILTRATION

Introduction

The term "gel filtration" was suggested by Porath and Flodin (10) for the chromatographic separation of molecules, on the basis of differences in molecular size, on columns of xerophilic granulated gels, i.e. gels that can swell reversibly. This procedure has also been designated as gel filtration chromatography (130), molecular sieve chromatography (131), exclusion chromatography (132) and gel permeation chromatography (133).

Although this method was seldom used until the present decade, its history can be traced back to more than a century ago. In 1861, Sir Thomas Graham introduced dialysis as a means of separating colloids from crystalloids. The method has subsequently become an indispensible tool for the contemporary biochemist and polymer chemist. We may consider a gel filtration column as the combination of many dialysis membranes. In the 1920's, a number of papers appeared on zeolites which had the interesting property of sorbing small molecules in preference to large ones, and which were given the name of "molecular sieves". The fundamental work of Barrer (134) has led to a thorough understanding of the structure and function of zeolites and has stimulated the preparation of synthetic zeolites on a technical scale. For example, nowadays, the Linde Molecular Sieves* are extensively used for the fractionation of hydrocarbons in the petrochemical industry. In ion-exchange chromatography, it has been observed that the capacity of a resin for an ion depended on the size of the ion (135). Moreover, the capacity of a resin for large ions is increased by decreasing

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* Manufactured and distributed by Union Carbide.

the degree of crosslinking, thus assuring a larger pore size (136). The explanation for this phenomenon may therefore be due to a discrimination induced by the three-dimensional matrix to which the fixed charges are connected. On passing non-electrolytes through a bed of an ion-exchange resin, effects are encountered which depend on the molecular sieve action of the resin network. Wheaton and Bauman (137) found that both ion-exchange and sieve effects were responsible for the separations obtained in crosslinked polystyrene ion-exchangers. With non-ionic crosslinked polystyrene gels, Vaughan (138) and Moore (133) achieved fractionation of high polymers in organic medium, and gel permeation chromatography, referred to as the GPC technique, is now widely used in the polymer industry.

The first publication on the use of water-swollen supporting media for the fractionation of hydrophilic compounds appeared in 1956 when Lathe and Ruthven (63) reported the graded penetration of solutes of different molecular sizes into columns packed with starch granules. A solution of hemoglobin, insulin and bacitracin A was thus fractionated into its three components. However, the great impetus to the field was provided in 1959 when Porath and Flodin (10) introduced the use of particulate dextran gels. Since that time, as described in Chapter I, other similar gels have been prepared and numerous applications have been reported.

Theory of Gel Filtration

Essentially two explanations have been proposed for the mechanism of gel filtration. Lathe and Ruthven (63), Flodin (139), Porath (92) and Pedersen (132) have attributed the difference of elution volumes of different molecules to the molecular sizes of the solutes. According to this concept, gel filtration is the result of exclusion due to dimensional heterogeneity of the gel interstices. Steere and Ackers (68,140), however, claimed that, at least for very loosely crosslinked gels like agar and Sephadex G-200, the

elution volume was controlled by a restricted diffusion process due to a steric and a frictional interaction of the solute molecules with the gel matrix. Both mechanisms certainly operate in gel filtration. It has been shown that the diffusion rate of a macromolecule is decreased in both polysaccharide gels (141) and in polysaccharide solutions (142), and that this decrease is a function of the size of the macromolecule. Furthermore, it has been shown, by equilibrium dialysis (143), osmometry (144) and solubility (145) studies, that polysaccharides such as hyaluronic acid and dextran exclude a certain volume of solvent from penetration for other solute macromolecules.

Both schools of thought recognize that the separation of solutes depends on differences in partition coefficients, that there is a mobile liquid phase and a stationary phase of imbibed liquid inside the gel, and that, in the absence of specific interaction effects such as adsorption and ion-exchange, the partition coefficient of the solute can be calculated from its elution volume and the volumes of the two phases.

Relationship Between Partition Coefficient and the Various Volumes (139)

One may designate the volume of the mobile liquid phase exterior to the gel as Vo (void volume) and the volume of the stationary imbibed liquid phase interior to the gel as Vi (imbibed volume). The resistance encountered by the flow of water in the gel phase is very high (compare, for example, the high flow resistance in polyacrylamide gels (146)). Therefore, it may be assumed that the flow of water through the column takes place primarily, if not exclusively, in the exterior liquid phase.

In the ideal case, a substance introduced into the system (the column) will equilibrate instantly between the liquid and the gel phases. Only a certain fraction, K_D, of the gel phase is, however, available for the substance. This means that the total volume in the gel phase available to the substance is $K_D \cdot Vi$. If the solute is completely excluded by the gel, it will be

eluted after passage of a volume, Ve, which is equal to the void volume. If, however, a fraction of the volume in the gel phase is available for the solute, only that part which is in the liquid phase at any given moment will move down the bed. The average speed of all solute molecules will be equal to the flow rate times the fraction of molecules which is present at each instance in the liquid phase. The fraction of solute molecules that moves at each moment will be Vo / (Vo + K_D· Vi). The elution volume of the substance will be, therefore, (Vo + K_D· Vi) / Vo times larger than that of a substance which is completely excluded from the gel phase. The equation for the elution volume of the substance will be

$$Ve = Vo + K_D \cdot Vi \qquad \text{Eqn. (1)}$$

The above equation can be readily rearranged for the computation of K_D, the partition coefficient:

$$K_D = \frac{Ve - Vo}{Vi} \qquad \text{Eqn. (2)}$$

Ideal Chromatogram and Separability

Under ideal conditions, that is, in the absence of zone spreading, the frontal analysis of a solution containing two solutes with partition coefficients K_D' and K_D'' yields an elution diagram as shown in Figure 1. The two solutes appear in the effluent at volumes Vo + K_D' Vi and Vo + K_D'' Vi respectively. It is apparent from this diagram that for complete separation of the two solutes, the volume of the sample, Vs, should conform to the following condition:

$$Vs < (K_D'' - K_D') Vi$$

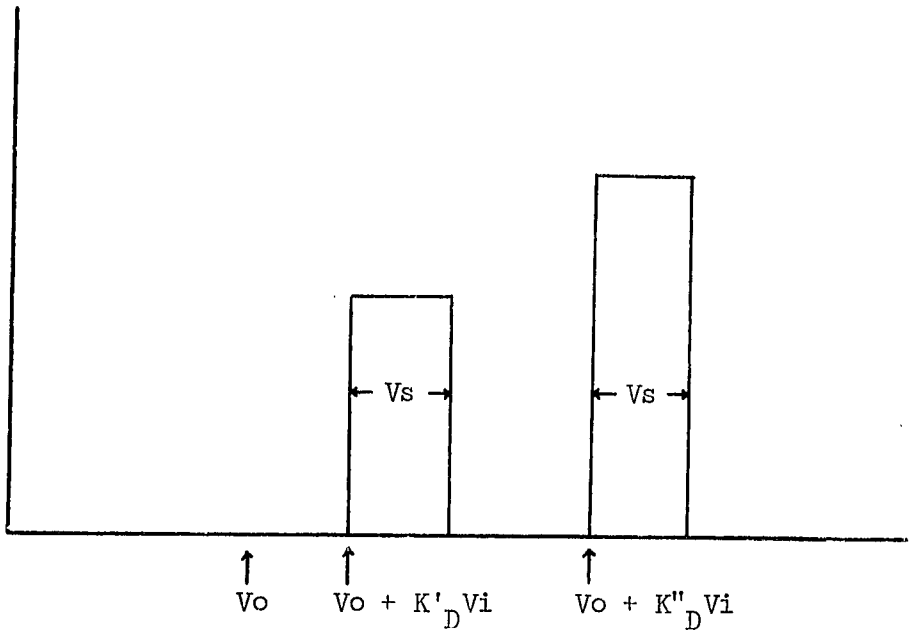

FIGURE 1 : Ideal gel filtration chromatogram of the fractionation of a sample containing two solutes.

Non-Ideal Conditions

In practice, ideal conditions for column operation are never attained and there is always some distortion of the ideal profile. Similar to other kinds of chromatography, the three most important factors causing deviations from ideal behaviour are also encountered in gel filtration. (i) Local non-equilibrium. A solute requires a finite time to diffuse into and out of the gel grain, that is, the diffusion equilibrium is not attained simultaneously and a local non-equilibrium condition arises. (ii) Longitudinal diffusion. Generally, this is of little importance in view of the time required for a gel filtration experiment, with the exception of the case when an extremely slow flow rate is being used. (iii) Irregular flow. Imperfect packing and lack of uniformity in particle size of the packing material usually lead to irregular flow of the eluant through the column.

This is practically the only important cause of deviation from ideal behaviour with a non-penetrating solute. For a penetrating solute, this effect is superimposed on the distortion of the profile resulting from local non-equilibrium.

For an elution profile having the shape of a Gaussian error function, the number of theoretical plates, N, for a solute in a column is given by Glueckauf's equation (17):

$$N = 8\left(\frac{Ve}{\beta}\right)^2 \qquad \text{Eqn. (3)}$$

where β is the width of the elution curve at the height $C_{max}/2.72$ over the base line, C_{max} being the maximum concentration.

Empirical Correlations

In their early chromatographic studies with starch columns, Lathe and Ruthven (63) pointed out the possibility of estimating molecular weights of solutes from gel filtration data. Without giving any justification, a number of workers have obtained linear relationships between the logarithm of the molecular weight of the solute and the partition coefficient or elution volume in gel filtration studies with agar (66), dextran (100,147), polyacrylamide (72) and even granulated latex rubber (148) columns. Although such relationships do not reveal any specific features of the mechanism of gel filtration, they provide a simple calibration method for the determination of molecular weights of the solutes.

The Restricted Diffusion Approach

Steere and Ackers' restricted diffusion mechanism (68,140) is based on the model in which the fluid regions within the gel particles are represented by uniform cylindrical channels. Each gel particle within the column presents a diffusion barrier to molecules in the exterior mobile phase. For a penetrating molecular species, this barrier can be characterized by two

types of interaction. (i) If the molecular radius, r_s, is smaller than the pore radius, r, the molecule can penetrate the gel only if its centre passes within a virtual pore of radius $r - r_s$. The probability of penetration of a given molecular species, being a function of $r - r_s$, is, therefore, less than the corresponding probability for a smaller species. (ii) If a molecule does enter the channel, it encounters increased hydrodynamic frictional resistance to motion and, therefore, has a lower diffusion coefficient than it would have in free solution. This restriction will be obviously greater for a larger molecular species. Partitioning of a molecular species between liquid phases interior and exterior to the gel is then governed by these two types of diffusional restriction.

To quantitate the above two effects, Steere and Ackers used Renkin's equation (149), i.e.

$$\frac{A_R}{A_o} = \left(1 - \frac{r_s}{r}\right)^2 \left[1 - 2.104\,\frac{r_s}{r} + 2.09\left(\frac{r_s}{r}\right)^3 - 0.95\left(\frac{r_s}{r}\right)^5\right] \quad \text{Eqn. (4)}$$

In this equation, the Stokes radius, r_s, of a macromolecule diffusing within a restrictive barrier of effective pore radius r is related to the equivalent free cross-sectional pore area, A_o, and the effective actual area, A_R, available for diffusion. The term $\left(1 - \frac{r_s}{r}\right)^2$ accounts for the steric effect as proposed by Ferry (150). The term in the square bracket was derived on theoretical grounds by Faxen (151) as a correction to Stokes' Law for the case where the dimensions of the chamber through which a particle is moving are of the same order of magnitude as those of the particle itself. Steere and Ackers proposed that the effective solute distribution ratio was governed by steric and frictional hindrances to diffusion and that K_D was equal to A_R/A_o. The validity of equation 4 was tested by a comparison of K_D values calculated and experimentally determined for solutes of known r_s.

It was found that experimental data fitted the equation well only when very porous gels were used (i.e. Sephadex G-200 and 2-12% agar) but poorly in the case of less porous gels, e.g., Sephadex G-75 and G-100.

The Molecular Exclusion Approach

In the following treatments, the penetrability of a gel for a certain solute was assumed to depend only on the geometry of the gel, and on the dimensions of the "cavities" in the gel. Difference of diffusion rates of the solute into and out of the gel phase was considered to be negligible. Implicitly, an instantaneous equilibrium partition of the solute between the liquid phase and the penetrable part of the gel phase was assumed.

Porath (92) assumed (i) that the cavities in the gel are conical in shape and of a certain average size with a diameter of A and a total depth of H, (ii) that A^3 and the volume of solvent in the gel, "solvent regain" S_r (c.f. water regain in Chapter III), bear a linear relationship, and (iii) that the radius of gyration of a given macromolecule is proportional to the square root of its molecular weight, M. On the basis of these assumptions, Porath arrived at the following expression,

$$K_D = k \left[1 - k_1 \frac{M^{1/2}}{(S_r - \alpha)^{1/3}} \right]^3 \qquad \text{Eqn. (5)}$$

where k, k_1 and α are constants. The experimental data on low molecular weight dextran fractions published by Granath and Flodin (100) were used to test the validity of this equation. When the square roots of the molecular weights of the solutes were plotted against the cubic roots of the corresponding partition coefficients, straight lines were obtained as expected from equation (5). From the slopes and intercepts of these plots, k, k_1 and α were obtained. These constants and equation (5) were then used to calculate the theoretical K_D values of six cellodextrins on Sephadex G-25.

Agreement between the experimental and theoretical values was very good.

Unaware of Porath's treatment, Squire (152) used a model consisting of cones, cylinders and rectangular parallelipipeds. Assuming the radius of gyration of a solute to be proportional to the cube root of the molecular weight, M, he derived the similar equation

$$\frac{Ve}{Vo} = \left[1 + g \left(1 - \frac{M^{1/3}}{C^{1/3}} \right) \right]^3 \qquad \text{Eqn. (6)}$$

where g is a constant and C is the molecular weight of the smallest protein that cannot enter the gel. Squire tested the validity of this equation with the appropriate chromatographic data obtained with four different Sephadex gels (i.e. G-200, G-150, G-100 and G-75) using a variety of proteins. The agreement between observed and theoretical values for Ve/Vo for Sephadex G-200 and G-150 gels was very good but serious deviations were observed for the other two more crosslinked gels. Surprisingly, however, the chromatographic data of Granath and Flodin (100) for the fractionation of dextrans on Sephadex G-85 agreed excellently with the values derived by Squire using equation (6).

In the author's opinion, the most elaborate approach was that of Laurent and Killander (153). Using a model of a uniform random suspension of straight and rigid fibres of definite length and negligible thickness, Ogston (154) derived an equation for the distribution of spaces in such a model network.

$$P = e^{-\pi L(r_s + r_r)^2} \qquad \text{Eqn. (7)}$$

In this equation, P is the probability of penetration or rotation of a spherical particle of radius r_s in the model network, L is the total fibre length per unit volume and r_r is the half-thickness of the fibre. Laurent and Killander (153) proposed that this model could be used to represent the

structure of the Sephadex gels and that K_D would be equal to P if r_s is taken as the radius of gyration of the solute in the gel filtration column. Thus, using reasonable but arbitrary values of L and r_r, they obtained the K_D values of a variety of proteins of known r_s. Very good agreement between theoretical and experimental K_D values observed with Sephadex G-200, G-100 and G-75 columns has been obtained in this manner. It should be noted that in addition to the rigorous mathematical treatment of Ogston, this approach does not require the assignment of specific geometrical shapes for the obviously highly complex internal structure of the gel, and does not assume any relationship between the molecular weight and the radius of gyration of the solute. In Chapter III, an extension to this method will be made by calculating the values of L from the physical and molecular properties of the polyacrylamide gels synthesized. The K_D values calculated will be compared with the corresponding results obtained in this investigation.

CHAPTER III

PREPARATION AND EVALUATION OF POLYACRYLAMIDE GELS FOR GEL FILTRATION

Introduction

According to Hermans (155), gels are defined as materials which satisfy the following conditions: (a) they are coherent, colloid, disperse systems of at least two components, (b) they exhibit mechanical properties resembling those of the solid states, and (c) both the dispersed component and the dispersion medium should extend continuously throughout the system. This definition would thus apply to a large number of systems, extending from low molecular weight substances, such as sodium chloride in alcohol provided by the reaction of gaseous hydrogen chloride with a strong solution of sodium alcoholate, to macromolecular systems of the type formed by cooling solutions of agar and gelatin.

For use as a molecular sieve in gel filtration, a swollen network possessing good mechanical and chemical stability and devoid of ionic groups gives the best results. For aqueous systems, most frequently encountered in protein chemistry, it would therefore be desirable to use polymers which contain hydrophilic groups of non-ionic character, and which are crosslinked by stable covalent bonds throughout their three-dimensional network.

The present study constitutes an extension of the work done in this laboratory on the preparation of a number of synthetic gels from well-defined vinyl and divinyl water-soluble monomers (69). Initially, N-vinyl pyrrolidone, vinyl ethyl carbitol and acrylamide were all used. As work progressed, the N-vinyl pyrrolidone system was discarded because of the undesirable adsorptive properties of the product polymer, and the vinyl ethyl carbitol system was abandoned due to the discontinuation of commercial production of the monomer. The system of acrylamide and N,N'-methylene bisacrylamide was found to satisfy

the criteria mentioned in the previous paragraphs and was adopted for the present study.

Materials

Acrylamide, N,N'-methylene bisacrylamide, ammonium persulphate, and β-dimethylaminopropionitrile were generously donated by Cyanamid Co. of Canada Ltd. Chlorogenic acid and caffeic acid were purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin. Histamine, 5-hydroxyindole-3-acetic acid, tryptophane and sodium chloride were reagent grade chemicals supplied by Fisher Scientific Co., Montreal. Bovine γ-globulin and lysozyme were purchased from Pentex Inc., Kankakee, Illinois. Bovine fibrinogen, bovine hemoglobin, bovine serum albumin, pepsin, ovomucoid, trypsin and ribonuclease were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Conjugates of bovine serum albumin with chlorogenic acid*, caffeic acid*, 5-hydroxyindole-3-acetic acid** and histamine** were prepared in this laboratory.

Experimental

Preparation of Polymers

Sixteen different crosslinked polymers were prepared by varying the relative and absolute concentrations of acrylamide and N,N'-methylene bis-acrylamide. For the sake of brevity, the different polymers will be identified with a code consisting of two numbers; the first number denotes the number of grams of acrylamide in 100 ml of the reacting solution and the second number denotes the number of grams of the crosslinking agent, N,N'-methylene bis-acrylamide, used with every 100 grams of acrylamide. The amount of each

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* Thanks are due to Mrs. S.H. Tenenhouse for these compounds.

** Thanks are due to Dr. N.S. Ranadive for these compounds.

component used in each preparation are listed in Table II. To illustrate the method of preparation, a detailed description of polymer "5-5" is given below.

Acrylamide (25.0 gm) and N,N'-methylene bisacrylamide (1.25 gm) were dissolved at room temperature in boiled distilled water. The solution was filtered, diluted to 500 ml with boiled distilled water, deaerated by a water pump for approximately five minutes, and then 0.5 ml of β-dimethylamino-propionitrile (DMAPN) and 0.5 gm of ammonium persulphate (AP) were cautiously added with gentle stirring. The reaction vessel was then placed into a constant temperature water bath at 50°C. The initiation of polymerization was evidenced by the appearance of turbidity within a few minutes. To ensure complete polymerization, the reaction was usually allowed to proceed overnight. The gel formed was cut into small pieces and washed exhaustively with distilled water to remove any unreacted monomers. It was then freeze-dried, pulverized in a mortar and separated into batches of uniform granular size by passage through a series of standard sieves. It was kept in the dry form before use.

Determination of Water Regain Values

A method similar to that of Pepper et al (156) was used. The polymer granules were allowed to swell in water for at least 24 hours. About 15 ml of the gel was then transferred into a weighed Lusteroid tube (100 x 16 mm), the bottom of which was perforated and covered with glass wool. The tube was suspended inside a glass centrifuge tube (120 x 35 mm), which was provided with a constriction (at about 30 mm from the bottom) so that the lower part acted as a reservoir for the filtered liquid. The water in the interstitial space of the gel was removed by centrifugation at 300-1000 g for about 20 minutes at 4°C. The Lusteroid tube containing the gel was weighed and then dried to constant weight in a vacuum desiccator at room temperature. From the difference of the two weights and after appropriate correction for the

TABLE II : Composition of 16 acrylamide-methylene bisacrylamide copolymers

Solvent:	boiled distilled water
Initiators:	0.5 ml β-dimethylaminopropionitrile + 0.5 gm ammonium persulphate
Reaction Volume:	500 ml
Reaction Temperature:	50° C
Reaction Time:	overnight

Gel	Acrylamide	Methylene Bisacrylamide
5 - 5	25.0 gm	1.25 gm
5 - 10	" "	2.50 "
5 - 15	" "	3.75 "
5 - 20	" "	5.00 "
10 - 5	50.0 "	2.50 "
10 - 10	" "	5.00 "
10 - 15	" "	7.50 "
10 - 20	" "	10.00 "
15 - 5	75.0 "	3.75 "
15 - 10	" "	7.50 "
15 - 15	" "	11.25 "
15 - 20	" "	15.00 "
20 - 5	100.0 "	5.00 "
20 - 10	" "	10.00 "
20 - 15	" "	15.00 "
20 - 20	" "	20.00 "

water absorbed by the glass wool and the Lusteroid tube, the water regain value (denoted as Wr) was calculated as grams of water imbibed per gram of dry gel.

Chromatography

The method used was essentially that described by Flodin and Kupke (157) for cellulose columns.

Preparation of Gels

The dry polyacrylamide powder used, unless otherwise mentioned, was the fraction passing through 100-200 mesh sieves. It was suspended in the appropriate eluant, usually 0.9% saline (w/v) to allow it to swell. After a sedimentation time of 30 to 60 minutes, the fines remaining in the supernatant were removed by decantation. This procedure was repeated at least three times. The volume of the swelling medium, i.e. the eluant, was chosen so that the ratio of the supernatant to sediment was at least 5:1.

Packing of Columns

The columns used were cylindrical glass tubes of 25 mm internal diameter and 500 mm in length, provided with a sintered glass plate joined to a fine capillary at the bottom of the column and with a female ground glass joint at the top for connection to an extension tube of identical bore. Before packing, the column with the extension tube was mounted vertically and filled with the eluant to a mark just above the ground glass joint. Care was taken that no air bubbles were present in the column. The well-stirred and swollen gel slurry was then added carefully through the extension tube avoiding disturbances due to convection. The suspended particles were allowed to sediment and once the "bed" was 1 to 2 cm high, the flow was started. A rising horizontal boundary of packed material was considered evidence of good packing. To compensate for the increasing resistance to flow with increasing

column height, the outlet tube was progressively opened. An attempt was always made to add the calculated amount of gel slurry for a desired height of bed; for best results, the number of additions of the gel slurry was kept to a minimum. After the column had been packed to the desired height, the extension tube was removed and the column was connected to the reservoir containing the eluant. For stabilization of the bed, it was percolated for at least the time necessary to collect a volume of eluate equivalent to the bed volume, or overnight, with the eluant. To avoid any change of bed volume, the hydrostatic pressure was maintained practically constant by provision of a large reservoir (2 l) placed approximately 2 to 3 feet above the volumn; elution was done at constant temperature, usually inside a cold room at $4^{o}C$.

Application of the Sample

First it was made certain that the column was well washed, i.e. no UV absorbing material was detectable in the eluate. Most of the liquid over the bed was carefully sucked off and the last few ml were allowed to pass into it. At the moment when the surface liquid was about to vanish, the sample, usually not larger than 1 ml and consisting of artificial mixtures of 1 to 4 components of the substances listed in Table III, was carefully added along the wall of the glass tube from a micropipette. Then, the flow was started and the sample allowed to enter the bed. As it was just about to disappear, a few ml of the eluant were added gently to wash the surface. Finally, the surface above the bed was filled with eluant and the column was connected to the reservoir.

Elution

The flow rate was maintained as constant as possible, usually at 20 ml/h, by varying the height of the hydrostatic head and by adjusting the Castaloy

hosecock clamps at the inlet and outlet tubes. In the event that such a rate was not attainable, the column was allowed to flow at its maximum rate. The eluates were collected in fractions of 2-5 ml with a Radi Rac LKB fraction collector.

Control of Packing

The homogeneity of packing was checked by passing a horizontal narrow coloured zone of o-nitrophenol through the bed. The column was repacked if the zone became skewed during the passage. Occasionally the state of the column was inspected by eluting a sample of bovine serum albumin, the volume of which was 5-10% of the bed volume. The condition of the column was considered satisfactory when 90% or more of the solute was eluted in a volume not exceeding 20% of the bed volume.

Preservation of the Beds

To avoid microbial growth when a packed column was not being used for a long time, a small amount of chloroform or toluene was added to the last portion of the eluant.

Analytical Methods

The optical densities of the effluent were recorded automatically with an LKB Uvicord Spectrophotometer at 254 mμ or manually with a Beckman DU Spectrophotometer at 280 mμ. In addition, bovine γ-globulin, bovine serum albumin and lysozyme were qualitatively identified by "ring" tests with the appropriate homologous rabbit antisera, by paper electrophoresis and by sedimentation velocity experiments. The identities of hemoglobin and the various protein-hapten conjugates were established by comparing their spectra over the range of 200-600 mμ with those of the pure compounds. The concentrations of these substances were determined spectrophotometrically at 490 and 280 mμ, respectively. Trypsin, ovomucoid and pepsin were identified by

their electrophoretic mobility on paper. Chlorogenic acid, caffeic acid, 5-hydroxyindole-3-acetic acid, histamine and tryptophane were measured spectrophotometrically at 280 mμ and compared with the spectra of the pure compounds in the ultraviolet spectral region. The antibody content of a serum or of a corresponding chromatographic fraction was determined by micro-hemagglutination and/or the precipitin method.

Preparation of Antisera*

Antisera were produced in rabbits against two different protein antigens, bovine serum albumin and lysozyme. Albino rabbits of 3 to 4 kilograms were injected into the marginal ear vein with 1 ml of a 1% solution of the antigen three times per week. Blood samples were collected periodically starting one week after the last injection and were tested by the "ring" test for antibody content. When a good titer had been reached, 50 to 70 ml of blood were collected from each rabbit from the margical ear vein three times in two weeks. The blood samples were allowed to clot at room temperature and were then placed in the cold to allow the clot to retract. The clotted bloods were centrifuged and the sera were decanted. The sera were filtered into sterilized vials through sterilized Seitz filters and stored at $4^{\circ}C$ or in a deep freezer until used.

"Ring Test"

About 0.5 ml of antiserum was pipetted into each of a series of culture tubes (6 x 50 mm). Then, 0.5 ml of the antigen solution at various concentrations was carefully layered above the antiserum. The tubes were incubated at room temperature for 5 to 30 minutes and were observed against an illuminating

*Thanks are due to Mr. M. Rabasse for the injection, care and bleeding of the animals.

neon light. The appearance of a white precipitin ring at the boundary of the layers indicated the presence of antibody in the serum.

Precipitin Experiment

The antibody content of whole antisera and of the corresponding chromatographic fractions was determined by quantitative precipitin tests performed according to the method of Heidelberger and Kendall (158). One ml volumes of the antiserum (or eluate) were added to a series of tubes each containing decreasing amounts of the homologous antigen in 1 ml volume. After mixing the two reactants, the solutions were incubated at 37°C for 2 hours, followed by incubation at 4°C for 24 to 48 hours. The precipitates were then collected by centrifugation and washed three times with cold saline. The washed precipitates were digested with sulphuric acid and analysed for nitrogen by the micro-Kjeldahl method (159). Alternatively, the washed precipitates were dissolved in 0.1 N hydrochloric acid and were analysed for total protein spectrophotometrically at 280 mμ. The precipitin curves were obtained by plotting the amount of specific precipitate against the amount of antigen added. The antibody content of the sample was calculated at the point of the curve where precipitation was maximal. A typical precipitin curve is shown in Figure 2.

Micro-Hemagglutination Test

The micro-hemagglutination method employed was that of Takatsy (160), as modified in this laboratory (161). Rabbit erythrocytes were used in this test. For this purpose, blood was collected from the marginal ear veins from "normal", i.e. non-immunized, rabbits, into a container with an equal volume of Alsever's solution (66) and stored at 4°C for not more than 24 hours. The serum used for the preparation of the "diluent" was obtained from the blood of the same rabbit which supplied the erythrocytes and the complement

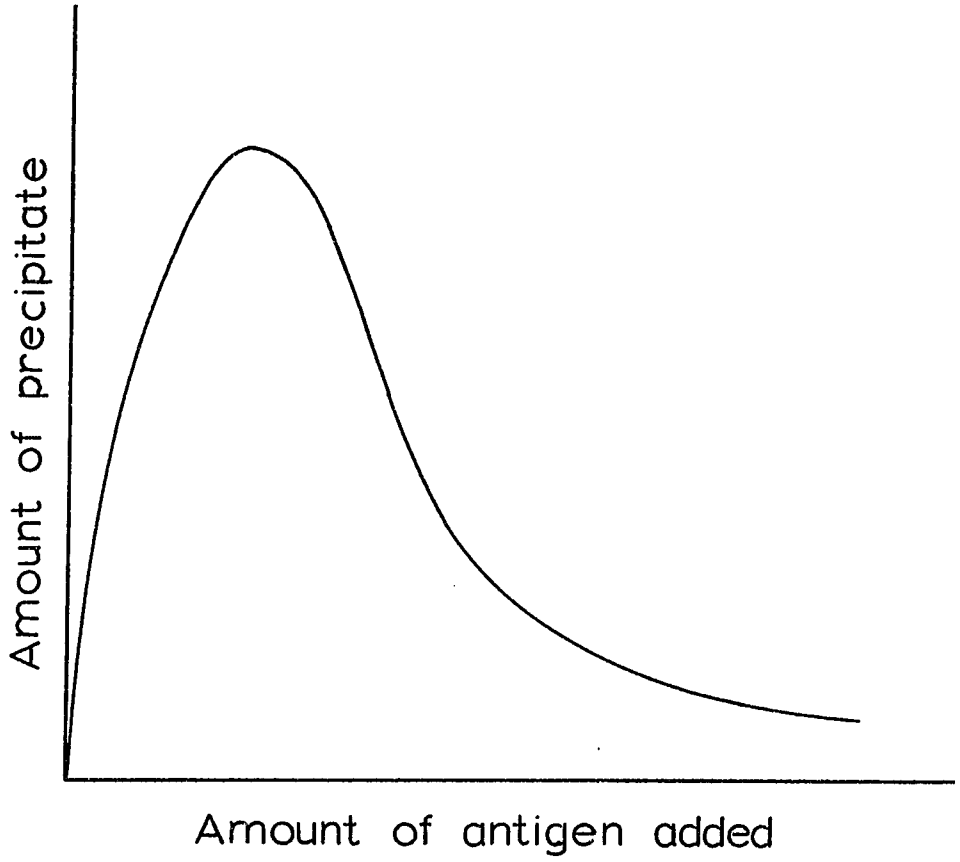

FIGURE 2 : Typical precipitin curve.

was inactivated by heating at 56°C for 30 minutes. The serum was then diluted 100-fold with phosphate buffer at pH 7.3.

The bis-diazotized-benzidene (BDB) used for coupling the antigens to the red blood cells was prepared according to the method developed previously in this laboratory (163) and was kept in small vials at -20°C until required. Before coupling the antigen to the erythrocytes, the frozen BDB was gently melted and then quickly diluted 15-fold with phosphate buffer at pH 7.3, which had been precooled to 4°C. It is recommended that this BDB-phosphate solution be used immediately due to the instability of the diazo compound.

Sensitization of the Erythrocytes

The packed rabbit red blood cells were washed three times by resuspension in cold physiological saline and centrifugation. The washed packed cells were then resuspended in an equal volume of saline. The optimal quantity of antigen was placed in a 15 ml centrifuge tube and mixed with 0.1 ml of the "50%" red blood cell suspension, i.e. red blood cell suspended in an equal volume of physiological saline. To this mixture was added the optimal amount of the freshly prepared BDB-phosphate solution. The reaction was allowed to proceed at room temperature for exactly 15 minutes with occasional stirring. The "sensitized" cells were separated by centrifugation and the brownish supernatant was discarded. The cells were washed with 3.5 ml of diluent, centrifuged and resuspended in diluent to a final volume of 5 ml to give a 1% (v/v) red blood cell suspension.

Standardization of the Method

To establish the optimal relative amount of BDB to antigen, the following procedure was used. With a constant amount of erythrocytes, (i) the BDB-phosphate volume was varied at constant antigen concentration, and (ii) the antigen concentration was varied at constant BDB-phosphate volume. Each batch of sensitized cells was then tested with normal rabbit serum and with

the appropriate antiserum. The proportion of BDB-phosphate volume to antigen concentration was considered optimal when the highest titer was obtained with the antiserum and no reaction resulted with the normal serum. The titer of a hemagglutination reaction is defined as the reciprocal of the highest dilution of the antiserum at which a positive agglutination reaction is detected. A negative reaction is manifested as a red compact button at the bottom of the well; in a positive reaction, the bottom of the well is covered with a layer of dispersed reddish yellow cells.

Performance of the Micro-Hemagglutination Test

Plexiglass plates (purchased from Metrimpex, Budapest, Hungary) provided with six rows of twelve wells each were used. The wells were provided with hemispherical bottoms with an effective capacity of 0.125 ml. A volume of 0.05 ml of diluent was pipetted into each well by means of a specially calibrated dropper. The absorbed serum was serially diluted in halving dilutions with a calibrated 0.05 ml stainless steel loop. Then 0.025 ml of the sensitized cell suspension was added to each well. The plates were gently shaken to ensure even dispersion of the cells, and covered to minimize evaporation. The reaction was allowed to proceed at room temperature and the titer was estimated four to twelve hours later.

Paper Electrophoresis

Whatman No. 3 filter paper was used. A paper strip (14 x 56 cm) was moistened in phosphate buffer (pH 7.0, $\Gamma/2$ 0.025) and gently blotted to remove excess buffer. It was then placed on a plexiglass support and approximately 0.05 ml of each sample and of the control protein were applied centrally (i.e. perpendicularly to the direction of ion migration) on a line about 2 cm wide. The paper was then placed on a glass tray in a horizontal position, serving as a bridge between two buffer chambers (filled with phosphate buffer pH 7.0 $\Gamma/2$ 0.025), which in turn were connected to the outer

electrode chambers (filled with 20% KCl solution) with wet paper bridges. Electrophoresis was then carried out by applying a constant D.C. voltage (110 volts, 7.5 milliamps), with the aid of a voltage regulator, for 24 hours at 4°C. At the end of the electrophoresis, the paper was removed and the excess moisture immediately blotted from the buffer soaked ends. It was then dried at 110°C and stained with a solution of 0.1% Brilliant Blue in 5% acetic acid. Removal of the background stain from the filter paper was achieved by repeated washings with 5% acetic acid.

Sedimentation Velocity Experiment

For identification of various proteins and chromatographic fractions, their sedimentation coefficients were determined by sedimentation velocity experiments in a Spinco Model E ultracentrifuge at 59,780 r.p.m. and 25°C.* The samples were dissolved in 0.9% saline and standard cells were used. Sedimentation coefficients were calculated with the classical Svedberg equation (163)

$$S = \frac{1}{\omega^2} \cdot \frac{d \ln x}{d t} \qquad \text{Eqn. (8)}$$

where S is the sedimentation coefficient, x the distance of the sedimenting boundary from the centre of rotation at time t, and ω the angular velocity of the rotor. Absolute sedimentation coefficient values were not necessary for this qualitative identification. Consequently, the sedimentation coefficients of the analysed samples were compared with those of pure protein of approximately the same concentration and under the same conditions of centrifugation.

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* Thanks are due to Mr. H. Sanderson for these determinations.

Calculation of Partition Coefficients from Chromatographic Data

The elution volume, Ve, of each compound was estimated from the measured volumes of the fractions as indicated by the recordings of the Uvicord. The void volume of a given gel, Vo, was determined by the elution of a sample of dilute India ink or of a high molecular weight protein such as fibrinogen. The imbibed volume, Vi, was calculated by multiplying the number of grams of the gel used for packing (denoted by b) by the water regain, W_r, of that particular gel, i.e. $Vi = b\ W_r$. For calibration purposes, the elution volume of each compound was established by passing the compound on its own through the chromatographic column. The partition coefficients of the various compounds eluted were calculated for each gel using the following equation (139):

$$K_D = \frac{Ve - Vo}{Vi} = \frac{Ve - Vo}{b\ W_r} \qquad \text{Eqn. (9)}$$

Calculation of Partition Coefficients from Gel and Solute Data

The calculations made in this section are based on the procedure proposed by Ogston, Laurent and Killander (153,154). In accordance with equation (7) (c.f. Chapter II), it would be possible to calculate K_D without chromatographic

$$K_D = e^{-\pi L(r_s + r_r)^2} \qquad \text{Eqn. (10)}$$

data, provided the appropriate molecular properties of the gel (L, total fibre length per unit volume, and r_r, half-thickness of the polymer fibre) and of the solute (r_s, radius of gyration of the solute) were known. An arbitrary value of 2 Å can be reasonably assigned for r_r. For common proteins, values of r_s are readily available in the literature. The variable remaining to be evaluated is L. Let us first define the symbols. Thus,

N_A = no. of monomeric acrylamide units per cm^3 of gel.

N_B = no. of monomeric methylene bisacrylamide units per cm^3 of gel.

l_A = segmental length per acylamide unit.

l_B = segmental length per methylene bisacrylamide unit.

A_v = Avogadro's number.

M_A = molecular weight of acrylamide.

M_B = molecular weight of methylene bisacrylamide.

V_B = bed volume of the column.

P_c = weight percent of methylene bisacrylamide in the copolymer.

Then,

$$L = N_A l_A + N_B l_B$$

$$= \frac{(1 - P_c)b}{V_B} \cdot \frac{A_v}{M_A} \cdot l_A + \frac{P_c\, b}{V_B} \cdot \frac{A_v}{M_B} \cdot l_B$$

$$= \frac{A_v\, Vi}{V_B\, W_r} \left[\frac{(1 - P_c) l_A}{M_A} + \frac{P_c\, l_B}{M_B} \right] \qquad \text{Eqn. (11)}$$

where b, W_r and Vi are defined as in the previous section.

The only unknown quantities in equation (11) are l_A and l_B. From the proposed chemical structure of polyacrylamide gels (Figure 3), the segmental length of an acrylamide unit may be considered as equivalent to two carbon-carbon single bonds and that of the methylene bisacrylamide unit to the sum of six carbon-carbon single bonds and two carbon-nitrogen single bonds. In the ideal case, l_A and l_B can therefore be calculated by using the published values of 1.54 Å and 1.47 Å for C-C and C-N bonds respectively. However, in crosslinked polymers like the present polyacrylamide gels, the fibres are never fully stretched. The chain lengths cannot be calculated by simple addition of bond lengths determined from small molecules. There is a "chain shortening" due to a variety of factors such as conjugation, mass aggregation, branching, crosslinking and imperfect swelling. It is, therefore, necessary to introduce a correction term, to be called the shortening factor S_f; thus

equation (11) becomes

$$L = \frac{A_v \; Vi \; S_f}{V_B \; W_r} \left[\frac{(1 - P_c) l_A}{M_A} + \frac{P_c \; l_B}{M_B} \right] \qquad \text{Eqn. (12)}$$

Reasonable values of S_f were obtained by the least square method by comparing values for L determined by equation (11) and by equation (10) using experimentally observed K_D values. Equations (12) and (10) were then used to calculate the theoretical partition coefficients for solutes of radii of gyration ranging from 0 to 100 Å. The computations were done with the aid of an IBM-7040 digital computer at the McGill Computing Centre.

Results

Structure

The probably structure of the network formed by the copolymerization of acrylamide and N,N'-methylene bisacrylamide shown in Figure 3 has been proposed on the basis of the known chemistry of the monomers and the vinyl polymerization process. A more detailed description of the copolymer network does not seem warranted. Obviously, slight modifications of the network could occur due to side reactions of the reactants. For example, the exothermic nature of the polymerization process and the close proximity of the hydrocarbon chains at high concentration of reactants, could have led to the intramolecular or intermolecular condensation of adjacent amide groups to yield an imide as shown schematically in Figure 4. This phenomenon has actually been observed in the mass polymerization of acrylamide alone by heating the monomer to approximately 150°C (164,165).

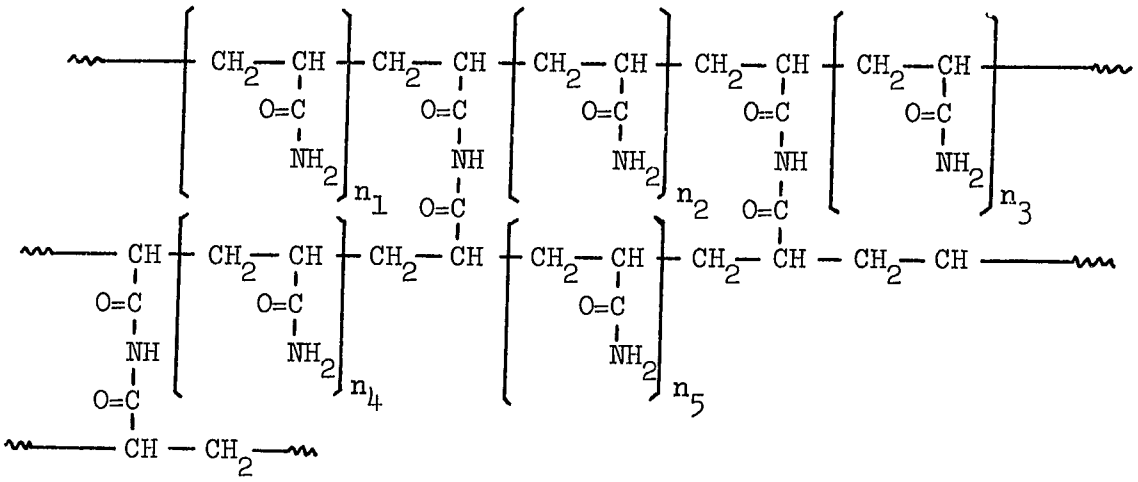
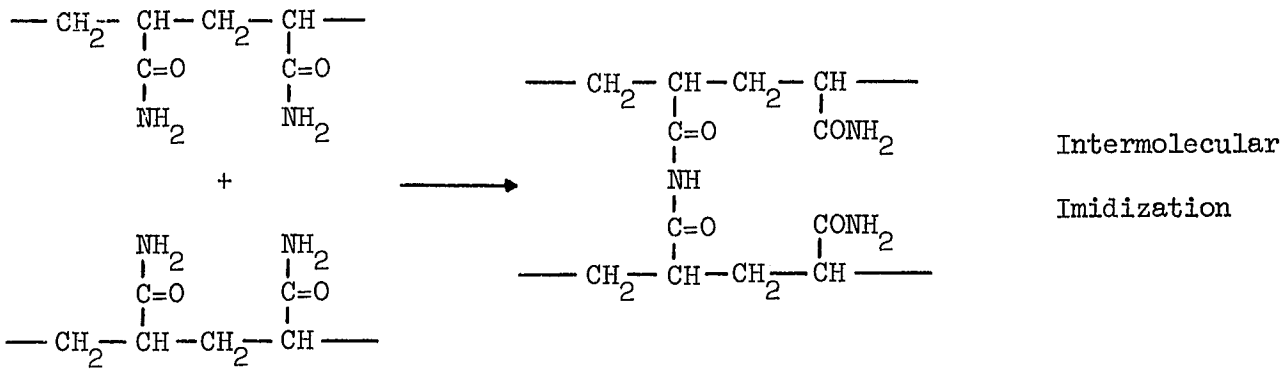
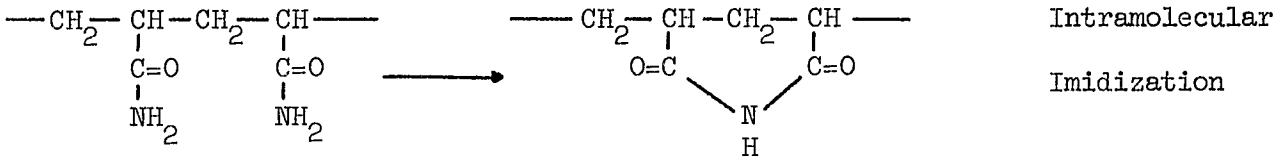


FIGURE 3 : Schematical structural formula of the acrylamide-methylene bisacrylamide copolymers. n_1, n_2, n_3, n_4 and n_5 are integral numbers.



Intermolecular Imidization



Intramolecular Imidization

FIGURE 4 : Inter- and intra-molecular imidization of adjacent amide groups in polyacrylamide

Physical Properties

When first prepared, the polyacrylamide polymers were in the form of a gel with a volume corresponding to the shape of the reaction vessel. In the dry and granular state, they were white, hard and tough powders. In the swollen state, however, they were brittle and disintegrated easily when crushed. The rigidity of the gels was found to increase with increasing concentrations of both mono- and divinyl monomers used in the copolymerization process and to depend particularly markedly on the degree of crosslinking at low monomer concentrations. This variation in rigidity, or rather in compressibility, affected considerably the maximally attainable flow rates of the column. Thus, in experiments with the more rigid gels (20-20, 20-15, 20-10, 20-5, 15-20, 15-15, 15-10, 15-5, 10-20, 10-15 and 10-10), flow rates were regulated to approximately 20 ml/hour, whereas in the case of the softer gels (10-5, 5-20, 5-15, 5-10 and 5-5), only much slower flow rates of the order of 2-10 ml/hour could be attained and often the columns became clogged and needed repacking.

The gels were found to be insoluble in water, aqueous salt solutions and common organic solvents. They could be used within the pH range of 2 to 11 and temperature range of 0 to 100°C. Lower pH could be tolerated for short periods, but very strong bases should be avoided due to the possibility of hydrolysing the amide group (166). Prolonged exposure of the gels to temperatures above 110°C was found to char the material.

Swelling

Water and aqueous buffer are the only common solvents in which the polyacrylamide gels swell. The water regain may be taken as a good indication of the degree of porosity of a gel. In Figure 5 are given the water regain values of the 16 gels prepared. It is evident that the amount of water accommodated within the gels depends on the concentration of both mono- and divinyl monomers; it increases with the decreasing concentration of the

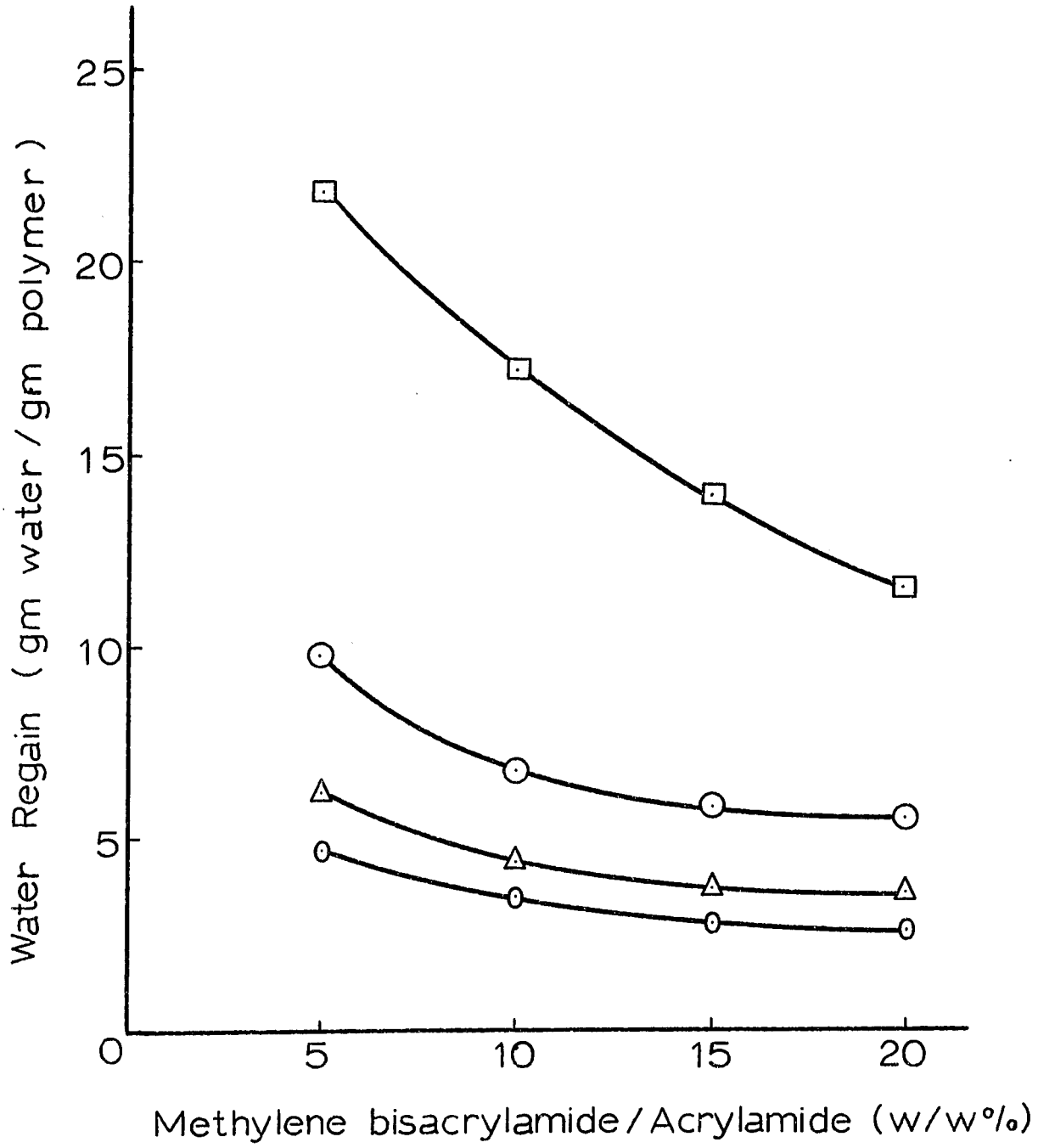


FIGURE 5 : Water regain values of polyacrylamide gels as a function of acrylamide concentration (⊡ 5%, ⊙ 10%, △ 15%, 0 20%) and of the relative concentrations of methylene bisacrylamide and acrylamide.

the reagents, this effect being enhanced by a decrease of the concentration of the crosslinking divinyl monomer at a constant concentration of acrylamide. As would be expected, these results demonstrate that on decreasing the ratio of methylene bisacrylamide to acrylamide, the resulting three-dimensional network becomes more loosely bound and more easily accessible to the clusters of water.

One arrives at a similar conclusion from a consideration of the partition coefficients for compounds of different molecular weights (c.f. Table III). Thus, for the same solute, the higher the partition coefficient, the larger is the accessible volume of the imbibed phase. From Table III, it is evident that a more crosslinked gel (e.g. gel 5-20) is less permeable to all solutes than is a less crosslinked one (e.g. gel 5-5) to all solutes. In the anomalous cases of lysozyme and tryptophane, the effect of adsorption might have overshadowed that of permeability.

The "water regain" values of the same 16 gels were determined also for saline, which was used as eluant in most experiments, and showed no significant differences from the values calculated for water.

Partition Coefficients and Degree of Separability

Although the values of the partition coefficients of many solutes listed in Table III are distinctly different, in practice, perfect separation of two solutes could not be accomplished unless their partition coefficients differed by 0.3 or more. This undesirable feature is due to the fact that the solutes were eluted in rather diffused bands, often spreading through 20-70 ml, the spreading being more pronounced with increasing elution volume. For example, to illustrate the degree of separation which can be achieved by gel filtration, a chromatogram of the fractionation of a mixture of bovine γ-globulin, bovine hemoglobin, ovomucoid and tryptophane with a 5-5 column is shown in Figure 6. As can be seen from Table III, these gels were found useful for the separation

TABLE III : Elution volumes (Ve) and partition coefficients (K_D) of various solutes t
0.9% saline (w/v) as eluant

Solutes	Log M	Gel 5-5 Vo = 68 ml Vi = 134 ml		Gel 5-10 Vo = 73 ml Vi = 129 ml		Gel 5- Vo = 8 Vi = 11
		Ve	K_D	Ve	K_D	Ve
Fibrinogen	5.763	68	0.000	73	0.000	85
Bovine γ-globulin	5.164	80	0.089	84	0.087	96
BSA-hapten	4.840	-	-	-	-	-
Bovine serum albumin	4.840	98	0.224	98	0.194	103
Hemoglobin	4.833	109	0.306	109	0.279	114
Pepsin	4.591	118	0.373	119	0.357	125
Ovomucoid	4.464	126	0.433	121	0.380	130
Trypsin	4.373	133	0.485	136	0.480	142
Lysozyme	4.230	158	0.671	169	0.744	178
Ribonuclease	4.104	150	0.614	152	0.613	157
Tryptophane	2.307	185	0.876	201	0.994	201
Caffeic acid	2.255	-	-	-	-	-
Histamine	2.017	-	-	-	-	-
Chlorogenic acid	2.549	-	-	-	-	-
5-OH-indole-3-acetic acid	2.243	-	-	-	-	-

olutes through 25 x 425 mm columns of polyacrylamide gels using

Gel 5-15		Gel 5-20		Gel 10-15		Gel 15-15		Gel 20-15	
Vo = 85 ml		Vo = 107 ml		Vo = 86 ml		Vo = 91 ml		Vo = 93 ml	
Vi = 116 ml		Vi = 97 ml		Vi = 115 ml		Vi = 109 ml		Vi = 108 ml	
Ve	K_D	Ve	K_D	Ve	K_D	Ve	K_D	Ve	K_D
85	0.000	107	0.000	86	0.000	91	0.000	91	0.000
96	0.095	118	0.113	86	0.000	91	0.000	93	0.000
-	-	-	-	-	-	91	0.000	-	-
103	0.155	122	0.155	92	0.052	91	0.000	93	0.000
114	0.250	131	0.248	100	0.122	94	0.028	93	0.000
125	0.345	140	0.340	107	0.183	95	0.037	93	0.000
130	0.387	143	0.371	112	0.226	96	0.046	95	0.019
142	0.491	153	0.475	125	0.339	122	0.285	99	0.056
178	0.802	188	0.835	170	0.729	180	0.726	183	0.763
157	0.594	166	0.609	139	0.461	134	0.394	121	0.259
201	1.083	217	1.134	204	1.037	205	1.045	210	1.083
-	-	-	-	-	-	194	0.946	-	-
-	-	-	-	-	-	211	1.102	-	-
-	-	-	-	-	-	232	1.295	-	-
-	-	-	-	-	-	251	1.470	-	-

of protein-hapten conjugates from the haptens themselves (e.g. BSA-chlorogenic acid conjugate from chlorogenic acid with a 15-15 column) and of high molecular weight proteins from smaller ones (e.g. BGG from lysozyme with a 15-15 column and BGG from ovomucoid, trypsin or lysozyme with a 5-5 column).

The recovery of solute in all cases was almost 100%. If a low yield of a pure component can be tolerated, even compounds of close molecular weights (e.g. BGG and BSA) can be separated in one or two repeated runs, each time discarding the tailing part of the faster moving component and the frontal part of the slower moving component. This principle was actually applied to the enrichment of the antibody content in an antibody preparation, by the partial removal of the albumin fraction and smaller molecular species (167) from the antiserum. The distribution of hemagglutinating antibodies among the chromatographic fractions is illustrated in Figure 7. The sedimentation coefficients of the two fractions were 7 S and 4 S respectively in the order of elution.

Solute-Solute Interaction

The partition coefficients in Table III were obtained from single component runs. In a multiple component system, interaction of the solutes may alter the degree of separation and the elution volume, which would be reflected as a change in partition coefficients. In Figures 8 and 9 are given the chromatograms for the separation of BSA from lysozyme and from ribonuclease, respectively, and for the pure components. For the systems used, therefore, it must be concluded that interaction between solutes was slight, since the "peak" volumes in single and multiple component runs were quite similar (within 3 ml) and reproducible.

Correlation Between Partition Coefficients and Logarithms of Molecular Weights

In Figures 10-16, the partition coefficients of eight proteins (BGG, BSA,

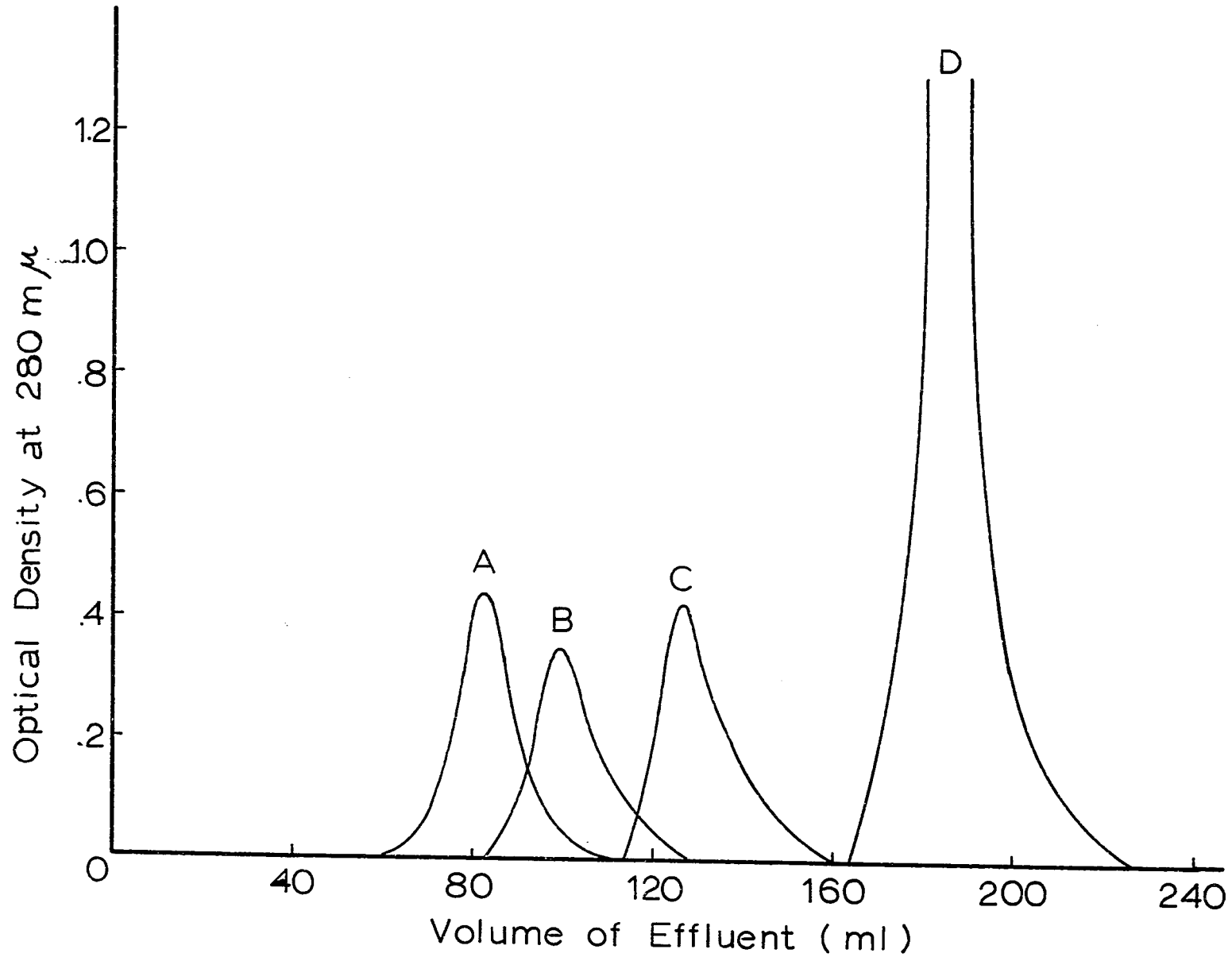


FIGURE 6 : Fractionation of bovine γ-globulin (A), hemoglobin (B), ovomucoid (C) and tryptophane (D); with a 25 x 425 mm column of gel 5-5 and 0.9% saline (w/v) as eluant.

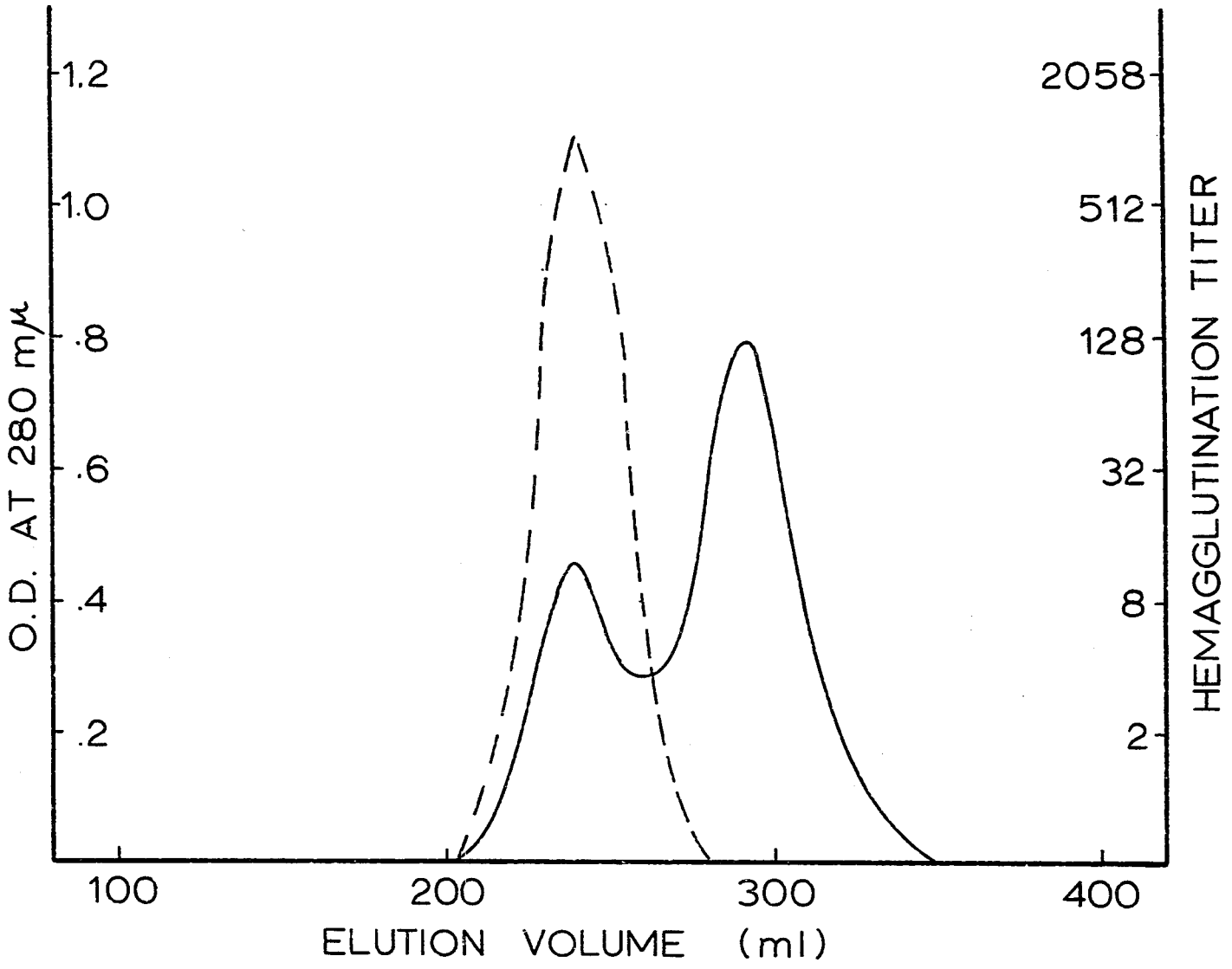


FIGURE 7 : Fractionation of anti-BSA antiserum with a 40 x 500 mm column of gel 5-5 and 0.9% saline (w/v) as eluant. The distribution of hemagglutinating antibodies among the fractions is illustrated by the broken line.

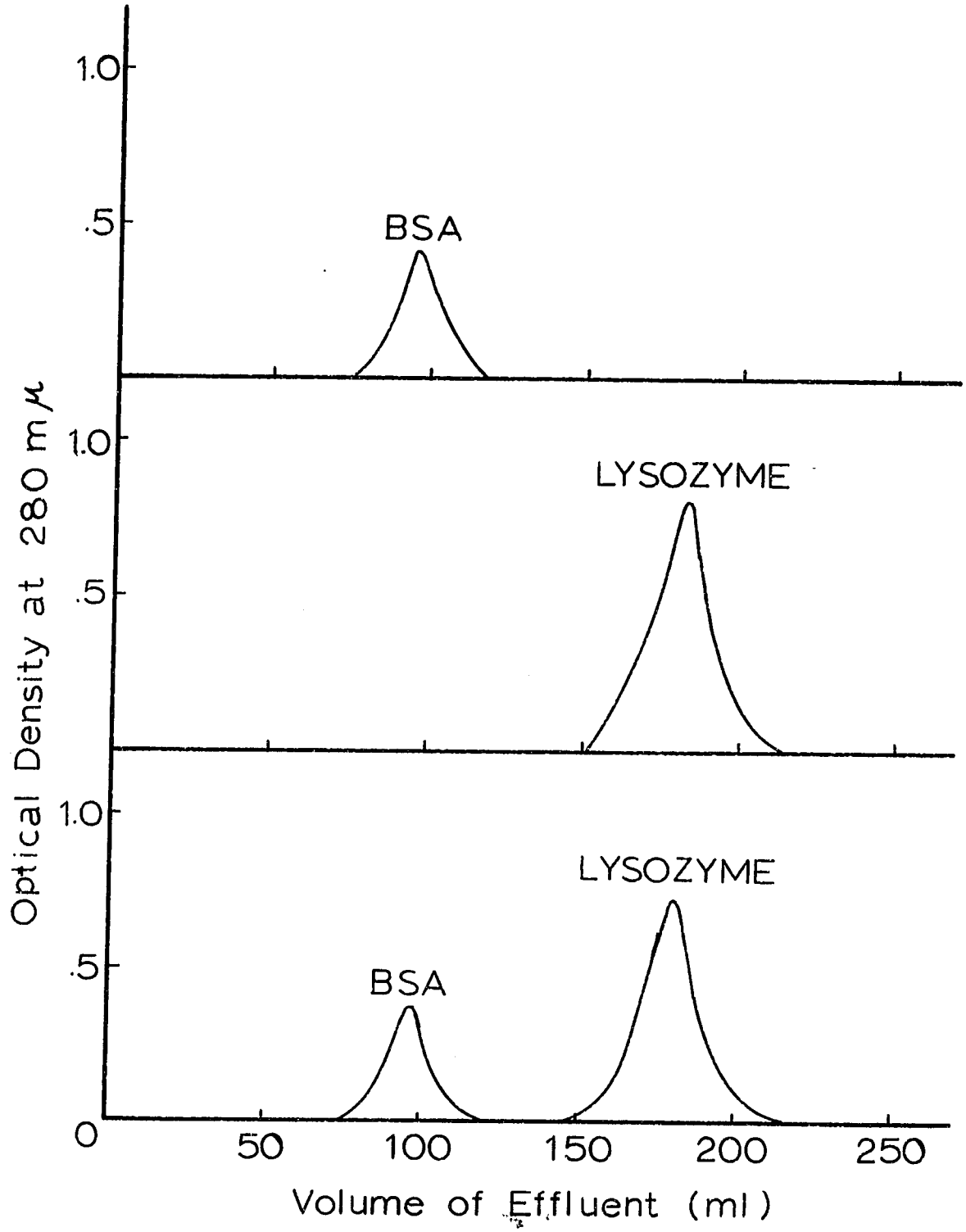


FIGURE 8 : Chromatogram of single and multiple component runs of bovine serum albumin and lysozyme through a 25 x 425 mm column of gel 15-15 using 0.9% saline (w/v) as eluant.

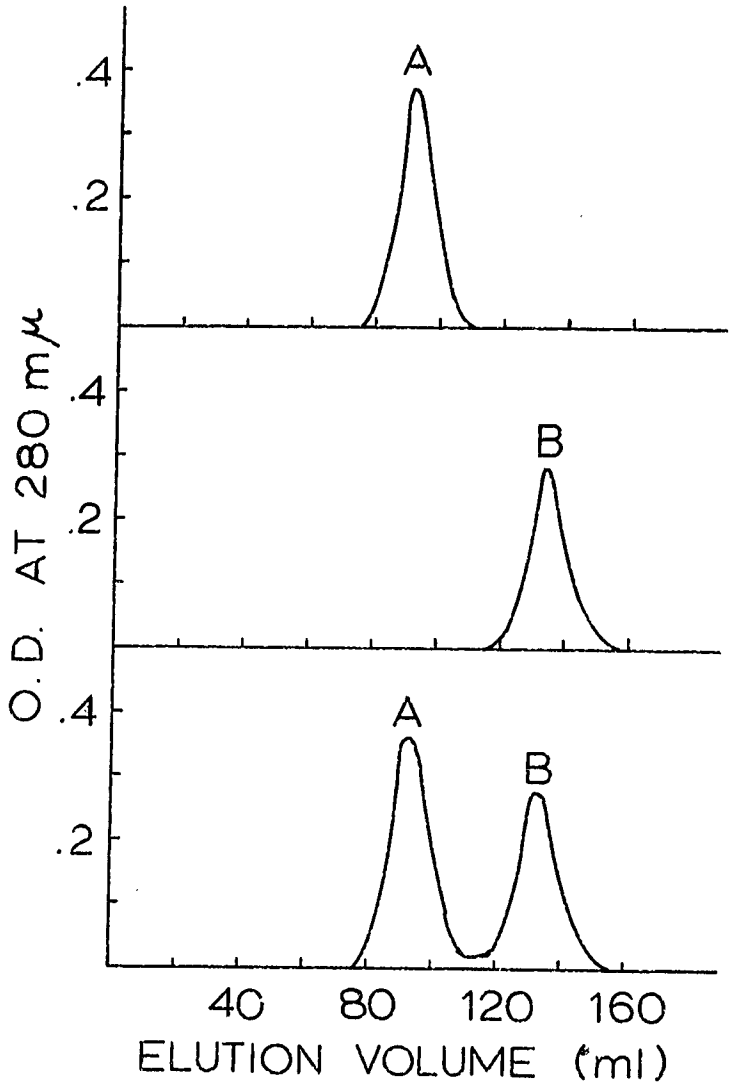


FIGURE 9 : Chromatogram of single and multiple component runs of bovine serum albumin (A) and ribonuclease (B) through a 25 x 425 mm column of gel 15-15 using 0.9% saline (w/v) as eluant.

hemoglobin, pepsin, ovomucoid, trypsin, lysozyme and ribonuclease) in seven gels are plotted against the logarithms of their corresponding molecular weights. It is evident that a simple linear relationship exists between the two parameters. The agreement is generally better for gels of high water regain values. However, in every case, hemoglobin and lysozyme show serious deviation from such correlation plots.

Correlation Between Partition Coefficients and Molecular Sizes

The partition coefficients of seven proteins calculated from chromatographic data and from molecular radii of gyration are listed in Tables IV to X, and plotted in Figures 17 to 23. Fairly good agreement was obtained between the experimental and theoretical values, especially for solutes with K_D values between 0.1 and 0.6 and for gels of high water regain values.

The S_f values used showed that the total chain lengths in the gels were two to four times shorter than the fully stretched linear chains. This is reasonable and comparable to the shortening by a factor of four to five observed in Sephadex (153). Furthermore, as expected, S_f decreases as the total monomer concentration and/or the degree of crosslinking increases, just like the water regain values.

TABLE IV : Theoretical and observed partition coefficients of 7 solutes for gel 5 - 5

Gel 5 - 5 W_r = 21.8 S_f = 0.4750

Solute	r_s	K_D		% Deviation
		Observed	Theory	
Ribonuclease	19.2	0.6143	0.5743	- 6.47
Trypsin	19.4	0.4850	0.5683	17.17
Ovomucoid	22.6	0.4330	0.4739	9.45
Pepsin	22.9	0.3730	0.4653	24.75
Hemoglobin	31.3	0.3060	0.2545	-16.82
BSA	36.1	0.2240	0.1668	-25.56
BGG	55.5	0.0890	0.0169	-81.00

TABLE V : Theoretical and observed partition coefficients of 7 solutes for gel 5 - 10

Gel 5 - 10		W_r = 17.2		S_f = 0.4150
Solute	r_s	K_D		% Deviation
		Observed	Theory	
Ribonuclease	19.2	0.6130	0.5412	-11.72
Trypsin	19.4	0.4800	0.5349	11.44
Ovomucoid	22.6	0.3800	0.4375	15.12
Pepsin	22.9	0.3570	0.4287	20.08
Hemoglobin	31.3	0.2790	0.2198	-21.21
BSA	36.1	0.1940	0.1376	-29.06
BGG	55.5	0.0870	0.0109	-87.45

TABLE VI : Theoretical and observed partition coefficients of 7 solutes for gel 5 - 15

Gel 5 - 15 W_r = 13.9 S_f = 0.3800

Solute	r_s	K_D		% Deviation
		Observed	Theory	
Ribonuclease	19.2	0.5940	0.5227	-12.01
Trypsin	19.4	0.4910	0.5163	5.15
Ovomucoid	22.6	0.3870	0.4174	7.86
Pepsin	22.9	0.3450	0.4086	18.43
Hemoglobin	31.3	0.2500	0.2017	-19.31
BSA	36.1	0.1550	0.1230	-20.65
BGG	55.5	0.0950	0.0085	-91.10

TABLE VII : Theoretical and observed partition coefficients of 7 solutes for gel 5 - 20

Gel 5 - 20	W_r = 11.4			S_f = 0.3600
Solute	r_s	K_D		% Deviation
		Observed	Theory	
Ribonuclease	19.2	0.6090	0.5225	-14.21
Trypsin	19.4	0.4750	0.5161	8.65
Ovomucoid	22.6	0.3710	0.4172	12.46
Pepsin	22.9	0.3400	0.4084	20.11
Hemoglobin	31.3	0.2480	0.2016	-18.73
BSA	36.1	0.1550	0.1229	-20.73
BGG	55.5	0.1130	0.0084	-92.54

TABLE VIII : Theoretical and observed partition coefficients of 7 solutes for gel 10 - 15

Gel 10 - 15	W_r = 5.8			S_f = 0.2750
Solute	r_s	K_D		% Deviation
		Observed	Theory	
Ribonuclease	19.2	0.4610	0.3277	-28.91
Trypsin	19.4	0.3390	0.3209	- 5.35
Ovomucoid	22.6	0.2260	0.2227	1.48
Pepsin	22.9	0.1830	0.2146	17.27
Hemoglobin	31.3	0.1220	0.0638	-47.73
BSA	36.1	0.0520	0.0272	-47.62
BGG	55.5	0.0000	-	-

TABLE IX : Theoretical and observed partition coefficients of 7 solutes for gel 15 - 15

Gel 15 - 15	W_r = 3.7			S_f = 0.2550
Solute	r_s	K_D		
		Observed	Theory	
Ribonuclease	19.2	0.3940	0.2150	-45.42
Trypsin	19.4	0.2850	0.2089	-26.72
Ovomucoid	22.6	0.0460	0.1260	174.4
Pepsin	22.9	0.0370	0.1200	224.3
Hemoglobin	31.3	0.0280	0.0225	-19.48
BSA	36.1	0.0000	-	-
BGG	55.5	0.0000	-	-

TABLE X : Theoretical and observed partition coefficients of 7 solutes for gel 20 - 15

Gel 20 - 15	W_r = 2.8		S_f = 0.2400	
Solute	r_s	K_D		% Deviation
		Observed	Theory	
Ribonuclease	19.2	0.2590	0.1505	-41.91
Trypsin	19.4	0.0560	0.1452	159.2
Ovomucoid	22.6	0.0190	0.0781	310.8
Pepsin	22.9	0.0000	-	-
Hemoglobin	31.3	0.0000	-	-
BSA	36.1	0.0000	-	-
BGG	55.5	0.0000	-	-

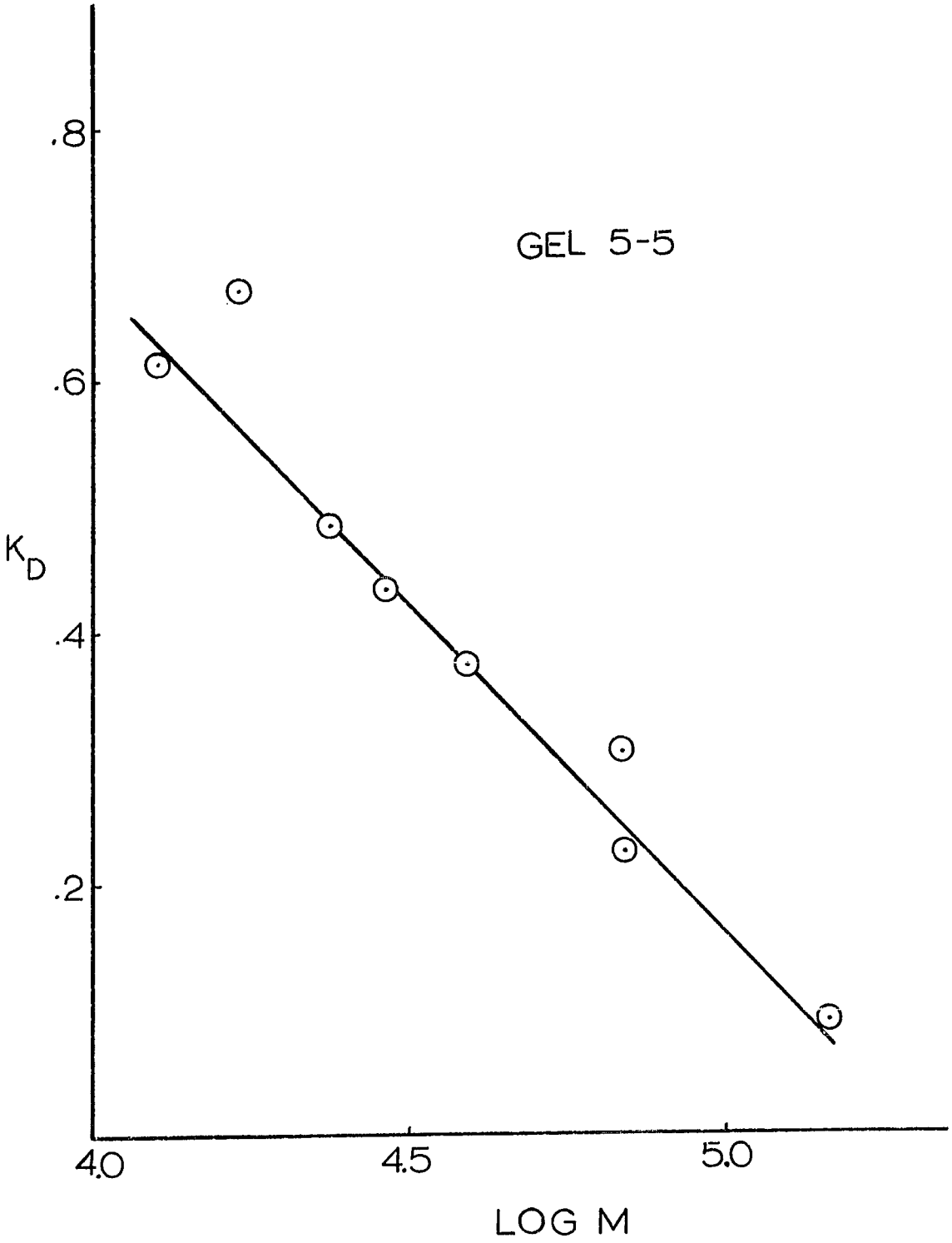


FIGURE 10 : Plot of the partition coefficients (K_D) of the solutes versus the logarithms of their molecular weights (log M) in gel 5-5.

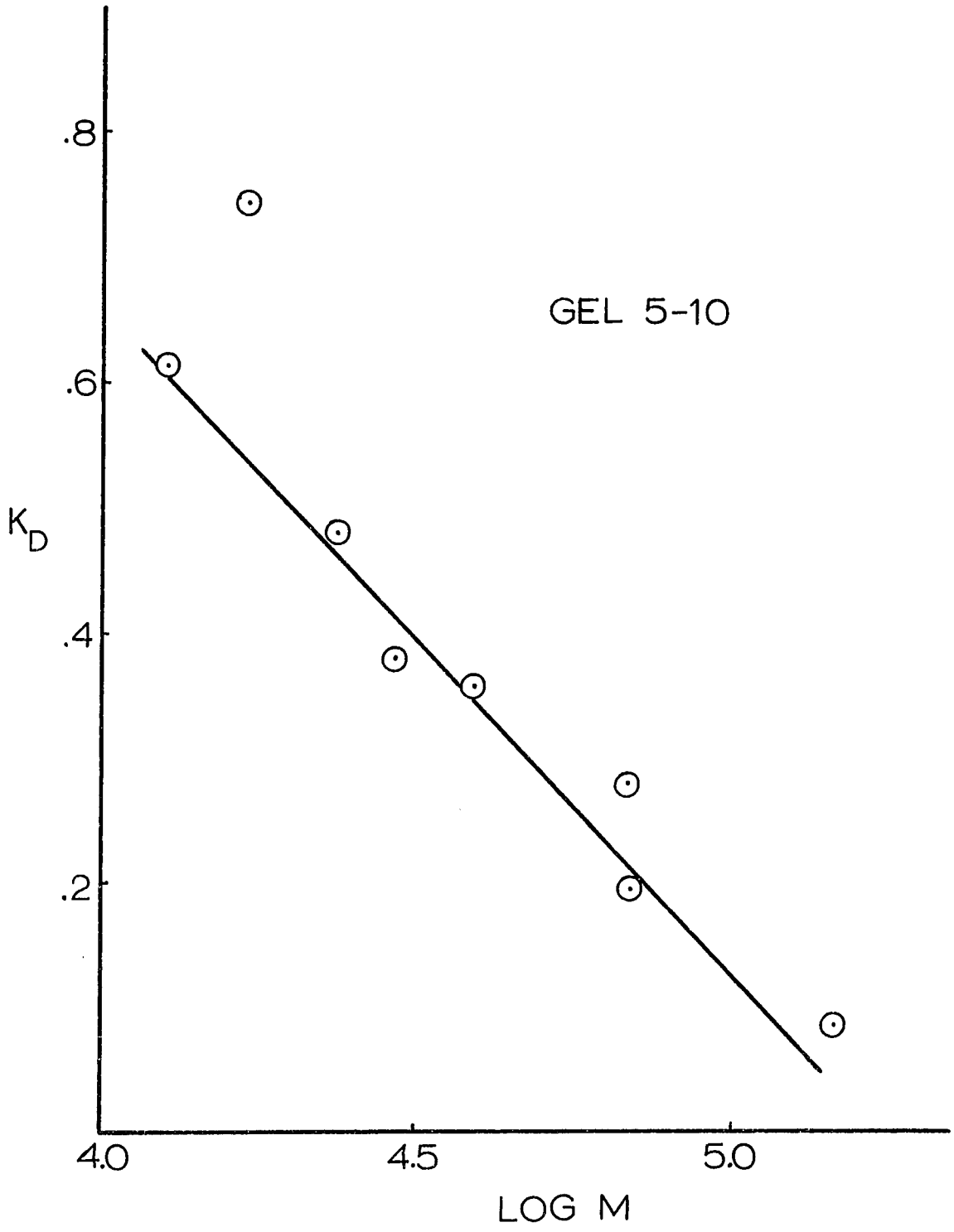


FIGURE 11 : Plot of the partition coefficients (K_D) of the solutes versus the logarithms of their molecular weights (log M) in gel 5-10.

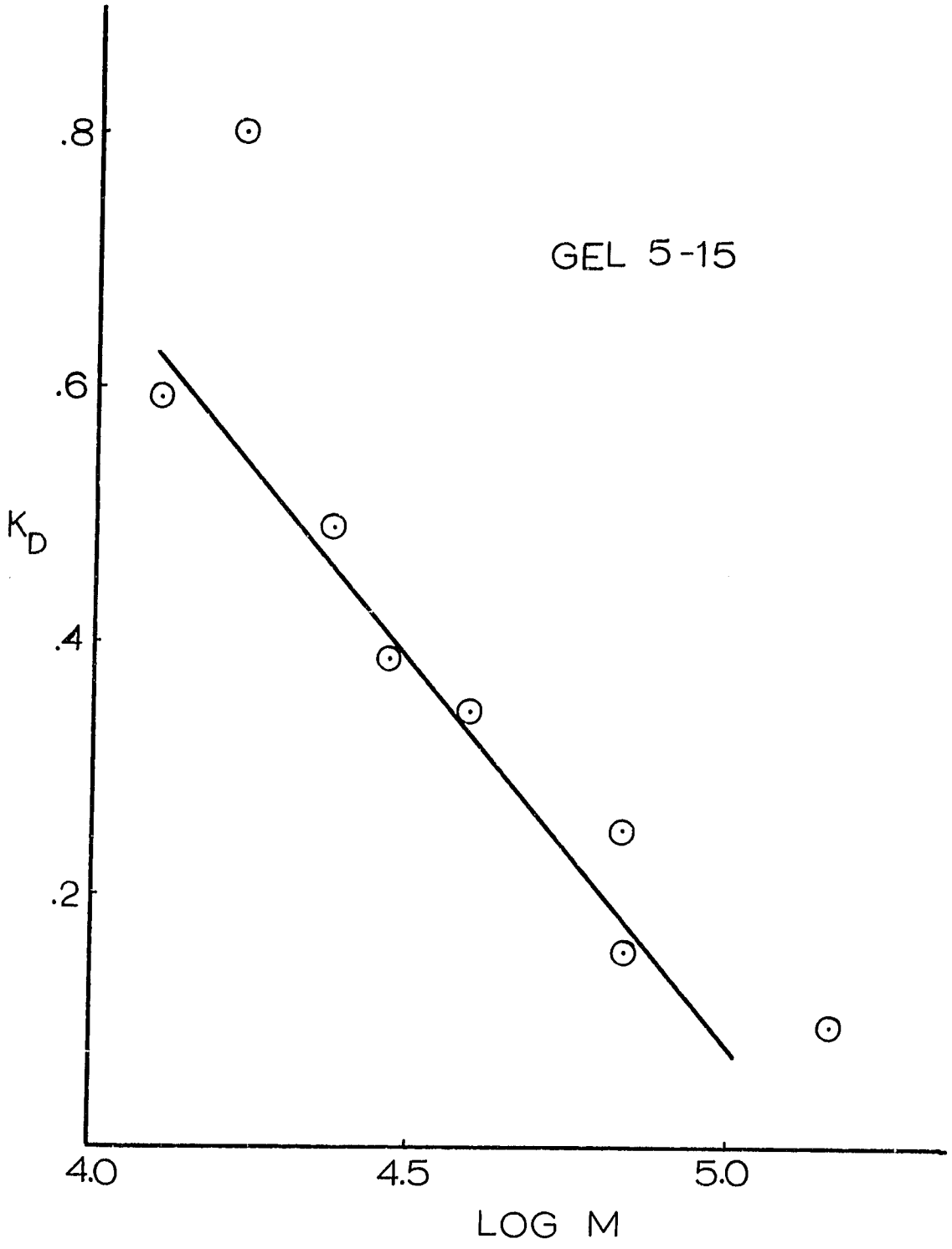


FIGURE 12 : Plot of the partition coefficients (K_D) of the solutes versus the logarithms of their molecular weights (log M) in gel 5-15.

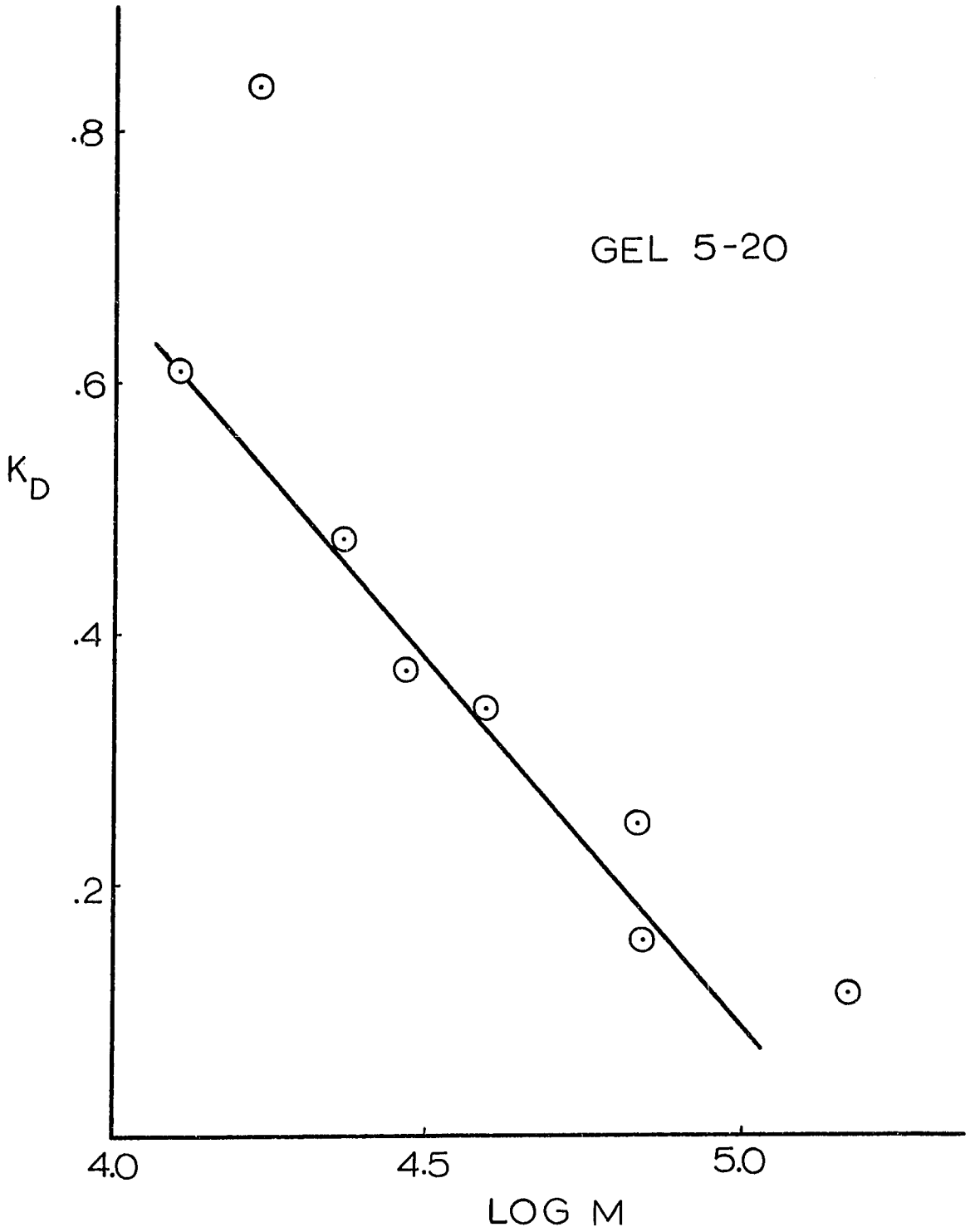


FIGURE 13 : Plot of the partition coefficients (K_D) of the solutes versus the logarithms of the molecular weights (log M) in gel 5-20.

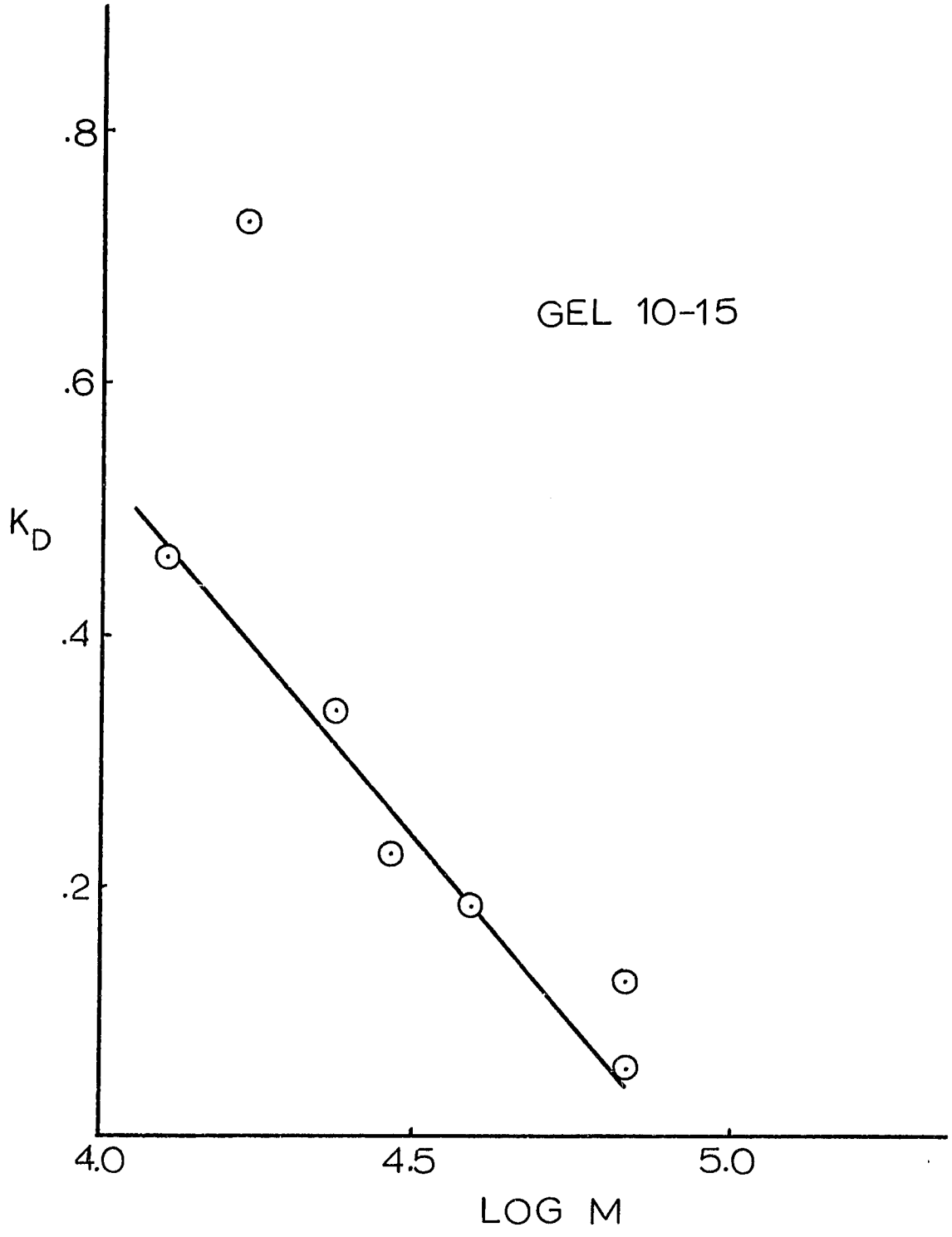


FIGURE 14 : Plot of the partition coefficients (K_D) of the solutes versus the logarithms of their molecular weights (log M) in gel 10-15.

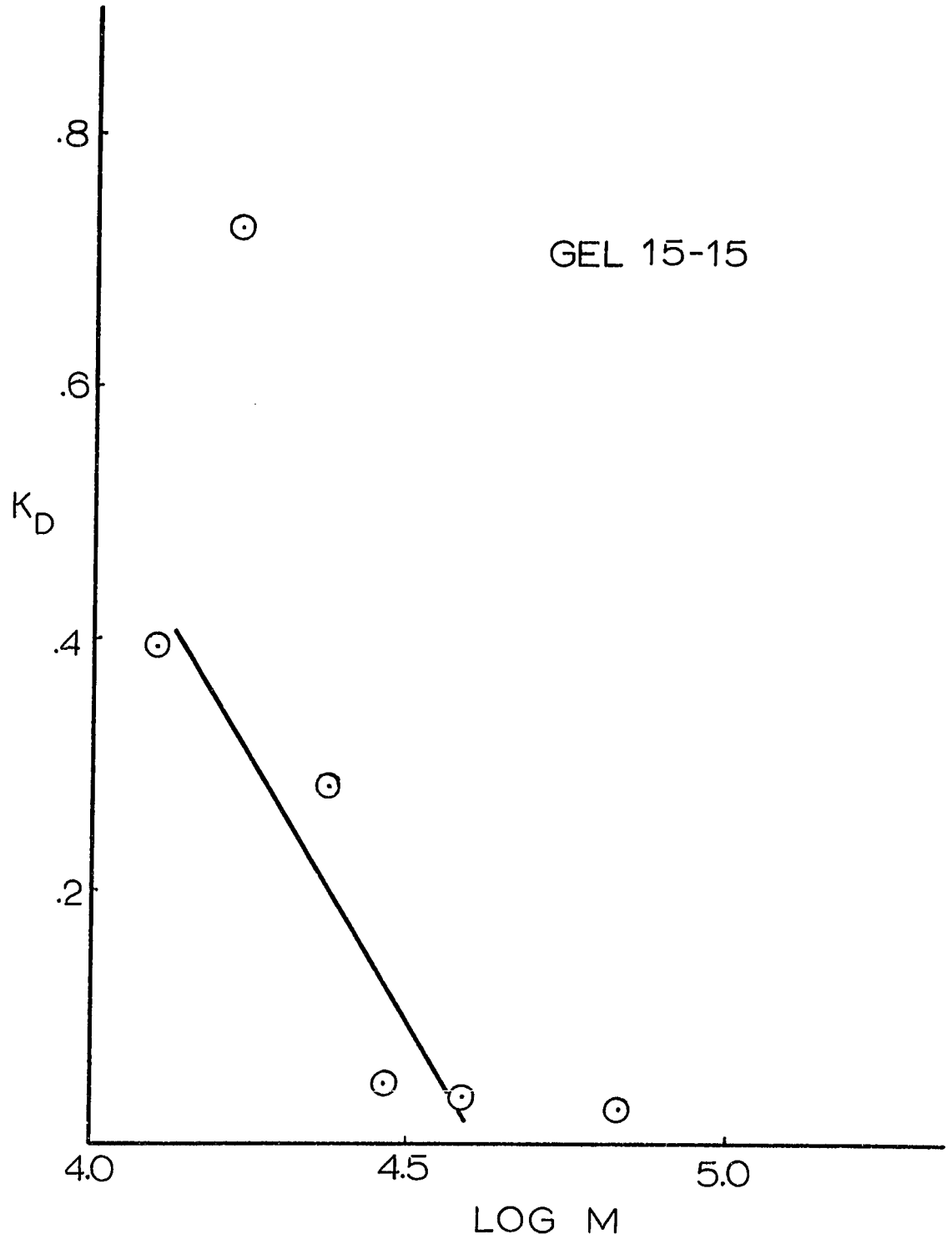


FIGURE 15 : Plot of the partition coefficients (K_D) of the solutes versus the logarithms of their molecular weights (log M) in gel 15-15.

FIGURE 16 : Plot of the partition coefficients (K_D) of the solutes versus the logarithms of their molecular weights (log M) in gel 20-15.

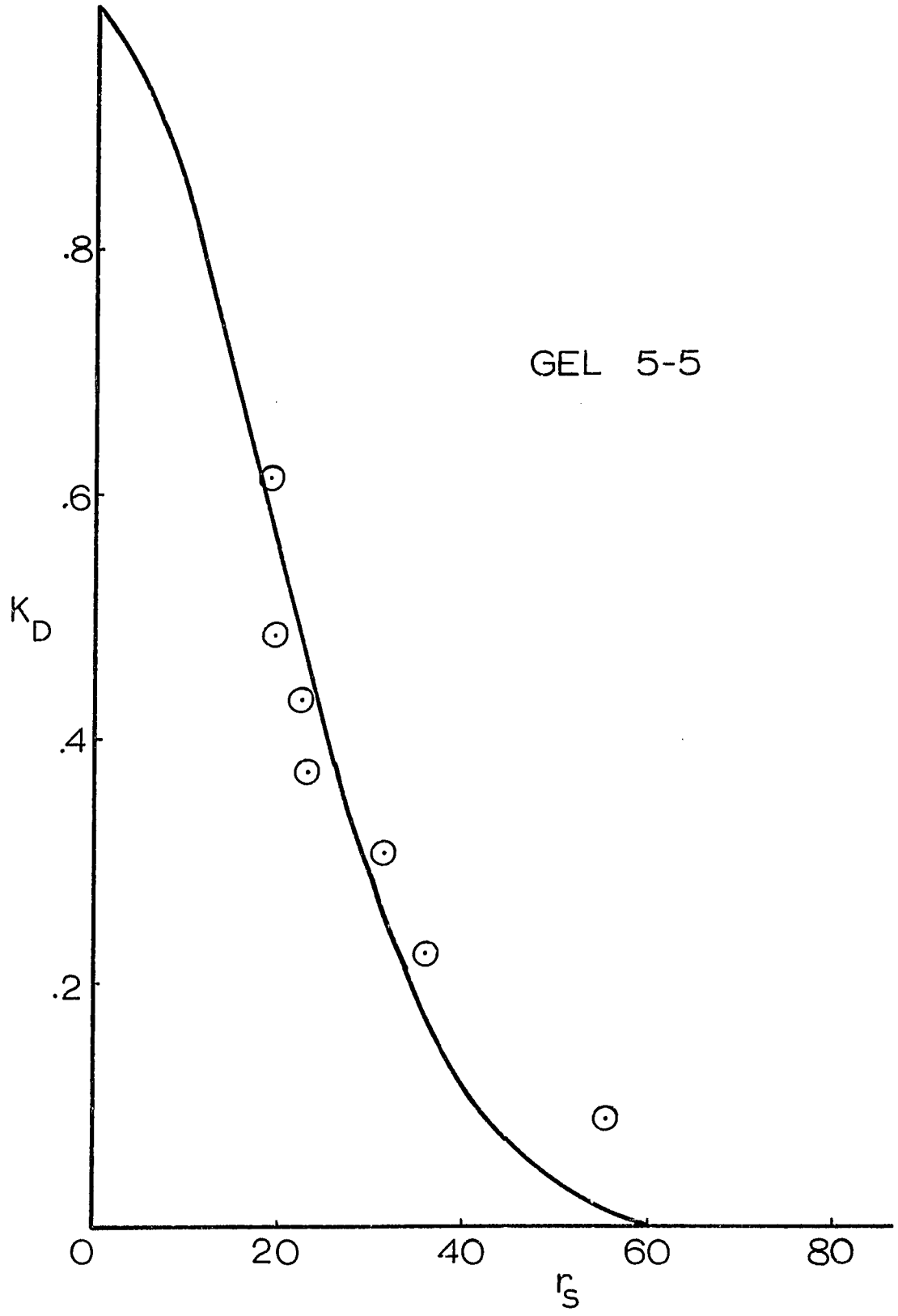


FIGURE 17 : Plot of the partition coefficients (K_D) of the solutes versus their radii of gyration (r_s) in gel 5-5.

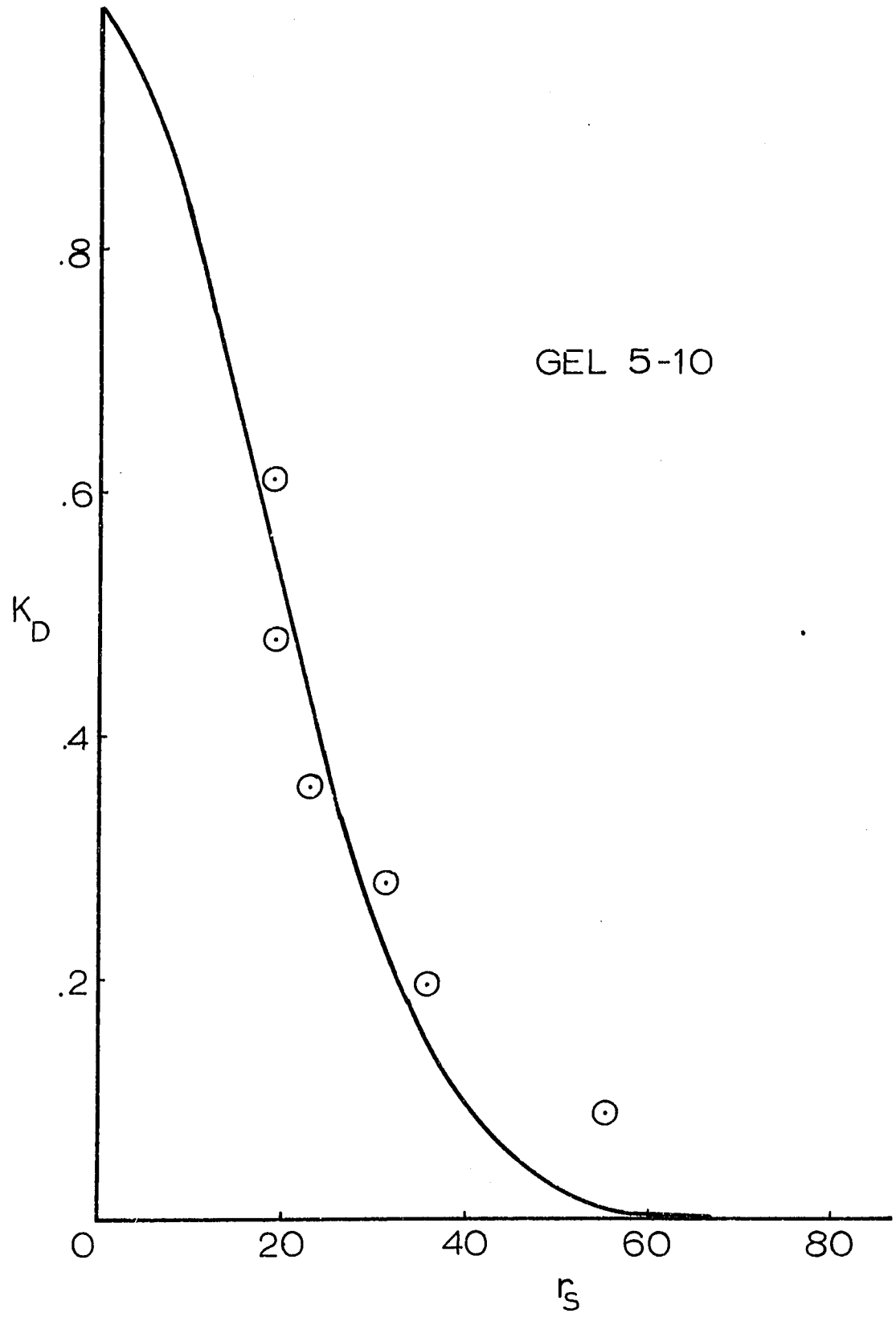


FIGURE 18 : Plot of the partition coefficients (K_D) of the solutes versus their radii of gyration (r_s) in gel 5-10.

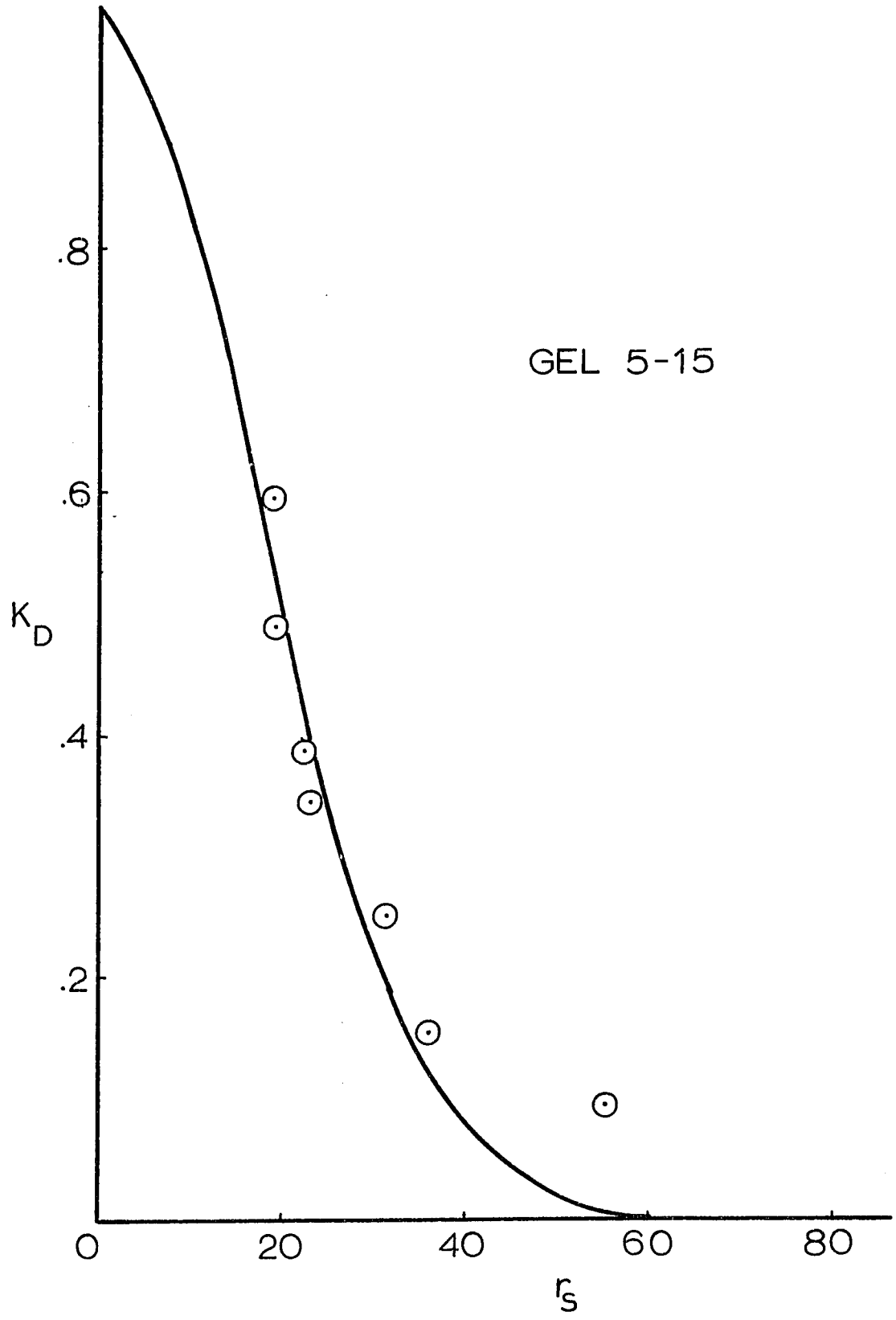


FIGURE 19 : Plot of the partition coefficients (K_D) of the solutes versus their radii of gyration (r_s) in gel 5-15.

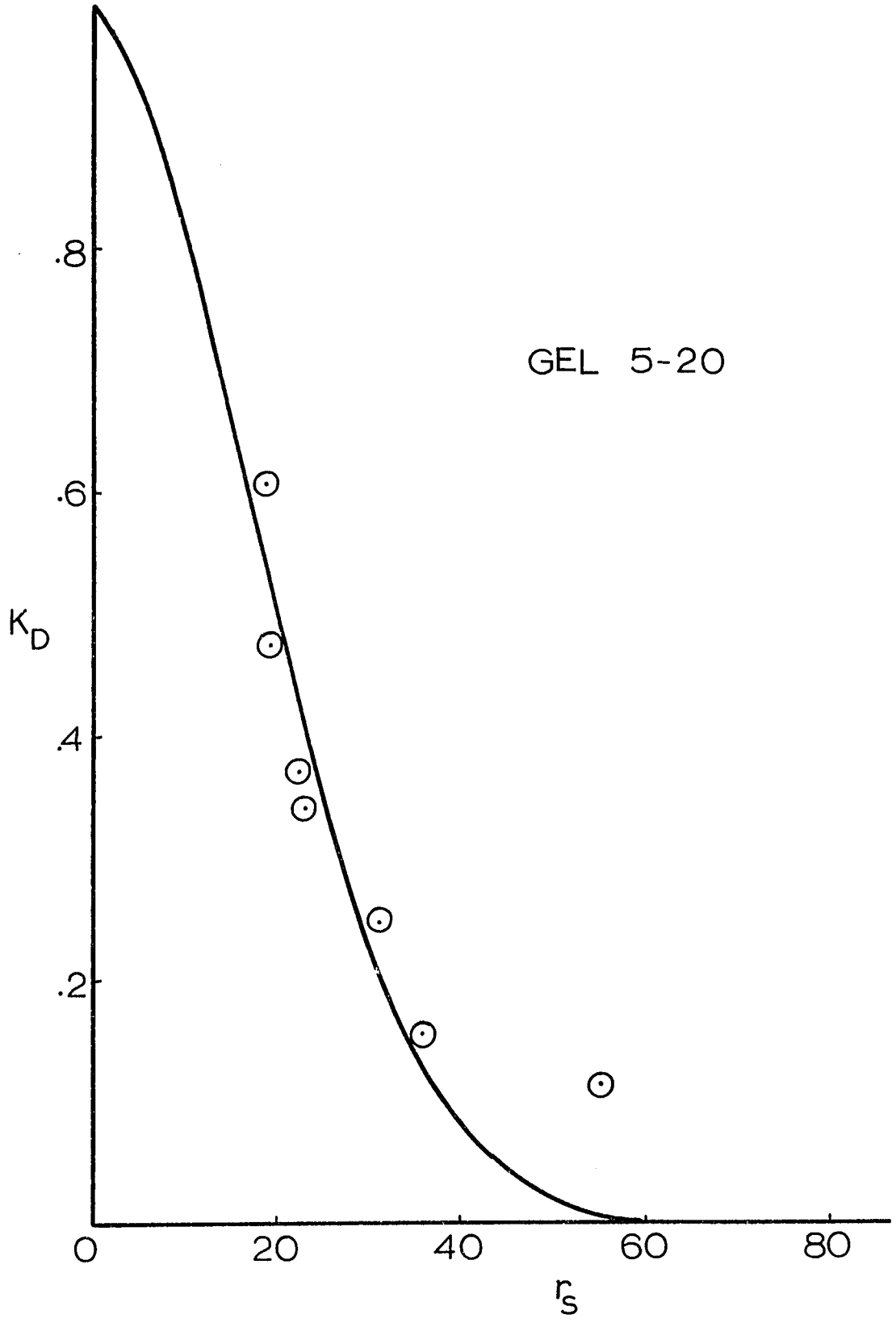


FIGURE 20 : Plot of the partition coefficients (K_D) of the solutes versus their radii of gyration (r_s) in gel 5-20.

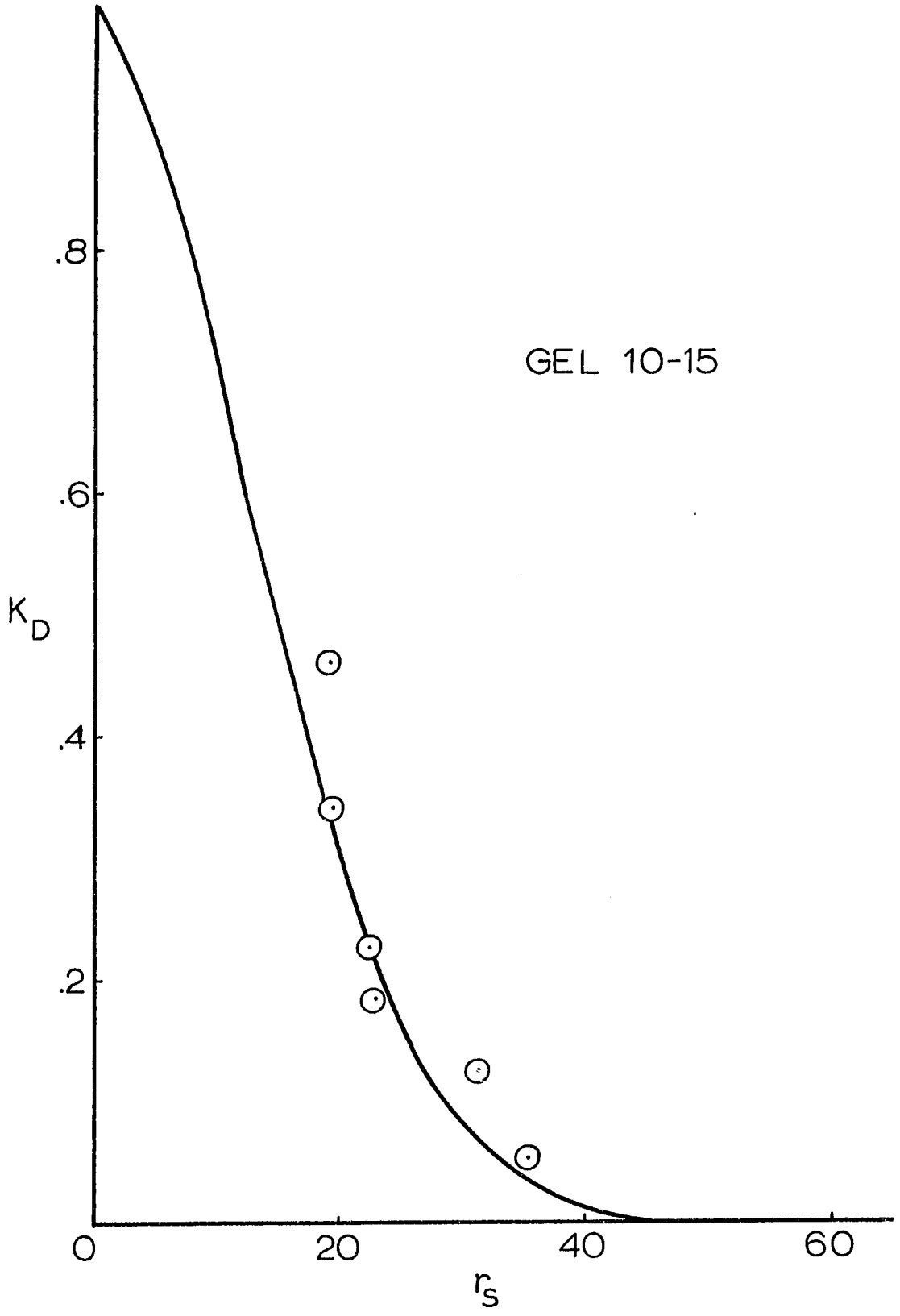


FIGURE 21 : Plot of the partition coefficients (K_D) of the solutes versus their radii of gyration (r_s) in gel 10-15.

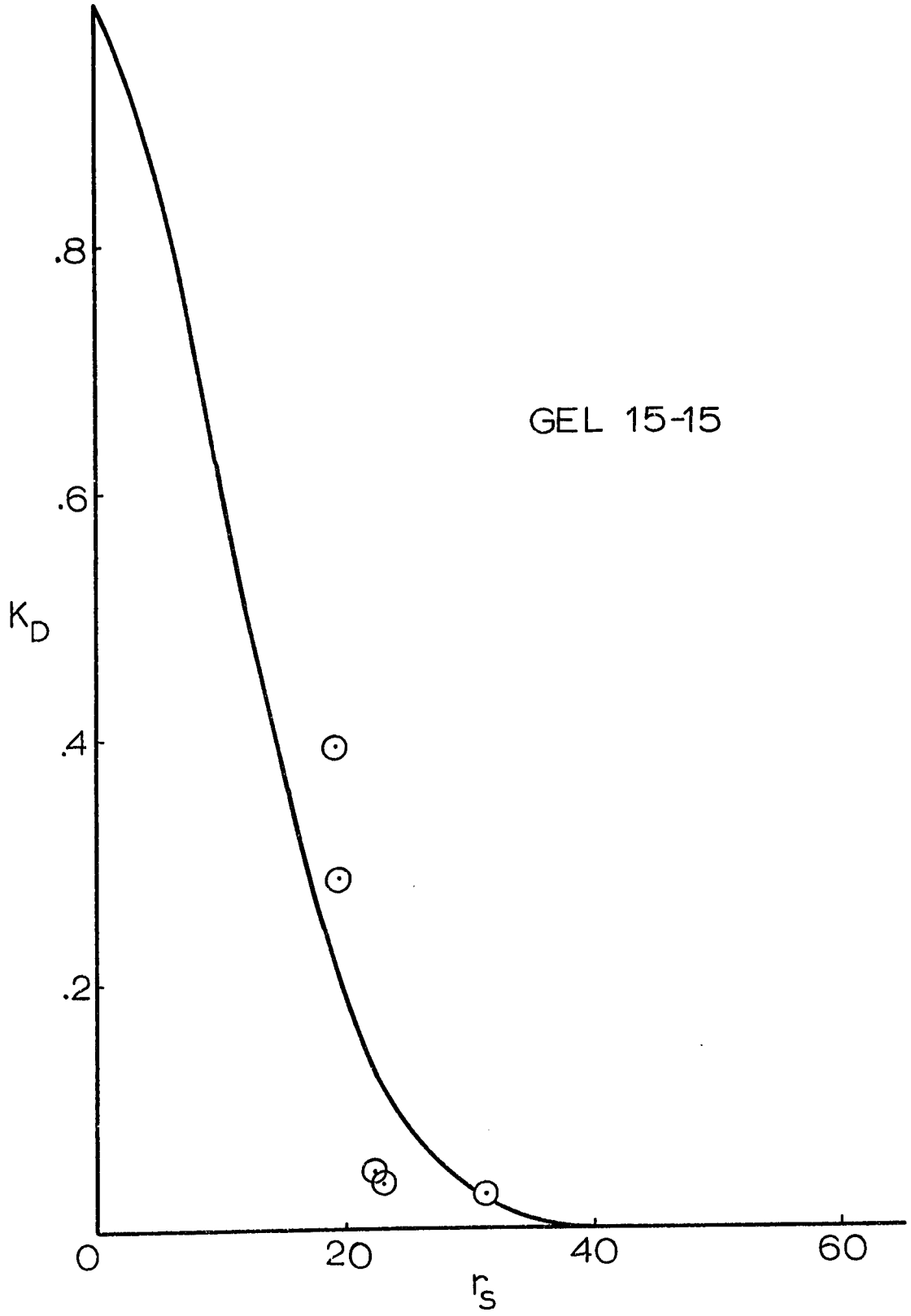


FIGURE 22 : Plot of the partition coefficients (K_D) of the solutes versus their radii of gyration (r_s) in gel 15-15.

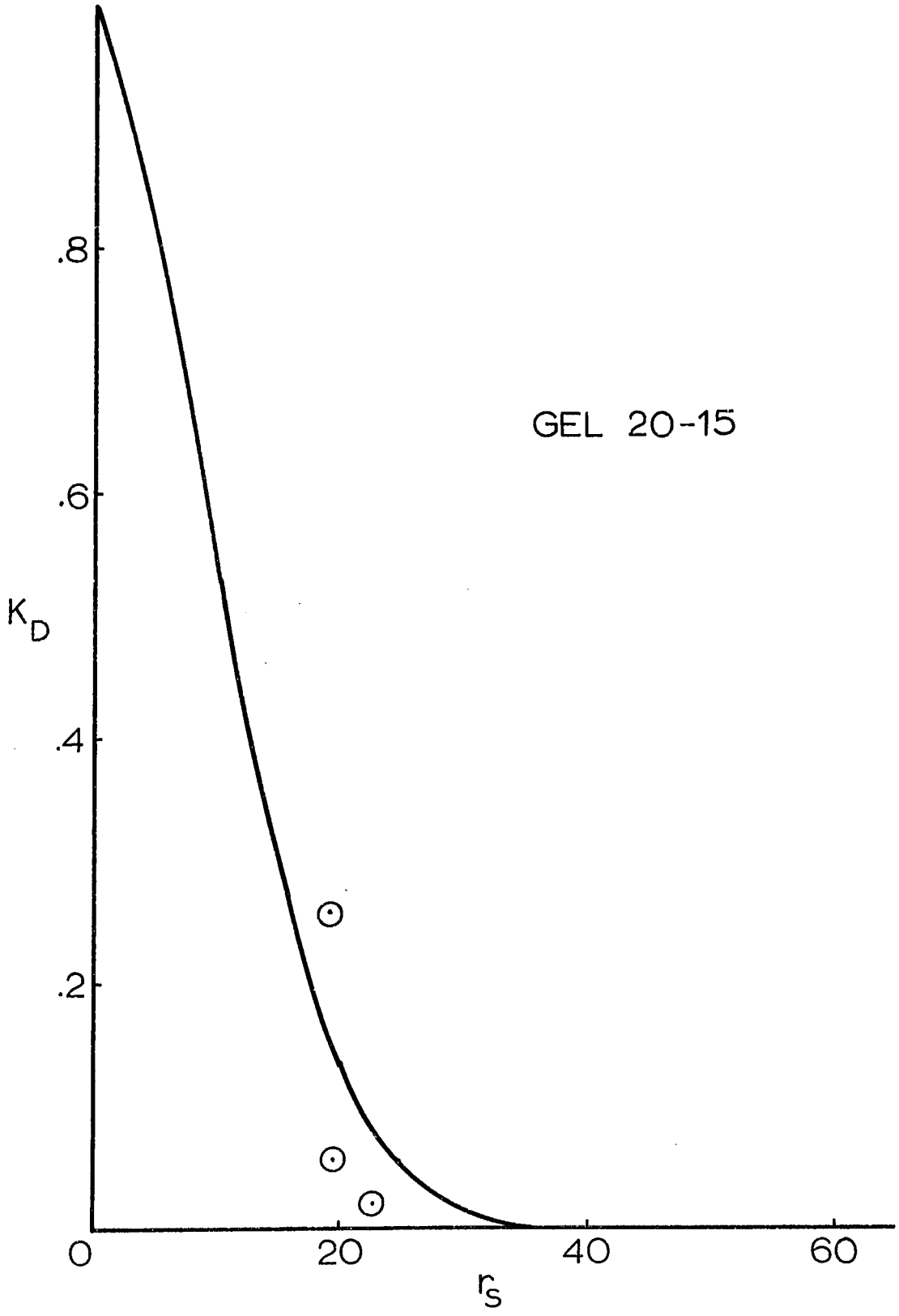


FIGURE 23 : Plot of the partition coefficients (K_D) of the solutes versus their radii of gyration (r_s) in gel 20-15.

Discussion

Physical Properties

In view of the crude method of pulverizing the copolymers with mortar and pestle, it was conceivable that the granules lacked uniformity in size and shape. This might have been an important factor controlling the flow rates and affecting the void volume of a given gel. It is obvious that for a better characterization of the gels, it would be advantageous to use granules prepared by an emulsion polymerization procedure in a non-aqueous medium leading to the formation of uniform beads.* Furthermore, when an exothermic reaction like the present polymerization is carried out in an emulsion, a more uniform temperature control throughout the reaction system can be achieved and hence probably a more homogeneous product.

Swelling

It can be seen from Figure 5 that the water regain value of a gel decreases as the total concentration of the monomers used in the polymerization process increases, even though the methylene bisacrylamide/acrylamide ratio is kept constant. Since the same amount of initiators were used in every preparation, it could be reasonably expected that the same number of chains were initiated. Therefore, the preparation involving a higher total monomer concentration would be expected to yield a higher molecular weight product which, according to Flory (168), would invariably lead to a decrease in swelling. The fact that the effect of methylene bisacrylamide/acrylamide ratio is more enhanced at lower total monomer concentration may be explained as follows. At high monomer concentration, the propagating chains were much

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*Different grades of bead-polymerized polyacrylamide gels are now commercially available under the trade name of Bio-gels manufactured by Bio-Rad Laboratories, Richmond, California.

closer to one another so that crosslinking could occur much more efficiently and there was a higher probability for chain entanglement. Both these effects would have led to a decrease in swelling.

It is not surprising that the water regain values of the gels determined in water and in saline do not differ significantly in view of the fact that the polyacrylamide gels prepared were not polyelectrolytes. However, it should be noted that the gel was under different stress in experiments designed to determine the water regain and during actual chromatography. In the former case, it was under a strong centrifugal force, whereas in the latter, it was subjected only to a few feet of hydrostatic pressure, the effect of which is conceivably much smaller. The calculated imbibed volumes might, therefore, be slightly smaller and the calculated partition coefficients consequently slightly greater than the real values, but probably by a constant factor and hence still correct on a relative scale. A conceivable remedy for such a situation would be to determine the void and imbibed volumes directly in an experiment with solutes that are completely excluded from and absolutely free to diffuse into all parts of the gel phase, respectively. Such solutes, moreover, should not have any interactions with the polymer backbone.

Diffused Chromatographic Bands

It is likely that the diffused bands observed in chromatography experiments were caused probably mainly by channelling and local nonequilibrium due to imperfect packing resulting from lack of uniformity in size and shape of the polymer granules. Among possible remedies one may suggest: (i) use of finely beaded polymer granules of close mesh size, (ii) coating of the walls of the column with an appropriate water repellent film such as 1% dimethyl-dichlorosilane in benzene (recommended by Bio-Rad Laboratories) to minimize the "wall effects" and, (iii) the use of an upward elution technique for

columns of extremely slow flow rates which, according to Morris (169), would yield sharper elution bands.

Anomalous Partition Coefficients

From Table III, it can be seen that a number of K_D values were greater than 1.0; this suggests that gel filtration was not the only operating mechanism. It should be noted, however, that all the solutes exhibiting this kind of behaviour were small aromatic compounds. In this connection it may be mentioned that similar observations were made in other studies in which it was shown that aromatic compounds became readily and strongly adsorbed onto hydrophilic matrices such as Sephadex (170), cellulose (171) and starch (63). From these findings it would appear that the planar benzene-like structure or extending systems of conjugated bonds of the solutes favour adsorption onto these matrices.

From the correlation diagrams of K_D and log M, lysozyme and hemoglobin also seemed to have much higher K_D values than expected from their molecular weights. The anomaly found in the case of lysozyme may also be attributed to adsorption in view of similar observations on polyacrylamide (169) and Sephadex (147) columns. The apparent low molecular weight of hemoglobin may, however, be explained by its tendency to dissociate into sub-units at high dilution (172) as has been observed in chromatography (66).

Highly Crosslinked Gels

Correlation of K_D with log M as well as with r_s showed greater deviation for highly crosslinked gels (15-15, 20-15). Three explanations can be offered here. (1) As the water regain decreases, more gel is needed to pack a column of the same dimension. As a consequence, the total surface area of the gel particles increases, and thus, if adsorption had been also operative in this process, one would have expected that the effect would have

been enhanced. (ii) Due to the difficulty of obtaining small proteins or polypeptides in the molecular weight range of 1-10,000, insufficient chromatographic data were collected in this range. This limitation would be expected to affect the assignments of the position of the straight line in the K_D vs log M plot and of the S_f values in the theoretical calculation. (iii) It must be relized that as the pore size of the gel gets smaller, the size of the penetrating solute must decrease correspondingly. Thus, in terms of the molecular exclusion model, r_r would be no longer much smaller than r_s, which would constitute a violation of the boundary conditions ($L \gg r_s \gg r_r$) in Ogston's equation. The mathematical derivation would, therefore, be no longer rigorous. In terms of the restricted diffusion model, the value of r is approaching that of a; this is expected to cause an unduly large steric hindrance and change in diffusion coefficient, when a solute migrates down a gel channel.

Small Partition Coefficients

A relatively large discrepancy was observed between experimental and theoretical values when K_D was smaller than 0.1. This can be explained with reference to equation (9). Since $K_D = (Ve - Vo)/Vi$ and in this case, Vo approaches Ve, a small change in Ve or Vo will result in a relatively much bigger change in K_D.

The K_D vs log M Plot

In spite of the simplicity and the usefulness of the K_D vs log M plots obtained in this and other studies, the correlation is strictly empirical. A plausible explanation is that, with some mathematical manipulation, Andrews' or Porath's, or Squire's equations or even the Laurent-Killander-Ogston equation (if one assumes some simple relationship between r_s and M), can be used to express K_D as a power series of M. It is obvious that with a suitable

choice of coefficients, these equations are bound to fit almost any set of data to a first approximation.

The primary process involved in gel filtration is the differential migration of various molecular species of different sizes into and out of the gel network. In the author's opinion, it is, therefore, more realistic to relate the partition coefficients with the radii of gyration of the solutes rather than with their molecular weights.

Molecular Exclusion or Restricted Diffusion?

The ideal situation for gel filtration is the sharp division of the eluant into a portion which is stationary within the gel particles and another portion which is mobile between them (Figure 24a). For the more porous gels, the mobile phase, as measured by the void volume, would be expected to be smaller. In Figures 24 (a), (b) and (c) is illustrated schematically the gradual narrowing of free flow channels, i.e. inter-particle space, between the gel grains as the porosity of the gel increases. In the case of the loose agar gels and Sephadex G-200 used by Steere and Ackers, one may imagine that these gels approach the system illustrated in Figure 24 (c), which is more uniform in pore size. Any solvent flow or transport of solute would thus be expected to occur primarily through the porous structure of the agar or Sephadex G-200. Consequently, the differential migration of molecules of various sizes would depend on the differences in their diffusion coefficients rather than on the partition coefficients between the two phases. However, the porosity of the polyacrylamide gels, at least of those used in this investigation, measured in terms of their exclusion limits, were smaller than those of the agar gels and of Sephadex G-200 used by Steere and Ackers. Thus, while it was reported that Sephadex G-200 and agar gels were capable of excluding solutes with molecular weights of the order of 10^6 (62), the polyacrylamide gels used in this investigation were found to exclude solutes with

molecular weights of the order of 10^5 (mol. wt. of BGG ~ 150,000). Therefore, the model illustrated in Figure 24 (a) is considered to represent these gels adequately. Judging from the results obtained in this investigation, it appears that molecular exclusion is the primary mechanism underlying gel filtration, though the participation of restricted diffusion cannot be completely excluded. The case when the radius of gyration of the solute approaches that of the pore size of the gel has already been discussed in a previous section dealing with highly crosslinked gels. Perhaps the same argument can be extended to explain the slightly higher K_D values observed than expected for BGG (c.f. Figures 17-20), i.e. the radius of gyration of BGG, in these cases, approaches the pore size of the gels.

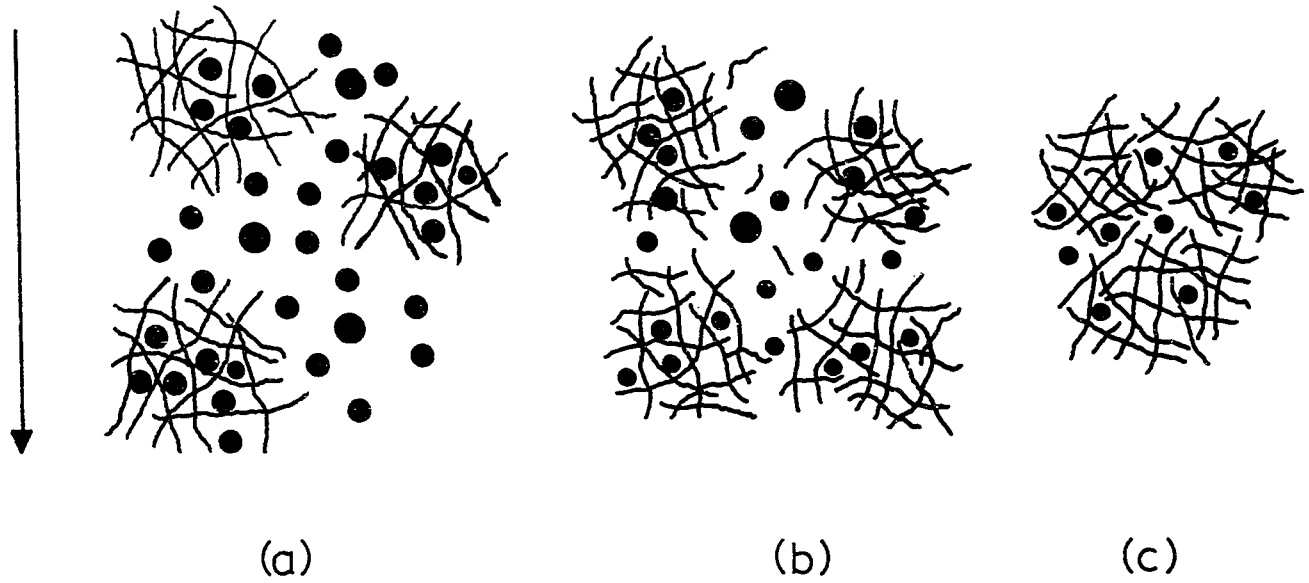


FIGURE 24 : Diagram illustrating the effect of the gradual narrowing of free flow channels between the gel grains on the mode of migration of molecules through a gel filtration column.

CHAPTER IV

THE ISOLATION AND PURIFICATION OF ANTIBODIES

Introduction

Ever since antibodies were identified as the factors responsible for the various immune manifestations, a great number of studies have been made to isolate them in a "pure" state. Availability of "pure"antibody preparations, i.e. free of "normal" globulins and other serum components, is of prime importance to many studies, particularly for those aiming at the elucidation of the factors underlying the mechanisms of antibody-antigen interactions and for investigations of the structure and formation of antibodies (173-175).

Procedures used for the isolation of antibodies may be divided into two groups: (a) non-specific methods, which involve the fractionation of antisera on the basis of physicochemical properties common to both antibody and non-antibody globulins, and (b) specific methods in which advantage is taken of the specific combination of antibodies with the appropriate antigens.

(a) Non-Specific Methods

Since non-specific methods of separation depend on physicochemical procedures which cannot distinguish between the closely similar properties of immune and other globulins, these methods lead at best to an increase in the ratio of antibody globulins to the total amount of proteins in the mixture. Purification in this sense would include all procedures which result in the separation of the globulin fraction from other proteins in the immune sera.

Column Chromatography

As mentioned in Chapter I, satisfactory fractionation of serum proteins has been achieved with some ion exchange resins, particularly cellulose derivatives, such as DEAE- CM- and TEAE- cellulose (48,176) and with gel

filtration columns, such as Sephadex and polyacrylamide gels (177,167). However, since conformational changes of the protein molecules may occur during adsorption and desorption, the possibility of denaturation by this procedure should be borne in mind, especially in the case of ion exchange chromatography.

Precipitation

Serum globulins are less soluble than albumins and can be isolated with appropriate salt concentrations (178-181), organic solvents (182-184) and complexing agents (185,186).

Electrophoresis

Serum proteins can be resolved at pH 7.5-9.0 into at least four electrophoretically distinct fractions, by the moving boundary electrophoresis method developed by Tiselius (187-191). A number of antisera have been fractionated in this way (188), but, in general, on account of the small volumes that can be handled by this technique, it has been used primarily for the analysis and characterization of proteins.

Fractionation of larger volumes of serum on a preparative scale can be achieved by zone electrophoresis (192,193) utilizing different supporting material, e.g., powdered cellulose, granular starch, glass beads, polyvinyl-chloride particles and gels. This method is relatively simple and mild, and leads, in general, to good recovery of antibodies, although some antibodies may be denatured or retained on the solid particles (189,194). However, this drawback can be minimized, or completely eliminated, by the use of continuous flow electrophoresis (195-197).

Electrophoresis-convection has proved to be effective for the separation of large volumes of serum yielding as many as eight electrophoretically distinct sub-fractions of γ-globulins (198,199). However, this method is

time-consuming; each fractionation step requires 24-48 hours and is preceded by dialysis.

Ultracentrifugation

It has been demonstrated that serum proteins possess sedimentation coefficients of the order of 4 and 19 Svedberg units and that antibody activities are found in the 7 S and 19 S globulins (191, 200-203). Ultracentrifugal fractionation procedures, with and without the use of a density gradient have been applied to the purification of antibodies.

In the first case, 19 S globulins have been separated from all the other serum components, but the 7 S globulins do not separate completely from the albumin (203,204). Recently, however, a class of antibodies with sedimentation coefficients intermediate between 7 S and 19 S have been discovered by this technique (205). In the second case, the antibodies of high molecular weight (macro-globulins) have been isolated in the form of a pellet after prolonged ultracentrifugation. These pellets are contaminated with more slowly sedimenting components, which can be eliminated, in principle, by multiple recycling processes. The drawback of this procedure is that it may involve significant losses of the heavy fraction and lead to denaturation and aggregation of the macro-globulins.

Generalization

It is obvious that in view of the closely similar physicochemical properties of antibodies and "normal" globulins, none of the non-specific methods leads to the isolation of pure antibody preparations. Nevertheless, fractions enriched in antibody by a factor of 10 to 20 can be readily isolated.

(b) Specific Methods

These methods exploit the property that antibodies combine specifically with their homologous antigens, and involve the following steps: (i) formation

of insoluble antibody-antigen complexes, (ii) isolation of these complexes from all the other serum proteins, (iii) dissociation of these complexes under mild conditions, and (iv) separation of the antibodies from the antigens. Since reactions of antibodies with antigens are associated with a considerable decrease in free energy (206-208), it would be expected that the dissociation of the complexes formed would require drastic conditions, which might lead to some denaturation of the antibody molecules. When the physical properties of antibody and antigen molecules are similar, their separation, after dissociation of the complexes, may involve considerable difficulty, which can be circumvented, however, by rendering the antigens insoluble during the dissociation process or by coupling the antigens to insoluble and inert supporting media.

Dissociation of Antibodies from Cellular Antigens by Heat

Since some antibody-antigen reactions are exothermic (209), increase in temperature results in partial dissociation of the antibody-antigen complexes. Thus, cold agglutinins adsorbed onto red cell stroma at $4^{\circ}C$ can be eluted from the washed stroma-antibody complexes at $37^{\circ}C$ to yield preparations of high purity (210). Further, antibodies differing in their affinity for the red-cell antigens have been sub-fractionated by elution at increasing temperatures (211). Antibodies to other tissues (liver, kidney, lung, spleen and tumours) have been prepared by elution at $60^{\circ}C$ from the insoluble tissue preparations; however, these specifically purified antibodies were found to cross-react with other tissues (212,213).

Isolation of Antibodies from Specific Complexes by Degradation of the Antigen

Selective enzymatic degradation of the antigen offers another approach to the isolation of antibodies from specific complexes. Pope and Healey (214) subjected dyphteria toxin-antitoxin floccules to the action of pepsin at

pH 3.0 and recovered 70% of the original antitoxin activity. Tetanus antitoxins were prepared by a similar procedure (215). However, the digestion of these horse antitoxins with proteolytic enzymes led to a decrease in molecular weight of the antibodies. Digestion of dextran-antidextran precipitates with dextranase resulted in the degradation of the dextran to dialysable oligosaccharides; the recovery of antibodies and their purity was 80-90%.

Dissociation of Antibody-Antigen Complexes

The presence of a charge on the antibody-combining site in juxtaposition to an opposite charge on the antigenic determinant has been demonstrated by Singer et al (216,217), and therefore it would appear that the forces underlying antibody-antigen interactions are, at least in part, electrostatic (218). Consequently, dissociation of such complexes can be brought about by changes of pH. Antibody-antigen complexes are stable within the pH range of 4.5 to 11; dissociation gradually increases outside this range and is complete at a pH of about 2.4 or 12.3 (219,220).

Purified antibodies to pneumococcal polysaccharides were prepared by treatment of specific precipitates with lime water or baryta since on dissociation of the complexes the polysaccharides formed insoluble calcium and barium salts (221). For the purification of antibodies to protein antigens, the latter were coupled to arsanilic acid and the modified antigen was used for precipitation of antibodies (222). On dissociation of the specific precipitates with calcium hydroxide, the modified antigen remained insoluble (yields were approximately 30%).

A general method for the isolation of antibodies based on the same principle has been developed by Singer et al (223). The protein antigen is first reacted with N-acetylhomocysteine thiolactone, and the polythiolated antigen is used for the precipitation of the antibodies. The antibody-antigen

complex is subsequently dissociated at pH 2.4 and the thiolated antigen is precipitated by crosslinking with 3,6-bis-(acetoxymercury-methyl)-dioxane. Rabbit anti-ovalbumin and anti-bovine serum albumin preparations were obtained with 90% purity. A lower degree of purity (69%) was recorded for anti-ribonuclease preparations. The yield of antibodies varied between 22% and 78%.

Isolation of Antibodies with Antigenically Specific Adsorbents ("Immunosorbents")

The common feature of the specific purification methods discussed in the previous paragraph is that they are applicable only to systems in which the antigen is insoluble or is rendered insoluble during the process of purification, depending on some special property of the particular antibody-antigen system. In such systems, the antibody, after dissociation from the antigens, is readily separated from the latter by centrifugation. A more general method for the insolubilization of soluble protein antigens involves the adsorption or attachment of the antigen through stable covalent bonds to some solid and inert supporting medium, thus forming an immunologically specific adsorbent (immunosorbent).

(i) Miscellaneous Supporting Media

Antigens were adsorbed on kaolin (224), charcoal (225) or glass beads (226), and these antigen-coated particles were capable of removing specifically antibodies. In these methods, the antigens were attached to the supporting media by non-specific adsorption forces and, therefore, it is conceivable that during dissociation some of the antigen may have become desorbed. To eliminate this risk, antigens have been coupled to insoluble supporting polymers via stable covalent bonds in all the later studies.

Landsteiner and van der Scheer (227) first demonstrated the feasibility of such a technique by coupling diazotized haptens to erythrocyte stroma and

using the resulting conjugates for absorption of anti-hapten antibodies. The antibodies were recovered by dissociation with dilute acetic acid and were 50% pure. Similar immunosorbents were used in this laboratory (228) for the isolation of anti-arsanilate antibodies, which were eluted with solutions of the hapten. The hapten was subsequently removed by dialysis. Both the purity and the yield of these antibody preparations was approximately 60%. A number of variations have been used by several workers (229-231), amongst which the method of Farah et al (230) deserves special mention. In their procedure, the free amino groups of the protein-hapten conjugate were blocked with 2,4-dinitrophenyl groups, thus conferring strongly anionic properties on the conjugate. The antibodies were then eluted from the specific precipitates with hapten in the presence of streptomycin, which precipitated the anionic conjugate. Using radioactive labelled haptens, it was shown that haptens could not be dissociated from all the antibody combining sites (230,231).

Antidextran antibodies were purified by absorption of the antibodies on Sephadex G-75 (which is an insoluble, crosslinked dextran), followed by batchwise and stepwise dissociation off the immunosorbent with small oligosaccharides (232). Recently, Hirata and Campbell (233) rendered bovine serum albumin insoluble, without the loss of antigenic determinants, by heat denaturation at pH 3.7. The adsorption of anti-BSA antibody on this material has been made the basis of a method for the estimation of the total antibody contained in the antiserum.

(ii) The Use of Cellulose as Supporting Medium

By far, cellulose is the most frequently used supporting medium for the preparation of immunosorbents. Weliky and Weetall (234) have recently prepared a comprehensive review on the chemistry and use of cellulose derivatives for the study of biological systems, including the preparation of immunosorbents.

Campbell et al (235) designed a general method for the isolation of anti-protein antibodies with adsorbents prepared by coupling the antigens to cellulose. Cellulose was reacted with p-nitrobenzyl chloride, the nitro groups were reduced, the resulting polyamino compounds were diazotized and then coupled to protein antigens. Antibodies were specifically absorbed with this immunosorbent and were subsequently eluted at pH 3.2 (yield and purity were approximately 90%).

Gurvitch et al (236) have modified this procedure and extended it for the quantitative determination of antibodies (237). The cellulose powder was pre-treated with acetyl chloride and reacted with m-nitrobenzyloxymethyl pyridinium chloride to give the corresponding m-nitrobenzyloxymethyl cellulose ether. The nitro groups were reduced and the "amino cellulose" was diazotized and coupled to protein antigens. After absorption, the antibodies were dissociated from the immunosorbent at pH 3.2. In some instances the antibody preparations were 100% pure; however, in other cases only 45-80% of the eluted protein was precipitable with the antigens.

Cellulose adsorbents were prepared in this laboratory according to the two earlier methods (235,236), but the yield and purity of the recovered antibodies were 35-50%. More recently, however, the high capacity of the immunosorbent and the high yield of purified antibodies, as reported by Gurvitch et al (238), have been confirmed (239).

A different chemical procedure was developed for the preparation of cellulose immunosorbents for the isolation of antibodies to classical haptens (240,241). Powdered cellulose was brominated by refluxing with phosphorus tribromide and the product was partially esterified with monosodium resorcinol. Diazotized haptens, such as p-aminobenzene arsonic acid (240,241), p-(p-amino-benzeneazo)-benzene arsonic acid (240) and p-aminobenzene sulphonic acid (240) and p-aminobenzene sulphonic acid (240) were coupled to the etherified cellulose

at pH 10. Desorption of specific anti-hapten antibodies was effected either by gradient elution (240) or stepwise elution (241), with the homologous hapten in increasing concentration; the hapten was subsequently removed by dialysis.

Efficient cellulose immunosorbents were recently prepared by reacting the carboxyl groups of CM-cellulose with the amino group of protein antigens (242,243) or of haptens, with the aid of N,N'-dicyclohexylcarbodiimide. The resulting CM-cellulose-antigen conjugates were shown to have a high capacity for absorbing specifically homologous antibodies which could be eluted at pH 2.3. The antibody preparations were about 95% pure and their yields were of the order of 75-80%. More recently, a cellulose immunosorbent for the isolation of purine-specific antibodies was synthesized (244) by coupling aminoethylcellulose to 6-trichloromethyl purine in a tetrahydrofuran water mixture at pH 10.0 to 10.5. Elution was done at pH 2.3; yield and purity of the antibody preparations were 80-90%.

(iii) The Use of Synthetic Polymers as Supporting Media

Isliker (33,245) converted carboxylated or sulphonated resins to the corresponding acyl or sulphonyl chlorides and reacted them with various antigens. The resulting conjugates absorbed antibodies specifically from the corresponding antisera. The recovery of antibodies was about 55% and their purity approached 75-90%.

Protein antigens have been coupled via azo bonds to polyaminopolystyrene in many laboratories (246-249). The resulting polystyrene-antigen conjugates removed antibodies specifically from appropriate antisera. Similarly, polystyrene-allergen conjugates removed specifically skin-sensitizing, blocking and hemagglutinating antibodies from sera to individuals allergic to ragweed (250) and grass (251) pollens. Precipitating antibodies could be readily eluted at pH 3. Some antibody preparations were 80% pure, as measured by their

precipitability with the appropriate antigens, but recoveries of antibodies were seldom higher than 35%.

Yagi et al (248) reported that the original polyamino-polystyrene had also the ability of binding protein antigens, presumably by physical adsorption through the formation of hydrophobic bonds, and the antigen-coated resin absorbed antibodies specifically. It is noteworthy that in all these studies the capacity of polystyrene-antigen conjugates was less than that of cellulose adsorbents by a factor of at least 30. It would appear, therefore, that hydrophilic polymers are more efficient than hydrophobic ones for reactions with proteins in aqueous solutions (249).

Recently, immunosorbents with a very high capacity for antibody adsorption were prepared by coupling protein antigens via bis-diazotized benzidine, or aryl-haptens directly via the corresponding azo bonds, to insoluble polymers of rabbit serum proteins (252). The insoluble protein framework was made by coupling mercaptosuccinyl groups to protein and then crosslinking the modified protein with tris (1-(2-methyl)aziridinyl) phosphine oxide. With an appropriate number of hapten groups incorporated, antibody was adsorbed with high efficiency; moreover, the weight of anti-hapten antibody adsorbed was greater than that of the adsorbent itself. The capacity of the adsorbent for anti-protein antibody was somewhat lower, but still much higher than that for adsorbents previously reported. The antibody content of the purified preparations was higher than 90%.

Another high capacity immunosorbent was recently developed in this laboratory (253). Protein antigens, human serum fractions, or aqueous ragweed pollen constituents were coupled to commercial samples of ethylene-maleic anhydride (EMA) copolymers. It was demonstrated that these immunosorbents had the capacity of absorbing 500 mg of antibodies per gram of EMA and the purity of the isolated antibodies was 91-97%.

CHAPTER V

PREPARATION AND EVALUATION OF POLYACRYLAMIDE-BSA CONJUGATES AS IMMUNOSORBENTS

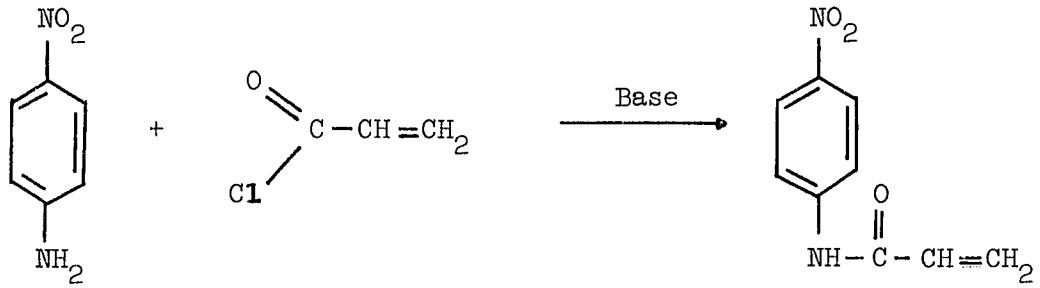
Introduction

A good immunosorbent must satisfy the following criteria: (i) the specificity and affinity of the antibody for the antigen must not be altered during preparation of the immunosorbent, (ii) the adsorbent must have a high capacity for antibodies without binding non-specifically other serum proteins, and (iii) all adsorbed antibodies ought to be elutable without denaturation, preferably leaving the specific adsorbent available for re-use.

Bovine serum albumin was chosen as the model antigen in this investigation simply because it has been frequently used in this and other laboratories and is commercially available in relatively pure form. Polyacrylamide was used as the supporting medium because of (i) the belief that a hydrophilic support was to be preferred to a hydrophobic one in investigations involving proteins and (ii) the author's experience with this polymer acquired in previous work.

Two general schemes were used for conjugating the protein to polyacrylamide:

(a) Various amino dyes, such as nitroanilines, 2,4-dinitroaniline and phenylene diamine, were reacted with acrylyl chloride in the presence of acetonitrile to obtain the vinyl derivatives of the dyes. The reaction is illustrated below with respect to nitroaniline:



These 'vinyl dyes' were reacted with acrylamide in the presence of free radical catalysts such as ammonium persulphate and β-dimethylaminopropionitrile or hydrogen peroxide, in the hope of obtaining an insoluble polymer with aromatic amino groups or with aromatic nitro groups which in turn could be reduced to amino groups. Such a polymer could then be diazotized and coupled with protein antigens to yield an immunosorbent. However, the results obtained were disappointing. According to infra-red analyses of the reaction products, copolymerization of the nitro dyes with acrylamide resulted in the formation of polymers with very low, if any, dye contents, presumably due to the solvent incompatibility of the monomers. The reaction products of phenylene diamine and acrylyl chloride had higher solubilities in the polymerization medium which was either water or a water-ethanol mixture. However, in addition to the heterogeneity of these products, they seemed to be rather unstable, as evidenced by the constant change in coloration of the dye solutions. In view of these difficulties, this scheme was abandoned.

(b) Khorana's reaction devised for the formation of peptide bonds was exploited by converting first the carboxyl groups of the polymer to the anhydride form, which was subsequently reacted with the free amino groups of protein antigens. The reaction mechanism as proposed by Khorana (254) is shown in Figure 25. Thus, copolymers of acrylamide, acrylic acid and methylene bisacrylamide were coupled to BSA with the aid of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide-metho-p-toluenesulphonate (CME-CD) by a "two-step" or "one-step" reaction as will be described in a subsequent section. By trial and error, it was found that the crosslinked copolymer of acrylamide and acrylic acid with a 5% (by wt) acrylic acid content was most suitable for this work. The non-crosslinked polymers were found unsatisfactory because the conjugated products obtained were either extremely fine colloidal dispersions, which were difficult to sediment, or sticky gels adhering to

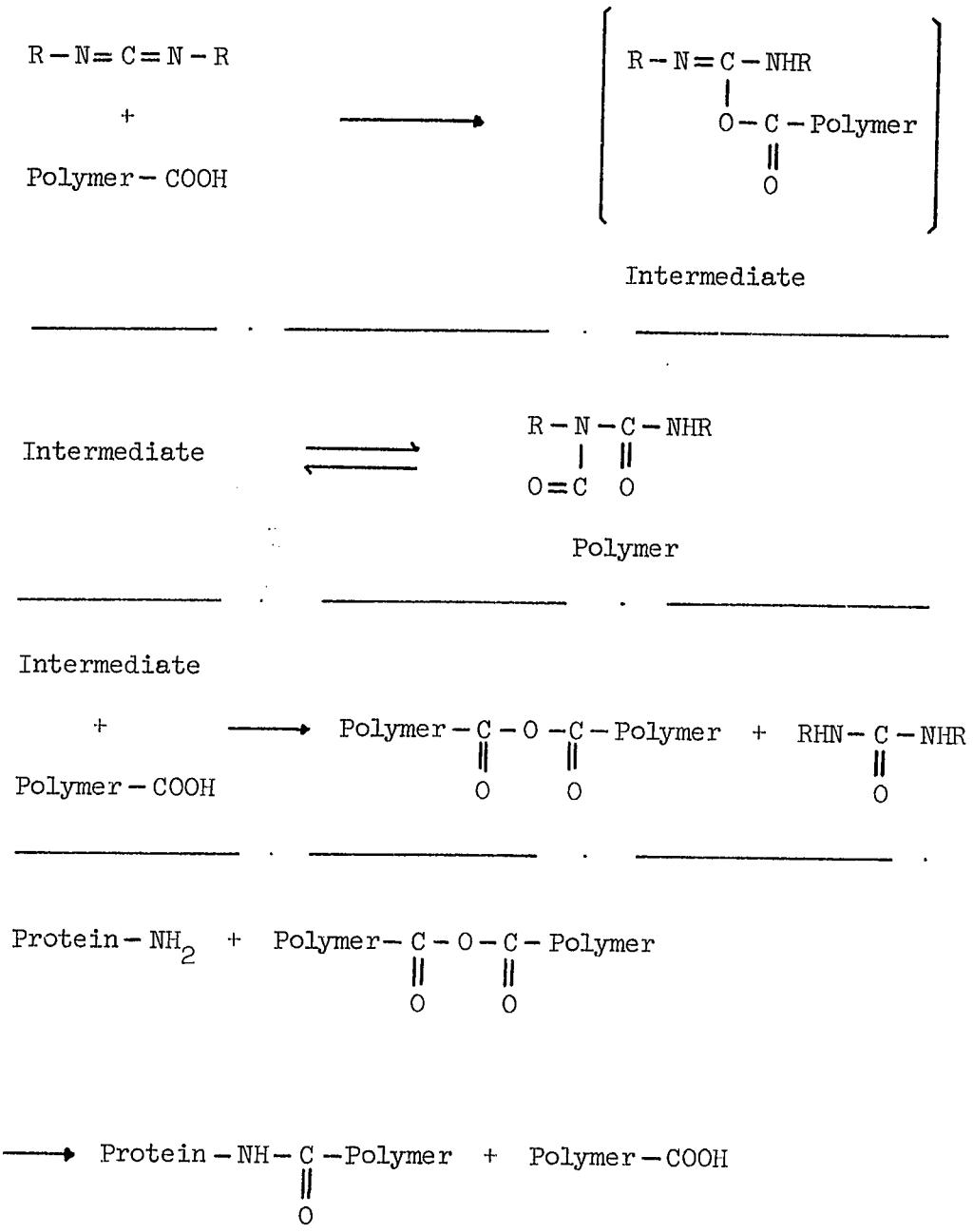


FIGURE 25 : Khorana's reaction mechanism for the reaction of carbodiimide with carboxyl and amino groups.

glassware. Copolymers with high acrylic acid contents showed undesirable non-specific adsorption properties, whereas those with low acrylic acid contents failed to bind significant amounts of BSA in the conjugation process.

Materials

Acrylic acid, ammonium sulphate, glycine, hydrochloric acid, sodium hydroxide, primary and secondary sodium phosphate, Folin-Ciocalteau reagent and N,N'-dimethyl formamide (DMF) were reagent grade chemicals purchased from Fisher Scientific Co., Ltd., Montreal. 1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide-metho-p-toluenesulphonate (CME-CD) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. I^{131}-labelled BSA was purchased from Charles E. Frosst and Co., Montreal. Sources of other chemicals were mentioned in Chapter III.

Experimental

Preparation of Acrylamide and Acrylic Acid Copolymers

Five copolymers of acrylamide and acrylic acid, and five copolymers of acrylamide, acrylic acid and methylene bisacrylamide were prepared by varying the proportions of the three monomers. The method adopted was the same as used for the preparation of acrylamide-methylene bisacrylamide copolymers described in Chapter III. The amounts of each component used in the various preparations are listed in Table XI.

Conjugation of BSA to Copolymer

Eleven different immunosorbents were prepared by conjugating BSA to copolymer A9.5AC0.5-5, using CME-CD as the coupling agent. Two procedures were used:

(i) "Two-step reaction". The copolymer A9.5AC0.5-5 was suspended in a solution of CME-CD in DMF and the reaction was allowed to proceed overnight to give the corresponding anhydride. The unreacted CME-CD was removed by washing

TABLE XI : Composition of 10 acrylamide-acrylic acid-methylene bisacrylamide copolymers

Reaction medium: 100 ml distilled water
Initiators: 0.1 gm ammonium persulphate + 0.1 ml β-dimethylamino-propionitrile
Reaction temperature: 50°C
Reaction time: overnight

Copolymer	Acrylamide	Acrylic Acid	Methylene Bisacrylamide
A8AC2-5	8.0 gm	2.0 gm	0.5 gm
A9AC1-5	9.0 "	1.0 "	" "
A9.5AC0.5-5	9.5 "	0.5 "	" "
A9.8AC0.2-5	9.8 "	0.2 "	" "
A9.9AC0.1-5	9.9 "	0.1 "	" "
A8AC2-0	8.0 "	2.0 "	0.0 "
A9AC1-0	9.0 "	1.0 "	" "
A9.5AC0.5-0	9.5 "	0.5 "	" "
A9.8AC0.2-0	9.8 "	0.2 "	" "
A9.9AC0.1-0	9.9 "	0.1 "	" "

with DMF and the modified polymer was added to an aqueous solution of BSA to achieve conjugation.

(ii) "One-step reaction". The three reactants were allowed to react simultaneously in water. The amounts of the various compounds used in the different preparations are listed in Table XII.

A typical "two-step" experiment is described below. To a mixture of 200 mg of A9.5AC0.5-5 and 100 mg of CME-CD, 10 ml of DMF was added with stirring. The reaction was allowed to complete overnight with stirring at $4^{o}C$. The resulting polymer was washed five times with 20 ml of DMF each time, by centrifugation and resuspension. The washed product was dispersed in 3 ml of DMF and the resulting suspension added dropwise to 400 mg of BSA dissolved in 10 ml of phosphate-buffered saline (pH 7.5, $\Gamma/_2$ 0.1), at $0^{o}C$ with vigorous stirring. The reaction mixture was stirred for an hour and the product was then washed exhaustively with saline solutions of pH 2.0, 10.0 and 7.0 (pH adjusted with 0.1 N HCl or 0.1 N NaOH).

A typical "one-step" experiment is described below. Four hundred milligrams of BSA were dissolved in 10 ml of phosphate-buffered saline (pH 7.5, $\Gamma/_2$ 0.1). To this solution were added 400 mg of copolymer A9.5AC0.5-5 gradually with stirring. The mixture was stirred for at least one hour to allow ample time for the copolymer to swell. Then 100 mg of CME-CD were added in small portions over a period of approximately five minutes. The reaction was allowed to proceed overnight at $4^{o}C$. The resulting cake was homogenized and the granules were exhaustively washed with saline solutions of pH 2.0, 10.0 and 7.0 before being used for reaction with antibodies.

For the sake of simplicity, the two types of product shall be denoted as Type I and Type II to designate that they were prepared by the "one-step" or "two-step" methods, respectively.

TABLE XII : Composition of 11 polyacrylamide-BSA immunosorbents

Polymer support: copolymer A9.5ACO.5-5
Antigen: BSA
Coupling agent: CME-CD
Reaction medium: 10 ml of phosphate-buffered saline, pH 7.5, $\Gamma/_2 = 0.1$
Reaction temperature: 0°C

Immunosorbent	A9.5ACO.5-5	BSA	CME-CD
II-1	200 mg	100 mg	100 mg
II-2	" "	200 "	" "
II-3	" "	300 "	" "
II-4	" "	400 "	" "
I-1	400 "	" "	" "
I-2	" "	" "	200 "
I-3	" "	" "	300 "
I-4	" "	" "	400 "
I-5	" "	100 "	200 "
I-6	" "	200 "	" "
I-7	" "	800 "	" "

The Folin-Ciocalteau Test

The amount of protein in a sample of immunosorbent or of a fraction eluted from the immunosorbent was qualitatively estimated by the Folin-Ciocalteau test (255). To a 1 ml sample in a 5 ml test tube, 0.5 ml of 20% sodium carbonate was added slowly with stirring. Then, two drops (~0.1 ml) of Folin-Ciocalteau reagent were added. The well-mixed sample was placed in a water bath at 37°C for five minutes. A blue coloration indicated the presence of protein in the sample.

Quantitative Determination of the Amount of BSA

Immunosorbent of Type II. The amount of BSA conjugated to the copolymer in each case was determined spectrophotometrically at 280 mμ, by the difference between the total amount used in the conjugation process and the amount left in the reaction washings (which were pooled and dialysed against saline).

Immunosorbent of Type I. For reasons not too well understood, to be discussed later, the washings of these immunosorbents were often turbid and gave erroneous spectrophotometrical readings. To overcome this difficulty, a radioactive tracer technique was resorted to using I^{131}-labelled BSA for conjugation to the polymer. The amounts of radioactivity in the control sample, the washed conjugate and the reaction washing were recorded with an RIDL 400 Channel Analyser*, the emission band with peak energy of .364 Kev being measured. The amount of BSA conjugated was calculted according to the following equation:

$$\frac{A_I}{A_T} = \frac{B_I}{B_T}$$

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* The author wishes to thank Dr. L. Yaffe of this department for permission to use the instrument and Mr. M.K. Dewanjee for technical advice.

where A_I = amount of radioactivity in the washed immunosorbent

A_T = total amount of radioactivity used, i.e. in the control sample

B_I = amount of BSA conjugated

B_T = total amount of BSA used in the preparation

Isolation of Antibody

In general, conjugates prepared with 400 mg of copolymer A9.5AC0.5-5 were capable of removing antibodies from 10 to 20 ml of the anti-BSA antiserum. To reduce the amount of non-specific adsorption, the "globulin fraction" of the antiserum was used, i.e. the serum fraction precipitated at 50% ammonium sulphate concentration.

Batchwise elution was used, instead of the preferred columnar operation, due to failure to obtain a reasonably fast flow rate through columns of immunosorbent. In this way, the length of time the antibodies had to spend at low pH was minimized. The polyacrylamide-BSA conjugate was placed in a 40 ml centrifuge tube and was exhaustively washed with saline solutions at pH 2.0, 10.0 and 7.0 until the washing had an optical density of 0.02 or less at 280 mμ. The antiserum was then added. The suspension was stirred at $4^{o}C$ for two to ten hours. Subsequently, it was washed by repeated suspension, centrifugation and decantation, with saline at pH 7.0, until no detectable amount of protein appeared in the washing. Elution was then started with a buffer of 0.5 M glycine acidified to pH 3.0 with 1.0 N HCl. The eluate was brought back to neutral pH by dialysis against phosphate-buffered saline. The pooled eluate and the pooled washing were concentrated to volumes approximately equal to the volume of the original antiserum, by dialysis against sucrose. The sucrose in these concentrated solutions was removed by subsequent dialysis against saline. The optical density of the dialysed eluate was determined and the antibody contents of the eluate and of the

washing were determined quantitatively by the precipitin experiment.

Calculations for Capacity, Recovery and Purity

In order to calculate the three parameters given under this heading, the experiment described in the previous section was performed with an excess amount of antiserum in the adsorption stage.

It was assumed: (i) that the non-specific adsorption was negligible and that, for practical purposes, only antibodies were adsorbed, (ii) that all the antibodies had the same extinction coefficient as bovine γ-globulin, and (iii) that 1 mg/ml solutions of BGG and BSA in saline had optical densities of 1.45 and 0.62, respectively. For easy comparison, the calculations were normalized to 1 gm of immunosorbent. Thus, if it was observed that:

no. of mg of antibody in sample = f
" " " " " " washing = g
" " " " " " eluate = h
" " " " protein " " = j

then,

antibody-binding capacity of the immunosorbent = (f-g) mg antibody/gm sorbent

percentage protein recovery = 100 j/(f-g)

percentage purity of the antibody preparation = 100 h/j

Non-Specific Adsorption

For this evaluation, the experiment was performed as described in the section "isolation of antibody", with the exception that normal rabbit serum was used instead of anti-BSA antiserum. The amount of protein eluted at pH 3.0 (j) per gm of immunosorbent was designated as the non-specific adsorption.

Immunoelectrophoresis

To study the progressive purification of antibody preparations, the whole anti-BSA antiserum, the 50% ammonium sulphate precipitated globulin fraction,

and the antibody preparation recovered from the immunosorbent were analysed by immunoelectrophoresis (256,257), using an LKB immunoelectrophoresis apparatus (model 6800 A).

The experiment consisted of two main stages, the first being an electrophoretic separation of the components of the test sample in agar gel and the second being a precipitin reaction in the gel of the separated components with the appropriate antiserum*. A detailed description of the apparatus and the technique is given in the LKB operation manual No. 1-6800A-E01.

Precipitin and Ultracentrifugation Experiments

The same procedures as described in Chapter III were employed.

Results

Conjugation of BSA to the Acrylamide-Acrylic Acid Copolymer

In the absence of CME-CD, BSA neither reacts with, nor becomes strongly absorbed onto the acrylamide-acrylic acid copolymer. This was evidenced by the quantitative recovery of BSA when a suspension of the copolymer in BSA solution was exhaustively washed with saline solutions of pH 2.0, 10.0 and 7.0. In the absence of the copolymer, BSA reacted with CME-CD to form a turbid suspension which, however, turned clear when the pH was lowered to 2.0. With the exception of preparation I-1, all other samples gave a blue coloration when treated with the Folin-Ciocalteau reagent at alkaline pH. These findings, in addition to the detection of radioactivity in the solid phase when I^{131}-labelled BSA was used in the conjugation, all strongly indicated that during the course of these preparations, BSA had been covalently bonded to the copolymer backbone. According to the mechanism of the reaction between

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* Thanks are due to Mr. F.T. Kisil for the generous supply of goat anti-rabbit serum used for the immunoelectrophoresis experiment.

carboxyl groups, carbodiimides and protein, postulated by Khorana (254), and protein-hapten conjugation studies investigated in this laboratory (258,259), it is highly probable that the link between the protein and the copolymer is a peptide bond. The amounts of BSA conjugated with the copolymer in each preparation are shown in Table XIII. Generally, it was found that as the ratio of BSA to copolymer was kept constant, the amount of BSA conjugated increased with increasing amount of CME-CD, which was used as the coupling agent. However, when the ratio of CME-CD to copolymer was kept constant, the amount of BSA conjugated increased first with increasing amount of BSA used, reached a maximum and then dropped with further increase of BSA. It should be noted, however, that the amount of BSA conjugated, when the "two-step" procedure was used, was considerably smaller than that when the "one-step" procedure was used.

Antibody-Binding Capacity

From Table XIII, it is obvious that the antibody-binding capacity of the immunosorbent depended very much on the method of conjugation; i.e. immunosorbents prepared by the "one-step" procedure had much higher capacities than those prepared by the "two-step" method. Furthermore, the capacity of a given immunosorbent increased as the BSA content of the immunosorbent increased, but reached a plateau value of ~90 mg antibody per gm sorbent when the BSA content was 93 mg BSA bound per gm sorbent or higher.

Non-Specific Adsorption

The method of preparation was found to determine the degree of non-specific adsorption. Thus, while those immunosorbents prepared by the "one-step" procedure had only a negligible capacity for adsorbing non-antibody proteins (~0.5 mg protein/gm sorbent), the immunosorbents prepared by the "two-step" procedure had a much higher capacity (~5 mg protein/gm sorbent).

TABLE XIII : Characteristics of polyacrylamide-BSA immunosorbents

Immunosorbent	BSA Content	Non-Specific Adsorption	Capacity	% Recovery	% Purity
	mg BSA/ gm sorbent	mg protein/ gm sorbent	mg antibody/ gm sorbent	mg protein eluted/ gm protein adsorbed	mg antibody/ 100 mg protein eluted
II-1	non-detectable	6.3	non-detectable	-	-
II-2	12.0	5.7	23	82	47
II-3	17.0	5.9	26	87	58
II-4	18.0	5.2	34	83	68
I-1	94.0	0.35	82	77	86
I-2	129.1	0.52	107	82	89
I-3	161.6	0.48	89	81	85
I-4	178.4	0.47	96	82	86
I-5	48.2	-	76	79	90
I-6	93.2	-	98	84	91
I-7	103.5	-	84	79	95

Protein Recovery

Regardless of the method of preparation and the composition of the immunosorbents, about 80% of the adsorbed proteins were recovered by elution at pH 3.0. It should be noted, however, that, to minimize the length of time the antibodies were maintained at low pH, this estimate represents only the amount recovered in the first four elutions. It ought to be pointed out that trace amounts of proteins were detected even in the tenth elution. Furthermore, during the stage of concentration of the eluate by dialysis against sucrose and saline a small amount of precipitate was always observed, which was discarded by centrifugation. Since the concentration of the eluted protein solution was determined after concentration and final dialysis against saline, and since during these procedures some material was precipitated, it was not surprising that the recovery was not quantitative.

Re-usability of the Immunosorbents

In principle, the immunosorbents would be expected to be re-usable if the antigenic determinant groups were not destroyed or blocked during successive experiments. In practice, however, it is advisable to use freshly prepared immunosorbents for each experiment provided the antigen is expendable. It was found, in an experiment with preparation I-2, that the capacity of the same immunosorbent decreased by 20-30% after each use. These findings indicated that (i) some antigen-antibody complexes might remain undissociated, under the elution conditions used, even after a considerable number of elutions, and (ii) some of the BSA on the immunosorbent might have been denatured during the process of elution at low pH. Higher recovery by employing more drastic elution conditions were not attempted because it would have inevitably led to more denaturation. The primary concern of this investigation was to obtain antibody in pure form without the loss of specific activity.

During storage, the immunosorbents were suspended in neutral saline without appreciable change in properties for at least two weeks.

Nature and Purity of Antibodies Isolated

From Table XIII, it can be seen that the purity of the antibody preparations isolated with immunosorbents prepared by the "one-step" and "two-step" procedures were of the order of 89% and 58%, respectively. This is a natural consequence of the low capacity and high non-specific adsorption obtained for the "two-step" immunosorbents. The so-called impurities could be either non-specifically adsorbed proteins or denatured antibodies. According to ultracentrifugal analyses, the whole anti-BSA rabbit serum consisted of three components with sedimentation constants of 18.1, 6.6 and 4.5 S; the ammonium sulphate precipitated fraction consisted of two components with S values of 17.6 and 6.4; whereas the purified fraction (from preparation I-2) showed a single symmetrical peak with an S value of 7.0 (Figure 26). The small differences in S values of the same component (e.g. 6.6, 6.4 and 7.0) could be attributed to differences in concentration in these samples. Immuno-electrophoresis experiments showed that there were at least seven distinguishable components in the whole antiserum, at least four components in the ammonium sulphate precipitated fraction but only one component for the purified fraction (Figure 27). The fact that the precipitin band formed by the purified fraction was a little blurred indicated that there were some impurities in the fraction, or that the antibody preparation had a lower degree of heterogeneity. However, the position of this band and the value of 7.0 S obtained for the sedimentation coefficient strongly suggested that the great majority of the antibodies isolated were IgG-globulins in nature.

Discussion

Conjugation

Two explanations may be given for the difference in the BSA content in the conjugates prepared by the two procedures:

(i) The anhydride group has a high affinity for water and would, therefore,

FIGURE 26 : Ultracentrifugal pattern of the purified antibody preparation obtained by treatment of anti-BSA antiserum with the polyacrylamide-BSA immunosorbent.

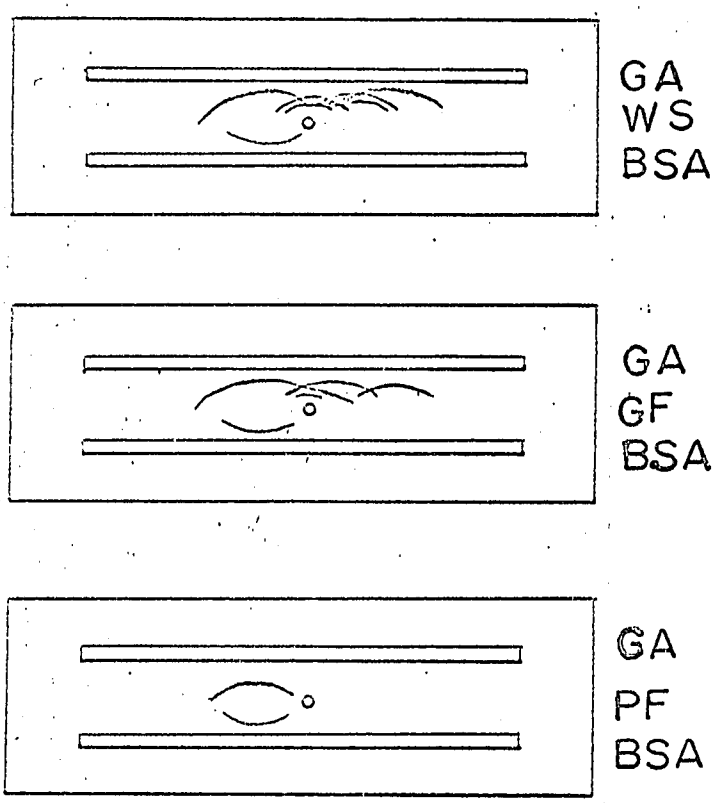

FIGURE 27 : Immunoelectrophoretic patterns of whole rabbit anti-BSA antiserum (WS), the globulin fraction (GF), the purified antibody preparation (PF) and the goat antiserum (GA).

react readily with water irrespective of the procedure. However, in the case of the "one-step" reaction, the carbodiimide was present in overwhelming excess so that the carboxyl groups resulting from the hydrolysis of the anhydride groups had repeated chances of reacting with more carbodiimide to regenerate the anhydride function and eventually of reaction with a free amino group of the antigen to form the conjugate. The same chance was not offered in the "two-step" procedure due to the removal of the carbodiimide between the two steps.

(ii) The addition of dimethyl formamide might have caused a change in the dielectric constant of the medium such that the free amino groups in the BSA molecule might have been involved in intramolecular salt-like linkages, and would then not have reacted with the anhydride group. Since the amino groups of the protein competed with water for reaction with the anhydride function, it is of paramount importance that the reaction be carried out at a condition when the free NH_2 form prevails.

Although much better results were obtained with conjugates prepared by the "one-step" method, there was one rather undesirable complication, viz. possible intramolecular peptide bond formation within the antigen molecule, since the carbodiimide could also react with the carboxyl groups on the protein molecule. Thus, since there are many carboxyl groups in a protein molecule like BSA, inter- as well as intra- molecular linking could have occurred in the "one-step" procedure. If the extend of the intra-molecular linking were excessive, the surface structure of the BSA molecule might undergo a considerable change. Consequently, many of the antigenic sites might have been destroyed or buried and this would result in an immuno-sorbent with a capacity lower than expected. Although the immunosorbents prepared in this study had a higher capacity than those of certain other immunosorbents such as the polystyrene-antigen type (260,249), they were inferior by comparison to conjugates of ethylene-maleic anhydride copolymer

with antigens(253).

It was expected from the law of mass action that while other factors were kept constant, increase of CME-CD (e.g. preparations I-1 to I-4) or BSA (preparations I-5, I-6, I-2 and I-7, and preparations II-1 to II-4) would result in an increase in the BSA content of the product. The deviation from this normal trend in preparation I-7 can probably be explained as follows. At the unusually high BSA concentration (80 mg/ml), the copolymer was probably not fully solvated and therefore the carboxyl groups on the copolymer were not as readily available for reaction as in other cases. Furthermore, the high BSA concentration would favour intramolecular linking and polymerization of the BSA molecules. Both of these would result in an effective decrease of CME-CD and thus a decrease in the extent of conjugation.

Non-Specific Adsorption

From the results of gel filtration experiments of serum proteins on polyacrylamide columns (Chapter III), one would expect hardly any non-specific adsorption from the present series of immunosorbents, if the carboxyl groups of the copolymer were all used up in the conjugation process. However, some of the carboxyl groups had probably not reacted and remained in their original form in the conjugated polymer. Furthermore, according to Khorana's reaction mechanism, one would expect that the reaction of an anhydride group with an amino group in the protein would result in the regeneration of a carboxyl group (in addition to the formation of a peptide bond). Theoretically, therefore, at least 50% of the original number of carboxyl groups would be expected to remain in the conjugated polymer prepared by the "two-step" procedure. This argument can probably be applied to explain the fact that much more non-specific adsorption was observed with the immunosorbents prepared by the "two-step" procedure than those prepared by the "one-step" procedure.

Capacity

From Table XIII, it may be concluded that an increase in the antigen content of the immunosorbent resulted in a corresponding increase in the antibody binding capacity, as expected, until the antigen content had reached ~90 mg BSA per gm sorbent. This observation suggests that when the latter condition was reached, the surface of the copolymer was fully coated with the antigen and that further conjugation (e.g. polymerization of BSA) would only have masked some of the previously conjugated ones. Since these masked antigens were not available for combination with the antibodies, there would not be an appreciable increase in the antibody binding capacity. A similar phenomenon was observed by Gurvitch (236) in his studies with cellulose immunosorbents.

Recovery

Although elution of antibodies was achieved at pH 3.0, the recovery was not quantitative. In addition to the part accounted for by the precipitates formed during the dialysis stage, some antibodies presumably remained strongly bound to the immunosorbent, while those with lower affinities for the antigen were easily eluted at pH 3.0. This interpretation, together with the finding that the band observed in immunoelectrophoresis was slightly diffuse, is in harmony with the accepted concept of heterogeneity of antibodies.

Purity

It should be noted that, in this discussion, the word "purity" is not equivalent to the concept used for description of simple, low molecular weight compounds. Rather, it refers to the percentage of the protein in the final "antibody preparation" which binds specifically with the antigen. The antibody preparations obtained with immunosorbents prepared by the "two-step" procedure were rather impure due to the higher extent of non-specific adsorption.

On the other hand, with the "one-step" immunosorbents, although the reported purity was ~80%, the amount of non-specific adsorption accounted for only ~0.5% of the total amount of protein adsorbed. This would likely suggest that the "impurities" of these preparations were mostly denatured antibodies.

The results of ultracentrifugal and immunoelectrophoretic analyses indicated that the majority of the antibodies isolated were of the IgG-globulin type. The inability to detect the presence of the other faster sedimenting immune globulins might have been due to (i) their relatively low concentration and (ii) their greater susceptibility to denaturation.

Other Immunosorbents

Although only the BSA-anti-BSA system was investigated in detail in this project, obviously, it is not the only antigen that can be coupled to the copolymer. In fact, preliminary investigations had shown that other proteins such as bovine γ-globulin, lysozyme and human serum albumin could also be similarly conjugated to the copolymer using the "one-step" procedure.

It must also be emphasized that the coupling reactions of some antigens to polymers may involve the antigenic determinants themselves, or other groups situated close to these determinants. This would result in a reduction or even in a complete loss of antibody-binding capacity. Thus, if the determinant group contains or is close to a tyrosyl or histidyl residue, coupling with a polymer bearing diazonium groups would lead to the blocking of, at least, some of the antigenic group. This limitation, however, could be overcome by the use of polymers possessing other groups capable of reacting readily with the reactive centres of the proteins. It is clear that the versatility of modern preparative polymer chemistry opens up unlimited possibilites in this area of research.

SUMMARY

Sixteen copolymers of acrylamide and N,N'-methylene bisacrylamide were synthesized, using ammonium persulphate and β-dimethylaminopropionitrile as initiators. The water regain values of these gels were determined and were found to increase with decreasing total monomer concentration and with decreasing N,N'-methylene bisacrylamide/acrylamide ratio.

The partition coefficients (K_D) of a variety of compounds with molecular weights between 104 and 580,000 were determined by normal elution chromatography for seven gels. The identities of the various solutes were established by spectrophotometry in the UV and visible regions, micro-hemagglutination and precipitin tests with homologous antisera, and paper electrophoresis and sedimentation velocity analyses. The K_D values of different solutes passed through the same column were found to vary linearly with the logarithms of their molecular weights. The K_D values of some of the haptens were greater than 1.0, which was interpreted to indicate that other mechanisms beside gel filtration might have been underlying these chromatographic processes. The retardation of hemoglobin on the column was explained by the tendency of this protein to dissociate into sub-units at high dilution, and the retardation of lysozyme and of the haptens was attributed to adsorption.

The molecular exclusion model for gel filtration, based on the Laurent-Killander-Ogston theory, was used for theoretical calculations. The total fibre length per unit volume of the gels was calculated from the physical and molecular properties of the gels. These values, in conjunction with the published data for the radii of gyration of the solutes and the arbitrarily assigned value of the half-thickness of the polymer fibre, were used to calculate the partition coefficients of the solutes in these gels. Good agreement between the theoretically calculated and experimentally determined

K_D values was obtained, and it was, therefore, concluded that the chromatographic processes involved primarily a gel filtration mechanism of the molecular exclusion type.

Four antigenically specific adsorbents were prepared by conjugating BSA to a copolymer of acrylamide, acrylic acid and methylene bisacrylamide, using 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide-metho-p-toluene-sulphonate (CME-CD) as the coupling agent in a "two-step" reaction, i.e. first converting the copolymer to its anhydride form and then reacting it with the protein antigen. These conjugates contained 12-18 mg of BSA per gm of immunosorbent, as determined spectrophotometrically by the difference between the total amount used and the amount left in the reaction washings. Their antibody-binding capacities were 23-34 mg of antibodies per gm of immunosorbent. However, they also showed a non-specific binding capacity of 5.2-6.3 mg of non-antibody proteins per gm of immunosorbents. The bound proteins and antibodies could be eluted with 0.5 M glycine acidified to pH 3.0 with 1.0 N HCl, in yields of 82-87% and a purity of 47-68%.

Seven other immunosorbents were prepared with the same reactants using a "one-step" procedure, i.e. the copolymer, CME-CD and BSA were allowed to react simultaneously. The BSA content of these immunosorbents, as determined by a radioactive tracer technique using I^{131}-labelled BSA, was 93-178 mg BSA per gm of immunosorbent. Generally, the amounts of BSA conjugated were found to increase with increasing amounts of both BSA and CME-CD used in the reaction. These immunosorbents combine specifically and firmly with anti-BSA antibodies at neutral pH and the non-specific adsorption was only negligible (0.35-0.52 mg of protein per gm of immunosorbent). The antibody binding capacity of these immunosorbents (76-107 mg of antibodies per gm of immunosorbent) increased with increasing BSA content in the adsorbent, and reached a plateau value of $\sim$100 mg of antibodies per gm of immunosorbent when about

90 mg of BSA was coupled per gm of the copolymer. Elution of the antibodies from the immunosorbent was also achieved with 0.5 M glycine acidified to pH 3.0 with 1.0 N HCl. Precipitating antibodies were recovered in yields of 77-84% with a purity of 85-91%. Immunoelectrophoretic and ultracentrifugal analyses of the antibody preparations showed that the isolated antibodies were mainly IgG-globulins.

Other protein antigens such as BGG, lysozyme and HSA were found to be capable of being conjugated to the acrylamide-acrylic acid-methylene bisacrylamide copolymer with the "one-step" procedure.

CLAIMS TO ORIGINALITY

1. Sixteen copolymers of acrylamide and N,N'-methylene bisacrylamide were synthesized, using ammonium persulphate and β-dimethylaminopropionitrile as initiators.

2. The water regain values of these gels were found to increase with decreasing total monomer concentration and with decreasing methylene bisacrylamide/acrylamide ratio.

3. The partition coefficients (K_D) of a variety of compounds of molecular weights between 104 and 580,000 were determined by normal elution column chromatography for seven gels.

4. The K_D values of a given solute through different gels were found to increase with decreasing total monomer concentration of the gel and with decreasing methylene bisacrylamide/acrylamide ratio.

5. For various solutes through the same column, the K_D values varied linearly with the logarithms of the corresponding molecular weights.

6. Hemoglobin, lysozyme and the various aromatic haptens were found to be eluted at volumes greater than would have been expected from their molecular weights.

7. Using a modification of the Laurent-Killander-Ogston theory, the K_D values of seven proteins through seven gels were calculated and were found to agree well with the experimentally observed values.

8. Eleven antigenically specific adsorbents were synthesized by the reaction of BSA with a copolymer of acrylamide, acrylic acid and methylene bisacrylamide, using 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide-metho-p-toluene-

sulphonate (CME-CD) as the coupling agent and using either a "one-step" or "two-step" procedure.

9. The BSA content of these immunosorbents were found to increase with increasing amounts of CME-CD or BSA used in the preparation.

10. These immunosorbents were found to combine specifically and firmly with the homologous antibodies, the binding capacities being higher for immunosorbents of high BSA content.

11. The antibodies were found to be elutable from the immunosorbents in yields of ~80% with 0.5 M glycine acidified to pH 3.0 with 1.0 N HCl.

12. The recovered antibodies were found to have a purity of as high as 91% and to consist mainly of IgG-globulins.

BIBLIOGRAPHY

1. Brit. Med. Bull. 22, No. 2 (1966).

2. Alexander, P. and Block, R.J., "A Laboratory Manual of Analytical Methods of Protein Chemistry", Pergamon Press, New York (1960).

3. Neurath, H. (ed.), "The Proteins: Composition, Structure and Function", Academic Press, New York (1965).

4. Zechmeister, L. in Heftmann, E. (ed.), "Chromatography", Chapter 1, Reinhold Pub. Corp., New York (1961).

5. Tswett, M., Proc. Warsaw Soc. Nat. Sci., Biol. Section, 14, No. 6 (1903).

6. Kuhn, R. and Lederer, E., Ber. 64, 1349 (1931).

7. Macek, K. and Hais, I.M., "Bibliografie papirove chromatografie (1944-1956)", Nakl. Csav, Prague (1960).

8. Macek, K., Hais, I.M., Gasparic, J., Kopecky, J. and Rabek, V., "Bibliography of Paper Chromatography", Nakl. Csav, Prague.

9. Martin, A.J.P. and Synge, R.L.M., Biochem. J. 35, 91, 1358 (1941).

10. Porath, J. and Flodin, P., Nature 183, 1657 (1959).

11. Consden, R., Gordon, A.H. and Martin, A.J.P., Biochem. J. 38, 224 (1944).

12. Hesse, G. and Tschachotin, B., Naturwiss. 30, 387 (1942).

13. James, A.T. and Martin, A.J.P., J. Biochem. 50, 679 (1952).

14. Van Deempter, J.J., Zuiderweg, F.J. and Klinkenberg, A., Chem. Eng. Sci. 5, 271 (1956).

15. Giddings, J.C., J. Chem. Educ. 35, 588 (1958).

16. Giddings, J.C., J. Chem. Phys. 26, 1755 (1957).

17. Glueckauf, E., in "Ion-Exchange and its Applications", Society of Chemical Industry, London (1955).

18. Feller, W., "Probability Theory and its Applications", Wiley, New York (1950).

19. Heftmann, E. (ed.), "Chromatography", Reinhold Pub. Corp., New York (1961).

20. Pecsok, R.L. (ed.), "Principles and Practice of Gas Chromatography", Wiley (1959).

21. Morris, C.J.O.R. and Morris, P., "Separation Methods in Biochemistry", Pitman, London (1964).

22. Tiselius, A., Arkiv. Kemi 7, 443 (1964).

23. Baddiley, J. and Neuhaus, F.C., Biochem. J. 75, 579 (1960).

24. Fredericq, E. and Oth, A., Biochim. et Biophys. Acta 29, 281 (1958).

25. Kellermann, C.M., Biochim. et Biophys. Acta 33, 101 (1959).

26. Massey, V., Biochim. et Biophys. Acta 37, 310 (1960).

27. Hjerten, S., Biochim. et Biophys. Acta 31, 218 (1959).

28. Clauser, H. and Li, C.H., J. Am. Chem. Soc. 76, 4337 (1954).

29. Willstätter, R., "Untersuchen über Enzyme", Springer, Berlin (1928).

30. White, W.F. and Fierce, W.L., J. Am. Chem. Soc. 75, 245 (1953).

31. Anastasi, A. and Erspamer, V., Archs Biochem. Biophys. 101, 56 (1963).

32. Morris, C.J.O.R., Brit. Med. Bull. 22, 168 (1966).

33. Isliker, H.C., Ann. N.Y. Acad. Sci. 57, 225 (1953).

34. Sehon, A.H., Brit. Med. Bull. 19, 183 (1963).

35. Boardman, N.K. and Patridge, S.M., Biochem. J. 59, 543 (1955).

36. Hirs, C.H.W., Moore, S. and Stein, W.H., J. Biol. Chem. 200, 493 (1953).

37. Peterson, E.A. and Sober, H.A., J. Am. Chem. Soc. 78, 751 (1956).

38. Porath, J. and Lindner, E.B., Nature 191, 69 (1961).

39. Commercial literature, Bio-Rad Laboratories, Richmond, California (1966).

40. Taborsky, G., J. Biol. Chem. 234, 2652 (1959).

41. Aquist, S.E.G. and Anfinsen, C.B., J. Biol. Chem. 234, 1112 (1959).

42. Keller, P.J., Cohen, E. and Neurath, H., J. Biol. Chem. 233, 344 (1958).

43. Press, E.N., Porter, R.R. and Cebra, J., Biochem. J. 74, 901 (1960).

44. Sober, H.A. and Peterson, E.A., Federation Proc. 17, 1116 (1959).

45. Gutter, F.J., Peterson, E.A. and Sober, H.A., Archs Biochem. Biophys. 80, 353 (1959).

46. Rhodes, M.B., Azari, P.R. and Feeney, R.E., J. Biol. Chem. 230, 399 (1958).

47. Sober, H.A., Gutter, F.J., Wykoff, M.M. and Peterson, E.A., J. Am. Chem. Soc. 78, 756 (1956).

48. Fahey, J.L. and Horbett, A.P., J. Biol. Chem. 234, 2645 (1959).

49. Ward, D.N., McGregor, R.F. and Griffin, A.C., Biochim. et Biophys. Acta 32, 305 (1959).

50. Friedman, S. and Munson, P.L., Biochim. et Biophys. Acta 35, 509 (1959).

51. Synston, L.K., Free, C.A. and Pierce, J.G., J. Biol. Chem. 235, 87 (1960).

52. Reisfeld, R.A., Bergenstal, D.M. and Hertz, R., Archs Biochem. Biophys. 81, 456 (1959).

53. Porath, J. Arkiv Kemi 11, 97 (1957).

54. Semenza, G., Helv. Chim. Acta 43, 1057 (1960).

55. Herbert, D. and Pinsent, A.J., Biochem. J. 43, 193 (1948).

56. Martin, A.J.P. and Porter, R.R., Biochem. J. 49, 215 (1951).

57. Porter, R.R., Biochem. J. 53, 320 (1953).

58. Porter, R.R., Brit. Med. Bull. 10, 237 (1954).

59. Porter, R.R. Biochem. J. 59, 405 (1955).

60. Andersen, W., Acta Chem. Scand. 8, 1723 (1954).

61. Determann, H., Angew, Chem. int. Edu. Engl. 3, 608 (1964).

62. Andrews, P., Brit. Med. Bull. 22, 109 (1966).

63. Lathe, G.H. and Ruthven, C.R.J., Biochem. J. 62, 665 (1956).

64. Polson, A., Biochim. et Biophys. Acta 50, 565 (1961).

65. Hjerten, S., Archs Biochem. Biophys. 99, 466 (1962).

66. Andrews, P., Nature 196, 36 (1962).

67. Steere, R.L. and Ackers, G.K., Nature 194, 114 (1962).

68. Steere, R.L. and Ackers, G.K., Nature 196, 475 (1962).

69. Lea, D.J. and Sehon, A.H., Can. J. Chem. 40, 159 (1962).

70. Hjerten, S. and Mosbach, R., Anal, Biochem. 3, 109 (1962).

71. Hjerten, S., Archs Biochem. Biophys., Suppl. No. 1, p. 147 (1962).

72. Sun, K. and Sehon, A.H., Can. J. Chem. 43, 969 (1965).

73. Flodin, P., J. Chromatog. 5, 103 (1961).

74. Bucovaz, E.T. and Davis, J.W., J. Biol. Chem. 236, 2015 (1961).

75. Ledvina, M., J. Chromatog. 11, 71 (1963).

76. Patrick, R.L. and Thiers, R.E., Clin. Chem. 9, 283 (1963).

77. Kisliuk, R.L., Biochim. et Biophys. Acta 40, 531 (1960).

78. George, W. and Walton, K.W., Nature 192, 1188 (1961).

79. Killander, J., Ponten, J. and Roden, L., Nature 192, 182 (1961).

80. Gordon, M.A., Edwards, M.R. and Tompkins, V.N., Proc. Soc. Expt. Biol. Med. 109, 96 (1962).

81. Tokumaru, T., J. Immunol. 89, 195 (1962).

82. Greenwood, F.C., Hunter, W.M. and Glover, J.S., Biochem. J. 89, 114 (1963).

83. Daisley, K.W., Nature 191, 868 (1961).

84. Bennich, H., Biochim. et Biophys. Acta 51, 265 (1961).

85. Guidotti, G., Hill, R.J. and Konigsberg, W., J. Biol. Chem. 237, 2184 (1962).

86. Lee, Y.C. and Montgomery, R., Archs. Biochem. Biophys. 97, 9 (1962).

87. Nolan, C. and Smith, E.L., J. Biol. Chem. 237, 446 (1962).

88. Press, E.M. and Porter, R.R., Biochem. J. 83, 172 (1962).

89. Carnegie, P.R., Nature 206, 1128 (1965).

90. Bagdasarian, M., Matheson, N.A., Synge, R.L.M. and Youngson, M.A., Biochem. J. 91, 91 (1964).

91. Porath, J. and Schally, A.V., Endocrinology 70, 738 (1962).

92. Porath, J., Pure Appl. Chem. 6, 233 (1963).

93. Rasmussen, H., Sze, Y.L. and Young, R., J. Biol. Chem. 239, 2852 (1964).

94. Folley, S.J. and Knaggs, W., J. Endocr. 33, 301 (1965).

95. Synge, R.L.M., Metabolism 13, 969 (1964).

96. Flodin, P. and Aspberg, K. in Goodwin, T.W. and Lindberg, O. (ed.), "Biological Structure and Function" vol. 1, p. 345, Academic Press, London.

97. Lundblad, A. and Bergard, I., Biochim. et Biophys. Acta 57, 129 (1962).

98. Flodin, P., Gregory, J.D. and Roden, L., Anal. Biochem. 8, 424 (1964).

99. Lee, Y.C. and Ballou, C.E., Biochemistry 4, 257 (1965).

100. Granath, K.A. and Flodin, P., Makromolek. Chem. 48, 160 (1961).

101. Andrews, P. and Roberts, G.P., Biochem. J. 84, 11 (1962).

102. Hummel, B.C.W. and Smith, D.C., J. Chromatog. 8, 491 (1962).

103. Nordin, P., Archs Biochem. Biophys., Suppl. No. 1, p. 157 (1962).

104. Heller, J. and Schramm, M., Biochim. et Biophys. Acta 81, 96 (1964).

105. Roberts, G.P. and Gibbons, R.A., Biochem. J. 98, 426 (1966).

106. Anderson, D.M.W., Dea, I.C.M., Rahman, S. and Stoddart, J.F., Chem. Commun. p. 145 (1965).

107. Campbell, J.H. and Papenheimer, A.M., Immunochem. 3, 195 (1966).

108. Campbell, J.H. and Pappenheimer, A.M., Immunochem. 3, 213 (1966).

109. Ingram, V.M. and Pierce, J.G., Biochemistry 1, 580 (1962).

110. Hohn, T. and Pollmann, W., Z. Natuf. 186, 919 (1963).

111. Hayes, F.N., Hansbury, E. and Mitchell, V.E., J. Chromatog. 16, 410 (1964).

112. Lipsett, M.N., J. Biol. Chem. 239, 1250 (1964).

113. Jones, O.W., Townsend, E.E., Sober, H.A. and Heppel, L.A., Biochemistry 3, 238 (1964).

114. Richards, E.G. and Gratzer, W.B., Nature 204, 878 (1964).

115. Schleich, T. and Goldstein, J., Proc. Nat. Acad. Sci. U.S.A. 52, 744 (1964).

116. Röschenthaler, R. and Fromageot, P., J. Molec. Biol. 11, 458 (1965).

117. Boman, H.G. and Hjerten, S., Archs Biochem. Biophys., Suppl. No. 1, p. 276 (1962).

118. Oberg, B., Bengtsson, S. and Philipson, L., Biochem. Biophys. Res. Commun. 20, 36 (1965).

119. Gelotte, B., Acta Chem. Scand. 18, 1283 (1964).

120. Downey, W.K. and Andrews, P., Biochem. J. 94, 642 (1965).

121. Roskes, S.D. and Thompson, T.E., J. Biol. Chem. 240, 198 (1965).

122. Killander, J., Biochim. et Biophys. Acta 93, 1 (1964).

123. Andrews, P., Biochem. J. 91, 222 (1964).

124. Andrews, P., Biochem. J. 96, 595 (1965).

125. Morris, C.J.O.R., J. Chromatog. 16, 167 (1964).

126. Andrews, P., Nature 209, 155 (1966).

127. Winzor, D.J. and Scheraga, H.A., J. Phys. Chem. 68, 338 (1964).

128. Rogers, K.S., Hellerman, L. and Thompson, T.E., J. Biol. Chem. 240, 198 (1965).

129. Steere, R.L., Science 140, 1089 (1963).

130. Strain, H., Anal. Chem. 32,

131. Fasold, H., Gundlach, G. and Turba, F. in Heftmann, E. (ed.), "Chromatography" p. 406, Reinhold Pub. Corp., New York (1961).

132. Pedersen, K.O., Archs Biochem. Biophys., Suppl. No. 1, p. 157 (1962).

133. Moore, J.C., J. Polymer Sci. Part A, 2, 835 (1964).

134. Barrer, R.M., Quart. Rev. Chem. Soc. 3, 293 (1949).

135. Helfferich, F., "Ionenaustauscher", Band I, Verlag Chemie GmbH, Weinheim/Bergstr. (1959).

136. Ayres, J.A., J. Am. Chem. Soc. 69, 2879 (1947).

137. Wheaton, R.M. and Bauman, W.C., Ann. N.Y. Acad. Sci. 57, 159 (1953).

138. Vaughan, M.F., Nature 188, 55 (1960).

139. Flodin, P., "Dextran Gels and their Application in Gel Filtration", Meijels Bokindustri, Halmstad, Uppsala (1962).

140. Ackers, G.K., Biochemistry 3, 723 (1964).

141. Ackers, G.K. and Steere, R.L., Biochim. et Biophys. Acta 59, 137 (1962).

142. Laurent, T.C., Björk, I., Pietruszkiewicz, A. and Persson, H., Biochim. et Biophys. Acta 78, 351 (1963).

143. Ogston, A.G. and Phelps, C.F., Biochem. J. 78, 827 (1961).

144. Laurent, T.C. and Ogston, A.G., Biochem. J. 89, 249 (1963).

145. Laurent, T.C., Biochem. J. 89, 253 (1963).

146. White, M.L., J. Phys. Chem. 64, 1563 (1960).

147. Whitaker, J.R., Anal. Chem. 35, 1950 (1963).

148. Brewer, P.I., Nature 190, 625 (1961).

149. Renkin, E.M., J. Gen. Physiol. 38, 225 (1955).

150. Ferry, J.D., J. Gen. Physiol. 20, 35 (1936).

151. Faxen, H., Arkiv. Mat. Astron. Fysik 17, No. 27 (1922).

152. Squire, P.G., Archs Biochem. Biophys. 107, 471 (1964).

153. Laurent, T.C. and Killander, J., J. Chromatog. 14, 317 (1964).

154. Ogston, A.G., Trans. Faraday Soc. 54, 1754 (1958).

155. Hermans, P.H. in Kruyt, H.R. (ed.), "Colloid Science" vol. 2, p. 483, Elsevier Pub. Co., Amsterdam (1949).

156. Pepper, K.W., Reichenberg, D. and Hale, D.K., J. Chem. Soc., 3129 (1952).

157. Flodin, P. and Kupke, D.W., Biochim. et Biophys. Acta 21, 368 (1956).

158. Heidelberger, M. and Kendall, F.E., J. Expt. Med. 50, 809 (1929).

159. McKenzie, H.A. and Wallace, H.S., Austr. J. Chem. 7, 55 (1954).

160. Takatsy, G., Acta Microbiol. Sci. Hungariae 3, 191 (1955).

161. Reiner, A., M.Sc. thesis, McGill University (1964).

162. Gordon, J., Rose, B. and Sehon, A.H., J. Expt. Med. 108, 37 (1958).

163. Svedberg, T. and Pedersen, K.O., "Die Ultrazentrifuge", Verlag von Theodor Steinkoff, Dresden (1939).

164. Jones, G.D., Zomlefer, J. and Hawkins, K., J. Org. Chem. 9, 500 (1944).

165. Staudinger, H. and Urech, E., Helv. Chim. Acta 12, 1107 (1929).

166. Kung, F.E., U.S. Patent 2,373,190 (1945).

167. Centeno, E.R., Sun, K. and Sehon, A.H., 13th Canadian High Polymer Forum, Ottawa (1965).

168. Flory, P., "Principles of Polymer Chemistry", Cornell University Press, Ithaca, New York (1953).

169. Fawcett, J.S. and Morris, C.J.O.R., Abstract II-54, 16th International Congress in Biochemistry, New York (1964).

170. Gelotte, B., J. Chromatog. 3, 330 (1960).

171. Giles, C.H. in Heftmann, E. (ed.), "Chromatography" p. 33, Reinhold Pub. Corp., New York (1961).

172. Gutfreund, H. in Roughton, F.J.W. and Kendrew, J.C. (ed.), "Hemoglobin", Butterworth, London (1949).

173. Porter, R.R. in Putnam, F.W. (ed.), "The Plasma Proteins" Vol. 1, p. 241, Academic Press, London (1961).

174. Nisonoff, A., Markus, G. and Wissler, F.C., Nature 189, 293 (1961).

175. Porter, R.R., Brit. Med. Bull. 19, 197 (1963).

176. Peterson, E.A. and Sober, H.A. in Putnam, F.W. (ed.), "The Plasma Proteins" vol. 1, p. 105, Academic Press, London (1961).

177. Rowe, D.S. and Fahey, J.L., J. Expt. Med. 121, 185 (1965).

178. Howe, P.E., J. Biol. Chem. 49, 93 (1921).

179. Cohn, E.J., McMeekin, T.L., Oncley, J.L., Newell, J.M. and Hughes, W.L., J. Am. Chem. Soc. 62, 3386 (1940).

180. Svensson, H., J. Biol. Chem. 139, 805 (1941).

181. Hermans, J., "Les globulines seriques du systeme gamma. Leur nature et leur pathologie", Masson, Paris (1960).

182. Cohn, E.J., Strong, L.E., Hughes, W.L., Mulford, D.J., Ashworth, J.N., Melin, M. and Taylor, H.L., J. Am. Chem. Soc. 68, 459 (1946).

183. Kekwick, R.A. and Mackay, M.E., Spec. Rep. Ser. Med. Res. Coun., Lond., No. 286 (1954).

184. Pennell, R.B. in Putnam, F.W. (ed.), "The Plasma Proteins" vol. 1, p. 9, Academic Press, London (1961).

185. Horejsi, J. and Smetana, R., Acta Med. Scand. 155, 65 (1956).

186. Isliker, H.C., Advanc. Protein Chem. 12, 387 (1957).

187. Tiselius, A., Trans. Faraday Soc. 33, 524 (1937).

188. Tiselius, A. and Kabat, E.A., J. Expt. Med. 69, 119 (1939).

189. Sehon, A.H., Harter, J.G. and Rose, B., J. Expt. Med. 103, 679 (1956).

190. Longsworth, L.G. in Bier, M. (ed.), "Electrophoresis" p. 137, Academic Press, New York (1959).

191. Cooper, G.R. in Putnam, F.W. (ed.), "The Plasma Proteins" vol. 1, p. 51, Academic Press, London (1961).

192. Kunkel, H.G. and Slater, R.J., Proc. Soc. Expt. Biol., N.Y. 80, 42 (1952).

193. Kunkel, H.G. and Trautman, R. in Bier, M. (ed.), "Electrophoresis" p. 137, Academic Press, New York (1959).

194. Fireman, P., Vannier, W.E. and Goodman, H.C., J. Expt. Med. 117, 603 (1963).

195. Brattsten, I., Ark. Kemi 8, 205, 227, 347 (1955).

196. Block, R.J., Durrum, E.L. and Zweig, G., "A Manual of Paper Chromatography and Paper Electrophoresis p. 333, Academic Press, New York (1955).

197. Hannig, K., Z. Anal. Chem. 181, 244 (1961).

198. Cann, J.R. in Najjar, V.A. (ed.), "Immunity and Virus Infection", p. 100, Wiley, New York (1959).

199. Cann, J.R., Brown, R.A. and Kirkwood, J.G., J. Biol. Chem. 181, 161 (1949).

200. Pedersen, K.O., "Ultracentrifugal Studies on Serum and Serum Fractions", Almquist and Wiksells, Uppsala (1945).

201. Schachman, H.K., "Ultracentrifugation in Biochemistry", Academic Press, New York (1959).

202. Wallenius, G., Trautman, R., Kunkel, H.G. and Franklin, E.C., J. Biol. Chem. 225, 253 (1957).

203. Kunkel, H.G. in Putnam, F.W. (ed.), "The Plasma Proteins" vol. 1, p. 279 (1961).

204. Kunke, H.G., Rockey, J.H. and Tomasi, T. in Heidelberger, M. and Plescia, O.J. (ed.), "Immunochemical Approaches to Problems in Microbiology", Rutgers University Press, New Brunswick, N.J. (1961).

205. Rockey, J.H. and Kunkel, H.G., Proc. Soc. Expt. Biol, N.Y. 110, 101 (1962).

206. Singer, S.J., J. Cell. Comp. Physiol. 50, Suppl. No. 1, p. 51 (1957).

207. Pressman, D. in Pauling, L. and Itano, H.A. (ed.), "Molecular Structure and Biological Specificity" p. 1, American Institute of Biological Sciences, Washington, D.C. (1957).

208. Talmage, D.W. and Cann, J.R., "The Chemistry of Immunity in Health and Disease", Thomas, Springfield, Ill. (1961).

209. Wurmser, R. and Filitti-Wurmser, S., Prog. Biophys. Biophys. Chem., 7, 87(1957).

210. Gordon, R.S., jr., J. Immunol. 71, 220 (1953).

211. Goodman, H.S., Nature 193, 350 and 194, 934 (1962).

212. Korngold, L. and Pressman, D., J. Immunol. 71, 1 (1953).

213. Day, E.D., Planinsek, J.A. and Pressman, D., J. Nat. Can. Inst. 27, 1107 (1961).

214. Pope, C.G. and Healey, M., Brit. J. Expt. Path. 20, 201 (1939).

215. Bolyn, A.E. and Moskowitz, M., J. Immunol. 75, 441 (1955).

216. Singer, S.J., Eggman, L. and Campbell, D.H., J. Am. Chem. Soc. 77, 4855 (1955).

217. Grossberg, A.L. and Pressman, D., J. Am. Chem. Soc. 82, 5478 (1960).

218. Sehon, A.H., Pure Appl. Chem. 4, 483 (1962).

219. Kleinschmidt, W.J. and Boyer, P.D., J. Immunol. 69, 247 (1952).

220. Singer, S.J. and Campbell, D.H., J. Am. Chem. Soc. 77, 4855 (1955).

221. Heidelberger, M. and Kabat, E.A., J. Expt. Med. 67, 181 (1938).

222. Sternberg, L.A. and Pressman, D., J. Immunol. 65, 65 (1950).

223. Singer, S.J., Fothergill, J.E. and Shainoff, J.R., J. Am. Chem. Soc. 82, 565 (1960).

224. Meyer, K. and Pic, A., Ann. Inst. Pasteur 56, 401 (1936).

225. Svensson, H., Ark. Kemi Min. Geol. 17, No. 5 (1943).

226. Sutherland, G.B. and Campbell, D.H., J. Immunol. 80, 294 (1958).

227. Landsteiner, K. and Scheer, J. van der, J. Expt. Med. 63, 325 (1936).

228. Froese, A. and Sehon, A.H., Canad. J. Biochem. Physiol. 39, 1067 (1961).

229. Karush, F. and Marks, R., J. Immunol. 78, 296 (1957).

230. Farah, F.S., Kern, M. and Eisen, H.N., J. Expt. Med. 112, 1195 (1960).

231. Velick, S.F., Parker, C.W. and Eisen, H.N., Proc. Nat. Acad. Sci. 46, 1470 (1960).

232. Schlossman, S.F. and Kabat, E.A., J. Expt. Med. 116, 535 (1962).

233. Hirata, A.A. and Campbell, D.H., Immunochem. 2, 195 (1965).

234. Weliky, N. and Weetall, H.H., Immunochem. 2, 293 (1965).

235. Campbell, D.H., Luescher, E. and Lerman, L.S., Proc. Nat. Acad. Sci. 37, 575 (1951).

236. Gurvitch, A.E., Kapner, R.B. and Nezlin, R.S., Biokhimiya 24, 144 (1959).

237. Gurvitch, A.E., Kuzovleva, O.B. and Tumanova, A.E., Biokhimiya 27, 246 (1962).

238. Gurvitch, A.E., Kuzovleva, O.B. and Tumanova, A.E., Biokhimiya 26, 934 (1961).

239. Reiner, A., Kisil, F. and Sehon, A., unpublished data.

240. Lerman, L.S., Nature 172, 635 (1953).

241. Kreiter, V.P. and Pressman, D., Immunochem. 1, 91 (1964).

242. Weliky, N., Weetall, H.H., Gilden, R.V. and Campbell, D.H., Immunochem. 1, 219 (1964).

243. Vannier, W.E., Bryan, W.P. and Campbell, D.H., Immunochem. 2, 1 (1965).

244. Weetall, H.H. and Weliky, N., Science 148, 1235 (1965).

245. Isliker, H.C., Advanc. Protein Chem. 12, 387 (1957).

246. Manecke, G. and Gilbert, K.E., Naturwiss. 42, 212 (1955).

247. Gyenes, L., Rose, B. and Sehon, A.H., Nature 181, 1465 (1958).

248. Yagi, Y., Engel, K. and Pressman, D., J. Immunol. 85, 375 (1960).

249. Manecke, G., Pure Appl. Chem. 4, 507 (1962).

250. Gyenes, L. and Sehon, A.H., Can. J. Biochem. Physiol. 38, 1249 (1960).

251. Frick, O.L., Gyenes, L. and Sehon, A.H., J. Allergy 31, 216 (1960).

252. Onoue, K., Yagi, Y. and Pressman, D., Immunochem. 2, 181 (1965).

253. Centeno, E.R. and Sehon, A.H., Fed. Proc. Abstr. 25, 729 (3044) (1966).

254. Khorana, H.G., Chem. Revs. 53, 145 (1953).

255. Kabat, E.A. and Meyer, M.M., "Experimental Immunochemistry", p. 556 (1948).

256. Grabar, P. and Williams, C.A. jr., Biochim. et Biophys. Acta 10,193 (1953).

257. Hirschfeld, J., Science Tools 7, No. 2, p. 18 (1960).

258. Tenenhouse, S.H., M.Sc. thesis, McGill University (1963).

259. Ranadive, N.S., Ph.D. thesis, McGill University (1965).

260. Gyenes, L. and Sehon, A.H., Canad. J. Biochem. Physiol. 38, 1235 (1960).