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ISOLATION AND CHARACTERIZATION OF IMMUNOGLOBULINS

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CONTENTS

I. INTRODUCTION

A. Isolation Parameters

With a view to the isolation of immunoglobulins, as well as of all other biopolymers, one must make use of those physicochemical properties or parameters that are peculiar to the polymer in question, and that are quantitatively different from the physicochemical properties of the other accompanying (but unwanted) polymers. There are five fundamentally different physicochemical parameters of biopolymers: solubility, electric charge, surface tension, size and shape, and ligand specificity.

In an earlier review on the separation of plasma proteins in general, written almost fifteen years ago for the first volume of the predecessor of this Series[1], only four of these five parameters were treated. It was only during the last few years that the surface tension of proteins became recognized as an intrinsic property[2] and as the parameter instrumental in their separability via, e.g., hydrophobic interaction chromatography[3].

B. Isolation Strategies

Different biopolymers may have one or two of these parameters in common, but they never quantitatively share all five of them. Thus the most successful strategy for isolating any given biopolymer, e.g., an immunoglobulin, from a mixture, consists of a combination of two or more methods, each one of which discriminates for 2 different physicochemical parameters. IgM in whole serum, for example, has its size in common with $\alpha 2$ macroglobulin, and its electric charge with many other β-globulins. Thus a two-step procedure, in which one step isolates the proteins of 800,000-900,000 Mw, whilst a second step selects the β-globulins, should in principle result in virtually pure IgM. In theory this is indeed so, but in practice a few more steps are desirable, e.g., an initial precipitating step, to eliminate 90% of the irrelevant proteins, as well as one or two ultrafiltration steps, to counteract the considerable dilution that accompanies practically all separation processes. A drastic improvement in purification can usually be obtained by selecting a different isolation parameter as a basis for

the next purification step. Repetition of the same procedure seldom leads to significantly enhanced purity.

II. SOLUBILITY

A. Precipitation by Protein-Protein Interaction

Antibodies, being proteins and thus amphoteres, have positively as well as negatively charged sites, which can cause them to bind to each other and form large insoluble complexes. Three conditions favor such protein-protein interactions: 1) a pH close to the isoelectric point of the protein (which creates a situation where the positive charges equal the negative charges of the protein molecule, leading to optimal protein-protein interaction); 2) a low ionic strength of the medium (which removes the ions that would otherwise shield the protein's charges, thus giving rise to protein-protein interaction, especially of "euglobulins"); 3) a low dielectric constant of the medium (which enhances the attractive Coulombic interaction energy between the protein dipoles), generally achieved through the addition of water-miscible organic solvents, such as ethanol or ether[4]. The major drawback of precipitation through protein-protein interactions lies in the (partly irreversible) protein denaturation and/or aggregation they give rise to. Thus human IgG, the major component of Cohn's Fraction II, produced on a large scale by precipitation from pooled plasma through the admixture of cold ethanol, comprises up to 15% of (10 S) dimers as well as a smaller proportion of higher polymers. Precipitation of immunoglobulins as euglobulins (e.g., a sizeable fraction of the

IgM) through lowered ionic strength also causes a certain degree of irreversible denaturation. Resolubilizing euglobulins, precipitated at low ionic strength, by the addition of neutral salts is alluded to as "salting in".

Precipitation induced by protein-complex formation through the addition of heavy metal (Zn^{++}, Cu^{++}, etc.) or organic ions (e.g. Rivanol)[4] also tends to give rise to some degree of denaturation.

B. Precipitation by Dehydration

Precipitation of proteins by dehydration, when done by the admixture of concentrated salt solutions, is also called "salting out". Salting out is most effective with completely ionized neutral salts with plurivalent co-ions (i.e. ions of the same sign of charge as that of the net charge of the proteins to be precipitated).

Sulfates fit these requirements most closely and mainly owing to its high degree of solubility, $(NH_4)_2SO_4$ is by far the salt most used for salting out. Salting out with $(NH_4)_2SO_4$ is a useful first step in the isolation of immunoglobulins from whole serum, as it can significantly reduce the total load of undesired proteins in one step. The immunoglobulins can all be precipitated in the presence of 1/3 saturated $(NH_4)_2SO_4$ (saturated $(NH_4)_2SO_4$ is $\approx$ 4M). Upon precipitation of this immunoglobulin-containing fraction, one eliminates 75% of the (irrelevant) proteins in the supernatant. The most soluble protein, serum albumin, only completely precipitates in the presence of 100% saturated $(NH_4)_2SO_4$[4]. Salting out of proteins is an unusually mild precipitation method, causing essentially no protein denaturation.

The principal drawback of the salting out step is the necessity for removing considerable amounts of salt still present in the redissolved precipitate, requiring relatively long periods of dialysis. Another more serious drawback is the following: When used for the isolation of soluble immune complexes or for the isolation of Ag-Ab complexes for affinity determination, the salting out method may cause the loss of a sizeable proportion of some complexes, as these salt concentrations favor the dissociation of certain (e.g., DNA/anti-DNA) Ag-Ab complexes of low to medium affinity[5,6].

One of the best non-ionic protein-precipitating solutes is polyethylene glycol, of a molecular weight 6,000 (PEG - 6000), which can be used for the selective precipitation of IgG[4,7], although it is somewhat less suitable than $(NH_4)_2SO_4$ for use as a first step in the isolation of immunoglobulins from whole serum. PEG-6000 however lacks the drawback of $(NH_4)_2SO_4$ of dissociating certain Ag-Ab complexes. Indeed, precipitation with PEG-6000 is most useful in the isolation of small immune complexes[8]. Whilst PEG-6000, like the other polyethylene glycols and $(NH_4)_2SO_4$, is a strong dehydrating agent, steric exclusion of protein molecules from the interstices between its coils also contributes strongly to the protein-precipitating effect[9].

III. ELECTRIC CHARGE

A. Ion Exchange Chromatography

As a γ-globulin, IgG is among the serum proteins with the lowest electric charge and thus especially easy to isolate by ion

exchange chromatography, without actually binding it to the ion exchange material. For that purpose, the positively charged diethyl aminoethyl (DEAE) cellulose, or DEAE Sephadex^{R} (cross-linked dextran with DEAE ligands) are the materials of choice. At pH 7.5 essentiall all IgG, and none of the other serum proteins, is positively charged, as is the anion exchanger. Thus all other serum proteins are bound to the column, and IgG passes through devoid of the other serum proteins (see Table II 13-2); for more details on the methodology see, e.g. 10. For other purposes (e.g., for the separation of immunoglobulin fractions, see below), negatively charged (cation) exchangers may be useful, such as carboxy methyl (CM) cellulose.

"Chromatofocusing" is a novel variety of ion exchange chromatography, first described by Sluyterman and Wijdenes[11]. It mainly differs from the ion exchange procedure used in separating many proteins from a mixture (employing a pH-gradient for elution), in the use of ampholyte buffers (see Section C, below) for elution, which gives rise to a certain localized concentrating effect of each fraction, hence the name "focusing" (see also "isoelectric focusing", Section C, below). There are as yet few published applications to this method of immunoglobulin isolation[12], but the method certainly should be considered as a likely improvement over ordinary ion exchange methods.

Whilst ion exchange is one of the simplest methods for isolating immunoglobulins (especially IgG), it is not the most efficient one, as aspecific adsorption still is an important source of immunoglobulin loss. The yield of IgG-class antibodies, obtained by DEAE-

cellulose ion exchange from a given antiserum usually is not more than 60 - 80%; somewhat better yields are obtainable when the more hydrophilic, but also more expensive DEAE-Sephadex^{R} is used.

B. Electrophoresis

Zone (or "block") electrophoresis is slabs of packed particles, such as starch[10], or polymer particles (e.g. of "pevikon", polystyrene or glass beads), or of (0.5%) agarose gels[13] is a simple and efficient method for separating serum globulins according to charge, from quantities of the order of 50 to 500 mg total protein. The least losses due to aspecific adsorption are obtainable with the most hydrophilic carrier materials i.e., starch blocks (with a 60 to 80% yield) or agarose gels (with a 70 to 80% yield). On account of considerable Jouleian heat development, the electrophoresis is best done in a cold room, at +4^{o}C, at fairly low field strengths, so that a separation takes 24 hours. At the conclusion of an electrophoretic run, the block is cut up in narrow (5-10 mm wide) slices and each slice placed into a sintered (coarse) glass funnel which in its turn stands in a ($\approx$50 ml) polypropylene centrifuge tube and spun at 1,000xG for 5 minutes[10]. When 0.5% agarose blocks are used, the individual slices are placed in centrifuge tubes, frozen to -20^{o}C and kept at that temperature overnight. After thawing the soluble protein fraction is separated from the agarose pellet by centrifugation[13]. In both cases the globulin yield can be much enhanced by additional washing and recentrifugation of the slurry in the sintered glass funnels, or of the agarose pellets.

Most free liquid electrophoretic methods have no advantage over the solid support methods (discussed above) for preparative immunoglobulin separations. Free flow electrophoresis between two closely spaced flat plates, endless belt electrophoresis, stable flow electrophoresis, and horizontal rotating cylinder electrophoresis[14] all are much more complicated to use than block electrophoresis and capable of processing only small amounts of protein. The same holds for static liquid column electrophoresis, stabilized by means of a density gradient. However, a number of these free liquid electrophoretic methods are useful for the separation of cells, for which solid supports cannot be used[14]. The only free liquid electrophoretic method that has advantages for the isolation of immunoglobulins is the vertical cylindrical rotating continuous flow method;by this process the γ-globulins of up to 1 L of serum can be separated per hour.

C. Other Electrokinetic Methods

Isoelectric focusing. While the electrophoretic transport of charged molecules in an electric field in a homogeneous buffer of a given pH is continuous and has to be stopped if one wishes to avoid losing the molecules through migration into the electrode compartment of the opposite charge, electromigration of a charged amphoteric molecule through a continuous pH-gradient automatically ceases when that amphoteric molecule has reached the place in the gradient where the pH is the same as its isoelectric point. Thus, isoelectric focusing is the sorting out of amphoteric molecules according to their different isoelectric points, by electromigration through a

pH-gradient. Generally speaking, prolonged electromigration of a mixture of amphoteric molecules in a pH-gradient tends to enhance the resolution of each of its constituents, hence the designation of isoelectric focusing. The development that made the general application of isoelectric focusing a practical reality was the synthesis of many different "carrier ampholytes", each having severa acidic and basic groups with closely spaced pK values per molecule with many different pH values. In protein separation, isoelectric focusing allows an improvement in resolution of about an order of magnitude, compared to electrophoresis. This is largely because, with time, bands separated by isoelectric focusing reach and maintain an optimal sharpness (due to the focusing effect) while with electrophoresis separated bands continuously tend to broaden because of diffusion. For the separation of proteins by isoelectric focusing, one must be mindful of the fact that the carrier ampholytes most used are isomers and homologues of aliphatic poly-amino-poly-carboxylic acids, and are thus difficult to distinguish from proteins by the most commonly used colorimetric or spectrophalometric methods. However, as these carrier ampholytes have an average molecular weight of about 800, while that of most proteins is above 10,000, proteins generally can be easily separated from the ampholyte molecules by means of gel filtration e.g., (with Sephadex^{R} G-50, or by ultrafiltration, see Section V, below.)

Preparative isoelectric focusing[16] is practiced in: liquid density gradients (stationary as well as continuously flouring), free fluid,[17] flat beds or cylinders stabilized with granulated

porous carriers, or with continuous gels[18]. In the latter case special attention should be paid to the various elution procedures[18]. See also Section X, below.

Isotachophoresis. Like isoelectric focusing, isotachophoresis is practiced in a buffer system of which the composition is non-constant with respect to location (see above), but while isoelectric focusing is best done in a buffer system consisting of an essentially continuous pH gradient, isotachophoresis needs a buffer system of which the components have markedly discontinuous properties. In isotachophoresis the sample mixture is placed in the sample compartment, "terminating electrolyte" in this cathode compartment, and the "leading electrolyte" in the anode compartment. The effective mobility of the leading electrolyte is the highest, and that of the terminating electrolyte the lowest, the effective mobilities of the components in the sample mixture should be intermediate between those extremes. Because of the possibility of achieving a total separation between different ionic species, once a steady state is reached, isotachophoresis affords an extremely high resolution[14,18,19]. Up to the present this method has been used more frequently for analytical purposes (see below, Section X) than for preparative isolations.

Molecular sieve electrophoresis, i.e. electrophoresis in gels with pore sizes of the same order of magnitude as the globulins that have to be separated[18] is mainly intended for the separation of proteins according to size, and is treated in Section V (below).

IV. SURFACE TENSION

A. Surface Tensions and van der Waals Interactions

The total van der Waals free energy of interaction[20] between two different particles or molecules 1 and 2, immersed in liquid 3 is:

$$\Delta F_{vdw} = \Delta F_{132} = -A_{132} / 12 \pi d_o^2, \tag{1A}$$

in which A_{132} is the effective Hamaker coefficient of the system and d_o the equilibrium distance between the two parallel particles or molecules. From Hamaker's combining rule[21]:

$$A_{132} = A_{12} + A_{33} - A_{13} - A_{23}, \tag{2}$$

and Berthelots combining rule:

$$A_{ij} = \sqrt{A_{ii} A_{jj}}, \tag{3}$$

where i or j stand for 1,2, or 3, it can be demonstrated[22] that A_{132} becomes negative when:

$$A_{11} > A_{33} > A_{22} \tag{4A}$$

and when:

$$A_{11} < A_{33} < A_{22} \tag{4B}$$

As, in analogy with eq. (1):

$$\Delta F_{ii} = - A_{ii}/12 \pi d_o \tag{1B}$$

and as:

$$\Delta F_{ii} = - 2 \gamma_{iv}, \tag{5}$$

where v stands for vapor, it is clear[20] that A_{132} also must be negative when:

$$\gamma_{1v} < \gamma_{3v} < \gamma_{2v} \quad (6A)$$

and when:

$$\gamma_{1v} > \gamma_{3v} > \gamma_{2v} \quad (6B)$$

The total free energy of interaction ΔF_{132} can in practice be obtained more easily via: $\Delta F_{132} = \gamma_{12} - \gamma_{13} - \gamma_{23}$ (7)

Whilst Hamaker coefficients A_{ii} are as yet only rarely easy to determine with any degree of accuracy, liquid-vapor (γ_{3v}) as well as solid-vapor (γ_{1v},γ_{2v}) surface tensions are readily obtained with appreciable accuracy by a variety of methods[23]. The Hamaker coefficient A_{132} of a system will therefore become negative (so that the total van der Waals interaction between components 1 and 2 immersed in liquid 3 becomes repulsive) when the surface tension of the liquid medium 3 acquires a value intermediate between the surface tensions of 1 and 2[20]. The van der Waals interaction between components 1 and 2 is attractive as long as the surface tension of the liquid medium is either higher or lower than the surface tensions of both 1 and 2.

It thus is possible, in a liquid medium, to make the van der Waals interaction between two different components attractive or repulsive at will, by adjusting the surface tension of the liquid. This principle is applied in practice in hydrophobic (and reversed phase) as well as in affinity chromatography (see below).

B. Hydrophobic and Reversed Phase Chromatography

"Hydrophobic interactions" are nothing but the van der Waals attraction between various macromolecules and/or particles with

lower surface tensions than water (i.e., they are more or less "hydrophobic"), in water; thus, under conditions[24] where:

$$(\gamma_{1v} \text{ as well as } \gamma_{2v}) < \gamma_{3v} \qquad (8)$$

After the attachment of proteins, which are rather hydrophilic, but which do have a slightly lower surface tension than water when not denatured[2], to fairly hydrophobic carriers in water, the proteins can be detached, by lowering the surface tension of the liquid medium to a value in between the surface tensions of the protein and the carrier (eqs. (6A or 6B)), by turning the van der Waals attraction into a repulsion, through the addition of water-miscible surface tension-lowering solvents, such as ethylene glycol or dimethyl sulfoxide. Upon a gradual lowering of the surface tension of the liquid effected in this manner, human serum proteins are eluted in the order of their decreasing surface tension, e.g.: first α2 macroglobulin, then serum albumin, α2 HS glycoprotein, β_1C globulin (complement component C3), IgG and finally transferrin.[3,20]

Attachment of very hydrophilic proteins to hydrophobic carriers can be enhanced by raising the surface tension of the aqueous medium: see eq. (8), e.g., by increasing the salt content of the water. Elution of such very hydrophilic proteins can then be effected with water of lower ionic strength[3]. This approach was especially successful in the isolation of monoclonal as well as of normal IgA.[25] It would seem that IgG3 and IgG1 are more hydrophobic than IgG2 and IgG4;[26,27] so that hydrophobic chromatography may be a promising approach for the fractionation of human IgG subclasses.

Reversed phase chromatogrphy has a mechanism that is essentially identical to that of hydrophobic chromatography.[20] The method (under the name "reversed phase", or "high performance liquid chromatography", or RP-HPLC) is used increasingly, with preparative columns, in "high performance liquid chromatography" devices.[28]

V. SIZE AND SHAPE

A. Gel Filtration Chromatography

Gel filtration (or pore-exclusion chromatography) is the method of choice for the preparative isolation of a narrow molecular weight range of proteins, from a complex biopolymer mixture with a wide range of molecular weights. The method consists of passing a polymer mixture (in solution) through a column packed with spherical gel beads, such that the larger polymers cannot penetrate the pores of the gel beads and emerge first from the columns, in the "void volume". The smaller polymers become trapped in the pores of the gel beads; the lower their molecular weight, the later they are eluted. Molecular symmetry or asymmetry also play a role in the entrapment of polymers in the pores of the gel-beads, so that the method normally should not be used for molecular weight determinations.

For immunoglobulin isolation Sephadex^{R} (Pharmacia, Piscataway, NJ) beads made of cross-linked dextran are used most.[10] Sephadex G-200 (which excludes globular proteins of a molecular weight > 200,000) (see Tables 13-3 and 13-4), or G-150 (excludes MW > 150,000) are used most, whilst for the isolation of immunoglobulin fractions,

G-100 (excludes MW >100,000) and G-50 (excludes MW > 50,000) are indicated. Sephadex G-100 for instance is useful for the fractionation of fragments with a molecular weight < 100,000. For special purposes (e.g., the isolation of IgD, see below), special gel-beads made of a three-dimensional polyacrylamide latice, filled with an interstitial agarose gel, can be used (LKB, Rockville, MD; IBF, Clichy, France - Fisher Scientific); e.g., Ultrogel AcA34. Sephadex G-25 is useful for desalting protein solutions. The yield of immunoglobulin-recovery, as far as the gel-filtration step is concerned, can, with care, be as high as 90%.

B. Molecular Sieve Electrophoresis

Molecular-sieve electrophoresis, i.e., electrokinetic transport of proteins in gels with pores that are only slightly larger than the proteins[18] aims at the separation of proteins according to their molecular size, but of course simultaneously effects a fractionation according to the electric charge of the proteins.[14] The only way to eliminate the influence of different electric charges of different proteins, and to use gel electrophoresis exclusively to separate proteins according to size, is to bestow an identical (and rather high) negative electrical charge to all proteins, great and small, by the admixture of sodium dodecyl sulfate (SDS) to the entire system.[29] For preparative purposes a drawback of SDS-gel electrophoresis is the difficulty of removing the SDS from the proteins after the separation. One way of removing the SDS is by an ion exchange chromatography, as a pH below that of the isoelectric

point of the protein, e.g., with DEAE cellulose or DEAE Sephadex, if necessary in the presence of 6 to 8 M urea, although this approach tends to give rise to loss of protein. A better way of SDS removal is to use gel electrophoresis or gel isoelectric focusing.[29]

C. Ultrafiltration

Ultrafiltration, with anisotropic ("skinned") membranes, is the method of choice for the re-concentration of intermediate and final fractions, after various protein fractionation steps, which always entail considerable dilution (see Tables II to IV). Various commercially available protein-stopping membranes with appropriate pore-sizes may be used (e.g., from: Amicon, Lexington, MA: Beckman, Anaheim, CA; Millipore, Bedford, MA). One can also rather easily (and much more cheaply) cast one's own (cellulose acetate) membranes,[30] which, partly because they do not need to be dried for shipment, have much faster flowrates than any of the commercial membranes. For normal immunoglobulin concentration the CA-50 cellulose acetate membrane is the best; membranes with smaller pore sizes may be more indicated for the concentration of smaller fractions such as L chains: the CA-35 membrane would be suitable for this.[30] The preparation of one membrane has been described (CNA-15) which retains all IgM, but passes a fair portion ($\approx$25%) of the IgG comprised in an immunoglobulin mixture.[30]

D. Sedimentation

One other method for the preparative separation of immunoglobulins according to size, i.e., mainly for the separation be-

tween IgG and IgM, is (generally sucrose-) density gradient ultracentrifugation. After 16 hours at ≈40,000 rpm, in tubes with a sucrose (10 - 40%) gradient, in a swinging bucket rotor, IgM is found at the bottom of the tubes and IgG in their middle region. The separation however is rarely complete, and not exquisitely reproducible.

In an analytical ultracentrifuge, with a Tiselius-cell (containing a porous partition), a proportion (30 to 50%) of the IgG present in a mixture can be obtained free from IgM. This process can be monitored by visual inspection so that one can determine with precision at which stage all the IgM has passed the porous barrier, leaving part of the IgG (now devoid of IgM), behind that barrier.

VI. LIGAND SPECIFICITY

Affinity chromatography, which is based upon ligand specificity, such as exists between antigen and antibody, is a generalized outgrowth of the older technique of immunoadsorption. For the isolation of immunoglobulins, variants of the old immunoadsorption technique are still very much applicable.

To begin with, immunoadsorbents are useful for the removal of, e.g., unwanted immunoglobulins (see Section VII, below, and Table I), especially in the purification of IgA, IgD and IgE.

Specific immunoadsorbents made with various insolubilized antigens are especially useful for the isolation of immunoglobulins, with one given antibody activity, see Table I.

TABLE I. PURIFICATION OF ANTIBODIES BY AFFINITY CHROMATOGRAPHY[38]

Principle: Antigen, covalently linked to a solid matrix, specifically combines with antibody in whole serum or immunoglobulin fractions. Examples of solid matrices are polymerized proteins, (Refs. 33,34) useful for batch-wise application, or agarose beads (Sepharose), useful for affinity chromatography. Immobilized antibodies can be used similarly to isolate antigens (Ref. 34).

Antibody	Immunoadsorbent	Eluant	Yield %	Reference
Anti-p-azophenyl-β-lactoside	Sepharose p-aminophenyl-β-lactoside	Lactose;p-nitrophenyl-β-lactoside	>90	38
Anti-hemoglobin A_1 (HbA_1)	Sepharose-HbA_1; -α-chain, β-chain	Acetic acid (1 M)	-	38
Anti-ρ-azobenzene-arsonate	Poly rabbit serum albumin (Poly RSA) -ρ-azobenzene-arsonate	Benzenearsonate	85	33,38
Anti-bovine serum albumin (BSA)	Poly RSA-BSA	Glycine HCl, pH 2.3	90	33,38
		Propionic acid, pH 3.5	>90	3,20
		50% Dimethylsulfoxide, pH 9.5	>90	3,20
Anti-IgG	Poly IgG	Glycine HCl, pH 2.8	90	34
Anti-dextran	Sephadex G-10 pH 7.2	60% Dimethylsulfoxide, pH 7.2	>90	32
Blood Group Antibodies (anti-A, anti-K, anti-D)	Human Red Cells	47% Dimethylsulfoxide, pH 9.5	>90	69

The specific attachment step in immunoadsorption is almost invariably quite readily achieved, under physiological conditions, in aqueous media. Exactly because of the (usually) rather pronounced strength of the bond between antigens and antibodies, the subsequent detachment or dissociation (i.e., the elution) is harder to achieve.

There are however, a number of approaches that favor a change of the antigen-antibody interaction into the direction of dissociation, e.g.:

1) Increase the temperature;[31]
2) Apply excess of low molecular weight hapten or hapten-like solutes, See Table I; [2,3,20,31-34]
3) Denature or deform the antigen and/or antibody molecules See Table I; [2,3,20,31-34]
4) Destroy (e.g. enzymatically) the carrier or the spacer;[31]
5) Apply considerable dilution;[31]
6) Apply an electric field;[35]
7) Dissociate by reversing both the Coulombic and the van der Waals attraction, see Table I.[2,3,20,31]

A particularly interesting ligand for immunoglobulin isolation is the lectin protein A from Staphylococcus aureus, in that columns with that ligand specifically bond to human IgG, except for IgG_3, see below. Protein A-columns are now increasingly used therapeutically, in extracorporeal hemoperfusion, for periodic removal of patients' IgG, in ex vivo treatment for various malignancies.[37] Such columns can also be used in the fractionation of some IgG sub-

classes.[37] In the same manner, Concanavalin-A ligands can be used for the removal of IgA and IgM.[37]

VII. ISOLATION OF IMMUNOGLOBULINS

A. Immunoglobulin G

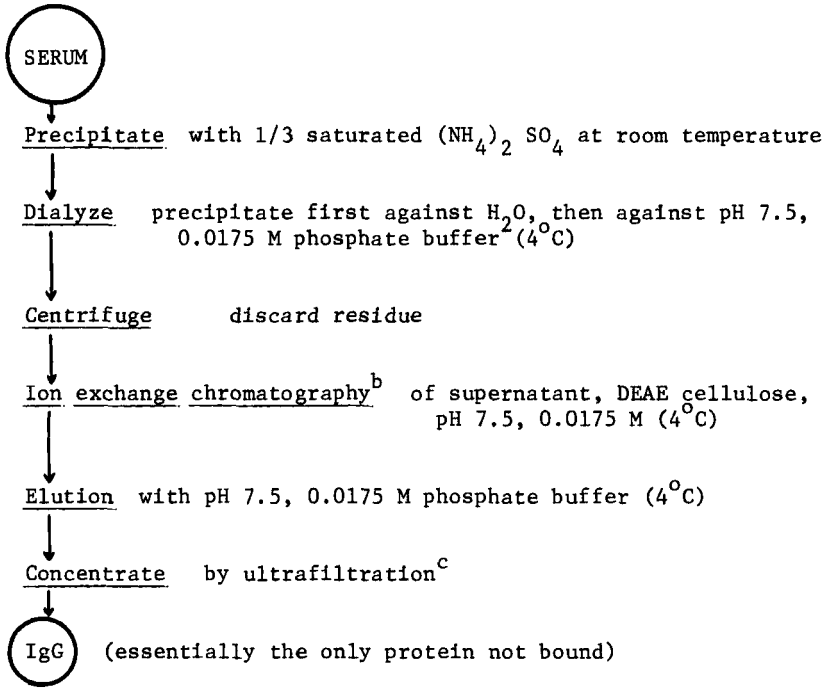
Isolation of human IgG from whole serum is best done by ion-exchange chromatography with the anion exchanger DEAE-cellulose (or DEAE Sephadex^{R}). In order to decrease the burden of non-IgG protein, and to eliminate any hemoglobulin that might be present in the serum, a preliminary salting-out step with 33% saturated $(NH_4)_2\ SO_4$ is recommended. IgG is the only serum protein not bound to DEAE at pH 7.5. The pH 7.5 eluate, which is virtually pure IgG, only needs to be concentrated by ultrafiltration; see Flow-chart (Table II).[10,38]

Subclasses of human IgG may be separated by affinity chromatography, with specific anti-subclass antibodies as ligands. (see Section VI, above), or use may be made of the fact that IgG3 and IgG1 are more hydrophobic than IgG2 and IgG4 (using hydrophobic chromatography, see Section IV, above), and of the fact that IgG3 does not bind to protein-A. Protein-A affinity chromatography can be used for the isolation of some of the rat and mouse IgG subclasses.[36] On an industrial scale IgG is recovered from fraction II of Cohn and Edsall's cold ethanol precipitation method No. 6.[4]

B. Immunoglobulin A

To quote the discoverer of IgA and of many of its functions, J. F. Heremans:[39] "The isolation of serum IgA from serum or exudates

TABLE II. FLOW SHEET FOR THE PREPARATION OF IgG FROM NORMAL HUMAN SERUM[a,b]



[a]The procedure is applicable to IgG myeloma sera with the provision that care is taken to ensure the resin is not overloaded. Alternatively a procedure employing preparative zone electrophoresis, followed by gel filtration of the γ spike thus obtained will frequently yield high purity IgG.

[b]Refs. 10, 38

[c]Ultrafilter membranes that may be used are Amicon UM-10, PM-10, or PM-30, or analogous commercially available membranes, or they may be prepared in the laboratory, such as the CA-50 membrane.[30]

is besieged by many difficulties". In the case of IgA isolation it is best not to start with a precipitation with $(NH_4)_2\ SO_4$, as this tends to enhance the formation of IgA dimers.

As IgA is exceptionally hydrophilic, it can also be isolated from other serum proteins by (salt-mediated) hydrophobic chromatography,[25] see Section IV, above. Secretory IgA can be isolated in the same manner as IgA (see Table III), but, in view of the higher molecular weight of sIgA the second (and third) gel filtration steps should be done with Sepharose 6B^R. For the isolation of J-chain and secretory component, see (G) below. IgA is found in fractions II and III of Cohn and Edsall's cold ethanol precipitation method No. 6.[4]

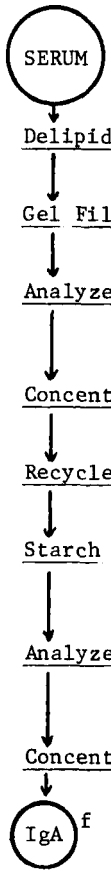
C. Immunoglobulin M

IgM is rather easy to isolate from serum, by first obtaining the macroglobulin fraction by gel filtration, followed by the removal of α2 macroglobulin by block electrophoresis (Table IV). Pure IgM has a strong tendency to form complexes (see Section II-B, above); to obviate complex (and even precipitate) formation of purified IgM preparations it is advisable to conserve pure IgM solutions at + 4°C, in the presence of, e.g., 0.1 to 1% serum albumin. Monomeric IgM (IgMs) can be obtained by mild reduction at neutral pH with 0.015M 2-mercaptoethylamine.[40]

D. Immunoglobulin D

IgD can be purified by gel filtration on Ultrogel Aca 34, from which it elutes in a position between IgG and IgM, followed by DEAE

TABLE III. FLOW SHEET FOR THE PREPARATION OF IgA FROM NORMAL HUMAN SERUM[a,b]



[a] Adapted from Heremans,[39] and van Oss.[38]

[b] The preparation of monoclonal IgA should proceed differently, involving primarily separation by charge (starch block electrophoresis or DEAE cellulose chromatography) followed by gel filtration (Sephadex, G-200).

[c] See Footnote c under Preparation of IgG, Table II.

[d] Refs. 10,38

[e] It still may be necessary to remove traces of IgD and IgE by affinity absorption.

[f] sIgA can be isolated in the same manner, but the second and third gel filtration steps should be done with Sepharose 6B.

TABLE IV. FLOW SHEET FOR THE PREPARATION OF IgM FROM NORMAL HUMAN SERUM[a]

SERUM

↓

Precipitate at 1/3 saturated $(NH_4)_2SO_4$ (room temperature (RT)) (discard supernatant)

↓

Wash precipitate three times with 1/3 saturated $(NH_4)_2SO_4$ (RT) (discard supernatant)

↓

Redissolve precipitate in distilled water equal to 1/10 of the starting volume (RT)

↓

Dialyze (4^oC) against 0.15 m NaCl until dialysis bath is free of SO_4, as determined with $BaCl_2$

↓

Gel filtration (4^oC), G-200 Sephadex^R in 0.15 M NaCl

↓

First protein peak collected (appears in void volume) as determined by UV absorption at 280 nm; contains IgM and α_2M, some β and α lipoprotein.

↓

Concentrate by ultrafiltration[10] (RT)

↓

Starch block electrophoresis[10] (4^oC) in 0.05 M, pH 8.6 barbital buffer

↓

Elution of peak cathodal to the point of application (RT)

↓

Concentrate by ultrafiltration (RT)[30]; add 0.1 to 1% serum albumin, for stability.

↓

IgM[b]

[a]Ref. 38. The preparation of monoclonal IgM from Waldenström's macroglobulinia patients sera starts directly at gel filtration; prior concentration of the globulin fraction by $(NH_4)_2SO_4$ precipitation is not needed.

[b]Product should be evaluated by immunoelectrophoresis and analytical ultracentrifugation. A repeat cycle (gel filtration) may be necessary. Small amounts of polymerized IgM will remain as a contaminant. This polymer may be removed by gel filtration on a sepharose column.)

ion exchange chromatography, where it elutes at pH 8.0, with a 0.035 M phosphate buffer.[41] The fractions should be monitored for IgD, and ultrafiltration steps used for reconcentration where appropriate see Table II footnote c.[30]

E. Immunoglobulin E

IgE is difficult to isolate, due to its exceedingly low concentration in plasma. Precipitation with 40% saturated $(NH_4)_2\ SO_4$ (at pH 7 at room temp.) should be the first step, followed by ion-exchange chromatography on DEAE-Sephadex A-50, with elution at pH 8 between 0.1 and 1M (tris-buffer), with a final series of thrice repeated gel filtrations on Sephadex G-150.[42] All fractions should be monitored for IgE, and ultrafiltration steps should be applied at regular intervals between steps to reconcentrate the active fractions, see Table II, footnote c.[30]

F. Monoclonal Immunoglobulins

All the above isolation procedures also are valid for monoclonal immunoglobulins. However, in all cases of monoclonal immunoglobulins, care should be taken that the chromatography columns used are not overloaded. The isolation of monoclonal IgA should be done somewhat differently from the separation of IgA from normal serum (see footnote b of Table III). In many cases of monoclonal immunoglobulins polymers may be encountered, so that especially gel filtration eluates should be monitored with the possibility in mind that active fractions may occur in several different molecular size

cut-offs. With monoclonal IgM the preliminary precipitation with $(NH_4)_2SO_4$ is not necessary (footnote a, Table IV).

VIII ISOLATION OF IMMUNOGLOBULIN CHAINS

A. Products of Enzymatic Cleavage

Fab and Fc fragments are obtained by papain digestion of IgG ($\approx$ 1 mg papain per 100 mg IgG), for 18 hrs. at 37°C (in the presence of 0.01 M cysteine, at neutral pH and with the addition of a drop of toluene to prevent spoilage). $F(ab')_2$ pieces are obtained by pepsin digestion of IgG at pH 4.5 (in 1M acetate buffer and 1 drop of toluene), for 8 hrs. at 37°; the reaction is stopped by increasing the pH to 8.

Undigested molecules should be removed from papain digest by gel filtration on Sephadex G-150. Fab fractions then are obtained by ion exchange on DEAE (cellulose or Sephadex) columns, whilst Fc fragments are best isolated by ion exchange on carboxy-methyl (cellulose or Sephadex).[43] Undesired pepsin fragments are best simply removed by dialysis, or the $F(ab')_2$ may be purified by precipitation with Na_2SO_4 or $(NH_4)_2\ SO_4$. Most recipes for enzymatic fragments of rabbit IgG also work for human IgG.

It is more difficult to obtain Fc fragments of IgA, but Fab and $F(ab')_2$ can be obtained with papain and pepsin respectively, much in the same manner as used for IgG.[40] With a bacterial endopeptidase (IgA protease), IgA as well as sIgA can be cleaved to form Fc and Fab fragments.[44] With IgM it is also rather difficult

to obtain Fc fragments, although with trypsin some success has been noted; trypsin also appears to be the enzyme of choice for obtaining Fab_{μ}.[40] Tryptic digestion also is the preferred way for obtaining Fab and Fc fragments of IgD.[45] IgE yields an Fc fragment upon digestion with papain which does, however, tend to degrade further. Fab is not easily obtained with IgE: short-term digestion is called for and even then the Fab obtained is labile. With pepsin an $F(ab')_2$ fragment is obtained which also still contains part of the Fc fraction.[40] With trypsin a slightly smaller $F(ab')_2$ fragment is obtained.[42]

B. H and L Chains and Domains

Dissociation of immunoglobulins into chains is achieved by reduction (with, e.g., 0.1 M 2-mercaptoethanol, or dithiothreitol) in the presence of 8 to 10 M urea at pH 8, followed by alkylation with 0.2 M iodoacetamide.[40,46] To prevent re-association and/or aggregation, the chains should be kept in the presence of 8 to 10 M urea, or 0.5 to 1M propionic acid. Separation of the chains can be done by CM-cellulose ion exchange (in urea), or by gel filtration with Sephadex G-100 in 0.5 M propionic acid.[46] Treatment of IgG with 0.1 M cercaptoethylamine, followed by addition of p-chloromercuribenzoate and lowering of the pM to 2.5 results in half-molecules of IgG.[46]

The greater part (98%) of IgA2, of the Am-group A2m (1) has no disulfide bond between H and L chains; these H and L chains can thus be separated without any other treatment, by gel filtration on Sephadex G-200, in the presence of 8 M urea.[47]

Separated H and L chains of antibodies with a given specificity, spontaneously and preferentially recombine, re-forming antibody molecules with most of the original binding activity.[48]

Some (but not all) of the immunoglobulin domains can be obtained by digestion with trypsin or plasmin of H or L chains or fragments at neutral pH, after prior treatment of these chains or fragments at pH 2.5.[49]

C. Secretory Component of IgA

Human secretory component of IgA (SC) can be prepared from human colostrum whey, by precipitation with 70% sat. $(NH_4)_2\ SO_4$, gel filtration on Sephadex G-200, re-precipitation with 70% sat. $(NH_4)_2\ SO_4$ and ion-exchange chromatography on DEAE-cellulose.[50]

D. J Chain of IgA and IgM

The isolation of J chain from IgM or from dimeric IgA or sIgA is not easy, on account of the fact that it represents only 1.5% of the total weight of IgM and 4.5 to 4.0% of dimeric IgA and sIgA respectively. From IgM it can be isolated after the reduction and alkylation needed to form IgMs (see above), after which it can be found in the 15,000 - 25,000 MW fraction by gel filtration on Sephadex G-100, and further separated from possible single L chains by electrophoresis or by ion exchange chromatography: J chain is more strongly negatively charged than L chains.[40] J chains can also be obtained from α chains (obtained via reduction and alkylation of dimeric IgA or sIgA that were isolated by gel filtration on Sephadex G-100),followed by further cleavage with 50 mM dithiothreitol

and alkylation with 120 mM iodoacetic acid and further gel filtration (in 1 M acetic acid) on Sephadex G-100. J chain is quite hydrophilic, asymmetrical (axial ratio ≈18/1) and has a molecular weight ≈15,000.[51]

IX DETERMINATION OF MOLAR ANTIBODY CONCENTRATION

A. Introduction

For all quantitative determinations pertaining to antigen-antibody interactions (and especially for determinations of thermodynamic parameters), it is necessary to know the (molar) antigen and antibody concentrations (usually bound as well as free). In most cases the determination of antigen concentrations is a simple matter, as one can (usually) start with the pure antigenic material and, if necessary, one can have it tagged with e.g., a radioisotope. Determinations of the molar concentration of a specific antibody, be it in whole antiserum or in an isolated immunoglobulin fraction is, due to the presence of a vast excess of irrelevant immunoglobulins and other proteins, a more complicated matter. Basically one can determine the specific antibody concentration: 1) through interaction with a known amount of antigen, and 2) after specific adsorption to and elution from the insolubilized antigen.

B. Interaction with a Known Amount of Antigen

Although antigens and antibodies can interact non-stoichiometrically, in the most diverse proportions, there are two conditions under which the antigen-antibody ratio is known, or can be

simply ascertained. These are: 1) at antigen excess, when all specific antibody-active sites are bound,[52] and 2) by precipitation done at the optimal antigen/antibody ratio (with neither free antigen nor free antibody in the supernatant), in which the total amount of antibody equals the total amount of precipitate minus the amount of antigen added. When determinations are done at antigen excess, in most cases soluble complexes are obtained. Here the amount of antigen added must be known, as well as the amount of antigen in the complexes, which can be isolated by, e.g., precipitation with $(NH_4)_2\ SO_4$, with polyethylene glycol 6000 (see Section II, above) or with anti-immunoglobulin antibodies.[52] Bound and free (radio-tagged) antigen can also be determined by gel-electrophoresis.[52] As indicated above (Section II-B), precipitation of soluble antigen-antibody complexes with $(NH_4)_2\ SO_4$ may cause the dissociation of certain types of complexes.[5,6] Finally, via radio-immunoassay and enzyme immunoassay, it also is possible to determine specific antibody concentrations, once antigen concentrations are known.

To obtain the molar antibody concentration, it is of course necessary to know the molecular weight of the antibody in question, and in case of antibody heterogeneity, to effect a further fractionation, see next section, below.

C. Affinity Procedures

The other way of knowing the exact concentration of the specific antibody one is working with, is to isolate it by immunoadsorp-

tion on (and subsequent elution from) the (insolubilized) antigen in question. In such a case the eluted protein consists solely of specific antibody, and the concentration of that protein is the concentration of the specific antibody in question. To obtain the molar antibody concentration of the preparation one must of course know of which immunoglobulin(s) it consists. If (dekavalent) IgM as well as other (divalent) immunoglobulin classes are present, an additional separation according to size, e.g., by gel filtration, see Section V-A, above, may be required.

X. CHARACTERIZATION OF IMMUNOGLOBULINS

A. Introduction

Basically, the characterization of immunoglobulins is a vast subject that easily deserves a chapter by itself. However, many aspects of immunoglobulins that essentially pertain to their characterization are treated elsewhere in great detail.[53]

The final section of the present chapter thus will be limited to: 1) The immunoglobulin characterization methods, of which the principles already have been touched upon above, under the treatment of the various isolation methods of immunoglobulins, see Sections B to E, below, and: 2) A few other methods, not usually treated elsewhere, that pertain to the characterization of the surface tension and of the primary, secondary and tertiary configurations of the immunoglobulin molecules, are treated in Sections F to H, below.

B, Electrokinetic Methods

Analytical Polyacrylamide Gel Electrophoresis give the best resolution when done with discontinuous buffers, or in "multiphasic buffer systems"; for the most advanced state of the art of this method;[54] see Section III-B.

Analytical Isoelectric Focusing however is fundamentally capable of even higher resolution (with respect to charge). The method has recently been well summarized by Braun et al.,[55] giving examples of multiclonal and oligoclonal antibody responses; see also Righetti.[16] There is however a limitation to the method, in that it is not possible to work with immunoglobulins that tend to precipitate at low ionic strength (i.e., with "euglobulins", see Section II-A). In such cases it is preferable to use:

Analytical Isotachophoresis. This technique has been well described by Bier and Allgyer,[19] and its application to immunoglobulin (especially "euglobulin") characterization by Ziegler and Köhler,[56] see also Section III-C.

Electrophoresis in Gels with Graded Porosity permits the distinction of a rather wide range of molecular weights.[57] This technique is especially powerful in the simultaneous discrimination between antigens, antibodies and soluble immune complexes.[53]

Bidimensional Combinations[57,58] of, e.g., isoelectric focusing and SDS-gel electrophoresis (see Section V-B), are among the characterization methods that permit the highest resolution at present attainable (see Section I-C). Even in monoclonal immunoglobulins there is some microheterogeneity, demonstrable by the "high reso-

lution bidimensional electrophoresis" method,[59] using gel isoelectric focusing in one direction, and SDS gel electrophoresis in the other. This permits the distinction between electric charge and molecular weight heterogeneities. In their study of monoclonal immunoglobulins Latner et al.[60] could thus show that microheterogenities reside only in the H and not in the L chains. The microheterogeneities of monoclonal γ, α and μ-chains are mainly based on charge and not on size differences, whilst in monoclonal δ chains the heterogeneities exist in both charge and size. The charge heterogeneities of monoclonal α, μ and δ chains (but not of γ chains) are partly due to sialic acid moieties. For actual molecular weight determinations in the SDS gel electrophoresis direction, as well as in graded porosity gel electrophoresis, one always must interpolate between two polymers of known molecular weights, and even then errors can arise when the asymmetries of the known and the unknown proteins are dissimilar. For accurate molecular weight determinations, see Sedimentation (Section D), below.

C. Chromatographic Methods

High Pressure Liquid Chromatography (HPLC) is, in a variety of modes, more and more applied to the characterization of small and large peptides, and of proteins.[28]

Reversed-phase (or hydrophobic) liquid chromatography (RPLC) is probably one of the most useful new techniques, as it separates proteins and peptides according to their relative hydrophobicity, which is not easily duplicated by other analytical methods (see Section IV-B, above).

Gel Permeation HPLC is a convenient approach to the estimation of the molecular weight of proteins. To that end one must have a number of polymers at one's disposal, to serve as molecular weight markers, and one must arrange these markers in such a way that the unknown protein(s) emerge from the column in between two or more known ones. This also holds for ordinary gel filtration (see Section V-A above) when used as a method for determining molecular size. In all these cases it must be remembered that a significantly greater (or lesser) deviation from symmetry of the unknown protein(s) than of the molecular weight markers, will lead to erroneous results, even if the interpolation method is properly adhered to. The ultimate criterion of molecular size determination of proteins still resides in the direct measurement of molecular weights and asymmetries by analytical ultracentrifugation, see the next section (D), below.

Ion Exchange HPLC, for the characterization of immunoglobulins and/or immunoglobulin chains is feasible, but in the face of the existence of electrophoretic, immunoelectrophoretic, gel electrophoretic and other electrokinetic methods (see Section B, above), this approach has little to commend it.

D. Sedimentation

With all the secondary methods for determining molecular weights of immunoglobulins, immunoglobulin chains and other polypeptides and proteins, that are used increasingly, such as gel filtration chromatography (or gel permeation HPLC), or SDS gel

electrophoresis (see above, Sections B and C), one must not lose sight of the fact that in all these cases control biopolymers of known molecular weights must be used, for interpolation and estimation of the molecular sizes of the unknown molecules or chains. Such interpolations indeed at best yield only estimations, as it is unlikely that the unknown has the same degree and type of asymmetry as the known molecules. And in the final analysis the molecular weights of the known molecules have been determined earlier by analytical ultracentrifugation. There thus are always reasons why one may want to (or have to) go back to a sedimentation technique for the precise and unequivocal determination of the molecular weight, as well as of the degree of asymmetry, of an immunoglobulin, an immunoglobulin chain, or any other protein or polypeptide.

It thus remains useful to recapitulate what can and what cannot be determined by various analytical sedimentation techniques.[61]

Sedimentation Rates have the considerable advantage that they even can be determined in heterogeneous mixtures (using, e.g., schlieren optics). The sedimentation constant(s) obtained have the drawback that the molecular weight (M) can only be obtained from them (via the Svedberg equation: $M = RTs/(1-\bar{v}\rho)D$,[61,62]) if the diffusion constant (D) also is known. However, nowadays diffusion constants of a given protein can also be determined in heterogeneous mixtures, as long as an antibody to that protein is available; double diffusion at right angles[63] will then yield D. Once both M and D, or both s and D, or both s and M, are known, the friction factor ratio f/f_o, expressing the ratio between the actual diffusion

coefficient, and the diffusion coefficient a molecule of the same molecular weight would have if it were completely spherical, and therefrom the asymmetry ratio can be determined.[61,62,64] If a protein is available in the purified state, M can also be directly determined (without knowing D) by sedimentation equilibrium, or by approach to equilibrium methods.[61] Sedimentation rate determination is a useful check for molecular weight homogeneity (provided at least 0.5 ml, containing 2 to 5 mg protein, is available). Small amounts of contaminants (<2%) however cannot be detected by that approach.

E. Surface Tension

From the order in which immunoglobulins and other plasma proteins emerge from a hydrophobic (or reversed-phase) chromatography column (see Section IV-B, above), one can obtain a qualitative estimation of the surface tension of these proteins. For the quantitative determination of the surface tension of proteins (γ_{PrV}), two methods may be used: contact angle measurement, or protein adsorption determination.

Contact Angle Measurement is by far the simplest method for obtaining γ_{PrV}.[2] One must however take great care not to denature the protein by exposure of too thin a layer to the air-interface.[65] To that effect, a thick, hydrated layer of concentrated protein must be deposited by ultrafiltration on an anisotropic membrane. The contact angle θ, made by a drop of saline water, deposited on top of the (only slightly dried) hydrated protein layer (attached

to the membrane) can then be measured, and from the contact angle θ γ_{PrV} may be obtained, e.g., by means of tables[23] based upon an equation of state.

Protein Adsorption from solutions, onto different polymer surfaces of various surface tensions, while immersed in liquids of the different surface tensions will also yield γ_{PrV}.[2] This is done by finding (by interpolation) the one liquid in which the degree of adsorption of a given protein (in $\mu g/cm^2$) is the same for all different polymer surface. The surface tension γ_{LV} of that liquid is equal to γ_{PrV}. This method is more laborious than the contact angle method, but it serves the useful purpose, whilst being totally different from and unrelated to the contact angle method, of nevertheless giving the same value of γ_{PrV} for the same protein (within $\pm$ 0.3 erg/cm^2).

Charge-Shift Electrophoresis is an electrophoretic method, using three types (anionic, nonionic and cationic) of surfactant as probes for hydrophobic moieties on protein molecules.[58] When the hydrophobic tail of surfactants can bind to a hydrophobic moiety of a protein, anionic surfactants will make the protein more negatively charged, cationic surfactants less negatively charged, and nonionic surfactants should have no influence. The use of all three kinds of surfactant is advised, to detect whether one of them attaches to the protein via an ionic (instead of a hydrophobic, or van der Waals) interaction, in which case one of the ionic ones would not cause a change in the protein's electrophoretic mobility. If none of the three types of surfactant causes a change in electrophoretic

mobility of a protein, that protein is unlikely to have a prominent hydrophobic moiety. The method has yielded interesting results in the study of cell membrane proteins, but does not show great promise in the characterization of the (very hydrophilic) plasma proteins. Still, for the further study of the relatively hydrophobic Fc tails of some immunoglobulins (e.g., of IgG1 and IgG3), the method might be useful.

Surface Tensions of Protein Solutions yield no information about the surface tension of the protein itself, but simply reflect the degree of adsorbtion of the protein at the air-liquid interface in the measuring device, and the concomitant denaturation of the protein.[65] However, changes in the surface tension of a solution of a synthetic polypeptide (poly-L-lysine), as a function of temperature, and as a function of pH, correlate well with changes in the secondary conformation of that polymer, as measured by circular dichroism. At the pK value of poly-L-lysine (pH 10.4) a minimum was found in the surface tension of the solution (at all temperatures), implying that at that pH the surface concentration of hydrophobic groups of the polypeptide molecule is at a maximum;[66] see also Section G, below.

The Surface Tensions of Proteins ($_{PrV}$) in the hydrated, native state, generally is the deciding factor in the degree to which a protein becomes aspecifically adsorbed to various surfaces (see Section IV-A, above). This explains the aspecific opsonization of hydrophobic bacteria by IgG1 and IgG3 (which have rather hydrophobic Fc tails). From the surface tension of a protein, its

van der Waals (or Hamaker) coefficient can be calculated, via equations (1A) and (7), see Section IV-A, above.[2]

F. Primary Configuration

The amino acid sequence of a peptide chain and the way in which two or more peptide chains are covalently bound together form the primary configuration of a polypeptide or protein. Several decades ago the determination of the amino acid sequence of peptides was an exceedingly laborious enterprise. With the advent of automatic protein sequencers (Beckman Instruments, Palo Alto, CA)[67] the determination of the primary configuration of even quite large proteins became a matter of routine. For the determination of the primary configuration of immunoglobulins, sequencing is only possible with monoclonal immunoglobulins. A number of amino acid sequences of human (monoclonal) γ, α, μ, δ, ε, κ and λ chains, showing the variable as well as the constant parts, compiled by A. L. Grossberg, are given by van Oss;[38] see also Nisonoff *et al.*[40] Automatic protein synthesizers, combined with protein sequencers, form increasingly important tools in the immunochemical study of the complete antigenic make-up of proteins. Knowledge of the primary configuration of protein antigens as well as of immunoglobulins remains of crucial importance in the understanding of their spatial (secondary and tertiary) configurations, as well as of their immunochemistry.[53]

G. Secondary Configuration

The secondary configuration of proteins alludes to their first-order spatial folding, e.g., in α-helices, β-pleated sheets or ran-

dom coil conformation.[68] The secondary configuration is usually determined by one of two related optical methods, i.e.: circular dichroism (CD) and optical rotatory dispersion (ORD). CD (usually the method of choice) measures the difference between right and left circularly polarized light, expressed as the difference in optical rotation in degrees (per mole of protein solution the light traverses), vs. the wavelength. In ORD the optical rotation of light passing through a column of protein solution is plotted vs. the wavelength. ORD spectra essentially depict the first derivative of CD spectra (of a given protein).[62] CD spectra allow the differentation between α-helices, β-sheets and random coils. Surface tension measurements of simple polypeptides (such as poly-L-lysine) also allow the measurement of conversion form α-helix to β-sheet in good agreement with CD determinations,[66] see Section E, above.

H. Tertiary Configuration

The actual folding of the α-helix and/or β-sheet of a protein into its final 3-dimensional structure results in the tertiary configuration. A variety of methods generally are used in conjunction with each other for the complete unraveling of the tertiary configuration. To begin with, the primary configuration of a protein must be known, and at least a fairly good knowledge of the secondary configuration. Then the method of choice is X-ray diffraction of crystals of the pure protein.[40,62] The high resolution of this method is due to the exceedingly small wavelength of X-rays ($\approx$1 Å). X-rays are scattered by the electrons within the atoms of the crystal, giving rise to diffraction patterns, due to

interference among the scattered radiations from the various atoms. The diffraction patterns are analyzed via Fourier transforms, to yield, after much trial and error, an electron density pattern, corresponding to the pattern corresponding to the atoms in the protein crystal, in one given layer. Many analyses of this type ultimately permit the construction of a three-dimensional representation of the atoms in the protein. For this it is essential to know not only the primary configuration of the protein, but also its real three-dimensional shape, obtained from, e.g., hydrodynamic sedimentation data (see Section D, above), or electron microscopy; see also Nisonoff et al.[40]

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