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Use of Gel Chromatography for the Determination of the Stokes Radii of Proteins in the Presence and Absence of Detergents. A Reexamination†

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ABSTRACT: In the course of a routine investigation of the complex between the erythrocyte membrane protein spectrin and sodium dodecyl sulfate, we observed a large discrepancy between the true Stokes radius (178 Å, measured by hydrodynamic methods) and the apparent value derived from gel chromatography (107 Å). In attempting to resolve this discrepancy, we have experiments that indicate that all large asymmetric particles may be subject to a similar discrepancy; e.g., native fibrinogen has a true Stokes radius of 108 Å, whereas the value derived by column chromatography after calibration with globular proteins is only 71 Å. The simplest interpretation is that end-on insertion of asymmetric particles into the gel pores contributes to their retardation. The phenomenon clearly limits the usefulness of gel chromatography as a quantitative measure of the hydrodynamic Stokes radius. Incidental data obtained in the course of this work indicate that spherical viruses may have weak chemical affinity for the porous gel. Chromatography of large proteins in the presence of detergents produced no effects ascribable to adsorption of the detergents, but the results suggest a need for further study of possible interaction between detergents and small gel pores.

The discovery by Porath and Flodin (1959) that cross-linked dextran gels can fractionate water-soluble molecules on the basis of molecular size has had a profound impact on protein chemistry, providing not only a practical tool for purification, but also an analytical tool for the measurement of relative molecular dimensions, and for the study of equilibria in reactions accompanied by a change in dimensions (Ackers, 1970). However, there have been no definitive studies relating the dimensional parameter that determines exclusion from the gel pores to actual physical measures of molecular size. An early proposal (Andrews, 1965) was that relative rates of elution of proteins are uniquely determined by molecular weight, but this holds true (even approximately) only when all proteins being compared belong to the same conformational class, e.g., if all are globular proteins or all are linear random coils (Fish et al., 1969). When proteins of different conformational types have been compared, it has been tacitly assumed that relative rates of elution are governed by the Stokes radius (R_s) of the protein particle. This radius is formally defined in terms of the frictional coefficient, $f \equiv 6\pi\eta R_s$ (where η is the viscosity of the solvent) and can be directly determined from the diffusion coefficient or by simultaneous measurement of molecular weight and sedimentation velocity (Tanford, 1961). The only serious test of this assumption (Laurent and Killander, 1964) is effectively limited to globular proteins with R_s at 60 Å or below. Warshaw and Ackers (1971) showed that the assumption is in fact false even for this class of proteins when elution characteristics are measured with high precision, but their work has had little impact because gel chromatography is usually used in circumstances where approximate answers (±10%) suffice. Work from this laboratory (Fish et al., 1970; Tanford et al., 1974) has indicated that the assumption is valid within a 10% uncertainty in the range of R_s = 30–80 Å. The comparisons in the latter studies involved proteins in three different conformational states: globular proteins in aqueous solution, random coils in guanidine hydrochloride, and complexes between proteins and sodium dodecyl sulfate. An additional conclusion implicit in the results was thus that drastic alterations in the solvent medium (including the presence of detergents) do not significantly alter the pore size distribution and elution characteristics of the chromatographic columns.

The reinvestigation of this problem described in this paper was prompted by discrepancies between observed and expected

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behavior in the gel chromatography of protein–detergent complexes of large size. The work required the use of large proteins of several kinds, both in the absence and presence of detergents, often under conditions where the molecular weight and R_s values could not be considered as completely established. These parameters were redetermined whenever any doubt existed, usually on fractions collected after chromatography, that had been used to establish the elution position of the protein.

Experimental Procedure

Protein. Human fibrinogen (Ab Kabi, Stockholm, Sweden) was donated by J. Abernethy; another sample, prepared in the laboratory of Dr. P. A. McKee had essentially the same elution properties. *Limulus* hemocyanin was a gift from Dr. J. Bonaventura: it contained a lower molecular weight impurity that separated from the principal fraction during chromatography. Rabbit skeletal muscle myosin was a gift from Dr. W. F. Harrington; NADPH[1]-sulfite reductase (*Escherichia coli*) was a gift from Dr. L. Siegel; bovine milk xanthine oxidase was donated by W. Waud; f2 virus was provided by Dr. R. E. Webster. Human erythrocyte spectrin and Ca^{2+} ATPase from rabbit muscle sarcoplasmic reticulum were prepared as described elsewhere (Schechter et al., 1976; le Maire et al., 1976). Other proteins were commercial preparations in routine use in this laboratory for standardization purposes.

Detergents. Sodium dodecyl sulfate for the experiments of Figure 3 was purchased from B.D.H. Chemicals, Ltd., and was >99% homogeneous by gas chromatographic analysis of the aliphatic alcohol produced by hydrolysis. Sodium dodecyl sulfate used for the data of Figure 2 was the highest purity grade from Schwarz/Mann: recent lots from this source have been found to be grossly contaminated with myristyl sulfate, but this does not affect the results because R_s values used in Figure 2 were measured directly and were thus not dependent on the purity of the detergent. Dodecyl octaethylene glycol monoether ($C_{12}E_8$) was a nominally pure compound from Nikko Chemicals Co., Tokyo. It was recrystallized from hexane before use.

Gel Chromatography. Sepharose 4B (Pharmacia) was used. Columns were 80–90 cm in length, with a diameter 0.8–1.5 cm, except that a column with dimensions 50 × 0.9 cm was used for the data of Figure 3. The columns were equilibrated and eluted with a variety of buffered salt solutions without observable effect on calibration characteristics. We used 0.5 M KCl, 0.5 M phosphate (pH 7.8), 0.01 M EDTA, 0.01 M NaN_3 for chromatography of native myosin, a medium designed to favor retention of the monomeric native state (Harrington et al., 1973). We used 0.6 M KCl, 0.005 M $CaCl_2$, 0.02 M Tris (pH 8) for hemocyanin, and included 10^{-4} M $CuSO_4$ in one run to guard against possible affinity of the resin for protein-bound Cu^{2+}. The native properties of fibrinogen were found to be best preserved at high ionic strength (1.2 M KCl, 0.005 M EDTA, 0.02 M Tris, pH 8), but the elution position observed at lower ionic strength was not significantly different. Catalase and f2 virus were used as controls whenever the column buffer solution was changed: no changes were observed on any column (in the absence of detergent), except where this is specifically indicated in the text. When detergent was used, it was washed out after use and the column was recalibrated in detergent-free buffer: the column used for Figure 2 is the only exception to this.

Direct Determination of Stokes Radii. R_s values were determined experimentally by combined sedimentation equilibrium and velocity measurements, employing a Beckman Model E ultracentrifuge, equipped with photoelectric scanner. The buoyant density factor is not needed for this determination (Tanford et al., 1974), and measurements in detergent solutions could therefore be made without knowledge of the amount of protein-bound detergent.

Results

Calibration with Globular Proteins. We have always found that chromatographic data for globular proteins in aqueous solution can be linearized if plotted according to the empirical[2] equation of Ackers (1967)

$$R_s = a_0 + b_0 \,\mathrm{erf}^{-1}(1 - K_d) \qquad (1)$$

where K_d is the distribution coefficient defined by Laurent and Killander (1964), and a_0 and b_0 are constants reflecting the pore size distribution of the gel. For Sepharose 4B, a_0 is generally close to zero and $b_0 \simeq 130$ Å. There are small variations between columns prepared at different times, which presumably reflect at least in part small differences between different lots of resin, and it is for this reason that the results presented in Figures 1–3 have not been combined into a single plot.

Before this investigation began we did not use any calibration standards with R_s between 52 and 100 Å because we found that the elution position of the spherical f2 bacteriophage (assigned R_s 105 Å) was highly reproducible and always lay precisely on a linear extension of the calibration plot derived from globular proteins with smaller R_s, as illustrated, for example, by Figure 1. The assigned R_s for this virus was based on the electron microscope measurements of Loeb and Zinder (1961) for f2 and the similar data of Vasquez et al. (1966) for R17, which is a virus of the same class and size as f2 (Boedtker and Gesteland, 1975). Recently, the true R_s for R17 has been measured from diffusion coefficients (Camerini-Otero et al., 1974) and found to be much larger than the assigned value we had used: the result obtained was $R_s = 140 \pm 2$ Å. We have carried out sedimentation equilibrium and sedimentation velocity measurements on the sample of f2 used by us, and have confirmed that the same value applies to this virus as well. With $R_s = 140$ Å, the point for f2 no longer falls on an extension of the calibration plot obtained with small globular proteins.

Measurements using globular proteins with larger R_s values were obviously required to resolve this difficulty. We used β-galactosidase (from *E. coli*), which can be assigned an R_s value of 69 Å on the basis of internally self-consistent sedimentation equilibrium and hydrodynamic measurements (Sund and Weber, 1963; Craven et al., 1965), NADPH-sulfite reductase, with an R_s value of 86–87 Å on a similar basis (Siegel et al., 1973), and *Limulus* hemocyanin. Reliable size measurements for the latter protein are not available, and a de novo determination was therefore made. Sedimentation velocity measurements on chromatographed protein yielded an accurate $s^0_{20,w}$ value of 57.0 S, but small amounts of both

[1] Abbreviations used are: cmc, critical micelle concentration; NADPH, reduced nicotinamide adenine dinucleotide phosphate; IgG, immunoglobulin; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

[2] The function $\mathrm{erf}^{-1}\, x$ is the inverse of the probability integral, $\mathrm{erf}\, x = (2/\pi^{1/2}) \int_0^x e^{-x^2} dx$. It should be mentioned that there is an error in the derivation of eq 1 (Ackers, 1967), such that the theoretical basis for the equation is correct only if $a_0 = 0$. This does not influence the use of the equation for linearization of experimental data, since no theoretical interpretation of a_0 and b_0 is involved. As it happens, a_0 is close to zero for the columns used in these studies.

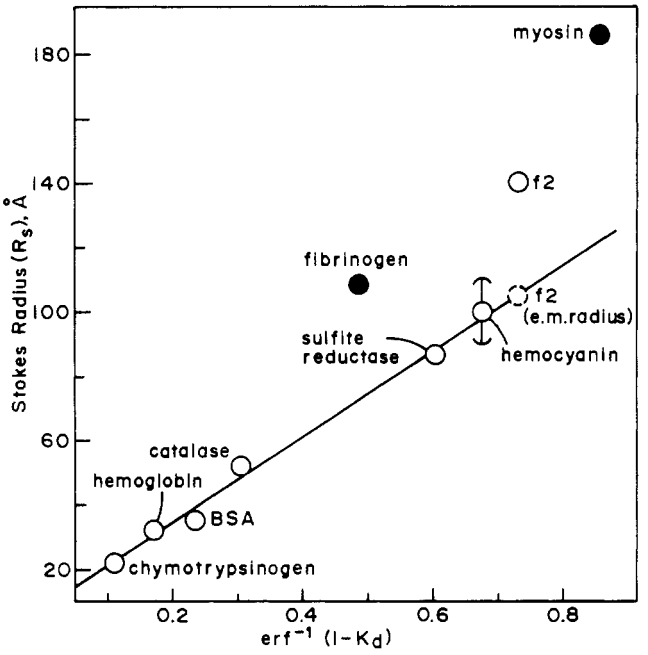

FIGURE 1: Results on Sepharose 4B column in aqueous solutions. Each experimental point represents between two and eight determinations on five different columns (all prepared from the same lot of Sepharose 4B). The maximum standard deviation for any one protein was ±0.020 in the observed value for the inverse error function. Filled circles are results for asymmetric proteins, discussed later in this paper. (Inclusion of one point for f2 on the basis of its electron microscopic radius is explained in the text.)

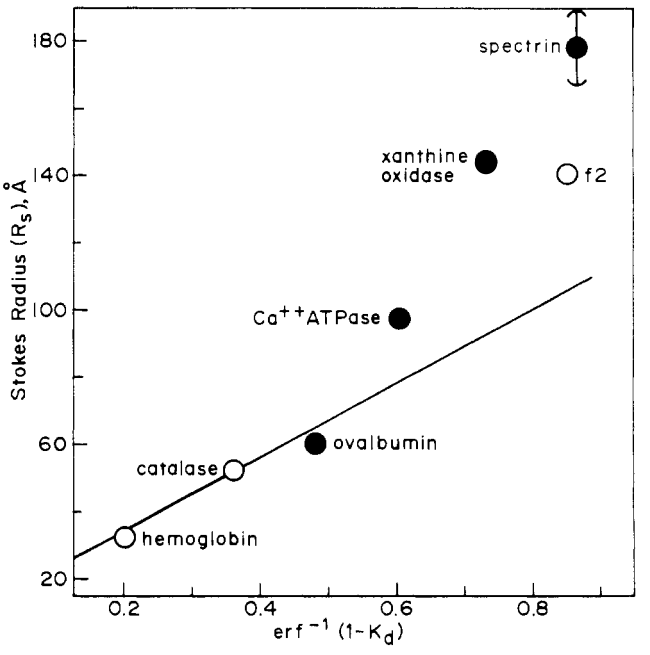

FIGURE 2: Comparison between results in aqueous buffer (open circles) and in 0.1% sodium dodecyl sulfate (filled circles) on Sepharose 4B. Extension of the calibration line in aqueous buffer to large R_s is discussed in the text.

slower- and faster-sedimenting material were present as well. This indicated a time-dependent deterioration of the native protein, since contaminants present in the initial sample would have become separated from the major fraction of the protein on the Sepharose column. The long time period required for sedimentation equilibrium measurements led to an increase in the amount of light and heavy material, and curved equilibrium plots were obtained, from which a somewhat unsatisfying molecular weight of about 2.6×10^6 for the major portion of the protein was deduced. The Archibald approach to equilibrium method (Tanford, 1961) was used in order to shorten the time of the experiment. A molecular weight of 2.1×10^6 was obtained from the upper meniscus, where contamination from the light degradation product would be expected to provide the major interference, and a value of 2.7×10^6 was obtained from the lower meniscus, where the heavy (presumably aggregated) degradation product would be expected to interfere. Our overall estimate for the molecular weight of the undegraded protein on the basis of these results is $(2.4 \pm 0.2) \times 10^6$, which, with $s^0_{20,w} = 57$ S, leads to $R_s = 100 \pm 8$ Å.

The chromatographic data for these proteins are shown in Figures 1 and 3. It is evident that no deviation from the linearity of the Ackers plots occurs for globular proteins up to $R_s = 100$ Å. (The results for fibrinogen and myosin will be considered later.) This conclusion leaves the observed result for the spherical f2 virus unexplained. It can be taken to indicate a sharp break in the Ackers plot at R_s above 100 Å, but there is strong evidence against this, based on previous measurements in this laboratory using Sepharose 4 B for the chromatography of randomly coiled polypeptide chains in concentrated guanidine hydrochloride. (The polypeptides in this state are swollen to large size, but maintain spherical symmetry.) As noted under the Introduction, chromatographic data for randomly coiled polypeptides in this solvent have coincided with calibration plots for globular proteins (on resins with a variety of pore sizes) whenever a direct comparison has been made. Because R_s values for random coils are much larger than for globular proteins, data in guanidine hydrochloride are readily extended to $R_s > 100$ Å, and in one study from this laboratory (Smith et al., 1972) a linear plot on Sepharose 4B was obtained, with no sign of any break, from lysozyme ($R_s = 32$ Å) to the heavy chain of myosin ($R_s = 135$ Å), the R_s values for all of the proteins being firmly established on the basis of viscosity measurements. There was no direct comparison with native globular proteins in this study, but it should be noted that the slope of the plot (b_0 of eq 1) is 130 Å, identical with the slope of the calibration plot in Figure 1. We therefore believe that the observed elution position for f2 virus represents an anomaly, possibly reflecting weak affinity between the virus and the gel matrix.

In the course of these experiments the same or duplicate columns were equilibrated with solvents covering a wide range of conditions, ranging from dilute buffered salt solutions of high ionic strength (e.g., 0.5 M KCl *plus* 0.5 M phosphate buffer). This was done, as explained under the Experimental Section to provide optimal media for the various proteins examined. Within experimental error, the linear plot defined by the globular proteins was unaffected by these changes, a result consistent with the observation that even 6 M guanidine hydrochloride does not appear to affect column characteristics.

Sodium Dodecyl Sulfate Complexes of Reduced Polypeptide Chains. The results that led to the present investigation are shown in Figure 2. Since no untoward behavior was anticipated, the globular protein calibration line was based on only three points, one of them being f2 virus with the assigned R_s value we now know to be incorrect. As Figure 1 shows, the correct calibration line is obtained in this way, and the slope and intercept in Figure 2 are within the range normally observed. An unexpected result was, however, obtained for the spectrin–sodium dodecyl sulfate complex, the point for which

falls far above the calibration line: the R_s determined from sedimentation equilibrium and sedimentation velocity data is 178 ± 11 Å, whereas the observed K_d value corresponds to an R_s of only 107 Å on the calibration line. A similar discrepancy was observed for the sodium dodecyl sulfate complex of the xanthine oxidase polypeptide chain, for which we measured an R_s of 144 Å. On the other hand, no discrepancy was observed for ovalbumin, which falls within the size range of sodium dodecyl sulfate complexes previously shown to have K_d values corresponding to the globular protein calibration line (Tanford et al., 1974). An R_s of 62 Å was measured, compared to the value of 58 Å estimated in the previous paper from viscosity data. Another large sodium dodecyl sulfate complex, that of reduced and alkylated sarcoplasmic Ca^{2+} ATPase, was measured on the same column by M. le Maire at a later date, and a deviation from the calibration line was again observed. The molecular weight of the ATPase chain is 119 000 and the R_s value of 97 Å was measured under the conditions used for the molecular-weight measurement (Rizzolo et al., 1976), at a lower sodium dodecyl sulfate concentration than that of the chromatographic experiment and at a binding level somewhat short of saturation. Thus, 97 Å is actually a minimal value for R_s under the conditions of the column experiment.

Figure 3 shows similar results obtained more recently, using a shorter column, where the experimental error in measuring K_d is somewhat larger. The R_s values used for the sodium dodecyl sulfate complexes were not measured experimentally, but were either taken from our previously published list (Tanford et al., 1974) or were estimated from a plot of log R_s vs. log molecular weight that includes measured R_s values for a large number of reduced sodium dodecyl sulfate–polypeptide complexes. Such a plot is linear, with a slope of 0.68, as originally reported by Fish et al. (1970). Subsequent data have produced no deviations from this plot that would be significant in relation to the accuracy with which K_d can be measured. Figure 3 includes new data for two additional sodium dodecyl sulfate complexes that are consistent with the results of Figure 2. It also shows that the presence of a large excess of sodium dodecyl sulfate micelles has only a small effect on the results. The cmc of the detergent at ionic strength 0.33 is well below 1 mM, so that most of the sodium dodecyl sulfate in the experiments at this ionic strength is in micellar form. The sodium dodecyl sulfate concentration used at the lower ionic strength is barely above the cmc and the concentration of sodium dodecyl sulfate in micellar form is of the order of 10^{-4} M. The small difference observed between the results at the two ionic strengths is barely significant experimentally, and may reflect a small effect of ionic strength on the dimensions of the highly charged sodium dodecyl sulfate complexes rather than being related to the presence or absence of sodium dodecyl sulfate micelles.

It may be noted that the aqueous calibration of the column used for Figure 3 was done before any sodium dodecyl sulfate was introduced and again after all sodium dodecyl sulfate had been washed out at the completion of the experiment. No significant difference was detected.

The results of Figures 2 and 3 show unambiguously that the anomalous elution on Sepharose 4B originally observed for the spectrin–sodium dodecyl sulfate complex is common to all large protein–sodium dodecyl sulfate complexes. There are two possible explanations: the anomaly may be due to the presence of detergent, reflecting an interaction between sodium dodecyl sulfate and the gel material; alternatively, the cause of the anomalous retardation may lie in the physical properties of the sodium dodecyl sulfate–protein particles per se. The complexes

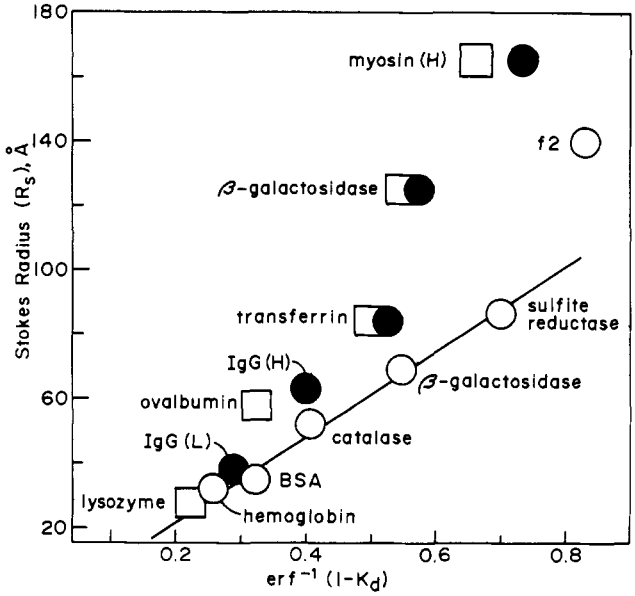

FIGURE 3: Comparison between results in aqueous buffer and in sodium dodecyl sulfate on another column. Data in aqueous buffer (phosphate, ionic strength 0.033, pH 8.3) were obtained with the freshly poured column before exposure to sodium dodecyl sulfate, and again after the experiments in sodium dodecyl sulfate had been completed: open circles are averages of the two determinations. Filled circles represent data in 3.47×10^{-3} M sodium dodecyl sulfate, in the same buffer. Squares represent data in 0.01 M sodium dodecyl sulfate in phosphate buffer at ionic strength 0.33, pH 8.3.

are rod-like with some flexibility, becoming increasingly asymmetric with increasing molecular weight (Reynolds and Tanford, 1970; Shirahama et al., 1974; Wright et al., 1975), and there have been no experimental studies of possible effects of such particle asymmetry on gel chromatography. Experiments related to both possibilities were carried out and are described below.

Effect of Detergents on Column Characteristics. In order to distinguish possible effects of detergents on column characteristics from effects on protein conformation, it is necessary to use a detergent that does not interact with the proteins being studied, so that the elution positions can be compared in the absence and presence of detergent while protein dimensions remain unchanged. It is desirable to work at detergent concentrations above the cmc, so as to maximize the thermodynamic activity of the detergent and to promote any possible cooperative binding of detergent to the gel resin. This dual requirement virtually dictates the choice of a nonionic detergent (Helenius and Simons, 1972; Makino et al., 1973), and we have selected dodecyl octaethylene glycol monoether ($C_{12}E_8$). This detergent has a cmc of about 9×10^{-5} M at room temperature in the buffer (0.02 M Tris, 0.1 M NaCl) that was used for chromatography. It forms micelles with about the same aggregation number as sodium dodecyl sulfate but with larger dimensions because of the large size of the octaethylene glycol head group. The cloud point for this detergent is well above room temperature and there is thus no tendency for phase separation or for formation of large "super-micelles".

The results obtained with $C_{12}E_8$ are shown in Figure 4. The column was equilibrated with 0.2 mM $C_{12}E_8$ (i.e., at approximately double the cmc), but was intermittently exposed to much higher detergent concentrations because studies of $C_{12}E_8$ micelles were being carried out within the same time period. (No change in the elution position of micelles was ob-

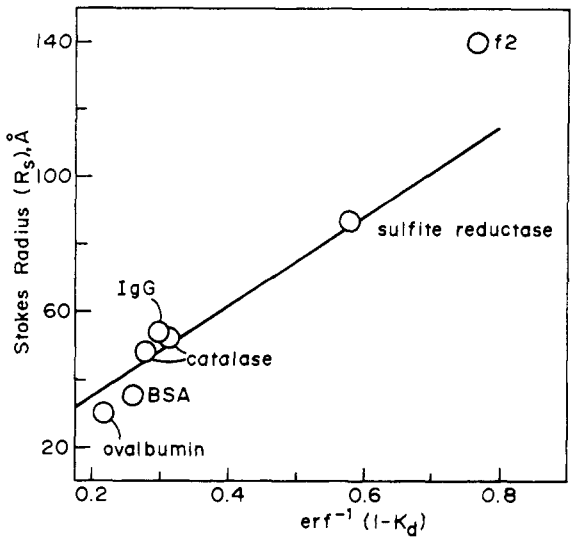

FIGURE 4: Effects of nonionic detergent. The column used was among those used to obtain the data of Figure 1, and the calibration line is taken from that figure. Circles represent results obtained with the column equilibrated and eluted with 0.2 mM $C_{12}E_8$; the two points for catalase are explained in the text.

served from the lowest concentration employed up to a loading concentration of 47 mM.) Proteins were usually dissolved in the column buffer without addition of excess detergent because no binding was anticipated, but an excess was added in a few experiments and had no influence on the elution position. Eluate fractions containing the protein were subjected to ultracentrifugation to verify that addition of the detergent did not alter the molecular dimensions. Such verification was obtained even for as complex a particle as f2 virus, for which we found a molecular weight of 3.7×10^6 and an $s_{20,w}$ of 75 S, identical within the accuracy of a single experiment to the values obtained by Camerini-Otero et al. (1974). Sulfite reductase was observed to dissociate slowly towards the end of a sedimentation equilibrium experiment, but there were no indications that significant alteration had taken place during chromatography. The catalase sample initially chromatographed (lower point in the figure) gave a slightly curved sedimentation equilibrium plot and a slightly lower than normal sedimentation coefficient: the weight-average R_s value was estimated as 48 Å, but the curvature of the sedimentation equilibrium plot indicated that dissociation should be suppressed if the protein concentration were increased tenfold. The upper point in the figure represents the result at the higher concentration, with R_s assigned its normal value of 52 Å (Tanford et al., 1974). Measured R_s values for serum albumin and ovalbumin were within 1 Å of the values for the native proteins. The R_s value for immunoglobulin (IgG) was measured as 54 Å, slightly larger than the assigned value of 51 Å for the native protein, but probably within the uncertainty with which the later assignment can be made. The overall conclusion from all the experiments is that $C_{12}E_8$ has no effect on column characteristics and that, apart from a tendency to cause some dissociation of multisubunit enzymes, it also has no effect on the dimensions of the proteins that were studied.

It may be noted that the two experimental points with smallest R_s in Figure 4 are slightly displaced from the calibration line in the direction of *smaller* K_d. A similar displacement has been observed for small sodium dodecyl sulfate–protein complexes ($R_s < 30$ Å) when chromatographed on Sephadex G-75, though Figure 3 does not indicate a similar phenomenon on Sepharose 4B. We have not pursued these findings to determine how reproducible they are, because we were chiefly interested in these studies in the behavior of large particles. The data, if verified, would suggest an interaction of detergent that is limited to gel pores of small diameter, blocking access of proteins to them. This would interfere with the retardation of protein particles of correspondingly small size, but would have no effect on the chromatography of larger particles. In any event, an adsorptive phenomenon of this kind clearly could not explain the deviations of large sodium dodecyl sulfate–protein complexes seen in Figures 2 and 3, which are in the opposite direction.

Large Asymmetric Proteins in Aqueous Solutions. The second of the two possible suggestions offered above to explain the anomalous behavior of large sodium dodecyl sulfate–protein complexes depends only on the particle shape and should therefore apply to the gel chromatography of any asymmetric particle in any solvent. To investigate this possibility we have used two fibrous proteins, fibrinogen and myosin, which are very asymmetric in their native states in aqueous solution. The R_s values for these proteins are readily evaluated from well-established data. For fibrinogen (Doolittle, 1973) $R_s = 108$ Å. For myosin we have used $R_s = 186$ Å on the basis of the recent results of Godfrey and Harrington (1970); earlier data (e.g., Holtzer and Lowey, 1959) lead to essentially the same value.

To make certain that fibrinogen was not degraded or otherwise altered during passage through the column, we showed that it retained the proper three-chain pattern on sodium dodecyl sulfate–gel electrophoresis, measured the sedimentation coefficient, and performed a standard clotting test (Blombäck and Blombäck, 1956). The latter indicated 69% clottability, which is satisfactory considering that the assay had to be carried out at a lower protein concentration (0.1 mg/ml) than is normally used. To maintain myosin in its native state proved more difficult. This protein undergoes a rapidly reversible dimerization even at high ionic strength, and higher aggregates are formed from the dimer (Harrington et al., 1973). The solvent system used in our experiments (see Experimental Section) was designed to minimize the association tendency, but chromatography was carried out at room temperature and aggregation could not be entirely prevented. The protein emerged from the column as two separate peaks, one near the position of the void volume marker, the other at the position shown in Figure 1. Material from both peaks was examined by sedimentation equilibrium, at 6 °C to avoid further aggregation. The first peak proved to be highly aggregated, but the second peak had a molecular weight of 456 000, essentially identical to the molecular weight of monomeric myosin (Godfrey and Harrington, 1970).

As Figure 1 shows, both fibrinogen and myosin were anomalously retarded in gel chromatography to about the same extent as the sodium dodecyl sulfate–protein complexes of comparable size.

Discussion

The results presented in this paper strongly indicate that anomalous retardation in gel chromatography is a general property of all large asymmetric particles. The simplest explanation is that end-on insertion into the gel pores contributes to retardation, to a greater degree than end-on motion contributes to the frictional properties in solution. A similar explanation has been offered by Laurent et al. (1975) to account for anomalously fast diffusion of asymmetric particles through hyaluronic acid gels: asymmetric particles were able to pene-

trate the gel network more readily than globular particles with comparable Stokes radius.

Some of our experiments suggest that detergents can sometimes interact with the gel resins used in chromatography so as to affect the column characteristics. Our observations on this subject are sparse, and much more work is needed to define the conditions under which the phenomenon occurs. It is, however, safe to say that interactions of this kind cannot provide a plausible alternative (to particle asymmetry) as an explanation for the results we obtained with reduced sodium dodecyl sulfate–protein complexes. Where effects directly attributable to the presence of detergents were observed, they prevented access of proteins to a portion of the pore volume, thereby decreasing K_d. It is difficult to visualize how detergents could produce the opposite effect, i.e., an increase in the available pore volume, leading to augmented retardation.

Recently published measurements of the hydrodynamic properties of spherical viruses (Camerini-Otero et al., 1974; Boedtker and Gesteland, 1975) have led to the discovery of another anomaly. A virus of this class (f2) that had previously appeared to have a K_d value consistent with its size and shape, does not do so when assigned its correct R_s value. The work of Huang (1969) provides what may be another example of the same phenomenon. Huang found that single-walled phosphatidylcholine vessels elute on Sepharose 4B at essentially the same position as Turnip Yellow Mosaic Virus. This virus has a radius of 140 Å on the basis of electron microscopy (Klug and Casper, 1960), and the true R_s value may be even larger to judge by the data for R17 cited above and a similar discrepancy between R_s and the electron microscope radius for Bushy Stunt Virus (Camerini-Otero et al., 1974). The true R_s value for the vesicles (consistent values have been obtained from diffusion, sedimentation and viscosity measurements) is close to 110 Å, i.e., either the virus or the vesicles must have behaved anomalously to produce the similarity in K_d. We have observed in at least one instance that phospholipid per se does not affect column calibration curves (Haberland and Reynolds, 1975), which suggests that the anomaly in Huang's experiments rests with the virus and not with the vesicles. It may be speculated that anomalous retardation results in these examples from chemical attraction between the viral surface and the gel matrix. Possibly viral surface sites intended for recognition of cell surface carbohydrates have some affinity for surface groups on the dextran resin.

The overall conclusion from these studies is that gel chromatography can be used as a quantitative measure of Stokes radius only under strictly limited conditions. The possible error for large asymmetric particles is not trivial. For fibrinogen, for example, the R_s that would be estimated by gel chromatography on the basis of calibration with globular proteins is 71 Å, whereas the true hydrodynamic R_s value is 108 Å; the corresponding figures for myosin are 121 and 186 Å. Another possible source of error arises from chemical affinity for the gel substance, and may be more common than has been generally realized. The data presented in this paper suggest that viruses have weak affinity for Sepharose beads, and so far unexplained anomalies observed for small proteins on Sephadex (e.g., Voordouw et al., 1974) may have a similar cause. Finally, the presence of detergents may introduce additional aberrations that may not always be within acceptable limits of error: a problem barely touched on in the present study, that merits further investigation. These limitations of course also affect derivative procedures, such as the determination of molecular weight from R_s and the sedimentation coefficient, as advocated, for example (in somewhat different guises) by Siegel and Monty (1966) and by a recent paper from this laboratory (Tanford et al., 1974).

Acknowledgments

We gratefully acknowledge gifts of proteins from the persons listed under the Experimental Section, and the accompanying advice on how best to maintain them. We also acknowledge helpful discussions with Dr. T. C. Laurent and Dr. K. van Holde.

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Ligand Interactions with the Acetylcholine Receptor from *Torpedo californica.* Extensions of the Allosteric Model for Cooperativity to Half-of-Site Activity†

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ABSTRACT: The solubilized acetylcholine receptor from *Torpedo californica* showed positive cooperativity in acetylcholine binding with a dissociation constant of 1.2×10^{-8} M. Blockade of acetylcholine binding by nicotine was competitive; blockade by *d*-tubocurarine appeared to result from an allosteric interaction that altered half of the acetylcholine binding sites to a lower affinity form; decamethonium blockade displayed properties of competitive and allosteric inhibition suggesting less specificity for decamethonium binding than seen with either nicotine or *d*-tubocurarine. The *d*-tubocurarine inhibition data were evaluated by several possible models involving either differential competitive inhibition or allosteric inhibition. The data were best described by the allosteric model.

Physiological evidence clearly indicates that there are at least two categories of agents that produce different effects upon the neuromuscular junction: agonists such as acetylcholine, nicotine, and decamethonium that depolarize; and antagonists such as *d*-tubocurarine and flaxedil that block depolarization caused by agonists. There is evidence for positive cooperativity in the actions of agonists (Rang, 1971; Higman et al., 1969; Changeux et al., 1970) and antagonists (Changeux and Podleski, 1968; Kasai and Changeux, 1971). However, such physiological evidence does not give direct information about competition for binding sites, nor the presence or absence of allosteric or cooperative effects that occur in the binding step, which is only the first part of the overall process.

The acetylcholine receptor from the electric organ of electric fishes has provided a convenient preparation for the study of ligand interaction (O'Brien et al., 1973; Eldefrawi et al., 1971; Weber and Changeux, 1974; Martinez-Carrion and Raftery, 1973) and oligomeric structure (Gibson et al., 1976; Carroll et al., 1973; Hucho and Changeux, 1973) of the acetylcholine receptor. This paper presents an analysis of the binding of [^{3}H]acetylcholine to solubilized preparations of acetylcholine receptor from *Torpedo californica* electroplax and the blockade of that binding by nicotine, *d*-tubocurarine, and decamethonium, interpreted in terms of a model for cooperative ligand binding. Although several models for cooperative ligand binding have been proposed (Monod et al., 1965; Koshland et al., 1966), the two-state model of Monod et al. has been applied with reasonable success to the evaluation of electrophysiological data (Karlin, 1967; Edelstein, 1972). Since the binding data indicated more than one class of binding sites, the data are described by extensions of the two-state model that include nonequivalent monomers.

Materials and Methods

Materials. [^{3}H]Acetylcholine (250 mCi/mmol), [^{3}H]nicotine (50 mCi/mmol), [^{3}H]decamethonium (50 mCi/mmol), and [^{14}C]dimethyl-*d*-tubocurarine (150 mCi/mmol) were purchased from Amersham/Searle. Nicotine was purchased from Aldrich; *d*-tubocurarine (dTC[1]) from Sigma, and decamethonium from Pfalz and Bauer.

Preparation of Acetylcholine Receptor. The acetylcholine receptor (AcChR) preparations were made from a heavy membrane preparation of *T. californica* electroplax. Live *Torpedo* were purchased from Pacific Biomarine Supply Co. (Venice, Calif.). The electric organs were removed and homogenized in 0.4 M NaCl (20% w/v) in a Waring blender at full speed for 2 min. The homogenate was filtered through two layers of cheesecloth and centrifuged at 10 000*g* for 30 min. The pellet was rehomogenized in a modified Ringer solution (116 mM NaCl, 4.6 mM KCl, 0.65 mM $CaCl_2$, 1.15 mM $MgSO_4$, and 0.016 M Na_2HPO_4 adjusted to pH 7.4) at 1 g of pellet/ml of Ringer solution and recentrifuged at 10 000*g* for 30 min. The pellet was then stored as a suspension of 1 g of pellet/ml of Ringer solution at −25 °C for less than 4 weeks before use. The AcChR suspension was solubilized by addition of Triton X-100 at a final concentration of 1% and the sus-

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[1] Abbreviations used are: AcCh, acetylcholine; AcChR, acetylcholine receptor; dTC, *d*-tubocurarine.