Physical Properties of Sponge Aggregation Factor. A Unique Proteoglycan Complex†

Charles B. Cauldwell,‡ Pierre Henkart,‡,§ and Tom Humphreys*

ABSTRACT: Physical properties of the aggregation factor isolated from the marine sponge *Microciona parthena* are described. The active molecular complex has an intrinsic viscosity of about 500 cm^3/g and a sedimentation constant of 62 S. These results suggest a large, fibrous molecule of about 21×10^5 daltons. Light scattering yields a similar molecular weight and a radius of gyration of 800 Å. These data, together with the electron microscopic images presented in the accompanying paper, indicate that the aggregation factor complex in solution has an open, sunburst structure with a diameter of 1600 Å or more. When Ca^{2+} ions are removed, the factor complex gradually dissociates into glycoprotein subunit molecules of 2×10^5 daltons plus a core.

The preceding paper (Henkart *et al.*, 1973) describes a proteoglycan macromolecular complex which is responsible for the species specific aggregation of dissociated sponge cells (Humphreys, 1963, 1967). This factor was prepared in a pure form and its chemical composition and basic structure as visualized in the electron microscope described. Here we examine the physical properties of the factor in order to establish its size and configuration in solution and discover the nature of its molecular organization. The physical measurements were especially required to show that the open, extended structure appearing in electron microscopic preparations (Henkart *et al.*, 1973; Humphreys *et al.*, in preparation) was not an artifact of the manipulation required to obtain the photographs but was an accurate indication of the configuration of the aggregation factor complex in solution.

Materials and Methods

Preparation and Purification of the Sponge Aggregation Factor. The aggregation factor (AF)[1] was prepared from the marine sponge, *Microciona parthena* (Henkart *et al.*, 1973), and had an activity of at least 30 units/mg.

Viscosity. All viscosity measurements were performed using a four-bulb Ubbelohde capillary viscometer having outflow times of 8–10 min for each bulb and kept at a constant temperature of 25.52 ± 0.02°. The outflow time for each bulb was measured with a stopwatch to 0.1 sec and at least three readings were made for each concentration in each bulb. The solvent was CaCMF unless specified. For EDTA studies, an aliquot of 0.1 M EDTA (pH 8) was added directly to a solution of AF in the viscometer and outflow times measured immediately.

Sedimentation. Sedimentation velocity measurements were made at 20° and at a speed of either 20,410 or 39,460 rpm in a Beckman Model E analytical ultracentrifuge employing an AN-D rotor with single aluminum cells and schlieren optics. A microcomparator was used to determine the radial distance, r, of the schlieren peak maxima. Plots of ln r *vs.* time could then be constructed for determination of the sedimentation coefficient. The microcomparator also allowed tracing of the schlieren pattern onto graph paper for area determination.

Sedimentation equilibrium measurements were performed using the high-speed method as described by Van Holde (1967). A double-sector-filled epon cell and an AN-H rotor were used, with interference optics. Results were analyzed with a microcomparator to give plots of the log of the vertical deflection *vs.* the radius squared (Chervenka, 1970).

A cesium chloride equilibrium density run, observed with monochromatic light at 275/nm, was performed using standard procedures (Chervenka, 1970). The bands were measured with a Joyce-Loebel microdensitometer.

Light Scattering. The angular dependence of the scattered light of solutions of AF was measured with a Brice-Pheonix Model 2000 photometer at 463 nm using a 15-ml cylindrical light-scattering cell. The data were corrected for reflection as described by Tomimatsu and Palmer (1963). Refractive index differences were determined on aliquots of the same solutions using a Brice-Pheonix differential refractometer. Concentration of the initial solution was determined by chemical analysis for protein (Lowry *et al.*, 1951). Accurate dilution by weight allowed calculation of the remaining concentrations.

Equilibrium Dialysis. Small plastic microculture slides with inlet and outlets ports were clamped together face to face on to a piece of dialysis membrane prepared to remove divalent cations and impurities (Hughes and Klotz, 1956). The chambers were filled and emptied with syringes and the volume was determined by weighing the syringe. Solutions of $^{45}Ca^{2+}$ were dialyzed against AF and aliquots were recovered after 6-hr dialysis and counted on a Beckman LS200 liquid scintillation counter. Calculations were performed as outlined in the bag binding method of Hughes and Klotz (1956).

Gel Filtration. Several gel filtration columns were employed for these studies. They were poured and eluted as recommended by the manufacturer, at 4°. A column of Sepharose 2B (4 × 60 cm) had a flow rate of 30 ml/hr, and was used to purify the factor. A Bio-Gel P300 column (0.9 × 30 cm) was used with a flow rate of 3 ml/hr. Another column of Se-

† From the Department of Biology, University of California, San Diego, California, and the Kewalo Marine Laboratory, Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96813. *Received January 10, 1973.* Supported by National Science Foundation Grants GB 18008 and 31618.

‡ National Institutes of Health postdoctoral fellows.

§ Present address: Immunology Branch, National Cancer Institute, Bethesda, Md. 20014.

[1] Abbreviations used are: AF, aggregation factor; CMF, Ca^{2+}- and Mg^{2+}-free artificial sea water (Humphreys, 1963); CaCMF, CMF with 0.001 M $CaCl_2$.

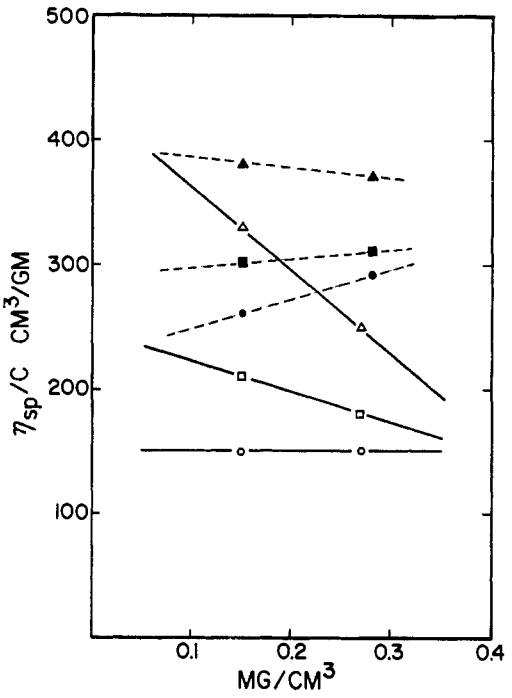

FIGURE 1: Reduced viscosity of native AF and AF after EDTA treatment. Three shear rates [(▲,△) lowest shear rate; (■,□) intermediate shear rate; (●,○) highest shear rate] were used to measure the viscosity of AF solutions before (- - - - - - -) and after (———) addition of EDTA. EDTA was added to viscometer reservoir to a final concentration of 5 mM.

pharose 2B (0.9 × 18 cm) had a flow rate of 2.5 ml/hr. All columns were washed with at least one column volume of solvent immediately prior to sample application.

Digestion with Papain and Pronase. AF (1.5 ml) was mixed with 7 μl of 0.5 M EDTA (pH 8) and left at room temperature for 6 hr. Following this treatment, 20 μl of 2 M thioglycolate (pH 8) and 5 μl of a 30-mg/ml suspension of papain in acetate buffer were added. This mixture was left in a 37° water bath overnight. Next morning, 5 μl of 1 M $CaCl_2$ and 20 μl of 7.5 mg/ml of Pronase in water were added. The digest was returned to the water bath for an additional 24 hr. Finally, 15 μl of 0.5 M EDTA (pH 8) was added, the slight precipitate was centrifuged out, and the solution was immediately chromatographed on a Bio-Gel P300 column.

Redigestion was performed by adding 10 μl of 1 M $CaCl_2$ to 3 ml of excluded material from the P300 column, then adding 20 μl of Pronase (7.5 mg/ml in water) and incubating at 37° for 24 hr. The redigested AF was then placed on the P300 column and eluted as before.

Results

Viscosity. The extended fibrous structure of the AF complex seen in the electron microscope (Henkart *et al.*, 1973; Humphreys *et al.*, in preparation) should give the factor a high intrinsic viscosity. In addition, the viscosity of the solution of AF should be sensitive both to shear rate due to the tendency of the molecules to collapse and orient in the direction of flow and to concentration due to altered probability of interactions occurring between the macromolecules.

The viscosity of AF solutions was examined at various concentrations and shear rates. The primary result is that the viscosity of AF solutions is reasonably high and shows shear rate dependence, indicating an extended fibrous structure (Figure 1). This general statement can be made somewhat more quantitative from the data although the viscosity behavior of the AF is unusual and will require more sophisticated experimentation for rigorous interpretation.

The intrinsic viscosity (limit of η_{sp}/c as $c \rightarrow 0$) at zero shear rate is the parameter which must be known for use in determining molecular structure. An approximation can usually be made by extrapolation of reduced viscosity curves at low shear rate to zero concentration. Such an extrapolation of our data suggests an intrinsic viscosity of 400–500 cm^3/g. We have obtained similar data from several preparations with the Ubbelohde viscometer and also with a very low shear rate rotating cylinder viscometer. The latter data extrapolated to a intrinsic viscosity of 550 cm^3/g. However, at low shear rates the curves have a negative slope and it is very unusual for reduced viscosity to decrease with increasing concentrations. Although one may make several physical models, including concentration-dependent association, to explain this type of viscosity behavior, we do not know of any case where such behavior has been examined carefully. Thus, extrapolation of the line to an intrinsic viscosity of about 500 cm^3/g is not rigorously justified and may be inaccurate. When compared to a value of 3.5 cm^3/g for a globular protein or 10,000 cm^3/g for a stiff, linear DNA molecule, the intrinsic viscosity of 500 cm^3/g for the aggregation factor provides a useful indication of the structure of the AF complex. It is evident, however, that the actual value could well fall any where in the range of 300–600 cm^3/g.

The AF was structurally stable under the shear forces applied in these experiments. Repeated passage through the capillary tubing of the viscometer or forcing it through a 27 gauge syringe needle did not change the viscosity appreciably.

Sedimentation behavior of the AF complex was examined in the analytical ultracentrifuge. As expected of an extended, fibrous molecule, it formed a hypersharp boundary during sedimentation and its sedimentation rate was highly concentration dependent. The hypersharp behavior allows measurements of boundaries at concentrations as low as 0.1 mg/ml, which is much lower than is normally possible with globular proteins. The sedimentation coefficient was determined from the sedimentation rate at various concentrations by extrapolating to zero concentration. A calculated sedimentation constant corrected to H_2O at 20° of 62 S was obtained.

We believe that the AF is somewhat heterodisperse in size. Because the schlieren boundary is hypersharp, due to the concentration-dependent sedimentation of the AF molecules, such heterodispersity cannot be detected from analytical sedimentation data (Tanford, 1961). In addition, the sedimentation rate of the AF varied as much as 7 S from preparation to preparation suggesting that the AF complexes were not identical in size in all preparations. The reason for this variation, which could be due to preparation technique, physiological condition of the sponge or other parameters, is not known. Its molecular basis seems to lie in the number and length of arms (Humphreys *et al.*, in preparation). The sedimentation of the AF as a well-defined band on a glycerol gradient indicates that the variation within a preparation is not extensive (Henkart *et al.*, 1973).

The AF is highly charged and requires Ca^{2+} for stability (Humphreys, 1967; Henkart *et al.*, 1973), suggesting the sedimentation of the AF might be highly sensitive to either the ionic strength or the Ca^{2+} concentration. This is not the case since AF in either CaCMF diluted 10-fold or in CMF with 10^{-4} rather than 10^{-3} M Ca^{2+} sedimented at the same rate as AF in CMF.

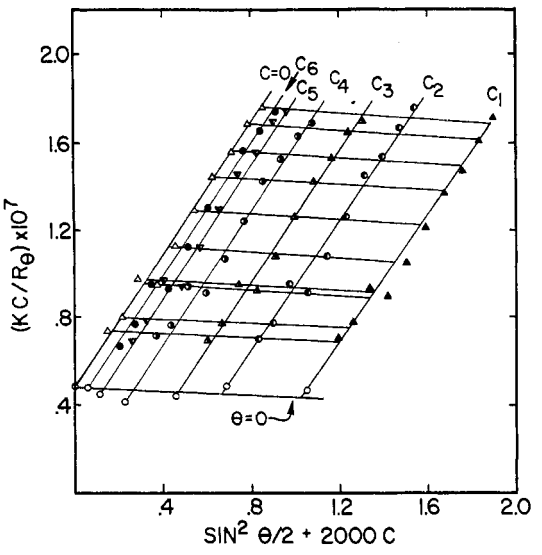

FIGURE 2: Light-scattering analysis of AF. Results are plotted according to Zimm (1948) as a function of both angle and concentration. Concentrations are 0.52, 0.34, 0.23, 0.11, 0.06, and 0.03 mg per ml for c_1–c_6, respectively. Angles in 10° increments from 135° (top) to 45° (bottom). Extrapolation to zero concentration (△) or to zero angle (○) made on best fit lines through each set of points, constant angle or concentration, as shown.

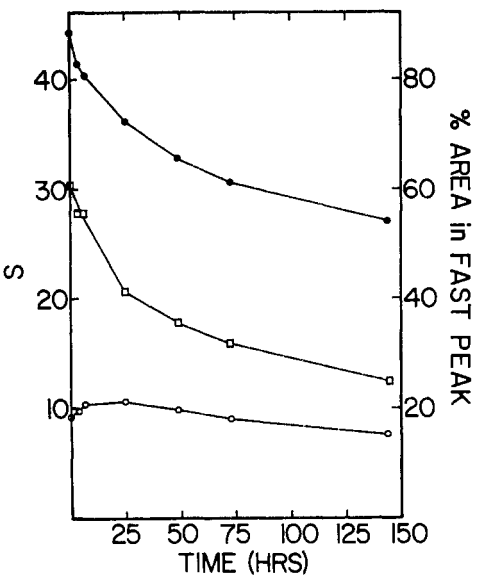

FIGURE 3: Sedimentation of AF at various times after EDTA treatment. (●,○) Sedimentation coefficients at 20° of respectively fast and slow components of EDTA-treated factor at a concentration of 1.1 mg/ml. (□) Per cent of total material under the fast peak. Area measurements, by trapezoidal approximation, were made from tracings of schlieren patterns on microcomparator. Zero time represents sedimentation immediately following treatment.

Light scattering provides direct estimates of the molecular weight and the radius of gyration of large macromolecules (Tanford, 1961). Comparison of the radius of gyration in solution with electron microscope pictures of the molecules should be especially useful. A Zimm (1948) plot of the data from a light-scattering experiment is presented in Figure 2. From the intercept on the ordinate the molecular weight is calculated to be 21 × 10^6 daltons. From the extrapolation to zero concentration, a radius of gyration of 800 Å is obtained. There is more scatter in the data than one would like, especially in the $\theta = 0$ extrapolation. From our data we believe that these estimates have an accuracy of ±20%, which is, as noted above, probably within the actual size variation from preparation to preparation. This large radius of gyration indicates that the complex in solution would have to be extended much as it appears in the electron micrographs (Henkart *et al.*, 1972; Humphreys *et al.*, in preparation).

Ca^{2+} Binding. Ca^{2+} is necessary for the stability as well as the activity of the AF. The reaction of Ca^{2+} with AF must be involved in the mechanism of action and the structure of the factor. Equilibrium dialysis was used to determine the binding of Ca^{2+} to AF. Small dialysis cells were constructed and tested. With ^{45}Ca dialyzed against CMF, equilibrium was reached within 6 hr. By employing the bag binding method of Hughes and Klotz (1956), aliquots from each side of the chamber could be recovered and counted for determination of the ion binding. Between 10^{-5} and 10^{-3} there appeared to be on the order of 1000–1300 Ca^{2+} binding sites/factor molecule of 20 × 10^6 daltons. At Ca^{2+} concentrations above 10^{-3} M another class of apparently weaker binding sites numbering several more thousand became evident. The nature of the two distinct groups of binding sites is unknown but may be related to the requirement of low Ca^{2+} concentrations for stabilization of the AF complex and of higher Ca^{2+} concentrations for the AF to bind cells together.

Organization of the AF Complex. The AF is a large, extended structure. It seems most likely that it is a complex composed of a number of subunit macromolecules. A number of standard methods for dissociating macromolecular aggregates without breaking covalent bonds were examined. Urea, sodium dodecyl sulfate, or mercaptoethanol had little effect as long as Ca^{2+} was present. EDTA, which causes an immediate and irreversible inactivation of AF (Humphreys, 1967), caused some of the factor to break down into smaller pieces and addition of urea, sodium dodecyl sulfate, or mercaptoethanol did not further dissociate the larger pieces. The dissociation of the factor complex in EDTA was examined in detail. The specific viscosity of a solution decreased 25–35% immediately after EDTA treatment (Figure 1) and continued to decrease still further with time. The single hypersharp boundary formed during sedimentation of native AF had become two peaks neither of which was hypersharp. At concentrations where the untreated factor sedimented at 41 S, there was a larger species sedimenting at approximately 44 S and a smaller species at 9 S immediately after the addition of EDTA. With time the size of the large species decreased and the amount of material in the 9–10S species increased (Figure 3). Complete dissociation of the larger species into the 9–10S component was never obtained.

The two components of EDTA-treated AF were separated by a Sepharose 2B column. Samples of AF were chromatographed on the 2B column at various times after EDTA treatment. The elution patterns confirmed the sedimentation studies (Figure 4); the large component decreased as the small one appeared. The separated fractions were removed from the column and their sedimentation behavior was analyzed. Their sedimentation velocities were identical with the components in the unchromatographed material at the same time after EDTA treatment. The molecular weight of the 9–10S component was measured by the high-speed method of sedimentation equilibrium and was 2 × 10^5 daltons. Both peaks have protein to sugar ratios equivalent to native factor, implying that they have similar chemical composition. The treated AF was centrifuged on a CsCl equilibrium density gradient. The pattern showed a single peak at a density of 1.48

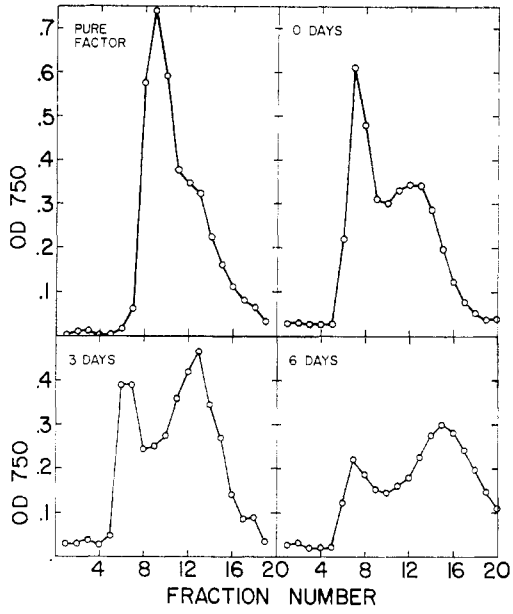

FIGURE 4: Gel filtration chromatography of EDTA-treated AF. A Sepharose 2B column (0.9 × 18 cm) was eluted with CaCMF plus 5 mM EDTA. Protein (Lowry *et al.*, 1951) was measured on the fractions. Pure factor was chromatographed first. EDTA was then added to an aliquot which was immediately placed on the column. AF plus EDTA were stored in the cold (4°), and aliquots were run again at the indicated times.

g/cm^3, very close to the density measured previously for the native factor (Henkart *et al.*, 1973). The EDTA is apparently breaking the AF complex down into glycoprotein subunit molecules plus a core.

These results suggest that the subunits may consist of glycoproteins with oligosaccharides or polysaccharides attached to a protein backbone. If the protein portions of the subunits are degraded enzymatically, saccharides should be released intact as individual chains. AF was digested exhaustively with the proteolytic enzymes, papain and Pronase, as described in the procedures section and chromatographed on Bio-Gel P-300 to obtain some idea of the size of the polysaccharide side chain (Figure 5). The polysaccharide containing digestion products, represented by the hexose and uronic acid curves run as two peaks, an excluded peak in fractions 6–10 and a partially retarded peak in fractions 13–19. The two peaks represent a major portion of the total carbohydrate added to the column. Most of the protein, representing the proteolytic enzymes as well as the protein of the AF, is completely retarded in fractions 20–30. However, a small peak of protein is coincident with the excluded fraction of polysaccharide and probably represents incomplete degradation of the AF. If these excluded fractions are redigested with Pronase and rechromatographed, further degradation is achieved and some of the polysaccharide now appears in the area of the partially retarded polysaccharide in fractions 13–19 of Figure 5.

Discussion

The AF viewed with the electron microscope (Henkart *et al.*, 1973; Humphreys *et al.*, in preparation) shows a unique sunburst configuration, with an inner circle of 800-Å diameter and 10–14 arms about 1000 Å long. Because this unusual structure could have been produced during preparation for microscopy, the properties of the molecule in solution were studied for comparison with the electron microscope image.

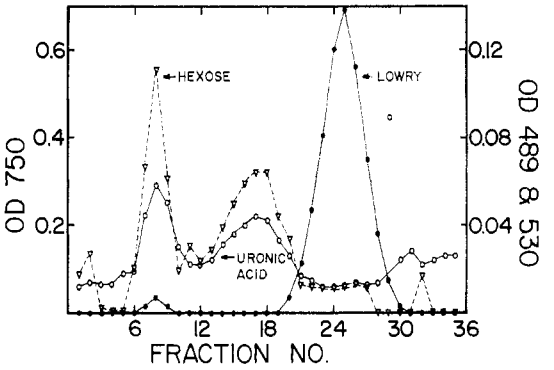

FIGURE 5: Gel filtration of papain- and Pronase-digested AF. The digest was eluted on a Bio-Gel P300 column (0.9 × 30 cm). Fractions were assayed for protein (●) (Lowry *et al.*, 1951), hexose (▽) (Dubois *et al.*, 1956), and uronic acid (○) (Gregory, 1960).

The two hydrodynamic methods used, viscometry and centrifugation, both indicate that the AF is a highly asymmetric structure. Its configuration could be compact and rigid, like an ellipsoid of revolution, open and flexible like a random coil or anything intermediate. One must look further into the data to obtain evidence for a more specific structure.

The numerical results of the hydrodynamic measurements can give an estimate for the molecular weight of the factor. Using Mandelkern and Flory's (1952) equation for a random coil, the molecular weight is calculated as 21 × 10^6 daltons. Scheraga and Mandelkern's (1953) equation for ellipsoids of revolution yields a molecular weight more than one order of magnitude higher. The former value is in excellent agreement with the molecular weight obtained from light-scattering results, and with the estimate from the electron micrographs (Henkart *et al.*, 1972). This agreement in molecular weight calculation based on the random-coil approximation of the structure supports the idea that the open fibrous structure proposed from the electron micrographs is an accurate representation of the molecule in solution.

It is difficult to interpret the light-scattering radius of gyration in terms of a specific structural model (Van Holde, 1971), but given the arrangements of fibers as visualized in the electron microscope and assuming the fibers approximate a random coil, this large radius of gyration indicates that the structure in solution is quite extended. We believe it probably appears as a three-dimensional sunburst with arms radiating to a diameter of 1600 Å or more.

The binding of Ca^{2+} ions to the factor indicates numerous binding sites. Using a molecular weight of 20 × 10^6 daltons, there is one strongly bound Ca^{2+} ion/20,000 daltons in the AF and several more which react above 10^{-3} M Ca^{2+} concentration. The relationship of the subunit structure to the number of Ca^{2+} binding sites is not clear. The polysaccharide chains alone are probably larger than 20,000 daltons and thus more than one Ca^{2+} ion must be bound per subunit.

EDTA causes the native factor to dissociate into two species, a 9–10S subunit and a core. These subunits appear to come from the arms; the circle does not break down in EDTA, but number and length of arms decreases (Humphreys *et al.*, in preparation). Assuming the length of the arms in the electron micrographs is a measure of their relative mass, it would take about 5–8 subunits of 200,000 daltons to make each arm. The subunits of the undissociated core, containing the circle, remaining after EDTA treatment, have not been examined. The core represents about 25% of the complex which is slightly more than the 15% one would predict from the con-

tour length of the circle assuming it had the same mass per length as the arms.

The studies on the polysaccharide side chains, although incomplete, show that they are probably fairly large. A theoretical calculation based on the elution properties of polyacrylamide gel columns and the elution properties of carbohydrates relative to protein, leads to an estimate of 20,000–40,000 daltons for the molecular weight of the polysaccharide unit. If a subunit is 2×10^5 daltons and is half polysaccharide, there would be only three or four polysaccharide molecules per subunit. In summary, these results suggest that the aggregation factor is a 20×10^6 dalton complex of smaller glycoprotein molecules arranged in fibers with an open, sunburst structure. The relationship of these physical properties of the factor and its various parts to its function as a material which makes cells aggregate remains to be elucidated.

References

Chervenka, C. H. (1970), A Manual of Methods for the Analytical Ultracentrifuge, Palo Alto, Calif., Beckman Instruments.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Gregory, J. D. (1960), *Arch. Biochem. Biophys. 89*, 157.

Henkart, P., Humphreys, S., and Humphreys, T. (1973), *Biochemistry 12*, 3045.

Hughes, T. R., and Klotz, I. M. (1956). *Methods Biochem. Anal. 3*, 265.

Humphreys, T. (1963), *Develop. Biol. 8*, 27.

Humphreys, T. (1967), *in* The Specificity of Cell Surfaces, Warren, L., and Davis, B., Ed., Englewood Cliffs, N. J., Prentice Hall, p 195.

Lowry, D. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mandelkern, L., and Flory, P. J. (1952), *J. Chem. Phys. 20*, 212.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Amer. Chem. Soc. 75*, 179.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley.

Tomimatsu, V., and Palmer, K. J. (1963), *J. Chem. Phys. 67*, 1720.

Van Holde, K. E. (1967), *Fractions 1*, 1.

Van Holde, K. E. (1971), Physical Biochemistry, Englewood Cliffs, N. J., Prentice Hall.

Zimm, B. H. (1948), *J. Chem. Phys. 16*, 1099.

Detection of Two Restriction Endonuclease Activities in *Haemophilus parainfluenzae* Using Analytical Agarose–Ethidium Bromide Electrophoresis†

Phillip A. Sharp,* Bill Sugden, and Joe Sambrook

ABSTRACT: A rapid assay for restriction enzymes has been developed using electrophoresis of DNA through 1.4% agarose gels in the presence of 0.5 μg/ml of ethidium bromide. The method eliminates lengthy staining and destaining procedures and resolves species of DNA which are less than 7×10^6 daltons. As little as 0.05 μg of DNA can easily be detected by direct examination of the gels in ultraviolet light. Using this technique, we have identified two different restricting activities in extracts of *Haemophilus parainfluenzae*. The two activities have different chromatographic properties on phosphocellulose and Bio-Gel A-0.5m, and they attack SV40 DNA at different sites. One activity (*Hpa* II) cleaves SV40 DNA at a single position situated 0.38 fractional genome length from the insertion point of SV40 sequences into the adenovirus SV40 hybrid $Ad2^{++}ND_1$. The other activity (*Hpa* I) cleaves SV40 DNA at three sites which appear to coincide with 3 of the 11 cleavage points attacked by a restriction system isolated from *H. influenzae* strain *Rd*.

Restriction enzymes have been isolated from a variety of strains of *Escherichia coli* and from various species of *Haemophilus* (see review by Meselson *et al.*, 1972; Yoshimori, 1971; Gromkova and Goodgal, 1972; Sack and Nathans, 1973). All of them are highly specific endonucleases which produce double-strand cleavages of native unmodified DNA. At least three of these enzymes (*Haemophilus influenzae* (Kelly and Smith, 1970), *E. coli* $R \cdot R_I$ (Hedgpeth *et al.*, 1972; Mertz and Davis, 1972), and *E. coli* $R \cdot R_{II}$) (H. W. Boyer, personal communication) attack base sequences that possess twofold rotational symmetry about an axis perpendicular to the axis of the DNA duplex; in other words, palindromic sequences of the type

$$\begin{array}{cccccc} A & B & C & C' & B' & A' \\ A' & B' & C' & C & B & A \end{array}$$

Because different enzymes attack different palindromes, each of them generates a characteristic set of cleavage products when reacted with DNA. For any particular enzyme the number of fragments obtained is a measure of the number of palindromic sites in the DNA specific to the enzyme, and the size of the fragments reflects the distribution of the sites along the DNA.

The two principal methods which have been used to analyze the fragments of DNA produced by restriction enzymes are velocity sedimentation and electrophoresis through poly-

† From Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724. *Received April 13, 1973.* Supported by Grant CA11432 from the National Cancer Institute, U. S. Public Health Service.