Characterization of Sponge Aggregation Factor. A Unique Proteoglycan Complex†

Pierre Henkart,‡ Susie Humphreys, and Tom Humphreys*

ABSTRACT: The aggregation factor from the marine sponge *Microciona parthena* has been purified and characterized chemically. It is a large, acidic proteoglycan complex of several million daltons. It sediments as a single component at about 70 S, bands at a density of 1.46 g cm^3 in CsCl, and behaves as a single, highly negative component during electrophoresis. Chemical analysis shows it is 47% amino acids and 49% sugars. The amino acid composition is not unusual except for a high amount of aspartic and glutamic acid residues. The major sugars are galactose, mannose, uronic acid, glucosamine, and galactosamine. Sulfates may be present in minor amounts. Examination with the electron microscope reveals that the complex is fibrous and that the fibers are arranged in a unique sunburst configuration with an inner circle and radiating arms.

Cellular recognition reactions mediated by cell surface molecules seem to play major roles in such diverse biological processes as morphogenesis, recognition of foreign antigens, and contact inhibition of growth. Biochemical approaches to these reactions have been rather difficult because little is known concerning the nature and function of the cell membrane and cell surface. We have studied the biochemistry of one such reaction, the species specific reaggregation of dissociated marine sponge cells. When sponges are dissociated by squeezing through a fine mesh cloth, the single cells produced will adhere to each other to form aggregates and in some cases complete functional sponges (Wilson, 1907). In a number of cases the aggregation process was found to be species specific when cells from sponges with natural pigments of contrasting colors were mixed (Wilson, 1910; Humphreys, 1970).

A molecular examination of this species specific aggregation behavior of marine sponge cells was begun with the isolation of an aggregation promoting factor from these cells (Humphreys, 1963). The factor was identified as a requirement for the aggregation of sponge cells which had been dissociated in Ca^{2+}- and Mg^{2+}-free sea water and then returned to normal sea water. The factor had been removed from the cells while they were being dissociated in the divalent cation-free sea water. Aggregation factors from different species reflected the same species specificity as the aggregation reactions (Humphreys, 1963, 1970).

A semiquantitative assay was developed for the factor (Humphreys, 1963) and has been used for a number of preliminary studies of the biochemical properties of the factor (Humphreys, 1965, 1967; Margoliash *et al.*, 1965; Gasic and Galanti, 1966; MacLennan and Dodd, 1967; MacLennan, 1970). In this paper we describe the purification and chemical characterization of the aggregation factor from the sponge *Microciona parthena*.

Experimental Section

Buffers. MBL formula artificial sea water (MBL)[1] and Ca^{2+}- and Mg^{2+}-free sea water (CMF) were prepared as previously described (Humphreys, 1963). Aggregation factor was stored in CMF with 1 mM $CaCl_2$ added (CaCMF).

Sponges. Sponges of various species were collected from the LaJolla, Calif., area and kept in running sea water for periods of up to 1 month before use. Aggregation factor was prepared from the encrusting sponge *Microciona parthena* (de Laubenfels, 1932).

Dissociation and Extraction of Aggregation Factor. Sponges were freed from foreign organisms and broken into pieces 5–10 mm in diameter. These pieces were blotted lightly and weighed. All subsequent operations were carried out at 5°. The sponge tissue was washed four times in CMF over a period of 20 min and dissociated by squeezing through nylon mesh (Nitex 130, Tobler, Ernst & Traber, Elmsford, N. Y.) into CMF in a beaker. The volume of CMF in milliliters was equal to five times the weight of the sponge tissue in grams. Generally 50–100 g of sponge were dissociated at a time. The skeletal matrix was discarded and the cell suspension was placed in an erlenmeyer or fernback flask on a gyratory shaker for 15 hr. The cells and the supernatant containing the dissolved factor were separated by centrifugation at 170*g* for 1.5 min. The pellet of cells was resuspended in the original volume of CMF and placed back on the shaker for 3 more hr of washing. They were then repelleted, suspended in MBL, and stored at 5° for up to several days for use in the factor assay. The supernatant which still contains many small cells was clarified by centrifugation at 27,000*g* for 0.5 hr. This supernatant, the crude factor extract, was light amber. It was decanted, adjusted to 1 mM in $CaCl_2$, and stored at 5° for periods up to several weeks.

Assay of Aggregation Factor. The aggregation factor was assayed by the serial dilution technique of Humphreys (1963) reduced to a total volume of 200 μl in 1-dram shell vials by using a modified gyratory shaker with a 2-in. radius of gyration (Henkart and Humphreys, 1970). For the assay 100 μl of a serial twofold dilution of aggregation factor in CaCMF was

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

[1] Abbreviations used are: MBL, Marine Biological Laboratory formula artificial sea water; CMF, Ca^{2+}- and Mg^{2+}-free artificial sea water; CaCMF, CMF with 10^{-3} M $CaCl_2$ added.

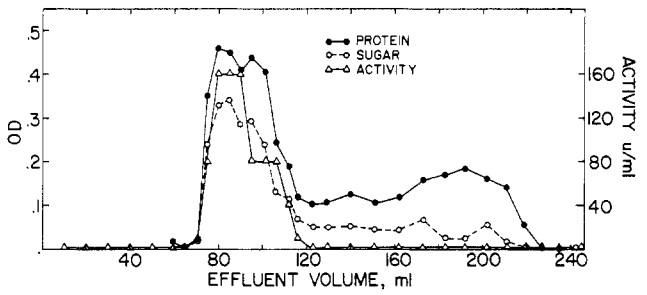

FIGURE 1: Sepharose 2B gel filtration of *M. parthena* aggregation factor. A solution of aggregation factor (4.0 ml) which had been partially purified by differential sedimentation was applied to a 2 × 70 cm column of Sepharose 2B and eluted with CaCMF at 15 ml/hr. Aliquots were removed for analysis of protein (Lowry *et al.*, 1951), OD_{750}), sugar (Dubois *et al.*, 1956), OD_{489}), and activity.

prepared in a series of vials. Then 100 μl of a suspension of 2 × 10^7 cells/ml in MBL was added to each vial and they were shaken at 70 rpm for 15 min. With *M. parthena* this assay was done at room temperature as these cells had negligible tendency to reaggregate at room temperature in the absence of aggregation factor. After shaking for 15 min or more the vials were examined with a dissecting microscope for the presence of aggregation relative to the control containing no factor. One unit per milliliter of activity was defined as the concentration which showed marginally enhanced aggregation.

Purification of Aggregation Factor. The differential centrifugation described for *Microciona prolifera* (Humphreys, 1965) was employed as the first purification step. The factor extract was centrifuged at 55,000*g* for 0.5 hr and the supernatant was then centrifuged at 100,000*g* for 90 min. The small amber pellet from the second centrifugation contained the activity and was dissolved in a small amount of CaCMF by stirring with a Vortex mixer. This was chromatographed on a column of Sepharose 2B (2 × 70 cm or 4 × 60 cm). The column was equilibrated and eluted with CaCMF at 15 ml/hr, at 5°. Figure 1 shows a typical result. Tubes containing 16 units/ml or greater were combined and centrifuged at 100,000*g* as before to concentrate the purified factor. The pellet, which was now colorless, was redissolved in a small volume of CaCMF. Factor purified in this way was used for all subsequent experiments. Between 10 and 20 mg of purified aggregation factor was obtained from 100 g of sponge tissue.

Chemical Analyses. Neutral hexoses were determined by the phenol–sulfuric acid method of Dubois *et al.* (1956), uronic acids were determined by the method of Gregory (1960), and methyl pentoses were analyzed by the procedure of Gibbons (1955).

The method of Lowry *et al.* (1951) was used to determine protein in column chromatography. Amino acid analyses were performed after hydrolysis for 24 hr in 6 N HCl at 110° in evacuated tubules. A Beckman amino acid analyzer was used with the chromatographic system of Spackman *et al.* (1958). Serine and threonine values were not corrected for destruction during acid hydrolysis and tryptophan was not determined. The content of cysteine plus cystine was determined by oxidation with performic acid by the method of Hirs (1967). Amino sugars were determined on the amino acid analyzer by the method of Walborg *et al.* (1963) after hydrolysis in 4 N HCl at 100° for 4 hr.

Analyses for dry weight, ash, and sulfur were performed by Elek Microanalytical Laboratories, Harbor City, Calif.

Neutral sugars were determined by paper chromatography after hydrolysis of 0.5-mg aggregation factor in 2 ml of 1 N H_2SO_4 at 100° for 4 hr. The hydrolysate was neutralized with $BaCO_3$ and evaporated to dryness, and the residue was redissolved in 50 μl of water. Aliquots of 5–50 μl were spotted on Whatman 3MM filter paper sheets along with standards of glucose, galactose, mannose, fucose, rhamnose, xylose, arabinose, and glucosamine. Descending chromatography was carried out for 53 hr using a buffer system of 1-butanol–ethanol–water (10:1:2) (Spiro, 1960). The paper was dried and stained with silver nitrate (Smith, 1960). This chromatographic system separates distinctly all the sugars used as standards except arabinose and mannose, which migrate together. These can be distinguished by the speed of appearance of the stain.

Zonal electrophoresis in a glycerol gradient was carried out using a vertical 1.5 × 25 cm glass tube with a polyethylene powder funnel fitted on top. The gradient was supported in the tube by a piece of dialysis membrane secured across the bottom end of the glass tube with a rubber band. The bottom end of the tube was immersed in the anode chamber, which consisted of a 150-ml beaker containing 50% glycerol–0.20 M NaCl–2 mM $CaCl_2$–0.20 M Tris-chloride (pH 8.0). A linear gradient of 20 ml of 10–50% glycerol in 0.10 M Tris-chloride–1 mM $CaCl_2$ (pH 8.0) was formed in the glass tube. The sample, 0.50 ml of a purified aggregation factor preparation containing 5% glycerol, was layered on top of the gradient, and the cathode buffer, anode buffer without glycerol (100 ml), was layered on top of the sample. The cathode buffer compartment was formed by the funnel on top of the glass tube.

Electrophoresis was carried out for varying lengths of time (2–7 hr) at 5° with a potential of 120 V. After pipetting off the cathode buffer, fractions were collected from the bottom of the gradient by a pump *via* a narrow stainless steel tube inserted through the gradient. Each fraction was dialyzed against CaCMF and then analyzed for protein, sugar, and activity.

Results

Activity and Specificity of M. parthena Aggregation Factor. The original extracts of aggregation factor from *M. parthena* have an activity of 16–32 units/ml on chemically dissociated homologous cells. This factor shows species specificity. It does not agglutinate chemically dissociated cells from several other sponge species we have tested, *e.g.*, *Haliclona* sp, *Hymeniacidon sinapium.* However, we have found sponges whose cells are agglutinated by *M. parthena* factor. For example, *Microciona prolifera* cells respond fully to the *M. parthena* factor and *Styletta estrella* cells respond, but with a lower apparent activity than the homologous cells.

Purification. The aggregation factor of *M. parthena* was purified by differential centrifugation and gel filtration on Sepharose 2B. As shown in Figure 1 the activity was found in the excluded and slightly included volume of the gel. This initial peak of material was concentrated by centrifugation to give the preparation of purified factor. The extent of purification is summarized in Table I. Because of the semiquantitative nature of the activity assay, both total activity and specific activity measurements have a factor of two uncertainty and give only rough estimates. However, the data shown are typical and indicate that the purification procedure results in a roughly 10-fold purification over the original extract with a high recovery of total activity. The cells remain intact during the dissociation procedure, and the original extract contains only soluble extracellular material and compounds released from the cell surface during treatment with CMF. The total

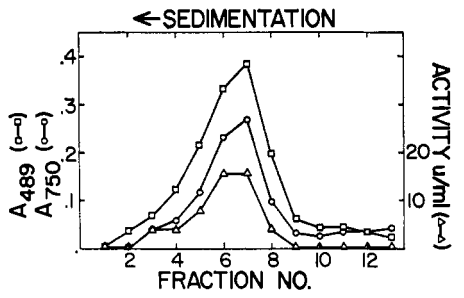

FIGURE 2: Zonal sedimentation of purified aggregation factor in a glycerol gradient. Purified aggregation factor (0.5 ml, 4 mg/ml) was layered on a 13-ml linear gradient, 10–40% glycerol in CaCMF, and centrifuged 3.5 hr at 25,000 rpm in a Beckman SW-27 rotor at 22°. Each fraction was dialyzed *vs.* CaCMF, and aliquots were removed for analysis of protein (Lowry *et al.*, 1951) (A_{750}), sugar (Dubois *et al.*, 1956), (A_{489}), and activity.

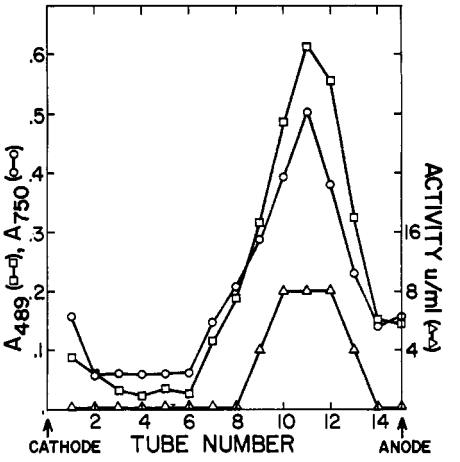

FIGURE 3: Zonal electrophoresis of purified aggregation factor in a glycerol gradient. Purified factor (2 mg in 0.5 ml) was applied at the position of fraction 2. Electrophoresis was carried out at 5° for 4 hr at 120 V. After dialysis, each fraction was analyzed for protein (A_{750}), sugar (A_{480}), and activity.

purification of factor from the whole sponge is several 1000-fold.

A number of other purification techniques were also attempted. During ion-exchange chromatography, the activity failed to bind to any of the cation-exchange resins, while it was irreversibly bound to anion exchangers, such as DEAE and Ecteola cellulose, hydroxylapatite, or methylated albumin kieselguhr. Since the aggregation factor appeared similar in some respects to mucopolysaccharides, precipitation with cetylpyridinium chloride was also carried out. Activity was lost and a precipitate formed at 0.1% detergent concentration, but neither activity nor the precipitate could be solubilized by using high concentration of salt.

Homogeneity. Because of the large size and fibrous nature (see later and accompanying paper; Cauldwell *et al.*, 1973) of the aggregation factor, standard analytical homogeneity criteria used for proteins could not be applied to this preparation. However, several preparative techniques applied to the factor in attempts to purify it further suggest that the factor is homogeneous. We have fractionated the factor by zonal sedimentation in a glycerol gradient, zonal electrophoresis in a glycerol gradient, and banding in a CsCl density gradient. While these separation methods do not have the resolving power of such techniques as disc gel electrophoresis and analytical ultracentrifugation, we have quantitatively measured protein, sugar and activity in the fractions obtained by these techniques and within experimental error these quantities are proportional across the peaks. Since these three methods separate macromolecules on the basis of their size, charge density, and buoyant density, respectively, and since all three separations indicate a single predominate component, we have concluded that our preparations are substantially free of impurities. Some heterodispersity of size of active factor molecules may be inferred from the spread of the peaks in Figure 1 and Figure 2, however, and the preparations are thus not homogeneous in the traditional sense.

In the glycerol gradient the aggregation factor sediments as a single peak at a rate estimated to be 70 S by comparison to markers in companion gradients (Figure 2). Electrophoresis of the factor in a glycerol gradient (Figure 3) revealed that the factor migrates in a single peak as a highly negatively charged molecule. The purified factor preparation bands in a single sharp peak in CsCl gradient at a density of 1.46 g/cm^3 (Figure 4).

Chemical Composition. The results of chemical analysis of purified aggregation factor are summarized in Table II. The aggregation factor contains protein and polysaccharide in about equal parts. A molecule with this ratio of protein to polysaccharide would have a buoyant density of about that observed, 1.46 g/cm^3. The sulfur analysis indicates that there are few, if any, sulfated sugar chains. While the total sulfur content was 1.51%, the sulfur in the protein accounts for at least 0.5%, leaving a maximum of 1.0% sulfur due to sulfate groups on the carbohydrate. This corresponds to about one sulfate per ten monosaccharides.

TABLE I: Purification of *Microciona parthena* Aggregation Factor.

Stage	Sp Act. (u/mg) Hexose	Sp Act. (u/mg) Protein	Total Units from 100 g of Sponge
Dissociation supernatant	256	40	8000
Differential centrifugation	670	270	4000
Sepharose column	840	420	5000

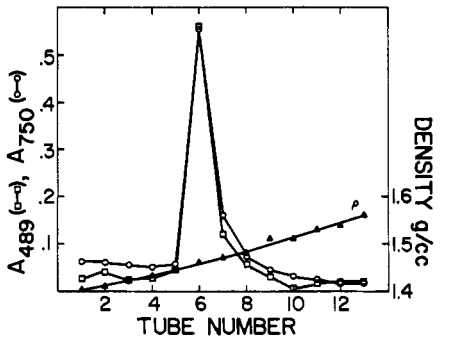

FIGURE 4: Isopycnic centrifugation of purified aggregation factor in a CsCl gradient. A sample of 1 mg of aggregation factor in 1.60 ml of CaCMF was layered over 7.3 ml of a 50% (w/v) CsCl solution in CaCMF and centrifuged at 35,000 rpm for 24 hr at 22° in a Beckman Ti50 rotor. Fractions of 0.70 ml were collected from the top of the tube with a pipet, and each fraction was dialyzed *vs.* CaCMF and analyzed for protein (A_{750}), sugar (A_{489}), and activity. All activity was recovered in the fraction containing the peak of protein and sugar.

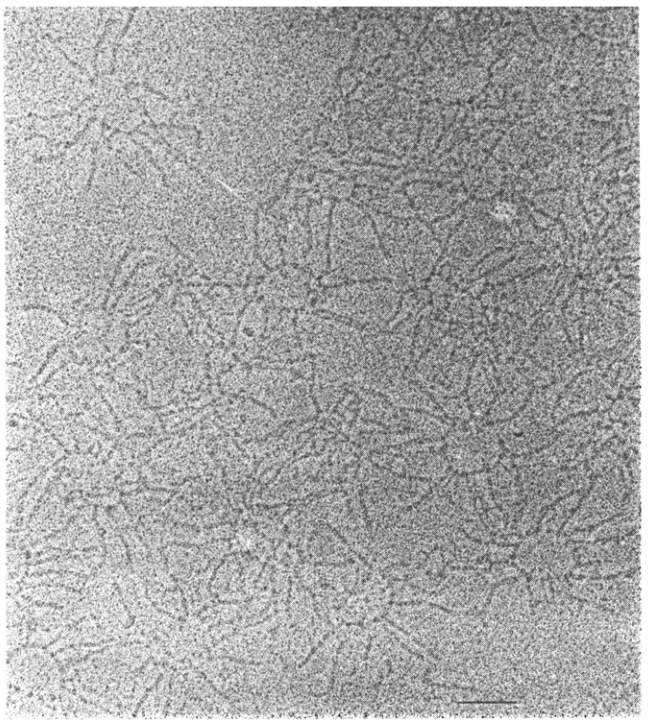

FIGURE 5: Uranium shadowed preparation of aggregation factor dried on a carbon film. Marker is 0.1 μm.

The polysaccharide appears to be a heteropolysaccharide containing 20% amino sugars, 20% uronic acid, and the other 60% neutral sugars. Paper chromatography of neutral sugars in hydrolysates showed one darkly staining spot, which had the R_F of galactose; most likely galactose is the predominant neutral sugar. Three lightly staining spots were also seen. One had the R_F and staining properties of mannose; another spot ran slightly behind fucose, but could not be assigned to any sugar; and a third spot corresponded to the amino sugars. It is estimated that the two minor neutral sugar components amounted to only 10–20% of the galactose present.

TABLE II: Chemical Composition of *Microciona parthena* Aggregation Factor.

Component	% by Wt[a]
Protein[b]	47.1
Neutral hexose[c]	26.0
Uronic acid, sodium salt	10.4
Galactosamine	5.8
Glucosamine	5.5
Methylpentose	1.2
Sulfur	1.5
Phosphate	<0.3
Sialic acid	<0.1
	96.0[d]

[a] Calculated as a per cent of the dry weight minus ash. As analyzed, the sample after exhaustive dialysis *vs.* distilled water contained 9.21% ash. [b] Determined by amino acid analysis. This value agrees to within 3% of that estimated by the Lowry method. [c] Determined by the phenol–sulfuric method and corrected for the uronic acid contribution, using glucuronic acid as a standard. [d] Not including sulfur.

TABLE III: Amino Acid Composition of *Microciona parthena* Aggregation Factor.

Amino Acid	Mol of Amino Acid/ 10^5 g of Protein
Lysine	9.2
Histidine	4.8
Arginine	35.1
Cysteine	25.5
Aspartic acid	147
Threonine	77.7
Serine	56.7
Glutamic acid	125
Proline	57.5
Glycine	85.6
Alanine	61.9
Valine	94.9
Methionine	11.0
Isoleucine	64.2
Leucine	78.8
Tyrosine	21.8
Phenylalanine	57.8

The amino acid analysis of aggregation factor is shown in Table III. All peaks found on the chromatograms corresponded to normal amino acids. The most notable aspect of this amino acid composition is the high proportion of acidic amino acids (some of which may be present as amides), and low amount of basic amino acids. This amino acid composition along with the 10% uronic acid content accounts for the high negative charge of the molecule observed in electrophoresis. Assays for phosphate and lipid failed to detect any in the purified factor preparations. This would seem to rule out the presence of membrane fragments. The buoyant density of the active factor is very close to that one would predict for a molecule containing about equal amounts of protein and polysaccharide, indicating that little lipid with its rather low buoyant density could be present.

Electron Microscopy. Preparations dried on a grid and shadowed with uranium were examined with the electron microscope. Large fibrous complexes were apparent (Figure 5). They were composed of 45-Å diameter fibers arranged in a 800-Å diameter circle with 11–15 arms 1100 Å in length radiating from the circle. The molecular weight of such a complex of 45-Å diameter fibers can be calculated to be 2.2×10^7 daltons using its buoyant density of 1.46 g/cm^3. This molecular weight is consistent with its sedimentation rate of 70 S in glycerol gradients and agrees well with its behavior on Sepharose 2B with an exclusion limit of 2×10^7 daltons and with calculations from analytical sedimentation and light-scattering data presented in an accompanying paper (Cauldwell *et al.*, 1973). A more complete electron microscopic examination of these complexes will be presented elsewhere (Humphreys *et al.*, in preparation).

Degradation of Factor. EDTA causes a rapid and irreversible inactivation of the aggregation factor (Humphreys, 1967). In 1 mM EDTA or 1 mM EGTA the 70 S peak slowly degrades into a very heterogeneously sedimenting mixture on a glycerol gradient. Even after long periods in EDTA there is still a substantial amount of rapidly sedimenting material and addition of 8 M urea and β-mercaptoethanol did not degrade the factor significantly more. Further studies on the EDTA-

treated aggregation factor are reported in the accompanying paper (Cauldwell *et al.*, 1973).

Treatment of the aggregation factor with 1% sodium dodecyl sulfate and subsequent sedimentation in a glycerol gradient containing 0.1% sodium dodecyl sulfate have carbohydrate and protein profiles similar to untreated factor. Biological activity was difficult to measure because of problems in removing sodium dodecyl sulfate; however, in one experiment this was accomplished and activity was recovered in the protein–sugar peak.

The intact aggregation factor did not appear to be affected by hyaluronidase. A preparation of factor in CaCMF was digested for 16 hr with 2.4 mg/ml of hyaluronidase and then layered on a glycerol gradient such as that of Figure 2; the resulting carbohydrate and activity profiles were identical to that of undigested factor.

Gel Formation. When the calcium concentration of CaCMF solution containing aggregation factor is raised to about 20 mM, a gel forms within a few minutes if the solution is gently swirled on a gyratory shaker. Gel formation is somewhat dependent on Ca^{2+} and factor concentrations, but with 25 mM Ca^{2+} all activity in solutions as dilute as 8 units/ml is quantitatively recovered in the gel when it is redissolved in CaCMF. This simple gel formation can be used to purify aggregation factor quickly from the original extract, but in some experiments factor prepared in this way showed signs of forming stable higher order aggregates. Magnesium ion shows little tendency to induce gel formation by aggregation factor. The calcium ion concentration in sea water is about 10 mM; gel formation is observed in sea water at high factor concentrations, and at concentrations of greater than 128 units/ml gelation is seen even in the 5 mM Ca^{2+} medium of our routine activity assays. Denaturation of aggregation factor activity by heat or EDTA also destroys its ability to form a gel even in high Ca^{2+} concentrations.

Discussion

The purification of the sponge aggregation factor is accomplished in a few steps and is based mainly on size and shape of the factor. However, its large size, fibrous nature, and high negative charge create difficulties with analytical homogeneity criteria and with the powerful fractionation procedures possible with chromatography. We believe the evidence presented is convincing that our preparations do not contain significant contamination of unrelated molecules without biological activity. However, because of the relatively crude techniques which had to be used, such a conclusion cannot be as satisfying as in the case of the more traditional globular protein. Indeed, active factor molecules apparently displayed some heterodispersity in size during chromatography on Sepharose and during sedimentation in glycerol gradients (see also accompanying paper, Cauldwell *et al.*, 1973).

Our results differ significantly from some work published concerning the aggregation factor from the sponge *M. prolifera* (Margoliash *et al.*, 1965). On the basis of our studies of *M. prolifera* aggregation factor (Humphreys, 1965, 1967, and unpublished data), we believe it is very similar to the *M. parthena* factor which we describe in this paper. The purification steps used by Margoliash *et al.* (1965) do not yield a pure preparation, and we believe most differences are due to such impurities. In particular we find no support for their conclusion that the active molecule is 25 Å sphere of about 22,000 molecular weight, or is made up of subunits this size (Cauldwell *et al.*, 1973).

The data in this paper clearly indicate that the active component in sponge aggregation factor is a large and unusual proteoglycan complex, rather different from others which have been described. On the basis of high uronic acid content, the large size of the carbohydrate chains (Cauldwell *et al.*, 1973), and the large size of the overall molecule, the aggregation factor fits the general description of proteoglycans. In such polysaccharides the sugar side chains contain uronic acid and are heavily sulfated. Except for heparin, no biological activity is known *in vitro* for the isolated molecules. The best studied proteoglycans are the protein–polysaccharide complexes from cartilage, which contain 10–15% protein. Recent work by Hascall and Sajdera (1969) indicates that this complex is made up of a 30 S proteoglycan subunit containing 97% of the total weight and more than 95% of the hexuronic acid, and also of a 5 S glycoprotein linker subunit which participates in the aggregation of the proteoglycan subunit. We have attempted to find evidence for a similar combination of subunits in sponge aggregation factor without success.

The striking shape of the aggregation factor molecule with its radiating arms is similar to the model for the somewhat larger first-order aggregates observed in the electron micrographs of fraction PPL5 of the cartilage proteoglycan (Rosenberg *et al.*, 1970). There are a number of specific differences, however. The short side chains on the radial arms of the PPL5 which the authors believe are 30,000 molecular weight carbohydrate side chains of the proteoglycan subunit do not appear on the arms of the sponge aggregation factor. Even preparations using the Kleinschmidt technique (Humphreys *et al.*, in preparation) show a uniformly dense fiber without side chains. The central piece in the model of the PPL5 is a core of the glycoprotein link subunit rather than a circle as in the sponge factor. The general similarity between sponge aggregation factor and cartilage proteoglycan in both shape and basic chemical composition is striking and may be significant in terms of related macromolecular activities. However, it seems likely to us that there are fundamental differences in the macromolecular organization of these two complexes.

In contrast to other proteoglycans, the aggregation factor has a biological function which can be assayed and which, because it can be easily inactivated, appears to depend on protein tertiary structure. At present there is little information available on the tertiary structure in other proteoglycans, but our results would suggest that such tertiary structure may exist and be responsible for important functional activities. The species specificity of the aggregation factor may depend on such tertiary structure. On the other hand, the extensive carbohydrate in the aggregation factor may mediate some of the specificity of the interactions. In either case, the large size of factor molecules means that they can be effective in promoting aggregation. In the case of antibodies it has been found that IgM antibodies are more effective agglutinins of red cells than IgG antibodies (Robbins *et al.*, 1965). This has been explained by the fact that the larger molecules do not have to overcome so much electrostatic free energy to bring two negatively charged cells together (Pollack *et al.*, 1965).

The demonstration that aggregation factor can form a gel in sea water and under assay conditions deserves careful attention, since a gel can physically trap cells and form an aggregate (Steinberg, 1963). Furthermore, there seems to be a loose correlation between gel formation and activity of the factor. The calcium dependence of factor activity roughly follows the calcium dependence of gel formation. In 1 mM Ca^{2+} gel formation has never been observed and aggregation factor cannot induce cell aggregation. In the 5 mM Ca^{2+} routine conditions of

the assay, gel formation occurs at high factor concentrations. Denaturation of factor activity by heat or EDTA is concomitant with a loss in gel formation ability. However, the possibility that aggregation factor activity is due to physical entrapment of cells is rigorously excluded by its species specificity (Humphreys, 1963). *M. parthena* factor can also show this specificity. Used in a standard assay system at 128 units/ml with *Hymeniacidon sinapium* cells, a gel is observed which does trap a few of the heterologous cells, but the great majority of cells remain free and single. The correlation of gel formation and activity may be interpreted as a reflection of the need for two or more factor molecules to interact in order for them to bring cells together into an aggregate.

While the simplicity of the sponge aggregation system makes it attractive for biochemical study, it is of obvious importance to know if similar aggregation factors play a role in the adhesion of the cells of higher animals. To date, no comparable factor has been isolated during dissociation of higher cells and it seems probable that the situation in higher animals is significantly more complex. Higher animal cells, even embryonic cells, cannot be easily dissociated into single viable cells without the use of enzymes such as trypsin. These enzymes may destroy intercellular molecules as well as their receptors on the cell surface. The tissue specificities shown during reaggregation of embryonic tissue imply that some specific molecular event may be operating at the cell surface (Roth and Weston, 1967; Roth, 1968). Histological techniques with the electron microscope (Rambourg and Leblond, 1967) indicate that the cells of most tissues are surrounded by carbohydrate-containing material, perhaps analogous to the aggregation factor we have described in sponges. Using the electron microscope, Overton (1969) has observed trypsin-sensitive fibrous material at the surface of reaggregating chick embryonic cells. It is possible that these specificities and materials are analogous to sponge aggregation factor.

References

Cauldwell, C. B., Henkart, P., and Humphreys, T. (1973), *Biochemistry 12*, 3051.

de Laubenfels, M. W. (1932), *U. S. Nat. Mus. Proc. 81*.

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

Gasic, G. J., and Galanti, N. L. (1966), *Science 151*, 203.

Gibbons, M. N. (1955), *Analyst 80*, 268.

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

Hascall, V. C., and Sajdera, S. W. (1969), *J. Biol. Chem. 244*, 2384.

Henkart, P., and Humphreys, T. (1970), *Exp. Cell Res. 63*, 224.

Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 59.

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

Humphreys, T. (1965), *Exp. Cell Res. 40*, 539.

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

Humphreys, T. (1970), *Nature (London) 228*, 685.

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

MacLennon, A. P. (1970), *Symp. Zool. Soc. 25*, 299.

MacLennon, A. P., and Dodd, R. Y. (1967), *J. Embryol. Exp. Morphol. 17*, 473.

Margoliash, E., Schenck, J. R., Hargie, M. P., Burokas, S., Richter, W. R., Barlow, G. H., and Moscona, A. A. (1965), *Biochem. Biophys. Res. Commun. 20*, 383.

Overton, J. (1969), *J. Cell Biol. 40*, 136.

Pollack, W., Hager, H. J., Reckel, R., Toren, D. A., and Singher, H. O. (1965), *Transfusion 5*, 158.

Rambourg, A., and Leblond, C. P. (1967), *J. Cell Biol. 32*, 27.

Robbins, J. B., Kenny, K., and Suter, E. (1965), *J. Exp. Med. 122*, 385.

Rosenberg, L., Hellman, W., and Kleinschmidt, A. K. (1970), *J. Biol. Chem. 245*, 4123.

Roth, S. (1968), *Develop. Biol. 18*, 602.

Roth, S., and Weston, J. A. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 974.

Smith, I. (1960), Chromatographic and Electrophoretic Techniques, London, Heinemann.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spiro, R. G. (1960), *J. Biol. Chem. 235*, 2860.

Steinberg, M. S. (1963), *Exp. Cell Res. 30*, 257.

Walborg, E. F., Jr., Cobb, B. F., Adams-Mayne, M., and Ward, D. N. (1963), *Anal. Biochem. 6*, 367.

Wilson, H. V. (1907), *J. Exp. Zool. 5*, 245.

Wilson, H. V. (1910), *Bull. Bur. Fisheries 30*, 1.