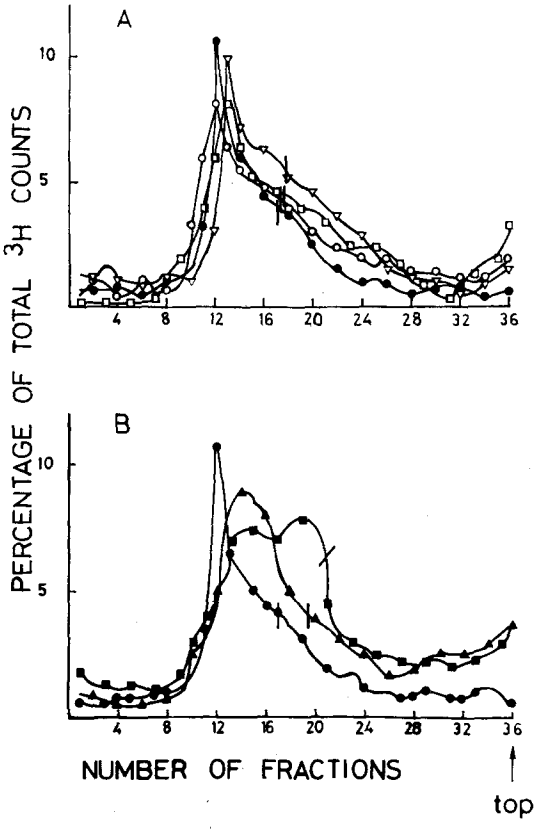

Effect of cysteine on the sedimentation profiles of DNA of *E. coli* cells in alkaline sucrose. Logarithmic phase *E. coli* K12 cells labelled in their DNA by ^{3}H-Me-thymine were incubated at 37 °C for 30 min in 'GS'-medium with and without cysteine and cysteamine. After incubation the sedimentation profiles were analyzed by the technique described by McGrath and Williams[9]. A) ●—●, untreated control; ▽—▽, treated with 0.5 m*M* cysteine; ○—○, treated with 10 m*M* cysteine; □—□, treated with 50 m*M* cysteine. Vertical bars indicate the 'S 1/2' values. B) ●—●, untreated control; ▲—▲, treated with 30 m*M* cysteamine; ■—■, treated with 50 m*M* cysteamine.

curve 3: 17.1; curve 4: 17.6. As the sedimentation pattern and the 'S 1/2' were similar at the other concentrations tested, they were not indicated in the figure.

Cysteamine used as a control at concentrations of 30 and 50 m*M* changed both the sedimentation pattern and 'S 1/2' values. These were found to be: curve 1: 17.0; curve 2: 19.5; curve 3: 21.5.

Present experiments were based on our previous results[11,12] according to which an asynchronous synthesis of macromolecules was induced by cysteine, i.e. after addition of cysteine, the net synthesis of RNA and of protein stopped immediately whereas the DNA synthesis preceeded for about 30 min. This effect of cysteine appeared at a concentration of 0.2 m*M* and it reached 100% at 10 m*M*.

It follows from this that the cysteine-treated cells are able to incorporate ^{3}H-thymine only for about 30 min. This fact becomes a source of error in experiments in which the incubation period takes 60–90 min or more. During the incubation period longer than 30 min, the untreated cells incorporate bigger quantity of radioprotective thymine due to their unaffected DNA synthesis.

The series of our experiments in which the incubation period was 30 min, showed that cysteine at concentrations of 0.5–50 m*M* did not change the sedimentation pattern of DNA, hence probably did not cause single-strand breaks of DNA in *E. coli* K12.

Zusammenfassung. Mit bekannter Methode wurde die Aktivität des Cysteins in verschiedenen Konzentrationen über das Sedimentationsprofil des DNS von *Escherichia coli* K12 Zellen geprüft und festgestellt, dass Cystein das Sedimentationsprofil des DNS nicht veränderte.

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Department of Pharmacology, University Medical School, Debrecen 12 (Hungary), 10 December 1973.

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Two Different Aggregation Principles in Reaggregation Process of Dissociated Sponge Cells (*Geodia cydonium*)

Species specific reaggregation of dissociated sponge cells seems to be restricted to a few binary combinations[1,2]. In an extensive study, John et al.[2] demonstrated that aggregation of dissociated sponge cells from different species initiates as a random process. In a later stage, a sorting out of the cells from one species occurs, uniting all homologous cells. In order to understand the basic mechanism of this observation, two different aggregation principles have to be postulated: a non-specific one, responsible for the early phase of aggregation of bi-specific cell mixtures, and another of higher specificity, causing species-specific aggregation in the late phase. In a previous paper[3] we were able to show that the aggregation process of dissociated sponge cells from the species *Geodia cydonium* Jam. occurs in 3 discrete steps, starting in an early phase with the initiation of primary aggregates, followed by a subsequent formation of secondary aggregates, and a late phase with a reconstitution of functional aquiferous systems by rearrangement in the secondary aggregates. This paper deals with the biochemical nature of the first two aggregation phases.

The primary aggregation phase. Washed cells from *Geodia cydonium*, chemically dissociated with calcium- and magnesium-free artificial sea water containing 20 m*M* EDTA and trypsin[3], reaggregate when placed into calcium- and magnesium-containing artificial sea water within 45 min, forming compact primary aggregates of

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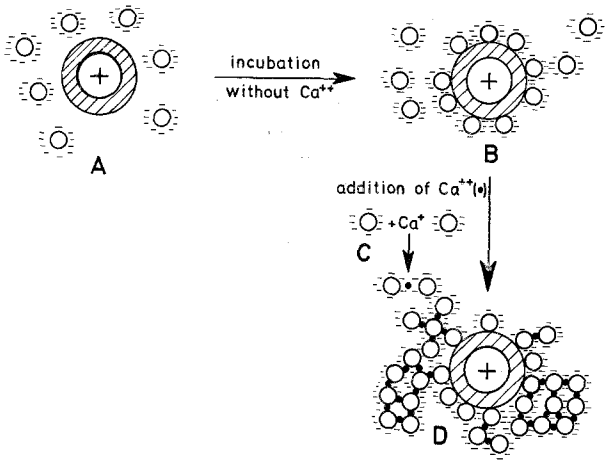

Fig. 1. Scheme of adhesion of single cells on DEAE-Sephadex (A-50) (Pharmacia) (A) in artificial sea water without and with calcium. In calcium-free sea water the single cells adhere by their negatively charged groups to the surface of the positively charged DEAE beads (B). Upon the addition of calcium ions to the incubation mixture to 20 mM, cell clumps are formed (C) by 'calcium-bridges'. Some of the clumps adhere to the cells initially sticking to DEAE bead (D); see also Figure 2.

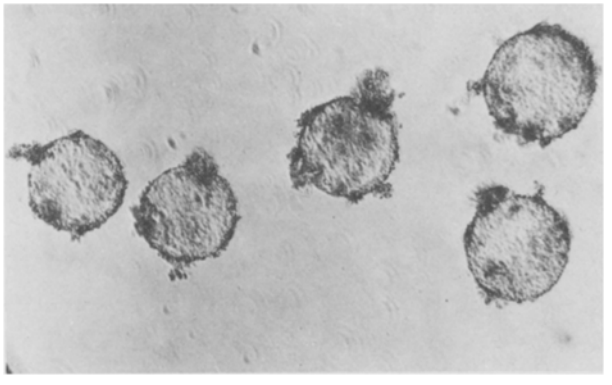

Fig. 2. Cell clumps adhering to the surface of DEAE beads. ×170.

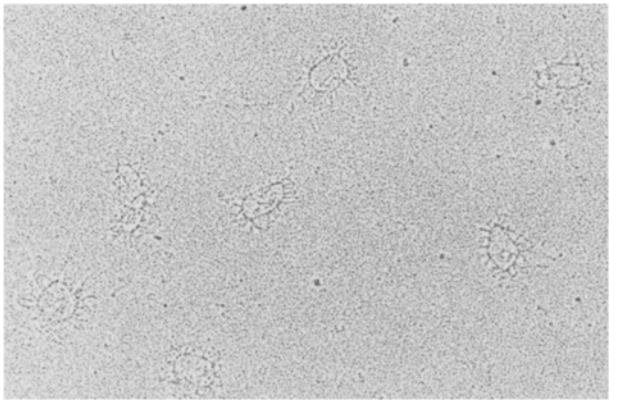

Fig. 3. Annular particles without secondary aggregation factor activity. The electron microscopic picture was taken from fraction V (Table I). Electronmicrographs were taken with a Siemens 101 (Germany) at an electronic magnification of 40,000. The molecules were mounted by the protein film procedure[10]. ×50,000.

68 μm diameter which contain 82 ± 15 cells[3]. Maximal aggregation was observed with calcium ions in a concentration of at least 18 mM, while at 3.8 mM Ca^{++} only 50% aggregation was found. Strontium and barium can replace calcium in our system, yielding 100% aggregation at concentrations above 25 mM Sr^{++} or Ba^{++}. The other divalent cations, magnesium and beryllium, did not support aggregation under the conditions of aggregation used in our studies. The important role of monovalent anionic groups on the plasmalemma surface could also be demonstrated by adhesion experiments (Figures 1 and 2) using positively charged DEAE-Sephadex beads.

Change in pH exerts a marked influence on aggregation in the assay. On raising the pH from 6.5 to 8.0, for each 0.7 pH units the activity increases 10 times. The optimum for aggregation is around pH 8.0. The cause of the strong pH effect may be related to cellular contraction[4].

We attempted to determine the chemical nature of the primary aggregation factor by preincubating dissociated cells with different enzymes. Proteolytic enzymes[3] and carbohydrate-hydrolyzing enzymes (α-amylase, amyloglycosidase, cellulase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucorunidase, hemicellulase, hyaluronidase, lysozyme, naringinase and neuraminidase) in concentrations of 0.5 mg/ml were without effect.

The secondary aggregation phase. In the test system used in our studies, chemically dissociated cells of small, primary aggregates do not grow any further. Addition of the cell-free calcium- and magnesium-free supernatant, from dissociated *Goedia* tissue containing 20 mM EDTA[3], to the primary aggregates initiates the secondary aggregation if Ca^{++} is present[3]. In this phase the diameter of the aggregates, now called secondary aggregates[3], increases from 68 μm to more than 1,000 μm. The required Ca^{++} ions can be replaced by Sr^{++} or Ba^{++}. Maximal secondary aggregation can be achieved at concentrations above 20 mM. Mg^{++} and Be^{++} did not bring about secondary aggregation. The secondary aggregation process is strongly pH-dependent, with an optimum around pH 8.0. In the range of pH 6.0 to pH 8.0, a 10-fold increase for each 0.9 pH units occurs.

The factor promoting secondary aggregation has been purified by 5 steps (Table I), to 500-fold. In the most purified fraction containing the aggregation factor, annular particles (Figure 3) with a contour length of 3,500 ± 100 Å have been detected[3,5]. These particles are surrounded by about 25 radially-arranged filaments, fixed on the peripheral region of the central rings. As shown below, from these large annular particles, the low molecular aggregation factor can be dissociated. The buoyant density of these particles was determined on a sucrose gradient from 5 to 80% in calcium- and magnesium-free artificial sea water in the 3×10 ml SW-rotor in a MSE 40 superspeed centrifuge. A buoyant density of 1.31 ± 0.03 g/cm³ has been calculated. Thus the aggregation factor from *Geodia* behaves like a protein and it differs markecly from the factor from *Microciona parthena*, with a buoyant density of 1.5 to 1.55 g/cm³[11].

The aggregation factor causing secondary aggregates is extremely sensitive towards different proteases[3]. From the exopeptidases tested, the carboxypeptidase B (Merck; hog pancreas; 60 units/mg) reduces the activity of the aggregation factor strongly, while the carboxy-

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Table I. Purification of the factor causing secondary aggregation

Fraction and step	Volume (ml)	Protein (mg/ml)	Total aggregation activity (10^5 AU)	Specific activity (10^5 AU/mg protein)
I. Crude extract	55	3.39	223.7	1.2
II. Ammonium sulfate	20	2.21	212.2	4.8
III. Acid precipitation	10	0.29	178.9	62.4
IV. Sepharose 4B	7.5	0.09	138.7	189.6
V. CM-Cellulose	18.0	0.01	90.6	604.8

The isolation (step I) and the purification (steps II–IV) have been described in detail[3]. Crude extract was obtained by collecting the cell-free supernatant from dissociated *Geodia* material (50 g) with calcium- and magnesium-free artificial sea water, containing 20 m*M* EDTA. The aggregation factor was purified by stepwise ammonium sulfate fractionation between 40% to 50% saturation. Dialysis was followed by a stepwise pH precipitation; the active fraction was obtained between pH 4 and pH 5. This was purified by 4B (Pharmacia) gel filtration. The active fraction elutes near the void volume (K_{av}value around 0.1; 7). Subsequently the fractions containing the aggregation factor were subjected to a CM-cellulose cation-exchange chromatography: The aggregation factor has been adsorbed with artificial sea water, containing 13 m*M* Ca^{++} and 50 m*M* Mg^{++}[11] and eluted with Ca^{++} and Mg^{++} free artificial sea water containing 10 m*M* EDTA. The protein content of the fractions has been determined[12]. The aggregation activity is expressed in aggregation units (AU) as described[3].

peptidase A (Merck; hog pancreas; 35 units/mg) is without effect[3]. Primarily carboxypeptidase B selectively hydrolyzes the basic amino acids lysine and arginine from proteins[6]. Aggregation factor containing these amino acids in radioactively labelled form has been used (Table II) in incubation experiments with carboxypeptidase B, 81% of the incorporated arginine and 50% of the lysine became acid soluble. Therefore we conclude that the 2 amino acids, lysine and arginine, are located in the functional groups causing secondary aggregation in *Geodia*.

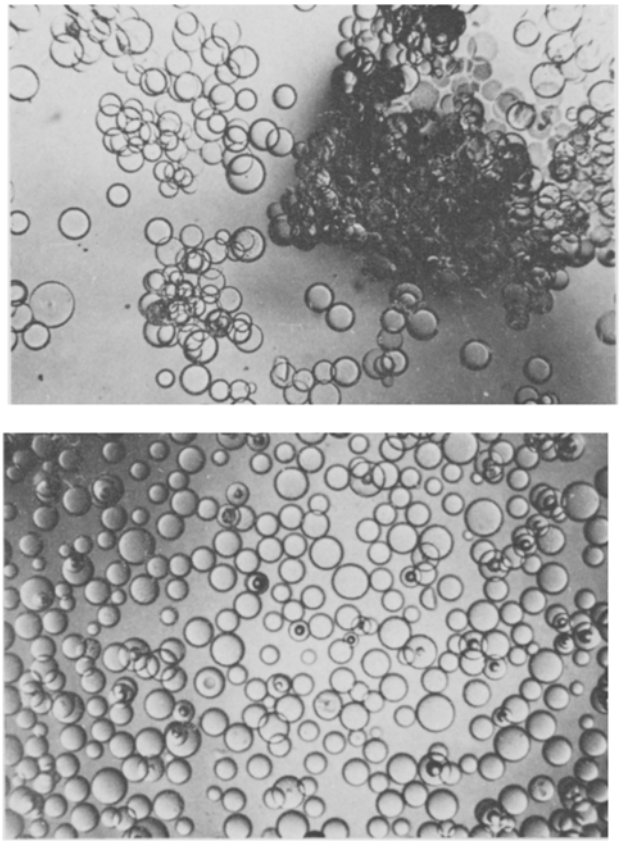

Fig. 4. Covalent attachment of 'small', secondary aggregation factor to AH-Sepharose 4B (Pharmacia). 'Small' aggregation factor was isolated as described in text (1% Nonidet NP-40 treatment followed by gel chromatography). The fractions (0.5 mg protein) containing the 'small', secondary aggregation factor, were used for the coupling procedure with N-cyclohexyl-N'-β-(N-methylmorpholine)- ethylcarbodiimide-p-toluenesulfonate (Merck) on 0.5 ml swollen AH-Sepharose 4B as described[9]. After washing with calcium- and magnesium-free artificial sea water, the beads were transferred into calcium- and magnesium-containing artificial sea water. a) Control: beads without aggregation factor; b) beads with covalently bound aggregation factor × 30.

Secondary aggregation factor per se. As described above, the secondary aggregation factor is associated with large annular particles. It can be dissociated from these compound structures by incubation with the non-ionic detergents Nonidet P-40 and Tween 80. The activity of the secondary aggregation factor is not influenced by these detergents up to concentrations of 3% (w/w). By gel chromatography on Sephadex G-100, the aggregation factor can be separated from the annular particles. Estimation of the molecular weight of the factor by gel filtration on Sephadex G-100 (Ve/Vo value of 2.03) amounts to 16,000[7]. The sedimentation coefficient of the factor has been determined in linear concentration gradients of 5 to 12% (w/w) sucrose in calcium- and magnesium-free artificial sea water and has been found to be 2.7 S, corresponding to 23,000 daltons[8]. The buoyant density (determination as described above) of the 'small' aggregation factor is 1.35 ± 0.04 g/cm³, compatible with a protein consisting mainly of amino acids.

We succeeded in coupling the 'small' aggregation factor to Sepharose beads by the carbodiimide method[9] (Figure 4). Thus it was possible to cause aggregation of the Sepharose beads via the aggregation factor isolated from *Geodia* tissue. Using this system, we hope to learn more about the biochemical events of the secondary aggregation phase, unimpeded by possible interference through the presence of cells or cellular membranes.

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Table II. Influence of carboxypeptidase B on the amount of lysine and arginine, incorporated into the fraction containing aggregation factor

Labelled amino acid	Aggregation factor		Aggregation factor incubated with carboxypeptidase B		Acid soluble radioactivity released by carboxypeptidase B	
	Total (cpm)	Acid insoluble (cpm)	Total (cpm)	Acid insoluble (cpm)	Absolute (cpm)	(%)
L-lysine	2,115	1,885	2,020	710	1,080	59
L-arginine	4,380	4,010	4,495	840	3,285	81

Sponge material (7 g) cut into cubes of 2 mm³ was incubated in 40 ml filtered sea water with 50 µCi ^{14}C-L-arginine (The Radiochemical Centre: spec. activity 318 mCi/mmol) and 50 µCi ^{14}C-L-lysine (The Radiochemical Centre: spec. activity 318 mCi/mmol) in an incubator[5]. After a period of 24 h at 18 °C aggregation factor has been isolated up to step IV (Table I) as described above. The fractions containing the aggregation factor were incubated with 200 µg carboxypeptidase B/ml. Acid insoluble fraction[13] and radioactivity[5] were determined as described.

Zusammenfassung. Chemisch dissoziierte Zellen des Kieselschwammes *Geodia cydonium* reaggregieren aufgrund zweier verschiedener Reaggregationsprinzipien. Der Aggregationsfaktor, auf den die Primäraggregation zurückgeht, ist membrangebunden und wird durch Proteasen nicht inaktiviert. Der sekundäre Aggregationsfaktor wurde 500fach angereichert. Das Molekulargewicht dieses Aggregationsfaktors beträgt etwa 20000 Daltons; er ist mit einem ringförmigen Makromolekül (2×10^9 Daltons) assoziiert.

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Free Amino Acid Composition of the Hemolymph of the Larval Blackfly *Simulium venustum* (Diptera: Simuliidae)

Recent attention has focused upon the possibility of using mermithid nematodes as biocontrol agents of blackflies[1]. However, field introductions of such mermithids cannot be made with probable success until procedures are devised for mass cultivating their infective stage(s). The lack of information concerning physiology (esp. hemolymph composition) of the simuliid hosts is a considerable hinderance to in vitro culture of these nematodes, because they derive nutriment from the host's hemolymph during parasitic development within the insect's hemocoel. The mermithid *Mermis nigrescens* Dujardin synthesizes proteins from amino acids available within the host hemolymph, but not from a dietary supply of dipeptides or proteins[2]. Therefore, this study was done to investigate the free amino acid composition of the hemolymph of the larvae of *Simulium venustum*, a blackfly species susceptible to mermithid parasitism[3].

Field-collected larval blackflies were held in an incubator at 10 °C until their hemolymph could be extracted. Insect larvae sampled for blood were primarily of 'maturing' and 'mature' developmental stages[4]. Using a stereomicroscope, hemolymph was obtained from surface-dried larvae by gently puncturing the insects in their proleg region with a fine insect pin. The fluid which exuded was drawn to fill a 10 µl capillary tube, expelled into a test tube containing the pooled blood sample and stored frozen at −20 °C. The pooled sample comprised blood taken from over 3,000 insects, because only a very small volume of hemolymph (0.3–0.5 µl) could be obtained from each blackfly larva. Therefore, the pooled sample was stored frozen throughout this protracted blood extraction process.

The pooled hemolymph sample was deproteinized by adding 30 mg sulphosalicylic acid, then centrifuged (6,500 g, 4 °C, 20 min). The volume of the supernatant was adjusted to 2.0 ml using a 0.2 *N* sodium citrate buffer (pH 2.2), then analyzed by the Beckman physiological fluids procedure[5] using a Beckman Model 121 amino acid analyzer. To determine total hemolymph levels of amino nitrogen, eight 5 µl aliquots of blood were collected from *S. venustum* larvae. Each aliquot was deproteinized by blowing it into 2 ml of 5% trichloroacetic acid. After centrifugation, 0.5 ml samples of the supernatant fluid were assayed colorimetrically for total amino nitrogen[6]. The mean free amino acid level of larval *S. venustum* hemolymph was found to be 39.3 ± 1.3 mg amino N per 100 ml hemolymph.

Consistent with findings for several other insect species[7–9], the larval *S. venustum* has high concentrations of

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