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Bivalve Mollusc Hemocyte Behaviors: Characterization of Hemocyte Aggregation and Adhesion and Their Inhibition in the California Mussel (*Mytilus californianus*)

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Abstract. Within minutes of removal from the California mussel, *Mytilus californianus,* hemocytes become sticky for one another and for foreign surfaces. We sought to understand the cell surface changes responsible for this altered state. Hemocyte aggregation and adhesion assays were used in experiments in which a variety of reagents potentially capable of interfering with aggregation were screened. Caffeine, nor-ethylmaleimide, cytochalasin B, and EDTA were completely or partially inhibitory towards aggregation and adhesion. However, RGD-containing peptides, glycosaminoglycans, protease inhibitors, heparin, or poly-L-lysine were without effect. Low temperature (4°C) slowed hemocyte adhesion and hemocyte cohesion. Based on the findings, it appears that (1) *Mytilus* hemocyte aggregation, *in vitro,* is a two-step process that requires metabolic energy and divalent cations (calcium and magnesium), and is temperature-sensitive; and (2) *Mytilus* hemocyte adhesion and hemocyte aggregation are two associated but different cell behaviors.

Introduction

Cell aggregation (cell clumping) in bivalves was first reported by Geddes (1880, cited by Narain, 1973). In molluscs, it has been presumed that clump formation is involved in hemostasis and wound healing (Bang, 1961; Sparks, 1972; Sminia, 1981). However, hemocyte aggregation in bivalves differs from blood clotting in vertebrates in that no extracellular fibers are formed. The aggregation of hemocytes in bivalves is reversible, and most aggregated cells later disperse, re-entering the circulatory system as wound repair progresses (Feng and Feng, 1974). Mussel hemocytes will spontaneously aggregate *in vitro.* When hemolymph is removed from a mussel, the free hemocytes form aggregates during the bleeding or immediately thereafter. When the aggregates and the free cells settle down on a foreign surface, they adhere (cell-substratum adhesion) and usually spread, and later migrate away. Although the phenomena of clump formation and hemocyte adhesion and spreading have been described both *in vivo* and *in vitro* in bivalves (Drew, 1910, cited by Narain, 1973; Dundee, 1953; Bang, 1961; Sparks, 1972; Cheng, 1981), few attempts have been made to unravel the operative mechanisms (Seifert, 1983). To study this rapid, spontaneous cell adhesion, it was first necessary to find effective inhibitors that could reversibly block the reaction. In this study, we used hemocytes of the California mussel, *Mytilus californianus,* to (1) identify inhibitors of hemocyte aggregation or adhesion *in vitro,* and then (2) investigate differences between hemocyte clump formation (cell-cell interaction) and hemocyte adhesion (cell-substratum interaction) *in vitro.*

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Abbreviations: NEM = *N*-ethyl maleimide; EDTA = ethylenediaminetetraacetic acid; RGD = arginine-glycine-aspartic acid; GAG = glycosaminoglycan; CMTBS = calcium- and magnesium-containing Tris-buffered saline; CMFTBS = calcium- and magnesium-free Tris-buffered saline; DMSO = dimethyl sulfoxide.

Materials and Methods

Chemicals

The following chemicals, tested at the indicated concentrations for their abilities to inhibit aggregation and adhesion of mussel hemocytes, were obtained from Sigma

Co. *Protease inhibitors:* phenylmethylsulfonylfluoride (PMSF, 20 μg/ml), soybean trypsin inhibitor (20 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), bovine α_2-macroglobulin (1 mg/ml); *glycosaminoglycans* (each at 1 mg/ml): hyaluronic acid, chondroitin sulfate (types A and C). *Others:* heparin (10 units/ml), protamine (1 mg/ml), poly-L-lysine (1 mg/ml), poly-L-glutamic acid (1 mg/ml). Ethylenediaminetetraacetic acid (EDTA), caffeine, cytochalasin B and nor-ethyl-maleimide (NEM) were tested at several concentrations stated in the results. Most of the suspected inhibitors were dissolved in CMTBS (Ca^{++}, Mg^{++} Tris-buffered saline: 10 m*M* $CaCl_2$, 60 m*M* $MgCl_2$, 50 m*M* Tris HCl, NaCl to 960 mOsm), but PMSF, pepstatin, aprotinin, leupeptin, and cytochalasin B were first dissolved in DMSO, then mixed with CMTBS. The final concentration of DMSO in the medium was <0.5%. Peptides containing the Arg-Gly-Asp (RGD) amino acid sequence were kindly provided by Dr. D. W. Barnes, Biochemistry, Oregon State University. BCA protein assay reagents were purchased from Pierce. All other chemicals were purchased from Sigma Co. The suspected inhibitors were dissolved in CMTBS (Ca^{++}, Mg^{++} Tris-buffered saline: 10 m*M* $CaCl_2$, 60 m*M* $MgCl_2$, 50 m*M* Tris HCl, NaCl to 960 mOsm). The EDTA was dissolved in calcium- and magnesium-free Tris-buffered saline (CMFTBS). Hydrophobic chemicals were first dissolved in DMSO, and then mixed with CMTBS. The final concentration of DMSO in treated hemolymph was lower than 0.5%. The pH value of solutions was adjusted to 7.4 with NaOH, and osmolarities were checked by freezing-point osmometer before they were mixed with hemolymph.

Animals

Individual *Mytilus californianus* larger than 8 cm in length were collected monthly from the rocky intertidal zone at Seal Rock State Park (15 miles south of Newport, Oregon). On the same day, these mussels were transferred to a filtered, recirculating, continuously aerated seawater system that was maintained at pH 7.6, nitrate < 10 ppm, nitrite < 0.2 ppm, 15°C, and close to normal salinity. Mussels were held in this system for at least three days before being used as a source of hemolymph.

Hemolymph collection

A plastic rod (3 mm diameter) was inserted between the two shells to prevent them closing, and seawater was drained from the mantle cavity. Animals were then transferred to a cold room (4°C). Hemolymph was collected from the posterior adductor muscle using a cooled sterile syringe with 18G 1½″ needle. With the needle removed, the colorless (slightly opalescent) hemolymph was immediately transferred to cooled sterile tissue culture tubes (Falcon) for further treatment. Hemolymph was collected from each animal only once.

Coating of surfaces for cell adhesion assay

Except for agarose, the coating process was as follows: the solution of coating material (see Table I) was loaded on the clean surface for 30 min at room temperature. Then the excess was washed and replaced by CMTBS. For agarose coating, melted agarose was added to washed eight-well slides (5 μl/well; Cel-Line, New Jersey), and then mounted by a cover slip which was held off the slide by two small pieces of coverslip. The coverslips were removed after keeping the slide at 4°C for a few minutes. The thin layer of agarose allowed satisfactory microscopic observation.

Inhibitor screening assay

The solutions of suspected inhibitors (see Results) were aliquoted into siliconized microtubes (PGC Scientifics, Maryland), and kept at 4°C. Slides with eight wells, cleaned by immersion in acid alcohol overnight, were dried and placed in a humidity chamber that was also kept at 4°C before use. As soon as the hemolymph had been transferred to a cooled sterile tissue culture tube, it was aliquoted and mixed (1:1) with test solutions by vortexing for about 3 s. The treated hemolymph was then loaded onto the undersurface of an inverted, cooled eight-well slide (50 μl/well). Each sample was loaded in duplicate onto the same position on a different slide. These were maintained as hanging-drops during gyration at 100 rpm performed by Gyrotory® Shaker-Model G2 (New Brunswick Scientific Co., Inc., New Jersey) at room temperature (22 ± 1°C). The purpose of using hanging-drops was to avoid cell adhesion to glass during gyration. Following 15 min gyration, the slide was carefully turned over. Two 1-mm-thick spacers were placed on the slide, followed by a coverslip (24 × 50 mm). Cell aggregation, cell adhesion, and cell spreading were observed under phase contrast microscopy.

Those inhibitors that interfered with cell aggregation or cell adhesion were selected for further studies. Due to occasional individual differences between mussels, each reagent was tested on at least three hemolymph samples taken from separate mussels at different times.

Assay for inhibition of cell aggregation

This assay followed the same protocol used in the screening test. After the slide was turned so the hemolymph was on the upper side, 100% formalin was added (1 μl/well) to fix the cells. The final concentration of formalin in hemolymph was 2%. Cells at the center of each preparation were then immediately dispersed *in situ* by

gentle pipetting five times. Samples were then mounted by a coverslip (24 × 50 mm) held on two 1-mm-thick spacers. The extent of cell aggregation was assessed by counting the total free cell numbers in five different areas in each well. To obtain values for "no aggregation," we proceeded as follows: fresh hemolymph was mixed with 20% formalin (1:1) to immediately block cell aggregation. The total (control) free cell number was counted in hemolymph fixed this way in 10% formalin as soon as it was taken from the mussel.

$$\text{The percentage of cells remaining free} = \frac{\text{Total free cell numbers in test treatment}}{\text{Total free cell numbers in fixed fresh hemolymph}} \times 100$$

Assay for inhibition of cell adhesion

After hemolymph had been mixed with test solutions, it was then loaded (50 μl/well) into wells of a pre-cooled (4°C), flat-bottom 96-well tissue culture plate (Corning, New York). Each treatment was loaded in triplicate. After 15 min at room temperature for cell adhesion to occur, the plasma and unattached cells were removed and each well was washed three times with CMTBS (100 μl/well). As a measure of the number of remaining (adherent) cells, protein concentrations were measured by means of the BCA protein assay (Pierce). The adherent cells were first lysed in 20 μl cell lysis solution (2% Na_2CO_3, 0.1 *M* NaOH) for at least 30 min at RT with occasional shaking by vortex, and then the fresh Pierce BCA protein assay reagent was added (100 μl/well). After 30 min incubation at 60°C, the plate was cooled to RT, and the absorbance of each well was read at 550 nm using a microtiter plate reader (Titertek Multiskan MCC/340).

The percentage of cell adhesion was calculated using the following equation:

$$[(A_i - A_b)/(A_c - A_b)] \times 100$$

A_i: Absorbance of inhibitor-treated well
A_b: Absorbance of background well (lysis solution and BCA reagent only)
A_c: Absorbance of CMTBS-treated well

According to this equation, the absorbance in background wells indicates 0% adhesion, and the absorbance in CMTBS-treated wells indicates 100% adhesion.

To correlate cell number with protein values, serial two-fold dilutions of hemolymph in CMTBS (50 μl each) were loaded in quintuplicate into wells of 96-well plates. After 15 min for adherence, all wells were washed three times with CMTBS. Then three were used for protein determinations as described above, and Hoechst 33342 (50 μl, 20 μg/mL CMTBS) was added to the other two. Using an inverted fluorescence microscope, nuclei were counted in the Hoechst-stained wells corresponding to those for which protein values were also obtained.

Data analysis

Each experiment was repeated at least three times, using fresh hemolymph samples from different animals. Data are presented as mean ± S.D. Statistical analysis of data was performed using paired or unpaired Student's *t*-test as appropriate. Differences were considered significant when $P < 0.05$.

Cell viability

Following the adhesion/spreading and aggregation assays, hemocyte viability was determined by visual observation of hemocyte spreading, and by cell exclusion of propidium iodide. This test was performed only on the samples in which cell aggregation or adhesion was interrupted. Treated hemolymph (40 μl) was gently removed from well slides or culture plates, and replaced by 40 μl CMTBS. If the remaining cells had not aggregated or spread in 10 min, two possibilities were considered; either the treated cells had been killed by the chemical or the inhibitory effect of the chemical was irreversible. To determine whether the treated cells were alive or not, the propidium iodide staining protocol was followed: 1 μl propidium iodide solution (500 μg/ml in CMTBS) was added to each sample (50 μl). In cells which are dead, the dye enters and binds to nuclear DNA with high affinity. Stained nuclei generate bright red fluorescence under epi-fluorescence using a Zeiss microscope with appropriate filter set.

Results

Normal hemocyte aggregation, adhesion, and spreading in vitro

Mytilus hemolymph contains three types of hemocyte (Moore and Lowe, 1977). Preliminary studies showed that hemocytes of distinct densities, separated on 60% Percoll, were all competent in adhesion and aggregation assays. All the following experiments were conducted with freshly taken, unseparated hemocyte populations.

When hemocytes were first removed from the mussels, they remained dispersed and were round (Fig. 1a). However, they changed from round to elongated in one minute or less (Fig. 1b). Sometimes aggregation occurred extremely rapidly during the bleeding, or while the hemolymph was being dispensed into the test tube. After initial cellular contact, cells were seen to pull together before the cells or aggregates began to spread. Aggregates formed this way without shaking were small (Fig. 1c), and the cell-cell binding strength was weak. The individual cells

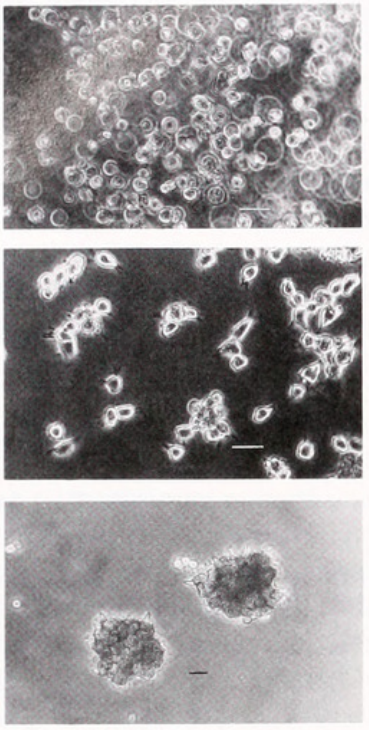

Figure 1. The sequential processes of *Mytilus californianus* hemocyte aggregation *in vitro*. (a) The naive free cell stage: these fresh hemocytes are approximately spherical in outline. (b) The initial stage of cell aggregation and adhesion: Within one minute, all of the cells form spikes, and some cells form small aggregates (cell number < 10). (c) Medium sized clumps: when these small aggregates collide, they can form bigger clumps. Each medium sized clump may be composed of about one hundred cells. Bars = 30 μm.

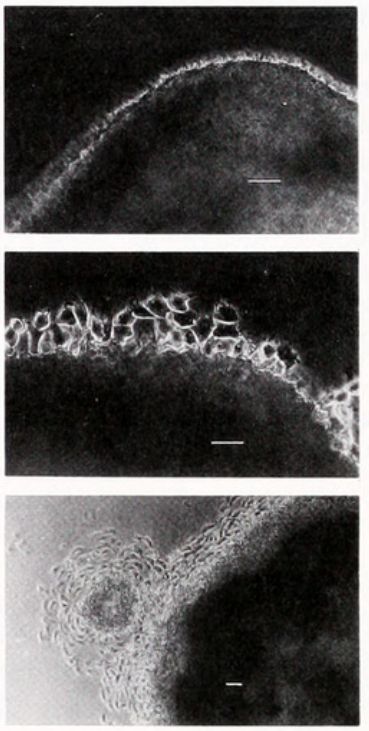

Figure 2. *Mytilus californianus* hemocyte spreading and migration *in vitro*. (a) Part of a large clump: each large aggregate like this may contain more than ten thousand cells. These cohere tightly together, and the individual cells are difficult to distinguish. Bar = 30 μm. (b) Early stage of hemocyte migration: cells at the edge begin to move out of the aggregate about 3 minutes after the aggregate settles down. Bar = 30 μm. (c) Late stage of hemocyte migration: cells have radially migrated from the aggregate after 10 min *in vitro*. Bar = 50 μm.

were distinguishable and could be easily dispersed from the aggregates by pipetting.

In contrast, clumps that formed during 10 or more minutes of shaking often contained more than ten thousand cells (Fig. 2a). This kind of clump was resistant to mechanical dispersion by pipetting or vortexing. When cells or aggregates were allowed to settle down onto glass or plastic, they attached, flattened, and developed pseudopodia. During subsequent incubation, cells located at the surface of the clump and in contact with the substratum adhered to the substratum and migrated radially out of the clump (Fig. 2b, c). The attached and spread cells moved by amoeboid locomotion. The cells possess a strong ability to adhere, spread, and migrate on foreign surfaces. Only on agarose substrata were such kinds of cell behaviors inhibited (Table I).

Assay of cell adhesion

The numbers of cells in individual wells and protein quantified in these wells were dependably correlated. When 50 μl of hemolymph (serially diluted twofold in CMTBS to $^1/_{16}$) was held in wells of flat-bottomed 96-well plates for 15 min at 22 ± 1°C, nuclear counts and protein values indicated 1215 cells yielding 69.5 pg protein in $^1/_{16}$ strength hemolymph, 1597 cells yielding 134 pg protein in $^1/_8$ strength hemolymph, and 6636 cells yielding 478.5 pg protein in $^1/_4$ strength hemolymph. These values, from one experiment representative of two runs, indicate a protein value of approximately 71 pg protein per 1000 cells.

Table I

Mytilus californianus *hemocyte behavior on various surfaces*

Surface	Adhesion	Spreading	Migration
Glass (well slide)	+++	+++	+++
Polystyrene (PS) (culture plate)	+++	+++	+++
Sigmacoted® glass	+++	+++	+++
Siliconized PS	+++	+++	+++
Poly-L-lysine (1 mg/ml) coated glass	+++	+++	+++
Poly-glutamate (1 mg/ml) coated glass	+++	+++	+++
BSA (1%) coated glass	+++	+++	+++
Fibronectin (1%) coated glass	+++	+++	+++
1% agarose coated glass	–	–	–

BSA: Bovine serum albumin.

+++: strong; ++: medium; +: weak; –: inhibition.

Fresh hemolymph was loaded onto different surfaces. After 15 min incubation at room temperature, the non-adherent cells were removed, and then cell adhesion, spreading, and migration were observed under a phase contrast microscope.

Table II

Chemicals tested for inhibition of aggregation and adhesion of Mytilus californianus *hemocytes*

	Chemicals (final concentration)
Protease inhibitors*	phenylmethylsulfonylfluoride (PMSF) (20 μg/ml), soybean trypsin inhibitor (20 μg/ml), pepstatin A (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), α_2-macroglobulin (1 mg/ml).
RGD peptides	RGDS, GRGDSP, GDSP, TGRG (2.5 mg/ml).
Glucosamino-glycans (1 mg/ml)	hyaluronic acid, chondroitin sulfate (type A, type C).
Others	heparin (10 unit/ml), protamine (1 mg/ml), poly-L-lysine (1 mg/ml), poly-L-glutamic acid (1 mg/ml).

* PMSF, pepstatin A, aprotinin, and leupeptin were dissolved in DMSO. The final concentration of DMSO was less than 0.5%.

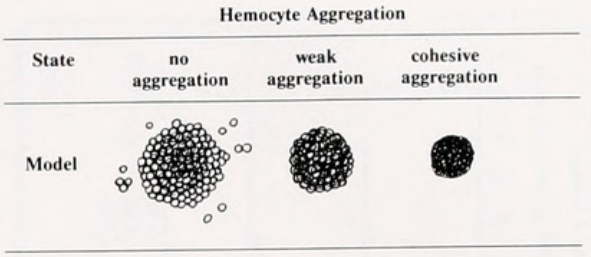

Figure 3. The appearances of *Mytilus californianus* hemocytes at three states of aggregation *in vitro*. The aggregation of California mussel hemocytes *in vitro* can be categorized into three levels, namely no aggregation, weak aggregation, and cohesive aggregation. No aggregation: cells accumulate at the central area of the well as a result of the gyratory force. A few small aggregates are sometimes seen, while in most cases the centralized cells just pile up, and no binding occurs between them. Such cells can be freely dispersed by gentle pipetting. Weak aggregation: individual cells are distinguishable, especially those located at the edge of the aggregate. Large clumps like this can be separated by pipetting to yield several small aggregates. Possibly due to the presence of mucous material (which is induced by certain inhibitors such as EDTA, $NaIO_4$), hemocyte contacts are sometimes restricted, and the morphology of weak aggregation varies. Cohesive aggregation: the compact nature of such aggregates prevents dispersion by pipetting or vortexing. The aggregated cells cannot be seen individually.

Influences of suspected inhibitors on hemocyte aggregation, adhesion, and spreading

From data obtained in screening assays, the tested reagents could be allocated into four categories. The first includes caffeine (25 m*M*) and *N*-ethyl maleimide (0.1 m*M*), which strongly inhibited both hemocyte aggregation and adhesion. The second category of chemicals poorly blocked hemocyte aggregation but significantly inhibited hemocyte adhesion. They include EDTA (60 m*M*), and poly-L-glutamic acid (MW 50,000–100,000; 1 mg/ml). Cytochalasin B (5 μg/ml) is the only chemical in the third category: it effectively inhibited hemocyte aggregation, but poorly blocked hemocyte adhesion. The fourth group of chemicals had no detectable inhibitory effect on either hemocyte aggregation or adhesion. They included RGD- (Arg-Gly-Asp-) containing peptides, glycosaminoglycans (GAGs), poly-L-lysine, heparin, protamine, protease inhibitors, and colchicine (Table II). Because inhibited hemocytes recovered their aggregation and adhesion competence after caffeine, EDTA, poly-L-glutamic acid, or cytochalasin B was replaced by CMTBS, the inhibitory effects of such chemicals, unlike NEM, were reversible (data not shown). The extent of hemocyte aggregation can be easily distinguished at three different states, namely no aggregation, weak aggregation, and cohesive aggregation (Fig. 3). Five different stages of hemocyte adhesion/spreading were scored (Fig. 4).

Hemocytes aggregated and adhered in 10 m*M* sodium azide (NaN_3). However, when the cells were co-incubated in 10 m*M* NaN_3 with 25 m*M* caffeine for 30 min, cell aggregation and adhesion were still affected after the caffeine/azide was replaced by azide solution. Such azide-poisoned cells aggregated weakly after the NaN_3 + caffeine solution was replaced by NaN_3. When the NaN_3 + caffeine was replaced by CMTBS, the cells recovered eventually and formed cohesive aggregates.

Low temperature (4°C) delayed cell adhesion for the first few minutes, but after 15 min no differences from controls could be seen (Fig. 5). In cell aggregation assays, cells still aggregated weakly at 4°C. With an increase in temperature, the aggregates contracted and cohered to form tight masses as if no interruption had occurred (Fig. 6).

Osmolarity seems not to be critical for *M. californianus* hemocyte aggregation and adhesion *in vitro*. There were

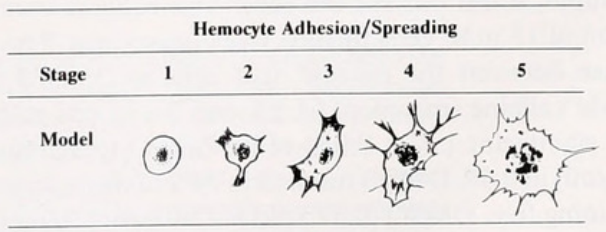

Figure 4. Illustration of the stages of *Mytilus californianus* hemocyte adhesion, and spreading *in vitro*. Hemocyte adhesion and spreading are continuous processes which can be divided into five stages from 1° to 5°. Normally, hemocytes *in vitro* can adhere and spread well (4°) in 5–10 minutes. 1° illustrates naive hemocytes in suspension; these resemble cells in which adhesion and spreading are inhibited. 2°: cell adhesion and spreading are blocked, but spike formation is not. 3°: cells adhere and flatten, but without extension of pseudopodia. 4°: cells extend small pseudopodia as the cells spread (5).

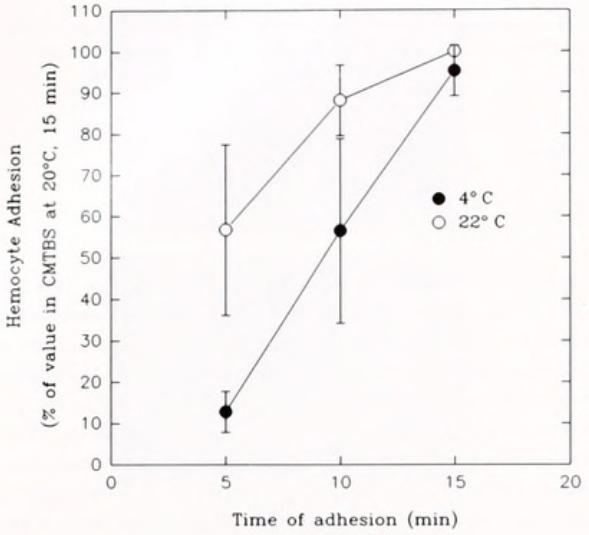

Figure 5. The influence of temperature on the adhesion of *Mytilus californianus* hemocytes *in vitro*. Hemolymph was loaded into 96-well culture plates (50 μl/well). These were then incubated at 4°C or 22 ± 1°C. At 5, 10, and 15 min, the non-adherent cells and plasma were removed. The wells were washed three times with CMTBS, and then the adherent cells were lysed for BCA protein assay. The protein values obtained for wells in which hemolymph was held at 22 ± 1°C for 15 min were used as 100% hemocyte adhesion. There is a significant difference ($P < 0.05$) at 5 min incubation at 4°C, and at 22°C.

no significant differences in hemocyte adhesion and aggregation when hemolymph was mixed (1:1) with CMTBS in the range of 800–1100 mOsm.

The inhibitory effects of caffeine, NEM, EDTA, and cytochalasin B on cell aggregation

Hemocytes exposed to 25 m*M* caffeine, 5 μg/ml cytochalasin B, or 0.1 m*M* NEM were strongly inhibited from aggregating (95%, 71%, and 72%, respectively, remained free). When hemocytes were treated with EDTA, the morphology of aggregates varied. Under the microscope, some colorless material was seen among the aggregates. Its presence seemed to restrict hemocyte contact. Though caffeine inhibition at 25 m*M* or 50 m*M* was reversible and rapid, it did not kill the cells. At a caffeine concentration of 15 m*M*, cells formed weak aggregates. The difference between the percent free cells in 25 m*M* and 15 m*M* caffeine (means of 61.5% and 7.1%) was statistically significant ($P < 0.05$) (Fig. 7a). Cytochalasin B (5 μg/ml) in 0.5% DMSO resulted in 70% of the hemocytes remaining free. DMSO (0.5%) did not influence hemocyte aggregation and adhesion (Fig. 7b). At 0.1 m*M*, NEM significantly inhibited hemocyte aggregation ($P < 0.05$) (Fig. 7c). EDTA at concentrations 15–60 m*M* significantly inhibited hemocyte aggregation (Fig. 7d). However, most cells were observed forming weak aggregates; in three experiments, values for percent free cells were 9.5, 18.2, and 21.8 at 60 m*M*; 8.0, 19.7, and 23.1 at 30 m*M*; and 8.4, 20.4, and 13.4 at 15 m*M*.

The inhibitory effects of caffeine, NEM, EDTA, and cytochalasin B on hemocyte adhesion and spreading

When hemolymph was treated with 5 μg/ml cytochalasin B or 0.1 m*M* NEM, the hemocytes remained round, thus resembling naive cells. Cytochalasin B (1 μg/ml) could retard hemocyte spreading, affect the morphology of spread hemocytes, and inhibit hemocyte migration. Cells exposed to 25 m*M* caffeine were irregular in outline (Fig. 8a). EDTA in the range 0.6–60 m*M* inhibited cell adhesion (Fig. 8b).

The concentration of caffeine necessary to inhibit cell adhesion was similar to that needed to inhibit aggregation. At 15 m*M* caffeine, cell adhesion was significantly greater relative to that in 25 m*M* caffeine (Fig. 9a). Cytochalasin B significantly inhibited cell adhesion at concentrations between 1 and 5 μg/ml ($P < 0.05$), though 60% of the

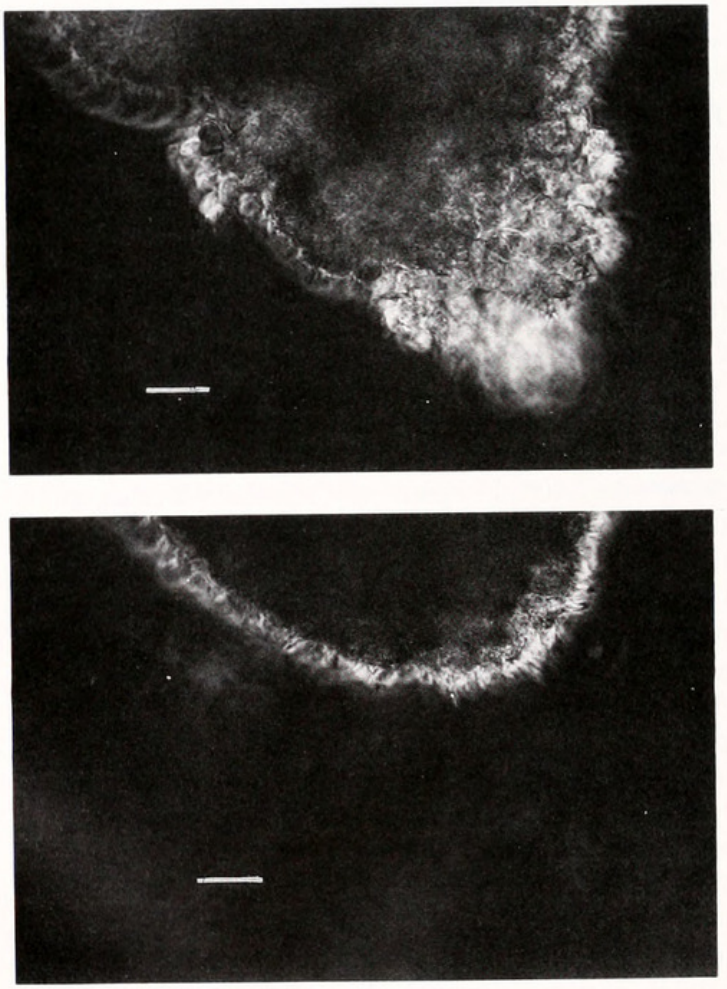

Figure 6. The effect of temperature on *Mytilus californianus* hemocyte aggregation *in vitro*. Weak aggregates like this one formed during 15 min gyration at 4°C. This photo (a) was taken immediately after the aggregate was removed from 4°C. Cohesive aggregates (b) formed after the incubation temperature was warmed up to 22 ± 1°C. The whole cell mass gradually shrunk when the incubation temperature increased. This contraction led to the formation of a tighter clump. Photograph (a) and (b) were taken from the same microscopic field at 1 and 15 min, respectively. Bars = 30 μm.

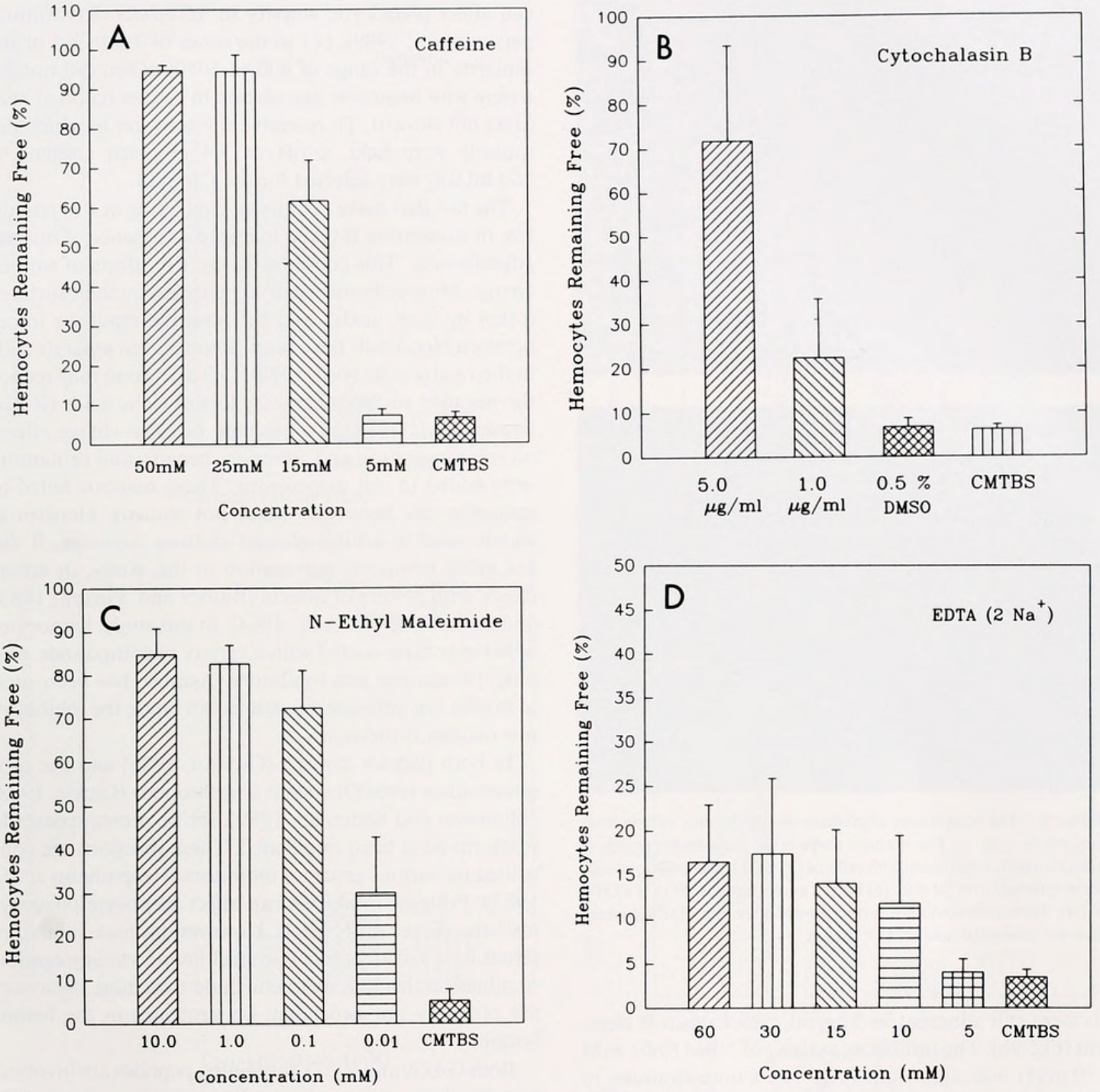

Figure 7. The influences of caffeine, cytochalasin B, NEM, and EDTA on *Mytilus californianus* hemocyte aggregation *in vitro*. Data are pooled from three separate experiments. In these figures, each value (%) is the mean number of free cells in inhibitor solution as a percent of free cells in 10% formalin. The counts in 10% formalin differed between individual bleeds; for the three caffein bleeds, for example, these were 1,480, 1,610 and 1,779. The highest count was 3,602 (2nd experiment with cyt B) and the lowest was 1,028 (3rd experiment with NEM). (a) At 25 m*M* or 50 m*M*, caffeine completely blocked hemocyte aggregation, thus most cells remained free. At 15 m*M*, caffeine still significantly inhibited hemocyte aggregation ($P < 0.05$). Like saline (CMTBS), 5 m*M* caffeine had no inhibitory effect. (b) Cytochalasin B (5 μg/ml in 0.5% DMSO) significantly inhibited hemocyte aggregation ($P < 0.05\%$). The vehicle, DMSO, did not have any inhibitory effect. (c) Hemocyte aggregation was strongly inhibited by 0.1 m*M* or higher concentrations of NEM ($P < 0.05$). (d) EDTA within the range of concentrations from 15 m*M* to 60 m*M* has significant influence on hemocyte aggregation ($P < 0.05$).

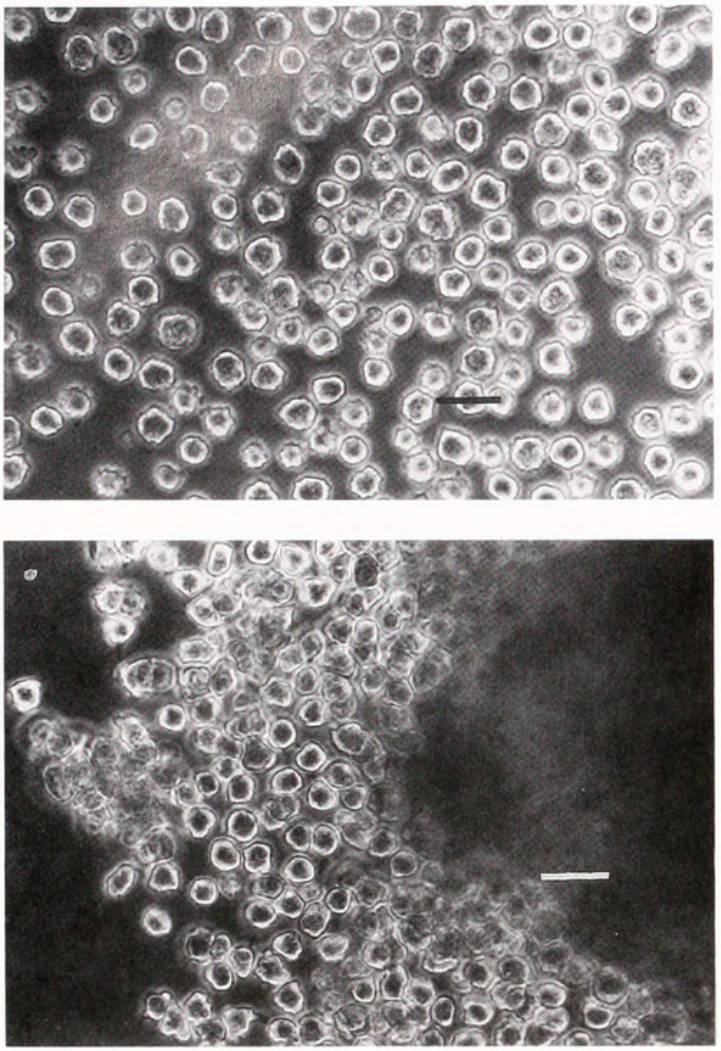

Figure 8. The morphology of inhibitor-treated *Mytilus californianus* hemocytes *in vitro*. (a) The irregular shape is the main characteristic of hemocytes which were treated with caffeine (25 m*M*). Such cells gradually become spherical over 30 min. (b) Part of a cell clump in 60 m*M* EDTA (pH 7.4). These cells could not further spread when the EDTA concentration was above 0.6 m*M*. Bars = 30 μm.

cells were still adherent in 5 μg/ml cytochalasin B treatment (Fig. 9b). The inhibitory activity of NEM (0.01 mM to 10 mM) was significant (Fig. 9c). Concentrations in this range inhibited 90% of cell adhesion on culture plates (polystyrene). EDTA ($\geq$0.6 m*M*) influenced cell adhesion on culture plates also (Fig. 9d). When EDTA-treated hemolymph was replaced by 20 m*M* or a higher concentration of buffered isotonic Ca^{++} or Mg^{++} solution, 80% of hemocytes recovered their adhesion competence (Fig. 10). Therefore, *Mytilus* hemocyte adhesion is Ca^{++} or Mg^{++}-dependent.

Discussion

A variety of buffers have been used in mussel hemocyte studies (Moore and Lowe, 1977; Renwrantz *et al.*, 1985; Dageförde *et al.*, 1986). Although the buffer osmolarity can affect phagocytic activity in *M. edulis* (Renwrantz, pers. comm., 1989), pH in the range of 7.4 to 8.4 or osmolarity in the range of 800 to 1050 mOsm did not interfere with hemocyte aggregation in studies reported here (data not shown). To resemble the seawater in which the mussels were held, a pH of 7.4 and salt content of 960 mOsm were selected for the CMTBS.

The fact that naive hemocytes circulating *in vivo* remain free in suspension is taken to imply an absence of mutual adhesiveness. This could be due to the effects of surface charge. Most cells are negatively charged on their surfaces (often by sialic acids), and the resulting repulsive forces between blood cells have been postulated to separate cells in their naive state (Bell, 1983). Cell activation may reduce the negative surface charge by loss of sialic acids (Rutishauser *et al.*, 1988). To evaluate possible charge effects on cell aggregation and adhesion, heparin and protamine were added to cell suspensions. These reagents failed to influence cell behaviors (data not shown). Heparin is widely used to inhibit platelet clotting, however, it did not affect hemocyte aggregation in this study, in accordance with results in insects (Ratner and Vinson, 1983) and *Limulus* (Bryan *et al.*, 1964). In this study, hemocytes adhered to glass coated with a variety of compounds, and only 1% agarose was inhibitory. Agarose has been used to inhibit cell adhesion of various cell types; the inhibitory mechanism is unknown.

In both platelet clotting (Guyton, 1986) and the prophenoxidase (proPO) system of arthropods (Lackie, 1988; Johansson and Söderhäll, 1989), serine-protease cascade reactions have been implicated. These reactions are controlled by various protease inhibitors (Hergenhahn *et al.*, 1987). Protease inhibitors can affect hemocyte phagocytosis also (Fryer *et al.*, 1991). However, protease inhibitors tested here failed to block mussel hemocyte aggregation or adhesion, leading us to conclude that these behaviors are probably not dependent on proteases in the hemolymph.

Both GAGs and RGD-containing peptides are involved in cell-substratum and cell-cell interactions (Underhill, 1982; Roseman, 1985). Furthermore, a sponge aggregation factor has been revealed that is a sulfated polysaccharide (Coombe and Parish, 1988). In our hemocyte aggregation and adhesion tests, none of the GAGs or RGD peptides had significantly inhibitory effects, though they did retard hemocyte spreading.

N-Ethyl-maleimide has been reported to irreversibly inhibit the aggregation of horseshoe crab hemocytes (Bryan *et al.*, 1964). Similarly, NEM interfered with *Mytilus* hemocyte aggregation and adhesion. Thus, it can be inferred that, in both these species, normal hemocyte behaviors require intact disulfide bond structures in cell proteins.

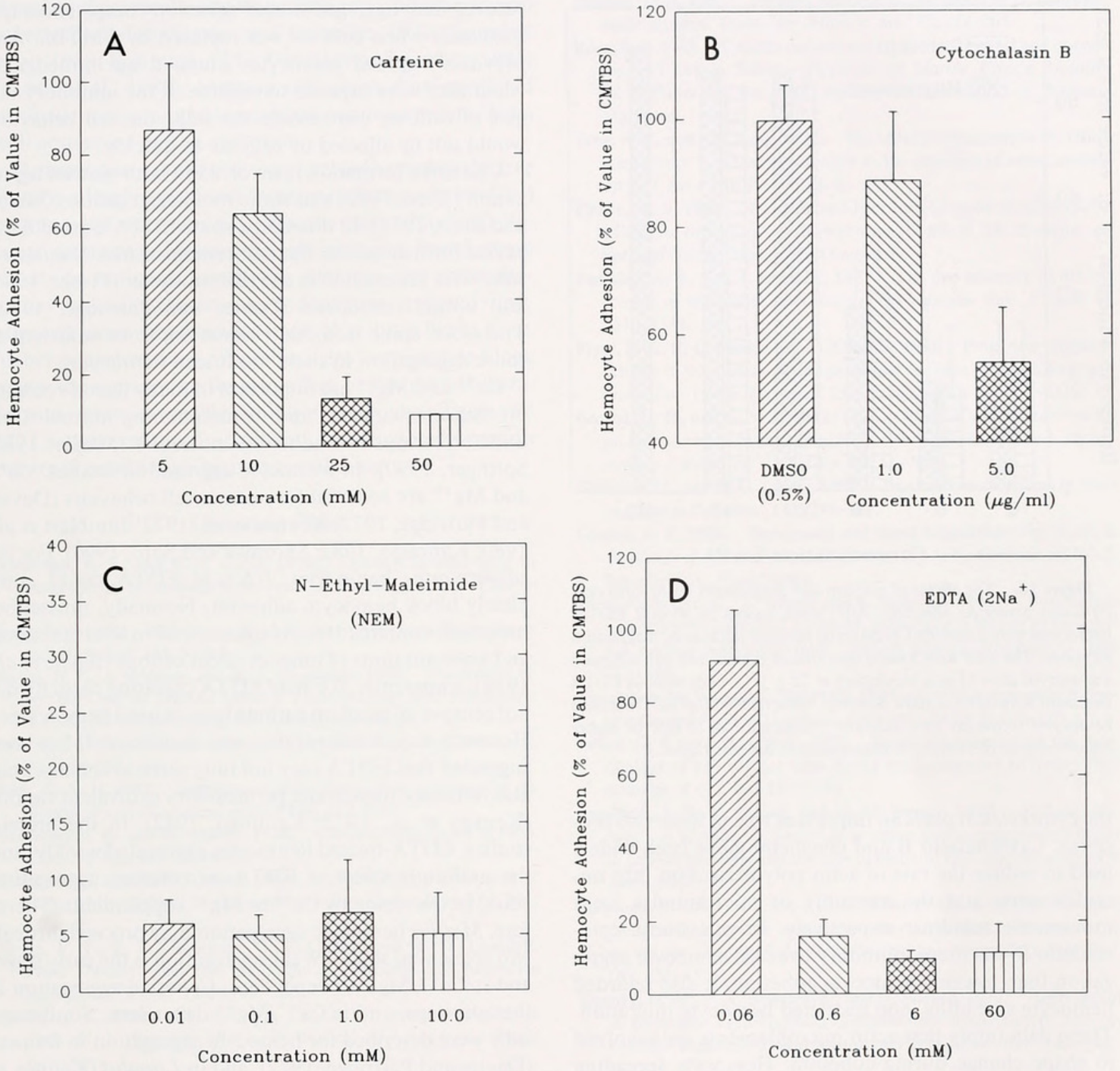

Figure 9. The influences of caffeine, cytochalasin B, NEM, and EDTA on *M. californianus* hemocyte adhesion *in vitro*. In these figures, each bar represents (%) the ratio of the protein value of adherent cells in inhibitor solution to the equivalent value in CMTBS. That is, CMTBS-mixed hemocyte adherence is taken as 100% adhesion competence. (a) At 25 m*M* or higher, caffeine significantly blocked hemocyte adhesion ($P < 0.05$). At 10 m*M*, caffeine was still partially inhibitory. (b) Cytochalasin B (5 μg/ml in 0.5% DMSO) significantly inhibited hemocyte adhesion ($P < 0.05$), but about 55% of hemocytes remained adherent. The vehicle, DMSO, did not have any inhibitory effect. (c) NEM strongly inhibited hemocyte adhesion. Less than 10% hemocyte adherence could be detected in 0.01 m*M* or higher concentrations of NEM ($P < 0.05$). (d) At 0.6 m*M*, EDTA significantly inhibited hemocyte adhesion ($P < 0.05$), but this agent was without effect at 0.06 m*M*.

Inhibition of cytoskeleton assembly has been reported to disrupt insect plasmatocyte encapsulation (Davies and Preston, 1987), and mollusc hemocyte chemotaxis and cell migration (Cheng and Howland, 1982), as well as cell surface receptor redistribution (Cheng and Howland, 1982; Dageförde *et al.*, 1986). Because bivalve hemocytes alter their shapes in both cell aggregation and cell adhesion (Jones and Gillett, 1976; Feng, 1988), it is inferred that

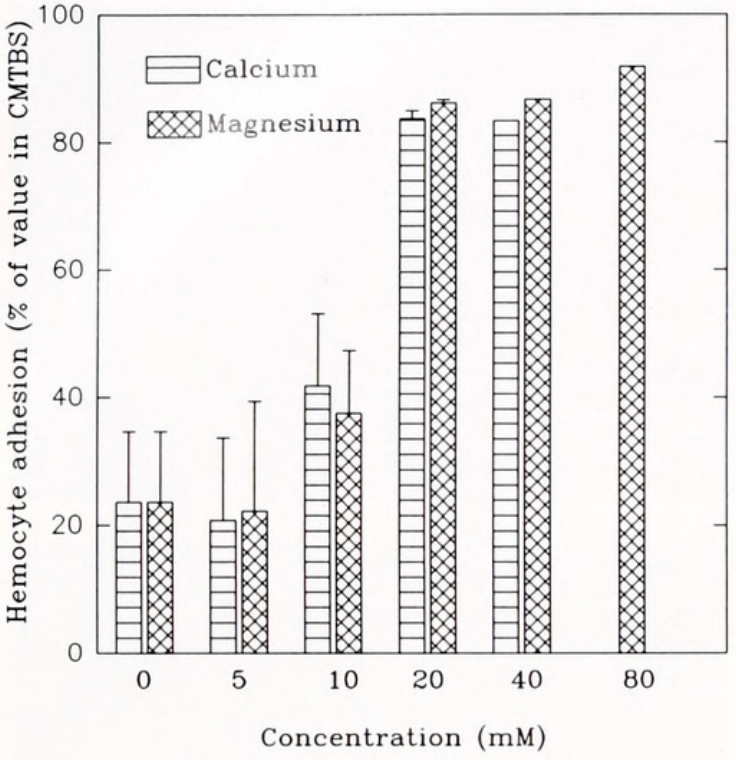

Figure 10. The effects of calcium and magnesium on *Mytilus californianus* hemocyte adhesion. After three washes in 30 m*M* EDTA, hemocytes were suspended in buffered isotonic calcium or magnesium solutions. The cells were loaded into culture plates, then cell adhesion was assayed after 15 min incubation at 22 ± 1°C. Cells without EDTA treatment were taken as 100% adherent. More than 80% of EDTA-treated hemocytes recovered their adhesion competence at 20 m*M* or higher concentrations of calcium or magnesium in isotonic buffer.

the cytoskeleton plays an important role in these two processes. Cytochalasin B and colchicine have been widely used to reduce the rate of actin polymerization into microfilaments and the assembly of microtubules from monomeric tubulins, respectively. In this study, cytochalasin B was more inhibitory towards hemocyte aggregation than towards hemocyte adhesion. It also retarded hemocyte spreading, and restricted hemocyte migration. These data imply that actin microfilaments are involved in shape change during cohesion. Hemocyte spreading and migration are actin-dependent, but hemocyte adhesion may not be associated with actin.

Caffeine has been used as an anticoagulant in slime molds (Brenner and Thoms, 1984) and in several invertebrates (Bertheussen and Seljelid, 1978; Ratner and Vinson, 1983), but its inhibitory mechanism is not clear. The pharmacological effect of caffeine is due to phosphodiesterase inhibition and the consequent increase in intracellular cAMP (Brenner and Thoms, 1984; Snyder, 1984). In *M. californianus*, inhibition by caffeine is a dose-dependent, reversible reaction that does not affect cell viability. We suspect that the inhibitory effect of caffeine on cell aggregation and adhesion is receptor-associated. Hemocytes treated with caffeine for 30 min were able to recover their aggregation and adhesion competences immediately when caffeine was replaced by CMTBS. And previously spread hemocytes rounded up immediately when they were exposed to caffeine. If the inhibitory targets of caffeine were inside the cells, the cell behaviors would not be affected by caffeine so quickly.

Cell spike formation is involved in both platelet aggregation (Born, 1962) and slime mold aggregation (Garrod and Born, 1971). In this study, some EDTA-treated hemocytes formed spikes, but still remained free. Similar results were reported with the eastern oyster (Fisher, 1986) and limpet hemocytes (Davies and Partridge, 1972). Therefore, spike formation seems not to be sufficient to cause aggregation in these molluscan hemocytes.

Ca^{++} and Mg^{++} are important in many ligand-receptor binding mechanisms, and in maintaining normal functions of various cell adhesion molecules (Müller, 1980; Springer, 1990). In hemocyte aggregation studies, Ca^{++} and Mg^{++} are essential for normal cell behaviors (Davies and Partridge, 1972; Kenney *et al.*, 1972; Jumblatt *et al.*, 1980; Kanungo, 1982; Shozawa and Suto, 1990). For *M. californianus* hemocytes, 0.6 m*M* EDTA could completely block hemocyte adhesion. Normally, mussel hemolymph contains 10 m*M* calcium, 60 m*M* magnesium, and trace amounts of other divalent cations (Bayne *et al.*, 1976). Apparently, 0.6 m*M* EDTA chelating capacity did not remove all divalent cations from mussel hemolymph. However, its inhibitory effect was significant. It has been suggested that EDTA may not only serve as chelator, but also influence membrane permeability to divalent cations (Kenney *et al.*, 1972; Kanungo, 1982). In the present studies, EDTA-treated hemocytes aggregated weakly, and the inhibitory effect of EDTA on cohesive aggregation could be overcome by Ca^{++} or Mg^{++} supplements. Therefore, *Mytilus* hemocyte aggregation may proceed through two sequential stages. Weak aggregation is the early stage, and is Ca^{++}/Mg^{++}-independent. Cohesive aggregation is the late stage, and is Ca^{++}/Mg^{++}-dependent. Similar results were described for hemocyte aggregation in limpets (Davies and Partridge, 1972), and in *Limulus* (Kenney *et al.*, 1972), as well as for coelomocyte aggregation in holothurians (Fontaine and Lambert, 1977), and in sea stars (Kanungo, 1982). All of these hemocyte or coelomocyte aggregations are two-stage reactions. One is Ca^{++}/Mg^{++}-independent, and the other is Ca^{++}/Mg^{++}-dependent.

Retardation of hemocyte aggregation has been observed at reduced temperature (4°C) in *Mytilus* (this study), *Limulus* (Kenney *et al.*, 1972), and limpets (Davies and Partridge, 1972). It remains to be determined if this is due to altered fluidity of cell membranes, altered kinetics of metabolic reactions, or alternative mechanisms.

In summary, *Mytilus* hemocyte aggregation is a two-step process that is retarded at low temperature, and requires endogenous metabolic energy and divalent cations.

Based on these results and on aggregation studies in several other cell models (Davies and Partridge, 1972; Jumblatt *et al.*, 1980; Kanungo, 1982; Gibralter and Turner, 1985; Pizzey *et al.*, 1988), two-step cell aggregation mediated by divalent cations and temperature appears to have been well conserved during evolution.

A major goal of this study was to characterize distinctive features of self recognition (hemocyte-hemocyte binding) and non-self recognition (hemocyte-matrix binding). Because no aggregation-specific inhibitor was found, these results do not constitute strong evidence that cell aggregation and adhesion are two independent cell activities. However, some observations imply that this is likely. First, higher concentrations of inhibitor are necessary to block hemocyte aggregation than to affect hemocyte adhesion. Second, when reversible inhibitors such as caffeine and EDTA were removed or diluted, hemocyte aggregation occurred earlier than hemocyte adhesion.

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