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CHEMICAL ECOLOGY: A NEW APPROACH TO THE STUDY OF LIVING BENTHIC EPIPHYTIC FORAMINIFERA

VLADIMIR BRESLER AND VALENTINA YANKO

Institute for Nature Conservation Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

ABSTRACT

Interactions of living benthic epiphytic foraminifera, *Pararotalia spinigera* (*Le Calvez*) and *Rosalina macropora* (Hofker), with unidentified natural organic compounds (UNOC) derived from decomposed seaweeds, heavy metal ions and three tracer xenobiotics, acridine orange (AO), neutral red (NR) and fluorescein (FLU), were investigated. Changes of redox state of nicotinamide adenine dinucleotide (NAD) in foraminifera, induced by UNOC, indicated that these compounds contained both metabolic substrates and toxic components (natural uncouplers). The presence of toxic components in UNOC was confirmed by results of a dye extrusion test with FLU with cadmium ions acting as an uncoupler.

The following defense mechanisms against xenobiotics were discovered in foraminifera studied: (1) mucopolysaccharide coat that forms additive diffusion barriers and binds some cationic xenobiotics, (2) plasma membrane that forms impermeable diffusion barrier against natural and anthropogenic anionic xenobiotics, (3) membrane carrier-mediated transport system for elimination of anionic xenobiotics from the cell, (4) active intralysosomal accumulation and isolation of some cationic xenobiotics, (5) peroxidases that protect foraminiferal cytoplasm against an excess of oxygen and peroxides, (6) haloperoxidases that protect the cytoplasm against Br^- and I^- penetration and produce brominated or iodinated xenobiotics, and (7) metallthioneine-like Cu^{2+}-binding tryptophan-containing proteins protecting the foraminifera against some toxic metals.

Presence of UNOC in seawater decreased acute toxicity of heavy metal ions for foraminifera. The state of the defense system against xenobiotics in benthic epiphytic foraminifera can serve as a very sensitive biomarker for monitoring and prediction of ecological consequences of anthropogenic pollution.

INTRODUCTION

The historical significance of foraminifera in global biogeochemical cycles of inorganic and organic compounds, which makes them one of the most important group of organisms, is well documented (e.g., Haynes, 1981; Lipps, 1983; Anderson, 1988; Lee and Anderson, 1991). Many ecological characteristics of foraminifera, such as feeding strategies, trophic interactions, population dynamics and production, are well studied (e.g., DeLaca and others, 1981; Lipps, 1983; Alexander and DeLaca, 1987; Murray, 1991; Langer and Gehring, 1993). However, chemical interactions between foraminifera and other members of their community have not been thoroughly investigated.

Chemical ecology investigates the origin, chemical structure, function, and ecological significance of natural xenobiotics that participate in such interactions. A xenobiotic is any chemical that is not used by an organism for its energetic and plastic metabolism (Parke, 1971; Barbier, 1976; Pianka, 1978; Harborne, 1993). Xenobiotics that are synthesized by bacteria, fungi, plants and animals for protection against parasites, predators and competitors are termed natural; those made by human activity, anthropogenic. Chemical ecology demonstrates the important role of chemical interactions within and between species for ecosystem structure and stability (Barbier, 1976; Pianka, 1978; Steinberg, 1984; Rice, 1985; Whitman, 1988; Harborne, 1993). These interactions induce formation of various defense mechanisms against natural xenobiotics in all organisms during coevolution (Parke, 1971; Yu and others, 1979; Dethier, 1980; Bresler and others, 1985, 1990; Distel and Provenza, 1991; Harborne, 1993; Counotte and others, 1993). The adaptive significance of such antichemical defense mechanisms is enhanced by their ability to protect the animals against various anthropogenic xenobiotics (Parke, 1971; Krieger and others, 1971; Bresler and Nikiforov, 1981; Bresler and others, 1985, 1990; Bresler, 1989; Bresler and Fishelson, 1994).

The chemical ecology of protozoans has been poorly understood. The presence of certain antichemical defense mechanisms was reported in some Ciliophora and Mastigophora (Khan and others, 1972; Murphy and others, 1982; Piccinni and others, 1985, 1992; Piccinni, 1989; Burton, 1991) but not in foraminifera. However, benthic foraminifera frequently inhabit more complicated chemical microenvironments than freshwater protozoans, and so must have more complicated defense mechanisms. Indeed, antichemical defense systems in foraminifera, such as a transport system for xenobiotic elimination and a system of enzymatic transformation, were recently discovered by Yanko and others (1994a).

All plants, including marine algae, protect themselves chemically against pests and herbivorous animals (Barbier, 1976; Pianka, 1978; Steinberg, 1984; Gschevend and others, 1985; Rice, 1985; Harborne, 1993). For example, algae of the genus *Fucus* produce and release volative halogenated xenobiotics (Gschevend and others, 1985; Matera and Lee, 1972). Furthermore, various seaweeds are in close contact with both seawater and bottom sediment that contain a variety of natural bioactive chemicals. The main part of dissolved organic carbon (DOC) occurs as humic acids (Perdue and Gjessing, 1990; Burgess and others, 1993). DOC also contains small alkyl carboxylic, simple aromatic, amino, nucleic, uronic and muramic acids and wide clusters of natural microbial, algal or animal toxins (Barbier, 1976; Tuschall and Brezonic, 1980; Fenical, 1982; Gschevend and others, 1985; Fuhrmann and Fergusson, 1986; Wotton, 1988; Perdue and Gjessing, 1990). Many of these natural compounds are generated by decomposition of microalgae as well as

benthic organisms, especially degrading parts of seaweeds (Perdue and Gjessing, 1990). Interaction between foraminifera and seaweeds is probably protocooperation. Seaweeds depend on grazing organisms like foraminifera that clean their leaf surfaces of dense microalgal cover (Langer, 1993). Foraminifera use seaweeds as a food source. In addition, seaweeds may protect foraminifera from the influence of some chemicals. Organisms living in permanent contact with these alien natural compounds must be adapted to their presence.

DOC and some other natural dissolved organic compounds can alter the toxicity of many anthropogenic xenobiotics, including heavy metals (Ryan and others, 1983; Fu and others, 1992; Versteeg and Shorter, 1992; Burgess and others, 1993). Our preliminary data showed that these seaweed-derived natural compounds may affect the acute toxicity of heavy metals to benthic epiphytic foraminifera (Bresler and Yanko, 1994).

The main goal of our research is to study the chemical ecology of benthic epiphytic foraminifera. This includes the action of natural organic compounds derived from the seaweed on foraminiferal metabolic processes (energetic metabolism, participation in trophic links, potential toxicity, influence on acute toxicity of some metal ions, etc.) and defense mechanisms. A new methodological approach to study cytophysiological and cytochemical parameters of living foraminifera in culturing experiments was developed by Bresler and Yanko (1994) and Yanko and others (1994a).

All fundamental normal or pathologic biological processes start at molecular and subcellular level. Therefore, the study of biochemical, molecular and subcellular parameters as indicators of environmental actions intensified over the past decade. However, many of these biomarker studies dealt with determination of the secondary effects rather than systems immediately involved in adaptation and defending the cell from environmental chemical or physical stresses (Bresler, 1989; Bresler and others, 1990; Dyer and others, 1993).

Generally accepted staining methods with Rose Bengal or with Sudan Black B (Walker and others, 1974) for distinguishing living from dead foraminifera can only show the presence or absence of cytoplasm, but not its functional state. However, parameters such as metabolic state of mitochondria, activity of enzymes and hemoproteins involved in metabolism of xenobiotics, level of proteins involved in binding or stress proteins of xenobiotics, and state of transport systems which eliminate xenobiotics from cells and organisms can serve as highly sensitive bioindicators of chemical interactions (Bresler and others, 1975, 1979, 1983, 1989, 1990; Nebert and Jansen, 1979; Nikiforov and Bresler, 1984; Fowler, 1986; Engel and Roesijadi, 1987; Steinberg and others, 1987; Di Virgilio and others, 1988, 1990; Sanders and others, 1991; Dyer and others, 1993; Bresler and Fishelson, 1994; Bresler and Yanko, 1994). This set of methods allows assessment of health of studied organisms like medical investigations allow assessment of human health.

MATERIAL AND METHODS

The benthic epiphytic foraminifera, *Pararotalia spinigera* (*Le Calvez*) and *Rosalina macropora* (Hofker), were used for our experiments. Seaweeds *Sargassum, Cystoseira* and *Jania* together with sediment were collected in an unpolluted site from 2–2.5 m depth near Mikhmoret, Israel (about 50 km north of Tel Aviv). These seaweeds were transported in plastic tanks filled with natural seawater to the laboratory within 2–3 hrs and placed in an aerated 60-liter aquarium continuously illuminated by a 100 W luminescent bulb. The water temperature and pH were 22–25°C and 8.1–8.4, respectively.

Seawater transported to the laboratory was functionally colorless at the start of the experiment, but as *Sargassum* and *Cystoseira* thalli began to die off and decompose during first 3 weeks, seawater in the aquarium was discolored to yellow-brown by various plant-derived unidentified natural organic compounds (UNOC). This color may be produced by various DOC, including pigment fucoxanthin produced by diatoms. This discolored water was used for the experimental study of UNOC-action on foraminiferal metabolism, antichemical defense and sensitivity to some metal ions.

Absorbance at 436 nm is usually used for DOC quantification (Perdue and Gjessing, 1990). Therefore, we used this technique to assess the concentration of plant-derived UNOC in the seawater. The optical density of colorless superficial seawater (seawater without UNOC) at 436 nm was 0.04 ± 0.017. The yellow-brown colored seawater (seawater with UNOC) from the bottom of aquarium had an optical density of 0.74 ± 0.08 (mean $\pm$ 95% confidence limits, $n = 10$ in both cases) after 3 weeks. We used seawater with UNOC as the experimental analog of natural plant-derived UNOC that is present around roots and rhizomes of seaweeds in natural bottom seawater.

Pieces of seaweed thalli 2–3 cm long were removed from the aquarium and placed in 100 mm glass Petri dishes. Both "intact" foraminifera (attached to the seaweed thalli) and, particularly, "detached" foraminifera (delicately removed by fine brush from their natural substratum) were used in our experiments. In some experiments we used "detached" foraminifera immediately after their detachment (freshly "detached" foraminifera). They lost some pseudopodia during their detachment and retracted the others. Vital cytotoxicity assay with neutral red (NR) showed marked signs of reversible cellular damage in these foraminifera: decrease or absence of NR accumulation in lysosomes of all specimens and slow diffuse staining of cytoplasm of some specimens. However, the normal level of intralysosomal NR accumulation was restored in adult specimens during the following 24 hrs (Yanko and others, 1994a). Therefore, we used mainly adult "detached" foraminifera that were detached 24 hrs before the start of the experiments (normal "detached" foraminifera). Twenty specimens of "detached" foraminifera and 10 small pieces of *Sargassum* and *Cystoseira* thalli (about 5×5 mm) together with "intact" foraminifera were transferred to 50 mm glass Petri dishes, and covered by filtered seawater. Seawater was filtered to remove sediment particles. We designated seawater as "clean" if no markers, substrates, inhibitors, or metals were added, and simply as "seawater" if some chemicals were added.

The number of foraminifera used in each experiment is given in figure legends. The total number of foraminifera used in this study was 2,000 specimens of *Pararotalia spinigera* and 300 specimens of *Rosalina macropora*. The fol-

lowing biophysical, cytophysiological and cytochemical methods were used in our study:

(1) Measurement of blue fluorescence of reduced nicotinamide adenine dinucleotide (NAD·H) (excitation at 365 nm, emission at 420–450 nm), was used to determine the metabolic state of mitochondria and their reaction to various interferences (Lehninger, 1972; Franke and others, 1980; Nikiforov and Bresler, 1984; Bailey and Ollis, 1987). Five typical metabolic states of mitochondria and corresponding redox states of NAD [flavine adenine dinucleotide (FAD), cytochroms c, b, a, in respiratory chain and level of ADP and substrate] are well studied (Lehninger, 1972; Franke and others, 1980). For example, in the anoxic state (state V), 100% of NAD is reduced and blue fluorescence is maximal; during the state of metabolic rest (state IV), 90% of NAD is reduced; during the state of active work (state III), about 50% of NAD is reduced and blue fluorescence is correspondingly decreased. Therefore, measurement of blue fluorescence coupled with known anoxic conditions and known inhibitors of the respiratory chain in substrates such as glucose, succinate and pyruvate allow investigation of the metabolic state of mitochondria in living foraminifera. Transitions from aerobic to anoxic and again to aerobic conditions as well as additions of glucose (a known substrate), 2,4-dinitrophenol (DNP) (a well known uncoupler), cadmium ions (potential uncoupler), or UNOC were used. Preliminary experiments demonstrated that mitochondria of both "intact" and "detached" foraminifera had a metabolic IV state.

For the experiments with transitions from aerated to anoxic and back to aerobic conditions, a flowing microcamera (that can be air-tight) was used. Small pieces of seaweed thalli with "intact" foraminifera were placed into a hermetically sealed microcamera; if the camera was lit before fluorescence measurements, oxygen was produced by photosynthesis, and aerobic conditions were supported. On the other hand, if the camera was incubated in darkness, photosynthesis was suspended, oxygen consumption was continued by plant and animal cells, and anoxic conditions were produced. Aerobic or anaerobic conditions were controlled in preliminary experiments according to Thorell and Chance (1959) by determination of typical spectral alteration in living erythrocytes (in their hemoglobin). Oxidized hemoglobin has two maxima of absorption at 540 and 580 nm, reduced hemoglobin has one maximum at 555 nm. We also observed formation of numerous small glittering bubbles of oxygen by seaweeds and microalgae during light irradiation, direct evidence of photosynthetic production of oxygen in this condition. In the same way, "detached" foraminifera with a drop of aerated "clean" seawater were placed in a hermetically sealed flowing microcamera, and in these the oxygen consumption by the animals produced anoxia during incubation. Flow of aerated water through the camera produced aerobic conditions following the anoxia.

(2) Inherent ultra-violet (UV) fluorescence of tryptophan-containing proteins (excitation at 253 nm, emission at 320–380 nm) in living foraminifera was used to measure their binding with copper ions (Engel and Broawer, 1987).

(3) Vital staining with a metachromatic fluorescent probe, acridine orange (AO), was used to expose mucopolysaccharides (glycosaminoglycans) and to determine both lysosomes and cell viability (Wittekind, 1973; Swanson, 1989; Bresler and Yanko, 1994). AO was used at 10 μM concentration in seawater.

(4) Vital staining with neutral red (NR) was used for measurement of the state of both lysosomes and cell viability (Crippen and Perrier, 1974; Dierickx and Van De Vyver, 1991; Saito and others, 1991; Jacobson and others, 1993 Bresler and Yanko, 1994). NR was added to the seawater to produce a final concentration of 0.002% (about 7.0 nM). The number of foraminifera with small red granules in the cytoplasm (living) and the number of unstained or intensive diffuse stained animals (dead) was calculated after 24 hrs incubation. In some cases, NR accumulation was measured by microspectrophotometry (Bresler and Yanko, 1994).

(5) Determination of plasma membrane permeability to acid organic dye fluorescein (FLU), the dye extrusion test, was used to distinguish "intact" foraminifera from damaged ones (Crippen and Perrier, 1974; Pieters and others, 1989; Bresler and Fishelson, 1994; Bresler and Yanko, 1994). FLU was added to the seawater at final concentration of 100 μM, and after 24-hrs incubation was determined in foraminiferal cytoplasm by fluorescent microscopy and microfluorometry.

(6) Measurement of the ability of intracellular esterase to hydrolyze fluorogenic substrates (fluorescein diacetate, FDA, or fluorescein dibutyrate, FDB), was used to study the liberated FLU run-out, or for determination of cell viability (Rotman, 1973; Janssen and Persoone, 1993; Bresler and Yanko, 1994). Both FDA and FDB were used at 1 μM concentration in the seawater. FLU, liberated into foraminiferal cytoplasm, was scored visually under a fluorescent microscope. Foraminifera were classified either as fluorescent (living) or non-fluorescent (dead) (Rotman, 1973; Yanko and Bresler, 1994). Both FDA and FDB showed similar results (Bresler and Yanko, 1994). Liberated FLU was measured by microfluorometry immediately after transferring foraminifera to the seawater and again one hour later.

Kinetics of free FLU run-out from foraminiferal cytoplasm were studied in special experiments with three equal groups of normal "detached" foraminifera. The first group was treated with FDB (1 μM) for 15 minutes, washed in "clean" seawater for 10 minutes, treated with FDB (1 μM) again, and then washed and placed again into "clean" seawater for microfluorometrical examinations. The second group was preloaded with 5 μM probenecid, a known inhibitor of organic anion transport (Di Virgilio and others, 1988, 1990; Bresler and others, 1989, 1990) for 2 hours, and then treated with FDB (1 μM) two times as before and placed again into "clean" seawater. The third group was incubated with probenecid (0.25 μM), then treated with FDB (1 μM) and placed into the "clean" seawater. The fluorescence intensity of the cytoplasm was examined immediately after washing and replacement of all three groups into the "clean" seawater. The measurement was repeated for the first group of animals five times: at 1, 2, 3, 4, and 24-hour intervals. Measurements of both the second and third groups were repeated three times: at 1, 2 and 3-hour intervals.

(7) Cytochemical determination of peroxidase activity with benzidine was used to expose the presence and location of this activity (Pearse, 1968).

(8) Haloperoxidase activity was measured by FLU bro-

moderivative determination in "detached" foraminifera before and after 4-hours contact with liberated FLU, as well as in crude homogenate from "detached" foraminifera. The activity was measured with the microspectrophotometrical plug method by manual scanning of the object (Bresler and others, 1990), and haloperoxidase activity was expressed as increase of optical density at 520 nm. A known peroxidase inhibitor, sodium azide (Pearse, 1968; Lehninger, 1972; Dixon and Webb, 1979), was used for the control determinations. FLU and Br^- (both at a final concentration 100 μM) were added to the crude homogenate. Hydrogen peroxide or hydrogen peroxide and sodium azide (all of them at the final concentration 1 mM) were also added during other experiments.

DEVICES

A special flexible microfluorometer, with a photomultiplier tube in the measuring head, a rectangular measuring diaphragm and two changeable excitation light sources, was constructed. A pencil-style, mercury-argon lamp and high-grade fused silica bundle for lateral (dark field) illumination during microfluorometry were used to measure blue and UV fluorescence of living foraminifera. The stabilized quartz-tungsten halogen lamp was used as an excitation light source for both fluorescent microscopy and microfluorometry in a visible region. A combination of dichroic mirror, excitation and barrier light filters cut off all background light and allowed only the light of fluorescence to the photomultiplier tube (Bresler and others, 1975, 1979, 1990; Nikiforov and Bresler, 1984). The technique of microfluorometry has been previously described by Bresler and others (1975, 1979, 1990). Data from microfluorometry were usually expressed in arbitrary units (a.u.), i.e., μA of photocurrent. For the standardization and calibration in the units of concentration, microcuvettes and fluorochrome solutions of known concentration were used (Bresler and Nikiforov, 1977; Bresler and others, 1990). The control measurements for FLU were taken in the microcuvette with 0.1 mm depth that corresponds to the mean thickness of the foraminifera. The luminescence intensity of FLU solution in this microcuvette depended linearly on its concentration in the range of 0.005–2 mM. Fluorescence intensity of 100 μM FLU solution in the microcuvette was adjusted to 75 ± 5 a.u. and it was constant during the entire working time.

The device for the microspectrophotometric plug method with manual scanning of the object was described previously by Bresler and others (1990). Four different cameras from each animal and the next vacant area (background) were alternatively combined with the measuring diaphragm for the determination of FLU transformation to its bromoderivatives by haloperoxidases. This was studied both in the cytoplasm of foraminifera and in the crude homogenate of foraminifera in a special optical microcuvette. For each pair of measurements, the value of the transmittance coefficient (τ) was determined at 520 nm. Also, the transmittance coefficient for the same foraminifera before bromoderivative formation was measured to make an allowance for light absorbed and dissipated by foraminiferal structures themselves.

Optical density was calculated from these values. The difference between optical density of the specimen before and after FLU transformation to its bromoderivatives (ΔD) was quantitatively proportionate to the dye amount in the cytoplasm. In the experiments with the crude homogenate, the optical densities before and after incubation were made from microcuvette. The difference between these data (D) was quantitatively proportionate to the amount of synthesized bromoderivatives of FLU. All data obtained by a microfluorometry and microspectrophotometry were computed and presented as a mean at the ±95% confidence level.

RESULTS

The study of thalli of living seaweeds *Sargassum, Cystoseira* and *Jania* under a dissecting microscope demonstrated the presence of a species-rich community that contained ciliates, nematodes, polychaetes, small crustaceans and their larvae, veligers and young mollusks as well as numerous epiphytic attached foraminifera such as *Pararotalia spinigera* (Le Calvez), *Rosalina macropora* (Hofker) and *Adelosina cliarensis* (Heron-Allen and Earland). These foraminifera usually dwell among the sediment particles, organic detritus, coralline red algae, green microalgae, bacteria and diatoms which cover the surface of the thalli.

Blue Fluorescence

Blue fluorescence of the living "intact" foraminifera *Pararotalia spinigera* was increased after incubation in darkness and decreased again after lighting (Fig. 1a). During lighting, numerous small glittering gas bubbles were produced by plant cells and often located in close contact with attached foraminifera. After dark periods no such bubbles were seen. Blue fluorescence of NAD·H in "intact" foraminifera was reversible when caused by addition of glucose, following a relatively long lag-period after washing (Fig. 1b). It was irreversibly decreased (also following a relatively long lag-period after washing) by DNP or cadmium ions (Fig. 1c, d). A marked reversible decrease of blue fluorescence in living foraminifera from "clean" seawater without UNOC was produced by the seawater with UNOC (Fig. 1e).

Blue fluorescence of NAD·H in living "detached" foraminifera was increased during incubation in the hermetically sealed microcamera, and was decreased again by flow of oxygenated water (Fig. 2a). Reversible decreases of blue fluorescence intensity after a relatively long lag-period were produced by glucose (Fig. 2b). DNP and cadmium ions produced irreversible decreases of NAD·H fluorescence (Fig. 2c, d). A marked irreversible decrease of blue fluorescence in freshly "detached" foraminifera was produced by seawater with UNOC; however, normal "detached" foraminifera showed a reversible decrease (Fig. 2e, f).

UV Fluorescence

Inherent UV fluorescence of the normal "detached" foraminifera, *Pararotalia spinigera,* was decreased in a dose-dependent manner between 1 and 10 μM Cu^{2+} dissolved in the seawater with UNOC, and it was stopped at 50% level to control at Cu^{2+} concentrations between 100 and 1000 μM (Fig. 3a). However, a similar (50%) decrease of UV fluorescence of normal "detached" foraminifera was produced

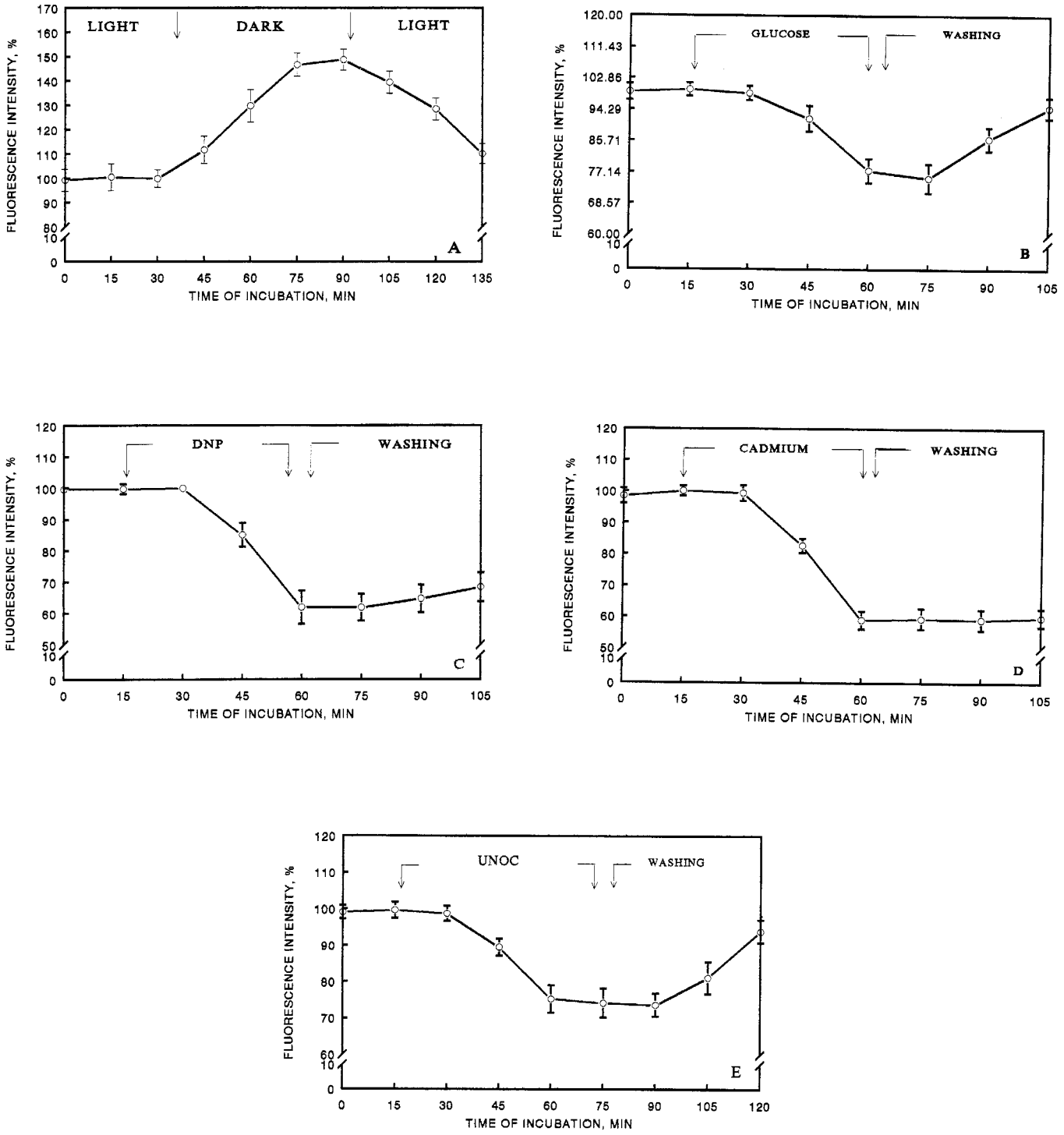

FIGURE 1. Dynamics of alterations of blue fluorescence in living "intact" *Pararotalia spinigera*: A. During light-dark-light incubation, i. e. under aerobic-anaerobic-aerobic conditions; B. After adding of glucose to final concentration of 100 μM and after washing; C. After adding 2,4-dinitrophenol (DNP) to final concentration of 10 μM and after washing; D. After adding of Cd^{2+} to final concentration of 0.6 μM and after washing; E. After action of seawater with UNOC and after washing. Note: Each point is a mean of separate measurements of 20 specimens, ±95% confidence limit. A total of 100 specimens were used in experiments A–E. Experiments A–D were performed in seawater without UNOC.

between 5 and 50 μM Cu^{2+} dissolved in the seawater without UNOC (Fig. 3b), which means that most of the 1,000 μM Cu^{2+} present in the seawater with UNOC was bound by UNOC, and only about 5 μM Cu^{2+} can interact with tryptophan-containing proteins in foraminiferal cytoplasm.

VITAL STAINING WITH ACRIDINE ORANGE

Vital staining with AO and fluorescent microscopy demonstrated the presence of a friable fur coat or extracellular matrix (ECM) with pale red fluorescence around pseudo-

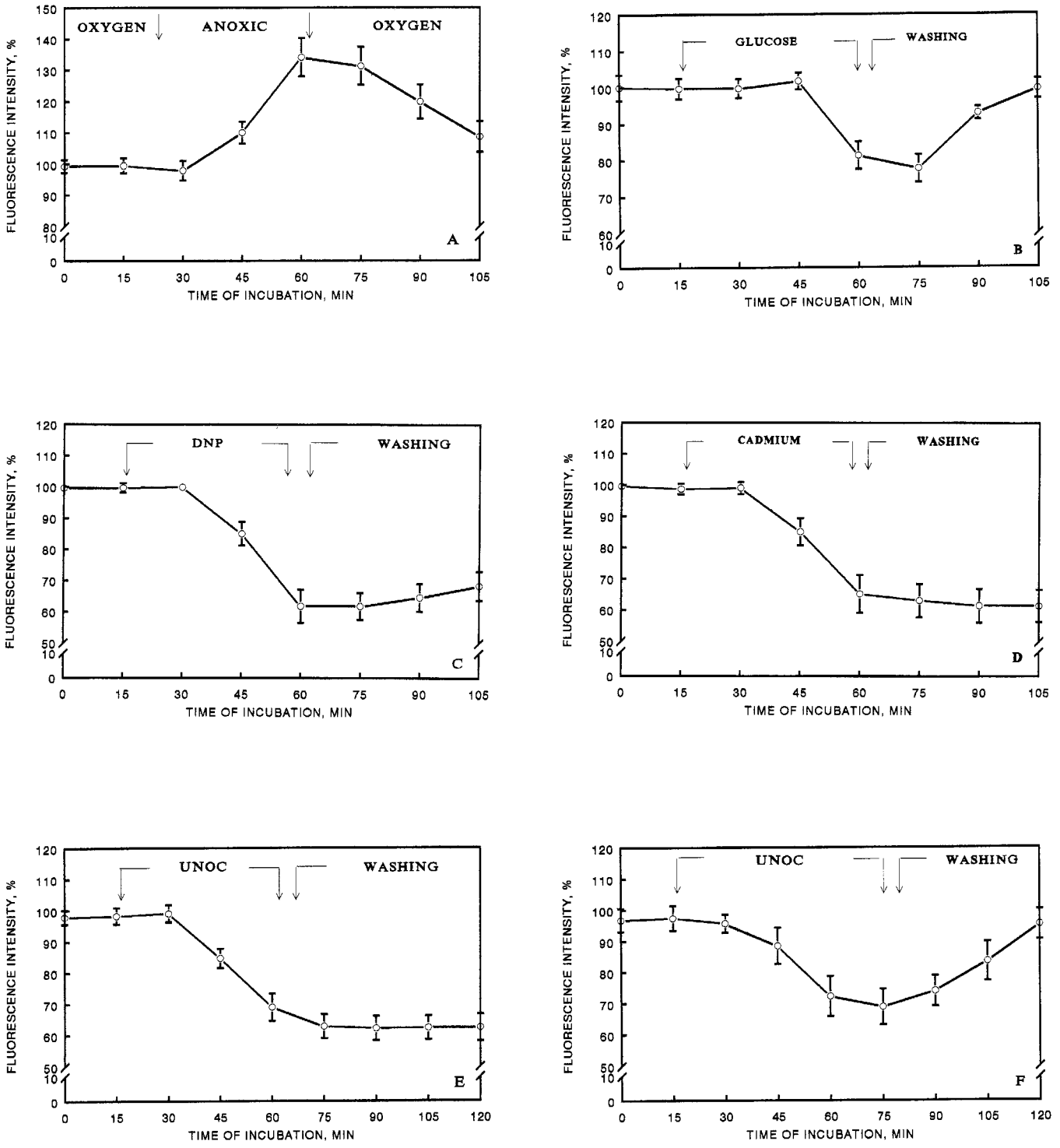

FIGURE 2. Dynamics of alteration of blue fluorescence in living "detached" *Pararotalia spinigera*: A. Under aerobic-anaerobic-aerobic conditions; B. After adding glucose to final concentration of 100 μM and after washing; C. After adding 2,4-dinitrophenol (DNP) to final of concentration 10 μM and after washing; D. After adding Cd^{2+} to final concentration of 0.6 μM and after washing; E. After action of seawater with UNOC on freshly "detached" foraminifera and after washing. F. After action of seawater with UNOC on normal "detached" foraminifera and after washing. Note: Each point is a mean of separate measurements of 20 specimens, ±95% confidence limit. A total of 120 specimens were used in experiments A–F. Normal "detached" foraminifera were used almost everywhere except experiment E where freshly "detached" foraminifera were investigated. Experiments A–D were performed in seawater without UNOC.

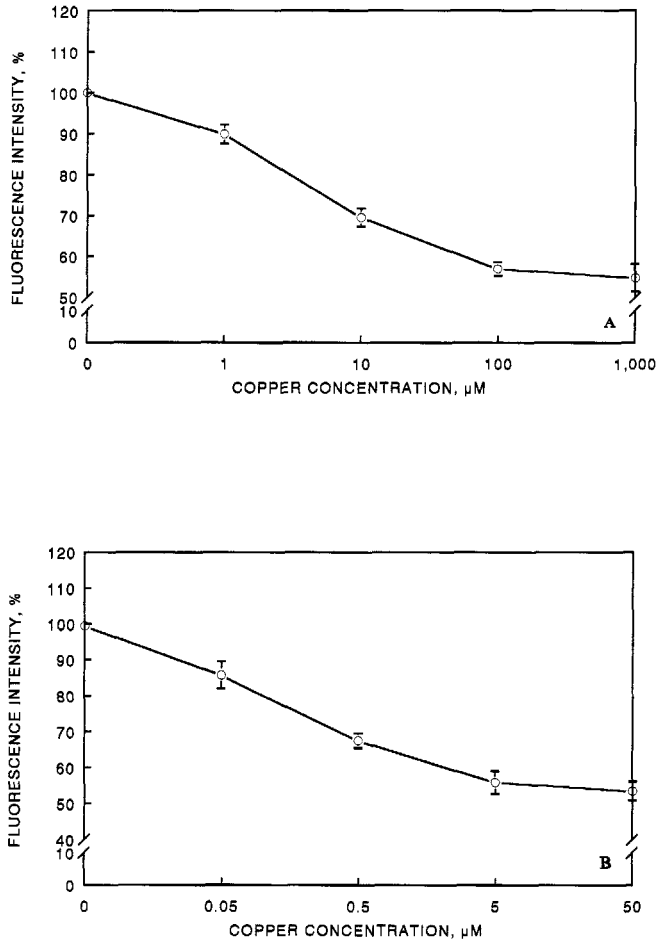

FIGURE 3. Action of Cu^{2+} dissolved in seawater on UV fluorescence of living normal "detached" *Pararotalia spinigera*: A. Cu^{2+} dissolved in seawater with UNOC. B. Cu^{2+} dissolved in seawater without UNOC. Note: Each point is a mean of separate measurements of 20 specimens, ±95% confidence limit. A total of 200 specimens were used in experiments A and B.

podia of "intact" foraminifera of both *Pararotalia spinigera* and *Rosalina macropora,* and a more compact similar substance that glues these animals to the thalli. This ECM often also glued and accumulated small mineral grains of sediment, bacteria, and algae.

Foraminiferal cytoplasm had green fluorescence with numerous small red granules. AO also stained other members of this community.

Normal "detached" foraminifera of both species exhibited this coat with red fluorescence after vital staining with AO dissolved in both seawater without UNOC and with UNOC. Some animals were glued to glass by a similar red substance. Cytoplasm of "detached" foraminifera had green fluorescence and contained numerous small red granules. However, freshly "detached" foraminifera incubated in the seawater with UNOC and with AO showed a marked decrease or absence of small red granules in their cytoplasm.

VITAL STAINING WITH NEUTRAL RED

"Intact" and "detached" foraminifera of both species demonstrated the presence of numerous small red granules

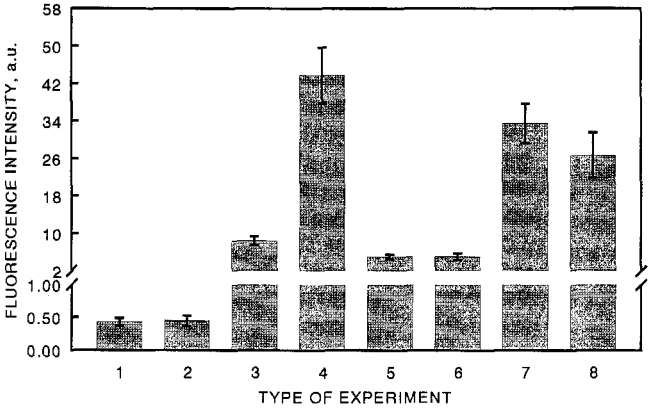

FIGURE 4. Permeation of fluorescein (FLU) into the cytoplasm of "intact" and "detached" *Pararotalia spinigera*: 1. "Intact" foraminifera incubated with FLU in seawater with UNOC; 2. Normal "detached" foraminifera incubated with FLU in seawater without UNOC; 3. Freshly "detached" foraminifera incubated with FLU in seawater without UNOC; 4. Freshly "detached" foraminifera incubated with FLU in seawater with UNOC; 5. Action of 1 μM Hg^{2+} dissolved in seawater with UNOC on FLU penetration into normal "detached" foraminifera; 6. Action of 10 μM Cd^{2+} dissolved in seawater with UNOC on FLU penetration into normal "detached" foraminifera; 7. Action of 0.07 μM Hg^{2+} dissolved in seawater without UNOC on FLU penetration into normal "detached" foraminifera; 8. Action of 0.6 μM Cd^{2+} dissolved in seawater without UNOC on FLU penetration into normal "detached" foraminifera. Note: In all these experiments FLU concentration was 100 μM, time of incubation 24 hrs. Each bar is a mean of separate measurements on 45 specimens, ±95% confidence limit. A total of 360 specimens were used in experiments 1–8.

in the cytoplasm after vital staining with NR dissolved both in the seawater with UNOC or without UNOC. Freshly "detached" foraminifera incubated for 24 hrs in seawater with UNOC and added NR demonstrated a marked decrease or absence of red granules in their cytoplasm. Some of these freshly "detached" animals demonstrated intensive diffuse staining of the cytoplasm and nuclei. Intensive diffuse staining of the cytoplasm and nuclei by NR signifies cell death. Therefore, UNOC contains toxic compound(s) that in freshly "detached" foraminifera can penetrate through the damaged plasma membrane and kill some specimens.

PLASMA MEMBRANE PERMEABILITY TO FLUORESCEIN

Permeability of the foraminiferal plasma membrane to FLU (dissolved in seawater with UNOC or without UNOC) was studied in both "intact" and "detached" specimens of *Pararotalia spinigera* and *Rosalina macropora.* After incubation with FLU for 24 hours, no marked fluorescence of foraminiferal cytoplasm was detected: the mean fluorescence intensity of foraminifera was in all cases <1 a.u. (Fig. 4). However, if freshly "detached" foraminifera were incubated with FLU for 24 hours, the mean fluorescence intensity of the cytoplasm was about 10 a.u. and more than 40 a.u. in the seawater without UNOC and with UNOC, respectively (Fig. 4).

ABILITY OF INTRACELLULAR ESTERASES TO HYDROLYZE FLUOROGENIC SUBSTRATES AND CYTOCHEMICAL DETERMINATION OF PEROXIDASE AND HALOPEROXIDASE ACTIVITY

Incubation of both "intact" and "detached" foraminifera of both species with FDA or FDB for 15 minutes in sea-

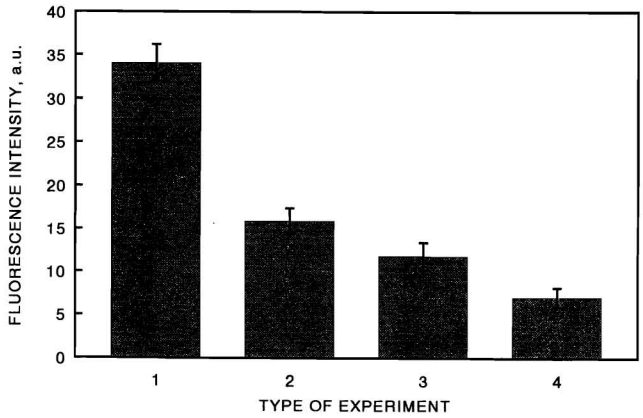

FIGURE 5. Enzymatic hydrolysis of fluorescein dibutirate (FDB) and future fate of liberated fluorescein in "intact" and "detached" *Pararotalia spinigera*: 1. "Intact" foraminifera incubated with FDB for 15 min, and then washed for 10 min in "clean" seawater; 2. "Intact" foraminifera incubated with FDB for 15 min, and then washed for 1 hr in "clean" seawater; 3. Normal "detached" foraminifera incubated with FDB for 15 min, and then washed for 10 min in "clean" seawater; 4. Normal "detached" foraminifera incubated with FDB for 15 min, and then washed for 1 hr in "clean" seawater. In all these experiments FDB final concentration was 1 μM. Each bar is a mean of separate measurements of 20 specimens, ±95% confidence limit. A total of 80 specimens were used in experiments 1–4.

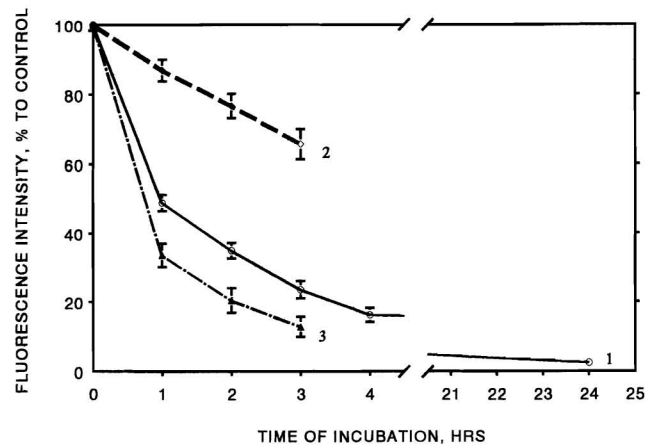

FIGURE 6. Dynamics of fluorescein run-out from cytoplasm of normal "detached" *Pararotalia spinigera*: 1. Incubation with FDB (1 μM), and then washing in "clean" seawater, each point representing a mean of separate measurements of 80 specimens, ±95% confidence limit; 2. Preincubation with probenecid (5 μM), then incubation with FDB (1 μM), and then washing in "clean" seawater, each point representing a mean of separate measurements of 60 specimens, ±95% confidence limit; 3. Incubation with FDB (1 μM), and then washing in "clean" seawater with 0.25 μM probenecid, each point representing a mean of separate measurements of 60 specimens, ±95% confidence limit.

water with UNOC or without UNOC induced the liberation of FLU into the cytoplasm of all the investigated animals. "Intact" foraminifera contained more free FLU than normal "detached" specimens; although about half of free FLU was eliminated from both groups during one hour (Fig. 5).

The kinetics of free FLU run-out in foraminifera from the first group is hyperbolic (Fig. 6). FLU run-out rate was significantly reduced in specimens from the second group (preloaded with probenecid), and slightly increased in animals from the third group (incubated with probenecid).

The control microscopic study revealed that foraminiferal cytoplasm was very weakly stained red after prolonged contact with liberated FLU. Microspectrophotometry showed a statistically significant increase of optical density in such foraminifera. If 1 mM sodium azide was added to the seawater, no increase of optical density was observed (Fig. 7).

The highest haloperoxidase activity in crude homogenates from foraminifera was detected in the probe that contained 100 μM Br^-, 100 μM FLU and 1 mM hydrogen peroxide. The activity decreased markedly if the hydrogen peroxide was absent, and in the presence of 1 mM sodium azide the activity was completely inhibited (Fig. 7).

The study of peroxidase activity in normal "detached" *Pararotalia spinigera* and *Rosalina macropora* showed that the former had more intensive peroxidases activity than the latter, although numerous small blue granules were detected in the cytoplasm of all investigated specimens. However, if 1 mM sodium azide was added to the incubation medium, such blue granules were absent in both species.

DISCUSSION

Vital microscopy shows that pseudopodia of epiphytic attached foraminifera *Pararotalia spinigera* and *Rosalina macropora* are partly covered by particles of sediment, organic detritus, diatoms and bacteria that resemble the canal systems in the rotaliid foraminifera (Röttger and other, 1984). Vital staining with AO shows that the surface of the pseudopodial net binds AO, which acquires under this condition red fluorescence. Such reaction is typical for so called acid mucopolysaccharides. Therefore, the cytoplasmic bod-

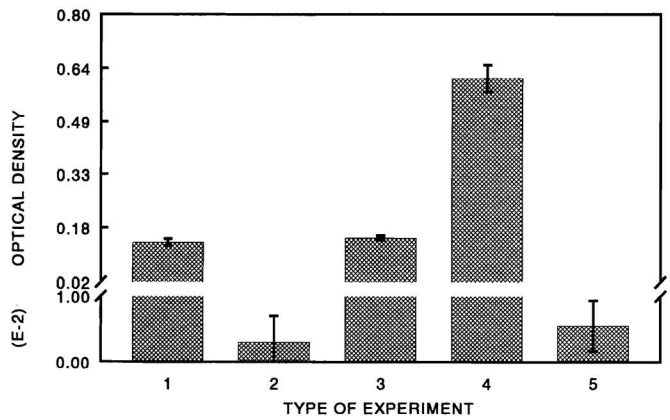

FIGURE 7. Haloperoxidases activity in living normal "detached" foraminifera *Pararotalia spinigera* and in crude homogenate from them: 1. Optical density at 520 nm of foraminifera after 4 hrs contact with liberated fluorescein; 2. Optical density at 520 nm of foraminifera after 4 hrs contact with liberated fluorescein in presence of 1 μM azide, each bar in (1) and (2) representing a mean of 80 separate measurements of 20 specimens, ±95% confidence limit; 3. Optical density at 520 nm of crude homogenate from 20 specimens after 4 hrs contact with 100 μM fluorescein in presence of 100 μM Br^-; 4. Optical density at 520 nm of crude homogenate from 20 specimens after 4 hrs contact with 100 μM fluorescein in presence of 100 μM Br^- and 1 μM hydrogen peroxide; 5. Optical density at 520 nm of crude homogenate from 20 specimens after 4 hrs contact with 100 μM fluorescein in presence of 100 μM Br^-, 1 μM hydrogen peroxide and 1 μM azide. Note: Time of incubation is 4 hrs. Each bar in 3–5 is a mean of 5 separate experiments, ±95% confidence limit. A total of 360 specimens were used in experiments 1–5.

ies of both species are covered by a mucopolysaccharide coat or ECM that glues their shells to the substrate and binds together numerous sediment particles, bacteria, organic detritus, and microalgae. Many foraminiferal species produce mucopolysaccharides (glycosaminoglycans) which play a significant role in their biology and ecology (Langer, 1992; Langer and Gehring, 1993). All eucaryotic cells have a mucopolysaccharide coat (glycocalyx) and/or extracellular matrix which forms an unstirred layer near the plasma membrane and can influence or transport solutes in the cell (Kotyk and Janacek, 1977; Counotte and others, 1993; Bresler and Fishelson, 1994). These structures in some eukariotic cells play a protective role (Counotte and others, 1993; Triebskorn and others, 1991; Bresler and Fishelson, 1994). Our data demonstrate the protective functions of such mucopolysaccharide coats in epiphytic attached foraminifera.

Both a relatively slow rate of AO or NR accumulation by foraminifera, together with a relatively long lag-period for the reactions of NAD·H on metabolic substrates and inhibitors, testifies that this coat forms an unstirred layer and an additional diffusion barrier. This barrier reduces the diffusion rate of various compounds across the plasma membrane.

Binding of AO by a mucopolysaccharide coat reflects the presence of numerous negatively charged groups which can also firmly bind some cationic organic compounds or metal ions. An important role of mucopolysaccharides in defense against inorganic and organic xenobiotics was described previously in various invertebrates and vertebrates (Segner and others, 1988; Arillo and Melodia, 1990; Triebskorn and others, 1991; Bresler and Fishelson, 1994).

Both AO and NR as well as other cationic xenobiotics (including some heavy metals) are accumulated by lysosomes (Wittekind, 1973; Swanson, 1989; Jenner and others, 1991; Bresler and Fishelson, 1994). This accumulation is determined by the pH gradient between lysosomes and cytoplasm (Swanson, 1989). Both decrease of cellular energetics and structure decrease proportionally lysosomal accumulation. Lysosomes play an important role in phagocytosis and exocytosis, cellular digestion, membrane recycling, intracellular regeneration, and cell pathology. Secondary lysosomes, especially in some protozoa, eliminate digested material from the cell and probably can eliminate accumulated xenobiotics (Swanson, 1989). Our data indicate that small lysosomes of foraminifera accumulate and isolate marker cationic xenobiotics, AO and NR. Cell damage decreases such intralysosomal accumulation. Therefore, the tests with both AO and NR suggest that incubation of freshly "detached" foraminifera with UNOC produces marked damage and even death to some of them.

Experiments with FDA and FDB demonstrated that foraminifera examined here have a clear esterase activity. The differences in the level of FLU liberation between "intact" and "detached" foraminifera might be attributed to diminished pseudopodial surface area in the "detached" foraminifera.

It is important to emphasize that liberated FLU was eliminated from foraminifera. Kinetics of free FLU run-out from cytoplasm is hyperbolic and can be satisfactorily described by the equation:

$$\mathrm{Ig}\ I_o/I_i = k \cdot t/2.303,$$

where I_o is mean fluorescence intensity immediately after loading with FDB, I_i is mean fluorescence intensity after washing in "clean" seawater for time t, and k is the rate constant for run-out process (which is equal to 0.693 for the first group). This equation is widely used for descriptions of kinetics of first-order reactions, including enzymatic reaction and carrier-mediated transport (Kotyk and Janacek, 1977; Dixon and Webb, 1979).

Inhibition of run-out by probenecid, a known inhibitor system of active transport of organic anions (SATOA) when present on the cys-side and slow stimulation when present on the trans-side, indicates that this run-out is a carrier-mediated transport process. Similar carrier-mediated run-out of another anionic fluorescent compound (Fura-2) was discovered in macrophages, pancreatic beta-cells and some other cell lines (Steinberg and others, 1987; Di Virgilio and others, 1988, 1990; Arkhammar and others, 1989). These cellular transport systems are similar to SATOA found in the renal proximal tubules, choroid plexus and liver of vertebrates, and in Malpighian tubules of insects and other organisms (Bresler and others, 1975, 1979, 1981, 1989, 1990; Bresler and Nikiforov, 1981; Bresler and Fishelson, 1994; Maddrell, 1977; Phillips, 1981). The presence of similar carrier-mediated transport systems for elimination of anionic xenobiotics in foraminifera, single mammalian cells and some organs of invertebrates and vertebrates testifies to the biological importance of such systems for ecological adaptation and defense (Bresler, 1989; Bresler and others, 1990).

Experiments with FLU demonstrate that both "intact" and "detached" foraminifera are impermeable for this anionic marker. However, freshly "detached" or, especially, freshly "detached" and incubated with UNOC specimens, have a clear increase of plasma membrane permeability. Mechanical damage of this membrane in freshly "detached" animals permits UNOC to penetrate into the cells and, therefore, unmasks toxicity of some UNOC. Heavy metal ions also produce a marked increase of permeability by both primary and secondary damage of plasma membrane. Presence of UNOC in seawater decreases dramatically the toxicity of heavy metal ions and the action of some of them on inherent UV fluorescence. We demonstrated previously that, at these increased UNOC concentrations, it kills 50% specimens (so called LC50) in a dose-dependent manner (Bresler and Yanko, 1994). Decrease of acute toxicity of heavy metals and some organic xenobiotics by DOC was described also by other investigators (Ryan and others, 1983; Fu and others, 1992; Versteeg and Shorter, 1992; Burgess and others, 1993). All these data indicate that DOC and other UNOC (that derived from decomposed seaweeds) might bind some xenobiotics, especially metal ions, and so decrease their real concentration in seawater.

We mentioned above that anionic dyes are widely used as a test for cell viability. Our data show that this test demonstrated the effects more weakly than might be expected. For example, both Hg^{2+} and Cd^{2+} in doses equal LC50 in seawater without UNOC produce effects that are less than 50% (see Fig. 4, and Bresler and Yanko, 1994). These results verify that systems for anionic xenobiotic elimination probably eliminate a certain part of the penetrated FLU.

Epiphytic attached foraminifera can transform some xenobiotics to their haloderivatives. Such transformation may occur in the presence of haloperoxidases in various marine invertebrates and algae (Theiler and others, 1978; Ahern and others, 1980; Van Pee and Lingens, 1985). Haloperoxidases can oxidize halogens to the powerful oxidants and free hypohalous acids (such as HOBr) that can react with thiols, thioethers, aromatics, amines, amino acids and other reactive molecules. These reactions can modify the biological activity of corresponding compounds (Theiler and others, 1978; Ahern and others, 1980; Van Pee and Lingens, 1985; Weiss and others, 1986). Thus, haloperoxidases were detected by us in benthic epiphytic foraminifera. Other investigators reported this in marine bacteria, algae, invertebrates and some mammalian cells (Van Pee and Lingens, 1985; Jong and others, 1981; Weiss and others, 1986). These haloperoxidases, together with products of their activity, are common ancient anti-pest and anti-predator mechanisms in both pro- and eukaryotic organisms (Barbier, 1976; Jong and others, 1981; Fenical, 1982; Van Pee and Lingens, 1985; Weiss and others, 1986). However, it is also possible that the primary function of haloperoxidases is to protect marine organisms against biologically active Br^- and I^- that can easily penetrate through plasma membranes.

Data presented here demonstrate that *Pararotalia spinigera* and *Rosalina macropora* have a high peroxidase activity in their cytoplasm. Peroxisomes were previously detected in some planktic foraminifera (Anderson and Tuntivate-Choy, 1984), and peroxidases are ancient, general defense enzymes that protect the cells against excess oxygen and peroxides, transferring peroxide oxygen to oxidized compounds (Dixon and Webb, 1979; Lehninger, 1972; Pearse, 1968). Benthic epiphytic foraminifera are in close contact with free oxygen produced by seaweeds and algae. Therefore, protection against oxygen and peroxides may be very important for their survival. The peroxidases in their cytoplasm may also metabolize some xenobiotics and modify their toxicity (Parke, 1971; Plewa and others, 1993).

Study of the redox state of NAD in epiphytic foraminifera demonstrates that the respiratory chains of both "intact" and "detached" foraminifera are sensitive to anoxia. Under anaerobic conditions a marked reduction of NAD (metabolic state V) was observed. The return to aerobic conditions brought oxidation of NAD·H to initial levels (metabolic state IV). A marked oxidation of NAD·H in both "intact" and "detached" foraminifera by added glucose or by UNOC reflects a transition of the respiratory chain from state IV to state III. This result indicates that both glucose and some UNOC penetrate into foraminiferal cytoplasm where these compounds are phosphorylated with production of ADP from ATP (Lehninger, 1972; Franke and others, 1980; Nikiforov and Bresler, 1984).

It appears that dissolved products of seaweed decomposition contain some trophic compounds that can be used by benthic epiphytic foraminifera. Accumulation of such trophic compounds from seawater was described previously in some benthic foraminifera (DeLaca and others, 1981).

Seawater with UNOC produces irreversible oxidation of NAD·H in freshly "detached" foraminifera; similar to the effect of the known uncoupler, DNP (Franke and others, 1980; Lehninger, 1972; Nikiforov and Bresler, 1984). Cadmium ions also produce uncoupling of respiration and oxidative phosphorilation (Nikiforov and Bresler, 1984). Therefore, dissolved products of seaweed decomposition contain some natural uncouplers and, probably, other toxic xenobiotics. However, these compounds do not penetrate into non-damaged foraminifera. These compounds are probably high molecular or anionic chemicals, such as dicoumarines, carbonylcyanid phenylhydrasones, salycilanilides, valinomycin, gramicidin, nonactin (Lehninger, 1972). Numerous defense mechanisms described above protect intact protists from these xenobiotics.

Decrease of inherent UV fluorescence of tryptophan-containing proteins by increased concentrations of Cu^{2+} reflects their interaction and quenching of tryptophan fluorescence. Engel and Broawer (1987) demonstrated that binding of apometallothioneins with Cu^{2+} produced quenching of UV fluorescence by these proteins. Our data demonstrate that not all tryptophan-containing proteins can bind with Cu^{2+}. Thus, there are some tryptophan-containing proteins with high and low affinity to Cu^{2+} in foraminiferal cytoplasm. Similar tryptophan-containing metal-binding proteins with high affinity were recently discovered in some freshwater Ciliophora and Metazoa (Piccinni and others, 1985, 1992; Engel and Broawer, 1987; Piccinni, 1989). It should be noted that Cu^{2-} can be used in metabolic processes of both invertebrates and vertebrates, so Cu^{2+}-binding proteins may have functions other than defense (Engel and Broawer, 1987). Cu is commonly present at low concentration in the organic matrix of foraminifera (Schwab and Plapp, 1983). However, excess Cu^{2+} can be eliminated by Cu^{2+}-binding proteins, oxidized, converted into sulphide derivates, or incorporated into the shell.

A similar quenching of UV fluorescence may be produced by various nominal concentrations of Cu ions dissolved in seawater without UNOC and with UNOC. Recently, we demonstrated that UNOC from decomposed seaweeds decreased acute toxicity of Cu^{2+}, Cd^{2+} and Hg^{2+} in a dose-dependent manner (Bresler and Yanko, 1994).

Thus, benthic epiphytic foraminifera have several defense mechanisms against xenobiotics which can protect them effectively in both normal and polluted environments. However, we cannot be certain that we know all defense mechanisms of foraminifera. Presence of DOC and other plant-derived natural compounds in seawater inside forests of seaweed can also play an important protective role for benthic epiphytic foraminifera. However, these defense mechanisms can be adversely affected in very polluted or stressed environments. The presence of various morphological abnormalities in the tests of foraminifera, found in anthropogenically polluted (Sharifi and others, 1991; Yanko and others, 1992, 1994 b) or naturally stressed environments (Boltovskoy and others, 1991), are evidence of dysfunction or damage of defense mechanisms. Therefore, further investigations of cellular defense mechanisms against xenobiotics in benthic foraminifera may be very useful for ecological monitoring and prediction in the marine environment.

CONCLUSIONS

The benthic epiphytic foraminifera *Pararotalia spinigera* and *Rosalina macropora* may contact routinely with various

plant-derived natural compounds that include toxic xenobiotics and metabolic substrates. This study illustrates the following defense mechanisms against xenobiotics within these foraminifera:

(1) A glycocalyx coat and ECM cover the entire cytoplasmic body, form a diffusion barrier for xenobiotics (especially acid) and bind some cationic xenobiotics.

(2) The cytoplasm membrane of intact foraminifera is impermeable to anionic compounds and protects them against widely distributed water-soluble xenobiotics, including plant-derived natural xenobiotics from DOC.

(3) A carrier-mediated transport system in the cytoplasmic membrane, that eliminates anionic xenobiotics from the cytoplasm, looks similar to a system of active transport of organic anions (SATOA) in some single cells of mammals and in some organs of vertebrates and invertebrates.

(4) There is active intralysosomal accumulation and isolation of some cationic compounds.

(5) Peroxidases protect against oxygen and peroxides and are able to metabolize some xenobiotics.

(6) Haloperoxidases produce halogenated derivatives from intracellular anthropogenic and natural xenobiotics. This common defense system protects against free Br^- and I^- and might be synthesized by anti-pests and anti-parasite xenobiotics.

(7) Metallothionein-like Cu^{2+}-binding tryptophan-containing proteins are very much like those in other protozoans and metazoans.

(8) Benthic epiphytic foraminifera can use dissolved trophic compounds present in DOC. These and other natural compounds from decomposed seaweeds can alter acute toxicity of some metal ions.

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