PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **147,** 171–176 (1974)

Plasma Membrane Marker Enzymes in Developing Sea Urchin Embryos (38306)

EBERHARD G. TRAMS, CARL J. LAUTER, GEORGE J. KOVAL, SABERA RUZDIJIĆ, AND VLADIMIR GLISIN

Laboratory of Neurochemistry, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014; Department of Molecular Biology, Center for Multidisciplinary Studies, University of Belgrade and Laboratory of Molecular Biology, Kotor, Yugoslavia

It is conceivable that the plasma membrane of the eucaryotic cell contains unique molecules which would be characteristic solely for that structure. Such compound(s) could also serve as biochemical marker(s) for preparative membrane isolation procedures. For some time now, certain enzymes have been suspected as subserving such function, but in reviewing the evidence, Solyom and Trams (1) concluded that a universal plasma membrane marker molecule has not been identified, though adenylate cyclase may well qualify.

The developing sea urchin embryo might provide a suitable model in which some aspects of plasma membrane marker molecules could be pursued. Following fertilization, the egg divides in rapid succession, and throughout the early divisions, until about a thousand cells are obtained, the biomass of the developing embryo remains constant. Conditions of culture can be adjusted for cells to divide synchronously and at a rate in which the cell number doubles in less than an hour. Therefore, the sum of the cell surfaces, or the plasma membrane mass of the culture, increases discontinuously during the initial divisions. If "quantum increases" of plasma membrane material occurred in synchronously dividing cultures (while biomass or total protein remained constant), then plasma membrane marker enzymes might show similar increases in total activity. Conversely, an increase in plasma membrane mass could be merely a phenomenon of differentiation of preformed (membrane) building blocks (2). Assays for membrane characteristic marker molecules in cultures at different stages then would yield similar results.

An experimental difficulty was taken into consideration: In unfractionated tissue homogenates the activity of nonmembrane enzymes may interfere with the assay of plasma membrane enzyme; for this reason we attempted to obtain membrane-enriched fractions. Also, since we were unable to assess membrane recovery during the fractionation procedure we have used adenylate cyclase activity as a rough index of membrane yield.

Materials and Methods. Arbacia lixula was obtained from the Adriatic Sea and *Lytechinus variegatus* from the Gulf of Mexico. Unfertilized eggs and embryos of various stages were kept at +4° or cultured at 20 ± 3°. For desalination, aliquots of the cultures were sedimented at 1000 rpm for 2 min through 30 ml of a solution containing 0.35 *M* sucrose and 0.1 *M* Tris–HCl (pH 8.0). For late stage cultures the density of the sucrose solution was adjusted down to 0.25 *M*. For the preparation of plasma membrane-enriched fractions, approximately 1 g wet wt cultures (or eggs) were homogenized in 20 volumes (w/v) of 10 m*M* Tris–HCl pH 8.0 in a Dounce homogenizer (clearance 0.1 mm) with eight strokes. The homogenate was centrifuged at 1500*g* for 10 min and the supernatant recentrifuged once at the same speed. The 1500*g* sediments were combined, suspended in 10 m*M* Tris buffer, and resedimented at 3000*g* for 10 min. Microscopic inspection of the various fractions indicated membrane enrichment in the final sediment. Since the experiments were carried out in the field, a more accurate (*i.e.*, electron-microscopic) description of the membrane fraction was not obtained.

Enzyme assays. Adenosinetriphosphatase (ATPase) activity (EC 3.6.1.3 & 3.6.1.4) was

determined according to Weil–Malherbe and Green (3) using ATP-γ-^{32}P as the substrate. Usually, membrane fractions were incubated for 20 min at 30° in a final volume of 0.4 ml containing 0.125 m*M* ATP, 40 m*M* KCl, 5 m*M* $MgCl_2$, 75 m*M* Tris–HCl (pH 7.5), 100 m*M* NaCl and from 140 to 300 μg of membrane protein.

5′-Nucleotidase (EC 3.1.3.5) was assayed as described previously (4) using from 0.5 to 5.0 m*M* 5′-adenosine monophosphate as the substrate and buffers ranging in pH from 7.5 to 9.5.

In *Arbacia* plasma membrane preparations, adenylate cyclase activity was estimated according to the method of Krishna and Birnbaumer (5), using 3.2 m*M* ATP-α-^{32}P (ICN; 12.5 Ci/mMole) as the substrate. Adenylate cyclase in whole homogenates of *Lytechinus* cultures was also measured by this method though with the following variations: In order to minimize interference by ATPases, 5′-adenylyl-imidodiphosphate-α^{32}P served as the substrate. At the end of a 10 min incubation period 0.5 μmoles unlabeled 3′:5′-cyclic AMP were added to each incubation mixture prior to heat inactivation. From the supernatant solution of the first $Ba(OH)_2$ precipitation, a 1.0 ml aliquot was dried and chromatographed on MN cellulose TLC plates (*n*-propanol/conc. NH_4OH/H_2O; 65/35/5, v:v:v). The spot containing 3′:5′-cAMP (R_f = 0.64) was scraped off, eluted with 1.0 ml of water, and the nucleotide and ^{32}P content determined. The yield of 3′:5′-cAM^{32}P during the incubation could be determined from this information.

p-Nitrophenylphosphatase (EC 3.1.3.1) was assayed with 5 m*M* *p*-nitrophenylphosphate (PNPP) as the substrate (4). Standard incubation mixtures also contained 120 m*M* Tris–HCl (pH 7.5), 10 m*M* $MgCl_2$ and 20 m*M* KCl in a final volume of 0.25 ml. After incubating 60 min at 30° the reaction was terminated by the addition of 0.75 ml 1 *M* NaOH; the mixture was centrifuged and the optical density of the supernatant determined at 410 nm.

Cholesterol content of unfertilized eggs or embryo cultures was estimated after TLC of the neutral lipid fraction according to Zlatkis *et al.* (6).

Protein was determined according to Lowry *et al.* (7) or alternately (for adjusting the starting homogenates before subfractionation) by the method of Warburg and Christian (8).

The experiments with *Arbacia* were conducted at the Laboratory of Molecular Biology, Kotor, Yugoslavia. The data from *Lytechinus variegatus* were obtained at the Mote Marine Laboratory, Sarasota, Florida.

Results. We have estimated the cell surface of an *Arbacia lixula* egg (r = 0.065 mm) and the sum of surfaces of ensuing generations after fertilization. Figure 1 illustrates the increases in total surface area from 1 to 2^{10} cells. We stipulated divisions into spherical daughter cells which introduced a moderate error for the initial F generations: Two spherical F_1 cells would have 1.21 times the surface of the egg while a more natural equatorial division (producing two hemispherical F_1 cells) would have 1.49 egg surfaces. We believe, however, that our estimate of a tenfold increase of total surface area for 2^{10} daughter cells is a good approximation.

Arbacia lixula divided first 75–90 min after fertilization. Subsequent cleavages occurred at about 35 min intervals for the first several hours. Cleavages of *L. variegatus* cultures occurred about every 50–55 min. We calculated that about 3 hr after fertilization the cell surface area had doubled. The thickness of the plasma membrane presumably does not change appreciably from cell to cell, or organism to organism; therefore plasma membrane mass of the sea urchin embryo should have doubled in that period. If the plasma membranes of sea urchin cultures at various stages had an obligatory (constant proportion) adenylate-cyclase content, and if the turnover number were to remain constant, then adenylate cyclase activity in whole homogenates should increase proportionately to the calculated increase in plasma membrane material. Our data on whole homogenates of *Lytechinus* cultures (Fig. 2) showed, however, that with the exception of an initial postfertilization rise, adenylate cyclase activity remained virtually constant over

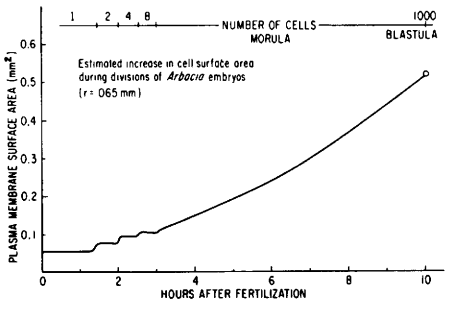

FIG. 1. Calculated increase in plasma membranes during early embryogenesis in *Arbacia lixula*.

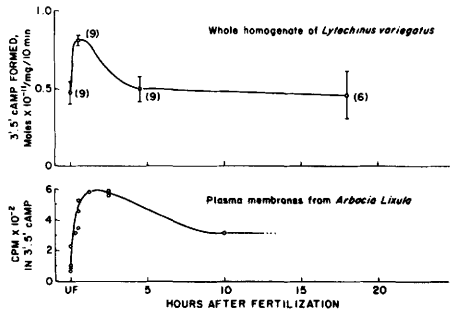

FIG. 2. Adenylate cyclase activity in whole homogenates of *Lytechinus variegatus* and in membrane-enriched fractions from *Arbacia lixula* cultures. Cyclase in *L. variegatus* assayed with 5.2 μM 5'-adenylylimidodiphosphate-α-^{32}P and in *A. lixula* with 3.2 m*M* ATP-α-^{32}P. Incubation mixtures contained 5 m*M* $MgCl_2$, 1 m*M* Na–EDTA, 12 m*M* KF, 10 m*M* theophylline and Tris–HCl buffer 45 m*M* pH 7.6; number of determinations shown in parentheses. Brackets = Standard error of the mean; UF = unfertilized eggs.

an 18 hr period. The postfertilization rise was also observed by Castañeda and Tyler (9) and seems to be restricted to the first 30 min after fertilization, *i.e.*, before the first division.

We attempted to obtain confirmatory data with partially purified plasma membrane preparations for *Arbacia* cultures. The starting homogenates of the latter always were adjusted to the same volume and protein concentration. The final pellet was suspended in buffer to make 1.0 ml, and adenylate cyclase activity was determined using a 25 μl aliquot as the enzyme source. The values shown in the lower half of Fig. 2 represent 3':5'-cAM^{32}P formed in such assays of *Arbacia* cultures. The similarity between the results obtained with whole homogenates and with partially purified membranes is evident.

For ATPase and *p*-nitrophenylphosphatase a unique association with plasma membranes was more questionable (1), and our data on the specific activities of these two enzymes fail to fit any meaningful pattern.

Our purified plasma membrane fractions were still crude and grossly contaminated with other subcellular elements. Plasma membrane (assuming a 100Å diameter) constitutes only about 0.05% of the biomass of an unfertilized egg and about 0.5% in a blastula. Therefore, a 10-20 fold purification, which we estimated to have obtained, may have been insufficient to clearly delineate membrane enzyme activity from that of nonmembrane enzymes.

ATPase (EC 3.6.1.3) activity in homogenates of *Lytechinus* cultures increased about 40% from the unfertilized egg to the 18 hr embryo (Table I). Since no significant changes were obtained by the addition of ouabain (or the omission of Na^+, not shown in Table I) we concluded that the Na^+, K^+-dependent, and probably membrane-specific, ATPase had been obscured by other ATPases. If these ATPases were predominantly membrane bound, a 40% increase in activity in the homogenate could reflect a manifold increase in membrane enzyme activity. Our data on the membrane-enriched fractions obtained from *Arbacia* cultures do not substantiate this (Fig. 3), since such an increase in membrane bound activity should have been magnified.

The neutral *p*-nitrophenylphosphatase, a K^+, Mg^{++}-stimulated phosphomonoesterhydrolase (EC 3.1.3.1) is considered to be functionally and structurally linked with the Na^+, K^+–ATPase (10). In the unfertilized egg or during early development, acid phosphomonoesterhydrolase activity is predominant. The pH curve shows only a minor inflection between pH 7.5 and 8, and activity of the neutral *p*-nitrophenylphosphatase is weak by comparison. Alkaline phosphatase activity (pH optimum 9–10) was not detected.

In the experiments illustrated in Fig. 4, a gradual increase in *p*-nitrophenylphosphatase activity will be noted. Assay of *Arbacia* cultures at the pluteus stage (70 hr) indicated that the activity of this enzyme increased—or was turned on—45 hr or later, following fertilization. Species differences in regard to this enzyme between *Lytechinus* and *Arbacia* seem minor; since specific enzyme activity was higher in homogenates than in membrane-enriched fractions, we have concluded that this enzyme is not a plasma

TABLE I. ATPase Activity in Whole Homogenates of *Lytechinus variegatus*. (μmoles ATP Hydrolyzed/mg Protein/hour[a].)

	Unfertilized eggs	Hours after fertilization		
		0.25	3.6	18
No ouabain	0.88	1.07	1.16	1.28
$5 \times 10^{-4}M$ ouabain	0.79	1.04	1.16	1.50

[a] Averages from six determinations each. Whole homogenate incubated in 25 m*M* KCl, 60 m*M* NaCl, 3 m*M* $MgCl_2$, 3 m*M* ATP-γ-^{32}P in 50 m*M* Tris–HCl buffer pH 7.5.

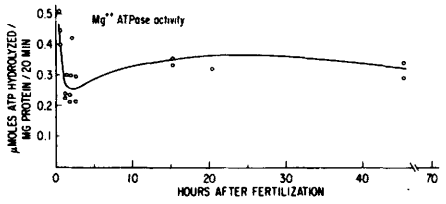

FIG. 3. ATPase activity in membrane-enriched fractions from *Arbacia lixula* cultures. (See text for method).

membrane marker in the developing sea urchin. 5′-Nucleotidase (EC 3.1.3.5) could not be detected in whole homogenates of *Lytechinus*, nor was it measurable in membrane-enriched fractions of *Arbacia* cultures.

We have measured the unesterified cholesterol content of unfertilized eggs and embryos at various stages of development. It has been argued occasionally that free cholesterol is a plasma membrane marker molecule (11), (though the evidence is by no means convincing). As shown in Fig. 5, the cholesterol content of sea urchin embryos remained rather constant, at least until the pluteus stage. The eventual decline in cholesterol content in *Arbacia* at the 70 hr stage may have been due to metabolic oxidation. The energy reserves of the embryo at that stage are critical, and cultures do not survive any longer without feeding them.

Our calculations indicated that 1 g wet wt of unfertilized *A. lixula* eggs (about 4×10^6 cells)

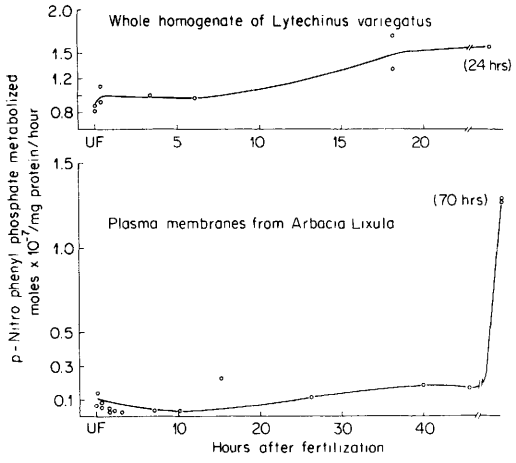

FIG. 4. Specific activities of *p*-nitrophenylphosphatase from *L. variegatus* homogenates and from *A. lixula* membrane-enriched fractions. Incubation mixture contained 5 m*M* PNPP, 10 m*M* $MgCl_2$, 20 m*M* KCl, and 120 m*M* Tris–HCl pH 7.5. UF = unfertilized eggs.

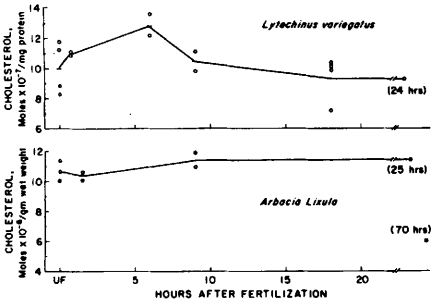

FIG. 5. Content of unesterified cholesterol in whole cultures of *Lytechinus variegatus* and *Arbacia lixula*. One gram wet weight of *Lytechinus* eggs contains about 60 mg of protein as determined by Lowry *et al.* (8); UF = unfertilized eggs.

would represent a lipid bilayer volume (100 Å diameter) of 2 mm^3. If the sea urchin cell contained an average amount of cholesterol (15%) in the lipid bilayer of the plasma membrane, we would expect approximately 0.8 μmoles of free cholesterol, provided that it were solely present in the plasma membrane. Our data showed that the unfertilized egg as well as the early stage embryos contained about 12 times that amount. Curiously, when the number of cells per embryo reached 2^{10}–2^{11}, the embryos contained just sufficient cholesterol to satisfy the requirements of the "average" plasma membrane.

Discussion. It can be argued that the plasma membrane composition (by relative abundance of molecular species) should be constant in a homogeneous cell population. The argument derives some support from analyses of lipid composition, which give fairly similar results for many different membrane preparations. The developing sea urchin conceptually seemed a good model to examine this hypothesis: If the first three divisions of the fertilized egg were accompanied by a doubling of cell surface (plasma membrane) area, did this rapid membrane expansion require that certain enzyme markers also double?

Ideally, the model should be interrogated by isolating a known proportion of the plasma membranes in highly purified form. The biochemical composition and enzymatic activity of the pure membranes could then be compared with the nonmembrane residues. Such an experiment with unfertilized eggs would require about a thousandfold purification and an accurate estimate of the preparative yield. We were unable to satisfy either requirement.

We have tried to examine this question by measuring the presence in membranes of molecular species which are thought to be exclusively found in this organelle.

We had postulated that of the parameters which were measured, only adenylate cyclase may be regarded as an exclusive plasma membrane constituent. The pattern of cyclase activity in whole homogenates corresponded satisfactorily with that in membrane-enriched subfractions. In whole homogenates of sea urchin cultures, the levels of this enzyme remained remarkably constant (disregarding the brief twofold increase immediately following fertilization). By comparison, adenylate cyclase activity in the membrane enriched fractions increased about threefold over a 10 hr period. Our interpretation of these observations was that the constant level of adenylate cyclase in whole homogenates and the increased activity in the membrane-enriched preparations signified an increase of plasma membrane material in the latter.

It also suggested that this enzyme, at least during the early stages of development, was of maternal origin. Apparently, it exists preformed in the unfertilized egg and is incorporated into newly formed plasma membranes when they differentiate during cleavage. Castañeda and Tyler (9), who studied adenylate cyclase in *Lytechinus pictus*, also concluded that the enzyme was membrane bound. If their and our conclusions are correct, then preformed enzyme is incorporated into the expanding plasma membrane mass by a distributive process. *De novo* biosynthesis of the enzyme, relying on new transcription and translation, would not be required. Moreover, an obligatory and constant molecular composition of the plasma membrane may not be required.

Our data on ATPase, *p*-nitrophenylphosphatase, and cholesterol left us with the impression that their value as plasma membrane markers in sea urchins was, at best, uncertain.

In concluding, we would like to dwell briefly on the underlying processes which allow for this rapid formation of new plasma membranes. Near instantaneous formation of a new membrane is not confined to periods of cellular divisions; it may also occur during exocytosis and it may involve the regeneration of an entire cell surface as the following experiment recounts. Chambers and Chambers (12) related that when an individual *Ameba dubia* was exposed to the air–water interphase in a suspended pond water droplet, the tension forces ruptured the plasma membrane, stripped it off, and left the denuded cytoplasma exposed to the environment. If, however, the protoplasmic mass was quickly withdrawn into the aqueous phase before the cytoplasm had dispersed, a new plasma membrane was regenerated immediately and "complete recovery of the ameba ensued".

To such processes we would like to attach the term "structuralization" as distinct from differentiation. In the dividing sea urchin egg we visualize this as a sequence of events during which individual, preformed macromolecules rapidly aggregate into a cellular structure which can be morphologically defined. The only requirement would be that an adequate supply of preformed molecular building blocks exists and that the directing force should be partially inherent in these building blocks and be expressed by intermolecular fit or cognition.

Summary. In sea urchin embryos during the first few hours after fertilization the number of cells increases from 1 to 2^{10} and plasma membrane volume should increase about tenfold. Since the increase in plasma membrane biomass might also be accompanied by a proportional increase in membrane marker molecules, presumptive plasma membrane "marker" enzymes were assayed in whole homogenates of sea urchin cultures as well as in plasma membrane-enriched fractions. Adenylate cyclase activity in whole homogenates remained constant (except for a brief postfertilization increase) from the unfertilized egg to the swimming blastula stage. In membrane-enriched fractions the activity of this enzyme increased. Reliable levels for Na^+-, K^+–ATPase activity were not obtained; 5′-nucleotidase activity could not be detected. Mg^{2+}–ATPase, *p*-nitrophenylphosphatase, and unesterified cholesterol apparently cannot be used as plasma membrane markers in sea urchins. It was concluded that the increase in plasma membrane biomass of one order of magnitude did not necessarily require an equivalent increase (or *de novo* synthesis) of constituent molecules *i.e.*, membrane enzymes. The latter may exist preformed in the unfertilized egg and thus would be of maternal origin during the early stages of development.

This work was supported in part by Grant No. 02-020-1 from the National Institutes of Health, U. S. Department of Health, Education and Welfare (PL-480).

1. Solyom, A., and Trams, E. G., Enzyme **13,** 329 (1972).

2. Crkvenjakov, R., Bajkovic, N., and Glisin, V., Biochem. Biophys. Res. Comm. **39,** 655 (1970).

3. Weil-Malherbe, H., and Green, R. H., Biochem. J. **49,** 286 (1951).

4. Lauter, C. J., Solyom, A., and Trams, E. G., Biochim. Biophys. Acta **266,** 511 (1972).

5. Krishna, G., and Birnbaumer, L., Anal. Biochem. **35,** 393 (1970).

6. Zlatkis, A., Zak, B., and Boyle, A. J., J. Lab. Clin. Med. **41,** 486 (1953).

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

8. Warburg, O., and Christian, W., Biochem. Z. **310,** 384 (1941).

9. Castañeda, M., and Tyler, A., Biochem. Biophys. Res. Comm. **33,** 782 (1968).

10. Albers, R. W., Ann. Rev. Biochem. **36,** 727 (1967).

11. De Duve, C., J. Cell Biol. **50,** 20D (1971).

12. Chambers, R., and Chambers, E. L., "Explorations into the Nature of the Living Cell," p. 102. Harvard Univ. Press, Cambridge, MA (1961).

Received May 2, 1974. P.S.E.B.M., 1974, Vol. 147.