Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 812–816, February 1988
Cell Biology

Enzyme stimulation upon fertilization is revealed in electrically permeabilized sea urchin eggs

(intracellular enzyme assays/metabolic activation/glucose-6-phosphate dehydrogenase/permeabilized cells)

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Communicated by Daniel Mazia, October 13, 1987

ABSTRACT **Sea urchin eggs and embryos subjected to high-voltage electric discharge in a medium mimicking the intracellular milieu retain their structural integrity and remain permeable, permitting substrates to enter the cytoplasm and thus assay of enzyme activity. At saturating concentrations of substrates, five of six enzymes assayed are more active (three to fifteen times) in permeabilized embryos than in permeabilized eggs, but no fertilization-related differences are seen in homogenates prepared from these same permeabilized cells. Furthermore, enzyme activity in homogenates always exceeds that in the permeabilized cell suspensions. This difference in enzyme reaction rates between unfertilized eggs and fertilized eggs is not due to differences in the diffusibility of substrates into the permeabilized cells. The activity of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP$^+$ 1-oxidoreductase, EC 1.1.1.49) in permeabilized cells was studied in greater detail and has the following characteristics. (*i*) Regulation of activity persists during early development. (*ii*) This regulation is not mediated by diffusible allosteric agents. (*iii*) Stimulation at fertilization is initiated by a rise in intracellular calcium and is further promoted by cytoplasmic alkalinization. (*iv*) The microenvironment experienced by this enzyme intracellularly differs from that of the enzyme in homogenates as evidenced by markedly different pH vs. activity profiles. These results indicate that the regulatory status of enzymes is preserved in electrically permeabilized cells and suggest that this regulation depends on some cell structural feature(s) that is (are) destroyed upon homogenization.**

Unfertilized sea urchin eggs are metabolically quiescent, and their activity increases dramatically at fertilization (1). The list of postfertilization changes includes structural alterations (2, 3), changes in ion flux (4), 3- to 15-fold increases in respiration and carbohydrate metabolism, increased protein synthesis, and ultimately initiation of DNA synthesis and cell division (1–10).

This increased cellular activity presumably results from increased enzyme activity *in vivo*, and here we report on the use of electrically permeabilized cells to examine these putative changes accompanying fertilization. Such permeabilized cells allow the investigator to control levels of low-molecular weight substances, such as substrates or regulatory ligands, that an enzyme is exposed to under conditions that maintain the macromolecular environment of cells—e.g., enzymatic compartmentalization, high concentration of protein, etc. (11, 12). Our analysis shows that in permeabilized eggs many enzymes are inhibited before fertilization, but when the cells are permeabilized after fertilization, the measured enzyme activities are 3- to 15-fold higher. These postfertilization changes are not seen in homogenates of these permeabilized cells; rather, homogenates from either unfertilized or fertilized eggs exhibit equal activity, which is higher than that seen in permeabilized cells. These findings suggest that permeabilized cells retain controls on enzymatic activity not seen in broken cell preparations. If this view is correct, our results also suggest a global change occurs at fertilization that simultaneously affects many enzymes. The nature of this change is not known but appears intimately linked to retention of cell structure.

MATERIALS AND METHODS

Cell Permeabilization Conditions. Gametes from the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* were obtained and processed as described previously (13). All chemicals were obtained from Sigma, except polyethylene glycol (Fluka), [^{3}H]thymidine monophosphate (Amersham), [^{14}C]sucrose (ICN), thioflavin S (Matheson Coleman and Bell), and KOH (VWR Scientific). Cell permeabilization was achieved by high-voltage electric discharge (electroporation; for reviews, see refs. 14 and 15) by a modification of the technique developed by Suprynowicz and Mazia (11, 12). This procedure produces two pores per discharge, is selective for the plasma membrane, and is reported to yield greater structural preservation than detergent-induced permeabilization of cells (14). Before permeabilization the eggs or embryos were washed twice in calcium-free seawater, then once in a permeabilization medium chosen to mimic some of the solution conditions of the intracellular milieu (300 mM glycine/175 mM potassium gluconate/185 mM mannitol/50 mM Pipes buffer/20 mM NaCl/5 mM $MgCl_2$/2 mM EGTA/2.5% (wt/vol) polyethylene glycol 20,000 M_r (PEG)/53 mM KOH, final pH 6.8 at 16°C), and then resuspended in this medium to a concentration of 1.5–3% (vol/vol). This suspension was then transferred to a chamber containing two parallel stainless steel plates separated by 5 mm, and the suspension was pulsed three times with an electric field across these plates of 2000 V/cm ($t_{1/2} \approx 25$ μsec per pulse).

Fluorimetric Assay of Glucose-6-Phosphate Dehydrogenase (G6PD). Permeabilized cells were suspended in a cuvette fitted with a paddle-stirring device to prevent cell settling. After monitoring the fluorescence of the sample at 460 nM (F_{460}; sample was excited with light at 350 nM) for 4–8 min, glucose-6-phosphate and NADP were added to the cuvette to 416 μM and 100 μM final concentrations, respectively. These concentrations approximate the physiological levels of these compounds (16, 17), and doubling the concentration of substrate or cofactor did not increase the reaction rate in permeabilized eggs, indicating that the system is operating at saturating substrate (i.e., V_{max}) concentrations. The rate of increase in F_{460} was then monitored, and this rate was

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Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; F_{460}, fluorescence at 460 nM; pH_i, intracellular pH; Ca^{2+}_i, intracellular calcium.

corrected for the depression in F_{460} of NADPH due to scattering of light by the permeabilized cells. (By adding known amounts of NADPH to the 1.5% cell suspensions used, we found measured rates must be increased by 65% to obtain the true rate of NADPH production.) A parallel batch of permeabilized cells was centrifuged, and the supernatant was assayed for G6PD (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) activity as described above. Subtracting G6PD activity in this supernatant from that in the permeabilized cell suspension corrects for any extracellular enzyme activity. (No appreciable increase in extracellular G6PD content occurs during the period that the permeabilized cell suspension is assayed.) After assaying the permeabilized cell suspension for G6PD activity, the cell suspension was then homogenized (Dounce, tight-fitting, 10–20 strokes), and an aliquot of the homogenate was then assayed in the permeabilization medium containing NADP and glucose-6-phosphate at the above concentrations; this rate was used to calculate the total activity in the samples, and the permeabilized cell measurements are expressed as a percentage of this total. Under our assay conditions (2.5 ml of 1.5% (vol/vol) egg suspensions) 100% activity corresponds to 0.248 ± 0.085 (n = 10) total units of G6PD activity, where one unit of activity produces one μmol of NADPH per min. All enzyme assays were conducted at 16°C.

Assay of Other Enzymes in Permeabilized Cells. For all other enzymes examined, the assay procedures are similar to those described above for G6PD—i.e., all enzyme measurements (suspension, supernatant, and homogenate) are done in permeabilization medium containing saturating levels of substrates (established empirically or taken from the literature), at a temperature of 16°C. The substrate concentrations used are as follows: 6-phosphogluconate dehydrogenase, 416 μM 6-phosphogluconic acid and 100 μM NADP; glutathione reductase, 1 mM glutathione disulfide and 100 μM NADPH; thymidylate kinase, 10 mM ATP and 10 μCi of [^{3}H]thymidine monophosphate per ml (1 Ci = 37 GBq; assay coupled with 1 unit of commercial nucleoside 5′-diphosphate kinase per ml); NAD kinase, 2 mM NAD and 5 mM ATP (assay coupled with 10 mM glucose-6-phosphate and 0.8 μg of commercial G6PD per ml; hexokinase, 10 mM glucose and 1 mM ATP (discontinuous assay coupled with 240 μM NADP and 4 μg of commercial G6PD per ml). Product formation was measured fluorimetrically in all assays except thymidylate kinase. In the latter case, the final product, thymidine triphosphate, was isolated by thin-layer chromatography on PEI-cellulose developed sequentially with 0.1 M acetic acid, then 1 M LiCl, and then quantified by liquid scintillation counting.

Influx and Efflux of [^{14}C]Sucrose in Permeabilized Cells. Eggs or embryos (20 min after insemination) from *L. pictus* were permeabilized, and at ±0 [^{14}C]sucrose was added to these suspensions to a concentration of 0.3 μCi/ml. The samples were incubated with swirling at 16°C; and at the times indicated in Fig. 1, 0.5-ml aliquots were removed, and the cells were separated from the bulk medium by centrifugation through an oil cushion [diisobutyl phthalate/diethyl phthalate, 2:1 (vol/vol)] as described (18).

RESULTS

Structural Integrity of Permeabilized Cells. Eggs permeabilized by this procedure exhibit a structural stability that depends on their developmental stage. Unfertilized eggs are mechanically fragile and will fall apart when kept in suspension by stirring unless PEG is included in the medium. Fertilized eggs that are permeabilized 10 min after insemination or later are stable whether PEG is present or not. However, fertilized eggs permeabilized during the first 10 min after insemination disintegrate rapidly; we have not yet found conditions that eliminate this problem.

Extent of Permeability Following Electric Shock. Cells permeabilized in this manner remain permeable for at least 30 min as assessed by the ability to stain these cells with a fluorescent dye (thioflavin S) that is excluded from untreated cells. Permeabilization was also assessed by measuring influx and efflux of small molecules. By 4 min after permeabilization, 92–94% of the ATP and NADP (assayed as in ref. 19) was released from permeabilized eggs. Diffusion of radioactive sucrose into or out of permeabilized eggs or embryos was rapid (Fig. 1) and at the time resolution of this experiment (1 min for influx; 2 min for efflux) there was no demonstrable difference between unfertilized and fertilized eggs in permeability to low-molecular weight compounds. Fig. 1 also shows the persistence of permeability in eggs or embryos, as evidenced by the rapid washout of [^{14}C]sucrose at the 15-min time point. (Control experiments with ^3H_2O indicate that the higher number of counts in permeabilized embryos can be accounted for by [^{14}C]sucrose trapped within the extracellular perivitelline space of the embryos.)

The permeabilized cells also exhibit limited and selective permeability to macromolecules. Rhodamine-labeled PEG (20,000 M_r) freely enters permeabilized eggs and embryos. Some proteins escape from the permeabilized cells, amounting to ≈10–12% of total cell protein. These proteins consist mainly of the low-molecular weight proteins of the cell (determined by gel filtration chromatography; data not shown), indicating a selective loss of protein. G6PD release from permeabilized cells is minimal (5–8% of the total in unfertilized eggs; 2% of the total in fertilized eggs).

Activity of G6PD in Permeabilized Eggs and Embryos. The pentose phosphate shunt is the major pathway of carbohydrate utilization in early embryos (20, 21), and in sea urchin gametes stimulation of this pathway might result from a change in the subcellular locale of G6PD, the first enzyme in this pathway (13, 22). When G6PD activity is assayed in permeabilized eggs (Table 1), almost all of its activity is inhibited (only 1–2% of the total activity is expressed), whereas G6PD activity in the permeabilized 20-min embryos (one-cell stage) is eight to fifteen times greater (15%). However, when the same cells used in the permeabilized cell assays are homogenized and assayed for activity (Table 1) we find that G6PD activity is identical in unfertilized eggs

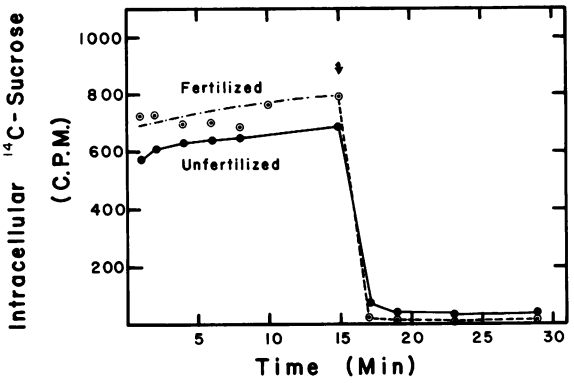

FIG. 1. Accumulation and loss of [^{14}C]sucrose in permeabilized eggs and embryos. *L. pictus* unfertilized eggs (●) or embryos 20 min after insemination (⊙) were permeabilized electrically, and at t_0 [^{14}C]sucrose was added to these cell suspensions. Radiolabel within these permeabilized cells was determined as described. At 15 min (arrow) the permeabilized cells were rapidly transferred to permeabilization medium lacking [^{14}C]sucrose with one wash. All data are corrected for adventitious binding of label, as determined by following these procedures on parallel batches of unpermeabilized eggs or embryos; such binding accounts for 15–20% of the total radioactivity in the experimental samples. C.P.M., counts per minute.

Table 1. G6PD activities in permeabilized sea urchin eggs and embryos

Assay condition	G6PD activity, % of total	
	S. purpuratus	*L. pictus*
Homogenized unfertilized eggs	100	100
Homogenized 20-min embryos	100	100
Permeabilized unfertilized eggs	1	2
Permeabilized 20-min embryos	15	15

These values are representative of data obtained in 10 experiments with *S. purpuratus* and 8 experiments with *L. pictus*.

and embryos. Modulation of G6PD activity in permeabilized cells continues during early cleavage; as seen in Fig. 2, activity remains at 15% for the first 30–40 min of development and then decreases over the next hour to a level of 8% of the total activity for the next 3 hr. G6PD activity in homogenates is constant throughout this period of development.

If small molecules that stimulate G6PD activity (e.g., allosteric regulators) are produced after fertilization, then removing endogenous low-molecular weight molecules from the incubation medium should inactivate G6PD in permeabilized embryos. We tested this hypothesis by washing permeabilized embryos three times and found that no changes in G6PD activity resulted from leaching cells of their low-molecular weight metabolites (Table 2). On the other hand, unfertilized eggs do not appear to contain a diffusible inhibitor of G6PD activity. When washed, permeabilized embryos were transferred to a permeabilization medium previously "conditioned" by allowing permeabilized unfertilized eggs to sit in the medium for 30 min, we found no inhibition of G6PD activity (Table 2). The data in Tables 1 and 2 therefore suggest that modulation of G6PD activity at fertilization is irreversible within the permeabilized cells and is not caused by production of diffusible stimulatory ligands or loss of inhibitory ligands.

G6PD Activity in Artificially Activated Eggs. Ionic signals initiating active metabolism at fertilization include elevations in intracellular Ca^{2+} levels (Ca_i^{2+}) and intracellular pH (pH_i) (1, 4). The influence of these ionic changes on G6PD activity in the permeabilized cells was examined with agents that

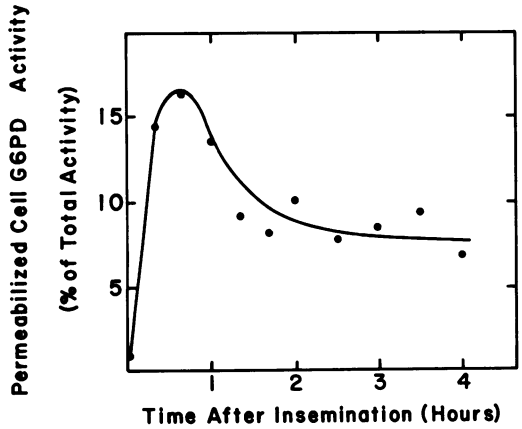

FIG. 2. G6PD activity in permeabilized sea urchin eggs during the first two cell cycles. A 1.6% suspension of *S. purpuratus* eggs in seawater was inseminated at ±0, and the embryonic culture was maintained at 16°C with gentle stirring. Shortly before the indicated times, samples were transferred to permeabilization medium, subjected to electric discharge, and G6PD activity was then determined as described. Data are representative of three such experiments. The ±0 point is for an unfertilized sample of these eggs. First cleavage occurred at ≈2 hr and 20 min after insemination, and second cleavage occurred between 3 and 4 hr after insemination.

Table 2. Effect of washing on G6PD activity in permeabilized embryos

Treatment of permeabilized embryos	Activity, % of total
Unwashed	13
3×-washed (fresh medium)	15
3×-washed (egg-conditioned medium)	13

Twenty minutes after fertilization, *S. purpuratus* embryos were permeabilized and then either assayed for G6PD activity immediately (line 1) or washed three times by centrifugation and resuspension in fresh permeabilization medium. Washed permeabilized cell pellets were then resuspended either in fresh permeabilization medium (line 2) or in permeabilization medium that had previously been conditioned 30 min by incubation with permeabilized unfertilized eggs (line 3). G6PD activity was measured as described. Permeabilized cell suspensions of 1.5% (vol/vol) were used.

raise Ca_i^{2+} (Ca ionophore A23187) or pH_i (NH_4Cl). Application of these agents preceded permeabilization of the eggs. Activation of eggs with A23187 (final concentration, 20 μM) in seawater for 20 min, which elevates both Ca_i^{2+} and pH_i (4), stimulated G6PD activity in permeabilized cells to 11%, comparable to fertilization-induced stimulation (12%); preventing the pH_i rise by eliminating extracellular Na^+ (22) reduced the extent of activation by greater than 50% (to 5%).

Artificially raising pH_i with ammonia-containing seawater (final NH_4Cl concentration, 15 mM, at pH 8.0 for 10 min) has no effect on G6PD activity in eggs permeabilized 30 min after addition of NH_4Cl. However, when eggs were permeabilized 60 min after NH_4Cl addition, the G6PD activity in the permeabilized cells had risen to 15% of the total, similar to the level of a fertilized sample of this batch of eggs (16%). This change in G6PD activity between 30 and 60 min after ammonia addition is not related directly to the pH_i increase, which occurs within 5 min of NH_4Cl addition (23). However, the change may be related to a Ca_i^{2+} increase, which occurs between 40 and 60 min after activation by NH_4Cl (24). These results then suggest that a rise in Ca_i^{2+} is involved in the activation of G6PD in the permeabilized cells and that cytoplasmic alkalinization (Na^+/H^+ exchange) further enhances this process.

Activity of Other Enzymes in Permeabilized Eggs or Embryos. Is the enzyme activation in permeabilized cells specific to G6PD or is this phenomenon shared with other enzymes? We compared five other enzyme activities in permeabilized cells with those in cell-free extracts before and 20 min after fertilization (Table 3). For all enzymes examined, homogenates were prepared from the assayed permeabilized cells, and the activities in the homogenates were identical in unfertilized and fertilized eggs. As seen, however, four of these five enzymes are severely inhibited in permeabilized eggs (only 2–17% of the potential activity is expressed), and a 3- to 11-fold increase is seen when the eggs are permeabilized after fertilization. The exception is NAD kinase, which exhibits similar activities before and after fertilization when assayed in the presence of its limiting activator, μM Ca^{2+} (25).

pH/Activity Profiles of G6PD. The regulation of enzyme activity in permeabilized cells and its absence in homogenates suggest that the local environment within the cell is affecting enzyme catalysis. In theory, this could influence the pH-dependence of enzymatic activities (26), and we therefore determined the pH vs. activity profile for G6PD in permeabilized embryos and compared this with the profile obtained from the enzyme in homogenates. The results (Fig. 3) show that G6PD activity in permeabilized cells is roughly constant from pH 6.4–7.6 but then decreases with further medium alkalinity. In contrast, the solubilized enzyme in homogenates evidences a biphasic trend of increased activity with increased pH. These results suggest (*i*) that the

Table 3. Changes in activities of other enzymes at fertilization

Enzyme assay (species)	Permeabilized cell activity, % of total		-fold increase
	Eggs	Embryos	
6-Phosphogluconate dehydrogenase (*S. purpuratus*)	2	23	11.5
Glutathione reductase (*S. purpuratus*)	4	26	6.5
Thymidylate kinase (*L. pictus*)	17	51	3.0
NAD kinase (*S. purpuratus*)	63	71	1.1
Hexokinase (*L. pictus*)	15	53	3.5

All assays contained saturating concentrations of substrates and cofactors (see *Materials and Methods*). For each enzyme data are representative of two experimental determinations. For 2.5 ml of a 1.5% (vol/vol) suspension of eggs or embryos the total (100%) units of enzyme activity (1 unit produces 1 μmol of product per min) are as follows: 6-phosphogluconate dehydrogenase, 9.93×10^{-2}; glutathione reductase, 2.19×10^{-2}; thymidylate kinase, 6.30×10^{-5}; NAD kinase, 8.45×10^{-3}; and hexokinase, 2.10×10^{-2}.

microenvironment of G6PD in the permeabilized embryos differs from that of the enzyme dissolved in the permeabilization medium and (*ii*) that alkalinization of the egg cytoplasm from pH 6.8 to 7.2 occurring after fertilization has little direct effect on G6PD activity.

DISCUSSION

It is perhaps not surprising to find no direct relationship between the enzyme activities measured in permeabilized cells and those measured after these permeabilized cells had been disrupted. Other workers have found that the kinetic properties of yeast citrate synthase (27) and *Escherichia coli* phosphofructokinase (28) determined in chemically permeabilized cells differed from those of the solubilized enzymes. If the current speculation about the relation between cell structure and enzyme function is correct—i.e., spatial organization of macromolecules *in vivo* effects metabolic regulation (29–31), then destroying this organization by cell homogenization is apt to perturb enzymatic properties.

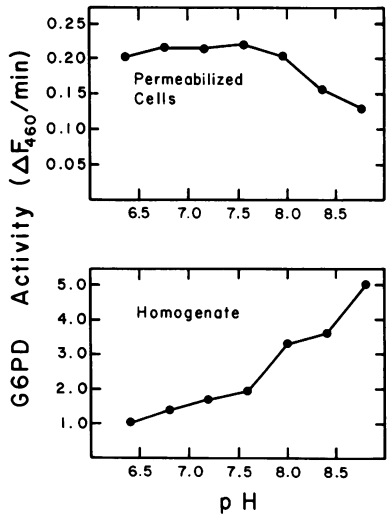

FIG. 3. pH dependence of G6PD activities in *S. purpuratus* permeabilized embryos and homogenates. G6PD assays were done as described. Pipes buffer was replaced with an equimolar amount of either Hepes buffer (pH 7.6 and 8.0) or Taps buffer (pH 8.4 and 8.8). The soluble enzyme assays (*Lower*) were done by diluting the permeabilized embryonic homogenates 100-fold in the assay mixture.

What is most surprising about the findings with eggs is how severely inhibited the enzymes are in terms of their potential activities. Suprynowicz and Mazia observed a similar phenomenon for the activity of the Ca^{2+}-sequestration system of sea urchin embryos [permeabilized cell activity was only 25% of that in homogenates of the same cells (11, 12)]. The release from inhibition of enzymatic activities reported here (15-fold for the first two enzymes of the pentose phosphate shunt and 3- to 6-fold for three of the four other enzymes assayed) corresponds to the increased shunt activity (20, 21) and phosphorylation of thymidine monophosphate (10, 32) demonstrated to occur *in vivo* after fertilization. (There are no *in vivo* measurements of fertilization-induced changes in the activities of hexokinase or glutathione reductase.)

Our data suggest that this inhibition of activity, and the changes seen after fertilization for five of these six enzymes, does not result from differences between permeabilized eggs and permeabilized embryos in substrate diffusibilities. As noted, ingress and egress rates for [^{14}C]sucrose are equal for permeabilized eggs and embryos, and in permeabilized eggs there is no enhancement of enzyme reaction rates upon doubling substrate concentration (done with G6PD, 6-phosphogluconate dehydrogenase, NAD kinase, and glutathione reductase), as would be the case if these rates were diffusion limited (33). Furthermore, if diffusional barriers to small molecules underlie differential enzyme activity after fertilization, then NAD kinase should have shown greater activity in permeabilized embryos than in permeabilized eggs. The data indicate that this is not the case. (This enzyme *in vivo* is most likely regulated solely by Ca_i^{2+}.)

The coordinated activation of many enzymes seen in the permeabilized cells suggests that these enzymes may share a common mechanism of regulation. Possibly the changes are related to cell architecture and its changes at fertilization. Sequestration of enzymes in organelles and their partial release at fertilization or homogenization are probably not involved because G6PD, 6-phosphogluconate dehydrogenase, and thymidylate kinase are fully soluble in iso-osmotic homogenates (refs. 13 and 34 and unpublished observations). However, at low ionic strength or high protein concentration these three enzymes bind to undefined structural elements of the unfertilized egg (13, 32). Such an association in the permeabilized cells could account for the difference between the pH profile for G6PD in permeabilized embryos and that in homogenates (26, 33). Major cell structural changes also accompany fertilization affecting membranes (3, 35), the cytoskeleton (3, 36, 37), and cytoplasmic viscosity (2), and such alterations could affect the intrinsic kinetic properties of numerous cytoplasmic enzymes by rearranging their interactions with other macromolecules.

Alternatively, activation at fertilization might involve modification of nondiffusible inhibitors—e.g., regulatory macromolecules, which cannot escape through the electrically generated pores, or small ligands whose binding to enzymes is enhanced by the high-protein content of the permeabilized cells (13, 38). These ligands would be free to diffuse away from their regulatory sites upon cell disruption, which would account for the higher activity in homogenates than in permeabilized cells; enzyme activation *in vivo* could then involve a (partial) modification of these ligands.

A final possibility is regulation by covalent modification—e.g., a phosphorylation of these enzymes to inhibit their activities; controlled phosphatase activity *in vivo* after fertilization could then account for the observed stimulation to levels of 15–50% of the potential activity, and uncontrolled hydrolysis as might occur upon homogenization could artifactually abolish all such modulation.

Our data support the contention (11, 12, 14, 15) that permeabilized cells may better represent the living state than do homogenized cells. However, whether electrically permeabilized cells do provide reliable information about the metabolism of living cells will require a direct comparison of the activities of enzymes in permeabilized cells with those obtained *in vivo* using intact cells.

We thank D. Mazia, F. Suprynowicz, C. Patton, T. Schmidt, R. Alberte, and J. Clegg for helpful discussions and G. Somero, V. Vacquier, D. Mazia, J. Oberdorf, and D. Larochelle for their critical reading of this manuscript. This work was supported by grants from the National Science Foundation and The March of Dimes to D.E. and by a training grant from the National Institutes of Health to R.R.S.

1. Epel, D. (1978) *Curr. Top. Dev. Biol.* **12,** 185–246.
2. Heilbrunn, L. V. (1915) *Biol. Bull.* **29,** 149–203.
3. Vacquier, V. D. (1982) *Dev. Biol.* **84,** 1–26.
4. Whitaker, M. J. & Steinhardt, R. A. (1982) *Quart. Rev. Biophys.* **15,** 593–666.
5. Warburg, O. (1908) *Z. Physiol. Chem.* **57,** 1–16.
6. Steinhardt, R. A. & Epel, D. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1915–1919.
7. Okabayashi, K. & Nakano, E. (1980) *Dev. Growth Differ.* **22,** 187–194.
8. Hino, A. & Yasumasu, I. (1979) *Dev. Growth Differ.* **21,** 229–236.
9. Yasumasu, I., Fujiwara, A., Shoger, R. L. & Asumi, K. (1975) *Exp. Cell Res.* **92,** 444–450.
10. McGwin, N. F., Morton, R. W. & Nishioka, D. (1982) *Exp. Cell Res.* **145,** 115–126.
11. Suprynowicz, F. (1984) Dissertation (Univ. California, Berkeley).
12. Suprynowicz, F. & Mazia, D. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2389–2393.
13. Swezey, R. R. & Epel, D. (1986) *J. Cell Biol.* **103,** 1509–1515.
14. Knight, D. E. & Scrutton, M. C. (1986) *Biochem. J.* **234,** 497–506.
15. Knight, D. E. & Baker, P. F. (1982) *J. Membr. Biol.* **68,** 107–140.
16. Epel, D. (1964) *Biochem. Biophys. Res. Commun.* **17,** 62–68.
17. Epel, D. & Iverson, R. M. (1965) in *Control of Energy Metabolism*, eds. Chance, B., Estabrook, R. W. & Williamson, J. R. (Academic, New York), pp. 267–272.
18. Dube, F. & Epel, D. (1986) *Exp. Cell Res.* **162,** 191–204.
19. Lowry, O. H. & Passonneau, J. V. (1972) *A Flexible System of Enzymatic Analysis* (Academic, New York), pp. 68, 151.
20. Isono, N. & Yasumasu, I. (1968) *Exp. Cell Res.* **50,** 616–626.
21. Krahl, M. E. (1956) *Biochem. Biophys. Acta* **20,** 27–32.
22. Isono, N. (1963) *J. Fac. Sci. Univ. Tokyo Sect. 4* **10,** 67–74.
23. Johnson, C. H. & Epel, D. (1981) *J. Cell Biol.* **89,** 284–291.
24. Poenie, M., Alderton, J., Tsien, R. Y. & Steinhardt, R. A. (1985) *Nature (London)* **315,** 147–149.
25. Epel, D., Patton, C., Wallace, R. W. & Cheung, W. Y. (1981) *Cell* **23,** 543–549.
26. Goldstein, L., Levin, Y. & Katchalski, E. (1964) *Biochemistry* **3,** 1913–1919.
27. Weitzman, P. D. J. & Hewson, J. K. (1973) *FEBS Lett.* **36,** 227–231.
28. Reeves, R. E. & Sols, A. (1973) *Biochem. Biophys. Res. Commun.* **50,** 459–466.
29. Clegg, J. S. (1984) *Am. J. Physiol.* **246,** R133–R151.
30. Srere, P. (1987) *Annu. Rev. Biochem.* **56,** 21–56.
31. Srivastava, D. K. & Bernhard, S. A. (1986) *Curr. Top. Cell. Regul.* **28,** 1–109.
32. Nonaka, M. & Terayama, H. (1977) *Dev. Biol.* **56,** 68–75.
33. Goldstein, L. (1976) *Methods Enzymol.* **44,** 397–443.
34. Nishioka, D. (1976) Dissertation (Univ. California, Berkeley).
35. Epel, D. & Johnson, J. (1976) *Biogenesis and Turnover of Membrane Macromolecules*, ed. Cook, J. S. (Raven, New York), pp. 105–120.
36. Balczon, R. & Schatten, G. (1983) *Cell Motil.* **3,** 213–226.
37. Cline, C. A. & Schatten, G. (1986) *Gamete Res.* **14,** 277–291.
38. Minton, A. P. (1983) *Mol. Cell. Biochem.* **55,** 119–140.