Observations on Intracellular pH during Cleavage of Eggs of *Xenopus laevis*

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ABSTRACT Direct measurement of intracellular pH was made with recessed-tip pH microelectrodes in fertilized eggs of the frog, *Xenopus laevis*, from ~1 h after fertilization to mid-blastula. The intracellular pH just before first cleavage was 7.65 ± 0.04 (SD; $n = 9$). By stage 5 to the middle of stage 6, average intracellular pH was 7.70 ± 0.06 (SD; $n = 16$). A statistically significant alkalization of 0.18 ± 0.03 pH unit (SD; $n = 5$) was observed beginning in early blastula. A cycle of ≤0.05 pH unit was occasionally observed during the pre-blastula period, but its significance is unknown. By exposing the early cleavage embryo to saline buffered with sodium propionate, pH 4.7–5.0, it was possible to lower intracellular pH with some degree of control. Apparently, normal cleavage continued to occur when intracellular pH had been forced as much as 0.3 unit below normal. We conclude that this implies no specific involvement of intracellular pH in mitosis and cytokinesis. If intracellular pH was lowered further, cell division ceased at about pH 7.2, and furrow regression began at about pH 7.0. Once furrow regression occurred, subsequent development was usually arrested or abnormal when the embryo was transferred back to normal saline.

After fertilization, the *Xenopus* egg begins a sequence of rapid cell division which, subsequent to the first, occur about every 0.5 h at room temperature and are nearly synchronous in the animal hemisphere at least until mid-blastula (11, 23). In combination with the relatively large size of the blastomeres, these properties make the *Xenopus* embryo a good system for a study of the physiology of mitosis and cytokinesis.

Our purpose in measuring intracellular pH (pH_i) in this system was two-fold. First, we wanted to see if there were pH_i changes associated with mitosis and cytokinesis. Gerson and Burton (5) found a change of 0.6 pH unit during the mitosis cycle of the slime mold *Physarum*, with the maximum of pH_i coincident with mitosis; and Gillies and Deamer (6) demonstrated two alkaline shifts of ~0.4 pH unit per cell cycle in *Tetrahymena*. There is no growth phase in the cell cycle of the early *Xenopus* embryo (8), so one could hope to detect the involvement of intracellular pH specifically in relation to mitosis and cytokinesis. We conclude that there is no such involvement.

Our second reason for looking at pH_i was to see if there are changes correlating with increased protein synthesis and changes in cell-cycle parameters. Recent work demonstrated a strong correlation between pH_i and the rate of protein synthesis in fertilized sea urchin eggs (9, 26, 38). Both the studies of Woodland (39) on *Xenopus* and Shih et al. (27) on *Rana pipiens* indicate an approximate doubling of the rate of protein synthesis from fertilization to two-cell stage, and another doubling from two-cell stage to blastula. Also, Graham and Morgan (8) have shown that it is not until mid-blastula that growth phases become an appreciable part of the embryonic cell cycle. Changes of pH_i at this time might reflect the recruitment of different control mechanisms by the cell. We report that such a change may occur.

MATERIALS AND METHODS

Procedures for Fertilized Eggs

Adult *Xenopus laevis* were purchased from the South African Snake Farm (Fish Hoek, South Africa) or Nasco Biological Science (Fort Atkinson, Wis.). Some fertilized eggs were obtained from induced matings by the method of Gurdon (10), but the majority of our material derived from in vitro fertilization. This was performed by exposing dry-stripped eggs to a sperm suspension in 20% Barth's solution as described by Thoman and Gerhart (33). Fertilized eggs were dejellied in 8–10 min of gentle swirling in freshly-made 1.2% or 2.5% (wt/vol) cysteine HCl, pH 7.8–8.0. The dejellied eggs were washed, cultured, and recorded in Steinberg's solution (20) or a modified Steinberg's solution with HEPES buffer (MSSH). Steinberg's solution contains NaCl, 60.0 mM; KCl, 0.7 mM; $Ca(NO_3)_2$, 0.3 mM; $MgSO_4$, 0.8 mM; Tris, 1.4 mM; pH 7.4, with HCl. The modified Steinberg's contains NaCl, 57.7 mM; KCl, 0.7 mM; $Ca(NO_3)_2$, 0.3 mM; $MgSO_4$, 0.8 mM; HEPES, 5.0 mM; NaOH, 2.4 mM, pH 7.4. There was no difference

THE JOURNAL OF CELL BIOLOGY · VOLUME 91 NOVEMBER 1981 414-419

between the two solutions in any recorded embryonic property, and both supported development at least to hatching. All salts were reagent grade. L-Cysteine, Tris, HEPES, and, where used, MES (2-(N-morpholino)ethanesulfonic acid) and sodium propionate were all obtained from Sigma Chemical Co. (St. Louis, Mo.). Experiment dish temperature was 19–22°C. Development of fertilized eggs was staged according to the Normal Table of Nieuwkoop and Faber (18).

Measurement of Intracellular pH

Measurements were made with conventional KCl and Thomas-type recessed-tip pH microelectrodes (34) as described previously (16). Conventional microelectrodes were filled with 3 M KCl, were typically of 20–25 Mohm resistance, and had been beveled (using a Sutter Instrument Model BV-10 beveler) to improve long-term stability. pH microelectrodes had a tip size of 0.5–1.0 μm, and the time required for full response to a unit pH change was 30–60 s. Electrode slopes were in the range 55–59 mV/pH unit. Recordings were made with the electrodes placed 200–300 μm apart in the animal hemisphere of an embryo confined in a depression of a plastic microtest dish (Falcon 3034; Falcon, Oxnard, Calif.).

Membrane potentials were recorded with a conventional electrophysiology preamplifier (design of P. Getting, University of Iowa), while the pH electrode went to an Analog Devices 311J varactor bridge electrometer (Analog Devices, Inc., Norwood, Mass.). Permanent records were made with a Linear Instruments Model 285 dual-channel chart recorder (Linear Instruments Corp., Irvine, Calif.) and Vetter Model A FM tape-recording system (A. R. Vetter Co., Rebersburg, Pa.). The indifferent electrode was normally provided by an agar bridge formulated in Steinberg's or MSSH and leading to a 3 M KCl reservoir, from which a Ag-AgCl wire was grounded. When very low pH variations of Steinberg's solution were employed, a standard glass calomel electrode (Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, Ill.) was used to ground the bath. For the most part, recordings were continuous through several cell divisions, the length of recording depending on electrode stability and the maintenance of good electrotonic coupling between the pH and conventional microelectrodes. Recordings were rejected if net drift of the electrodes exceeded 5 mV. Intracellular pH was generated electronically by subtracting the potential recorded by the conventional microelectrode from that of the pH microelectrode at the differential input of the chart recorder during playback of the tape-recorded experiment (as in Figs. 1 and 3), or manually from the real-time chart record (as in Fig. 2).

Although we usually initiated a measurement with both microelectrodes in the same animal blastomere, no effort was made to reposition them as they became separated by cell division. This assured continuity of recording while minimizing damage due to multiple impalement. For pH values obtained by this method to be accurate, the membrane potential seen by both electrodes must be the same at all times. Although equality of the membrane potential of blastomeres has not been demonstrated directly for early cleavage stages in *Xenopus*, evidence from later stages (30) and other amphibians (2, 13, 15) is strongly in favor of equipotential blastomeres. It has also been demonstrated that all surface cells on the animal hemisphere of the *Xenopus* embryo are electrotonically coupled at least into mid-blastula (20). When impalement of one cell of an embryo caused damage, this was reflected in depolarization of the other impaled cell, and the "healing" of each was similar (20, and our own observations).

RESULTS

Intracellular pH—Short-term Changes

Figs. 1 and 2 are examples of the continuous recording of intracellular pH during cleavage under normal conditions. The first five cleavages were the most useful to look for pH changes related to mitosis and cytokinesis, since the cycling of the cytokinesis-dependent membrane potential (E_m) makes it easy to gauge the period of the cell cycle. In most experiments, pH_i was almost constant during this period, and we could detect no changes of pH_i associated with either mitosis or furrowing (see Fig. 1). In two of seven continuous recordings of early cleavage stages, we observed a clear cycle of pH_i of ≤0.05 pH unit with the same period as the E_m cycle, but slightly out of phase with it (see Fig. 2). These pH_i changes do not appear to be passive since they are generally in a direction opposite to that which would occur if pH_i were influenced by E_m. In no experiment could we resolve an unequivocal pH_i cycle after stage 6.

Lowered Intracellular pH

To assess the importance of the pH_i cycle seen in Fig. 2 and

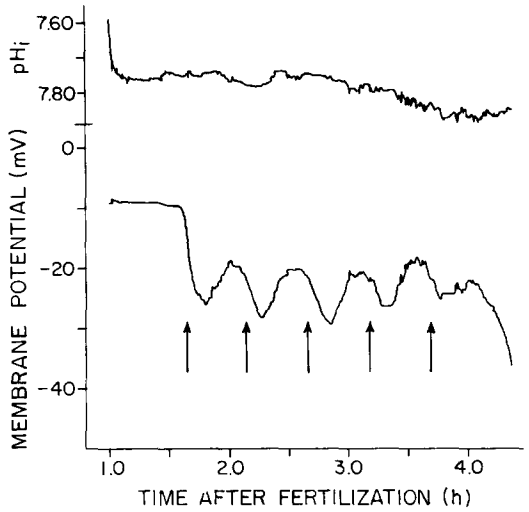

FIGURE 1 Membrane potential and intracellular pH in a fertilized egg from before first cleavage. The arrows indicate the approximate times at which new cleavage furrows were first visible. The live data were recorded on tape and played back in this format, as described previously (16). Recorded in MSSH.

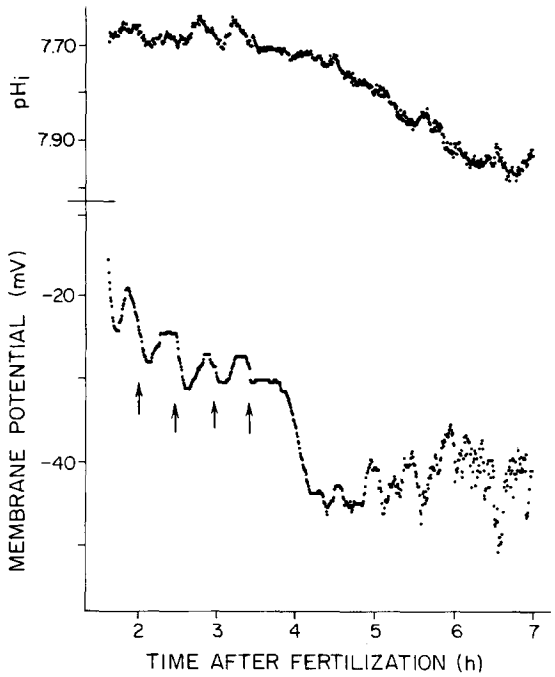

FIGURE 2 Membrane potential and intracellular pH from 2-cell stage to mid-blastula. The arrows indicate approximate times of new furrow formation. A tape record was unavailable, so this figure was generated by manually digitizing the real-time chart record at each 0.5-min interval and plotting the resultant data with the aid of an Apple II computer and NEC 5515 Spinwriter printer. E_m was very erratic towards the end of the experiment. Recorded in Steinberg's solution.

the role, if any, of pH_i in cytokinesis, we decided to try to lower pH_i 0.1–0.2 unit during cleavage. The *Xenopus* embryo is relatively insensitive to externally applied agents (28), and we similarly found a requirement for surprisingly strong treatments to effect the desired changes.

Our most successful procedure was to expose the embryo to a modified Steinberg's solution buffered at pH 4.7–5.0 with sodium propionate. (NaCl concentration was adjusted such that total Na remained near 60 mM.) Since the pK_a of pro-

pionic acid is 4.873 at 20°C (17), ~50% of the propionate in these solutions was in the uncharged form and would be expected to be membrane permeable. Fig. 3 is our best example of the effect of this procedure on a cleaving embryo. An increasing concentration of propionic acid at pH 4.75 induced a relatively slow drop of pH_i. Lowering pH_i to as low as ~7.3 from its normal value of 7.66 ± 0.06 (SD; stage 1–6 average, based on the data of Table I) did not delay or morphologically alter cleavage; but additional cell division was not observed when pH_i dropped to ~7.2, and regression of the most recent cleavage furrow was observed when pH_i was ~7.0 (Table II). Furrow regression occurred in two other experiments in which pH_i was forced to 7.0 or less by exposing the experiment dish to a short pulse of CO_2 gas. However, this procedure caused precipitous decreases of pH_i and was not useful for imposing smaller changes. On the whole, it was difficult to control pH_i in the range 7.0–7.3, even with propionate.

If the propionate was diluted out, or no further CO_2 was added, embryo pH_i would recover to normal values in 45–60 min. Where cleavage had been stopped, it would reinitiate when pH_i reached 7.2–7.5. However, regressed furrows rarely recovered, and subsequent development in those embryos which had suffered furrow regression was almost always abortive or abnormal. In one experiment in which pH_i was dropped to 6.46 with CO_2 before first cleavage, the recorded zygote and the two others in the experiment dish with it never cleaved, even when transferred back to normal MSSH.

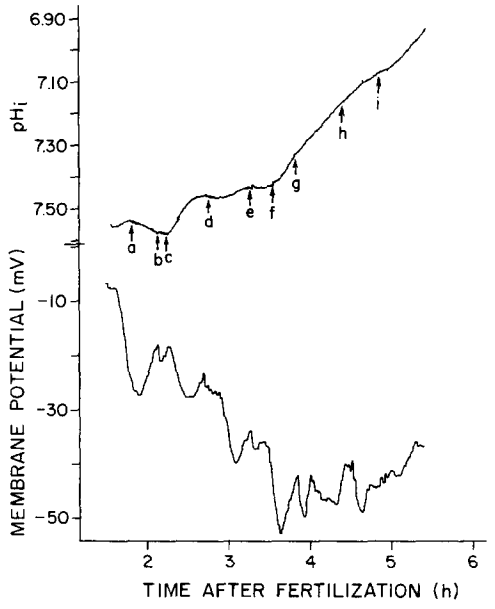

FIGURE 3 An example of a cleaving egg exposed to low pH, propionate-containing Steinberg's solution. This tape-playback record begins just at first cleavage. The egg was impaled before this, and initial pH_i was 7.63 in MSSH. Then a MES-buffered Steinberg's was added to the experiment dish to lower extracellular pH (pH_e) to ~5.0. This dropped pH_i ~0.1 unit. In other experiments, pH_i would recover from this in a few minutes, but this embryo was further challenged with propionate before complete recovery. (*a*) Propionate-buffered Steinberg's solution added to the experiment dish to a final propionate concentration of 5.4 mM; electrodes are in the same blastomere. (*b*) Early second cleavage, coincident with controls; electrodes now in separate cells. (*c*) Propionate added to 7.9 mM; from here on, pH_e is nearly constant at 4.75. (*d*) Early third cleavage, with controls; to 9.1 mM propionate. (*e*) Early fourth cleavage, with controls; to 10.8 mM propionate. (*f*) To 12.3 mM propionate. (*g*) Early fifth cleavage, with controls. (*h*) Perhaps slight relaxation of the fifth cleavage furrow; controls are beginning sixth cleavage. (*i*) Embryo shows no change from *h*. At the end of the experiment, the fifth cleavage furrow is regressing; pH_e is 4.73. Because of incomplete mixing in the experiment dish, the stated propionate concentrations may only approximate the actual initial concentrations seen by the embryo.

TABLE I

Average Intracellular pH and Membrane Potential during Cleavage

Nieuwkoop stage (18)	Intracellular	Membrane potential	No. of records
	pH ± SD	*mV ± SD*	
1 Egg	7.65 ± 0.04	−8.5 ± 1.7	9
2 2-cell	7.64 ± 0.05	−22.2 ± 4.2	3
3 4-cell	7.64 ± 0.06	−24.4 ± 1.0	4
4 8-cell	7.66 ± 0.02	−25.2 ± 3.3	3
5 16-cell	7.69 ± 0.05	−25.7 ± 2.5	4
6 32-cell	7.69 ± 0.08	−25.5 ± 2.2	6
Morula	7.70 ± 0.07	−36.8 ± 6.0	6
7 Early blastula	7.73 ± 0.07	−45.3 ± 6.8	6
High potential only	7.74 ± 0.04	−50.4 ± 2.1	3
Low potential only	7.71 ± 0.10	−40.2 ± 5.7	3
8 Mid-blastula	7.84 ± 0.05	−44.8 ± 10.4	5
High potential only	7.85 ± 0.00	−55.9 ± 0.04	2
Low potential only	7.84 ± 0.07	−37.4 ± 2.9	3

TABLE II

Cleavage at Lowered Intracelular pH *

pH_i range	No. of cleavages observed	No. of embryos	No. other embryos in recording dish with similar morphology
7.50–7.59	9	5‡	22
7.40–7.49	4	3	15
7.32–7.39	3	2	6
6.92–7.08	Furrow regression	2	12

* All lowered with propionate. Five batches of eggs, each from a different donor. Initial pH_i all ≥7.62.

‡ One of these delayed 15 min vs. controls. All others showed no significant delay.

Electrode impalement itself did not impair the development of normally reared embryos. Low extracellular pH and propionate itself have no effect inasmuch as embryos reared in Steinberg's solution buffered with MES, pH 4.7–5.1, or with 10 mM sodium propionate, pH 7.3 (equal to 0.04 mM uncharged form), developed normally through hatching. Other experiments showed that embryos exposed continuously to ≤0.4 mM of the uncharged form would still develop normally. Embryos exposed to >4.0 mM uncharged form ceased dividing and exhibited furrow regression up to two cleavages, while those cultured at intermediate propionic acid concentrations developed abnormally or arrested in late blastula (Table III).

Intracellular pH—Long-term Changes

Table I lists the average pH_i of fertilized eggs through mid-blastula (stage 8). Fig. 2 is a typical example of the pattern we observed. The initial intracellular pH (measured 1 to 1.25 h after fertilization) was 7.65 ± 0.04 (SD; range 7.56–7.70) and rose slowly to 7.70 ± 0.06 at stage 5 to midway through 6

TABLE III

Effect of Propionate on Development

Concentration of uncharged form	No. of embryos¶	No. of egg donors	Effect on Development**
mM			
0.0, Low pH*	14	3	None
0.04–0.43‡	18	3	None
0.85–3.04§	18	2	Abnormal or arrested in late blastula
4.27–17.95‖	33	6	Arrested 0–2 divisions after exposure

* pH 4.7–5.1 MES-buffered Steinberg's solution.

‡ Range of total propionate from 1.0 mM, pH 5.0, to 10.0 mM, pH 7.3.

§ Range of total propionate from 2.0 mM, pH 5.0, to 8.3 mM, pH 5.11; intermediate concentrations at pH as high as 5.5.

‖ Range of total propionate from 10.0 mM, pH 5.0, to 30.0 mM, pH 4.7; intermediate concentrations at pH as high as 5.2.

¶ Continuously exposed to indicated concentration from division 0–5 onward.

** As gauged through at least one day's development, and usually through hatching. Development of dejellied embryos in MSSH was morphologically normal at a rate of at least 90%.

(morula). During stage 7, pH_i would inflect and alkalize to a level 0.18 ± 0.03 pH unit higher (five experiments, each from a different batch of eggs). This change is statistically significant ($P < 0.01$). The inflection occurred an average 5.2 ± 0.5 h after fertilization, and pH_i required 0.9 ± 0.4 h to reach a new stable point sometime during stage 8. This higher value was 7.87 ± 0.06 (five experiments; average pH_i at the stage-7 inflection point was 7.69).

Beginning in stage 7, we could distinguish two classes based on a difference in membrane potential. Those embryos with a stage-7 E_m that was larger in magnitude than −45 mV continued to hyperpolarize into stage 8. Those with an E_m that was <−45 mV stayed the same or depolarized slightly. According to the data of Palmer and Slack (20), the higher potentials are more typical of stage-8 embryos. Our lower values may reflect membrane damage, especially since these embryos showed greater scatter for both E_m and pH_i. Neverthees, there was no difference in average pH_i between the high and low E_m embryos at stages 7 and 8.

Membrane Potential Changes

Figs. 1 and 2 are representative of the membrane potential changes seen in all experiments. Table I gives the average E_m at each stage. There was a marked hyperpolarization at first cleavage and then little change in average E_m through the next four, although E_m cycling was characteristic of this period. Beginning midway through stage 6, E_m hyperpolarized ~20 mV, and cycling became erratic. The hyperpolarization began an average 4.0 ± 0.4 h after fertilization and was complete by early stage 7. There was no significant change in average E_m into stage 8, although E_m fluctuations often increased.

DISCUSSION

The Level of Intracellular pH

Our data correspond well to other accounts of intracellular pH in the *Xenopus* embryo. The pH_i which we measured for the fertilized egg just before first cleavage is equivalent to the values found by Webb and Nuccitelli (37) using a pH microelectrode and by Nuccitelli et al. (19) using ^{31}P-NMR (nuclear magnetic resonance). In our experiments, average pH_i in the cleavage embryo through stage 7 was 7.68 ± 0.06 (SD); from 4-cell to stage 7, it was 7.69 ± 0.07. This is not statistically different from the 7.74 ± 0.02 (SE) for 4-cell to early blastula reported by Turin and Warner (36). Because Turin and Warner were careful to impale a single blastomere with both pH and reference microelectrodes, the similarity of our results supports the assumption that blastomeres are equipotential. If it were true that the blunter pH microelectrode had induced a depolarization confined primarily to its impaled blastomere—as appears to be the case in Fig. 3 of Turin and Warner (36)—then our value for pH_i would indeed be low; but the discrepancy between 7.69 and 7.74 can be accounted for by a membrane potential difference of <3 mV, which is probably within the inherent limitations of the technique (30).

The early intracellular pH of 7.65 is almost exactly intermediate between the final pH_i of matured oocytes from hormonally stimulated and nonstimulated females (16). How this might relate to the hormonal history of egg donors is unclear because no uniform protocol was used in these experiments. Many of the donors had received no hormone for a number of months before induced ovulation, and oocytes from such donors tended to be variable (16). This contrasts with the reports of Webb and Nuccitelli (19) and Nuccitelli et al. (37) that pH_i is ~0.3 unit less before fertilization. It is possible that the frog lowers pH_i in the oviduct, as suggested by Smith and Ecker for *Rana* (31), or that in vitro maturation with progesterone does not accurately mimic all aspects of the in vivo process.

Mitosis and Cytokinesis

In the majority of our experiments, we were unable to detect any cycling of pH_i with mitosis, and certainly nothing of the magnitude reported by Gerson and Burton (5) or Gillies and Deamer (6). In other experiments from this laboratory we were able to confirm a cycle of pH with mitosis in *Physarum*, although our values of pH_i 7.0–7.4 with a peak just before mitosis differ considerably from the values reported by Gerson and Burton (M. Morisawa and R. A. Steinhardt, manuscript in preparation). This suggests that the pH_i shifts observed in these other systems reflect changes associated with cell growth but not mitosis or cytokinesis per se. Gillies and Deamer suggest that increase in pH_i are specifically related to DNA synthesis in *Tetrahymena*, but this would appear not to be the case in *Xenopus*, because pH_i during meiotic maturation in the oocyte (16) can be as high as in the cleaving egg.

In two recordings we did observe a very small pH cycle, as has recently been reported by Webb and Nuccitelli (37) for early cleavage of *Xenopus* embryos which retain their jelly. Because these two recordings involved electrodes which had been partitioned into separate blastomeres by cell division, it is possible that the pH "cycle" is an artifact caused by the electrodes being in partially uncoupled cells with slightly different membrane potential cycling. In two control experiments we measured membrane potential with two conventional microelectrodes continuously over the period from before first cleavage to the middle of stage 6, and from stage 5 to stage 8. The membrane potentials measured by the electrodes were equal on the average and within ±1.5 mV of each other 90% of the time, but transient differences of up to 4 mV were observed. However, as far as we can tell, all variation between the electrodes was random, not cyclical, in nature. Hence, we cannot in our own data rule out the possibility of artifact producing the pH cycle; but it is just as reasonable to us that our inability to resolve a pH cycle more consistently could

have been due to such random membrane potential variation obscuring a true pH oscillation.

In any case, it seems unlikely that distinct cytoplasmic events could be regulated by pH_i changes as small as this. A possibility is that the change observed at the pH microelectrode reflects a localized pH change of greater magnitude in the deep cytoplasm. Arguing against this are those experiments in which the cytoplasm was held at artificially low pH values without disrupting cell division. It seems more likely that any pH change is a secondary effect related to the cycle of intracellular sodium activity (4, 29) or the insertion of highly K-permeable new membrane during furrowing (28, 40).

Neither did we observe anything to suggest a pH "trigger" for furrowing. If this were a localized change it might be missed, but the result was the same even when the pH electrode was very close to the developing furrow. Moreover, experiments in which the cytoplasm was acidified with propionate as much as 0.3 pH unit below normal without affecting cytokinesis strongly suggests that pH is not involved in the regulation of this process.

The working hypothesis of many investigators of cytokinesis is that the contractile ring is analogous to a muscle sarcomere, including probable regulation by free Ca^{2+} levels (24). It is clearly true that treatments which increase membrane permeability to Ca^{2+} (7) or release it from intracellular stores (25) will induce surface contractions in frog eggs, as will the direct iontophoretic injection of Ca^{2+}—but not Mg^{2+}, K^+, Na^+, or Cl^-—immediately beneath the cell membrane (7).

However, other work involving the use of Ca^{2+} buffering agents in frog eggs (1, 22) and a mammalian cell model (3) has suggested that, while a certain threshold level of free Ca^{2+} is required for cytokinesis, this level is not measurably different from the probable resting level and need not change during cytokinesis. Efforts to detect increases in the Ca^{2+} level during cleavage with the Ca-dependent photoprotein aequorin have been inconclusive in frog and fish eggs (1, 21) and completely unsuccessful in the sea urchin egg (14). A second investigation of Ca^{2+} in the frog egg with a Ca-sensitive microelectrode also found no changes in Ca^{2+} levels (22).

Although other experiments suggest that Ca^{2+} may play a role in determining the site of the cleavage furrow (12, 35), it seems clear that Ca^{2+} does not regulate force production in the contractile ring as it does in the sarcomere. Moreover, the experiments reported here would appear to eliminate H^+, the last ionic candidate for this regulator.

Lowering pH_i to ~7.2 arrested cell division. The nature of this effect is unclear, although it may reflect a general repression of cellular metabolism and/or block mitosis. In amphibian embryos it has been demonstrated that low pH_i will uncouple electrotonically coupled cells in both *Xenopus* (36) and *Ambystoma* (32), and the pH_i required to effect this is similar to that needed to stop cleavage. However, it seems unlikely that uncoupling per se would disrupt cleavage because dissociated *Ambystoma* blastomeres continue to cleave (11).

Long-term Changes of Intracellular pH

We have observed two phases of alkalization in fertilized *Xenopus* eggs. The first of these occurs gradually during the first few divisions. It is quite small and may not be statistically meaningful. A change of pH_i reflecting the reported increase of protein synthesis at fertilization would be expected before first cleavage, as has been reported (19, 37).

The second increase in pH_i occurs in early blastula; it is dramatic and statistically significant. It suggests a correlation with the doubling of the rate of protein synthesis reported to occur between 2-cell stage and blastula. Although Shih et al. (27) do not describe the kinetics of this change in *Rana*, the *Xenopus* data of Woodland (39) imply that the change occurs after stage 6, which is consistent with our observations.

Membrane Potential Changes

The increase of E_m with development has been reported for a number of systems, most completely for *Xenopus* by Palmer and Slack (20). Our data are in good agreement with theirs except for stage 8, where our average value is lower. The cycling of E_m during early cleavage stages has also been reported (29). Slack and Warner (28) suggest that the increase of E_m during development is due to the increasing amount of new membrane facing on the developing blastocoel. The marked hyperpolarization midway through stage 6 to 7 would seem to reflect a more deliberate alteration of membrane and/or blastocoel properties. It might relate to the formation of the double-layered embryo in early blastula (18).

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