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^{31}P NMR reveals increased intracellular pH after fertilization in *Xenopus* eggs

(pH regulation/egg activation/phosphocreatine)

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ABSTRACT **^{31}P NMR spectra of mature eggs of the frog (*Xenopus laevis*) were taken prior to and after both fertilization and activation by a Ca^{2+}/H^+ ionophore (A23187). The eggs were constantly perfused with fresh well-buffered solution during the experiments, and the intracellular pH (pH_i) was determined from the pH-dependent chemical shift of the internal P_i peak. The detection of this P_i peak in the presence of overlapping yolk phosphoprotein signals was accomplished by a T_2 experiment which discriminated against the broader yolk phosphoprotein peak. The average pH_i of the unfertilized, fertilized, and activated eggs was 7.42, 7.66, and 7.64, respectively. Thus, a cytoplasmic alkalinization of 0.24 pH unit occurs within 90 min after fertilization. These values are practically identical to pH_i measurements made in this laboratory on *Xenopus* eggs by using pH-sensitive glass microelectrodes. These ^{31}P NMR studies also indicate that extracellular pH changes as large as 3 pH units had no effect on pH_i. We also found that phosphocreatine levels are very sensitive to metabolic perturbations such as oxygen depletion or metabolic inhibitor application. These treatments resulted in a rapid decrease in the phosphocreatine concentration; the ATP concentration declined only slowly after the phosphocreatine peak had disappeared.**

It has long been suspected that changes in the egg's intracellular ion concentrations must be important steps in the activation of development because simple manipulations of the ionic environment are effective in stimulating artificial parthenogenesis (1). Although most of the earlier data pointed to the involvement of intracellular Ca^{2+} (1–4), the most successful parthenogenetic treatments often included NH_3 which was known to change intracellular pH (pH_i) (1, 5–8). Subsequent attempts to measure pH_i during fertilization by using antimony electrodes (9) or injected indicator dyes (10, 11) were inconclusive due to technical problems. Recently, the involvement of pH_i changes in activation has been postulated for the sea urchin egg (12–14), and advances in pH-sensitive microelectrodes have allowed reliable direct measurements of the pH_i increase that is triggered at fertilization (15). Application of this same technique in this laboratory has revealed a similar pH_i increase after fertilization in the egg of the freshwater vertebrate *Xenopus laevis* (16). However, because microelectrode impalement may cause some injury to the eggs, it seemed desirable to confirm this finding with the noninvasive technique of ^{31}P NMR.

Here we report that the ^{31}P NMR technique also indicates a pH_i increase after both fertilization and artificial activation.

MATERIALS AND METHODS

Animals and Eggs. Ovulation was induced by injecting a priming dose of 35 international units of pregnant mare's serum gonadotropin (National Institutes of Health, National Pituitary Agency) into the dorsal lymph sac 72 hr before eggs were needed, followed about 65 hr later by 1000 international units of human chorionic gonadotropin (Sigma). Ten to 20 *X. laevis* each were injected for morning and for afternoon experiments so that a constant supply of eggs was available over an 8-hr period. Eggs were squeezed out of the females into modified F1 solution without the phosphate [31.25 mM NaCl, 1.25 mM KCl, 0.25 mM $CaCl_2$, 0.0625 mM $MgCl_2$, 10 mM *N*-tris(hydroxymethyl)methylglycine, 1.9 mM NaOH, 2 mM $NaHCO_3$ (17)] at pH 7.8 and inspected under a stereoscope for lysis and uneven pigmentation blotches. Only healthy-looking eggs were used in the experiments, and each run required about 30 ml of eggs before dejellying, usually provided by three to six females. After each NMR experiment, the eggs were again inspected visually and scored for pigmentation blotches and lysis. If any deterioration had occurred, those NMR results were discounted.

Fertilization was accomplished by mincing one-fourth of a testis in F1 solution and adding it to eggs being agitated in a 15.25-cm Petri dish. Each dish was then scored for rotation before the eggs were placed in the NMR tube. The data presented here were taken from batches exhibiting >80% rotation. These eggs proceeded to cleave normally in the NMR tube throughout our experiments and, when removed, looked identical to control embryos growing in a shallow Petri dish. Ionophore activation was accomplished in a similar manner by adding a small amount of A23187 (Calbiochem; 1 mg/ml in ethanol) to the Petri dish so that the final A23187 concentration was 2 μM and the final ethanol concentration was <0.1%.

Xenopus eggs are surrounded by three jelly layers which expand greatly upon hydration so that these 1.3-mm diameter eggs typically pack 4 mm center to center. In order to increase the packing density, the eggs were partially dejellied by combining 100 μl of mercaptoethanol with 100 ml of F1 plus 30 ml of eggs at pH 8.4. After 5–10 min of mild swirling, the packing density was approximately doubled, after which the mercaptoethanol was quickly washed off. Most eggs were still separated from their neighbors by jelly after this treatment. This is important because totally dejellied eggs pack so tightly that the perfusion of fluid around them is uneven and erratic. We used an 18-mm inner-diameter sample tube, a 24-mm-high spectrometer detector window, and partial egg dejellying to a 2-mm center-to-center packing density, providing spectra representing signal contributions from approximately 1000 eggs.

Using a peristaltic pump, we continuously perfused the eggs by adding aerated F1 at the bottom of the NMR tube and drawing off medium from the top of the tube. Typically we used 15 ml of cells with a 15-ml column of fluid above them and perfused

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Abbreviations: Gro*P*Cho, glycerophosphorylcholine; *P*Cr, phosphocreatine; pH_i, intracellular pH; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FID, free induction decay; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid.

at 2–4 ml/min. This exchanged the 27 ml of extracellular fluid in 7–14 min. *Xenopus* eggs are ideal for this perfusion because, due to their size and density, they quickly settle to the bottom of the tube and remain there undisturbed by the perfusion. This continuous flow also allowed us to change the medium and directly monitor extracellular pH while collecting spectra.

Egg Extract Preparation. The egg extract solutions were prepared by first totally dejellying the eggs in 0.3% mercaptoethanol and washing them several times with an extract buffer described below. Next, they were gently lysed by drawing them into a 1-mm disposable pipette and then combined with an equal volume of extract buffer. The extract buffer was designed to match closely the intracellular ionic environment when combined with an equal volume of lysed eggs. Both the total and free intracellular ion concentrations are listed in Table 1, and substantial sequestering or binding of Mg^{2+} and Ca^{2+} was evident. To ensure similar low free Ca^{2+} and Mg^{2+} levels in the extract, the divalent chelators EDTA and ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) were used to make two separate buffer solutions. We did not measure the total Ca^{2+} and Mg^{2+} released during our gentle lysing procedure but would expect it to be lower than the total divalent cation store in the egg because some ions would be sequestered inside cellular organelles and some would be bound to proteins and membranes. Extract buffer 1 had EGTA at a final concentration of 1.5 mM which would hold the free Ca^{2+} down to low levels if the total Ca^{2+} were less than 1 mM. Extract buffer 2 was designed to buffer both the Ca^{2+} and Mg^{2+} by containing 5.5 mM EDTA. According to the stability constants of Martell and Smith (ref. 22; 0.1 ionic strength corrected) and estimates of 5 mM total Mg^{2+} and 1 mM total Ca^{2+}, the free Mg^{2+} and Ca^{2+} levels would be 0.5 mM and 1.5 μM, respectively. Perchloric acid extracts were made by adding 3 ml of 50% $HClO_4$ to approximately 27 ml of eggs at 0°C for 10 min before titration back to pH 7.0 with KOH. After 12 hr at 4°C, the solution was centrifuged for 2 hr at 12,100 × *g* and the supernatant was used for NMR.

Recording and Treatment of Spectra. ^{31}P NMR spectra were recorded at 81 MHz on a Nicolet Magnetics NT 200 spectrometer operating in the Fourier transform mode. For the single-pulse experiments, a 54° tipping pulse of 15 μsec was applied every 30 msec. All spectra in this paper have been plotted using 7-Hz line broadening. Two standards were used: (*i*) a sealed capillary containing 85% phosphoric acid, assigned 0 ppm chemical shift; and (*ii*) 0.6 mM extracellular glycerophosphorylcholine (Gro*P*Cho) (Sigma, grade 1), assigned 0.49 ppm. It has been shown that the Gro*P*Cho chemical shift is independent of pH (23) and we confirmed this over the pH range 7–8. All experiments were performed at 20°C in the absence of ^2H_2O.

Table 1. Extract buffer solution composition and cytoplasmic ion concentrations in *Xenopus* eggs

	Concentration, mM*			
	Free cytoplasmic	Total in egg	Extract buffer 1	Extract buffer 2
Na^+	19 (18)	58 (18)	12	19
K^+	52 (18)	87 (18)	52	52
Cl^-	54 (18)	62 (18)	53	28
Mg^{2+}	≤0.5†	22 (19)	0.5	—
Ca^{2+}	10^{-4} (20)	2–7 (19, 21)	—	—
EGTA	—	—	3	—
EDTA	—	—	—	11
Hepes	—	—	—	10

* Numbers in parentheses are references.
† Sherwin Lee and Rick Steinhardt, personal communication.

Therefore, field-frequency locking was not used, but our reference standard, Gro*P*Cho, was always present and our spectrometer drift was negligible over these short experimental periods (<1 hr).

In order to pull the P_i peak out from under the broad yolk phosphate peak, we used a two-pulse T_2 experiment. This discriminates between the two peaks on the basis of line width. The free induction decay (FID) signals from broad lines such as the yolk phosphates decay more quickly than do signals from narrow lines such as P_i. Therefore, by applying a second pulse after most of the yolk contributions have died out, the refocused FID will contain mainly P_i contributions. Lines experiencing homonuclear decoupling such as ATP peaks are not refocused properly and occur with anomalous phase in the transformed spectra (24). This T_2 experiment resolved the P_i peak much better than did convolution difference experiments.

RESULTS

^{31}P NMR Spectra of Unfertilized Eggs. The ^{31}P NMR spectra of unfertilized *Xenopus* eggs are shown in Fig. 1 *A* and *B*. The single-pulse experiment (Fig. 1*A*) produced essentially the same spectra as observed by Colman and Gadian (19), with a large, broad, yolk phosphoprotein peak obscuring the P_i peak. By using the two-pulse T_2 experiment we were able to discriminate against broad line signals and pull out the P_i peak (Fig. 1*B*). This peak was identified as P_i by observing an increase in peak height when P_i was added to the extracellular medium adjusted to the same pH. It is also the only narrow line phosphate compound in that region of the egg extract spectrum. After studying six different batches of approximately 1000 unfertilized, partially dejellied *Xenopus* eggs each, we found that the average P_i chemical shift was 2.63 ppm (Table 2). The T_2 spectra exhibit anomalous phasing for the ATP peaks because the phosphates are closely coupled (24).

^{31}P NMR Spectra of Fertilized Eggs. Groups of fertilized eggs in which more than 80% of the eggs had rotated so that the pigmented animal hemisphere was uppermost were dejellied to the same extent as were the unfertilized eggs and their ^{31}P NMR spectra were collected. Scoring for rotation and partial dejellying delayed the beginning of the NMR studies by 40 min, and 20–40 min of experiment time was needed to achieve a satisfactory signal-to-noise ratio. Because cleavage occurs between 60 and 90 min after fertilization, the eggs usually underwent first cleavage during our measurements. Fig. 1*C* shows the spectrum from about 1000 fertilized eggs originally taken from the same group as the eggs in Fig. 1 *A* and *B*. This spectrum is practically identical to the unfertilized egg spectrum; however, a distinct difference is revealed by the T_2 experiment (Fig. 1*D*). This difference is best illustrated by overlaying expanded versions of Fig. 1 *B* and *D* as shown in Fig. 1*E*. The Gro*P*Cho and phosphocreatine (*P*Cr) peaks coincide quite well, but there is a distinct shift of 0.2 ppm in the P_i peak. This shift was observed in all five groups of fertilized eggs and all three groups of ionophore-activated eggs studied. The average P_i chemical shifts are listed in Table 2.

In order to convert these P_i chemical shifts into pH_i indicators, we studied the P_i shift dependence on pH in cell extract solutions. The extract pH was adjusted and measured with a pH-sensitive glass electrode before and after determination of the P_i chemical shift with NMR. Fig. 2 shows this pH dependence with extract buffer 2 which chelates both Ca^{2+} and Mg^{2+} to physiological levels. Fig. 2 *Inset* shows the data taken with extract buffer 1 at two different temperatures and after perchloric acid precipitation of the protein. No significant temperature dependence (0–20°C), protein dependence, or Mg^{2+} (0.5–10

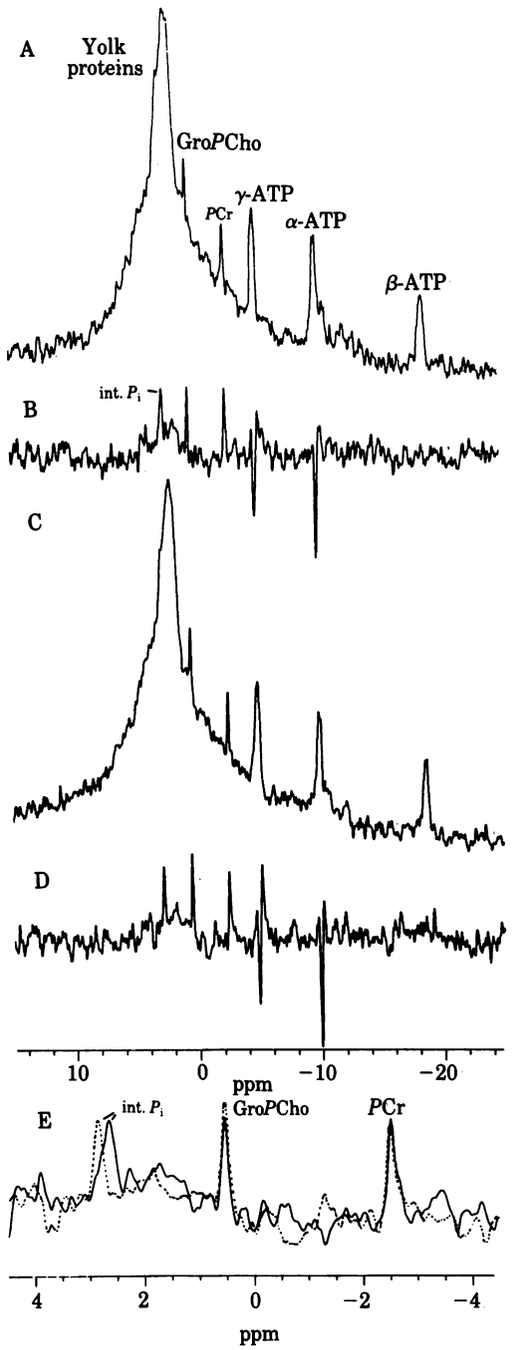

FIG. 1. ^{31}P NMR spectra of approximately 1000 *Xenopus* eggs before and after fertilization. (*A*) Average of 1000 single-pulse (54°) scans repeated every 300 msec on unfertilized eggs. The peaks were identified by adding indicated substances to egg extract solution. Peak assignments using Gro*P*Cho as a reference are (in ppm): yolk proteins, 2.18; Gro*P*Cho, 0.49; phosphocreatine (*P*Cr), −2.53; γ-ATP, −5.02; α-ATP, −9.96; β-ATP, −18.65. (*B*) Average of 2000 two-pulse (90°–180° with 15-msec τ) scans repeated every 800 msec on the same eggs as in *A*. Intracellular P_i peak is now visible at 2.62 ppm. The other peak assignments are within 0.02 ppm of the values in *A*. (*C*) Average of 1000 single-pulse scans on fertilized eggs from the same group of females as in *A* and *B*. Peak assignments (in ppm): yolk protein, 2.14; Gro*P*Cho, 0.49; *P*Cr, −2.53; γ-ATP, −4.94; α-ATP, −10.09; β-ATP, −18.65. (*D*) Average of 2000 two-pulse (90°–180° with 15-msec τ) scans on same eggs as in *C*. P_i peak is now visible at 2.82 ppm. Other peak assignments are within 0.02 ppm of the values in *C*. (*E*) Portions of Fig. 2 *B* (solid line) and *D* (dotted line) are overlaid on an expanded scale so that the 0.2 ppm shift of the P_i peak is clear.

Table 2. P_i chemical shifts from T_2 experiments and corresponding pH_i in *Xenopus* eggs

Egg condition	P_i chemical shift, ppm	pH_i	
		^{31}P NMR	Microelectrode
Unfertilized	2.63 ± 0.04 (6)	7.42 ± 0.04 (6)	7.39 ± 0.11 (11)
Fertilized	2.81 ± 0.04 (5)	7.66 ± 0.06 (5)	7.69 ± 0.05 (6)
A23187 activated	2.80 ± 0.01 (3)	7.64 ± 0.02 (3)	7.70 ± 0.1 (14)

Results are shown as mean (±SD) from the number of experiments in parentheses. The spectra in each NMR experiment were contributed by approximately 1000 eggs.

mM) dependence was found. The only way we could shift this curve was by making large changes in ionic strength. When sea urchin egg extract solution (25) with a 5-fold higher ionic strength was used, the curve was shifted approximately 0.2 ppm toward higher values. Thus, pH 7.4 corresponded to a P_i chemical shift of 2.62 in our *Xenopus* extract medium but to a shift of 2.80 in sea urchin egg extract. Therefore, the cytoplasmic ionic strength must be known to generate accurate pH_i data. The pH_i values indicated by our P_i chemical shift measurements are listed in Table 2. These measurements indicate that the pH_i of the fertilized eggs is 0.24 unit more alkaline than that of the unfertilized egg. Similarly, eggs that had been activated with 2 μM A23187 showed an intracellular alkalinization of 0.22 pH unit.

pH_i Regulation. During the above experiments we held the extracellular pH (pH_o) constant at 7.8. However, during other experiments we found that pH_o changes as large as 3 pH units (pH 6–9) had no effect on pH_i. In one example, shown in Fig. 3, pH_o was changed from 7.9 to 8.95 but pH_i remained at 7.44 even after 2 hr in the high pH_o medium. This suggests that H^+ is not passively distributed across the plasma membrane because pH_i should then follow pH_o according to: $pH_i = (E_m/58.8\ mV) + pH_o$, in which E_m is the membrane potential.

Phosphate Metabolite Levels. While monitoring the effect

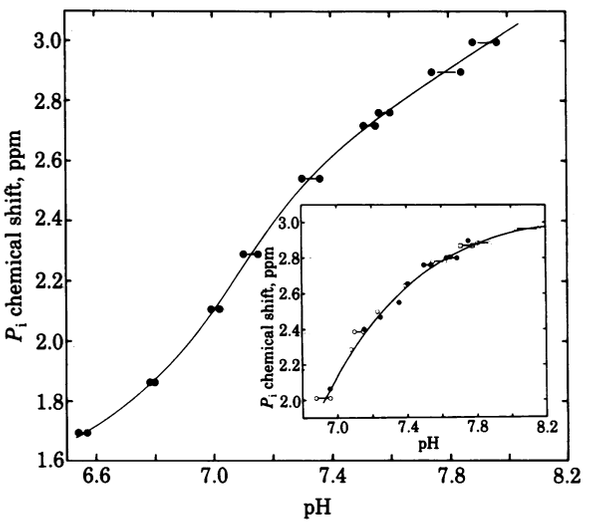

FIG. 2. Chemical shift of P_i as a function of pH in the frog egg extract solution made with buffer 2 at 20°C. A pH electrode was inserted into the NMR tube before and after the chemical shift measurement and both values are plotted with a horizontal line drawn between them. The curve was drawn to the data points by eye. (*Inset*) Plot of same relationship in extract solution made with buffer 1 while varying temperature and protein conditions: ○, at 0°C on extract supernatant after centrifugation for 1 hr at 12,000 × *g*; ●, at 20°C on supernatant of perchloric acid-precipitated extract after centrifugation for 2 hr at 13,000 × *g*; □, at 15°C on extract supernatant after centrifugation for 1 hr at 12,000 × *g*.

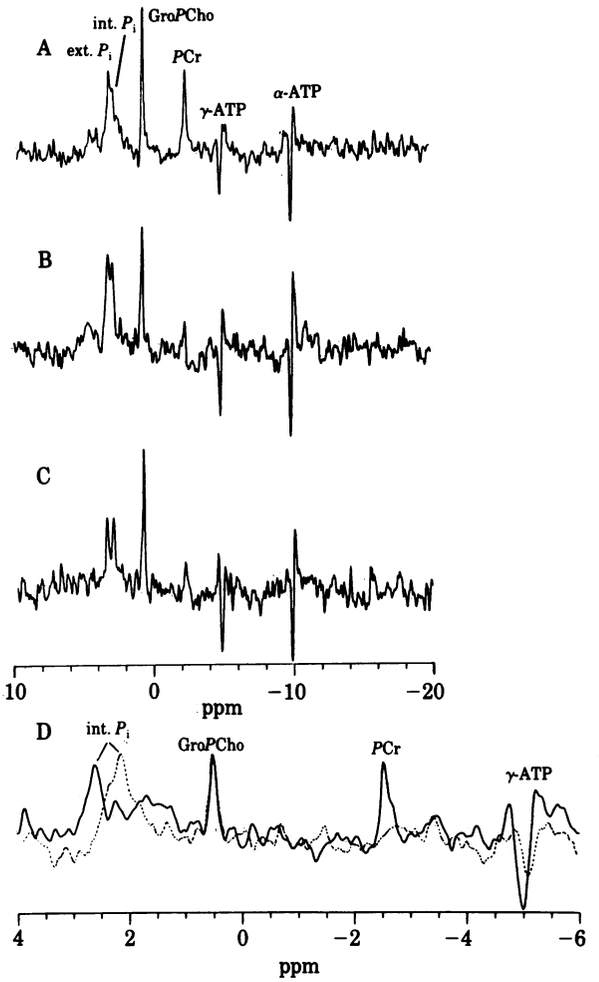

FIG. 3. ^{31}P NMR spectra of approximately 1000 unfertilized *Xenopus* eggs in a two-pulse (90°–180° with 15-msec τ) sequence; 0.5 mM P_i and 0.6 mM Gro*P*Cho were added to the extracellular medium in *A–C*. (*A*) Average of 1728 two-pulse scans over a 30-min period on eggs perfused with aerated medium at pH 7.9. Peak assignments: ext. P_i, 2.92; int. P_i, 2.65 (pH 7.44); Gro*P*Cho, 0.49; *P*Cr, −2.53. (*B*) Average of 1512 two-pulse scans over a 26-min period on the same eggs as in *A* perfused with N_2-bubbled medium at pH 8.2. This sequence was begun after 40 min of perfusion with N_2 medium. Peak assignments: ext. P_i, 2.94; int. P_i, 2.64 (pH 7.44); Gro*P*Cho, 0.49; *P*Cr, −2.53. (*C*) Average of 1000 two-pulse scans over a 17-min period on the same eggs as in *B* perfused with N_2-bubbled medium at pH 8.95. This sequence was begun after 92 min of N_2 medium perfusion. Peak assignments: ext. P_i, 3.11; int. P_i, 2.65 (pH 7.44); Gro*P*Cho, 0.49; *P*Cr, −2.54. (*D*) The solid line is the average of 2000 scans over a 43-min period on eggs perfused with aerated medium with 0.6 mM Gro*P*Cho added (no P_i was added). Peak assignments: int. P_i, 2.61 (pH 7.39); Gro*P*Cho, 0.49; *P*Cr, −2.54; γ-ATP, −4.99. The dotted line is an average of 1200 scans over a 26-min period on the same eggs beginning 27 min after the start of perfusion (6 ml/min) with 1 mM dinitrophenol in F1 at pH 7.82. Peak assignments: int. P_i, 2.12 (pH 7.01); Gro*P*Cho, 0.49; γ-ATP, −5.03.

of various metabolic inhibitors on pH_i, we noted that *P*Cr levels were sensitive to any changes in metabolism whereas the ATP levels remained constant. Specifically, oxygen removal or addition of 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or 1 mM dinitrophenol resulted in a rapid fall in *P*Cr level (faster than our 10-min time resolution) and this was usually accompanied by an increase in P_i. Oxygen removal, by either perfusing with N_2-bubbled medium or stopping the perfusion completely, decreased the *P*Cr level. The ATP levels, on the other hand, remained unperturbed initially and only began falling after the *P*Cr peak had completely disappeared. Two examples of this *P*Cr dependence on metabolism are shown in Fig. 3. While the pH_o was being increased as described above, the eggs were also perfused with N_2-bubbled medium as shown in Fig. 3 *B* and *C*. A sharp decrease in the *P*Cr peak is evident, and a small increase in the P_i peak occurred. Fig. 3*D* shows the effect of 1 mM dinitrophenol. The *P*Cr peak completely disappeared and the P_i peak shifted 0.4 pH unit down. This is in good agreement with our pH microelectrode measurements which indicate that dinitrophenol acts as a weak acid and causes an intracellular acidification which begins a few minutes after application and peaks about 1 hr later. Dinitrophenol is also a metabolic uncoupler which causes a slow decline in the ATP level, and this also could change pH_i if active H^+ transport mechanisms were at work.

DISCUSSION

The combination of partial dejellying for increased egg density and utilizing the T_2 experiment for discriminating against the yolk phosphoproteins allowed us to uncover the P_i peak that was hidden in the *Xenopus* egg spectra (19). The position of this peak shifts within an hour after fertilization, indicating an alkalinization of 0.24 pH unit. Due to the low level of P_i in the egg, it was necessary to signal average about 2000 pulse pairs, which limited our time resolution to about 40 min.

These experiments also revealed rapid changes in the *P*Cr level upon metabolic perturbation. Reduction in O_2 supply or the presence of the uncouplers FCCP or dinitrophenol caused a decrease in *P*Cr whereas the ATP level remained fairly constant during at least an hour of these treatments.

Cytoplasmic or Intracellular Compartment pH? Because ^{31}P NMR detects the P_i signal from all regions of the egg, P_i in organelles such as mitochondria, yolk platelets, and endoplasmic reticulum can contribute along with cytoplasmic P_i. Indeed, in some cells, both mitochondrial and cytosol P_i peaks have been detected (26). However, we think that it is unlikely that mitochondrial P_i is being detected here for three reasons. (*i*) In this large (1.3-mm diameter) yolky egg, the mitochondrial volume is only a small fraction (<1%) of the cytoplasmic volume and the P_i will distribute between these two compartments as a weak acid. Because mitochondria are only about 0.2 pH unit more alkaline than the cytoplasm (26), the mitochondrial P_i concentration will be only slightly greater than that of the cytoplasm, so at least 99% of the P_i should be nonmitochondrial. (*ii*) When FCCP or N_2-bubbled medium was applied, no shift in the P_i peak occurred. Both of these treatments have been shown to change mitochondrial pH, so this insensitivity again suggests that we are not detecting mitchondrial P_i. (*iii*) We find only a single P_i peak in our gently lysed cells. Here the mitochondria and yolk platelets are almost certainly intact, yet the P_i peak shifts as we change the extramitochondrial pH. Here again we can detect no mitochondrial P_i component. Although it is more difficult to rule out a P_i contribution from the endoplasmic reticulum, we note below that our intracellular pH microelectrode data agree quite closely with these NMR results, and it is unlikely that these electrodes routinely penetrated the endoplasmic reticulum.

Good Agreement Between Intracellular pH Microelectrodes and ^{31}P NMR. Recent measurements of pH_i in *Xenopus* have been made in this laboratory by using the Thomas-type recessed-tip pH microelectrode (16). The average values of the pH_i data collected in the same period, May through September 1980, are listed in Table 2. These values obtained from single egg measurements are almost identical to the measurements reported here from populations of eggs and the noninvasive NMR technique. The microelectrode technique has a much

better time resolution and indicates the slightly larger pH_i, 0.30 pH unit, increase after fertilization compared to 0.24 unit indicated by ^{31}P NMR. This excellent agreement between the two techniques should be reassuring to both NMR and pH microelectrode enthusiasts.

Previous Investigations with Similar Observations. The sensitivity of *P*Cr level to metabolic perturbations also has been observed in muscle cells (27–32). Here again, both O_2 depletion and metabolic inhibitor addition result in a rapid decline of the *P*Cr level with only slow changes in the ATP level.

An intracellular alkalinization has been associated with the activation of metabolism or the cell cycle in a wide range of organisms. The list includes activating sea urchin (15) and frog eggs (16), the release from dormancy of bacteria (33), yeast (34), and shrimp (W. Busa and J. Crowe, personal communication); the activation of glycolysis in human erythrocytes (35) and frog muscle (36); and cell cycle activation in yeast (37), ciliates (38), and slime molds (39). Therefore, this cytoplasmic alkalinization after fertilization in frog eggs may represent one application of a general cellular regulation mechanism important for the control of the cell cycle and metabolic rate.

We thank Dr. J. A. Den Hollander for suggesting the T_2 experiment to uncover the P_i peak and Dr. Robert Gillies for suggesting the use of Gro*P*Cho as a pH-independent reference. We also thank Dr. Matthew Winkler for many stimulating discussions of this data and Dr. Jerry Hedrick for his generosity in sharing his *Xenopus* colony and encouraging his technician (S.T.L.) to work on this project. Dr. Sherwin Lee kindly shared his $[Mg^{2+}]_i$ data with us and calculated the proper EDTA concentration for extract buffer 2. This investigation was supported by National Science Foundation Grant PCM 78-26022 and a University of California Faculty Research Grant to R.N. and National Institutes of Health Grant HD-04906 to Dr. Jerry Hedrick. The Nicolet Magnetics NT-200 NMR spectrometer was purchased in part by a National Science Foundation grant to the Department of Chemistry, and the spectrometer was made available to us through the University of California Davis NMR Facility.

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