Eur Biophys J (1989) 17: 191–199

European Biophysics Journal

Measurement of an intracellular pH rise after fertilization in crab eggs using ^{31}P-NMR

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Received January 20, 1989/Accepted in revised form June 12, 1989

Abstract. The effect of fertilization upon the intracellular pH, pH_i, in crab ovulated eggs was examined by ^{31}P-NMR. The pH_i values were obtained from the chemical shift differences between the phosphoarginine PA resonance and the inorganic phosphate P_i resonance. The detection of the P_i peak was accomplished by Hahn spin-echo experiments in order to cancel the broad signal arising from phosphoproteins which overlaps the P_i signal. The average pH_i of the unfertilized unactivated eggs was 6.55 and a rise of 0.12 pH unit occurred after fertilization.

Key words: Crab eggs fertilization, ^{31}P-NMR, intracellular pH, ^{31}P transverse relaxation in cells

Introduction

^{31}P nuclear magnetic resonance is a non-invasive and therefore useful technique in the study of intracellular pH, pH_i, by following the chemical shift of the intracellular inorganic phosphate P_i resonance. ^{31}P NMR has been applied for the determination of pH_i of various cells: intact normal erythrocytes (Henderson et al. 1974; Moon and Richards 1973; Labotka and Kleps 1983), abnormal erythrocytes (Lam et al. 1979; Tehrani et al. 1982; Swanson et al. 1983), isolated frog skins (Lin et al. 1985), muscles (Hoult et al. 1974; Burt et al. 1976), living animals (Ackerman et al. 1980), *Escherichia coli* cells (Ogawa et al. 1978), yeast (Navon et al. 1979), plant cells (Martin et al. 1982), amoebae (Martin et al. 1987). Variations in pH_i were also followed during changes of the physiological state of the cell. Meiotic maturation and fertilization of oocytes are physiological situations where variations of pH are involved but at the present time only sea urchin eggs (Winkler et al. 1982) and amphibian eggs, *Xenopus* frog eggs (Nuccitelli et al. 1981; Colman and Gadian 1976), *Rana* frog eggs (Morrill et al. 1983, 1984, 1985) have been extensively investigated.

Gamete fusion at fertilization implies complex mechanisms leading to changes in membrane electrical properties and intracellular ion concentrations. In most invertebrate (Steinhardt and Mazia 1973; Johnson et al. 1976; Johnson and Epel 1981; Shen and Steinhardt 1978) and vertebrate (Nuccitelli et al. 1981; Webb and Nuccitelli 1981; Morrill et al. 1984) eggs, cytosolic Ca^{2+} concentration rises and a pH_i increase occurs after fertilization. Decapod crustacean eggs of crab and lobster display unusual electrical and morphological responses to fertilization: a hyperpolarization of the egg plasma membrane (Goudeau et al. 1984; Goudeau and Goudeau 1985, 1986) in place of the usual membrane depolarization reported for eggs of many species (Gould-Somero and Jaffe 1984; Jaffe and Gould-Somero 1985) and a long lasting cortical reaction (Goudeau and Becker 1982). For a further understanding of these responses, we have undertaken in the present NMR study a measurement of pH_i on ovulated eggs of crabs which were unfertilized and were in vitro fertilized. A measurement was also possible on eggs fertilized in vivo i.e. eggs collected from natural spawning.

Morphological tests were performed on an aliquot of the eggs for the determination of the percentage of eggs resuming meiosis in each experimental situation and of the percentage of fertilized eggs in the in vivo and in vitro experiments.

Material and methods

Animals, preparation of samples

Ripe female crabs (*Carcinus maenas*) were obtained from the Station Biologique at Roscoff in Brittany, during the winter and spring breeding seasons. Ripe

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ovaries contain about 4–6 cm^3 of packed ovulated oocytes. Crab oocyte diameter is about 300–350 μm.

Females selected just before or at the onset of the act of spawning were dissected and the ovulated oocytes were put into artificial sea water (ASW) at 2 °C with a composition in m*M* of: 475 NaCl, 12 KCl, 12 $CaCl_2$, 30 $MgCl_2$, 10 *Tris* HCl at pH 8.2. The low ASW temperature was chosen to maintain oocytes in a low metabolism condition and to avoid any activating effect of divalent cations in the ASW before measurements. Indeed a separate study (Goudeau et al. 1989) shows that incubation of crab oocytes in sea water at 20 °C involves a meiosis resumption and a rise in intracellular pH_i whereas this spontaneous activation is blocked at low temperature.

Th oocyte mass from one female was usually divided in two batches and treated as follows:

i) Unfertilized unactivated oocytes: UFA-oocytes. Oocytes simply collected and kept in ASW at 2 °C served as control for the present fertilization experiments and for the separate activation experiments (Goudeau et al. 1989). Thereby the control oocytes were referenced to unfertilized unactivated oocytes: UFA-oocytes.

ii) In vitro fertilized eggs: F-eggs. The sperm was collected from 6–8 spermatheca of the female genital duct (Anghelou-Spiliotis and Goudeau 1982) and in vitro fertilization was accomplished by dispersing the sperm into a Petri dish containing an UFA-oocyte suspension in ASW at 20 °C. The Petri dish was shaken for 30–60 min. Eggs were then washed several times with ASW to eliminate the sperm. These in vitro fertilized eggs were transferred to a NMR tube filled with ASW at 2 °C.

In addition, in vivo fertilized eggs were obtained from natural spawning and collected from the breeding pouch constituted by the convex abdomen folded back to the sternal plates of the female about 40 min after egg laying. They were tested in the same conditions as in vitro fertilized eggs.

In the following, the number of tested females was indicated by *N*.

Typically for each biological situation, 2–2.5 ml of eggs were loosely packed in a 10-mm diameter NMR tube filled with 5 ml ASW at 2 °C. A 5-mm diameter tube filled with D_2O for both shimming and locking the magnetic field was positioned coaxially within the sample tube.

^{31}*P-NMR spectra*

^{31}P-NMR spectra were recorded at 121.5 and 36.4 MHz on a Bruker MSL300 and a Bruker WH90, respectively. We used the MSL300 spectrometer for a complete identification of the various resonances of oocytes and a determination of the corresponding transverse relaxation rate $1/T2$, whereas fertilization experiments were performed with the Bruker WH90. This electro-magnet spectrometer was more convenient for technical reasons (perfusion) and for spectroscopic reasons which are discussed below.

Single-pulse spectra were obtained with a 45° tipping pulse and a recycling time of 1.5 s. A sweep width of 5000 Hz and a 8 k memory size were used. The spin-echo spectra were recorded using the Hahn sequence ($90-\tau-180-\tau-$Acq). The temperature of the probe was maintained at 2 °C. In order to prevent a temperature increase of the sample, a two level broadband proton decoupling was employed.

All chemical shifts were referenced in parts per million (ppm) to 85% inorganic phosphate acid as external reference. As discussed above, the intracellular phosphoarginine PA was used as an internal reference.

Elementary analysis of inorganic phosphate transverse relaxation in the presence of cells

Since the intracellular inorganic phosphate (P_i) resonance and spin-echo experiments are largely concerned in this work, we decided to analyse the P_i transverse relaxation rate ($1/T2$) on large lipidic unilamellar vesicles (LUV) which are model cells. LUVs were formed by reverse phase evaporation under reduced pressure (Szoka and Papahadjopoulos 1978). The lipids used were L-α-phosphatidylcholine (PC) and phosphatidic acid (PA) in a molar ratio of PC/PA 9 : 1. The composition of the aqueous medium was 0.4 *M* NaH_2PO_4 sodium phosphate (Prolabo) with or without 1 m*M* EDTA (Prolabo) dissolved in 40% D_2O pH = 5.5. The presence or absence of EDTA was used to test the effect of paramagnetic ions on $1/T2$. After vesicle formation, suspensions were diluted in 0.4 *M* Na_2SO_4 to obtain a final lipid concentration of 18 m*M* and were filtered through Nuclepore membranes of 0.8 μm pore size. Finally, suspensions were brought to pH = 7.5. Thereby, a pH difference between the internal (pH = 5.5) and the external (pH = 7.5) medium allowed us to distinguish the internal (P_i) and external (P_e) inorganic phosphate resonances. P_i and P_e transverse relaxation rates were determined by performing Hahn spin-echo experiments at 36.4 and 121.5 MHz in the presence and absence of EDTA (Table 1).

Examination of Table 1 indicates that, in the absence of EDTA, the paramagnetic effect is the major relaxation mechanism at 36.4 MHz (95% and 92% of the total relaxation rate for the P_e and P_i resonances respectively). This is also true at 121.5 MHz for the P_e

Table 1. LUV internal P_i and external P_e inorganic phosphate relaxation rate $1/T2$ values (s^{-1}) determined from Hahn spin-echo sequences at 36.4 and 121.5 MHz

$1/T2$ (s^{-1})	36.4 MHz		121.5 MHz	
	With EDTA	Without EDTA	With EDTA	Without EDTA
P_e	1.2	22	2.3	37
P_i	2.8	34	15	67

resonance (94%). In contrast, for the P_i relaxation rate, the other relaxation mechanisms, i.e., chemical shift anisotropy, dipole-dipole interaction and exchange process are far from negligible at 121.5 MHz: they represent 22% of the total P_i relaxation rate. This difference between P_i and P_e relaxation rates at two frequencies can be explained by a difference between the apparent viscosities (correlation times) of the internal and external medium via the difference between their respective volumes and/or by a difference between the 36.4 and 121.5 MHz NMR time scales experienced by the exchange rates. Anyway, one can predict a large $1/T2$ value of about 67 s^{-1} for the intracellular inorganic phosphate resonance at 121.5 MHz. Such a value appears as a serious limitation for a study of intracellular pH values in biological samples at 121.5 MHz when it is necessary to use spin-echo experiments in order to cancel the signal arising from phosphoproteins which overlaps the P_i resonance.

For this reason and for technical reasons, the measurements of the P_i chemical shifts were carried out at 36.4 MHz.

Calibration curve

In order to convert the P_i chemical shift into internal pH (pH_i) values, a calibration curve was determined by studying the pH dependence of the P_i chemical shift in an UFA-oocyte extract preparation. For this purpose approximately 2 cm^3 of oocytes were homogenized in a glass potter and diluted with an equal volume of buffer (2 °C) of composition in m*M*: KCl: 400, NaCl: 50, $MgCl_2$: 12, $CaCl_2$: 2, EDTA: 27, Hepes: 100. We estimated from electrophysiological experiments (unpublished results) that the intracellular activity of the major cations K^+ and Na^+ in UFA-oocytes are in the range 280–300 m*M* and 20–30 m*M*, respectively. From this, we chose a buffer containing (in m*M*) 400 KCl, 50 NaCl and 100 Hepes, which brought the ionic strength of the final calibration curve close to the intracellular ionic strength of UFA-oocytes, when mixed with an equal volume of egg extract.

Checking the physiological state of eggs

The viability of eggs packed in the NMR tube at 2 °C was examined by measuring the P_i chemical shift value every 5 min. Five tests were done on UFA-oocytes during 30 min and four tests on *F*-eggs during 25 min. Under these conditions the chemical shift values remained unchanged except for one experiment on *F*-eggs for which the value was slightly smaller after 22 min.

Tests of egg perfusion were carried out at 36.4 MHz (one experiment for UFA-oocytes and four experiments for *F*-eggs). The sample in the NMR tube was continuously perfused by circulating aerated cold (2 °C) ASW via a peristaltic pump. Perfusion without promoting dispersion of eggs in the tube needed a 3 ml/min flow rate. Spectra identical to those recorded without perfusion were obtained with the same P_i chemical shift confirming *a)* the recording of only the intracellular phosphate compounds *b)* the viability of oocytes or eggs without perfusion.

Examination of the NTP and NDP peaks on the single-pulse spectra showed that the NTP and NDP peaks kept the same intensities on *F*-eggs spectra compared to the signals on UFA-oocytes spectra. From this observation we may deduce that fertilization does not produce noticeable changes in the NTP, NDP and PA intensities. After a period of 30 min, we noticed a decline in NTP for both types of experimental conditions. This remark was also applicable for the PA signal intensity.

In all the tests, the stability of the PA signal was constantly verified.

Morphological tests

The single criterion used to attest that fertilization occurred is the presence of one sperm nucleus in the cortex of both in vitro inseminated and naturally spawned eggs. This was visualized by the whole mount technique of Zalokar and Erk (1977).

Eggs resumed meiosis when their nuclear apparatus, previously arrested at the first metaphase, underwent the subsequent meiotic stages.

Results

Unfertilized unactivated oocytes

1) Signal identification and transverse relaxation rate measurements. Signal identification and $T2$ measurements were performed on UFA-oocytes spectra at 121.5 MHz. Figure 1 A shows a representative ^{31}P-NMR spectrum of UFA-oocytes ($N=4$).

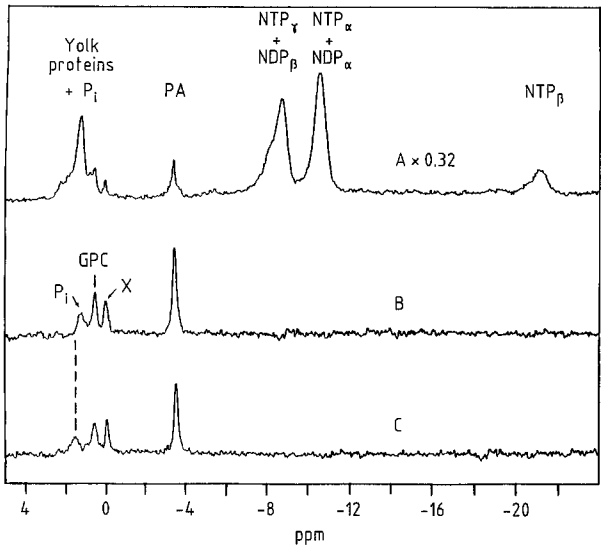

Fig. 1 A–C. ^{31}P-NMR spectra of unfertilized unactivated oocytes (UFA-oocytes) obtained at 121.5 MHz (2 °C), with a 8.5 min accumulation. A Single-pulse spectrum or Hahn spin-echo spectrum ($90-\tau-180-\tau-$Acq) with $\tau=0.1$ ms (vertical scale × 0.32). B Hahn spin-echo spectrum with $\tau=15$ ms. C Hahn spin-echo spectrum ($\tau=15$ ms) after addition of 10 m*M* NH_4Cl to **B** to increase the internal pH and to produce a shift of the P_i peak from $\delta=1.16$ ppm to 1.63 ppm. Peak assignments (ppm): P_i (**B**), +1.16; P_i (**C**), 1.63; PA, −3.52; GPC, +0.49; *X*, −0.07; γNTP, βNDP, −6.88; αNTP, αNDP, −10.76; βNTP, −21.30

Similar spectra were observed by Colman and Gadian (1976), Nuccitelli et al. (1981) and Morrill et al. (1984) for amphibian oocytes. These spectra display a broad signal containing the resonances of the yolk phosphoprotein, the phosphoarginine PA signal and the NTP, NDP resonances. In the *Crustacea* eggs the phosphagen is phosphoarginine rather than phosphocreatine (Morrison 1973). The resonance of intracellular inorganic phosphate P_i is unobservable: it is buried under the large yolk phosphoprotein signal. Therefore, we used a Hahn spin-echo sequence ($90°-\tau-180°-\tau-$Acq) which enables us to discriminate the peaks according to their *T*2 transverse relaxation times. Successive spectra with τ values varying from 0.1 ms (same spectrum as the single pulse one, Fig. 1 A) to 30 ms were recorded. Figure 1 B shows that, using a τ value of 15 ms, the spectrum contains four narrow lines. Addition of 3 m*M* $MnCl_2$ does not modify the spectrum so that all the four resonances are due to intracellular phosphate compounds.

Figure 1 C shows that addition of 10 m*M* NH_4Cl pH = 8.1 in the sample tube induces a shift of the peak previously located at $\delta=1.16$ ppm to 1.49 ppm ($N=1$) or to 1.63 ppm ($N=2$). This experiment confirms that the peak located at $\delta_i=1.16$ ppm corresponds to the intracellular P_i: in the external solution, the equilibrium $NH_4^+ \rightleftharpoons NH_3+H^+$ is established and NH_3 (but not NH_4^+) rapidly diffuses into the cell where it combines with protons resulting in a rise of pH_i (Winkler and Grainger 1978).

Table 2. Values of crab oocyte phosphorus relaxation rate $1/T2$ (s^{-1}) determined from Hahn spin-echo sequences at 121.5 MHz

Compounds	$1/T2$ (s^{-1})
NTPβ	625
NDPα, NDPβ	257
P_i	65
PA	46
GPC	43
X	35

In addition to the PA peak ($\delta=-3.52$ ppm) and the P_i peak, two other signals appear: the resonance at $\delta=0.49$ ppm is assigned to glycerophosphorylcholine GPC since an increase in peak height is observed when a final 5 m*M* GPC solution is added to a perchloric acid extract of oocytes prepared according to Navon et al. (1979) and the resonance at $\delta=-0.07$ ppm ($=X$) has not been identified.

The values of $1/T2$ relaxation rates of the various peaks are given in Table 2. The value of 65 s^{-1} obtained for the P_i resonance is very close to the value obtained for the large unilamellar lipidic vesicles containing no EDTA: 67 s^{-1} (Table 1).

2) pH Measurement: calibration curve. The chemical shift of the P_i peak is suitable for an accurate determination of pH_i (Burt et al. 1976; Pringent et al. 1980). However, the chemical shift difference between two peaks arising from intracellular compounds is free from magnetic susceptibility effects which can alter absolute resonance positions (Burt et al. 1976). Therefore, the pH_i was determined by measuring the difference Δ (in ppm) between the chemical shift of the P_i peak and that of PA since the chemical shift of the PA peak is pH independent above a value of about 6 ($pK_a=4.26$) (Vogel and Bridger 1983). In order to convert these chemical shift differences Δ to pH_i, we studied the pH dependence of Δ (Fig. 2) in oocytes extract solution made with simulating intracellular fluid described in Methods. During the preparation of the extract solution, PA and yolk proteins were degraded and the spin-echo spectra of this solution exhibits two narrow peaks corresponding to the P_i resonance and to a degradation product (*V*). Hence it was necessary to add phosphoarginine (10 m*M*) to this solution for the determination of the calibration curve (Fig. 2 inset).

No significant temperature dependence (2°–23 °C) for the spectra was found, as mentioned by Nuccitelli et al. (1981).

In all cases, the chemical shift of the PA peak was constant ($\delta=-3.32$ ppm) above pH = 5.8. We fitted

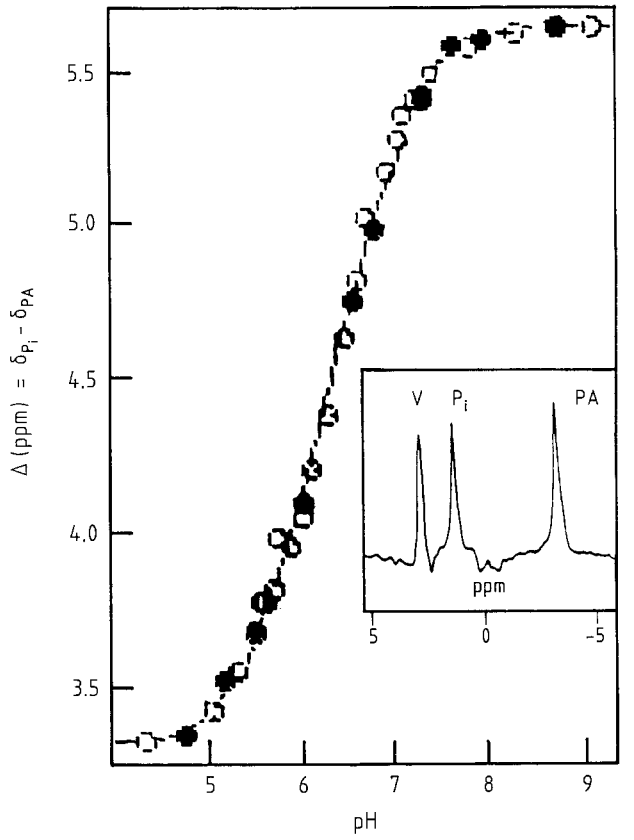

Fig. 2. Calibration curve of the difference Δ (in ppm) between the chemical shift of P_i (δ_i) and that of PA (δPA) as a function of pH. The pH titrations were performed by adding HCl or NaOH. pH values were measured with a glass electrode before and after the direct determination from NMR spectra of δi and δPA. Empty and filled symbols correspond to calibration curve obtained at 2 °C and 23 °C respectively. *Inset:* Hahn spin-echo spectrum ($\tau = 15$ ms) recorded at 36.4 MHz of the oocyte extract solution made with a simulating intracellular fluid to which 10 m*M* PA solution was added

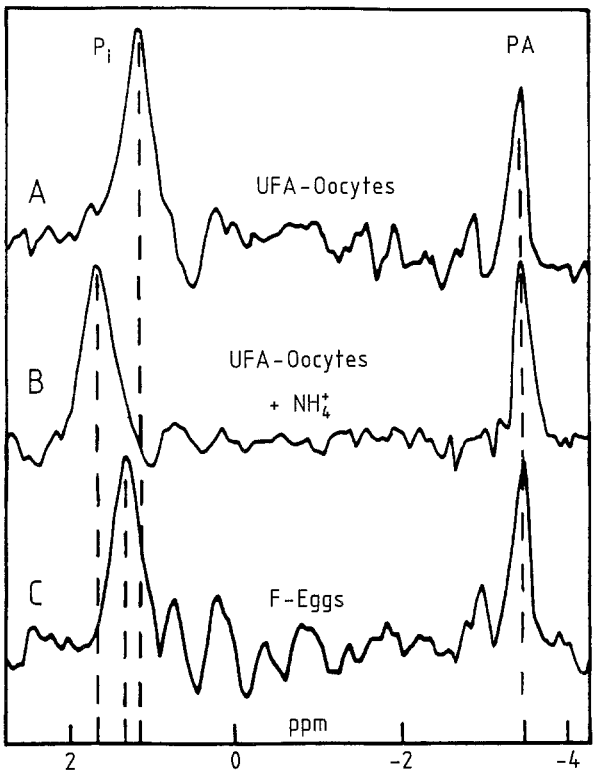

Fig. 3A–C. ^{31}P-NMR spectra of unfertilized unactivated oocytes (UFA-oocytes) and fertilized eggs (*F*-eggs) obtained at 36.4 MHz (2 °C) and showing internal phosphate (P_i) and phosphoarginine (PA) resonances. (**A**) P_i signal corresponding to UFA-oocytes; (**B**) P_i signal corresponding to UFA-oocytes after addition of 10 m*M* NH_4Cl (UFA-oocytes + NH_4^+); (**C**) P_i signal corresponding to *F*-eggs. Peak assignments (ppm): PA = −3.52, P_i (**A**) = +1.16; P_i (**B**) = +1.63; P_i (**C**) = +1.30

the experimental data to the generally used relationship which would theoretically describe the titration data for pH values between 4.5 and 8:

$$pH_i = pK_a + \log \frac{(\delta_i - \delta PA) - (\delta HP - \delta PA)}{(\delta H_2P - \delta PA) - (\delta_i - \delta PA)},$$

where δHP represents the observed P_i chemical shift at pH = 8 and δH_2P represents the one at pH = 4.5.

The least-square fit gave the values:

$$\delta HP - \delta PA = 3.33; \quad \delta H_2P - \delta PA = 5.63; \quad pK_a = 6.40$$

3) Intracellular pH. The measurement of the P_i chemical shift was performed on 24 females using Hahn spin-echo spectra ($\tau = 15$ ms) recorded at 36.4 MHz.

For 22 experiments the corresponding spectra were identical. The PA peak was found at −3.52 ppm and the P_i peak was located at 1.16 ± 0.03 ppm (Fig. 3A and Table 3).

For two experiments, the P_i peak was located at 1.10 ± 0.03 ppm (Table 3) whereas the PA peak was still observed at $\delta = -3.52$ ppm.

From the calibration curve these values of δ_i correspond to a pH_i of 6.55 ± 0.03 for the UFA-oocytes (Table 3). This value of 0.03 pH unit corresponds to the accuracy on the chemical shift since the standard error of the mean is very low (0.002 pH unit).

Morphological examinations showed that these oocytes did not resume meiosis (Table 3) and remained arrested at first metaphase.

These experiments showed a remarkable reproducibility of the pH_i value (Fig. 4) carried out on females obtained during different breeding periods.

In vitro fertilized eggs

The Hahn spin-echo spectra of in vitro *F*-eggs revealed a noticeable shift of the P_i peak compared to spectra

Table 3. Pi chemical shifts (δ_i), corresponding intracellular pH (pH_i) and the associated egg physiological states. ([a] In vivo fertilized eggs collected from one natural spawning)

UFA-oocytes				*F*-eggs							*N* ♀
RMN		Morp. tests		RMN				Morp. tests			
δ_i (ppm)	pH_i	%r.m.	*n*	δ_i (ppm)	$\Delta\delta_i$ (ppm)	pH_i	ΔpH_i	%r.m.	%fert.	*n*	
–	–			1.30 [a]		6.66		100	100	189	1
1.16	6.55	0	63 (1 ♀)	1.23	0.07	6.61	0.06	0	93	26 (1 ♀)	5
1.16	6.55	0	45 (2 ♀)	1.30	0.14	6.66	0.11	51	93	15 (1 ♀)	8
1.16	6.55	0	15 (1 ♀)	1.37	0.21	6.74	0.19	100	100	16 (1 ♀)	3
1.10	6.51			1.30	0.20	6.66	0.15	50	93	15 (1 ♀)	2
				(1.10)		(6.51)					
1.16	6.55			1.36	0.20	6.72	0.17				1
				(1.16)		(6.55)					
1.16	6.55										5

UFA-oocytes: unfertilized unactivated oocytes kept at 2 °C; *F*-eggs: in vitro fertilized eggs at 20 °C.
$\Delta\delta_i$ and ΔpH_i, differences in δ_i or pH_i calculated from the values of δ_i or pH_i in fertilized eggs minus the values of δ_i or pH_i in unfertilized unactivated oocytes; %r.m. and %fert., percentage of eggs resuming meiosis or fertilized eggs, calculated from morphological observations (Morp. tests); *n*, number of oocytes or eggs morphologically examined; *N*, number of females. Data were obtained from ^{31}P-NMR spin-echo spectra at 36.4 MHz

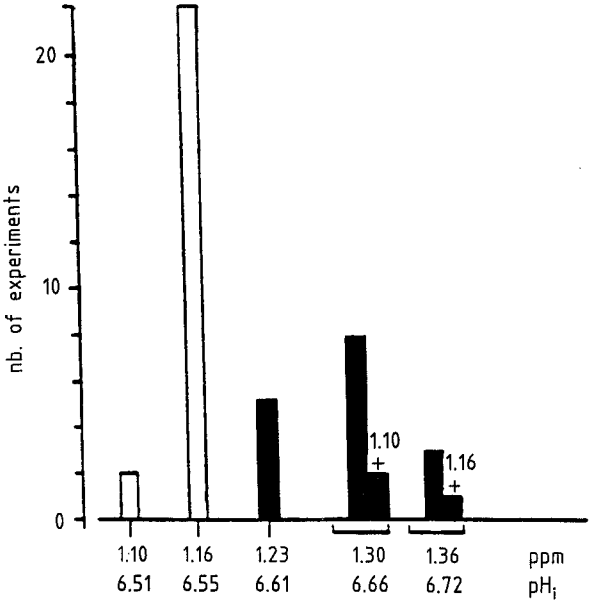

Fig. 4. Histogram of values of P_i chemical shift (δ_i) (expressed as ppm) and the corresponding intracellular pH, pH_i for unfertilized unactivated oocytes (*empty bars*) and in vitro fertilized eggs (*filled bars*). *Crosses + number* indicate the unchanged values in P_i chemical shift when the P_i resonance was splitted in two peaks for some in vitro fertilization experiments

of UFA-oocytes (Table 3 and Fig. 3 C) while the PA peak was found at the same position ($\delta = -3.52$ ppm).

Sixteen experiments on in vitro *F*-eggs were performed and the P_i resonance was observed between $\delta_i = 1.23$ ppm and $\delta_i = 1.37$ ppm.

Over these 16 experiments, five showed a δ_i of 1.23 ppm which corresponds to a very low increase of the chemical shift ($\Delta\delta_i = 0.07$ ppm) compared to that of UFA-oocytes, i.e., to a very low increase in the pH_i ($\Delta pH = 0.06$). A morphological test performed on one of these five samples showed that although 93% of the eggs were fertilized, none resumed meiosis. Eight experiments displayed a $\delta_i = 1.30$ ppm corresponding to a pH_i of 6.66. A morphological test performed on one of these samples showed that 93% of the eggs were fertilized but that only 51% resumed meiosis. For the remaining three experiments, the pH_i value increased to 6.74 ($\delta_i = 1.37$ ppm) and one morphological test showed that 100% of the eggs were fertilized and resumed meiosis.

These experiments suggest that the increase in pH_i is correlated with the percentage of eggs that resumed meiosis which, in turn, could be related to the degree of maturity of the oocytes at the beginning of the experiment.

Finally, for three other in vitro fertilization experiments, the P_i resonance exhibited two peaks corresponding to two different populations of eggs: UFA-oocytes [$\delta_i = 1.10$ ppm ($N = 2$); $\delta_i = 1.16$ ppm ($N = 1$)] and *F*-eggs [$\delta_i = 1.30$ ppm ($N = 2$); $\delta_i = 1.36$ ppm ($N = 1$)] respectively (Table 3). Morphological data showed that 50% of eggs resumed meiosis although 93% of them were fertilized.

All the data are presented on the histogram shown in Fig. 4. The mean value of pH_i is 6.66 ± 0.03 for the in vitro *F*-eggs. Thus the in vitro fertilization produces an intracellular alkalinisation of 0.12 ± 0.03 ($N = 19$).

In addition one experiment was carried out on eggs collected from a natural spawning (in vivo fertilized eggs). The pH_i was 6.66 ($\delta_i = 1.30$ ppm); the fertiliza-

tion produces a pH_i increase of 0.11; 100% of these eggs were fertilized and resumed meiosis (Table 3).

The same NH_4^+ experiment carried out on UFA-oocytes (Fig. 3 B) was performed on three samples of in vitro *F*-eggs (not shown). A shift of the P_i peak was observed from 1.32 ppm $\pm$ 0.03 ppm to 1.72 ppm $\pm$ 0.05 ppm ($N=3$). This shift is similar as the one observed on UFA-oocytes.

Discussion

^{31}P-NMR spin-echo experiments give a pH_i value for unfertilized unactivated crab oocyte with a noticeable reproducibility: for 22 females out of 24, a P_i peak is observed at 1.16 ppm corresponding to a pH_i value of 6.55.

This value is slightly lower than that already obtained on untreated cells such as amphibian embryos *Xenopus laevis* ($pH_i = 6.8 \pm 0.2$) (Colman and Gadian 1976) using ^{31}P-NMR and sea urchin eggs of *Lytechinus pictus:* $pH_i = 6.86 \pm 0.02$ using DMO (dimethyloxazolidinedione) method (Johnson and Epel 1981), $pH_i = 6.84 \pm 0.02$ using the H^+-sensitive microelectrodes (Shen and Steinhardt 1978). Using microelectrodes, freshly prepared germinal vesicle stage oocytes had a pH_i value ranging from 6.6 to 6.8 whereas aged oocytes had a pH_i value in the range 7.0 to 7.2 (de Santis et al. 1987). Greater pH_i values were found using ^{31}P-NMR on *Xenopus* eggs ($pH_i = 7.42$) (Nuccitelli et al. 1981), *Rana* eggs ($pH_i = 7.1$ to 7.2) (Morrill et al. 1984) and sea urchin eggs ($pH_i = 7.12$) (Winkler et al. 1982).

It could be argued that the eggs were not maintained at physiological conditions in the NMR tube and the NMR spectra recorded over 16 min accumulation might be affected by these conditions. We believe these objections to be unfounded for the following reasons: in the absence of perfusion and at 2 °C, the spectra were remarkably stable over periods as long as 30 min and were identical in ASW perfusion conditions, without any reduction in signal intensity of the phosphoarginine or shift of the P_i peak.

It has to be pointed out the pK_a value of 6.40 obtained from the calibration curve is smaller than those usually found, which vary from 6.60 to 7.18 for a 1 m*M* sodium phosphate solution containing different ionic concentrations (Seo et al. 1983). This low pK_a value can be due to a high concentration of divalent ions such as Mg^{2+} or Ca^{2+} or to the effect of ionic strength of the intracellular medium: an increase in ionic strength by 0.15 or 0.30 decreases the pK_a value by about 0.4 or 0.5 respectively (Seo et al. 1983). Any error in intracellular pK_a, originating from the accuracy of the intramedium simulating, produces an identical error in pH_i. Exact knowledge of intracellular ionic strength and free Mg^{2+} levels would reduce the uncertainty to approximately 0.1 pH unit-as these factors appear to exert the most significant effects (Roberts et al. 1981). It is worthing to notice that the apparent pK_a of P_i was found to be as low as 6.5 (Christen et al. 1983) using the sea water as calibration solution for the ^{31}P-NMR study of sea urchin sperm. Interestingly, the authors of this study found the same apparent pK_a of P_i by calibrating the P_i signal in situ with nigericin and monensin or in sea water which presumably has an ionic strength not very different from that of our calibration solution.

The occurrence of a rise in intracellular pH_i after fertilization has only been reported in the eggs of sea urchins (Johnson et al. 1976; Johnson and Epel 1981; Shen and Steinhardt 1978; Winkler et al. 1982) and *Anuran amphibia* (Nuccitelli et al. 1981; Webb and Nuccitelli 1981; Morrill et al. 1984). Up to the present time there was no information concerning variations of pH_i in any arthropodan eggs at fertilization. Our ^{31}P-NMR results indicate that fertilization in crab eggs is associated with a permanent pH_i rise which amounts to 0.12 ± 0.01 pH unit. In a separate paper (Goudeau et al. 1989) we showed an identical increase in pH_i measured by H^+-sensitive microelectrodes.

The pH_i rise induced by fertilization of crab eggs appears smaller than that observed in sea urchins and *Anuran amphibia* eggs (Johnson et al. 1976; Johnson and Epel 1981; Shen and Steinhardt 1978; Winkler et al. 1982; Nuccitelli et al. 1981; Webb and Nuccitelli 1981; Morrill et al. 1984).

The small variation of pH_i, measured after fertilization can be related to the slowness of the early development of the yolky egg of crab, since the two blastomere stage is only reached in about 12 h at the physiological temperature.

It is worth noting that addition of 10 m*M* NH_4^+ pH = 8.1 in the external medium produced a P_i shift of 0.42 ± 0.05 ppm for either unfertilized unactivated oocytes or in vitro fertilized eggs. This shift corresponds to an increase in pH_i of 0.37 ± 0.04 which is greater than that caused by fertilization.

The cytoplasmic alkalisation observed at fertilization in *Xenopus* eggs is reported to be independent of the activity of an Na^+/H^+ exchanger in the plasma membrane (Nuccitelli et al. 1981). For *Rana* eggs, Morrill et al. (1984) reported that pH_i is a function of cytosolic free Ca^{2+} levels and/or related to an Ca^{2+}/H^+ exchange across the oocyte plasma membrane. From the H^+-sensitive microelectrode experiments on crab eggs (Goudeau et al. 1989), we demonstrated that the suppression of external sodium induced a slight cytoplasmic acidification and consequently ruled out the intervention of an Na^+/H^+ exchanger in the plasma membrane. At the present time we have no experimental evidence of a relationship

between intracellular Ca^{2+} and pH_i values, but the occurrence of Ca^{2+}/H^+ exchange mechanism in the plasma membrane, as was suggested for Rana eggs (Morrill et al. 1984) is an attractive hypothesis which can explain a permanent rise in Ca_i^{2+} (Jaffe 1983) necessary to induce the long lasting peculiar cortical reaction observed in crab eggs (Goudeau and Becker 1982).

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