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Phosphorous-31 nuclear magnetic resonance studies of cellular systems

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PHOSPHORUS-31 NUCLEAR MAGNETIC RESONANCE STUDIES OF
CELLULAR SYSTEMS

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Phosphorus-31 nuclear magnetic resonance

studies of cellular systems

by

Pierre-Marie Luc Robitaille

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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Zoology

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In Charge of Major Work

For the Major Departments

For the Graduate College

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DEDICATION

Dedicated to my wife, Patti.

LIST OF ABBREVIATIONS

A, absorbance

A, angstrom

A_{447}, absorbance at 447 nanometers

ADP, adenosine-5'-diphosphate

1-AEP, 1-aminoethylphosphate

2-AEP, 2-aminoethylphosphonate

AMP, adenosine-5'-monophosphate

ASW, artificial sea water

ATP, adenosine-5'-triphosphate

BIS-TRIS, [bis(2-hydroxyethyl)imino-tri(hydroxymethylmethane)]

°C, degrees Celcius

cAMP, cyclic adenosine-3',5'-monophosphate

CDP, cytidine-5'-diphosphate

CTP, cytidine-5'-triphosphate

D_2O, deuterium oxide

2,3-DPG, 2,3-diphosphoglycerate

EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid

GDP, guanosine-5'-diphosphate

GPC, glycerylphosphocholine

GTP, guanosine-5'-triphosphate

HEPES, [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]

hr, hours

Hr, hemerythrin

Hz, Hertz

$^\circ$K, degrees Kelvin

K_f, binding constant

l, liter

μl, microliter

M, molar

μM, micromolar

methr, methemerythrin

MHz, megahertz

min, minutes

ml, milliliter

mm, millimeter

mM, millimolar

MW, molecular weight

NAD^+, nicotinamide adenine dinucleotide

NADH, nicotinamide adenine dinucleotide (reduced form)

NDP, nucleotide-5'-diphosphate

nm, nanometers

NMP, nucleotide-5'-monophosphate

NMR, nuclear magnetic resonance

NTP, nucleotide-5'-triphosphate

oxyhr, oxyhemerythrin

P_{50}, half saturation pressure

PA, phosphoarginine

PCr, phosphocreatine

PDE, phosphodiester

O-PEA, O-phosphorylethanolamine

PEP, phosphoenolpyruvate

pH, hydrogen ion concentration

P_i, intracellular inorganic phosphate

PIPES, piperazine-N,N'-bis[2-ethane sulfonic acid]

PME, phosphomonoester

^{31}P-NMR, phosphorus-31 nuclear magnetic resonance

P_o, extracellular inorganic phosphate

ppm, parts per million

rpm, revolutions per minute

s, seconds

μs, microseconds

SMI, sperm motility initiator

TDP, thymidine-5'-diphosphate

TTP, thymidine-5'-triphosphate

UDP, uridine-5'-diphosphate

UDPG, uridine-5'-diphosphoglucose

UTP, uridine-5'-triphosphate

UV, ultraviolet

GENERAL INTRODUCTION

In 1973, R. B. Moon and J. H. Richards utilized phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) to examine the human erythrocyte and monitor its intracellular pH (Moon and Richards, 1973). Ever since this classic report, the field of _in vivo_ NMR has virtually exploded. The growth of this technique can be attributed to its non-invasive nature and its ability to yield complete, real-time, metabolic profiles at a relatively low cost. In these two respects, nuclear magnetic resonance remains unchallenged by other physical techniques.

The use of nuclear magnetic resonance in medical image reconstruction has initiated a fury of developments in this field over the past ten years (Lauterbur, 1973). Rapid imaging techniques (Frahm _et al._, 1985; Haase _et al._, 1986) and NMR microscopy (Aguayo _et al._, 1986) are now coming of age. As a result, the NMR phenomenon is really just beginning to be exploited to its full potential.

In this dissertation, ^{31}P-NMR spectroscopy was applied to the study of 1) sipunculan erythrocytes (part 1), 2) spermatozoa isolated from several vertebrate and invertebrate species (parts 3-5), and 3) unfertilized eggs isolated from the blue crab and the horseshoe crab (part 6). Part 2 deals with the determination of P_{50} values for the oxygen-hemerythrin interaction in the presence or absence of effectors. This study was performed utilizing uv-visible spectroscopy.

^{31}P-NMR results (parts 1, 3-6) center on the identification of key metabolites and on the determination of intracellular pH. In studies involving fish spermatozoa, emphasis was also placed on examining changes in metabolic profiles following 1) an anaerobic insult, 2) motility initiation, or 3) short-term storage. This dissertation also captures several difficulties in spectral interpretation which a spectroscopist is likely to encounter.

EXPLANATION OF DISSERTATION FORMAT

The chapters of this dissertation are modified manuscripts that have been or will be submitted for publication in the following scientific journals: Biochemistry, Comparative Biochemistry and Physiology, Journal of Biochemistry and Cell Biology, Biological Bulletin, and Biochemical and Biophysical Research Communications. Each part is self-contained and has its own abstract and/or introduction, materials and methods, results, discussion, references section, and acknowledgements.

With the exception of the electron micrograph in part 5 and the oxygen uptake experiments in parts 4 and 5, the candidate performed at least 90% of the research presented in this dissertation.

PART 1: ^{31}P-NMR STUDIES OF ERYTHROCYTES ISOLATED FROM THE

SIPUNCULIDS, Phascolopsis gouldii AND Themiste zostericola

^{31}P-NMR studies of erythrocytes

isolated from the sipunculids,

<u>Phascolopsis</u> <u>gouldii</u> and <u>Themiste</u> <u>zostericola</u>

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ABSTRACT

Erythrocytes containing the oxygen storage protein hemerythrin were

isolated from the sipunculid Phascolopsis gouldii and examined by ^{31}P-

NMR at 5°C. These cells were shown to possess phosphorylethanolamine

(PEA), 2-aminoethylphosphonic acid (2-AEP), mannose-1-phosphate, and

nucleotide triphosphates (NTP) as the major soluble phosphorus

metabolites. The concentration of PEA in these cells was established to

be greater than 20 mM. The intracellular pH was measured to be 7.2±0.1

in fully aerobic cells and 6.5±0.1 in anaerobic cells. The free

magnesium concentration in aerobic cells was 0.34 mM and 85% of the ATP

was complexed to Mg^{+2}. PEA was found to bind to P. gouldii

methemerythrin (K_D=7.0±0.4M^{-1}). Scatchard and Hill plots revealed that

this interaction was best characterized in terms of negative

cooperativity or non-specific binding. In addition, PEA was found to be

a potent inhibitor of azide binding to methemerythrin (metHr) at pH 7.2.

No significant effect of 2-AEP on azide binding at pH 7.2 was observed.

Erythrocytes from the sipunculid Themiste zostericola were also

subjected to analysis by ^{31}P-NMR and were found to possess a resonance

corresponding to phosphomonoesters in addition to the metabolites

mentioned previously for P. gouldii. Erythrocytes from Themiste

zostericola contained unusually high levels of 2-AEP. For aerobic

erythrocytes, an intracellular pH of 7.1±0.1 was determined. The

percentage of ATP complexed to Mg^{+2} was estimated to be 43 to 51%, and

the free magnesium concentration was shown to range from 0.05mM to

0.06 mM.

INTRODUCTION

The central role of hemoglobin in nature has served as the focal point for numerous research efforts on the isolated protein and on the cells in which it is contained. Scientific endeavors have also been concerned with oxygen carrying proteins which are functional analogues of hemoglobin, namely hemocyanin and hemerythrin (Mangum, 1985). These proteins have been extensively studied in order to gain a better understanding of the thermodynamic and kinetic factors involved in O_2 binding to metal centers.

For most sipunculids, octameric hemerythrin is obtained from erythrocytes contained in ceolomic fluids. The molecule is comprised of eight identical or nearly identical monomers (MW=13,500) each of which contains a binuclear, non-heme oxygen binding site (Kurtz, 1985). The active site of the protein can exist at various oxidation levels. Thus, in deoxyhemerythrin, both irons are in the +2 oxidation state. In binding oxygen and formation of oxyhemerythrin, an electron is transferred from each of the iron ions to the oxygen molecule resulting in the formation of a peroxide and leaving both iron ions in the +3 oxidation state. Methemerythrin defines another state of the active site where both iron ions are in the +3 oxidation state and oxygen is not bound to the active site. An intermediate oxidation state exists where one of the iron ions is in the +2 oxidation state and the other is in the +3 oxidation level. This species is known as semimethemerythrin

(Kurtz, 1985; Bradic and Wilkins, 1983; Klotz et al., 1976).

Unlike hemoglobin, O_2 binding in hemerythrin is not known to exhibit any cooperativity. The presence of 2,3-DPG has been established in human erythrocytes and this molecule is known to lower the oxygen affinity of human hemoglobin. No corresponding natural effector of hemerythrin is known although small ions such as perchlorate are known to be allosteric effectors of hemerythrin (Garbett et al., 1971; Stenkamp et al., 1978; and Bradic and Wilkins, 1983). Some effect of perchlorate on oxygen affinity has been reported (DePhillips, 1971). Unfortunately, this perchlorate effect on hemerythrin is of no biological importance. However, in the case of several sipunculids, the oxygen affinity of hemerythrin is reported to be lower in erythrocytes than when isolated (Weber and Fange, 1980; Mangum and Kondon, 1975; Manwell, 1960). In this light, we have utilized ^{31}P-NMR to study sipunculan erythrocytes in order to better understand the intracellular conditions which surround native hemerythrin.

Human erythrocytes were first studied with ^{31}P-NMR by Moon and Richards as reported in their classic article (Moon and Richards, 1973). Since that time, numerous NMR studies of this cell type have been performed (Henderson et al., 1974; Brown et al., 1977; Gupta et al., 1978b; Lam et al., 1979; Brindle et al., 1982; Fabry and San George, 1983; Labotka and Kleps, 1983; Labotka, 1984; Mitsumori, 1985; Brauer et al., 1985). NMR studies along these lines have been reviewed by Friedman (1983). The human erythrocyte ^{31}P-NMR spectrum is characterized by two sharp resonances due to 2,3-DPG in addition to

resonances corresponding to ATP, and at times, inorganic phosphate.

In this paper are reported the results of ^{31}P-NMR analyses of erythrocytes isolated from two sipunculid species, Phascolopsis gouldii and Themiste zostericola. The presence of a newly discovered allosteric effector of hemerythrin, the phosphate PEA, in these erythrocytes has been established. In addition, the presence of 2-AEP in the sipunculan erythrocyte has been demonstrated. The intracellular pH, the percentage of ATP complexed as MgATP, and the free magnesium concentrations in the erythrocytes of these two sipunculids have also been determined.

MATERIALS AND METHODS

Preparation of erythrocyte samples

Fully aerated erythrocytes   Erythrocytes from Phascolopsis gouldii were isolated from live worms obtained from Marine Biological Laboratory, Woods Hole, Mass. The coelomic fluid from these worms was then strained through cheesecloth and the erythrocytes isolated by centrifugation in a clinical centrifuge for 5 min. The supernatant was decanted, artificial sea water (ASW) was added and the erythrocytes were centrifuged as before. The supernatant was once again discarded and the erythrocytes were washed two more times with ASW (Jungle Labs Corp., Comfort, Texas). These erythrocytes were then placed in a 10 mm NMR tube on ice and the NMR spectrum was immediately recorded. Erythrocytes from specimens of Themiste zostericola, obtained live from Pacific Bio Marine Labs, Venice, California, were prepared in the same manner.

Anaerobic erythrocytes   Two dozen live specimens of Phascolopsis gouldii were placed in a jar of ASW. The jar was placed in a 15°C constant temperature bath. N_2 was bubbled through the ASW for 16 hr. After this time period, the eythrocytes were isolated as described previously and placed in a 10 mm NMR tube. All of the isolation steps were performed under argon. Spectra were immediately accumulated at 278°K.

Erythrocyte extract   Freshly isolated erythrocytes were placed

on ice for 15 min at which time a 1:1 volume of 6% $HClO_4$ was added.

Extraction was allowed to continue on ice for at least 30 min. This

mixture was centrifuged at 10,000 rpm for 10 min in a Sorval centrifuge

(16,300g) to remove cellular debris. The supernatant was then

neutralized with either K_2CO_3 or 2 M KOH. This product was centrifuged

once again and the supernatant was subjected to NMR analysis.

^{31}P-NMR: collection of spectra and identification of resonances

Spectra were collected using a Bruker WM300 spectrometer operating

at 121.49 MHz. A 20.0 µl capillary containing a solution of 0.1 M

methylenediphosphonic acid in D_2O was used as an external standard.

This standard possessed a chemical shift of approximately 18.4 ppm

relative to an 85% phosphoric acid standard which is customarily

assigned a shift of 0.00 ppm. The external standard was placed in a

10 mm NMR sample tube and held in place by a vortex suppressor. The

accumulation temperature was maintained at 278 or 283±1°K. The

collection parameters included a sweep window of 13888.889 Hz, a pulse

time of 13.0 µs (78°), a 0.500 s receiver delay, and an acquisition time

of 0.590 s resulting in a cycling time of 1.09 s. The number of cycle

repetitions ranged from 25 to approximately 24,000. Resonances were

identified by the direct addition of known standards to the extract.

The identification of the resonances was confirmed by the titration of

extracts in the presence or absence of standards. The pH was varied from 1 to 13 by the addition of 2 M HCl or 2 M KOH.

Measurement of intracellular pH

Erythrocytes were isolated from Phascolopsis gouldii, strained through cheesecloth, and washed twice with ASW (at 22°C) as described previously. Cells were then placed on ice and 3 ml aliquots of packed erythrocytes were added to 15 ml centrifuge tubes. A 0.05 M PIPES buffer pH 6.46 (50 ml) containing 1 mM 2,4-dinitrophenol (1 mM) was adjusted to various pH values by addition of KOH or HCl and was then added to each centrifuge tube in 5 ml portions. The tubes were permitted to equilibrate on ice for at least 30 min. The tubes were then centrifuged and the pH was recorded while the solutions were kept on ice. The Beckman pH meter was first standardized using certified buffer solutions. The supernatant was decanted and the cells were transferred to 10 mm NMR tubes. These were kept on ice until the NMR spectra could be collected as described previously. Immediately after each acquisition, the pH was re-recorded. This second pH value was used for all further calculations.

A standard curve was generated from this whole cell titration data. The chemical shift difference (Δ) between the 2-AEP and mannose-1-phosphate resonances was plotted as a function of the corresponding pH value. The erythrocyte pH was calculated by noting the

value of Δ in aerobic or anaerobic erythrocytes and then comparing them to the standard curve.

Determination of free magnesium concentration

The separation between the α and β resonances of ATP was utilized to measure the ratio of free ATP to total ATP and free Mg^{+2} according to a method developed by Gupta (Gupta et al., 1978a,b; Gupta and Moore, 1980; Gupta and Yushok, 1980; Gupta et al., 1983a,b; Resnick et al., 1984). A stock solution containing 0.14 M KCl, 0.01 M NaCl, 0.02 M BIS-TRIS, and 0.004 M ATP was prepared and adjusted to a pH of 7.2 by addition of HCl. The NMR spectrum of this solution was then recorded at 10°C. This procedure was repeated for 4 mM ATP and 10 mM Mg^{+2} in the prepared stock solution (pH 7.2). The separation in Hz, $\delta_{\alpha\beta}$, between the α-ATP and β-ATP resonances were determined in these two cases and in the whole aerobic (or anaerobic) erythrocyte. The ratio of free ATP to complexed ATP could be calculated directly as follows (Resnick et al., 1984):

$$\phi = \frac{[ATP]_f}{[ATP]_T} = \frac{\delta_{\alpha\beta} - \delta_{\alpha\beta}^{MgATP}}{\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP}}$$

where $\delta^{cell}_{\alpha\beta}$ is equal to the separation between the α and β resonance for ATP in the cell and $\delta^{MgATP}_{\alpha\beta}$ and $\delta^{ATP}_{\alpha\beta}$ correspond to the same distance in a model solution containing 10 mM Mg^{+2} or in which Mg^{+2} was absent. The free magnesium concentration could then be calculated according to the following equation:

$$[Mg]_f = K_d^{MgATP}\left(\frac{1}{\phi} - 1\right)$$

where K_d^{MgATP} was assumed to have a value of 60 µM at pH 7.2 and 10°C (Gupta and Yushok, 1980).

UV-visible studies

Experiments were performed on a Perkin-Elmer diode array spectrophotometer (lambda array model 3840) using 1 cm pathlength cells. All of these studies utilized metHr. MetHr was prepared from oxyHr (Klotz et al., 1957) which had been stored in crystalline form in a liquid nitrogen bath. The oxyHr was warmed to room temperature and centrifuged at high speeds in a clinical centrifuge for approximately 5 min. The resulting pellet was coated by a white layer of denatured protein. This layer was discarded and the remaining red oxyHr crystals were dissolved in 8-10 ml of 0.15 M HEPES buffer at pH 7.2. A few grains of potassium fericyanide $[K_3Fe(CN)_6]$ were added and this solution was allowed to stand for 3-4 hr at room temperature. The excess

$Fe(CN)_6^{-3}$ was then removed by dialysis against a large excess of

0.15 M HEPES buffer (pH 7.2) using 12,000 exclusion limit dialysis

tubing.

Stock solutions of 1 M O-PEA, 1 M $NaClO_4$, 1 M 2-AEP, and 0.1 M NaN_3

in 0.15 M HEPES buffer pH 7.2 were prepared. A 200 or 1,000 μl

micropipet was used to transfer 50 or 500 μl portions of each of these

stocks as needed to 1 ml volumes of stock metHr. Solutions of metHr

containing ClO_4, PEA, or 2-AEP were permitted to come to equilibrium for

up to 12 hr, 15 hr, and 3 hr, respectively, prior to addition of an

excess (10-50 μl) of 0.1 M NaN_3. The rate of azide binding was

monitored by noting the absorbance change at 447 nm versus time (Garbett

et al,. 1969). Final maximal concentrations of 0.045 M ClO_4, 0.045 M O-

PEA, 0.333 M O-PEA, and 0.045 M 2-AEP were present in the equilibrated

solutions. The pH was recorded after each measurement. To measure the

K_f for O-PEA and metHr, 1 ml aliquots of metHr stock solution were

placed in 10 ml vials. 1 M O-PEA was added in aliquots of 500 μl,

200 μl, 100 μl, 50 μl, and 20 μl and 0.2 M O-PEA in 25 μl, 10 μl, 5 μl

and 0 μl aliquots giving final PEA concentrations of 0.333 M, 0.133 M,

0.066 M, 0.033 M, 0.0133 M, 0.0066 M, 0.0033 M, 0.0013 M, 0.00066 M, and

0 M, respectively, once total volume was adjusted to 1.5 ml with

0.15 M HEPES buffer pH 7.2. Appropriate blanks were prepared and UV-

visible spectra were collected. A binding constant was calculated by

monitoring the absorbance change at 377.5 nm. Concentrations of metHr

were determined from absorbance of the azide adduct (Garbett et al.,

1969). The hydrogen ion concentration was measured using a Beckman pH

meter immediately following each measurement. These studies were performed at $22\pm1^{\circ}$C.

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RESULTS

In Figure 1, the ^{31}P-NMR spectrum of aerobic erythrocytes isolated from P. gouldii is shown. An informative spectrum could be obtained in less than 25 pulses, and spectra of considerable quality was achieved in approximately 100 scans. No changes in chemical shift or peak intensity could be detected in acquiring up to 2,500 scans at 10°C. A similar spectrum was also acquired at 5°C. Figure 1 reveals the presence of 2-AEP (2) other phosphonates (3), PEA (4), NTP (7, 8, and 11), inorganic phosphate and mannose-1-phosphate (5), phosphoenolpyruvate (6), NAD/NADH (9), and UDPG (9 and 10) in these cells. Resonances corresponding to NTP/NDP are principally comprised of ATP/ADP with minor contributions from UTP/UDP, GTP/GDP, CTP/CDP, and TTP/TDP. One should note that inorganic phosphate is the minor component of resonance (5). Peaks have been identified by the addition of known standards to a 6% $HClO_4$ extract of these cells. These extracts were titrated (pH 1 to 13) in the presence or absence of the standards. Identical limiting shifts and pK_a's of standard and unknown were assumed to be sufficient for assuring proper assignment of resonances. The ^{31}P-NMR spectrum of a 6% $HClO_4$ extract of P. gouldii erythrocytes neutralized to pH 7.63 is shown in Figure 2. The presence of 2-AEP (2) and PEA (4) in these cells has been well established by these methods. In addition to 2-AEP, other phosphonates (3) have been detected. From their chemical shifts (14.93 and 14.40 ppm), however, it is likely that one of these resonances

Figure 1. ^{31}P-NMR spectra of freshly isolated aerobic

P. gouldii erythrocytes at 10°C. [Spectra were

acquired using 25, 100, 250, and 2,500 scans. Resonances

have been assigned as follows: 1) methylenediphosphonic

acid standard in a concentric capillary (18.48 ppm),

2) 2-aminoethylphosphonic acid (17.38 ppm), 3) other

phosphonates (14.93 and 14.40 ppm), 4) O-phosphoryl-

ethanolamine (4.09 ppm), 5) inorganic phosphate and

mannose-1-phosphate (2.08 ppm), 6) phosphoenolpyruvate

(-0.38 ppm), 7) γ-NTP (-5.34 ppm), 8) α-NTP

(-10.24 ppm), 9) NAD/NADH and UDPG (-10.77 ppm),

10) UDPG (-12.37 ppm), and 11) β-NTP (-19.04 ppm).]

25 scans

100 scans

250 scans
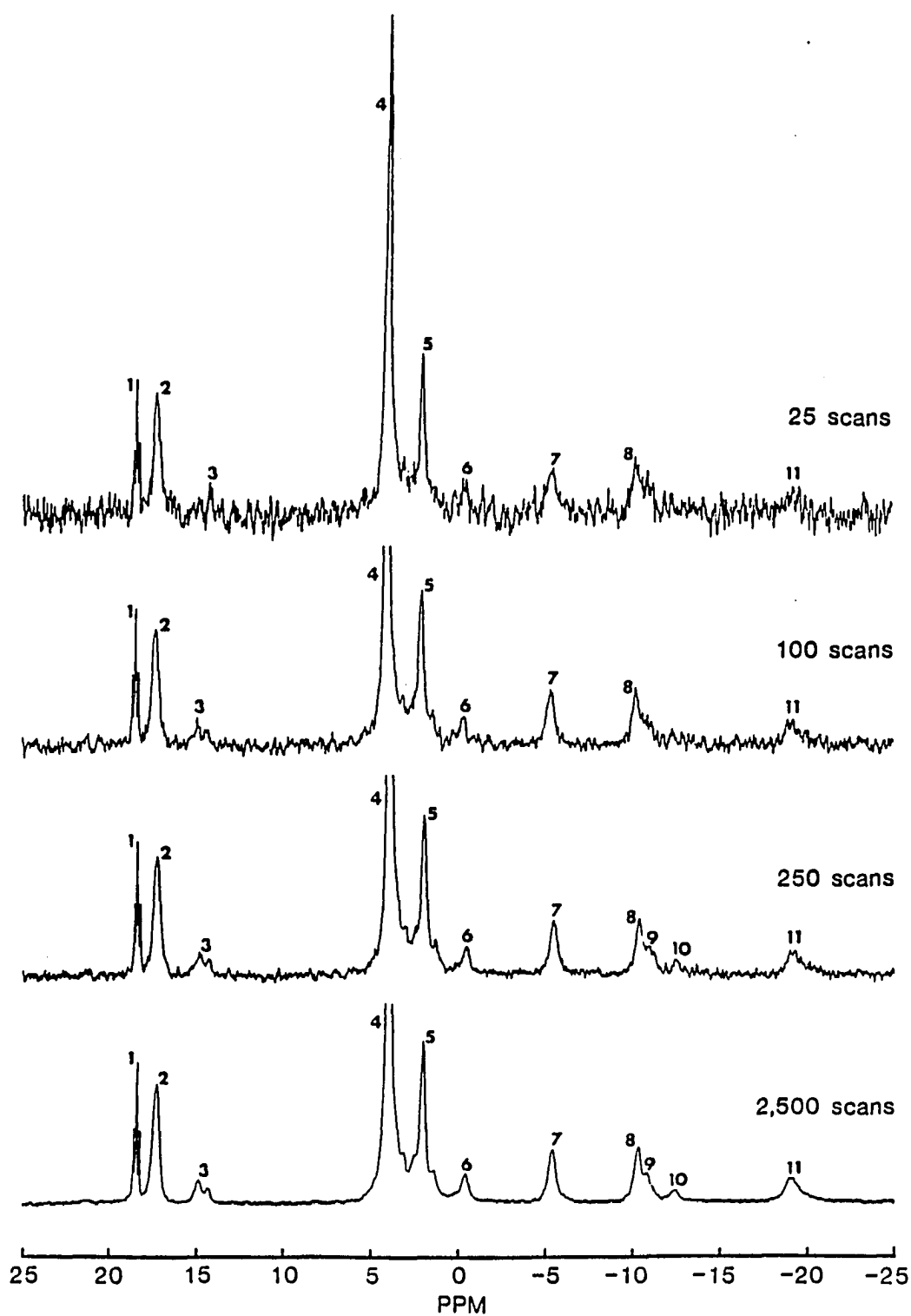
2,500 scans

Figure 2. ^{31}P-NMR spectrum (24,000 scans) of a 6% perchloric acid extract of P.

gouldii erythrocytes neutralized to pH 7.63. [Resonances correspond to

the following: 1) methylenediphosphonic acid standard in a concentric

capillary (18.45 ppm), 2) 2-aminoethylphosphonic acid (17.16 ppm),

3) other phosphonates (14.64 ppm), 4) O-phosphorylethanolamine

(4.25 ppm), 5) inorganic phosphate and mannose-1-phosphate (2.31 ppm),

6-7) unidentified resonances (3.09 ppm, 1.43 ppm),

8) phosphoenolpyruvate (0.21 ppm), 9) a phosphogen (-3.30 ppm),

10) γ-NTP (-5.05 ppm), 11) α-NTP (-10.13 ppm), 12) β-NTP (-18.66 ppm),

13) β-NDP (-5.55 ppm), 14) α-NDP (-9.66 ppm), 15) NAD/NADH and UDPG

(-10.77 ppm), and 16) UDPG (-12.40 ppm).]
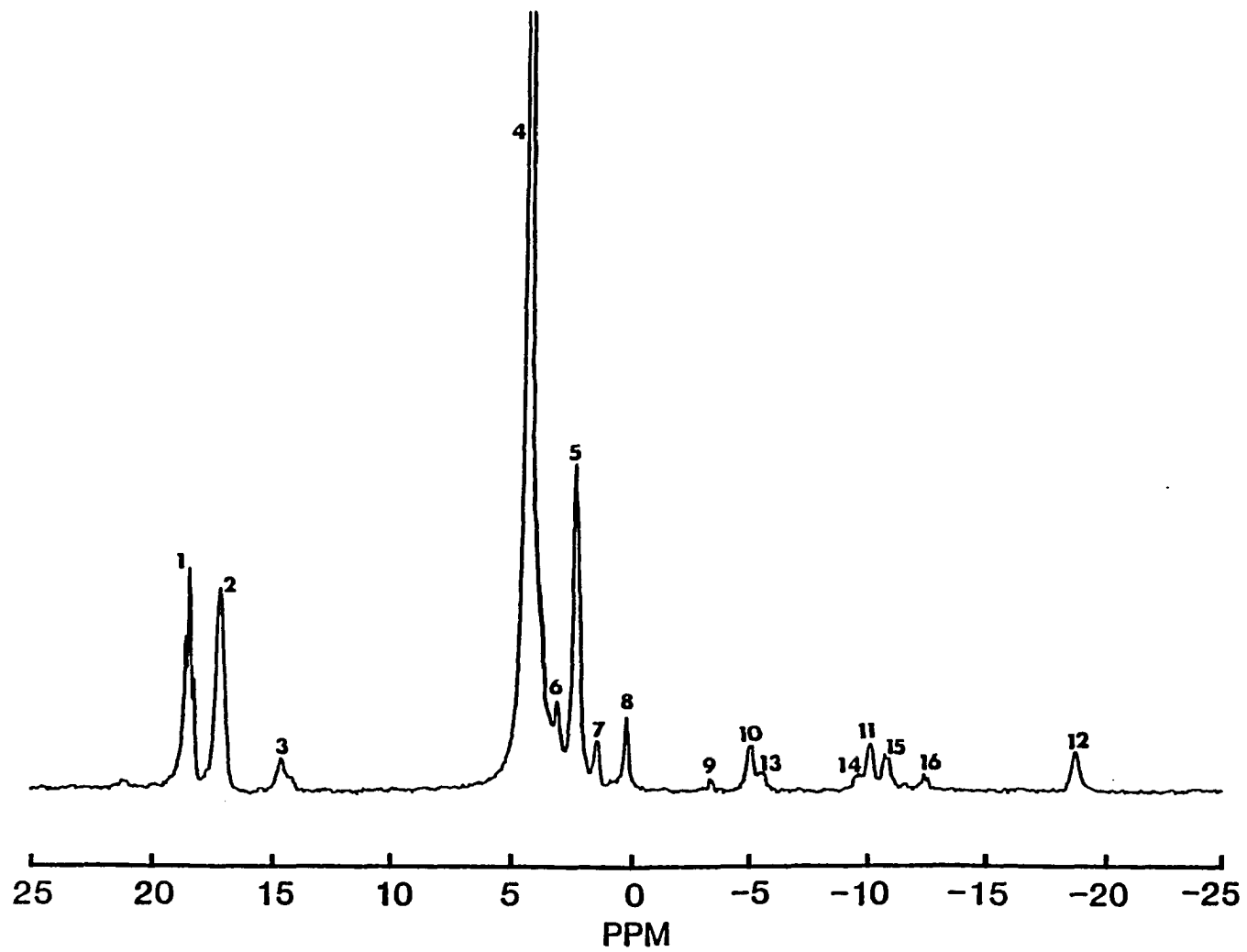
corresponds to 1-hydroxy-2aminoethylphosphonate (Deslauriers et al.,
1980). This compound has previously been identified in Acanthamoeba
castellanii (Korn et al., 1973). Moreover, we have established that
these phosphonates do not correspond to the isomer of 2-AEP, that is
1-AEP. The titration profile of peaks corresponding to 2-AEP, PEA,
mannose-1-phosphate, and phosphoenolpyruvate in these extracts is shown
in Figure 3. This titration profile was useful in the identification of
resonances. In addition, from a 6% $HClO_4$ extract, the concentration of
PEA within the P. gouldii erythrocytes has been estimated to be greater
than 20 mM.

From a difference of 15.3 ppm in the chemical shifts of 2-AEP and
mannose-1-phosphate at 5°C, an intracellular pH of 7.2±0.1 could be
calculated for aerobic P. gouldii erythrocytes by use of the standard
curve shown in Figure 4. The value of the [ATP] versus [MgATP] and of
free Mg^{+2} within the aerobic P. gouldii erythrocyte could be calculated
from the difference in the chemical shifts for the α and the β
resonances of ATP. From these calculations, it was determined that 85%
of the ATP is complexed as MgATP in these cells at 10°C. In addition, a
free Mg^{+2} concentration of 0.34 mM was found.

In Figure 5, the ^{31}P-NMR spectrum of anaerobic P. gouldii
erythrocytes reveals considerable peak broadening due to greater
paramagnetism within these cells from increased levels of
deoxyhemerythrin. Resonances are the same as those identified in
Figure 1. In the same manner as described previously, an intracellular
pH of 6.5±0.1 could be determined for anaerobic P. gouldii erythrocytes

Figure 3. ^{31}P-NMR titration profile of a 6% perchloric acid extract of P. gouldii erythrocytes. [Curves correspond to the following: 1) 2-AEP, 2) O-PEA, 3) mannose-1-phosphate and 4) phosphoenolpyruvate.]
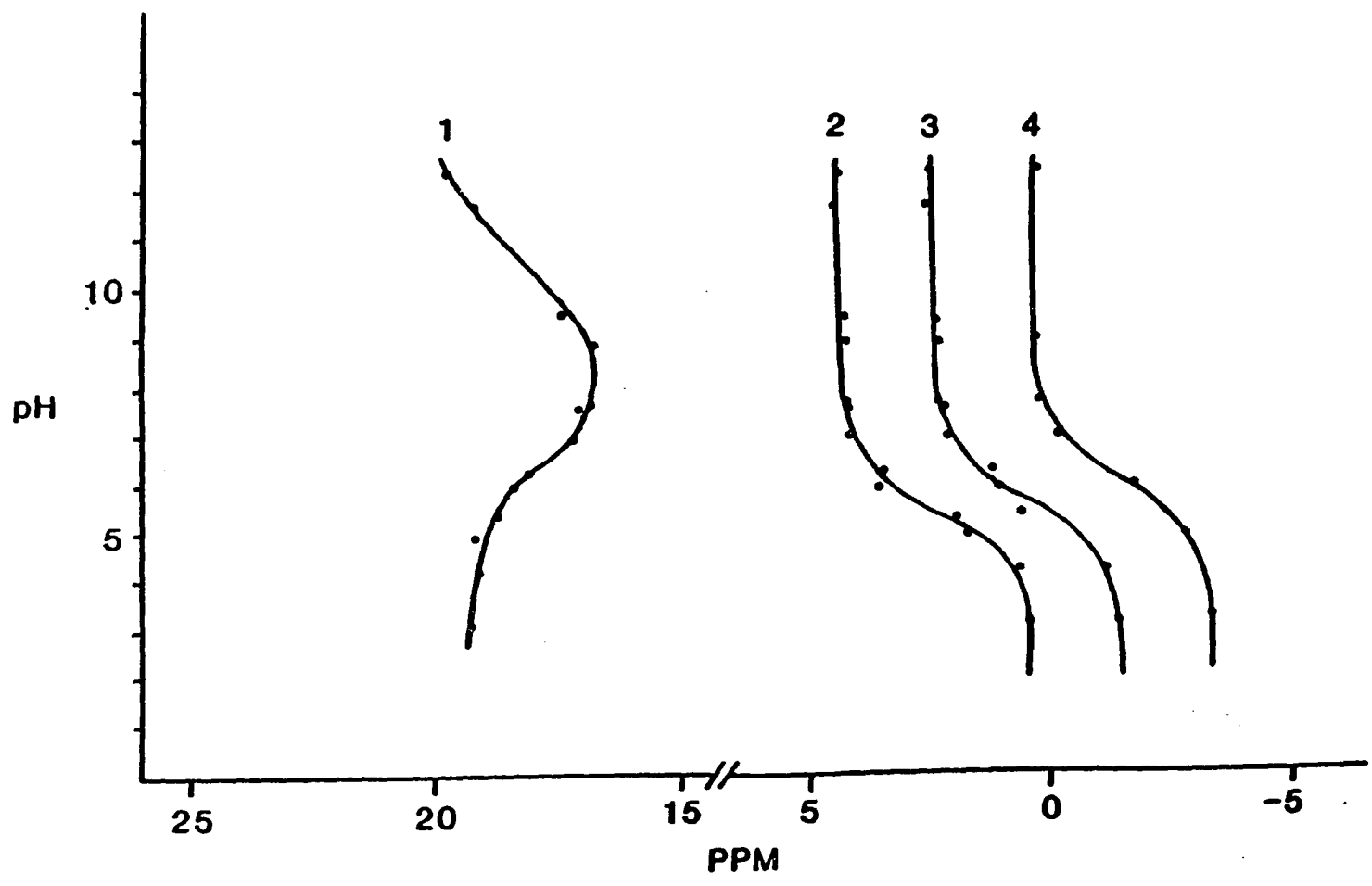
Figure 4. Plot of the relative separation (in ppm) between the
2-aminoethylphosphonic acid peak and the mannose-1-phosphate
peak (resonances 2 and 5 in figure 1) as a function of pH.
[This calibration curve for intracellular pH determination
was of whole P. gouldii erythrocytes. These cells had been
uncoupled with a proton ionophore, 2,4-dinitrophenol, and
then allowed to reach equilibrium for at least one hour in
buffers of varying pH at 5°C.]
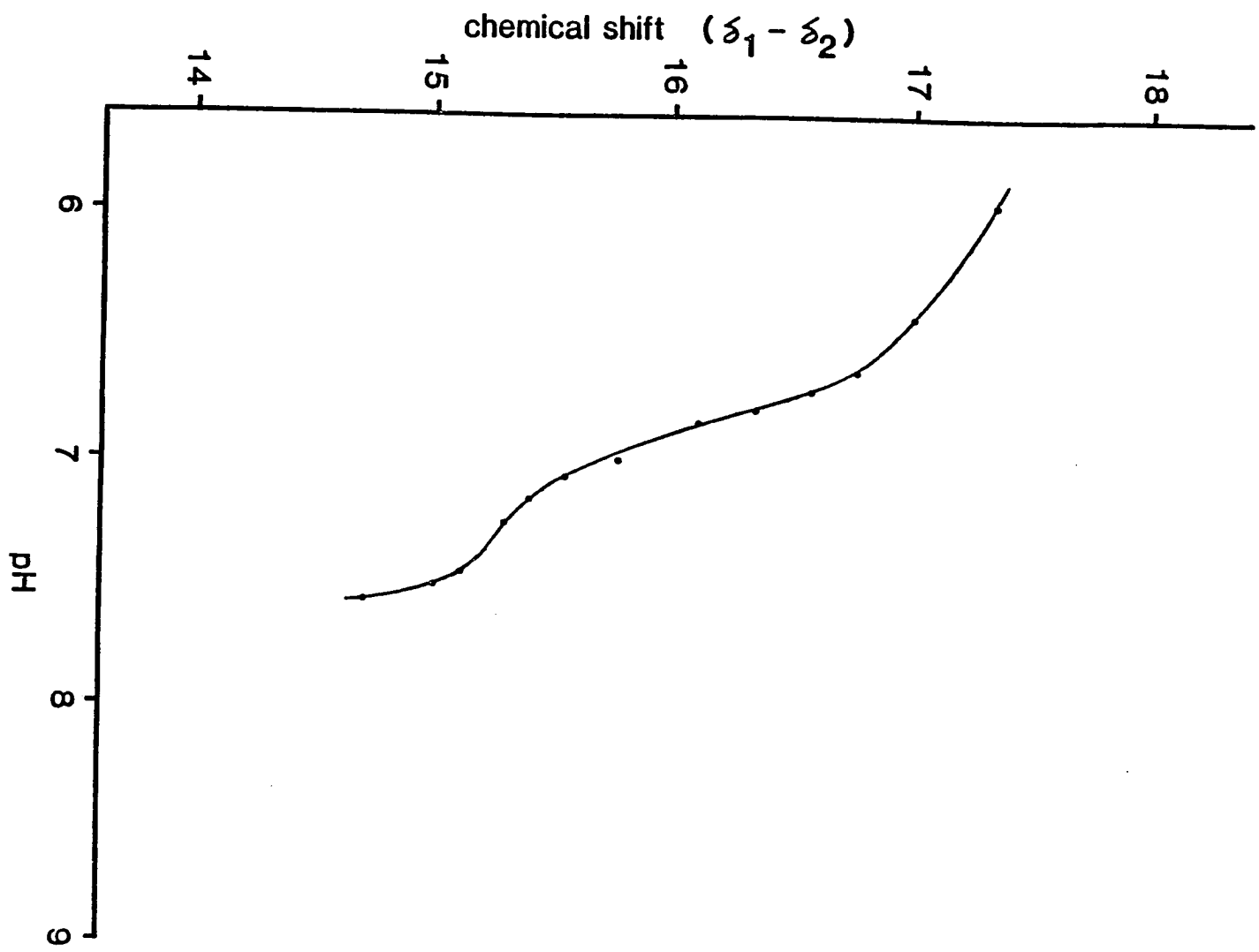
Figure 5. ^{31}P-NMR spectrum (5,300 scans) of anaerobic P. gouldii erythrocytes at

5°C. [Resonances have been assigned as follows:

1) methylenediphosphonic acid in a concentric capillary (18.44 ppm),

2) 2-aminoethylphosphonic acid (19.04 ppm), 3) other phosphonates (16.45

and 16.22 ppm), 4) O-phosphorylethanolamine (4.39 ppm), 5) inorganic

phosphate and mannose-1-phosphate (2.12 ppm), 6) phosphoenolpyruvate

(-0.70 ppm), 7) γ-NTP (-4.97 ppm), 8) α-NTP (-9.91 ppm), 10) UDPG

(-11.56 ppm), and 11) β-NTP (-18.42 ppm).]
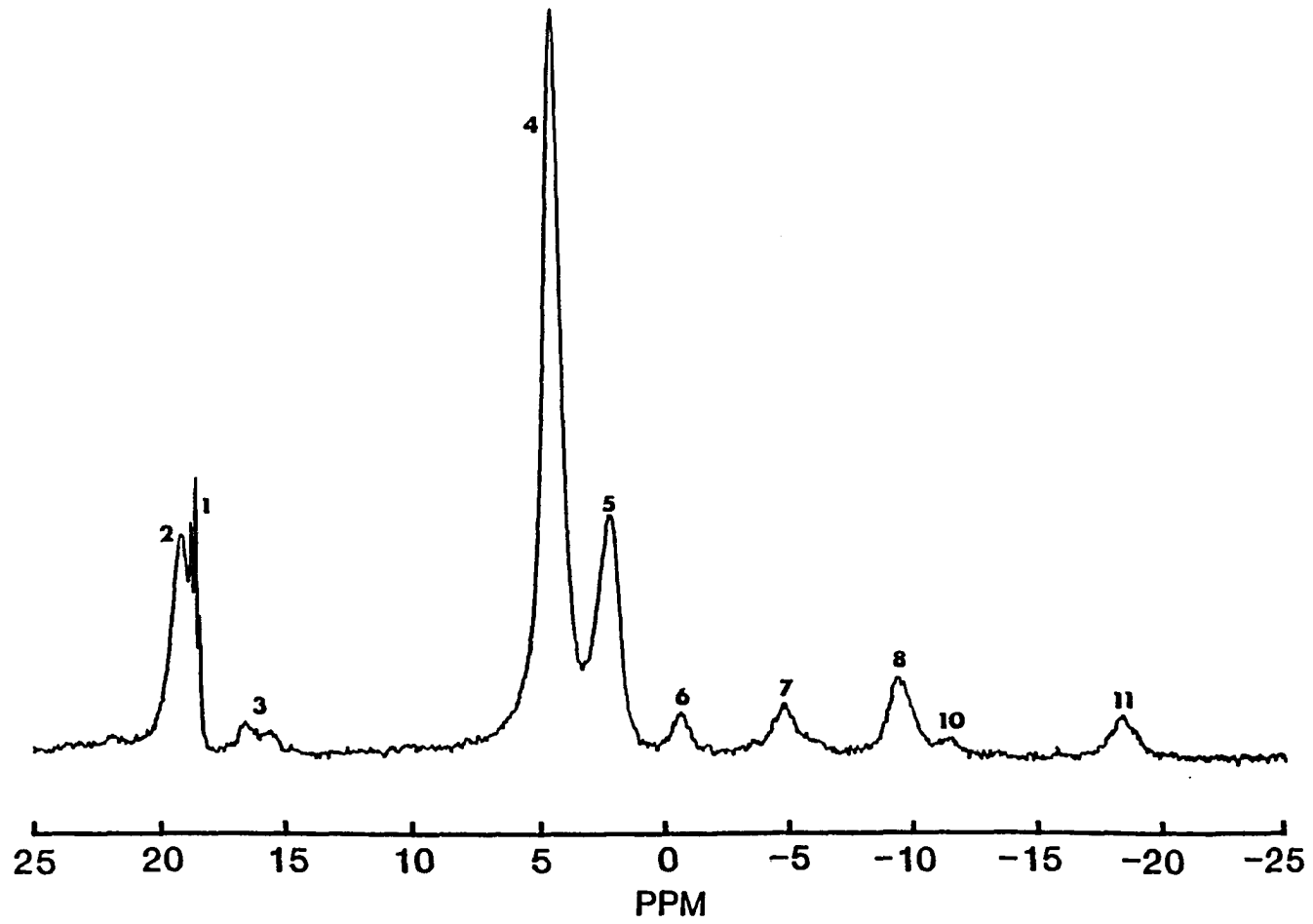
(see Figure 4). In this case the free Mg^{++} concentration and the percentage of ATP complexed as MgATP was not calculated since the intracellular pH was found to deviate significantly from neutrality where a reliable binding constant for MgATP is known.

The ^{31}P-NMR spectrum of aerobic erythrocytes isolated from a second sipunculid, T. zostericola, is shown in Figure 6. A surprisingly large 2-AEP (2) peak is found along with a much decreased PEA (5) resonance in comparison to Figure 1. A new resonance corresponding to a phosphomonoester (4) is also observed. The intracellular pH was calculated to be 7.1±0.1 by assuming that the curve in Figure 4 generated for P. gouldii could also be used for T. zostericola. This assumption is reasonable since the cellular environment for these two cell types should be very similar. Mannose-1-phosphate has been found in T. zostericola by titration of extracts. Resonances corresponding to NTP in rapid exchange with Mg-NTP (7, 8, and 9,) have also been identified. These resonances are very broad. However, with this in mind the percentage of ATP existing as MgATP and the concentration of free Mg^{+2} within these cells was calculated to be 51% and 0.06 mM, respectively. We have also observed slow exchange between Mg-NTP and NTP in this cell type (Figure 7). By integrating the β-NTP resonance corresponding ATP and MgATP the percentage of NTP existing as MgNTP could be calculated directly from this spectrum as 43%. The concentration of free Mg^{++} in this case was calculated as 0.05 mM. Peak assignments were made after the preparation of a T. zostericola extract (see Figure 8).

Figure 6. ^{31}P-NMR spectrum (500 scans) of freshly isolated T. zostericola

erythrocytes at 5°C. [Resonances correspond to the following:

1) methylenediphosphonic acid in an external capillary (18.34 ppm),

2) 2-aminoethylphosphonic acid (17.69 ppm), 3) other phosphonates (15.32

and 14.61 ppm), 4) a hexose-6-phosphate (4.18 ppm), 5) O-

phosphorylethanolamine (3.65 ppm), 6) inorganic phosphate and

mannose-1-phosphate (2.12 ppm), 7) γ-NTP (-5.21 ppm), 8) α-NTP

(-10.07 ppm), and 9) β-NTP (-19.64 ppm).]
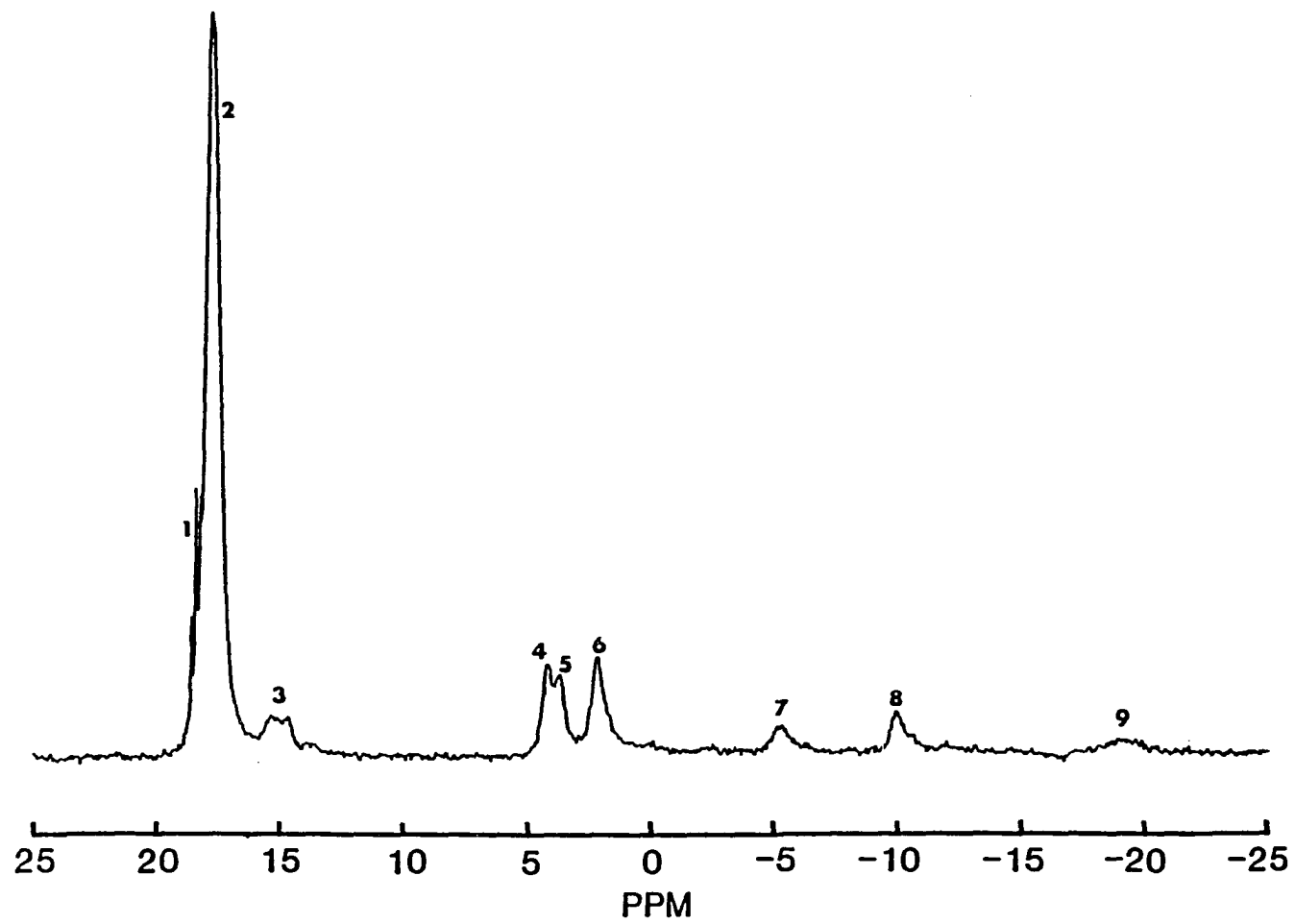
Figure 7. ³¹P-NMR spectrum (2,000 scans) of freshly isolated T. zostericola

erythrocytes at 22°C. [Resonances correspond to the following:

1) 2-aminoethylphosphonic acid (17.69 ppm), 2) other phosphonates

(15.16 ppm), 3) a hexose-6-phosphate and O-phosphorylethanolamine

(4.15 ppm), 4) inorganic phosphate and mannose-1-phosphate (2.30 ppm),

5) a phosphogen (-2.49 ppm), 6) γ-NTP (-5.24 ppm), 7) α-NTP

(-10.02 ppm), 8) β-MgNTP (-16.31 ppm), and 9) β-NTP.]
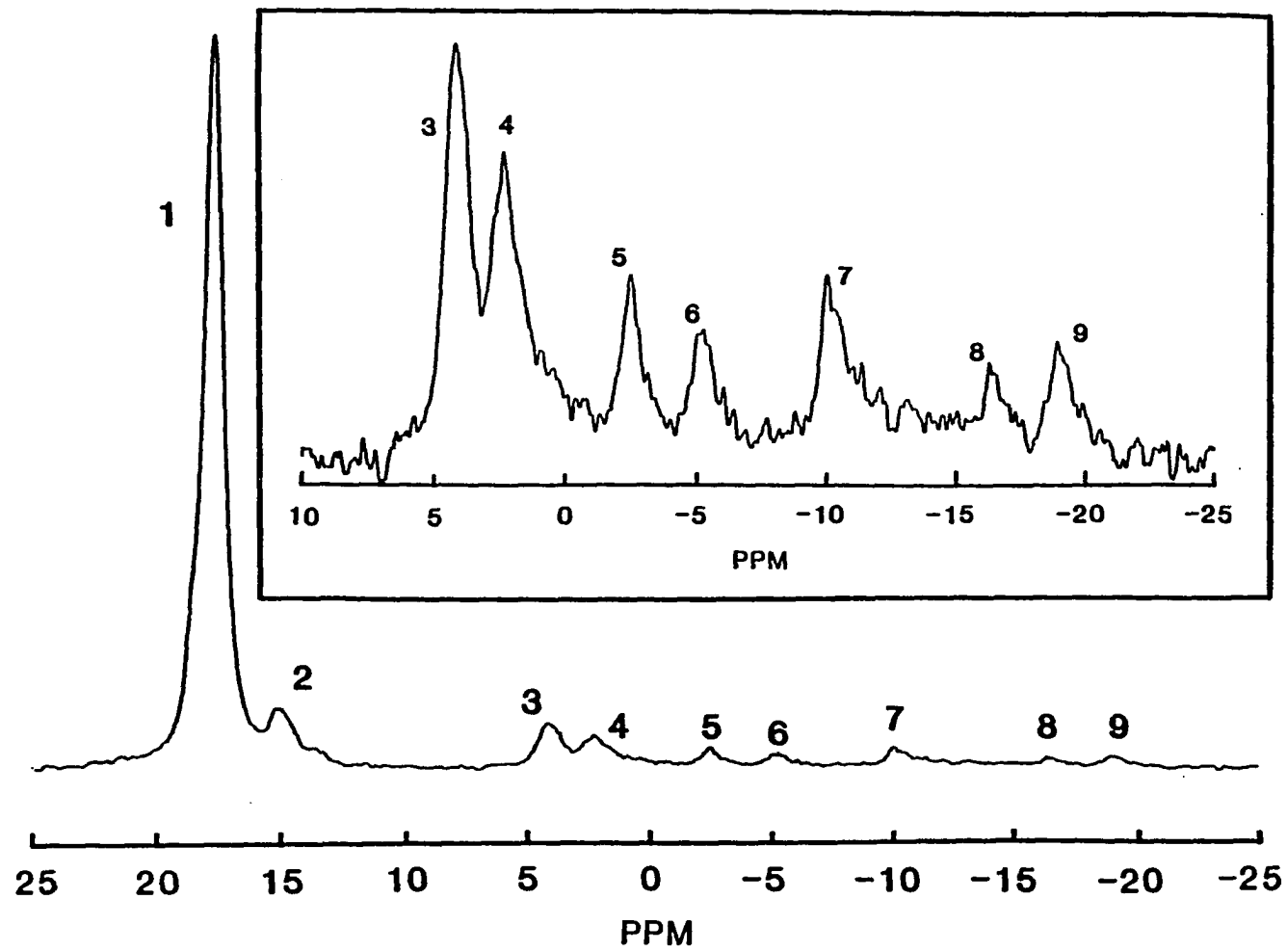
Figure 8. ^{31}P-NMR spectrum (4,700 scans) of a 6% perchloric acid extract of

freshly isolated T. zostericola erythrocytes. [The pH was adjusted to

12.7 with 2 M KOH. Resonances correspond to the following:

1) methylenediphosphonic acid standard in a concentric capillary

(18.39 ppm), 2) 2-aminoethylphosphonic acid (19.88 ppm), 3) other

phosphonates (15.98 and 14.33 ppm), 4) a hexose-6-phosphate (5.12 ppm),

5) O-phosphorylethanolamine (4.27 ppm), 6) mannose-1-phosphate

(3.59 ppm), 7) ɤ-NTP (-5.51 ppm), 8) α-NTP (-10.35 ppm), 9) β-NTP

(-21.05 ppm), 10) a phosphodiester (1.36 ppm), and 11) a phosphagen

(-2.62 ppm).]
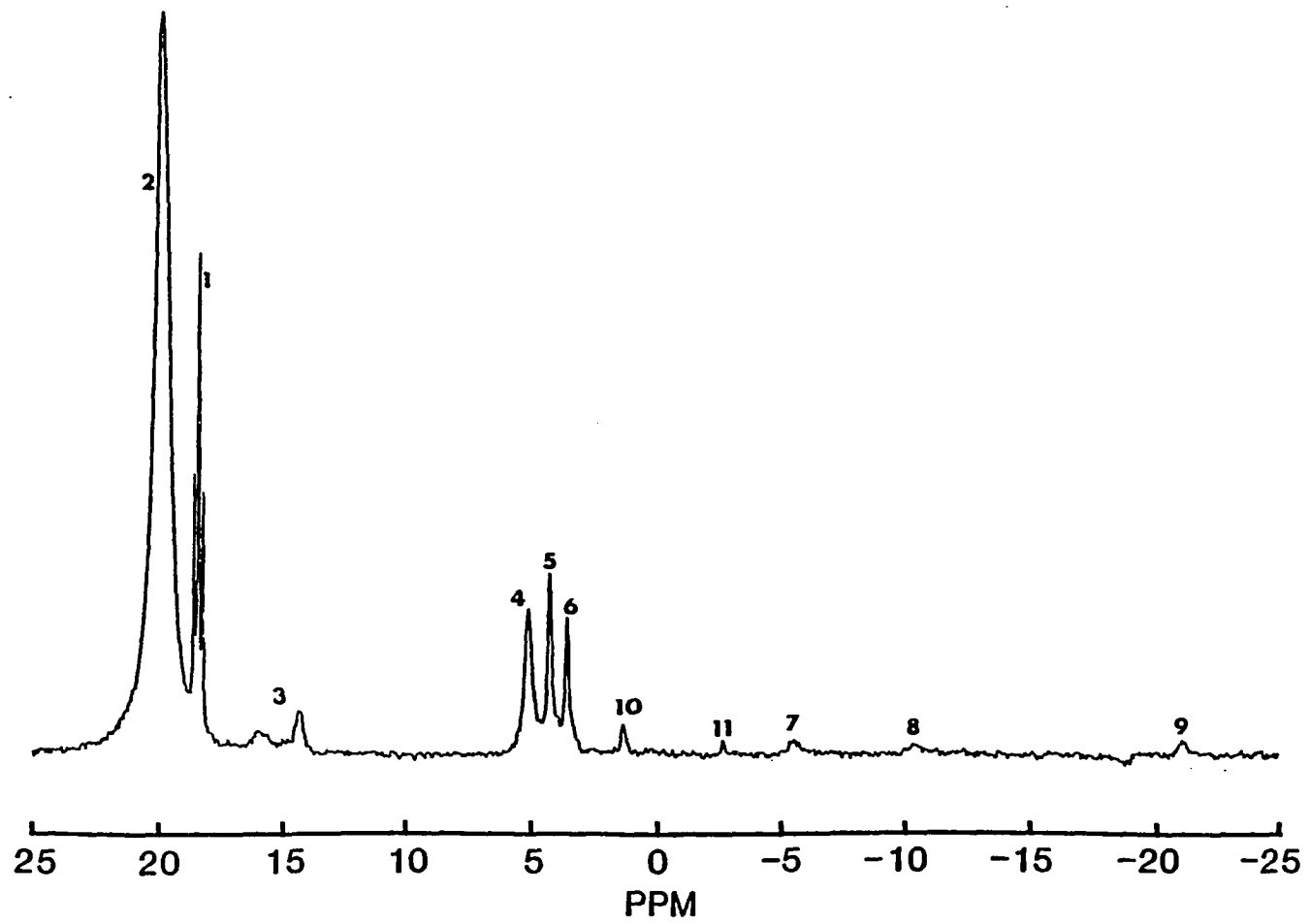
The binding of O-PEA and 2-AEP to metHr was investigated by UV-
visible spectroscopy. The effect of PEA on metHr at neutral pH can be
seen in Figure 9. Scatchard analysis has revealed that this interaction
is best described in terms of negative cooperativity or non-specific
binding (see Figure 10). A binding constant (K_f) value of 7.0 ± 0.4 M^{-1}
was determined through the use of a Hill plot assuming n=1. This plot
had a slope of approximately 0.8 (see Figure 11). The interaction of
metHr and 2-AEP has been investigated at neutral pH. No changes in the
UV-visible spectrum of metHr upon addition of 2-AEP up to a
concentration of 0.45 M were detected.

In addition, PEA has been found to be a potent inhibitor of azide
binding to metHr. Rate constants at 22°C and pH 7.1 were determined by
curve fitting the ΔA_{447} versus time data, measured under pseudo-first
order conditions, with single exponentials. The second order rate
constant for azide binding in the presence of 0.33 M PEA has been
measured ([Hr]=0.064 mM, $[N_3^-]$=0.0032 M) and its value has been
established at 0.048 ± 0.010 $M^{-1}s^{-1}$ at 22°. Measurements of azide binding
to metHr in the presence of perchlorate ($[ClO_4^-]$=0.045 M, [Hr]=0.088 mM,
$[N_3^-]$=0.0032 M), 2-AEP ([2-AEP]=0.045 M, [Hr]=0.080 mM, $[N_3^-]$=0.0045 M),
and control (no effector) ([Hr]=0.081 mM, $[N_3^-]$=0.0048 M) gave second
order rate constants at 22° with values of 1.13 ± 0.15 $M^{-1}s^{-1}$,
3.68 ± 0.15 $M^{-1}s^{-1}$, and 4.10 ± 0.15 $M^{-1}s^{-1}$, respectively. From these data
as well as from conventional ln A/A_o versus time plots (Figure 12), it
can be readily observed that the rate constant for azide binding to
metHr is 85-fold lower than the control in the presence of PEA. Azide

Figure 9. The uv-visible spectrum of metHr (0.10 mM) in the presence of an

effector, O-phosphorylethanolamine (50 mM). [Spectra were recorded 0,

15, 30, 45, 60, 90, 105, 120, and 180 minutes after the addition of O-

phosphorylethanolamine (50 mM). This solution had a pH value of 7.14.

The arrows indicate directions of absorbance changes with time.]
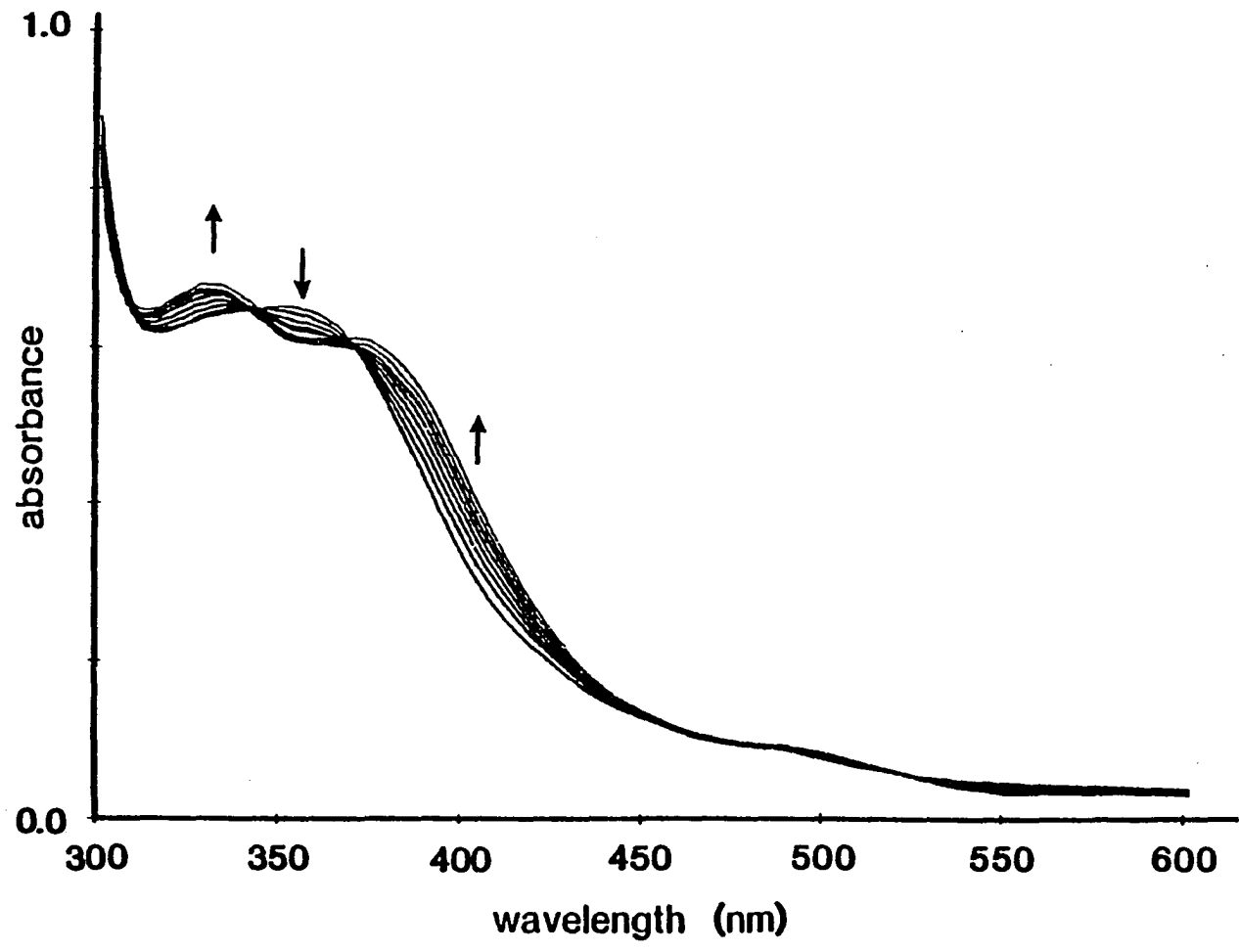
Figure 10. Scatchard plot used to analyze the binding of O-PEA to 0.068 mM methemerythrin at a pH value of 7.1. [Points correspond to varying levels of O-PEA (0.333 M, 0.133 M, 0.066 M, 0.033 M, 0.0133 M, 0.0066 M, 0.0033 M, 0.00066 M).]
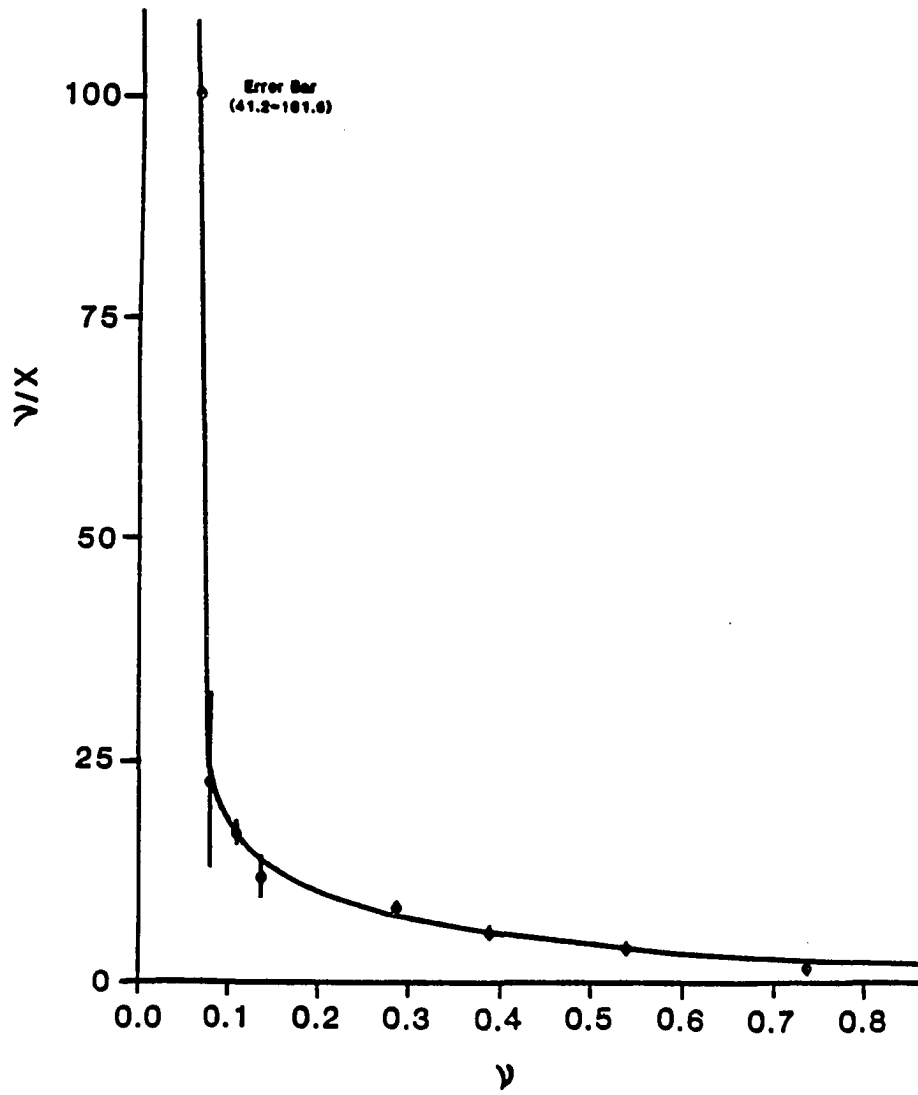
Figure 11. Hill Plot used to analyze the binding of O-PEA to 0.068 mM

methemerythrin at pH=7.1. [Points correspond to varying

levels of O-PEA (0.333 M, 0.133 M, 0.066 M, 0.033 M,

0.0133 M, 0.0066 M, 0.0033 M, 0.00066 M).]
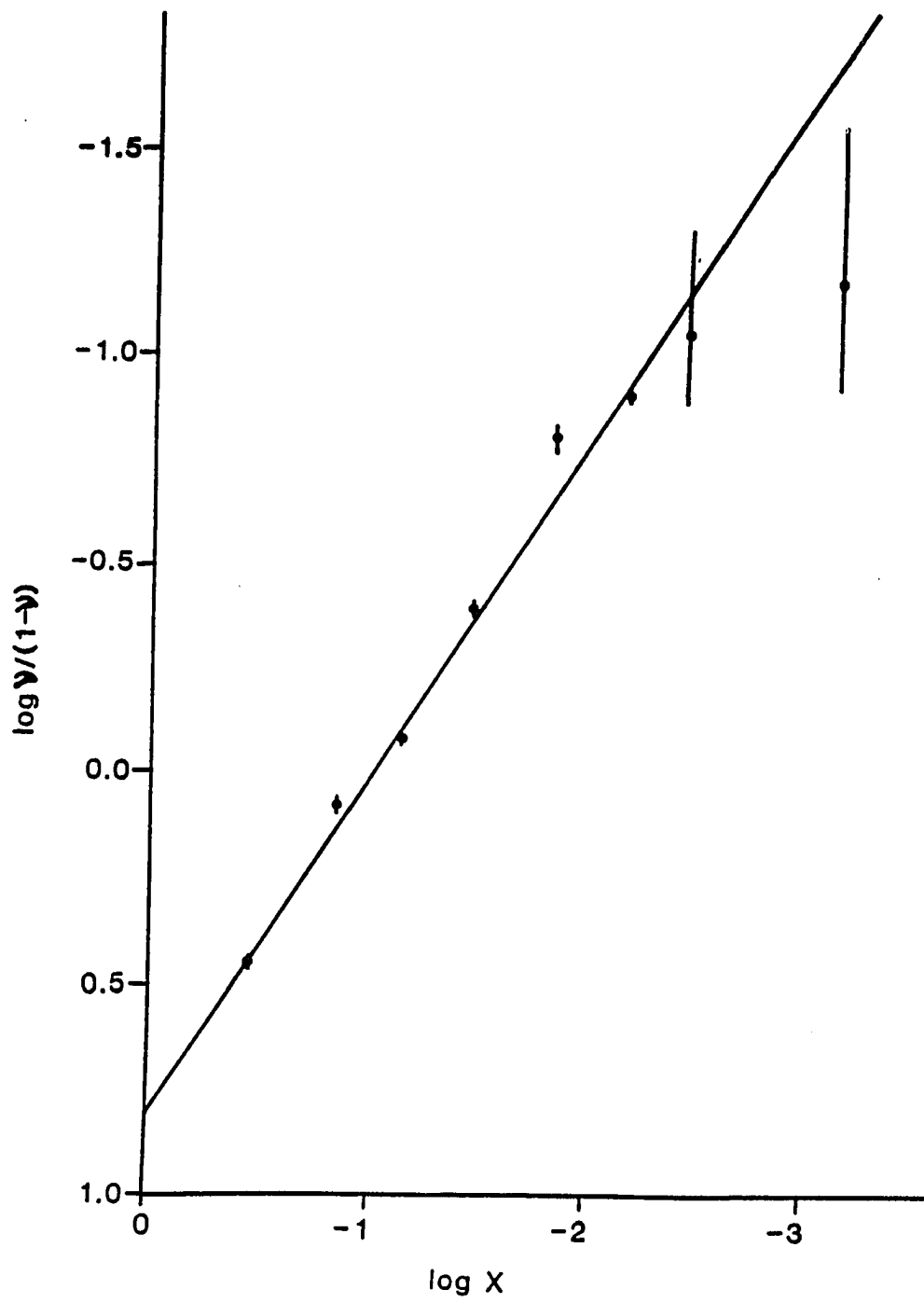
Figure 12. Plot illustrating changing rates of azide binding to methemerythrin in the presence or absence of effectors. [Curves correspond to 1) control ([Hr]=0.081 mM, [N_3^-]=0.0048 M), 2) perchlorate ([ClO_4^-]=0.045 M, [Hr]=0.088 mM, [N_3^-]=0.0032 M), 3) O-PEA ([O-PEA]=0.33 M, [Hr]=0.064 mM, [N_3^-]=0.0032 M). Data obtained with 2-AEP was not plotted as it was almost superimposable with the control ([2-AEP]=0.045 M, [Hr]=0.080 mM, [N_3^-]=0.0045 M). This work was performed at a pH value of 7.1 and a temperature of 22°C.]
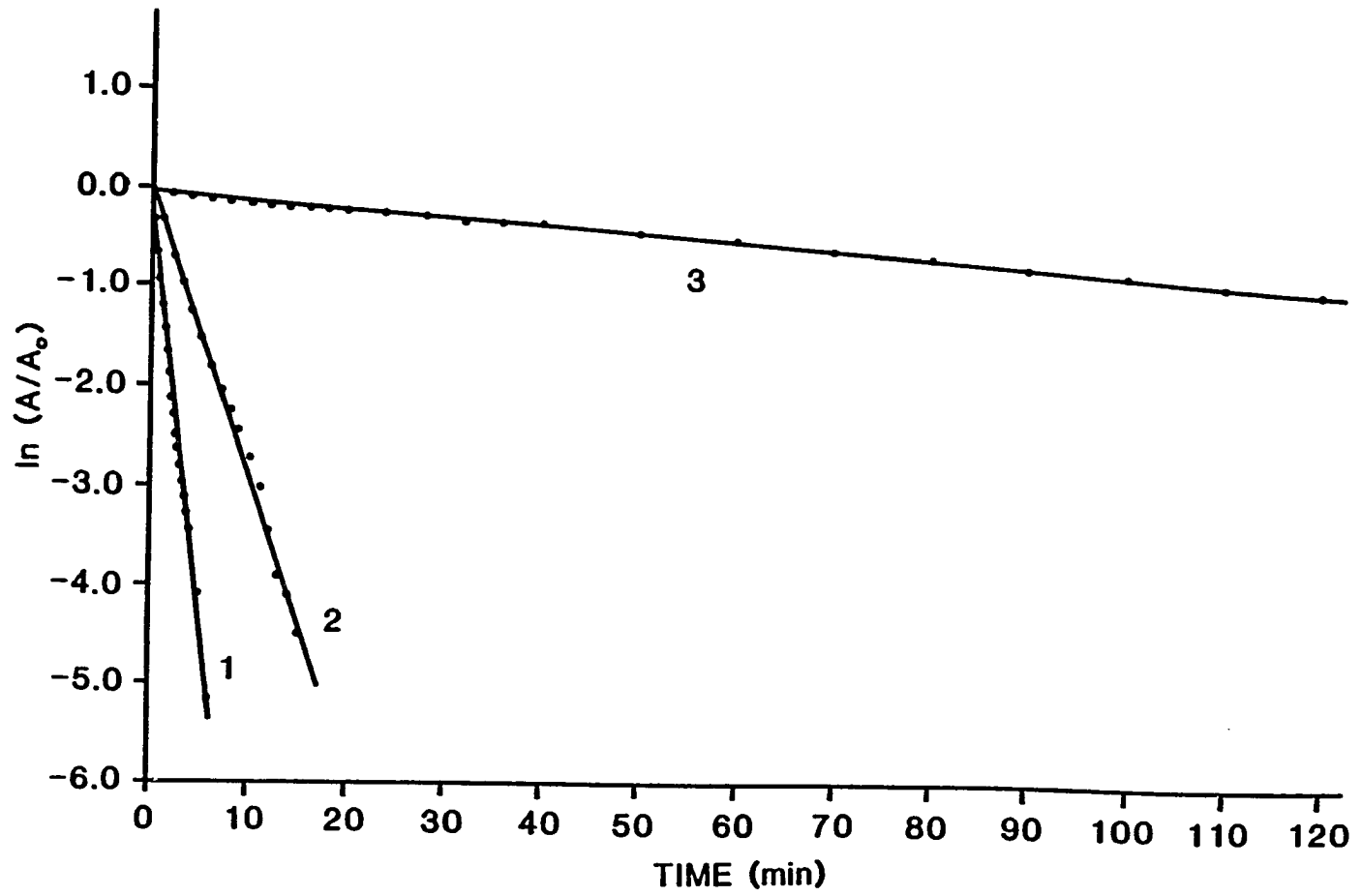
binding to methemerythrin has also been studied in the presence of these

same effectors but with a lower ratio of azide (0.91 mM) to

methemerythrin (0.11 mM). Although lower O-PEA concentrations (0.045 M)

were used in this case the decrease in the rate of azide binding is

readily observable (see Figure 13). Similar results were found at

higher azide concentrations ($[N_3^-]$=0.0045 M, [Hr]=0.086 mM, [O-

PEA]=0.045). In these studies, results for the control, with

perchlorate and with 2-AEP could be treated with a single exponential.

However, the O-PEA data at the lower concentration (0.045 M), required

two exponentials to achieve a good fit. These two exponentials were

found to generate second order rate constants which corresponded roughly

to those of the control (3.64±0.15 $M^{-1}s^{-1}$) and those of O-

PEA (0.034±0.010 $M^{-1}s^{-1}$) obtained at the higher O-PEA levels (0.33 M).

Figure 13. Time course of the binding of azide (0.91 mM) to methemerythrin

(0.11 mM) in the presence of effectors as monitored by noting the

increase in absorbance at 447 nm. [Individual curves correspond to the

following effectors 1) control (no effector), 2) 2-AEP (0.045 M),

3) perchlorate (0.045 M), and 4) O-PEA (0.045 M).]
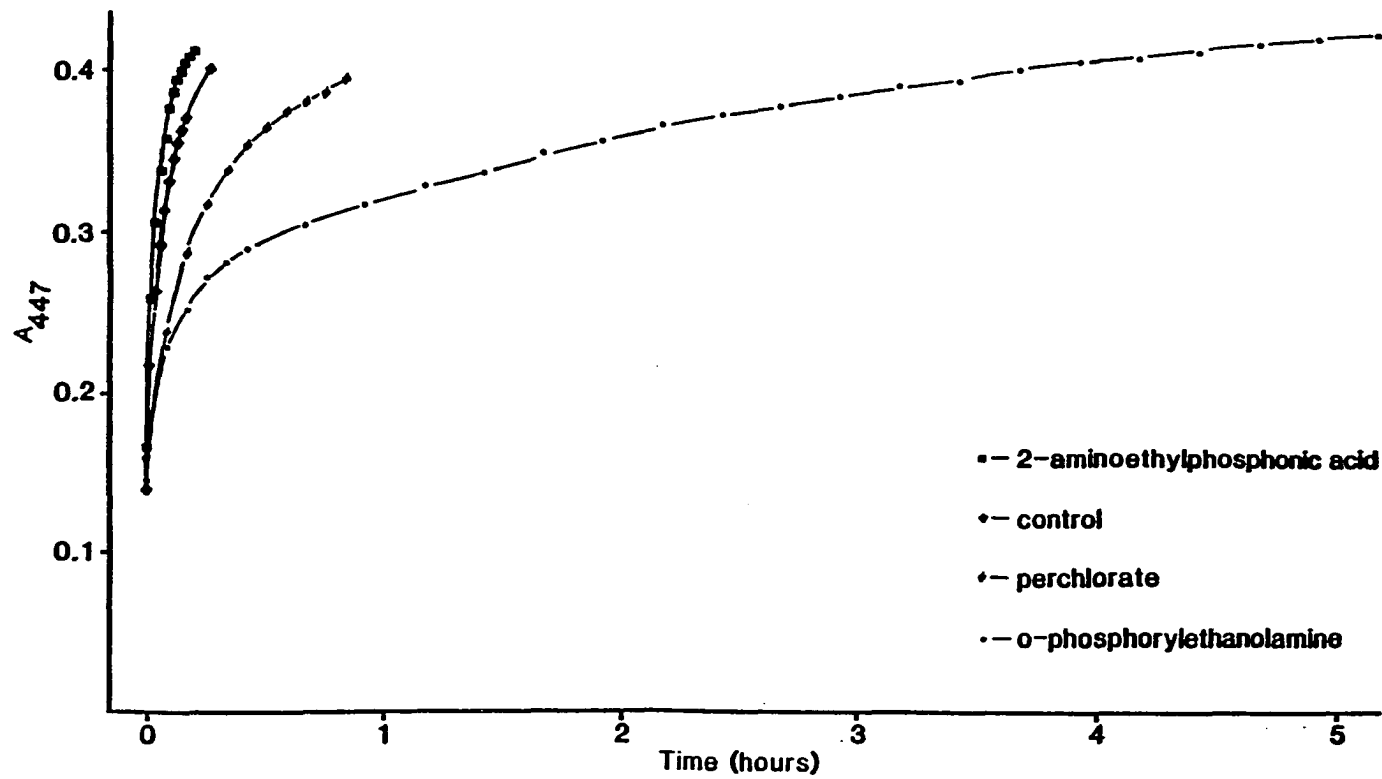
DISCUSSION

Using ^{31}P-NMR we have examined the erythrocytes of P.gouldii and T. zostericola. The spectrum of P. gouldii has been reported in Figure 1 and that of T. zostericola in Figures 6 and 7. From titration of the cellular extracts shown in Figures 2 and 8, these cells were found to share several metabolites, the most prominent of which are 2-AEP, PEA, NTP, and NDP. Other common constituents include inorganic phosphate and mannose-1-phosphate. Erythrocytes isolated from P. gouldii also revealed the presence of phosphoenolpyruvate, NAD$^+$/NADH, and UDPG. In addition, cells isolated from T. zostericola were found to possess a sharp phosphomonester resonance (4.18 ppm) which is probably a hexose-6-phosphate. This resonance was not present in P. gouldii erythrocytes. Perchloric acid extracts of both cell types and whole T. zostericola erythrocytes also revealed traces of phosphagens. Phosphoguanidotaurine has been previously identified in sipuncilids (Thoai et al., 1953 and Roche et al., 1957). Therefore, it is likely that this compound would give rise to a phosphogen signal in these cells. The principal difference in ^{31}P-NMR spectra of the erythrocytes from the two species lies in the relative abundance of 2-AEP and PEA. Relatively high levels of PEA and low levels of 2-AEP were found in erythrocytes from P. gouldii, whereas the reverse was the case in cells isolated from T. zostericola. ^{31}P-NMR has previously been utilized to study biological phosphonates in solution (Glonek et al., 1970 and

Henderson et al., 1972). ^{31}P-NMR studies of the planorbid snail Helisoma sp. have revealed the presence of aminoethylphosphonic acid in this organism (Miceli, 1978). In vivo ^{31}P- NMR studies have also established the presence of free phosphonates in whole cells, namely in the protozoans Acanthamoeba castellanii (Deslauriers et al., 1980; 1982a) and in Tetrahymena (Deslauriers et al., 1982b).

The ^{31}P-NMR spectrum of erythrocytes from T. zostericola also showed considerable broadening of the NTP resonances. Such extensive broadening was not observed in either aerobic or anaerobic P. gouldii cells. This result would seem to rule out the presence of relatively higher levels of deoxyHr in T. zostericola erythrocytes as the explanation for broadening of the NTP resonanes. It is more likely that this broadening of the NTP resonances is caused by relatively slower exchange of ATP with MgATP within these cells or by differences in levels of MgATP within the erythrocytes of these two species of sipunculids. In this respect, it should be noted that the ^{31}P-NMR spectrum of anaerobic erythrocytes from P. gouldii did show some broadening of the phosphorus signals and that in this case, the broadening observed was experienced by all resonances to a similar extent and the NTP resonances were not in any way singled out (Figure 5). However, it may also be possible that this broadening is caused by the interaction of NTP with a macromolecule, possibly hemerythrin. It should by noted that hemerythrin itself is not known to be phosphorylated and therefore is unable to lead to any yet unassigned phosphorus signals. (Purified P. gouldii oxyHr gave no ^{31}P-NMR

spectrum.)

Using the curve depicted in Figure 4, the intracellular pH of these cells were calculated as previously explained. Intracellular pH values of 7.2±0.1, 6.5±0.1 and 7.1±0.1 were calculated,for aerobic and anaerobic erythrocytes of P. gouldii, and aerobic erythrocytes from T. zostericola, respectively. The pH of human erythrocytes has been extensively examined by ^{31}P-NMR. The first such study by Moon and Richards (1973) revealed intracellular pH values 6.86±.05 from DPG and 6.89±.06 from P_i. More recent and reliable studies (Labotka, 1983; 1984) established the pH of oxygenated human erythrocytes at 7.15, rising to 7.29 upon deoxygenation. In direct contrast to this we found that the intracellular pH in P. gouldii erythrocytes fell by 0.7 pH units from 7.2±0.1 to 6.5±0.1. Such a drop in pH upon anaerobiosis has previously been observed in the coelomic fluid of the sipunculid Sipunculos nudus (Porter et al., 1984). However, the present results represent the first measurements of pH within sipunculan erythrocytes.

The extent of Mg^{+2} complexed to ATP in these cells was calculated as 85% and 43-51% for aerobic P. gouldii and T. zostericola erythrocytes, respectively. Gupta et al. (1978a) have obtained values of 84% and 78% in aerobic and anaerobic human eythrocytes, revealing that complexation of ATP to Mg^{+2} decreases in these cells in anaerobic conditions. Free Mg^{+2} concentrations of 0.34 mM and 0.05-0.06 mM were found in this study for aerobic P. gouldii and T. zostericola erythrocytes, respectively. Flatman and Lew (1977) have measured the free Mg^{+2} concentration in human erythrocytes as 0.4 mM. Moreover,

Gupta et al. (1978b) have performed similar measurements yielding values

of 0.24 mM and 0.67 mM in aerobic and anaerobic human erthrocytes,

respectively. We do not report the free Mg^{++} values for anaerobic

sipuculan erythrocytes, since no reliable value of the dissociation

constant of MgATP is known at the acidic pH value we have detected.

The wide range of free Mg^{+2} in the two types of sipunculan

erythrocytes we have examined may help support our hypothesis that free

Mg^{+2} levels may be closely linked to the metabolic state and the

function of hemerythrin in these cells (see below).

In studying T. zostericola erythrocytes we have found that slow

exchange between NTP and MgNTP can sometimes be detected. Slow exchange

has been considered theoretically (Vasavada et al., 1984) and has been

previously detected in model systems (Sontheimer et al., 1986).

As shown in Figures 9, 10, and 11, PEA was found to bind to

methemerythrin at a pH value of 7.1. PEA was found to bind to

methemerythrin in a manner indicative of negative cooperativity or non-

specific binding. This conclusion was supported by a concave Scatchard

plot (Figure 10) and a Hill plot (Figure 11) with a slope of

approximately 0.8. These plots are consistent with either negative

cooperativity or with non-specific binding. From this Hill plot, a K_f

value of 7.0 ± 0.4 M^{-1} was calculated. Since we have demonstrated that

the concentration of PEA in the P. gouldii erythrocytes is greater than

20 mM, this K_f value translates to a ratio of [Hr-PEA]/[Hr] of at least

1.4 within the cell where [Hr] is expressed in terms of subunits. That

is to say, at least 58% of the hemerythrin present in the cell exists as

a PEA-Hr complex, if one assumes that the interaction of PEA with oxyHr is roughly the same as that with metHr.

Perchlorate is also known to bind to methemerythrin. The exact location of the perchlorate binding site on hemerythrin has been determined by difference maps and x-ray diffraction (Stenkamp et al., 1978). In these studies, two perchlorate binding sites were found in the octamer. We propose that PEA binds to one of these sites with relative ease. The binding of a second PEA to the next ClO_4^- binding site is inhibited by the presence of the first PEA molecule. This could explain the behavior that we have observed for the binding of PEA to methemerythrin.

In addition, PEA was found to significantly lower the rate of azide binding to methemerythrin (0.048 ± 0.010 $M^{-1}s^{-1}$) versus that for 2-AEP (3.68 ± 0.15 $M^{-1}s^{-1}$) or for the control (4.10 ± 0.15 $M^{-1}s^{-1}$). The second order rate constant obtained in the presence of PEA was significantly lower than that obtained with perchlorate (1.13 ± 0.15 $M^{-1}s^{-1}$), a known effector of hemerythrin (Figure 12). The rate for the control determined in this work at pH 7.1 agrees well with previously published values at pH 6.3 and 8.2 (Wilkins and Harrington, 1983).

When lower concentrations of PEA (0.045 M) were used in azide binding studies, the resulting binding curves showed biphasic behavior and required two exponentials to achieve a good fit (Figure 13). The first of these exponentials was roughly the same as that used in fitting the control (no effector). The second of these two exponentials was roughly the same as that used to fit the azide binding data at the

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higher PEA concentration (0.33 M). These data indicate that when
working at the lower PEA concentration (0.045 M), the PEA binding sites
on methemerythrin are not fully saturated.

In this study, ^{31}P-NMR spectroscopy has proved useful for analyzing
the metabolic state of erythrocytes isolated from both P. gouldii and T.
zostericola. These cells were found to contain O-phosphorylethanolamine
and a phosphonate, 2-aminoethylphosphonic acid. The presence of PEA has
been previously established in erythrocytes isolated from the Southern
Fiddler Ray Trygonorhina fasciata guanerius (Coates et al., 1979). In
these cells PEA accounted for at least 20% of the total acid soluble
phosphate. However, the function of PEA in these erythrocytes has
apparently not been established. The presence of the biological
phosphonate 2-AEP was first reported in rumen protozoa in 1959
(Horiguchi and Kandatsu, 1959) and was later found to be present in sea
anemone by Kittredge et al. (1962). Early reviews on biological
phosphonates have been written by Quin (1965, 1966) and by Kittredge and
Roberts (1969). Since that time, this compound and its derivatives have
been found in many other species including mammals (Alhadeff et al.,
1972; Curley and Henderson, 1972; and Hasegawa et al., 1976). The
compound has also been found in human brain, muscle, heart, and liver
(Alhadeff and Daves, 1970; 1971). The metabolism of 2-AEP and that of
other phosphonates has been extensively studied during the past two
decades revealing that these compounds are inherently linked to lipid
metabolism (Hori et al., 1964; Itasaka et al., 1969; De Koning, 1970;
Smith and Law, 1970; Mason, 1972 and Baraud and Maurice, 1982). 2-AEP

has also been found in proteins (Quin, 1964 and Smith and Lepak, 1982)

and polysaccharides (Korn et al., 1973).

The synthesis and breakdown of 2-AEP and other phosphonates remains

an area of keen interest. It has been established that [^{32}P] inorganic

phosphate is incorporated into 2-AEP in bacteria and rat liver

(Rosenberg, 1964; Kittredge and Hughes, 1964; Kandatsu et al., 1965)

although no enzyme capable of synthesizing the P-C bond has yet been

found. Kittredge and Hughes (1964) proposed that 2-AEP was formed from

2-amino-3-phosphonopropionic acid through decarboxylation. This

hypothesis has been supported by studies in rat liver cells (Horigane et

al., 1979). However, dephosphorylation of 2-phosphonoacetaldehyde to

give acetaldehyde and P_i seemed to be the principal metabolic pathway

taken by this compound, rather than amination to give 2-AEP (Scheme 1).

Segal (1965) proposed that 2-AEP could be produced from

phosphatidylethanolamine through a series of rearrangements (Scheme 2).

It may be possible that the interconversion of these two compounds in

vivo may regulate O_2 binding in this protein. In light of this,

however, we should emphasize that we did not detect appreciable relative

concentration changes in PEA and 2-AEP in aerobic versus anaerobic

sipunculan erythrocytes.

Glycolytic intermediates such as phosphoenolpyruvate (PEP) have

been proposed (Warren, 1968) to be possible precursors of 2-AEP through

rearrangement (Scheme 3). Intermediates in this mechanism were also

encountered in Scheme 1. It has also been proposed that

phosphatidylenolpyruvate is the actual precursor to 2-AEP (Liang and

Scheme 1. Proposed steps in the metabolism of 2-AEP
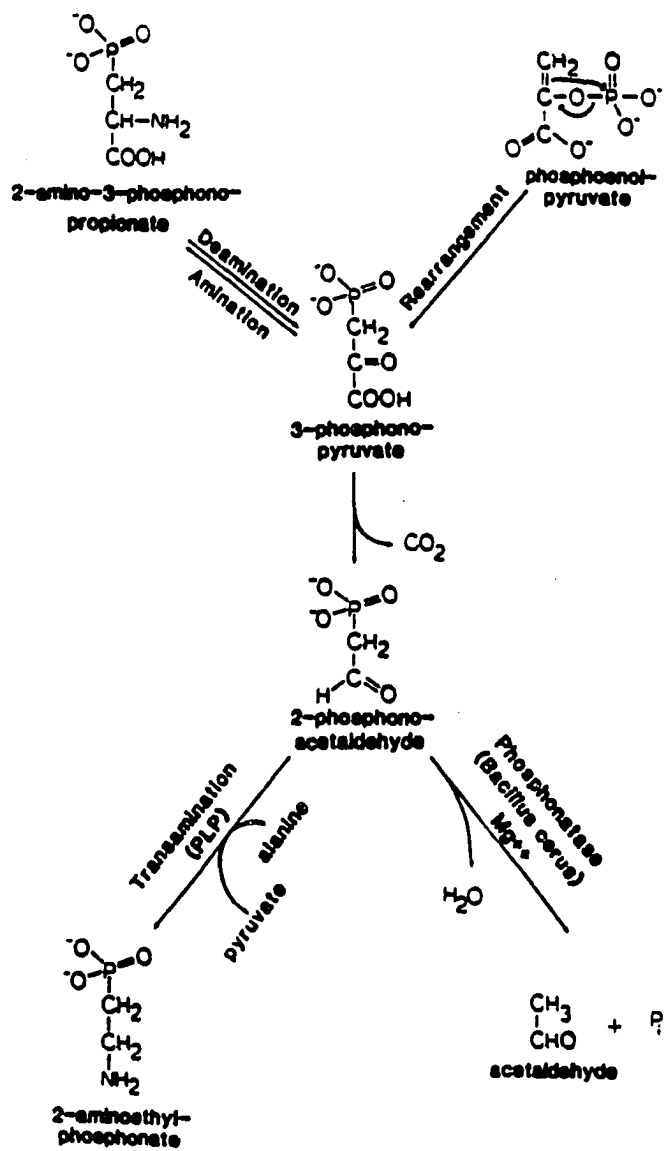
Scheme 2. Proposed mechanism for the formation of

2-AEP from lipid-bound PEA

RO–P(O⁻)(=O)–O–CH₂CH₂NH₃⁺ —**Proton Transfer**→ RO–P⁺(O⁻)(OH)–O–CH₂CH₂N̈H₂ ——→ ROH + [cyclic O⁻, P⁺, HO, O, NH–CH₂CH₂] —**+H₂O**→

ROH + HO–P⁺(O⁻)(O⁻)–NHCH₂CH₂OH ——→ RO–P⁺(O⁻)(OH)–NHCH₂CH₂OH —**–H₂O**→ RO–P⁺(O⁻)(OH)–NH–CH₂=CH₂ ——→

RO–P⁺(O⁻)(OH)–CH₂CH₂=NH —**Reduction**→ RO–P⁺(O⁻)(OH)–CH₂CH₂NH₂ —**+H₂O**→ ROH + HO–P(=O)(O⁻)–CH₂CH₂NH₂

R = CH₂OCOR'
    CHOCOR'
    CH₂OH
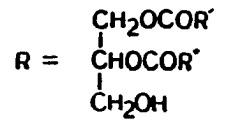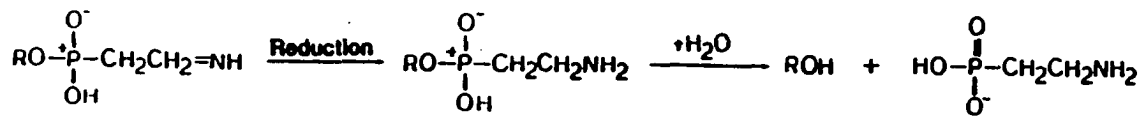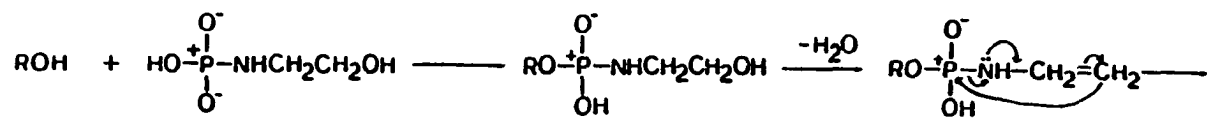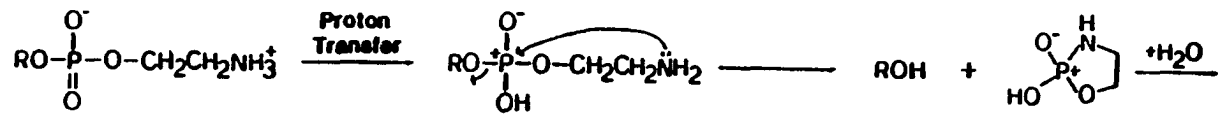
Scheme 3. Proposed mechanism for the formation of 2-AEP from PEP
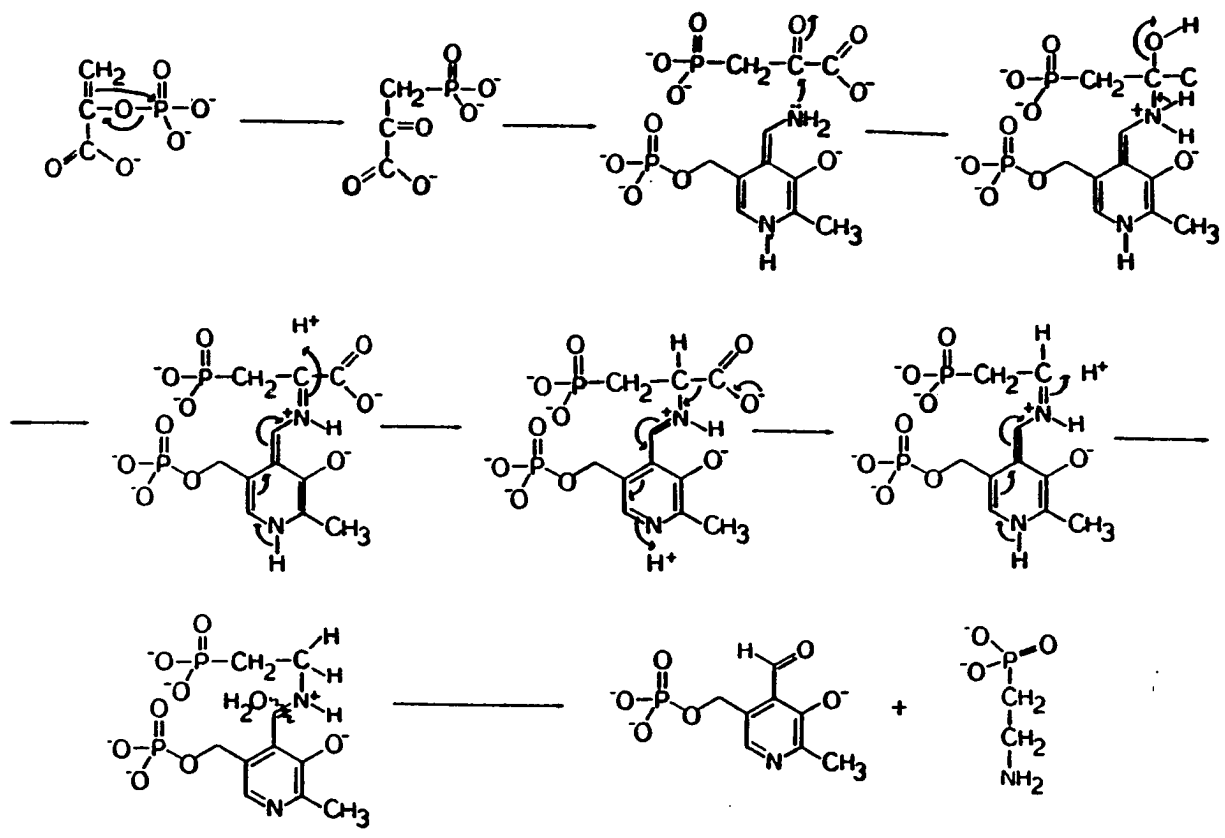
Rosenberg, 1968). This proposal is supported by the observation that phosphatide-bound 2-AEP is synthesized prior to free 2-AEP in tetrahymena (Rosenberg, 1964; Liang and Rosenberg, 1968).

La Nauze and Rosenberg (1967) have established that the bacterium Bacillus cereus can utilize 2-AEP as their sole source of phosphate. The release of P_i from 2-AEP occurred only when adequate levels of pyridoxal phosphate (PLP) was provided in the presence of an amino group acceptor such as pyruvate or oxaloacetate. This tends to indicate the presence of a transamination reaction whereby 2-AEP is modified to 2-phosphonoacetaldehyde and pyruvate is altered to alanine (Scheme 1). La Nauze and Rosenberg have shown that 2-phosphonoacetaldehyde is a degradation product in the metabolism of Bacillus cereus (La Nauze and Rosenberg, 1968). The 2-phosphonoacetaldehyde produced has then been shown to be broken down into P_i and acetaldehyde. La Nauze et al. (1970) have established the presence of a phosphonatase which is responsible for the cleavage of 2-phosphonoacetaldehyde into acetaldehyde and P_i in Bacillus cereus. This enzyme is a dimer (MW = 88,000) which requires Mg^{+2} for activity and is inhibited by orthophosphite. The enzyme has optimal activity between pH 8 and 9, has a K_m of 40 μM for the substrate and was shown to possess 0.4 and 0.3 g atom of zinc and copper per dimer molecular weight. However, the authors have shown that this is not a heavy metal metalloenzyme. In light of this report, it may be possible that the very high levels of 2-AEP in erythrocytes isolated from T. zostericola may be linked to the low activity of an analogous 2-phosphonoacetaldehyde phosphonatase.

This may be related to the very low levels of Mg^{+2} (0.06 mM) we have reported in this cell type. Transamination of 2-AEP and other phosphonates (as shown in Scheme 1) have also been established in several cellular extracts (Horiguchi and Kittredge, 1968) and non-enzymatic transamination in the presence of PLP has been established (Cassaigne et al., 1971). ^{13}C-NMR studies may someday help shed some light into the biochemical origin and function of 2-AEP.

It has been proposed by Rosenberg (1964) that the role for phosphonates in nature is to store inorganic phosphate. In addition, Rosenberg (1973) has also proposed that since these phosphonates are resistant to enzymatic attack, they may serve to provide environmental protection to these cells. Interestingly, it has been reported that 2-AEP uptake in Bacillus cereus is dependent on O_2 supply (Rosenberg and La Nauze, 1967).

Moreover, it has recently been established that synthetic aminoalkylphosphonic acids are capable of removing the ferric ion from transferrin (Harris, 1984). It is interesting to speculate that 2-AEP may be involved in iron uptake by and release from hemerythrin within the sipunculan erythrocyte. In addition, we have qualitatively observed that 2-AEP appears to increase the solubility of deoxyHr. Such a role for 2-AEP may help maintain high concentrations of hemerythrin within the sipunculan erythrocyte. This may in turn help an individual cell to carry a greater oxygen load.

ACKNOWLEDGEMENTS

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PART 2: EFFECTS OF PHOSPHORYLETHANOLAMINE AND 2-AMINOETHYLPHOSPHONATE

ON OXYGEN AFFINITY OF HEMERYTHRIN FROM Phascolopsis gouldii

Effects of phosphorylethanolamine and 2-aminoethylphosphonate

on oxygen affinity of hemerythrin from Phascolopsis gouldii

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INTRODUCTION

Analysis of $\underline{P}$. $\underline{gouldii}$ and $\underline{T}$. $\underline{zostericola}$ erythrocytes by ^{31}P-NMR
has revealed the presence of high levels of phosphorylethanolamine (PEA)
and 2-aminoethylphosphonate (2-AEP). Moreover, UV-visible spectroscopy
has shown that PEA is an effector of methemerythrin, and that in the
presence of PEA, the rate of azide binding to methemerythrin was found
to decrease by 85-fold. Several anions, amongst which perchlorate is
the most prevalent, have also been shown to be effectors of $\underline{P}$. $\underline{gouldii}$
hemerythrin (Garbett $\underline{et}$ $\underline{al}$., 1971; Stenkamp $\underline{et}$ $\underline{al}$., 1978; Bradic and
Wilkins, 1983). It has also been reported that perchlorate has some
effect on the oxygen affinity of hemerythrin (DePhillips, 1971). As a
result, it seems logical to investigate the possible role of PEA and
2-AEP on the hemerythrin-O_2 equilibrium.

The oxygen affinity of hemerythrin has been previously
investigated. Hemerythrins have P_{50} values on the order of 3-5 mm Hg
(DePhillips, 1971; Mangum and Kondon, 1975; Manwell, 1960a; Wells and
Dales, 1974; Richardson $\underline{et}$ $\underline{al}$., 1983). However, hemerythrin within
coelomic fluid or within erythrocytes are known to possess slightly
higher P_{50} values (Weber and Fange, 1980; Mangum and Kondon, 1975;
Manwell, 1960b). Generally, hemerythrins have much higher oxygen
affinities than hemoglobins which have P_{50} values on the order to
20-40 mm Hg and have Hill coefficients (n) on the order of 2.8
indicative of cooperative behavior (Rossi-Fanelli $\underline{et}$ $\underline{al}$., 1964; Ho,

1982; Antonini and Brunori, 1971; Lamy and Lamy, 1981). By contrast, hemerythrins have Hill coefficients on the order of 1.2-1.4 revealing little or no cooperativity (Bonaventura et al., 1977; Klotz et al.,1976; Garbett et al., 1969; Okamura and Klotz, 1973; Manwell, 1960a; DePhillips, 1971; Klippenstein, 1980; Kurtz et al., 1977; Wells and Dales, 1974; Weber and Fange, 1980). Some notable cooperativity (n=2) has been observed in Lingula reevii hemerythrin (Richardson et al., 1983).

The half-saturation point of many oxygen binding proteins is known to be affected by changes in pH values. This behavior has been termed as the Bohr effect. Bohr effects in hemoglobin and hemocyanin have been well established (Wyman, 1948 and 1964; Bonaventura et al., 1975). The hemerythrins are a diverse group in this respect since several coelomic hemerythrins have Bohr effects, whereas others do not. Amongst the group not affected by pH, the hemerythrin from P. gouldii is the most important member (Kubo, 1953 Florkin, 1933; Bonaventura et al., 1977; Okamura and Klotz, 1973; Manwell, 1960a,b; Klippenstein, 1980; Kurtz et al., 1977).

MATERIALS AND METHODS

Oxyhemerythrin was isolated using established procedures (Klotz et al., 1957; Nocek, 1986). The crystalline form of the protein was dissolved in 0.15 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH=7.2). This solution was then centrifuged in a clinical centrifuge for approximately 5 minutes to remove any denatured protein. Part of this resulting solution (pH=7.2) was used directly and the remaining was kept in liquid N_2 and thawed out as needed. Deoxyhemerythrin was prepared from 50 ml portions of oxyhemerythrin following the procedure outlined by Nocek (1986). The oxyhemerythrin was placed in dialysis bags (exclusion limit = 13,000) and dialyzed anaerobically for 12 hr against 1 l of a degassed 0.15 M HEPES solution (pH=7.2) containing 2 mM sodium dithionite (BDH Laboratories). To remove excess sodium dithionite following conversion to deoxyhemerythrin, the protein was dialyzed anaerobically against five 1 l portions of degassed 0.15 M HEPES (pH=7.2) for a total of 2.5 days. The concentration of deoxyhemerythrin used in this study ranged from 0.16 to 0.23 mM determined by measuring the absorbance of oxyhemerythrin at 500 nm (Nocek, 1986).

The prepared deoxyhemerythrin was then transferred anaerobically using a gas-tight syringe, a vacuum line, and argon gas to 2 dram vials which had been sealed with rubber septa and copper wire. These vials, each containing 4 ml of deoxyhemerythrin, were then filled with argon

and wrapped in parafilm and insulation tape. These vials were then kept in a vacuum desicator, under vacuum at 5°C, for a period of time not exceeding 5 days. In monitoring the effect of PEA and 2-AEP (Sigma) on the half-saturation point of hemerythrin, solutions of deoxyhemerythrin (2 ml) were equilibrated for at least 3 hours with 1 ml of 1M PEA or 1 ml of 1M 2-AEP, in a sealed 2 dram vial under argon. Each transfer of solution was made with 5 or 2 ml gas-tight syringes which had previously been flushed with argon. Final concentrations of PEA and 2-PEA were 0.33 M. This concentration of PEA was previously shown to give an 85-fold decrease in rate of N_3-binding to metHr.

The solution of deoxyhemerythrin to be examined (3 ml) was transferred to the tonometer as detailed in Appendix I. The filled tonometer was attached to a vacuum manifold and the lower chamber was placed under a vacuum initially while the upper chamber was filled with 100% oxygen at ambient pressure. The barometric pressure was recorded immediately following this step. This pressure was corrected by 0.5 torr to take into account the displacement in the Hg column. Oxygen aliquots were transferred from the upper chamber to the lower one by turning the partially bored stopcock, or in the end, by opening the tonometer sidearm (stopcock 2 in Figure A1 of Appendix 1). The volume of dissolved oxygen in solution was considered to be negligible (Keyes et al., 1967). The partial pressure of oxygen in the lower chamber could be calculated at any point from the number of turns of the partially bored stopcock and knowledge of the volumes of the bore in this stopcock, the lower chamber, and the amount of hemerythrin used.

The change in pressure in the upper chamber resulting from turning the partially bored stopcock 3 was considered to be negligible.

UV-visible spectra were collected with a dual beam Perkin-Elmer 554 spectrophotometer using 1 cm cells. The cell compartment was kept at 24°C. All measurements were made in triplicate at 24±1°C. The absorbance was also taken digitally at 500 nm. Following each turn of the stopcock, the protein solution was allowed to reach equilibrium with the oxygen aliquot. This equilibration time ranged from 5 to 60 minutes depending on the particular experiment. Thorough checking of proper equilibration time was ensured including waiting up to 3 hours to insure equilibration for the last data point. The pH of each solution was recorded following measurement using a Chentrix 61 pH meter calibrated with standard buffer at pH values of 7.00 and 10.04. Measurements of oxygen affinity were made at pH values of 7.1±0.1 for all hemerythrin solutions.

Sample calculation

$$P_u = P_A + P_{Hg} \qquad P_L = \frac{NV_pP_U}{V_L - V_S}$$

where: 'P_U' is the initial pressure of oxygen in the upper chamber

'P_A' is the atmospheric pressure

'P_{Hg}' is the amount of Hg that must be displaced by O_2

when flowing through the capillary column attached to the

vacuum manifold (0.5 mm Hg)

'P_L' is the partial pressure of oxygen in the lower chamber

'N' is the number of turns given to the partially bored stopcock

'V_P' is the volume of the partially bored stopcock (0.056 ml)

'V_L' is the volume of the lower chamber (46.0 ml)

'V_S' is the volume of solution placed in the lower chamber

    (3.0 ml)

Using the data for Figure 1:

$$P_U = 734.0 + 0.5 = 734.5 \text{ mm Hg}$$

$$P_L = \frac{N(0.056 \text{ ml})(734.5 \text{ mm Hg})}{(46.0 \text{ ml}) - (3.0 \text{ ml})} = N(0.957 \text{ mm Hg})$$

Thus, the partial pressure of oxygen in the lower chamber, P_L,

after each turn of the partially bored stopcock (stopcock 3 in

Appendix 1) can be calculated directly. Note that this procedure does

not take into account the change in the pressure of the upper chamber

with each turn of the partially bored stopcock. This results in a

maximal error of 1.3% in the pressure within the lower chamber after 15

turns. This error was considered to be negligible.

The absorbance at 500 nm was then plotted against P_L and the data

fitted to a single exponential using the program EXPSUM. The half-

saturation pressure of O_2, P_{50}, value could then be calculated directly

from the exponential obtained.

$$P_{50} = \frac{\ln 0.50}{EXP}$$

where: EXP is the value of the exponential curve used to fit the data.

For example, using the exponential obtained in fitting the data for

Figure 2:

$$P_{50} = \frac{\ln 0.50}{-0.168} = 4.12 \text{ torr}$$

RESULTS

The affect of oxygen addition to deoxyhemerythrin is shown in Figure 1. The addition of oxygen to deoxyhemerythrin resulted in an increased absorbance at 500 nm indicating the presence of rising amounts of oxyhemerythrin. These data were collected in triplicate and and each data set was fitted to a single exponential (see Figure 2). The average of these fits resulted in a half-saturation point (P_{50}) of 3.65±0.50 mm Hg. Representative UV-visible spectra illustrating the change in the absorbance of hemerythrin in the presence of 0.33 M 2-AEP and 0.33 M PEA and increasing amounts of O_2 are shown in Figures 3 and 4. These data were also collected in triplicate and each data set was fitted to a single exponential. Distortions in the spectra in the presence of 0.33 M 2-AEP and 0.33 M PEA as compared to the control could be explained by the fact that baseline corrections, which were performed air to air, did not account for some degree of absorbance by both PEA and 2-AEP in the visible range. As an example, the absorption spectrum of 0.33 M 2-AEP is shown as an inset in Figure 4. For deoxyhemerythrin in the presence of 0.33M PEA or 0.33M 2-AEP, average P_{50} values of 3.70±0.20 and 3.74±0.50 mm Hg, respectively, were obtained. These values were obtained with single exponentials as shown in Figures 5 and 6.

Figure 1. UV-visible spectra illustrating the conversion of
deoxyhemerythrin to oxyhemerythrin (0.19 mM) with increasing
partial pressures of oxygen. [From bottom to top, the
spectra represent equilibration with oxygen at 0, 0.96,
1.92, 2.83, 3.84, 4.80, 5.76, 6.72, 7.68, 8.64, 9.60, 14.40,
and 461.57 torr at pH=7.12 and 24°C.]
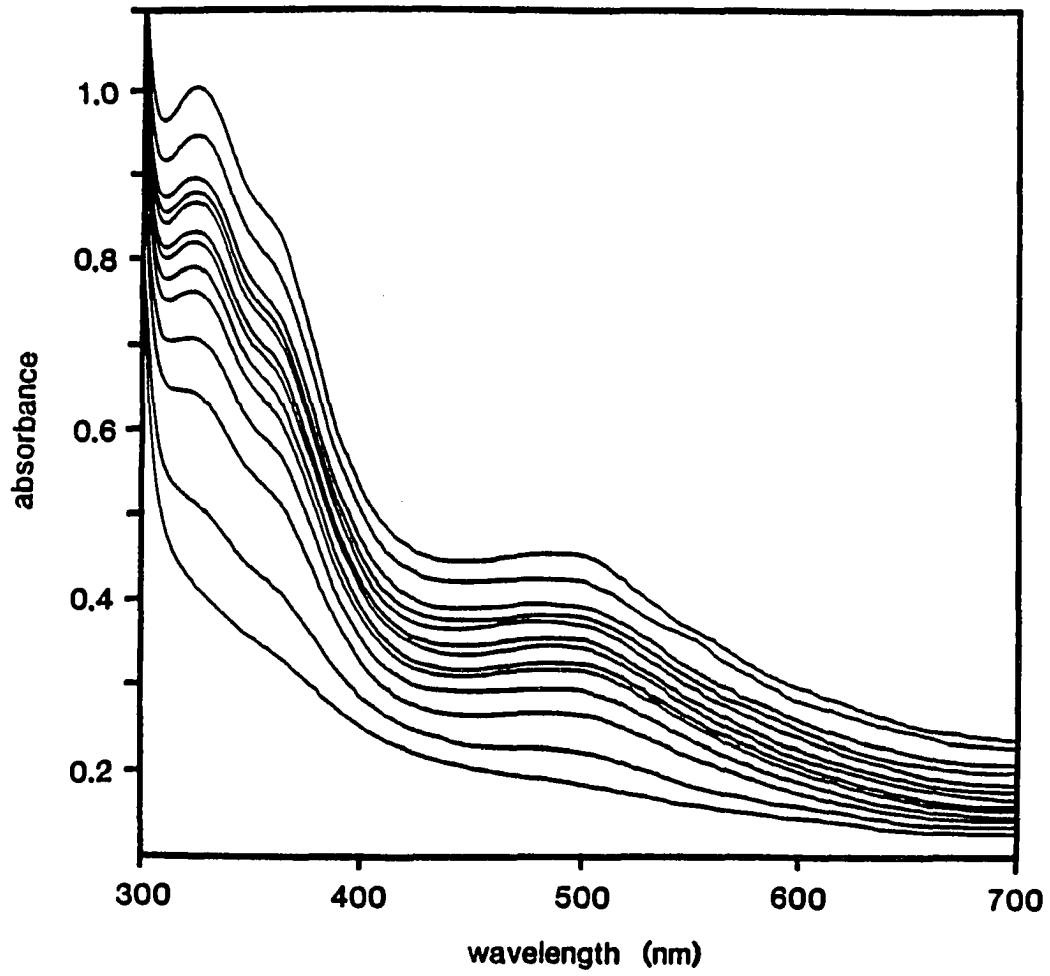
Figure 2. Plot of A_{500} versus partial pressures of oxygen for

data shown in Figure 1. [The region between 0 and 30 torr

has been expanded and is shown in the inset. These data

were fitted with a single exponential (see Appendix II).]
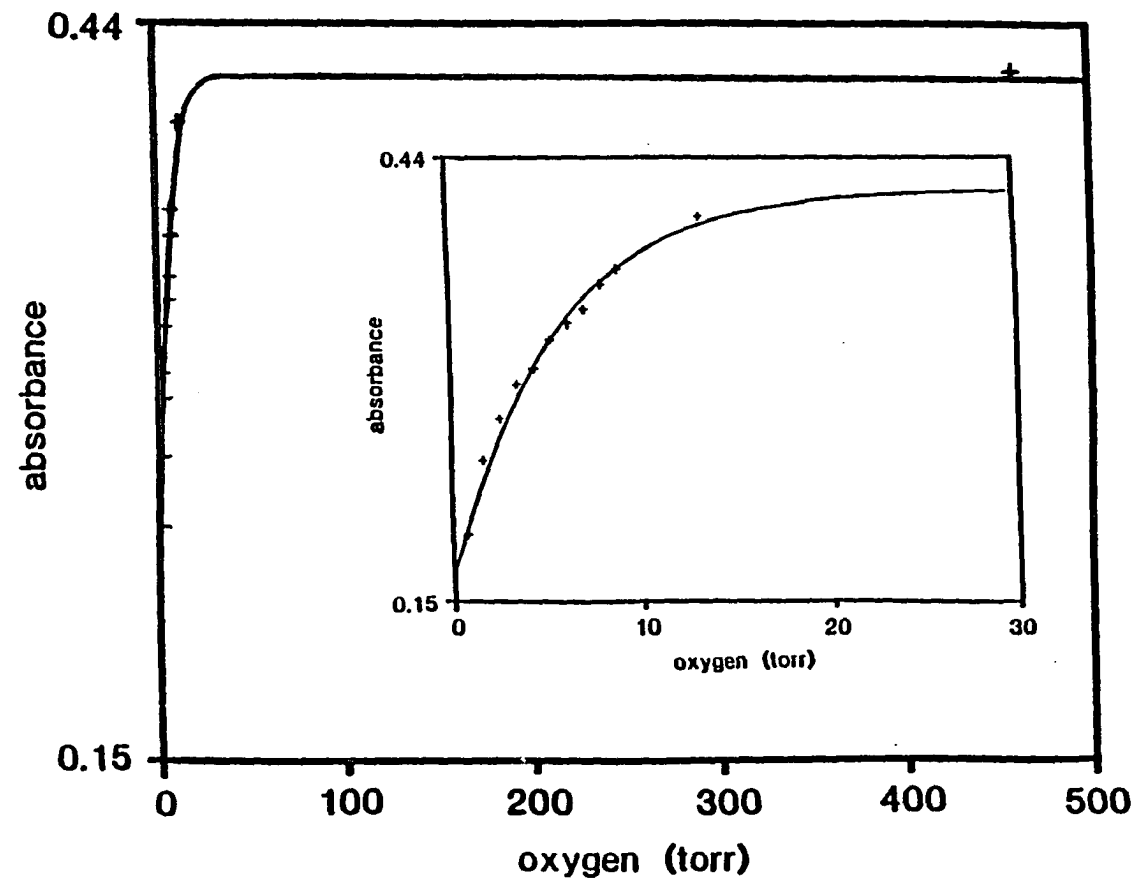
Figure 3. UV-visible spectra illustrating the conversion of deoxyhemerythrin to oxyhemerythrin (0.19 mM) in the presence of 0.33 M PEA with increasing partial pressures of oxygen. [From the bottom to the top, the spectra represent equilibration with oxygen at 0, 0.96, 1.92, 2.88, 3.84, 5.58, 7.67, 9.59, 14.39, and 461.20 torr at pH=7.04 and 24°C.]
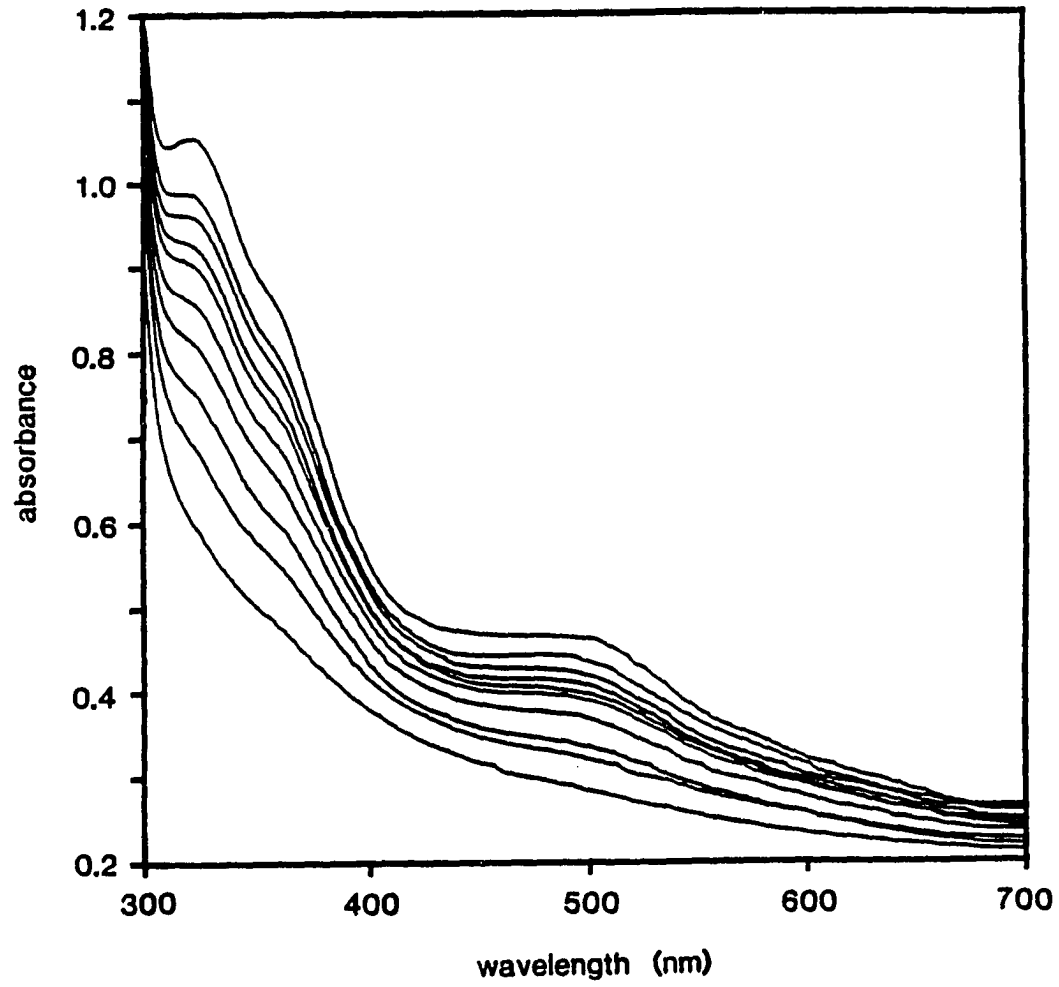
Figure 4. UV-visible spectra illustrating the conversion of

deoxyhemerythrin to oxyhemerythrin (0.19 mM) in the presence

of 0.33 M 2-AEP with increasing partial pressures of oxygen.

[From the bottom to the top, the spectra represent eqili-

bration with oxygen at 0, 0.97, 1.93, 2.90, 3.86, 5.79, 8.69,

11.59, 14.48, and 464.28 torr at pH=7.18 and 24°C.

The inset illustrates the absorption spectrum of 0.33 M 2-AEP

in this range.]
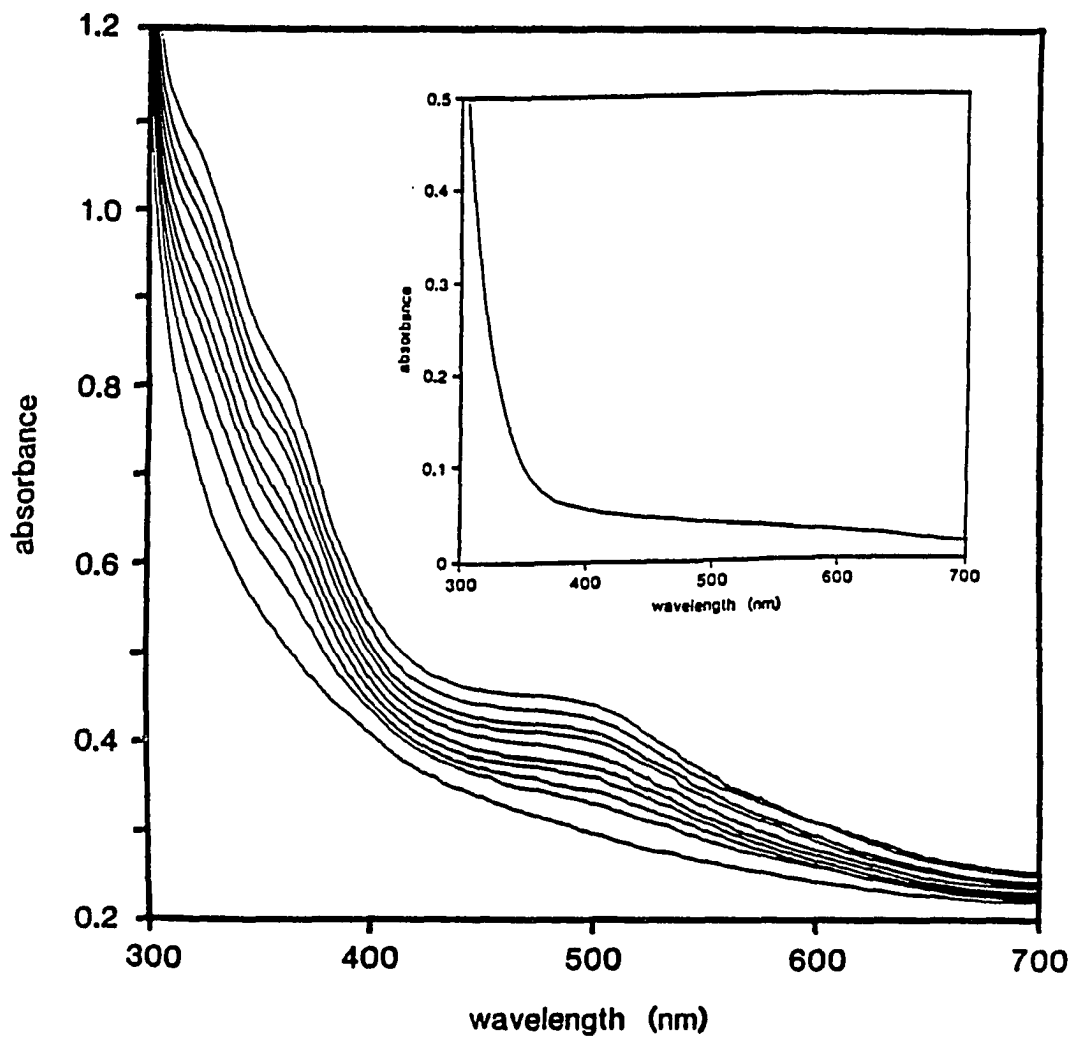
Figure 5. Plot of A_{500} versus partial pressures of oxygen for

data shown in Figure 3. [The region between 0 and 30 torr

has been expanded and is shown in the inset. These data

were fitted with a single exponential (see Appendix II).]
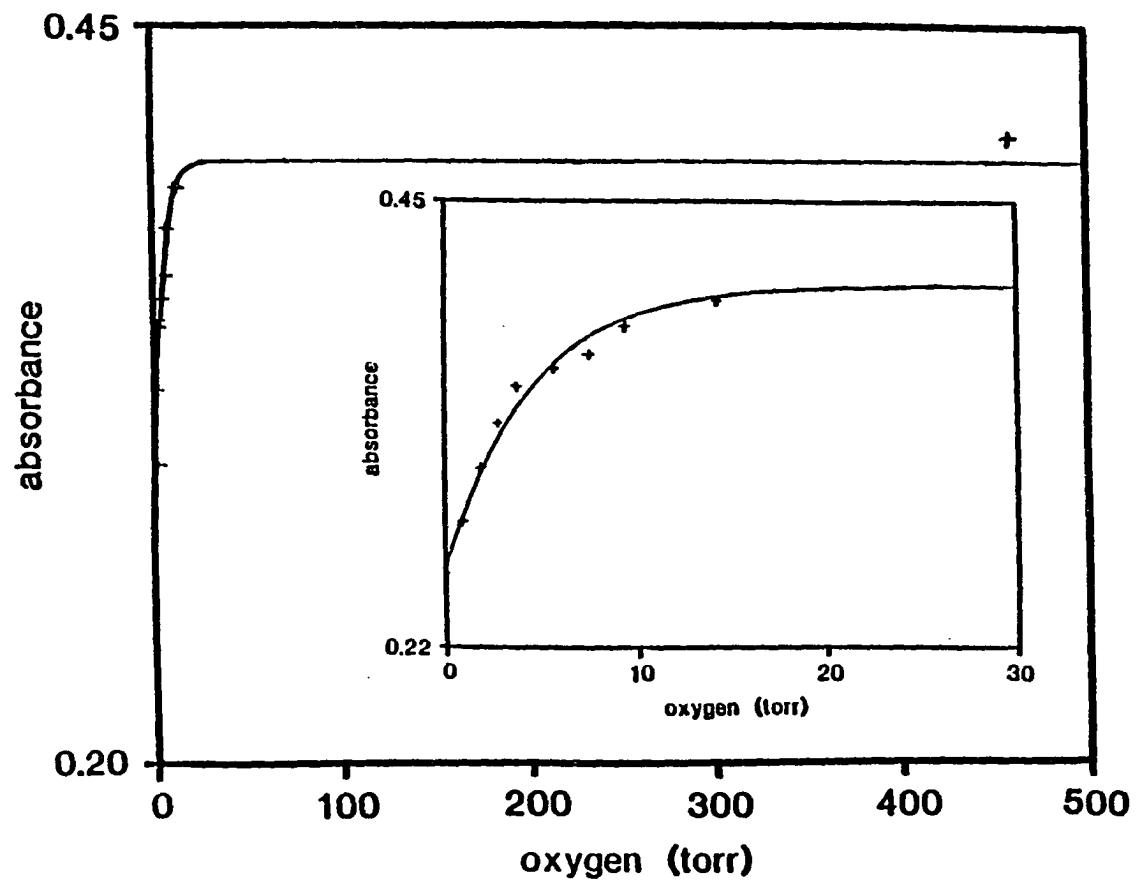
Figure 6. Plot of A_{500} versus partial pressure of oxygen for data

shown in Figure 4. [The region between 0 and 30 torr has

been expanded and is shown in the inset. These data were

fitted with a single exponential (see Appendix II).]
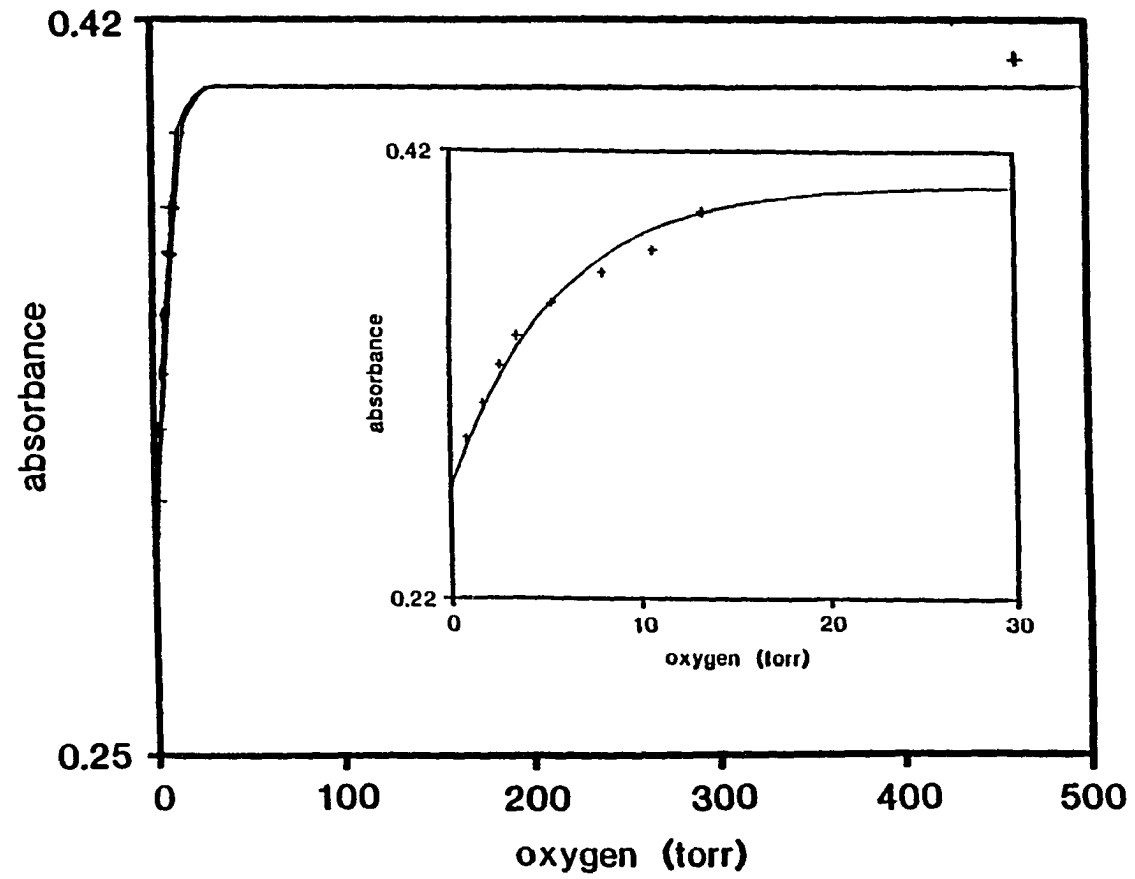
DISCUSSION

The binding of oxygen to $\underline{P}$. gouldii deoxyhemerythrin has been

extensively studied in the presence or absence of 2-AEP and PEA. This

investigation revealed a P_{50} value of 3.65 ± 0.50 mm Hg for $\underline{P}$. gouldii

hemerythrin in the absence of an effector. The error range corresponded

to the variability in the data. This variability resulted principally

from uncertainties in the amount of grease in the partially bored

stopcock and from fluctuations in the actual volume of solution

delivered using the gas tight syringe. P_{50} values of 3.74 ± 0.50 mm Hg

and 3.70 ± 0.2 mm Hg were obtained for hemerythrin when 0.33 M 2-AEP and

0.33 M PEA were present, respectively. Thus, within experimental

uncertainty, there is no detectable change in the P_{50} value of

hemerythrin in the presence of high concentrations (0.33 M) of either

2-AEP or PEA.

Moreover, no evidence for cooperative behavior for hemerythrin in

the presence or absence of PEA and 2-AEP was detected. It has been

reported that there appears to be a difference in P_{50} between

hemerythrin in coelomic cells versus purified hemerythrin (Weber and

Fange, 1980; Mangum and Kondon, 1975; Manwell, 1960b). As shown in this

study, however, this difference in P_{50} values, at least for $\underline{P}$. gouldii

hemerythrin, does not seem to be induced by the presence of either PEA

or 2-AEP. Thus, the role of PEA and 2-AEP in coelomic cells of

sipunculids may not be an alteration of the P_{50} value of hemerythrin.

Nonetheless, these two compounds may very well serve other functions in hemerythrin chemistry such as insertion of the iron into the protein backbone. In this light, it sould be pointed out that aminoalkylphosphonates were reported to he capable of removing the ferric ion from transferrin (Harris, 1984). We have qualitatively observed in this study that 2-AEP seems to enhance the solubility of deoxyhemerythrin. Therefore, another possible function for PEA and 2-AEP could be to increase the solubility of the hemerythrin octamer in sipunculan erythrocytes. In this respect, it should be noted that the concentration of hemerythrin within these cells approaches the 10 mM range (Utecht, 1986). Purified P. gouldii dexoyhemerythrin is not soluble to this extent. The presence of PEA and 2-AEP in sipunculan erythrocytes may help alleviate this problem.

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PART 3: PHOSPHORUS-31 NUCLEAR MAGNETIC RESONANCE STUDIES OF

SPERMATOZOA FROM THE BOAR, RAM, GOAT AND BULL

Phosphorus-31 nuclear magnetic resonance studies of

spermatozoa from the boar, ram, goat and bull

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ABSTRACT

Phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR) was utilized to examine semen and spermatozoa isolated from goats, rams, bulls, and boars. Untreated semen, washed spermatozoa, and extracts of washed spermatozoa were observed. In each of these species untreated semen was found to contain high but variable levels of glycerylphosphocholine (GPC), most of which could be removed by washing spermatozoa with extender or saline buffer.

Concentrated spermatozoa were found to contain resonances for inorganic phosphate, free nucleotide triphosphates, and phosphomonoesters which included both fructose-1,6-bisphosphate and glucose-6-phosphate. Also observed was a broad resonance from 30 to -25 ppm, part of which has been shown to originate from nucleotide tri- and diphosphates (NTP/NDP) determined by extraction of washed spermatozoa with cold 6% perchloric acid ($HClO_4$). No high energy molecules such as phosphoarginine (PA) or phosphocreatine (PCr) which could act as energy shuttles were observed.

From these results we have concluded that a phosphagen shuttle does not exist within the flagellum of mammalian spermatozoa, and that instead ATP is in rapid exchange between a bound state and a free state. In addition, resonances corresponding to the phosphate anhydride, nicotinamide dinucleotide (NAD^+/NADH), were not detected in the boar, the bull or the goat.

Intracellular pH values of 7.0, 7.0, 6.7, and 6.3 were calculated for the boar, the ram, the goat and the bull, respectively. Free magnesium concentrations were not reported since the acidic pH values obtained preclude such calculations.

INTRODUCTION

The use of ^{31}P-NMR has grown immensely in its application to living

systems since the first landmark studies of erythrocytes by Moon and

Richards (1973) and Henderson et al. (1974). The principal strength of

this technique centers upon its ability to measure non-invasively the

levels of phosphorus metabolites and the intracellular pH of cells (for

reviews see Hollis, 1980; Gillies et al., 1982; Barany and Glonek, 1982;

Iles et al., 1982; Shulman, 1983).

As with erythrocytes and other cell types, spermatozoa can be

readily studied with ^{31}P-NMR. Spermatozoa are particularly suited

because: 1) they are plentiful and high concentrations can be obtained

easily, 2) their functions are highly specialized (motility, acrosome

reaction, sperm-egg attachment) and can be studied separately, and

3) their environment can be controlled without disrupting the cells.

However, despite these advantages only a few ^{31}P-NMR studies have been

performed on semen or spermatozoa. For convenience, these studies are

separated into two groups depending on the presence or the absence of

phosphagens. In the former case, resonances for nucleotide

triphosphates (NTP), nucleotide diphosphates (NDP), inorganic phosphate

(P_i), phosphomonoesters, phosphodiesters, and phosphagens such as

phosphocreatine (PCr) or phosphoarginine (PA) were observed in

spermatozoa of two sea urchin species, Strongylocentrotus purpuratus

(Christen et al., 1983; Winkler et al., 1982) and Lytechinus pictus

(Johnson et al., 1983), the horseshoe crab, Limulus polyphemus (Robitaille et al., 1986); and in several fish species (Robitaille et al., see part 4). The presence of phosphagens is particularly important because of their proposed role in an energy shuttle system (Christen et al., 1983). Such roles have been supported by Tombes and Shapiro (1985) who described the presence of a phosphocreatine shuttle in sea urchin.

In the second group of spermatozoa, phosphagens are not presence and nucleotides are difficult to resolve. For example, in ^{31}P-NMR studies of rooster semen (Burt and Chalovich, 1978) and human semen (Arrata et al., 1978) resonances corresponding to NTP, NDP, PCr, or PA were not found. However, phosphorus resonances which correspond to phosphodiesters such as glycerolphosphocholine (human), phosphocholine, and serine ethanolamine (rooster) were identified. Preliminary studies in our laboratory focusing on porcine spermatozoa also did not reveal the presence of free NTP/NDP peaks. However, in a recent ^{31}P-NMR study of bovine and hamster epididymal spermatozoa, Smith et al. (1985) did established the presence of free NTP/NDP. Because of this discrepancy in observing NTP/NDP peaks and a presumed different system in regards to energy supply and demand we have performed ^{31}P-NMR analysis on the spermatozoa of four mammals, namely the boar, ram, buck and bull.

MATERIALS AND METHODS

Animals

<u>Boars</u>   The three cross-bred boars used in this study were two to five years of age, weighed 190-330 kg, and were known to be fertile.

<u>Rams</u> <u>and</u> <u>bucks</u>   The five rams used in this study were three to six years of age, weighed 90-200 kg and were routinely used in reproductive studies.

The five bucks (goats) used in this study were about 5-12 years of age, weighed 25-50 kg and were involved in breeding. The breeds of the rams and bucks used in this study were not known.

<u>Bulls</u>   The three bulls in this study were two to four years of age, weighed 700-1100 kg, and were known to be fertile. The bulls were Aberdeen Angus and Hereford breeds.

Seminal collection

Porcine semen was collected in a 1/2 liter thermos bottle using the gloved-hand technique. To remove the gelatinous portion, each ejaculate (100-400 ml; 5-10 x 10^8 spermatozoa/ml) was strained through a double layer of cheesecloth. Semen was collected during a 12 month period and usually at least one week apart. Bovine semen (5-10 ml; 5-15 x 10^8

spermatozoa/ml) was collected using an artificial vagina and an estrogenized cow. Semen was collected in a 15 ml plastic centrifuge tube (5-10 ml; 5-15 x 10^8 spermatozoa/ml) and then transferred to plastic bags. Samples were collected about once a week over a two month period so as many as five samples were obtained from each individual. Ovine and caprine semen (3-12 ml; 3-15 x 10^8 spermatozoa/ml) was collected by electroejaculation into 15 ml plastic centrifuge tubes. Semen was collected over a four month period.

Seminal preparation

Following collection, the seminal samples were either allowed to cool to room temperature (20-22°C) or placed on ice and then prepared for ^{31}P-NMR analysis in one or more of the following procedures: 1) whole semen, 2) seminal fluid, 3) unwashed spermatozoan pellet, 4) washed spermatozoan pellet, and 5) washed spermatozoan pellet that was frozen and thawed or 6) washed spermatozoan pellet that was extracted with 6% perchloric acid. Whenever possible motility was examined before and after each step of treatment. Each of these procedures was performed on porcine semen, whereas only procedures 1, 3 and 6 were performed with the ruminants. In each case semen was pooled for procedures 3 and 6.

<u>Whole semen</u>   A 2.5 ml aliquot of freshly collected semen was added to a 10 mm NMR tube and subjected to NMR analysis at 22°C.

<u>Seminal fluid</u>   A 3 ml aliquot of freshly collected semen was centrifuged at 2000 rpm in a Sorval RC2-B centrifuge (650 g) for 10 min at room temperature. The supernatant was removed, examined under the microscope for the presence of remaining spermatozoa, and subjected to ^{31}P-NMR analysis at 22°C.

<u>Unwashed spermatozoan pellet</u>   Twenty to 100 ml of freshly collected semen were centrifuged at 2000 rpm in a Sorval RC2-B centrifuge (650 g) for 10 min at room temperature or at 5°C. Approximately 2.5 ml (or 10 ml) of the spermatozoan pellet was transferred to a 10 mm (or 20 mm) NMR tube for ^{31}P-NMR analysis at 22°C or 5°C.

<u>Washed spermatozoan pellet</u>   An equal volume of Kiev extender (22°C) was added to the seminal sample. Kiev extender was prepared by dissolving the following compounds into 1 L of distilled water: 60 g glucose monohydrate, 3.75 g citric acid (trisodium salt), 1.20 g sodium bicarbonate, 3.70 g EDTA, 0.3 g sodium penicillin G (1670 IU/mg), and 1.00 g dihydrostreptomycin (Johnson <u>et</u> <u>al.</u>, 1982). A 200 ml aliquot of extended semen was centrifuged at 2000 rpm (650 g) in a Sorval RC2-B centrifuge for 10 min at room temperature. The resulting supernatant was discarded and the pellet was either used directly in ^{31}P-NMR analysis or resuspended and washed. In the latter case, the spermatozoan pellet was gently resuspended into 40 ml of Kiev extender (pH=7.2-7.3) or a saline solution (1.78 g HEPES, 4.06 g NaCl, 50 ml

ddH_2O, pH=7.3). The centrifugation procedure was repeated three times and each time the supernatant was discarded and replaced with fresh extender or saline solution except after the final centrifugation when the pellet was transferred to a 10 or 20 mm NMR tube and subjected to ^{31}P-NMR analysis at 22 or 5°C.

Frozen and thawed spermatozoa   Porcine spermatozoa were washed and concentrated as explained previously. The resulting pellet was frozen in liquid nitrogen (-196°C) and thawed before it was analyzed by ^{31}P-NMR at 5°C.

Extracted pellet   Freshly collected semen (10-100 ml) was washed and concentrated as described previously. The final pellet was cooled on ice and mixed with an equal volume of ice cold 6% $HClO_4$. The reaction was allowed to continue on ice for 15 min and then the extracted sample was centrifuged at 10,000 rpm (16,300 g) for 10 min. The supernatant was neutralized with K_2CO_3 (pH=8.7) and recentrifuged at 16,300 g for 10 min. The supernate was saved, frozen (-70°C), and lyophilized to a final volume of 2.5 ml. The lyophilized sample was then warmed to room temperature, and transferred to a 10 mm NMR tube for ^{31}P-NMR analysis at 22°C.

^{31}P-NMR collection

Spectra were collected using a Bruker WM300 spectrometer operating at 121.49 MHz. The prepared sample (2.5 ml) was placed in a 10 mm NMR tube. A capillary containing methylenediphosphonic acid in D_2O (δ=18.72) was calibrated against 85% phosphoric acid and used as an external standard. Accumulation temperature was maintained at 22°C or 5°C. Spectra were acquired with sample rotation. Collection parameters included a sweep width of 13888.9 Hz, a 40° (5 μs) to 78° (13.6 μs) pulse, and an acquisition time ranging from 0.22 to 0.59 s. The number of scans ranged from 200 to 30,000 with a resulting total length of accumulation ranging from 45 s to 10 hr. In using a 20 mm tube, the same general protocol was followed, however, the sample volume was increased to 10 ml, and 80 μs were required to generate a 78° pulse. Spectral collection was usually initiated 15-30 min after sample preparation. Baseline corrections were not employed in plotting the data. Sperm morphology and motility were examined after each ^{31}P-NMR analysis.

^{31}P-NMR area calibration

A 10 mM solution of glycerylphosphocholine (GPC) in Kiev extender was prepared and the pH was adjusted to 7.2. A 2.5 ml aliquot of this solution was then transferred to a 10 mm NMR tube and an external standard was inserted. The ^{31}P-NMR spectrum of the resulting sample was collected. A 2.5 ml aliquot of fresh semen was then added to a 10 mm NMR tube and the same external standard was inserted. The ^{31}P-NMR spectrum was collected using the same parameters as for the sample of 10 mM GPC. Peaks in each spectrum were integrated and the concentration of GPC in semen was then calculated.

Intracellular pH measurements

A standard curve was prepared by titrating a 5 mM solution of inorganic phosphate (P_i) using ^{31}P-NMR. This solution (100 ml) was prepared from 85% phosphoric acid and also contained 0.3 M glycine, 0.15 M potassium acetate, 40 mM NaCl, 20 mM HEPES, 10 mM EGTA, and 20 mM glycerol. This curve had limiting shifts of 0.72 and 3.18 for the second dissociable proton of inorganic phosphate, with a pKa of 6.63. The intracellular pH was obtained by noting the chemical shift for P_i in the NMR spectrum and then using the standard curve for obtaining an approximate pH measurement.

RESULTS

Figure 1A shows the ^{31}P-NMR spectrum of whole porcine semen. Besides the reference, the only visible peak is the glycerylphosphocholine (GPC, 0.49 ppm) and its concentration was calculated to range from 1 mM to 14 mM depending on the particular boar examined. The spectra of whole bovine, ovine and caprine semen was similar to that shown in Figure 1A. GPC was found to be located primarily in the seminal fluid and not within the spermatozoa. This is illustrated in Figure 1B which represents a ^{31}P-NMR spectrum of porcine seminal fluid following the removal of the spermatozoa by centrifugation. This study revealed that the concentration of GPC in the seminal fluid was on the order of that found in the whole seminal samples examined. GPC concentrations have also been monitored in semen from the bull, the ram and the buck. GPC levels of 2 to 35 mM and 4 to 25 mM were found in the bull and the ram respectively, and from 2 to 39 mM in the buck. The GPC levels in six bucks and three bulls were monitored for two to five weeks and these results have been summarized in Table 1. Reduced motility in caprine spermatozoa seemed to be correlated with decreasing levels of GPC in buck semen.

Figures 2A-C show the spectra of porcine semen diluted 1:1 with Kiev Extender, concentrated by centrifugation, and monitored for 1600 (30 min), 3100 (1 hr) and 36,000 scans (11 hr). These spectra have been amplified (see Figures 2E-G). The principal resonances in Figure 2

Figure 1. ^{31}P-NMR spectra of porcine semen and seminal fluid.

[A] ^{31}P-NMR spectrum of fresh porcine semen collected

at 22°C using a 78° pulse, receiver delay of

0.5 s, and 200 scans (15 min). Resonances correspond to

methylenediphosphonic acid, an external standard (16.08 ppm),

and glycerylphosphocholine (0.57 ppm). The concentration of

this metabolite was found to range from 1-14 mM. B) ^{31}P-NMR

spectrum of spermatozoa-free porcine seminal fluid.

Resonances are as shown in Figure 1A. This spectrum was

collected at 22°C using a 78° pulse, a 0.5 s receiver

delay, and 300 scans (20 min).]
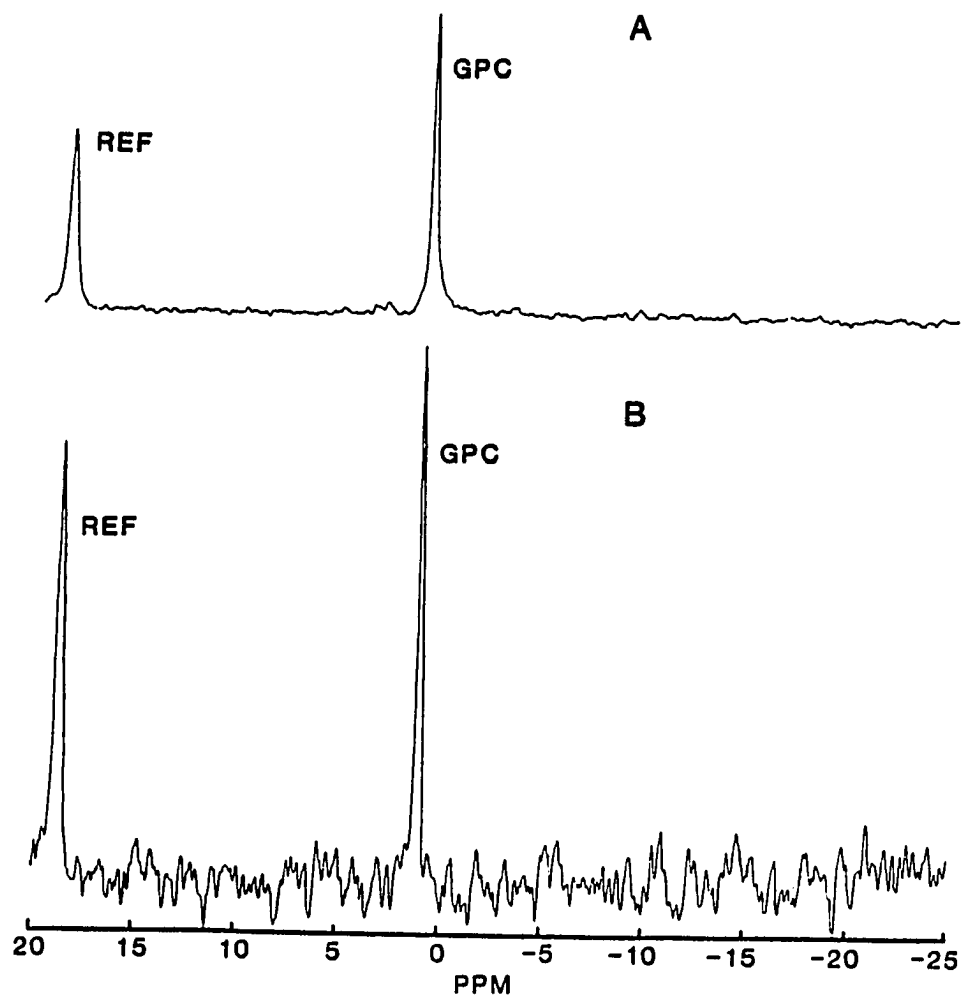
Table 1. The concentration of glycerylphosphocholine in whole
semen collected from individual goats and bulls over
a two to five week period

Species			Week		
Goats	1	2	3	4	5
1	15.6[a]	7.4	6.3	3.8	--
2	--	14.1	13.5	23.1	--
3	--	25.1	14.4	13.5	--
4	35.6	19.0	39.3	24.4	--
5	--	6.2	--	--	--
6	2.8	1.5	--	--	--
Bulls					
1	--	--	29.1	35.3	--
2	5.2	6.8	8.2	20.1	20.7
3	3.8	1.7	4.3	18.3	--

[a]All values are in mM.

Figure 2. ^{31}P-NMR spectra of concentrated porcine spermatozoa at

5°C after 0.4 (A), 0.8 (B) and 9.1 hr (C). [The spectra

have been amplified (10.7x) in the vertical direction as

shown in insets D, E, and F. Spectra were acquired with a

1.089 s cycling time.]
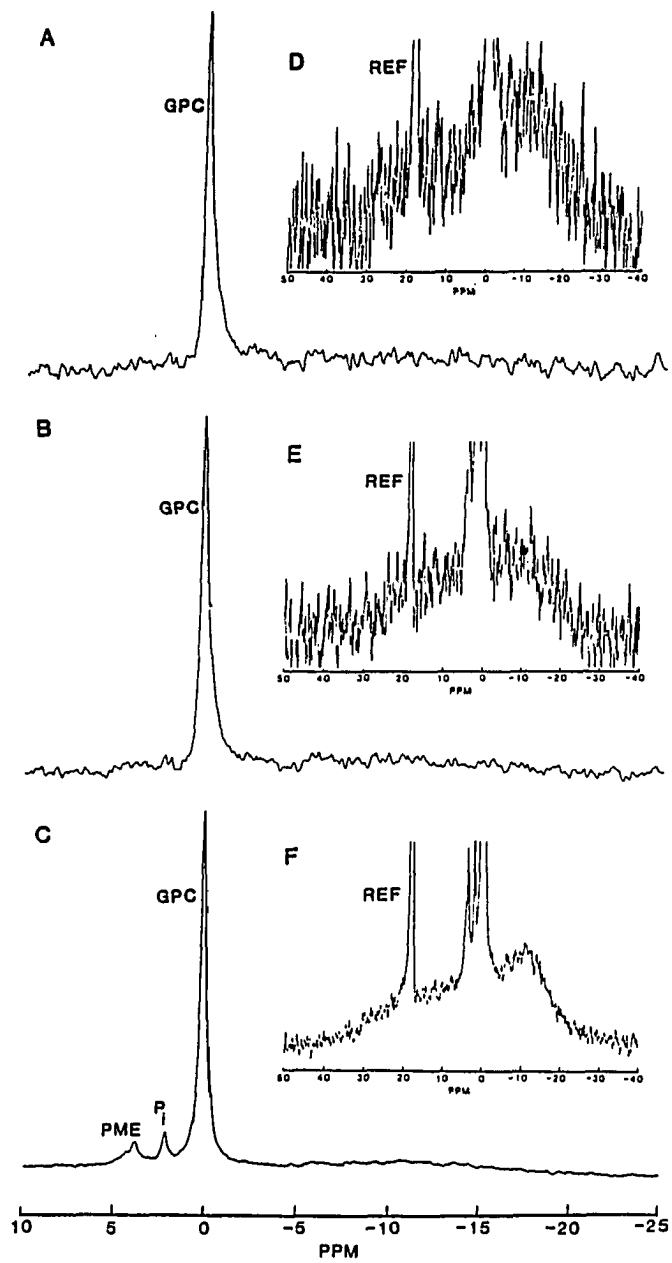
include phosphomonoesters (4.09 ppm), inorganic phosphate (P_i, 2.48 ppm), GPC (0.49 ppm), and a broad peak (30 to -25 ppm). With these procedures, free NTP/NDP peaks were not observed in the procine spermatozoa. From the chemical shift of P_i (2.48 ppm), the intracellular pH was estimated to be 7.0±0.2. Although the data are not shown, similar results were also obtained with shorter sampling times (5-15 min). The spectrum of untreated, concentrated porcine spermatozoa is similar to the one represented in Figure 2A with the exception that a higher GPC peak was obtained since the GPC has not been removed (spectrum not shown).

In order to determine the orgins of the broad peak, the three following procedures were performed. Firstly, concentrated porcine spermatozoa were isolated from whole semen and subjected to ^{31}P-NMR analysis with faster pulse repetition (3 pulses/s). This spectrum is shown in Figure 3A and required less than 10 min to acquire. In addition to the resonances reported previously, free NTP/NDP peaks were detectable at -5.03 and -10.30 ppm. In the second procedure porcine spermatozoa was frozen in liquid nitrogen and thawed prior to ^{31}P-NMR analysis (1 pulse/s). This result is shown in Figure 3B and has been amplified in Figure 3D. These spectra are similar to those shown in Figures 2A,B,E,F with the exception that the GPC peak is of lower intensity. In the third procedure, porcine spermatozoa were extracted with 6% $HClO_4$. Figure 3C thus reveals peaks corresponding to fructose-1,6-diphosphate, P_i, GPC, NTP, and NDP. These resonances were assigned by standard addition and by following changes in this spectrum

Figure 3. ^{31}P-NMR spectra of concentrated porcine spermatozoa.

[A] ^{31}P-NMR spectrum of concentrated porcine spermatozoa

at 22°C, acquired for 11 min using a 0.295 s cycling time.

B) ^{31}P-NMR spectrum of concentrated porcine spermatozoa frozen

in liquid nitrogen, thawed to room temperature and acquired

for 1.7 hr at 5°C. This spectra has been amplified

(10.7x) in the vertical direction as shown in inset D.

C) ^{31}P-NMR spectrum of porcine spermatozoa washed three times

with extender, extracted with 6% $HClO_4$, and neutralized with

K_2CO_3 to pH=8.7. Accumulation time was 7 hr.

Assignments correspond to inorganic phosphate (3.23 ppm),

fructose-1,6-bisphosphate (4.55 and 4.41 ppm), glyceryl-

phosphocholine (0.34 ppm), δ-NTP (-4.95 and -5.11 ppm),

β-NDP (-5.26 and -5.44 ppm), α-NDP (-9.61 and -9.72 ppm),

α-NTP (-10.06 and -10.21 ppm), and β-NTP (-21.10 ppm).]
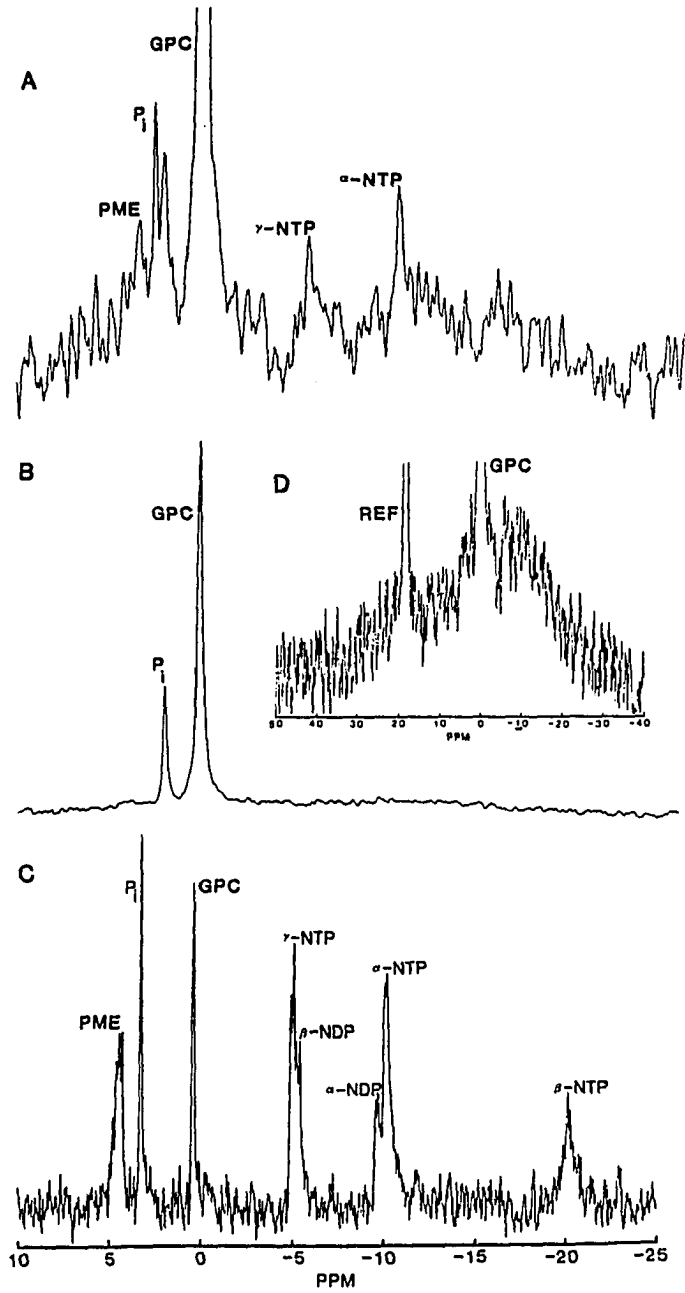
with pH changes. Interestingly, this procedure freed the NTP/NDP. Similar results were also obtained with spermatozoan extracts of ram and buck.

The ^{31}P-NMR spectra of an unwashed pellet of ovine spermatozoa acquired at 5°C using 500, 6,400, and 30,000 scans are shown in Figures 4A-C. Resonances have been shown to correspond to phosphomonoesters (4.42 and 3.93 ppm), P_i (2.46 to 2.36 ppm), GPC (0.49 ppm), and nicotinamide dinucleotides (NAD$^+$/NADH, -10.50 ppm). In addition, a broad resonance (30 to -25 ppm) was detected in each of these spectra. This can be seen in the amplified spectra (see Figures 4D-F). It should be noted that free NTP peaks were not detected in our studies with the ram. An important feature of this study with ovine spermatozoa is the presence of a resonance coresponding to NAD$^+$/NADH. A change in this spectrum with time has been detected, namely an increase in the intensity of the phosphomonoester resonance at 4.42 ppm and in the P_i. A 0.10 ppm change in the chemical shift of the latter was also noted resulting in a change in the calculated intracellular pH from 7.0 to 6.9 ±0.2.

The ^{31}P-NMR spectra acquired at 5°C of an unwashed pellet of caprine spermatozoa along with its amplification are shown in Figures 5A,B. In addition to the resonances corresponding to phosphomonoesters (3.67 ppm), P_i (2.11 ppm), GPC (0.49 ppm) and a broad peak from 30 to -25 ppm, free NDP/NTP peaks was detected at approximately 5.2 and 10.1 ppm. This spectrum was expanded (see Figure 5B). From the chemical shift of P_i an intracellular pH of

Figure 4. ^{31}P-NMR spectra of an ovine spermatozoan pellet acquired

for 0.13 (A), 1.6 (B) and 7.9 hr. (C) at 5°C. [These

spectra have been amplified (10.7x) in the vertical direction

as shown in insets D, E, and F, respectively. Similar

results were obtained at an accumulation temperature of

22°C.]
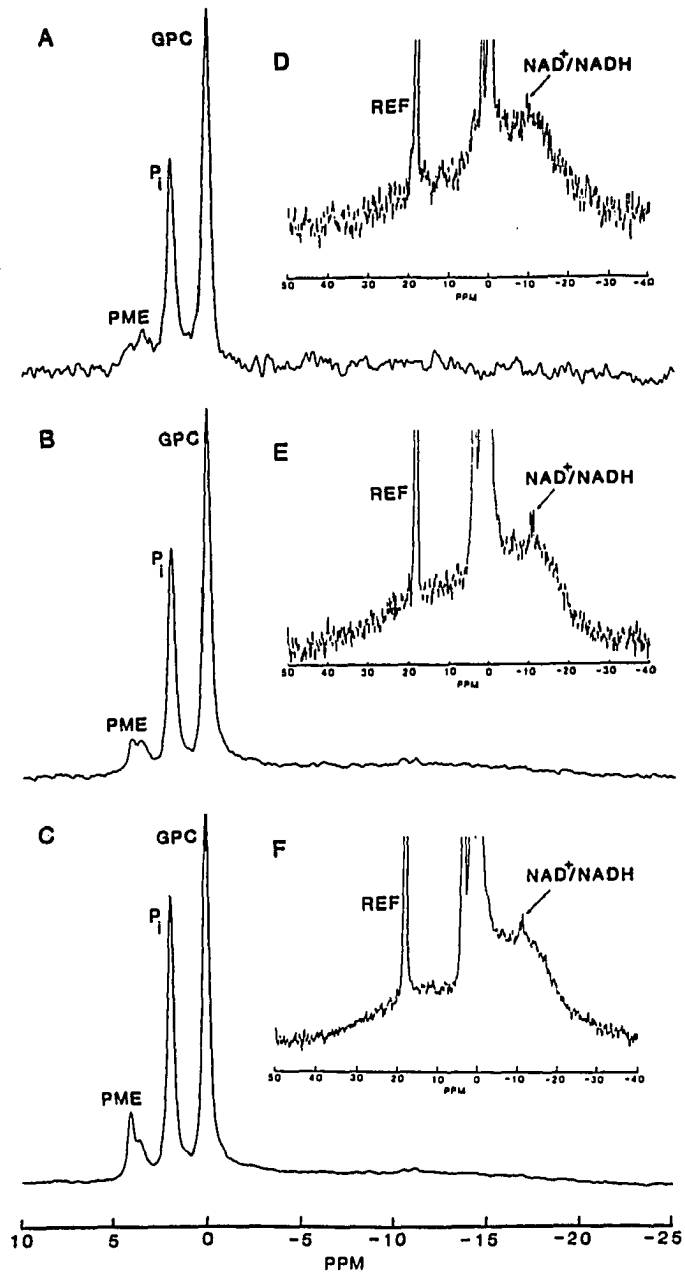
Figure 5. ^{31}P-NMR spectrum of a caprine spermatozoan pellet at

5°C acquired for 0.14 hr using a 1.089 s cycling time.

[This spectra has been amplified (7.1x) in the vertical

direction as shown in Figure 5B. Similar results were

obtained at an accumulation temperature of 22°C.]
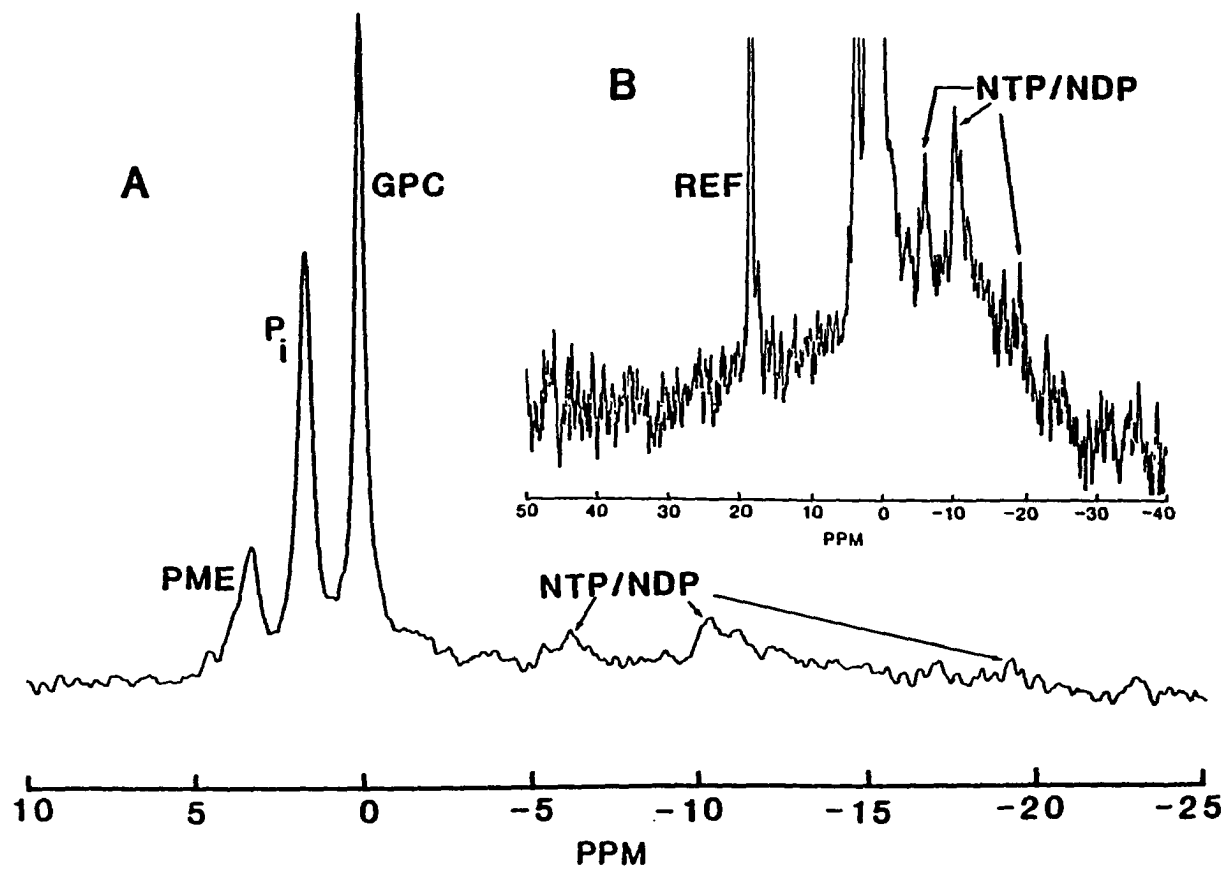
6.7±0.2 was calculated.

The ^{31}P-NMR spectra of unwashed pellets of bovine spermatozoa acquired using 128, 320, 1100, and 15,500 scans are shown in Figures 6A-D. These spectra were acquired at 5°C using an increasing number of scans, and were characterized by the presence of phosphomonoesters (3.54 ppm), P_i (1.51 ppm), GPC (0.49 ppm), γ-NTP/β-NDP (-5.30 ppm), α-NTP/NDP (-10.09 ppm), β-NTP (-18.99 ppm), and a broad resonance ranging from 30 to -25 ppm. From the inorganic phosphate chemical shift an intracellular pH value of 6.3 ±0.2 was calculated. These spectra have been amplified in Figures 6E-H. The presence of glucose-6-phosphate and fructose-1,6-biphosphate was established by extracting bovine spermatozoa through the use of a 6% $HClO_4$ extract (data not shown).

In general, the morphology and motility of spermatozoa appeared mormal after centrifugation and after other procedures at room temperature. However, reduced motility was observed at times in the boar, particularly after cold shock.

Free Mg^{++} concentrations, and the percentage of ATP complexed to Mg^{++} were not calculated in this study since the intracellular pH values we have obtained preclude such calculations.

Figure 6. ^{31}P-NMR specra of a bovine spermatozoa pellet acquired at

5°C using a 1.089 s cycling time for 0.03 (A), 0.08 (B),

0.26 (C), and 3.95 (D) hr. [These spectra have been amplified

(10.7x) in the vertical direction as shown in insets E, F,

G, and H, repectively. Similar results were obtained at an

accumulation temperature of 22°C.]
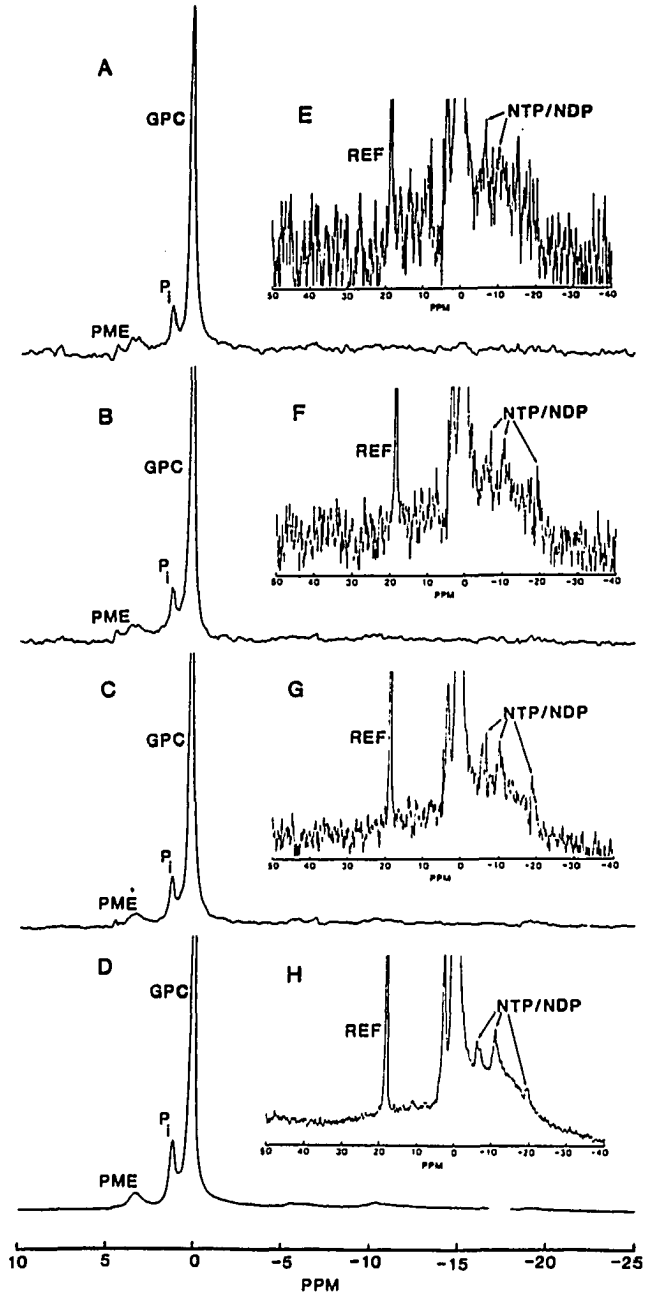
DISCUSSION

Mammalian semen has received little attention from NMR
spectroscopists. This is surprising in light of the broad scientific
interest which surrounds this tissue and the exceptional suitability of
spermatozoa for NMR analysis. Previous ^{31}P-NMR studies have involved
examination of human semen (Arrata et al., 1978) and the more recent
study by Smith et al. (1985) on bovine and hamster epidydimal
spermatozoan samples. The present ^{31}P-NMR study, compares spectra of
semen and spermatozoa from four mammalian species and emphasizes the
differences in the motility mechanisms between mammals and that found in
some lower animals, particularly the sea urchin, Strongylocentrotus
purpuratus, and the horseshoe crab, Limulus polyphemus.

In this study, porcine, ovine, caprine and bovine seminal samples
were found to contain high but variable concentrations of
glycerylphosphocholine (GPC) which was shown to exist primarily in the
seminal fluid (Figures 1A-B). In Table 1 the variation of GPC levels as
monitored in six bucks and three bulls respectively is reported. As a
rule in the buck, low GPC levels seemed to be correlated with poor
seminal quality. That is, a lower concentration of spermatozoa and less
than optimal motility. Indeed, studies with bucks 5 and 6 which had low
GPC levels were discontinued due to very poor seminal quality. In
addition, during the study period semen from buck 1 showed a progressive
decrease in seminal quality. This was also correlated with a decrease

in GPC concentrations. Such trends were not observed in the bull.

GPC has also been found in high levels in the semen of humans (Arrata et al., 1978; Frenkel et al., 1974) and other mammals (Dawson and Rowlands, 1959). Arrata et al. (1978) have proposed that the presence of high levels of GPC in human semen could be linked to higher levels of sperm motility and have suggested that GPC may act to protect spermatozoa from thermal changes or proteolysis. This idea is analogous to the role of glycerol in freezing spermatozoa and is indirectly supported by the observation that nearly 50% of the GPC may be in contact with the spermatozoan exterior within the seminal fluid (Frenkel et al., 1974). It has also been suggested that GPC may be important in spermatozoan maturation (Riar et al., 1973).

The spermatozoa of the four species examined in this study reveal very similar spectra, although some interesting differences are demonstrated. The principal features of the spectra for all spermatozoa are the resonances for phosphomonoester, inorganic phosphate (P_i), GPC, and the broad peak. Differences involve peak heights, intracellular pH, and the presence of free nucleotides. The series of spectra on ram (Figures 4A-F) reveal an increase in the phosphomonoester and the inorganic phosphate peaks with higher number of scans. It is likely that this increase results from the gradual change in the adenylate charge of the cell, resulting in the production of AMP and P_i as noted by increases in resonances at 4.42 ppm and 2.36 ppm and in the lowering of the pH by a 0.1 pH unit. Studies with caprine spermatozoa (Figures 5A-B) and bovine spermatozoa (Figures 6A-F) showed the presence

of phosphomonoesters, P_i, GPC, but more notably free nucleotide di- and triphosphates (NDP/NTP). It is of interest to note that in much more extensive studies the resonances for free nucleotides were not generally ovserved in the spermatozoa of the boar and the ram. However, by using faster pulsing techniques, we were able to establish the presence of free NTP peaks within procine spermatozoa (Figure 3A). The presence of free NDP/NTP resonances in bovine spermatozoa has been previously reported (Smith et al., 1985). This puzzling species difference may help to provide importnat clues regarding the energetics of motility in mammalian spermatozoa.

The nature of these differences between spermatozoa of various mammalian species may be explained by re-examining some of the published work. In addition to the dynein ATPases, other membrane-bound, ATP-dependent enzymes have been found in mammalian spermatozoa. At least two phosphatases have been found in guinea pig spermatozoa (Gordon et al., 1978). The presence of the Na^+/K^+ ATPase in the mid-piece tail region, has been well established in mammalian spermatozoa (Voglmayr et al., 1969a,b; Chulavatnatol and Yindepit, 1976; Gordon et al., 1978). Several enzymes including adenylate cyclase, guanylate cyclase, c-AMP dependent protein kinase are also known to be present (Johnson et al., 1985; Mann and Lutwak-Mann, 1981). As a result, it seems surprising that earlier ^{31}P-NMR studies on human semen (Arrata et al., 1978) and work on chicken semen (Burt and Chalovich, 1978) did not reveal the presence of ATP. This could be explained by noting that these research efforts minimized the spectral collection period and centered on the

examination of whole semen and not concentrated spermatozoa. As was found by Smith et al. (1985) with epididymal spermatozoa on isolated spermatozoa and by our group, we expect upon concentration of human or rooster spermatozoa and with the use of longer sampling times, free NTP/NDP peaks or a broad resonance corresponding to NTP/NDP may also be observed.

The most significant result of this study is the identification of the broad peak (Figures 2-6, 30 to -25 ppm) as principally originating from nucleotide di- and triphosphates (NDP/NTP). Initially, we unsuccessfully attempted to release these nucleotides by rupturing porcine spermatozoa through slow freezing (-70°C) and rapid thawing, or through sonification (data not shown). In addition, rapid freezing in liquid nitrogen (-196°C) followed by rapid thawing did not result in any noticable spectral changes (see Figure 3B). This indicates that the damage caused to porcine spermatozoa during cryopreservation does not seem to involve the release of bound ATP from the dynein arms within the flagellum. With 6% $HClO_4$ extraction we were able to demonstrate that the presence of NDP/NTP in porcine spermatozoa was indeed responsible for the majority of the broad resonance (see Figure 3C). A similar broad resonance has now been observed in this laboratory in a study of trout and walleye spermatozoa (Robitaille et al., see part 4). It should be noted that phosphodiesters within the plasma membrane may also contribute to this broad peak. Greater insight into the specific nature of this broad peak could probably be gained with solid state ^{31}P-NMR (Cholli et al., 1985).

The broadening of part of the ATP peaks detected in this study can be explained by considering the spin-spin relaxation process in the NMR experiment and the binding of these nucleotides to proteins within the spermatozoa. In collecting an NMR spectrum, the nuclei within the sample are subjected to a radiofrequency pulse. As a result, the nuclei under observation (in this case, ^{31}P) absorb energy and undergo a transition from a lower energy state to a higher energy state and are then permitted to relax back to the lower energy state. In these spermatozoa, the ^{31}P relaxation rate within ATP could be altered if some of the ATP were bound to ATPases located on the dynein arms of the flagellum. Because of this binding, several modes of relaxation available to the atoms of the bound ATP molecules would be altered. The ^{31}P-NMR signal which is observed for such bound ATP would then be modified, since the bound ATP molecules would no longer be able to tumble rapidly in solution. This situation normally leads to a decrease in the spin-spin relaxation time (T_2), resulting in line broadening of those resonances due to the bound ATP. The equation relating T_2 values to band width at half height ($w_{\frac{1}{2}}$) in units of hertz is as follows: $w_{\frac{1}{2}} = 1/(\pi T_2)$. Note that as T_2 decreases, $w_{\frac{1}{2}}$ increases. In contrast, the free ATP molecules exhibit "normal" relaxation and should result in distinct, sharp NTP peaks.

The presence of sharp NTP peaks in addition to a broadened NTP resonance in our spectra indicates that there are at least two NTP pools in mammalian spermatozoa in slow exchange with one another. We propose that one pool (pool A) is comprised of free NTP and that the other pool

(pool B) is comprised of bound NTP/NDP or free NTP and bound NTP/NDP in rapid exchange with one another. This free NTP pool (pool A) is believed to be morphologically isolated possibly in the spermatozoan head. Our inability to detect the presence of free NTP/NDP in ovine and porcine spermatozoa using normal sample acquisition is somewhat surprizing in light of the fact that using the same procedures their presence was easily detected in caprine and bovine spermatozoa. If this is not an experimental artifact it raises several important questions regarding energetics in mammalian spermatozoa. It would appear that the presence of NMR detectable free NTP may not be required for spermatozoan motility in mammals since both the porcine and ovine spermatozoa examined were motile. Indeed, the presence of a free NTP pool (pool A), if morphologically isolated, would not be expected to be required for motility. The absence of easily detectable free NTP peaks in porcine spermatozoa may be due to the inability of this cell type to maintain its free NTP levels (pool A) in anaerobic conditions. This is a logical assumption since mammalian spermatozoa are known to produce lactic acid as an end product of glycolysis and since it has been observed that porcine spermatozoa are known not to do well when subjected to anaerobic conditions (Aalbers et al., 1961; Nevo et al., 1970). In this respect, it is interesting that the two species which did not easily reveal the presence of free NTP peaks also displayed the highest intracellular pH values. Thus, we have also shown that the intracellular pH in procine, ovine, caprine and bovine spermatozoa where 7.0±0.2, 7.0±0.2, 6.7±0.2 and 6.3±0.2 respectively. The somewhat large margin of error cited is a

reflection of the nature of the standard curve used to determine intracellular pH.

A comparative approach to the study of mechanisms for motility between mammalian spermatozoa and some invertebrates is of particular interest when the energy transport systems are considered. The role of a phosphocreatine shuttle in spermatozoa is currently an area of scientific controversy. In sea urchin (Christen et al., 1983; Winkler et al., 1982; Johnson et al., 1983), as well as in the horseshoe crab (Robitaille et al., 1986), the trout and the walleye (Robitaille et al., see part 4), high energy phosphorus compounds which could act as energy shuttles have been detected by ^{31}P-NMR, namely phosphocreatine or phosphoarginine. It has been suggested for sea urchin spermatozoa that phosphocreatine could act as a shuttle mechanism transporting chemical energy from the mitochondria into the flagellum where high energy compounds are required for motility (Christen et al., 1983). The existence of a phosphocreatine shuttle has recently been given added credence in sea urchin (Tombes and Shapiro, 1985). In invertebrates and the lower vertebrates the presence of an energy shuttle may have a second function, i.e., to help direct energy usage within the spermatozoa. Since invertebrate spermatozoa are characterized by possessing relatively few mitochondria, the shuttle may serve to direct the energy through the tail when it is required for motility. Christen et al. (1983) have suggested that a phosphagen is present in mammalian spermatozoa and thus could provide a simple method for assaying spermatozoan function prior to artificial insemination. However, in

this study as well as that of Smith et al. (1985), no phosphagens were found at detectable levels in the spermatozoa (or in their perchloric acid extract) of five species of mammals indicating that a high energy shuttle in the form of phosphocreatine is not likely to be present in mammalian sperm. This conclusion is also supported by chemical analysis (Brooks, 1970).

In seeking to understand the absence of a phosphagen in this study, and consequently the likely absence of a phosphagen shuttle, one may gain insight by noting the morphological differences between selected invertebrate and mammalian spermatozoa. Many of the so-called "typical" invertebrate spermatozoa (Afzelius, 1972) possess only a few mitochondria and these are located in the spermatozoan head at the proximal end of the flagellum. Consequently, these spermatozoa may have to depend on some kind of mechanism to efficiently transport high energy molecules toward the distal end of the flagellum. In addition, motility in such invertebrate spermatozoa is usually characterized by short duration time. In contrast to the invertebrates, mammalian spermatozoa are characterized by prolonged motility and a mitochondrial sheath which surrounds the middle piece, a significant length of the tail. As a result, in mammalian spermatozoa, a tremendous amount of ATP necessary for flagellar motion is being synthesized in large quantity in close proximity to a major portion of the flagellum. This proximity may explain the absence of a phosphoarginine or phosphocreatine shuttle system. Thus, simple diffusion of mitochondrial ATP along the flagellum may be adequate for maintaining the energy demands of the dynein ATPase.

This hypothesis is supported by several theoretical papers (Adam and Wei, 1975; Nevo and Rikmenspoel, 1970; Brokaw, 1975; Brokaw and Benedict, 1968). Maximal energy output could be obtained for a particular diffusional event in this model resulting in maximal energetical economy by invoking the action of adenylate kinase. With the function of each ATPase, one ATP molecule is cleaved and one ADP and one P_i are produced. It may be possible that the ADP produced could then be used to phosphorylate a second ADP through the action of adenylate kinase according to the reaction 2ADP $\rightarrow$ ATP + AMP. The resulting AMP could then diffuse to the mitochondrial sheath to be rephosphorylated to ATP. Such a mechanism would optimize the diffusional event. More weight must be given to the diffusional model in light of the fact that neither phosphocreatine nor phosphoarginine were found in the mammalian spermatozoa examined by our group and by Smith et al. (1985).

Smith et al. (1985) have recently invoked the presence of acetylcarnitine in mammalian spermatozoa to replace the energy storage function of phosphocreatine found in spermatozoa of lower animals. This is a logical assumption. However, it should be clarified that acetylcarnitine acts in storing substrates prior to their utilization for energy production in the mitochondria. Phosphocreatine is already a high energy compound. This is an important distinction.

Another important feature of this study is the frequent lack of a resonance corresponding to free nicotinamide dinucleotides. This peak was expected at approximately 10.50 ppm in the spermatozoan suspensions of four species examined in this study. However, a resonance corresponding to free NAD^+/NADH was detected only in the case of the ram. This is an unexpected result because this resonance is readily identifiable in the ^{31}P-NMR spectrum of Limulus spermatozoa (Robitaille et al., 1986) and a large variety of other cell types (Navon et al., 1977; Henderson et al., 1974; Ugurbil et al., 1982; Shulman, 1983). Since this resonance has only been observed in the ram, this difference may perhaps be accounted for by the varying levels of NAD^+/NADH in mammalian spermatozoa. The concentration of NAD^+/NADH in boar has been estimated at 9 nmole/10^9 spermatozoa and is lower than the concentration of NAD^+/NADH in spermatozoa of the bull (12.7 nmole), ram (24.6 nmole), and stallion (16.8 nmole) (Brooks, 1970; Brooks and Mann, 1972). Since NAD^+/NADH is known to be present in mammalian spermatozoa, the determination of its concentration by ^{31}P-NMR would be significant. The lower concentration of NAD^+/NADH in porcine spermatozoa is also accompanied by the absence of the NAD^+/NADH dependent enzyme, sorbitol dehydrogenase (Mann and Lutwak-Mann, 1981). The low concentration of NAD^+/NADH may be responsible for the inability to detect these compounds in whole and extracted mammalian spermatozoa by ^{31}P-NMR as a result of masking of this resonance by the much larger and significant NTP/NDP resonance. This should be especially true for the bull and the buck.

The exact means of energy transport through the mammalian flagellum is an interesting problem, which will require a considerable amount of further research. The trout sperm may prove to be the ideal subject of such investigations, since its ^{31}P-NMR spectrum reveals the presence of phosphocreatine, bound NTP and free NTP. Preliminary NMR studies on mammalian spermatozoa have opened several important scientific avenues. Future ^{31}P-NMR and ^{13}C-NMR studies should provide greater insight into questions surrounding the energetics of motility in this cell type.

ACKNOWLEDGEMENTS

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PART 4: ^{31}P-NMR STUDIES OF TROUT SPERMATOZOA AT REST, AFTER

MOTILITY, AND DURING SHORT-TERM STORAGE

^{31}P-NMR study of trout spermatozoa at rest,

after motility, and during short-term storage

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ABSTRACT

^{31}P-NMR was utilized to study respiration in spermatozoa isolated from the rainbow trout. The spectrum obtained under aerobic conditions revealed the presence of phosphomonoesters, intracellular inorganic phosphate, phosphodiesters, phosphocreatine, and free nucleotide triphosphates. The anaerobic spectrum was characterized by an increase in the inorganic phosphate resonance and by the disappearance of the phosphagen and free nucleotide triphosphate peaks. Upon re-aeration, the aerobic spectrum was once again observed.

Initiation of motility using fish ringer or fresh water resulted in a decrease in phosphocreatine and nucleotide triphosphate levels along with an increase in inorganic phosphate. The presence or absence of a phosphagen peak seemed to be correlated with motility. Preliminary studies on short-term storage of spermatozoa were also performed. These findings are analyzed in terms of the phosphocreatine shuttle and its role in the maintenance of motility in the spermatozoa of fish and some invertebrates.

INTRODUCTION

With the present state of knowledge, the energetics associated with motility in spermatozoa can best be described in terms of two separate hypotheses, represented by two groups of organisms. The first group is thought to possess spermatozoa which utilize an energy shuttle system, whereby a phosphagen, phosphoarginine (PA) or phosphocreatine (PCr), acts as a high energy carrier and directs chemical energy towards the distal end of the flagellum. This situation parallels that proposed in muscle and heart tissues (Bessmann and Geiger, 1981; Meyer et al., 1984). The second group of organisms is thought to possess spermatozoa which utilize simple diffusion of ATP from the mitochondria to the flagellum (Mann and Lutwak-Mann, 1981). In this model any phosphagen present would only serve in energy storage. The latter hypothesis is presently represented by mammalian spermatozoa, whereas the former, if present, is represented by the spermatozoa of several invertebrates.

Tombes and Shapiro (1985) have examined the existence of a shuttle mechanism in the spermatozoa of sea urchins and have demonstrated the presence of two creatine kinase isoenzymes which are involved in the phosphorylation of dinucleotides and creatine respectively. The production of phosphocreatine (PCr) is therefore a key feature in the maintenance of motility since this metabolite is important in energy transfer or storage (Tombes et al., 1984). As a result, the concentration of PCr is relevant to the functional state of the

spermatozoa. In supporting studies utilizing phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR), this importance of PCr for motility has been demonstrated in the spermatozoa of sea urchins (Christen et al., 1983a,b; Winkler et al., 1982; Johnson et al., 1983). In a more recent study using spermatozoa of Limulus polyphemus, similar results were obtained with the exception that the phosphagen was phosphoarginine (PA) (Robitaille et al., 1986a). Besides the phosphagens, all of these ^{31}P-NMR studies identified resonances corresponding to nucleotide triphosphates (NTP; principly ATP), nucleotide diphosphates (NDP; principally ADP), inorganic phosphate (P_i), phosphomonoesters (PME), and phosphodiesters (PDE). It should be noted that the baselines of the ^{31}P-NMR spectra collected in these studies were generally flat, an observation, as discussed in the next paragraph, represents one of the major differences between the two groups of spermatozoa mentioned above.

^{31}P-NMR studies of mammalian spermatozoa have been limited to only a few investigations (Smith et al., 1985; Robitaille et al., 1986b). In these studies, resonances observed in studies on invertebrate spermatozoa have also been demonstrated with the exception of phosphagens which are either at low levels or absent in mammalian spermatozoa. For example, low levels of PCr in bovine spermatozoa have been supported by chemical analysis (Brooks, 1970). As reported for sea urchin spermatozoa, two creatine kinase isoenzymes have also been found in human and chicken spermatozoa (Wallimann et al., 1984, 1986). However, along with these enzymes, adenylate kinase has also been

reported in the same species (Wallimann et al., 1986). Such low levels of PCr and high levels of adenylate kinase make the shuttle hypothesis an unlikely mechanism for sperm motility in the mammals, even if the presence of a possible PCr shuttle enzymatic system has been demonstrated. In light of this, in our ^{31}P-NMR studies on the spermatozoa of boar, bull, ram, and goat, we have described a broad resonance not found in the examined invertebrate spermatozoa (Robitaille et al., 1986b). The presence of this broad resonance has been explained by invoking an exchange between two distinct ATP pools, one of which is the bound ATP located on the ATPases of the dynein arms within the flagellum and the other of which is comprised of free nucleotides. The high numbers of mitochondria and the diffusion of ATP in these spermatozoa thus provides sufficient energy to support continuous motility. Such a hypothesis helps to explain in mammalian spermatozoa the mechanisms involved in motility and the reason for the absence of a phosphagen resonance (Robitaille et al., 1986b).

Rainbow trout spermatozoa are excellent material for ^{31}P-NMR analysis particularly since they are not motile upon collection and can be rendered motile by the addition of fresh water or fish ringer, an event which provides a well-defined metabolic change for analysis by ^{31}P-NMR. Previous studies on sperm metabolism and high energy phosphorus compounds of trout spermatozoa are limited even though numerous investigations of short-term storage and cryopreservation have been performed (Scott and Baynes, 1980). Actual measurements of ATP and cAMP on trout spermatozoa were performed by Benau and Terner (1980)

during a seasonal period and before and after motility. A major

contribution has been the identification of cAMP as an intracellular

initiator of motility in trout spermatozoa (Benau and Terner, 1980).

This work has also been supported by Morisawa and Okuno (1982). To our

knowledge phosphocreatine has not yet been reported in fish spermatozoa.

Other metabolic studies have involved oxidative and biosynthetic

reactions of glucose, pyruvate, acetate (Terner, 1962; Mounib, 1967;

Terner and Korsh, 1963a), and lipids (Terner and Korsh, 1963b; Minassian

and Terner, 1966).

One of the objectives of the present paper is to demonstrate a

system which seems to incorporate some features of both groups as

described above. Specifically, the high energy phosphorus compounds of

trout spermatozoa represent an intermediate between the two groups, in

that a phosphagen is present along with two distinct pools of ATP. As a

result, this system presents a greater challenge for interpreting the

mechanism of motility.

Besides examining metabolic profiles the activation of motility in

fish spermatozoa has stimulated considerable interest. The effect of

metal ions on motility has been extensively studied in salmonid

spermatozoa. Potassium ions are known to inhibit motility when added in

concentrations higher than 5-10 mM (Scheuring, 1925; Schlenk and

Kahmann, 1938; Kusa, 1950; Stoss et al., 1977; Baynes et al., 1981;

Benau and Terner, 1980). Moreover, it is known that Na^+ or the divalent

cations, Ca^{++} and Mg^{++}, reduce the inhibitory effect of K^+ (Scheuring,

1925; Schlenk and Kahmann, 1938; Stoss et al., 1977; Baynes et al.,

1981). The relationship of the activation of rainbow trout spermatozoa with pH has also been studied (Baynes et al., 1981; Schlenk, 1933). In general, motility and fertility levels are enhanced by pH values similar to or more alkaline than the pH of seminal fluid (Petit et al., 1973; Billard et al., 1974; Billard, 1981).

The specific objectives of this study are: 1) to implement ^{31}P-NMR to evaluate mechanisms governing sperm metabolism and motility, 2) to examine sperm metabolism during short-term storage, and 3) to compare the ^{31}P-NMR analysis of trout semen to that of other species. Specifically, relative changes in the levels of phosphorus containing compounds were determined before and after motility, as a result of anaerobic conditions, and during short-term storage.

MATERIALS AND METHODS

Animals

Adult male specimens of rainbow trout, _Salmo gairdneri_, were obtained from the Manchester Fish Hatchery in Decorah, Iowa. They were either stripped at the hatchery or shipped via aquarium tank trucks and stripped immediately upon arrival. In both cases, the milt (semen) was immediately cooled and transported to our laboratory. Approximately 60-70 animals were used for collection during a period of 9 months and included Fall breeders (August - December, 1985) and winter breeders (January - April, 1986).

Gamete collection

Male trout were anesthesized with tricane methanesulfonate (MS 222) in order to control the collection of semen. Semen was expressed out by gentle pressure on the abdomen and collected in plastic bags which were stored on ice (approximately 1°C) until used. Samples in bags were stored with large volumes of air or oxygen. Approximately 5-20 ml of whole semen ($5-16 \times 10^9$ spermatozoa/ml) were obtained from each male. Some samples were stored as long as 3-4 weeks at 1°C to 4°C and examined periodically.

Motility or activation

At collection, trout spermatozoa were non-motile. By diluting with fresh water (dechlorinated tap water) or fish ringer (50:1:1; 0.133 M NaCl:0.133 M KCl:0.099 M CaCl$_2$), motility was stimulated and lasted for approximately 30 s. Generally, motility was tested by placing a very small drop of semen on a coverslip and adding a drop of the diluent (fresh water or ringer), at an approximate 10-20 fold dilution. Usually, a ten-fold dilution was adequate to cause 100% motility. Although not exactly measured, sperm quality was rated 100%, >50%, 10-50%, 1-10%, or 0% motility.

Sperm sample preparation

Spermatozoa were collected as whole semen and used directly or were subjected to one or more of the following treatments: 1) washed and concentrated, 2) made anaerobic, 3) activated and reactivated, and/or 4) extracted with 6% perchloric acid. Sperm samples treated in step 1 were usually aerated and maintained on ice prior to observation or NMR analysis. Following analysis, all samples were examined at room temperature and checked for quality of motility.

<u>Washed</u> <u>and</u> <u>concentrated spermatozoa</u>   As much as 5-6 ml of whole

semen was centrifuged at 2000 rpm in a Sorval RC2-B centrifuge (650 g)

for 10 min at 4°C. The seminal fluid was used directly for ^{31}P-NMR

analysis. The sperm pellet was also analyzed directly.

<u>Anaerobic</u> <u>conditions</u>   After ^{31}P-NMR analysis of 3 ml of freshly

collected semen, the sample was warmed to 10°C or higher and was placed

in a plastic bag which was sealed in such a manner to exclude all air.

Alternatively, nitrogen gas was bubbled through the semen at room

temperature. After such treatment for 1 hr or more, samples were cooled

to 1°C and analyzed. By replacing the same samples into plastic bags,

spermatozoa could then be easily re-aerated by opening the bag and

flushing several times with air over a period of 15-60 min. These re-

aerated samples were then cooled to 1°C and re-analyzed.

<u>Activated</u> <u>and</u> <u>reactivated spermatozoa</u>   Approximately 3 ml of

semen were placed in each of five 100 ml beakers in a waterbath at 13°C.

Then, 27 ml of fish ringer were added to each of two beakers and 27 ml

of fresh water were added to each of two other beakers. These were

rapidly mixed with the semen in each beaker. The semen in the fifth

beaker was untreated. The ratio of diluent to semen was 9:1. This was

the lowest ratio at which 100% sperm motility was consistently

demonstrated. The sperm suspension was immediately examined for

motility and then allowed to sit with some agitation for 10 min. After

this period, sperm suspensions were centrifuged at 2000 rpm for 10 min

at 0-1°C. The pellets were resuspended at the same temperature to 3 ml

of the same solution in which they were initially suspended (fish ringer

or fresh water), transferred to NMR tubes, and analyzed as soon as possible (usually within 1 hr). The control or 3 ml of untreated whole semen passed through the above procedure except was not diluted. This experiment was repeated three times with similar results.

Reactivation of spermatozoa was performed by centrifuging previously activated spermatozoa and resuspending the pellet into seminal fluids. After 15-30 min, such treated spermatozoa could be activated for another period of motility.

Perchloric acid extract of spermatozoa   Approximately 0.8 ml of whole semen was diluted to 12 ml with 0.13 M KCl in a 15 ml centrifuge tube. This diluted semen was concentrated by gentle centrifugation at 5°C. The supernate was discarded and the centrifuge tube containing the sperm pellet was placed on ice. Five hundred μl of ice cold 6% $HClO_4$ were mixed with the pellet. The lysing reaction was allowed to continue for 15 min and the resulting mixture was then centrifuged. The pellet was discarded and the supernate was neutralized by the addition of K_2CO_3 crystals. The supernate was then centrifuged to remove excess K_2CO_3 and was used for subsequent ^{31}P-NMR analysis. After analysis, various standard compounds such as phosphocreatine, phosphoarginine and ATP were added to the extract mixture to help establish the identity of each resonance in the spectrum.

NMR spectra

For NMR analysis, 3 ml of the prepared sample was pipetted into a 10 mm NMR tube. This tube was inserted into the super conducting magnet of a Bruker WM300 spectrometer operating at 121 MHz for ^{31}P observation. An external capillary containing 0.1 M methylene diphosphonic acid (Alfa Products), a chemical shift reference (δ = 18.69 ppm) in 99.8% D_2O (Columbia), was added to the tube to provide a lock signal. All shifts were reported such that 85% phosphoric acid is assigned a chemical shift of 0.00 ppm. Spectra were collected while spinning at 1°C. Since the concentration of spermatozoa in whole semen is quite high (10-15 x 10^9 spermatozoa/ml) relatively good spectra could be obtained in less than 2 min of acquisition. Spectra were observed using a 78° pulse (10.6 μs), a delay of 0.5 s, a 13,889 Hz sweep width and an aquisition time of 0.59 s without proton decoupling. Spectra were also obtained with a delay of 0.0 s and an aquisition time of 0.037-0.074 s. The number of scans range from 10 to 2,200.

Measurement of oxygen uptake

To supplement studies with NMR, oxygen uptake of whole semen was measured with a Gilson Differential Respirometer. Approximately 1-2 ml of whole semen were added to each 20 ml flask. The oxygen uptakes were measured either in a series at 1, 5, 10, and 20°C or at a selected temperature for varying periods up to 3 hr. In other experiments, 1.5 ml of fish ringer or fresh water was added to the side arm and mixed (3:1) with the whole semen (0.5 ml) after 95 min of collecting data on oxygen uptake. Data were then collected for an additional 40 min. In all cases, the quality of motility was measured after completing the observations.

Intracellular pH measurements

A standard curve was prepared by titrating a 5 mM solution of inorganic phosphate (P_i) using ^{31}P-NMR. This solution (100 ml) was prepared from 85% phosphoric acid and also contained 0.3 M glycine, 0.15 M potassium acetate, 40 mM NaCl, 20 mM HEPES, 10 mM EGTA, and 20 mM glycerol. This curve had limiting shifts of 0.72 ppm and 3.18 ppm with a pKa of 6.63 for the second dissociable proton of P_i. The intracellular pH was obtained by noting the chemical shift for P_i in the NMR spectrum and then using this standard curve for obtaining an approximate pH measurement.

Determination of MgNTP/NTP ratio

The separation between the α-ATP and β-ATP resonances was utilized to measure the ratio of free NTP to total NTP according to the method developed by Gupta (Gupta and Moore, 1980; Gupta et al., 1983a,b). A stock solution containing 0.14 M KCl, 0.10 M NaCl, 0.20 M BISTRIS, and 0.004 M ATP was prepared and adjusted to a pH of 7.2. The NMR spectrum of this solution was then recorded at 10°C. This was repeated for 4 mM ATP and 10 mM Mg^{+2} in the prepared stock solution (pH 7.2). The separation (in Hz) between the α-ATP and β-ATP resonances was determined in these two cases as 1306 Hz and 1040 Hz, respectively.

RESULTS

^{31}P-NMR has allowed us to identify metabolites and to establish their relative intensities in rainbow trout spermatozoa and seminal fluid. Trout seminal fluid was found to contain small (<5 mM) and variable quantities of extracellular inorganic phosphate (P_o).

In Figure 1 the ^{31}P-NMR spectrum of freshly collected whole trout semen is shown. These spectra were collected at 1°C using 155, 320, 1000 and 2,275 scans (Figures 1A-D). The spectrum in Figure 1E is identical to Figure 1D with the exception that methylenediphosphonic acid, a chemical shift reference, can now be seen at 18.69 ppm. This series of spectra reveal the presence of NTP (-5.14, -10.07, and -19.02 ppm), PCr (-2.59 ppm), P_i (2.67 ppm), and PME (4.25 ppm) within these spermatozoa. Following spectra collection, the acquisition was halted. Acquisition was then reinitiated on the same sample for 200 scans to determine if resonances had changed during the accumulation. Under these conditions, no significant changes in spectral features were detected. However, spectra collected at 10°C showed a rapid decrease in PCr and NTP along with a corresponding increase in P_i (data not shown).

Generally, samples were collected in the mid-morning and were not analyzed until late afternoon. Therefore, to determine if this period of time had any detrimental effects on the metabolic status of the cells, some male trout were confined near the spectrometer and stripped approximately 10 min prior to NMR analysis. The results of this study

Figure 1. ^{31}P-NMR spectra of whole trout semen acquired at 1°C

using a 0.500 s receiver delay and a 0.589 s acquisition

time. [Spectra in Figures 1A-D display the increase in

signal to noise with longer sampling times of 2.4 min

(155 scans), 4.9 min (320 scans), 15.3 min (1,000 scans),

and 34.8 min (2,275 scans), respectively. Figure 1E is

identical to 1D with the exception that the external

standard, methylene diphosphonic acid, is shown at 18.69 ppm.

Other resonances correspond to phosphomonoesters (PME,

4.25 ppm) intracellular inorganic phosphate (P_i,

2.67 ppm), phosphocreatine (PCr, -2.59 ppm), γ-NTP (-5.14

ppm), α-NTP (-10.07 ppm), and β-NTP (-19.02 ppm).]
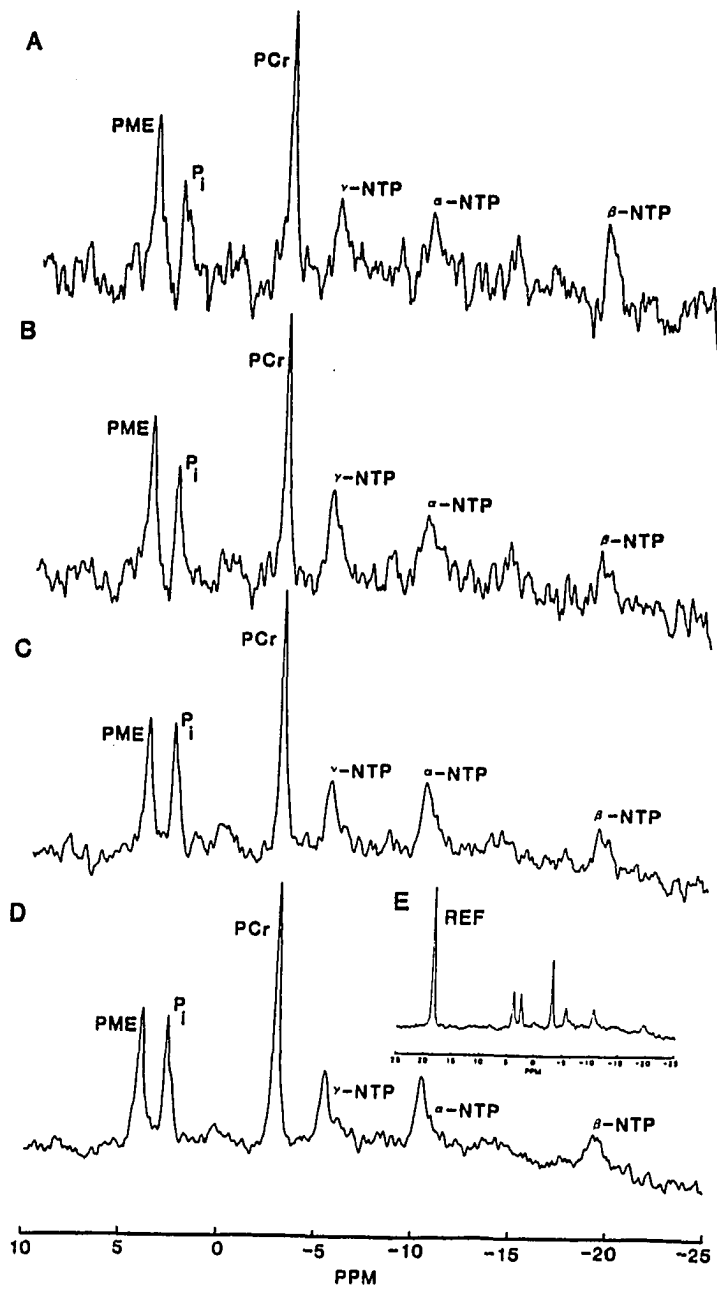
are shown in Figures 2A-D. This series of spectra confirms the observations demonstrated in Figure 1. From the chemical shift of P_i in these spectra the intracellular pH of resting trout spermatozoa was established as 7.3±0.2 (6 determinations). In addition, further work showed that 81% of the NTP was found complexed to Mg^{++}.

As shown in Figure 3 we have found that trout spermatozoa are rapidly affected by anaerobic conditions. In Figure 3A the aerobic spectrum of trout spermatozoa is shown. Resonances correspond to PME (4.26 ppm), P_i (2.81 ppm), PCr (-2.59 ppm) and NTP (-5.13, -10.09, and -19.0 ppm). If these spermatozoa are then exposed to a totally anaerobic environment by removing the oxygen, or by replacing it with nitrogen, at 10-20°C, the spectrum in Figure 3B is obtained. In this spectrum a total decrease in PCr and NTP is observed along with simultaneous increase in P_i. Phosphomonoesters seem to remain essentially unchanged at 4.20 ppm. From the P_i chemical shift (2.33 ppm), a drop in pH of 0.5 units from 7.4±0.2 to 6.9±0.2 was also observed. If these spermatozoa are then re-aerated (Figure 3C), recovery from anoxia occurs, as indicated by the reappearance of the PCr (-2.59 ppm) and NTP/NDP peaks (-5.19 ppm, -10.06 ppm and -19.21 ppm). Since the chemical shift of P_i has now risen back to 2.58 ppm a change in pH back to 7.1 is indicated. This decrease and increase of the level of PCr can be repeated several times by the above procedures and was repeated in several samples. To support these observations oxygen uptake in trout spermatozoa was also studied with a Gilson Differential Respirometer. Semen (15.5 x 10^9 spermatozoa/ml) was found to take up

Figure 2. ^{31}P-NMR spectra of whole trout semen where acquisition

was initiated within 10 min of semen collection. [Spectra

were acquired as stated for Figure 1. Spectra in Figures 2A-D

display the increase in signal to noise with longer sampling

times of 0.8 min (50 scans), 3.2 min (210 scans), 8.8 min

(575 scans), and 23.0 min (1,500 scans). Resonances

correspond to PME (4.27 ppm), P_i (2.75 ppm), PCr

(-2.59 ppm), γ-NTP (-5.11 ppm), α-NTP (-10.15 ppm),

and β-NTP (-19.07 ppm).]
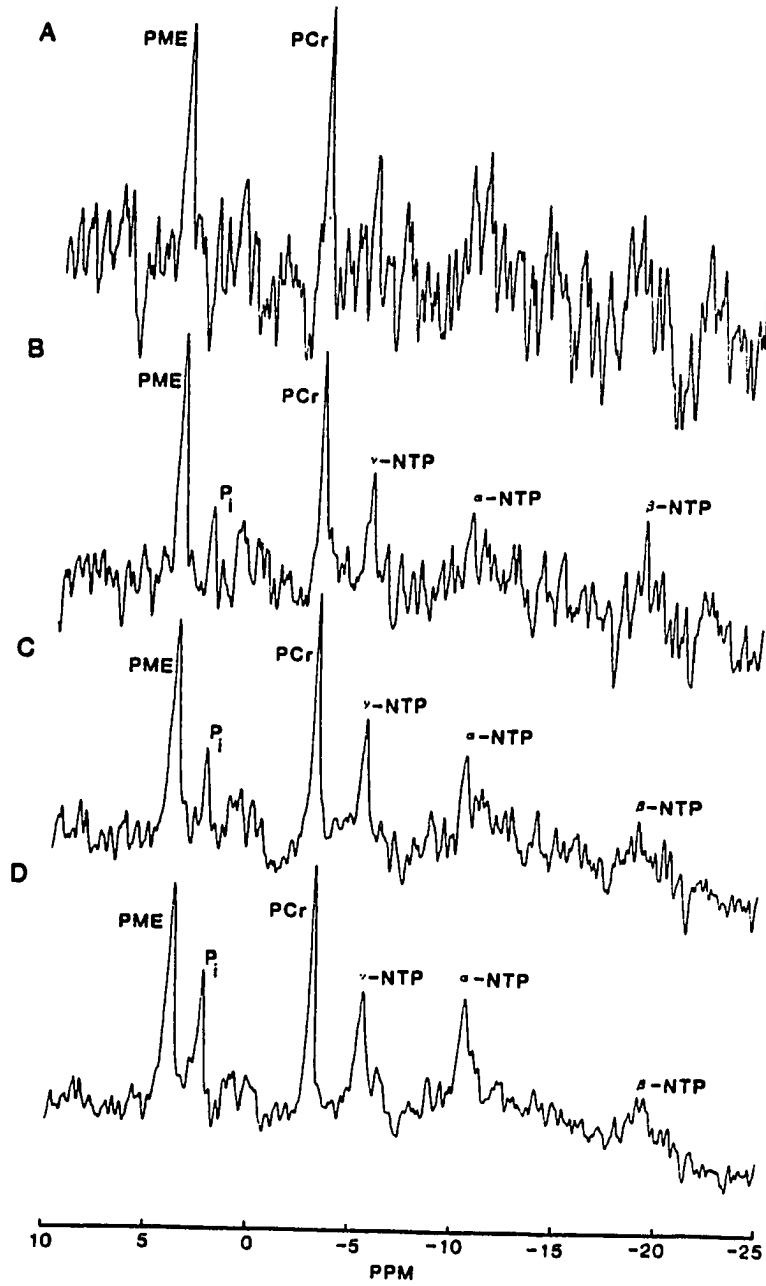
Figure 3. ^{31}P-NMR spectra of trout spermatozoa prior to and

following anoxia. [Spectra were acquired as stated for

Figure 1. A) Aerobic trout spermatozoa acquired for 30.6 min

(2,000 scans). Resonances correspond to PME (4.26 ppm),

P_i (2.81 ppm), PCr (-2.59 ppm), γ-NTP (-5.13 ppm),

α-NTP (-10.09 ppm), and β-NTP (-19.0 ppm). B) Anaerobic

trout spermatozoa acquired for 13.0 min (850 scans).

Resonances correspond to PME (4.20 ppm) and P_i (2.33 ppm).

C) Re-aerated trout spermatozoa acquired for 40.7 min (2,660

scans). Resonances correspond to PME (4.23 ppm), P_i

(2.58 ppm), PCr (-2.59 ppm), γ-NTP (-5.19 ppm), α-NTP

(-10.06 ppm), and β-NTP (-19.21 ppm).]
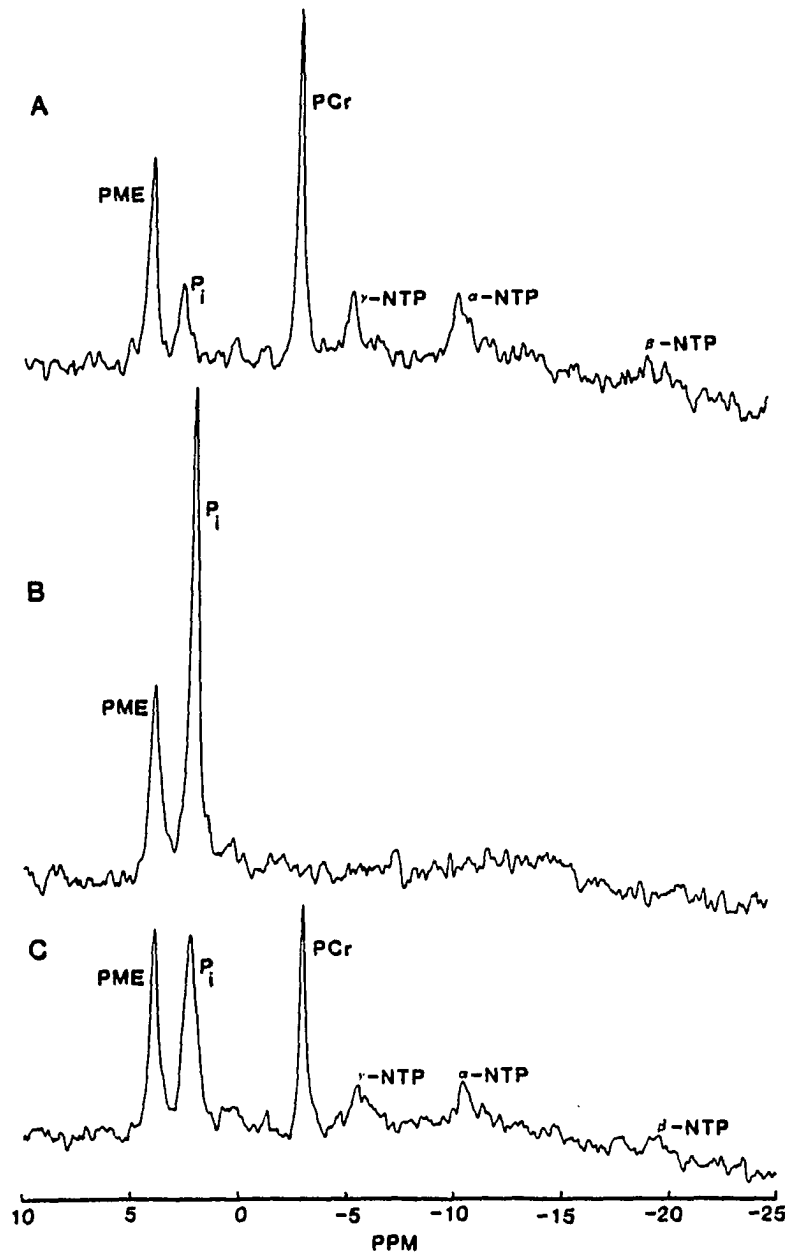
oxygen at 40-45µl/h at 20°C during 95 min test periods. In other test experiments, approximate oxygen uptake at 10°C, 5°C, and 1°C was 11-16, 5-6, and 3-4µl/h of semen, respectively. Because of this oxygen uptake, even when placed on ice, sperm samples were frequently aerated by changing the air in the plastic bag. In addition, they were analyzed at 1°C, a temperature where respiration is minimized.

Motility in trout spermatozoa was activated using fish ringer or fresh water (9:1; diluent:whole semen). When spermatozoa were activated with ringer, vigorous motility was usually observed (100%) for less than 1 min and sometimes followed by a twitching of spermatozoa (<5%) for approximately 5 min. Motility could not be reactivated by addition of further ringer. However, if spermatozoa were centrifuged out of the ringer and resuspended in seminal fluid at room temperature for 15 min or more, reactivation of motility could be achieved by adding ringer. In this case motility ranged from 50 to 100%. This could be repeated several times. In contrast, spermatozoa initially activated with fresh water could not be made to undergo a second burst of motility even after prolonged storage in seminal fluid. These results could be expected after considering the ^{31}P-NMR results obtained with spermatozoa activated with ringer and fresh water.

In Figure 4 the effect of motility activation in trout spermatozoa is shown. The control (Figure 4A) reveals, as expected, the presence of high levels of PCr (-2.59 ppm) and NTP (-5.06, -10.11 and -19.06 ppm) along with PDE (0.53 ppm and 0.11 ppm), PME (4.25 ppm), P_i (2.76 ppm) and P_o (2.93 ppm). From the chemical shift of P_i and P_o, intracellular

Figure 4. ^{31}P-NMR spectra of trout spermatozoa at rest and

following motility activation. [Spectra were acquired as

stated for Figure 1. A) Trout spermatozoa which have not

undergone motility activation (control), acquired for

23.8 min (1,550 scans). Resonances correspond to PME

(4.25 ppm), P_o (2.93 ppm), P_i (2.76 ppm), PDE (0.53

and 0.11 ppm), PCr (-2.59 ppm), γ-NTP (-5.06 ppm), α-NTP

(-10.11 ppm) and β-NTP (-19.06 ppm). This spectrum has

has been expanded in the vertical direction (7.1x) as

shown in Figure 4D. A broad resonance can now be observed

from 30 to -25 ppm. B) Trout spermatozoa following

motility activation with ringer, acquired for 23.0 min

(1,500 scans). Resonances correspond to PME (4.02 ppm),

P_i (1.89 ppm), PDE (0.47 and -0.02 ppm), PCr

(-2.61 ppm), γ-NTP (-5.18 ppm), and α-NTP (-10.23 ppm).

C) Trout spermatozoa following motility activation with

fresh water acquired for 23.0 min (1,500 scans).

Resonances correspond to PME (4.24 and 4.07 ppm) and P_i

(2.50 ppm).]
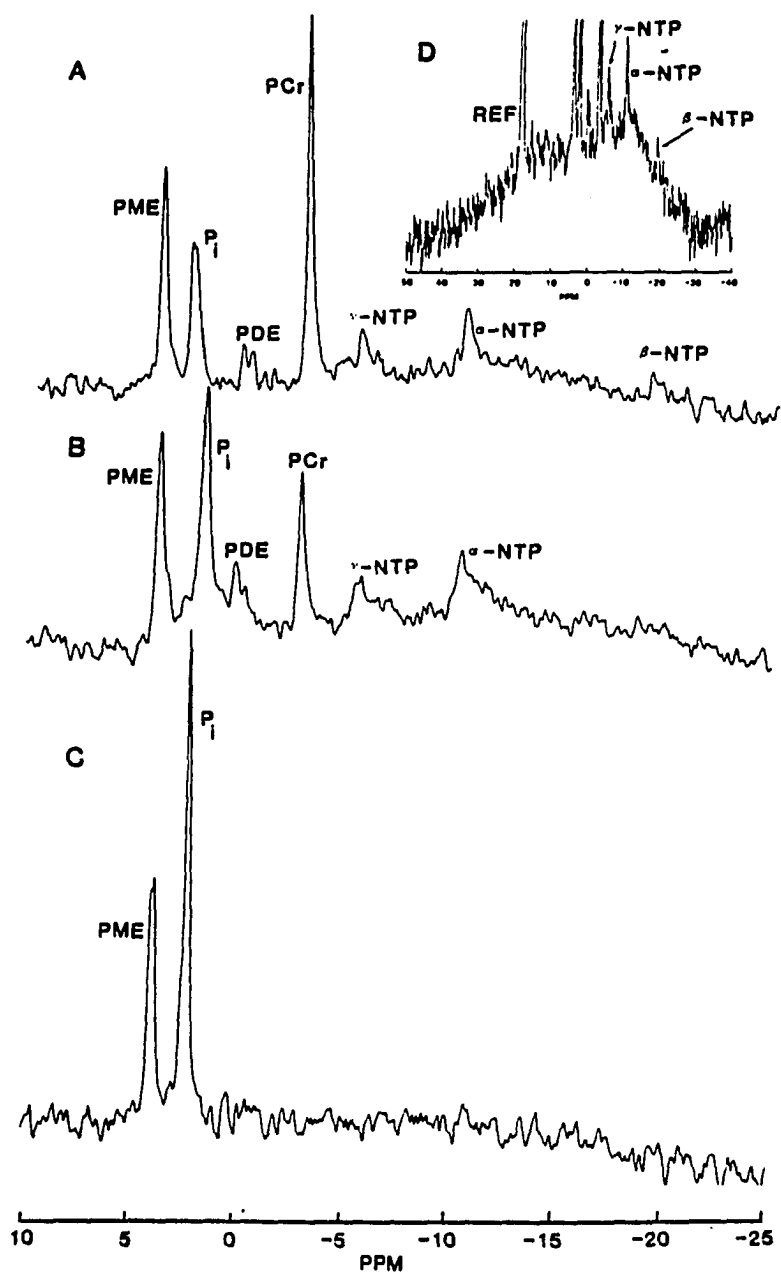
and extracellular pH values of 7.3±0.2 and 7.6±0.2 respectively could be estimated. When motility was initiated with ringer (9:1 dilution) a partial decrease in PCr (-2.61 ppm) accompanied with a subsequent increase in P_i (1.89 ppm) is observed (Figure 4B). The intracellular pH is now observed to decrease by 0.7 units to 6.6±0.2. The NTP/NDP ratio appears to drop as seen by the disappearance of the β-NTP resonance. With the respirometer, an increase of approximately 50% in oxygen uptake was observed during the 40 min following motility activation with ringer (3:1 dilution) indicating an increased respiration rate. When motility was activated with fresh water (9:1 dilution), a total decrease in PCr and NTP/NDP was detected (Figure 3C). Surprisingly, from the chemical shift of P_i (2.50 ppm) the intracellular pH was shown to decrease by only 0.2 pH units from 7.3±0.2 to 7.1±0.2. In addition, a new PME resonance can now be detected at 4.07 ppm. Oxygen uptake was found to be drastically reduced (approximately 75%) after fresh water (3:1 dilution) was added.

We have also attempted to look at the effect of short-term storage (0-14 days at 1°C) of trout spermatozoa with ^{31}P-NMR analysis. As shown in Figure 5, when trout spermatozoa are stored, an initial decrease occurs in NTP/NDP levels, followed by a slower decrease in PME and PCr levels. This is accompained by a rise in P_i and PDE levels. No change in intracellular pH (7.5±0.2) was detected. Furthermore, the presence or absence of PCr was found to be a much better indication of potential for motility in a sample than were NTP/NDP levels. In this series of spectra (5A-C) spermatozoa were found to display 100%, 80-100%, and 25%

Figure 5. ^{31}P-NMR spectra of stored trout semen. [Spectra were

acquired as stated for Figure 1. A) Freshly collected trout

semen acquired for 3.9 min (256 scans). Resonances

correspond to PME (4.23 ppm), P_i (2.87 ppm), PDE

(-0.63 ppm), PCr (-2.59 ppm), ɤ-NTP (-5.20 ppm), α-NTP

(-10.11 ppm), and β-NTP (-18.8 ppm). B) Seven day old trout

semen acquired for 5.4 min (356 scans). Resonances

correspond to PME (4.27 ppm), P_i (2.88 ppm), PDE

(-0.63 ppm), and PCr (-2.59 ppm). C) Fourteen day old

trout semen acquired for 3.9 min (256 scans). Resonances

correspond to PME (4.26 ppm), P_i (2.90 ppm), and PDE

(-0.63 ppm).]
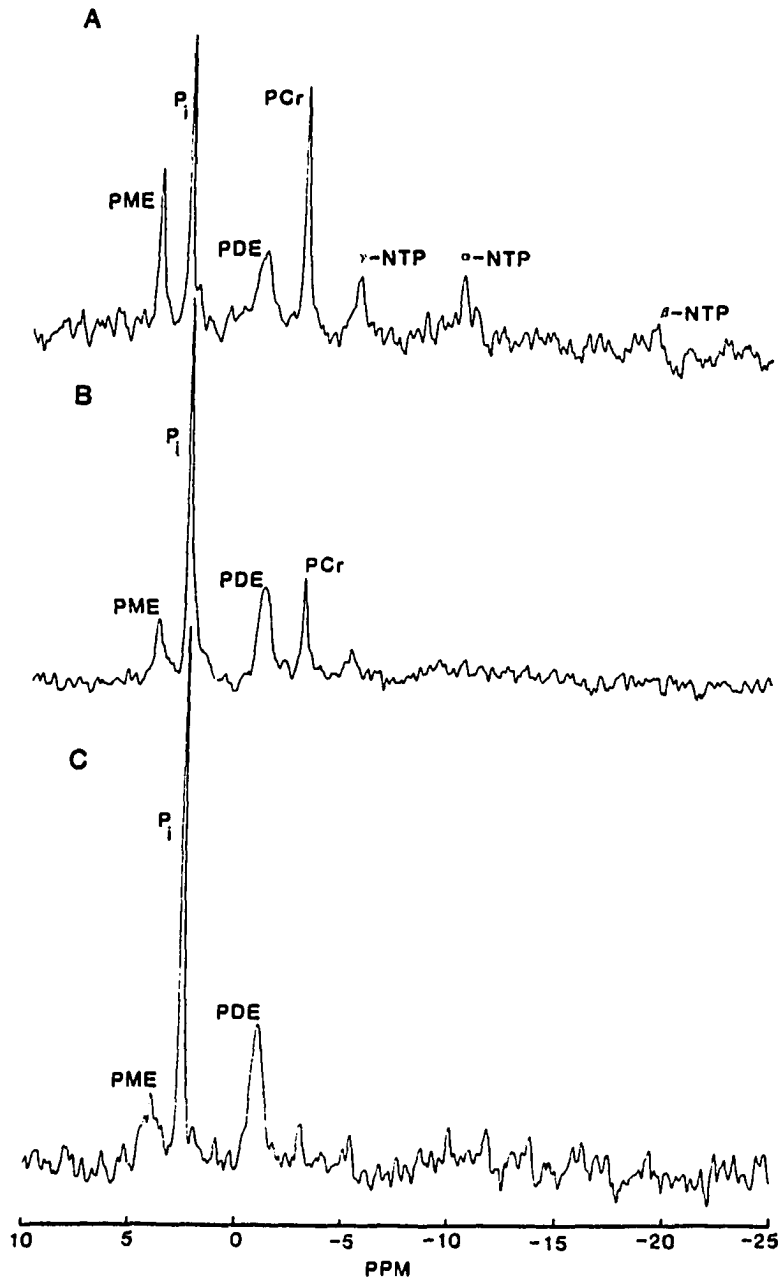
motility, respectively. The presence of a strong phosphodiester resonance (-0.63 ppm) in this series of spectra may reflect the use of a different trout strain since its presence was much more frequent in the winter spawner. However, it should be noted that the intensity of this resonance seems also to vary between individual animals.

DISCUSSION

During the last few months we have performed some preliminary work on rainbow trout semen using ^{31}P-NMR. Although other investigators (Stoss, 1983) have performed some interesting studies on trout sperm metabolism, to our knowledge no studies have used NMR on trout semen or for that matter on any fish semen. In addition, we have demonstrated that trout semen is an excellent material for NMR analysis and that high energy phosphorus compounds can be readily observed (Figure 1), particularly phosphocreatine (PCr), a compound heretofore not described in fish spermatozoa. We have found that these spermatozoa are not fragile and can withstand many washings and centrifugations. They can be obtained in high concentrations (5-16 x 10^9 spermatozoa/ml) and in large volumes (2-20 ml), two aspects necessary for NMR studies.

The pH of trout seminal fluid has been examined in reference to sperm activation (Baynes et al., 1981). It has been suggested that low extracellular pH values (below 7.0) may be present in normal seminal fluid and may be important in preventing sperm activation, however, such a function has not been established. In our study, the pH of freshly collected seminal fluid was determined as 7.6±0.2, a value not only higher than the above suggestion but also higher than the intracellular pH by 0.3 units.

With ^{31}P-NMR analysis, the level of PCr is observed to decrease rapidly during anaerobic conditions, but can be restored by aeration. This decrease is accompanied intracellularly by a corresponding fall and rise in P_i and in pH, precisely what is expected under such conditions. How often such behavior can be repeated would be an interesting experiment and would probably depend on the endogenous nutrients.

Immediately following dilution in fish ringer, trout spermatozoa were observed to undergo vigorous motility which lasted for up to 1 min. This period was sometimes followed by less pronounced and variable levels of motility. Such observations are supported by several studies where trout spermatozoa mixed in various isotonic media were found to be motile between 1 and 5 min (Ginsburg, 1963; Scheuring, 1925; Baynes et al., 1981). We have also found, by ^{31}P-NMR analysis, that such a burst of motility with ringer reduces the level of PCr in addition to increasing the NDP/NTP ratio and the levels of P_i (Figure 4B). At the same time intracellular pH decreases 0.7 units to 6.6±0.2, a result contrary to that found in activated spermatozoa of sea urchins (Christen et al., 1983a,b) and of Limulus (Robitaille et al., 1986a). Moreover it is interesting to note that motility in trout spermatozoa is reported to double in duration when these cells are placed in the ovarian fluid released with the eggs (Ginsburg, 1963; Billard, 1980). This enhancement was attributed to astaxanthine, beta carotene, or an unspecified substance (Hartmann et al., 1947; Yoshida and Nomura, 1972). This situation is reminiscent of the effect of the sperm motility initiating peptide (SMI) on the spermatozoa of Limulus polyphemus

(Clapper and Brown, 1980a,b; Clapper and Epel, 1982a,b). In addition, we have found that motility could be reactivated in spent spermatozoa by treatment with seminal fluids. Reactivation is supported by several other works (Nomura, 1964; Billard and Jalabert, 1974; Guest et al., 1976; Kusa, 1950; Ginsburg, 1963). Along these lines it should be noted that Schlenk and Kahmann (1938) were able to reactivate motility in trout spermatozoa after a period of rest in high K^+ solution followed by exposure to a solution of lower K^+ content.

Recent studies have shown cessation of motility in spermatozoa of Salmo gairdneri after only 15 s in natural pond water (Cosson et al., 1985). It has been proposed that swelling and lysis of the cell due to the hypotonic water is the result of the termination of motility (Schlenk and Kahmann, 1938; Huxley, 1930; Billard, 1978). The ^{31}P-NMR results we have obtained would tend to support such a hypothesis.

Studies on oxygen uptake by fish spermatozoa are comprised of only a few investigations. Spermatozoa of a sunfish, Lepomis (sp.), and the sucker, Catostomus commersonnii, use 110-140 μl O_2 per 10^{10} cells/h, whereas spermatozoa of Atlantic salmon, Salmo salar, and cod, Gadus morhua, are reported to use 20-40 μl O_2 per 10^{10} cells per hr (Terner, 1962; Terner and Korsh, 1963a; Mounib, 1967). These values are in good agreement with a value of 25-29 μl O_2 per 10^{10} cells per hr obtained in this study. Oxygen uptake was also measured before and after mixing of ringer or fresh water with semen. Due to the limiting capacity of our system, this 3:1 dilution with ringer was not an adequate ratio to cause 100% motility since we were still able to induce motility with further

dilution after the experiments. Nevertheless, we did observe an increase in oxygen uptake with the ringer. With the ^{31}P-NMR studies, we used the adequate ratio of 9:1 and obtained complete motility. By cooling rapidly, centrifuging and analyzing as soon as possible we were able to detect decreases in NTP and PCr levels and in intracellular pH. More than likely, we are observing these decreases near the lowest levels, since the recovery period during increased oxygen uptake should be minimum.

We have performed preliminary studies of short-term storage. Typical results include an initial total decrease in NTP/NDP levels followed by the subsequent gradual decrease in PCr. Motility could be initiated even in the absence of free NTP resonances, but lower levels of motility were observed when PCr levels began to decrease. The fact that during short-term storage PCr levels remain relatively high while NTP/NDP levels are found to drop significantly is a very interesting phenomenon, which to our knowledge has not been previously observed. Moreover, the behavior is in direct contrast to other creatine kinase systems such as muscle and heart where PCr levels have been established to drop prior to any significant change in NTP/NDP levels (Schmidth and Smith, 1983; Ellington, 1983; Barany and Glonek, 1982; Neurohr et al., 1983; Hoult et al., 1974; Ingall, 1982; Osbakken and Briggs, 1984; Brooks and Willis, 1983; Malloy et al., 1985).

Since, thermodynamic equilibrium for the creatine kinase catalysed reaction usually lies towards the breakdown of PCr and the formation of ATP from ADP, the result which we report is somewhat unusual (Lawson and Veech, 1979; Watts, 1973). However, if one considers the morphology of spermatozoa in reference to the PCr shuttle hypothesis the results which we report are not overly bizzare. Tombes and Shapiro (1985) have recently reported the discovery of two creatine kinase isoenzymes in sea urchin spermatozoa. One of these enzymes was located in the mitochondria whereas the other was isolated from sperm tails. A parallel enzymatic system has been found in studies of chicken and human spermatozoa (Wallimann et al., 1986). A similar system in trout could readily account for the results we observed. That is, the presence of a transmembrane mitochondrial creatine kinase could result in the creation of an enzymatic system where only the forward rate (ATP to PCr) is of importance. Thus, a decrease in NTP levels could not be supplemented by the conversion of PCr to NTP since the formation of PCr, as described above would be essentially irreversible. This would result in the buildup of PCr over NTP if the second creatine kinase in the flagellum remained inactive. In this light, it should be remembered that it has been reported that intracellular alkalinization is required for activation of dynein ATPases in sea urchin sperm (Christen et al., 1983a). Our results on short term storage in trout spermatozoa would thus tend to support the PCr shuttle hypothesis.

The effect of short-term storage has also been studied in sea urchin spermatozoa (Christen et al., 1983b). However, it should be noted that in this study a total decrease in PCr was noted prior to a similar decrease in NTP. Because of this difference between sea urchin and trout spermatozoa we feel that a significant amount of further work will be required in order to understand fully the energetics within spermatozoa during short-term storage.

Spectra of trout spermatozoa have also revealed the presence of a broad resonance (30 - -25 ppm). The presence of this resonance which is particularly prominent from 0 - -25 ppm, has been illustrated in Figure 4D, although all other spectra collected in this also displayed this feature. In previous studies on spermatozoa of bull, goat, ram and boar, a similar resonance was observed (Robitaille et al., 1986b). In these spermatozoa, this broad resonance was assigned principally to bound ATP, or free and bound ATP in rapid exchange with one another, where the bound ATP is located on the ATPases of the flagellar dynein arms. A similar situation may be occurring in trout spermatozoa. This feature along with the presence of a small PCr resonance in these spermatozoa may help explain why these cells are still capable of motility in the absence of readily detectable free NTP peaks.

At this stage of investigation, it is impossible to deduce from our data whether a PCr shuttle is present in trout spermatozoa or whether motility results from the simple diffusion of ATP from the mitochondria to the dynein arms of the flagellum.

It may well be, however, that both of these systems are important for motility in the fish. This is not unlikely, in light of the evolutionary position of these animals.

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PART 5: ^{31}P-NMR ANALYSIS OF THE NON-MOTILE SPERM OF THE BLUE

CRAB, <u>Callinectes</u> <u>sapidus</u>

^{31}P-NMR analysis of the non-motile sperm of the

blue crab, <u>Callinectes</u> <u>sapidus</u>

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ABSTRACT

The sperm of the blue crab, .Callinectes sapidus is atypical because
it has no flagellar apparatus or any other means of motility. These
spermatozoa display a diverse morphology, are packaged in spermatophores
and can be stored for several months. As a result, it seemed resonable
to examine with phosphorus-31 nuclear magnetic resonance the energy
make-up of this cell type and compare to energy systems found in the
typical flagellar type of spermatozoa. Resonances corresponding to
nucleotide triphosphates, inorganic phosphate, phosphomonoesters and
phosphodiesters were found. However, the presence of phosphagens which
are found in many motile spermatozoa was not demonstrated. In addition,
the stored C. sapidus sperm appears to be inert metabolically.

INTRODUCTION

Phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR) studies have been performed on spermatozoa of several species of animals (for reveiw, Robitaille et al., 1986a,b). These studies have revealed the relative concentrations of high energy phosphorus containing compounds and established that changes in the concentrations of these compounds occur during motility. However, in all of these reports the typical flagellar spermatozoa were examined. In addition, since phosphagens have been invoked in an energy shuttle system in spermatozoa (Tombes and Shapiro, 1985), the presence or absence of a phosphagen has stimulated much scientific interest. From a metabolic point of view it is of interest to qualify the energy required by a sperm which lacks a flagellum or any other obvious means of motility. The spermatozoa of decapod crustaceans are non-motile (Binford, 1913) and are appropriate for such investigations.

For this study, the blue crab, Callinectes sapidus, was chosen since it is abundant, the seasonal reproductive cycle is well understood (Cronin, 1947), and spermatozoa can be obtained in quantities sufficient for NMR analysis. The spermatozoa of C. sapidus and other brachyurans have been extensively examined morphologically (Brown, 1966; Langreth, 1968; Pochon-Masson, 1968; Goudeau, 1982), however, practically nothing is known about the metabolic machinery. A peculiar feature of spermatozoa of C. sapidus and other brachyurans is the absence of

mitochondria, although numerous intracellular membranes or lamellae are present (Brown, 1966; Pearson and Walker, 1975; Goudeau, 1982). This is also true for spermatozoa of most other groups of decapods, particularly penaeids (Clark et al., 1973; Clark et al., 1981), palinurans (Talbot and Summers, 1978), and astacideans (Lopez-Camps et al., 1981; Talbot and Chanmanon, 1980). At least one exception to this generality in the decapods is in some anomuran spermatozoa which do have typical mitochondria (Pochon-Masson, 1968; Hinsch, 1973).

For comparative purposes, results obtained with the spermatozoa of C. sapidus are compared to data collected on the flagellated sperm of the horseshoe crab, Limulus polyphemus. In a previous paper on L. polyphemus spermatozoa (Robitaille et al., 1986a), we reported the relative levels of high energy phosphorus containing compounds in resting spermatozoa and the changes in the levels occurring during sperm motility. Resonances corresponding to phosphomonoesters (PME), phosphodiesters (PDE), phosphoarginine (PA), nucleotide di- and triphosphates (NDP/NTP), and nicotinamide adenine dinucleotide (NAD$^+$/NADH) were reported. The nucleotides are primarily adenosine di- and tri-phosphates (ADP/ATP). These are typical resonances which with some exceptions have also been observed in spermatozoa of other species (sea urchins, Christen et al., 1983; mammals, Smith et al., 1985; Robitaille et al., 1986b; fish; Robitaille et al., see part 4).

MATERIALS AND METHODS

Animals

Mature males and females of the blue crab, <u>Callinectes</u> <u>sapidus</u>, were collected from the Chesapeake Bay in the York River in the proximity of Gloucester Point, Virginia and were shipped to Ames, Iowa. Two shipments were received: September, 1985, and May, 1986. In each case, animals were shipped on the day caught and were used for investigation on the day received, a time lapse of 24-36 hr. The males had not recently copulated at either period of time and had fully distended vas deferens. Their reproductive stage was determined by examining the color of the flipper. Approximately 75 males and 15 females were used in this study. Animals were kept in the laboratory at 17-18°C in a 150 gallon Instant Ocean aquaria containing artificial seawater (ASW) from Jungle Laboratories Corporation, Comfort, TX. The crabs were fed squid and kept on a 14-10 light/dark cycle.

Gamete collection

Spermatozoa are packaged in spermatophores which are formed and collected in the anterior vas deferens of the male. Spermatophores are also usually found in seminal receptacles of females which have copulated but have not spawned. Thus, in order to obtain spermatozoa, spermatophores were collected. With the male, the carapace was removed, and the spermatophoric region of each vas deferens was removed and placed in a 9 cm Petri dish containing 25-30 ml of ASW either at 0°C or room temperature (20-22°C). These regions were teased apart with watchmaker forceps freeing the spermatophores which were then concentrated by swirling the dish. These were then collected with pipets and placed in 15 ml centrifuge tubes. Spermatophores were then washed 6-8 times with ASW at the collected temperature. From 9-12 males, approximately 3 ml of spermatophores were collected. Spermatophores are approximately 500 µm in diameter and when packed are in a concentration of 150,000/ml. Since 4.6×10^6 spermatozoa are estimated to be in each spermatophore, the approximate sperm concentration was calculated to be 700×10^9 spermatozoa/ml of spermatophores. Samples from males were prepared in the Fall and in the Spring.

For collection of spermatophores from the female, the carapace was removed and the seminal receptacles were dissected out. The spermatophoric masses were removed from the seminal receptacles and placed in a 15 ml centrifuge tube and washed several times with ASW.

This was performed in the Fall and only one sample was prepared from approximately 20 females.

NMR spectra collection

For NMR analysis, 3 ml of the prepared sample of spermatophores was pipetted into a 10 mm NMR tube. This tube was inserted into the super conducting magnet of a Bruker WM300 spectrometer operating at 121 MHz for ^{31}P observation. An external capillary containing 0.1 M methylene diphosphonic acid (Alfa Products), a chemical shift reference (δ = 18.69 ppm), in 99.8% D_2O (Columbia) was added to the 10 mm NMR tube to provide a lock signal. All shifts were reported such that 85% phosphoric acid is assigned a chemical shift of 0.00 ppm. Spectra were collected at 1, 5, or 20°C depending on the particular study. Since the concentration of spermatozoa in each sample was very high, relatively good spectra were obtained in less than 2 min of acquisition. Spectra were observed using a 78° pulse (10.6 μs), a 0.5 s receiver delay, a 13,889 Hz sweep width and an aquisition time of 0.59 s without proton decoupling. The number of scans range from 50 to 1,500.

Measurement of oxygen uptake

To supplement studies with NMR, oxygen uptake was measured with a Gilson Differential Respirometer. Approximately 2 ml of spermatophores were added to each 20 ml flask. The uptakes were measured at 20°C for 3 hr.

Intracellular pH measurements

A standard curve was prepared by titrating a 5 mM solution of inorganic phosphate (P_i) using ^{31}P-NMR. This solution (100 ml) was prepared from 85% phosphoric acid and also contained 0.3 M glycine, 0.15 M potassium acetate, 40 mM NaCl, 20 mM HEPES, 10 mM EGTA, and 20 mM glycerol. This curve had limiting shifts of 0.72 ppm and 3.18 ppm with a pKa of 6.63 for the second dissociable proton of P_i. The intracellular pH was obtained by noting the chemical shift for P_i in the NMR spectrum and then using this standard curve for obtaining an approximate pH measurement.

Determination of free magnesium concentration

The separation between the alpha and beta resonances of NTP was utilized to measure the ratio of free NTP to total NTP according to the method developed by Gupta and others (Gupta and Moore, 1980; and Gupta et al., 1983a,b). A stock solution containing 0.14 M KCl, 0.10 M NaCl, 0.20 M BISTRIS, and 0.004 M ATP was prepared and adjusted to a pH of 7.2. The NMR spectrum of this solution was then recorded at 10°C. This was repeated for 4 mM ATP and 10 mM Mg^{+2} in the prepared stock solution (pH 7.2). The separation (in Hz) between the α-ATP and β-ATP resonances were determined in these two cases as 1306 Hz and 1040 Hz, respectively.

Electron microscopy

Spermatophores were fixed in 5% glutaraldehyde (phosphate buffer) and postfixed in 1% osmium tetroxide. Free spermatozoa were obtained from seminal receptacles, fixed and embedded in 2% agar. Swiss Araldite (Durcupan, Fluka) was used for embedding medium. Uranyl acetate and lead citrate were used for counterstaining. Ultrasectioning was performed on an LKB Ultrotome I yielding sections with thickness varying between 400-1000°A. Examination was carried out on a Siemens Elmiskop IA or a Philips 100B electron microscopes.

RESULTS

Figure 1A illustrates the ^{31}P-NMR spectra of $\underline{C}$. $\underline{sapidus}$ spermatozoa
(encased in spermatophores) which are examined at 1°C using 1500 scans
(22.9 min). Resonances correspond to PME (4.22 ppm), P_i (2.66 ppm), γ-
NTP (-5.35 ppm), α-NTP (-10.24 ppm), β-NTP (-20.0 ppm), and uridine
diphosphoglucose (UDPG) (-10.24 and -11.97 ppm). Identical results were
obtained using only 50 scans (0.7 min). From the chemical shift of P_i
an intracellular pH of 7.2±0.2 was calculated. Moreover, the separation
between the alpha and beta resonances of NTP indicates that 53% of the
NTP is complexed to Mg^{++}. Although this spectrum was obtained from
spermatophores collected from vas deferens of males in the Spring,
similar results were obtained from spermatophores collected from males
and from seminal receptacles of females in the Fall (data not shown).
The ^{31}P-NMR spectrum of $\underline{C}$. $\underline{sapidus}$ spermatozoa which have been allowed
to stand anaerobically for 48 hr at room temperature is shown in
Figure 1B. This spectrum was collected using 500 scans (7.7 min).
Resonances correspond to PME (3.81 ppm), P_i (2.33 ppm), γ-NTP
(-5.39 ppm), α-NTP (-10.19 ppm), β-NTP (-19.82 ppm), and UDPG
(-12.04 ppm). From the chemical shift of P_i a drop in intracellular pH
by 0.3 units to 6.9±0.2 could be calculated. In this case 52% of the
NTP was calculated to be complexed to Mg^{++}.

Figure 1. ^{31}P-NMR spectra of <u>Callinectus</u> <u>sapidus</u> spermatozoa.

[A] ^{31}P-NMR spectrum of freshly .isolated <u>Callinectes</u> <u>sapidus</u> spermatozoa collected at 1°C using 1500 scans (22.9 min). Resonances correspond to phosphomonoesters (PME, 4.22 ppm), intracellular inorganic phosphate (P_i, 2.66 ppm), nucleotide triphosphate (𝛾-NTP, -5.35 ppm; 𝛼-NTP, -10.24 ppm; 𝛽-NTP, -20.0 ppm) and uridine diphosphoglucose (-10.24 and -11.97 ppm). B) ^{31}P-NMR spectrum of <u>C</u>. <u>sapidus</u> spermatozoa which had been kept under anaerobic conditions for 2 days collected at 1°C for 500 scans (7.7 min). The resonance positions of PME and P_i have shifted to 3.81 and 2.33 ppm indicating a pH change of 0.3 units to a pH value of 6.9±0.2.]
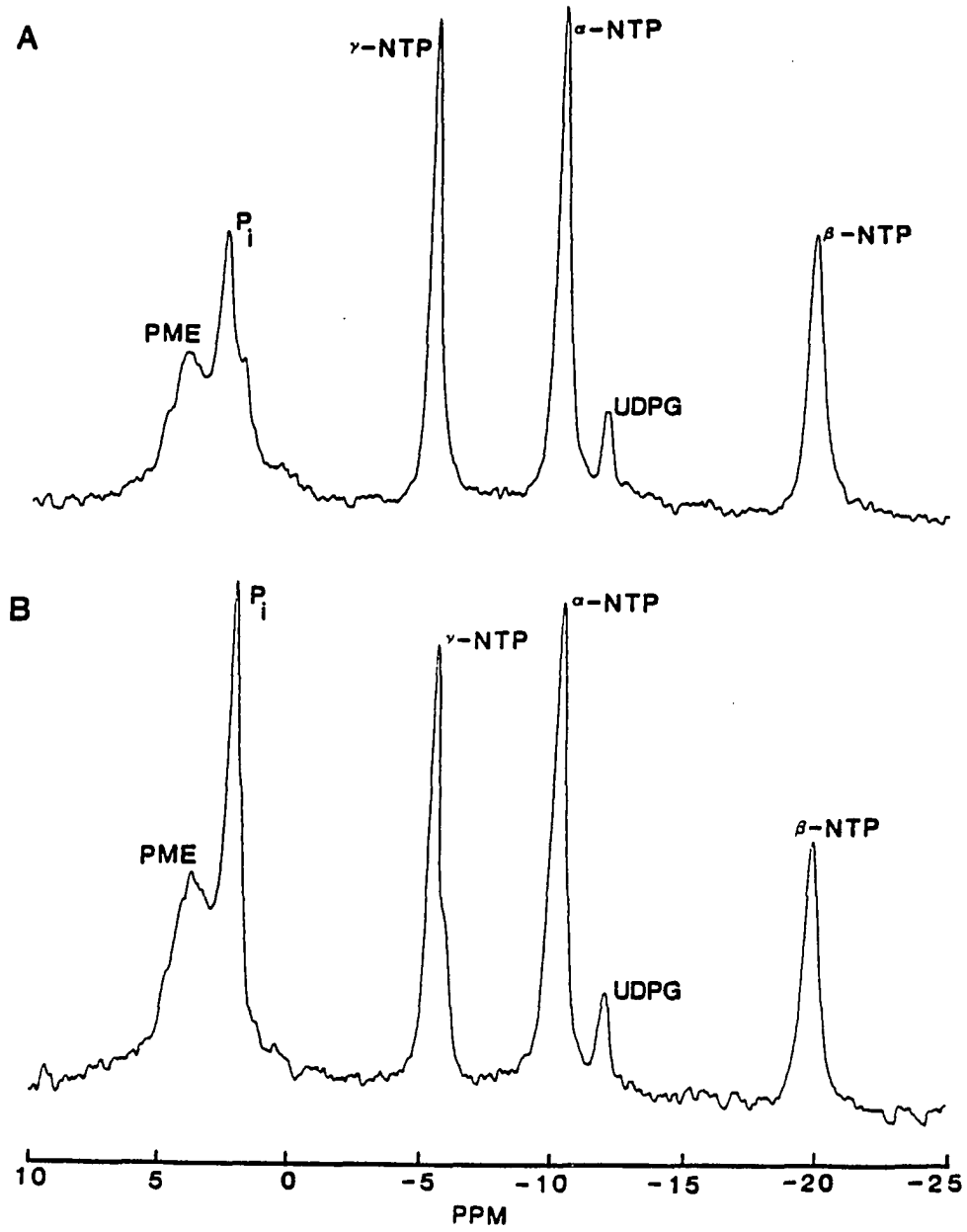
Oxygen uptake of C. sapidus spermatozoa revealed very low, if any, oxygen consumption. During a period of 1-3 hr, the amount of oxygen taken up was insignificant.

Figure 2 shows the ^{31}P-NMR spectra of spermatozoa collected from Limulus polyphemus. These represent a typical flagellar invertebrate sperm and are shown for comparison purposes. The spectra shown were obtained by methods similar to those outlined in a previous paper (Robitaille et al., 1986a), however, these spectra were obtained by using larger samples and much shorter scanning periods.

The C. sapidus sperm is aflagellate and does not have recognizable mitochondria although extensive lamellae are present (Figure 3A). These lamellae are located in the central region which is very closely associated with the acrosomal region. In the spermatophores the spermatozoa are tightly packed together (Figure 3B) leaving very little space for extracellular material. The spermatophoric wall (not demonstrated) contains homogenous material and is definitely extracellular (for review, Uma and Subramoniam, 1979).

Figure 2. ^{31}P-NMR spectra of <u>Limulus</u> <u>polyphemus</u> spermatozoa
examined at 5°C using: A) 128 scans (2.0 min),
B) 300 scans (4.5 min), and C) 900 scans (13.8 min).
[Resonances correspond to PME (4.55 ppm and 4.13 ppm),
P_i (2.48 ppm), PDE (0.63 ppm), PA (-3.12 ppm),
γ-NTP (-5.17 ppm), α-NTP (-10.18 ppm), β-NTP (-19.03 ppm),
and NAD^+/NADH (-10.61 ppm). From the chemical shift of
P_i, an intracellular pH value of 7.0±0.2 was
calculated. Approximately 87% of the NTP was complexed
as MgNTP. Procedures for sperm isolation and parameters
for collection of spectra are similiar to those described
in Robitaille <u>et</u> <u>al</u>. (1986a) except that the prepared
sample was larger and the number of scans was considerably
smaller.]
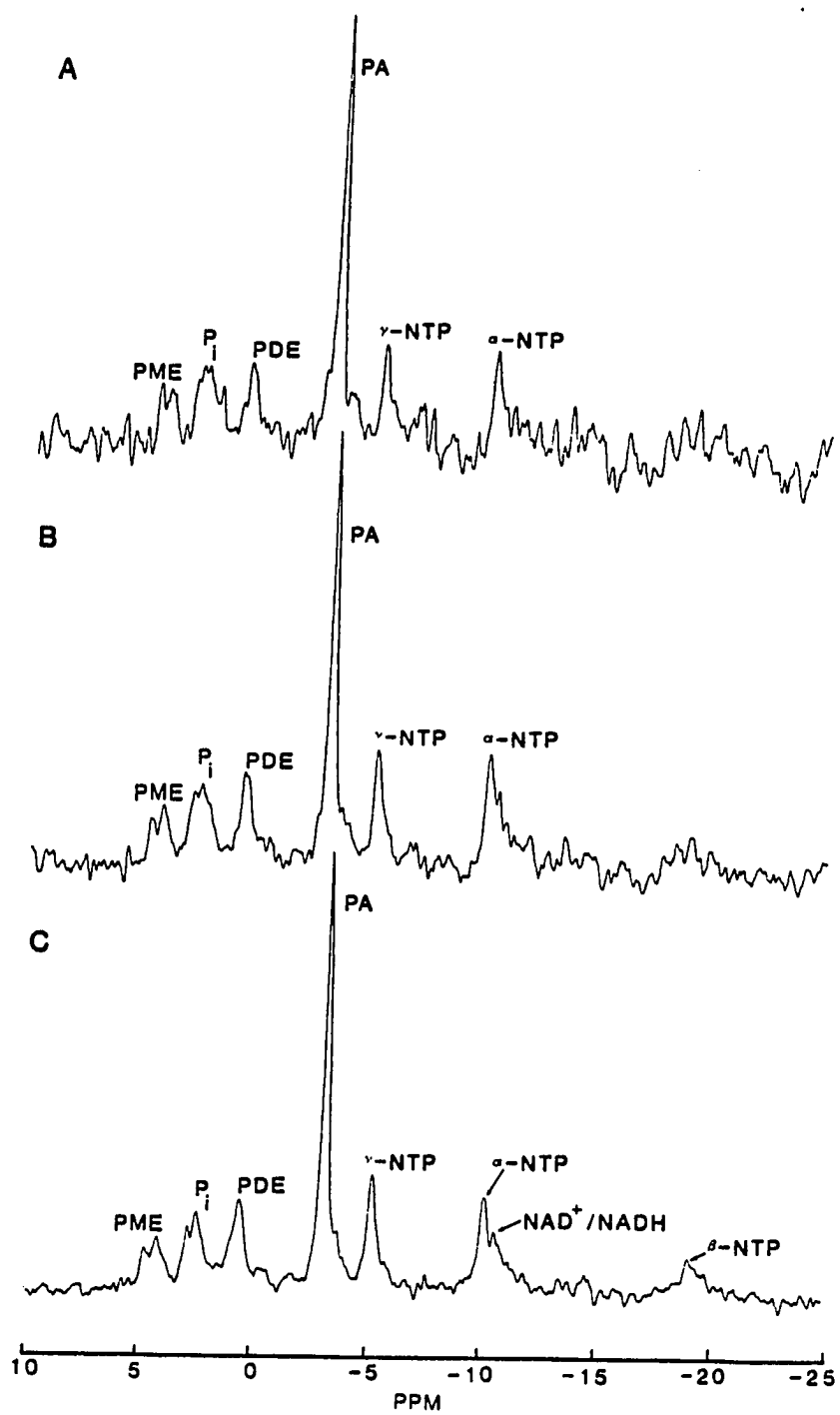
Figure 3. Electron micrograph of <u>Callinectus sapidus</u> spermatozoa.

[A) Electron micrograph of a mature <u>C</u>. <u>sapidus</u> sperm which

has been freed from a female seminal receptacle. The acrosomal

region (AR) consists of the acrosomal vesicle and subacrosomal

tubule. The central region (CR) is primarily composed of

lamellae. The nuclear region (NR) consists of diffuse material

which extend into radial arms. B) Section through a

spermatophore. Spermatozoa are tightly packed together with

very little material in the extracellular space (arrows).

In this micrograph are sections of at least six spermatozoa.]
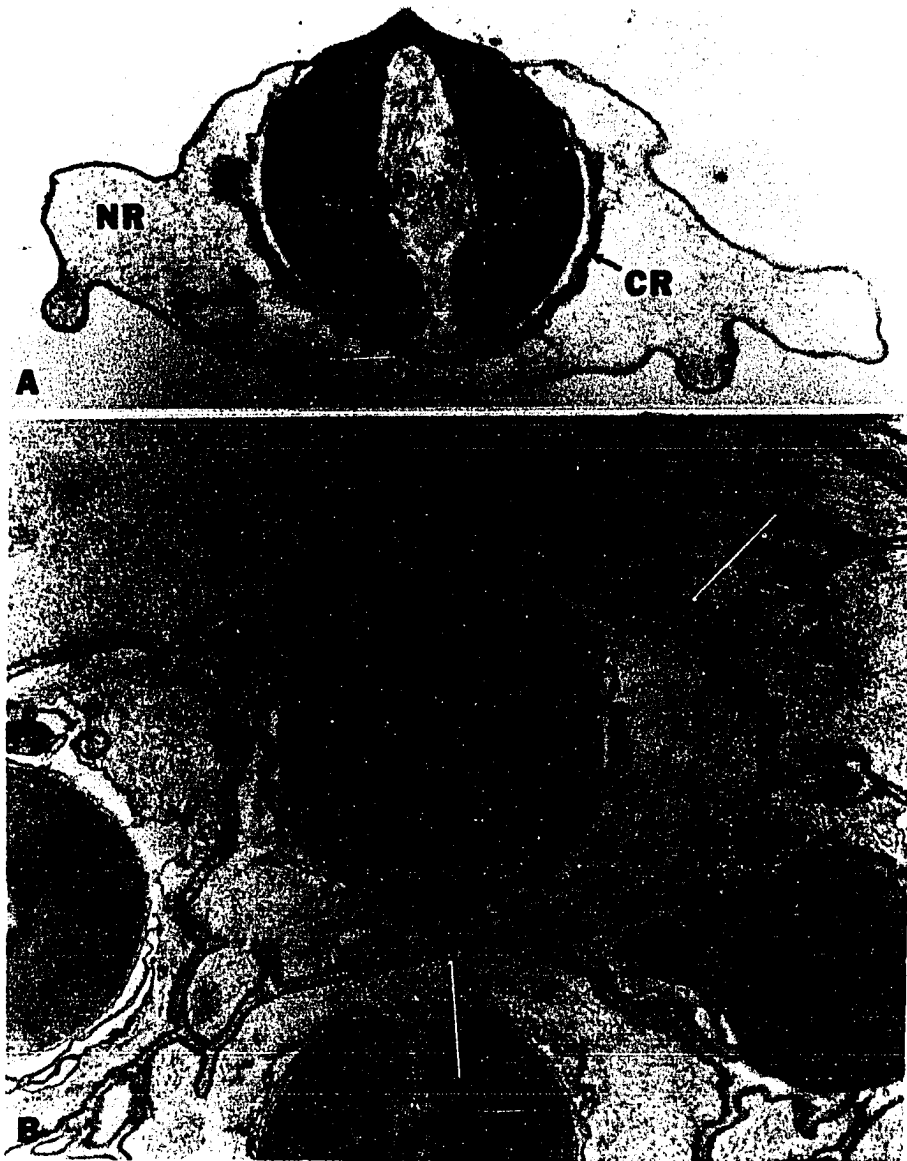
DISCUSSION

Spermatozoa in the spermatophores of C. sapidus were found to possess high levels of NTP, PME, and UDPG and were found to be insensitive to anaerobic conditions revealing only modest spectral changes after 2 days of anoxia. Since they also do not take up detectable amounts of oxygen, these spermatozoa are probably metabolically inert. Because of their long storage period (as long as 1-2 years) both in the vas deferens and the seminal receptacle, such conservation of energy has probably been a selective evolutionary feature. Also, as demonstrated in the electron micrographs, these mature spermatozoa lack typical mitochondria.

In sperm studies on other species of decapods, mitochondria have been observed to degenerate during spermiogenesis and to be absent in the mature spermatozoa (Moses, 1961; Anderson and Ellis, 1967; Langreth, 1969; Pearson and Walker, 1975; Lopez-Camps et al., 1981; Goudeau, 1982). Pearson and Walker (1975) in their studies were unable to demonstrate the presence of cytochrome c oxidase activity of the electron transport system in the mature sperm of Carcinus maenas, indicating a probable lack of oxidative activity. Therefore, high energy compounds would need to be synthesized during spermiogenesis and stored in the mature sperm. Presumably the NTPs are stored in the lamellae of the central region. Wherever located, they are free nucleotides as is indicated by the sharp resonance we observe and are

not bound to another macromolecule.

It is interesting to speculate on why significant levels of ATP are found in the $\underline{C}$. $\underline{sapidus}$ sperm since these high energy compounds are apparently not used for maintenance and since these cells are devoid of motility. However, judging from studies on brachyuran sperm penetration (Brown, 1966; Goudeau, 1982), considerable energy is probably being expended during these events. Since the sperm attaches to the egg chorion and undergoes an activation process (acrosomal reaction) before entering the egg cytoplasm, a great deal of energy could be consumed without being replaced. High levels of UDPG in these spermatozoa were also unusual since this resonance was not observed in ^{31}P-NMR studies with spermatozoa of other species (Christen $\underline{et}$ $\underline{al}$., 1983; Smith $\underline{et}$ $\underline{al}$., 1985; Robitaille $\underline{et}$ $\underline{al}$., 1986a,b). UDPG functions as a coenzyme and is found where rapid transfer of glucose would be necessary. This could possibly happen at the time the sperm nucleus comes into contact with the egg plasma membrane. May be a mechanism in light of the absence of mitochondria to provide a local source of energy at the penetration site.

Moreover, it is of interest to note that no phosphagen was observed in $\underline{C}$. $\underline{sapidus}$ spermatozoa. In the comparison with $\underline{L}$. $\underline{polyphemus}$ spermatozoa, one of the major differences is the presence of the phosphagen, phosphoarginine, in $\underline{L}$. $\underline{polyphemus}$ sperm. Phosphagens have also been described in the spermatozoa of sea urchins (Christen $\underline{et}$ $\underline{al}$., 1983) and the trout (Robitaille $\underline{et}$ $\underline{al}$., see part 4). There is some debate that the phosphagens are serving as a shuttle for the flagellated

spermatozoa, however, they are notably absent in mammalian spermatozoa

(Smith et al., 1985; Robitaille et al., 1986b). The lack of a

phosphagen in the non-motile C. sapidus spermatozoa may give indirect

support to the shuttle hypothesis as described for the flagellated

spermatozoa of several invertebrates and the lower vertebrates.

However, it should be mentioned that serious diffusional arguments exist

against the shuttle hypothesis (Meyer et al., 1984). It is likely that

further ^{31}P-NMR studies may shed some light on this issue.

ACKNOWLEDGEMENTS

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PART 6: ^{31}P-NMR STUDY OF UNFERTILIZED EGGS ISOLATED FROM THE

HORSESHOE CRAB, <u>Limulus</u> <u>polyphemus</u> L. AND THE BLUE CRAB,

<u>Callinectus</u> <u>sapidus</u>

^{31}P-NMR study of unfertilized eggs isolated from

the horseshoe crab, Limulus polyphemus L. and

the blue crab, Callinectus sapidus

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ABSTRACT

Unfertilized eggs were isolated from the horseshoe crab, Limulus polyphemus and the blue crab, Callinectes sapidus, and examined by phosphorus-31 nuclear magnetic resonance. Eggs of both species displayed resonances corresponding to phosphomonoesters, phosphodiesters, inorganic phosphate, phosphoarginine, and nucleotide di- and triphosphates. In addition, L. polyphemus eggs also possessed resonances corresponding to uridine diphosphoglucose and nicotinamide adenine dinucleotide. Eggs of C. sapidus displayed an increasingly acidic intracellular pH with egg maturation falling from 6.9 ± 0.2 to 6.4 ± 0.2. Maturation was not examined Limulus polyphemus eggs. Fertilization did not result in significant changes in that species.

INTRODUCTION

Fertilization and the steps which precede and follow it are perhaps the most fascinating phenomena in the biological world. Consequently, it is not surprising that these events have been the focus of numerous studies. In <u>Limulus polyphemus</u> and <u>Callinectus sapidus</u> considerable attention has been given to egg morphology prior to, during, and following fertilization (Shoger and Brown, 1970; Brown and Barnum, 1983; Barnum <u>et al</u>., 1985; and Brown, 1966). Morphological studies have also centered on the ovary of the lobster and on fertilization events in the decapod <u>Carcinus maenas</u> (Talbot, 1981; Goudeau and Beeker, 1982). However, little attention has been given to metabolic changes in these species with regard to fertilization with one notable exception (Bannon <u>et al</u>., 1981).

Echinoderm eggs have been the subject of several ^{31}P-NMR studies (Inoque and Yoshioka, 1980; Winkler <u>et al</u>., 1982) and the developing embryos of <u>Xenopus laevis</u> have been the focus of a classic NMR report (Colman and Gadian, 1976). The ova of <u>Xenopus laevis</u> have also been examined using NMR imaging microscopy (Aguayo <u>et al</u>., 1986). ^{31}P-NMR has also been used to monitor the intracellular pH in <u>X</u>. <u>laevis</u> eggs (Nuccitelli <u>et al</u>., 1981). An increase in intracellular pH of 0.24 units was noted in this study following fertilization. In sea urchin a pH increase of 0.43 units was observed by ^{31}P-NMR following fertilization (Winkler <u>et al</u>., 1982). The developing egg and larvae of

plaice have also been examined by ^{31}P-NMR (Grasdalen and Jorgensen, 1985). To our knowledge the reports listed above constitute the only applications of ^{31}P-NMR to the analysis of oocytes and embryos. In sea urchin there is considerable indication that an increase in pH following fertilization is important in triggering increased rates in protein and DNA synthesis, resulting in egg activation (Shen and Steinhardt, 1978; Epel et al., 1974; Johnson et al., 1976; Grainger et al., 1979; Winkler and Steinhardt, 1981). In this study, we have selected two species of arthropods for further analyses of Limulus polyphemus and Callinectus sapidus egg metabolism. Particular attention was made to the distribution of high energy phosphorus compounds and intracellular pH values. These two species were selected since 1) eggs are readily available, 2) egg morphology has been examined, 3) life cycles are understood, and 4) the maturation process in the unfertilized eggs have been previously studied. It should also be noted that L. polyphemus tends to have mature eggs year round while C. sapidus is seasonal.

Objectives of this study are: 1) to examine the metabolic profiles in unfertilized eggs, 2) to compare with profiles of other species, 3) to examine possible seasonal changes, 4) to determine application of methods to maturity of animals.

MATERIALS AND METHODS

Animals

Specimens of blue crabs, <u>Callinectes</u> <u>sapidus</u>, were obtained from the Chesapeake Bay, York River region, and shipped to Ames, Iowa. Female crabs were obtained in October, 1985, and May 1986. For the Fall shipment, the crabs had mated and were in the primary molt. For the Spring shipment, the crabs were clean and shiny indicating a recent molt. In both cases, the ovaries of all crabs used in this study were fully extended. Also, the seminal receptacles contained seminal material in various stages of insemination. In general, in the Fall, most seminal receptacles examined were distended. In the Spring, the spermatophoric mass had been absorbed, but the spermatozoa were not freed. Approximately 12 animals were used in this study.

Specimens of mature <u>Limulus</u> <u>polyphemus</u> females were obtained in May, 1985 from the Gulf Coast Specimens. They were collected directly from the breeding areas near the Oklocknee River Mouth area. Approximately 6 animals were used in this study.

All animals were kept at 17-18°C in 150 gal aquaria using artificial sea water (ASW) from Jungle Laboratories Corporation, Comfort, TX. Animals were fed squid and kept on a 14-10 light/dark cycle.

Collection of eggs

Eggs of C. sapidus were collected by dissecting the ovaries. The carapace was removed and the entire ovaries were dissected out at 20°C and placed in a 9 cm Petri dish with ASW. The ovaries were dissected with tweezers and eggs were freed into medium. After dissection, the large particles were removed and the whole milieu was passed through a sieve 25 (707 μm) into a 12 cm Petri dish. Eggs were pipetted into a 100 ml beaker and washed with ASW 1-3 times. Eggs were examined for damage. Only egg batches which were not swollen were used in these studies. Using this method, approximately 7 ml of packed eggs could be obtained from each female. In general, eggs had an orange color, were 200-250 μm in diameter, and were fairly opaque. Eggs were collected at room temperature (20-22°C) since collection on ice caused a high percent of swollen eggs. In addition, for NMR studies, portions of the undissected ovary was used.

Eggs of L. polyphemus, were collected by brief electrical stimulation (3-4 V, 0.2-1.0 mA, AC) to a region proximal to the genital pores. Eggs were collected using wooden applicators and immediately placed in ice cold sea water in 9 cm Petri dishes to prevent activation (Brown and Clapper, 1980). Eggs are approximately 2-3 mm in diameter.

NMR spectra collection

For NMR analysis, 3 ml of the prepared sample was pipetted into a 10 mm NMR tube. This tube was inserted into the super conducting magnet of a Bruker WM300 spectrometer operating at 121 MHz for ^{31}P observation. An external capillary containing 0.1 M methylene diphosphonic acid (Alfa Products) a chemical shift reference (δ = 18.69 ppm) in 99.8% D_2O (Columbia) was added to the 10 mm NMR tube to provide a lock signal. All shifts were reported such that 85% phosphoric acid is assigned a chemical shift of 0.00 ppm. Spectra were collected at 1, 5, 10, or 22°C depending on the particular experiment. Spectra were observed using a 78° pulse (10.6 µs), a delay of 0.5 s, a 13,889 Hz sweep width and an aquisition time of 0.59 s without proton decoupling. Spectra were also obtained with a delay of 0.0 s and an aquisition time of 0.037-0.074 s. The number of scans range from 10 to 2,200.

Measurement of oxygen uptake

To supplement studies with NMR, oxygen uptake under various conditions were measured with a Gilson Differential Respirometer. Approximately 2 ml of packed eggs of C. sapidus were added to a 25 ml flask. The uptakes were measured at 20°C for periods of 1 to 3 hr. Oxygen uptake of eggs of L. polyphemus was not measured because eggs will activate approximagely 5-10 minutes after collection initiating an

abortive development.

Intracellular pH measurements

A standard curve was prepared by tritrating a 5 mM solution of inorganic phosphate (P_i) using ^{31}P-NMR. This solution (100 ml) was prepared from 85% phosphoric acid and also contained 0.3 M glycine, 0.15 M potassuim acetate, 40 mM NaCl, 20 mM HEPES, 10 mM EGTA, and 20 mM glycerol. This curve had limiting shifts of 0.72 ppm and 3.18 ppm with a pKa of 6.63 for the second dissociable proton of P_i. The intracellular pH was obtained by noting the chemical shift for P_i in the NMR spectrum and then using this standard curve for obtaining an approximate pH measurement.

RESULTS

The ^{31}P-NMR spectrum of $\underline{C}$. sapidus eggs examined in the Fall at 1°C is shown in Figure 1. Spectra in Figures 1A-D were acquired using collection periods of 0.15 min, 0.77 min, 3.06 min, and 30.61 min respectively. Resonances correspond to P_i (2.12 ppm), PDE (0.79 and -0.50 ppm), ɣ-NTP (-5.90 ppm), α-NTP (-10.04 ppm), β-NTP (-19.53 ppm), β-NDP (-6.60 ppm), and α-NDP (-10.04 ppm). By noting the chemical shift of P_i an intracellular pH value of 6.8±0.2 was calculated. The ratio of NTP/NDP could be calculated directly through integration as 1:3.4.

The ^{31}P-NMR spectra of unfertilized $\underline{C}$. sapidus eggs examined in the Spring is shown in Figure 2. Figure 2A shows the spectrum of blue crab eggs in early Spring collected at 1°C for 44,600 scans (11.4 hr). Spectra were also collected using 50, 100, 250, and 500 scans and identical results were obtained. This spectrum reveals that two distinct pools of eggs (referred to as I and II) are found at this time of year. Presumably these two pools of eggs correspond to eggs at different stages in the maturation process. Resonances correspond to $P_{i(I)}$ (2.27 ppm), $P_{i(II)}$ (1.61 ppm), P_o (1.11 ppm), and PDE (0.25 and -0.36 ppm). From the chemical shift of $P_{i(I)}$ and $P_{i(II)}$ intracellular pH values of 6.9±0.2 and 6.4±0.2 respectively could be calculated for pools I and II, respectively. Further resonances corresponding to the more alkaline pool (I) include the NTP peaks at -5.49, -10.02, and -19.34 ppm and the NDP peaks at -6.02 and -9.69 ppm. Further resonances

Figure 1. ^{31}P-NMR spectra of blue crab eggs examined in the Fall

at 1°C. [These spectra (A-D) reveal an improvement

in signal to noise (S/N) with increasing number of scans

corresponding to 9 scans (0.15 min), 50 scans (0.77 min),

200 scans (3.06 min), and 2,000 scans (30.6 min).

Resonances correspond to intracellular inorganic phosphate

(P_i), phosphodiesters (PDE), and nucleotide di- and

triphosphates (NDP/NTP).]
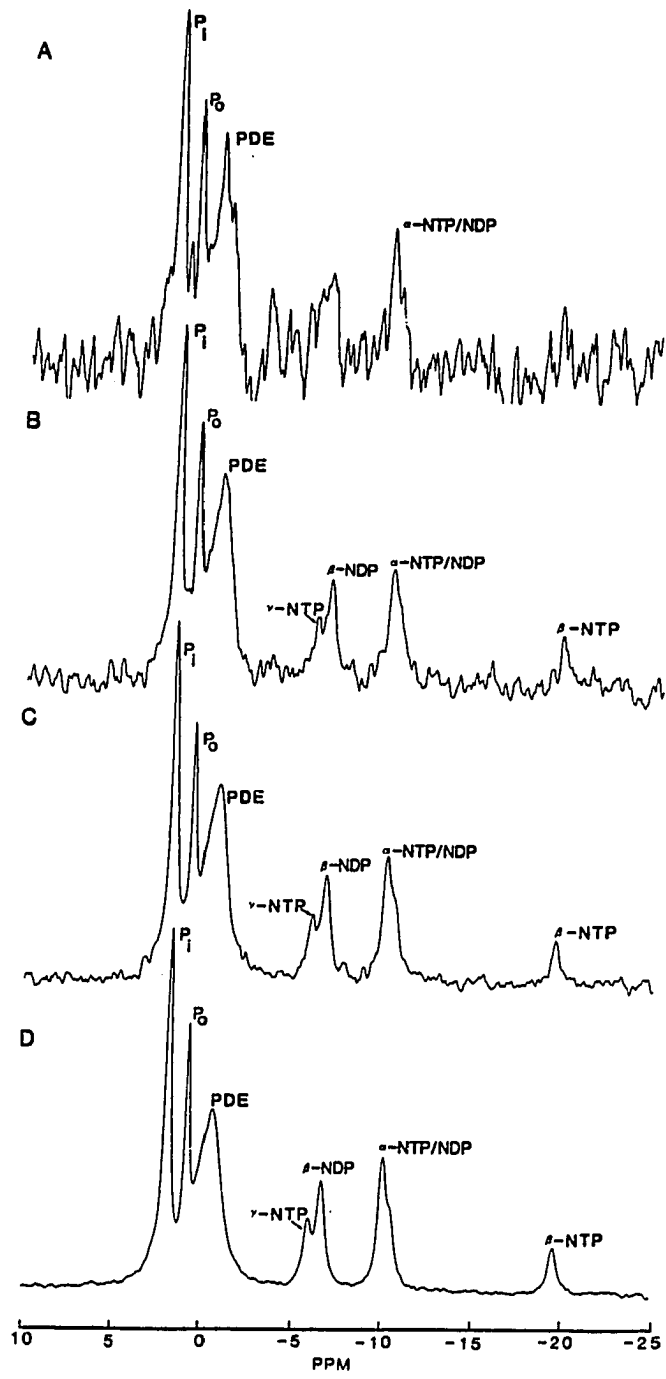
Figure 2. ^{31}P-NMR spectra of blue crab eggs and ovaries observed

during the Spring. [A) Blue crab eggs examined in early

Spring at 1°C. Resonances arise from two distinct pools

of eggs (I and II) having intracellular pH values of

6.9±0.2 and 6.4±0.2, respectively. Resonances

correspond to intracellular inorganic phosphate ($P_{i(I)}$,

$P_{i(II)}$), extracellular inorganic phosphate (P_o),

and nucleotide di- and triphosphates from both pools of

eggs (NDP/NTP$_{(I)}$; NDP/NTP$_{(II)}$). B) Blue crab eggs

isolated and examined three weeks later (late Spring).

Resonance arise principally from pool II. C) Blue crab

ovaries examined four weeks (late Spring) after sample

shown in Figure 2A. D) Blue crab eggs (early Spring) washed

with 10 mM NH$_4$Cl such that the external pH was 8.30.

Resonances in Figures 2B-D are assigned as in Figure 2A.

The resonance labeled with an X has not been identified.]
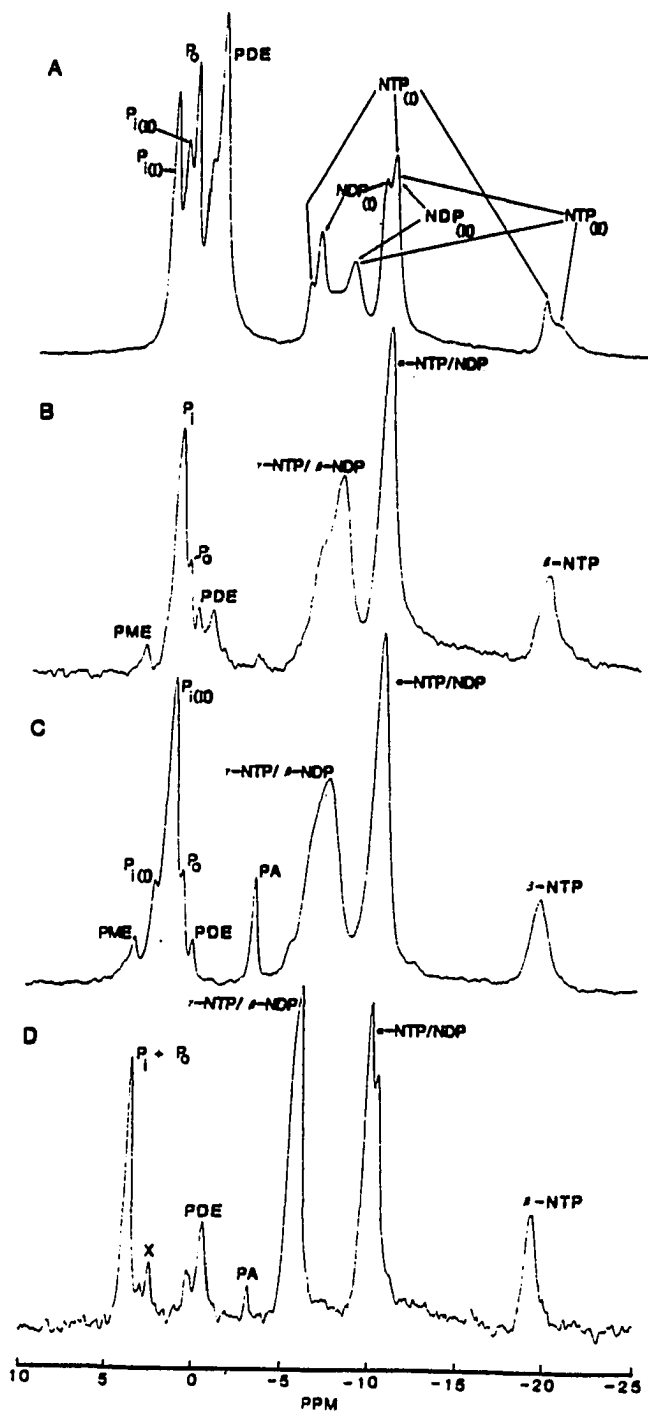
corresponding to the more acidic pool (II) of eggs include NTP peaks at approximately -8.0, -10.02, and -20.0 ppm and overlapping NDP peaks at -8.0 and -10.02 ppm. From the chemical shift of P_o in this spectra an extracellular pH of 5.9±0.2 was calculated. This coincided with the extracellular pH measured directly in this case, although the extracellular pH was found to range from about 6.5 to 5.3 depending on the time of observation.

In Figure 2B the ^{31}P-NMR spectra of fresh eggs taken approximately 3 weeks later. These cells are obviously acidic as seen by the chemical shifts of P_i (1.65 ppm) and NTP/NDP at -7.58, -10.12, and -19.61 ppm. The chemical shift of P_i corresponds to an intracellular pH of 6.4±0.2. Additional resonances correspond to PME (3.39 ppm), P_o (1.03 ppm), PDE (0.44 and -0.38 ppm), and PA (-3.04 ppm). The extracellular pH in this case corresponded to 5.8±0.2.

The ^{31}P-NMR spectra of C. sapidus ovary examined four weeks after sample in Figure 2A is shown in Figure 2C. Less than 1 min had elapsed from the beginning of sample collection to the beginning of NMR acquisition at 22°C. Resonances correspond to PME (3.79 ppm), P_i (1.81 ppm), P_o (1.12 ppm), PDE (0.48 ppm), PA (-3.01 ppm), and NTP (-7.01, -10.00, and -19.29 ppm). The chemical shift of P_i and P_o correspond to intracellular and extracellular pH values of 6.5±0.2 and 5.9±0.2 respectively. Fertilized eggs were not examined since free spermatozoa were not isolated. Figure 2D shows the ^{31}P-NMR spectrum of eggs which had been washed with 10 mM NH_4Cl such that the external pH was 8.30. Prior to washing, these eggs had a ^{31}P-NMR spectrum similar

to that displayed in Figure 2A. These cells were examined at 22°C using 1000 scans (15.3 min) although shorter acquisitions of 50, 100, 250 and 500 scans showed identical results. This spectra displays resonances corresponding to P_i and P_o (3.86 ppm), an unidentified resonance (X, 2.58 ppm), PDE (0.41 and -0.40 ppm), PA (-3.01 ppm), NTP (-5.34, -10.20, and -19.07 ppm), and NDP (-5.71 and -9.70 ppm). Resonances corresponding to NTP/NDP display the expected pattern for alkaline pH values. The unidentified resonance is likely to be buried under the inorganic phosphate peaks in Figures 2A-C.

The spectrum of L. ployphemus eggs acquired at 22°C using 4,000 scans (1.0 hr) is shown in Figure 3. Resonances correspond to PME (3.99 ppm), P_i (2.64 ppm), PDE (0.50 ppm), PA (-3.01 ppm), γ-NTP (-4.94 ppm), α-NTP (-10.09 ppm), β-NTP (-18.72 ppm), UDPG (-10.69 and -12.33 ppm), and NAD^+/NADH (-11.46 ppm). Similar results were obtained using sampling times of less than 10 min. From the chemical shift of P_i an intracellular pH of 7.2±0.2 was calculated (4 determinations). Similar results were also obtained with fertilized eggs and early embryos. Moreover, fertilized and unfertilized eggs showed no significant changes in their ^{31}P-NMR spectra for the first 24 hr following activation.

Figure 3. ^{31}P-NMR spectrum of horseshoe crab eggs acquired for

4,000 scans (1 hr) at 22°C. [Resonances correspond to

phosphomonoesters (PME), intracellular inorganic phosphate

(P$_i$), phosphodiesters (PDE), nucleotide triphosphates

(NTP), uridine diphosphoglucose (UDPG), and nicotinamide

adenine dinucleotide (NAD$^+$/NADH).]
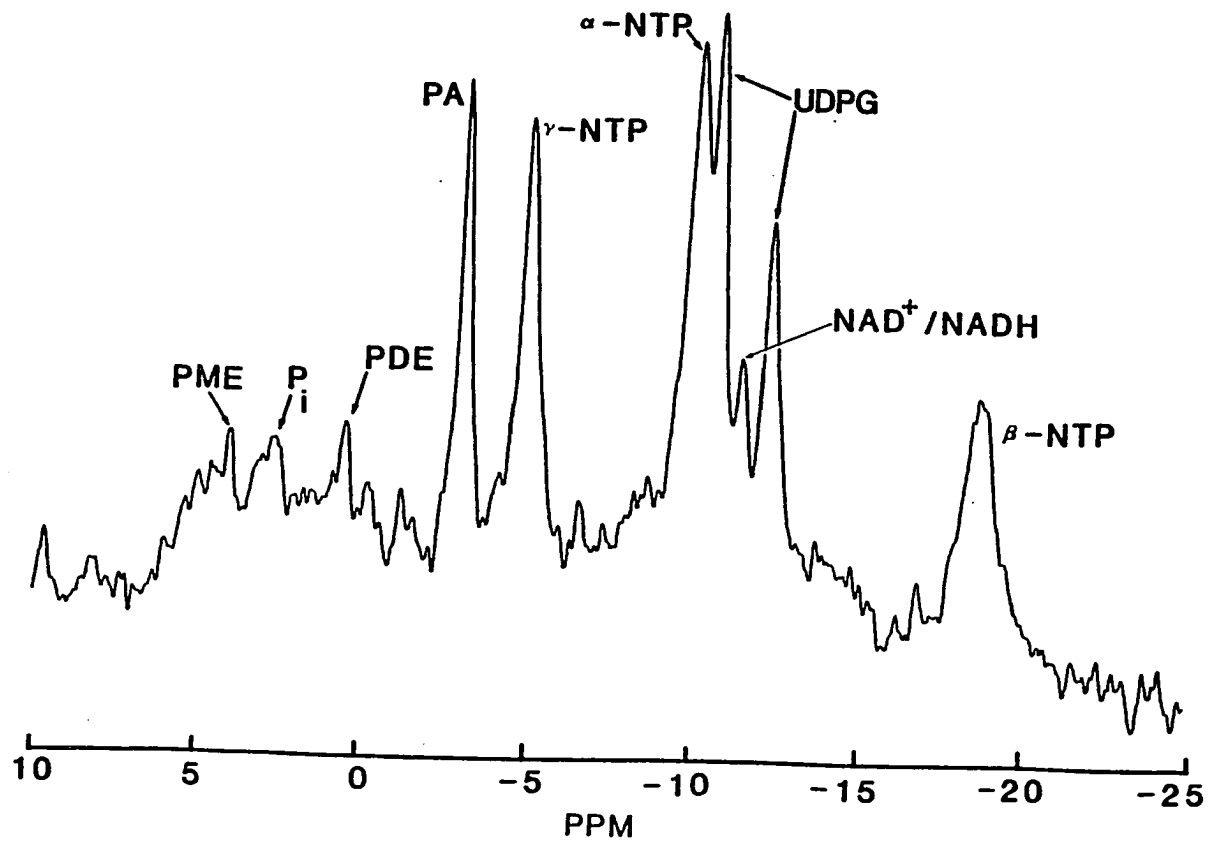
DISCUSSION

We have examined the egg maturation process in <u>Calinectus</u> <u>sapidus</u>. Our results indicate that this process is associated with a drop in intracellular pH of approximately 0.4-0.5 pH units. Such a drop in pH is indicated by a change in P_i chemical shift.

From the ^{31}P-NMR spectra it would appear that the intracellular acidification of blue crab eggs with maturation does not occur gradually, but rather in one step. This conclusion stems from the fact that only two distinct P_i peaks are detected with no intermediatly shifted resonances. Moreover, the increased protonation of γ-NTP results in a shift in the resonances of β and γ-NTP. The lower pH detected with increased egg maturation accounts for the unusual NTP pattern observed in Figures 2A-C. The possibility the resonances at -8.0, -10.02 and -20.0 ppm arise from some metabolite other than NTP has been eliminated. Thus, 6% $HClO_4$ extracts did not reveal the presence of unusual resonances. Moreover, by raising the intracellular pH of eggs displaying these resonance with NH_4Cl, the familiar pattern of resonance for NTP at neutral pH was observed. Note also that the possibility that these signals arose from mitochondrial compartments has been eliminated by valinomycin treatment. To our knowledge, we are the first group to observe such a significant effect of protonation on NTP resonance <u>in</u> <u>vivo</u>.

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SUMMARY DISCUSSION

The focus of this dissertation has been on the application of ^{31}P-NMR spectroscopy to three major cellular systems: 1) the erythrocyte, 2) the spermatozoan, and 3) the ovum.

In part 1, ^{31}P-NMR analysis of sipunculan erythrocytes revealed the presence of O-phosphorylethanolamine, 2-aminoethylphosphonic acid, and nucleotide di- and triphosphates within this cell type. The large concentrations of 2-AEP in T. zostericola erythrocytes were somewhat surprising, re-emphasizing the possible importance of phosphonates in cells. As a result, the T. zostericola erythrocytic system may be ideal for the study of phosphonate metabolism through the use of ^{13}C-enriched pyruvate and ^{13}C-NMR. Such studies would surely lead to a better understanding to phosphonate metabolism in cells.

Sipunculan erythrocytes also displayed the presence of mannose-1-phosphate. This constitutes the first NMR report of the presence of mannose-1-phosphate in a biological system. From a spectroscopic point of view, this finding is of key importance since mannose-1-phosphate resonates at the position expected for inorganic phosphate and titrates in a somewhat parallel fashion to the latter in the neutral range. Thus, the elimination of mannose-1-phosphate as a possible cellular component in in vivo ^{31}P-NMR studies is vital to the proper assignment of inorganic phosphate.

The finding that O-PEA was a potent effector of azide binding to methemerythrin establishes the existance of the first naturally occuring effector of hemerythrin. Since O-PEA was found to such a large extent within sipunculan erythrocytes, this is an exciting result. Unfortunately, no effect on the P_{50} values of the oxygen-hemerythrin interaction were found in the presence of high levels of O-PEA (part 2). However, it is possible that O-PEA, or for that matter 2-AEP, have other hemerythrin-related functions within the sipunculan erythrocyte such as controlling the rates of auto-oxidation of oxyhemerythrin or helping to solublize the octomer within the cells.

In parts 3-5, an extensive ^{31}P-NMR survey of spermatozoa was undertaken. The study of mammalian spermatozoa (part 3) revealed that these cells varied in their display of NMR-detectable free NDP/NTP resonances. Interestingly, cells which did not display free NDP/NTP peaks were still capable of undergoing motility. This finding was explained by invoking the presence of a broad component in the NMR spectra of these cell types. This component was found by perchloric acid extraction to be principally comprised of bound NDP/NTP or free NDP/NTP and bound NDP/NTP in rapid exchange. Moreover, mammalian sperm cells were found to be devoid of any detectable phosphocreatine in either intact cells or within perchloric acid extracts. These findings thus helped to support the ATP diffusional model for motility in mammalian sperm cells at the expense of the phosphocreatine shuttle hypothesis.

In part 4, ^{31}P-NMR was utilized to study fish spermatozoa. This system is ideal for NMR studies since 1) fish spermatozoa are very concentrated and can be obtained in large amounts, 2) the activation process can be triggered specifically by the addition of fresh water or ringer solution, and 3) fish spermatozoa reveal the presence of phophocreatine, free NDP/NTP peaks, and bound (or free and bound) NDP/NTP peaks. As a result, in trying to further study the existance of a phosphocreatine shuttle in striated muscle, cardiac muscle, and spermatozoa, the use of fish spermatozoa may provide a good model. In this regard, NMR diffusion measurements and the use of creatine analogs may be appropriate studies to conduct.

The study of blue crab eggs (part 6) proved to be an extremely fruitful one, from a cellular and spectroscopic point of view. In this respect, not only were free NDP/NTP peaks for the alkaline pool of eggs observed, but the corresponding pattern for the acidic pool was also clearly seen. To our knowledge, no similar spectra has been reported in the literature. Thus, the appearance of two distinct pools of eggs in early Spring and the NMR spectra produced was truly an unexpected result. The fertilization process in this cell type would probably constitute an interesting avenue for future research.

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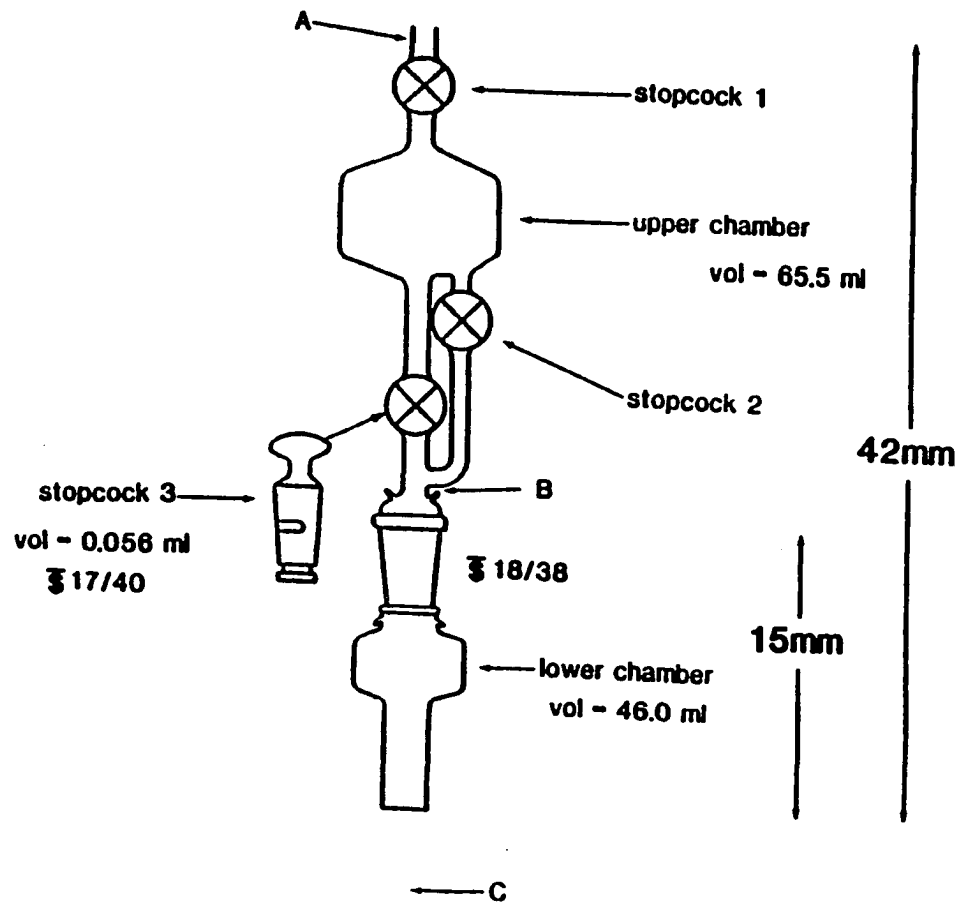
APPENDIX I

Use of tonometer

The tonometer used is essentially that used by Keyes, Mizukami, and Lumry (1967). This tonometer is shown in Figure A1. The stopcocks were purchased from Ace Glass and included a solid stopcock (special order). The solid stopcock (5) was bored using a diamond tip 1/8" drill. The volume of the partially bored stopcock was determined to be 0.056 ml using mercury metal (d=13.5939 g/ml) and weighing by difference as described by Keyes et al. (1967). The volumes of the upper and lower chambers were determined to be 65.5±0.1 ml and 46.0±0.1 ml, respectively. These volumes were determined using distilled H_2O (d=1.00 g/ml) and weighing by difference.

Here are some important guidelines to follow when using this tonometer.

1. Clamp the tomometer at points A and B. In addition, place a partially opened clamp at point C below the lower chamber as a safety measure.

2. Use high quality grease (Apeizon-N) for the partially bored stopcock.

Figure A1. Schematic representation of the tonometer used to determine the oxygen affinity of hemerythrin in the presence or absence of O-PEA or 2-AEP

A

stopcock 1

upper chamber

vol = 65.5 ml

stopcock 2

stopcock 3

vol = 0.056 ml

$\overline{S}$ 17/40

B

$\overline{S}$ 18/38

42mm

15mm

lower chamber

vol = 46.0 ml

C

3. When inserting the partially bored stopcock, place the hole such that it is opened to the upper chamber. Turn the stopcock several times to insure a good seal. Use a 6" Q-tip to clean out extra grease from the channel leading to the lower chamber and the bore of the partially bored stopcock.

4. Grease the partially bored stopcock thoroughly. If streaks are seen, gas will be able to flow from the upper chamber to the lower one. Avoid greasing too closely to the bore of the partially bored stopcock.

5. Place plenty of vacuum grease on the joint linking the upper and lower chambers. Put the lower chamber into position. Pull a vacuum on the system and remove any excess grease from the joint. Then use two elastic bands to secure the lower chamber to the rest of the tonometer.

6. Make sure that the tonometer is set up such that stopcock 2 is not too far open. For the best results, close the stopcock, pull a vacuum, and then open the stopcock just enough to hear the pitch of the vacuum pump fall. At that point, stopcock 2 is sufficiently opened. This will insure good argon flow into the lower chamber during addition of deoxyHr to the lower chamber.

7. When adding deoxyHr to the lower chamber, make sure that there is an adequately strong flow of argon through stopcocks 1 and 2. Add the deoxyHr using a gas-tight syringe. A 3-4 ml volume will usually be enough. Immediately upon addition of deoxyHr, close the bottom chamber and pull a vacuum. Flush with argon and pull a vacuum at least twice more. Leave under vacuum and close the side arm stopcock. Record the volume of solution delivered to the tonometer.

8. The ground glass joint linking the lower and upper chamber can leak. To prevent this, wrap it with parafilm and then with duct tape.

9. To fill the upper chamber with oxygen, the argon line must be disconnected from the manifold. The 100% oxygen line is then attached and a vacuum is pulled on the manifold in order to flush out the remaining argon. Make sure that the 100% oxygen tank will not be open to the vacuum. Then fill the manifold with 100% oxygen and repeat the evacuation step twice. The upper chamber of the tonometer can then be filled and the top stopcock closed. During these operations, the top stopcock (stopcock 1) should always remain open.

10. Tape the side arm stopcock (stopcock 2) to prevent it from being opened accidently.

11. Do NOT break the barrel of the partially bored stopcock by tightening it too much.

12. While equilibrating the system, do not bring the hemerythrin solution near the partially bored stopcock. This avoids picking up some of the hemerythrin solution into the bored stopcock the next time that the bolt is turned into the lower chamber.

13. Repeated turns of the partially bored stopcock will cause it to become tighter and more difficult to turn. Once again, make sure that the stopcock barrel does not become broken by force.

Reference

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APPENDIX II

Data for Figure 2.

Best line: Y=a(1)*exp(-.168053 +/- 7.76238E-03 *t)+a(inf)

DATA:
wavelength = 0 nm
a(1) =-.254982

X	Y	Y(calc)	Diff
0	.16	.163558	-3.55822E-03
.96	.194	.201548	-7.54795E-03
1.92	.242	.233878	8.12243E-03
2.803	.269	.259344	9.65628E-03
3.839	.292	.284782	7.21827E-03
4.8	.302	.30473	-2.72954E-03
5.76	.321	.321686	-6.86199E-04
6.719	.331	.336103	-5.10266E-03
7.68	.341	.348397	-7.39685E-03
8.639	.356	.358837	-2.83748E-03
9.599	.366	.367733	-1.73265E-03
14.4	.401	.395866	.0051336
461.573	.42	.418541	1.45936E-03
a(inf)		.418541	

Data for Figure 5.

Best line: Y=a(1)*exp(-.222001 +/- .0191275 *t)+a(inf)

DATA:
wavelength = 0 nm
a(1) =-.161921

X	Y	Y(calc)	Diff
0	.24	.24204	-2.03972E-03
.96	.269	.273119	-.0041191
1.918	.299	.298186	8.13901E-04
2.878	.325	.318489	6.51133E-03
3.836	.346	.334864	.0111364
5 755	.356	.358833	-2.83316E-03
7.673	.364	.374481	-.0104812
9.592	.38	.384708	-4.70752E-03
14.387	.395	.39732	-.0023202
461.203	.412	.403961	8.03936E-03
a(inf)		.403961	

Data for Figure 6.

Best line: Y=a(1)*exp(-.165689 +/- .0149008 *t)+a(inf)

DATA:
wavelength = 0 nm
a(1) =-.136287

X	Y	Y(calc)	Diff
0	.26	.268156	-8.15573E-03
.966	.292	.288313	3.68693E-03
1.931	.308	.305473	2.52736E-03
2.897	.325	.320111	4.88934E-03
3.862	.338	.332572	5.42826E-03
5.793	.352	.352251	-2.50787E-04
8.69	.366	.372147	-6.14718E-03
11.587	.376	.384459	-.0084587
14.484	.393	.392077	9.23127E-04
464.28	.41	.404442	5.55757E-03
a(inf)		.404442	

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