BIOLOGY OF REPRODUCTION 41, 907–920 (1989)

Intracellular pH Regulates Bovine Sperm Motility and Protein Phosphorylation[1]

DANIEL W. CARR[2] and TED S. ACOTT

Departments of Ophthalmology and Biochemistry and Molecular Biology
Oregon Health Sciences University
Portland, Oregon 97201

ABSTRACT

Bovine sperm in neat caudal epididymal fluid become motile in response to either pH elevation or dilution of the fluid. Buffers containing permeant weak acids at physiologic concentrations are able to mimic these effects of caudal fluid. These observations lead to the hypothesis that a pH-dependent epididymal fluid quiescence factor regulates bovine sperm motility by modulating sperm intracellular pH (pH_i). Here, we report that sperm pH_i, measured with the fluorescent pH probe carboxyfluorescein, increases by ~0.4 units in response to either of these motility-initiating manipulations.

At least 26 discrete phosphoprotein bands are distinguishable by sodium dodecylsulfate-polyacrylamide gel electrophoresis after incubation of intact caudal sperm with $^{32}PO_4$. A prominent phosphoprotein, with M_r ~255,000 (pp255) and a relatively high specific radioactivity, is reversibly dephosphorylated in response to elevations in pH_i that initiate sperm motility. Unlike most of the sperm phosphoproteins, the extraction of pp255 requires reducing agents. This phosphoprotein cosediments with the sperm heads but not the tail, midpiece, soluble, or plasma membrane fractions. No other pH_i-dependent phosphorylation changes are apparent in gels of whole sperm extracts. However, subcellular fractionation allows the detection of increased phosphorylation of two plasma membrane phosphoproteins (M_r ~105,000 and 97,000) and decreased phosphorylation of another plasma membrane phosphoprotein (M_r ~120,000) in response to increasing pH_i. This is the first report describing changes in endogenous phosphoproteins from intact motile and nonmotile bovine sperm that are regulated by pH_i.

INTRODUCTION

Several modulators of mammalian sperm motility have been identified and studied, but their exact mechanisms of action in normal sperm physiology have been difficult to establish. Although many motility activators (i.e. caffeine or forskolin) seem to employ cyclic adenosine 3′,5′-monophosphate (cAMP) as a second messenger (for reviews, see Garbers and Kopf, 1980; Tash and Means, 1982; Brokaw, 1987), the physiological first messenger remains elusive. The events involved in the motility initiation of sea urchin sperm are perhaps the best understood. Upon release into sea water, an increase in sodium ions activates an Na^+/H^+ pump that increases internal pH (pH_i) and stimulates motility (Christen et al., 1982, 1983; Lee et al., 1983). But even in sea urchin sperm, a cAMP-dependent phosphorylation is thought to be involved in the regulation of motility (Brokaw, 1987). A relationship between pH_i and cAMP-dependent phosphorylation reactions has been suggested, but has yet to be fully elucidated (Brokaw, 1987; Goltz et al., 1988).

A change in pH_i at the time of ejaculation has been hypothesized to regulate the initiation of bovine sperm motility (Acott and Carr, 1984). Mature bovine sperm are stored in the cauda epididymidis prior to ejaculation and, when examined in undiluted caudal fluid, exhibit minimal motility. The pH of this fluid is 5.8; if the pH is elevated to approximately 7.0 (without dilution) or the fluid is diluted approximately 1:10 (without pH change), the sperm become vigorously motile (Acott and Carr, 1984; Carr and Acott, 1984). Both of these manipulations (dilution at same pH and an elevation of fluid pH) would be expected to increase internal pH if there were permeant weak acids in the fluid (Babcock et al., 1983). Both dilution and an elevation of pH occur during ejaculation; either is sufficient to produce reversible motility initiation. These observations lead to the conclusion that bovine sperm are quiescent due to a pH-dependent motility inhibitor in cauda epididymal

Accepted July 3, 1989.
Received March 9, 1989.
[1]Supported by NIH Grant #H17429.
[2]Reprint requests: D. W. Carr, Department of Ophthalmology, L105, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201.

fluid. Lactate, at concentrations measured in bovine caudal fluid (8 – 10 mM), will mimic the concentration-dependent and pH-dependent effects of the motility quiescence factor (Carr et al., 1985) and appears to be the quiescence factor. Babcock et al. (1983) have also reported changes in bovine sperm motility and respiration under conditions that produce changes in pH_i. During epididymal maturation, bovine sperm develop the capacity for progressive motility (Acott and Hoskins, 1978; Hoskins et al., 1978), and the pH_i of washed caudal sperm is higher than that of washed caput sperm (Vijayaraghavan et al., 1985). However, changes in bovine sperm pH_i have not been reported under conditions appropriate to the initiation of motility at ejaculation. To address this question, a pH-dependent chromophore was used to monitor pH_i under conditions where motility was initiated either by increasing external pH or by dilution using buffered lactate at physiologic levels.

There is an abundance of evidence indicating that protein phosphorylation is involved in motility regulation (Garbers and Kopf, 1980; Tash and Means, 1982; Brokaw, 1987), but the mechanism by which a phosphoprotein affects motility has yet to be determined. An obstacle to the study of protein phosphorylation in mammalian spermatozoa has been the inability to incorporate sufficient amounts of $^{32}PO_4$ into cellular adenosine triphosphate (Babcock et al., 1975). Recently, methods for increased $^{32}PO_4$ uptake into the nucleotide pool of bovine sperm have been described (Noland et al., 1987; Schoff and Lardy, 1987), which facilitates intact sperm protein phosphorylation studies. To evaluate the relationship between sperm pH_i, motility, and protein phosphorylation, we have conducted studies of changes in protein phosphorylation in intact bovine sperm in response to manipulation of sperm pH_i.

MATERIALS AND METHODS

Materials

Bovine epididymides were obtained from local slaughterhouses. Carboxyfluorescein (CF) and carboxyfluoresceindiacetate (CFDA) were from Molecular Probes, Inc. (Junction City, OR); bovine serum albumin (BSA, type V), digitonin, and lactate were from Sigma Chemical Co. (St. Louis, MO); carrier-free [^{32}P]orthophosphate ($^{32}PO_4$) was from Amersham (Arlington Heights, IL).

Sperm Collection and Motility Measurements

Mature bovine sperm were extruded from caudal epididymides by retrograde flush of the vas deferens and were washed once before loading with either CFDA or $^{32}PO_4$. Motility units, defined as the percentage of motile sperm plus their flagellar intensity from 0 to 100 divided by 2, were measured as described by Carr and Acott (1984).

Measurement of pH_i

Sperm pH_i was measured essentially as previously described (Babcock, 1983; Babcock et al., 1983). Briefly, sperm were loaded with CF by incubating for 20 min at room temperature in a buffer containing 2 μM CFDA, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM fructose, and 10 mM (2-[N-morpholino]ethanesulfonic acid) (MES) (pH=6.2). Free CFDA was removed by centrifugation for 3 min at 2000 × *g*, and sperm were resuspended with or without BSA (5 mg/ml) at the indicated pH in a buffer containing 105 mM NaCl, 45 mM KCl, and 20 mM MES. The relative fluorescence intensities were measured at an emission wavelength of 515 nm with excitation at 495 nm with a Farrand Mark I spectrofluorometer and a strip chart recorder. Excitation and emission slits were 10 nm pregrating and 5 nm postgrating. Samples were placed in 3-ml cuvettes and relative fluorescence intensities were measured at room temperature with continuous, gentle stirring. Intracellular pH values were estimated by null-point curve analysis (Babcock, 1983) using digitonin (50 μM, final concentration) to equilibrate the extra- and intracellular spaces. To correct for small differences in CF concentration between tubes, the pH was elevated to 10 (driving all of the chromophore into the high pH form), after the final readings were made, and the fluorescence was measured again. BSA interacts with CF after permeabilization with digitonin and affects fluorescence intensity in a pH-dependent manner. When necessary, corrections were made for this effect by using the BSA quench curves plotted in Figures 3B and 3C. To control for quenching artifacts due to dye-dye interactions within the cell at high CF concentrations, fluorescence measurements were made with sperm that were loaded for different times and at different concentrations of extracellular CFDA. All of the studies presented were made at concentrations below those at which this artifact becomes significant. Solution pH values were determined with a glass combination microelectrode (Micro-electrodes, Inc., Londonderry, NH) inserted directly

into the cuvette using an Ion 85 pH meter (Radiometer, Copenhagen, Denmark).

Protein Phosphorylation Studies

Intact sperm were loaded with $^{32}PO_4$ by a modification of the method of Noland et al. (1987). Washed sperm were resuspended at 1×10^9 sperm/ml in loading buffer containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 20 mM (3-[N-morpholine]propanesulfonic acid) (MOPS) (pH=7.4) and 1 mCi/ml $^{32}PO_4$. Except as indicated, these sperm were incubated for 2 h at 37°C under aerobic conditions. The free $^{32}PO_4$ was then removed by diluting the sperm 10-fold in buffer without $^{32}PO_4$ and centrifuging for 5 min at $1100 \times g$; the sperm were then resuspended in buffer at the pH values indicated in the text. The phosphorylated sperm proteins were prepared for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis by adding 75 μl of sperm to 200 μl of boiling 2% SDS, 50 mM (2-[N-cyclohexylamino]aminoethanesulfonic acid) (CHES) (pH=9.5), and 5% β-mercaptoethanol (SDS/CHES/β-ME). The samples were then spun at $13,000 \times g$ to sediment the DNA. To solubilize under nonreducing conditions, the sperm were boiled in buffer containing 100 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH=6.8), 3% SDS, and 10% glycerol and spun at $13,000 \times g$ for 10 min. The supernatant was then removed, mercaptoethanol (5%) was added, and the samples were reboiled before they were electrophoresed.

$^{32}PO_4$ Uptake and Incorporation

Washed sperm were incubated with $^{32}PO_4$ for various times in loading buffer (MOPS, pH=7.4, same as above) or buffer in which 10 mM sodium bicarbonate was substituted for MOPS. Sperm uptake and macromolecular incorporation of $^{32}PO_4$ were determined by layering an aliquot (100 μl) of this sperm suspension on a step gradient of 100 μl glycerol and 100 μl of silicone oil in a microfuge tube. The sperm were centrifuged for 5 min at $13,000 \times g$, and the interface between the silicone oil and the glycerol (which contained the sperm) was cut out and radiation was measured by Cerenkov counting to determine uptake or added to 0.5 ml of 10% trichloroacetic acid (TCA), centrifuged, washed three times by resuspension in TCA and counted to determine macromolecular incorporation.

Electrophoresis and Data Analysis

SDS-PAGE was conducted on 4 – 12% linear gradient acrylamide slab gels by the method of Laemmli (1970). Dried, Coomassie Brilliant Blue-stained gels were autoradiographed on X-Omat AR5 or OMC film (Kodak, Rochester, NY). Autoradiographs and Coomassie Brilliant Blue-stained gels were scanned and analyzed with a Hoefer densitometer and GS365 software. Individual peak areas were determined by Gaussian integration (Bevington, 1969) of densitometric scans and were corrected for the relative amount of protein applied to each lane, as determined by integration of the whole lane. The ratio of ^{32}P-labeled phosphoprotein per Coomassie Brilliant Blue-stained protein (relative specific radioactivity) was determined by dividing the digitized autoradiography scan by the stained protein scan of the same lane.

Subcellular Fractionation

Sperm were loaded with $^{32}PO_4$ for 2 h at pH 7.4, then resuspended for 30 min at pH 7.4 or 5.5 with 10 mM lactate added. Subcellular fractionation was carried out using two different procedures. The first was a method for separation of heads and tails after sonication as described by Calvin (1976) and the second was a method for isolation of sperm plasma membrane after nitrogen cavitation as described by Noland et al. (1983). In both cases, just before cellular disruption, the sperm were spun down and resuspended in buffer designed to freeze the phosphorylation state of the proteins and inhibit proteases. The buffer contained 100 mM NaF, 15 mM ethylenediaminetetraacetic acid (EDTA), 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 50 mM NaCl, 10 mM benzamidine-HCl, 50 mM MOPS (pH=7.4). The samples were also kept at 4°C after cellular disruption until they were solubilized in boiling SDS/CHES/β-ME. For the sonication method, the sperm were resuspended in 3 ml of buffer, sonicated for 20 s, layered on a sucrose step gradient (3 ml each of 1.6 M, 2.05 M, and 2.2 M sucrose), and spun for 1 h at $91,000 \times g$. The cytosolic fraction remained at the top of the tube (the top 2 ml were collected and concentrated 20:1 before use), membranes were collected from the first interface, tails and midpieces were collected from the 1.6/2.05 M interface, and the heads were in the pellet. The membranes and tails were collected with an 18-g needle attached to a syringe, diluted 1:5 in buffer without sucrose, and centrifuged at $27,000 \times g$. The fractions were resuspended in SDS/CHES/β-ME and boiled for 3 min. The nitro-

gen-bomb method for isolating the plasma membranes was essentially the same as that of Noland et al. (1983). The membrane fractions shown in Figure 7 are equivalent to their band 2. For both separation methods, microscopic analysis of fractions was used to determine contents and purity.

RESULTS

Changes in pH_i and Motility with Changes in Extracellular pH

The motility of bovine caudal sperm, resuspended in either undiluted bovine cauda epididymal fluid or 10 mM lactate in buffer, have identical pH dependencies (Acott and Carr, 1984; Carr and Acott, 1984; Carr et al., 1985). The pH dependence of sperm motility in the presence and absence of 10 mM lactate are quite different (Fig. 1, Panel A). The pH-dependent inhibition of

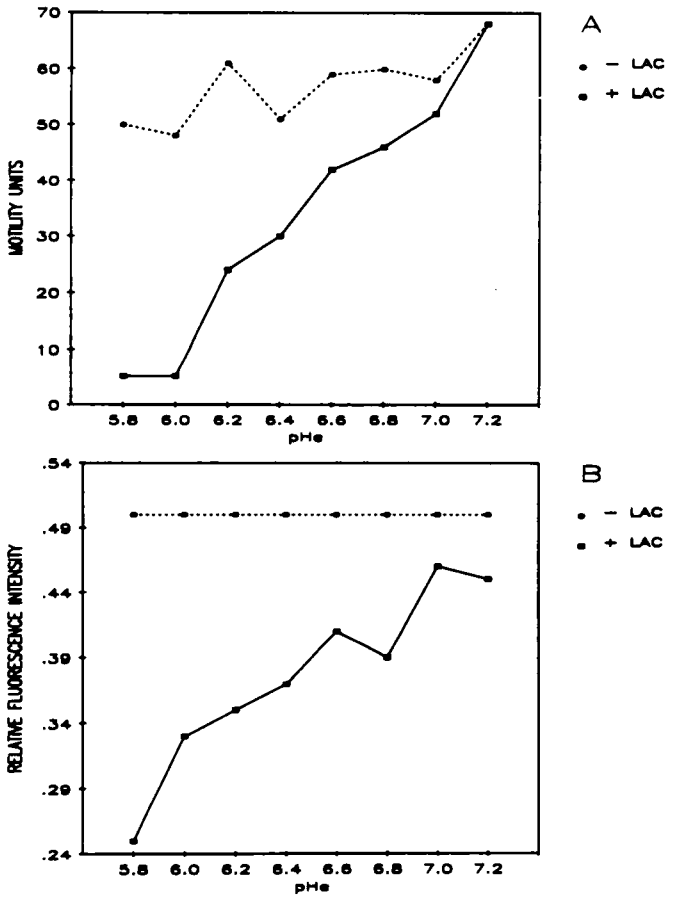


FIG. 1. Dependence of sperm motility and pH_i on extracellular pH with and without lactate present. Sperm motility (*A*) and relative fluorescence intensity of carboxyfluorescein (*B*) were measured at the indicated extracellular pH values in the absence (*dashed lines*) and presence (*solid lines*) of 10 mM lactate. All samples contained BSA (5 mg/ml) and were preincubated for 10 min before measurements were taken. Relative fluorescence intensities were normalized to a common control value.

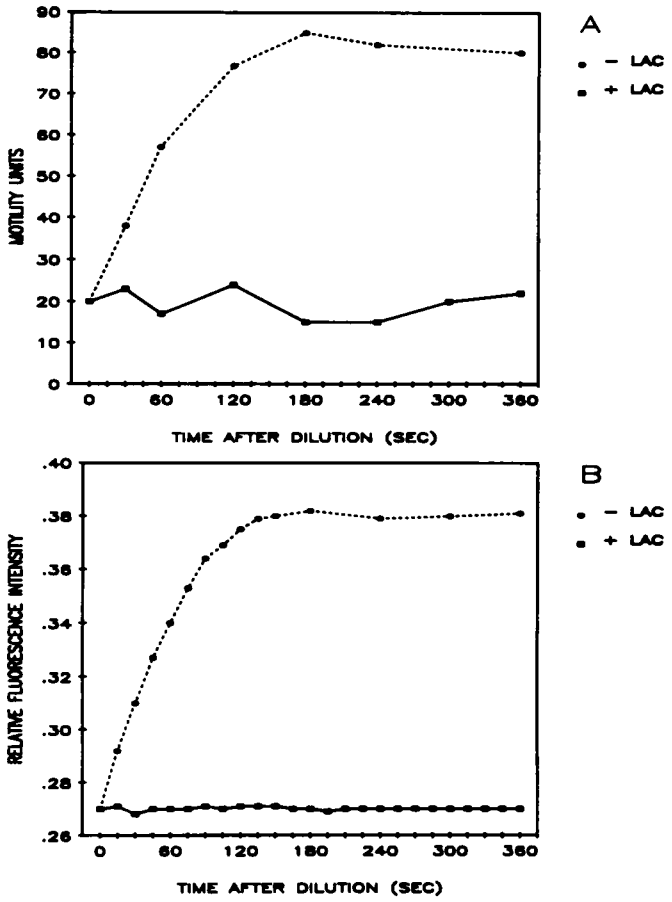
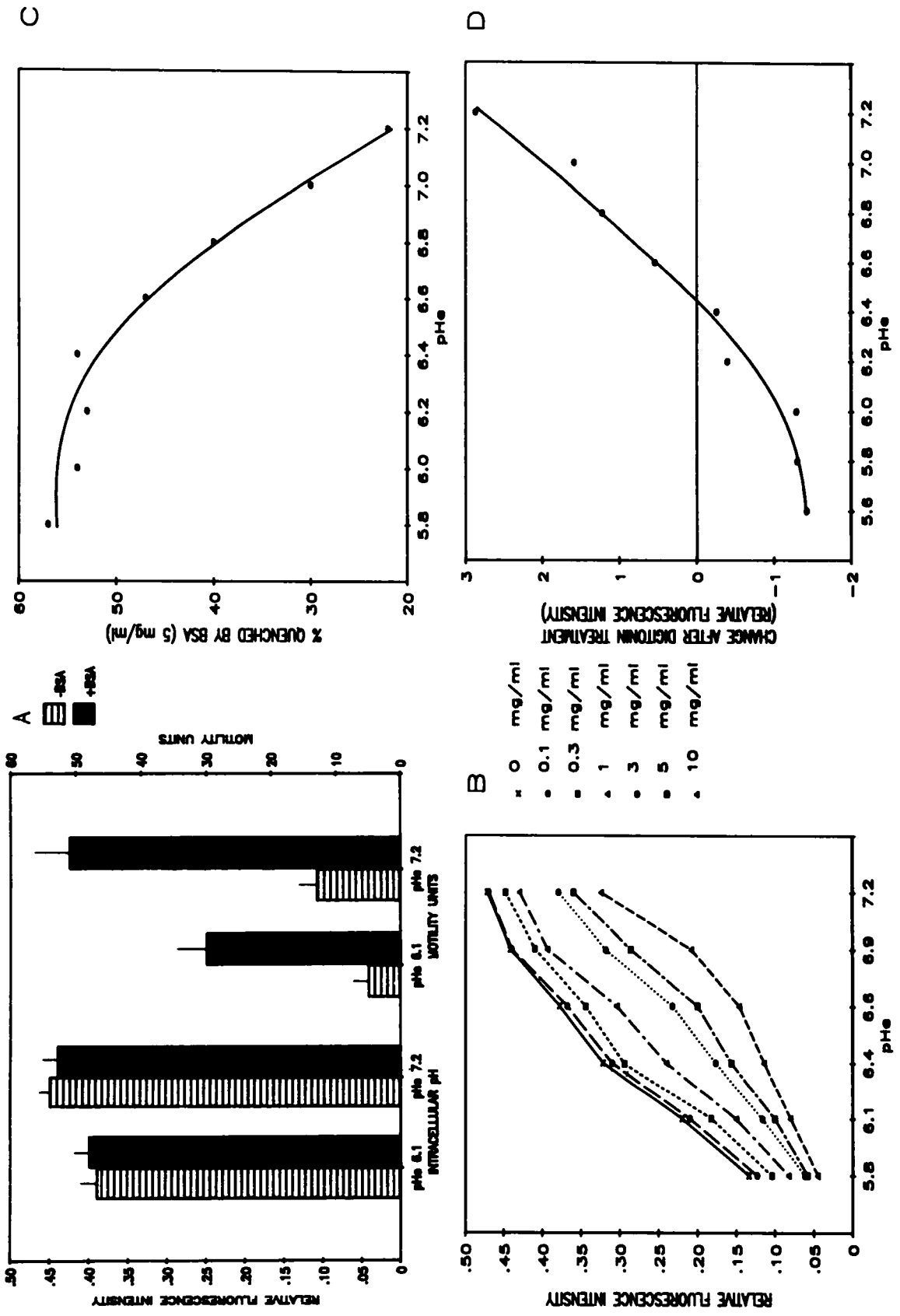


FIG. 2. Time course of sperm pH_i and motility changes following dilution. Sperm in buffer containing 10 mM lactate and BSA (5 mg/ml) at pH 5.8 were diluted 1:10 into the same buffer with (*solid line*) or without (*dashed line*) 10 mM lactate. Motility (*A*) and relative fluorescence intensity (*B*) were determined at the times indicated.

FIG. 3. Effects of BSA on motility and upon absolute pH_i measurements. *A*) Relative fluorescence intensity and motility were measured at pH 6.1 and pH 7.2 in the absence and presence of BSA (5 mg/ml) in buffer containing 10 mM lactate. Values are mean ± SEM. *B*) Relative fluorescence intensity was determined for buffered solutions containing 0.1 μM CF and the indicated concentrations of BSA over the pH range indicated. *C*) Correction curve for BSA effects on absolute pH_i measurements at 5 mg BSA/ml. The percent quenching of the relative fluorescence intensity of CF by 5 mg/ml BSA, when compared to the relative fluorescence intensity measured in the absence of BSA, is shown at the pH values indicated. *D*) Typical null-point curve showing the change in relative fluorescence intensity of CF-loaded sperm that is produced by the permeabilization of the membranes by digitonin (50 μM), thereby allowing equilibration of the intracellular and extracellular compartments.



motility in the presence of lactate is fully reversible (Acott and Carr, 1984). This differential pH dependence of motility in the presence and absence of lactate was correlated with the lowering of the pH_i of sperm, which was reflected in the lowering of the relative fluorescence intensity of carboxyfluorescein-loaded sperm (Fig. 1B). In the absence of lactate, the sperm pH_i and motility were nearly independent of fluid pH. However, in the presence of lactate, both motility and pH_i were reduced at the lower pH.

Changes in Motility and pH_i with Fluid Dilution

A 1:10 dilution of nonmotile sperm suspended in either cauda epididymal fluid or pH 5.8 buffer containing 10 mM lactate into pH 5.8 buffer without lactate allows full motility initiation (Carr and Acott, 1984; Carr et al., 1985). To determine if dilution of the lactate was effecting pH_i, sperm loaded with CF were suspended in pH 5.8 buffer containing 10 mM lactate. At Time 0, the sperm were diluted 1:10 into the same buffer or into pH 5.8 buffer without lactate (Fig. 2). Both motility (Panel A) and relative fluorescence intensity of the CF (Panel B) were monitored from 0 to 360 s. Dilution of the sperm in the presence of lactate produced no change in motility or pH_i. Dilution into buffer without lactate was followed by an increase in motility and a significant intracellular alkalinization.

Effects of BSA and Determination of Absolute pH_i Values

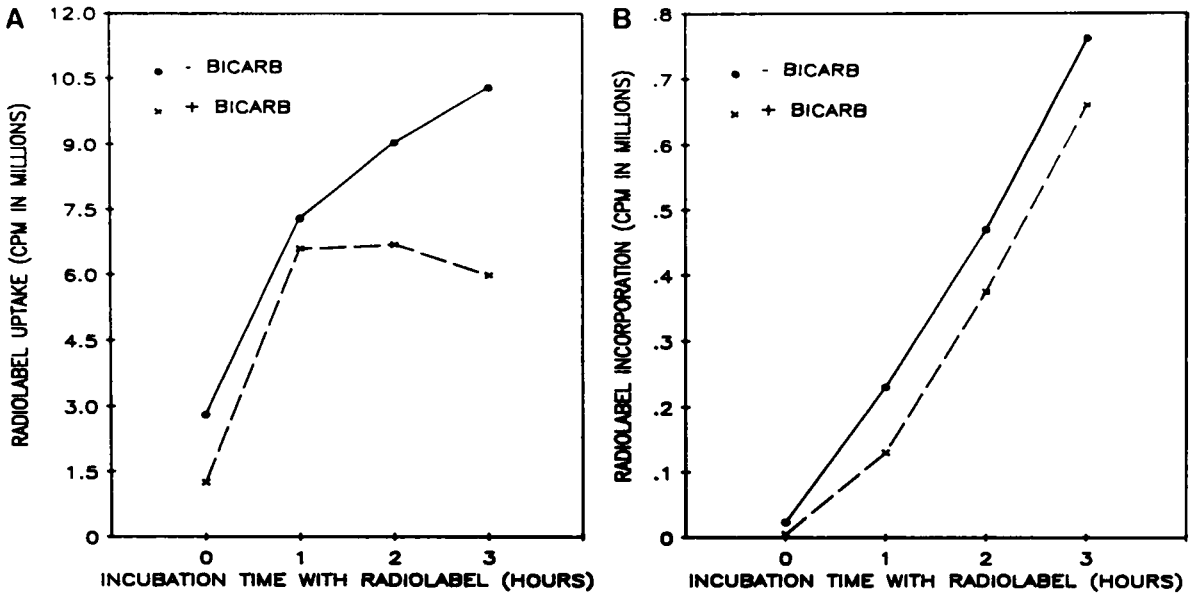
Adding BSA (5 mg/ml) to sperm loaded with CF did not affect the relative fluorescence intensity (pH_i) but it did significantly change sperm motility (Fig. 3A). BSA is known to bind fluorescein (Andersson et al., 1971) and would therefore alter the measurements used in determining the pH_i by the null-point method. Prior to digitonin treatment, BSA did not change intracellular CF fluorescence intensity. However, after the membranes were permeabilized, BSA interacted with and quenched the fluorescence of the extracellular chromophore. Previous studies of sperm pH_i have been conducted in the absence of BSA (Babcock, 1983; Babcock et al., 1983; Vijayaraghavan et al., 1985) to avoid its effects on absolute pH_i measurements. However, the presence of BSA is desirable for motility measurements, partly because it keeps the sperm from sticking to the glass slide and coverslip. Therefore, we quantified the pH-dependent effect of BSA on the fluorescence intensity of CF (Fig. 3B). These curves were used to correct for the quenching by BSA. The percent of quenching caused by 5 mg/ml BSA at various pHs (Fig. 3C) was used to determine the null-point values plotted in Figure 3D. The pH_i value of 6.44 determined by this method was not significantly different from values we obtained using the null-point method without BSA present and was similar, although slightly lower, than values reported for bovine sperm pH_i (6.5 – 6.6, Babcock, 1983; Smith et al., 1985). If the null-point curve had not been corrected for BSA quenching, the apparent pH_i value would have been ~6.9. From null-point determinations, corrected for BSA, the change in pH_i with motility initiation (Figs. 1 or 2) was 0.40 ± 0.05 pH units.

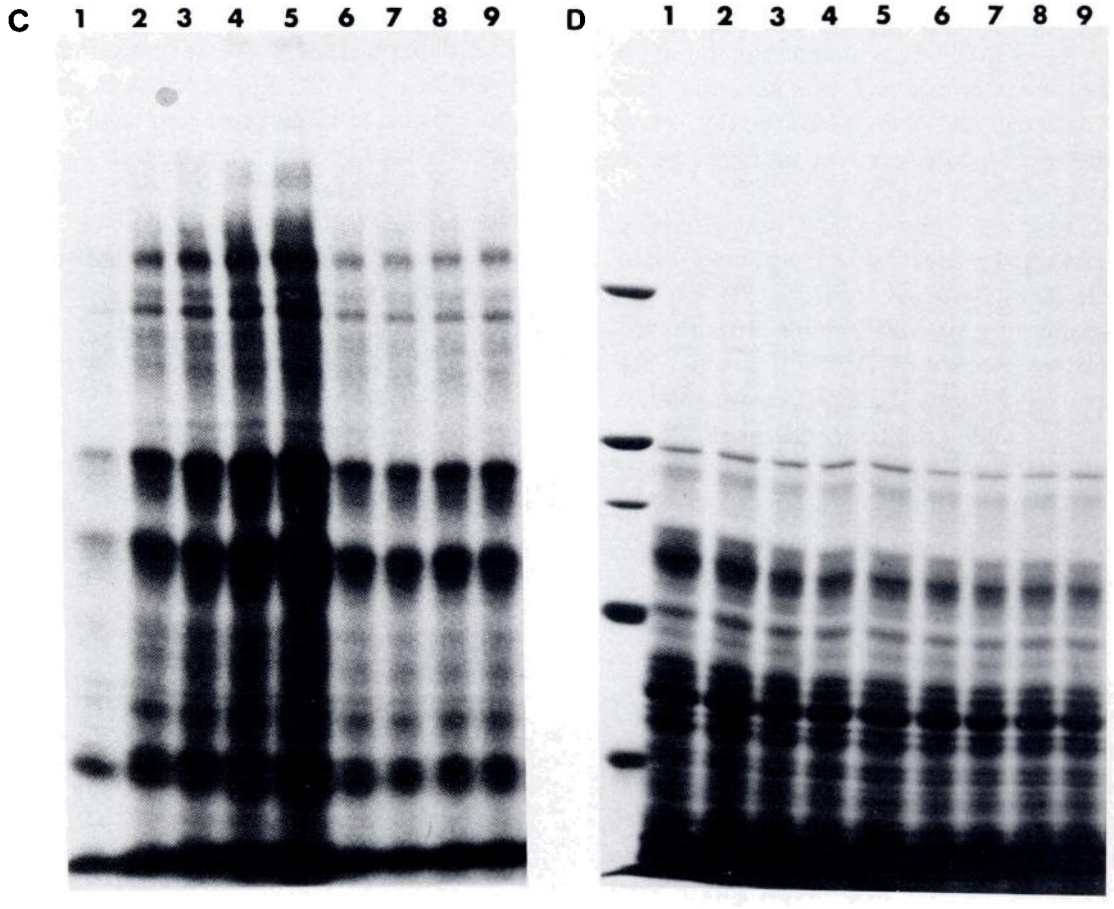
Equilibration of $^{32}PO_4$ into Phosphoproteins

Noland et al. (1987) suggested that bicarbonate facilitates the uptake of $^{32}PO_4$ by intact sperm. To evaluate this suggestion, we measured the uptake of $^{32}PO_4$ by intact sperm in the presence and absence of bicarbonate (Fig. 4A). In the absence of bicarbonate, uptake continued to increase up to at least 3 h, in the presence of bicarbonate, uptake leveled off after 1 h. The addition of 10 mM glucose increased $^{32}PO_4$ uptake both in the presence and absence of bicarbonate, but 25 mM glucose was less effective because it caused a lowering of the pH (data not shown). Incorporation of radiolabel into trichloroacetic acid-precipitable macromolecules also increased for at least 3 h (Fig. 4B), but was slightly less efficient in the presence of bicarbonate than in its absence. After 3 h of continuous exposure, radiolabel incorporation into macromolecules was ~8% of total uptake.

Sperm were incubated for various times in $^{32}PO_4$ and then were solubilized and analyzed by SDS-PAGE.

FIG. 4. Time courses for ^{32}P uptake by sperm and for incorporation into sperm macromolecules. Sperm were incubated in either MOP buffer (pH=7.4) or 10 mM bicarbonate buffer (pH=7.4) for the times indicated. The sperm were then washed and the amount of total radiolabel taken up by the sperm (*A*) or the amount of total radiolabel incorporated into TCA-precipitable material (*B*) was determined. *Panel C* is an autoradiograph of an SDS-PAGE gel (*D*, Coomassie Brilliant Blue-stained). Intact sperm were incubated 1 mCi/ml $^{32}PO_4$ for 1, 2, 3, 4, and 5 h (*Lanes 1 – 5*, respectively) before being solubilized in boiling SDS/CHES/β-ME and applied to the gel. Sperm exposed to radiolabel for 2 h were washed and resuspended in the same buffer without radiolabel, and aliquots were solubilized after 5, 15, 30, or 60 min (*Lanes 6 – 9*, respectively). The unlabeled *left lane* in *D* contains molecular weight markers; M_r = 200,000, 116,000, 97,000, 66,000, and 43,000 from *top* to *bottom*; phospholipids and nucleotides that migrated beyond the marker dye are not shown.





Lanes 1 – 9 of the Coomassie Brilliant Blue-stained gel (Fig. 4D) correspond to equivalent lanes in the autoradiograph (Fig. 4C). Sperm, continuously exposed to radiolabel, showed increasing incorporation of radiolabel into phosphoproteins for at least 5 h (Fig. 4C, Lanes 1 – 5). If sperm are loaded with $^{32}PO_4$ for 2 h, washed, and resuspended in buffer without radiolabel for 5, 15, 30, or 60 min and then analyzed, there was little or no change in the level of protein phosphorylation (Fig. 4C, Lanes 6 – 9). The following studied used this protocol to achieve radiolabel equilibration of the phosphoproteins.

Protein Phosphorylation Patterns

Analysis of autoradiographs (Fig. 5A) and the associated Coomassie Brilliant Blue-stained SDS-PAGE slab gels (Fig. 5B) allowed the identification of at least 26 discrete and reproducible phosphoprotein bands; different exposure times for the autoradiographs were necessary to optimize the identification of all 26 bands. Phosphoproteins were numbered from the top of the gel, as bands 1 through 26 (see numbering of major bands in Fig. 5A) for reference purposes here and later. A larger band of small radiolabeled molecules, phospholipids, and free ^{32}P-phosphate was observed below the dye front (not shown).

Densitometric scans of the lanes of a typical autoradiograph (Fig. 5A) and of the same lane of the dried, Coomassie Brilliant Blue-stained gel (Fig. 5B) showed the level of phosphorylation and staining of each protein band. A relative specific radioactivity profile (Fig. 5C), was obtained by dividing the digitized densitometric scan of the autoradiograph by the scan of the Coomassie Brilliant Blue-stained gel. This allowed comparisons (within one autoradiograph) of the phosphoprotein bands in lanes to which slightly different amounts of protein may have been added. Note the high relative specific radioactivity of band 5 (Fig. 5C).

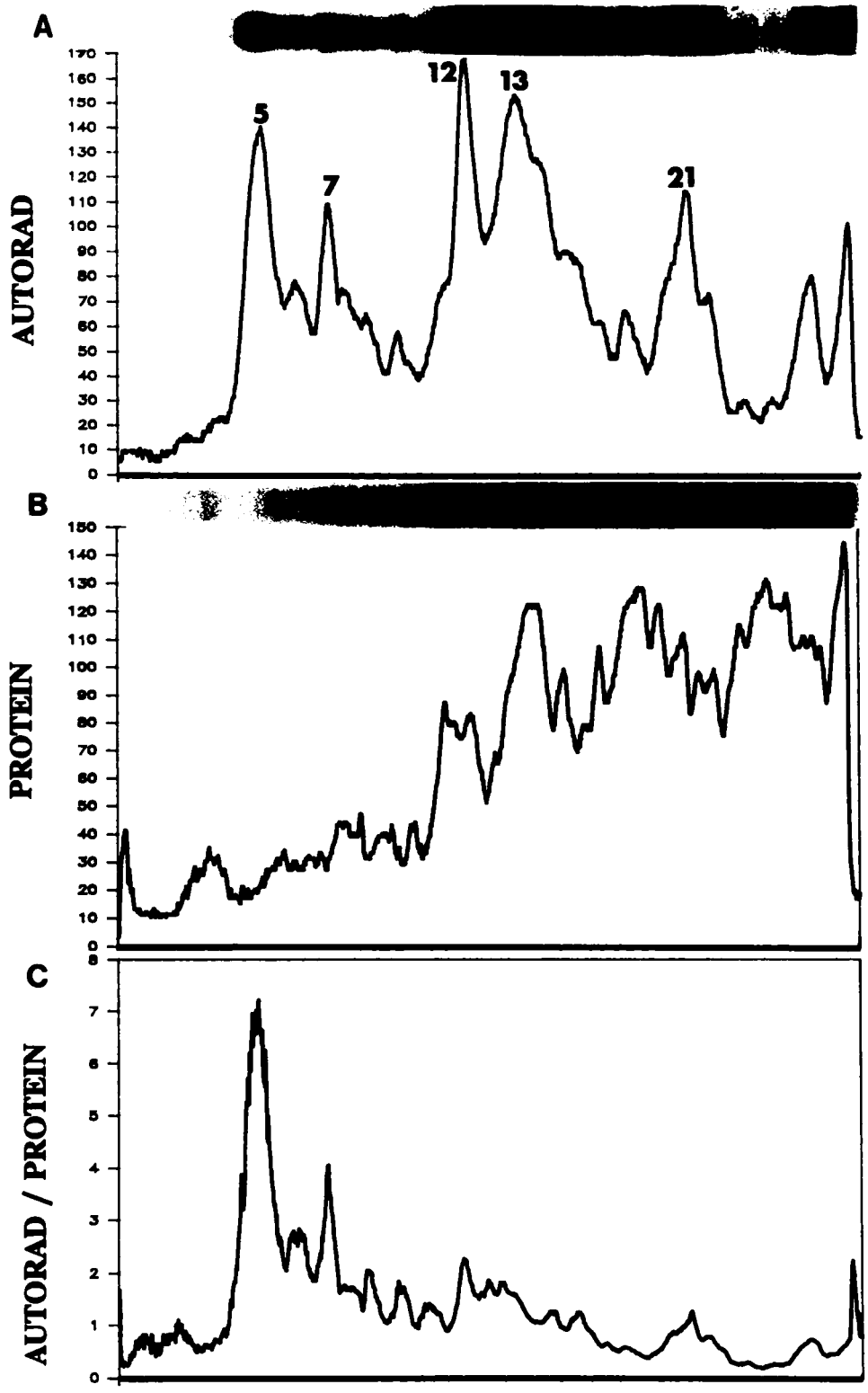
Effect of Sperm pH_i on Protein Phosphorylation

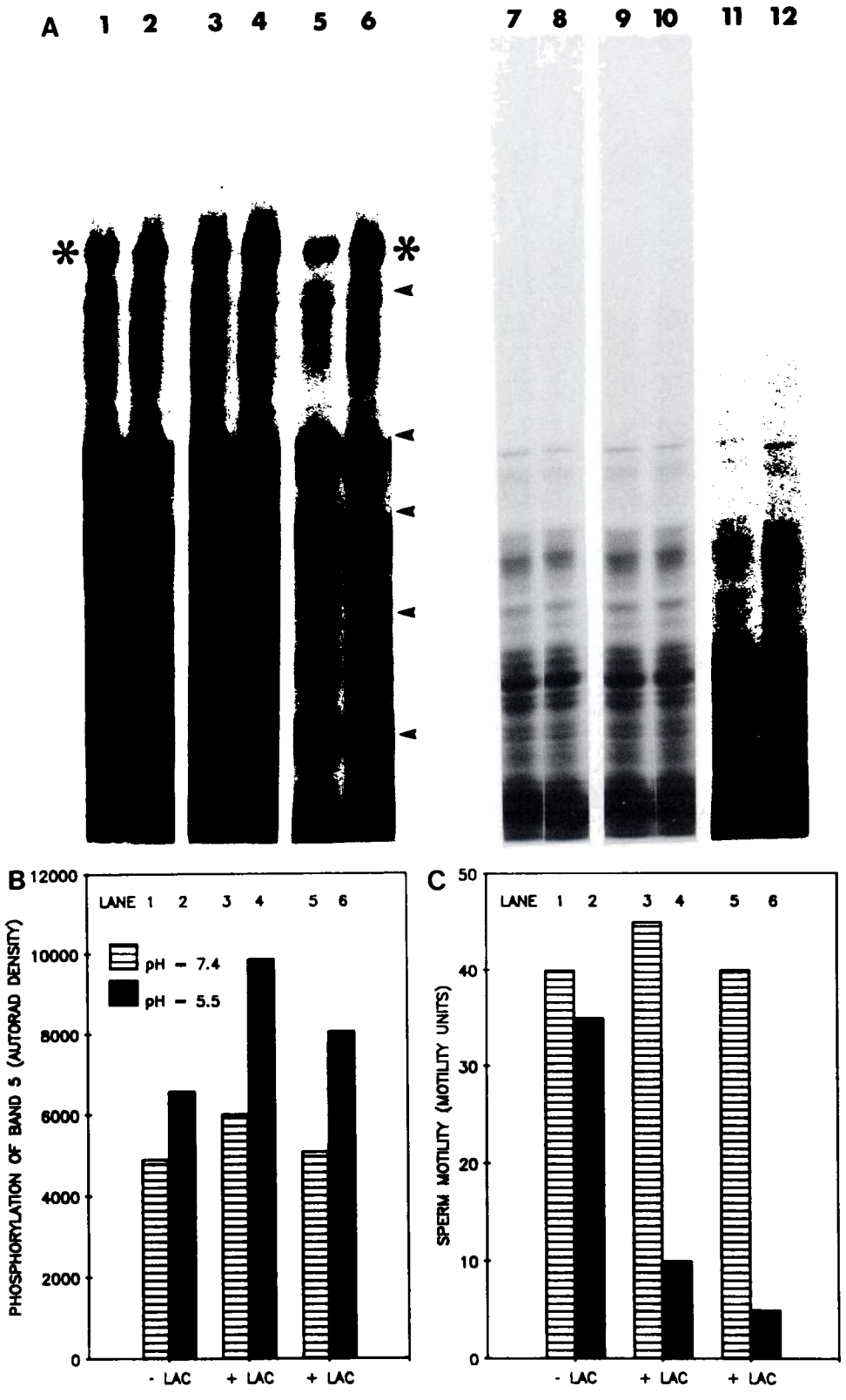
To determine if the phosphoprotein profile of motile sperm was the same or different from that of nonmotile sperm, the sperm were loaded with $^{32}PO_4$ and then resuspended in various buffers designed to modulate their motility by changing their pH_i. When sperm were resuspended at either pH 7.4 or 5.5 in the absence of lactate, there was little difference in either pH_i or motility (Fig. 6C, Lanes 1 and 2, respectively), or in phosphoprotein profiles (Fig. 6A, Lanes 1 and 2, respectively); a small difference in band 5 (between asterisks) was detectable. If sperm were resuspended at either pH 7.4 or 5.5 in the presence of lactate, pH_i was much lower at pH 5.5 and motility was reduced at pH 5.5 (Fig. 6C, Lanes 3 and 4, respectively), and the phosphorylation of band 5 was increased at pH 5.5 (Fig. 6A, Lanes 3 and 4, respectively).

These differences were all reversible. When the sperm initially at high pH_i with lactate (Lane 3) were resuspended at low pH_i with lactate (Lane 6), band 5 phosphorylation increased. When sperm initially at low pH_i with lactate (Lane 4) were resuspended at high pH_i with lactate (Lane 5), band 5 phosphorylation was decreased. Motility also was reversed (Fig. 6C, Lanes 5 and 6) as was sperm pH_i (data not shown). The slightly higher levels of motility observed in this experiment (Fig. 6C), compared to earlier studies (Fig. 3), was due to the different temperatures (37°C compared to room temperature).

These phosphorylation differences were compared (Fig. 6B) by quantitating the autoradiograph densities of band 5 from lanes 1 – 6. In the absence of lactate, resuspension at pH 5.5 increased phosphorylation slightly compared to resuspension at pH 7.4, but in the presence of lactate the increase was more than 60%. This phosphorylation difference was reversible (Fig. 6C) by reversing the pH of the buffer. The values in Panel 6B were normalized to correct for small differences in the total amount of protein applied to each lane. The total protein per lane was determined by integrating the densitometric scan of the entire Coomassie Brilliant Blue-stained lane (Fig. 6A, Lanes 7 – 12). An inverse correlation between phosphorylation of band 5 (pp255) and sperm motility was apparent when Figures 6B and 6C were compared. No other significant

FIG. 5. Identification of discrete phosphoprotein bands and determination of relative specific radioactivity of each band. An extract of whole sperm, loaded for 2 h with radiolabel, was applied to a 4 – 12% linear gradient SDS-polyacrylamide gel. *Panel A* is the autoradiograph of the Coomassie Brilliant Blue-stained proteins in *Panel B* with densitometric scans of both lined up underneath the lanes (units are relative absorbance). The top of the gel is on the *left side* of the *panel*. The digitized scan of the autoradiograph was divided by the scan of the protein to produce the ratio of $^{32}PO_4$ per stain protein (*C*). Approximately 26 discrete phosphoprotein bands were consistently identifiable (using several autoradiographic exposure times) and were labeled 1 – 26 from the *top* to the *bottom* of the gel. The numbers associated with the 5 major phosphoprotein bands are shown (*A*). The M_rs for bands 5, 7, 12, 13, and 21 are 255,000, 190,000, 105,000, 82,000, and 43,000, respectively.





differences in phosphorylation were detectable under these conditions.

Subcellular Localization of pp255 and other Phosphoproteins

To determine where pp255 was located, sperm were loaded with $^{32}PO_4$ and then resuspended under motile (Fig. 7; pH 7.4, odd-numbered lanes) or nonmotile (Fig. 7; pH 5.5 with 10 mM lactate, even-numbered lanes) conditions, followed by sonication and fractionated by sucrose density gradient centrifugation. Panel A shows the autoradiograph of the corresponding proteins in Panel B. The fractions were collected as described in Materials and Methods: whole sperm (Lanes 1 and 2), supernatants from high-speed centrifugation (Lanes 5 and 6), plasma membranes (Lanes 7 and 8), tail fragments and midpieces (Lanes 11 and 12), and heads (Lanes 13 and 14). The pp255 (large asterisks) was found only in the fraction containing the sperm heads.

Fractionation of these phosphoproteins prior to electrophoresis allowed the identification of other pH-dependent phosphorylation changes that were masked in the whole sperm extraction gels. The membrane fractions, obtained by either the sonication method (Fig. 7, Lanes 7 and 8) or by the nitrogen bomb method (Lanes 9 and 10) contained two phosphoproteins (M_r ~105,000 and 97,000; bottom two asterisks next to Lane 8) that were more heavily phosphorylated at high pH_i and one phosphoprotein (M_r ~120,000; top asterisk next to lane 8) that was less heavily phosphorylated at high pH_i. Although other minor pH_i-dependent changes were detectable, these were the only significant differences observed after correction for protein concentration and that showed consistent changes in three separate experiments.

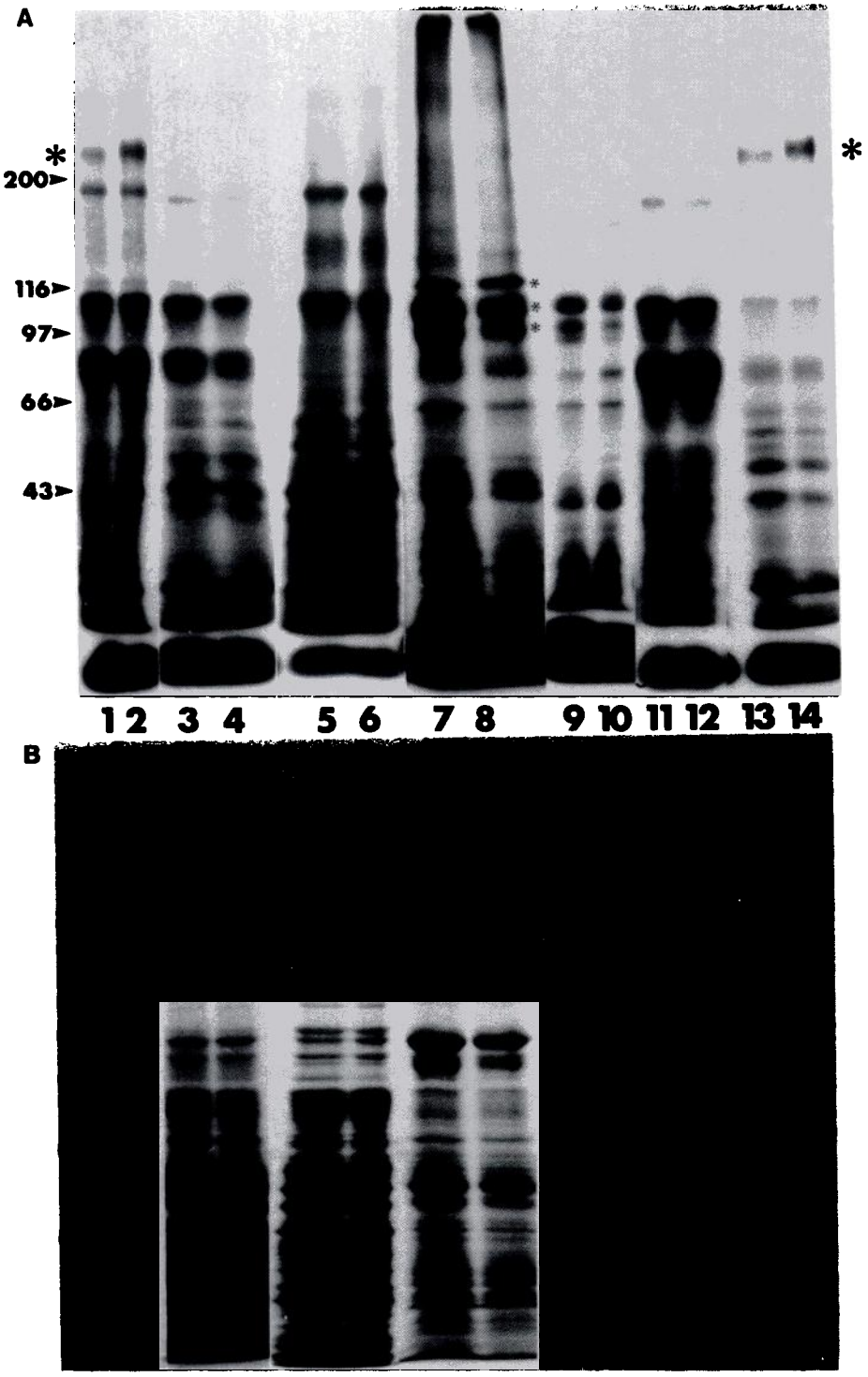
During differential extraction experiments, we observed that pp255 was extracted only in the presence of reducing agents. When whole sperm were extracted with (Fig. 7, Lanes 1 and 2) or without (Lanes 3 and 4) β-mercaptoethanol, this difference was apparent (large asterisks). Although almost all of the other phosphoproteins were solubilized in the absence of reducing agents, pp255 was totally absent under these conditions.

DISCUSSION

The first objective of these studies was to monitor sperm pH_i during motility initiation under conditions previously shown to mimic the initiation of mammalian sperm motility that occurs at ejaculation (Acott and Carr, 1984; Carr and Acott, 1984; Carr et al., 1985). Although motility/pH_i relationships have been reported for bovine sperm (Babcock et al., 1983; Vijayaraghavan et al., 1985), the conditions used in these studies were not selected to address this specific question. The results presented in Figure 1 and 2 provide support for our hypothesis that the initiation of bovine sperm motility at the time of ejaculation is mediated by cytoplasmic alkalinization. The quiescence of sperm prior to ejaculation is due to their acidic pH_i, which in turn is caused by the low pH of the surrounding caudal fluid and a quiescence factor (probably lactate produced by the sperm). The motility of some invertebrate sperm, before and after release, also appears to be regulated via changes in pH_i, although the extracellular modulation appears to be different (Christen et al., 1982, 1983; Lee et al., 1983).

Macromolecular changes associated with pH_i-mediated alterations in motility include changes in phosphorylation levels of at least 4 proteins. Two show a positive correlation with motility (105,000 and 97,000) and two show a negative correlation (120,000 and 255,000). These changes in phosphorylation are probably not due to changes in cAMP levels, since we have previously shown that increasing pH_i does not increase cAMP levels in bovine sperm (Carr and Acott, 1984), and Babcock et al. (1983) have shown that increasing sperm pH_i does not change the activation state of cAMP-dependent protein kinase. Since the pH dependence of sperm protein kinase(s) and phosphoprotein phosphatase(s) with endogenous sperm substrates has not been determined, these activities are likely candi-

FIG. 6. Reversible effects of changes in sperm pH_i on phosphorylation profile and sperm motility. Washed sperm, which had been incubated with $^{32}PO_4$ (1 mCi/ml) for 2 h, were resuspended for 15 min in buffer without radiolabel in the absence of lactate at pH 7.4 (*Lane 1*) or pH 5.5 (*Lane 2*); or in the presence of lactate pH 7.4 (*Lane 3*) or pH 5.5 (*Lane 4*). Sperm from Lanes 3 and 4 were then centrifuged and resuspended with lactate at the other pH to test for reversibility of the changes, i.e. Lane 3 sperm were resuspended at pH 5.5 (*Lane 6*) and Lane 4 sperm were resuspended at pH 7.4 (*Lane 5*). *Lanes 7 – 12* are the proteins stained with Coomassie Brilliant Blue that correspond to the autoradiographs in Lanes 1 – 6. Band 5, pp255, is seen in *Panel A* (between the *asterisks*); the *arrows* indicate the positions of molecular weight standards with M_r = 200,000, 116,000, 97,000, 66,000, and 43,000, respectively from top to bottom. The area under the peak of band 5 (M_r = 255,000) was calculated for each lane by Gaussian integration and corrected for protein concentration (*Panel B*). Motility measurements were performed with aliquots of the same sperm samples that were applied to the various lanes. All incubations were for 15 min at 37°C without BSA.



dates for the point of action of the pH_i change. This difference in phosphorylation could be due to the pH dependence of either enzyme's activity or to pH-induced changes in the charge or conformation of the protein substrates.

Although the co-sedimentation of pp255 with the head fraction may suggest that it is not involved in motility regulation, this role has not been ruled out yet. It is possible that the protein is located at the base of the head or in the neck region and thus would be in a position to exert control over axonemal movement. Also, co-sedimentation studies do not provide positive proof that the protein is located only in the head. We are currently pursuing more detailed localization and characterization of pp255.

A reciprocal relationship between motility and phosphorylation is not surprising for a protein that would regulate flagellar beating. If phosphorylation of the 255,000 protein initiated a flagellar wave (~20 beats/s for mature caudal sperm; Acott et al., 1983) with a dephosphorylation required between beats, direct inhibition by low pH_i of the phosphoprotein phosphatase or of the motility apparatus would be expected to produce a build-up of the phosphorylated protein, but phosphate turnover would be inhibited. Other agents that activate the protein kinase would also be expected to produce a net increase in phosphorylation, but the turnover rate of the phosphate would also be expected to increase. Our method of analysis provides no information on phosphorylation turnover.

In addition to pp255, three membrane-associated phosphoproteins change with pH_i modulation of motility. Because the plasma membrane fraction that we isolated (by either method) is from the whole sperm, one or more of these phosphoproteins may be on the neck, midpiece, or tail. Subsequently, any or all of these phosphoproteins may be involved in the regulation of sperm motility by pH_i. Recently, the pH dependence of cAMP modulation of motility in modeled, demembranated bovine sperm was reported (Goltz et al., 1988); their permeabilization may or may not have removed the membrane phosphoproteins that we have identified.

Much attention has been given to phosphoproteins at M_r ~56,000 that were reported to be involved in motility regulation (Brandt and Hoskins, 1980; Tash and Means, 1982; Tash et al., 1984, 1986; Noland et al., 1987; Paupard et al., 1988). In our system, we can detect a phosphoprotein band at this molecular weight, although it is not a major band. It localizes mostly to the soluble fraction and its phosphorylation state is not significantly changed with modulation of motility by pH_i.

In summary, pH_i is a strong candidate for the intracellular regulator of bovine sperm motility initiation at ejaculation. We find four proteins (pp97, pp105, pp130, and pp255) that show phosphorylation changes with pH_i modulation and suggest that any of these may be causally involved in regulating the bovine motility apparatus. Additional studies are necessary to define these relationships in detail.

FIG. 7. Subcellular localization of sperm proteins and phosphoproteins. Radiolabel-loaded sperm were incubated with 10 mM lactate at pH 7.4 (*odd-numbered lanes*) or pH 5.5 (*even-numbered lanes*) prior to direct extraction or subcellular fractionation (see *Materials and Methods*). An autoradiograph (*A*) was taken of the gel stained with Coomassie Brilliant Blue (*B*); exposure times were optimized individually for each pair of lanes. Whole sperm were extracted in the presence of β-ME (*Lanes 1* and *2*), or in the absence of reducing agents (*Lanes 3* and *4*). The rest of the lanes are subcellular fractions: supernatant from high speed sonication method (*Lanes 5* and *6*), plasma membranes from sonication method (*Lanes 7* and *8*), plasma membranes from nitrogen bomb method (*Lanes 9* and *10*), tails and midpieces from the sonication method (*Lanes 11* and *12*), and heads from the sonication method (*Lanes 13* and *14*). The large *asterisks* indicate the location of band 5, (M_r = 255,000) and the three smaller *asterisks* next to *Lane 8* indicate three plasma membrane bands that change phosphorylation levels in response to change in pH_i.

ACKNOWLEDGMENTS

We thank Dr. Donner Babcock for suggestions and discussions relating to this work and Dr. Charles Lindeman for providing a preprint of his manuscript (Goltz et al., 1988).

REFERENCES

Acott TS, Carr DW, 1984. Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. Biol Reprod 30: 926 – 35

Acott TS, Hoskins DD, 1978. Bovine sperm forward motility protein: partial purification and characterization. J Biol Chem 253:6744 – 50

Acott TS, Katz DF, Hoskins DD, 1983. Motility characteristics of bovine epididymal spermatozoa: effects of forward motility protein and epididymal maturation. Biol Reprod 29:389 – 99

Andersson L-O, Rehnstrom A, Esker DL, 1971. Studies on "nonspecific" binding. The nature of the binding of fluorescein to bovine serum albumin. Eur J Biochem 20:371 – 80

Babcock DF, 1983. Examination of the intracellular ionic environment and of ionophore action by null point measurements employing the fluorescein chromophore. J Biol Chem 258:6380 – 89

Babcock DF, First NL, Lardy HL, 1975. Transport mechanism for succinate and phosphate localized in the plasma membrane of bovine spermatozoa. J Biol Chem 250:6488 – 95

Babcock DF, Rufo GA Jr, Lardy HA, 1983. Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. Proc Natl Acad Sci USA 80:1327 – 31

Bevington PR, 1969. Data Reduction and Error Analysis for the Physical Sciences. New York: McGraw-Hill

Bloom W, Fawcett DW, 1975. A Textbook of Histology. Philadelphia: W. B. Saunders

Brandt H, Hoskins DD, 1980. A cAMP-dependent phosphorylated motility protein in bovine epididymal sperm. J Biol Chem 255:982 – 87

Brokaw CJ, 1987. Regulation of sperm flagellar motility by calcium and cAMP-dependent phosphorylation. J Cell Biochem 35:175 – 84

Calvin HI, 1976. Isolation and Subfractionation of Mammalian Sperm Heads and Tails. Methods in Cell Biol. Vol. 13. New York: Academic Press, pp. 85 – 104

Carr DW, Acott TS, 1984. Inhibition of bovine spermatozoa by caudal epididymal fluid: I. Studies of a sperm motility quiescence factor. Biol Reprod 30: 913 – 25

Carr DW, Usselman MC, Acott TS, 1985. Effect of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. Biol Reprod 33:588 – 95

Christen R, Schackmann RW, Shapiro BM, 1982. Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, Strongylocentrotus purpuratus. J Biol Chem 257:14881 – 90

Christen R, Schackmann RW, Shapiro BM, 1983. Metabolism of sea urchin sperm: interrelationships between intracellular pH, ATPase activity, and mitochondrial respiration. J Biol Chem 258:5392 – 99

Garbers DL, Kopf GS, 1980. The regulation of spermatozoa by calcium and cyclic nucleotides. In: Greengard P, Robison GA (eds.), Advances in Cyclic Nucleotide Research, Vol. 13. NY: Raven Press, pp. 251 – 306

Goltz JS, Gardner TK, Kanous KS, Lindemann CB, 1988. The interaction of pH and cyclic adenosine 3′,5′-monophosphate on activation of motility in Triton X-100 extracted bull sperm. Biol Reprod 39:1129 – 36

Hoskins DD, Brandt H, Acott TS, 1978. Initiation of sperm motility in the mammalian epididymis. Fed Proc 37:2534 – 42

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680 – 85

Lee HC, Johnson C, Epel D, 1983. Changes in internal pH associated with initiation of motility and acrosome reaction of sea urchin sperm. Dev Biol 95:31 – 45

Noland TD, Abumrad NA, Beth AH, Garbers DL, 1987. Protein phosphorylation in intact bovine epididymal spermatozoa: identification of the type II regulatory subunit of cyclic adenosine 3′,5′-monophosphate-dependent protein kinase as an endogenous phosphoprotein. Biol Reprod 37: 171 – 80

Noland TD, Olson GE, Garbers DL, 1983. Purification and partial characterization of plasma membranes from bovine epididymal spermatozoa. Biol Reprod 29:987 – 98

Paupard M-C, MacLeod J, Wasco W, Orr GA, 1988. Major 56,000-Dalton, soluble phosphoprotein present in bovine sperm is the regulatory subunit of a type II cAMP-dependent protein kinase. J Cell Biochem 37:161 – 75

Saacke RG, Almquist JO, 1964. Ultrastructure of Bovine Spermatozoa. II. The neck and tail or normal, ejaculated sperm. Am J Anat 115:163 – 84

Salisbury JL, Aebig KW, Coling DE, 1986. Isolation of the calcium-modulated contractile protein of striated flagellar roots. Methods Enzymol 134: 408 – 14

Salisbury JL, Floyd GL, 1978. Calcium-induced contraction of the Rhizoplast of a Quadriflagellate Green Alga. Science 202:975 – 77

Schoff PK, Lardy HA, 1987. Effects of fluoride and caffeine on the metabolism and motility ejaculated bovine spermatozoa. Biol Reprod 37:1037 – 46

Smith MB, Babcock DF, Lardy HA, 1985. A ^{31}P NMR study of the epididymis and epididymal sperm of the bull and hamster. Biol Reprod 33:1029 – 40

Tash JS, Hidaka H, Means AR, 1986. Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for activation of sperm flagellar motility. J Cell Biol 103:649 – 55

Tash JS, Kakar SS, Means AR, 1984. Flagellar motility requires the cAMP-dependent phosphorylation of a heat-stable NP-40-soluble 56kd protein, axokinin. Cell 38:551 – 59

Tash JS, Means AR, 1982. Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol Reprod 26:745 – 62

Vijayaraghavan S, Critchlow LM, Hoskins DD, 1985. Evidence for a role for cellular alkalinization in the cyclic adenosine 3′,5′-monophosphate-mediated initiation of motility in bovine caput spermatozoa. Biol Reprod 32: 489 – 500