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Comparison of Phosphorylated Proteins in Intact Rat Spermatozoa from Caput and Cauda Epididymidis[1]

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ABSTRACT

Spermatozoa from rat epididymis were incubated with [^{32}P] orthophosphate and the radioactively labeled proteins were solubilized for analysis by electrophoresis in SDS-gels or in two-dimensional gels by isoelectric focusing and SDS electrophoresis. Three major phosphorylated protein bands of M_r 42,700, 56,200, and 76,200 were identified together with several minor phosphorylated proteins. The phosphorylated proteins of M_r 42,700 and 76,200 were more heterogeneous in charge than the one of M_r 56,200. The major phosphorylated proteins were not found in the isolated heads or cytosol derived from sperm sonicate. They were not solubilized by 1% Triton X-100 and 2 mM DTT, which removed the plasma membrane and mitochondria, but they were solubilized by 6 M urea and 5 mM DTT away from the insoluble fibrous sheath which contained no appreciable radioactivity. Most of the major phosphorylated bands were solubilized by 2% SDS and 4 mM DTT, leaving the insoluble outer dense fiber-connecting piece (ODF-CP) complex with some of the proteins. The ODF-CP complex of the spermatozoa from the cauda epididymis contained more of the major phosphorylated bands than did that of the spermatozoa from the caput region. Treatment with 1% SDS alone can solubilize about half of the major phosphorylated bands from the spermatozoa of the caput region and essentially none from the spermatozoa of the caudal part. The latter required 1% SDS and 13 mM DTT to achieve solubilization, suggesting the formation of disulfide bonds holding the three major phosphorylated proteins to some intracellular structure during sperm maturation.

INTRODUCTION

Biological roles of protein kinases are usually mediated by their phosphorylated protein products in intact cells (Greengard, 1978). Thus, to begin assigning the roles of protein kinases present in spermatozoa (Hoskins et al., 1972; Garbers et al., 1973; Hoskins et al., 1974; Lee and Iverson, 1976), it is essential first to identify the sperm proteins that are phosphorylated by these enzymes in intact spermatozoa and second to know the distributions of these phosphoproteins in various cellular compartments. Because live spermatozoa can incorporate adequate isotopic inorganic phosphate from incubation medium into proteins (Babcock et al., 1973), two studies were conducted on the identification of phosphorylated proteins in the intact spermatozoa. First, Huacuja et al. (1977) showed that three proteins of M_r 50,000 80,000 and 105,000 of human sperm membrane were phosphorylated, suggesting the role of protein kinase in membrane function. Second, Tongkao and Chulavatnatol (1979) showed the phosphorylation of microtubules of rat spermatozoa from cauda epididymis. Other studies using demembranated sperm models (Morton, 1973; Tamblyn and First, 1977; Lindemann, 1978) did not show significant phosphorylation of proteins during ATP-reactivation. Brandt and Hoskins (1980) identified in sperm homogenate a putative cAMP-dependent protein kinase substrate of M_r 55,000 associated with cAMP stimulation. However, protein kinases are known to show broad specificity towards proteins and can phosphorylate several proteins in homogenate, some of which may normally be compartmentalized away from the enzymes and thus may not be bona fide substrates for the enzymes. For this reason, the phosphorylated proteins observed using cell homogenate may have little, if any, physiological relevance.

Therefore, this study was designed to

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identify the proteins phosphorylated in intact spermatozoa. To achieve this aim, a technique of total protein solubilization from intact cells after phosphorylation was used, and the analysis of soluble proteins was carried out by two-dimensional (2-D) gel electrophoresis which is the protein analysis technique of highest resolution to date. Distribution of phosphoproteins in different compartments of the spermatozoa was also studied using differential chemical extraction and head-tail fractionation. Comparisons were made between findings in the spermatozoa from the caput epididymis and those of cells from the caudal region.

MATERIALS AND METHODS

Spermatozoa from rat cauda epididymis were freshly extruded into Hank's balanced salt solution containing 70 μM phosphate and 4% bovine serum albumin, pH 5.9, at room temperature by the method described previously (Chulavatnatol et al., 1977). The cells from the caput portion were similarly prepared. Prior to incubation with radioactive inorganic phosphate, the spermatozoa were pelleted using a bench-top centrifuge at 400 × g for 10 min. The supernatant fluid was removed and replaced by fresh buffer of the same composition, and the spermatozoa concentration was determined using a hemocytometer and adjusted to 2×10^8 cells/ml. This was incubated with 1 mCi/ml [^{32}P]orthophosphate (Amersham) at 37°C for 1 h with occasional shaking. During incubation, the spermatozoa showed good motility as judged by inspection under a light microscope. After the incubation, the spermatozoa were again pelleted by centrifugation, and the supernatant fluid was removed. Unless stated otherwise, the packed spermatozoa were then resuspended in 50 mM Tris HCl, pH 7.4, and kept at 0°C for further processing.

Prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the spermatozoa or isolated fractions thereof in 50 mM Tris-HCl, pH 7.4, were completely solubilized with 1.25% SDS 13 mM DTT at 37°C for 15 min. One milliliter of the solubilized preparation was then boiled for 3 min with 0.25 ml of electrophoretic sample buffer (final concentrations: 2% glycerol, 1% 2-mercaptoethanol, 3% SDS, 12.5 mM Tris-HCl, pH 6.8) (Mujica et al., 1978). The resulting clear but viscous solution was further treated with deoxyribonuclease (40 μg/ml, Sigma) at 37°C for 1 h to reduce the viscosity. The proteins in the solution were subjected to the SDS-PAGE according to the method of Laemmli (1970) using 7.5–15% acrylamide (linear gradient) to form slab gels or 7.5% acrylamide to form rod gels. The protein bands were visualized by Coomassie blue staining. The slab gels were dried using a Biorad slab gel dryer prior to autoradiography using medical x-ray film (NS-2T). Rod gels were cut with a razor blade into 2 mm cross sections. Each slice was solubilized by treating overnight with 0.2 ml of 35% hydrogen peroxide at 80°C in a scintillation vial. The radioactivity from the solubilized slice was determined in a Packard scintillation counter after addition of 10 ml of Triton X-114 in xylene (Anderson and McClure, 1973). The following proteins were used as molecular weight markers: bovine serum albumin (M_r 66,000), ovalbumin (45,000), trypsin (23,000), β-lactoglobulin (18,000), myoglobin (17,000), and lysozyme (14,000).

For two-dimensional electrophoresis, the packed spermatozoa after incubation with radioactive inorganic phosphate were resuspended in 0.1 M Tris-HCl containing 0.1 mM EDTA, pH 7.0, to the concentration of 3×10^7 cells/ml and boiled immediately for 5 min to inactivate proteases. The boiled suspension was further incubated with 1% SDS, 0.1 M DTT, and 0.1 mM EDTA, pH 7.0, at 37°C for 1 h. Deoxyribonuclease (50 μg/ml) was added, and the incubation was continued for 1 h more. At this point, the spermatozoa were totally solubilized and the solution was clear but not viscous. Nine volumes of ice-cold ethanol were then added and the solution was left at −20°C for 1 h to allow protein precipitate to form. The precipitate was recovered by centrifugation at 12,000 × g for 10 min in a Sorvall RC-2B centrifuge and further washed with 5 ml of ethanol, followed by 5 ml of methanol: $CHCl_3$ (1:3), and dried under vacuum. The protein precipitation step was designed to remove salts to obtain good resolution in isoelectric focusing gels. The dried protein pellet was redissolved in 9.5 M urea, 2% NP 40, 2% ampholytes (LKB), 5% β-mercaptoethanol. The electrophoresis was carried out according to the method of O'Farrell (1975) with the following modifications. In the first dimension, the isoelectric focusing gel contained ampholytes (20% pI 3–10, 30% pI 6–8, and 50% pI 4–6). The second dimension was SDS-PAGE using a linear gradient of 10–15% acrylamide. As molecular weight markers, the following proteins were included in the second dimension: bovine serum albumin (M_r 66,000), ovalbumin (45,000), concanavalin A (27,000), and myoglobin (17,000). The protein spots in the gel were visualized by Coomassie blue staining. After the gel slab was dried using a Biorad gel dryer, autoradiography was performed using medical x-ray film.

To prepare heads, tails, and cytosolic fraction, the labeled spermatozoa were suspended at 5×10^7 cells/ml in 50 mM Tris-HCl, pH 7.4, containing 2 mM phenylmethylsulfonyl fluoride (PMSF) to prevent proteolysis. The suspension was sonicated at 4°C for 1 min using a sonicator (Heat-Systems Ultrasonics, model W-225) with pulse at 50% duty cycle. The condition was predetermined to achieve complete separation of heads and tails with minimal damage of the latter. The sonicated sample was then centrifuged at 27,000 × g for 20 min in a Sorvall centrifuge. The supernatant fluid was used as cytosolic fraction. The pellet containing heads and tails was resuspended in 50 mM Tris HCl, pH 7.4. Heads and tails were then isolated by centrifugation in a discontinuous sucrose gradient according to Calvin (1979). Each fraction was later solubilized and subjected to SDS-PAGE as described above.

The chemical fractionation of the labeled spermatozoa was carried out at room temperature according to published methods. Plasma membrane and mitochondria were solubilized from the packed labeled spermatozoa by treating with 1% Triton X-100, 2 mM DTT, and 0.1 M NaCl in 25 mM Tris-HCl, pH 9.0, twice, 15 min each (Olson and Sammons, 1980). The preparation of outer dense fiber-connecting piece

TABLE 1. Molecular weights of the three major phosphorylated bands analyzed by SDS-PAGE using rod gels.

Band	n[a]	Mean ± SEM ($M_r \times 10^{-3}$)	Range ($M_r \times 10^{-3}$)
A	14	42.7 ± 2.6	39 – 46
B	20	56.2 ± 2.4	52 – 61
C	13	76.2 ± 3.1	68 – 79

[a]Number of determinations from gels of whole spermatozoa that showed the three bands and from gels of fractionated samples that showed one, two, or three bands.

(ODF-CP) was obtained by treating a suspension of the labeled spermatozoa with an equal volume of 2% SDS/4 mM DTT in 50 mM Tris-HCl, pH 9.0, for 10 min (Olson and Sammons, (1980). The ODF-CP complex was then pelleted by centrifugation at 400 × g for 15 min, and the supernatant fluid was removed. The pellet was washed once with 50 mM Tris-HCl, pH 9.0. Isolation of the fibrous sheath was carried out according to the method of Olson (1979). After removing the plasma membrane and mitochondria as described above, the extracted pellet was treated with 6 M urea, 5 mM DTT in 50 mM Tris-HCl, pH 8.0, for 1–2 h. The fibrous sheath could then be pelleted by centrifugation at 700 × g for 30 min and washed once with the Tris-HCl buffer. Since heads were not removed from the labeled spermatozoa prior to the fractionation, some heads were found contaminating in the preparations of ODF-CP complex and of the fibrous sheath. As would be evident later, the head contamination would not interfere with the study. During the fractionation steps, samples were monitored under a phase-contrast microscope to ensure the completion of the process and the correct appearance of the desired components. Each preparation was solubilized for SDS-PAGE according to the above mentioned procedure.

To study the disulfide linkages of the phosphorylated proteins, a portion of the packed, labeled spermatozoa (10^8 cells) was extracted sequentially with the following solutions, 1 ml each: 0.1% Triton X-100; 1% SDS; 1% SDS and 2.7 mM DTT; 1% SDS and 6.5 mM DTT; and 1% SDS and 13 mM DTT. Each solution was made up in 50 mM Tris HCl, pH 7.4. Treatment with each solution took 5 min at room temperature. The supernatant fluid was separated from the pellet by centrifugation in a bench-top centrifuge at 400 × g for 10 min. The pellet was then treated with the next solution. The process was repeated until the last solution was used to dissolve the pellet from the preceding extraction completely. Again, monitoring the disintegration of the spermatozoa after each treatment was carried out under a phase-contrast microscope. Each fraction was processed further as described above for SDS-PAGE.

RESULTS

Sizes of Phosphorylated Proteins

Upon analysis by SDS-PAGE using rod gels, three major radioactive bands appeared in the total extract of intact spermatozoa for either caput or cauda epididymis after incubating with ^{32}Pi (inorganic phosphate) at 37°C for 1 h. From gels of extracts of whole spermatozoa as well as those of cells fractionated by various chemical methods described in Materials and Methods, the molecular weights of these three bands (A, B, and C) were estimated (Table 1). The experimental values for each band usually fell within 10% of its mean which was considered acceptable for this technique. Among the three bands, band B was consistently observed to be most intensively labeled while the other two bands were about equally labeled. When equal numbers of cells were used, no significant difference in the intensities of the three bands was consistently found between the spermatozoa of the caput region and those of the cauda.

Two-Dimensional Gel Electrophoresis

To analyze more fully the number of phosphorylated proteins in intact spermatozoa, two-dimensional gel electrophoresis of high resolution was employed. From the typical protein staining patterns (Fig. 1A,B), it was evident that there was great similarity between the total solubilized proteins of spermatozoa from the caput epididymis and those of the cells from the caudal. However, five protein spots (marked by arrows in Fig. 1A) were more intensively stained in the pattern of the spermatozoa of the caput region than were those present in the pattern of the spermatozoa of the cauda. One of these proteins had M_r 62,000, three 54,000, and one 52,000.

As seen in the autoradiographic patterns (Fig. 1a,b) of the protein patterns (Fig. 1A,B), several phosphorylated proteins were present in the total extract of intact spermatozoa from both parts of the epididymis. A major radioactive spot (marked X) of M_r 52,000 appeared

to be a single polypeptide and should correspond to band B in SDS-PAGE (Table 1). Also highly radioactive were two streaks of M_r 43,000 and 76,000, corresponding to bands A and C in SDS-PAGE, respectively (Table 1). In addition, there were several proteins of less radioactivity, among them, five (marked by arrows) were found only in the spermatozoa of the caput epididymis. Although it can be seen that the intensities of the spots from the spermatozoa of the cauda epididymis (Fig. 1b) were relatively stronger in this experiment, they cannot be consistently reproduced. By superimposing the autoradiographic pattern on its protein pattern (Fig. 2), it can be seen that most of the radioactive spots or streaks did not superimpose on the stained protein spots or streaks. This suggests that the phosphorylated proteins existed in minute amounts below the sensitivity of detection by the dye staining.

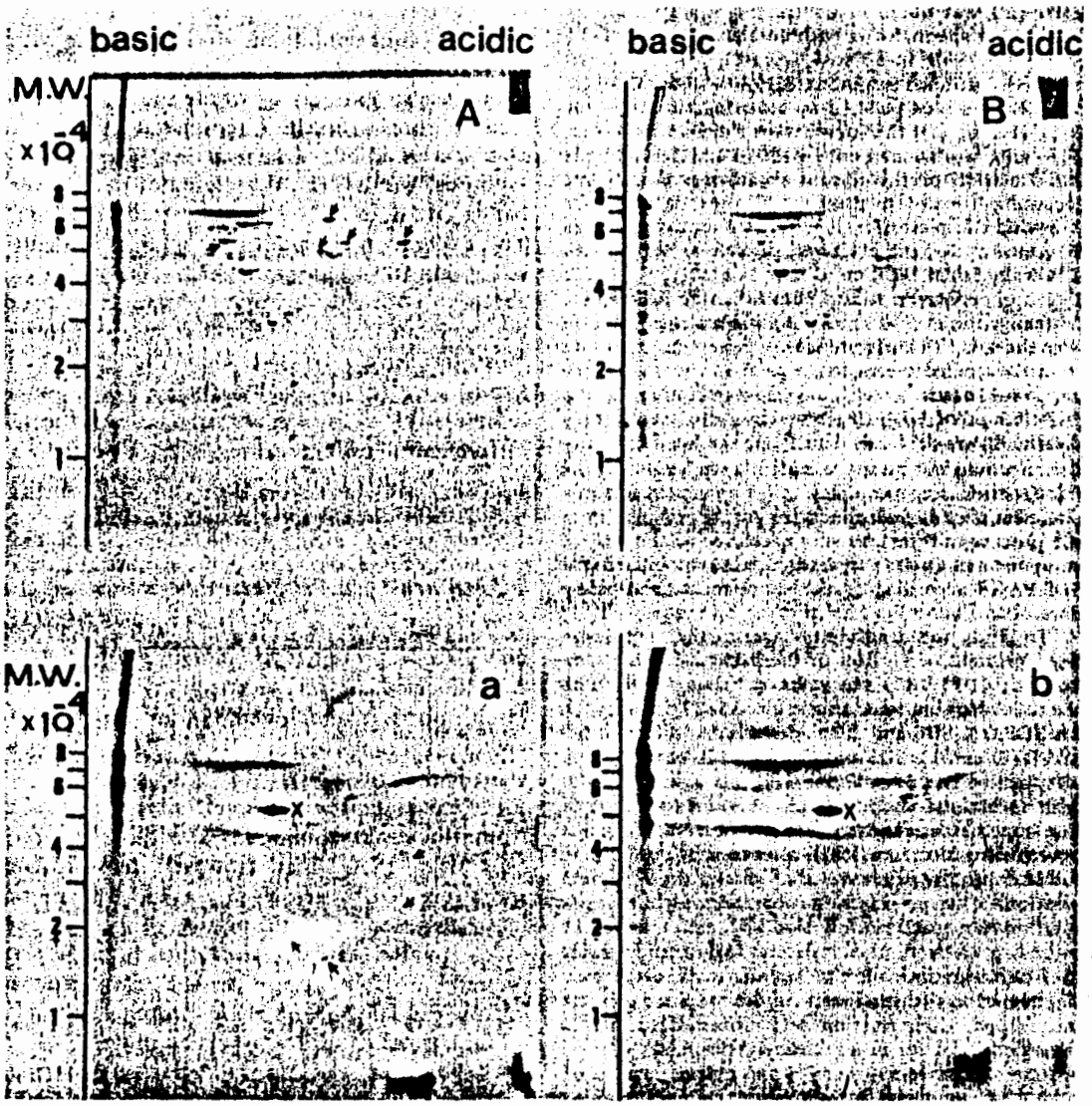

FIG. 1. Two-dimensional electrophoretic (upper) and autoradiographic (lower) patterns of phosphorylated proteins of rat spermatozoa from the caput (A, a) and cauda (B, b) epididymidis. Each gel represents proteins from 12 X 10^6 cells. The autoradiographic plates were developed after exposure for 5 days. Arrows indicate spots showing difference between the patterns of the cells from the caput and cauda epididymidis.

Analysis of Head, Tail, and Cytosolic Fractions, and Effect of Caffeine

By SDS-PAGE using slab gels for convenience of subsequent autoradiography, it can be easily shown that the protein pattern of the extract from complete solubilization of whole spermatozoa was similar to that of the preparation of the tails (Fig. 3A,B). However, the head and the

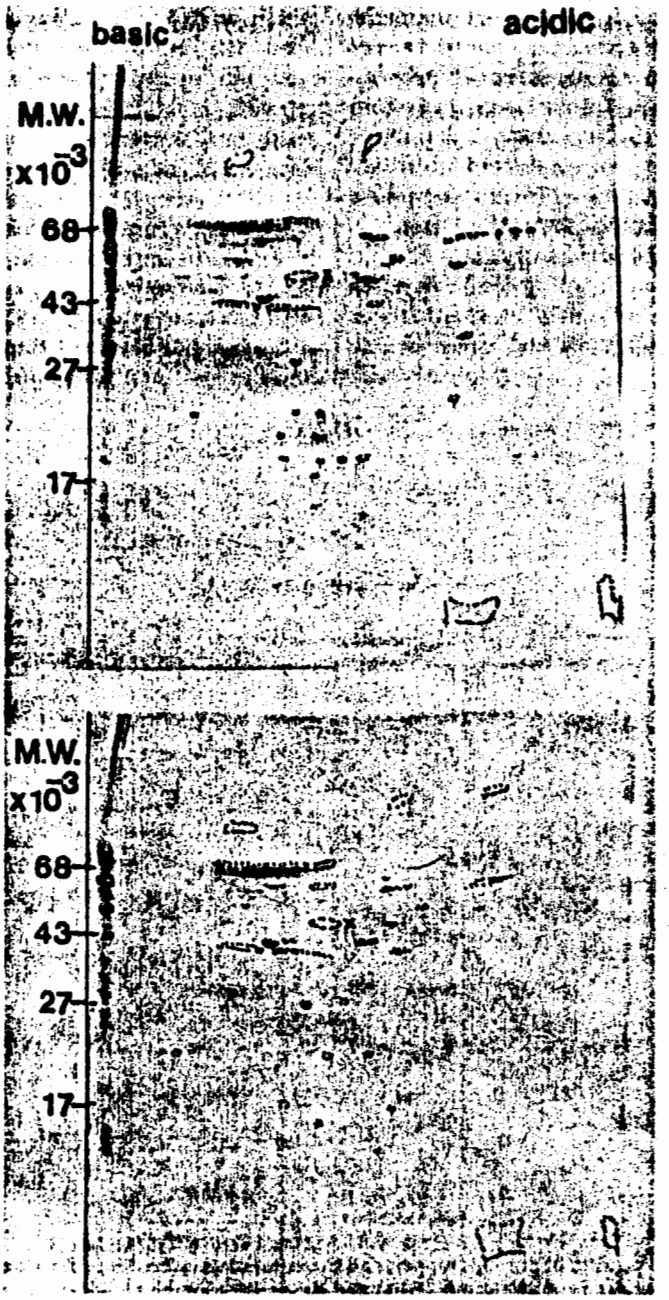

FIG. 2. Superimposition of the autoradiographic pattern (dotted) onto the corresponding protein pattern shown in Fig. 1. The upper panel represents the patterns of the spermatozoa of the caput epididymis. The lower panel represents those of the cauda epididymis. The superimposition was guided by the positions of three radioactive ink spots asymmetrically placed on the side of gel but not included in the figure.

cytosolic fractions contained different proteins from those in the tails. When spermatozoa of the caput were compared with those of the cauda epididymis, the protein patterns of corresponding fractions were identical.

Autoradiographic patterns of the gels (Fig. 3a,b) showed clearly the three major radioactive bands A, B, and C present in the extract from intact spermatozoa and in the tail preparations but not detectable in the preparations of heads and cytosol. Upon 5–10-fold concentration of the cytosolic fraction, a phosphorylated band of M_r 15,000 can be detected (result not shown). In addition, a few bands of less radioactivity were also detected in the whole cell and tail preparations. No obvious or consistent difference can be found between the phosphorylated products of spermatozoa from the caput epididymis and those of the cauda. Since caffeine can raise the intracellular cAMP, it was of interest to test its effect in our incubation. The phosphorylation was found to be unaffected by the presence of 10 mM caffeine in the incubation mixture.

To ascertain that the head proteins did not participate in the phosphorylation, protamine which was the main class of proteins in sperm heads (Calvin, 1976) and could be phosphorylated by protein kinase in vitro (Soontaros and Panyim, unpublished observation) was extracted with 0.25 M HCl after the 30 min incubation with 1.6 M guanidine hydrochloride and 10 mM DTT according to the method of Puwaravutipanich and Panyim (1975) from the labeled spermatozoa of both caput and cauda epididymidis. The preparations were found to contain no detectable radioactivity by acid-urea PAGE using slab gels (data not shown).

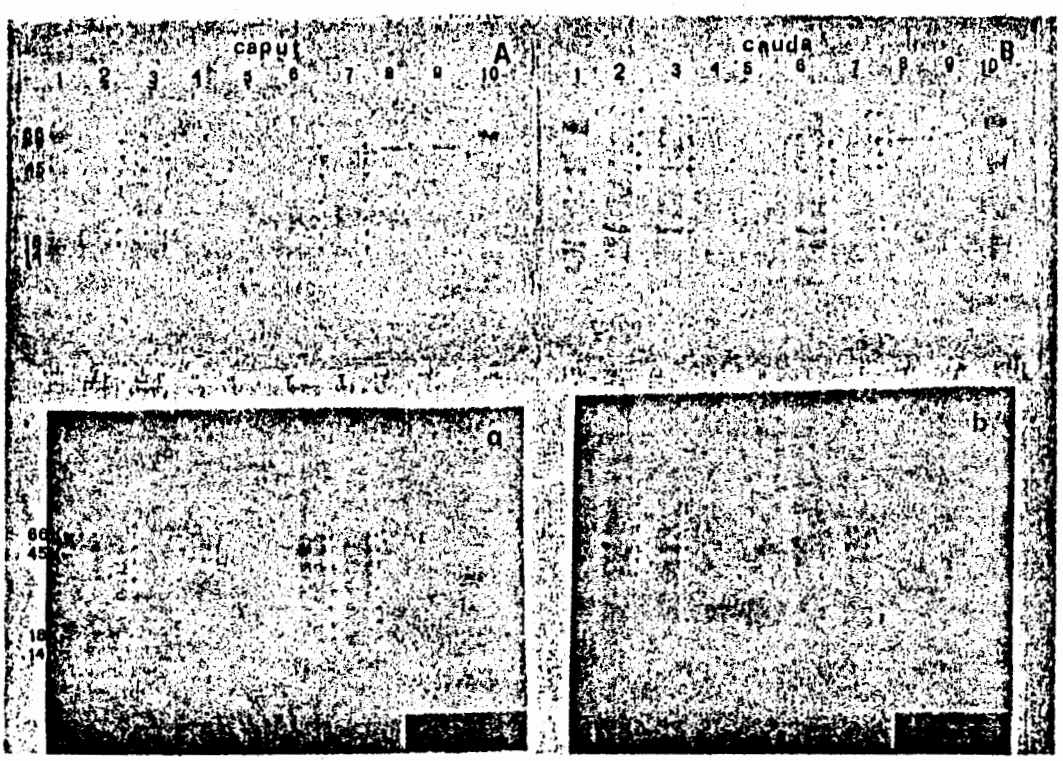

FIG. 3. SDS-PAGE profiles of proteins (upper) and corresponding autoradiography (lower) of phosphorylated proteins of rat spermatozoa from the caput (A, a) and cauda (B, b) epididymidis. Lanes 2 and 3 represent proteins from whole cells; lanes 4 and 5 represent proteins from sperm heads; lanes 6 and 7 represent proteins from sperm tails; and lanes 8 and 9 represent cytosolic proteins. Lanes 1 and 10 show the molecular weight markers: bovine serum albumin, ovalbumin, β-lactoglobulin, and lysozyme. The spermatozoa were incubated with ^{32}Pi in the absence (lanes 2, 4, 6, and 8) or in the presence (lanes 3, 5, 7, and 9) of 10 mM caffeine. The autoradiographs were developed after exposure for 2.5 days.

Analysis of Preparations of Plasma Membrane, Mitochondria, Fibrous-Sheath, and Outer Dense Fiber-Connecting Pieces

To gain some insight into the cellular localization of these major phosphorylated proteins in the tails, selective solubilization of the structural components of spermatozoa by certain chemicals was employed. The rationale of this approach was based on the fact that covalent bonds between phosphoryl groups and proteins can not be cleaved by Triton X-100, SDS, urea, or DTT, singly or in combinations, while these reagents can selectively dissolve components of rat spermatozoa (Olson, 1979; Olson and Sammons, 1980). Treatment of the labeled spermatozoa with 1% Triton X-100 and 2 mM DTT, which was known to solubilize mainly the plasma membrane and mitochondria (Olson and Sammons, 1980), could only remove some of band B from the spermatozoa (Fig. 4). Further solubilization of the Triton/DTT-extracted spermatozoa with urea/DTT yielded insoluble fibrous sheath (Olson, 1979) which did not possess the three radioactive proteins (Fig. 4). Thus this experiment suggested that most of the three major phosphorylated proteins were not part of or attached to the plasma membrane, mitochondria, or fibrous sheath. The preparation of ODF-CP complex according to the method of Olson and Sammons (1980) from the labeled spermatozoa of the cauda epididymis by treatment with 2% SDS/4 mM DTT showed that some radioactive bands B and C were associated with the ODF-CP complex (Fig. 5). However, the same treatment of the labeled spermatozoa from the caput epididymis yielded curled bundles of the ODF which possessed only a limited amount of band B (Fig. 5).

Disulfide Linkages of Phosphorylated Proteins

As sulfhydryl groups in sperm proteins were known to be gradually oxidized into disulfide linkages during the epididymal transit of the spermatozoa (Calvin et al., 1973), it was of interest to determine if the three major phos-

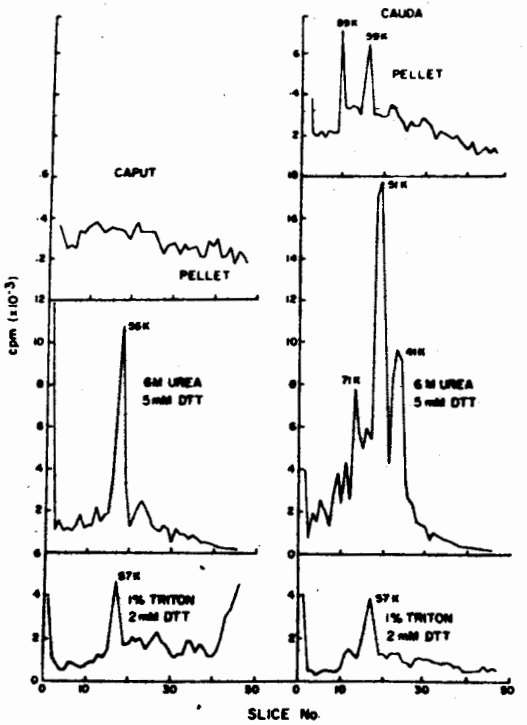

FIG. 4. Radioactive profiles of SDS-PAGE using rod gels of phosphorylated proteins extracted from the labeled spermatozoa of the caput (left panels) and the cauda (right panels) epididymidis. The cells were first extracted with 1% Triton X-100/2 mM DTT (lower panels) and then with 6 M urea/5 mM DTT (middle panels). The final pellets (upper panels) were verified by light microscopy as fibrous sheath.

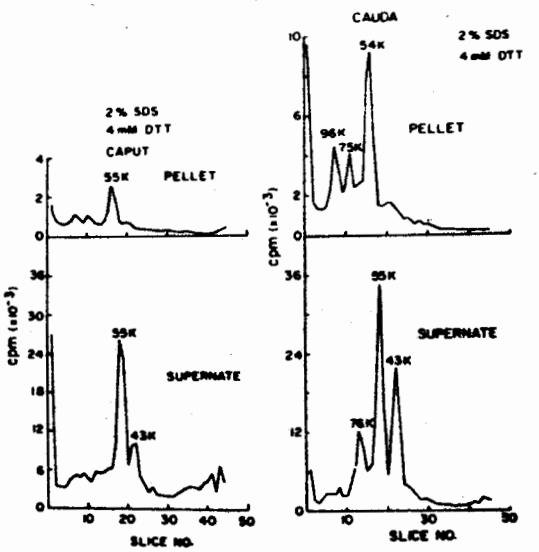

FIG. 5. Radioactive profiles of SDS-PAGE using rod gels of phosphorylated proteins solubilized by 2% SDS/4 mM DTT (supernate) from the labeled spermatozoa of the caput (left panel) and the cauda (right panel) epididymidis. The pellet fractions were verified by light microscopy as ODF-CP complex.

phorylated proteins would become increasingly linked to the axonemes by disulfide bonds as the spermatozoa matured. This was done by first treating the labeled spermatozoa with nonionic detergent, 0.1% Triton X-100, to solubilize the plasma membrane partially. This would then allow ionic detergent, SDS (1%), which was used in the subsequent treatment of the Triton-treated cells, to pass through the plasma membrane and to reach the intracellular structures. The SDS treatment solubilized most of bands A and C and about half of band B from the spermatozoa of the caput region (Fig. 6). The remainder of band B could mainly be solubilized by further treating the spermatozoa with 1% SDS/6.5 nM DTT. In contrast, only minute amounts of these bands could be solubilized by 1% SDS from the spermatozoa of the cauda epididymis (Fig. 7). Only when 6.5 mM DTT was included in 1% SDS in the subsequent extraction, could band A and half of bands B and C be dissociated from the spermatozoa. The rest of bands B and C could be dissolved when an even higher concentration of the sulfhydryl reagent (1% SDS/13 mM DTT) was employed. This last reagent would completely dissolve the spermatozoa.

DISCUSSION

Based on molecular weights, band B is similar to the smallest phosphorylated protein reported in human spermatozoa by Huacuja et al. (1977), phosphotubulin in rat spermatozoa (Tongkao and Chulavatnatol, 1979), or the phosphorylated protein in bovine spermatozoa (Brandt and Hoskins, 1980). However, size similarity alone is insufficient to suggest that they are the same protein. Phosphorylation of band A has never been reported before. Although band C appears similar in size to the phosphorylated protein of M_r 80,000 in the plasma membrane of human spermatozoa (Huacuja et al., 1977), it is probably a different protein because it is not found in the plasma membrane fraction (Fig. 4). The use of two-dimensional gel electrophoresis not only gives a better resolution of the sperm proteins but can also demonstrate that the degrees of heterogeneity of bands A and C are much more than that of band B (Fig. 1). The apparent heterogeneity can arise either from the varying degrees of phosphorylation of the same protein, giving products of varying negative charges, or from phosphorylation of various proteins of the same size but differing in their charges. The present study cannot distinguish the causes of heterogeneity of bands A and C. The two-

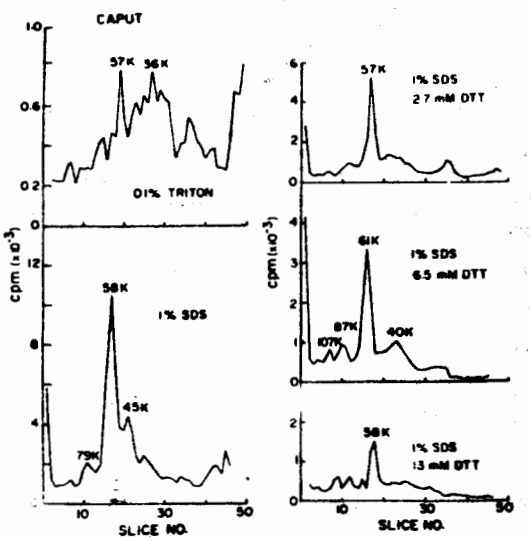

FIG. 6. Radioactive profiles of SDS-PAGE using rod gels of phosphorylated proteins extracted from the labeled spermatozoa of the caput epididymis. The cells were sequentially extracted with 0.1% Triton X-100; 1% SDS; 1% SDS/2.7 mM DTT; 1% SDS/6.5 mM DTT, and 1% SDS/13 mM DTT.

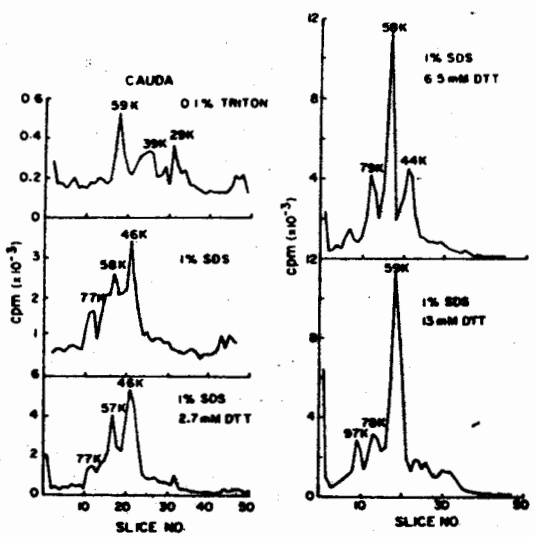

FIG. 7. Radioactive profiles of SDS-PAGE using rod gels of phosphorylated proteins extracted from the labeled spermatozoa of the cauda epididymis. The cells were extracted sequentially as described in Fig. 6.

dimensional gels also show that the amounts of proteins phosphorylated (bands A, B, and C) are small and below the sensitivity of the dye staining (Fig. 2). This would mean the phosphorylation is specific and the phosphorylated proteins are specifically modified by protein kinase action. Alternatively, the phosphorylated form, having additional phosphoryl group(s) and hence being more negatively charged, can possibly move toward more acidic region relative to its nonphosphorylated form of the same molecular weight. This means only a fraction of the protein is phosphorylated and moves to more acidic pH. This phenomenon has been verified in our two-dimensional gel system using bovine serum albumin and ovalbumin phosphorylated in vitro with purified catalytic subunit of protein kinase (Panyim, unpublished observation).

Before their physiological role can be logically proposed, the localization of these three major phosphorylated proteins should be investigated. The absence of these proteins in the isolated heads (Fig. 3) has greatly simplified our efforts in using chemical fractionations of whole spermatozoa without prior removal of the heads. Since cytosolic fraction prepared by brief sonication of labeled spermatozoa does not contain any appreciable amount of the major radioactive bands (Fig. 3), our attention was then directed to structural components of the mid and principal pieces. The availability of the established methods (Olson and Sammons, 1980) to solubilize the plasma membrane and mitochondria and to isolate the fibrous sheath from rat spermatozoa (Fig. 4) allows us to rule out these structures quickly as the components possessing most of the three major phosphorylated bands. However, a relatively small fraction of band B detected in the membrane mitochondria fraction should be noted. The detection of some of these bands in the preparation of ODF-CP complex of spermatozoa from the cauda epididymis (Fig. 5) would suggest that some of the phosphorylated proteins are part of or linked to ODF-CP complex. By logical elimination, the only major structure that can contain the remainder of the three major phosphorylated proteins should be the axonemes. Although selective solubilization of axonemes from guinea pig spermatozoa is known (Friend et al., 1979), its applicability has not been established in rat spermatozoa. Thus, the above conclusion has not yet been confirmed by specific solubilization of the rat axonemes. However, microscopic appearance and the protein patterns in SDS-PAGE of each extraction agree well with the finding in the literature (Olson, 1979; Olson and Sammons, 1980). We are, therefore, assured that the conclusion is based on firm experimental data and should be valid. By becoming increasingly linked by disulfide bonds either among themselves or to other proteins during epididymal transit (Figs. 6, 7), the three major phosphorylated proteins may be more tightly interacting with the motile apparatus (axonemes/ODF) and hence can be expected to exert more effective action on the function of the structure. Whether their action is part of the motility mechanism or of its control will have to await more study on their identities and definitive localization within the motile apparatus.

Our suggestion that three proteins of the motile apparatus but not the plasma membrane of rat spermatozoa are the major phosphorylated components does not agree with the findings in human spermatozoa in which three proteins of the plasma membrane are phosphorylated (Huacuja et al., 1977). We have not tried to resolve the discrepancy. It may be due to species difference or dissimilarity in the experimental conditions.

The lack of effect of caffeine in the phosphorylation of bands A, B, and C (Fig. 3) cannot indicate if the phosphorylation is dependent or independent on cAMP since the assays have shown no alteration of the sperm cAMP content in the presence of caffeine. Furthermore, unlike its action in other species, caffeine has been found to be ineffective in stimulating motility or metabolism of rat spermatozoa (Paz et al., 1978). If band B is the same as the phosphorylated protein identified in bovine spermatozoa (Brandt and Hoskins, 1980), since they are similar in size, its phosphorylation should be dependent on cAMP in the same manner as the bovine protein.

The nature of the minor phosphorylated proteins is still unknown. Since they appear to be extracted by Triton DTT (Fig. 4), they are probably associated with the plasma membrane, mitochondria, or cytoplasm. The presence of phosphorylated proteins in mitochondria and cytoplasm would be anticipated since several enzymes involved in carbohydrate and lipid metabolism can exist as phosphorylated forms in other tissues.

The absence of any detectable phosphorylated proteins in the heads can be due to the

lack of active protein kinase, or [γ-^{32}P] ATP or of suitable protein substrate in the heads. Since protamine is the major protein in the heads, and can generally serve as substrate for protein kinase in vitro, it should be phosphorylated in the intact spermatozoa. We have not explored further whether the lack of protamine phosphorylation is because the protamine in the sperm chromatin is inaccessible to protein kinase or because it is already fully phosphorylated before spermatozoa enter the epididymis.

Both the major and the minor phosphorylated proteins detected in intact spermatozoa should generally be formed by the action of protein kinases and are not immediately dephosphorylated by phosphoprotein phosphatase known to be present in spermatozoa (Tang and Hoskins, 1975). Therefore, their phosphorylation should be of physiological importance. However, the use of intact cells will not permit detection of the phosphorylated proteins that are immediately dephosphorylated. Such phosphorylated proteins are probably too short-lived to exert any biological action in the phosphorylated forms. The findings in this study clearly suggest that further work on the three major phosphorylated proteins would lead to a better understanding of the role of protein kinases in spermatozoa.

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