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Localization of Zinc in a Dense Fiber-Connecting Piece Fraction of Rat Sperm Tails Analogous Chemically to Hair Keratin

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ABSTRACT

The intracellular distribution of zinc in mature rat sperm was examined by subcellular fractionation and atomic absorption spectrophotometry. The results indicate that >90 percent of this trace metal is located in the tail, *ca.* 85 percent in -S-S- crosslinked tail structures which do not disintegrate in 1 percent sodium dodecyl sulfate (SDS). Association of zinc with -SH is supported by the primary localization of the latter within the same SDS-insoluble material. The concentrations of both zinc and -SH decrease in the heads and tails of rat sperm during passage through the epididymis. Of the minor fraction of zinc in the rat sperm head (5–10 percent), >75 percent is not extracted with SDS and therefore appears also to be associated with -S-S- bonded structures.

Over 75 percent of the zinc in isolated tails is retained by a subfraction which consists mainly of dense fibers, with connecting pieces present as a minor constituent, and is largely non-dialyzable upon solubilization of these structures. The major component isolated from the solubilized product, following aminoethylation, is of 35,000 mol. wt. Polyacrylamide gel electrophoresis in SDS also reveals significant components of *ca.* 75,000, 25,000 and 15,000 mol. wt. and trace components of 90,000, 70,000 and 50,000 mol wt. The amino acid composition of this protein mixture includes 11–12 percent cysteine and is found to be strikingly similar to the overall compositions of wool and guinea pig hair, which also possess sizeable contents of stably-bound zinc. The possibilities that the proteins of hair and sperm keratin (i.e.-*kerateines*) share a common phylogenetic origin and undergo similar interactions with zinc during macromolecular assembly are therefore noted.

INTRODUCTION

A keratin, according to Mercer (1969), may be defined as "a mixture of proteins rendered insoluble by the disulfide crosslinking of some of its components."[1] The presence of such complexes in the heads and tails of mammalian spermatozoa has been recognized for many years (Zittle and O'Dell, 1941). It may therefore be of related significance that zinc, a trace element present in relatively high concentrations within keratins and necessary for normal keratogenesis (see Underwood, 1971), is also essential for spermatogenesis in mammals (see Mann, 1964; Gunn and Gould, 1970; Underwood, 1971), during which it becomes firmly associated with the sperm (Gunn and Gould, 1970).

Evidence has accumulated recently that a major fraction of zinc in mammalian sperm is, in fact, located within -S-S- linked (i.e.-keratinous) structures, largely in association with sulfhydryl groups. In the rat, incorporation of zinc into the developing spermatid occurs during the most advanced stages of spermiogenesis (Wetterdal, 1958; Parizek et al., 1966), concurrent with the elaboration of the tail, where most of the zinc in the fully-differentiated sperm is concentrated (Millar et al., 1961; Calvin and Bleau, 1974). Similarly, most of the sulfhydryl groups in rat sperm are associated with the tail (Nelson, 1960; Calvin et al., 1973; Calvin, 1975), which contains a variety of structures stabilized by -S-S- bonding (Bedford and Calvin, 1974). Following release of the sperm from the testis, during maturation in the epididymis, its contents of zinc (Gunn and Gould, 1970; Calvin and Bleau, 1974) and -SH groups (Calvin et al., 1973; Calvin and Bleau, 1974) both decrease, as the latter are oxidized

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[1] In the present context this definition will be adhered to, and the term *kerateine* (Goddard and Michaelis, 1935) will be employed to designate the cysteine-rich molecules which become cross-linked to form keratins.

to form -S-S- crosslinks (Calvin and Bedford, 1971). In mature sperm, further oxidation of -SH may be retarded by zinc, for when added *in vitro*, this trace element can inhibit both oxidation and alkylation of sperm sulfhydryls (Calvin et al., 1973), presumably by forming stable complexes with these groups. The natural occurrence of such complexes in rat sperm is supported by the demonstration that only a minor fraction of its endogenous zinc can be removed with EDTA (Saito et al., 1969), unless the cell is first treated with iodoacetamide, p-mercuribenzoate or Ag^+ (Calvin and Bleau, 1974), all of which react preferentially with -SH groups.

The probable association of most of the zinc in rat sperm with -SH groups is consistent with its localization within -S-S- bonded structures. This notion is strengthened by observations that relatively little zinc is released from mature sperm either by moderate sonication or by extraction with sodium dodecyl sulfate (SDS) (Calvin and Bleau, 1974), neither of which is able to disintegrate those sperm structures which are cross-linked tightly by -S-S- bonds. Further information on the site of the zinc-binding moiety in rat sperm has been furnished by a preliminary report from this laboratory (Calvin, 1975), which assigned over 70 percent of the zinc in mature rat sperm to a tail fraction comprised of dense fibers and connecting pieces, whose proteins display an overall amino acid composition resembling that of wool. A zinc-binding keratin has also been isolated from bull sperm tails by Baccetti et al. (1973). The latter product, which likewise consists predominantly of dense fibers, has been partially fractionated, and two major protein components, which contain respectively 6 and 19 percent cysteine, have been identified.

In the present communication, the ultrastructural identity of the major zinc-binding fraction in rat sperm is confirmed and its heterogeneity with respect to protein composition is examined. Its amino acid analysis, which reveals a higher percentage of cysteine than that determined previously (Calvin, 1975), continues to suggest a close homology with the keratins of hair.

MATERIALS AND METHODS

Sperm Samples

Sperm were expressed at 0–5°C from the caput or cauda epididymides of 400–600 g Sprague-Dawley rats into 0.02 M NaH_2PO_4, buffered to pH 6.0 (Buffer P), and freed of debris by filtration through nylon mesh and low speed centrifugation (Calvin et al., 1973). The designations immature or mature refer respectively to sperm derived from the caput or cauda of the epididymis. Sperm suspended in Buffer P were either sonicated directly in this medium for the separation of heads and tails, or were centrifuged at 1500 g for 10 min and resuspended in other appropriate media for the procedures used in this study. It has been found that intact sperm retain the same amount of zinc in Buffer P as in isotonic media (Calvin and Bleau, 1974).

Separation of Heads and Tails

All operations were performed at 0–5°C. Sperm were decapitated by sonication in Buffer P (Calvin et al., 1973) and the heads separated from the tails on sucrose density gradients, which contained 13 ml each of: 1.80 M sucrose (sample layer), 2.05 M sucrose, and 2.20 M sucrose. The sample layer was made up by mixing sperm suspension in Buffer P with a suitable volume of 2.20 M sucrose. All sucrose solutions were prepared gravimetrically and included Buffer P. Centrifugation was carried out in nitrocellulose tubes with a Beckman L3-40 centrifuge, equipped with an SW-27 rotor, at 22,500 rpm (67,000 g, r_{av}) for 60 min. Tails were recovered from the 1.80 M/2.05 M sucrose interface and heads from the bottom of the gradient. The tail fraction was diluted in Buffer P and concentrated by recentrifugation at 67,000 g for 20 min. Additional details concerning this gradient procedure, modified from that described previously (Calvin et al., 1973), will be published elsewhere (Calvin, in press). In some experiments, it was necessary to obtain heads or tails essentially free of cross-contamination. For this purpose, the isolated fractions were resuspended in Buffer P and recentrifuged in the above gradients *(double gradient purification)*. To assure even suspension for subsequent quantitative experiments and minimize losses on test tube walls, heads and tails were resuspended by brief sonication at 55 w in a buffer with mild detergent properties: 0.02 M Tris-HCl, pH 7.5 – 0.15 M NaCl – 0.2 mg/ml bovine serum albumin (Buffer TSA). To further minimize surface adsorption, head fractions were kept in small nitrocellulose tubes (Calvin, in press).

Cell Concentrations

Concentrations of sonicated sperm, sperm heads or sperm tails, suspended in Buffer TSA, were determined by dilution of aliquots into 0.02 M Tris-HCl (pH 7.5) – 0.15 M NaCl – 0.02 percent SDS, at concentrations between 1.5 and 2.5 × 10^6/ml, and counting with a hemocytometer. Scoring was performed as described elsewhere (Calvin et al., 1973; Calvin, in press).

Extraction and Assay of Zinc

Suspensions of sonicated sperm, sperm heads or sperm tails in Buffer TSA were assayed for total zinc by mixture of an aliquot with an equal volume of 2 M HCl, resonication at 55 w for 30 sec, incubation for at least 1 h, and atomic absorption spectrophotometry of the supernatant obtained by centrifugation at 1500 g for 10 min (Calvin and Bleau, 1974). In selected

experiments, the completeness of extraction with 1 M HCl was verified by solution of the sperm residue in 30 percent (w/w) HNO_3 − 35 percent (w/w) $HClO_4$. Further localization of the zinc was achieved by treatment of intact sperm, sperm heads or sperm tails (50–100 × 10^6/ml) with SDS, in some cases supplemented with a low concentration of dithiothreitol (DTT) and mild sonication. *SDS extraction* was performed by incubation in 1 percent SDS − 0.05 M Tris-HCl, pH 7.5 at 25 C for 15 min and centrifugation (Sorvall RB2 centrifuge, HB4 rotor) at 10,000 g (r_{max}). *SDS-DTT sonication* was performed by overnight incubation of sperm or sperm fractions at 25°C in 1 percent SDS − 0.2 mM DTT − 0.05 M Tris-HCl, pH 7.5, followed by sonication for 2 min at 55 w and centrifugation as above. Zinc contents of supernatants and 1 M HCl extracts of the residues were then compared by atomic absorption spectrophotometry, as described previously (Calvin and Bleau, 1974). In two experiments, affinity for zinc of sperm tail fractions which survive the above treatments was evaluated by solubilizing the residues in 5 M guanidine-HCl − 0.01 M DTT − 0.20 M Tris-HCl, pH 8.6, and dialysis overnight against water. The total zinc content of the resulting suspensions was determined following incubation in 1 M HCl and centrifugation, by assay of the resulting supernatants.

Assay of SH Groups

Duplicate sets of assays of -SH in sperm or subcellular fractions were performed in quadruplicate, either with or without preincubation at pH 2. The suspension of sperm or sperm fraction (0.1 ml in Buffer TSA at a concentration of 20–40 × 10^6/ml) was either diluted with 0.02 M HCl (0.1 ml) and incubated at 25°C for 30 min, or else diluted with water and kept in ice. Determination of -SH was then carried out by addition of 0.3 ml of a solution which contained: 50 μmoles of Tris-HCl, pH 7.5; 0.5 μmoles of sodium EDTA; 80 μg of bovine serum albumin; and 2.5 μmoles of [1-^{14}C] iodoacetamide (New England Nuclear Corp.), diluted to a specific activity of 2.90 × 10^5 dpm/μmole. This composition was modified for detection of the relatively low content of -SH in mature sperm heads by increasing the specific activity of the tracer to 2.90 × 10^6 dpm/μmole, with the total dpm kept constant (i.e., only 0.25 μmoles of iodoacetamide present in each assay). Following incubation for 90 min at 25°C, 1 ml of 10 percent trichloroacetic acid and 0.1 ml of 0.2 percent bovine serum albumin were added and the contents were homogenized by vortex mixing, followed by brief sonication at 55 w. The suspension was then collected on a Whatman GF/C filter, which was washed and assayed for radioactivity as described previously (Calvin et al., 1973). Counting efficiency was 85–90 percent. The specificity of the above assay was tested with unfractionated sperm by incubation with 5.0 μmoles of Ellman's reagent (1959) for 15 min, in a total volume of 0.4 ml in the presence of all ingredients except [^{14}C] iodoacetamide, before addition of the tracer (2.5 μmoles/0.1 ml). Under these conditions, 95 percent inhibition of [^{14}C]-incorporation was observed.

Isolation and Analysis of Dense Fiber-Connecting Piece Fraction

Sperm tails isolated by double gradient purification were suspended at concentrations between 50 and 100 × 10^6/ml in 1 percent SDS − 0.2 mM dithiothreitol (DTT) − 0.05 M Tris-HCl, pH 7.5, and incubated at 25°C overnight. The suspension was chilled to less than 5°C for ensuing procedures. (Because of the inclusion of Tris in the medium, no precipitation of SDS was observed at this point.) After disruption of the tails by sonication at 55 w for 2–3 min, the surviving structures were collected by centrifugation at 10,000 g (r_{max}). The residue of dense fibers and connecting pieces was then washed at least twice with water by resuspension and recentrifugation and solubilized at 25°C in 5 M guanidine-HCl − 0.01 M DTT − 0.20 M Tris-HCl (pH 8.6). To 10 vol of the dissolved keratin, 3 vol of 3 M Tris-HCl, pH 8.6 were added and the proteins were aminoethylated at 25°C by 3 additions of 0.2 vol ethylenimine at 10 min intervals (Cole, 1967). When -SH was no longer detectable with the reagent of Ellman (1959), the reaction mixture was cooled in ice, acidified with concentrated HCl to a pH below 3.0 and dialyzed against 3 changes of 0.001 M HCl (100 vol) for a total period of at least 30 h. Molecular weight distribution of proteins in the dialyzed product was examined by electrophoresis in gels containing 10 percent polyacrylamide and 0.1 percent SDS, according to Weber and Osborn (1969). The following standards, whose molecular weights are indicated in parentheses, were electrophoresed as references: rabbit muscle phosphorylase a (94,000), bovine serum albumin (68,000), ovalbumin (43,000), bovine α-chymotrypsinogen A (26,000) and horse heart cytochrome c (12,000). For amino acid analysis, samples were lyophilized, dissolved in 6 M HCl, equilibrated with N_2, sealed under a vacuum of less than 25 μ, incubated for 24 h at 110°C–112°C, lyophilized again and analyzed with a Beckman Model 120 analyzer on 1-cm × 50-cm columns of Beckman AA-15 resin, using the 2-column system of Spackman et al. (1958).

RESULTS

Intracellular Distribution of Zinc and -SH

The intracellular distribution of zinc and -SH in rat sperm has been dealt with in previous communications from this laboratory. It has been reported that over 90 percent of the zinc in mature rat sperm is localized within the tails and that very little of this trace element can be released from the sperm by incubation in SDS (Calvin, 1975; Calvin and Bleau, 1974), a treatment which spares only those structures stabilized by -S-S- bonding (Calvin and Bedford, 1971). From this it was concluded that the zinc in rat sperm resides primarily within an -S-S-bonded (i.e. keratinous) fraction of the sperm tail. Similarly, -SH is localized predominantly within the tail, also within structures stable in

SDS (Calvin, 1975).

Studies on the distribution of these two constituents within rat sperm have since been repeated, following the development of improved procedures for the handling of dilute suspensions of sperm and sperm fractions to minimize losses by adsorption to glass. To maximize alkylation of -SH groups by [^{14}C]iodoacetamide, it was necessary to preincubate samples at pH 2 for 30 min before reaction with iodoacetamide. As shown in Table 1, this pretreatment stimulated [^{14}C]-incorporation in all samples tested, except the heads of immature (i.e., caput) sperm. Under these optimal assay conditions, specificity of the reagent for -SH was confirmed by 95 percent inhibition of [^{14}C]-incorporation with Ellman's reagent. Additional incorporation obtainable by prolonging the period of incubation and by increasing the incubation temperature to 37°C was largely not inhibitable with Ellman's reagent and could not therefore be ascribed with any certainty to -SH groups.

Estimates of -SH in mature (i.e., cauda) rat sperm ranged between 4.9 and 5.7 μmoles per 10^9 cells, whereas determinations of zinc varied between 0.58 and 0.77 μmoles per 10^9 cells. The localization of both of these primarily within the tail is illustrated by the two experiments quoted in Table 1, where it is also demonstrated that SDS removes hardly any zinc and only a minor fraction of -SH from cauda sperm. Estimates of -SH and zinc in unfractionated caput sperm and caput sperm heads are also documented in Table 1. These confirm that in immature cells as well, both -SH and zinc are located primarily within the tail. Comparison of the data presented respectively for immature and mature cells supports the conclusion that -SH and zinc decrease in both heads and tails during the final stages of sperm maturation in the epididymis.

Stimulation of -SH alkylation by preincubation at pH 2 in all fractions except immature sperm heads (Table 1) indicates that a significant percentage of sulfhydryls did not react with iodoacetamide unless they were exposed in some way by acid. It has been proposed that the major inhibitor removed by acid from these cells is zinc (Calvin and Bleau, 1974), which has been shown to interfere with the alkylation of rat sperm -SH by iodoacetamide (Calvin et al., 1973). In support of this, it was determined during the development of the present assay that only 40–50 percent of the zinc in sonicated rat sperm is released by incubation for 90 min in 5 mM iodoacetamide – 1 mM EDTA – 0.1 M Tris-HCl, pH 7.5, in the absence of

TABLE 1. Intracellular distribution of zinc and sulfhydryls in rat spermatozoa.

Experiment	Sample	Acid preincubation	μmoles/10^9 cells SH	Zn	SH/Zn
1	Caput sperm	+	13.7	1.23	11.1
		–	12.3		
	Caput sperm heads	+	1.08	0.095	11.3
		–	1.08		
	Cauda sperm	+	5.15	0.76	6.8
		–	3.19		
	Cauda sperm heads[a]	+	0.073	0.046	1.6
		–	0.047		
2	Cauda sperm	+	5.04	0.58	8.7
		–	3.27		
	Cauda sperm,[b] SDS-treated	+	3.89	0.56	6.9
		–	3.04		
	Cauda sperm tails	+	4.24	0.56	7.6
		–	2.76		

[a]This sample was incubated with ^{14}C-iodoacetamide of 10-fold the specific activity used for all of the other samples (see Materials and Methods).

[b]Intact cauda sperm were incubated for 15 min at 25°C with 1% SDS – 0.05 M Tris-HCl (pH 7.5). The residue was washed once with buffer TSA, resuspended in this medium and sonicated as usual.

TABLE 2. Stability of zinc complexes in rat sperm.

Source	Fraction	Treatment[a]	No. of detn.	% Retained
Caput	Whole sperm	SDS	2	64
Cauda	Whole sperm	SDS	3	92
		SDS-DTT, sonication	3	80
	Tails	SDS	2	94
		SDS, solubilization	2	72
		SDS-DTT, sonication	4	77
		SDS-DTT, sonication, solubilization	2	62
	Heads	SDS	2	76

[a]As described in Materials and Methods.

preincubation at pH 2, whereas such preincubation alone removes ca. 90 percent of the zinc. The possibility that pretreatment in 0.01 N HCl increases -SH reactivity by alternative or additional mechanisms is of course not precluded.

Localization of Zinc in -S-S- Linked Structures

The inability of SDS to extract an appreciable fraction of the zinc from mature rat sperm (Table 1) confirms its localization within stable structures. As indicated in Table 2, over 90 percent of the zinc is retained following extraction of cauda epididymal sperm or sperm tails by SDS. On the assumption that 90–95 percent of the zinc in rat sperm resides in the tail (Table 1), it may be estimated that ca. 85 percent of the total zinc in the cell is situated within tail structures which are stable in SDS. These components, which are crosslinked by -S-S- bonds, include the connecting piece, dense outer fibers, fibrous sheath and outer mitochondrial membranes (Bedford and Calvin, 1974). In contrast to their behavior in mature sperm, the same structures in caput epididymal sperm disintegrate partially in SDS, as a consequence of their relative deficiency of -S-S- crosslinks, whose full complement is not established until sperm have reached the cauda epididymidis (Bedford and Calvin, 1974). Therefore, it is not surprising that only 64 percent of the zinc in caput sperm was retained following SDS extraction (Table 2).

Rat sperm heads isolated by double gradient purification contain only 5–10 percent of the zinc originally present in the sperm (Table 1, exp. 1). Nevertheless, attempts have been made to gain information concerning the nature of the zinc binding material in the head (Table 2). Extraction of the head with 1 percent SDS, which solubilizes all structures except chromatin, perforatorium and remnants of the post-acrosomal sheath (Calvin and Bedford, 1971), releases approximately 25 percent of the zinc. Thus, most of the zinc in the head is located within one or more of these -S-S- crosslinked structures.

Protein-Bound Zinc in the Dense Fiber-Connecting Piece Assembly

Further localization of the major zinc-binding material in mature rat sperm has been achieved by sonication of unfractionated sperm

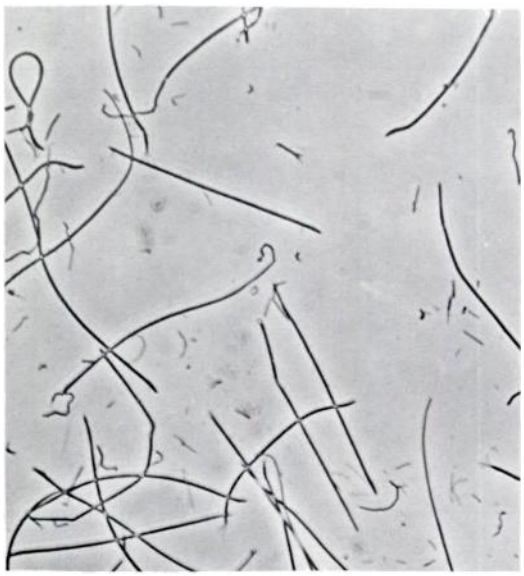

FIG. 1. Rat sperm tails isolated by sonication and sucrose density gradient centrifugation. ×330.

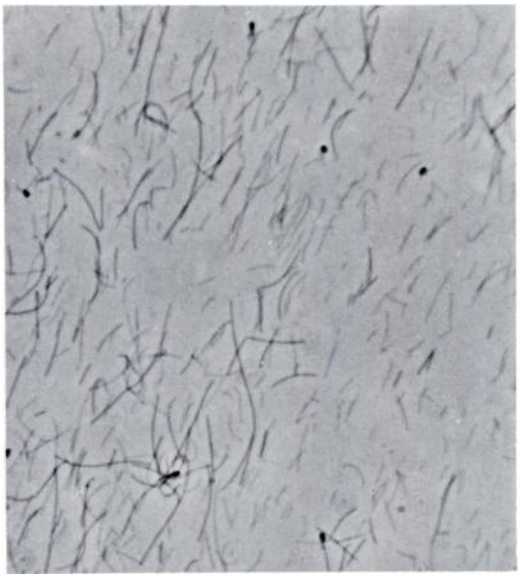

FIG. 2. Dense fibers and connecting pieces isolated by centrifugation, following sonication of tails in 1 percent SDS – 0.2 mM DTT – 0.05 M Tris-HCl (pH 7.5). ×500.

or tails after incubation in 1 percent SDS – 0.2 mM DTT. The residues isolated by centrifugation at 10,000 g for 10 min contain over 75 percent of the zinc originally present (Table 2). The consequences of such disruption of sperm tails are illustrated, first of all, by comparison of Figs. 1 and 2. When sonicated tails (Fig. 1) are treated in this way, the residue (Fig. 2) consists of fragmented fibers and small dense bodies of uniform size. These have been identified respectively as dense outer fibers and connecting pieces (Fig. 3), with the dense fibers by far the predominant component. Other sperm structures, although detectable, are present in only trace amounts.

In the electron micrograph which appears in Fig. 3, both the dense fibers and the connecting piece display some loss of structural integrity, as indicated by the fraying and mottled staining which are most evident at their periphery. The largest of the electron-dense spots are of abnormally high density and are characteristically found along the borders of the dense fibers, although occasionally they are seen as detached elements. Similar peripherally-localized intensely hyperchromatic bodies have been observed following treatment of a crude fraction of bull sperm dense fibers with thiols or with EDTA (Baccetti et al., 1973), and might therefore

FIG. 3. A field of dense fibers containing one connecting piece (cp), as viewed in thin section in the electron microscope, following preparation as described previously (Bedford and Calvin, 1974), modified by substitution of 0.1 M NaH_2PO_4, buffered to pH 7.4, for borate buffer during fixation. Disintegration of the fibers is especially evident along their periphery (arrows), where prominent hyperchromatic bodies (h) are also seen. (Courtesy of Dr. G. W. Cooper.) ×18,000.

involve some form of rearrangement of dense fiber substructure. The possibility that other denatured sperm components may contribute to the hyperchromatic bodies cannot, however, yet be excluded.

The affinity of sperm tail keratins for zinc has been found to persist even in solubilized material. When residues from SDS extraction or SDS-DTT sonication of tails were dissolved in 5 M guanidine-HCl – 0.01 M DTT – 0.20 M Tris-HCl (pH 8.6) and dialyzed against water, the proteins reprecipitated gradually. Assay of the resulting suspensions for zinc revealed that the majority of the zinc in the original residues was retained following solubilization and dialysis (Table 2), supporting its stable association with protein.

Composition of the Dense Fiber-Connecting Piece Fraction

The dense fiber-connecting piece fraction, which retains most of the zinc in the rat sperm tail, is heterogeneous with respect to protein content. Following solution of the material in guanidine-HCl – DTT and aminoethylation, polyacrylamide gel electrophoresis of the dialyzed derivative in SDS reveals a strong band whose position corresponds approximately to 35,000 molecular weight (Fig. 4). In addition, 3 minor components of molecular weights 15,000, 25,000 and 75,000 and 3 faint bands whose positions suggest molecular weights of 50,000, 70,000 and 90,000 are present.

The amino acid composition of this mixture of proteins is presented in Table 3. Resemblance to the overall composition of samples of wool (O'Donnell and Thompson, 1962) and guinea pig hair (Steinert and Rogers, 1973), listed in the same table for comparison, is evident. Similarly prominent percentages of cysteine, serine, proline and arginine, as well as correspondingly low percentages of phenylalanine, histidine and methionine, seem more than coincidental. Moreover, the total percentages of the hydrophobic residues, alanine, isoleucine, leucine, methionine, phenylalanine, tyrosine and valine in the sperm keratin is within the typical range of 25–30 percent suggested by these two varieties of hair. Finally, the sperm sample yields a similar total percentage of aspartic and glutamic acid residues upon acid hydrolysis. The fraction of these originally present as amides has not been determined.

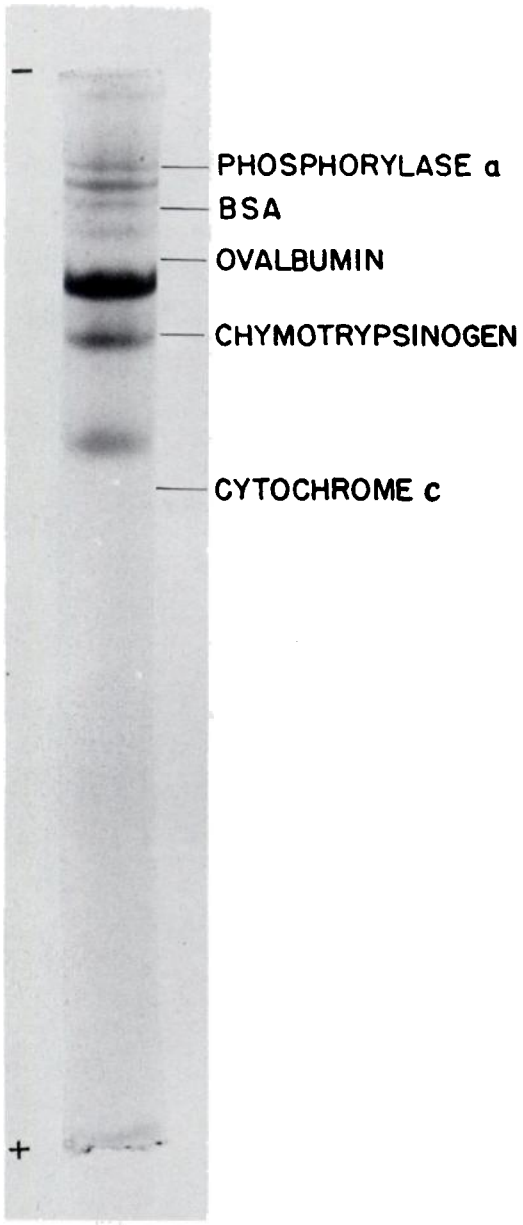

FIG. 4. Analysis of solubilized dense fiber-connecting piece fraction by electrophoresis in 10 percent polyacrylamide gels, containing 0.1 percent SDS, and staining with 0.25 percent Coomassie blue. The sample shown was run in parallel with standards, for 2 h in 0.5 cm × 6 cm gels, at a current of 6 ma per gel. Preincubation with 2-mercaptoethanol, which did not affect the results, was omitted. Migration was towards the anode (+). The distance from the origin (–) of the leading edge of each band was used to determine mobility. The relative migration of 5 proteins of known molecular weight (see Materials and Methods) is indicated. BSA = bovine serum albumin.

TABLE 3. Amino acid analyses of rat sperm tail fibers, wool and guinea pig hair.

Amino acid	Residues/100 residues		
	Rat sperm tail fibers[b]	Wool[c]	Guinea pig hair[d]
Alanine	3.1	5.5	4.9
Arginine	7.5	6.6	7.6
Aspartic acid[a]	9.3	6.5	6.0
Cysteine[a]	11.6	11.4	10.8
Glutamic acid[a]	7.8	11.3	12.6
Glycine	6.0	8.8	9.2
Histidine	1.1	0.8	0.6
Isoleucine	3.5	3.4	3.2
Leucine	10.2	7.8	7.0
Lysine	6.2	3.0	2.6
Methionine	1.0	0.5	0.6
Phenylalanine	1.5	2.9	2.3
Proline	8.6	6.0	7.2
Serine	9.4	9.6	11.3
Threonine	2.9	6.1	7.0
Tyrosine	5.6	4.1	3.8
Valine	4.7	5.9	4.8

[a]Because of the procedures employed for solubilization and hydrolysis, these analyses do not distinguish between cysteine and ½-cystine, or between aspartic and glutamic acids and their respective amides.

[b]Analysis based on two 24-hr hydrolyses, uncorrected for losses during hydrolysis. Cysteine residues analyzed as aminoethylcysteine.

[c]Adapted from the data of O'Donnell and Thompson (1962). Cysteine residues analyzed as cysteic acid.

[d]Adapted from the data of Steinert and Rogers (1973). Cysteine residues analyzed as carboxymethylcysteine. The trace content of citrulline has been omitted.

DISCUSSION

From the results presented here, it appears likely that the major fraction of zinc in mature rat spermatozoa is associated with the dense outer fibers. The preparation described in Figs. 2 and 3 retains more than 75 percent of the zinc originally present in the tail (Table 2), and therefore over 70 percent of the zinc in the sperm. It consists primarily of dense fibers. Connecting pieces, although readily visible in such a preparation, constitute only a minor fraction of its content. Contamination by other structures is relatively insignificant. Since both the dense fibers and connecting pieces were visibly damaged during their isolation (Fig. 3), percent recoveries of zinc in the isolated material may represent underestimations of the amounts actually present in these structures.

The precise location and relative distribution of the remainder of zinc in rat sperm remain open to conjecture. Besides the dense fibers and connecting pieces, two other -S-S- bonded tail structures, namely the fibrous sheath and outer mitochondrial membranes, are resistant to SDS (Bedford and Calvin, 1974). The lower percentage of zinc in the dense fiber-connecting piece fraction, in comparison with that retained in the residue of SDS extraction (Table 2), implies that the two structures eliminated by sonication in SDS-DTT may contain significant concentrations of zinc. In addition, the minor fraction of zinc in rat sperm tails solubilized by 1 percent SDS is probably associated, at least in part, with the axial filament complex, which is soluble in SDS (Bedford and Calvin, 1974), and has been shown in the sea urchin to contain appreciable concentrations of zinc (Morisawa and Mohri, 1972). A small percentage of zinc in rat sperm is associated with the head (Table 1). Here too, -S-S- linked structures appear to bind the trace element, for when extracted with SDS, the isolated sperm heads retain approximately 75 percent of their zinc.

Localization of zinc almost entirely within the tail of rat sperm is in accord with earlier autoradiographic evidence obtained with sections of rat testis and epididymis, following *in vivo* administration of ^{65}Zn (Millar et al., 1961), and is further supported by similar findings in the bull (Baccetti et al., 1973).

Moreover, kinetic and developmental studies on zinc uptake by rat testis (Wetterdal, 1958; Parizek et al., 1966) and evaluation of the primary effects of zinc deprivation on spermatogenesis (Millar et al., 1960; Orgebin-Crist et al., 1970; Diamond et al., 1971) suggest that the most quantitatively significant requirement for zinc in rat spermatogenesis occurs during elongation of the spermatid, and may therefore be involved directly in differentiation of the tail. On the other hand, staining of rat testis sections with dithizone has indicated that zinc is primarily located in the heads of testicular sperm (Timm and Schulz, 1966). It is not clear at present to what extent this apparent contradiction of our findings with epididymal sperm reflects unreliability of dithizone for the cytochemical determination of zinc, the actual existence of a loosely-bound fraction of zinc in testicular sperm which is nearly absent in the more mature cells, or the removal of such a fraction by suspension of sperm in buffer, as in the present experiments. It has also been reported, on the basis of X-ray microprobe analysis of unwashed sperm, that the heads of human sperm are the major site of zinc (Hall, 1966). However, attempts to repeat this finding, using sperm which had been carefully washed to remove contamination from the zinc-rich seminal plasma, failed to detect zinc in the human sperm head (Friberg and Nilsson, 1974). Thus, the distribution of stably-bound zinc between heads and tails of human sperm remains to be established.

Facilitation of zinc release by sulfhydryl reagents has indicated that the major fraction of zinc in rat sperm is associated with -SH groups (Calvin, 1975; Calvin and Bleau, 1974). The 12 percent cysteine content of the dense fiber-connecting fraction (Table 3), which confirms earlier observations of the high content of -SH in rat sperm dense fibers (Nelson, 1960), is consistent with this interpretation. Moreover, the relatively low content of histidine, the only other of the 20 common amino acids which binds zinc with comparable avidity (Hallman et al., 1971; Giroux and Henkin, 1972), favors cysteine residues as the major zinc ligands in this fraction. A protein fraction of even higher cysteine content (19 percent), whose approximate molecular weight is 30,000, has been isolated from crude preparations of bull sperm dense fibers, which also contained connecting pieces and remnants of the fibrous sheath (Baccetti et al., 1973). The concentration of zinc per mg of these fibrous preparations was nearly 3-fold that of the whole cell. Since the 30,000 molecular weight component was the predominant one therein, it has been proposed that this cysteine-rich protein is the primary zinc-binding ligand in bull sperm dense fibers. By analogous reasoning, the aminoethylated component of 35,000 molecular weight isolated from rat sperm dense fibers is most likely to be the major zinc-binding ligand. Its cysteine content has not yet been determined. However, the 11–12 percent estimate for the total dense fiber-connecting piece fraction in which it predominates appears to be on firm ground. Submitting the solubilized aminoethylated dense fiber fraction to a repeat sequence of reduction in guanidine-HCl – DTT and aminoethylation has failed to increase this value. Likewise, the estimate has remained unchanged when the rat dense fibers were solubilized and carboxamidomethylated according to the procedure of Baccetti et al. (1973).

In a preliminary communication, Price (1973) has described the isolation from rat sperm of a dense fiber fraction whose contents of cysteine (15 percent) and of glycine (12 percent) were higher than those listed in the present communication. Since details concerning this preparation are not yet available, it is not possible to evaluate these discrepancies between amino acid analyses.

On the other hand, as illustrated in Table 3, striking similarities exist between the overall amino acid composition of the dense fiber-connecting piece proteins isolated in the present study and the overall compositions of either wool (O'Donnell and Thompson, 1962) or guinea pig hair (Steinert and Rogers, 1973), which fall into the category of substances known as hard keratins. Such similarities are consistent with the possibility of common phylogenetic origin. The stable, relatively prominent content of zinc in hair and other hard keratins (e.g. – nail, horn) and the essential role of this trace metal in the formation of keratinous tissue are, moreover, well established (Underwood, 1971). Therefore, the evidence discussed here that stable zinc-sulfhydryl complexes are intrinsic to the development of certain -S-S- linked sperm tail structures may be of relevance to its role in other keratinous cells.

By contrast to the evidence for the importance of zinc-sulfhydryl interactions in the sperm tail, there is no clear indication, as yet, of whether -SH groups play a major role in

binding zinc within the rat sperm head. In the mature sperm head, the primary localization of this trace metal within -S-S- linked (i.e. SDS-insoluble) structures (Table 2) and the stimulation of -SH alkylation by preincubation at pH 2 (Table 1) suggest that this is a possibility. However, assay of -SH in the immature sperm head is not measurably affected by acid preincubation (Table 1). Furthermore, the apparent content of -SH in this structure decreases during epididymal maturation to a far greater extent that that of zinc, so that the ratio of -SH to zinc has diminished from >10 in the immature sperm head to <2 in that of the mature sperm. This at least indicates a poor developmental correlation between the respective levels of these two constituents in the head, in contrast with that observed in the tail.

The comparable decreases in -SH and zinc during maturation of the tail may be interpreted on the basis of evidence that: 1) the great majority of both -SH and Zn is located in the tail (Table 1), 2) the -SH which is lost during maturation becomes converted to -S-S- (Calvin and Bedford, 1971; Bedford and Calvin, 1974), and is therefore localized within those structures which become -S-S- linked during maturation, 3) zinc is also localized within -S-S- stabilized (i.e. SDS-insoluble) structures (Tables 1 and 2), 4) the ratio of -SH to zinc in both immature and mature sperm (Table 1) is well in excess of the coordination number of 3 reported for zinc-mercaptide complexes (Kagi and Vallee, 1961), and 5) the percent decrease of -SH during epididymal maturation is somewhat greater than that of zinc (Table 1).

The following model has been deduced to explain these findings. First of all, certain -S-S- linked structures (e.g. – the dense outer fibers) are assembled as complexes of sulfhydryl-rich proteins and zinc, in which the majority of -SH is not associated with zinc. Secondly, although nearly all of the zinc in these structures may be interacting with -SH groups, not all of the resulting complexes are equally stable. During maturation, most of the -SH groups which are not protected by zinc oxidize to -S-S-. At the same time, zinc which is only weakly complexed with sulfhydryls is largely released, and concomitantly, the exposed sulfhydryls are available for oxidation to disulfides. Finally, in the mature sperm, the great majority of zinc and a significant fraction of sulfhydryls exist as zinc-mercaptide chelates, the most stable of which contain 3 sulfur atoms and 1 zinc atom (Kagi and Vallee, 1961).

Since such complexes are known to resist alkylation (Kagi and Vallee, 1961), it is quite understandable that preincubation of sperm at pH 2, which removes zinc, enhances the reaction of -SH with [^{14}C] iodoacetamide (Table 1) (Calvin and Bleau, 1974). In addition, it is likely that the -SH groups shielded by zinc are largely protected from oxidation, and that therefore this trace metal regulates the extent and arrangement of -S-S- crosslinking in the sperm tail, a factor which must have important consequences for sperm motility. It has in fact been suggested that zinc and copper, both present in mammalian sperm tails (Mann, 1964), and recently shown to bind competitively to human sperm (Maynard et al., 1975), may exert opposite effects on -S-S- crosslinking, on the basis of studies which indicate that Zn^{2+} can inhibit the oxidation of rat sperm -SH to -S-S- by Cu^{2+} *in vitro* (Calvin et al., 1973). Opposing effects of these two cations on the oxidation of labile functional groups such as -SH have been proposed to occur in other systems, e.g., the cell membrane (Chvapil et al., 1972). If such antagonism between Cu^{2+} and Zn^{2+} does occur *in vivo* in the sperm tail, the analogy between hair and sperm tail keratins would be reinforced, for it has long been suspected, as a result of nutritional deficiency studies in sheep, that trace amounts of Cu^{2+} are necessary for the normal oxidation of -SH to -S-S- in wool (Marston, 1946; Underwood, 1971). In addition, the affinity of the -SH groups in reduced wool for Zn^{2+} has been demonstrated by differential studies on the uptake of this cation both before and after reduction of -S-S- bonds (Masri and Friedman, 1974).

The possible function of zinc during the differentiation and maturation of keratin-like structures in mammalian sperm may be relevant to the development of similar elements which occur in sub-mammalian species, including invertebrates (Bedford and Calvin, 1974), even when phylogenetic kinship to the keratins of mammalian sperm or hair seems unlikely. Thus, it is worthy of mention that the tails of octopus sperm, which possess -S-S- linked outer fibers (Bedford and Calvin, 1974), are unusually rich in zinc (Martin et al., 1973). Wherever -S-S- crosslinked auxiliary elements do occur in sperm tails, it would be of interest to establish if zinc is concentrated in these structures.

At the same time, it is recognized that the

prominence of zinc in the axial filament complex of sea urchin sperm (Morisawa and Mohri, 1972), in prostatic and vesicular secretions (Mawson and Fisher, 1953; Mann, 1964; Boursnell et al., 1972) and in the epithelium of the human epididymis (Schell and Hornstein, 1974), the stimulatory or inhibitory effects on sperm metabolism and motility which have been claimed for zinc in a variety of species (Fujii et al., 1955; White, 1955; Saito et al., 1967; Lindholmer and Eliasson, 1974; Maynard et al., 1975), its marked affinity for proteins of the sperm membrane (Blank et al., 1974), and its ability to counteract the harmful effects of Cd^{2+} on the testicular vasculature of rodents (Parizek, 1957; Gunn et al., 1963; Gunn and Gould, 1970) suggest additional important functions for this trace metal in sperm and testis.

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