of iron porphyrin, thus leading to inactivation of cytochrome P-450 in ecdysteroid synthesis.

Discussion. Present studies have shown that the inhibitory action of ES-X on ecdysone biosynthesis is species-nonspecific among crabs; it was also found that the inhibitory action of **1** and **2** occurs at the site of the Y-organ. However, **2** does not suppress the 20-hydroxylation of ecdysone in the body fluid although we have shown the latter to contain an ecdysone-20-hydroxylase (by ecdysone → 20-hydroxyecdysone conversion, scheme). Experiments with insect tissues [23] and ecdysone 20-monooxygenase from insect sources [24] have also demonstrated that **1** and **2** do not inhibit the subsequent 20-hydroxylation of ecdysone. In addition, injection of **1** and **2** into lobsters [25] neither lowered the circulating ecdysteroids nor prolonged the molt cycle of eyestalk ablated lobsters. It is noteworthy that insects also release endogenous inhibitors of microsomal oxidations [26], though their biological function in vivo is unknown. Ongoing investigations include endocrinological studies of **1** and **2**, clarification of their modes of inhibitory action in ecdysone synthesis, identification of a further MIH-active fraction (if any) in ES-X, in vivo assays with crabs and lobsters, and clarification of the functional relationships between the recently isolated neuropeptide [18] and **1** and **2**.

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Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males

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Summary. Protamines were extracted from the sperm of fertile and infertile human males and the relative proportion of protamines 1, 2, and 3 were determined by scanning microdensitometry following electrophoresis of total protamine in polyacrylamide gels. The proportion of the three protamines was found to be similar in sperm obtained from different normal males. The distribution of protamines in sperm obtained from a select group of infertile males producing an elevated level of large sperm heads, in contrast, was different from that of the fertile males.
Key words. Human sperm; human protamine; protamine 1; protamine 2; protamine 3; infertility; gene expression.

Numerous studies have demonstrated that the DNA of human sperm is packed into the sperm nucleus by three different protamine molecules [1–7]. As in other placental mammals [2, 8–11], human protamines are synthesized and deposited onto DNA late in spermiogenesis, replacing the majority of the somatic histones and other transition proteins. This repackaging of DNA appears to 'deprogram' the bulk (85–90%) of the male genome and condense the DNA into a highly compact, biochemically inert nucleoprotamine complex. Only a small proportion (10–15%) of the DNA sequences remain packaged by a special group of sperm specific histones [12].

Each of the three human protamines has recently been characterized by several different laboratories [4–7, 13]. The primary sequence of protamine 1 (HP 1) was found to be similar to sequences obtained for bull [14], boar [15], ram [16] and mouse [17] protamine 1. The amino terminal sequence of each protamine 1 begins with the conserved sequence ala-arg-tyr-arg-cys-cys, the proteins contain precisely 50 amino acids, and the bulk of the arginine residues are concentrated in the

center of the molecule. Protamines 2 (HP 2) and 3 (HP 3) are structurally different from protamine 1. Human protamine 2 and 3 are not only larger than protamine 1, but they also contain significantly more histidine residues. As others have observed with mouse protamine 2[18], the arginine residues are also distributed throughout the length of the molecule. Human protamine 2 and 3, on the other hand, only differ from each other by an additional tripeptide sequence located on the amino-terminus of protamine 2.

While protamine 1 appears to be the predominant protein in the nucleus of most mammalian sperm, protamine 2 has been identified as a major component of mouse[19, 20] and hamster[21] sperm chromatin. Hybridizations with a cDNA probe for the mouse protamine 2 gene[22] have suggested that the protamine 2 gene may be present in other mammals, but that it is not always expressed. Analyses of the relative contents of protamines 1 and 2 (or 2 + 3) in human, mouse and hamster sperm have also demonstrated that the proportion of these two proteins is constant within a given species[23], while it varies widely among different species[24]. Mouse sperm, for example, contain twice as much protamine 2 as protamine 1; hamster sperm contain only half as much protamine 2 as protamine 1. In humans, the ratio of protamine 1 to protamine 2 (2 + 3) is 1 : 1.

A high proportion of abnormally large sperm heads is often observed in the semen of infertile males[25]. In this study, we examine the distribution of protamines 1, 2 and 3 in fertile males and a select group of infertile males producing an unusually high percentage of sperm with large heads. The results reveal an apparent uncoupling of the expression of protamines 1 and 2 in the sperm produced by the infertile males.

Materials and methods. Semen samples were collected from two separate sources of fertile individuals, a group of volunteer (Northern California) donors and a Thai group from Bangkok. Sperm counts varied from 75–186 × 10^6 sperm per ml. Infertile samples were collected from Thai infertility clinic patients with spouses of known fertility. The infertile individuals used in this study also produced semen in which 50% or more of the total sperm had abnormally large sperm heads. Sperm counts on these samples all fell between 16 and 49 × 10^6 sperm per ml. All samples exhibited 70% or better motility. Individual samples were frozen after collection and stored at − 20 °C until the nuclear proteins could be isolated. Thawed semen samples were diluted with 0.01 M Tris-HCl, 0.15 M sodium chloride, pH 8 (Tris-saline), sonicated briefly with a Branson sonifier to suspend the cells, and centrifuged at 10,000 rpm for 3 min to pellet the sperm. The pellet was washed a second time with Tris-saline and then treated with 10 mM dithiothreitol, 0.01 M Tris pH 8 containing 1% mixed alkyltrimethylammonium bromide (Sigma Chemical Co, St. Louis, MO) to remove the acrosome, tails and nuclear membrane[20]. Total sperm basic proteins were extracted from the amembraneous nuclear pellet as described previously for mouse sperm[20] and the protamines resolved by electrophoresis in 10 cm Panyim-Chalkley acid-urea polyacrylamide gels[26]. The gels were stained overnight in 0.1% Naphthol Blue Black, 0.9 N acetic acid, 20% ethanol and destained electrophoretically.

The amount of protamine 1, 2 and 3 in each sample was determined by scanning the protamine region of each gel with a Model SL 504 Zeineh Soft Laser Microdensitometer (Biomed Instruments, Inc). The protein (Naphthol Blue Black-protein complex) content of each peak was quantified either by determining the areas under the protamine 1 and combined protamine 2 + 3 peaks or by fitting a series of electronically generated Gaussian curves to each of the three peaks using a DuPont Curve Resolver.

Results and discussion. Upon electrophoresis of total human sperm basic proteins in acid-urea gels, the human protamines resolve into three distinct electrophoretic species (fig. 1). A minor group of proteins that migrate coincident with somatic (calf thymus) histones, the human sperm specific histones, are also resolved. While the three protamine bands appear to be well separated, the microdensitometer scans of protamine 2 and 3 (fig. 1) are not resolved sufficiently to permit direct integration of the areas under all

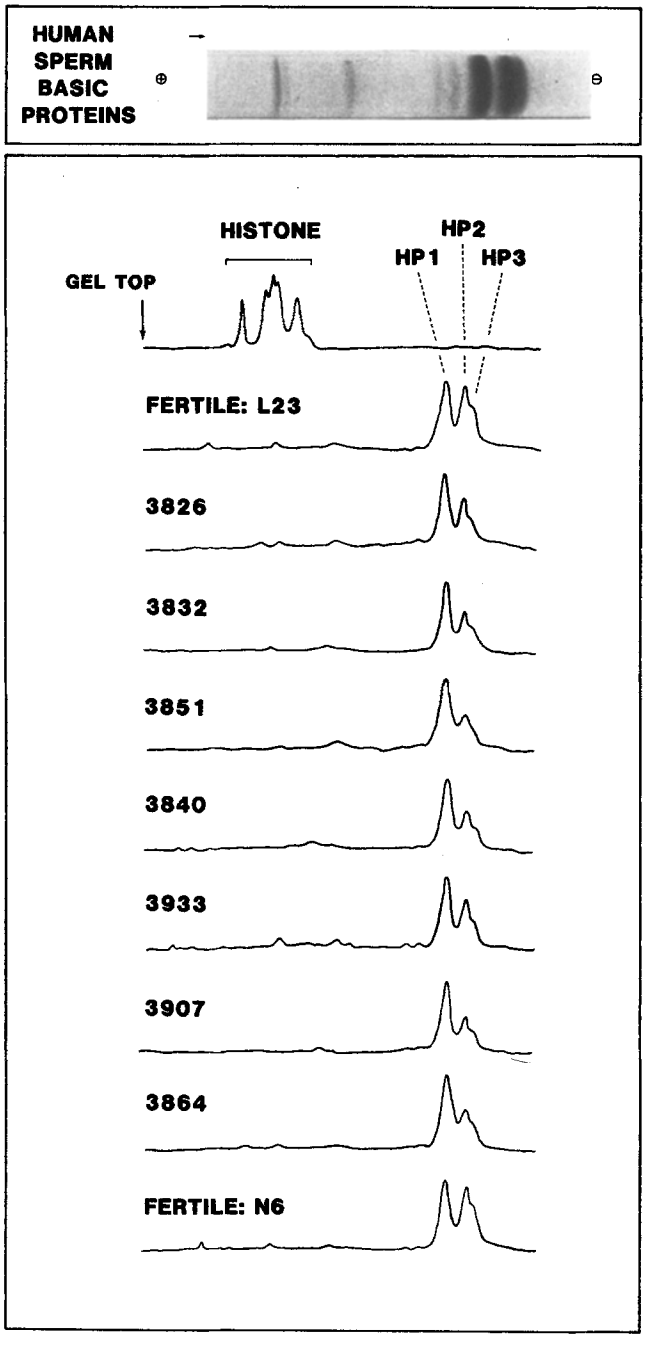

Figure 1. Electrophoretic pattern and microdensitometer scans of total human sperm basic proteins isolated from fertile and infertile males. Electrophoresis was performed in 10-cm gels at 120 V, 20 mA for 1.5 h. Gels were stained, destained and scanned as described in the Materials and Methods section. Top Panel: Stained gel showing electrophoretic separation of HP 1, 2 and 3 in an acid-urea gel. Bottom Panel: Microdensitometer scans of calf thymus histone (top scan, for reference), two representative protamine samples (L 23 and N 6) from fertile males, and the protamines of all seven infertile individuals (3826, 3832, 3851, 3840, 3933, 3907, 3864).

three peaks. Protamine 3, which differs from protamine 2 by only three amino terminal amino acids, appears only as a small shoulder on the leading edge of the protamine 2 peak. Previous experiments with mouse protamine 1 and 2 have shown that both proteins bind equivalent amounts of Naphthol Blue Black in stained acid-urea gels[11]. Similar results have been obtained for the human protamines[24]. Microdensitometry measurements performed on mixtures of the two protamines stained with Naphthol Blue Black, therefore, provide results that relate directly to the protein content of gel bands. The linear range over which the Naphthol Blue Black-protein complex absorbs light at 600 nm was determined by scanning a series of stained gels containing increasing amounts of total human protamine. Plots of the areas under the protamine 1 and protamine 2 + 3 peaks are shown in figure 2. In each case the protein content determined by microdensitometry measurements increases linearly over a range of 1–10 µg protamine per band. Above 10 µg protamine, absorption measurements of Naphthol Blue Black binding begin to plateau. These results were used to set the upper and lower limits of protein applied to gels for all subsequent experiments.

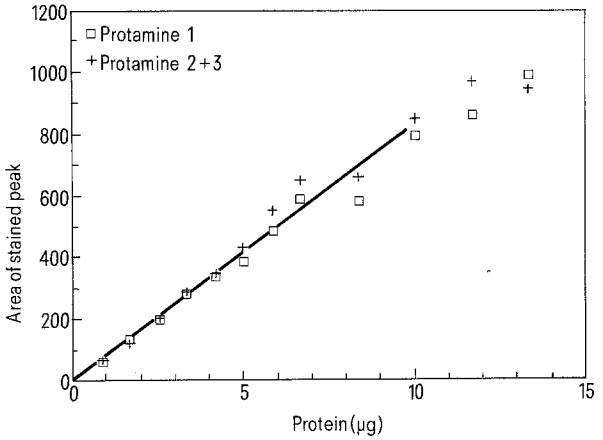

Figure 2. Concentration range over which Naphthol Blue Black absorption is linearly related to the protein content of stained human protamine 1 (HP 1) and protamine 2 + 3 (HP 2 + 3). Protamine samples were electrophoresed in acid-urea gels, the gels stained with Naphthol Blue Black, and the areas under the peaks determined as described in the text.

Quantitative microdensitometry measurements of areas under the protamine 1 and protamine 2 + 3 peaks show that sperm obtained from both local and Thai fertile males contain equal proportions of protamine 1 and protamine 2 + 3 (table). While it is recognized that the total number of individuals examined in this study is small, the mean values obtained for the two geographically distinct populations do not differ significantly. In contrast, the proportion of protamine HP 2 + 3 in all but one of the infertile samples (# 3933) was found to fall outside the range of values obtained for the normal (fertile) samples (table, fig. 3). Analyses of the protein content of all three protein bands by Gaussian curve fitting strongly suggests that the apparent decrease in protamine 2 + 3 in these samples cannot be explained by an increase in the relative proportion of protamine 1 alone. While the protamine 3 content of the infertile

Quantitation of human protamines HP 1, HP 2 and HP 3 in sperm of fertile and infertile males.

Individual (code No.)	Percent total protamine Microdensitometry[a]		Curve resolution[b]		
	HP 1	HP 2 + 3	HP 1	HP 2	HP 3
Fertile controls:					
L19	53	47	50.5	37.5	12
L21/22	48	52	47.5	40.5	12
L23	49	51	47	38.5	14
L26	46	54	47.5	37	15.5
L27	51	49	50	36.5	13.5
C1	47	53			
C2	48	52			
Mean	48.9	51.1	49	38	13
SD	2.4	2.4	2	2	2
N1	56	44	55	34	11
N2	47	53	50	36	14
N3	50	50	2	35	13
N4	47	53	48	39.5	12.5
N5	44	56	46	40	14
N6	49	51	47	38	15
N7	49	51	47	42	11
N8	49	51	46.5	41	12.5
N9	54	46	51	40.5	8.5
N10	50	50	49	41.5	9.5
Mean	49.5	50.5	49	39	12
SD	3.4	3.4	3	3	2
Infertile:					
3826	60	40	59	26	14
3832	58	42	57	30.5	13
3840	63	37	61	28	11
3851	66	34	65	25	10
3864	62	38	61	28	11
3907	63	37	61	28	11
3933	55	45	57	30	13
Mean	61	39	60	28	12
SD	3.7	3.7	3	2	2

[a] Area integration; [b] Fit electronically generated curves and analyze areas, DuPont Curve Resolver.

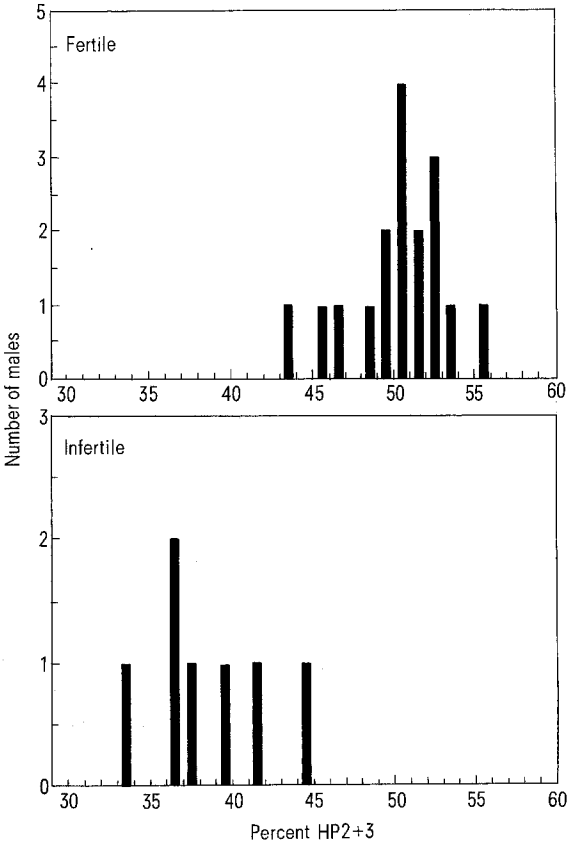

Figure 3. Distribution of the protamine HP 2 + 3 content of sperm obtained from fertile and infertile individuals. Values plotted are those taken from the table (microdensitometry).

samples is indistinguishable from that observed in normal samples (12–13% of the total), the mean content of protamine 2 in the infertile samples is only 28%. The sperm of normal males contain 38–39% protamine 2. This change appears, therefore, to reflect a reduction in the level of expression of protamine 2. An actual decrease in protamine 2 of this magnitude would appear to be compensated for by a slight increase in protamines 1 and 3. The level of expression of both protamine 1 and 3 would have remained unchanged, but each would constitute a greater proportion of the total protein complement.

It is possible, on the other hand, that the relative level of protamine 2 expression has not changed, but that the expression of protamines 1 and 3 have increased slightly in the infertile samples. Methods that permit the absolute quantitation of each of the three protamines relative to DNA at a level of precision that would be required to support or discount this possibility are not currently available. This change could also reflect a reduction in the level of protamine 2 expression in a population of sonication resistant 'abnormal' sperm or arrested late-step spermatids, since the observed values reflect an average protamine composition for all the sperm and sonication resistant spermatids in the sample.

Although the correlation between the observed decrease in protamine 2 in sperm obtained from these infertile males and infertility is intriguing, this link should be considered tenuous until the results can be substantiated by increasing the size of the populations examined. Perhaps a more important implication of this study, however, is the suggestion that the expression of the genes for protamines 1 and 2 may actually be uncoupled in the developing spermatids of certain human males. This coupling appears to be rigorously controlled within other species containing protamine 2, as in *Mus musculus*. Comparisons of the ratio of protamine 1 and 2 in different strains of the laboratory mouse have shown the ratio of the two proteins to be invariant[23]. In contrast, expression of the two protamine genes definitely differs among species. The protamine 2 gene is not expressed in the bull and rat, two species that appear to contain the gene[22]. It is expressed at various levels in the Syrian hamster (36.5% HP 2), human (50% HP 2 + 3), and mouse (70% HP 2).

We have learned a great deal about the structure and function of the mammalian protamines in the last decade. Perhaps one of the more puzzling discoveries in recent mammalian protamine research is the observation that the sperm of certain mammalian species require two very different protamine molecules to package their DNA, while many other species need only one. Although it is tempting to assume that protamine 1 and 2 bind to and package DNA in a similar fashion and that they may function interchangeably, the recent discovery that protamine 2 is synthesized as a precursor (while protamine 1 is not)[18] argues against this notion. Protamine 1 and the precursor to protamine 2 are synthesized and deposited onto DNA concurrently in mouse spermatids (submitted for publication, 1987). The precursor is subsequently processed over a 24-h period to mature protamine 2 by removal of 40% of the amino terminus of the molecule while protamine 1 remains unaltered. The results of this current study suggest the possibility that protamine 2 may perform a unique function in sperm chromatin and that this 'function' may be essential for fertility. Whether this deficiency of protamine 2 is actually responsible for the observed infertility remains to be determined.

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