1 week at 4 °C, developed in Kodak D-19, stained with hematoxylin and eosin, and mounted with another coverslip.

Slides were scored by counting silver grains over the nuclei of non-S-phase cells, excluding as S-phase cells those with greater than 40 grains per nucleus. Mean grain counts of the non-S-phase cells, corrected for background, were determined on 3 groups of 150 cells at each dose point. The SEM from an average count of 3 such replicate groups of 150 cells at each dose point was 14%. The presence of UDS is demonstrated in the figure by the dose-dependent increase in silver grains over the nuclei of non-S-phase cells, indicating replacement of damaged sites in DNA. For both the X-ray (solid line) and UV (dashed line) curves, there appears to be no significant difference in mean grain count between the resistant (squares) and sensitive (circles) lines. The percentage of cells in the S-phase, i.e., those cells containing >40 grains/nucleus, remained relatively constant ($32.6\pm3.6\%$) in unirradiated and irradiated cultures from both lines.

Positive correlations between radiosensitivity and DNA-repair replication have been made in 2 lines derived from human cervical cancer[10] and 2 L5178Y lines[11]. A similar correlation between radiosensitivity and UDS was not seen in our experiments. One must bear in mind, however, the distinction between apparently 'normal' levels of UDS and the integrity of the repair system. UDS measures only the incorporation of labeled DNA precursors into DNA of non-S-phase cells and not necessarily the correction of radiation-induced lesions. The possibility remains that radiosensitive cells may possess defective repair systems which appear in quantitative terms similar to the functional repair systems possessed by the more radioresistant cells.

1 The assistance of Mirriam M. Posilero in the preparation of this manuscript is gratefully acknowledged.
2 R.E. Rasmussen and R.B. Painter, J. Cell Biol. *29*, 11 (1966).
3 S.R. Ayad, M. Fox and B.W. Fox, Mutat. Res. *8*, 639 (1969).
4 R.B. Painter and J.E. Cleaver, Radiat. Res. *37*, 451 (1969).
5 J.E. Cleaver, Proc. nat. Acad. Sci. USA *63*, 428 (1969).
6 J.E. Cleaver, J. invest. Derm. *54*, 181 (1970).
7 A. Norman, R.E. Ottoman, P. Chan and I. Klisak, Mutat. Res. *15*, 358 (1972).
8 J. Shaeffer, A.M. El-Mahdi and T. Merz, Cancer *29*, 581 (1972).
9 A.M. El-Mahdi, J. Shaeffer and W.C. Constable, Acta Radiol. *13*, 478 (1974).
10 J.E. Byfield, Cancer Chemother. Rep. *58*, 528 (1974).
11 I. Korner, M. Walicka, W. Malz and J.Z. Beer, Stud. Biophys. *61*, 141 (1977).

The formation of disulfide bonds in human protamines during sperm maturation

W. Saowaros and S. Panyim[1]

Department of Biochemistry, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 4 (Thailand), 28 March 1978

Summary. The disulfide contents of human sperm heads, as measured by reduction to the sulfhydryls and subsequent alkylation with ^{14}C-iodoacetamide, increase about 2-fold during the sperm passage from the caput to caudal epididymides. Majority of the increased disulfides reside in the human protamine fractions.

Sperm maturation is a process whereby testicular sperm acquire fertilization potential during passage through the epididymis[2,3]. The maturation process requires the epididymal environments[3] and androgen[4]. In addition to morphological changes, several biochemical changes have been shown to accompany the maturation process including increases in disulfide content, metabolic rate and surface negative charges[5]. In rats, disulfide formation has been shown to occur in the head as well as in the tail of the sperm[6]. Since mammalian protamines have high contents of half-cystine[7,8], they are likely to be involved in this epidymal disulfide formation. This has been confirmed in rats, where the protamines isolated from sperm of the cauda epididymis contain more disulfides per molecule than those obtained from the sperm of the caput epididymis[9]. However, from these studies, it is still not clear whether protamins represent the major class of proteins forming disulfide bonds during sperm maturation. Furthermore, in view of the species specificity of the reproductive process, it is difficult to extrapolate studies in other species to the humans. Studies in our laboratory[7,10] and in others[11] have shown that human protamines consist of several components, which exhibit a different electrophoretic pattern and amino acid composition to the protamines of other species. In this report we have investigated the occurrence of disulfide bond formation in human sperm, with special reference to the relative importance of the protamines in such a process.

Materials and methods. Epididymal sperm were isolated from accident victims within 12 h post mortem following the method of Calvin et al.[6], except that 5 mM iodoacetamide was present throughout. The sperm suspension (in 0.2 M phosphate, 1 mM EDTA, 5 mM iodoacetamide, pH 7.3) was kept in the dark at 4 °C for 1 h to block completely free sulfhydryl groups, and was then sonicated for 30 sec at 70–80% of the maximum output of a Branson Sonicator, model J-17A. Ejaculated sperm were treated in a similar manner and then sonicated for 2 min. The sonicated sperm suspensions were layered on top of 40% sucrose and centrifuged at $700\times$ g for 30 min to pellet sperm heads[10].

The content of unblocked half-cystine, which should be in the disulfide form, was determined by reduction of the above sperm heads is an O_2-free solution containing 5 mM dithiothreitol, 0.1 M Tris. HCl, pH 7.3, 1 M guanidine hydrochloride for 1 h, followed by alkylation with 0.02 M ^{14}C-iodoacetamide (1 μCi/μmole) in the dark at 25 °C for 1 h. The reaction was terminated by adding excess unlabelled iodoacetamide. One portion of the mixture was taken to quantitate the ^{14}C-carboxymethylated products by precipitation with trichloroacetic acid (TCA) and filtration on Whatman GF/A filters, followed by extensive washing with 5% TCA and acidified acetone (39 :1 acetone :1 N HCl). The dried GF/A paper was counted in toluene cocktail with a counting efficiency of 47–52%. Another portion of the ^{14}C-carboxymethylated mixture was extracted with 0.25 N HCl[10] and samples from this acid-soluble

fraction were assayed for radioactivity. The remainder of this fraction was precipitated with 20% TCA and then subjected to electrophoresis, following the method of Panyim and Chalkley[12]. The destained gels were longitudinally cut into halves, dried and radioautographed for 2 weeks using Kodak medical X-ray film.

Results and discussion. The results (table) show that the ^{14}C-iodoacetamide incorporation into human sperm heads increase approximately 1-fold during passage of sperm from the caput to the cauda epididymis. This incorpotation represents only the sulfhydryl groups obtained by reduction of disulfide bonds, present in vivo, since free sulfhydryl groups present in vivo should have been previously blocked by the unlabelled iodoacetamide, to which the sperm were initially exposed. Similarly, prior treatment with unlabelled iodoacetamide should prevent other nucleophilic side-chains from taking up ^{14}C-iodoacetamide. Therefore, increased iodoacetamide incorporation signifies the formation of disulfide bonds in sperm heads during maturation.

Since iodoacetamide was incorporated into TCA precipitable material, the disulfide bonds present should reside in protein, most likely protamine, since it is the major protein in sperm head and is also rich in half-cystine[7]. When sperm heads were extracted with 0.25 N HCl, the usual procedure for protamine isolation[10], approximately 86% ^{14}C-carboxymethylated products were obtained in the acid-soluble fraction (table). Upon electrophoresis at pH 2.7, as shown in the figure, the electrophoretic pattern obtained was highly characteristic of the protamines, containing all the subfractions[10]. Since there is no observable difference among the protamine patterns extracted from caput, cauda and ejaculated sperm (figure, a, c and e), there appears to be no change in protamine components during human sperm maturation, in contrast to the change of basic proteins observed in the house cricket[13].

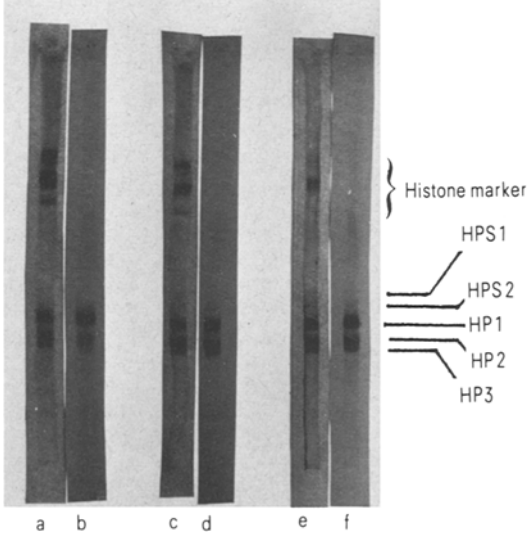
The autoradiographs (figure, b, d and f) clearly show that the HP1, HP2 and HP3 protamines are all carboxymethylated, demonstrating that all these protamine subfractions formed disulfide bonds during sperm passage through the epididymis. Since electrophoresis was performed at pH 2.7, all proteins should carry net positive charges and should be able to penetrate into the gel matrix. Thus the electrophoretic bands observed should represent all the HCl-soluble ^{14}C-carboxymethylated products present. Thus protamines are major proteins involved in forming disulfide bonds during epididymal sperm maturation.

The physiological significance of disulfide formation in the human protamines can only be speculated on at present. For example, such disulfide cross-links may make chromatin adopt a more compact structure. This idea is supported by the report that bull sperm DNA binds less actinomycin D as the sperm travels from the caput to the cauda epididymis[14]. The compactness of chromatin structure may be essential in keeping DNA undamaged when exposed to hostile environments, such as acidity in the vaginal tract[15]. Since the sperm nucleus is metabolically inert[16], damage to the DNA cannot be repaired and may be harmful to the survival of the species.

^{14}C-iodoacetamide incorporation and its distribution in the 0.25 N HCl soluble fraction

Sources of sperm heads*	Incorporation per 10^6 heads (nmoles)	0.25 N HCl soluble fraction (nmoles)	Percent of total
Experiment I			
Caput epididymis	0.51	-	-
Corpus epididymis	0.56	-	-
Cauda epididymis	1.15	-	-
Ejaculated sperm	1.37	-	-
Experiment II			
Caput epididymis	0.71	0.57	80.3
Corpus epididymis	0.81	0.77	95.1
Cauda epididymis	1.10	0.94	85.5
Ejaculated sperm	1.27	1.21	88.2
Experiment III			
Caput epididymis	0.68	0.57	83.8
Corpus epididymis	0.70	0.62	88.6
Cauda epididymis	1.20	1.04	86.7
Ejaculated sperm	1.35	1.15	85.2

* The number of sperm heads used in each assay was 1-5 millions.



Electrophoretic and autoradiographic patterns of the ^{14}C-alkylated products of the sperm heads from caput (*a, b*) and cauda (*c, d*) epididymides and from ejaculated sperm (*e, f*). Electrophoresis was performed in 15% polyacrylamide gels, pH 2.7, at 13 V/cm for 2 h. *a, c* and *e* are patterns obtained by amido black staining. *b, d* and *f* are patterns obtained by autoradiography. Calf thymus histone was used as the marker protein in the electrophoresis.

1 We thank Drs Jisnuson Svasti and Nongnuj Tanphaichitr for their valuable suggestions. This work was supported by the Faculty of Science, Mahidol University, and the Rockefeller Foundation.

2 W.C. Young, J. exp. Biol. *8*, 151 (1931).
3 M.C. Orgebin-crist, Biol. Reprod., suppl. *1*, 155 (1969).
4 M.C. Orgebin-crist, B.J. Danzo and J. Davies, in: Handbk Physiol. *5*, 313 (1975).
5 J.M. Bedford, Handbk Physiol. *5*, 303 (1975).
6 H.I. Calvin, C.C. Yu and J.M. Bedford, Exp. Cell Res. *81*, 333 (1973).
7 P. Pongsawasdi and J. Svasti, Biochim. biophys. Acta *434*, 462 (1976).
8 H.I. Calvin, Biochim. biophys. Acta *434*, 377 (1976).
9 Y. Marushige and K. Marushige, J. biol. Chem. *250*, 39 (1975).
10 T. Puwaravutipanich and S. Panyim, Exp. Cell Res. *90*, 153 (1975).
11 A.H.J. Kolk and T. Samuel, Biochim. biophys. Acta *393*, 307 (1975).
12 S. Panyim and R. Chalkley, Arch. Biochem. Biophys. *130*, 337 (1969).
13 R. McMaster-Kaye and J.S. Kaye, Exp. Cell Res. *97*, 378 (1976).
14 B.L. Gledhill, J. Reprod. Fert., suppl. *13*, 77 (1971).
15 W.H. Masters, Ann. N.Y. Acad. Sci. *83*, 301 (1959).
16 E. Premkumar and P.M. Bhargava, Nature, New Biol. *240*, 139 (1972).