Effect of Glycerol and Freezing on Some Staining Reactions of Human Spermatozoa. (20583)

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Recently we(1) observed that human spermatozoa in glycerol treated semen showed no obvious changes in motility or morphology after surviving freezing and thawing. It was decided, however, to test for more subtle alterations which may occur within the spermatozoa as a result of such treatment. This was suggested by the observation of Keilin and Hartree(2) that freezing of spermatozoa changes the absorption spectrum of their cytochrome and Tyler's(3) report that it may increase their release of a protein lysin. Moreover, Lovelock(4) reported modifications in salt concentration during the freezing of red blood cells and indicated the general application of his results to other cells as well.

The purpose of this communication is to present the results of an experiment designed to detect possible differences between the protein of spermatozoal nuclei in untreated and glycerol treated semen before freezing and after thawing. The principle underlying the technic employed is that "staining intensity varies with changes in the protein and, consequently, is a sensitive criterion of the modification to which the protein was previously subjected." (Singer(5)).

Methods. Dyes. Seven oxidation-reduction potential indicator dyes were employed (Table I). A 0.5% solution in appropriate buffers was prepared for each dye at pH 4, 5, 6, 7, 8, 9, and 10 (when solubility permitted). Acetate buffer was used for pH 4, 5, and 6, phosphate buffer for pH 7 and borate buffer for pH 8, 9, and 10. *Semen.* Semen specimens, considered normal by the usual seminological tests, (Farris(6)), were obtained from 2 donors and processed separately. After liquefaction, a portion of the semen was treated with glycerol, 9 parts semen to one part absolute glycerol. Four 12 x 75 mm bacteriological test tubes were then prepared, 2 with 0.5 cc portions each of untreated semen and 2 with the same quantity of treated semen. One tube each of untreated and treated semen was then frozen by placing them in an insulated box containing dry ice (—70°C) and were subsequently thawed 24 hours later in a 37°C water bath. The semen from these 4 tubes was processed as follows: *Staining technic.* Smears were made on ordinary 3" x 1" slides and fixed overnight with equal parts of 95% alcohol and absolute ether. The slides were then washed in two 5 minute changes of absolute alcohol containing silica gel and dried for 10 minutes in a 37°C oven. The dried smears were placed in the dye solution for one minute and then dipped 5 times in first 50% then 80% alcohol to remove excess dye. This was followed by dehydrating in 2 absolute alcohol baths, one minute each, and by clearing in xylol for 2 minutes prior to mounting in a synthetic resin.

Microscopic examination. Using the same microscope and illumination, each author separately rated the intensities of nuclear staining. In some areas there were minor disagreements on intensity. These were resolved by re-examination. The intensity classification employed is shown in Table I.

Results. The results of our study are summarized in Table I and were identical for the 2 semen specimens examined. No differences in staining intensities were noted between: 1) Fresh semen, untreated and treated with glycerol, 2) Frozen-thawed semen, untreated and treated with glycerol, 3) Fresh and frozen-thawed semen, untreated and treated with glycerol.

Discussion. Knowledge of the relative staining intensities achieved with a number of oxidation-reduction potential indicator dyes at different pH ranges probably gives information concerning changes at the sites of dye binding on the proteins which are stained. Such information may also indicate changes in the isoelectric points of these proteins. However, Klotz(7,8), among others, presents chemical evidence to cast doubt upon the pre-

TABLE I. Staining Intensities of Spermatozoal Nuclei, of Untreated and Glycerol Treated Semen before Freezing and after Thawing, Following Exposure to Dyes Indicated.* Intensity is designated as 0 = unstained, ± = faint, + = light, 2+ = moderate, and 3+ = dark. I = dye insoluble at given pH.

Dye	E_0^1 pH 7	pH 4	5	6	7	8	9	10
Sodium 2,6, dichlorobenzenone indophenol	+.217	I	I	0	0	0	0	0
Brilliant cresyl blue	+.047	+	+	+	2+	+	0	0
Methylene blue chloride	+.011	+	+	2+	3+	3+	3+	3+
K_4 indigo tetrasulfonate	—.046	+	+	±	±	0	0	0
Cresyl violet	—.167	2+	3+	3+	3+	+	I	I
Safranin bluish	—.421	2+	2+	2+	3+	3+	3+	3+
Phenosafranin	—.525	+	2+	2+	3+	2+	3+	3+

* Since pattern of staining, with reference to various dyes at given pH's, was the same for all semen samples, a single table here represents the data obtained.

cision of measurements of either the isoelectric points or the oxidation-reduction potentials of proteins through staining technics. Our conclusions, however, concern only the detection of *relative* modifications in those proteins which are stained under the conditions of the experiment and no such *relative* alterations were detected in spermatozoal samples treated and frozen as described.

Summary. No differences were observed between the staining intensities of spermatozoal nuclei in untreated and glycerol treated semen, either before freezing or after thawing, when they were stained at pH 4, 5, 6, 7, 8, 9, or 10 with certain oxidation-reduction potential indicator dyes. On the basis of this criterion, it has been demonstrated that treating 9 parts of liquefied semen with one part absolute glycerol, placing it in an insulated box containing dry ice (—70°C) for 24 hours, and then thawing it in 37°C water bath does not modify those proteins of the spermatozoal nucleus which are stained.

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