treatment, disappeared under the influence of estrogen. In the other rabbits the only effect noted was a decrease in the concentration of the –S 35 fraction. The absence of a concomitant increase in alpha$_1$-lipoproteins in rabbits contrasts with the results of Barr *et al.* (3), obtained by alcohol fractionation and confirmed by ultracentrifugation at a density of 1.21(7), in patients receiving estrogens. Nava and Zilli(8) also observed an increased concentration of serum alpha-globulin following treatment of normal healthy men and women with estradiol or diethylstilbestrol. This variation in response to estrogens is associated with difference of species and difference in lipoprotein pattern of rabbit and human serum. The most significant finding in the present experiments is the increase in the low density lipoproteins (–S (40-70) and –S > 70) following treatment with testosterone; it explains the sex difference and may offer a partial explanation for the greater susceptibility of male than female to atherosclerosis.

Summary. The serum lipoprotein patterns of immature rabbits were determined by ultracentrifugation at a density of 1.21. Two main peaks were resolved: one at –S 35 (20-40) and another at –S 7 (1-10). After castration, these animals were treated with diethylstilbestrol and with testosterone. Only testosterone altered significantly and regularly the lipoprotein pattern by the appearance of two abnormal components; one, –S > 70 and the second, –S (40-70); both disappeared on cessation of treatment.

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Observations on Preservation of Human Spermatozoa at Low Temperatures. (20219)

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The survival of human spermatozoa, after freezing at low temperatures, is not a new observation. Mantegazza(1), as recorded by Davenport(2), was perhaps the first to report this phenomenon. Jahnel(3), Shettles(4), and Hoagland and Pincus(5) have since shown, by various technics, that human spermatozoa will survive temperatures of –79°C to –269.5°C. In an attempt to evaluate the merits of rapid freezing and to reconcile the varied results of these reports, Parkes(6) showed that survival is far better when 1.0 cc of semen is frozen in ampoules rather than tiny amounts in fine capillary tubes. He states it was fairly certain that, under optimum conditions, a large number of human spermatozoa could survive prolonged freezing, but expressed considerable doubt that the speed of freezing and thawing was the primary factor

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in their survival. However, his observation of increased survival with increased volume gave us evidence for the importance of slow freezing, as greater volume extends the freezing time. Further indication of this trend was given in the data of Hoagland and Pincus(5), where greatest survival resulted through the use of foamed semen produced by air bubbles which certainly insulated much of the semen and therefore prolonged its freezing time. Moreover, Luyet(7) noted that the air cushion formed about an object immersed in liquid nitrogen at −196°C (the apparently preferred freezing medium) made it a method of freezing 5 times slower than isopentane at −150°C and even slower than a dry ice bath at −77°C. A comparison of different freezing methods seemed logical and, since glycerol had been shown to increase the resistance of human spermatozoa to freezing, Polge, Smith, and Parkes(8), it was decided to employ this protective substance in this study.

The purpose of this communication is to present some findings concerning the conditions necessary for the maximum survival of human spermatozoa after freezing and storage at low temperatures.

Methods. Collection of semen. Semen was obtained on 3 occasions from each of 5 different donors. Collections were made after 5 days of abstinence to minimize production variation and to coincide with the spermatozoal production peak. These semen specimens were considered normal by the usual seminological tests, Farris(9). *Pretreatment of semen.* Preliminary tests indicated that 1 part of absolute glycerol added to 9 parts of liquefied semen, maintained semen quality with a minimum dilution. The glycerol was added immediately after liquefaction and, depending upon the volume of semen, 0.5 ml or 1.0 ml portions of the treated semen were placed in 12 mm x 75 mm bacteriological test tubes. The tubes were then corked and allowed to stand for 30 minutes at room temperature prior to freezing. *Freezing of semen.* In the probable order of fastest to slowest freezing methods, isopentane (IP) cooled to −150°C by liquid nitrogen, dry ice in acetone slush (DIA) at −79°C, liquid nitrogen (LN) at −196°C, and dry ice in an insulated box (DI) at −70°C were compared as refrigerants. Luyet's(7) and our own gross observations gave evidence that this is the actual order of freezing time. Since initial experiments had shown that glycerol increased survival in all 4 freezing methods, only glycerol treated semen was employed. Each sample was distributed in 4 tubes. The first three tubes were immersed for 5 minutes in their respective baths of IP, DIA, and LN. These 3, along with the fourth, were then placed in DI for periods of one to 3 months, at which time they were quickly thawed in a 37°C water bath. *Tests of semen quality.* In previous observations, objective measurements of spermatozoal survival were either lacking or limited to motility counts. It was decided to make use of additional valid criteria of measurement, both before freezing and after thawing. The usual blood hemacytometer method was employed to compute the per cent motility while the eosin-nigrosin staining technic of Blom(10) provided information concerning viability and morphology. The speed and type of movements were noted as well as the length of time spermatozoa were motile at room temperature.

TABLE I. Average % Survival in 15 Series of Glycerol Treated Human Spermatozoa after Freezing by Methods Indicated, then Stored in DI, 1 to 3 Months and Quickly Thawed in a 37°C Water Bath. % viability based upon eosin-nigrosin test.

Speed of freeze	Method of freeze	% viability Before freeze	% viability After thaw	% survival
Fastest	IP	64	15	23
Fast	DIA	64	27	42
Slow	LN	64	9	14
Slowest	DI	64	43	67

Results. 1) The results of our study are summarized in Table I. The greatest per cent survival was obtained in specimens which were frozen and stored in dry ice (DI). The results obtained from 15 semen samples which were frozen in dry ice (DI) are summarized in Table II.

2) The per cent survival, as measured by the eosin-nigrosin method, was usually identical (within 2%) with the per cent motility as computed by hemacytometer counts. This agrees with the findings of Williams and Pollak(11).

3) Several semen samples were examined

TABLE II. Breakdown of Those Data in Table I Which Concerns DI as a Freezing Method.

Donor	Sample	% viability: Before freeze	After thaw	% survival
A	1	73	50	69
	2	68	50	74
	3	69	52	75
B	1	50	34	68
	2	61	39	64
	3	53	37	70
C	1	65	49	76
	2	63	48	76
	3	69	53	77
D	1	67	42	62
	2	68	39	58
	3	61	19	31
E	1	68	46	68
	2	62	43	70
	3	60	41	69
Avg		64	43	67

after storage for one, 2, and 3 months, and no loss in per cent survival was noted.

4) No significant difference was observed between the results with 0.5 cc and 1.0 cc of semen.

5) There was no apparent difference between the type, speed, and duration of motility of glycerol treated spermatozoa before and after preservation. No morphologic alterations were obvious in our observations.

Discussion. If the order of freezing speeds assumed for the refrigerants employed in this study is correct, the results obtained with liquid nitrogen (LN) are not consistent with the trend toward increased survival with slower methods of freezing (Table I). This problem, as well as the one concerning the protective action of glycerol, is now being attacked experimentally by us.

There is evidence under our experimental conditions that, except for one sample from donor D (Table II), the per cent survival is fairly constant in semen samples from the same donor. There seems to be some variation, though, from donor to donor. Shettles (4) maintains there is a marked individual variation in the sensitivity of human spermatozoa to extremely low temperatures. We feel, however, that a greater number of observations are necessary before any definite statements can be made on either score.

Clinical application of practical storage banks for human spermatozoa in infertility problems is now in progress. Artificial inseminations to test the ability of frozen human spermatozoa to fertilize and induce normal embryonic development are underway in this laboratory.

Summary. Initial experiments indicate that when 9 parts of liquefied human semen are treated with 1 part of absolute glycerol and placed in an insulated box containing dry ice, it can be stored for at least 3 months and, after quick thawing in a 37°C water bath, it will show an average spermatozoal survival of 67%, a figure superior to those realized with faster freezing methods. Such revived spermatozoa show no detectable alterations in motility or morphology.

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