REVIVAL OF MAMMALIAN SPERM AFTER IMMERSION IN LIQUID NITROGEN

By HUDSON HOAGLAND and GREGORY PINCUS*

(From the Physiological Laboratories, Clark University, Worcester)

(Received for publication, October 9, 1941)

I

It has been shown (Hoagland, 1933) that we estimate clock or "public" time as longer the higher the internal body temperature. Experimentally we systematically count more subjective seconds to the clock minute the higher the internal temperature and this makes clock time appear to drag. The Arrhenius equation was found to describe the human time sense surprisingly well. The rate of passage of "private" time (counting estimated seconds) yields a μ value, or temperature characteristic, of 24,000 calories, a value associated in several studies with cellular respiration.

It is well known that some living organisms can recover after being subjected to the very low temperatures of the liquified gases such as air, hydrogen, and helium. For such organisms at these very low temperatures, with metabolism virtually stopped, time, as a reciprocal rate of some aspect of metabolism, must pass very rapidly as far as the organism is concerned. Since life, and its concomitant aging processes, is a series of chemical events proceeding irreversibly towards death, these temperatures must essentially stop aging processes. Such organisms are, for all intents and purposes, projected forward into the future at these low temperatures. A stay of a century at -270°C., the temperature of liquid helium, with subsequent warming and recovery, would, if it were possible to effect, be precisely equivalent to projecting the organism forward a century into the future—a sort of Wellsian time machine idea.

Luyet, who has made a number of contributions to the field of low temperature effects on organisms, has recently reviewed with Gehenio (1940) the subject of life and death at low temperatures. Some 120 studies, beginning with those of Pictet in 1893, have demonstrated that a variety of small organisms can withstand the temperatures of the liquified gases. These include some bacteria, protozoa, plant cells, and a few metazoans. The procedure in general involves rapid chilling and warming so as to vitrify the organisms and not permit their water to crystallize. Crystallization of water takes time and colloidal solutions with their large particles are relatively slow to crystallize.

* Fellow of the John Simon Guggenheim Memorial Foundation.

The crystallization range for protoplasmic systems, according to Luyet, extends for only some 30–40° below 0°C. Rapid passage through this range is, for most organisms, essential if they are to survive extremes of chilling. Moreover, the length of time spent at temperatures below the freezing or crystallization range usually, as we should expect, has no effect on the per cent of revivable organisms (Luyet and Gehenio, 1940; Luyet and Hartung, 1941). Organisms of more than a millimeter or so in size cannot survive this treatment since heat cannot be conducted from and to their interiors fast enough on chilling and on warming to prevent internal crystallization and death. The water content of the organisms also is important. In general, organisms with a low water content best withstand vitrification.

The germ plasm of mammals seemed to us to be of special interest in connection with vitrification since the storage of sperm would be of value in connection with the fertilization to produce offspring perhaps generations after the donor's death.

Luyet and Hodapp (1938) vitrified and revived an appreciable number of frog sperm after removing some of their water content by plasmolysis with hypertonic sucrose solutions. They were, however, unable to revive any rat sperm. Shettles (1940) in the course of a study of the physiology of human sperm had been able to vitrify and revive a few per cent of seminal human sperm immersed in capillary tubes in the liquified gases and later rapidly warmed.

II

Experiments with Human Sperm

During the past winter we have confirmed Shettles' findings and extended them. We have been able to revive as many as 67 per cent of sperm after exposure to liquid nitrogen at a temperature of −195°C. We have used seminal sperm always from the same human donor in a series of experiments attempting to improve the viable yield. Luyet and Gehenio (1940) described immersing small organisms in liquid air by entrapping them in a film on a wire loop 2 to 3 mm. in diameter. In this way there is only a small amount of surrounding liquid and contact is favored with the refrigerant. This method of immersion with certain types of pretreatment of the sperm produced occasional small yields of motile sperm after liquid nitrogen (see Table I). With human sperm our best results came by producing an air emulsion, or foam, of the seminal fluid by bubbling it through a capillary pipette and plunging it, trapped on a very fine wire screen into the liquid nitrogen. The solidified foam is afterwards warmed by quickly plunging it into a small volume of isotonic Locke-Ringer solution, glucose phosphate solution, or blood serum at approximately 35°C. The sperm, motile and dead, are then counted with an hemocytometer. The revived sperm show motility indistinguishable from that of

TABLE I

Experiments with Human Sperm

Date	Method of pretreatment	Method of immersion in liquid nitrogen	Results: Per cent motile after liquid N_2	Comments
1940				
Nov. 23	None	In film on wire loop	Negative	Warming solution is isotonic glucose phosphate unless otherwise stated. The warming solution is always tested for its effect on controls
	"	Semen in capillary tubes	"	
	Plasmolysis to immobility in 2 M glucose phosphate	In films on loop	"	
	Plasmolysis with 1 M glucose phosphate for 2 min.	" " " "	3–18 per cent	About the same number revivable after this plasmolysis alone and without chilling
Dec. 7	Isotonic glucose phosphate	" " " "	Negative	Dipped in toluol and dry ice mixture at −79° C.
Dec. 8	Various solutions glucose phosphate ranging from isotonic to many times hypertonic	In capillary tubes	"	
		In films on loop	"	
Dec. 13	Very fresh sample no pretreatment	" " " "	"	
	Plasmolyse 1 M glucose for 10 min.	" " " "	"	
	Plasmolyse 1M glucose for 2 min.	" " " "	0.05 per cent	Found none alive with < 2′ plasmolysed
		In capillary tube	Negative	Found none alive with > 3′ plasmolysed
Dec. 14	Pretreated as follows; none, plasmolyse with various concentrations of glucose phosphate for from ½ to 40 min.	In film on loop	"	

TABLE I—*Continued*

Date	Method of pretreatment	Method of immersion in liquid nitrogen	Results: Per cent motile after liquid N_2	Comments
1940				
Dec. 22	Plasmolyse with Ringer × 5	In film on loop	None	Sperm ½ hr. old
	Plasmolyse with Ringer × 3.3 For 15 sec.	" " " "	1 per cent	
	Plasmolyse with Ringer × 3.3 For 5 min.	" " " "	2 per cent	
	Plasmolyse with Ringer × 3.3 For 13 min.	" " " "	4 per cent	
	Plasmolyse with 1 M glucose phosphate no pretreatment	" " " " Bubbles dropped in	Negative 1 per cent	Note no pretreatment here.
Dec. 25	None	" " "	2 per cent	
	Plasmolyse with 3.3 × Ringer for 2 to 7 min.	" " "	0.1 per cent	
	None	In film on loop	Negative	
	None	Bubbles and foam	15–30 per cent	Note curious effect of bubble immersion
1941				
Jan. 16	Human sperm in rabbit serum	" " "	20.4 per cent	Controls always taken in conjunction with each test. (See Fig. 1)
	" "	" " "	28.8 per cent	
	None	" " "	12.2 per cent	
	None	" " "	9.2 per cent	
	Semen sperm with isotonic glucose phosphate	" " "	7.0 per cent	
	" "	" " "	3.5 per cent	
	" "	" " "	Negative	
	" "	" " "	1.9 per cent	
	Sperm in rabbit serum again	" " "	12.1 per cent	
Mar. 23	4 min. air drying of smear	Fine mesh copper screen	3.1 per cent	Controls 75 per cent motile
	None	Smear on cellophane	50 per cent	" "
	Sperm in beef serum	Cellophane smear	Negative	Controls 67 per cent motile

TABLE I—*Concluded*

Date	Method of pretreatment	Method of immersion in liquid nitrogen	Results: Per cent motile after liquid N_2	Comment
1941				
Mar. 23 (*Cont.*)	Sperm in beef serum	Bubbles and foam	5.0 per cent	Controls 67 per cent motile
	" "	" " "	15.0 per cent	" "
	None	" " "	20.6 per cent	
	None	" " "	4.0 per cent	
	Sperm in beef serum concentrated × 4	" " "	7.2 per cent	The sperm are all immediately reversibly immobilized by this concentrated serum.
	None	" " "	15.0 per cent	Controls 52 per cent motile
	None	" " "	15.0 per cent	

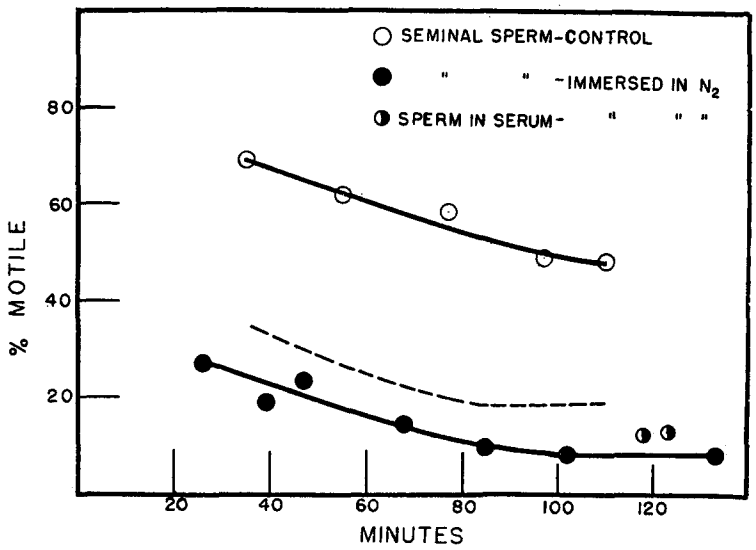

FIG. 1. Upper curve shows effect of age after emission on motility of human seminal sperm. Lower curve shows, for the same sample, per cent motile sperm after vitrification in liquid nitrogen with subsequent warming as a function of age after emission. Middle dashed curve shows the per cent of *living* sperm before vitrification which survived liquid nitrogen and subsequent warming.

normal controls. We have tried a wide variety of plasmolysing solutions as pretreatment to increase the yield. These will be considered later in connection with our experiments with rabbit sperm. In the case of human sperm pretreatment of this kind usually tends to decrease the yield. With the foam

method we typically get yields of 20 to 40 per cent with fresh sperm samples. There is a certain amount of variation from day to day. Table I summarizes our work with human sperm.

Freshness of the samples of human sperm is important. This was also emphasized by Shettles' experiments. Fig. 1 shows a typical curve of per cent recovery of motile sperm after chilling to −195°C. as a function of the sample's age since emission. The recovery curve parallels a control curve treated the same way except for chilling. Table II shows further data from another sample of sperm indicating the effect of age *in vitro* on the sperms' ability to withstand the temperature of liquid nitrogen.

We do not know how long sperm would remain viable at −195°C. but we would expect them to do so indefinitely. Shettles kept samples of human sperm at −79°C. up to 70 days. His data show a small decrease in viability

TABLE II

Experiments with Human Sperm

Age of sperm *in vitro*	Total motile after liquid nitrogen immersion	Motile sperm in unchilled controls	Living sperm motile after liquid nitrogen immersion
min.	*per cent*	*per cent*	*per cent*
40	50.0	75	67
65	20.6	—	—
88	—	50	—
130	15.0	52	29
140	15.0	52	29

over this period. But with his small yields and with the wide variability in samples showing these low yields we do not regard this apparent trend in a few experiments as significant.

III

Animal Sperm

Human sperm withstand vitrification and revival better than do those from other animals that we have examined. Our preliminary experiments with sperm from rat, mouse, guinea pig, rabbit, and bull yielded completely negative results. We accordingly decided to investigate rabbit sperm systematically. We have studied sperm samples from the vas deferens of 31 rabbits and also 8 samples of ejaculated rabbit sperm. Luyet and Hodapp were unable to revive frog sperm smeared in films on mica and dipped in liquid air without first plasmolysing them in sucrose solutions. After 3 minutes of plasmolysis in 2 M sucrose solution, about 40 per cent of their frog sperm ceased motion but the remainder survived immersion in liquid air if followed by very rapid warming. These revived sperm were motile as long afterwards as were controls.

We have found that this same procedure does not permit any revival of mammalian sperm just mentioned. Human sperm occasionally give by this procedure a small viable yield (Table I). We have plasmolysed rabbit sperm for lengths of time varying from 2 seconds to 30 minutes in many solutions. These include Ringer solution varying from isotonic to ten times isotonic. We have varied the Na, Ca ratio and K, Ca ratios of our pretreating solutions. We have plasmolysed with rabbit, human, and beef serum ranging from isotonic to five times isotonic. We have pretreated the sperm with glycerine, mineral oil, olive oil, citrate, gelatin, petroleum ether, and hypertonic solutions containing butyric acid. All of our experiments were controlled in that the effect on sperm of each pretreating agent was first determined not to produce irreversible immobilization. We have also pretreated rabbit sperm with sterile human seminal fluid.

Following a method of Luyet we have immersed the pretreated sperm samples into the liquid nitrogen by exposing them as films on fine wire loops, by smearing them on mica and cellophane, and by our foaming technique described above in connection with human sperm. We have tried dehydration by the air drying of films and of foam at various rates and have also vacuum dried the sperm at −79°C. after liquid nitrogen immersion in order to sublime off excess water. In some of the experiments we have used a machine for transferring the sperm into the liquid nitrogen and then retransferring them to the warming medium. This machine effects the transfers in approximately 0.01 second but this does not produce appreciably better results than rapid transfers by hand. All of our experiments have been repeated several times, usually on sperm from different rabbits. Our warming medium has usually been an isotonic glucose phosphate solution experimentally demonstrated to be a good medium for rabbit sperm. Isotonic Locke-Ringer is about equally effective. Rabbit serum maintains motility a little better than either of these media.

Positive results have occurred only as follows: Occasionally we have had approximately 0.1 per cent revival of bull and of rabbit sperm after liquid nitrogen immersion by pretreating them for several minutes with Ringer solution of from 1 to 2 times isotonicity containing double the normal amount of Ca. Very occasionally we have had a comparable yield of motile rabbit sperm after plasmolysing for 5 to 10 minutes in a solution of 5 times the normal Ringer concentration. Our best consistently reproducible results on recovery of motile rabbit sperm after immersion at −195°C. have been obtained by first drying thin smears of otherwise untreated vas or ejaculated sperm *nearly* to completion on cellophane in air. These, after the chilling, yield from 0.1 per cent to as much as 1.0 per cent motile sperm when rapidly warmed. Pretreatment of rabbit sperm for from 2 to 10 minutes with a solution made up of 0.01 N butyric acid in 3–4 times the normal Ringer or serum concentration also

yields positive and repeatable results to the extent of about 0.1 per cent recoverable motile sperm after liquid nitrogen. All of the plasmolysing solutions that are effective produce complete immobilization on their own account, but this is reversible when the sperm are put in isotonic Ringer solution, serum, or glucose phosphate solution.

SUMMARY

1. A wide variety of procedures was used to test the motility of mammalian sperm after plunging them into liquid nitrogen at −195°C. and later rapidly warming them to 35°C. by plunging them into a suitable balanced and isotonic medium.

2. Using seminal fluid sperm from the same human donor, maximal numbers of motile sperm survived vitrification when the samples were (*a*) very fresh, (*b*) untreated with plasmolysing solutions, (*c*) plunged into the refrigerant in the form of a foam. The maximum yield of motile human sperm recoverable from the liquid nitrogen was 50 per cent. Since in this sample only 75 per cent of the sperm were alive before immersion, 67 per cent of the living sperm survived vitrification.

3. Experiments with sperm from 31 rabbits were made with a variety of conditions of pretreatment to obtain maximal yields of recoverable, motile sperm after vitrification by liquid nitrogen. (*a*) A consistent recoverable yield of about 0.5 per cent was obtained when the untreated suspension of sperm was smeared on cellophane and *partially* dried in air before immersing in liquid nitrogen. (*b*) On a few out of many occasions plasmolysis for several minutes with hypertonic Ringer solution gave a recoverable yield of 0.1 per cent as did (*c*) pretreatment with hypertonic Ringer and butyric acid.

REFERENCES

Hoagland, H., 1933, *J. Gen. Psychol.*, **9**, 267.

Luyet, B. J., and Hodapp, E. L., 1938, *Proc. Soc. Exp. Biol. and Med.*, **39**, 433.

Luyet, B. J., and Gehenio, P. M., 1940, Life and death at low temperatures, Normandy, Missouri, Biodynamica. 341 pp.

Luyet, B. J., and Hartung, M. C., 1941, *Am. J. Physiol.*, **133**, 368.

Pictet, R., 1893, *Arch. sc. phys. et nat.*, **30**, sér. 3, 293.

Shettles, L. B., 1940, *Am. J. Physiol.*, **128**, 408.