is clear that a considerable fraction of the collagen produced fails to precipitate in the cell layer, and passes out into the medium. A similar phenomenon appears to occur *in vivo,* permitting soluble collagen to circulate in the blood(16). Ascorbic acid had no effect extracellularly on the precipitation of collagen fibers in culture, as the fraction of total collagen escaping from the cell layer to the medium was not reduced.

Keloid fibroblasts did not appear to have as great a capacity for growth or collagen synthesis under these culture conditions as fetal lung or adult skin fibroblasts. This is surprising, in view of their behavior *in vivo.* They did, however, respond to ascorbic acid by a relatively marked increase in collagen formation.

Summary. Human diploid fibroblast strains derived from fetal lung, adult skin and keloids, produce collagen *in vitro* when allowed to grow to confluence and remain without transfer. This property may be retained throughout most of their culture life. Keloid fibroblasts produced the smallest quantities of collagen and had the lowest growth potential. All strains produced more collagen when ascorbic acid was added to the medium. Up to 3% of the total protein being made in the presence of ascorbic acid consisted of collagen. An appreciable fraction of the collagen failed to precipitate in the cell layer and escaped to the medium both in the presence and in the absence of added ascorbate.

1. Rothfels, K. H., Kupelwieser, E. B., Parker, R. C., 5th Canadian Cancer Congress (Honey Harbor) New York, Academic Press 1963, p191.

2. Todaro, G. J., Green, H., *J. Cell Biol.,* 1963, v17, 299.

3. Goldberg, B., Green, H., Todaro, G. J., *Exp. Cell Res.,* 1963, v31, 444.

4. Green, H., Goldberg, B., *Nature,* 1963, v200, 1097.

5. Hayflick, L., Moorhead, P. G., *Exp. Cell Res.,* 1961, v25, 585.

6. Todaro, G. J., Wolman, S. R., Green, H., *J. Cell. and Comp. Physiol.,* 1963, v62, 257.

7. Eagle, H., Oyama, V. I., Levy, M., Freeman, A. E., *J. Biol. Chem.,* 1957, v226, 191.

8. Prockop, D. J., Udenfriend, S., *Anal. Biochem.,* 1960, v1, 228.

9. Myhill, D., Jackson, D. S., *ibid.,* 1963, v6, 193.

10. Chinard, F. P., *J. Biol. Chem.,* 1952, v199, 91.

11. Gross, J., *J. Biophys. and Biochem. Cytol.,* 1956, v2 (Suppl.), 261.

12. Revel, J. P., Hay, E. D., *Z. fur Zellforsch.,* 1963, v61, 110.

13. Goldberg, B., Green, H., *J. Cell Biol.,* 1964, v22, 227.

14. Jackson, F. S., Smith, R. H., *J. Biophys. and Biochem. Cytol.,* 1957, v3, 897.

15. Gould, B. S., *Int. Rev. Cytol.,* 1963, v15, 301.

16. Keiser, H., LeRoy, E. C., Udenfriend, S., Sjoerdsma, A., *Science,* 1963, v142, 1678.

17. Green, H., Goldberg, B., *Nature,* in press.

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Dimethyl Sulfoxide As a Protective Agent During Freezing and Thawing of Human Spermatozoa.* (29552)

J. K. SHERMAN

Department of Anatomy, University of Arkansas School of Medicine, Little Rock

The survival of most types of cells during freezing and thawing depends upon pretreatment with a protective substance. Although the mechanism of freeze-thaw protection largely is unknown, the colligative properties of the widely used simply polyhydric alcohol, glycerol, seem ideally suited for this purpose. Pretreatment with glycerol has made possible preservation by freezing of a wide variety of cells and tissues(1). Lovelock and Bishop introduced the water-miscible organic solvent, dimethyl sulfoxide (DMSO), as a freeze-thaw protective agent(2). They found that DMSO compared favorably with glycerol in protecting bovine and human erythro-

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cytes. They also reported that less DMSO and a shorter pretreatment time was required, presumably because it penetrated cells faster than glycerol. Bovine spermatozoa, however, were not protected as well with DMSO as they were with glycerol during freezing and thawing.

Since 1959 DMSO has been used successfully for preservation of chick, mouse and human tissue cultures(3,4,5). Recent studies by the writer have revealed that DMSO is at least as effective as glycerol during freezing and thawing of full thicknesses of mouse skin. DMSO has been reported to be superior to glycerol in freeze-thaw protection of bone marrow cells(6), trypanosomes(7), antibody-producing cells(8) and rabbit spermatozoa (9). There are conflicting reports, however, concerning possible toxicity of DMSO with hematopoietic cells *in vitro*(6,10), while *in situ* it appears to be far less toxic than glycerol(11).

Seven to 10% glycerol by volume currently is used in methods for preservation of human spermatozoa by freezing. There is need for improving freeze-thaw protection of these cells as about 30% loss still occurs in spite of the presence of glycerol(12). This report concerns the evaluation of DMSO as a substitute for glycerol with human spermatozoa in terms of relative freeze-thaw protection as well as toxicity.

Materials and methods. The study involved pooled and split-sample comparisons with spermatozoa from one to 3 ejaculates obtained from each of 16 individuals. The number of progressively motile cells, represented as percent motility, was used as the criterion of functional survival. Motility measurements were made in triplicate on samples treated as unknowns in a cross-check by two observers.

DMSO and glycerol, at a concentration of 10% by volume, were compared as to their ability to protect spermatozoa during freezing in liquid nitrogen vapor at an overall average rate of 16°C/min to −196°C and thawing to room temperature in a water bath (22°C) at an overall average rate of 60°C/min. One ml portions of semen were placed in glass ampules and sealed by flame, after the usual stepwise addition of the protective substance(13) at 22°C. Freezing and thawing of semen in ampules was accomplished by methods described previously(12). Rates of temperature change were followed and recorded on a continuous pen thermograph potentiometer through a copper-constantine thermocouple positioned in the center of the fluid mass of a "lead" ampule. Relative toxicity of the freeze-thaw protective substances was determined during 24 hour storage at 22°C of unfrozen as well as frozen-thawed cells by measuring percent motility at intervals of 1, 4, 8, 12, and 24 hours. Concentrations by volume of 0, 5, 10, 15 and 20% of DMSO were evaluated in a manner similar to that described above for comparison of DMSO and glycerol, as to protective and toxic action.

Results. Comparison of 10% glycerol with 10% DMSO revealed a comparable effect in that: 1) There was a slight but statistically significant ($P<0.5$) average drop of 7% (range of 0 to 12%) in percent motility which was due to pretreatment with each substance prior to exposure to freezing conditions, and 2) Motility after thawing was the same (44%) in the presence either of glycerol or DMSO.

Motility loss at 4, 8, 12 and 24 hour intervals during 22°C storage with 10% DMSO was significantly greater than with 10% glycerol, both before freezing and after thawing (Fig. 1).

In comparing 5, 10, 15 and 20% DMSO, an interaction between protective and toxic levels was observed (Table I). Greatest freeze-thaw survival was shown at the 10% level, least at 20% while 5% and 15% were equal. Toxicity of DMSO increased with increase in concentration and was observed within one contact hour during 22°C storage both before freezing and after thawing. A concentration of 10% reflected the most favorable balance between protection and toxicity among the concentrations evaluated.

Discussion. These findings suggest caution in using DMSO and other substances solely on the basis of freeze-thaw protective capa-

TABLE I. Interaction of Protective and Toxic Levels of DMSO in Freeze-Thawing and 22°C Storage of Human Spermatozoa.

	% Motility*										
	Pre-freeze storage (hr)						Post-thaw storage (hr)				
% DMSO	1	4	8	12	24	% DMSO	1	4	8	12	24
0	61	57	51	46	31	0	—	—	—	—	—
5	57	53	46	38	19	5	33	29	18	13	6
10	50	39	30	24	10	10	40	30	18	11	4
15	36	18	8	6	2	15	33	19	8	7	1
20	31	12	5	2	0	20	15	5	3	1	0

* Data represents averages of triplicate measurements involving 21 semen samples.

city. Evaluation of freeze-thaw protection should be supplemented by toxicity studies to determine and balance their possible interaction. The importance of latent freeze-thaw injury may be pertinent in this regard. Samples of frozen-thawed bull spermatozoa often lose one-third to two-thirds of their motile cells within 18 hours after thawing, while unfrozen samples show no loss under identical storage conditions(14). When expression of natural physiological function requires an appreciable time interval after thawing, as in fertilization, toxicity of a freeze-thaw protective agent thus may accentuate the loss due to latent injury.

Knowledge of whether toxic loss occurs during pretreatment before freezing or in time after thawing may facilitate efforts to minimize it and to improve survival. The writer has found that usually all the loss of frozen-thawed mouse skin transplants, as tested by auto-transplantation, occurs in pretreatment with glycerol or DMSO before cooling and freeze-thawing. Addition of phospholipids, lipoproteins, sugars and the like (1), may minimize toxicity of protective substances and/or facilitate their beneficial action in preservation by freezing.

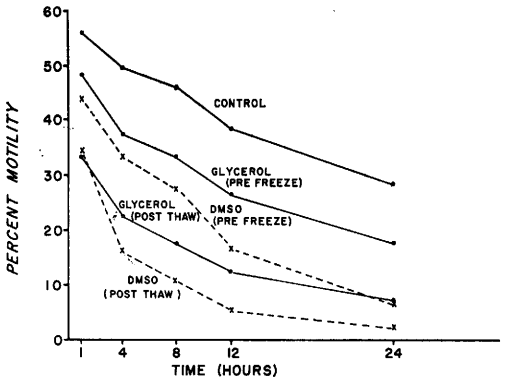

FIG. 1. Comparative toxic effect of 10% glycerol and 10% DMSO as shown motility loss of human spermatozoa during storage at 22°C before freezing to −196°C and after thawing from −196°C. Each value represents an average of triplicate measurements made on each of 38 semen samples. Differences between post thaw as well as pre freeze motility values of DMSO and glycerol treated samples at 4, 8, 12, and 24 hours are statistically significant ($P < 0.01$).

Varied results reported with DMSO may be due in part to the toxicity of traces of impurities which remain in some commercial grades of DMSO or to methods of evaluation. We found no differences between commercial and research grades of DMSO made by Crown Zellerbach Corp., in toxicity or freeze-thaw protection with human spermatozoa. Concentration of protective substance, method of addition, time and temperature of pretreatment, constituents of medium, use of dialysis or dilution, methods of freezing, storage and thawing, animal source, and the like, are common variables in this kind of research. DMSO is reported to be superior to glycerol in freeze-thaw protection of rabbit spermatozoa(9), but though this probably will prove correct, the percentage of protective substance as well as the constituents of the medium used with each substance differed considerably in the experimental comparison. It is important, also, to use split-sample comparisons of the same sample to avoid inconsistencies due to biological variation in spermatozoa from different individuals.

Summary. Dimethyl sulfoxide (DMSO) was evaluated, in terms of motility, as a freeze-thaw protective agent with human spermatozoa. DMSO was found to be equal

to glycerol in this protective capacity but proved more toxic during storage at 22°C both before freezing and after thawing. An interaction of protective and toxic effects was noted in a comparison of 5, 10, 15 and 20% DMSO which suggested the 10% level as the most favorable. On the basis of findings, DMSO is not recommended as a substitute for glycerol in the preservation of human spermatozoa by freezing with the methods described.

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1. Smith, A. U., *Biological Effects of Freezing and Supercooling,* Williams & Wilkins Co., Baltimore, 1961.

2. Lovelock, J. E., Bishop, M. W. H., *Nature,* 1959, v183, 1394.

3. Porterfield, J. S., Ashwood-Smith, M. J., *ibid.,* 1962, v193, 548.

4. Dougherty, R. M., *ibid.,* 1962, v193, 550.

5. Perry, V. P., *Fed. Proc.,* 1963, v22, 102.

6. Pyle, H. M., Boyer, H. F., *Vox Sanguinis,* 1961, v6, 199.

7. Walker, P. J., Ashwood-Smith, M. H., *Ann. Trop. Med. and Parasit.,* 1961, v55, 93.

8. Albright, J. F., Makinodan, T., Mazur, P., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 489.

9. Sawada, Y., Chang, M. C., *Fertil. & Steril.,* 1964, v15, 222.

10. Persidsky, M., Richards, V., *Nature,* 1963, v197, 1010.

11. Ashwood-Smith, M. J., *Int. J. Radiation Biol.,* 1961, v3, 44.

12. Sherman, J. K., *Fertil. & Steril.,* 1963, v14, 49.

13. ———, *Am. J. Physiol.,* 1957, v190, 281.

14. ———, *J. Dairy Sci.,* 1958, v42, 94.

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Liver UDPG-Transglucosylase and Phosphorylase in Fasted, Refed and Nephrotic Rats. (29553)

ALISA GUTMAN AND ELEAZAR SHAFRIR
(Introduced by Felix G. Sulman)
Laboratory of Clinical Biochemistry, Department of Biochemistry, Hebrew University—Hadassah Medical School and Hadassah University Hospital, Jerusalem, Israel

Selective changes in the activity of enzymes concerned with liver glycogen metabolism occur following dietary and hormonal influences(1,2). Changes in the activity of transglucosylase (UDPG glucose: α-1,4 glucan α-4-glucosyl transferase) and phosphorylase (α-1,4-glucan phosphorylase) effected by fasting and refeeding were the subject of this study as a background for understanding the alterations in carbohydrate metabolism of nephrotic animals. Decreased liver glycogen levels reported by Drabkin and Marsh(3) in nephrotic animals were ascribed to shunting of glucose for the support of increased protein and fat synthesis, characteristic of the nephrotic syndrome, or possibly due to changes in food intake.

Experimental. Male albino rats weighing about 150 g were used. Aminonucleoside or Kidney Antiserum nephrosis was induced as described previously(4). All treated rats had severe proteinuria, hypoalbuminemia and hyperlipidemia. The rats were investigated after a period of fasting or refeeding and on sacrifice the fasted, refed or nephrotic animals were interspersed with normal controls and the enzyme assays performed within a short span of time, to minimize the chance of fluctuations in enzyme activity or in glycogen content. The activity of transglucosylase was measured by a modification of the procedure of Leloir and Goldemberg(5), in which the evolution of UDP from UDPG and the interaction of UDP with phosphoenolpyruvate and pyruvic kinase proceeded in the same reaction mixture(6). Phosphorylase was measured according to Sutherland(7). Units of enzyme activity refer to μmoles of the respective substrate utilized per mg of liver protein per hr. Liver glycogen was isolated according to Good *et al*(8) and determined by the anthrone reaction. Liver pro-