TABLE II. Mean Difference between Initial and Final Blood Glucose Concentration in Control and Test Rats.

Group	n*	Mean difference, mg %
Control	8	85 ± 14.2†
Test	8	51 ± 4.8

* Blood glucose analyses were not obtained in one exp. pair included in Table I.
† Mean ± stand. error.

trations is shown in Table II. The increase is significantly greater ($t = 2.24$; $p<0.05$) by group comparison analysis(5) in the control group. For all the rats in which blood glucose concentrations were obtained, mean initial value was 94 mg %. Although not quantitatively measured, the peristaltic activity as reflected by oscillations in the fluid level in the distal syringe barrel appeared to be about the same in both control and test systems.

Discussion. It is apparent that under the test conditions in the rat, both tolbutamide and glucose are readily absorbed from the same solution. The net glucose absorption data support this directly in the case of glucose. The significantly smaller increment of blood glucose rise over the period of equivalent net glucose absorption is indirect evidence that tolbutamide was absorbed in sufficient quantity to exert its typical effect on blood glucose in normal rats. The apparent inhibition in absorption of glucose in the experiments with carbutamide previously cited(1) may be due to prolongation of gastric emptying time but this awaits direct proof. Structural differences in this family of compounds are too well known to be associated with widely varying physiological action to allow conclusions to be drawn by analogy from tolbutamide to carbutamide.

Summary. In rats glucose absorption from the small intestine was not affected by the presence of tolbutamide in the glucose solution used. The tolbutamide absorbed in these experiments was sufficient to produce a relative suppression of the alimentary hyperglycemia present at the end of the absorption period.

1. Friedlich, T. L., Ashworth, M. A., Hawkins, R. D., Haist, R. E., *Canad. M. A. J.,* 1956, v74, 973.
2. Haist, R. E., Hawkins, R. D., Ashworth, M. A., *Diabetes,* 1957, v6, 21.
3. Kleibel, F., *Lancet,* 1954, v1, 882.
4. Somogyi, M., *J. Biol. Chem.,* 1952, v195, 19.
5. Snedecor, G. W., *Statistical Methods,* The Iowa State College Press, Ames, 1946.

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Survival of Unfertilized Mouse Eggs During Freezing and Thawing.* (24224)

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Preservation by freezing has been possible only with certain cells, others being susceptible to lethal factors, usually associated with ice formation. Long-term storage of germ cells at sub-freezing temperatures was given impetus by introduction of glycerol as a protective agent(1). Today, many of our dairy cattle are bred artificially with semen so preserved(2), and the technic has had successful clinical application in insemination of humans(3).

Similar success has not been realized with the mammalian female gamete, although reports on fertilized rabbit eggs and eggs of lower forms are encouraging. Eggs of the sea urchin reportedly were fertilized upon rewarming from a medium of sea water plus glycerol which had been frozen at −10°C(4). Formation of ice in the jelly coats of fertilized eggs of the frog did not prevent their subsequent normal development into tadpoles(5). Some hydrated eggs or early embryos of the brine shrimp proceed to hatch into free-swimming larvae after thawing from their frozen

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salt water environment at –196°C(6). Smith (7) reported survival, on the basis of cleavage *in vitro,* of some (20-30%) fertilized rabbit eggs exposed to –20°C in frozen glycerolated medium. Also, upon rewarming from –190°C, 1% cleaved. However, it is not known whether fertilized rabbit eggs so treated are capable of developing into progeny when transferred into a foster mother.

We recently reported that unfertilized eggs of the mouse can be cooled to –10°C in an ice-free medium without alteration of their functional capacity(8). The purpose of this communication is to report their capability to be fertilized and to develop into progeny after the medium is frozen at this temperature.

Materials and methods. The technic of transplanting genetically labeled eggs from immature brown C57 females to mated, fertile, white BALB/c foster mother recipients, was employed in evaluating survival(9). Recipients were autopsied on the 19th day of gestation for detection of transplanted eggs, which developed as dark-eyed fetuses. Per cent survival was computed by dividing the number of transplanted eggs which developed, by the total number of eggs transplanted in pregnant recipients, and multiplying quotient by 100. Modified Locke's solution containing 5% glycerol by volume, was used as a medium(8). Ten to 12 eggs were placed in 1.5 ml glass ampuls containing 0.3 ml of the medium. Sealed by flame and clipped to fiberglass canes, the ampuls were passed through a bath at 5°C and transferred to a –10°C alcohol bath for 15 minutes. The ampuls were then lifted above the bath surface and the supercooled content frozen by seeding with a stick of solid carbon dioxide by contact with the side of the ampul. The frozen mass (Fig. 1A) was then reimmersed in the –10°C bath, before thawing in a 5°C water bath. Thermograph recording through a thermocouple in a test ampul with eggs and 0.3 ml medium, showed that average rate of temperature changes was 8°C/min from 21 to 5°C, 2°C/min from 5 to –10°C and 5°C/min from –10 to 0°C. After thawing, the eggs were removed from the ampuls with a small amount of medium and 6 from each transferred into recipients in tests of progeny development(9).

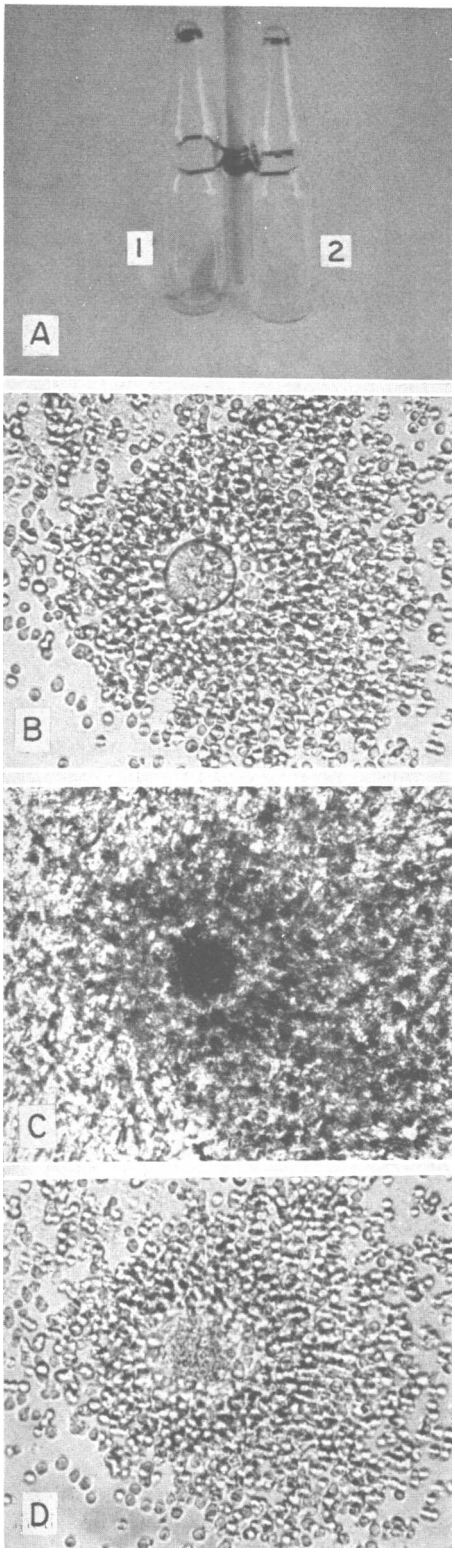
TABLE I. Survival of Unfertilized Mouse Eggs in Frozen Medium at –10°C.

Exposure time (hr)	No. of recipients		No. of eggs transplanted in pregnant recipients		
	Total	Pregnant	Total	Developed	% survival
½	7	6	36	7	19
3½	14	10	60	7	12

Freezing in the ampul did not favor accumulation of more detailed information on the relationship between site of ice formation and egg survival. To accomplish this, a special cooling chamber† was used which permits microscopic observation during freezing and thawing. Cover slip preparations of eggs with medium were supercooled to and seeded at –10°C. Because of differences in conditions in the ampul and between 2 cover slips (volume of fluid, rates of cooling and rewarming, velocity of ice formation, etc.), observations were also made on several types and sizes of hanging and inverted hanging drop preparations which more closely resembled conditions in the ampul. Since, under imposed conditions, no internal ice formations were seen as distinct structures with ordinary and polarized light, intracellular crystallization was considered as characterized by "blacking out" of these cells. Ice formation produces this opacity(10).

Results. Eggs in ampuls thawed from medium frozen at –10°C were indistinguishable from controls. Survival of eggs, as tested by transplantation, after exposure to frozen medium at –10°C in ampuls for ½ and 3½ hours (Table I) agrees well with that range of values (11-17%) found for several control series. Without exception, eggs thawed from medium seeded at –10°C in cover slip preparations were damaged, their contents being disorganized and vitelline membrane unrecognizable (Fig. 1D). These eggs were observed, also without exception, to freeze internally (Fig. 1C) after the supercooled condition (Fig. 1B) was seeded. Observations on the

† This chamber was designed and constructed by Mr. Maxim Persidsky in the biophysics laboratory at this foundation.



hanging and inverted hanging drop preparations of eggs during freezing to and thawing from −10°C, reveal the following: a) With occasional exception, eggs frozen internally suffer severe damage, b) Frequently, intracellular ice forms in some eggs but not in others of the same preparation, c) At times, it appears that intracellular ice forms in the surrounding cumulus cells but not in the eggs, and d) Very often, there is a delay of one to as many as 15 seconds between the internal freezing of different eggs which freeze in the same preparation.

Discussion. To our knowledge, intracellular ice has never been demonstrated in mammalian spermatozoa or any other cells during procedures employed in their successful preservation by freezing. Our results, although not ruling out the remote possibility suggested by occasional maintenance of structure following internal crystallization, indicate that unfertilized mouse eggs, treated as described, do not survive detectable intracellular ice formation. Probably, eggs that survived exposure to frozen medium in the ampul had not frozen but remained in a supercooled condition. Our recent work (unpublished observations) reinforces the notion that most, or all, unfertilized mouse eggs are physically injured by detectable internal crystallization. Over 90% of eggs cooled slowly to −20°C at a rate of 0.7°C/min remain unaltered structurally while those cooled rapidly at 12°C/min to the same temperature are almost always destroyed. The proposed explanation is that, in association with little or no penetration of glycerol(9), such slow cooling permits further cellular dehydration during the extracellular hypertonicity accompanying extracellular ice formation, obviating or minimizing the subsequent internal ice formation, while rapid cooling prevents this dehydration, hence favoring appreciable internal crystallization with damage to the cytoplasm.

We may now add unfertilized mouse eggs to

FIG. 1. (A) Ampuls with unfertilized mouse eggs in 0.3 ml modified Locke's + 5% glycerol, at −10° C, (1) unfrozen, (2) frozen; (B) egg in unfrozen medium at −10°C in cover slip preparation within cooling chamber; (C) same egg at −10°C after freezing; (D) same egg after thawing.

the growing list of mammalian cells which survive "freezing" in presence of glycerol. However, "freezing" may only mean survival in presence of extracellular ice without intracellular ice formation. Evidence for egg survival after intracellular ice formation would have to be based on observation of ice formation under the microscope, followed by transplantation of the same individual intact eggs and their normal reproductive performance.

Summary. This investigation has established the possibility of survival of unfertilized mouse eggs with extracellular ice formation in glycerolated modified Locke's solution at –10°C for periods up to 3½ hours. Survival of eggs so treated was evaluated by progeny development after transplantation into foster mothers.

1. Polge, C., Smith, A. U., Parkes, A. S., *Nature,* 1949, v164, 666.

2. Herman, H. A., *Hoard's Dairyman,* Oct. 10, 1956, 988.

3. Bunge, R. G., Keettel, W., Sherman, J. K., *Fertil. and Steril.,* 1954, v5, 520.

4. Hultin, E., Hagstrom, B., *Arkiv. Zool.,* 1954, v6, 523.

5. Schenk, S. L., *Sitzunsb. Wien. Akad.,* 1870, v60, 25.

6. Sherman, J. K., *The Physiologist,* 1957, v1, 77.

7. Smith, A. U., in *Mammalian Germ Cells,* Little Brown and Co., Boston, 1953, 217.

8. Sherman, J. K., Lin, T. P., *Nature,* 1958, v181, 785.

9. Lin, T. P., Sherman, J. K., Willett, E. L., *J. Exp. Zool.,* 1957, v134, 275.

10. Smith, A. U., Polge, C., Smiles, J., *J. Roy. Micr. Soc.,* 1951, v71, 186.

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Plasma Cells and Serum Proteins in Marine Fish.* (24225)

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There is evidence that in human beings and other mammals plasma cells may participate in the production of gamma globulins and antibodies(1,2). Studies on plasma cells and gamma globulins in lower forms were initiated(3-5) to determine whether or not they are associated. This report describes observations on occurrence of plasma cells and on electrophoretic properties of blood serum proteins in some elasmobranchs and teleosts.

Materials and methods. From one to 10 members of each species were examined shortly after capture. The fish were trapped in the region of Woods Hole, Mass., the majority from Buzzard's Bay. Blood was obtained from living animals by cardiac puncture with silicone-treated equipment. Collection of blood was greatly facilitated by anesthetizing the fish in a tank of sea water containing 1 part per thousand of M.S. 222§ before obtaining the sample. The blood was transferred to plastic tiubes, was allowed to clot, was centrifuged, and the serum withdrawn. It was either analyzed on the day of withdrawal or stored at –10°C for a few days. One specimen, serum of the lemon shark, *Negaprion brevirostris,* was obtained from the Serological Museum, Rutgers University.‖ This sample had been sterilized by filtration and stored at 4°C for several months. The serum proteins were analyzed by zone electrophoresis in starch gel. Soluble starch was produced by acetone-HCl treatment of po-

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‡ Serological Museum, Rutgers Univ., New Brunswick, N. J. Aided by grant from National Science Fn.

§ Meta-aminobenzoic acid-ethylester methane sulfonate obtained from Sandoz Pharmaceutical Co., Hanover, N. J.

‖ We are indebted to Dr. Alan Boyden, for making this specimen available.