tion with the attendant advantages of light microscopic examination, ability to use fixed material previously embedded in paraffin, and permanent preparations. We have demonstrated that neoplasia may result in the loss of differentiated function. This is a variable but not uncommon finding in tumors[16]. We have also shown the lack of ectopic production of amylase in transformed ductal cells. Ductal cells are embryologically one step less differentiated than acinar cells. While some tissues that undergo malignant degeneration begin to elaborate amylase (lung, ovary) it is of interest that ductal cells of parotid gland do not.

Conclusion. We have demonstrated the ability to localize amylase in normal human tissues. We utilized this technique to study differentiation in parotid neoplasia. This technique can be used in various developmental studies and in the study of the evolution of neoplasia.

1 Acknowledgment. This is publication No. 80-27 and was supported in part by National Institutes of Health research grant GM-19178.
2 To whom reprint requests should be addressed.
3 D.J. Stiefel and P.J. Keller, Biochim. biophys. Acta *302*, 345 (1973).
4 A.D. Merritt and R.C. Karn, Adv. human Genet. *8*, 135 (1977).
5 M.J. Weiss, H.A. Edmondson and M. Wertman, Am. J. clin. Path. *21*, 1057 (1951).
6 R.L. McGeachin and M.R. Adams, Cancer *10*, 497 (1957).
7 N. Ende, Cancer *13*, 283 (1960).
8 R.W. Ammann, J.E. Berk, L. Fridhandler, M. Ueda and W. Wegmann, Ann. intern. Med. *78*, 521 (1973).
9 K. Sudo and T. Kanno, Clin. chim. Acta *73*, 1 (1976).
10 F.W. Kraus and J. Mestecky, Arch. oral Biol. *16*, 781 (1971).
11 K. Yasuda and A.H. Coons, J. Histochem. Cytochem. *14*, 303 (1966).
12 J.J. Geuze, J.W. Slot and K.T. Tokuyasu, J. Cell Biol. *82*, 697 (1979).
13 J.G. Streefkerk, J. Histochem. Cytochem. *20*, 829 (1972).
14 E. Kurstak, Meth. Virol. *5*, 423 (1971).
15 L. Sternberger, Immunocytochemistry. Prentice-Hall Inc., Englewood Cliffs, New Jersey 1979.
16 T.A. Bonfiglio and M.R. Feinberg, Arch. Path. Lab. Med. *100*, 307 (1976).

Effects of the temperature of ice-seeding on survival of frozen-and-thawed mouse morulae

H. Miyamoto and T. Ishibashi

Department of Animal Science, College of Agriculture, Kyoto University, Kyoto 606 (Japan), 8 April 1980

Summary. Mouse morulae were frozen to −196 °C in the presence of 1.2 M ethylene glycol or 1 M glycerol. After ice-seeding in embryo samples at −10 °C or below, the survival rates in vitro were lower than those obtained by ice-seeding between −4° and −8 °C. The proportion morulae developing into live young in vivo after ice-seeding at −13 °C and freezing was lower than that of embryos seeded at −4 °C.

Since the first significant report by Whittingham et al.[1] on the survival of mouse embryos after freezing and thawing, inducing extracellular ice formation (ice-seeding) in embryo samples before slow cooling has always been done[2,3]. The temperature of ice-seeding affected the survival of 8-cell mouse embryos[2] and morulae and blastocysts of sheep[3,4] during freezing and thawing in the presence of dimethyl sulphoxide (DMSO). In the present paper we have examined the effects of the temperature of ice-seeding on the survival of mouse morulae after freezing and thawing in the presence of 1.2 M ethylene glycol or 1 M glycerol as the cryoprotectant.

Materials and methods. Female ICR mice were induced to superovulate, and mated[1]. Morula embryos were recovered from the reproductive tracts with a modified Dulbecco's phosphate-buffered salt solution (PBS)[1] at 80–84 h after the injection of HCG. The embryos were washed in several changes of PBS and 14–22 embryos were transferred to each test-tube containing 0.1 ml PBS. The tubes were cooled to 0 °C at 2 °C/min in a Dewar flask containing ethanol, and the cryoprotectant in PBS was added to samples at 0 °C in 2 increments of 0.05 ml at 10-min intervals. The final concentration of ethylene glycol and glycerol used as the cryoprotectant was 1.2 and 1 M, respectively. The samples were equilibrated at 0 °C for 10 min and cooled to the temperatures used for ice-seeding at 0.5–1 °C/min. They were seeded at between −4 and −13 °C by placing a cooled hypodermic needle in PBS. They were held at the ice-seeding temperature for 5 min and then cooled to −79 °C at 0.5−1 °C/min by adding dry ice to ethanol in a Dewar flask. The Dewar flask containing the frozen samples was placed in liquid nitrogen to be cooled from −79 °C to −120 °C at 1−2 °C/min, and then samples were transferred directly to liquid nitrogen. After being frozen at −196 °C for 1–24 h, the frozen samples were thawed at approximately 15 °C/min in air at room temperature.

After thawing, the embryos were cultured by the microdrop method in a modified Krebs-Ringer bicarbonate medium[1] under paraffin oil at 37 °C for 36 h in 5% CO_2 in air. The survival of frozen-and-thawed morula embryos was assessed by their ability to develop into expanded blastocysts during culture in vitro. Experiments were replicated 4–5 times and data were analyzed for statistical significance by the χ^2-test. The further potential for survival of frozen-and-thawed and unfrozen morulae was tested by transferring the blastocysts, which had expanded after 36 h in culture, to the uterine horns of females (5–10 blastocysts/horn) on day 3 of pseudopregnancy (day 1 is the day on which the copulating plug is found).

Results and discussion. As shown in table 1, the temperature of ice-seeding in embryo samples was important for the survival of frozen-and-thawed mouse morulae to the blastocyst stage in vitro, regardless of the cryoprotectant used. There were no apparent differences between the ice-seeding temperatures of −4 to −8 °C in the survival rate of mouse morulae after freezing and thawing in the presence of 1.2 M ethylene glycol or 1 M glycerol. After seeding at −10 °C or below, however, the survival rates of frozen-and-thawed embryos were lower ($p < 0.05$) than those obtained by ice-seeding at −4 to −8 °C.

As shown in table 2, the developmental rate for morulae to live young in vivo after ice-seeding at −13 °C and freezing to −196 °C was lower ($p < 0.01$) than that for morulae seeded at −4 °C.

The present results showed that the survival of mouse morulae frozen in the presence of ethylene glycol or glycerol was affected by the temperature of ice-seeding as

Table 1. Effects of temperature of ice-seeding on survival of mouse morulae frozen to −196 °C in the presence of ethylene glycol or glycerol

Cryoprotective agent (conc.)	Temperature of ice-seeding (°C)	No. of embryos frozen	No. of embryos recovered	No of embryos morphologically normal at recovery (%)*	No. of expanded blastocysts after culture (%)*
Ethylene glycol (1.2 M)	−4	97	92	78 (85)	72 (78)
	−5	80	73	68 (93)	60 (82)
	−6	91	88	70 (80)	67 (76)
	−8	92	91	85 (93)	73 (80)
	−10	97	92	63 (68)	56 (61)
	−12	91	85	65 (76)	51 (60)
	−13	90	89	64 (72)	56 (63)
Glycerol (1 M)	−4	90	88	75 (85)	65 (74)
	−5	91	91	79 (87)	64 (70)
	−6	93	86	72 (84)	62 (72)
	−8	87	83	70 (84)	53 (64)
	−10	87	83	50 (60)	39 (47)
	−11	95	95	62 (65)	43 (45)

The embryos recovered include normal, damaged and degenerate embryos. * Percentage of the number of embryos recovered.

Table 2. Effects of temperature of ice-seeding on development of frozen-and-thawed mouse morulae to live young after culturing for 36 h and transferring to pseudopregnant females

Cryoprotective agent (conc.)	Temperature of ice-seeding (°C)	No. of blastocysts transferred	No. of recipients	No. of pregnant recipients	No. of blastocysts transferred to pregnant recipients	No. of live young (%)
	Control (unfrozen)*	66	7	4	41	29 (44)
Ethylene glycol (1.2 M)	−4	58	6	2	20	14 (24)
	−13	75	6	1	14	6 (8)
Glycerol (1 M)	−4	50	5	2	19	14 (28)
	−13	48	4	0	0	0 (0)

* Unfrozen morulae were cultured for 36 h before transfer to the recipient females.

in case of mouse[2] and sheep[3,4] embryos frozen with DMSO, and also indicated that ice-seeding should be done at temperatures of −8 °C or above under the present experimental conditions. The low survival of frozen-and-thawed embryos after transfer to the recipient females may be due to: a) Deficiencies in the transfer technique. b) The fact that the embryos in this study were equivalent to 120 h post injection of HCG after culture and at the time of transfer to day 3 recipients. This may have had an adverse effect on post-implantation survival. c) Ethylene glycol and glycerol may afford less effective protection during freezing of embryos than DMSO. If the procedures associated with the freezing and fransfer techniques could be improved, survival of frozen-and-thawed embryos would be enhanced.

Intracellular freezing generally causes great damage to living cells and the cooling rate must be slow enough to permit cellular dehydration during the extracellular hypertonicity accompanying extracellular ice formation[5]. The likelihood of intracellular freezing in unfertilized mouse eggs increased with increasing cooling rate and the decrease in survival of unfertilized mouse eggs was related to intracellular ice formation[6,7]. When samples of embryos suspended in DMSO were cooled at the optimum rate in the bath, the greater the degree of supercooling the higher the cooling rate of the sample immediately after extracellular ice formation, i.e., the cooling rate became far higher than that optimal for embryo survival[2,8].

By extracellular supercooling of sea urchin eggs, deformation was caused and their developing ability was lost, and it has been suggested that a structural change may occur in the protoplasm of the cortex in sea urchin eggs by supercooling[9]. Extracellular supercooling in human erythrocytes reduced the extent of cellular dehydration even if the normal cooling rates were unmodified, and thereby the probability of intracellular freezing was increased[10]. As in the case of human erythrocytes[10] and 8-cell mouse embryos[2], extracellular supercooling may produce configurational changes in the cell membranes and inadequate dehydration may be caused during the supercooling of mouse morulae in the presence of ethylene glycol or glycerol, and thereby the probability of intracellular ice formation in mouse morulae may be increased during freezing and thawing.

1 D.G. Whittingham, S.P. Leibo and P. Mazur, Science *178*, 411 (1972).
2 D.G. Whittingham, in: Ciba Foundation Symposium on The Freezing of Mammalian Embryos, p.97. Ed. K. Elliott and J. Whelan. Elsevier, Amsterdam 1977.
3 N.W. Moore and R.J. Bilton, in: Ciba Foundation Symposium on The Freezing of Mammalian Embryos, p.203. Ed. K. Elliott and J. Whelan. Elsevier, Amsterdam 1977.
4 S.M. Willadsen, in: Ciba Foundation Symposium on The Freezing of Mammalian Embryos, p.175. Ed. K. Elliott and J. Whelan. Elsevier, Amsterdam 1977.
5 P. Mazur, Science *168*, 939 (1970).
6 J.K. Sherman and T.P. Lin, Proc. Soc. exp. Biol. Med. *98*, 902 (1958).
7 S.P. Leibo, J.J. McGrath and E.G. Cravalho, Cryobiology *15*, 257 (1978).
8 S.P. Leibo and P. Mazur, in: Methods in Mammalian Reproduction, p.179. Ed. J.C. Daniel, Jr. Academic Press, New York 1978.
9 Y. Ohyama and E. Asahina, Cryobiology *9*, 22 (1972).
10 K.R. Diller, Cryobiology *12*, 480 (1975).