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Re-establishment of breeding stocks of mutant and inbred strains of mice from embryos stored at −196 °C for prolonged periods

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SUMMARY

Breeding stocks were re-established from embryos of various mutant and inbred strains of mice after prolonged storage in liquid nitrogen at the 8-cell stage even with strains where only a proportion of the progeny were of the desired type, i.e. in the Mo^{dp} and XO strains. Gonadotrophin treatment failed to produce superovulation consistently in any of the strains tested. Although the initial survival of embryos after thawing and culture to the morula and blastocyst stage was highest for embryos from XO mothers (61 %), these embryos suffered the heaviest early post-implantation loss after transfer (61 %). The proportion of embryos, originally frozen, developing into foetuses and offspring was variable (13 % HT, 14 % PT, 20 % Mo^{dp}, 14 % XO, 21 % CBA and CBA-T6) and lower than previously reported for hybrid 3H1 embryos (20–30 %). The sex ratio of the liveborn young was within the normal expected limits except for the Mo^{dp} strain, where it differed significantly from the expected 2♀:1♂ ratio. The proportion of young of the desired type from the frozen embryos of Mo^{dp} and XO females was less than expected (17 and 3 % respectively). In all cases a normal breeding stock was re-established whose performance was within normal limits for each strain. Even without further improvements in embryo collection and the freezing technique *per se*, the storage of embryos in liquid nitrogen is an extremely economic way of preserving mouse genetic stocks.

1. INTRODUCTION

Since mouse embryos can survive storage at −196 °C (Whittingham, Leibo & Mazur, 1972; Wilmut, 1972) the possibility arises of conserving valuable and unique genetic material in embryo banks, thus providing an important adjunct to mammalian genetical research (Whittingham, 1974; Lyon, 1975). To realize this goal, it is necessary to determine (*a*) the viability of frozen embryos after

extended storage times, (*b*) the factors which might affect survival during the storage process, and (*c*) the feasibility of re-establishing breeding stocks from the frozen embryos of mutant and inbred strains of mice.

Data on the survival of embryos after very prolonged periods of storage are unavailable since the first successful method of storage was only developed in 1972 (Whittingham *et al.* 1972), but so far, there is no evidence for a decrease in the survival of 2-cell and 8-cell mouse embryos stored at −196 °C for 4 years (Whittingham, 1977). Since no DNA repair occurs at −196 °C, the cumulative effect of background radiation might lead to a build-up of mutations during storage. We have recently studied the effect of continuously exposing 8-cell mouse embryos to increased levels of γ-radiation at −196 °C (Whittingham, Lyon & Glenister, 1977). While the freezing technique *per se* significantly reduced embryonic survival compared with unfrozen control embryos, there was no further reduction in viability even after 29 months storage, and although at the highest radiation dose (100× background) survival was slightly impaired, it was unrelated to storage time. All progeny bred normally, showing no chromosomal anomalies or increase in mutation rate when mated with mutation testing stocks (Lyon, Whittingham & Glenister, 1977).

The animals used in this earlier work were F_1 hybrids between two inbred strains and might therefore have been expected to be particularly vigorous. However, in embryo banks it would be necessary to store samples from mutant or inbred strains which, from one cause or another, might have impaired fertility or embryonic viability. Therefore, in the present study we have examined the feasibility of re-establishing breeding stocks from the frozen embryos of various mutant and inbred strains of mice. The following stocks were chosen for investigation.

1. *HT and PT strains.* These are both homozygous for a combination of recessive genes (mutation-testing stocks) and were chosen to test whether the cumulative debilitating effect of the recessive genes might affect embryonic survival.

2. *Mo*^{dp} *strain.* This carries the X-linked mutant gene, dappled. This is lethal in the male and therefore only $\frac{1}{3}$ of the surviving offspring carry the mutation in the heterozygous state, and a proportion of the frozen embryos would be genetically inviable.

3. *XO stock.* The reproductive capacity of XO females is low (Lyon & Hawker, 1973), and their embryos develop poorly (Burgoyne & Biggers, 1976). Some embryos are genetically inviable, and only a small proportion of the surviving offspring will be the desired XO females (Morris, 1968).

4. *CBA/CaH and CBA/H-T6T6 strains.* These were chosen as examples of inbred strains of mice.

Comparisons were made between the various strains to determine whether there were differences in (*a*) the number of embryos obtained following superovulation, (*b*) the sensitivity of the embryos to freezing, thawing and storage time, (*c*) embryonic development *in vitro*, (*d*) post-implantation development

following transfer to foster mothers, and (*e*) the re-establishment of breeding colonies from the resulting progeny.

2. MATERIALS AND METHODS

(i) *Source and collection of embryos*

The strains of mice used in this study have already been listed above. In an attempt to increase the number of embryos obtainable from each female, sexually mature females were treated with gonadotrophins. Intraperitoneal injections of between 5 and 10 i.u. PMSG and HCG were given 40–48 h apart, females were caged with males after the HCG injection and successful mating was indicated by the presence of a vaginal plug on the following morning – day 1 of pregnancy. Embryos were flushed from the oviducts on the morning of the third day of pregnancy when the majority of embryos were at the 8-cell stage. In some strains a small proportion of the embryos was still at the 4-cell stage (PT, XO, CBA/CaH and CBA/H-T6T6 – see Table 1). This was probably due to slower rate of fertilization or development (Whitten & Dagg, 1961). The 4- to 8-cell stage is considered to be the most suitable for storage, as discussed previously (Whittingham, 1975). Therefore, only the embryos considered to be at the correct developmental stages were collected; fragmented, lysed, 1-, 2- and 3-cell ova were all discarded. After the number of normal embryos from each female had been recorded, all the embryos of one strain were pooled, then washed through 2 changes of medium (2 ml/wash) and between 20 and 30 embryos were transferred to each plastic freezing ampoule (2 ml screw-capped ampoules – Sterilin) containing 0·1 ml medium. Embryos damaged during washing were discarded. The ampoules were carefully labelled in order to prevent any confusion concerning the origin of embryos at thawing. The medium used for collection and for suspending the embryos during freezing and storage was a modified Dulbecco's phosphate-buffered saline designated PB1 (Whittingham, 1974).

(ii) *Freezing and thawing*

The procedures for freezing and thawing are fully described elsewhere (Whittingham *et al.* 1972; Whittingham, 1974) and also the use of plastic ampoules for storing the embryos (Whittingham, 1976; Whittingham, Lyon & Glenister, 1977). The embryos were frozen in the presence of 1·5 M-dimethylsulphoxide (DMSO) as the cryoprotective agent at rates ranging between 0·45 and 0·80 °C/min (mean $0{\cdot}64 \pm 0{\cdot}02$ °C/min) to -80 °C when they were transferred to liquid nitrogen for storage. After various intervals of storage, samples were thawed at rates ranging between 6·9 and 15·0 °C/min (mean $10{\cdot}90 \pm 0{\cdot}33$ °C/min).

(iii) *Embryo recovery and culture*

The number of samples thawed at any one time depended upon the number of pseudopregnant females available for embryo transfer. After thawing, the samples were placed in an icebath at 0 °C, where the DMSO was diluted out before the

embryos were recovered and further washed free of DMSO through several changes of medium as described previously (Whittingham *et al.* 1972). The number of embryos recovered from each ampoule and their morphological appearances were recorded, the morphological categories scored being normal; damaged with 1 or more lysed blastomeres; swollen; or totally degenerated. Loss of or damage to the zona pellucida was noted too. Finally, the embryos were cultured (for details see Biggers, Whitten & Whittingham, 1971) in a mouse embryo culture medium (No. 16, Whittingham, 1971). After 20 to 24 h incubation, the numbers of blastocysts and morulae that were normal in appearance were recorded and then the normal embryos were transferred to PB1 medium and held at room temperature until transfer to recipient females.

(iv) *Embryo transfer*

Mature females (of the outbred R stock) were used as recipients for frozen-thawed embryos from all the different strains. The females were mated naturally with sterile males (carrying the translocation T145H) and 4–6 embryos were transferred to each uterine horn on the third day of pseudopregnancy. Each female received embryos from one strain only and for each strain the females were divided randomly into two groups. The one group were killed and examined on day 14 or 15 of gestation and the number of normal live embryos and resorbing implantation sites (moles) recorded. The other group were allowed to litter and the resulting progeny examined for any abnormalities.

(v) *Re-establishment of breeding stocks*

When the liveborn progeny had reached breeding age, a sample from each stock were paired in a manner which would have been appropriate for normal maintenance of that stock. Animals of the CBA/CaH and CBA/H-T6 strains were sib-mated, those of the HT and PT strains were non-sib mated within the strain, and XO or Mo^{dp} females were mated to normal wild-type males which had not undergone frozen storage. The pairs were allowed to breed normally.

(vi) *Statistical analysis*

Student's t test, following the angular transformation of the data obtained from each individual sample or ampoule, was used to compare the recovery, normality and *in vitro* development of embryos from the different mutant and inbred strains. χ^2 analyses were considered to be more appropriate for comparing the proportions of transferred embryos implanting or developing to live foetuses or live offspring, since the data were accumulated over a considerable period in time.

3. RESULTS

(i) *Superovulation and embryo collection*

The overall response of all strains to superovulatory treatment with gonadotrophins is summarized in Table 1. The 3H1 stock has been included for it repre-

Table 1. *The total numbers of females mating, embryos recovered at the 8-cell stage and embryos stored after the attempted superovulation of the various strains of mice*

Strain	No. ♀♀ treated	No. ♀♀ with vaginal plugs	% ♀♀ mating	No. normal embryos recovered	No. embryos per mated ♀	No. embryos per treated ♀	No. embryos frozen (% of recovered embryos)
HT	57	42	73·7	610	15·3	10·7	545 (89·3)
PT	60	39	65·0	423*	10·9	7·1	387 (91·5)
Mo^{dp}	76	72	94·7	1303	18·1	17·1	1007 (77·3)
XO	70	46	65·7	677*	14·7	9·7	558 (82·4)
CBA, CBA-T6	110	72	65·5	893*	12·4	8·1	699 (78·3)
Total	373	271	72·7	3906	14·4	10·5	3196 (81·8)
3H1†	477	431	90·4	9999	23·2	21·0	7644 (78·3)‡

* A small proportion of embryos at 4-cell stage.
† Data from Whittingham *et al.* (1977).
‡ 232 normal embryos were cultured fresh.

Table 2. *Recovery and development* in vitro *of frozen-thawed 8-cell mouse embryos from various mutant stocks and inbred strains*

Source	Length of storage at −196 °C (months)	No. of samples thawed*	Total no. of embryos thawed	Mean % embryos recovered/sample (± S.E.)	Mean % embryos normal at recovery/sample (± S.E.)	Mean % morulae and early blastocysts/ samples after 20–24 h culture (± S.E.)
HT	15–17	13	321	78·39 ± 4·99	44·61 ± 3·88	45·62 ± 3·28
PT	15–17	4	95	96·50 ± 1·19†	48·13 ± 3·40	46·46 ± 3·99
Mo^{dp}	6–18	11	320	91·06 ± 2·23†	41·61 ± 7·57	40·21 ± 5·48
XO	9–18	18	366	90·11 ± 2·06†	62·41 ± 3·64‡	61·03 ± 3·44§
CBA, CBA-T6	12–21	7	180	87·85 ± 3·43	54·68 ± 9·28	44·33 ± 6·47

* 20 or 30 embryos/sample.
† Significantly different from HT at $P < 0·05$ level.
‡ Significantly different from HT at $P < 0·01$ level and Mo^{dp} at $P < 0·02$ level.
§ Significantly different from HT and Mo^{dp} at $P < 0·01$ level and CBA at $P < 0·05$ level.

sents a normal crossbred wild-type stock (F_1, C3H/HeH × 101/H). It may be considered as a control for comparative purposes, especially since the females were superovulated at approximately the same time as the other strains in order to obtain embryos for the radiation studies (Whittingham *et al.* 1977). A high proportion of the 3H1 females mated following superovulation (over 90 %) and an average of approximately 25 8-cell embryos were recovered from each female. A similar mating response was obtained with the Mo^{dp} strain, which has largely a 3H1 background, but the number of embryos recovered per female was significantly lower ($t_{(501)} = 3{\cdot}16$; $P < 0{\cdot}01$). Females from all the other strains mated less well than the 3H1 females ($P < 0{\cdot}001$ for all χ^2 comparisons with 3H1 ♀♀) and the number of embryos recovered per mated female was significantly lower (*t*-test comparisons from egg collection data, $P < 0{\cdot}001$ in all cases). These other strains did not differ significantly from each other in mating response, and although the numbers of normal embryos recovered per female was variable at least within the range of gonadotrophin doses used in this study, they were not clearly higher than after natural mating.

The total number of embryos frozen for each strain is given in Table 1. A reduction in the number of normal embryos occurred between collection and freezing (varying from 10 to 25 % for the different strains). This resulted from the elimination of embryos with blastomeres which were damaged probably during the manipulative procedures of washing the embryos after collection.

(ii) *Recovery and development* in vitro

The recovery and development *in vitro* of frozen-thawed 8-cell embryos from the various mutant and inbred strains is summarized in Table 2. For each strain the storage times have been combined, as there were too few samples thawed at each time to test accurately the effect of length of storage at −196 °C on the subsequent viability of the embryos. Only enough samples were thawed to re-establish the mutant or inbred strains as breeding colonies. Moreover, in a contemporary study no deterioration in the viability of frozen 3H1 embryos was observed following storage for $2\frac{1}{4}$ yr (Whittingham *et al.* 1977).

The recovery of frozen HT embryos was significantly lower than recovery from PT, Mo^{dp} and XO strains at $P < 0{\cdot}05$ level ($t_{(15)} = 2{\cdot}29$; $t_{(22)} = 2{\cdot}15$; $t_{(29)} = 2{\cdot}33$ respectively). Whether this is due to increased fragility of the HT embryos, causing complete disintegration of the embryos during freezing and thawing, is unknown. The overall survival rate at recovery, i.e. the proportion of embryos appearing normal, was low (range between 41 and 62 %) and was probably due in part to storage in plastic instead of glass ampoules (see discussion). The number of normal embryos from XO females was significantly higher than for HT and Mo^{dp} embryos ($t_{(29)} = 3{\cdot}28$, $P < 0{\cdot}01$; $t_{(27)} = 2{\cdot}74$, $P < 0{\cdot}02$ respectively) and also higher than for PT and CBA embryos, but insufficient samples of the latter embryos were thawed to establish a real difference. The numbers of embryos developing to morulae and early blastocysts were similar to the numbers scored as morphologically normal in all strains except CBA, where development

Table 3. *Postimplantation development of frozen-thawed 8-cell mouse embryos from various mutant and inbred stocks*

Source of embryos	Length of storage at −196 °C (months)	Total no. embryos transferred (no. recipients)	No. of embryos transferred to pregnant recipients (no. pregnant ♀♀)	Examination on day 14 or 15 of gestation: No. implantation sites (%)	Examination on day 14 or 15 of gestation: No. live foetuses (%)	No. live young at birth (%)	% live foetuses and liveborn
HT	15–17	128 (12)	56 (5)	36 (64)	17 (30)	—	35
			62 (6)	—	—	25 (40)	
PT	15–17	40 (4)	20 (2)	11 (55)	6 (30)	—	33
			20 (2)	—	—	7 (35)	
Mo^{dp}	6–18	122 (9)	69 (5)	60 (87)*	33 (48)	—	46
			53 (4)	—	—	23 (43)	
XO	9–18	217 (21)	94 (9)	51 (54)	20 (21)†	—	27
			93 (9)	—	—	31 (33)	
CBA, CBA-T6	12–21	85 (8)	55 (5)	28 (51)	20 (36)	—	45
			30 (3)	—	—	18 (60)‡	

* Significantly different from HT and PT at $P < 0{\cdot}01$ level and XO and CBA at $P < 0{\cdot}001$ level.
† Significantly different from Mo^{dp} at $P < 0{\cdot}001$ level and CBA at $P < 0{\cdot}05$ level.
‡ Significantly different from XO at $P < 0{\cdot}01$ level.

after culture was lower. The survival of embryos from XO females to the morula and early blastocyst stage was significantly higher than for HT, Mo^{dp} and CBA strains ($t_{(29)} = 3{\cdot}13$, $P < 0{\cdot}01$; $t_{(27)} = 3{\cdot}38$, $P < 0{\cdot}01$; $t_{(23)} = 2{\cdot}47$, $P < 0{\cdot}05$ respectively), but again, although the number of PT embryos surviving was lower it was not statistically significant. No differences were found in all other strain comparisons, i.e. for recovery, normality at recovery, and development *in vitro*.

(iii) *Development* in vivo *after transfer*

The post-implantation development of morulae and blastocysts derived from the frozen 8-cell embryos of the different strains following transfer to pseudopregnant recipients is summarized in Table 3. Embryos were transferred to a total of 54 pseudopregnant recipients and 50 subsequently became pregnant with transferred embryos (92·6 %). The lowest number of recipients becoming pregnant occurred with the transfer of embryos originating from XO females (18/21 = 85·7 %). This may be due to the overall lower post-implantation survival of frozen embryos from XO females. The proportion of embryos implanting from the Mo^{dp} strain was significantly higher than for all the other strains (χ^2 comparison – see Table 3). The proportions of embryos developing into live foetuses and live offspring were similar for all strains except those derived from XO females where foetal development at 15 days gestation was significantly lower than in the Mo^{dp} strain ($\chi^2_1 = 12{\cdot}78$, $P < 0{\cdot}001$) and both foetal development and live births were lower than in the CBA strain ($\chi^2_1 = 4{\cdot}02$, $P < 0{\cdot}05$; $\chi^2_1 = 6{\cdot}73$, $P < 0{\cdot}01$, for live foetuses and live offspring respectively). The highest proportions of stored embryos developing to foetuses and liveborn were obtained with embryos from the Mo^{dp} and CBA strains (46 and 45 % respectively). A valid statistical comparison of early post-implantation loss between the different strains was not possible because of insufficient data. However, early post-implantation loss of embryos was highest with those derived from XO females (62 %) and lowest with CBA embryos (29 %). For HT, PT and Mo^{dp} strains the early post-implantation mortality was similar (53, 45 and 45 % respectively.)

(iv) *Breeding data*

In considering the re-establishment of breeding stocks after freeze-preservation of embryos, it is first necessary to consider the suitability of the liveborn frozen-thawed young as parents. The animals should be healthy and the sex ratio normal. In those stocks where only a proportion of the young are expected to be of the type desired for breeding the expected proportion of such young should be found.

Table 4 shows that the sex ratios in the samples of liveborn young obtained in the present work were in general within normal limits. The most unexpected ratio was in the Mo^{dp} stock. Here, since Mo^{dp}/Y males die prenatally, a 2:1 ratio of females to males would be expected. In fact, there were only 10 females and 13 males, which is significantly different from a 2:1 ratio ($\chi^2 = 5{\cdot}57$, $P < 0{\cdot}02$). In view of the small sample, little importance is attached to this.

In the two stocks Mo^{dp} and XO, in which only a proportion of young were

expected to be of the desired type, the observed proportion was in both cases less than expected. In Mo^{dp} the discrepancy was relatively small and statistically insignificant. In the XO stock, only one of 20 females born was XO, whereas typically about 15% of females born in a conventionally maintained XO stock are of the XO type. Further work is necessary to test whether this apparent poor response of XO embryos to freezing and thawing is a real effect.

Table 4. *Sex and genotype of liveborn young from frozen-thawed mutant stocks*

Strain	No. transferred	No. born	(%)	Sex		Mutant (%)
				Female	Male	
HT	62	25	(40)	13	12	—
PT	20	7	(35)	4	3	—
Mo^{dp}	53	23	(43)	10	13	4 (17)
XO	93	31	(33)	20	11	1 (3)
CBA, CBA-T6	30	18	(60)	9	9	—

Table 5. *Breeding performance of liveborn animals from frozen-thawed mutant stocks*

Strain	No. mated pairs	No. litters (per pair)	Young born (per pair)	Young weaned (per pair)	Mean litter size
HT	3	10 (3·3)	68 (22·7)	68 (22·7)	6·8
PT	2*	16 (8·0)	89 (44·5)	84 (42·0)	5·6
Mo^{dp}	4	34 (8·5)	132 (33·0)	112 (28·0)	3·9
XO	1	5	11	3	2·2
CBA, CBA-T6	4	9 (2·25)	40 (10·0)	38 (9·5)	4·4

* Three pairs were mated but one male had to be killed through ill health after one litter. Data presented relate to remaining two pairs.

The breeding results from the samples of frozen-thawed animals which were mated to re-establish the stocks are shown in Table 5. One PT male had to be killed soon after pairing because of a skin lesion, but all other animals were fertile and the breeding performance was within normal limits for each strain. Since the samples of parents were small we cannot say any more precisely whether the breeding performance had been affected by the freezing and thawing procedure. It is theoretically possible that the use of uterine foster mothers from a different strain may have affected the breeding. However, we can say that in all cases a normal breeding stock was re-established, even in the case of XO, where only one suitable female was available for breeding. This female reared only one litter, but this included an XO daughter, which was in turn successfully used for breeding. Thus, the samples of embryos transferred to uterine foster mothers were numerically adequate, but it would be prudent in future to transfer larger samples of the XO stock.

4. DISCUSSION

This work has clearly demonstrated the feasibility of preserving mutant and inbred stocks by storing 8-cell embryos in liquid nitrogen. Breeding stocks were re-established from stored embryos in all cases, including those stocks in which only a proportion of the progeny from frozen embryos were of the desired type, i.e. in the Mo^{dp} and XO strains.

Table 6. *The number of progeny obtained per female used for embryo collection*

Strain	No. embryos thawed for transfer	Foetuses and liveborn (%)	Embryos collected per donor female	Foetuses and liveborn per donor female
HT	321	13	10·7	1·4
PT	95	14	7·1	1·0
Mo^{dp}	290	20	17·1	3·4
XO	366	14	9·7	1·4
CBA, CBA-T6	180	21	8·1	1·7
3H1*	1290	23	21·0	4·8

* Controls and 2× background series from Whittingham *et al.* (1977).

However, there is clearly room for improvement in the technique, since we obtained only between 1 and 3 live offspring per female used to provide embryos in the mutant and inbred strains, compared with approximately 5 per female in the hybrid 3H1 strain (Table 6). One problem concerns the response of females from the various strains to the gonadotrophins used for superovulation. The proportion of treated females mating and the number of embryos collected per female were much lower than previously reported for 3H1 females (Whittingham *et al.* 1977), except for the Mo^{dp} females, whose genetic background was similar to the 3H1 strains. This probably results mainly from the known variation in sensitivity of mice of various strains and ages to treatment with gonadotrophins (Gates, 1965, 1971). In addition, one should bear in mind that in this study the eggs were not scored at ovulation, but at the 8-cell stage, and hence any variations among strains in viability of early embryos could have affected the results. In particular the YO embryos from XO females fail to develop to the 8-cell stage (Morris, 1968), and hence only about 75 % of the fertilized eggs of this strain would have been collected in our work.

As at present operated the actual process of freezing embryos is relatively time-consuming and laborious, owing to the need for 'seeding' and to the long period of cooling at *c.* 0·5 °C/min. A good response to superovulation is therefore very valuable in providing a large number of embryos to be frozen at one time. The variable response of the mutant and inbred strains was therefore disappointing. However, although the gonadotrophin treatment did not produce superovulation consistently, it was advantageous to have an adequate number of mated females

at one time, and to give relatively synchronized ovulation so that the embryos were of more uniform developmental age at collection than would be expected with naturally ovulating females. Based upon the previous findings of Gates (1965, 1971) we have assumed throughout this study that the developmental potential of embryos from superovulated and naturally mated mice is similar. However, this may not be true for all mutant and inbred strains of mice, and such treatment may have contributed to the differences in the overall viability after storage.

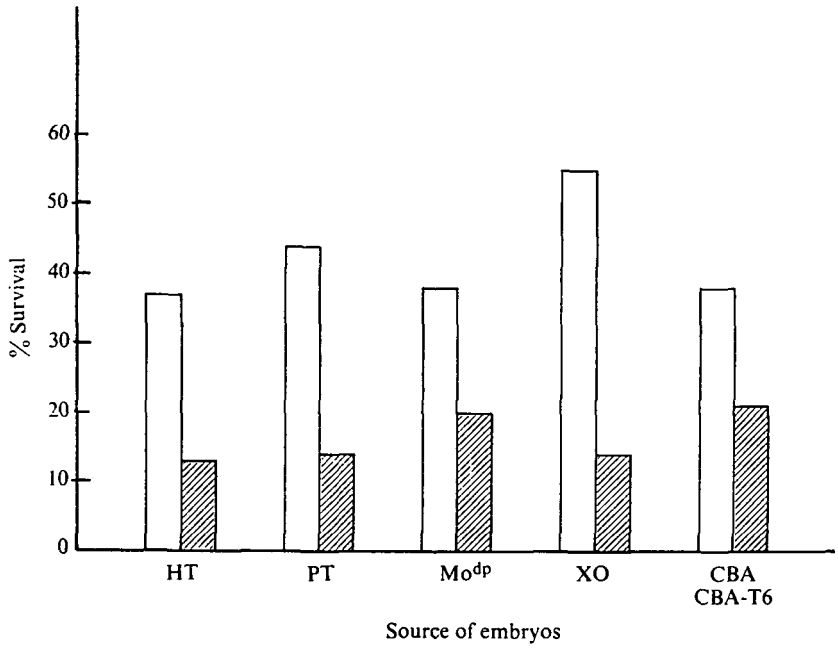

Fig. 1. Survival of 8-cell mouse embryos from various mutant and inbred strains to morulae and early blastocysts ☐ and foetuses and liveborn ▨, expressed as a percentage of the number of embryos originally frozen.

A further problem in the freezing and thawing of mutant and inbred strains concerns the numbers of live foetuses and offspring obtained from the samples thawed. As Fig. 1 shows, between 13 and 21% of the embryos originally frozen developed to foetuses and liveborn, a definitely lower value than obtained with 3H1 embryos used in the radiation studies (Whittingham *et al.* 1977). There appeared to be differences between strains in the overall viability, but we did not compare the viabilities of unfrozen cultured embryos of each strain. Hence, it is not clear whether there were true differences between strains either in general viability of embryos, or in resistance to freezing and thawing. The greatest post-implantation mortality occurred in embryos from XO females. Burgoyne & Biggers (1976) observed that embryos from XO females developed poorly from the 2-cell to blastocyst stage *in vitro*. Hence, although we found that the development of frozen-thawed eggs of XO animals from 8-cell to morula or early

blastocyst stage was unimpaired, the excess early post-implantation loss may have been an effect of the XO condition in the mother.

Thus, there are two main ways in which the technique might be improved. Firstly, the number of embryos for freezing obtained from each female might be increased, by further study of methods of superovulation. Secondly, the survival of embryos after freezing and thawing might be improved. The use of glass ampoules in place of plastic is an obvious possibility here, which would probably increase the embryonic survival by 20–30 % (Whittingham *et al.* 1977; Whittingham, 1976).

With present techniques and levels of embryonic survival, the method of freeze preservation of genetic stocks is already a practicable one provided relatively large samples of embryos are preserved. We plan to freeze approximately 500 embryos per stock. If, in accord with the present work, about 20 % of these are recovered as liveborn young, this gives potentially 100 animals for re-establishing the stock. Since a breeding nucleus of 10 animals would in general be adequate to establish a stock, this gives a safety factor of about 10 ×. In the case of inbred strains, since it would be highly laborious to keep the embryos of full sibs together in the freezing and thawing processes, we plan to pool embryos from a single generation in the preserved sample. This means that one generation of sib-mating will be lost, but this should not cause any major impairment of the genetic homogeneity of the strain.

This work provides further evidence on the economics of freeze preservation. We estimated earlier (Lyon, 1975) that the cost of freezing and thawing a mouse genetic stock, if a sample of 500 embryos were frozen, would be equal to that of $\frac{1}{4}$–$\frac{1}{2}$ year's normal stock maintenance. We can now confirm that the suggested sample size of 500 seems a reasonable one. Rarely used mutant stocks, such as might be frozen, are usually kept in a space of 6–15 cages each. We had established previously that the cost of freezing embryos, in terms of producing females to provide embryos, was roughly equivalent to $\frac{1}{4}$ year's normal breeding. The cost of thawing mainly involves the production of uterine foster mothers, and the care of them and their frozen-thawed progeny until the latter have reached breeding age. Since in the present work about 40 % of transferred embryos survived to term, our earlier estimate that thawing also would cost the equivalent of $\frac{1}{4}$ year's normal breeding (i.e. the care of 6 foster mothers and young for 3 months) seems a reasonable one. Even without further improvement the method of freezing in liquid nitrogen seems a highly economic method of preserving mouse genetic stocks.

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