Death of mouse embryos that lack a functional gene for glucose phosphate isomerase

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Summary

A null allele of the *Gpi-1s* structural gene, that encodes glucose phosphate isomerase (GPI-1; *E.C.* 5.3.1.9), arose in a mutation experiment and was designated $Gpi-1s^{a-m/H}$. The viability of homozygotes has been investigated. No offspring homozygous for the null allele were produced by intercrossing two heterozygotes, so the homozygous condition was presumed to be embryonic lethal. Embryos were produced by crossing $Gpi-1s^a$/null heterozygous females and $Gpi-1s^b$/null heterozygous males. Homozygous null embryos were identified at different stages of development by electrophoresis and staining either for GPI-1 alone or GPI-1 plus phosphoglycerate kinase (PGK) activity. At $6\frac{1}{2}$ and $7\frac{1}{2}$ days *post coitum* homozygous null embryos were present at approximately the expected 25% frequency (37/165; 22·4% overall) although at $7\frac{1}{2}$ days the homozygous null embryos tended to be small. By $8\frac{1}{2}$ days most homozygous null embryos were developmentally retarded and had not developed significantly further than at $7\frac{1}{2}$ days; some were dead or dying. By $9\frac{1}{2}$ days the homozygous null conceptus was characterised by a small implantation site that contained trophoblast and often a small amount of extraembryonic membrane. Surviving trophoblast tissue was also detectable at $10\frac{1}{2}$ days. Previous studies have shown that oocyte-coded GPI-1 persists only until $5\frac{1}{2}$ or $6\frac{1}{2}$ days. Survival of homozygous null embryos to $7\frac{1}{2}$ or $8\frac{1}{2}$ days and survival of certain extraembryonic tissue to $10\frac{1}{2}$ days suggests that the homozygous null condition may not be cell-lethal although it is certainly embryo-lethal. Mutant cells that are deficient in glycolysis may use the pentose phosphate shunt to bypass the block in glycolysis created by the deficiency of glucose phosphate isomerase, and/or might be rescued by the transport, from the maternal blood, of energy sources other than glucose (such as glutamine). Either strategy may only permit slow cell growth that would not be adequate to support normal embryogenesis. Transport of maternal nutrients would be more efficient to the trophoblast and extraembryonic membranes and this may help to explain why these tissues survive for longer than the embryo itself. The morphological similarity between homozygous nulls and androgenetic conceptuses, where the trophoblast also survives better than the embryo, is discussed.

Introduction

The glucose phosphate isomerase enzyme (GPI-1; *E.C.* 5.3.1.9) present in normal mouse embryos is exclusively oocyte-coded until the embryonic *Gpi-1s* genes are activated at $2\frac{1}{2}$ to $3\frac{1}{2}$ days *post coitum* (*p.c.*). Embryo-coded GPI-1 is readily detected from $3\frac{1}{2}$ days and oocyte-coded enzyme is exhausted by $5\frac{1}{2}$ to $6\frac{1}{2}$ days (Chapman, Whitten & Ruddle, 1971; Brinster, 1973; West & Green, 1983; Duboule & Burki, 1985;

Gilbert & Solter, 1985; West, Leask & Green, 1986; West & Flockhart, 1989).

The discovery of 'null alleles' of *Gpi-1s*, that lack detectable GPI-1 activity, (Soares, 1979; Peters & Ball, 1986) provides the opportunity to investigate, not only when the mouse embryo synthesises GPI-1, but when it needs this enzyme. In this study we have produced embryos homozygous for a null allele of *Gpi-1s* and determined at what stage of development these usually die. These embryos inherit GPI-1 from the oocyte but are genetically incapable of producing their own enzyme.

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Materials and methods

Mice

The *Gpi-1s* null allele arose in the offspring of a 101/H strain male mouse that had been treated with 250 mg/kg ethylnitrosourea so that induced mutations would have occurred in spermatogonia (Peters & Ball, 1986). The mutant null allele originated from the *Gpi-1s^a* allele and was designated *Gpi-1s^{a-m1H}*. (The gene symbol is abbreviated to '*m*' in this paper.) A congenic stock (C57BL/Ola-*Gpi-1s^{a-m1H}*) that carries the null mutation was produced by crossing a *Gpi-1s^a/Gpi-1s^{a-m1H}* (*a/m*) heterozygote with an inbred C57BL/Ola mouse (homozygous for *Gpi-1s^b*). Heterozygous *Gpi-1s^b/Gpi-1s^{a-m1H}* (*b/m*) offspring were crossed with C57BL/Ola-*Gpi-1s^a* congenic mice (West & Fisher, 1984) and subsequent generations crossed alternately to C57BL/Ola and C57BL/Ola-*Gpi-1s^a* strains (14 generations). C57BL/Ola-*Gpi-1s^{a-m1H}* strain mice are heterozygous for the null mutation and either the *Gpi-1s^a* or the *Gpi-1s^b* allele. They are designated *a/m* (*a*/null) or *b/m* (*b*/null) respectively. Heterozygous *a/m* females were mated either with heterozygous *b/m* males (experimental matings) or C57BL/Ola (*b/b*) males (control matings) in the Centre for Reproductive Biology. Additional crosses were set up at the MRC Radiobiology Unit with mice on a mixed genetic background of C3H/HeH and 101/H. These were used for the analysis of heterozygous 12½ day embryos (Fig. 1) but not for the analysis of homozygous null embryos earlier in gestation. Results from both sets of animals are included in the breeding data shown in Table 1.

Embryo Collection

Ovulation was induced by intraperitoneal injections of 5 IU pregnant mares' serum gonadotrophin (PMS) at approximately 12 noon followed 48 hours later by 5 IU human chorionic gonadotrophin (hCG). Females were then housed with males and mating verified the next morning by the detection of a vaginal plug. The day of the vaginal plug was designated 0·5 days *post coitum* (*p.c.*) because mating was assumed to occur during the preceding night. Postimplantation embryos were dissected from the decidual swellings in isotonic saline, phosphate buffered saline or M2 medium (Quinn, Barros & Whittingham, 1982), classified as normal, small or dead and dissected free of maternal tissue. No rigid criteria for small versus large embryos were used because there was considerable variation between litters, particularly at 6½ and 7½ days. Classifications were made subjectively with reference to other embryos in the litter. Individual 6½, 7½ and the smaller 8½ day embryos were stored in Pasteur pipettes as described by West & Green (1983). The larger 8½ day embryos and small samples of tissue (such as trophoblast) from 9½ day and later conceptuses were

stored in 10 µl of M2 (or M2 mixed with an equal volume of glycerol) in a multi-well plate. Normal embryos at or beyond 9½ days were stored in larger volumes of media in 1·5 ml plastic tubes. All samples were stored at −20 °C and frozen and thawed three times before electrophoresis.

Blood collection

Mice of weaning age or older were bled from the orbital sinus while under ether anaesthesia.

GPI-1 Electrophoresis

GPI-1 electrophoresis of red cells or whole blood was carried out as described, respectively, by Peters & Ball (1990) and West & Fisher (1984). Samples of embryos were applied to 76 × 60 mm Helena, Titan III cellulose acetate electrophoresis plates. Electrophoresis and staining for GPI-1 activity was done as previously described (West *et al.* 1986) in order to identify embryos of the four *Gpi-1s* genotypes (Fig. 2*a*).

Combined GPI-1 and PGK-1 electrophoresis

Some embryos were simultaneously assayed for the presence of GPI-1 and phosphoglycerate kinase-1 (PGK-1) in order to check that those with no GPI-1 activity were homozygotes for the null mutation rather than missing samples. Samples were again run on the Titan III plates but the running buffer (suggested by Dr J. D. Ansell of the Department of Zoology, University of Edinburgh) was a 1:1 mixture of pH 8·1–8·5 Tris-glycine buffer used for GPI-1 (3·0 g Tris base plus 14·4 g glycine per litre as described by Eicher & Washburn, 1978) and the pH 8·6 buffer described by Bucher *et al.* 1980 (20 mM sodium barbital, 10 mM sodium citrate, 2 mM disodium ethylenediaminetetra-acetic acid and 5 mM magnesium sulphate). The samples were applied to the centre of the electrophoresis plate and 12 milliamps current passed per plate for 30 minutes.

PGK-1 activity was identified by the 'backwards' reaction (Oelshlegel Jr & Brewer, 1972; Bucher *et al.* 1980), using a modification of the method described by Bucher *et al.* (1980) and Ansell & Micklem (1986). For each plate, 0·8 ml of 0·1 M triethanolamine pH 7·6 buffer, containing 20 mM MgCl$_2$, was mixed with 1·5 ml glycerol. To this was added the enzymes: 10 µl glucose-6-phosphate dehydrogenase (Sigma G8878, 312 U/ml), 10 µl aldolase (Sigma A1893, 300 U/ml), 5 µl glyceraldehyde phosphate dehydrogenase (Boehringer 105686, 800 U/ml), 5 µl glycerol dehydrogenase (Sigma G6751, 417 U/ml) and 5 µl hexokinase (Sigma H5625, 476 U/ml). This was followed by 100 µl indicator stock [270 mg anhydrous glucose, 112·5 mg ADP, 306 mg NADP, 320 mg MgSO$_4$.7H$_2$O per 10 ml 100 mM triethanolamine buffer (185·4 mg triethanolamine per 10 ml); pH adjusted to pH 7·3

Table 1. *Breeding results with mice heterozygous for the GPI-1 null allele*

Cross (female × male)*	Phenotypes (and expected genotypes) at weaning			
	AB (*a/b*)	A (*a/m* or *a/a*)	B (*b/m*)	0 (*m/m*)
1. *a/a × b/m*	167	163	0	0
2. *b/m × a/a*	173	154	0	0
3. *b/b × a/m*	108	0	107	0
4. *a/m × b/b*	157	0	137	0
5. *a/m × b/m*	45	35	35	0
6. *b/m × a/m*	69	50	70	0
7. *a/m × a/m*	0	23	0	0

* The genotype symbols are abbreviated: *a* is $Gpi-1s^a$, *b* is $Gpi-1s^b$ and *m* represents the null mutation $Gpi-1s^{a \cdot m1H}$. Phenotype '0' indicates no GPI-1 activity as expected for the homozygous null genotype (*m/m*). In crosses 1–6, phenotypes A and B would be produced by *a/m* and *b/m* heterozygotes rather than *a/a* and *b/b* homozygotes but in cross 7 the A phenotype could be either *a/m* or *a/a*.
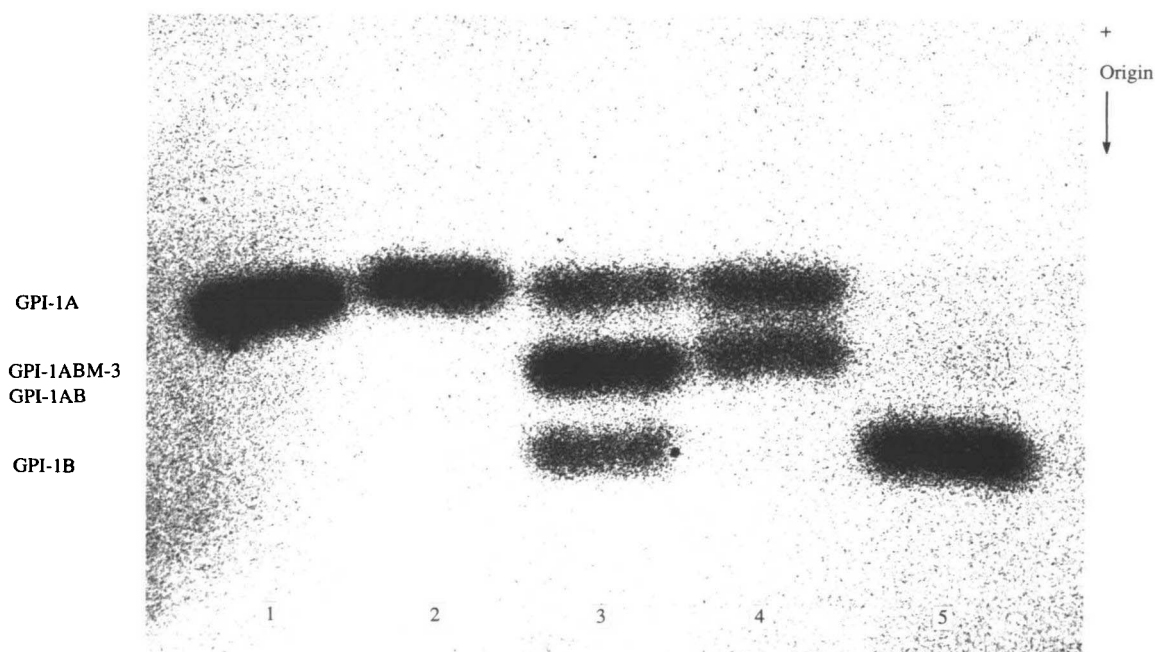

Fig. 1. Electrophoresis and staining for GPI-1 activity in $12\frac{1}{2}$ day conceptuses of different *Gpi-1s* genotypes. Track 1, *a/a*; track 2, *a/m*; track 3, *a/b*; track 4, *a/bm-3*; track 5, *b/m*. The *bm-3* mutation is discussed more fully by Peters & Ball (1990).

with 2 N NaOH], 400 μl assay stock (368 mg $K_2HPO_4 \cdot 3H_2O$, 32 mg NAD and 650 mg fructose diphosphate per 40 ml of pH 8·6 barbital buffer), 300 μl nitroblue tetrazolium (27 mg/ml in water) and 20 μl phenazine methosulphate (2·5 mg/ml in water).

For the combined GPI/PGK stain 170 μl fructose-6-phosphate (20 mg/ml in water) was added to the stain mixture. After the electrophoresis run was complete the plates were carefully blotted and the stain mix poured directly onto the plates (3·5 ml per plate). The inclusion of glycerol makes the mixture more viscous so that a supporting medium (e.g. agar or cellulose acetate membrane) is unnecessary.

In most cases the different GPI-1 phenotypes could not be distinguished by this combined GPI/PGK method but the embryos were classified for the presence or absence of GPI-1 and PGK-1 activity, which both stained as blue bands with the tetrazolium-based stain. Both enzymes migrated towards the anode, under the conditions used, but GPI-1 moved more slowly than PGK-1 and remained close to the application point (Fig. 2b). Control samples were stained for GPI-1 and PGK-1 separately as well as with the combined GPI/PGK stain in order to verify the identity of the bands of staining. In some cases the relative staining intensity of the GPI-1 and PGK-1 bands was estimated by scanning densitometry.
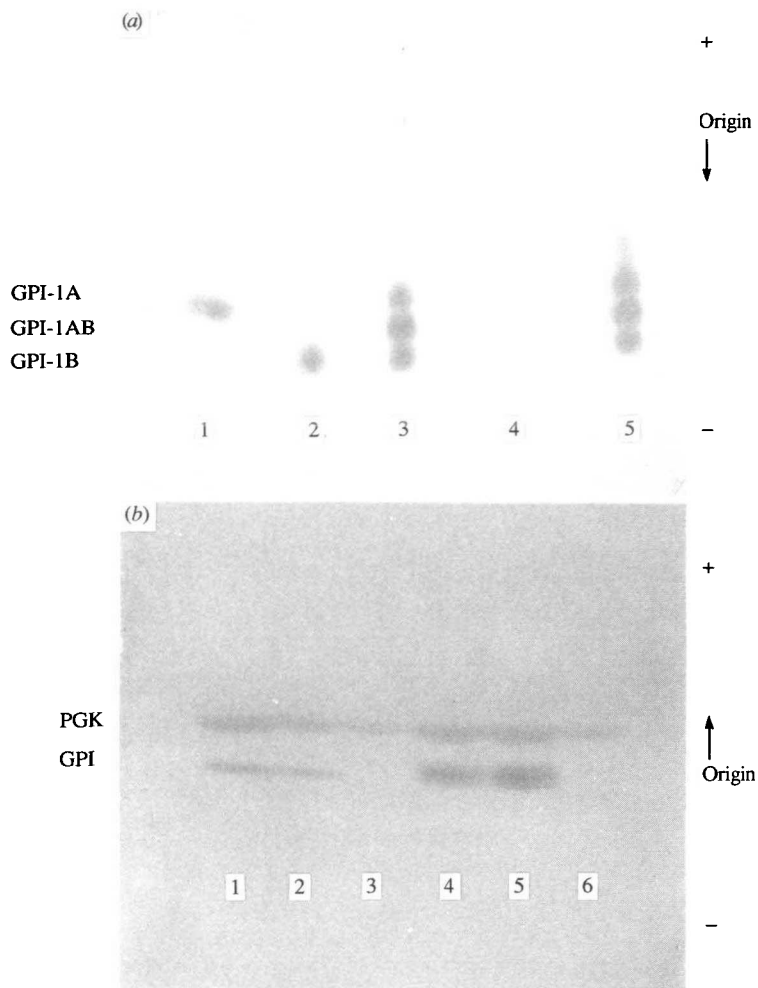
Fig. 2. (a) Electrophoresis and staining for GPI-1 activity. Tracks 1 to 4 were each loaded with single $7\frac{1}{2}$ day embryos from the same $(a/m \times b/m)$ F1 litter. Track 5 was loaded with diluted blood from an adult a/b heterozygote and shows all three GPI-1 allozyme bands (GPI-1A, GPI-1AB and GPI-1B) produced by heterozygotes. The absence of GPI-1 activity from track 4 suggests that this embryo was a m/m null homozygote. The genotypes of the embryos in the other tracks are assumed to be a/m (track 1), b/m (track 2) and a/b (track 3). (b) Electrophoresis and simultaneous staining for GPI-1 activity and PGK activity. Tracks 1 to 6 were each loaded with samples from single $8\frac{1}{2}$ day conceptuses from the same $(a/m \times b/m)$ F1 litter. Samples 1, 4 and 6 were embryos; 2, 3 and 5 were samples of trophoblast. Samples 1 and 2 and samples 4 and 5 were paired embryo and trophoblast samples from the same conceptuses. The absence of GPI-1 activity, in the presence of PGK activity, from tracks 3 and 6 shows that these conceptuses were m/m null homozygotes.

Results

Breeding results

Reciprocal crosses between mice heterozygous for the Gpi-1s null allele and mice homozygous for either Gpi-1s^a or Gpi-1s^b showed that approximately equal numbers of AB and either A or B progeny were produced (Table 1, crosses 1–4). However, intercrosses between two null heterozygotes failed to produce any homozygous null offspring (Table 1, crosses 5 and 6), thus confirming that the homozygous null genotype is lethal. Three experiments were undertaken to discover when these homozygotes die.

Biochemical characterisation of the null mutation

The mutant allele appears to be a complete null because in a/m and b/m heterozygotes (Fig. 1, tracks 2 and 5) a single isozyme is found and there is no evidence of a heterodimeric band, although another mutant allele, b-$m3H$, does determine some GPI activity and a heterodimer (Fig. 1, track 4).

Experiment 1: GPI analysis of $6\frac{1}{2}$–$8\frac{1}{2}$ day conceptuses

The frequencies of the four GPI-1 phenotypes (Fig. 2a) among the embryos produced in the experimental and control crosses in experiment 1 are shown in Fig. 3. At $6\frac{1}{2}$ days, 5/31 (16·1 %) embryos analysed had no GPI-1 activity. Although this is lower than the 25 %
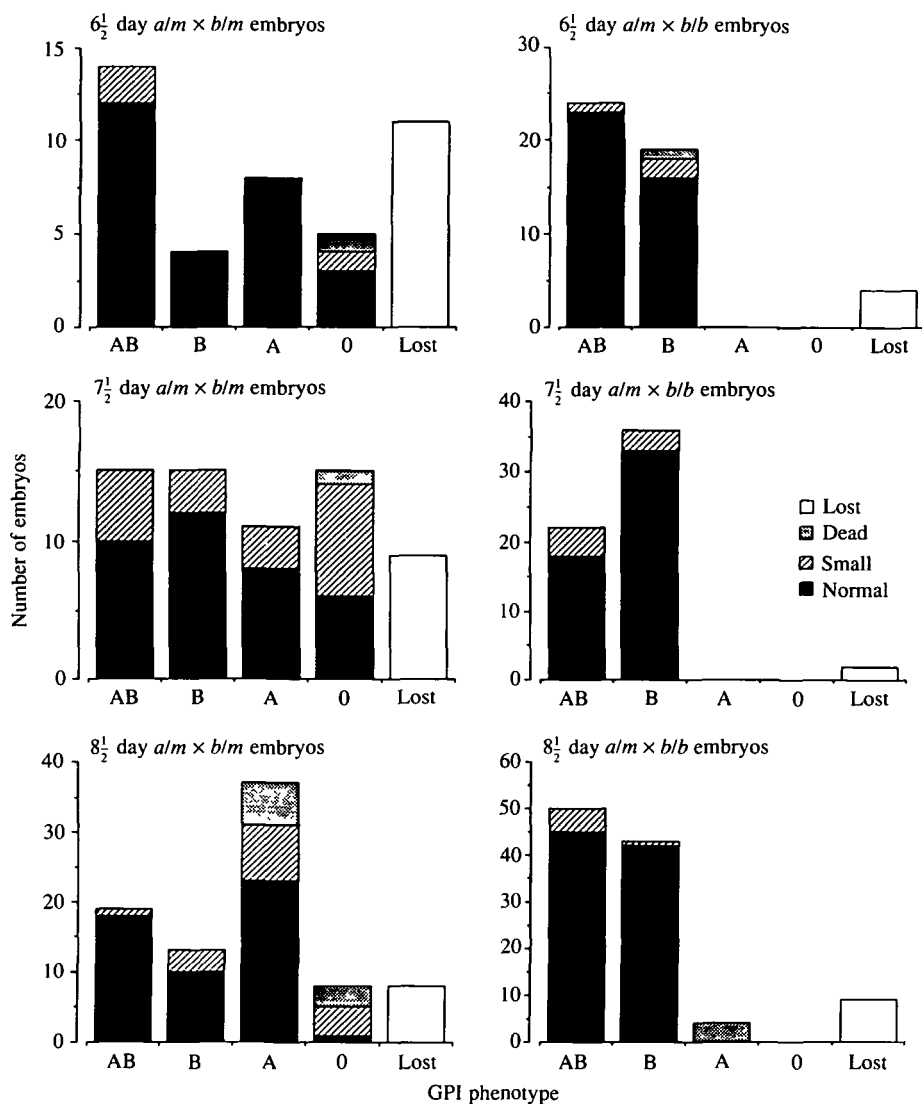
Fig. 3. Distribution of four GPI-1 phenotypes among $6\frac{1}{2}$, $7\frac{1}{2}$ and $8\frac{1}{2}$ day embryos from the experimental and control crosses in experiment 1. The embryos recorded as 'lost' include empty decidua and losses due to technical difficulties during dissection and analysis.

expected if all homozygous null (m/m) embryos are recognisable at this stage, the total number of embryos is small and the frequency of B embryos $(b/m$ genotype) is also lower than expected. The control $6\frac{1}{2}$ day embryos were all either AB or B and the percentage in each group (56%:44%) was close to the expected 50:50 ratio.

At $7\frac{1}{2}$ days the pattern was similar, although the 56 embryos in the experimental cross (15 AB, 15 B, 11 A, and 15 nulls or 27%:27%:20%:27%) were distributed more closely to the expected 25:25:25:25 ratio. Of the viable embryos, the proportion of homozygous nulls that were classified as small embryos (8/14, 57·1 %) was higher than the proportion of small embryos in the other three groups (11/41, 26·8 %). This difference was statistically significant in a one-tailed test (by Fisher's exact test, $P = 0.043$). This suggests that by $7\frac{1}{2}$ days many of the homozygous null embryos are developmentally retarded. Although

the only dead embryo at this age was a presumptive null homozygote, the ratio of the four genotypes suggests that most homozygous null embryos are still alive at $7\frac{1}{2}$ days.

At $8\frac{1}{2}$ days four of the 97 control embryos had a GPI-1A phenotype. No a/m embryos were expected in this cross but since all four embryos were dead (1 in one litter and 3 in another) it seems likely that the GPI-1 activity was entirely due to contaminating maternal blood on the resorbing embryos. (The cause of death is unknown but sporadic embryonic loss is not unexpected.) Some of the 6 dead embryos scored as GPI-1A (a/m) in the experimental cross may also have been m/m nulls that were contaminated with maternal blood because the total percentage (37/77, 48·1%) of GPI-1A embryos was higher than the expected 25%. This could also be true of some of the small embryos scored as GPI-1A but, because none of the GPI-1B embryos had any detectable GPI-1A
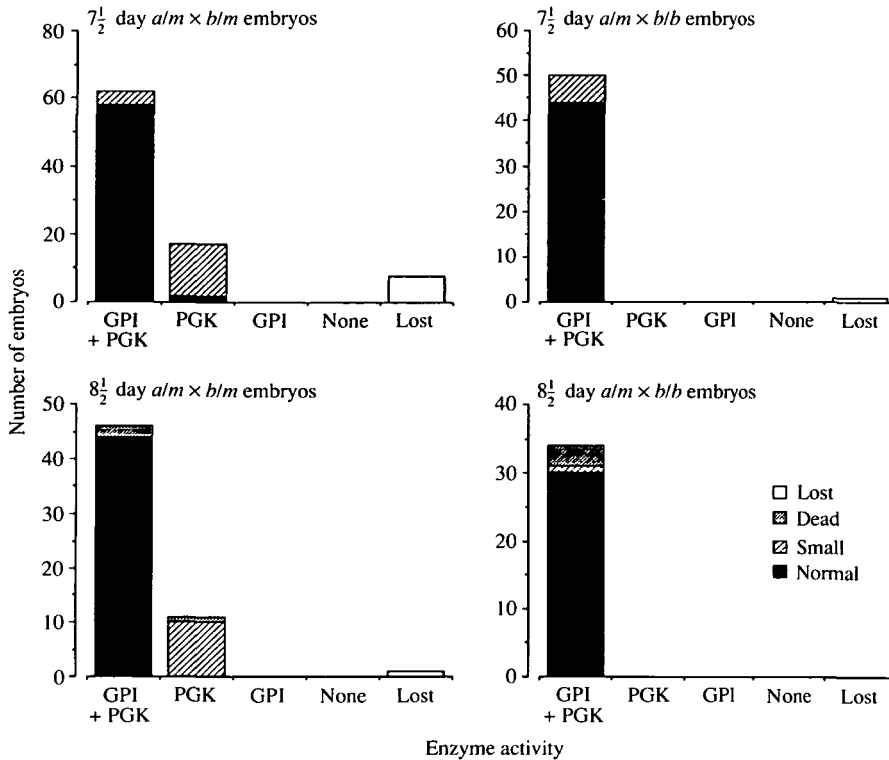
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Fig. 4. Distributions of $7\frac{1}{2}$ day embryos (top) and $8\frac{1}{2}$ day embryos (bottom) with either GPI plus PGK activity or only PGK activity in the experimental and control crosses of experiment 2. PGK activity was detected in all the embryos analysed so none had only GPI or no detectable enzyme activity. The embryos recorded as 'lost' include empty decidua and technical losses during dissection and analysis.

enzyme, maternal contamination is likely to be rare, at least in larger embryos. It is also possible that some of the first $8\frac{1}{2}$ day, GPI-1B embryos to be collected were misclassified as GPI-1A because initially each $8\frac{1}{2}$ day embryo was stored, whole, in a fine pipette and the whole embryo was applied to the electrophoresis plate. This resulted in overstaining and some loss of resolution but such misclassification would have been limited to a few samples. Subsequently, all of the larger $8\frac{1}{2}$ day embryos were stored in a multi-well plate as described in the Materials and Methods section.

Despite the uncertainties about some of the GPI-1A embryos, eight homozygous null embryos were identified at this age and the majority (7/8) were classified as either small or dead. This is significantly higher than the corresponding proportion for AB (1/19) and B (3/13) embryos (Fisher's 1-tailed exact tests: $P = 0.000093$ and $P = 0.0067$ respectively) and implies that most homozygous null embryos are dead, dying or developmentally retarded by $8\frac{1}{2}$ days.

Experiment 2: combined GPI/PGK analysis of $7\frac{1}{2}$ and $8\frac{1}{2}$ day conceptuses

In order to confirm that the 25% (14/55) of the viable $7\frac{1}{2}$ day samples in the experimental cross without GPI-1 activity (Fig. 3) were homozygous null embryos and not simply samples that had lost all enzyme activity, another set of $7\frac{1}{2}$ day embryos was collected. These were tested for both GPI-1 and PGK activity (Figs. 2b and 4). As expected, all embryos from the control $a/m \times b/b$ cross had both GPI-1 and PGK activity. Embryos from the experimental $a/m \times b/m$ cross either had both GPI-1 and PGK activity or just PGK activity. In about 90% of the $7\frac{1}{2}$ day embryos with both enzyme activities, the GPI-1 was stained more intensely than PGK and the staining was similar in the remaining 10%. No attempt was made to use the GPI-1/PGK ratio to identify embryos heterozygous for the *Gpi-1s* null allele because the GPI-1/PGK ratio may have varied for technical reasons associated with the two reactions and the GPI-1 activity was more resistant to freezing and thawing than PGK. However, the lower activity of PGK in this series of embryos makes it virtually certain that those embryos with only PGK activity were homozygous for the *Gpi-1s* null allele because any GPI-1 should have been detected more readily than PGK.

The frequency of viable $a/m \times b/m$ embryos with only PGK activity (putative homozygous *Gpi-1s* nulls) was 17/79 (21.5%) which is close to the 25% expected if all of the homozygous nulls were alive at $7\frac{1}{2}$ days. Although they were alive, the majority of the putative homozygous nulls were small (15/17) whereas few of the embryos with both PGK and GPI-1 activity were classified as small (4/62). This difference is statistically highly significant (by Fisher's exact test, $P = 8.7 \times 10^{-11}$) and more clear-cut than the results of the
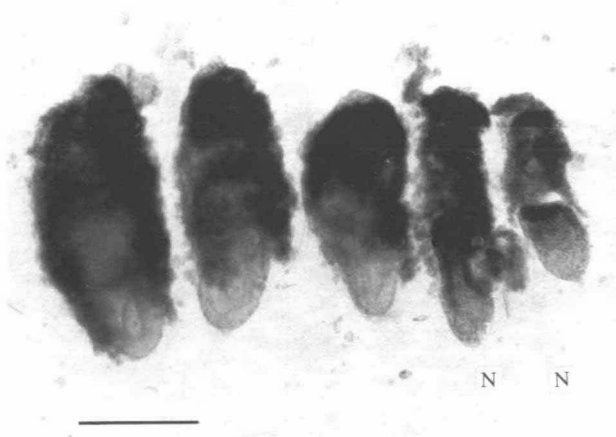
Fig. 5. Five $7\frac{1}{2}$ day embryos from a large $(a/m \times b/m)$ F1 litter (20 embryos from 21 implantation sites). The top half of each embryo is covered with trophoblast tissue (appears dark in the photograph) and this was dissected off before electrophoresis. The two smaller embryos (labelled 'N') on the right of the photograph produced PGK activity but no GPI-1 activity, implying that these were both m/m null homozygotes. The other three embryos all produced both GPI-1 and PGK activity and so were not homozygous for the null allele. The scale bar is 0·5 mm.
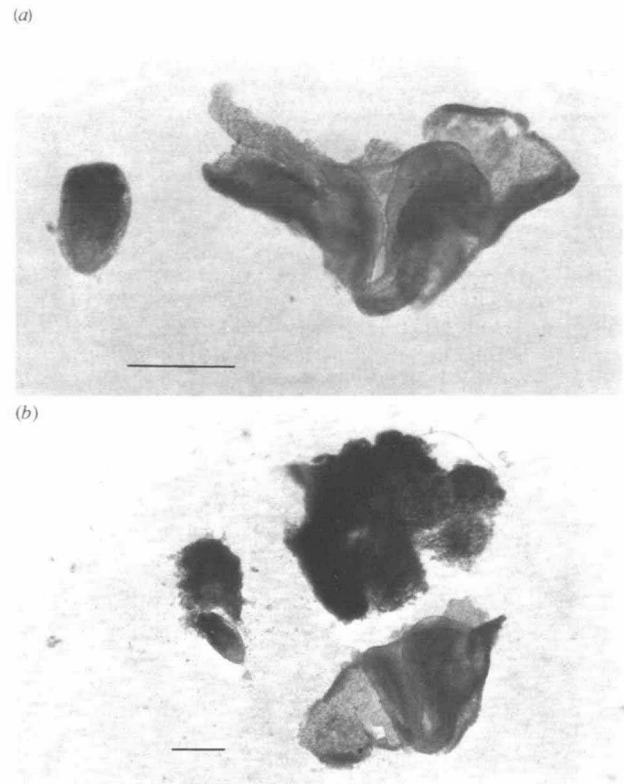

Fig. 6. Two pairs of littermates at $8\frac{1}{2}$ days, from the $a/m \times b/m$ experimental cross. Each pair consists of one normal embryo (on the right in each photograph) and one small embryo (on the left). In each case the morphologically normal (head fold stage) embryo had both GPI-1 and PGK activity whereas the small, retarded (egg cylinder stage) embryo produced PGK activity but no GPI-1 activity (m/m null homozygote). The overlying trophoblast tissue has been dissected off all four embryos. It is not shown in photograph (a) but is the dark mass of tissue above each embryo in (b). The scale bars are 0·5 mm.

first experiment (Fig. 3). Typical morphological differences between small and normal embryos are shown in Fig. 5. These results confirm that the homozygous *Gpi-1s* null embryos are alive but developmentally retarded at $7\frac{1}{2}$ days.

A second group of $8\frac{1}{2}$ day embryos from the experimental cross was also examined by combined electrophoresis of GPI-1 and PGK (Fig. 2b). Trophoblast samples were usually analysed in addition to the embryo in order to confirm the phenotype classification. Trophoblast samples of some putative m/m homozygotes had a trace of GPI-1 activity (much weaker than PGK) which was assumed to be a result of maternal contamination. In the other conceptuses, PGK staining was usually equal to and sometimes slightly stronger than GPI-1. So, although putative m/m homozygotes were not the only conceptuses with GPI-1 staining weaker than PGK they were still readily identified by the relative enzyme activities (Fig. 2b). Apart from 3 dead embryos, all of the embryos in the experimental cross were easily classified as normal (head-fold stage) or small and grossly retarded (egg cylinder stage) as shown in Fig. 6. The results (Fig. 4) were again more clear cut than those from Experiment 1. All of the 34 embryos in the control group had both GPI-1 and PGK activity, although one was classified as small (egg cylinder stage) and three as dead. (The small and dead embryos were all found in one large litter of 19 embryos.) In the experimental group, 57 embryos were recovered and 1 decidual swelling was empty. Of the 11 putative homozygous m/m embryos with only PGK activity,

10 were classified as small, 1 dead and none normal. Of those with both GPI-1 and PGK activity, 44/46 were normal and the remaining 2 were classified as dead. Disregarding the three dead embryos, all of the retarded embryos produced only PGK (putative homozygous m/m embryos) and all of the morphologically normal embryos produced both GPI-1 and PGK. The morphological difference between the normal and small embryos (Fig. 6) was very striking at $8\frac{1}{2}$ days and the putative m/m homozygotes appeared not to have developed significantly between $7\frac{1}{2}$ and $8\frac{1}{2}$ days. This second series of $8\frac{1}{2}$ day embryos confirms the conclusion from experiment 1 that most homozygous null embryos are dead, dying or developmentally retarded by $8\frac{1}{2}$ days, although in this series only 1/11 was classified as dead.

Experiment 3: analysis of $9\frac{1}{2}$ and $10\frac{1}{2}$ day conceptuses

At $9\frac{1}{2}$ and $10\frac{1}{2}$ days most of the implantation sites were readily classified into large (normal) or small. (Four exceptions are noted below.) The large implantation
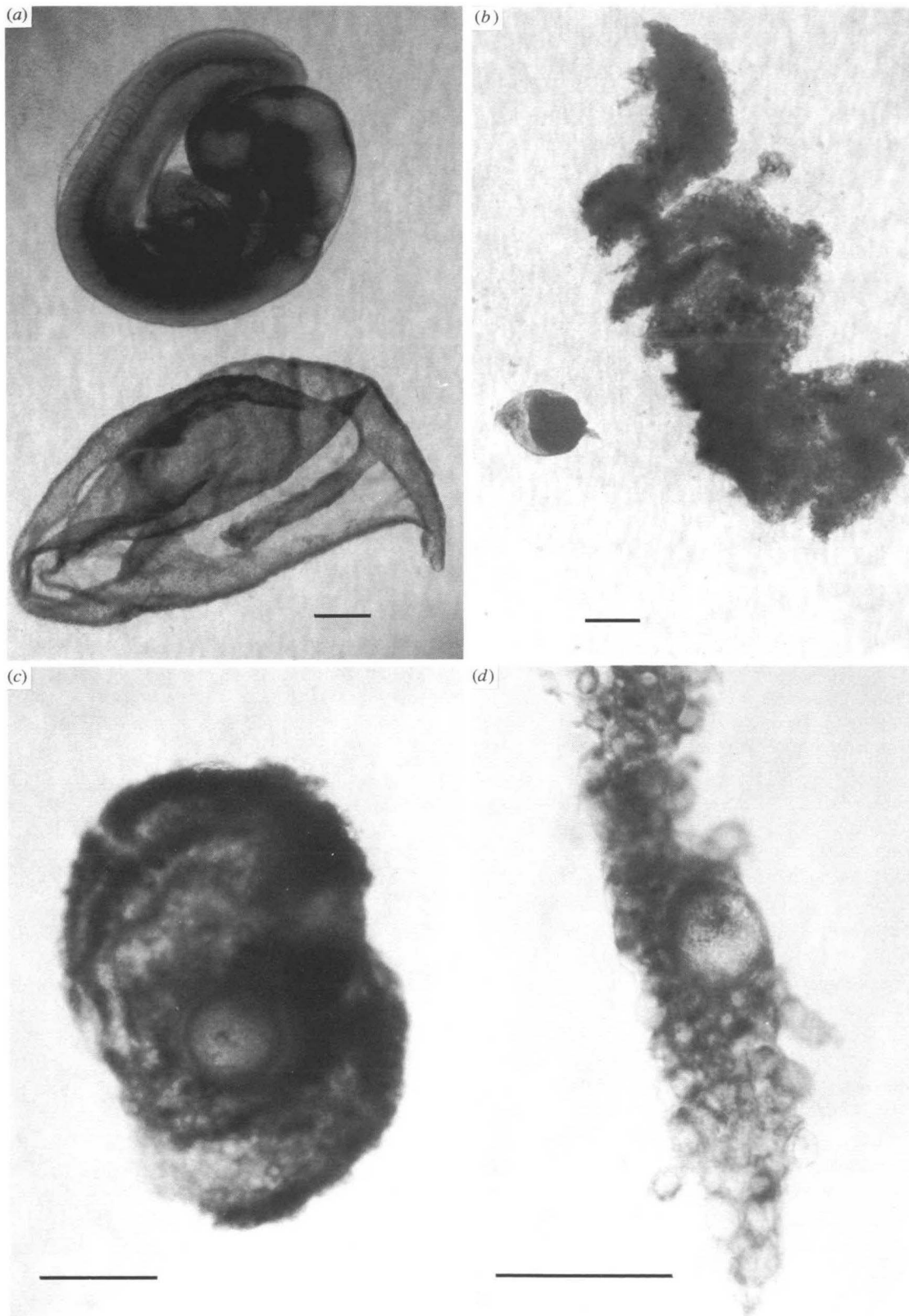
Fig. 7. Normal and small conceptuses at $9\frac{1}{2}$ days from the $a/m \times b/m$ experimental cross. (*a*) Morphologically normal embryo with visceral yolk sac below; GPI-1AB phenotype. (*b–d*) Trophoblast (note trophoblast giant cells) and vesicles of extraembryonic tissue dissected from 'moles'. These three conceptuses were each classified as m/m homozygotes after analysis of GPI-1 and PGK (see text). The conceptus shown in (*b*) appears to have been arrested at the egg cylinder stage although the trophoblast has proliferated enormously (compare with Figs. 5 and 6). Scale bars are 0·5 mm.

sites contained a normal conceptus but the small implantation sites were usually 'moles' comprising maternal decidual tissue, blood, trophoblast giant cells and often a vesicle-like membrane (Fig. 7c, d), which could have been either Reichert's membrane or visceral yolk sac. In order to avoid losing samples required for enzyme analysis, the membrane samples were not dissected so their identity remains uncertain and it is not known whether they contained other tissues. One conceptus (Fig. 7b) comprised a large mass of trophoblast and a much smaller vesicle of extraembryonic tissue. In this instance, the outer membrane looked like Reichert's membrane and appeared to contain other tissue. The overall appearance of this conceptus was similar to an egg cylinder enclosed in Reichert's membrane (see $7\frac{1}{2}$ day embryos in Fig. 5 and small $8\frac{1}{2}$ day embryos in Fig. 6). So, this conceptus may have remained relatively unchanged since $7\frac{1}{2}$ days, apart from a dramatic proliferation of trophoblast.

Two samples were usually taken from the normal implantation sites: the embryo was cleaned of trophoblast and maternal tissue (although the amnion and yolk sac were not always removed) and a separate sample of trophoblast tissue was usually also analysed. The trophoblast sample was sometimes visibly contaminated with maternal tissue. Trophoblast (usually contaminated with blood) was also dissected from the small implantation sites. When other extraembryonic tissue was present, this was either taken as a separate

sample, or if it was too small, it was included with the trophoblast sample.

All samples recovered at $9\frac{1}{2}$ and $10\frac{1}{2}$ days were analysed in two ways. GPI-1 electrophoresis was used to identify the *Gpi-1s* genotype and combined GPI/PGK electrophoresis was used to confirm the identity of homozygous null samples (expected to have PGK but no GPI-1 activity). In practice, all trophoblast samples at these stages had some GPI-1 activity but some had low levels of GPI-1A and the PGK activity appeared to be greater than GPI. Normal sized conceptuses could readily be classified as a/b, b/m or a/m genotypes according to their GPI-1 allozymes. All of the morphologically normal embryos recovered at these stages had high levels of GPI-1 activity so none was a putative m/m homozygote. The homozygous m/m moles that had both trophoblast and membrane samples were identified by the absence of GPI-1 (but presence of PGK) in the membrane sample and a weak GPI-1A band (significantly weaker than PGK) staining in the trophoblast sample. Moles that had only trophoblast tissue and produced only a single GPI-1A band were classified as putative m/m homozygotes (GPI-1 staining weaker than PGK) or a/m heterozygotes (GPI-1 staining equal to or stronger than PGK). This classification was less certain because PGK may have been stronger than GPI-1 staining in some a/m heterozygotes, as it was in some embryos at $8\frac{1}{2}$ days (e.g. tracks 1 and 2 of Fig. 2b). Other moles had trophoblast (and sometimes additional extra-
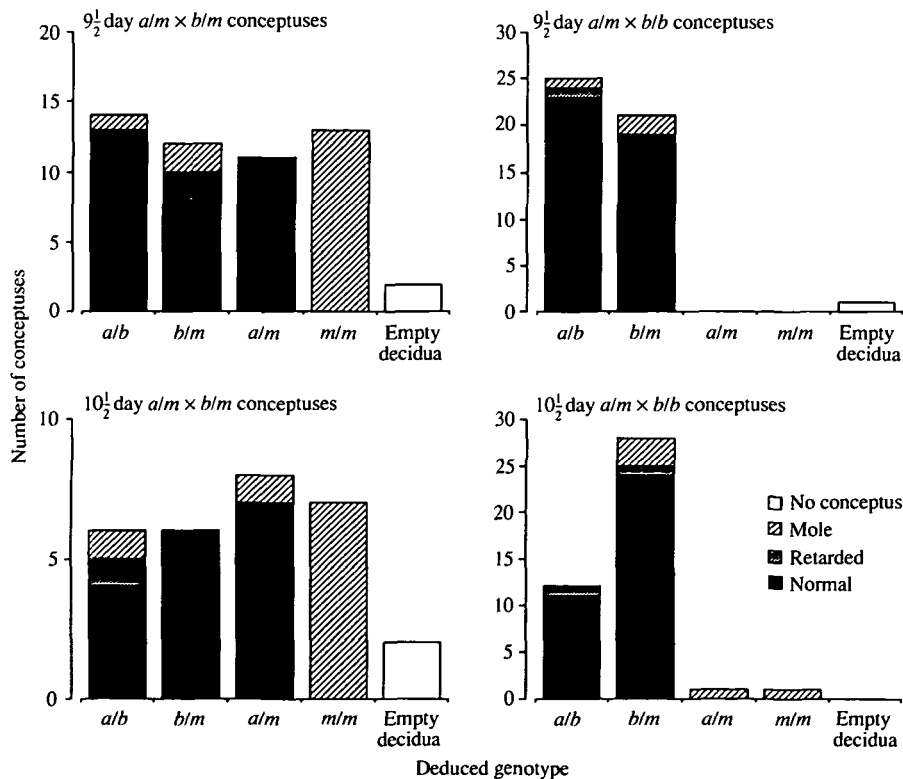
Fig. 8. Distribution of four GPI-1 genotypes among $9\frac{1}{2}$ and $10\frac{1}{2}$ day embryos from the experimental and control crosses in experiment 3 (see text for classification of genotypes). The embryos recorded as 'lost' include empty decidua and technical losses during dissection and analysis.

embryonic membrane tissue) that produced all three GPI-1 bands (A, AB and B; *a/b* genotype) or only a B band or a strong B and a weaker A band (*b/b* genotype, with or without maternal contamination).

The results from control and experimental crosses are shown in Fig. 8. At $9\frac{1}{2}$ days one AB conceptus in the control group was judged to be approximately one day retarded. At $10\frac{1}{2}$ days one AB conceptus in the experimental group was recorded as a recent death and one implantation site in the control group contained two embryos. One was about two days retarded and was phenotypically AB and the other was dead, also about two days retarded but phenotypically GPI-1B. These four conceptuses were classified as retarded embryos (Fig. 8) but all other conceptuses were either normal embryos or moles.

Moles were more frequent in the experimental cross than the control cross at both ages. At $9\frac{1}{2}$ days 16/50 (32.0%) of the experimental conceptuses and 3/46 (6.5%) of the controls were moles. At $10\frac{1}{2}$ days 9/27 (33.0%) of the experimental conceptuses and 5/42 (11.9%) of the controls were moles.

Approximately 25% of the experimental conceptuses (all moles) were classified as homozygous (*m/m*) nulls. At $9\frac{1}{2}$ days the incidence of conceptuses classified as nulls was 13/50 (26.0%) and at $10\frac{1}{2}$ days the frequency was 7/27 (25.9%). At $9\frac{1}{2}$ days 8/13 of the presumed *m/m* moles had some extraembryonic membrane in addition to trophoblast tissue but all three of the $9\frac{1}{2}$ day moles, that were not putative *m/m* homozygotes, had only trophoblast tissue. At $10\frac{1}{2}$ days 3/7 of the presumed *m/m* moles had some extraembryonic membrane in addition to trophoblast tissue but both of the $10\frac{1}{2}$ day moles, that were not *m/m* homozygotes, had only trophoblast tissue. It therefore seems that trophoblast cells from homozygous *m/m* conceptuses were still present at $10\frac{1}{2}$ days but that other extraembryonic tissues were seen in only a proportion of these conceptuses.

Two of the control conceptuses (both moles with blood, a little trophoblast but no extraembryonic membranes) recovered at $10\frac{1}{2}$ days produced only GPI-1A glucose phosphate isomerase activity; one had approximately equal levels of PGK and GPI and the other had stronger staining PGK. These, therefore, were classified as *a/m* and *m/m* respectively, according to the criteria discussed above, although it is clear that the GPI-1A activity must have been maternal in origin because this cross could not have produced either of these genotypes. Similar maternal contamination was noted above for four $8\frac{1}{2}$ day conceptuses in experiment 1 (Fig. 3). The three other moles in this cross were classified as *b/m*. Each had both trophoblast and extraembryonic membrane tissue present; each membrane sample produced a single B band but the trophoblast samples produced respectively only B, B plus A and only A.

Although two control moles were obviously misclassified, the much higher frequency of moles in the experimental crosses supports the conclusion that, at $9\frac{1}{2}$ and $10\frac{1}{2}$ days, most of the *m/m* conceptuses, produced by the experimental cross, were moles. Also, some of these putative experimental *m/m* moles had extraembryonic membrane tissue that was not contaminated with maternal tissue and produced only PGK activity (8/13 of the $9\frac{1}{2}$ day putative *m/m* moles and 3/7 at $10\frac{1}{2}$ days). It is unlikely that these moles would have been misclassified, so it seems safe to conclude that trophoblast and extraembryonic tissues survive at least until $10\frac{1}{2}$ days in some homozygous *m/m* null conceptuses.

Discussion

Studies of embryos produced by crossing null heterozygotes showed that most mouse embryos that were homozygous for the $Gpi\text{-}1s^{a\text{-}m/H}$ null allele survived to the egg cylinder stage at $7\frac{1}{2}$ days *post coitum* although by this time they tended to be smaller than their littermates. By $8\frac{1}{2}$ days they had progressed little further and were grossly retarded compared to their littermates which had reached the headfold stage. Some $8\frac{1}{2}$ day homozygous embryos were dying or already dead. Trophoblast tissue continued to proliferate beyond this stage and although other extraembryonic cells also survived only the trophoblast appeared to grow. By $9\frac{1}{2}$ and $10\frac{1}{2}$ days only trophoblast and small pieces of extraembryonic tissues were found but since no dissection or histology was performed on these small samples it remains uncertain which tissues survived. No systematic analysis was carried out beyond $10\frac{1}{2}$ days but it seems likely that biochemical identification of *m/m* homozygotes, at later stages, would be made increasingly difficult by the contaminating maternal cells.

It is already established that the early mouse embryo inherits high levels of oocyte-coded GPI-1 activity and that this is exhausted by $5\frac{1}{2}$ or $6\frac{1}{2}$ days (West & Green, 1983; Duboule & Burki, 1985; Gilbert & Solter, 1985; West, Leask & Green, 1986; West & Flockhart, 1989). In *b/a* heterozygous embryos from *b/b* homozygous mothers, about 20–35% of GPI-1 activity was oocyte-coded at $4\frac{1}{2}$ days and by $5\frac{1}{2}$ days this proportion had declined to 0–4% (West, Leask & Green, 1986; West & Flockhart, 1989). The activity of oocyte-coded enzyme remaining in embryos from females that are heterozygous for the null allele is likely to be similar or lower since the relative GPI-1 activity in oocytes from heterozygous *a/m* mothers is only about 50% of that in oocytes from homozygous *a/a* mothers. (The GPI-1 activities were 0.41 ± 0.01 and 0.85 ± 0.04 arbitrary units respectively; West & Flockhart, 1989).

It was, therefore, expected that, if GPI-1 deficiency was a cell-lethal condition, the embryos would die when the supply of oocyte-coded GPI-1 fell below a critical threshold or certainly soon after it was exhausted at around $5\frac{1}{2}$ days. Until this activity was depleted, the embryo would be rescued by oocyte-

GLYCOLYSIS   Glucose   PENTOSE PHOSPHATE PATHWAY

                                    G6PD
                 Glucose-6-P ─────────────► 6-Phospho-
GPI   gluconate ──────────┐
                 Fructose-6-P   │
                                                          Ribulose
                                                          5-phosphate

                 Glyceraldehyde-3-P

                 1,3-Diphosphoglycerate

PGK   ├──────► ATP

                 3-Phosphoglycerate

                                 ├──────► ATP

                 Pyruvate ───────────► Lactic acid

                 Acetyl CoA

Glutamine

Glutamate ──► α-ketoglutarate   TCA
                                       CYCLE
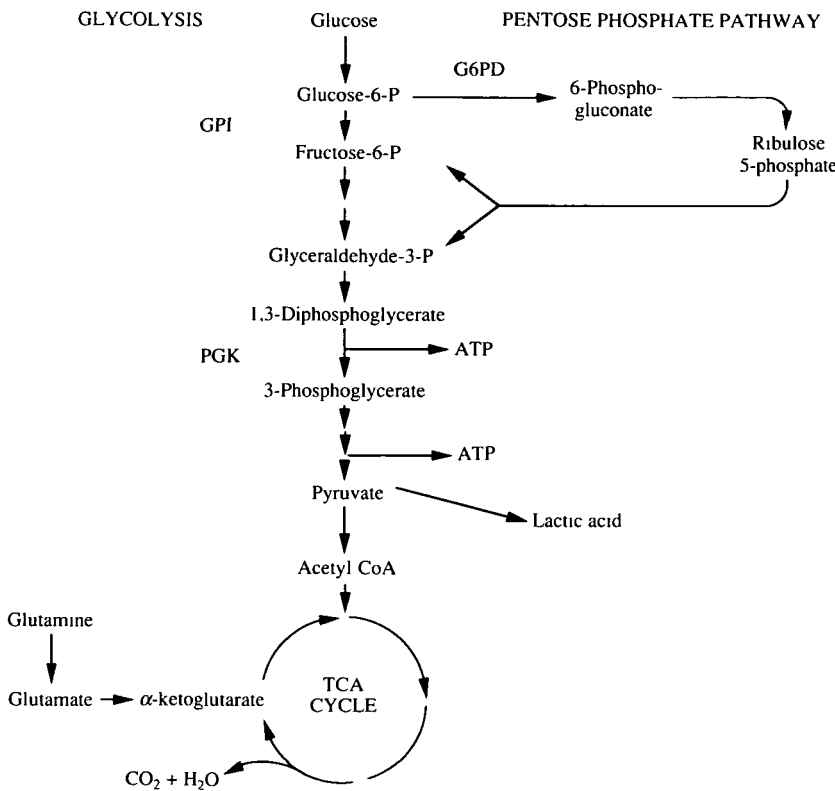
     $CO_2 + H_2O$

Fig. 9. Simplified metabolic pathways showing relationships between glycolysis, the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle. The pentose phosphate pathway by-passes GPI by converting glucose-6-phosphate to ribulose-5-phosphate and then produces fructose-6-phosphate and glyceraldehyde-3-phosphate. Glutamine can also act as an energy source; it is converted first to glutamate and then to alpha-ketoglurate which enters the TCA cycle (see Reitzer *et al.* 1979).

coded enzyme and its phenotype would not accurately reflect its genotype. (This survival of a lethal genotype, would be equivalent to the phenomenon of 'per-durance', which was defined by Garcia-Bellido & Merriam (1971) as 'the persistence of a phenotype after the removal of the conditioning genotype' and discussed with respect to somatic recombination.) However, our results show that embryos, that are genetically incapable of producing GPI-1, survive for two or three days after oocyte-coded GPI-1 activity is exhausted. It seems that, although *Gpi-1s* genes are expressed by $2\frac{1}{2}$ to $3\frac{1}{2}$ days (see Introduction), this gene expression is not needed for embryo survival until several days later. Cells from some extraembryonic tissues of these homozygous null (*m/m*) conceptuses survive for a further two days and trophoblast tissue appears to continue to proliferate, at least to $9\frac{1}{2}$ days. This seems longer than would be predicted for a cell-lethal condition.

A homozygous null embryo might survive for a few days in the absence of maternally-inherited GPI-1 for several reasons. First, the *Gpi-1s^{a-m1H}* mutation could produce a low level of enzyme that is sufficient for cell survival and growth for a few days. Peters & Ball (1990) found residual GPI-1 activity from two other putative *Gpi-1s* null mutations, in conceptuses heterozygous for either mutation *Gpi-1s^{b-m3H}* or mutation

Gpi-1s^{b-m4H}, but no activity in those heterozygous for the *Gpi-1s^{a-m1H}* mutation used in the present study. The residual activity in *Gpi-1s^a/Gpi-1s^{b-m3H}* heterozygotes is illustrated in Fig. 1. The present study revealed no evidence that the *Gpi-1s^{a-m1H}* allele produced any GPI-1 enzyme. Some overstained electrophoresis plates with $10\frac{1}{2}$ day *b/m* conceptuses produced a strong GPI-1B band and a weak anodal band but this was also seen in some *b/b* conceptuses, from *b/b* × *b/b* crosses (data not shown) and so could not represent the product of the *Gpi-1s^{a-m1H}* null allele. Similar sub-bands were found occasionally by West & Flockhart (1989) and may be the 'pseudo-isozymes' reported by Blackburn *et al.* (1972). The observation that oocytes from heterozygous *a/m* mothers had only about 50% as much GPI-1 activity as oocytes from homozygous *a/a* mothers (above) also supports the conclusion that the mutant allele produces no enzymatically active gene product.

A second reason for prolonged survival of homozygous null embryos could be the use of an alternative metabolic pathway. The pentose phosphate pathway by-passes GPI (Fig. 9) and may be sufficient for cell survival if glycolysis is blocked by the absence of glucose phosphate isomerase but this may only permit slow cell growth that would not be adequate to support normal embryogenesis. Experiments with null

mutations of GPI in other species are instructive. Although mutant yeast (*Saccharomyces cerevisiae*) that lack GPI do not grow on glucose (Maitra, 1971; Herrera & Pascual, 1978), similar bacterial (*Escherichia coli*) mutants survive and grow at 33% of the normal rate by utilising the pentose phosphate pathway (Fraenkel & Levisohn, 1967; Fraenkel & Vinopal, 1973). It may be possible to test whether the involvement of the pentose phosphate pathway is beneficial to homozygous null mouse embryos by testing whether homozygous embryos, trophoblast and other extraembryonic cells, from homozygous null conceptuses, die earlier if they have additional genetic defects of the pentose phosphate pathway enzymes, such as the low activity mutant of glucose-6-phosphate dehydrogenase (Pretsch, Charles & Merkle, 1989).

Mutant Chinese hamster ovary (CHO) cells, that lack both GPI and PGK grow *in vitro* on glucose at almost the normal rate (Morgan & Faik, 1980, 1981, 1986) but glutamine and pyruvate are provided in the culture medium and provide alternative energy sources (via respiration) without the need for glycolysis (Zielke *et al.* 1976, 1978; Reitzer *et al.* 1979; Morgan & Faik, 1981; Morgan, Bowness & Faik, 1981, 1983; McKeehan, 1986). In contrast to wild type CHO cells, which metabolise at least 98% of the glucose that they consume by glycolysis, the mutant cells used only about 10% as much glucose and at least 80% of this was metabolised by the pentose phosphate pathway. However the majority of the energy production was provided by oxidation of exogenous glutamine and pyruvate, that was supplied in the culture medium (Morgan *et al.* 1981, 1983).

Pouyssegur *et al.* (1980) isolated a mutant Chinese hamster lung fibroblast cell line which was null for GPI but not for PGK. This grew on glucose at about 66% of the wild type rate and again used glucose at a low rate and only via the oxidative pentose phosphate pathway. Of particular significance is the observation that these mutant cells were able to form tumours in homozygous nude (*nu/nu*) mice. Tumour formation took longer with the GPI mutant cells (4–8 weeks instead of the normal 2 weeks) and the tumours grew more slowly than normal but this report clearly demonstrates that, even *in vivo*, the absence of GPI is not a cell lethal condition. Tumour formation was also less efficient than with wild type cells. The authors suggested that the mutant cells' dependence on respiration for energy production made them more dependent on a good oxygen supply and some tumours might have died of hypoxia before the blood supply was established. Establishment of a GPI-deficient tumour in a nude mouse is an interesting parallel with a homozygous null conceptus growing in a heterozygous mother.

A homozygous *Gpi-1s* null conceptus *in vivo* may also have access to alternative energy sources if nutrients were transported from the maternal circulation. Sherman & Chew (1972) showed that maternal esterase could be detected in the trophoblast and visceral yolk sac in mid-gestation ($9\frac{1}{2}$ and $10\frac{1}{2}$ days) mouse conceptuses but not in the amnion or fetus. Glutamine is known to be present in blood at high concentrations (approximately 0·4–0·5 mM) and could be transferred in a similar way, perhaps with other nutrients. (There is also evidence for utilization of plasma glutamine by the rat fetus and metabolism of glutamate by human trophoblast cells; reviewed by McKeehan, 1986). This transfer might be limited to the trophoblast and outer extraembryonic membranes (as in the case of esterase) or might provide insufficient energy for normal embryogenesis in larger embryos. It seems unlikely that GPI-1 itself is transferred from the maternal circulation because evidence of maternal GPI-1A was only found in trophoblast and dead embryos that were visibly contaminated with blood.

It is also possible that GPI (or at least the *Gpi-1s* gene) may have a role in embryogenesis in addition to carbohydrate metabolism. It has been shown that the DNA sequence of the mouse *Gpi-1s* gene is identical to the published sequence of the mouse gene encoding neuroleukin (Faik *et al.* 1988). There is also 90% sequence homology between the mouse neuroleukin sequence and that for pig GPI (Chaput *et al.* 1988). Neuroleukin is a neurotrophic factor that supports the survival of embryonic spinal neurones, skeletal motor neurones and sensory neurones and also induces secretion of immunoglobulin (Gurney, 1984, Gurney *et al.* 1986*a, b*). According to Gurney (1988), it is unlikely that the biological activity of neuroleukin is attributable to GPI enzymatic activity because monoclonal antibodies that block the neuroleukin bioassay do not affect GPI activity. This suggests that if the neuroleukin and enzymatic activities reside on the same molecule they involve different epitopes. Further studies may clarify whether the dimeric GPI enzyme molecule has the neurotrophic and immunomodulating properties ascribed to neuroleukin, whether neuroleukin is identical to GPI dimers, GPI monomers or a peptide fragment processed from the *Gpi-1s* gene. The published molecular weight of neuroleukin is 56000 Daltons (Gurney, 1984) which is approximately half that of the GPI dimer. The molecular weight of rabbit GPI has been estimated as 108000 (Scopes & Penny, 1971) and 132000 (Pon *et al.* 1970), and that of pig GPI as 120000 (Muirhead & Shaw, 1974).

It is not yet clear whether the absence of neuroleukin activity in homozygous *Gpi-1s* null conceptuses is relevant to our present observation, that certain extraembryonic cells from such conceptuses survive for longer than the embryos, but this appears unlikely. It seems most likely that cells of homozygous null embryos survive but grow more slowly because of disturbed carbohydrate metabolism and less efficient energy production. The mutant embryos would rely on a combination of the pentose phosphate pathway, to by-pass the block in glycolysis, and oxidation of

alternative energy sources. From the experiments with CHO cells in culture, oxidation of alternative energy sources (such as glutamine) would probably provide significantly more energy than the pentose phosphate pathway. Transfer of glutamine (or other nutrients) from the maternal circulation is perhaps the most probable explanation for the survival of homozygous *m/m* cells. Death of the homozygous embryos may occur around $8\frac{1}{2}$ days either because the complex interactions of growth and development involved in normal embryogenesis require maximum energy production or because transfer of maternal nutrients (energy sources or oxygen) is more difficult in larger embryos and is insufficient to maintain cell viability in the absence of a normal glycolytic pathway. This would be analogous to the explanation of the lower incidence of tumour production from GPI-deficient fibroblasts (Pouyssegur *et al.* 1980; discussed above). The longer survival of trophoblast and extraembryonic membrane cells could then be explained either by a less stringent requirement for optimal energy production (slower growth rate being compatible with tissue survival) or, more likely, more ready access of the outer extraembryonic tissues to maternal nutrients, as previously noted for maternal esterase activity (see above).

The analysis of genetically determined embryonic death caused by deficiencies in genes encoding known protein products (in this case glucose phosphate isomerase) may provide insights into the cause of death when the gene product is unknown but the timing and morphological characteristics of the lethal effect show a consistent pattern. The proliferation of trophoblast and the failure of embryonic development in homozygous *Gpi-1s* null conceptuses is similar to that seen in androgenetic diploid mouse conceptuses (Barton, Surani & Norris, 1984; McGrath & Solter, 1984) and some androgenetic human hydatidiform oles (Kajii & Ohama, 1977; Wake, Takagi & Sasaki, 1978; Jacobs, Wilson, Sprenkle, Rosenshein & Migeon, 1980). It seems likely that androgenetic conceptuses have abnormal gene expression as a result of unbalanced genomic imprinting (exclusively paternal imprinting pattern). It is possible that the deficient gene expression could result in a simple metabolic deficiency in all cells of the conceptus but that the effect is ameliorated in the trophoblast tissue by access to maternal nutrients.

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