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Pigmentation and lysosome function in mice homozygous for both pale ear and Beige-J pigment genes

By EDWARD K. NOVAK, RICHARD T. SWANK
Department of Molecular Biology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, N.Y. 14263

AND MIRIAM H. MEISLER
Department of Human Genetics, The University of Michigan, 1137 East Catherine Street, Ann Arbor, Michigan 48109

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SUMMARY

We have examined mice doubly homozygous for both pale ear (*ep*/*ep*) and beige (bg^J/bg^J) mutations in order to detect genetic interactions between these 2 loci affecting pigmentation and lysosome physiology. The doubly homozygous mouse has a new pigmentation phenotype consistent with independent effects of *ep* and *bg*. The beige (Brandt, Elliott & Swank, 1975) and pale ear (Novak & Swank, 1979) genes have abnormal kidney lysosomal enzyme accumulation caused by defective secretion into urine. No cumulative effect on these functions was observed in the new double mutant phenotype. The new phenotype has giant lysosomes typical of the beige mutation. Unexpectedly, the beige gene corrects the effect of the pale ear on serum lysosomal enzyme concentration. There is also a gene dosage effect of the beige gene on this serum lysosomal enzyme phenotype. The results suggest that the beige and pale ear genes affect the same pathway(s) of lysosome biosynthesis and/or processing. The action of the beige gene may precede that of the pale ear gene in lysosome physiology.

1. INTRODUCTION

Melanosomes and lysosomes share several structural features and may be synthesized by similar mechanisms. It is therefore, of interest that several mutations affecting pigmentation in the mouse contain increased concentrations of kidney lysosomal enzymes and decreased secretion of lysosomal enzymes into urine (Brandt *et al.* 1975; Meisler, 1978; Swank *et al.* 1978; Novak & Swank, 1979). Of 33 pigmentation mutants studied to date, 8 are characterized by lysosomal abnormalities. Since these mutations are encoded by separate genes and have distinctive effects on pigmentation and on lysosome function, they probably control different gene products. The effects on lysosome function of the pigmentation genes of this report, beige-J and pale ear, have been partially characterized (Brandt *et al.* 1975; Swank & Brandt, 1978; Meisler, 1978; Brandt *et al.* 1978; Novak & Swank, 1979).

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Beige-J, a spontaneous mutation in strain C57BL/6J, is an animal model for the human Chediak–Higashi Syndrome. Affected humans and animals contain diluted pigmentation, giant lysosomes in many tissues and increased susceptibility to infection (Brandt *et al.* 1978). Lysosomal enzymes accumulate in the kidney of the beige mouse because of decreased secretion of lysosomal enzymes into urine (Brandt *et al.* 1975). Depressed secretion has also been reported in two other cell types, the polymorphonuclear leukocyte (Boxer *et al.* 1976) and the platelet (Boxer *et al.* 1977) in Chediak–Higashi Syndrome. Furthermore, subcellular organelles, including lysosomes (Oliver & Essner, 1973), melanosomes (Windhorst *et al.* 1968), platelet dense granules (Holland, 1976), and mast cell granules (Chi & Langunoff, 1975), have altered morphology or function in beige mice.

The *pale ear* mutation originally appeared in strain C3HeB/FeJ (Lane & Green, 1967) and was subsequently transferred to C57BL/6J by repeated backcross matings. *Pale ear* has no pigment in the ear and tail. This mutant also exhibits decreased secretion of lysosomal enzymes into urine (Novak and Swank, 1979). The giant lysosomes characteristic of the beige mutation are not associated with the pale ear mutation (Piccini, unpublished).

In order to test whether the beige-J and pale ear mutations interact in both pigmentation and lysosomal enzyme function, we have studied animals homozygous for the beige and pale ear genes.

2. MATERIALS AND METHODS

(i) *Animals*

C57BL/6J $\pm/\pm$, C57BL/6J bg^J/bg^J, and C57BL/6J *ep*/*ep* were obtained from the Jackson Laboratories, Bar Harbor, Maine. The mice were then bred at the animal facilities of Roswell Park Memorial Institute. The crosses used to construct the new phenotype and all backcrosses (Table 1) were also conducted at this facility.

(iii) *Testosterone induction and tissue preparation*

Testosterone pellets (35 mg) prepared by B. B. Sheth, Department of Pharmaceutics, University of Tennessee, were implanted into 8- to 16-week-old female mice for 20 days (Brandt *et al.* 1975). Excised tissues and sera were prepared and stored at -20 °C (Novak & Swank, 1979).

(iii) *Enzymes assays*

β-Glucuronidase (E.C. 3.2.1.31) was assayed fluorometrically using the fluorescent substrate 4-methylumbelliferyl β-D-glucuronide (Brandt *et al.* 1975). β-Galactosidase (E.C. 3.2.1.23) was assayed as previously described (Tomino & Meisler, 1975) with the substrate p-nitrophenyl β-D-galactoside. Serum β-galactosidase was assayed with 4-methylumbelliferyl β-D-galactoside (Brandt *et al.* 1975). α-Mannosidase was assayed using 4-methylumbelliferyl-α-D-mannopyranoside as the substrate (Novak & Swank, 1979).

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(iv) *Urine collections*

Each metabolism cage housed three mice and urine was collected at 24 h intervals in tubes containing mineral oil to prevent evaporation.

(v) *Histology*

Glucuronidase was stained in kidney sections 8 μm thick using napthol AS-Bl glucuronide as substrate (Hayashi, 1964).

3. RESULTS

(i) *Construction of double homozygotes*

When mice homozygous for *ep* and *bg* were crossed, the F_1 offspring had the black phenotype of the C57BL/6J normal mouse. This is consistent with the recessive expression of mutant pigmentation in *ep*/± mice and *bg*/± mice and with the known fact that the mutations are at separate loci. In the F_2 generation,

Table 1. *Results of mating pale ear* (*C57BL/6J* ep/ep), *beige-J* (*C57BL/6J* bg^J/bg^J) *and normal* (*C57BL/6J* ±/±) *mice*

Cross	Progeny observed	Progeny expected	Coat colour	Presumed genotype
ep/ep, ±/± × *bg/bg*, ±/±	40	—	Black	*ep*/±, *bg*/±
ep/±, *bg*/± × *ep*/±, *bg*/± (F_2)	177	176	Black	*ep*/±, *bg*/± ; ±/±, *bg*/± ; *ep*/±, ±/±
	49	58	Pale ear	*ep/ep*, *bg*/± ; *ep/ep*, ±/±
	65	58	Beige	*ep*/±, *bg/bg*; ±/±, *bg/bg*
	21	20	Pale beige	*ep/ep*, *bg/bg*
ep/ep, *bg/bg* × *ep/ep*, *bg/bg*	> 100	—	Pale beige	*ep/ep*, *bg/bg*
ep/ep, *bg/bg* × ±/±, *bg/bg*	29	—	Beige	*ep*/±, *bg/bg*
ep/ep, *bg/bg* × *ep/ep*, ±/±	29	—	Pale ear	*ep/ep*, *bg*/±
ep/ep, *bg/bg* × ±/±, ±/±	7	—	Black	*ep*/±, *bg*/±

Figures for progeny expected in the F_2 generation were calculated for Mendelian segregation of unlinked genes among 312 progeny.

312 progeny segregated with the expected Mendelian proportions of black pale ear and beige individuals, as well as a new phenotype (Table 1). The new phenotype was diluted in coat colour in comparison to either mutant parent with a steel-grey coat colour that darkened on the crest of the nose. The ears and tail lacked pigmentation, as in the pale ear mutation. The eyes were also altered in colour and appeared light red while both beige and pale ear mice had normal black eyes. The mice with the new pigmentation phenotype had normal litter sizes and life-spans. For convenience, we will refer to this new phenotype as pale beige (Plate 1, fig. 1).

The results of several breeding studies suggest that the genotype of pale beige is indeed *ep/ep, bg/bg* (Table 1). Self-matings of the pale beige phenotype have produced more than 100 offspring with this phenotype. When pale beige mice were

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backcrossed to beige-J, only offspring with beige phenotype were observed. Alternatively, backcrossing of pale beige to pale ear yielded only offspring with pale phenotype. Finally, when pale beige mice were crossed to C57BL/6J normal, only mice of normal black phenotype were observed. Males and females were equally affected by the pigment genes.

In beige mice (Brandt *et al.* 1975) and pale ear mice (Meisler, 1978; Novak & Swank, 1979), the concentration of lysosomal enzymes in kidney is elevated, and lysosomal enzyme secretion into urine is reduced. The quantitative effects of the two mutations are similar. The beige mouse, unlike pale ear, has giant lysosomes in many tissues, including kidney. The pale ear mouse, unlike beige, has a two- to threefold increased lysosomal enzyme concentration in mouse serum (Novak & Swank, 1979). In order to determine whether these mutations interact in lysosome function we tested the expression of these phenotypes in pale beige mice.

Table 2. *Kidney and liver lysosomal enzymes*

	Chromosome location	β-glucuronidase (U/g)	β-galactosidase (U/g)	α-mannosidase (U/g)
Kidney				
C57BL/6J Normal	—	120 ± 4·0	90·7 ± 4·2	23·8 ± 1·2
Pale ear (*ep/ep*)	19	295 ± 13****	230 ± 4·5****	35·1 ± 1·7***
Beige-J (bg^J/bg^J)	13	304 ± 26****	191 ± 23***	44·4 ± 2·4***
Pale beige (ep/ep, bg^J/bg^J)	13, 19	362 ± 22****	249 ± 21****	50·6 ± 1·1****
Pale ear × beige-J ($ep/\pm$, $bg^J/\pm$)		119 ± 10	112 ± 11	26·0 ± 0·4
Liver				
C57BL/6J Normal	—	34·2 ± 2·7	57·2 ± 0·9	15·7 ± 0·5
ep/ep, *bg/bg*	13, 19	29·8 ± 0·9	48·5 ± 1·0**	16·4 ± 0·8

Female mice were treated 20 days with testosterone. Values represent the mean ± S.E.M. of 3–6 mice.

** $P < 0·02$, *** $P \leqslant 0·01$, **** $P \leqslant 0·001$ when compared to C57BL/6J normal.

Kidney lysosomal enzyme concentrations are shown in Table 2. The F_1 mice are not different from C57BL/6J normal. This was expected, since $bg/\pm$ (Brandt *et al.* 1975) and $ep/\pm$ (Novak, unpublished) heterozygotes do not differ from wild type. The mice with new phenotype, pale beige, had two- to threefold increased concentrations of kidney β-glucuronidase, β-galactosidase and α-mannosidase when compared with C57BL/6J. This effect is quantitatively equivalent to the effect of either mutation alone.

As shown in Table 2, liver lysosomal enzyme concentrations are essentially normal in the pale beige mouse, as previously found in the beige and pale ear mutants (Brandt *et al.* 1975; Novak & Swank, 1979). Likewise, the kidney concentrations of two non-lysosomal enzymes, alcohol dehydrogenase (cytosolic) and D-amino acid oxidase (peroxisomal), did not differ from normal mice (not shown). This indicates the increased enzyme concentration is specific for lysosomal enzymes,

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as found in pale ear and beige, respectively (Novak & Swank, 1979; Brandt *et al.* 1975).

The elevated concentration of kidney lysosomal enzymes in beige and pale ear is the result of decreased enzyme secretion into urine (Brandt *et al.* 1975; Novak & Swank, 1979). To determine whether the beige and pale ear genes had a cumulative effect on this process, lysosomal enzyme secretion was measured in the pale beige mutant. As shown in Table 3, the pale beige mouse had abnormally low secretion of two lysosomal enzymes, β-glucuronidase and β-galactosidase. However, when the pale beige mutant was compared to pale ear or beige, no significant difference in secretion was observed for either enzyme. This is consistent with kidney lysosomal enzyme data, since urinary enzymes are derived from kidney by secretion.

Table 3. *Lysosomal enzyme secretion into urine*

	β-glucuronidase (U/mouse/day)	β-galactosidase (U/mouse/day)
C57BL/6J normal	17·8 ± 1·5	6·62 ± 0·8
Pale ear	1·02 ± 0·04****	1·28 ± 0·06****
Beige-J	2·39 ± 0·12****	1·53 ± 0·18***
Pale beige	1·59 ± 0·07****	1·16 ± 0·08****

Values represent the mean total enzyme activity collected ± S.E.M. Collections were made for days 17–20 after testosterone treatment. Three female mice were housed per metabolism cage.

*** $P \leqslant 0·01$, **** $P \leqslant 0·001$.

Recently, β-glucuronidase in mouse liver, kidney and spleen was reported to have a half-life of 2–3 days (Smith & Ganschow, 1978). However, β-glucuronidase in rat liver has been reported to have a very long intracellular half-life of 15–19 days (Wang & Touster, 1975). Since there is little secretion of kidney β-glucuronidase in beige and pale ear mutants, most of the enzyme may be expected to turn over via intracellular degradation. If this intracellular degradation is characterized by the long half-life described by Wang and Touster for rat liver, a long exposure to testosterone might be required for detection of a difference between the pale beige mice and the mice homozygous for a single mutation. Therefore, mice were treated for as long as 50 days with testosterone, as shown on Table 4. Even after 50 days, the accumulation of kidney lysosomal enzymes in the double mutant did not differ from that in *ep/ep* or *bg/bg* mice.

The effects of long-term testosterone exposure were also examined in untreated 6-month-old male mice (Table 5). β-Galactosidase and α-mannosidase concentrations were significantly increased in all mutants, when compared with wild type. β-Glucuronidase concentrations were not significantly different from normal. The α-mannosidase concentration in pale beige was not higher than for the two single homozygotes, but β-galactosidase was 15–30% higher than in either homozygote. This result differs from that in testosterone-treated female mice, even after 50 days treatment with testosterone. However, in another experiment using

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Table 4. *Long-term testosterone effects*

	30 days	40 days	50 days
	β-glucuronidase (U/g kidney)		
C57BL/6J Normal	117 ± 5·7	128 ± 2·6	109 ± 9·3
Pale ear	234 ± 11	250 ± 7·8	231 ± 11
Beige	—	251 ± 0·6	—
Pale beige	268 ± 3·5	267 ± 26	262 ± 1·0
	β-galactosidase (U/g kidney)		
C57BL/6J Normal	83·3 ± 4·8	82·5 ± 5·5	95 ± 15
Pale ear	204 ± 14	252 ± 3·8	244 ± 14
Beige	185 ± 18	182 ± 7·0	174 ± 7·5
Pale ear, beige	260 ± 20	265 ± 11	248 ± 40

Female mice were treated with testosterone. Values represent the mean ± S.E.M. for 3–4 mice.

only normal and pale beige mice, kidney β-galactosidase concentrations were only 3–4 times greater than normal, similar values to those typically found for beige or pale ear mice alone. Normal concentrations of liver lysosomal enzymes were observed in pale beige males (Table 5), as in females (Table 2). The pale beige mutant males were also low in lysosomal enzyme secretion into urine (Table 6), but did not differ significantly from *bg/bg* or *ep/ep* mice.

The pale beige mutant was then tested for those properties unique to beige (giant lysosomes) and pale ear (elevated serum lysosomal enzyme concentrations). Kidney sections of mice treated 20 days with testosterone and stained with naphthol AS-Bl-glucuronide, a specific substrate for glucuronidase, are shown in Figs 2–4 (Plates 1, 2). The giant lysosomes typical of the beige phenotype were found in the pale beige mutant in the cortico-medullary region of the kidney. Thus, in lysosome morphology, the pale beige mutant has giant lysosomes typical of beige mice rather than the normal-appearing granules found in pale ear mice.

Table 5. *Lysosomal enzymes in males*

	β-glucuronidase (U/g)	β-galactosidase (U/g)	α-mannosidase (U/g)
	Kidney lysosomal enzymes		
C57BL/6J Normal	16·2 ± 0·9	98·2 ± 3·5	15·6 ± 0·3
Pale ear	22·3 ± 1·8	489 ± 8·4****	25·9 ± 2·6**
Beige	20·5 ± 1·7	400 ± 9·8****	21·9 ± 2·0*
Pale beige	21·3 ± 1·3	573 ± 7·4****	23·1 ± 1·3***
	Liver lysosomal enzymes		
C57BL/6J Normal	30·1 ± 3·4	79·2 ± 6·8	13·9 ± 0·6
Pale ear	39·7 ± 1·2	92·7 ± 8·5	12·6 ± 1·5
Beige	31·5 ± 2·0	65·2 ± 5·0	8·9 ± 1·0*
Pale beige	27·2 ± 1·0	67·5 ± 3·4	7·8 ± 0·4****

Values represent the mean ± S.E.M. of four mice 6 months old. * $P \leqslant 0·05$, ** $P \leqslant 0·01$, *** $P \leqslant 0·02$, **** $P \leqslant 0·001$.

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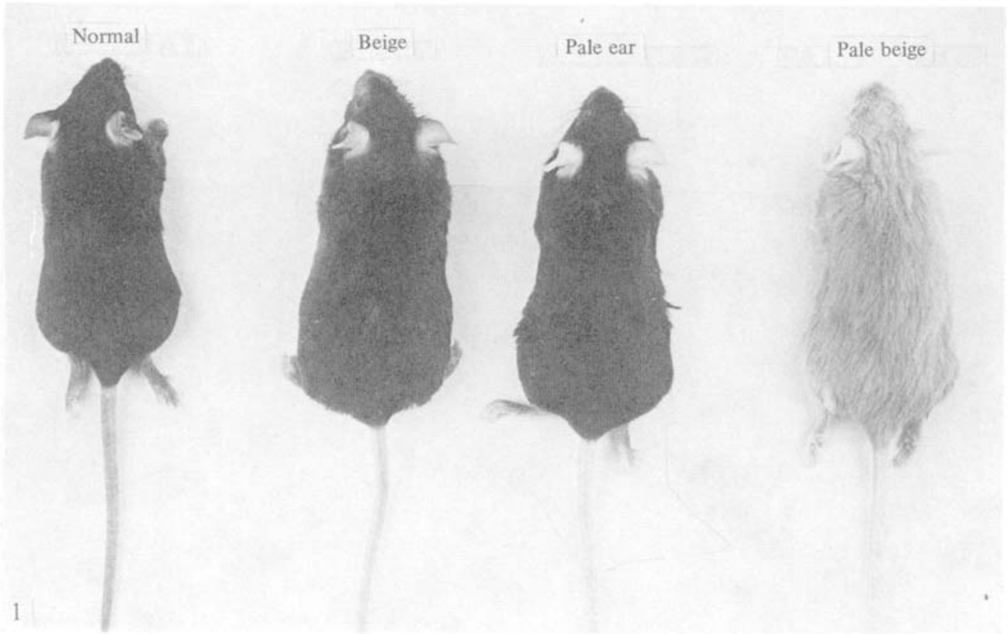

Fig. 1. Degree of pigmentation in normal, beige, pale ear, and double homozygous pale beige mice.

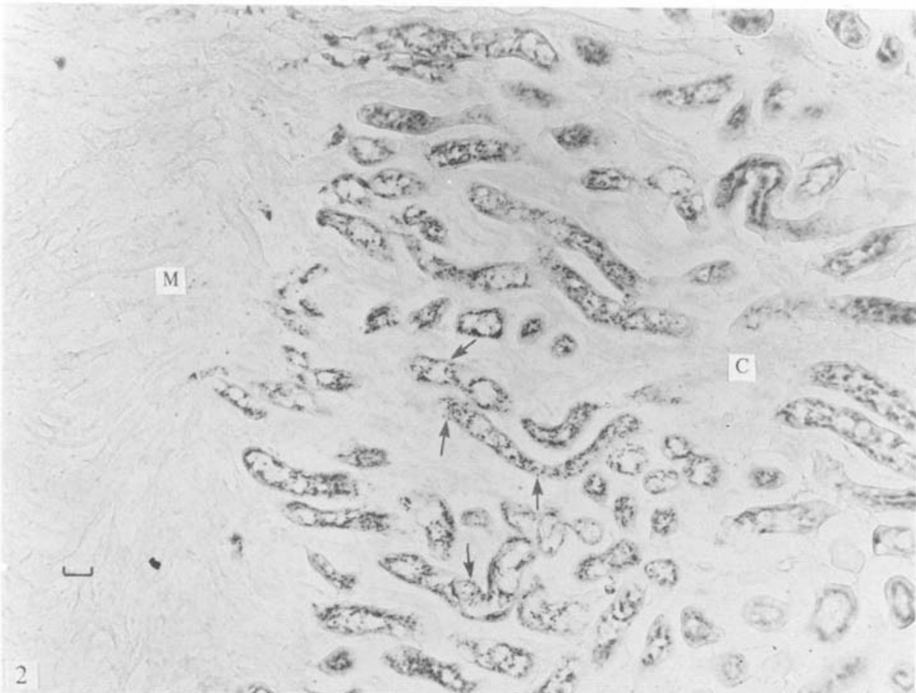

Fig. 2. See plate 2 for legend.

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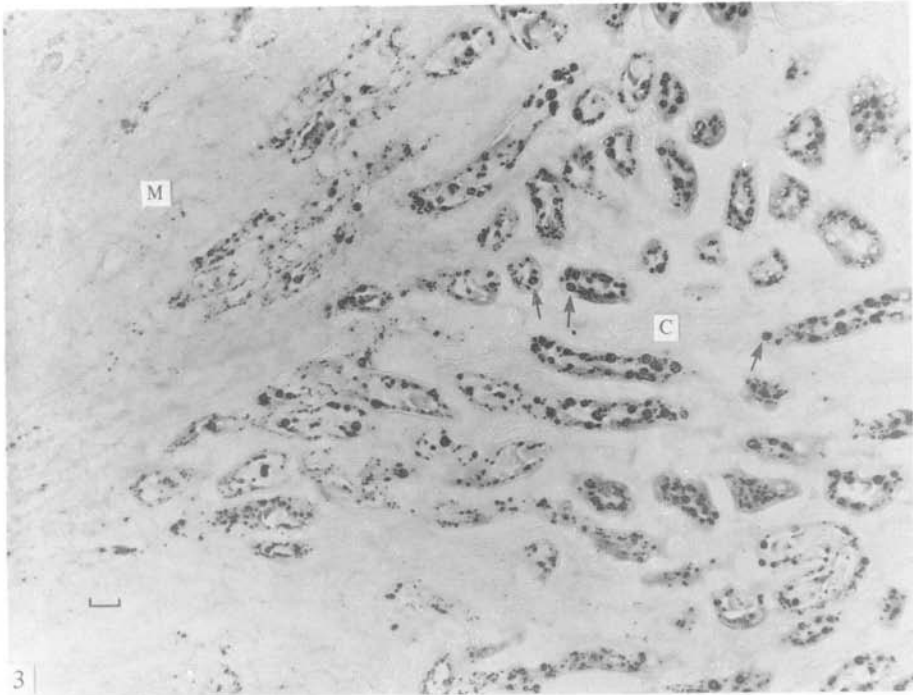

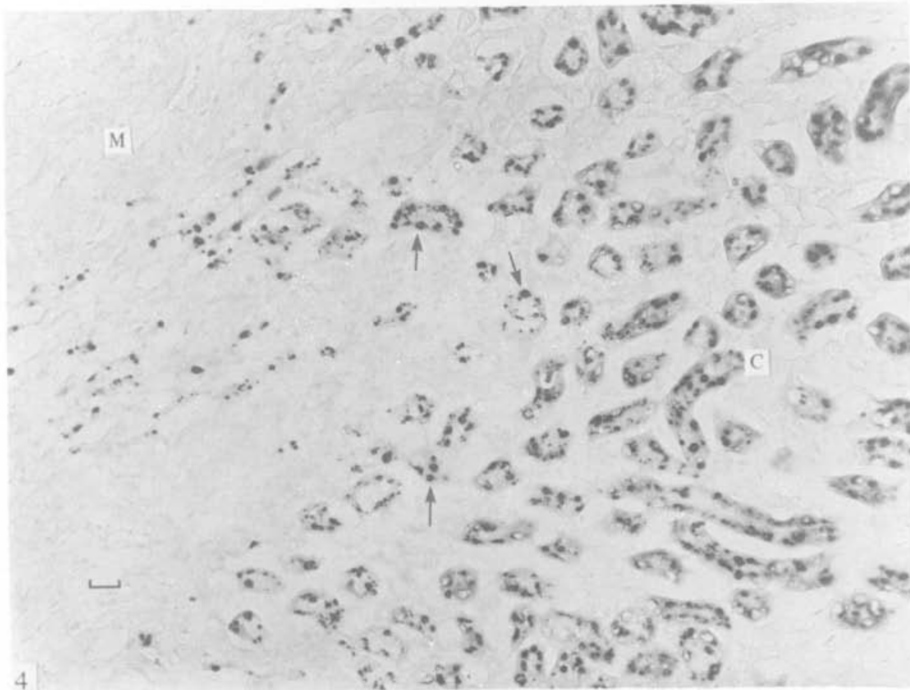

Figs. 2–4. Kidney corticomedullary region of mice treated 20 days with testosterone. Kidney sections were specifically stained with napthol AS-Bl glucuronide as substrate ($\times$ 160). M, Medulla; C, cortex (C). Bar = 25 μm, arrows denote a typical lysosome. Fig. 2: normal; Fig. 3: beige; Fig. 4: pale beige.

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Table 6. *Lysosomal enzyme secretion into urine in male mice*

	β-glucuronidase (U × 10)	β-galactosidase (U × 10)
C57BL/6J normal	6·7 ± 0·8	2·2 ± 0·3
Pale ear	2·1 ± 0·4***	0·76 ± 0·1**
Beige	1·3 ± 0·3***	0·90 ± 0·1**
Pale beige	1·9 ± 0·08***	0·66 ± 0·1***

Lysosomal enzyme secretion into urine. Values represent the mean ± S.E.M. of enzyme activity in urine collected for 4 consecutive days.

** $P \leqslant 0.02$, *** $P \leqslant 0.01$.

Serum lysosomal enzymes are increased two- to threefold over normal in pale ear mice while beige mice have normal lysosomal enzyme levels (Novak & Swank, 1979). Unexpectedly, pale beige mice had normal serum lysosomal enzyme levels (Table 7). Mixing experiments from sera of normal and mutant mice were additive, indicating no inhibitor was present in the pale beige serum (not shown). The serum of (*ep*/ ±, *bg*/ ±) mice, having only one copy of the pale ear gene was normal. Interestingly, there was a dosage effect of the beige gene, since (*ep*/*ep*, ± / ±), (*ep*/*ep*, *bg*/ ±) and (*ep*/*ep*, *bg*/*bg*) mice (having 0, 1 and 2 beige gene copies, respectively) have progressively and statistically significant ($P \leqslant 0.01$) lower concentrations of serum lysosomal enzymes.

Table 7. *Serum lysosomal enzyme concentrations*

	β-glucuronidase (U/ml × 10^2)	β-galactosidase (U/ml × 10^2)
C57BL/6J normal	5·42 ± 0·70	5·30 ± 0·80
Pale ear (*ep*/*ep*, ± / ±)	12·6 ± 0·10****	15·8 ± 0·60****
Beige-J (± / ±, *bg*/*bg*)	5·06 ± 0·36	5·76 ± 0·41
Pale beige (*ep*/*ep*, *bg*/*bg*)	4·0 ± 0·10	4·65 ± 0·12
ep/ ±, *bg*/ ±	6·25 ± 1·0	4·95 ± 1·4
ep/*ep*, *bg*/ ±	8·6 ± 0·8	9·35 ± 0·60*

Values represent the mean serum activity ± S.E.M. for 4–6 mice. For *ep*/ ± *bg*/ ±, the values represent two mice. β-galactosidase was assayed using 4-methylumbelliferyl-β-D-galactosidase as substrate.

* $P \leqslant 0.05$, **** $P \leqslant 0.001$.

4. DISCUSSION

We have bred a mouse with a new pigmentation phenotype (pale beige), homozygous for both the pale ear and beige pigment genes, in order to determine the effects on lysosomes and melanosomes of the simultaneous presence of both genetic lesions. The phenotypic expression in double mutants could indicate whether the beige and pale ear mutations affect the same pathway of biogenesis and/or processing of subcellular organelles. If the mutant alleles at these two loci block different subcellular processes, we would expect additive or interactive effects in the double-mutant animals to produce unique phenotypes. If the two genes affect the same pathway, it is likely that the resultant phenotype would be

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common to that of the parental mutants and would be determined by the relative sequence of action of the mutant genes on that pathway.

Among the phenotypes of the doubly homozygous mutant studied in this report, four general classes can be distinguished (Table 8). These in turn can be divided into 2 classes characterized by (*a*) no obvious interaction of the parental genes so that the phenotype of the offspring resembles that of one or both parental mutants or by (*b*) those in which there is some interaction of parental genes to give a new phenotype.

With respect to kidney dysfunction, the double mutant was indistinguishable from either parent (Table 8). In this case, the loss of either normal gene product appears to produce complete loss of function; both genes are required for normal

Table 8. *Phenotypes in parental mutant mice and in double-mutant-offspring*†

	bg/bg	*ep/ep*	*bg/bg, ep/ep*
(A) Double-mutant phenotypes common to both parental mutants			
Kidney dysfunctions affecting lysosomal enzymes			
β-galactosidase in male	4–5 × †	4–5 ×	4–5 ×
Enzyme induction in female	2–3 ×	2–3 ×	2–3 ×
Urinary secretion‡	0·1–0·15 ×	0·1–0·15 ×	0·1–0·15 ×
(B) Double-mutant phenotypes common to one parental mutant			
Kidney lysosome morphology	Enlarged	Normal	Enlarged
Ear and tail colour	Slight dilution	No pigment	No pigment
(C) New phenotypes in double-mutant			
Coat colour	Slightly diluted	Normal§	Extreme dilution
Eye colour	Black	Black‖	Light red
(D) Phenotype unique to one mutant corrected in double-mutant			
Serum lysosomal enzymes	Normal	2–3 ×	Normal

† Note: all values expressed relative to values in C57BL/6J

‡ Based on percentage of total kidney enzyme secreted per mouse per day.

§ Slight dilution of coat colour in very young animals; adult coat colour is identical to C57BL/6J.

‖ Pigmentation dilute in neonates.

lysosome secretion and no interaction was observed. Likewise, kidney lysosome morphology and ear and tail colour mimic either one or the other mutant parent so that no interaction was observed. The presence of the typical beige giant type lysosomes in the double mutant suggests that the action of the chromosome 7 beige gene may precede that of the pale ear gene in lysosome physiology.

Interactions of the *bg* and *ep* gene products were evident with respect to two phenotypic traits: hair and eye pigmentation and serum enzyme concentration. The extreme dilution of colour in the double mutant demonstrates interaction of the products of these two genes during melanosome maturation. The interaction of pale ear mice with other coat colour loci resulting in dilution of eye colour while maintaining the characteristic ear, tail, and coat colour of pale ear mice have been described (Silvers, 1979). Interaction is also suggested by the correction in the double mutant of the abnormality in serum glycosidase concentrations. It may be that the *bg* mutation acts to prevent the accumulation of a normal intermediate

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responsible for the serum abnormality in *ep* mice. However, the gene-dosage effect in heterozygotes (*ep/ep, bg/* ±) suggests a stoichiometric rather than catalytic role for the *bg* product. The stiochiometric relationship in *ep/ep, bg/* ± heterozygotes suggests in fact that the primary product of the *bg* locus is responsible for this phenotype (Paigen, 1971). Without more information regarding thc source of the serum enzymes, however, the correction phenomenon is difficult to interpret. Definitive evidence for the mechanism of action of the pale ear and beige gene products awaits identification of each primary gene product.

The molecular mechanisms responsible for the lysosomal dysfunctions described in this report are unknown. Some studies on Chediak–Higashi patients have suggested that the molecular mechanisms involve abnormally high ratios of cyclic AMP to cyclic GMP (Boxer *et al.* 1976) accompanied by decreased polymerization of microtubles (Oliver, 1973; Hinds & Dane ,1976). These observations have however not been confirmed in more recent studies (Gallin *et al.* 1979; Frankel *et al.* 1978). Other studies implicate abnormal fusion of lysosomes to give giant lysosomes (Oliver & Essner, 1973; Davis *et al.* 1971). Our observations could be explained if *bg* and *ep* encode abnormal proteins localized in the membranes of melanosomes and lysosomes. Another possibility is that altered lipid metabolism may be involved (McCluer *et al.* 1979; Haak *et al.* 1979). Studies on double mutants of beige and other pigmentation genes known to affect lysosome function (Meisler, 1978; Novak & Swank, 1979) should help in establishing interactions and relative sequence of actions of genes which affect organelle processing and function.

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