DEFECTIVE LYSOSOMAL ENZYME SECRETION IN KIDNEYS OF CHEDIAK-HIGASHI (BEIGE) MICE

E. J. BRANDT, ROSEMARY W. ELLIOTT, and RICHARD T. SWANK

From the Roswell Park Memorial Institute, Department of Molecular Biology, Buffalo, New York 14263

ABSTRACT

The beige mouse is an animal model for the human Chediak-Higashi syndrome, a disease characterized by giant lysosomes in most cell types. In mice, treatment with androgenic hormones causes a 20-50-fold elevation in at least one kidney lysosomal enzyme, β-glucuronidase. Beige mice treated with androgen had significantly higher kidney ß-glucuronidase, ß-galactosidase, and N-acetyl-ß-D-glucosaminidase (hexosaminidase) levels than normal mice. Other androgen-inducible enzymes and enzyme markers for the cytosol, mitochondria, and peroxisomes were not increased in kidney of beige mice. No significant lysosomal enzyme elevation was observed in five other organs of beige mice with or without androgen treatment, nor in kidneys of beige females not treated with androgen.

Histochemical staining for glucuronidase together with subcellular fractionation showed that the higher glucuronidase content of beige mouse kidney is caused by a striking accumulation of giant glucuronidase-containing lysosomes in tubule cells near the corticomedullary boundary. In normal mice lysosomal enzymes are coordinately released into the lumen of the kidney tubules and appreciable amounts of lysosomal enzymes are present in the urine. Levels of urinary lysosomal enzymes are much lower in beige mice than in normal mice.

It appears that lysosomes may accumulate in beige mice because of defective exocytosis resulting either from decreased intracellular motility of lysosomes or from their improper fusion with the plasma membrane. A similar defect could account for characteristics of the Chediak-Higashi syndrome.

Humans with the Chediak-Higashi syndrome have diluted pigmentation, increased susceptibility to infections and, most pertinent in regard to this study, abnormally large lysosomes in most cell types (25). The disease is rare, but lethal; in the human population most affected individuals die by their teen years. Lysosomes in granulocytes of affected individuals appear to be impaired in their ability to fuse with phagocytosed bacteria (32, 42) but the molecular basis for the defect remains unknown.

Thus far, the disease has been described in man, mink, cattle, and mice (24, 25). In all species it is inherited as an autosomal recessive trait, and in the mouse its genetic locus has been precisely mapped on chromosome 13 (17).

In the mouse the homologous mutation, first called slate and then beige (28, 29), arose spontaneously in the C57BL/6J strain (16). The beige mutant, C57BL/6J (bg/bg), probably differs from the parental C57BL/6J (+/+ ) line only in the beige gene on chromosome 13, making the parental strain an ideal control for experimental purposes. The phenotype of beige mice resembles that of Chediak-Higashi humans including lighter coat color, decreased bactericidal activity of granulo-
enzymes (9) and enlarged lysosomes in a wide variety of cells (21). Despite the presence of morphologically abnormal lysosomes in Chediak-Higashi disease, there are contradictory reports concerning lysosomal enzyme levels. Some workers have found altered lysosomal enzyme activity (15) but these reports have not been confirmed by other investigators (23, 41).

A major increase (20–50-fold) occurs in the kidney level of glucuronidase after administration of androgens to female mice (8). The increase in enzyme is accompanied by a considerable hypersecretion of androgens to female mice (8). The increase in enzyme in that 30–40% of the kidney enzyme is apparently abnormal lysosomes in Chediak-Higashi disease, there are contradictory reports concerning lysosomal enzyme levels. Some workers have found altered lysosomal enzyme activity (15) but these reports have not been confirmed by other investigators (23, 41).

A major increase (20–50-fold) occurs in the kidney level of glucuronidase after administration of androgens to female mice (8). The increase in enzyme is accompanied by a considerable hypersecretion of androgens to female mice (8). The increase in enzyme in that 30–40% of the kidney enzyme is approximately 200-fold increase in secretion of the enzyme into urine (31). Subsequent studies (36) have shown that induction results from an increase in de novo enzyme synthesis. The rate of glucuronidase synthesis after androgenic treatment is under the control of the Gur locus, a cis-acting gene in the region of the glucuronidase structural gene on chromosome 5 (27, 36).

Glucuronidase is an unusual acid hydrolase enzyme in that 30–40% of the kidney enzyme is attached to endoplasmic reticulum (26, 35). Binding of the enzyme to endoplasmic reticulum is effected through a noncovalent association with a 64,000 mol wt protein, egasyn (37). This process is under the genetic control of the Eg locus on chromosome 9 (10).

The beige mutation afforded the opportunity to test the effect of an inherited morphological alteration of lysosomes on lysosomal physiology in the mouse kidney. In proximal tubule cells of the mouse kidney, the vacuolar apparatus is actively involved in the processes of phagocytosis of extracellular proteins from the newly filtered urine (19, 33, 34) and synthesis (36) and secretion of at least one lysosomal enzyme, β-glucuronidase (EC 3.2.1.31). An ideal genetic control is available for the C57BL/6J (bg/bg) mouse through its normal chromosome 5 (27, 36).

This paper presents our initial observations on measurements of the effects of the beige mutation on three lysosomal enzymes, β-glucuronidase, β-galactosidase (EC 3.2.1.23), and N-acetylglucosaminidase (hexosaminidase) (EC 3.2.1.30). We have found that in normal mice a major fraction of the kidney content of each of these lysosomal enzymes is secreted daily from kidney proximal tubule cells after male mice are treated with androgen. Under the same conditions there is a markedly lowered secretion of all three lysosomal enzymes in the beige mouse, resulting in two- to fourfold higher kidney enzyme levels. The beige effect appears to be lysosome specific in that other androgen-inducible, but nonlysosomal, enzymes are not affected. Similarly unaffected are enzymes of three other subcellular compartments.

**MATERIALS AND METHODS**

**Animals**

Female inbred C57BL/6J (+/+), C57BL/6J (+/bg), and C57BL/6J (bg/bg) mice were obtained from the Jackson Laboratory, Bar Harbor, Me., and were utilized at 8–16 wk of age. The animals were maintained on a 12-h light, 12-h dark schedule, and fed ad libitum on Rockland Mouse Breeder Diet (Teklad, Inc., Monmouth, Ill.) containing 17% minimum crude protein, 10% minimum crude fat, and 2.5% maximum crude fiber.

**Induction and Preparation of Tissues**

Dihydrotestosterone suspended at 50 mg/ml in olive oil was administered by subcutaneous injection (36). Alternatively, 75-mg testosterone pellets were implanted subcutaneously and remained throughout the induction period. Mice were killed by exposure to carbon dioxide gas or by cervical dislocation. Excised tissues were weighed and homogenized for 1 min in approximately 10 vol (wt/vol) of ice-cold 0.25 M sucrose containing 0.02 M imidazole-HCl, pH 7.4, with a 13-mm Polytron homogenizer (Kinematica GMBH, Lucerne) at setting 4.8. Homogenates were stored at –20°C except where otherwise specified.

**Assays**

A fluorimetric assay with 0.4 mM 4-methylumbelliferyl-β-D-glucuronide (final assay concentration) as substrate was used for glucuronidase assays. Substrate was added at 0°C to homogenate or urine diluted in 0.1 M sodium acetate buffer, pH 4.6, containing 0.2% Triton X-100 to give a final volume of 0.1 ml. The mixture was incubated at 37°C for 10–30 min after which the reaction was terminated by immersion in an ice bath followed by adding 1 ml 0.1 M sodium carbonate. Fluorescence, corrected for substrate blank, was determined relative to a standard 4-methylumbelliferone solution, using an Aminco Fluoro-Colorimeter (American Instrument Co., Silver Spring, Md.) equipped with an excitation interference filter of peak wavelength 360 nm and an emission filter passing wavelengths above 415 nm.

β-Galactosidase was assayed with 0.22 mM 4-methylumbelliferyl-β-D-galactoside as the substrate. Substrate was added at 0°C to tissue homogenate or urine diluted in 0.1 M sodium citrate buffer, pH 3.5, containing 0.05% Triton X-100 to give a final volume of 0.9 ml. The mixture was incubated at 37°C for 10–30 min after which the reaction was terminated by immersion in an ice bath followed by adding 1 ml 0.1 M sodium carbonate. Fluorescence, corrected for substrate blank, was determined relative to a standard 4-methylumbelliferone solution, using an Aminco Fluoro-Colorimeter (American Instrument Co., Silver Spring, Md.) equipped with an excitation interference filter of peak wavelength 360 nm and an emission filter passing wavelengths above 415 nm.

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the reaction was terminated by adding 0.2 ml of 2.8 M sodium carbonate. Fluorescence was determined against a substrate blank and standard as described for the glucuronidase assay.

A spectrophotometric assay with 4.0 mM paranitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside as the substrate was used to assay hexosaminidase. Substrate was added at 0°C to homogenate or urine diluted in 0.1 M sodium citrate buffer, pH 4.5, containing 0.1 M NaCl to give a final volume of 1 ml. The mixture was incubated at 37°C for 30 min after which the reaction was terminated by immersion in an ice bath followed by adding 0.2 ml 30% trichloroacetic acid. After mixing, the tubes were centrifuged at 1,000 g for 10 min. Supernatant solutions were transferred to tubes containing 0.2 ml of 5.0 M 2-amin-2-methyl-1,3 propanediol. The absorbance of released p-nitrophenol was measured at 415 nm ($E_{1cm} = 14,000$).

Succinate dehydrogenase (EC 1.3.99.1) was assayed by the spectrophotometric assay described by Veeger et al. (38). Freshly prepared tissue homogenates were used. The reaction was proportional to both time and homogenate concentration.

A spectrophotometric assay for d-amino acid oxidase (EC 1.4.3.3) with d-phenylalanine as substrate was utilized according to the method of Wellner and Lichtenberg (40). Freshly prepared kidney homogenates were used. D-amino acid oxidase activity was determined from its absorbancy at 300 nm ($E_{1cm} = 9,360$ for the product phenylpyruvate) in 1 M borate-arsenate solution.

For alcohol dehydrogenase (EC 1.1.1.2) assays, fresh kidney extracts were centrifuged at 100,000 $g$ for 1 h to remove endogenous substrates, aliquots (0.4 ml) were rapidly chromatographed at room temperature on Sephadex G-25 columns of 6 ml bed vol which had been equilibrated with 0.033 M pyrophosphate buffer, pH 8.8. The void volume was used for assay. The reaction mixture contained 30 mM sodium pyrophosphate buffer, pH 8.8, 20 mM ethanol, and 8 mM NAD$^+$. Solutions were first incubated for 10 min at 25°C minus ethanol to remove residual traces of endogenous substrates. The reaction, in a constant-temperature cell housing maintained at 25°C, was started by adding ethanol and was followed by measuring the fluorescence of NADH in an Amino Fluoro-Colorimeter attached to a recorder and containing filters identical to those described for the glucuronidase assay. The amount of NADH produced was calculated using a standard NADH solution.

For all enzyme assays, one unit of activity is defined as the amount of enzyme required to form 1 μmol of product per hour.

**Analytical Methods**

Protein concentrations were determined using the microbiuret procedure of Gooa (11). The phenol-sulfuric acid colorimetric method described by Dubois et al. (2) was used for analyzing carbohydrates and reducing sugars. Total lipid content was determined according to the procedure of Bligh and Dyer (1).

**Electrophoresis**

Polyacrylamide gel electrophoresis of tissue homogenates and subcellular fractions was done by the method described by Swank and Paigen (35) and the gels were stained specifically for glucuronidase (13). Urine samples were dialyzed at 4°C overnight against 0.25 M sucrose containing 0.02 M imidazole-HCl, pH 7.4, before electrophoresis.

**Subcellular Fractionation**

The procedure was a modification of that used by Ganschow and Paigen (10). Kidneys were added to 10 vol of chilled 0.25 M sucrose containing 0.02 M imidazole-HCl buffer, pH 7.4, and homogenized at 0°C using eight full strokes with a Potter-Elvehjem homogenizer. The homogenate was filtered through four layers of gauze. An aliquot of the filtrates (whole homogenate) was centrifuged at 100,000 $g$ for 30 min. The cytosol-soluble fraction was saved and the pellet was resuspended in 0.02 M imidazole-HCl buffer, pH 7.4. The suspension was incubated at 4°C for 30 min to rupture the osmotically sensitive lysosomes. After centrifugation at 100,000 $g$ for 30 min, the supernate (lysosomal extract) was removed and 0.1 vol of 2.5 M sucrose was added to the lysosomal extract to prevent clumping after freezing and thawing. The pellet (microsomes) was washed by resuspending in 0.02 M imidazole-HCl, pH 7.4, and centrifuging at 100,000 $g$ for 30 min. The supernate (microsomal wash) was saved and the pellet containing the microsome fraction was resuspended to the whole homogenate volume with 0.25 M sucrose containing 0.02 M imidazole-HCl buffer, pH 7.4.

**Urine Collection**

Mouse metabolism cages equipped with food and water dispensers and containing six mice each were used. Urine was collected over several consecutive 24-h periods into 50-ml flasks containing 5 ml mineral oil but no preservative. Samples were clarified by centrifuging at 1,000 $g$ for 10 min and stored at -20°C.

**Histology**

Kidneys were fixed in 10% buffered Formalin and embedded in paraffin for sectioning and routine hematoxylin and eosin staining.

The histochemical staining of glucuronidase in frozen sections of kidney was demonstrated by the naphthol AS-BI glucuronide and hexazonium-pararosanilin method described by Hayashi (13).

**Immunochemical Quantitation of Glucuronidase**

Single radial immunodiffusion was utilized to quantitate the enzyme in kidney homogenates and urine.
according to the procedure of Lanzerotti and Gullino (18). A monospecific goat antibody prepared against purified mouse glucuronidase was the gift of Dr. Shiro Tomino.

Chemicals

β-Nitrophenyl-2-acetamido-2-deoxy β-D-glycopyranoside, and 4-methylumbelliferyl galactoside were obtained from Koch-Light Laboratories, Ltd., Colnbrook, England. Naphthol AS-BI β-D-glucuronide, 5α-androst-17β-ol-3-one (dihydrotestosterone) and 4-methylumbelliferyl-β-D-glucuronide, NAD+, FAD, and 2,6-dichlorophenol-indophenol were from Sigma Chemical Co., St. Louis, Mo. Testosterone (75 mg Oretton pellets) was generously provided by the Schering Corp., Kenilworth, N.J. The 2-amino-2-methyl 1:3 propanediol was obtained from Commercial Solvents Corp. All chemicals utilized were reagent grade.

RESULTS

Lysosomal Enzyme Levels in Beige and Normal Mice

To determine whether any change in lysosomal enzyme activity accompanies the presence of morphologically altered lysosomes in beige mice, the activities of glucuronidase, galactosidase, and hexosaminidase were measured in organs of control females and females treated for 23 days with androgen.

Table I shows that there are no significant differences between control beige and normal mice for the three acid hydrolases in all the organs tested with the single exception of kidney hexosaminidase. This similarity is somewhat surprising in view of the fact that enlarged granules have been reported in all of these organs of beige mice but is consistent with the reports of other workers (23, 41).

Enzyme levels were also determined in organs of mice treated with testosterone for 23 days. This treatment is known to increase maximally the rate of synthesis of glucuronidase in mouse kidney proximal tubules (36). Table I shows that in liver, spleen, lung, heart, and brain the treated mutant and normal mice do not differ from each other. Comparison of the data in Table I shows that testosterone treatment caused no significant increases in these organ enzyme levels. However, in kidney the activity of glucuronidase is greatly increased in both normal and beige mice after treatment with androgen, and the small increase seen in hexosaminidase is statistically significant.

| Table I | Activities of Lysosomal Enzymes in Organs of Untreated and Androgen-Treated C57BL/6J Normal (+/+ ) and Beige (bg/bg) Mice |
|----------|----------------------------------------------------------------------------------------------------------------------------------|
| Organ | Enzyme activity (U/g) |
|       | Glucuronidase | Galactosidase | Hexosaminidase |
|       | (+/+ )        | (bg/bg)       | (+/+ )        | (bg/bg)       |
| Untreated |                  |               |               |               |
| Kidney  | 7.38 ± 0.65    | 10.4 ± 1.3    | 26.7 ± 3.4    | 33.4 ± 4.9    | 282 ± 13     | 365 ± 26     |
| Liver   | 22.9 ± 1.2     | 25.0 ± 2.2    | 14.3 ± 2.0    | 9.1 ± 2.0     | 294 ± 13     | 364 ± 48     |
| Spleen  | 27.3 ± 1.7     | 30.6 ± 2.4    | 30.5 ± 3.7    | 23.9 ± 2.4    | 917 ± 71     | 934 ± 57     |
| Lung    | 10.2 ± 0.3     | 10.8 ± 0.65   | 3.3 ± 0.1     | 3.6 ± 0.5     | 214 ± 22     | 286 ± 35     |
| Heart   | 0.75 ± 0.02    | 0.96 ± 0.10   | 1.6 ± 0.2     | 1.5 ± 0.1     | 38.4 ± 0.8   | 49.8 ± 6.1   |
| Brain   | 1.2 ± 0.07     | 1.3 ± 0.06    | 2.7 ± 0.1     | 2.4 ± 0.2     | 206 ± 12     | 232 ± 6.4    |
| Androgen-treated |                  |               |               |               |
| Kidney  | 121 ± 6.5      | 353 ± 14      | 26.4 ± 0.3    | 40.8 ± 1.1    | 345 ± 16     | 492 ± 27     |
| Liver   | 34.0 ± 1.4     | 30.4 ± 1.1    | 13.7 ± 1.7    | 9.3 ± 0.02    | 272 ± 20     | 208 ± 6.2    |
| Spleen  | 32.3 ± 4.5     | 34.3 ± 3.5    | 30.9 ± 1.8    | 19.8 ± 1.3    | 874 ± 105    | 820 ± 85     |
| Lung    | 8.45 ± 0.36    | 9.18 ± 0.9    | 4.6 ± 0.4     | 2.9 ± 0.05    | 213 ± 8      | 239 ± 23     |
| Heart   | 1.13 ± 0.04    | 1.28 ± 0.04   | 2.1 ± 0.3     | 1.5 ± 0.1     | 53.0 ± 1.6   | 50.0 ± 2.3   |
| Brain   | 1.40 ± 0.05    | 1.38 ± 0.08   | 3.0 ± 0.3     | 2.0 ± 0.05    | 217 ± 6.2    | 221 ± 19     |

Values are mean ± SEM for three or more animals. Treated mice were induced for 23 days with testosterone. Untreated normal and beige mice have significantly different (P < 0.05) kidney hexosaminidase levels. Kidney activities are significantly higher in treated beige mice for glucuronidase (P < 0.001), galactosidase (P < 0.001) and hexosaminidase (P < 0.01).
Comparison of the values for induced beige and induced normal mouse kidney shows that for all three enzymes, levels in the beige mutant are significantly elevated over those found in the normal mouse.

The major difference that we have detected in lysosomal activity between normal and beige mice is the markedly higher enzyme content after induction in the kidneys of beige mice. Because this increase is most pronounced for glucuronidase, and because the mechanism of induction for this enzyme in mouse kidney is by far the best understood among the lysosomal enzymes (27), we chose to further characterize this enzyme in the kidneys of beige and normal mice.

There was little difference in the time course of glucuronidase induction in beige and normal mice before day 7 (Fig. 1). However, after this time the rate of increase of glucuronidase in beige mice accelerates, until by day 23 the kidney glucuronidase activity in these animals is three times that of their normal counterparts. Thus, the higher glucuronidase activity in kidney of androgen-stimulated beige mice is recessive as are all other known phenotypic characteristics of the beige mutation.

The higher glucuronidase-specific activity in beige kidney results from an accumulation of three times the number of enzyme molecules and not from activation of pre-existing molecules. The amount of enzyme antigen relative to enzyme activity was tested using single radial immunodiffusion analysis with a monospecific antibody to mouse glucuronidase (Fig. 2). Identical ring diameters were obtained at three different levels of applied enzyme activity, proving that the same number of enzyme units react per unit of antibody in beige and normal mice. Thus, the elevated glucuronidase level in the tubule cells of induced beige mice must be due to excess accumulation of glucuronidase molecules, not to activation of pre-existing enzyme moieties or synthesis of different species of enzyme with glucuronidase activity.

Specificity of Beige Gene Action on Cellular Organelles and Molecular Components

To test whether the beige alteration is specific for lysosomal enzymes of kidney, we quantitated D-amino acid oxidase, succinate dehydrogenase, and alcohol dehydrogenase, markers for peroxisomes, mitochondria, and cell sap, respectively. In addition, we tested for accumulation of total...
TABLE II
Activities of Nonlysosomal Enzymes in Kidneys of Normal and Beige Mice

| Genotype            | Androgen treatment | D-Amino acid oxidase | Succinate dehydrogenase | Alcohol dehydrogenase |
|---------------------|--------------------|----------------------|-------------------------|-----------------------|
|                     | U/g kidney         | U/g kidney           | U/g protein             |
| C57BL/6J (+/+)      | -                  | 187 ± 9              | 52.8 ± 4.8              | 14.9 ± 1.7            |
| C57BL/6J (bg/bg)    | -                  | 166 ± 7              | 62.4 ± 3.3              | 20.2 ± 2.2            |
| C57BL/6J (+/+)      | +                  | 351 ± 11             | 46.5 ± 3.4              | 137 ± 11              |
| C57BL/6J (bg/bg)    | +                  | 389 ± 20             | 52.8 ± 3.4              | 102 ± 8               |

Values are mean ± SEM for four or more animals. Activities of the three enzymes are not significantly different in normal and beige mice whether untreated or treated with androgen.

TABLE III
Carbohydrate, Lipid, and Protein in Kidneys of Normal and Beige Mice

| Genotype            | Androgen treatment | Carbohydrate | Lipid | Protein |
|---------------------|--------------------|--------------|-------|---------|
|                     |                    | mg/g kidney  | mg/g kidney | mg/g kidney |
| C57BL/6J (+/+)      | -                  | -            | -     | 202 ± 4.0 |
| C57BL/6J (bg/bg)    | -                  | -            | -     | 198 ± 1.7 |
| C57BL/6J (+/+)      | +                  | 7.71 ± 0.27  | 16.7 ± 0.25 | 206 ± 2.9 |
| C57BL/6J (bg/bg)    | +                  | 8.58 ± 0.15  | 19.7 ± 2.0 | 216 ± 11.0 |

Androgen treatment was for 23 days. Values are mean ± SEM for four animals.

carbohydrate, lipid, and protein in kidneys of beige mice.

The data show that there is no significant difference between beige and normal mice in the kidney levels of any of the three nonlysosomal enzymes (Table II). Like glucuronidase, D-amino acid oxidase and alcohol dehydrogenase are inducible by androgens, 2- and 10-fold, respectively. However, unlike glucuronidase, the hormone-elevated levels are identical in beige and normal mice. Also, unlike the lysosomal enzymes, none of these enzymes could be detected in urine of control or treated mice.

The beige mutation has no effect on carbohydrate, lipid, or protein concentrations in kidney of androgen-treated mice (Table III). While kidney protein concentration does not differ in control and androgen-treated beige and normal mice, the total weight of kidney is significantly higher in beige mice than in normal mice after androgen treatment. Fig. 3 shows that normal kidneys hypertrophy from 0.2 g to 0.35 g during the 23 days of hormone treatment, while beige mouse kidneys hypertrophy to 0.45 g. In contrast, liver weights of normal and mutant mice (Fig. 3) change in parallel throughout the hormonal treatment.

Because of the larger kidney size of androgen-treated beige mice, the increase in total kidney content of each lysosomal enzyme in beige mice is actually 20–30% greater than indicated by the data of Table I.
Glucuronidase in Induced Beige Mouse Kidney is Lysosomal

Glucuronidase has been reported to be present in both lysosomes and endoplasmic reticulum in mouse kidney (35). In order to determine the subcellular location of the increased glucuronidase in kidneys of androgen-treated beige mice, both fractionation and histochemical studies were undertaken.

Mouse kidney glucuronidase includes at least six electrophoretically separable forms of the enzyme (Fig. 4) which are specifically associated with different subcellular fractions (35). In normal mice electrophoretic component L is concentrated in lysosomes and soluble fraction (not shown), while bands X and M₁, M₂, M₃, and M₄ are found only in the microsomal fraction. Consistent with the capacity of kidney to extrude lysosomal enzymes, band L is the only electrophoretic component found in urine. Electrophoretic analysis of urine and of subcellular fractions of kidney prepared by osmotic shock proved that this distribution likewise holds for beige mice. Furthermore, as evidenced by the tracings of Fig. 4, no new molecular forms of glucuronidase occur in beige mouse kidney despite a threefold elevation in enzyme level.

Quantitative densitometry of these electrophoretic bands in extracts of induced kidney of beige and normal mice (Table IV) showed that beige mice contain 280 U/g kidney in band L as opposed to only 80 U/g kidney in band L of normal mice, a highly significant difference. However, differences between the two types of mice in the specific activity of the various microsomal forms X, M₁, M₂, M₃, and M₄ were not significant.

Figs. 5–7 illustrate some morphological characteristics of normal and beige mouse kidney and document that, after androgen treatment, most of the cellular glucuronidase activity in the beige mice is located within the giant granules.

**TABLE IV**

| Glucuronidase activity (Units/g Kidney) | C57BL/6J (+/+) | C57BL/6J (bg/bg) |
|---------------------------------------|---------------|-----------------|
| L                                    | 80.0 ± 1.5    | 280 ± 7.1       |
| X                                    | 6.0 ± 1.2     | 6.3 ± 1.1       |
| M₁                                   | 20.0 ± 2.2    | 23.7 ± 0.21     |
| M₂                                   | 6.7 ± 0.59    | 10.0 ± 0.19     |
| M₃                                   | 2.7 ± 0.21    | 3.0 ± 0.59      |
| M₄                                   | 2.7 ± 0.12    | *               |
| Total                                | 118 ± 2.3     | 323 ± 6.4       |

Whole kidney homogenates prepared from 21-, 22-, and 23-day dihydrotestosterone-treated normal and beige mice were electrophoresed. The gels were scanned at 550 nm after staining for glucuronidase activity with naphthol AS-BI glucuronide. Units of glucuronidase electrophoresed were 0.025 and 0.024 for normal and beige mice, respectively. Values are mean ± SEM for three experiments.

* Detectable but too low to quantitate.

FIGURE 4 Subcellular localization of glucuronidase electrophoretic components. Homogenates were prepared from kidneys of 23-day induced normal C57BL/6J (+/+) (—) and beige C57BL/6J (bg/bg) (——) mice. Urine was from 27-day induced mice. Osmotic shock fractions and urine, prepared as described in Materials and Methods, were subjected to polyacrylamide gel electrophoresis and then scanned at 550 nm after staining for glucuronidase activity with naphthol AS-BI glucuronide. Units of glucuronidase electrophoresed for normal and beige mice, respectively, for each fraction were whole homogenate: 0.037 and 0.030, lysosomal: 0.023 and 0.024, microsomal: 0.014 and 0.014, and urine: 0.007 and 0.007.
The morphological alteration in kidneys of beige mice is readily demonstrated by hematoxylin and eosin staining. While few enlarged clear granules are visible in sections of kidney from untreated beige mice (Fig. 5 a), after androgen treatment large numbers of giant granules (up to 11 μm diameter) are visible in the tubule cells, especially in the corticomedullary region (Fig. 5 b). Many cells in this region, in fact, appear to be engorged with these granules, with some single granules apparently occupying most of the cellular volume. In contrast, we have not detected such enlarged granules in kidney of normal mice before (Fig. 5 c) or after (Fig. 5 d) androgen treatment. The tubule cells of hormone-treated normal and beige mice are noticeably enlarged when compared with control mice. The increased cellular size is especially apparent in treated beige mice whose tubules have very little visible lumen. This swelling is apparently due to the presence of the huge granules and may explain the measurably larger increase in kidney weight of beige mice found after treatment with androgens (Fig. 3).

In tubule cells of androgen-treated normal mice specifically stained for glucuronidase activity (Fig. 6 a, b), granules, presumably lysosomes, are concentrated at the apical portion of the cell near the tubule lumen and all lysosomes are approximately the same size (about 1.8 μm maximum diameter). No enzyme activity was detected in cells of the medulla. Lysosomes in cells of the outer cortex are small, being equal in size to the lysosomes of normal mouse kidney cortex. Cells in the corticomedullary region, however, contain much larger lysosomes (up to 11 μm) which showed very intense glucuronidase staining. In general, the lysosomes in tubules of beige mice are concentrated at the apical side of the cell, although in many instances the enlarged lysosomes occupy most of the cellular volume. Comparison of Figs. 6 a and 7 a likewise shows that the tubules of androgen-treated beige mice have a less conspicuous lumen than comparable cells of normal mice, in confirmation of the hematoxylin and eosin results.

Table V lists confirmatory data on the subcellular localization of glucuronidase in induced beige kidney from experiments in which fractionation is accomplished by the osmotic shock technique. In normal mouse kidney, about 30% of the enzyme is present in a soluble (cytosol) fraction. Approximately the same percent is found in the osmotically sensitive particle fraction (lysosomes), and about 20% cannot be washed off the remaining particles (microsomes) by hypotonic buffer. In beige mice almost all of the increased activity of whole homogenate can be accounted for by increased activity in the soluble and lysosomal fractions which comprise 60% and 20%, respectively, of the recovered homogenate activity. There is, likewise, a doubling of units of glucuronidase in the microsomal fraction in beige mice, but the percent microsomal enzyme in beige is less than in normal mice. Also, densitometry (not shown) of the isolated microsomal fraction revealed that 90% of the increase in microsomal fraction activity in kidney of beige mice is accounted for by the increase in component L. The increased level of soluble enzyme probably is a reflection of the 5–10-fold increased size of beige lysosomes which may render them more labile during homogenization. Neither very gentle homogenization nor prior in vivo administration of lysosomal stabilizing agents such as cortisol has reduced the percent enzyme released into the soluble fraction of kidney extracts of cells of beige mice (Swank, unpublished observations).

Abnormal Secretion of Lysosomal Enzymes by Beige Mice

A possible basis for the higher lysosomal enzyme content in kidney tubule cells of beige mice is that these cells are defective in extrusion of lysosomal contents into the tubule lumen. Secretion is a major mechanism of removal of lysosomal enzymes from mouse kidney, especially after administration of androgen (Table VI). In androgen-treated normal mice, for example, 41%, 97%, and 12% of the total kidney content of glucuronidase, galactosidase, and hexosaminidase, respectively, are secreted daily. The relatively small percentage of kidney hexosaminidase secreted may reflect the fact that a large fraction of the kidney content of this enzyme is known to be in the medulla rather than in actively secreting proximal tubule cells (12).
FIGURE 5  Hematoxylin- and eosin-stained sections of the kidney corticomedullary region of beige and normal mice (× 400). (a) Untreated beige mouse kidney. There are very few large clear granules (arrow) in tubule cells. The tubules are not swollen and have readily visible lumens. (b) Kidney of a beige mouse treated 14 days with testosterone. All tubule cells are engorged with large clear granules (arrow) which often are much larger than the darkly staining nuclei. The cells show extensive hypertrophy and lumens are obscured. (c) Untreated normal mouse kidney. Darkly staining granules are nuclei. Note that tubules (arrow) contain no clear granules, are not hypertrophied, and lumens are readily visible. (d) Kidney of a normal mouse treated for 14 days with androgen. Tubule cells (arrow) contain no visible granules, but are very much hypertrophied when compared to tubules from untreated mice.
FIGURE 6 Localization by histochemical staining with napthol AS-BI β-D-glucuronide of glucuronidase in kidney of normal mice treated for 23 days with testosterone. (a) Medulla with no glucuronidase activity is to the left (× 400). The small granules (filled arrow) are clustered in the apical region of the tubule cells, surrounding the lumen. Open arrow shows a nucleus. (b) Localization of glucuronidase in kidney of normal mice treated for 23 days with testosterone (× 200). Glucuronidase activity is found in small discrete granules in proximal tubules (arrow) throughout the cortex.
Figure 7. Localization of glucuronidase in corticomedullary region of beige mice treated for 23 days with testosterone. (a) Kidney sections were specifically stained for glucuronidase activity using napthol AS-BI \( \beta \)-D-glucuronide (\( \times \) 400). Medulla with no glucuronidase activity is to the left. Most tubules contain giant lysosomes (filled arrows), larger in size than nuclei (open arrow). (b) Glucuronidase activity is throughout the cortex in discrete granules (\( \times \) 200). However, the granules are much larger in tubules of the corticomedullary region (filled arrows) than in tubules of outer cortex (open arrow).
Beige mice secrete significantly fewer units of the three lysosomal enzymes than their normal counterparts with the sole exception of hexosaminidase of untreated animals. Most noteworthy is the finding that in androgen-treated mice, where secretion is a major mechanism of removal of these enzymes, beige mice secrete only one-third to one-fourth as much of all three lysosomal enzymes as normal mice.

Enzyme assays on various dilutions of beige and normal mouse urine and of mixtures of the two have not revealed the presence of inhibitors or activators. Furthermore, by single radial immunodiffusion analyses, equal glucuronidase activity units from beige and normal mouse urine produced equal ring diameters after specific enzyme staining, indicating that the lower glucuronidase activity in beige mouse urine is due to the presence of fewer glucuronidase molecules than are found in normal mouse urine.

The urinary protein analyses (Table VI) show that in both types of mice androgen treatment causes a four- to fivefold increase in proteinuria. Much of this urinary protein is known to be a serum-protein complex of average mol wt 17,800, whose synthesis is induced in liver by androgen (6, 7). Unlike the lysosomal enzymes, there appears to be little difference between normal and beige mice, whether control or induced, in total urinary protein content. Beige mice, therefore, are not altered in the general processes of glomerular filtration and excretion of proteins.

**CONCLUSIONS**

The principal conclusion of our studies is that the beige mutant is defective in the normal process of extruding lysosomal enzymes through the plasma membrane of the kidney tubule cells. As a result, there is a significant accumulation of lysosomal enzymes in the kidney, especially after enzyme synthesis has been stimulated by androgen. Al-

**Table V**

| Glucuronidase Activity Distribution Among Subcellular Fractions Prepared by Osmotic Shock Treatment |
|--------------------------------------------------------------------------------------------------|
| **Subcellular fraction**  | **C57BL/6J (+/+)** | **C57BL/6J (bg/bg)** |
| Whole homogenate           | 118 ± 2.3          | 322 ± 6.4          |
| Soluble                    | 32.7 ± 0.9         | 172 ± 8.7          |
| Lysosome                   | 42.1 ± 1.2         | 59.9 ± 1.2         |
| Microsome wash             | 2.79 ± 0.1         | 5.68 ± 0.4         |
| Microsome                  | 20.4 ± 0.8         | 43.6 ± 1.8         |
| Recovery (%)               | 83.2 ± 0.8         | 87.2 ± 1.8         |

Homogenates were prepared from kidneys of normal and beige mice induced for 23 days with dihydrotestosterone. Values are mean ± SEM for three experiments.

**Table VI**

| Daily Secretion of Lysosomal Enzymes and Protein by Normal and Beige Mice |
|---------------------------------------------------------------|
| **No androgen**                                               | **Androgen**               |
| **C57BL/6J (+/+)**                                           | **C57BL/6J (bg/bg)**       |
| **Gluconuronidase**                                         | **Gluconuronidase**        |
| Secreted (U)                                                | Secreted (U)               |
| 0.068 ± 0.007                                               | 0.029 ± 0.003              |
| Secreted (%)                                               | 4.2                         |
| 1.2                                               | 1.0                         |
| Galactosidase                                              | Galactosidase              |
| Secreted (U)                                                | Secreted (U)               |
| 0.327 ± 0.037                                               | 0.079 ± 0.003              |
| Secreted (%)                                               | 5.6                         |
| 1.0                                               | 1.0                         |
| Hexosaminidase                                             | Hexosaminidase             |
| Secreted (U)                                                | Secreted (U)               |
| 2.78 ± 0.38                                                | 2.00 ± 0.15                |
| Secreted (%)                                               | 4.5                         |
| 2.3                                               | 2.3                         |
| Protein                                                   | Protein                    |
| Excreted (mg)                                              | Excreted (mg)              |
| 5.26 ± 0.58                                                | 3.88 ± 0.12                |
| * Percent of total kidney activity. Values are mean ± SEM for 24-h urine samples collected on 4 consecutive days starting on day 23 of treatment for androgen-treated mice. Uninduced control levels are significantly different for glucuronidase (P < 0.01) and galactosidase (P < 0.01) but are not different for hexosaminidase or protein. Induced values for all three enzymes but not protein are different (P < 0.001) for normal and beige mice.
demonstrated that the rate of synthesis of kidney 
does not affect the physiology of other subcellular 
through the plasma membrane.

A second conclusion is that the beige mutation 
does not affect the physiology of other subcellular 
organelles. Kidneys of beige mice had normal 
levels of marker enzymes for the cytosol, peroxi-
somes, and mitochondria. Also, other androgen-
induced enzymes, D-amino acid oxidase and alco-
hol dehydrogenase, that are not present in lyso-
somes, are at equal levels in kidneys from andro-
gen-treated normal and beige mice. Since these organs are not known to synthesize and 
release large amounts of lysosomal enzymes, it 
appears that the beige mutation causes a signifi-
cant elevation of lysosomal enzymes only in tissues 
where secretion or excretion is a major mechanism 
of lysosomal enzyme turnover. In kidneys of 
androgen-stimulated beige mice the secretion of all 
three lysosomal enzymes is lowered by approxi-
mately the same factor when compared to normal 
mice. The simplest, though not the only, interpre-
tation of this observation is that all three enzymes 
are enclosed in the same lysosome, which then 
under normal conditions undergoes exocytosis 
through the plasma membrane.

A second conclusion is that the beige mutation 
also reduces the synthesis of other lysosomal 
enzyme, since the beige mutant does not have 
elevated glucuronidase microsomal component.

The subcellular fractionation showed a doubling 
in microsomal glucuronidase activity in beige 
mice, but by electrophoretic analysis practically all 
this doubling was due to increase in the L compo-
nent which is found in lysosomes. This fact sug-
gests that the higher microsomal glucuronidase in 
beige kidney is a contaminant from broken lyso-
somes rather than a microsomal form of the 
enzyme which is affected by the beige mutation.

After osmotic shock treatment a large fraction 
of glucuronidase is found in the soluble fraction. 
However, by electrophoretic analysis, soluble glu-
curonidase has the same molecular properties as 
normal lysosomal enzyme, supporting the view 
that soluble glucuronidase in kidneys of beige mice 
arises from rupture of the giant lysosomes during 
homogenization. That glucuronidase in the soluble 
fraction arises from broken lysosomes is supported 
by the fact that a large fraction of \( \beta \)-galactosidase, 
another marker enzyme for lysosomes, is found in 
the soluble fraction after homogenization under 
the same conditions used in these experiments (A. 
Lusis, G. Breen, and K. Paigen, manuscript in 
preparation). Also, in mink with the Chediak-
Higashi syndrome, an unusually large amount of 
acid phosphatase is solubilized during homogeni-
zation (41), further emphasizing the fragility of 
lysosomes in animals with this disease.

Together with the biochemical measurements, 
the histological studies show that in the beige 
mice there is a massive accumulation of glucu-
ronidase-containing giant lysosomes after andro-
gen treatment. The majority of the cellular glucu-
ronidase appears concentrated in these granules. 
It also is notable that there is an abrupt change in 
size of these lysosomes in beige kidney across the 
cortex, with the lysosomes near the corticomedul-
ary boundary (S3 region) reaching 10–11 \( \mu \)m in 
diameter. This region contains the distal portion of 
the proximal convoluted tubule. Essner and Oliver 
(4) have recently reported that this same region 
of kidney of beige mice contains numerous large 
polysaccharide-rich granules not present in normal 
mice. One possible reason the lysosomes in kidney 
of beige mice are particularly large here might be 
that cells at the distal portion of the tubule actively 
phagocytose lysosomal enzymes released from 
cells nearer the glomerulus. Alternatively, cells in 
the S3 region may have different rates of turnover 
of lysosomal enzymes.
Our data also suggest that the rate of synthesis of lysosomal enzymes in addition to glucuronidase may be increased in mouse kidney after stimulation by androgens. It is now established that the rate of glucuronidase synthesis is elevated 20- to 50-fold by androgen treatment (36). Because kidney levels of other lysosomal enzymes show little or no increase after androgen administration, it has been assumed that these lysosomal enzymes were not induced. However, the data in Table VI show that the loss by secretion of galactosidase increases about 30-fold and that of hexosaminidase or no increase after androgen administration, it has been assumed that these lysosomal enzymes were not induced. However, the data in Table VI show that the loss by secretion of galactosidase increases about 30-fold and that of hexosaminidase about 5-fold after implantation of testosterone pellets. The most likely mechanism for balancing these greatly increased secretion rates and maintaining constant tissue enzyme levels is a corresponding increase in the rate of enzyme synthesis. The probability that androgens induce a general increase in biosynthesis of many lysosomal enzymes makes mouse kidney an attractive system for studying the molecular aspects of the biosynthesis of this very important organelle.

The molecular abnormality resulting in lowered lysosomal enzyme extrusion from beige kidney cells remains unknown. At least two general alterations are possible: a lowered mobility of the giant lysosomes within the cell, or an abnormal fusion of lysosomes with the plasma membrane during exocytosis. Recent studies have demonstrated that capping of concanavalin A plasma membrane receptors occurs more readily in beige polymorphonuclear leukocytes than in normal leukocytes (22). This phenomenon is consistent with the lysosomal secretion defect we have observed in that it could be due either to an alteration of the plasma membrane in beige mice or to alterations in the microtubular system which presumably controls the intracellular mobility of both the concanavalin A receptors and lysosomes (3, 39). Two studies have shown that intravenously injected horseradish peroxidase is pinocytosed at normal rates in kidney of beige mice but is subsequently hydrolyzed at a much slower rate (5, 30). The slow rate of hydrolysis in beige mice may not be a result of defective protein hydrolysis per se. Rather, there may be a problem of delivery, i.e. either migration of phagosomes and lysosomes within the cell, or fusion of the two types of granules may be defective in the same manner in which we observe a defect in delivery of lysosomal enzymes through the plasma membrane to the kidney tubule lumen.

A decreased rate of delivery of the content of phagocytic vesicles to lysosomes has been described in leukocytes of patients with Chediak-Higashi syndrome (23, 42). It is possible that the increased susceptibility to infection seen in Chediak-Higashi patients and in beige mice results from such a defect. This abnormality may have the same molecular basis as the lowered secretion of lysosomal enzymes from the kidneys of beige mice.

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