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T C Douglas
K A Kimmel
P E Dawson

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GENETICALLY CONTROLLED VARIATION OF "ACID"
β-GALACTOSIDASE DETECTED IN Rattus norvegicus
BY ISOELECTRIC FOCUSING

TOMMY C. DOUGLAS, KATHRYN A. KIMMEL1 AND PATTI E. DAWSON

Medical Genetics Center, The University of Texas
Graduate School of Biomedical Sciences,
P.O. Box 20334, Astrodome Station, Houston, Texas 77025

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ABSTRACT

Two genetically variant forms of rat “acid” β-galactosidase were found to differ in isoelectric point and pH dependence, but not in thermostability or sensitivity to inhibition by p-mercuribenzoate (PMB). The results of two backcrosses and an intercross indicated that the isoelectric focusing phenotypes are controlled by two codominant alleles at a single autosomal locus, for which we propose the name Glb-1. No significant linkage between Glb-1 and albino (LG I), brown (LG II), or hooded (LG VI) was observed. Strain-specific differences in total levels of kidney β-galactosidase were detected, but it is not yet known whether the variation is controlled by genes linked to Glb-1. Experiments in which organ homogenates were incubated with neuraminidase indicated that the genetically variant forms do not result from differences in sialylation, though sialylation does appear to be largely responsible for the presence of multiple bands within each phenotype and for differences in the banding patterns of β-galactosidases derived from different organs. The β-galactosidase present in the bands used for Glb-1 typing resembles human G_{M_1} gangliosidase (GLB1) with respect to pH optimum, substrate specificity, and susceptibility to inhibition by PMB. It also appears that Glb-1 is homologous with the Bgl-e locus of the mouse. In rats as in mice the genetically variant bands of β-galactosidase are active at acid pH and have relatively high isoelectric points. In both species these bands are readily detectable in kidney homogenates, and can be revealed in homogenates of liver or spleen following treatment with neuraminidase. The presence of the same β-galactosidase bands in homogenates of rat kidney and small intestine as well as in neuraminidase-treated homogenates of liver and spleen suggests that the Glb-1 variants differ by one or more point mutations in the structural gene for “acid” β-galactosidase.

ENZYMES capable of hydrolyzing β-galactosidic linkages are widely distributed among mammalian tissues where they play a role in the metabolism of carbohydrates, glycoproteins, glycolipids, and possibly in fertilization (Ma-jumder and Turkington 1974; Gray 1978; O’Brien 1978; Suzuki and Suzuki 1978). The laboratory rat has been used extensively in studies of the

1 Present address: Department of Microbiology and Immunology, The University of Michigan Medical School, Ann Arbor, Michigan 48109.

tissue distribution, subcellular localization, and developmental expression of \( \beta \)-galactosidase, but until now no genetic polymorphism for \( \beta \)-galactosidase has been reported in this species. In this paper we describe the detection of two distinct forms of rat \( \beta \)-galactosidase by isoelectric focusing. Evidence is presented that these variants affect the "acid" lysosomal \( \beta \)-galactosidase and that they are controlled by codominant alleles at a single autosomal locus for which we propose the name \( Glb-1 \).

\section*{Materials and Methods}

\textit{Animals:} Rats were obtained from several sources: Charles River Breeding Laboratories, Wilmington, Massachusetts (BN/Crl), Microbiological Associates, Walkersville, Maryland (ACI/MA, BN/MA, LEW/MA and MAXX/MA inbreds, LEF \( \times \) BN hybrids, and MA Wistar outbreds), Sprague-Dawley, Inc., Madison, Wisconsin (F344 and WF inbreds and Sprague-Dawley outbreds), and Timco Breeding Laboratories, Houston, Texas (BUF/Tex and Wistar/Munich/Tex (WM/Tex) inbreds and Tex: (SD) randombreds). Animals of strain BS were the generous gift of Dr. EBERHARD GUNTHER, Freiburg, West Germany.

Thin layer isoelectric focusing (IEF): Samples were prepared either from freshly dissected tissues or from tissues which had been stored at \(-70^\circ\text{C}\). In early experiments including our initial strain survey, Potter-Elvehjem tissue grinders were used to prepare 50\% W/V tissue homogenates in distilled water. In later experiments kidneys were homogenized using a Brinkmann Polytron homogenizer or sonicated for a total of approximately 30 sec using a Branson model W140D sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) equipped with a micro tip. Homogenates were centrifuged for 2–3 min at 8700 \( \times \) g and titrated approximately to pH 5 (BREEN, LUSIS and PAIGEN 1977) by the addition of 1 M citric acid. Following one hr incubation at 37\(^\circ\text{C}\), precipitated material was removed by centrifugation at 8700 \( \times \) g for 2 min. Thin layer IEF gels (total gel volume 61.5 ml) contained acrylamide (5.58\%), N,N’-methylene bisacrylamide (0.28\%), glycerol (9.9\%), Bio-Rad technical grade carrier ampholytes pH 4.9 (1.76\%) and pH 7-10 (0.2\%), and ammonium persulfate (1.07 mM). Gels were cast on horizontal 125 \( \times \) 260 \( \times \) 1 mm glass plates (LKB Instruments, Inc., Rockville, Md.) under an atmosphere of humidified nitrogen and allowed to polymerize at room temperature for 2 hr. Samples prepared as described above were applied to pieces of Whatman 3MM filter paper which were then placed near the anodal end of the gel. Isoelectric focusing was carried out at 10\(^\circ\text{C}\). Gels were focused across their short dimension for 21/2–3 hr at a constant power of approximately 18 watts for routine typing, or along their long dimension overnight at a constant power of 5 watts for photography or more accurate measurement of isoelectric points. The anodal and cathodal electrode solutions were 1 M H\(_3\)PO\(_4\) and 1 M NaOH respectively. At the termination of the run the pH gradient was measured with a flat surface pH electrode.

Staining: In early experiments, gels which had been soaked for 30 min in 0.5 M citric acid-NaOH buffer, pH 3.5 were stained by overlaying with 5 ml of 0.1 M citrate-NaOH buffer, pH 3.5, containing 1 mg/ml of 4 methylumbelliferonyl-\( \beta \)-D-galactoside (4MUGal; Sigma Chemical Co., Saint Louis, Mo.). When these gels were viewed under long-wave ultraviolet light, fluorescent bands were visible against a dark background after 30 min to one hr of incubation at 37\(^\circ\text{C}\) (BREEN, LUSIS and PAIGEN 1977). Pretreatment of homogenates at pH 5 to remove acid-precipitable material was especially useful in conjunction with this fluorescent staining method. Samples that had not been treated in this way contained large amounts of material that precipitated when the gels were soaked in pH 3.5 buffer and thus interfered with the observation of fluorescent bands.

In later experiments gels were stained using the substrate 5-bromo-4-chloro-3-indolyl-\( \beta \)-D-galactoside (BCIGal; FELTON, MEISLER and PAIGEN 1974). To determine the best conditions for the use of this stain we devised a test tube assay (LOJDA et al. 1973) in which the insoluble reaction product was pelleted by centrifugation, washed in cold distilled water, redis-
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solved in N,N'-dimethyl formamide, and quantitated by spectrophotometry at its absorption maximum of 625 nm. We found that the optimal pH for the use of this substrate is 4.5–5.0, probably because of the pH dependence of the two oxidation reactions required to convert the 5-bromo-4-chloroindoxyl aglycone to the final colored product (HOLT 1958). Based on these results gels were soaked for 30 min in 0.5 M citrate-NaOH buffer, pH 4.5, then stained by overlayering with 10 ml of a solution containing 2.5% (W/V) BCIGal dissolved in N,N'-dimethyl formamide (0.22 ml), McILVAINE's (1921) citrate-phosphate buffer, pH 4.5 (8.84 ml), 0.85% (W/V) NaCl (0.11 ml), spermidine HCl (2.2 mg), and an aqueous solution of 0.05 M potassium ferricyanide and potassium ferrocyanide (0.83 ml). Blue bands appeared after approximately 30 min incubation at 37° and became more intense overnight.

Three experiments in which we attempted to stain gels for β-glucosidase activity by substituting the substrate BCIGlu (5-bromo-4-chloro-3-indolyl-β-D-glucoside) for BCIGal were not successful, probably due to the relative insensitivity of the BCIGlu substrate. The BCIGlu test tube reaction, judged in terms of OD units of product per molecule of enzyme, is approximately 20–25 times less sensitive than the standard PNPGlu assay (see below) whereas the BCIGal reaction has ½ to ¾ the sensitivity of the standard PNPGal assay. The optimal pH for the hydrolysis of BCIGlu was found to be 5.0–5.5.

Photographs of stained thin layer IEF gels were used to measure the distance of each band from the cathodal end of the plate. Isoelectric points (pI) were then determined graphically from a plot of the pH gradient for that particular gel. To estimate the pI values of the major bands of phenotypes A and B, duplicate samples of the two phenotypes were run on each of three thin layer IEF gels. The average pI value for each band, as determined from a single gel, was treated as a single data point. The mean and standard error for each pI value were then calculated.

Neuraminidase treatment: Tissue homogenates (50% W/V in distilled water) were centrifuged for 3 min at 8700 × g, then brought to a concentration of 0.05 M citrate-phosphate buffer by the addition of 10X concentrated buffer. To 0.95 ml of buffered homogenate was added 0.05 ml of citrate-phosphate buffer, pH 5.0 containing 1 unit of Clostridium perfringens neuraminidase (Sigma type IX). This mixture was incubated for 3 hours at 37°, then dialyzed against distilled water overnight at 4°. It was not necessary to add calcium to the reaction mixture since Clostridium perfringens neuraminidase does not have a divalent metal ion requirement. In each experiment a control sample was incubated at 37° in 0.05 M citrate-phosphate buffer, pH 5.0 without neuraminidase. Acid-precipitable material was removed from all samples by centrifugation at 8700 × g for 2 min prior to analysis by thin layer IEF.

Preparative granulated gel isoelectric focusing: Granulated gel flat bed IEF was done essentially as described in LKB Instruments, Inc. Application Note No. 198. The electrode solutions, carrier ampholyte composition and concentration, position of sample application, and method of pH gradient measurement were the same as for thin layer gels. In each run a 1 ml sample of kidney homogenate was applied to an approximately 70 ml bed of washed Sephadex G-75 (“Ultrodex”, LKB Instruments, Inc.). After overnight isoelectric focusing at a constant power of 6 watts and a temperature of 10° the granulated gel bed was divided into sections with a metal grid. Fractions corresponding to the desired pH ranges were pooled, collected into syringe barrels containing plugs of glass wool, and eluted with 0.25 M sucrose, 0.05 M tris(hydroxymethyl)aminomethane (“tris”) buffer, pH 7.5. Eluates were dialyzed overnight against tris-sucrose, pH 7.5, and sterilized by passage through 0.22 micron membrane filters (“Millex”, Millipore Corp., Bedford, Mass.).

Enzyme assays: Assays for β-galactosidase were run for one hour at 37° according to the method of MEISLER and PAIGEN (1972). Control tubes, which were incubated at 37° but to which enzyme was added immediately before termination by TCA, were included for each determination. Results were expressed as OD$_{415}$ (experimental) minus OD$_{415}$ (control) = “corrected OD$_{415}$”. All samples were tested at a dilution such that the resulting OD$_{415}$ values fell into a range in which the assay was linear with respect to enzyme concentration and time. Enzyme activities were calculated as nanomoles of p-nitrophenol liberated per hr per mg protein assuming a molar extinction coefficient of 14,000 for p-nitrophenol (MEISLER and
Protein determinations were made by the method of Lowry et al. (1951) using bovine serum albumin as the standard. To determine the pH dependence of the β-galactosidase present in a given fraction, the pH 3.5 citrate-NaOH buffer which is used in the standard PNPGal assay was replaced with McIlvaine’s citrate-phosphate buffer of the desired pH. Triplicate assays were run at each pH value tested.

Conditions for the assay of β-glucosidase were the same as those for β-galactosidase except that p-nitrophenyl-β-D-glucoside (PNPGlu) was substituted for PNPGal and McIlvaine’s citrate-phosphate buffer, pH 6.0, was substituted for citrate-NaOH, pH 3.5. This assay was also linear with respect to enzyme concentration and time.

**Inhibition of β-galactosidase by p-mercuribenzoate:** Parahydroxymercuribenzoate (PMB) was purchased from Sigma Chemical Co., Saint Louis, Mo. This inhibitor and the related compound p-chloromercuribenzoate which is often employed in studies of β-galactosidase may be used interchangeably because the latter is converted to the hydroxy form by the conventional practice of dissolving it in dilute NaOH. Accordingly, we have adopted the designation p-mercuribenzoate as suggested by Boyer (1954). When PMB was being tested for its ability to inhibit the assay for PNPGal β-galactosidase, it was included in the reaction mixture prior to addition of the enzyme. In PMB inhibition experiments with thin layer IEF gels the inhibitor was present during both the soaking and staining procedures.

**Thermostability:** Thermostability analyses were carried out in dilute phosphate buffer, pH 7.0 as described by Lx and Daniel (1976) except that kidney homogenates were centrifuged at 13,000 x g for one hr. Diluted 13,000 x g supernatants (0.4% W/V) were incubated at 47.5° for 0–30 min with samples being taken at 5 or 6 min intervals. Regression lines were fitted to semilogarithmic plots of the percent remaining activity versus time. The slopes of these lines were then compared by analysis of covariance.

**RESULTS AND DISCUSSION**

**Detection of β-galactosidase variation by isoelectric focusing:** In screening different stocks of rats for β-galactosidase variants we selected kidney as an enzyme source because of the high level of β-galactosidase activity in this organ (Conchie, Findlay and Levey 1959) and the success with which kidney has been used to define an electrophoretic variant of β-galactosidase in the mouse (Breen, Lusis and Paigen 1977). We initially attempted to separate rat

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**FIGURE 1.—**Band patterns of rat kidney and small intestine β-galactosidase following thin layer IEF and staining with BCIGal as substrate. G1b-1 phenotypes are shown at the bottom of the figure. A) The numbers at right indicate the pH gradient in the gel. Mean values ± standard errors for the isoelectric points of the phenotype A bands are 8.05 ± 0.01, 7.85 ± 0.02, 7.52 ± 0.02, 7.26 ± 0.01, and 7.07 ± 0.03. The pI values for bands of phenotype B are 7.98 ± 0.04, 7.71 ± 0.03, 7.35 ± 0.03, 7.12 ± 0.01, and 6.93 ± 0.05. Kidney donors were LEW/MA (A), BN/MA (B), and (LEW × BN) F₁, hybrids (AB). Because of differences in staining intensity on the original gel the images of tracks 3, 4, and 5 were intentionally underexposed during printing. B) The principal band of type A intestine β-galactosidase activity aligns with band 1 of type A kidney (tracks 1 and 2), and the principal band of type B intestine aligns with band 1 of type B kidney (tracks 5 and 6). The focusing patterns of type A and B intestine are contrasted in tracks 3 and 4. Minor bands corresponding to bands 2 and 3 of the kidney pattern are visible in track 5, and bands corresponding to kidney bands 4 and 5 could also be seen with some intestine homogenates. Organ donors were of strains WM/Tex (tracks 1–3) and BN/MA (tracks 4–6).
kidney β-galactosidase isozymes by using polyacrylamide gel electrophoresis procedures similar to those employed by Tomino and Paigen (1970) and by Breen, Lusis and Paigen (1977) for mouse β-galactosidase, but were unable to obtain clear and reproducible banding patterns. In contrast, thin layer IEF in polyacrylamide gels readily resolved a large number of isozyme bands. These bands were separated into two groups, one with isoelectric points between 5.7 and 6.7, and the other with more basic pI values of approximately 6.9 to 8.1. The latter group, which was resolved more clearly in this system, has been used for typing as described below.

Kidney samples from the inbred rat strains ACI/MA, BS, LEW/MA, F344, MAXX/MA, WF, BUF/Tex, and WM/Tex produced similar patterns with five major bands in the basic region of the gels (phenotype A, Figure 1A). Additional minor bands could be observed in some cases. Similar results were also obtained with samples prepared from outbred MA Wistar, Sprague-Dawley, and Tex:(SD) rats. In contrast, the pattern seen with samples from strain BN/MA or BN/Crl (phenotype B, Figure 1A) was shifted so that the major bands were staggered relative to those of phenotype A. Both sets of bands were exhibited by (LEW × BN) F, hybrids.

The five bands used for typing appear to be part of a spectrum of molecular species which have similar enzymatic activities but different isoelectric points. The variable spacing of the major bands is largely an artifact of the pH gradient formed during isoelectric focusing; the gradient produced by this ampholyte mixture tends to be approximately linear from pH 9.2 down to 7.1, shallower from pH 7.1 to 6.7 (causing wider spacing of bands), and steeper from pH 6.7 to 5.8 (causing closer spacing of bands). In such gradients band 5 of phenotype A (pI 7.07 ± 0.03) usually maintains the regular spacing of bands 1-4, but band 5 of phenotype B (pI 6.93 ± 0.05) is often displaced toward the group of bands with more acidic isoelectric points as in Figure 3A. Gels in which the linear portion of the pH gradient extends down as far as pH 6.7 show relatively even spacing of the 5 bands used for typing plus the emergence of a probable "band 6" from the acidic group as in Figure 3B. In some cases a faint band of β-galactosidase activity is also visible above band 1 in both the A and B phenotypes. It is possible that major bands with isoelectric points higher than those of the bands used for typing exist, but are inactivated by exposure to high pH during isoelectric focusing.

The group of five bands that could be seen in virtually all kidney samples was not observed with similarly prepared homogenates of brain, erythrocytes, heart, salivary gland, skeletal muscle, testis, or thymus. These tissues either failed entirely to stain or showed β-galactosidase activity only in the acidic region of the pH gradient. Some samples of liver, lung, and spleen exhibited weak staining in the positions of bands 4 and 5, and homogenates of small intestine had a strong band of activity corresponding to band 1 with much weaker bands at positions 2, 3, 4, and 5 (Figure 1B). As can be seen from Figure 1B, the strong band of activity present in phenotype A intestine aligned with band 1 of type A kidney (tracks 1 and 2) whereas the strong
band of activity in phenotype B intestine aligned with band 1 of type B kidney (tracks 5 and 6).

Identification of the variant as "acid" β-galactosidase: When aqueous homogenates of rat kidney are prepared, the degradative enzymes which are confined within lysosomes in vivo (SHIBKO and TAPPEL 1965; PATEL and TAPPEL 1970) are released due to osmotic shock and mechanical shearing forces. The predominant form of β-galactosidase which is detected in such homogenates with the synthetic substrates 4MUGal or PNPGal is "acid" lysosomal β-galactosidase (FURTH and ROBINSON 1965). This enzyme is maximally active at pH 3-4, does not attack synthetic β-glucosides, and is strongly inhibited by the organic mercurial compound PMB (FURTH and ROBINSON 1965; ROBINSON, PRICE and DANCE 1967; PATEL and TAPPEL 1970). A second kidney lysosomal enzyme, galactosylceramide β-galactosidase (BOWEN and RADIN 1968, MIYATAKE and SUZUKI 1974), is also most active at acidic pH (approximately 4.5), but it accounts for only a small fraction of the total β-galactosidase which is measured with 4MUGal or PNPGal. A third enzyme present in rat kidney and designated "neutral" β-galactosidase is extralysosomal in origin, has a pH optimum of approximately 6, and attacks β-glucosides as well as β-galactosides (ROBINSON, PRICE, and DANCE 1967).

In order to characterize the β-galactosidase present in the bands used for typing as described above, kidney homogenates were fractionated by preparative granulated gel flat bed IEF. Material that had focused in the relatively basic region of the pH gradient was eluted, dialyzed overnight, and sterilized by membrane filtration. The pH-activity curve for the β-galactosidase present in this fraction had an optimum at pH 3-4 and showed no indication of a shoulder at higher pH values which might indicate the presence of an appreciable amount of "neutral" β-galactosidase. Neither this fraction nor a second fraction which had been similarly eluted from the acidic region of the same preparative IEF gradient differed significantly in its pH-activity profile from the unfractionated kidney homogenate prepared from the same donor. This was true whether the fractions were isolated from a rat of type A (Figure 2A) or type B (Figure 2B).

The β-galactosidase present in these high pI fractions also resembled "acid" β-galactosidase with respect to its specificity for the β-galactosidic linkage of PNPGal as opposed to the β-glucosidic linkage of PNPGlu. The ratio of β-galactosidase to β-glucosidase activity in the high pI fractions was greatly increased in comparison with that for crude kidney homogenates (Table 1).

Finally, we found that the β-galactosidase present in the high pI fractions is strongly inhibited by PMB (Figure 2C, D), a compound which inhibits "acid" lysosomal β-galactosidase prepared from rat kidney, liver, or testis (PATEL and TAPPEL 1970; BACCINO, ZURETTI, and PERNIGOTTI 1975; MAJUMDER and TURKINGTON 1974), but not the neutral β-galactosidase ("lactase")

1 Human patients who have a selective deficiency of galactosylceramide β-galactosidase (globoid cell leukodystrophy) maintain normal β-galactosidase levels as determined with 4MUGal or PNPGal whereas patients who have a selective deficiency of "acid" β-galactosidase (G31 gangliosidosis) are deficient with respect to 4MUGal and PNPGal β-galactosidase even though they have normal levels of the galactosylceramide enzyme (SUZUKI and SUZUKI 1978).
which is localized in the intestinal brush borders (Koldovský, Asp, and Dahlqvist 1969; Seetharam et al. 1977). The apparent quantitative difference in PMB inhibition between the crude homogenates and the restricted PI fractions is probably due to the presence in the unfractionated homogenates of higher concentrations of non-β-galactosidase components which also react with PMB.

Effect of neuraminidase treatment on the banding pattern: One possible explanation for the differences in the isoelectric points of the type A and B enzymes is differential sialylation. If this were the case, it should be possible to convert the more heavily sialylated form (lower PI) to the less heavily sialylated form

Figure 2.—pH dependence and PMB inhibition of rat kidney β-galactosidase using PNPGal as substrate. Circles: unfractionated kidney homogenates; triangles, low PI fractions isolated by preparative granulated gel IEF; squares: high PI fractions isolated by preparative granulated gel IEF: closed symbols, Glb-1 phenotype A; open symbols: Glb-1 phenotype B. A) PI ranges of the two fractions were 5.1–6.5 and 7.5–8.1. Maximal activities for the 3 samples represent corrected OD₄₁₅ values of 1.09 (●), 0.61 (▲), and 0.63 (■). B) PI ranges of the two fractions were 4.8–6.6 and 7.4–8.7. Maximal activities for three samples represent corrected OD₄₁₅ values of 1.17 (○), 0.34 (△), and 0.44 (□). C) Restricted PI fractions same as in panel A. Control activities for the three samples represent corrected OD₄₁₅ values of 1.18 (●), 0.67 (▲), and 0.84 (■). D) Restricted PI fractions same as in panel B. Control activities for the three samples represent corrected OD₄₁₅ values of 1.52 (○), 0.34 (△), and 0.35 (□). Kidney donors for these experiments were phenotype A progeny from the LEW × (LEW × BN)F₁ backcross and phenotype B progeny from the BN × (LEW × BN)F₁ backcross. All assay times were 60 minutes.
RAT $\beta$-GALACTOSIDASE

TABLE 1

$\beta$-galactosidase and $\beta$-glucosidase activities of restricted pH fractions isolated from rat kidney by preparative granulated gel IEF

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$\beta$-Galactosidase (X)</th>
<th>$\beta$-Glucosidase (Y)</th>
<th>Ratio (X/Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>3290</td>
<td>699</td>
<td>4.7</td>
</tr>
<tr>
<td>Acidic region (pI 4.5-6.6)</td>
<td>82.7</td>
<td>19.2</td>
<td>4.3</td>
</tr>
<tr>
<td>Basic region (pI 7.4-8.8)</td>
<td>79.4</td>
<td>0.2</td>
<td>397.</td>
</tr>
</tbody>
</table>

Enzyme activities are stated as nanomoles of p-nitrophenol liberated per mg protein per hour. The kidney donor for this experiment was a female of strain WM/Tex. In a second experiment the ratio of $\beta$-galactosidase to $\beta$-glucosidase activity was 36.0 for the high pH (7.5-8.6) fraction as compared with a value of 3.6 based on unfractionated homogenates prepared from donors of the same strain.

(higher pI) by treatment with neuraminidase. Contrary to this explanation, experiments such as the ones shown in Figure 3A showed that treatment of kidney homogenates with Clostridium perfringens neuraminidase did not convert the type B pattern to the type A pattern or vice versa. In comparison with control samples, which had been incubated in the absence of neuraminidase, the treated samples usually produced relatively less staining in the low pH region. In some cases the intensity of band 5 in the high pH region was also reduced. These findings suggested that it might be possible to generate the standard high pH bands from homogenates of organs such as liver or spleen which normally lack them.

The effect of neuraminidase treatment on the banding pattern of liver $\beta$-galactosidase is illustrated in Figure 3B. All five of the characteristic high pH bands which are present in kidney could be seen in neuraminidase-treated liver but none of them was present in the sham-treated liver control. Bands 4 and 5 tended to predominate in neuraminidase-treated liver samples of type A whereas bands 3 and 4 predominated in neuraminidase-treated samples of type B. We presume that this distribution of activity reflects the incomplete removal of sialic acid residues.

A second effect of neuraminidase treatment, which was seen with both type A and B liver samples, was to increase the total amount of staining on the gel. (Compare track 2 in Figure 3B with track 3, which received an identical amount of liver homogenate.) It is possible that neuraminidase treatment releases $\beta$-galactosidase molecules from complexes which would be precipitated out during the low pH treatment or fail to enter the IEF gel. Treatment of spleen homogenates with neuraminidase also generated the full complement of five high pH bands, but it had little effect on the total amount of staining visualized on the gel.

In each case neuraminidase-treated liver or spleen homogenates that had been prepared from rats of phenotype A produced the characteristic A banding pattern whereas neuraminidase-treated liver or spleen homogenates from phenotype B animals produced the B banding pattern. These results demonstrate
FIGURE 3.—The effect of neuraminidase treatment on β-galactosidase banding patterns. Samples marked "N" were treated with neuraminidase; those marked "−" were incubated in the same buffer without neuraminidase. Organ donors were LEW/MA (A) or BN/MA (B). A) The banding patterns of neuraminidase-treated kidney homogenates (tracks 2 and 4) resembled those of control homogenates of the same phenotype (tracks 1 and 3) in the basic region of the pH gradient, but showed weaker staining in the acidic region. B) The neuraminidase-treated LEW/MA liver homogenate in track 3 produced all 5 of the high PI bands characteristic of phenotype A, but the control sample of the same homogenate in track 2 produced none of them. Band 1, which is weak in track 3, may not be visible in the photograph. The strong band near the origin of track 3 and all of the apparent activity in track 2 are due to precipitated protein (opalescent brown), and not to the indigo stain (clear blue) which is found as a result of the hydrolysis of BCIGal. Tracks 1 and 4 are control homogenates of type A kidney.

the effectiveness of the neuraminidase treatment and reinforce the conclusion that the differences between the two phenotypes are not due to differential sialylation.

We have not determined fully the nature of the molecular differences responsible for the occurrence of multiple bands within each β-galactosidase phenotype, though our neuraminidase experiments suggest that this is due largely to differences in sialic acid content. The relative depletion of the low PI bands in neuraminidase-treated samples suggests that these molecules are being converted to forms with higher isoelectric points. Also, the β-galactosidases present
in the low pI and high pI fractions are similar with respect to their pH optima and inhibition by PMB. The differences in the β-glucosidase activities of these two fractions (Table 1) can be readily explained by assuming contamination of the low pI fraction with "neutral" β-galactosidase.

In addition to the processes of glycosylation, deglycosylation and proteolysis to which "acid" β-galactosidase and other lysosomal glycoproteins are subjected in vivo (Goldstone and Koenig 1974a, Needleman, Koenig and Goldstone 1975), other factors may contribute to the formation of multiple isozyme bands within a single β-galactosidase phenotype. Further shifts in isoelectric point, caused largely by the release of degradative lysosomal enzymes, are induced in vitro during the preparation and handling of tissue homogenates. It is highly likely that these autolytic processes are occurring in our experiments since they take place most rapidly under approximately the same conditions which we have employed for the removal of acid-precipitable material (Goldstone and Koenig 1974b). Another factor which could be expected to affect the β-galactosidase banding pattern is the degree of polymerization of the enzyme subunits. The effect of isoelectric focusing on the quaternary structure of β-galactosidase, a variable which is known to depend upon pH and ionic strength (Asp 1970; Lusis, Breen and Paigen 1977; Kuo and Wells 1978), has not yet been determined. Also unknown is the degree to which the β-galactosidase subunits may form complexes with other components, thus causing an apparent change in their isoelectric point.

The isoelectric focusing differences that distinguish the A and B phenotypes can be most simply explained as resulting from one or more point mutations in the β-galactosidase structural gene. The occurrence of the same set of polymorphic bands in neuraminidase-treated homogenates of liver and spleen as well as in untreated homogenates of kidney and small intestine favors this hypothesis. Based upon the banding patterns of kidney homogenates it appears that the primary gene product of phenotype A has a higher isoelectric point than that of phenotype B, but it is difficult to make a definitive judgment about the direction of this shift due to the presence of multiple strong bands within each phenotype. This distinction is clearer when homogenates of small intestine are compared, because most of the β-galactosidase activity extracted from this organ is concentrated in a single band (Figure 1B). The major band of type A intestinal β-galactosidase aligns with band one of the type A kidney IEF pattern (pI 8.05 ± 0.01) whereas the major band of type B intestinal β-galactosidase aligns with band one of the type B kidney pattern (pI 7.98 ± 0.04). The relatively high isoelectric points of the major intestinal bands suggest that these may consist of unsialylated β-galactosidase molecules.

Alternatively, it could be argued that the primary structural difference between the two phenotypes actually affects some other component with which the β-galactosidase subunits form complexes, or that initially identical β-galactosidase subunits can undergo either of two different proteolytic processing steps (Skudlarek and Swank 1979). In the latter case it might be expected that the shorter polypeptide would predominate in phenotype AB animals, a
prediction which is in agreement with our finding that the B bands are often stronger than the A bands in homogenates of type AB kidneys.

Other comparative properties of the phenotype A and B enzymes: To test for possible differences in β-galactosidase thermostability, diluted kidney supernatants prepared from type A or B animals were incubated at 47.5° for 0–30 minutes, then assayed for residual enzymatic activity. As can be seen from Table 2, the type B enzyme of strain BN/MA did not differ from that of the type A enzyme from strains LEW/MA, BUF/MA, WM/Tex, F344, or BS. Comparisons made among two pairs of type A strains, BUF/MA vs WM/Tex and F344 vs BS, also failed to detect any significant differences. Under the same test conditions the thermolabile BGL-EA β-galactosidase variant of the mouse was readily distinguished from the thermostable BGL-EB variant (Table 2). It is possible that other assay conditions might detect thermostability differences between the type A and B rat β-galactosidases (Felton, Meisler and Paigen 1974; Li and Daniel 1976; Lusis, Breen and Paigen 1977; Berger and Lusis 1978), but this is not suggested by the available data.

The β-galactosidases present in type A and B kidney homogenates were also

<table>
<thead>
<tr>
<th>Experiment</th>
<th>First strain</th>
<th>Second strain</th>
<th>Slope</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BN/MA (B)††</td>
<td>LEW (A)</td>
<td>12.0 ± 0.3</td>
<td>13.3 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>BN/MA (B)</td>
<td>LEW (A)</td>
<td>16.1 ± 1.2</td>
<td>16.7 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>BN/MA (B)</td>
<td>LEW (A)</td>
<td>13.7 ± 0.7</td>
<td>13.7 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td>BN/MA (B)</td>
<td>LEW (A)</td>
<td>14.0 ± 1.1</td>
<td>12.8 ± 1.7</td>
</tr>
<tr>
<td>5</td>
<td>BN/MA (B)</td>
<td>BUF (A)</td>
<td>19.0 ± 1.7</td>
<td>20.9 ± 2.2</td>
</tr>
<tr>
<td>6</td>
<td>BN/MA (B)</td>
<td>BUF (A)</td>
<td>17.2 ± 2.6</td>
<td>17.0 ± 1.6</td>
</tr>
<tr>
<td>7</td>
<td>BN/MA (B)</td>
<td>WM (A)</td>
<td>17.2 ± 2.6</td>
<td>12.9 ± 3.4</td>
</tr>
<tr>
<td>8</td>
<td>BUF (A)</td>
<td>WM (A)</td>
<td>17.0 ± 1.6</td>
<td>12.9 ± 3.4</td>
</tr>
<tr>
<td>9</td>
<td>BN/MA (B)</td>
<td>F344 (A)</td>
<td>10.8 ± 0.7</td>
<td>14.0 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>BN/MA (B)</td>
<td>F344 (A)</td>
<td>15.9 ± 0.7</td>
<td>13.0 ± 1.4</td>
</tr>
<tr>
<td>11</td>
<td>BN/MA (B)</td>
<td>BS (A)</td>
<td>15.9 ± 0.7</td>
<td>15.8 ± 1.2</td>
</tr>
<tr>
<td>12</td>
<td>BN/MA (B)</td>
<td>BS (A)</td>
<td>13.0 ± 1.4</td>
<td>15.8 ± 1.2</td>
</tr>
<tr>
<td>13</td>
<td>BN/MA (B)</td>
<td>BS (A)</td>
<td>13.0 ± 0.8</td>
<td>13.6 ± 1.1</td>
</tr>
<tr>
<td>14</td>
<td>C3H/HeJ (A)</td>
<td>BS (A)</td>
<td>13.6 ± 1.1</td>
<td>11.5 ± 2.2</td>
</tr>
</tbody>
</table>

Control experiments with mouse kidney “acid” β-galactosidases

<table>
<thead>
<tr>
<th>Experiment</th>
<th>First strain</th>
<th>Second strain</th>
<th>Slope</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>CBA/J (B)</td>
<td>C3H/HeJ (A)</td>
<td>11.1 ± 1.8</td>
<td>24.7 ± 2.2</td>
</tr>
<tr>
<td>11</td>
<td>CBA/J (B)</td>
<td>C3H/HeJ (A)</td>
<td>13.1 ± 0.7</td>
<td>30.8 ± 1.7</td>
</tr>
<tr>
<td>12</td>
<td>CBA/J (B)</td>
<td>MA/MyJ (A)</td>
<td>12.6 ± 0.9</td>
<td>31.4 ± 1.6</td>
</tr>
<tr>
<td>13</td>
<td>CBA/J (B)</td>
<td>MA/MyJ (A)</td>
<td>11.6 ± 0.9</td>
<td>27.8 ± 2.3</td>
</tr>
<tr>
<td>14</td>
<td>C3H/HeJ (A)</td>
<td>MA/MyJ (A)</td>
<td>24.4 ± 3.7</td>
<td>27.8 ± 2.3</td>
</tr>
</tbody>
</table>

* In each experiment the amount of PNPGal β-galactosidase activity remaining after 0–30 min incubation at 47.5° was determined. Regression lines were fitted to semilogarithmic plots of the percent remaining activity versus time. The slopes of these lines, initially expressed as —log%/minute, were multiplied by 1000 for ease of presentation.

† The Gb-1 phenotypes of the inbred rat strains and BGL-E phenotypes of the inbred mouse strains are indicated in parentheses.
tested for susceptibility to inhibition by PMB (Figure 2C, D). No consistent differences were found.

In reviewing the pH activity curves which are also shown in Figure 2, we noted that the portion of the curves between pH 5 and 7 for samples of phenotype B was shifted to the left by approximately 0.3 pH unit relative to the corresponding portion of the curves for samples of phenotype A. The same pattern was apparent whether the data for crude homogenates, high pI fractions, or low pI fractions were compared. To determine whether this pH shift was reproducible, pH dependence was measured using unfractionated kidney homogenates from six additional donors, three of phenotype A and three of phenotype B. To minimize possible effects of sex or of genes unlinked to those determining the β-galactosidase isoelectric focusing phenotypes we selected (LEW × BN)F₂ females as kidney donors for this experiment. The average magnitude of the shift was 0.2 unit in the second experiment, with the direction of the shift being the same as before. At each pH value tested in the range five to seven all of the phenotype A samples possessed relatively higher activity than did those of phenotype B (Figure 4). We presume that the difference in the pH-activity profiles of the type A and B enzymes results from the same molecular alteration which causes the two β-galactosidase IEF phenotypes to have different isoelectric points.

Type A and B rat strains were also tested for possible differences in their total kidney β-galactosidase levels (Table 3). Adult females of the type B strain BN/Crl had reproducibly higher enzyme levels (p<0.001) than did those of the type A strains WM/Tex or LEW/MA. Preliminary data indicate that strain BN/MA resembles BN/Crl. A smaller but still significant difference (p<0.01) was also seen between the two type A strains. We do not yet know whether rat kidney β-galactosidase levels are controlled by genetic factors linked with those which determine the IEF phenotypes as is the case in the mouse (BREEN, LUSIS and PAIGEN 1977), though preliminary data from the six (LEW × BN)F₂ animals described in Figure 4 are consistent with this hypothesis.

**Genetic control of the β-galactosidase isoelectric focusing phenotypes:**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Pooled</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM/Tex (A)</td>
<td>1250 ± 40(5)</td>
<td>1130 ± 30(5)</td>
<td>1190 ± 30(10)</td>
</tr>
<tr>
<td>LEW/MA (A)</td>
<td>1410 ± 90(4)</td>
<td>1450 ± 90(5)</td>
<td>1430 ± 60(9)</td>
</tr>
<tr>
<td>BN/Crl (B)</td>
<td>1900 ± 150(5)</td>
<td>1980 ± 40(5)</td>
<td>1940 ± 70(10)</td>
</tr>
</tbody>
</table>

* Enzyme activities are stated as nanomoles of p-nitrophenol liberated per mg protein per hour. Probability values given in the text were determined using Student's t test as modified by COCHRAN for the case in which σ₁ and σ₂ are not necessarily equal (SNEDECOR and COCHRAN 1967).

† The Glb-I phenotypes of the inbred rat strains are indicated in parentheses.
order to determine the mode of inheritance of the $\beta$-galactosidase IEF polymorphism (LEW × BN)$F_1$ males were backcrossed to LEW and BN females and intercrossed to (LEW × BN)$F_1$ females. The kidney $\beta$-galactosidase IEF phenotypes of the resulting progeny were then determined (Table 4). Only members of the first litter born to each female were tested. The results of the two backcrosses are in good agreement with the 1:1 ratio and those of the intercross with the 1:2:1 ratio expected if the three $\beta$-galactosidase phenotypes are determined by two codominant alleles at a single autosomal locus. In keeping with the recently propounded recommendations regarding comparative gene nomenclature (SHOWS et al. 1979) we suggest that this locus be desig-

### Table 4

**Segregation of Glb-1 alleles**

<table>
<thead>
<tr>
<th>Mating</th>
<th>Progeny phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\varphi$ $A$ $\sigma$</td>
</tr>
<tr>
<td>A × AB</td>
<td>13 18</td>
</tr>
<tr>
<td>B × AB</td>
<td>0 0</td>
</tr>
<tr>
<td>AB × AB</td>
<td>5 6</td>
</tr>
</tbody>
</table>

The animals mated to produce the above progeny were LEW/MA (A), BN/MA (B), and (LEW × BN)$F_1$ (AB). By chi-square analysis there were no significant deviations from the Mendelian ratios expected on the basis of two alleles at a single autosomal locus.
nated Glb-1 with alleles Glb-1<sup>a</sup> and Glb-1<sup>b</sup> corresponding to the phenotypes A and B, respectively.

Because the LEW strain is homozygous for c, B, and h<sup>o</sup>, and BN is homozygous for C, b, and h<sup>i</sup> (Festing 1978), the crosses shown in Table 4 could also be used to test for linkage between Glb-1 and the genes albino (c, Linkage Group I), brown (b, Linkage Group II), and hooded (h<sup>o</sup>, Linkage Group VI). Chi-square analysis showed that none of the recombination frequencies observed between Glb-1 and albino, brown, or hooded differed significantly (p<0.05) from those expected on the basis of independent assortment (Table 5). Tests for Y chromosome linkage were also negative. The recombination frequencies shown in Table 5 for Glb-1 and hooded were calculated according to the convention that the Irish allele (h<sup>i</sup>) is fully dominant over the hooded allele (h<sup>j</sup>) (Curtis and Dunning 1937). Because we felt that the h<sup>i</sup>h<sup>i</sup> and h<sup>i</sup>h<sup>j</sup> classes could be distinguished in the progeny of the backcross to strain BN and of the intercross, linkage calculations were also made using the assumption that h<sup>i</sup> was behaving as semidominant over h<sup>j</sup>. No significant linkage between Glb-1 and hooded was detected in either case. We therefore lack positive linkage data that would indicate the chromosomal location of Glb-1.

Comparison of the rat β-galactosidase variants with those in other species:
The enzyme that is affected by the Glb-1 polymorphism in rats is clearly homologous with human G<sub>M1</sub> ganglioside β-galactosidase (GLB1). Both of these enzymes have acidic pH optima, specifically attack β-galactosides but not β-glucosides, and are inhibited by PMB (Asp, Dahlqvist and Koldovský 1970; O'Brien 1978). The synthetic substrate BCIGal which we have used for staining thin layer IEF gels is also hydrolyzed by human G<sub>M1</sub> ganglioside β-galactosidase and has in fact been used as a histological stain in the prenatal diagnosis of G<sub>M1</sub> gangliosidosis (Kaback et al. 1973). Unlike the Glb-1 variants of the rat, the known human variants have drastically reduced enzymatic activities. The presence in liver samples from G<sub>M1</sub> gangliosidosis patients of material which lacks enzymatic activity but which is immunologically cross-reactive

### Table 5

<table>
<thead>
<tr>
<th>Mating</th>
<th>Recombination frequency&lt;sup&gt;*&lt;/sup&gt; ± std. error (&lt;i&gt;n&lt;/i&gt;) for Glb-1 and</th>
<th>Recombination frequency&lt;sup&gt;*&lt;/sup&gt; ± std. error (&lt;i&gt;n&lt;/i&gt;) for Glb-1 and</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>albino</td>
<td>brown</td>
</tr>
<tr>
<td>A × AB</td>
<td>54.9±7.0%(51)</td>
<td>——</td>
</tr>
<tr>
<td>B × AB</td>
<td>——</td>
<td>59.1±10.5%(22)</td>
</tr>
<tr>
<td>AB × AB</td>
<td>61.1±8.8%(44)</td>
<td>45.3±10.4%(34)‡</td>
</tr>
</tbody>
</table>

* Recombination frequencies and their standard errors were calculated using the maximum likelihood (Green 1963).
† If h<sup>i</sup> is assumed to be semidominant rather than completely dominant over h<sup>j</sup>, the recombination frequency for Glb-1 and hooded is 54.5±10.6% (<i>n</i>=22) for the B × AB backcross and 52.8±9.1% (<i>n</i>=30) for the intercross.
‡ Due to the occurrence of albino individuals only a portion of the progeny from these crosses could be scored for hooded.
§ There was a significant excess (p<0.05) of brown progeny in the intercross (19 black: 15 brown). This was the only case of distorted segregation observed in these crosses.
with the normal enzyme suggests that a mutation in the structural gene for β-galactosidase has occurred (O'BRIEN 1978). This interpretation is supported by the finding of NORDEN and O'BRIEN (1975) that the electrophoretic mobility of the residual "acid" β-galactosidase activity was altered in one GM1 gangliosidosis patient.

In the mouse two forms of "acid" β-galactosidase which can be identified by either electrophoresis or isoelectric focusing are controlled by codominant alleles at the autosomal locus Bgl-e (BREEN, LUSIS and PAIGEN 1977; PAIGEN 1979). Several observations suggest that Bgl-e and Glb-1 are homologous. In both species the variant β-galactosidases are active at acid pH and are most readily detected in kidney. The isozyme bands used for typing have relatively high isoelectric points, i.e., 6.9–8.1 in the rat and 7.0 and 7.5 in the mouse. These characteristic high PI bands are not detectable in conventionally-prepared spleen or liver homogenates from either species, but can be visualized following treatment of these homogenates with neuraminidase (LUSIS, BREEN and PAIGEN 1977).

An apparent difference between the mouse and rat genetic variants is that the fast and slow Bgl-e variants differ in isoelectric point by an integral number of bands whereas the Glb-1 variants are shifted by a one-half band difference so that the positions of the bands in the A and B phenotypes are staggered relative to one another. However, it is difficult to know what significance to attach to this result due to the uncertainty regarding the molecular differences among the isozyme bands as described above. A second difference is that the Bgl-e variants differ in thermal stability (LI and DANIEL 1976; BERGER and LUSIS 1978) but the rat Glb-1 variants, tested under identical conditions, do not (Table 4).

Although some exceptions have been observed (YOSHIDA 1978), the linkage maps of the rat and mouse are probably very similar (WOMACK and SHARP 1976). Thus, the known linkage relationships of Bgl-e probably offer the best guide for mapping Glb-1. Our preliminary experiments with the rat stocks described above have not detected any clear-cut genetic polymorphisms for the possible rat homologues of the mouse genes Lap-1 (leucine arylaminopeptidase), Thy-1 (thymus cell antigen-1), Mpi-1 (mannosephosphate isomerase) or Mod-1 (malic enzyme, supernatant) which are known to be linked to Bgl-e on chromosome 9 (FELTON, MEISLER and PAIGEN 1974; BREEN, LUSIS and PAIGEN 1977; DOUGLAS, MEO and SKARVALL 1978). VAN ZUTPHEN et al. (1981) have reported a possible rat homologue of Lap-1, though there are indications that the organ distribution of the enzyme is different in the two species. A rat serum protein variant described by BENDER and GÜNThER (1978) initially appeared to be homologous to the chromosome 9 marker Trf (trans-
ferrin) of the mouse, but subsequent data indicate that this is not the case (Bender, Cleve and Günther 1981). Of particular interest are the murine loci Bgl-s and Bgl-t which appear to regulate the level of β-galactosidase by controlling its rate of synthesis, and which are closely linked to Bgl-e (Berg-er, Paigen and Meisler 1978). It is not yet known with certainty whether genetic factors homologous with Bgl-s and Bgl-t are present in the rat or whether such factors will prove to be part of a β-galactosidase genetic complex (Paigen 1979). Experiments along this line are currently in progress.

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