Speckhard, D. C., Wu, F. Y.-H., & Wu, C.-W. (1977) *Biochemistry 16*, 5228.
Summers, A. O., & Silver, S. (1972) *J. Bacteriol. 112*, 1228.
Wu, C.-W., Wu, F. Y.-H., & Speckhard, D. C. (1977) *Biochemistry 16*, 5449.
Wu, F. Y-H., & Wu, C.-W. (1973) *Biochemistry 12*, 4343.
Wu, F. Y.-H., & Wu, C.-W. (1981) *Adv. Inorg. Biochem. 3*, 143.
Wu, F. Y.-H., & Wu, C.-W. (1983) *Met. Ions Biol. Syst. 15*, 157.
Yarbrough, L. R., & Wu, C.-W. (1974) *J. Biol. Chem. 249*, 4079.

Elevated Levels of Erythrocyte Hypoxanthine Phosphoribosyltransferase Associated with Allelic Variation of Murine *Hprt*†

Gerald G. Johnson,*,‡ Teresa A. Larsen,‡,§ Patricia Blakely,‡ and Verne M. Chapman∥

Department of Biology and the Molecular Biology Institute, San Diego State University, San Diego, California 92182, and Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, New York 14263

Received January 8, 1985

ABSTRACT: Murine stocks with wild-derived hypoxanthine phosphoribosyltransferase (HPRT) A alleles (*Hprt a*) have erythrocyte HPRT activity levels that are approximately 25-fold (*Mus musculus castaneus*) and 70-fold (*Mus spretus*) higher than those of laboratory strains of mice with the common *Hprt b* allele (*Mus musculus*: C3H/HeHa or C57Bl/6). Since the purified HPRT A and B enzymes have substantially similar maximal specific activities (64 and 46 units/mg of protein, respectively), we infer that these HPRT activity levels closely approximate the relative levels of HPRT protein in these cells. Red blood cells of HPRT A and B mice have similar levels of adenine phosphoribosyltransferase activity (APRT; EC 2.4.2.7) and reticulocyte percentages, which suggests that the elevated levels of HPRT in erythrocytes of HPRT A mice are not secondary consequences of abnormal erythroid cell development. The HPRT activity levels in reticulocytes of HPRT B mice are approximately 35-fold higher than the levels in their erythrocytes and approach the HPRT activity levels in reticulocytes of HPRT A mice. Thus, the marked differences in the levels of HPRT protein in erythrocytes of HPRT A and B mice result from differences in the extent to which the HPRT A and B proteins are retained as reticulocytes mature to erythrocytes. The substantial and preferential loss of HPRT B activity from reticulocytes is paralleled by an equivalent loss of HPRT immunoreactive protein (i.e., CRM) from that cell, and we infer that the HPRT B protein is degraded or extruded as reticulocytes mature to erythrocytes. In studies to be reported elsewhere, we provide evidence that the differences in the levels of HPRT in erythrocytes of HPRT A and B mice are specified by the HPRT structural gene (G. G. Johnson and V. M. Chapman, unpublished results). Thus, the HPRT protein structure is identified as an important factor in determining its susceptibility to turnover in murine erythroid cells.

There is a single hypoxanthine phosphoribosyltransferase (HPRT)[1] structural gene in the mammalian X chromosome (Seegmiller et al., 1967; Miller et al., 1971; Chapman & Shows, 1976). This locus has been widely used as a model system for studies to detect factors that alter gene expression in mammalian somatic cells in culture [Sharp et al., 1975; reviewed by Caskey & Kruh (1979)], since only a single copy of the HPRT gene is expressed in mammalian somatic cells (Rosenbloom et al., 1967; Migeon et al., 1968; Salzmann et al., 1968) and cell culture media are available that can select for either HPRT-plus or HPRT-minus phenotypes in cells (Hakala & Taylor, 1959; Brockman, 1960; Szybalski et al., 1962; Littlefield, 1964).

Few studies, on the other hand, have examined factors that lead to the estimated 200-fold variation in the specific activities of this enzyme in the diverse normal somatic cell types in vivo (Kelley et al., 1969). The reason for the rarity of studies of somatic cell determinants of HPRT expression appears to be that most recognized variants of HPRT represent mutations of the HPRT structural gene which result in the virtual absence of HPRT activity in all cell types (e.g., Lesch–Nyhan syndrome in humans) (Wilson et al., 1983).

We detected electrophoretically distinct forms of HPRT in samplings of feral murine populations which appear to differ from the enzyme deficiency variants described in humans, in that they are common in the populations in which they occur and they are not associated with any obvious deleterious phenotypic effect (Chapman & Shows, 1976; Chapman et al., 1983; G. G. Johnson and V. M. Chapman, unpublished results). In this report, we describe results of studies that compare the levels of HPRT in tissues of mice expressing the wild-derived murine *Hprt a* allele(s) (from *Mus musculus*

† Supported by Grants GM-32471 (to G.G.J.) and GM-24125 (to V.M.C.) from the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service, and by a Biomedical Research Grant to San Diego State University.

‡ San Diego State University.
§ Present address: Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90024.
∥ Roswell Park Memorial Institute.

[1] Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); *Hprt*, the structural gene of HPRT; APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); EDTA, ethylenediaminetetraacetic acid; P-Rib-PP, 5-phosphorylribose 1-pyrophosphate; PEI, poly(ethylenimine); SDS, sodium dodecyl sulfate.

0006-2960/85/0424-5083$01.50/0

castaneus, a feral house mouse from Thailand, and *Mus spretus*, an aboriginal *Mus* species of Europe) to that in tissues of mice expressing the *Hprt b* allele common in inbred strains of mice.

Materials and Methods

Buffers. The buffers used are identified by the following letters in the text: buffer A, 10 mM potassium phosphate, pH 6.8, 0.25 M NaCl, 0.01% Triton X-100, 15% sucrose, 10 mM dithiothreitol, and 1 mg/mL bovine serum albumin (crystalline); buffer B, 20 mM imidazole hydrochloride, pH 7.4, and 0.25 M sucrose; buffer C, 1 mM potassium phosphate, pH 7.9, 0.1 mM EDTA, and 0.1 mM dithiothreitol; buffer D, 10 mM potassium phosphate, pH 6.5, 1 mM EDTA, and 1 mM dithiothreitol; buffer E, 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM dithiothreitol, and 0.25 M NaCl.

Mice. Mice of the inbred strains C57Bl/6J and C3H/HeHa, which carry the *Hprt b* allele, were from colonies maintained at the Jackson Laboratory (Bar Harbor, ME) or at the Roswell Park Memorial Institute. *Mus musculus castaneus* is a feral house mouse from Southeast Asia. The stock used in this study was derived from mice trapped in Bangkok, Thailand, and shipped to Roswell Park in 1973. They have been maintained as an outbred stock avoiding brother–sister matings since that time with 10–15 breeding pairs per generation. Each breeding pair contributes only two individuals to the next generation to minimize founder effects and inbreeding. *M. spretus* is an aboriginal mouse species from Southern France, Spain, and Morocco. The stock used in this study was derived from samples trapped in France by Francois Bonhomme (Labatoire d'Evolution des Vertebres, Paleontologie et Genetique, Faculte des Sciences, Montpellier, France) and in Spain by Richard Sage (Department of Biochemistry and Museum of Vertebrate Zoology, University of California, Berkeley, CA). They have been in the laboratory since 1978 and maintained as an outbred stock similar to *M. m. castaneous*.

In addition to these stocks, we have analyzed males in which the *M. m. castaneus Hprt a* allele has been introduced into the C3H/HeHa genetic background ($N = 5$) and in which the *M. spretus Hprt a* allele has been introduced into the C57Bl/6J background ($N = 4$). The viability and fertility of progeny obtained from matings of these wild-derived mice with the inbred strains of mice has been obtained (G. G. Johnson and V. M. Chapman, unpublished results).

Normal and Reticulocyte-Rich Whole Blood Samples and Analyses. Whole blood samples, typically 50 μL, were collected from the retroorbital plexus into 40 μL of 0.25 M EDTA(Na), pH 7.5, or into heparinized capillaries. Hemoglobin concentrations were determined by the formation of cyanomethemoglobin (using an extinction coefficient of 0.689 mg/(L·cm^2) at 540 nm; Drabkin & Austin, 1935), and reticulocytes were detected by using New Methylene Blue (Davidson & Nelson, 1974). Reticulocyte percentages were based on counts of 1000 cells. The whole blood samples were diluted in buffer A and were used directly for assays of HPRT and APRT activity in erythroid cells (see later).

Reticulocyte-rich whole blood samples were obtained on day 7 following intraperitoneal injections of 0.8 mg of neutralized phenylhydrazine (0.4% in 50 mM $NaHCO_3$) on day 1 at 5 p.m. and on day 2 at 9 a.m. and 5 p.m. (Spivak et al., 1972). For mice 10 weeks of age or older, the quantity of phenylhydrazine administered was increased to 1.2 mg/dose.

Tissue Samples. Homogenates of brain, kidney, liver, and spleen tissues, approximately 5–10% (w/v) in buffer B, were prepared by using a polytron homogenizer.

HPRT and APRT Enzyme Assays. Assays for HPRT and APRT activities in whole blood samples and in tissue homogenates were carried out in 50 μL of a solvent containing 0.1 M sodium glycine, pH 10, 30 mM $MgCl_2$, 5 mM dithiothreitol, 0.1% Triton X-100, 1 mM 5-phosphorylribose 1-pyrophosphate (P-Rib-PP), and either 80 μM [^{14}C]hypoxanthine (for HPRT) or [^{14}C]adenine (for APRT) (10 μCi/μmol). The incubations were initiated by addition of 10 μL of the enzyme fraction diluted in buffer A and were carried out at 37 °C for 15–30 min. The reactions were terminated by the addition of 10 μL of 0.25 M EDTA, and labeled nucleotide product was collected on DE-81 filter disks (Whatman) as previously described (Johnson & Littlefield, 1979). The assays of these enzymes in the immunotitration studies were identical with the conditions described above except that the $MgCl_2$ concentration was 5 mM. One unit of activity is defined as the quantity of enzyme that converts 1 μmol of its purine base substrate to nucleotide per minute.

The plasma fraction of murine whole blood contains no detectable HPRT or APRT activity or inhibitors or stimulators of either enzyme, and therefore, the assays of these enzymes in whole blood samples directly reflect the levels of the enzymes in erythroid cells (Blakely, 1980).

Isoelectric Focusing in Agarose Gels. Gels, 10 × 10 × 0.1 cm, containing 1% agarose (IsoGel agarose, FMC Corp.), 10% sucrose, and 1% pH 5–7 and 0.25% pH 3.5–10 ampholytes (LKB), were cast onto GelBond film as described by the manufacturer (FMC Corp., Rockland, ME). Following overnight storage at 4 °C, the gels were warmed to room temperature, blotted with Whatman No. 1 paper, and prefocused for 10 min at 200 V.

HPRT samples were diluted in a solution containing 10 mM potassium phosphate, pH 6.8, 0.25 M sucrose, 10 mM dithiothreitol, 2% pH 5–7 ampholyte, and 5 mg/mL bovine serum albumin. The diluted whole blood samples were frozen and thawed (4 times), and then all samples were incubated at 37 °C for 2 h. Samples containing approximately 10 microunits of HPRT activity in 5 μL were applied to the horizontal slab gel with an applicator mask (LKB) and electrofocused at 500 V for 90 min with cooling to 4 °C.

Following electrofocusing, the gel was blotted with a sheet of Whatman No. 1 filter paper and the gel surface covered with 1 mL of a solution containing 0.5 M sodium glycine, pH 10, 50 mM $MgCl_2$, 1% Triton X-100, 10 mM dithiothreitol, 10 mM P-Rib-PP, and 50 μM [^{14}C]hypoxanthine (50 μCi/μmol). The gel and overlying incubation solution were then placed in contact with a thin-layer sheet of cellulose–PEI (Baker) and incubated at 37 °C for 30 min. The cellulose–PEI sheet was then rinsed in tap water, and radioactively labeled inosine monophosphate product was detected by radioautography.

Acrylamide Gel Analyses. Analyses in acrylamide gels containing sodium dodecyl sulfate and in gels containing 8 M urea were performed as previously described (Laemmli, 1972; Johnson et al., 1982).

HPRT Purification. Mice were sacrificed by cervical dislocation, and the brain tissue was added to 9 volumes of buffer B on ice and homogenized in a polytron homogenizer. The homogenates from brains of C3H/HeHa (122 animals; HPRT B) and *M. m. castaneus* (47 animals; HPRT A) were separately pooled and stored frozen at −20 °C. All subsequent purification procedures were carried out at 0–4 °C unless otherwise specified.

The homogenates were thawed and centrifuged at 18000*g* for 60 min, and the pelleted material was discarded. The

Table I: HPRT Activities in Tissue Homogenates of HPRT A and B Mice[a]

stock	HPRT allele	HPRT activity (microunits/mg of tissue) brain	kidney	liver	spleen
C3H/HeHa	*b*	544	336	391	606
	b	576	314	389	652
M. m. castaneus	*a*	1184	413	662	476
	a	1165	491	666	584
M. spretus	*a*	1411	494	611	923
	a	1400	425	576	846

[a] Tissues were disrupted with a polytron homogenizer at approximately 5–10% tissue wet weight in 50 mM imidazole hydrochloride, pH 7.4, with 0.25 M sucrose. Values are reported as HPRT activities per wet weight of tissue for two animals of each stock.

supernatant fraction was adjusted to pH 5 with 1 M CH_3C-OOH and centrifuged at 1500*g* for 5 min, and the pelleted material was discarded. The pH 5 supernatant fraction was adjusted to pH 7.9 with 1 N KOH. These preliminary fractionation steps follow closely the methods developed for purification of HPRT from murine liver (Hughes et al., 1975).

For each 100 mL of pH 5 supernatant fraction, approximately 10 mL of packed DEAE-Sephacel (equilibrated in buffer C) and sufficient additional buffer C (approximately 100 mL) was added to achieve adsorption of approximately 90% of the HPRT activity. This mixture was stirred for 60 min and the DEAE-Sephacel collected by centrifugation at 1000*g*. The DEAE-Sephacel was rinsed with buffer C, recentrifuged, and then poured into an appropriate size column (a disposable plastic syringe). The HPRT activity was eluted from this column with a linear salt gradient of 0–0.2 M NaCl in buffer C (100 mL of gradient per 10-mL column). The HPRT activity peak fractions from the DEAE-Sephacel eluate were brought to 70% saturation with solid ammonium sulfate [472 mg of $(NH_4)_2SO_4$/mL], and the precipitated material was collected by centrifugation at 1500*g* for 10 min. The ammonium sulfate pelleted material was dissolved in 3 mL of buffer D and dialyzed against that buffer.

The dialyzed samples were diluted in buffer D to approximately 0.2 unit of HPRT/mL, $MgCl_2$ and P-Rib-PP were added to final concentrations of 5 and 1 mM, respectively, and the samples were incubated at 37 °C for 15 min followed by heating to 85 °C. The samples were maintained at 85 °C for 15 min and quickly cooled in an ice–water bath. The precipitated material was removed from this fraction by centrifugation at 10000*g* for 30 min. $MgSO_4$ was added to the supernatant fractions to achieve a final concentration of 20 mM, and HPRT was precipitated by the addition of ethanol to a final concentration of 40%. These samples were centrifuged at 1500*g* for 10 min and the supernatants discarded. The ethanol pellets were extracted with 0.1–0.2 mL of buffer E (at least 3 times), and those fractions with high HPRT activity levels were pooled. HPRT in the pooled extracts were reprecipitated by the addition of an equal volume of ethanol, and this pellet was extracted twice, first with 0.2 mL and then with 0.1 mL of buffer E. The extracts of the second ethanol precipitation were combined and centrifuged through linear 5–20% sucrose gradients in buffer E as described previously (Johnson et al., 1982). Fractions from the sucrose gradients were collected from the bottoms of the centrifuge tubes and were stored at −70 °C.

Immunization and Immunotitration Studies. Immunization followed the procedure of Wahl et al. (1975). For the initial immunization, 23 μg of purified murine HPRT B protein (fraction VIII, Table III) in 0.4 mL of complete Freund's adjuvant was injected into the rear footpads of a female rabbit. Two months later, a single booster dose of 37 μg of HPRT [containing approximately equal quantities of purified HPRT A and B proteins (fraction VIII, Table III)] in 0.75 mL of incomplete Freund's adjuvant was injected into the rear footpads. Peak anti-murine HPRT was detected 2 weeks following injection of the booster dose, and remained at comparable levels for an additional 6 weeks. The serum sample used in this study was fractionated over a DEAE-Sephacel column, which enriches for the γ-globulin fraction of antibody and removes rabbit HPRT activity (Elder & Johnson, 1983).

Immunotitration experiments were conducted as described previously, except that the incubations of murine HPRT with rabbit serum were shortened to 30 min, while the incubations with goat antibody to rabbit γ-globulin were shortened to 60 min (Elder & Johnson, 1983). The specificity of the immune serum used in these studies was indicated by the following: (1) absence of HPRT precipitating activity in the preimmune serum; (2) absence of APRT precipitating activity in the immune serum (data not shown). For comparisons of HPRT activity and immunoreactivity (i.e., CRM), the quantity of HPRT activity precipitated per volume of immune serum was estimated by averaging points from a titration curve where the quantities of HPRT activity precipitated are approximately proportional to the volume of antiserum added (see legend to Figure 3).

Results

We compared the activities of HPRT in tissues of mice expressing *Hprt a* and *b* alleles to ascertain whether tissue-specific differences in expression of these alleles might be evident. As shown by the data summarized in Table I, the activities of HPRT appear slightly higher in tissues of HPRT A mice, yet the increases are not large, ranging from the nearly indistinguishable or slightly elevated levels in some tissues (kidney, liver, and spleen) to 2–3-fold higher levels in brain. In striking contrast, the erythrocyte HPRT activities in mice that express the *Hprt a* allele are approximately 25-fold (*M. m. castaneus*) and 70-fold (*M. spretus*) greater than in mice expressing the *Hprt b* allele (*M. musculus*: C57Bl/6 and C3H/HeHa) (Table II).

We have previously shown that the murine HPRT A and B enzymes are separable by electrophoresis, and we have provided evidence that these enzymes are encoded as structural gene alleles in the murine X chromosome (Chapman et al.,

Table II: HPRT and APRT Activities in Whole Blood Samples of HPRT A and B Mice[a]

stock	*N*	HPRT (allele)	HPRT activity	APRT activity	reticulocytes
C57Bl/6J	8	*b*	105 (81–126)	44 (39–51)	1.2 (0.2–4.3)
C3H/HeHa	6	*b*	105 (82–132)	100 (93–109)	1.7 (0.4–3.6)
M. m. castaneus	6	*a*	2542 (2327–2906)	45 (35–60)	0.9 (0.3–1.7)
M. spretus	8	*a*	7087 (4873–8646)	41 (16–69)	3.1 (0.8–7.0)

[a] HPRT and APRT activities (units of microunits per milligram of hemoglobin) were determined as described and are reported relative to hemoglobin concentrations in the whole blood samples (Materials and Methods). Reticulocytes were determined as described (Materials and Methods), with percentages based on counts of 1000 cells. Values are reported as averages, with the ranges for the individual determinations in parentheses. *N* = number of animals analyzed.

Table III: Purification of HPRT from Murine Brain Tissue[a]

fraction		HPRT B vol (mL)	HPRT B activity (units/mL)	HPRT B sp act. (units/mg)	HPRT A vol (mL)	HPRT A activity (units/mL)	HPRT A sp act. (units/mg)
I	homogenate	520	0.070	0.0054	178	0.136	0.012
II	S18	425	0.062	0.014	149	0.105	0.023
III	pH 5 supernatant	435	0.034	0.025	161	0.061	0.047
IV	DEAE-Sephacel	40	0.208	0.219	40	0.190	0.704
V	70% $(NH_4)_2SO_4$	15	0.760	0.384	15	0.467	0.805
VI	heated supernatant	52	0.184	4.60	51	0.133	≥13.3
VII	40% ethanol	0.3	27.5	8.3	0.3	20.4	14.0
VIII	sucrose gradient	2.0	3.3	46.1	2.0	2.51	64.2

[a] Brain tissue was obtained from C3H/HeHa (HPRT B) and *M. m. castaneus* (HPRT A) mice and purified as described (Materials and Methods). HPRT activity is reported in units of micromoles per minute, with specific activities based on units of activity per milligram of protein determined by the method of Lowry et al. (1951).

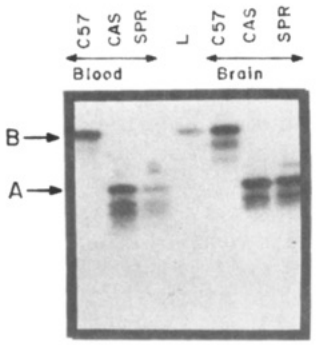

FIGURE 1: Isoelectric focusing of HPRT from erythrocytes and brain tissue of HPRT A and B mice. Whole blood samples were freshly obtained in heparinized capillaries. The brain tissue samples were supernatant fractions (40000*g* for 30 min) of homogenates (10% wet weight per volume in 10 mM potassium phosphate, pH 6.8, 0.25 M sucrose, and 10 mM dithiothreitol) stored at −70 °C. The sample for the mouse cell culture line [L(TK)−] was prepared as the tissue samples, except that cell lysis was achieved by freezing and thawing. These samples were then diluted, preincubated, electrofocused, and assayed for HPRT activity as described (Materials and Methods). The arrows identify the major isoelectric forms of HPRT observed in HPRT A and B mice. We have previously shown that there is a single isoelectric form of HPRT in L cells that electrofocuses at a pH of approximately 6.6 (Johnson et al., 1979), and this is indistinguishable from the major form of the enzyme in tissues of HPRT B mice. The HPRT A enzymes electrofocus at a pH of approximately 6.3, slightly anodally to the human enzyme (Johnson et al., 1979; Johnson et al., 1982; unpublished observations). The reasons for the occurrence of minor satellite bands of HPRT activity in the tissue samples has not been investigated. Samples from kidney, liver, and spleen tissues of these mice have isoelectric focusing patterns that are indistinguishable from those observed for the enzyme from brain tissue (data not shown). The samples are designated C57 (C57Bl/6J), CAS (*M. m. castaneus*), and SPR (*M. spretus*). Only the area of the gel with detectable HPRT activity is shown.

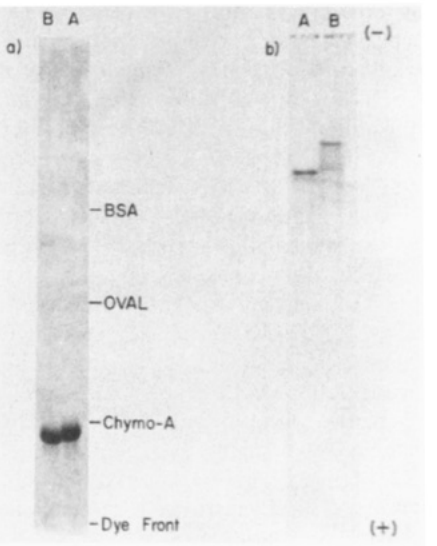

FIGURE 2: Sodium dodecyl sulfate– and 8 M urea–isoelectric focusing acrylamide gel analysis of purified HPRT A and B proteins. (a) Approximately 15 μg of each of purified HPRT A and B proteins (fraction VIII, Table III) were analyzed in SDS–acrylamide slab gels as described previously (Laemmli, 1970; Johnson et al., 1982). Molecular weight standards analyzed in adjacent lanes were bovine serum albumin (BSA), ovalbumin (OVAL), and chymotrypsinogen A (Chymo-A). (b) Approximately 7 μg each of the purified HPRT A and B proteins (fraction VIII, Table III) were analyzed in 7.5% acrylamide gels containing 8 M urea and 2% pH 6–8 and 0.25% pH 3.5–10 ampholytes (LKB) as described previously (Johnson et al., 1982). The protein from the purified fractions was precipitated with 2 volumes of ethanol and dissolved in a solution containing 8 M urea, 10 mM EDTA, pH 7.5, and 10 mM dithiothreitol prior to electrofocusing. The 0–19-cm segment of the gel is displayed.

1983). We show here that there are single major isoelectric forms of HPRT in erythroid cells of HPRT A and B mice and that these major isoelectric species are the same as those observed in the other tissues of these mice (Figure 1; see the legend to Figure 1). This result is consistent with previously published genetic and molecular evidence that there is a single HPRT structural gene in the mouse X chromosome (Chapman & Shows, 1976; Melton et al., 1984) and argues against an interpretation that the HPRT A mice differ in the occurrence of structural gene copies which are uniquely expressed in erythroid cells.

Further evidence that these differences in erythrocyte HPRT activity levels are specifically associated with the expression of the *Hprt a* and *b* alleles rather than genetic differences that affect erythroid cell development is provided by the additional observations that erythroid cells of these mice have (1) comparable levels of activity of another purine salvage enzyme, APRT, and (2) similar percentages of reticulocytes (Table II).

Two lines of evidence suggest that the increases in HPRT activity in erythroid cells of HPRT A mice are due to increases in the numbers of enzyme molecules rather than catalytic differences between the HPRT A and B enzymes. First, as indicated previously, the levels of HPRT activity in tissues other than erythroid cells of HPRT A and B mice differ by a factor of 3-fold or less (Table I). More directly, the HPRT enzymes purified to apparent homogeneity from brain tissue of HPRT A and B mice (Figure 2) are observed to have substantially similar maximal specific activities (Table III).

The purified HPRT enzymes have an estimated subunit molecular weight of approximately 25,000 daltons, as expected for murine HPRT (Hughes, Wahl and Capecchi, 1975; Konecki et al., 1982), and the HPRT A and B subunits appear indistinguishable by this criterion (Figure 2). On the other hand, the difference in the isoelectric charges of the HPRT A and B subunits, revealed in the 8 M urea–acrylamide gels

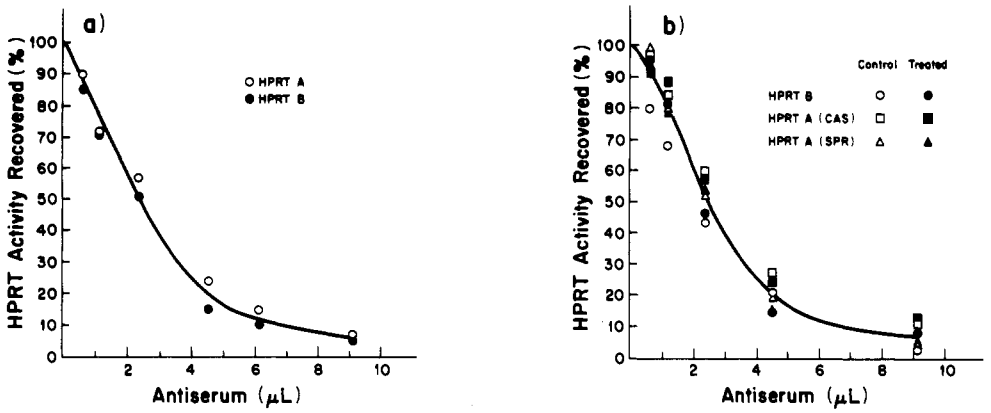

FIGURE 3: Immunotitrations of HPRT. Immunotitration experiments were conducted by using equivalent quantities of HPRT activity (28 microunits) for the samples to be analyzed (Materials and Methods). (a) Titrations of purified HPRT A and B proteins (fraction VIII, Table III). (b) Titrations of HPRT from normal blood samples [control (open symbols)] and reticulocyte-rich whole blood samples [phenylhydrazine-treated mice (filled symbols)] of mice expressing the *Hprt b* (C57Bl/6J), *Hprt a* (CAS) (*M. m. castaneus*), and *Hprt a* (SPR) (*M. spretus*) alleles (Table IV). The immunoreactivities for the purified enzymes were estimated to be 6.5 and 6.9 microunits of HPRT activity precipitated per microliter of antiserum for the HPRT A and B enzymes (part a), respectively, and did not differ substantially from the erythroid cell samples (part b). Additional titrations of two to three erythroid cell samples of each mouse stock and for each condition (control and reticulocyte rich) gave comparable estimates of the immunoreactivities of HPRT (data not shown).

(Figure 2), is consistent with the observed isoelectric point differences between the catalytically active forms of the HPRT A and B enzymes (Figure 1).

The HPRT A and B enzymes retain nearly complete catalytic activity following heating at 85 °C for 15 min in the presence of the Mg-P-Rib-PP cofactor (Materials and Methods; Table III), and this relatively high thermal stability is exploited in their purification. It is not known at this time whether these enzymes have differential thermal stabilities or whether such differences might relate to differences in the turnover rates of these proteins in vivo.

Finally, we note that the maximal specific activity of the HPRT B enzyme purified from brain tissue in the present study appears higher than that reported for the HPRT B enzyme purified from mouse liver (46 vs. ≥3 μmol min^{-1} mg^{-1}) (Hughes et al., 1975). As evidence indicates that these authors had also obtained a homogeneous preparation of HPRT, we believe that this discrepancy may be reconciled as differences in the enzyme or protein assays or as a reduced activity resulting from the reported instability of HPRT purified by affinity chromatography (Hughes et al., 1975). The purified HPRT A and B enzymes appear stable as isolated (Table III, fraction VIII) (with no apparent loss of activity in 1 year) and are estimated to have maximal catalytic activities slightly higher than the human HPRT enzyme when assayed under identical conditions (46 vs. 25 μmol min^{-1} (mg of protein)$^{-1}$; Johnson et al., 1982).

To explore the possibility that these differences in HPRT activity in erythroid cells of HPRT A mice are due to increased levels of the enzyme in precursors of the circulating erythrocytes or due to differential losses of the HPRT A and B enzymes as erythroid cells mature, we compared the levels of HPRT activity in reticulocyte-rich sample of these mice. As summarized in Table IV, the levels of HPRT activity in reticulocyte-rich samples of HPRT B mice are approximately 35-fold higher than in normal whole blood samples and approach the levels of HPRT activity in reticulocyte-rich samples of HPRT A mice (differing by a factor of 2-fold or less when compared to that of *M. m. castaneus*). Thus, the major differences in the HPRT activity levels in erythrocytes of HPRT A and B mice must result from differences in the extents to which the HPRT A and B enzymes are retained as reticulocytes mature to erythrocytes. The persistence of the approximate 2–3-fold higher levels of HPRT activity in reticulocytes of *M. spretus* when compared to that of *M. m. castaneus* parallels the differences in their levels in erythrocytes (Tables II and IV; see Table IV footnote), and is consistent with an interpretation that HPRT is expressed at higher levels in nucleated erythroid cell precursors of reticulocytes in *M. spretus* than in *M. m. castaneus*. However, this difference between the two HPRT A stocks could be accounted for by alternative explanations, and this subject will be considered further under Discussion.

Finally, we have compared the levels of HPRT activity and immunoreactivities (i.e., CRM) using an anti-murine HPRT antibody prepared against the purified HPRT A and B proteins (Materials and Methods). As indicated by the data shown in Figure 3a, the immunoreactivities of the purified HPRT A and B enzymes are indistinguishable. Moreover, the im-

Table IV: HPRT and APRT Activities in Normal and Reticulocyte-Rich Whole Blood Samples[a]

mouse stock	enzyme activities: control HPRT	control APRT	treated HPRT	treated APRT	reticulocytes (%)
C57Bl/6J	104	35	4042	195	81
	103	26	3325	179	87
			4065	213	87
C3H–*Hprt a* (*M. m. castaneus*)	2512	27	5512	162	57
	2789	27	6377	158	53
C57–*Hprt a* (*M. spretus*)	9248	21	13544	141	67
	9081	24	15002	148	63
			15718	126	72

[a] The activities of the HPRT and APRT enzymes in the whole blood samples are reported relative to the concentrations of hemoglobin (Materials and Methods). Reticulocyte precentages for the reticulocyte-rich samples are based on counts of 1000 cells. The mice used in this experiment were C57Bl/6j, C3H–*Hprt a* (*M. m. castaneus Hprt a* allele in the C3H/HeHa genetic background) (N = 5), and C57–*Hprt a* (*M. spretus Hprt a* allele in the C57Bl/6j genetic background) (N = 4). The levels of HPRT activity in the control samples of C57–*Hprt a* are approximately 90-fold higher than that of the C57Bl/6 strain, while the levels of HPRT activity in erythroid cell samples of the *M. spretus* stock are only 70-fold higher than C57Bl/6j (Table II). This is a consistent change observed upon introduction of the *M. spretus Hprt a* allele into the C57Bl/6j genetic background (G. G. Johnson and V. M. Chapman, unpublished results). Control = normal whole blood samples. Treated = whole blood samples from phenylhydrazine-treated mice (Materials and Methods).

munoreactivities of the HPRT A and B enzymes in reticulocyte-rich and normal whole blood samples are similar to each other and to those observed for the purified enzymes (Figure 3). This indicates that the levels of HPRT activity in erythroid cells of HPRT A and B mice directly reflect the levels of HPRT protein in these cells (i.e., CRM) and that the decrease in HPRT activity between the reticulocyte and erythrocyte stage in HPRT B mice is paralleled by an equivalent loss of HPRT protein. Although our studies cannot exclude the possibility that the HPRT B enzyme protein is retained in the erythrocyte in a catalytically inactive and immunologically undetectable form, the most simple interpretation is that the HPRT B enzyme in reticulocytes is degraded or extruded as reticulocytes mature to erythrocytes.

Discussion

We have shown that feral and aboriginal murine stocks expressing *Hprt a* alleles have erythrocyte HPRT levels that are approximately 25-fold (*M. m. castaneus*) and 70-fold (*M. spretus*) higher than the levels in mice that express the *Hprt b* allele (*M. musculus*; C3H/HeHa or C57Bl/6J). Available evidence supports the interpretation that these increases in erythrocyte HPRT activity levels in HPRT A mice result from corresponding increases in the numbers of HPRT A enzyme molecules per cell: the purified HPRT A and B enzymes have substantially similar maximal specific activities (Table III) and immunoreactivities (Figure 3), and the increased HPRT activity levels in erythrocytes of HPRT A mice are paralleled by equivalent increases in HPRT immunoreactive protein (i.e., CRM) (Figure 3).

Further studies have shown that the levels of HPRT activity and protein differ by a factor of 2-fold or less in reticulocytes of HPRT A (*M. m. castaneus*) and B mice (Table IV) and thus that the 25-fold difference in the levels of HPRT in their erythrocytes must result largely from differences in the extent to which the HPRT A and B enzymes are retained during the maturation of reticulocytes to erythrocytes. The additional, approximate 3-fold higher level of HPRT in *M. spretus* compared to that in *M. m. castaneus* is apparent in reticulocytes and in erythrocytes (Tables II and IV) and may be explained simply as an increased level of the *M. spretus* HPRT A enzyme in nucleated precursors of the circulating reticulocytes (e.g., erythroblasts). Alternatively, these species are estimated to have diverged approximately 2 million years ago, as judged by the criteria of frequency of base substitutions in mitochondrial DNA (Ferris et al., 1983) and by isoenzyme differences (Bonhomme et al., 1984), and it is possible that the primary structures of the HPRT A proteins of *M. spretus* and *M. m. castaneus* differ even though the HPRT proteins of these mice are indistinguishable by the criterion of isoelectric point (Figure 1). If differences were present, they could potentially affect the extent of turnover of these proteins in murine erythroid cells in a manner which is similar to the HPRT A vs. HPRT B difference. Further studies will be required to distinguish between these alternatives.

We have extended our studies of these allelic variants of murine HPRT to provide evidence that both the *Hprt a* and *Hprt b* alleles are common in feral and aboriginal murine populations and that there is a strict association between expression of the *Hprt a* allele(s) and the occurrence of elevated erythroid cell HPRT activity levels in these mice (G. G. Johnson and V. M. Chapman, unpublished results). Furthermore, the elevated levels of erythrocyte HPRT activity remain associated with the *Hprt a* allele in genetic crosses, with no recombinants observed either among several hundred back-cross progeny or in the construction of two parallel congenic strains which were each back-crossed for nine generations and carry the *Hprt a* allele on an inbred strain background (G. G. Johnson and V. M. Chapman, unpublished results). These observations suggest that the *Hprt a* and *b* alleles are normal variants and that the elevated level of HPRT in erythroid cells of HPRT A mice is explicable solely on the basis of this HPRT structural gene variation. The further implication that normal structural gene variants simultaneously affect the enzyme levels in diverse somatic cell types (e.g., elevated levels in erythroid cells and brain tissue of HPRT A mice; Tables I and II) will be considered in a subsequent publication (G. G. Johnson and V. M. Chapman, unpublished results).

Our suggestion that the changes in HPRT in murine erythroid cells accompany reticulocyte maturation rather than erythrocyte aging is based on observations of changes in HPRT in humans. There, the levels of HPRT activity (and protein) decrease from approximately 6300 to 1300 microunits/mg of protein (units of μmol/min) as reticulocytes mature to erythrocytes, while there is virtually no change in the enzyme in erythrocytes (Johnson et al., 1982; Elder & Johnson, 1983). In addition, we have interpreted the changes in HPRT in murine erythroid cells as involving differences in protein turnover, although we recognize that our experiments do not specifically exclude the possibility that the differences result from differential stabilities of HPRT A and B mRNAs. However, as the levels of HPRT in reticulocyte-rich samples of HPRT A (*M. m. castaneus*) and B mice are similar (Table IV), there is no evidence to suggest any substantial differences in HPRT A and B mRNA stabilities through the reticulocyte stage of erythroid cell development. Further, it appears unlikely that even complete stabilization of HPRT A mRNA at this late stage of erythroid cell development could account for the high HPRT levels in erythrocytes of HPRT A mice, since reticulocytes are relatively inefficient in protein synthesis (Lajtha & Oliver, 1960; Borsook et al., 1962) and HPRT protein turnover approaches 97% (i.e., from approximately 3500 microunits/mg in reticulocytes to 100 microunits/mg in erythrocytes of HPRT B mice; Table IV).

Turnover in mammalian erythroid cells is extensive and entails elements of extrusion and of degradation. Externalization is evident in the extrusion of the nucleus from the erythroblast and may be active in the loss of other organelles and membrane components from the maturing reticulocyte (Kent et al., 1966; Simpson & Kling, 1968; Kornfeld & Gregory, 1969; Bessis, 1973; Gasko & Danon, 1974; Pan & Johnstone, 1983). Protein degradative activities (or systems) have also been demonstrated in reticulocytes, and these activities have been implicated in the degradation in vivo of the components of cellular organelles (Muller et al., 1980) as well as normal proteins and unstable protien variants [Morris et al., 1963; Rabinovitz & Fisher, 1964; Rieder et al., 1965; Etlinger & Goldberg, 1977; Morelli et al., 1978; Hershko et al., 1979; Boches & Goldberg, 1982; review by Hershko & Ciechanover (1982)]. While turnover in erythroid cells is thus rather extensive, it appears equally to be specific. Thus, while entire organelles and several cellular proteins are virtually eliminated from these cells, the concentrations of other proteins, including the normal globins, are unchanged (Lajtha, 1965; Marks et al., 1958; Bishop & Van Gastel, 1969). The most direct implication of our observations is that the HPRT A and B proteins have substantially different sensitivities to the factors that are involved in effecting their turnover in maturing erythroid cells. In analogy to the use of protein structural variants for elucidation of mechanisms that are

active in protein turnover in vivo (Bukhari & Zipser, 1973), the murine HPRT variants may be useful to studies that seek to identify factors that are directly involved in effecting HPRT turnover.

ACKNOWLEDGMENTS

We thank Dr. Muriel Nesbitt for very generous help in the development of studies of HPRT and APRT in mice and Dr. Kirby D. Smith for comments and suggestions regarding the manuscript.

Registry No. Hypoxanthine phosphoribosyltransferase, 9016-12-0.

REFERENCES

Bessis, M. (1973) *Living Blood Cells and Their Ultrastructure* (Weed, R. I., *Transl.*) pp 1–767, Springer-Verlag, New York.

Bishop, Ch., & Van Gastel, C. (1969) *Haematologia 3*, 29–41.

Blakely, P. (1980) M.S. Thesis, San Diego State University.

Boches, F. S., & Goldberg, A. L. (1982) *Science (Washington, D.C.) 215*, 978–980.

Bonhomme, F., Catalan, J., Britton-Davidian, J., Chapman, V. M., Moriwaki, K., Nevo, E., & Thaler, L. (1984) *Biochem. Genet. 22*, 275–303.

Borsook, H., Lingrel, J. B., Scaro, J. L., & Millette, R. L. (1962) *Nature (London) 196*, 347–350.

Brockman, R. W. (1960) *Cancer Res. 20*, 643–653.

Bukhari, A. I., & Zipser, D. (1973) *Nature (London) New Biol. 243*, 238–241.

Caskey, C. T., & Kruh, G. D. (1979) *Cell (Cambridge, Mass.) 16*, 1–9.

Chapman, V. M., & Shows, T. (1976) *Nature (London) 259*, 665–667.

Chapman, V. M., Kratzer, P. G., & Quarantillo, B. A. (1983) *Genetics 103*, 785–795.

Davidson, I., & Nelson, D. (1974) in *Clinical Diagnosis by Laboratory Methods* (Davidson, I., & Henry, J. B., Eds.) pp 177–179, Saunders, Philadelphia.

Drabkin, D. L., & Austin, J. H. (1935) *J. Biol. Chem. 112*, 51–65.

Elder, A. L., & Johnson, G. G. (1983) *Biochem. Genet. 21*, 227–238.

Etlinger, J. D., & Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 54–58.

Ferris, S. D., Sage, R. D., Prager, E. M., Ritte, U., & Wilson, A. C. (1982) *Genetics 105*, 681–721.

Gasko, O., & Danon, D. (1974) *Br. J. Haematol. 28*, 463–470.

Hakala, M. T., & Taylor, E. (1959) *J. Biol. Chem. 234*, 126–128.

Hershko, A., & Ciechanover, A. (1982) *Annu. Rev. Biochem. 51*, 335–364.

Hershko, A., Ciechanover, A., & Rose, I. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3107–3110.

Hughes, S. H., Wahl, G. M., & Capecchi, M. R. (1975) *J. Biol. Chem. 250*, 120–126.

Johnson, G. G., & Littlefield, J. W. (1979) *Anal. Biochem. 92*, 403–410.

Johnson, G. G., Eisenberg, L. R., & Migeon, B. R. (1979) *Science (Washington, D.C.) 203*, 174–176.

Johnson, G. G., Ramage, A. L., Littlefield, J. W., & Kazazian, H. H., Jr. (1982) *Biochemistry 21*, 960–966.

Kelley, W. N., Greene, M. L., Rosenbloom, F. M., Henderson, J. F., & Seegmiller, J. E. (1969) *Ann. Int. Med. 70*, 155–206.

Kent, G., Minick, O. T., Volini, F. I., & Orfei, E. (1966) *Am. J. Pathol. 48*, 831–857.

Konecki, D. S., Brennand, J., Fuscoe, J. C., Caskey, J. T., & Chinault, A. C. (1982) *Nucleic Acids Res. 10*, 6763–6775.

Kornfeld, S., & Gregory, W. (1969) *Biochim. Biophys. Acta 177*, 615–624.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lajtha, L. G. (1965) *Ser. Haematol. 2*, 26.

Lajtha, L. G., & Oliver, R. (1960) *Haemopoiesis: Cell Prod. Its Regul., Ciba Found. Symp.*, 289–314.

Littlefield, J. W. (1966) *Exp. Cell res. 41*, 190–196.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marks, P. A., Johnson, A. B., & Hirschberg, E. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 529–536.

Melton, D. W., Konecki, D. S., Brennand, J., & Caskey, C. T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2147–2151.

Migeon, B. R., der Kaloustian, V. M., Nyhan, W. L., Young, W. J., & Childs, B. (1968) *Science (Washington, D.C.) 160*, 425–427.

Morelli, A., Benatti, U., Gaetani, G. F., & De Flora, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1979–1983.

Morris, A., Arlinghaus, R., Favelukes, S., & Schweet, R. (1963) *Biochemistry 2*, 1084–1090.

Muller, M., Dubiel, W., Rathmann, J., & Rapoport, S. (1980) *Eur. J. Biochem. 109*, 405–410.

Pan, B.-T., & Johnstone, R. M. (1983) *Cell* (Cambridge, Mass.) *33*, 967–977.

Rabinovitz, M., & Fisher, J. M. (1964) *Biochim. Biophys. Acta 91*, 313–322.

Rieder, R. F., Zinkham, W. H., & Holtzmann, N. A. (1965) *Am. J. Med. 39*, 4–20.

Rosenbloom, F. M., Kelley, W. N., Henderson, J. F., & Seegmiller, J. E. (1967) *Lancet 1967-II*, 305–306.

Salzmann, J., DeMars, R., & Benke, P. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 545–552.

Seegmiller, J. E., Rosenbloom, F. M., & Kelley, W. N. (1967) *Science (Washington, D.C.) 155*, 1682–1684.

Sharp, J. D., Capecchi, N. E., & Capecchi, M. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3145–3149.

Simpson, C. F., & Kling, J. M. (1968) *J. Cell. Biol. 36*, 103–109.

Spivak, J. L., Marmor, J., & Dickerman, H. W. (1972) *J. Lab. Clin. Med. 79*, 526–540.

Szybalski, W., Szybalska, E. H., & Ragni, B. (1962) *Natl. Cancer Inst. Monogr. 7*, 75–89.

Wahl, G. M., Hughes, S. H., & Capecchi, M. R. (1975) *J. Cell. Phys. 85*, 307–320.

Wilson, J. M., Young, A. B., & Kelley, W. N. (1983) *N. Engl. J. Med. 309*, 900–910.