Disparate Enzyme Activity in Erythrocytes and Leukocytes

A VARIANT OF HYPOXANTHINE PHOSPHORIBOSYL-TRANSFERASE DEFICIENCY WITH AN UNSTABLE ENZYME

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ABSTRACT A family is reported in which each of two sisters has a son with no detectable hypoxanthine phosphoribosyltransferase (HPRT) (EC 2. 4. 2. 8) in his erythrocytes, a finding considered pathognomonic of Lesch-Nyhan disease. However, neither has the stigmata of the disease. One boy is neurologically normal, and the other is moderately retarded. There was only a slight increase in urinary uric acid, but the amounts of hypoxanthine and xanthine, and their ratios, were similar to those found in Lesch-Nyhan disease, strongly indicating that excesses of these last two oxypurines are not responsible for the symptomatology in that disease. In contrast to the nondetectable HPRT activity in the red blood cells, leukocyte lysates from the two boys have 10–15% of normal activity, possibly reflecting continuing synthesis of an unstable enzyme. This hypothesis is supported by the demonstration that at 4°C HPRT activity was rapidly lost in the propositus while the activity increased in control subjects. The mother's cells were intermediate between the two. The intact and disrupted leukocytes of the hemizygote, in the absence of added phosphoribosyl pyrophosphate, converted as much hypoxanthine to inosinate as the normal cell, and appropriate tests indicated that under these circumstances enzyme concentration is not rate limiting whereas the concentration of the cosubstrate, phosphoribosyl pyrophosphate, is. The capacity for normal function in the intact mutant cell is more representative of in vivo conditions than the lysate, which may explain the important modification of clinical symptomatology, the relatively mild hyperuricosuria, and the presence of mosaicism in the circulating blood cells of the heterozygotes. A similar explanation may apply to other genetic diseases in which incomplete but severe enzyme deficiencies are found in clinically normal individuals.

An associated deficiency in glucose-6-phosphate dehydrogenase in this family permitted confirmation of previous observations on linkage with hypoxanthine phosphoribosyltransferase.

INTRODUCTION

Lesch-Nyhan (LN) [1] syndrome is an X-linked recessive disease characterized by mental retardation, spasticity, and a bizarre type of compulsive self-mutilation. Biochemically, there is a greatly increased production of hypoxanthine and an excessive excretion of uric acid associated with a primary deficiency in hypoxanthine phosphoribosyltransferase (HPRT) (EC 2. 4. 2. 8). The enzyme defect is readily demonstrated in the erythrocyte.

Among the gout population patients have been found with partial deficiencies of HPRT (1–3). The correlation of symptomatology with the amount of residual HPRT activity as measured in erythrocytes has not always been good. In some instances, the level of HPRT activity has been extremely low, approaching

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[1] *Abbreviations used in this paper:* APRT, adenine phosphoribosyltransferase; G6PD, glucose-6-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase; LN, Lesch-Nyhan; PRPP, phosphoribosyl pyrophosphate.

that in LN disease, and yet the patients have been normal neurologically.

We have recently had the opportunity to study two cousins with no detectable HPRT activity in their erythrocytes. One of these boys is clinically normal and the other has moderate neurological damage. Investigation of HPRT activity in lysates of leukocytes and in intact leukocytes has provided an explanation for this apparent discrepancy. It also explains the presence of mosaicism in the blood cells of the heterozygote, differing in this respect from LN disease in which the blood cells of the heterozygote have normal enzyme activity (4, 5). An associated glucose-6-phosphate dehydrogenase (G6PD) deficiency in the same family has permitted observations concerning linkage between these two genetic loci.

METHODS

Case reports. Ho. Ro., a 14-yr old black male, was under care because of mental retardation and seizures. He was the product of a full-term pregnancy. He sat at 1 yr, walked at 15–16 mo, spoke his first word at 15 mo. The patient was considered normal until 5 yr of age when seizures began. *Grand mal* seizures have been incompletely controlled despite intensive anticonvulsive therapy. At present, he is moderately retarded mentally, has indistinct speech, and has a slightly incoordinate gait. There is no choreoathetosis or compulsive self-mutilation. Routine studies revealed a serum uric acid of 10 mg/100 ml.

Ro. McN., 15-yr old, is the son of Mrs. Ro.'s sister. He is in 10th grade in school, 1 yr ahead of his age level, and is considered a good student. He participates in physical sports. Neurological examination was negative. HPRT deficiency was discovered during investigation of the family of propositus Ho. Ro. His serum uric acid was 6.2 mg/100 ml.

HPRT assay. Erythrocyte lysates or leukocytes that had been disrupted by repeated freeze-thawing were incubated with [8-^{14}C]hypoxanthine alone and with excess phosphoribosyl pyrophosphate (PRPP) (3).

A 1:50 lysate of packed red cells in 0.01 M potassium phosphate buffer, pH 7.0, was clarified by centrifugation. The assay mixture containing 10 μl 1 M Tris buffer, pH 8.0, 50 μl 0.02 M $MgCl_2$, 50 μl 10^{-3} M PRPP (mixed just before the assay), and 50 μl lysate. The reaction was started by adding 50 μl [8-^{14}C]hypoxanthine, 5×10^{-4} M. In the white cell assay, adenylate was added to the radioactive substrate to protect the product against nucleotidases. Incubation was for 10 min at 37°C in a water bath with shaking. The incubation was stopped by immersing the tubes for 1 min in a boiling water bath. The precipitated protein was removed by centrifuging, and 25 μl of supernate were spotted on 1-inch square diethylaminocellulose paper. The papers were dried at room temperature, washed for 10 min, twice with water and twice with ethanol, successively, dried in air, and assayed for radioactivity in a scintillation counter with toluene scintillant. Controls from one large lot of red blood cells and of boiled red cells were run concurrently. Activities as low as 0.1% of normal (0.1 nmol/mg protein/h) can be detected by this procedure. Intact leukocytes were also incubated with radioactive hypoxanthine, and the product was isolated after disrupting the cells (6).

[8-^{14}C]Hypoxanthine, 100 nmol/ml containing $2–5 \times 10^6$ cpm/ml, was added to $1–5 \times 10^7$ cells. Incubation was for 30 min at 37°C in a shaking water bath, terminated by chilling in an ice bath. The cells were washed twice with buffered salt solution at 4°C, then suspended in 0.2 ml water, and heated in a boiling water bath for 2 min. After centrifugation, 0.1 ml supernate was spotted onto squares of diethylaminocellulose paper and washed as described above.

PRPP concentration. PRPP concentration was measured by a modification of the method of Flaks (7). Fresh cells were washed twice with 2 vol of Hanks's balanced salt solution and centrifuged at 1,500 rpm for 10 min. Approximately 10^7 cells were lysed by addition of 0.5 ml of chilled distilled water and 3 cycles of freezing and thawing. The lysate was centrifuged at 2,500 rpm for 10 min, and aliquots of 50 μl of the supernate were assayed for PRPP by incubating for 1 h at 37°C with 50 μl [8-^{14}C]adenine, 0.5 mM, inosinate at a final concentration of 1 mM, 10 μl 20 mM $MgCl_2$, and 10 μl partially purified adenine phosphoribosyltransferase (APRT) in excess (sufficient to convert 100 nmol/h of substrate). Inosinate prevents distruction of labeled product, adenylate, by nucleotidases. The product was isolated as described in HPRT assay, and the amount of PRPP calculated was from the micromoles of adenylate formed. Results were confirmed with radioactive hypoxanthine and excess of purified HPRT.

Autoradiography. Venous blood was drawn with a syringe containing approximately 0.1–0.2 ml of heparin (Liquemin), and the erythrocytes were separated by gravity for 1 h. Aliquots of 0.75 ml of plasma containing leukocytes were dispersed into plastic centrifuge tubes, and the cells were collected by centrifuging for 10 min at 600 rpm in a clinical centrifuge. Plasma was decanted, and the cells were resuspended in Robinson's buffer (8) containing 100 μCi of [^{3}H]hypoxanthine (3.14 Ci/mmol). The cell suspension was incubated in a water bath shaker at 37°C for 90 min. Cells were collected by centrifuging for 10 min at 600 rpm and were washed by centrifugation three times with 2 ml of ice-cold Hanks's balanced salt solution. The cell pellet was resuspended in two drops 1% polyvinyl pyrolidone, smeared on clear glass slides, and dried rapidly in a stream of hot air. The slides were prepared for autoradiography as previously described (4) and after a 2 wk exposure were developed and stained with May-Grünwald-Giemsa.

Urinary metabolites. 24-h urine collections were made on the patients; only casual morning specimens were studied on other members of the family. Uric acid and creatinine were determined by standard colorimetric procedures (9, 10). Hypoxanthine and xanthine were separated by column chromatography and assayed enzymatically (11).

G6PD assay and electrophoretic identification. The assay was performed on lysates by measuring the reduction of NADP as previously described (12). Electrophoresis was performed on starch gel, according to Kirkman and Hendrickson (13).

RESULTS

The results of HPRT and G6PD assays are presented in Table I. The HPRT assays were performed on lysates of erythrocytes and leukocytes in the presence of excess PRPP. The hemizygotes, Ho. Ro. and Ro. McN., have no detectable HPRT in the erythrocytes but 14 and

TABLE I
HPRT and G6PD Activity

	HPRT			G6PD
	Erythrocytes (lysate +PRPP)	Leukocytes (lysate +PRPP)	Leukocytes (intact)	Erythrocytes
Ro. family				
Mother, Cl.	23	35	0.40	0.70
Daughter, Ju.	126	80	—	2.57
Son, Ho.*	<0.1	11	0.36	0.82
McN. family				
Mother, Ed.	11	23	0.46	3.63
Daughter, An.	122	80	0.35	5.23
Son, Be.	97	76	0.48	0.56
Son, Ro.*	<0.1	7.5	0.47	6.41

HPRT activity in nanomoles product per milligram protein per hour. G6PD activity in international units per grams hemoglobin. HPRT assays were performed on erythrocyte and leukocyte lysates with excess PRPP (2.5×10^{-2} M). Intact leukocytes were incubated with hypoxanthine without added PRPP. G6PD assay was performed on lysates as previously described. (8) Normal values: HPRT in erythocytes 107 nmol ± 26/mg protein/h; in leukocytes, 67 nmol/mg protein/h; in the intact leukocytes 0.4 nmol/mg protein/h. G6PD in erythrocytes: normal value is 6 U/g hemoglobin; in leukocytes, 0.01 U/10^6 leukocytes. G6PD in mutant leukocytes was normal.
* Hemizygotes.

10% of normal activity, respectively, in the leukocytes. The rest of the siblings in both families are normal.

Both mothers, who are clinically obligatory heterozygotes, have a greatly reduced enzyme activity in the erythrocytes suggesting, in accordance with the Lyon hypothesis, that Cl. Ro. has approximately 18% normal erythrocytes and Ed. McN. about 10%. The results with the leukocyte lysates indicate a roughly similar distribution of $HPRT^+$ and $HPRT^-$ cells. The mosaicism in the leukocytes with a disproportion of abnormal cells was confirmed by autoradiography (Table II).

TABLE II
Autoradiography of Leukocytes

	Labeled cells (%)
Controls	
Jo. Da.	91
Su. Tr.	82
Hemizygote	
Ho. Ro.	8
Heterozygotes	
Cl. Ro.	26
Ed. McN.	22

Leukocytes were incubated with [^{3}H]hypoxanthine and prepared for autoradiography as described in the text. 200 leukocytes (lymphocytes and mononuclear leukocytes) were scored. Cells with more than 10 grains in the nucleus were rated as labeled.

Inosinate synthesis was assayed in intact leukocytes by incubating with [8-^{14}C]hypoxanthine and then disrupting the cell and measuring the conversion to radioactive inosinate. By this technique, there was no difference in activity among the normal, the heterozygote and the hemizygote (Table I). When the assay was repeated with lysed cells without additional PRPP, the results were essentially the same. These data do not appear in the table. The addition of purified HPRT (14) in amounts that would be sufficient to increase conversion 100-fold in the presence of adequate amounts of PRPP did not increase the conversion to inosinate. From these experiments the amount of PRPP could be estimated and converted into concentration using available figures for the volume of leukemic lymphocytes (15). The volume (250 μm^3) is about half that of previous crude estimates of normal lymphocytes so that the real concentration of PRPP may be lower than calculated. The calculated concentration of PRPP, 0.8×10^{-5} M, was considerably below the K_m for the enzyme (2.4×10^{-4}) (16).

The stability of the enzyme was investigated by maintaining the intact leukocytes at 4°C for 96 h and periodically measuring HPRT and APRT activity (Table III). HPRT activity fell rapidly in the propositus (Ho. Ro.), but APRT activity remained stable, indicating that the former did not reflect an overall loss in vitality of the cells. In the normal, the HPRT activity actually

TABLE III
Stability of Enzyme in Leukocytes

Age of extracted cells	HoRo		ClRo (mother)		Normal	
	APRT	HPRT	APRT	HPRT	APRT	HPRT
Fresh	3,800	2,000	3,000	6,300	5,600	11,000
24 h	5,100	610	3,600	4,300	6,600	18,000
96 h	5,800	50	4,200	3,400	7,200	19,000

Leukocyte lysates were maintained at 4°C in buffer. At indicated times aliquots were removed and enzyme activity determined. The size of the aliquot was constant for each subject. The assay mixture contained 10 µl 1 M Tris buffer, pH 8.0, 50 µl 0.02 M $MgCl_2$, and 50 µl 10^{-3} M PRPP (mixed just before assay; 110 µl of the mixture used per tube), 50 µl of 1:50 lysate. The reaction was started by adding [8-^{14}C]-hypoxanthine, 5×10^{-4} M. Results in counts per minute per assay.

rose, similar to results previously obtained with erythrocytes (17). The stability curve of the mother's cells, Cl. Ro., was intermediate between the two, reflecting a mixed population of normal and abnormal cells.

Examination of purine metabolites in the urine (Table IV) revealed an elevated uric acid in the hemizygotes but at levels far below that seen in LN disease. However, the amount of hypoxanthine and xanthine and the ratios of these two oxypurines were in the same range as those in LN disease (11).

In Fig. 1 are presented the family tree and the genotypes. Concentrating first on the three males, Be. and Ro. McN. and Ho. Ro., there are three genotypes represented. Be. McN. is HPRT$^+$, G6PD^{A-}; Ro. McN. is HPRT$^-$, G6PD^B; and Ho. Ro. is HPRT$^-$, G6PD^{A-}. Unfortunately the fathers and grandparents could not be studied. However, it is evident from the available data that the HPRT and G6PD loci, both on the X chromosome, must have crossed over at least once.

The assignment of the gene loci to a specific X chromosome in the heterozygote is speculative. The genotypes as depicted have the advantage of economy in that one crossover involving Ed. McN. is sufficient to explain the genotypes in the remaining members of the family. An. McN. must be considered only tentatively HPRT^{++}. An exhaustive attempt using either hair root assay (18, 19) or selective media with skin fibroblasts (20, 21) was not made at this time to exclude a small population of HPRT$^-$ cells, a possibility suggested by the presence of electrophoretically identifiable G6PD^{A-} and a total G6PD activity in the normal range.

TABLE IV
Urinary Excretion of Oxypurines

	Uric acid	Hypoxanthine	Xanthine	Hypoxanthine / Xanthine
		μmols/mg creatinine		
Ho. Ro.	4.82	1.01	0.662	1.52
Ro. McN.	5.04	1.17	0.560	2.09
Cl. Ro.	2.76	0.56	0.076*	High*
Be. McN.	2.99	Trace	Trace	—
Normal (children)	3.8	0.041	0.130	0.31
Normal (adults)	2.1	0.010	0.190	0.05
Lesch-Nyhan	15.0	0.575	0.218	2.64

Normal values and those of Lesch-Nyhan patients are taken from a previous study. (7) Points to be noted: Uric acid excretion in hemizygotes Ho. Ro. and Ro. McN. are only slightly elevated and far less than LN subjects. However, hypoxanthine and xanthine excretion and hypoxanthine: xanthine ratio in hemizygotes and in heterozygote Cl. Ro. are very similar to LN.

* Xanthine value was at lower limits of accuracy; only qualitative estimate of ratio is indicated.

DISCUSSION

The presenting complaint of Ho. Ro. was convulsions with moderate mental retardation. Laboratory studies

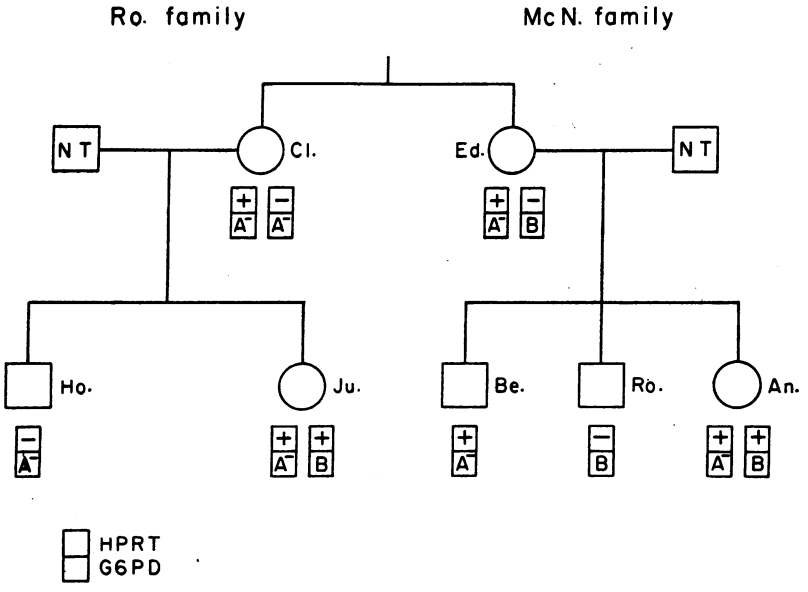

FIGURE 1 The genotypes of the hemizygotes Ho. Ro., Be. McN., and Ro. McN. indicate at least one crossover between the loci for HPRT and G6PD. The assigned genotypes for the heterozygotes are somewhat tentative as explained in the text. NT, not tested.

revealed an elevated serum uric acid, which led to an investigation of HPRT activity in his peripheral erythrocytes. There was no detectable activity, an observation considered pathognomonic of LN disease. Survey of the family revealed a son of Mrs. Ro.'s sister who also had no HPRT activity but was neurologically normal. It is possible that this represents poor "penetrance" or the presence of modifier genes. However, the possibility that the neurological damage arose from unrelated causes must be seriously considered. Ho. Ro. was considered normal until he was 5 yr old by a mother who is intelligent and observant. Neurological symptoms were ushered in by a series of convulsions requiring prolonged anticonvulsive therapy, suggesting an acute insult. As the investigation of patients with neurological symptoms increases, the exposure of unrelated biochemical deviations from normal will also increase. This problem has been discussed briefly as it relates to hyperlysinemia (22). The propositus had symptoms of neurological disease; however, two siblings and a cousin with the same biochemical defect appear normal. It is now uncertain as to whether hyperlysinemia causes neurological damage.

An associated G6PD deficiency in the family was identified. Since the genetic loci for G6PD and HPRT are on the X chromosome, observations on linkage were possible (Fig. 1). The evidence for at least one crossover between these two loci in the present family confirms previous suggestions based on tissue culture studies (23) and clinical experience with another similar family (5) that the loci are not closely situated.

The discordance between the clinical picture and the absent HPRT activity in the erythrocyte led to a more thorough study of the family. Measurement of enzyme activity in leukocyte extracts of the hemizygotes revealed easily detectable levels that were 10–15% of normal. Studies with the intact leukocytes suggested that enzyme levels of this magnitude may not be rate-limiting in the conversion of hypoxanthine to inosinate in vivo. Enzyme activities measured with whole cells resulted in similar values in the normal, the heterozygote, and the hemizygote. The activity approximated only 1% that achieved in the standard assay. Similar results were obtained with disrupted cells excluding such potentially limiting factors as transport. Appropriate studies with lysed leukocytes to which excesses of enzyme or excesses of PRPP (the standard assay technique) were added indicated that in the whole cell with 10–15% of normal enzyme activity, PRPP concentration is the limiting factor. Estimates of PRPP concentration were possible from the studies with lysed leukocytes and excesses of enzyme. The figure of 0.8×10^{-5} is well below the K_m of the enzyme (2×10^{-4} M) (16). Analogous studies with APRT using the normal intact cell yielded similar results. Under these circumstances, the endogenous PRPP appears to be rate limiting.

It is commonly stated that a fraction of the normal enzyme complement is sufficient to maintain normal function. However, it is not often that a kinetic explanation can be offered for the observation. The present experience demonstrates the superiority of the leukocyte over the anucleate erythrocyte in providing an index of the enzymatic performance of other tissues. Furthermore, assays with the intact cells may more closely parallel the in vivo situation than the customary enzyme assays which use lysates or extracts in the presence of excesses of cofactors and cosubstrates. Thus, the normal conversion of hypoxanthine to inosinate by the whole cell in the hemizygote is consistent with the clinical picture. Ro. McN. is neurologically normal, and the symptoms in Ho. Ro. are not at all suggestive of LN disease and may be entirely unrelated to HPRT deficiency. The relatively moderate increase in uric acid excretion is compatible with a minor reduction in overall performance in HPRT. The deficiency in erythrocyte HPRT activity accounts for part of the reduction. There may also be deficiencies in other tissues not readily available for biopsy. The hypoxanthine and xanthine excretion and their ratio are distinctly abnormal (11) indicating that they may provide a more sensitive index of HPRT function than uricosuria.

The cause of the neurological damage in LN disease is unknown, but it does not appear to result from excesses of oxypurines. The failure of allopurinol to relieve or prevent symptomatology has exonerated the hyperuricemia. The present observations make it clear that abnormalities in hypoxanthine and xanthine production of a magnitude similar to that found in LN disease may be tolerated in the absence of the characteristic neurological picture.

The probable explanation for the higher enzyme activity in the leukocyte than the erythrocyte in the hemizygotes is the continuing synthesis of an unstable enzyme by the former. In the anucleated erythrocyte, no longer capable of protein synthesis, the levels of enzyme activities are determined by the rates of degradation. The normal half-life of HPRT in the erythrocyte is 34 days (24). In the present subjects no enzyme activity was detected even in the "young" fraction of erythrocytes obtained by centrifugation, suggesting a very rapid rate of degradation. An unstable HPRT could be demonstrated in vitro (Table III). Further studies on half-life using skin fibroblasts are in progress. In G6PD^A deficiency, a similar explanation has been offered for an analogous situation (12, 25).

Another interesting observation is the presence of mosaicism in the peripheral blood of the heterozygotes. This differs from the situation in the LN heterozygote where the enzyme activity in the blood cells is not reduced, indicating that only normal cells are represented. Attention was first called to the selection of $HPRT^+$ blood cells in LN disease in an autoradiographic study of the peripheral leukocytes. It was suggested that $HPRT^-$ cells might suffer a propagative disadvantage early in embryogenesis (4). A similar observation concerning the erythrocyte was made in a family with combined HPRT and G6PD deficiency, in which the latter mutation permitted definitive demonstration that only one X chromosome was active in the heterozygote (5).

It had been suggested previously that, in families with a partial deficiency of HPRT, mosaicism may be evident in the blood cells of the heterozygote (*26*). These speculations were based on the demonstration of reduced HPRT activity in the red blood cells. In one reported family, one of three possible heterozygotes had two electrophoretically identifiable forms of HPRT, one of which was considered normal and the other was felt to be mutant (*27*). The present family provides convincing supportive evidence of mosaicism. HPRT activity is very low in the erythrocytes and leukocytes of the two obligatory heterozygotes, Ed. McN. and Cl. Ro., a finding consistent with a mixed population of $HPRT^+$ and $HPRT^-$ cells with a preponderance of the latter (Table I). Autoradiographic studies of the leukocytes with [^{8}H]hypoxanthine also indicated a mixed population with a high proportion of mutant cells (Table II). Independent confirmation of mosaicism in the erythrocytes of Ed. McN. was derived from G6PD studies (Fig. 1).

It would appear that sufficient HPRT activity is maintained in the mutant stem cell to prevent any selective advantage of $HPRT^+$ cells such as occurs in LN disease. The predominance of $HPRT^-$ cells is compatible with random inactivation of the X chromosome, if this had occurred early in embryogenesis among a relatively limited number of stem blood cells. The similar distribution of wild and mutant cells in erythrocyte and leukocyte is consistent with the event involving a common stem cell.

The results with autoradiography in which only 8% of the hemizygotes cells were labeled and mosaicism was demonstrable in the heterozygote appear to be in conflict with the normal inosinate synthesis observed in the mutant intact cells. Restating the observation in enzymological terms under the conditions of enzyme assay with intact cells, the physiologically low levels of PRPP is the rate-limiting factor obscuring differences in HPRT activity; however, under the conditions of autoradiography, the enzyme level is rate limiting so that inosinate synthesis in the mutant cell is visibly less than in the normal cell. Inosinate synthesis in the intact cell was measured under relatively physiological conditions—a short incubation period preventing exhaustion of endogenous PRPP and a level of hypoxanthine (10^{-4} M) similar to that in extracellular fluids. In autoradiography the incubation period was 90 min, and the substrate level was 10^{-6} M. The differences that were observed are probably best explained by a consideration of the enzyme kinetics involved in the two procedures. It is probably directly related to the differences in substrate concentration. However, the subject was not further investigated. The 8% of labeled cells in the hemizygote may result from higher levels of unstable enzyme in very young cells.

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