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Protein variations associated with Lesch–Nyhan syndrome

(genetic screening/two-dimensional electrophoresis/polymorphism/lymphocyte)

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ABSTRACT Patients having Lesch–Nyhan syndrome were studied by using enzymatic, immunologic, and two-dimensional electrophoretic techniques. Four hundred proteins were analyzed on each two-dimensional electrophoretogram for positional or quantitative variation. In autoradiograms of lymphocytes stimulated with phytohemagglutinin, there were 11 quantitative differences found in all patients that were significant at the $2P < 0.01$ level. A significant quantitative difference was also found in an analysis of silver-stained gels of unstimulated lymphocytes. Patients had trace amounts of erythrocyte hypoxanthine phosphoribosyl transferase (HPRT) activity and trace or no immunoprecipitable HPRT. However, HPRT was observed in silver-stained erythrocyte electrophoretograms and in autoradiograms from phytohemagglutinin-stimulated lymphocytes. Unstimulated lymphocytes contained 65% of the control HPRT concentration. Currently, the technology of two-dimensional electrophoresis detects a fraction of the total cellular proteins and defective proteins may not show electrophoretic alterations. However, specific secondary changes in other polypeptides may be observed and, when catalogued, will serve as an aid in the diagnosis and understanding of the pathophysiology of metabolic diseases.

Human behavioral disorders are particularly difficult to investigate at the molecular level. The Lesch–Nyhan syndrome is one of the few neuropsychiatric diseases for which the metabolic origin is known (1). For this reason, it was chosen as a model system to find markers and uncover pathophysiologic protein changes by using quantitative two-dimensional electrophoretic techniques.

Lesch–Nyhan disease is characterized by self-mutilation, spasticity, and hyperuricemia. The failure of azathioprine to inhibit excessive uric acid production in these patients (2, 3) led to the discovery that they are deficient in hypoxanthine phosphoribosyltransferase (HPRT; IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8).

Erythrocyte adenine phosphoribosyltransferase (APRT) was found to be elevated when it was used as a control enzyme for HPRT assays (4). Inosinic acid dehydrogenase activity was also elevated. These elevated activities appear to be due to increases in specific activity (4–9). Other enzymes found to be increased in activity are orotate phosphoribosyltransferase and orotate decarboxylase in erythrocytes, but not in leukocytes or fibroblasts (10), and phosphoribosylpyrophosphate glutamine amidotransferase in cultured fibroblasts (11), and lymphoblasts (12).

A systematic search for protein alterations is now possible by using two-dimensional electrophoresis (13). Only a fraction of the total cellular protein can be detected and alterations in enzyme activity that are not associated with variations in electrophoretic mobility or concentration are not visible. However, hundreds of proteins can be surveyed for alterations in mass, charge, or concentration. We have examined 400 proteins in two-dimensional electrophoretograms of stimulated and unstimulated lymphocytes and studied erythrocyte HPRT by using enzymatic, immunologic, and two-dimensional electrophoretic techniques.

MATERIALS AND METHODS

Lymphocytes and Erythrocytes. Heparinized blood was collected in sterile fashion. Lymphocytes were separated from whole blood by using a Ficoll/Hypaque gradient. Lymphocytes and erythrocytes were washed three times with phosphate-buffered saline and frozen at −70°C.

^{14}C-Labeling of lymphocyte proteins was accomplished after incubation at 2×10^6 cells per ml in chromosome medium/phytohemagglutinin (PHA; GIBCO) for 24 hr at 37°C in 5% CO_2/95% air. Cells were then centrifuged at $500 \times g$ for 10 min and suspended in 2 ml of Dulbecco's modified Eagle's medium (GIBCO) supplemented 10% fetal calf serum containing 50 μCi of [^{14}C]leucine (341.0 Ci/mol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear). Labeling was carried out for 3 hr at 37°C, and cells were then washed twice with phosphate-buffered saline and stored at −70°C.

Enzyme Assays. Equal volumes of packed erythrocytes and lysis buffer [10 mM Tris·HCl, pH 7.6/20 mM KCl/0.1 mM phosphoribosyl pyrophosphate/0.2 mM dithiothreitol/10% (vol/vol) glycerol/0.5% Nonidet P-40 (Bethesda Research Laboratories, Rockville, MD)] were freeze-thawed three times. Lysates were centrifuged at $90,000 \times g$ for 10 min and the supernatants stored at −70°C until assayed for HPRT and APRT activity as described (14). The sensitivity of the assays was 2 pmol·mg^{-1} · min^{-1} HPRT and 128 pmol · mg^{-1}·min^{-1} for APRT. Protein concentrations were determined by method of Lowry *et al.* (15).

Enzyme Purification and Antibody Production. Normal human erythrocyte HPRT was purified from outdated blood by the method of Olsen and Milman (16) and used to make antibody in a goat. Activity of the purified HPRT was 17 μmol of IMP formed per mg of protein per min (at 37°C). Antibody precipitates were made from human erythrocyte lysates that had been centrifuged at $90,000 \times g$ for 1 hr. The supernatant was immunoprecipitated as described (17).

Electrophoresis. Two-dimensional electrophoresis was performed as described by O'Farrell (13) with minor modifications (18).

Staining. Gels were stained with silver as described (18, 19). However, we now use a photochemically derived silver stain (20, 21), with some modifications: gels are fixed in 20% (wt/vol) trichloroacetic acid for 20 min and then washed three times for 10 min each with 10% ethanol/5% acetic acid. The next steps

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Abbreviations: HPRT and APRT, hypoxanthine and adenine phosphoribosyltransferase, respectively; PHA, phytohemagglutinin.

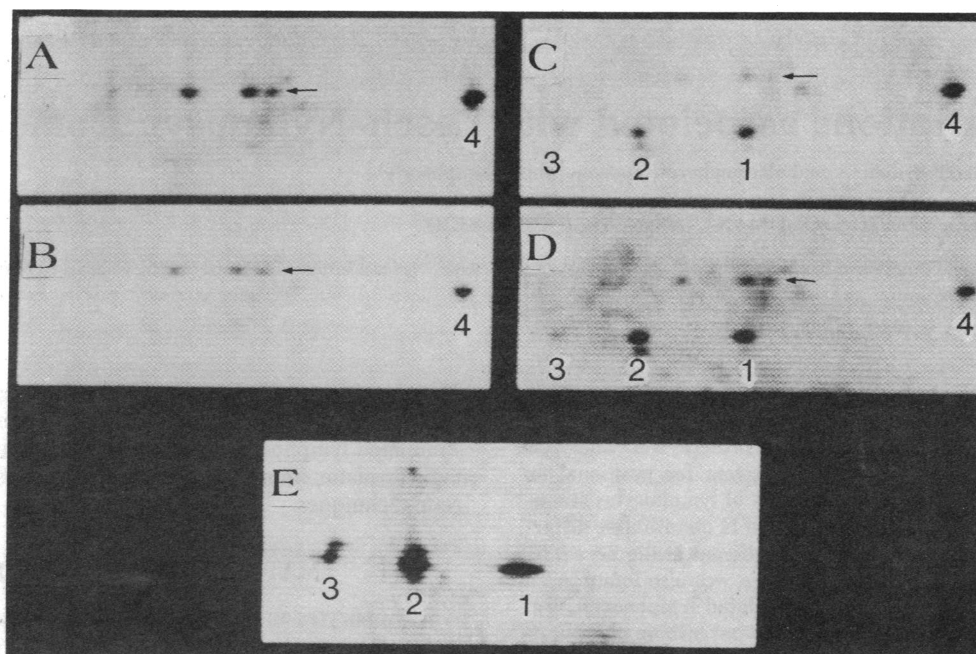

FIG. 1. Pertinent regions of two-dimensional electrophoretic gels of immunoprecipitated erythrocyte HPRT from controls (*C* and *D*) and Lesch–Nyhan patients (*A* and *B*) and of purified human HPRT (*E*). Purified HPRT had a M_r of 26,000 with three species having pI values of 6.5, 6.3, and 6.1. Little, if any, patient HPRT was immunoprecipitated. ←, IgG light chains solubilized from the immunoprecipitates; 1, 2, and 3, HPRT species; 4, carbonic anhydrase present as a contaminant. Isoelectric focusing was done by using a 4:1 mixture of pH 5–7/pH 3–10 Biolyte (Bio-Rad) while the second dimension used 10% polyacrylamide. Proteins were visualized by using a silver stain. The images in this figure were photographed from a computer video terminal.

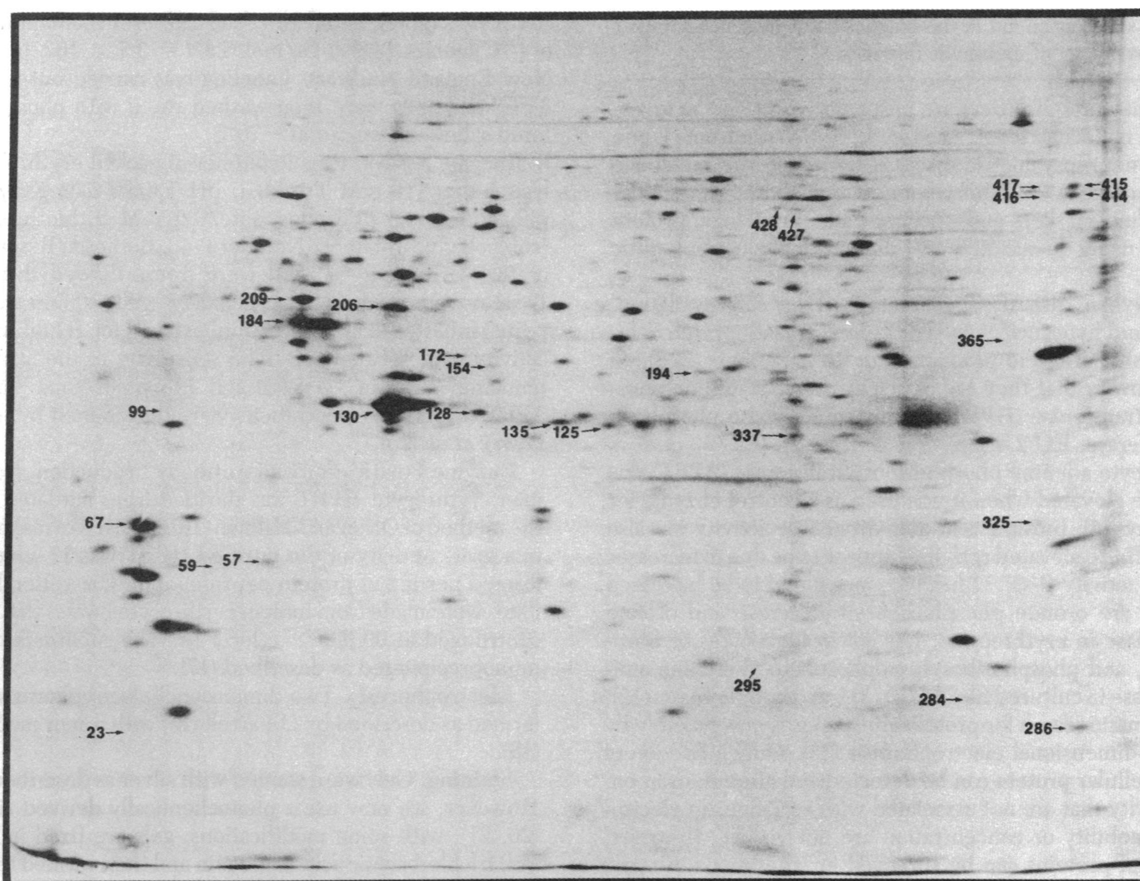

FIG. 2. Two-dimensional autoradiographic pattern of PHA-stimulated lymphocytes of a Lesch–Nyhan patient. High molecular weight proteins are at the top and acidic proteins are on the left. Data on the protein species shown in this figure and in Fig. 3 are given in Table 1.

are performed under subdued or amber light. Gels are soaked for 5 min in 3.4 mM potassium dichromate/3.2 mM nitric acid and then for 20 min in 12 mM silver nitrate. Images are developed while agitating by rinsing with at least two changes of 0.28 M sodium carbonate containing 0.5 ml of 37% formaldehyde per liter. Development can be stopped with 1% acetic acid. Gels are washed twice with water before storage.

Autoradiography. Gels were soaked in methanol/H_2O/glycerol, 70:27:3 (vol/vol) for 1 min and dried on filter paper. Dried gels were placed in film cassettes (Kodak X-omatic) with Kodak XR-2 film and exposed at −70°C for 4 months.

Gel Analysis. Stained gels were photographed with 120-mm Tri-X (Kodak) film next to a calibrated density standard. Photographic negatives and autoradiograms were digitized at 100-μm resolution with an Optronics 1000 HS scanning densitometer (Optronics, Chelmsford, MA). Image densities of stained gels were converted to optical density units by using the calibrated standard. Gel comparisons and density measurements were made with a PDP 11/60 computer (Digital Equipment, Maynard, MA) equipped with a DeAnza IP5000 image processor (DeAnza Systems, San Jose, CA). To normalize density measurements, nonsaturating protein densities in each gel were compared with those in a control gel to derive a slope and y intercept by linear regression analysis.

RESULTS

Enzyme Assays. Patients' erythrocytes showed decreased HPRT and increased APRT activities. Patient HPRT activities were 18, 240, and <2 units (1 unit = 1 pmol of phosphoribosylated purine formed per mg of protein per min). Control activities were 1568, 1910, and 1768 units. There were 450, 246, and 632 units of APRT activity in the patients' erythrocyte lysates while controls had 140, 174, and 212 units.

Purified HPRT and Immunological Studies. Electrophoretic analysis of purified human HPRT and of immunoprecipitates prepared from control erythrocytes with HPRT-specific antibody showed three major species with pI values of 6.5, 6.3, and 6.1 and M_r of 26,000 (Fig. 1). Little, if any, HPRT was observed after immunoprecipitation and electrophoresis of patients' erythrocytes. However, two-dimensional electrophoretic patterns of both patient and control erythrocytes and lymphocytes showed proteins in the position expected for HPRT as determined by coelectrophoresis with purified HPRT.

Computer Analysis. The position of lymphocyte HPRT in an autoradiogram obtained from PHA-stimulated cells is shown in Fig. 2 (protein 295). Four hundred proteins were quantitated from autoradiograms of PHA-stimulated lymphocytes and from unstimulated lymphocytes whose proteins were silver stained. Protein index numbers were assigned by using local protein pattern geometries as guides. Correlation coefficients for protein densities in gel-to-gel comparisons were 0.91–0.99 and were higher (r = 0.970) for within-class comparisons than for control vs. patient comparisons (r = 0.936). Most proteins did not vary significantly in position or quantity (Fig. 3). Of 400 proteins analyzed, 3 varied positionally but did not correlate with the disease. The average level of positional variation for pairwise gel comparisons was 0.3%. This is close to the 0.5% rate of charge polymorphism observed in two-dimensional elec-

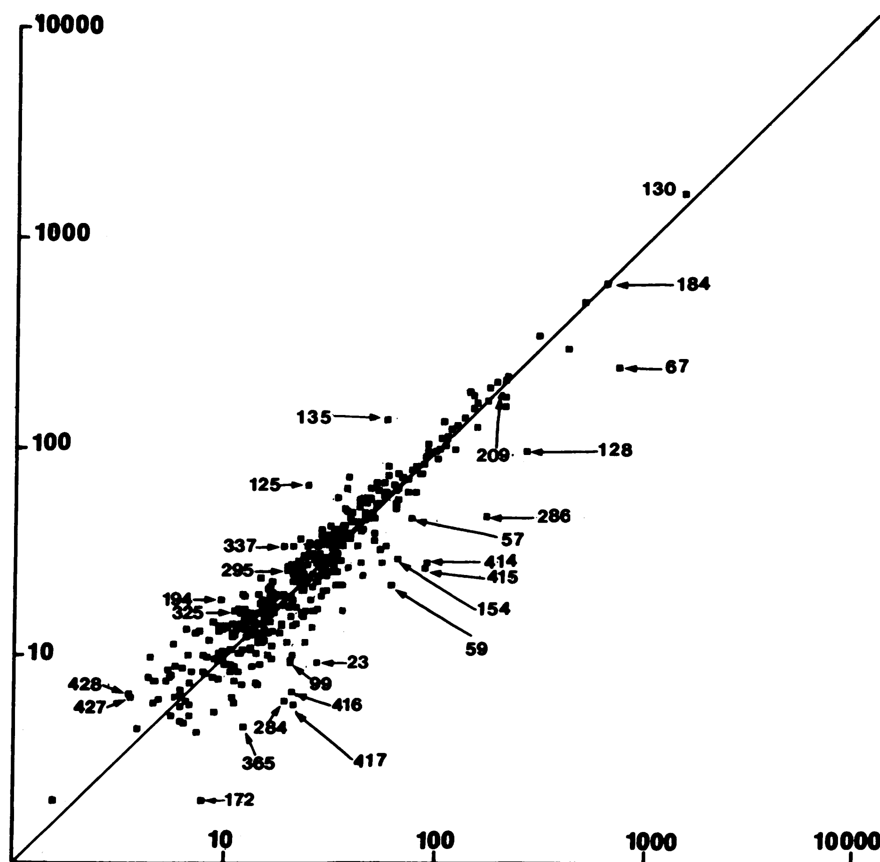

FIG. 3. Densitometric comparison of proteins from Lesch–Nyhan patients and controls. Densities were measured from autoradiograms of PHA-stimulated lymphocyte gels. Background values were derived from the modal density obtained from a histogram of all the intensities in the vicinity of each protein. Protein data are given in Table 1.

Table 1. Data for Figs. 2 and 3

Index no.	Protein	Density		t
		Control	Lesch–Nyhan	
23	*	23.1 ± 7.9	8.7 ± 3.6	−1.649
57	***	65.3 ± 0.3	40.7 ± 3.5	−7.007
59	**	54.4 ± 7.0	19.4 ± 4.4	−4.256
67	***	596.4 ± 56.2	217.8 ± 33.4	−5.798
99	**	17.0 ± 2.0	8.4 ± 0.7	−4.165
125	*	33.5 ± 2.1	65.2 ± 16.3	1.934
128	***	231.0 ± 4.1	84.9 ± 19.0	−7.508
130	Actin (β and γ)	1235.3 ± 69.1	1427.6 ± 184.2	0.977
135	*	50.9 ± 12.7	123.7 ± 29.7	2.252
154	**	57.0 ± 7.5	26.6 ± 1.7	−3.939
172	**	6.8 ± 1.3	1.8 ± 0.6	−3.549
184	β-Tubulin	533.9 ± 67.1	550.3 ± 26.3	0.228
194	***	10.5 ± 0.9	17.7 ± 0.4	7.479
206	α-Tubulin	168.0 ± 26.4	184.2 ± 11.0	0.568
209	IFP	181.1 ± 5.7	156.3 ± 15.9	−1.475
284	**	16.0 ± 3.6	5.5 ± 0.6	−2.897
286	***	147.3 ± 15.3	42.7 ± 10.2	−5.697
295	HPRT	19.0 ± 3.3	18.2 ± 3.7	−0.166
325	***	10.7 ± 0.1	13.2 ± 0.3	8.527
337	***	20.3 ± 1.3	10.6 ± 2.6	−5.298
365	***	10.4 ± 0.4	4.1 ± 0.4	−10.771
414	*	73.4 ± 20.4	25.7 ± 3.2	−2.311
415	*	71.8 ± 20.3	24.4 ± 5.4	−2.257
416	**	17.4 ± 2.3	6.2 ± 1.7	−3.830
417	***	17.8 ± 2.1	5.3 ± 1.4	−4.923
427	***	3.1 ± 0.1	5.7 ± 0.2	11.185
428	***	3.0 ± 0.1	6.0 ± 0.4	6.744

Results are mean ± SEM. One density unit equals optical density × 0.01 mm^2. Densities were measured from autoradiograms of PHA-stimulated lymphocytes by using computerized microdensitometry. ***, $2P < 0.01$ by Student's t test; **, $2P < 0.05$ and 2-fold difference seen between controls and patients; *, significant only as outliers from the normal distribution of the ratio of Lesch–Nyhan protein-density mean to control mean protein-density; IFP, intermediate filament protein.

trophoretograms of human fibroblasts (22), but the present study was not designed to detect normal positional variants. There were no significant variations in the densities of five identified proteins (actin α- and β-tubulin, intermediate filament protein, and HPRT) in the autoradiographic patterns (see Figs. 2 and 3). However, other proteins distinguished patients from controls. One of these is shown in Fig. 4. For proteins that varied significantly in the patient vs. control comparison (Figs. 2 and 3), 11 quantitative variations (observed in all patients) were significant at the $2P < 0.01$ level, and 6 were significant at the $2P < 0.05$ level and were more than 2-fold different. When the data were regrouped by mixing the patients and the controls so that one set contained two patients and one control and the other set contained two controls and one patient, there were only six proteins whose density variations were significant at the $2P < 0.01$ level.

The most dramatic of the protein variations found in unstimulated lymphocytes is shown in Fig. 5. This protein was increased in patients, being present at 3.37 times the concentration in controls. HPRT was decreased from 38.6 ± 3.2 in controls to 25.1 ± 1.2 in patients, but did not vary in autoradiograms of PHA-stimulated lymphocytes.

DISCUSSION

In initial studies, it was found that some Lesch–Nyhan patients had undetectable levels of HPRT. However, in later studies,

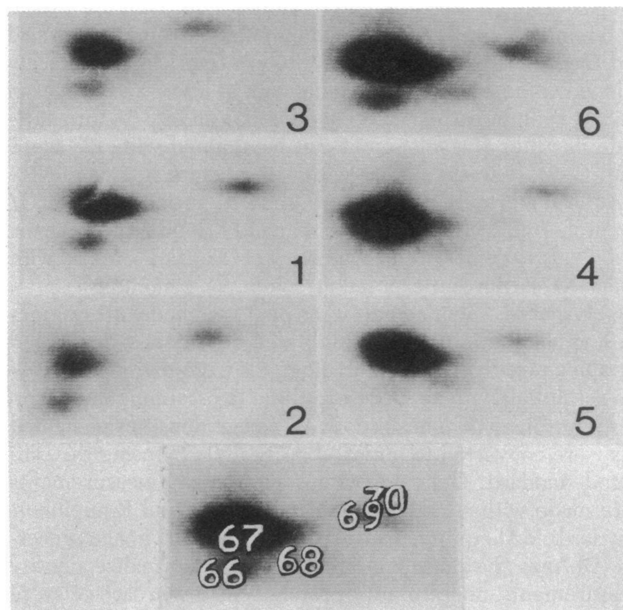

FIG. 4. Subregions from Lesch–Nyhan (*Left*; 1, 2, and 3) and control (*Right*; 4, 5, and 6) gels illustrate a difference observed in autoradiograms of PHA-stimulated lymphocytes. Protein 67 differed by a factor of 2.7, and the difference was significant at the $2P < 0.01$ level. This figure is a composite of gel subregions photographed from a computer video terminal.

which used large amounts of erythrocyte hemolysate, all cases showed some residual amount of HPRT activity. Cells other than erythrocytes contained still higher residual HPRT activity

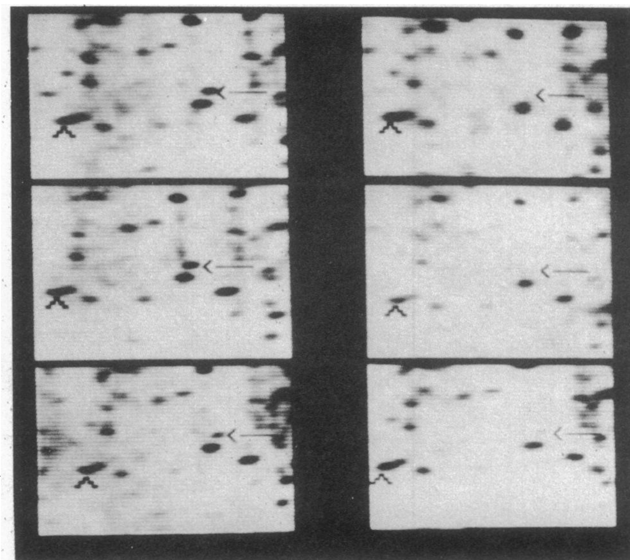

FIG. 5. Subregions from silver-stained gels from unstimulated lymphocytes of patients (*Left*) and controls (*Right*). ∧, HPRT; ←, protein 460, a variant protein that correlates with Lesch–Nyhan disease.

Index no.	Protein	Density		T
		Lesch–Nyhan	Control	
295	HPRT	25.1 ± 1.2	38.6 ± 3.2	3.955
460		28.0 ± 6.0	8.3 ± 2.1	3.121

Results are mean ± SEM. $2P < 0.05$. Subregions were photographed from a computer video terminal after contrast enhancement.

(23). Bakay and Nyhan (24) found that each of six patients had trace enzyme activities that migrated in the position of HPRT on disc gels. Studies of mutant HPRT proteins showed a wide range of enzyme activities, differences in thermal stability, and altered kinetic behavior, suggesting that a variety of structural mutations may occur (23).

The first reports using immune techniques found material that crossreacted with antibody to normal HPRT (25–32). Arnold and Kelley (25) found that all 14 patients studied had normal amounts of crossreacting material, and Rubin *et al.* (26) made a similar observation. However, with the advent of more highly purified human HPRT and more specific HPRT antibodies, most patients did not appear to have immunoreactive material (33–35). Of 16 patients studied by Ghangas and Milman, 4 had HPRT activities >0.1% of normal, but only one was crossreactive material-positive by their antibody method (33). In a study by Bakay *et al.* (35), none of 15 patients had crossreactive material; however, two patients had 2–5% of normal HPRT activity. Two other male patients, having 4.5% and 50% of normal HPRT activity, had disproportionate levels of crossreactive material (35). Despite the lack of crossreactive material in the three patients in this study (Fig. 1), protein that comigrated on electrophoresis with purified human HPRT was observed in lymphocyte (Fig. 5) and erythrocyte lysates and residual HPRT activity was detected in the erythrocytes of two patients. We found normal quantities of HPRT in autoradiograms of PHA-stimulated lymphocytes. Decreased levels of protein in the HPRT position in silver-stained gels of unstimulated lymphocytes could reflect an increased lability of mutant enzyme. Zannis and coworkers observed HPRT in two-dimensional electrophoretograms of erythrocytes from a Lesch–Nyhan patient (36).

Ghangas and Milman (33) have suggested that a defect in enzyme structure could result in loss of affinity to an antibody prepared against normal enzyme. Antibodies used in earlier studies may have had broader specificity. Narrow antibody specificity may result in lack of crossreactivity in the presence of a mutant enzyme detectable enzymatically or electrophoretically.

Although structural alterations in an enzyme may be undetectable, alterations in a metabolic pathway will often cause specific secondary metabolic changes. Increases in the activities of six enzymes have been described in the Lesch–Nyhan syndrome. Our survey showed a number of protein alterations, some of which may be characteristic. Extensive clinical studies will be required to establish their specificity. The protein surveying methods used in this study may be of use in understanding the pathophysiology of the Lesch–Nyhan syndrome and in searching for molecular alterations in diseases that have proved to be biochemically more elusive.

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