Enzyme Defect Associated with a Sex-Linked Human Neurological Disorder and Excessive Purine Synthesis

Abstract. *A sex-linked familial neurological disease consisting of cerebral palsy, mental retardation, choreoathetosis, and compulsive aggressive behavior is associated with a loss of an enzyme that participates in purine metabolism, namely, hypoxanthine-guanine phosphoribosyltransferase. The production of excessive uric acid in this disorder implies that the enzyme is involved in the normal regulation of purine biosynthesis. This is the first example of a relation between a specific enzyme defect and abnormal compulsive behavior. It is also the first enzyme defect in purine metabolism demonstrated in a neurological disease.*

A familial neurological disorder consisting of choreoathetosis, spasticity, mental retardation, aggressive behavior, and a compulsive biting resulting in mutilation of the lips and fingers was first described by Lesch and Nyhan (*1*). These findings on two young brothers were associated with hyperuricemia and excessive uric acid synthesis. Subsequently other cases have been described (*2, 3*). The original patients excreted in their daily urine three to six times the amount of uric acid found in urine from control subjects of similar body size and age. They incorporated between 100 and 200 times as much uniformly labeled glycine-^{14}C into urinary uric acid as nonaffected control subjects did. This degree of overproduction of uric acid is far greater than that encountered in adults with clinical gouty arthritis, yet development of clinical gouty arthritis is a relatively late manifestation of their disease. Gouty nephropathy, however, has been a contributing factor to the death of some of these patients in early puberty (*4*). The apparent familial distribution and limitation to males is compatible with X-linked inheritance (*3*).

The drug azathioprine [6-(1′-methyl-4′-nitro-5′-imidazolyl)thiopurine] (*5*), a purine analog used clinically as an immunosuppressive agent, has been reported to inhibit the excessive purine synthesis found in some gouty adults (*6*). We compared the effectiveness of this drug in inhibiting uric acid production in two children affected with the neurological disease and in two gouty men who also produced abnormally large quantities of uric acid. Both of the children were 14 years of age, unrelated, and had the complete clinical syndrome. Purine synthesis was assessed by the daily excretion of uric acid (while the four patients were on a purine-free diet) and the incorporation of 5 μc of orally administered glycine-1-^{14}C into urinary uric acid (*7*). As shown in Table 1, azathioprine at a dose of 4 mg per kilogram of body weight per day markedly suppressed purine synthesis in the two gout patients but did not diminish it in the children. Sorensen (*8*) found similar results.

Table 1. Effects of azathioprine on purine metabolism in patients manifesting overproduction of uric acid, showing the cumulative incorporation of isotopic glycine into urinary uric acid in 7 days (expressed as percentage of administered dose).

Subject	Drug	Serum urate (mg/100 ml)	Excretion, uric acid (mg/day)	Glycine incorporation (%)
	Adult overproduction—Gout			
B. P.	None	9.7	1241	1.24
B. P.	Azathioprine	6.5	740	0.22
T. B.	None	10.5	1529	1.11
T. B.	Azathioprine	6.8	922	0.28
	Childhood overproduction with neurological disease			
D. F.	None	12.4	659	2.49
D. F.	Azathioprine	11.8	719	2.70
F. H.	None	10.0	828	3.16
F. H.	Azathioprine	10.9	926	3.15

Since some of the pharmacological actions of azathioprine may result from its degradation to 6-mercaptopurine, the effect of the latter drug on purine synthesis by fibroblasts grown in vitro from skin biopsies obtained from a child with the neurological disorder was compared with its effect on cells from a normal subject. Cells were grown in Eagle's minimal medium supplemented with 10 percent fetal calf serum, nonessential amino acids, and neomycin (50 μg/ml), for bacteriostasis. Purine synthesis by fibroblasts growing in monolayer was blocked at the stage of formylglycinamide ribonucleotide (FGAR) by use of azaserine (*9*), and the activity of the first three enzymes of purine biosynthesis was assessed by determining the incorporation of sodium formate-^{14}C into FGAR (*10*). As shown in Table 2, $10^{-5}M$ 6-mercaptopurine greatly inhibited purine biosynthesis in normal cells but had no effect on cells derived from a child with the neurological disorder.

Resistance to the action of 6-mercaptopurine, ascribed to a deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (E.C. 2.4.2.8), has been described in mutant mammalian tumor cells selected in vitro (*11*) and in leukemic leukocytes selected in vivo during the course of treatment of leukemia with 6-mercaptopurine (*12*). These considerations led us to examine the activity of this enzyme in fibroblasts grown in vitro as well as in dialyzed hemolyzates of washed erythrocytes obtained from children affected with this neurological disease and from spastic quadriplegic, gouty, and normal subjects. This enzyme converts the free bases hypoxanthine or guanine to their respective ribonucleotides by reaction with 5-phosphoribosyl-1-pyrophosphate (PRPP) (Fig. 1).

The incorporation of hypoxanthine-8-^{14}C into inosinic acid, of guanine-8-^{14}C into guanylic acid, and of adenine-8-^{14}C into adenylic acid was determined after separation of these substances in the incubated reaction mixture by high-voltage electrophoresis. Both fibroblasts and erythrocytes (Table 3) from unrelated children manifesting the complete syndrome were unable to synthesize inosinic acid or guanylic acid from the respective bases but were able to synthesize adenylic acid from adenine. We conclude that the activity of the enzyme in erythrocytes from these chil-

Table 2. Effects of 6-mercaptopurine on incorporation of formate-^{14}C into formylglycinamide ribonucleotide in fibroblasts in tissue culture in the presence of azaserine. Growth medium (6 ml) deficient in glutamine and containing the appropriate concentration of 6-mercaptopurine and azaserine (10 μg/ml) was overlayered on cells in stationary monolayer culture (the cells were not confluent). After 30 minutes at 37°C, glutamine (0.5 mg/ml) and sodium formate-^{14}C (50 μc/flask, specific activity 52 mc/mmole) were added. Eighteen hours later the reaction was stopped by washing away the labeled substrate with 25 volumes of cold isotonic saline. The cells were treated with trypsin and harvested; the nucleotide was extracted by the addition of five volumes of ethanol and separated from other radioactive compounds by high voltage electrophoresis on 3-MM Whatman paper in 0.05*M* borate buffer, *p*H 9.0, at 2000 volts for 45 minutes. The portion of the paper containing radioactive FGAR was identified by radioautography cut out, and counted in a liquid scintillation counting system at 52 percent efficiency of counting.

6-Mercaptopurine (mole/liter)	FGAR-^{14}C accumulation in the soluble ethanol fraction*	
	Normal	Mutant
0	100†	100‡
10^{-6}	101	109
10^{-5}	19	99
10^{-4}	18	86

* Expressed as percentage of control without 6-mercaptopurine. † 7,500 count/min. ‡ 12,550 count/min.

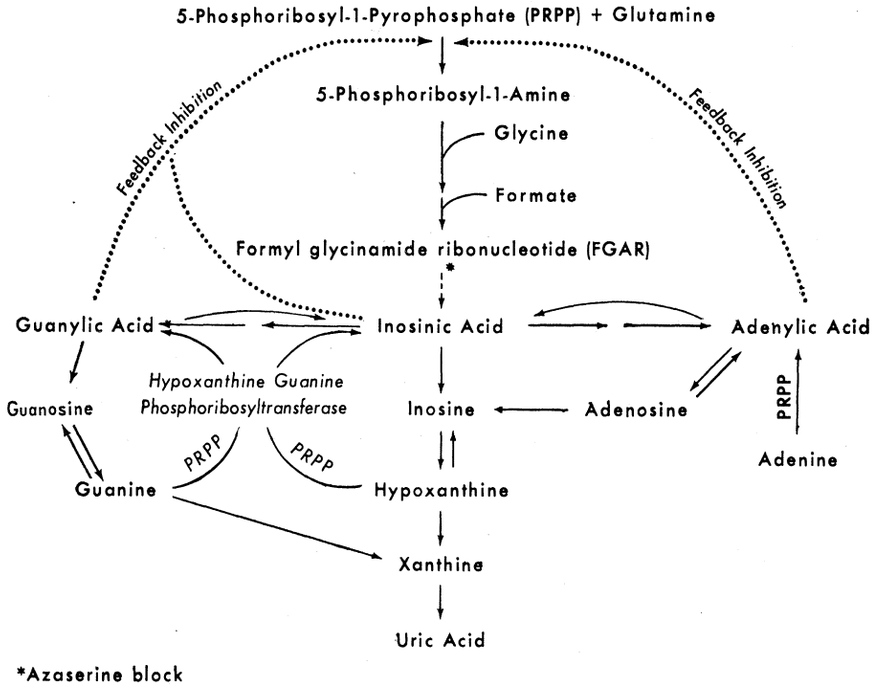

Fig. 1. Purine biosynthesis and interconversions.

Table 3. Specific activities of hypoxanthine, guanine, and adenine phosphoribosyltransferase from erythrocyte hemolyzates and fibroblast extracts. The erythrocyte hemolyzate assay system was made up as follows. Incubation mixtures contained 55 m*M* tris buffer, *p*H 7.4; 5 m*M* $MgCl_2$; 1 m*M* 5-phosphoribosyl-1-pyrophosphate (PRPP); 0.6 m*M* hypoxanthine-8-^{14}C (4.1 mc/mmole); 0.6 m*M* adenine-8-^{14}C (3.7 mc/mmole), or 0.14m*M* guanine-8-^{14}C (12.6 mc/mmole); and 0.6 to 1.2 mg of protein from dialyzed erythrocyte hemolyzate in a final volume of 200 μl. Each sample was incubated for 20 minutes at 38°C. The reactions were terminated by the addition of 4 μmole of neutralized ethylenediaminetetraacetate (EDTA) and chilling on ice. The fibroblast extract assay system was made as follows. The reaction mixture (total volume 120 μl) contained 330 μg of crystalline bovine albumin; 0.27 m*M* adenine-8-^{14}C (3.7 mc/mmole), or 0.27 m*M* hypoxanthine-8-^{14}C (6.6 mc/mmole); 1.67 m*M* PRPP; 9 m*M* $MgCl_2$; 6 m*M* tris buffer, *p*H 7.4; and between 150 to 225 μg of protein of cell extract consisting of the supernatant after centrifugation at 19,000*g* of disrupted washed fibroblasts. Incubation was at 25°C for 1 hour.

Source	Sex	Age	Phosphoribosyltransferase activity (count/min) per milligram of protein per hour as:		
			Inosinic acid	Guanylic acid	Adenylic acid
		Erythrocyte			
Normal adults					
J.E.S.	M	46	639	1188	141
J.S.,Sr.	M	28	511	1881	146
N.F.	F	51	574	1560	158
J.A.	F	32	467	1548	107
Spastic quadriplegia					
S.S.	M	6	653	1656	200
C.L.	F	4	526	1515	164
B.R.P.	M	2	443	1638	132
Adult overproduction—gout					
B.P.	M	27	592	1104	105
A.P.	M	48	477	1194	136
Childhood overproduction with neurological disease					
J.S.*	M	3	< 0.2	< 0.2	322
M.W.†	M	8	< 0.2	< 0.2	295
F.H.‡ (NIH 069353)	M	15	< 0.2	< 0.2	426
		Fibroblast			
Normal adult					
F.R.	M	30	13		31
Childhood overproduction with neurological disease					
D.F.§ (NIH 067235)	M	15	< 0.2		8

* Patient has been reported by Shapiro *et al.* (*3*). † This patient is the propositus of the original case description of Lesch and Nyhan (*1*). ‡ This patient was one of two affected siblings described in family A by Hoefnagel, *et al.* (*3*). § Case is being reported (*14*).

dren is less than 0.05 percent of normal activity. Hemolyzate from affected children added to the hemolyzate of a normal subject did not alter normal enzyme activity. From this we conclude that the presence of an inhibitor does not explain the absence of enzyme activity.

The biochemical function of the enzyme hypoxanthine-guanine phosphoribosyltransferase has been assumed until now to be solely that of allowing utilization of preformed purines. The precise mechanism by which the absence of this enzyme gives rise to overproduction of purines is not yet clear. The association of an excessive formation of a metabolic end product (uric acid) with an enzyme deficit rather than with a primary increase in activity or amount of an enzyme is of considerable interest. To our knowledge this is a unique finding among human mutants. These findings suggest that the deficient enzyme may be concerned with some aspect of the normal regulatory mechanism of purine synthesis (Fig. 1).

The absence of hypoxanthine-guanine phosphoribosyltransferase in these patients readily explains their failure to respond in the normal manner to azathioprine and to 6-mercaptopurine, since the latter compound must be converted to its ribonucleotide by this enzyme before it can function as a pseudo feedback inhibitor of purine synthesis (*11*).

The sex-linked pattern of inheritance in this disorder suggests that the genetic locus for this enzyme may be on the X chromosome, and suspected female carriers should therefore show mosaicism for this enzyme defect similar to that shown for glucose-6-phosphate dehydrogenase deficiency (*13*).

The association of a specific enzyme defect with a neurological disease, mental retardation, and a characteristic compulsive aggressive behavior may serve to reorient our fundamental approach to other behavioral disorders.

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Temperature Measurements from Oxygen Isotope Ratios of Fish Otoliths

Abstract. *Measurements have shown that the temperature of a fish's habitat can be deduced from the oxygen isotope ratio of its otoliths (ear bones). Isotope ratios obtained from fossil otoliths indicate a water temperature which agrees wiht that found by isotope measurements on associated benthonic foraminifera.*

Oxygen isotope measurements on calcium carbonate laid down by certain marine animals in the form of a shell or skeleton can give the temperature of the water in which the animals lived. Since the original work in oxygen isotope measurements by Urey *et al.* (*1*) many different forms of marine fossil carbonate, including belemnite guards, molluscan shells, and foraminiferal tests, have been used for this purpose.

Although fish form no suitable skeleton, their otoliths (ear bones) are composed of calcite and they have often been preserved as fossils in sediments. Otoliths from both living and fossil fish were studied, and the results given in this report indicate that fish form their otoliths in isotopic equilibrium with the sea water in which they live and that the temperature of their habitat can be deduced from the oxygen isotope ratio of these otoliths.

Suitably sized living fish that had lived under conditions of controlled temperature were not available for study, so samples were obtained of species that had known habitats, where the temperature range was also known. The isotopic "temperature" determined from an otolith is an average of the temperature fluctuations from winter to summer—possibly a weighted average if the calcite was not laid down at an even rate all year round.

Results of oxygen isotope measurements on fossil otoliths contained in Tertiary sediments from New Zealand gave temperatures close to the bottom temperature, based on similar measurements on associated mollusk shells and benthonic foraminifera tests (*2*).

Fossil otolith samples were cleaned in an ultrasonic bath for 2 minutes; they were then dried and ground to a powder. Otoliths of living fish were powdered and soaked in a 5 percent aqueous solution of sodium hypochlorite for 3 days to remove any organic material incorporated in the calcite. (I found that sodium hypochlorite treatment is unnecessary with fossil otolith material.) After soaking, the samples were rinsed with distilled water and dried. Powdered samples were reacted with 100 percent phosphoric acid under vacuum in a glass reaction tube at 25°C. Oxygen and carbon isotope ratios of the resulting carbon dioxide were measured on a Nuclide Analysis Associates 60°, 6-inch (15-cm), double collector mass spectrometer.

The results are given in Tables 1 and 2. Oxygen isotope ratios are given in the δ notation, that is,

$$\delta O^{18} = \left[\frac{(O^{18}/O^{16})_{sample}}{(O^{18}/O^{16})_{PDB}} - 1 \right] 1000$$

with respect to PDB (carbon dioxide prepared by phosphoric acid evolution, Peedee belemnite carbonate from the University of Chicago; it is an international standard for carbon and oxygen isotope measurements). The analytical error in each δO^{18} measurement is $\pm$ 0.2 (*3*). The temperature was calculated from the result of the oxygen isotope measurement by using the equation of Epstein *et al.* (*4, 5*). The expected temperature for living fish is the mean of summer and winter temperatures of the water in the area where the fish lived (*6*).

The fossil otoliths that I examined were from various Pliocene and Miocene sediments and were thought to have been deposited at depths of a few hundred meters. Unsuccessful attempts were made to identify the fossil otoliths from published data and by comparison with otoliths from Recent species. However, before the powdered samples were prepared the otoliths from each sediment sample were grouped arbitrarily on the basis of their shape. Each group was clearly distinct but may have contained otoliths from several species of fish. Each powdered sample was made up of one complete otolith, and this normally weighs 5 to 10 mg. A typical series of results for one sediment sample is given in Table 2.

Results (Table 2) obtained from measurements on benthonic foraminifera indicate that the bottom water temperature was 8° to 9°C. The otolith

Table 1. Results of oxygen isotope measurements on otoliths from living fish. Analytical error in each δO^{18} measurement is $\pm$ 0.2 (*3*).

Sample	δO^{18} (with respect to PDB)	Temperature (°C)	
		Calc. isotopic	Expected
Tarakihi (Cheilodactylus macropterus)			
1st specimen			
1st otolith	2.1	8.3	8 to 10
2nd otolith	2.1	8.3	8 to 10
2nd specimen			
1st otolith	2.0	8.7	8 to 10
Butterfish (Coridodax pullus)			
1st specimen			
2 otoliths combined	1.4	10.9	12 to 13
2nd specimen			
2 otoliths combined	1.1	12.0	12 to 13
Snapper (Chrysophrys auratus)			
1st specimen			
1st otolith	0.2	15.7	15 to 16

Table 2. Results of oxygen isotope measurements on fossil otoliths contained in an Upper Miocene sediment from Bells Creek, Wairarapa, New Zealand. Analytical error in each δO^{18} measurement is $\pm$ 0.2 (*3*).

Sample	δO^{18} (with respect to PDB)	Calc. isotopic temp. (°C)
Benthonic foraminifera		
Miliolidae, mixed species	2.2	8.0
Robulus species	1.8	9.4
Otoliths		
Group A		
1st specimen	2.2	8.0
2nd specimen	2.0	8.7
3rd specimen	2.0	8.7
4th specimen	1.7	9.7
Group B		
1st specimen	1.4	10.9
2nd specimen	1.3	11.3