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Purine Metabolism in Normal and Thioguanine-Resistant Neuroblastoma

(cell culture/hypoxanthine phosphoribosyltransferase/Lesch–Nyhan syndrome)

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ABSTRACT **Purine metabolism has been examined in a clonal line of mouse neuroblastoma cells resistant to the cytotoxic effects of 6-thioguanine. Comparative studies in the resistant and parental lines indicate that the former cells have less than 1% of normal hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) activity. The activities of other enzymes important in the *de novo* and salvage pathways of purine biosynthesis were not significantly different in the two lines. Hypoxanthine phosphoribosyltransferase deficiency in this neuroblastoma line was associated with increased intracellular concentrations of 5-phosphoribosyl-1-pyrophosphate, an increased rate of purine biosynthesis *de novo*, and failure to incorporate hypoxanthine, but not adenine, into nucleotides. There are essentially the same alterations in purine metabolism that occur in hypoxanthine phosphoribosyltransferase-deficient fibroblasts or lymphoblasts derived from individuals with the Lesch–Nyhan syndrome. Clonal lines of hypoxanthine phosphoribosyltransferase-deficient neuroblastoma cells may therefore be of use in elucidating the mechanisms by which the enzyme defect leads to the neurologic dysfunction seen in children with this disease.**

The Lesch–Nyhan syndrome (1) is a rare, X-linked disease whose primary biochemical defect is a severe deficiency of hypoxanthine phosphoribosyltransferase (HPRT) (EC 2.4.2.8) activity (2). This enzyme deficiency results in a characteristic increase in rate of purine nucleotide synthesis in fibroblasts (3) and lymphoblasts (4) derived from these patients. In addition to abnormal metabolism of purines, patients with the Lesch–Nyhan syndrome exhibit in varying degrees severe neurological abnormalities characterized by mental retardation, spasticity, choreoathetosis, and compulsive self-mutilation. The pathophysiology interrelating the biochemical and neurological abnormalities has not been elucidated.

Animal models of this disease are not yet available. However, cells of the nervous system cultured *in vitro* and expressing the mutant phenotype offer an opportunity for the study and potential delineation of the neurological abnormalities. Relevant material from the human nervous system is difficult to obtain and also represents a complex mixture of cell types. In contrast, clonal lines of mouse neuroblastoma cells grown *in vitro* represent homogeneous neuroectodermal cell populations which exhibit several neural properties including neurites (5–8), electrically excitable membranes (8), acetylcholine receptors (9), and enzymes for the synthesis and degradation of neurotransmitter (5, 6, 10, 11). In this report, purine metabolism in a thioguanine-resistant clonal line of mouse neuroblastoma cells is described. The cells are shown to be deficient in HPRT activity and show the increased rate of purine nucleotide synthesis characteristic of cells from patients with the Lesch–Nyhan syndrome.

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); PP-ribose-P, 5-phosphoribosyl-1-pyrophosphate; APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); FGAR, α-*N*-formyl-glycinamide ribonucleotide; DPBS, Dulbecco's phosphate-buffered saline; DME, Dulbecco's modified Eagle's medium 6-SGua, 6-thioguanine; PP-ribose-P amidotransferase, glutamine phosphoribosyl pyrophosphate amidotransferase (EC 2.4.2.14).

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EXPERIMENTAL PROCEDURES

Materials. [8-^{14}C]Adenine (60 mCi/mmol), [*U*-^{14}C]adenine (287 mCi/mmol), [8-^{14}C]hypoxanthine (59 mCi/mmol), and sodium [^{14}C]formate (56 mCi/mmol) were obtained from Amersham-Searle, and [*U*-^{14}C]glutamine (218 mCi/mmol) from New England Nuclear. Other materials were purchased as follows: unlabeled purine bases, nucleosides, and nucleotides from Sigma; azaserine and 6-thioguanine (6-SGua) from Calbiochem; dimagnesium phosphoribosyl-1-pyrophosphate (PP-ribose-P) from P-L Laboratories; cellulose thin-layer chromatography sheets from Eastman Kodak; polyethyleneimine-cellulose thin-layer chromatography sheets from Baker; and DEAE-cellulose paper discs (DE-81) from Whatman. Dulbecco's modified Eagle's medium (DME) was obtained from Grand Island Biological. All other chemicals were of reagent grade and purchased commercially.

Origin and Maintenance of Cells. Clone N4TG1 is a murine neuroblastoma line derived from Cl300 clone N4 by treatment with ethyl methanesulfonate and sequential selection in 10^{-6} M and 10^{-4} M 6-SGua. The conditions used in mutagenesis, selection, and propagation of this clone have been described (12, 13). At biweekly intervals, the N4TG1 cells were grown for several generations in media containing 10^{-4} M 6-SGua to eliminate any cells that may have reverted to the parental phenotype.

Enzyme Assays. Cells in late logarithmic phase growth were harvested by gentle tapping in fresh media, washed three times in Dulbecco's phosphate-buffered saline (DPBS), and resuspended in a minimal volume of 0.29 M sucrose, 0.01 M Tris·HCl, pH 7.4 at a cell density of about 10^8 cells/ml. After disruption by freeze-thawing three times in liquid nitrogen, cellular debris and mitochondria were collected by centrifugation at 30,000 × *g* for 30 min at 4° and discarded. HPRT and adenine phosphoribosyltransferase (APRT) (EC 2.4.2.7) activities were determined in the cell-free ex-

tracts by a described (4) modification of the method of Fujimoto and Seegmiller (14). Glutamine phosphoribosyl pyrophosphate amidotransferase (PP-ribose-P amidotransferase) (EC 2.4.2.14) activity was assayed by determining the PP-ribose-P-dependent hydrolysis of glutamine to glutamic acid by the method of Martin (15) described in detail elsewhere (4). PP-Ribose-P synthetase (EC 2.7.6.1) activity was determined by a described modification (4) of the two-step procedure of Hershko *et al.* (16). For this assay, the cells were washed and disrupted as described above except that the final buffer was 15 mM sodium phosphate, pH 7.4, with 5 mM reduced glutathione and 1 mM EDTA.

Estimation of the Rate of de novo Purine Synthesis. The rate of the early steps of purine synthesis was estimated by the measurement of the incorporation of sodium [^{14}C]formate into α-*N*-formylglycinamide ribonucleotide (FGAR) in the presence of azaserine, a glutamine analog which blocks further conversion of this intermediate in *de novo* purine synthesis by inhibiting the enzyme phosphoribosyl-formylglycineamidine synthetase (EC 6.3.5.3). A cellulose thin-layer chromatographic method (17) for separation of FGAR and FGAR polyphosphate from other labeled compounds was used after incubation and ethanol extraction, as described (3).

Determination of PP-ribose-P Concentration. Intracellular concentration of PP-ribose-P was determined by the conversion of [^{14}C]adenine to [^{14}C]adenylic acid in the presence of an excess of highly purified human erythrocyte APRT (M. L. Greene, J. A. Boyle, and J. E. Seegmiller, in preparation). Complete experimental details have been described (4).

Incorporation of Adenine and Hypoxanthine into Acid-Soluble Nucleotides. Cells were harvested, washed three times in DPBS, and resuspended in fresh media at a concentration of 2 $\times$ 10^6 cells per ml. One million viable cells were incubated with 50 μl of 4.2 mM [8-^{14}C]hypoxanthine (59 mCi/mmol) or the same volume of 0.8 mM [8-^{14}C]adenine (60 mCi/mmol) for 60 min at 37°. The reaction was then terminated by the addition of 20 μl of 42% perchloric acid (0°). After 10 min at 0°, 30 μl of 4.45 M KOH was added to neutralize the reaction mixture and the precipitate was removed by centrifugation at 1200 $\times$ *g*. An aliquot of the supernatant fluid was applied to sheets of polyethyleneimine-cellulose and developed in methanol–water (50:50 v/v) for 16 hr, as described by Crabtree and Henderson (18). The purine nucleotides that remained at the origin were cut out and counted for radioactivity without further resolution.

RESULTS

HPRT Activity. Fig. 1 shows the relationship between HPRT activity and protein concentration and time of incubation in cell-free extracts of the parental (N4) and thioguanine-resistant (N4TG1) cell lines. Under the assay conditions described, inosinic acid formation in lysates of normal cells was proportional to protein concentration up to 100 μg of protein and to incubation time for at least 50 min. The specific activity of HPRT in the N4 line was 200 nmol/hr per mg of protein, while enzyme activity of N4TG1 cells grown in the absence of 6-SGua for at least 72 hr was less than 1% of this value. When extracts from parental and 6-SGua-resistant lines were mixed, neither an inhibitor of HPRT activity in N4TG1 cell extracts nor an activator of enzyme activity in the N4 cell extract was detected.

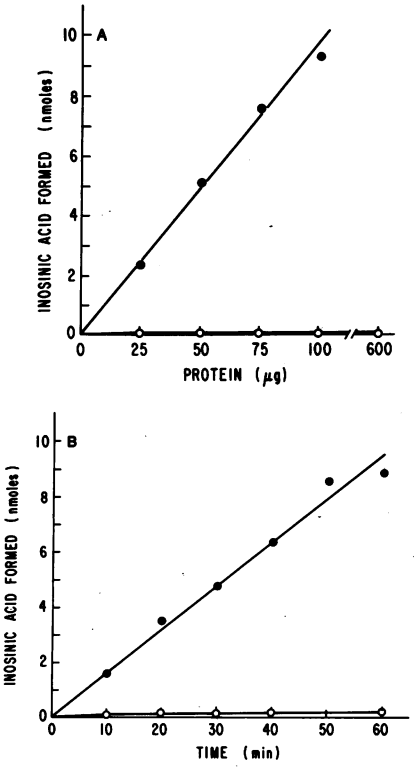

FIG. 1. Activity of HPRT as a function of protein concentration (*A*) and time of incubation (*B*) in normal (●) N4 cell extracts and in 6-SGua-resistant (○) N4TG1 cell extracts. Assays were done as described in *Experimental Procedures* and the legend to Table 1. Time of incubation in *A* was 30 min; protein concentration in *B* was 50 μg/assay.

Other Enzymes of Purine Synthesis. APRT catalyzes the formation of AMP from adenine and PP-ribose-P by a reaction analogous to the HPRT reaction. PP-Ribose-P is also a substrate for glutamine PP-ribose-P amidotransferase, the first and presumed rate-limiting reaction of *de novo* purine biosynthesis. PP-Ribose-P itself is synthesized from ATP and ribose-5-phosphate by PP-ribose-P synthetase. Table 1 illustrates the specific activities of these enzymes in N4 and N4 TG1 cell extracts. Except for the loss of HPRT activity, thioguanine-resistant cells showed no significant alterations in the enzyme activities examined.

Intracellular PP-Ribose-P Concentration and Rate of Initial Reactions of de novo Purine Biosynthesis in Intact Cells. Previous studies in fibroblasts (3) and lymphoblasts (4) have shown that loss of HPRT activity is associated with an increased intracellular concentration of PP-ribose-P and an increased rate of purine biosynthesis *de novo*. The data in Table 2 show that N4TG1 cells deficient in HPRT have a five-fold elevation in PP-ribose-P concentration and an almost three-fold increase in FGAR synthesis. Addition of hypoxanthine to the incubation mixture in a final concentration of 1 $\times$ 10^{-4} M results in a 40% inhibition of FGAR synthesis in N4 cells, but no significant change in FGAR synthesis in N4TG1 cells. Adenine at the same concentration completely suppresses FGAR synthesis in both cell lines. Purine base inhibition of *de novo* purine biosynthesis is presumably mediated through inhibition of PP-ribose-P amidotransferase by the nucleotide form of the purine base and by depletion of intracellular PP-ribose-P. The loss of HPRT activity in the

TABLE 1. *Comparative activities of PP-ribose-P-metabolizing enzymes in lysates of neuroblastoma cells*

Cell Line	Enzyme activity* nmol/hr per mg of protein			
	HPRT†	APRT†	PP-ribose-P amido-transferase‡	PP-ribose-P synthetase§
N4	200	1050	100	600
N4TG1	2	1132	89	610

* All enzymes were assayed under optimal conditions. Values represent the average of at least two separate experiments performed in duplicate.

† Phosphoribosyltransferase reaction mixtures contained in a final volume of 0.10 ml: 50 μmol Tris·HCl (pH 7.4); 5 μmol $MgCl_2$; 1.0 μmol PP-ribose-P; and either 0.8 μmol [8-^{14}C]hypoxanthine or 0.4 μmol [8-^{14}C]adenine (5 mCi/mmol). Incubation time was 30 min at 37° and amount of protein assayed was 50 μg.

‡ Reaction mixture contained in a final volume of 0.10 ml: 50 μmol Tris·HCl (pH 6.8); 5 μmol $MgCl_2$; 2 μmol PP-ribose-P; and 4μmol L-[U-^{14}C]glutamine (2 mCi/mmol). Incubation time was 30 min at 37° and amount of protein assayed was 400 μg.

§ PP-ribose-P was generated in a 2.0-ml reaction mixture, final pH 7.4, containing: 100 μmoles Tris·HCl; 2 μmol EDTA; 0.10 μmol $MgCl_2$; 5 μmoles reduced glutathione; 60 μmoles sodium phosphate; 1.0 μmol ATP; 0.7 μmoles ribose-5-phosphate; and 30–65 μg protein from dialyzed cell extract. Incubation time was 20 min at 37°. The reaction was stopped by addition of 0.2 ml of 0.1 M EDTA. After deproteinization by heating for 1 min at 98°, PP-ribose-P was determined by conversion of [8-^{14}C]-adenine to adenylic acid catalyzed by purified APRT (4).

TABLE 2. *Purine metabolism in normal and HPRT-deficient neuroblastoma cells*

	Normal cells (N4)	HPRT-deficient cells (N4TG1)
A. PP-ribose-P (pmoles/10^6 cells)	76	425
B. FGAR synthesis (cpm/10^6 cells)		
No addition	121,000	330,000
10^{-4} M adenine	<500	<500
10^{-4} M hypoxanthine	72,600	323,000
C. Soluble purine nucleotide from adenine (cpm/10^6 cells)	301,000	260,000
D. Soluble purine nucleotide from hypoxanthine (cpm/10^6 cells)	108,000	100

A. Cells were harvested and washed twice in fresh DME medium. Immediately before immersion in a 98° water bath for 45 sec, cells were collected by centrifugation and resuspended in 0.3 ml of 50 mM Tris·HCl, 1 mM EDTA, pH 7.4. PP-ribose-P was determined as described (4). Values represent the average of five replicate determinations.

B. Rate of [8-^{14}C]formate incorporation into FGAR was determined in cells washed twice in fresh DME and resuspended in a final volume of 0.6 ml which contained 6×10^5 neuroblastoma cells in 0.5 ml fresh DME, 150 μg glycine, 30 μg azaserine, and 0.42, μmol sodium [^{14}C]formate (56 mCi/mmol). All reagents were dissolved in DPBS. Incubation at 37° was for 60 min.

C. and D. Purine nucleotide synthesis from labeled adenine or hypoxanthine was determined as described in *Experimental Procedures*.

thioguanine-resistant cells thus explains the failure of hypoxanthine to inhibit FGAR production.*

Purine Base Incorporation into Nucleotides. A direct assessment of the relative abilities of N4 and N4TG1 cells to form acid-soluble nucleotides from purine bases is shown in Table 2. Adenine uptake and incorporation into total nucleotide in N4TG1 is 90% of that found in the parental line (*C*, Table 2), while incorporation of hypoxanthine into nucleotide is less than 1% of that in the parental line (*D*, Table 2). The marked deficit in the ability of the HPRT-deficient cells to incorporate hypoxanthine into nucleotide provides a functional demonstration of the basis of their resistance to the toxic effects of 6-SGua which is converted to the pharmacologically active nucleotide by HPRT. The 10% difference in adenine incorporation in N4 and N4TG1 cells may be due to the breakdown of labeled AMP to hypoxanthine [by way of adenosine and inosine (19)] and the inability of HPRT-deficient cells to reutilize the base for nucleotide formation.

DISCUSSION

While HPRT is distributed throughout the body, the highest levels of enzyme activity are found in the brain, particularly in the basal ganglia, where levels are ten-fold higher than in other tissues (20, 21). HPRT activity was grossly deficient in brain tissue obtained at autopsy from a child affected with the Lesch–Nyhan syndrome as well as from two affected fetuses (21–23). Despite the severe impairment of neurological function in affected individuals, no distinctive morphologic or histologic changes in the central nervous system have been observed, and the role of HPRT in normal neuronal function is not yet understood. Three subpopulations of HPRT-deficient individuals can be identified on the basis of neurological impairment. The most severe deficiencies of HPRT activity result in the classic neurological features of the Lesch–Nyhan syndrome. Individuals with 1% or more of normal erythrocyte HPRT activity have no apparent neurological abnormalities, but exhibit excessive production of uric acid. An intermediate group possesses some neurological abnormalities, varying from individual to individual. The extent of neurological impairment is inversely related to the residual HPRT activity (20).

Girls heterozygous for this X-linked defect have no neurological manifestations despite the predicted enzyme absence in that fraction of cells undergoing X inactivation of the chromosome bearing the normal allele (24). If X inactivation is a general phenomenon throughout the body, this latter observation is particularly puzzling, given the ability to detect clinically defective function of a small number of neurons. A possible explanation for the failure to detect neurological impairment in heterozygotes may be the ability of normal cells to supply critical metabolites or the information for their synthesis to cells deficient in HPRT. Such metabolic cooperation has been demonstrated in fibroblast cultures in which normal and HPRT-deficient cells are grown together (14, 25, 26). An alternative possibility would be a selective elimination of HPRT-deficient neuronal cells very early in embryonic development. Although no evidence of such a selection of

* A molecular model for these interactions has recently been presented by the elegant studies of Holmes, Kelley, and Wyngaarden (1973), *J. Biol. Chem.* **248**, 6035–6040.

neuronal cells has been found, an analogous loss of HPRT-deficient precursors of hematopoietic cells has been proposed to account for the normal content of HPRT generally found in erythrocytes and lymphocytes from girls heterozygous for severe HPRT deficiency (27, 28). Since the two populations of cells predicted by the random inactivation of one of the two X chromosomes in women by the Lyon Hypothesis (24) are present in fibroblasts (29–31) and hair follicles (32–34) of heterozygous women, the selective loss of HPRT-deficient cells occurs only in certain tissues.

A major question in understanding the pathophysiology of the Lesch–Nyhan syndrome is whether the enzyme defect must be expressed within the nervous system (autonomous) or in some other organ system. A related question is whether the defect must be expressed in neurons or glia, either one or both cell types. Because human nervous system material is difficult to obtain, two general experimental approaches are possible. First, induction of the mutation in whole animals, either vertebrate [e.g., mouse as recently outlined by DeMars (35)] or invertebrate (e.g., drosophilia), would appear useful. The use of gynandromorphs would appear particularly well suited to the study of the autonomy of the defect (36). Second, culture of nervous tissue either from patients bearing the defect, or of specially created mutant neural and glial lines is possible.

This paper reports a mutant mouse neuroblastoma line (N4TG1), deficient in HPRT, that was selected by virtue of resistance to 6-SGua. In addition to a deficiency of HPRT, the mutant line is characterized biochemically by increased intracellular PP-ribose-P concentration, an increased rate of purine biosynthesis *de novo* unaffected by exogenously added hypoxanthine, and failure to incorporate hypoxanthine into nucleotides. As expected, loss of HPRT activity is not associated with any significant differences in other enzymes important for purine nucleotide synthesis. These findings, which represent the first direct demonstration of increased purine production in neural tissue deficient in HPRT, are in accordance with data on fibroblasts and lymphoblasts derived from patients with the Lesch–Nyhan syndrome (3, 4) and, thus, the mutant neuroblastoma cells biochemically mimic the cells of these patients. Whether the defect in the neuroblastoma cells represents a mutation in a structural gene or an inherited change in regulation of HPRT activity has yet to be resolved.

Is HPRT deficiency associated with any defect in neural properties? To date, 6-SGua-resistant neuroblastoma clones, including N4TG1, have shown no qualitative differences from parental lines in activity of acetylcholinesterase, neurite formation, silver impregnation, electrical excitability (12, 13), or synthesis of acetylcholine (37). It should now be possible to study neurotransmitter metabolism and membrane chemosensitivity in HPRT-deficient mutant clones derived from parent neuroblastoma lines of the appropriate phenotype (37). The possible role of HPRT deficiency in glial cell functions and neuronal-glial interactions may now also be explored with a 6-SGua-resistant clone derived from rat glioblastoma lines (37).

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1. Lesch, M. & Nyhan, W. L. (1964) *Amer. J. Med.* **36**, 561–570.
2. Seegmiller, J. E., Rosenbloom, F. M. & Kelley, W. N. (1967) *Science* **155**, 1682–1684.
3. Rosenbloom, F. M., Henderson, J. F., Caldwell, I. C., Kelley, W. N. & Seegmiller, J. E. (1968) *J. Biol. Chem.* **243**, 1166–1173.
4. Wood, A. W., Becker, M. A. & Seegmiller, J. E. (1973) *Biochem. Genet.* **9**, 261–274.
5. Augusti-Tocco, G. & Sato, G. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 311–315.
6. Schubert, D., Humphreys, S., Baroni, C. & Cohn, M. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 316–323.
7. Olmsted, J. B., Carlson, K., Klebe, R., Ruddle, F. & Rosenbaum, J. (1970) *Proc. Nat. Acad. Sci. USA* **65**, 129–136.
8. Nelson, P., Ruffner, W. & Nirenberg, M. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 1004–1010.
9. Harris, A. J. & Dennis, M. J. (1970) *Science* **167**, 1253–1255.
10. Seeds, N. W., Gilman, A. G., Amano, T. & Nirenberg, M. W. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 160–167.
11. Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R. & Nirenberg, M. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 786–792.
12. Minna, J., Nelson, P., Peacock, J., Glazer, D. & Nirenberg, M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 234–239.
13. Minna, J., Glazer, D. & Nirenberg, M. (1972) *Nature New Biol.* **235**, 225–231.
14. Fujimoto, W. J. & Seegmiller, J. E. (1970) *Proc. Nat. Acad. Sci. USA* **65**, 577–584.
15. Martin, D. W., Jr. (1972) *Anal. Biochem.* **46**, 239–243.
16. Hershko, A., Razin, A. & Mager, J. (1969) *Biochim. Biophys. Acta* **184**, 64–76.
17. Boyle, J. A., Raivio, K. O., Becker, M. A. & Seegmiller, J. E. (1972) *Biochim. Biophys. Acta* **269**, 179–183.
18. Crabtree, G. W. & Henderson, J. F. (1971) *Cancer Res.* **31**, 985–991.
19. Sorenson, L. B. (1970) *J. Clin. Invest.* **49**, 968–978.
20. Kelley, W. N., Greene, M. L., Rosenbloom, F. M., Henderson, J. F. & Seegmiller, J. E. (1969) *Ann. Intern. Med.* **70**, 155–206.
21. Rosenbloom, F. M., Kelley, W. N., Miller, J., Henderson, J. F. & Seegmiller, J. E. (1967) *J. Amer. Med. Ass.* **202**, 175–177.
22. Boyle, J. A., Raivio, K. O., Astrin, K. H., Schulman, J. D., Graf, M. L., Seegmiller, J. E. & Jacobson, C. B. (1970) *Science* **169**, 688–689.
23. Van Heeswijk, P. J., Blank, C. H., Seegmiller, J. E. & Jacobson, C. B. (1972) *Obstet. Gynecol.* **40**, 109–113.
24. Lyon, M. F. (1961) *Nature* **190**, 372–373.
25. Subak-Sharpe, H., Bürk, R. R. & Pitts, J. D. (1966) *Heredity* **21**, 342–343.
26. Friedmann, T., Seegmiller, J. E. & Subak-Sharpe, J. H. (1968) *Nature* **220**, 272–274.
27. Dancis, J. P., Berman, P. H., Jansen, V. & Balis, M. E. (1968) *Life Sci.* **7**, 587.
28. Nyhan, W. L., Bakay, B., Connor, J. D., Marks, J. F. & Keele, D. K. (1970) *Proc. Nat. Acad. Sci. USA* **65**, 214–218.
29. Rosenbloom, F. M., Kelley, W. N., Henderson, J. F. & Seegmiller, J. E. (1967) *Lancet* **2**, 305–306.
30. Salzmann, J., DeMars, R. & Benke, P. (1968) *Proc. Nat. Acad. Sci. USA* **60**, 545–552.
31. Migeon, B. R., Der Kaloustian, V. M., Nyhan, W. L., Young, W. J. & Childs, B. (1968) *Science* **160**, 425–427.
32. Gartler, S. M., Scott, R. C., Goldstein, J. L., Campbell, B. & Sparkes, R. (1971) *Science* **172**, 572–573.
33. Silvers, D. N., Cox, R. P., Balis, E. M. & Dancis, J. (1972) *N. Engl. J. Med.* **286**, 390–395.
34. Francke, U., Bakay, B. & Nyhan, W. L. (1973) *J. Pediat.* **82**, 472–478.
35. DeMars, R. (1971) *Fed. Proc.* **30**, 944–955.
36. Hotta, Y. & Benzer, S. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1156–1163.
37. Minna, J. D. (1973) "Genetic Analysis of the Mammalian Nervous System Using Somatic Cell Culture Techniques," in *Tissue Culture of the Nervous System*, ed. Sato, G. (Plenum Publishers, New York, N.Y.), Vol. 1 in press.