To the Graduate Council:

I am submitting herewith a thesis written by Daniel Grey Hatcher III entitled "Characterization of a V-79 Mutant Deficient in HPRT Activity." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

Robert E. Bryant
Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

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# CHARACTERIZATION OF A V-79 MUTANT DEFICIENT IN HPRT ACTIVITY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Daniel Grey Hatcher III

June 1980

3041830

ABSTRACT

A mutant Chinese hamster cell line, designated trMH-19, is 99% deficient in the uptake of hypoxanthine when compared to the parent cell line, V-79. The aim of my thesis was to test the hypothesis that trMH-19 had a genetically altered hypoxanthine phosphoribosyl transferase (HPRT) activity.

Plating efficiencies in media containing selective and counterselective inhibitors were performed. HAT medium contains hypoxanthine, aminopterin, and thymidine. In IAT medium inosine is substituted for the hypoxanthine of HAT medium. Cells viable in HAT and IAT media must be able to utilize exogenous purines via HPRT. V-79A cells exhibited normal viability in HAT and IAT media while trMH-19 cells did not. Eight-azaguanine is lethal to cells that can metabolize the compound via HPRT. V-79A cells were not viable in 8-azaguanine while trMH-19 cells were. These results are consistent with the hypothesis that trMH-19 does not have an in vivo functioning HPRT.

The metabolism of hypoxanthine was monitored in V-79A and trMH-19 by incubating the cells with $^{3}$H-hypoxanthine and then extracting the $^{3}$H-products and separating them by thin-layer chromatography. The results of these experiments led to the conclusion that trMH-19 cells did not convert

hypoxanthine to any form of phosphorylated product. This was a strong indication of defective HPRT in trMH-19.

HPRT enzyme from V-79A and trMH-19 was assayed in cell extracts to determine the apparent $K_m$'s of the enzyme from each line for hypoxanthine and for phosphoribosyl pyrophosphate (PRPP). A twelve-fold difference in $K_m$ for PRPP was found in the mutant enzyme. This difference in $K_m$ indicates a structural change in the HPRT enzyme. This twelve-fold decrease in the affinity of HPRT from trMH-19 for PRPP probably causes the 99% reduction in hypoxanthine uptake.

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# CHAPTER I

## INTRODUCTION

The plating techniques for mammalian cells introduced by Puck (4) in the mid-1950s have made these cells useful for the quantitative study of the genetics of mammalian cells (3). Cultured cells have been extensively used to study chromosomal damage resulting from mutagen treatment (3). As soon as it was statistically proven that chemical agents could induce mutations in cultured cells, numerous new lines of research involving the effects of mutagens on cells were opened (3). Chemical mutagens were used to produce stable phenotypes of such diverse types as auxotrophs, drug-resistant mutants and temperature-sensitive mutants in cultured cell lines (2).

One of the most intensively studied loci in cultured cells is the HPRT locus. This locus produces hypoxanthine phosphoribosyl transferase (HPRT: E.C. 2.4.2.8) (1). The HPRT locus is one of the first markers ever studied. Szybalski (13) isolated a human cell line in 1958 that was resistant to 8-azaguanine (8-AG). Other analogs such as 6-thioguanine and 6-mercaptopurine were found to select for cells deficient in HPRT activity (1). HAT medium (containing hypoxanthine, aminopterin, and thymidine) was developed as a counterselective medium for HPRT deficient cells (11) and

Littlefield (27) used it to develop cell-cell hybriization techniques. The identification of the link between HPRT deficiency and Lesch-Nyhan syndrome in humans by Seegmiller et al. (28) aroused further interest in HPRT. These studies indicated that HPRT is an x-chromosome linked gene, hence hemizygotic in male cells and functionally hemizygotic in females.

Irene Schauer, working in Dr. Bryant's laboratory, isolated a mutant from V-79 cells (Chinese hamster lung cell line) that is deficient in the uptake of the purine base hypoxanthine. Schauer's work, which has not yet been published, will be reviewed here. Uptake is defined here, as by Plagemann (5), as the taking in and subsequent metabolization of a substrate to a form usable in the cell. Mutagenesis was induced in a stable clone of V-79 cells, V-79A, with ethylmethanesulfonate (EMS) at a concentration of 400 µg/ml. EMS is probably a point mutagen (3). The isolated mutant, designated trMH-19, was selected by a tritium-suicide technique. The procedure allowed cells with the ability to take up tritium-labeled hypoxanthine ($^{3}$H-hypoxanthine) to die from accumulated radiation poisoning, while leaving cells lacking the ability to take up the labeled substrate alive. The trMH-19 mutant line appeared normal when compared to the parent V-79A except for a greater than 99% reduction in uptake of hypoxanthine.

Subsequent experiments in which the acitivity of HPRT was assayed in whole cell extracts indicated that the apparent activities from both V-79A and trMH-19 were approximately equal. Defective hypoxanthine uptake and normal HPRT activity were consistent with the hypothesis that trMH-19 was a hypoxanthine transport mutant. This hypothesis was tested by Irene Schauer and Paul Naylor, using membrane vesicles prepared from both V-79A and trMH-19. Counter-transport measurements showed equal rates of transport in trMH-19 and V-79A. This result suggested that trMH-19 was not a transport mutant. Schauer also studied the rapid transport rates for hypoxanthine in whole cells by Plagemann's methods (8). There was no apparent difference in hypoxanthine transport rate for V-79A and trMH-19. Further work was necessary to determine the nature of the deficiency in hypoxanthine uptake.

An altered HPRT could significantly affect the uptake of hypoxanthine, since unmetabolized substrate concentration inside the cell would be dependent upon the concentration of hypoxanthine in the external medium (5). Numerous agents have been shown to induce mutations at the HPRT locus and EMS is in this group (9,10). Since the work of Schauer and Naylor outlined above indicated that trMH-19 was probably not a transport mutant, the next most likely hypothesis was that trMH-19 exhibited defective hypoxanthine uptake because

of defective HPRT. My thesis was devoted to testing this hypothesis.

# CHAPTER II

# MATERIALS AND METHODS

## A. Cell Lines

All experiments were done on two cell lines, one designated V-79A, the other trMH-19. V-79A is a stable clone of V-79 (originally derived from Chinese hamster lung fibrolasts) with twenty-one chromosomes and was isolated by Irene Schauer. The trMH-19, also isolated by Irene Schauer, was derived by mutagenesis with EMS and selection with tritium-labeled hypoxanthine by the tritium-suicide method of Finkelstein, Slaymann, and Adelberg (21). Both cell lines were grown as monolayers on 60, 100, and 150 millimeter diameter tissue culture dishes (Corning or Falcon) at 37° in an atmosphere of 95% air and 5% $CO_2$ in LAC medium. LAC medium consists of Hank's Minimal Medium (MEM) (Gibco or Flow) supplemented with 10% fetal calf serum, lactalbumin hydrozylate (Gibco), sodium bicarbonate (1.3 grams/liter), trypsin (105 grams/liter) and L-glutamine (.058 grams/liter). Both lines grow with a generation time of approximately fourteen hours. No apparent gross morphological differences exist between them as determined by microscopic observation.

## B. Plating Efficiencies

Experiments were done to determine the plating efficiency of both the V-79A and trMH-19 cell lines in the presence of various inhibitors. HAT medium (Littlefield, 1964) consisted of LAC medium supplemented with dialyzed fetal calf serum, with added hypoxanthine, aminopterin, and thymidin thymidine at concentrations of 0.1 mM, 0.4 μM, and 16 μM, respectively. In IAT medium inosine at 0.1 mM is substituted for the HAT medium with the same concentrations of aminopterin and thymidine in IAT medium as in HAT medium. LAC medium containing 7.5 μg/ml of 8-azaguanine (8-AG) was also used for these experiments. Concentrations of trypsinized cells in suspension were determined by haemocytometer counting. Serial dilutions were used to plate known numbers of cells from both lines in 100 millimeter culture dishes containing LAC, HAT, IAT, or 8-AG medium.

After plating, the cells were placed in a 37° incubator (95% air, 5% $CO_2$) and allowed to grow undisturbed for 4-7 days so that individual colonies would become visible. At the end of the growth period, the medium was decanted from each plate and the individual colonies representing growth from single cells were stained with Wright's stain and counted.

## C. Determination of the Fate of $^{3}$H-Hypoxanthine in Cells

In order to determine whether hypoxanthine could be taken up and metabolized by trMH-19 cells, experiments monitoring the uptake and metabolic fate of hypoxanthine were done. Cells from both lines were grown in LAC medium on 60 mm plates to a density of 1.5-3.0 × $10^5$ cells per plate (actively dividing, logarithmic growth). The medium was then replaced with LAC medium containing 10% dialyzed fetal calf serum (FCS) and 80 µCi/ml (30 µM) tritium-labeled hypoxanthine. The cells were allowed to take up hypoxanthine from this medium for twenty minutes, then stopped by quickly washing with 1 ml per plate cold phosphate buffered saline (PBS). The cells were immediately extracted by adding 100 µl of 5% TCA (trichloroacetic acid) to each plate and scraping the cells with a 200 µl pipette tip. This extract was then centrifuged for ten minutes at 2000 rpm in a Sorvall type GLC-1 desk top centrifuge. Ten µl of the supernatant was applied to replicate PEI-cellulose thin layer chromatography plates that had been previously spotted with unlabeled GTP, ATP, ADP, AMP, IMP, and hypoxanthine standards. Fifty µg of the standards, at 5 mg/l in 5% TCA, were spotted on the plates.

The spotted plates were then developed a distance of 16 cm from the origin in one of two solvent systems (23).

The methanol system used two solutions, which were: (a) pure methanol and (b) a mixture of methanol, n-propanol. 25% ammonia, and 1% ethylenediaminetetraacetic acid (EDTA) in a 3:9:2:6 ratio. Development in this system was done by (a) allowing the pure methanol to travel 16 cm from the origin, then drying; (b) allowing the 3:9:2:6 mixture to travel 8 cm from the origin, then drying; and (c) allowing the 3:9:2:6 mixture to travel 16 cm from the origin, with a final drying step. The LiCl system required successive development of the plate (a) for 2 minutes in 0.2 M LiCl, (b) for 6 minutes in 1.0 M LiCl, and (c) allowing 1.6 M to travel the remainder of 16 cm from the origin.

After development, the plates were marked at 0.5 cm intervals, and the lanes for the cell extracts were cut into 32 fractions. These fractions were placed in scintillation fluid made from Fisher Scientific Co.'s Scintiprep (25x solution containing PPO-POPOP) and Scintanalyzed toluene, and counted in a Beckman Liquid Scintillation Counter. The positions of the standards were determined with UV light. The positions and compositions of the labeled products from the cell extracts were determined from the positions of the standards. Using the known specific radioactivity of the labeled hypoxanthine medium and known numbers of cells, the specific radioactivity of incorporated label in the cells was calculated and expressed as CPM per cell.

## D. Enzyme Studies

### 1. Harvest of Cells and Preparation of Extract

The method used was basically that of Tiemier and Milman (25). Both V-79A and trMH-19 cells were grown in LAC medium on 150 ml plates (Falcon) to three-quarters confluency. Approximately $2 \times 10^8$ cells were harvested by trypsinization with subsequent centrifugation at 1500 rpm in the Sorvall GLC-1 centrifuge for 5-7 minutes. The cell pellet was then resuspended and washed in tris-saline buffer (10 mM tris, 0.85% NaCl), pH 7.4 and pelleted again at 1500 rpm for 5-7 minutes. The cells were next resuspended in 4 ml distilled water to a final concentration of $5 \times 10^7$ cells/ml and lysed either by rapid freeze-thawing in a dry-ice ethanol bath followed by a 37° water bath or by breaking in a Dounce homogenizer fitted with a tight (A) pestle. Microscopic examination was used to monitor cell lysis. The lysed cell suspension was centrifuged in a Sorvall RC2B centrifuge fitted with a SS-34 rotor at 30,000 g for 20 minutes. The supernatant from this spin was spun at 110,000 g in a Beckman L3-50 centrifuge using a Ti50 rotor. The final supernatant was used as cell extract for the assays of HPRT and could be stored at -70° indefinitely.

2. Basic Assay Procedure

HPRT was assayed as cell extract in a final volume of 50 µl containing Tris buffer (100 mM), $MgCl_2$ (10 mM), 5-phosphoribosyl-1-pyrophosphate (PRPP) (2.0 mM), $^3$H-hypoxanthine (0.4 mM, 25 uCi/ml) and 10-50 µg of extract protein, as determined by Lowry's method, at pH 7.4. This mixture was incubated in a 37° water bath for 15 minutes, then the reaction was stopped by addition of EDTA to 60 µM. Forty microliters of the reaction mixture were subsequently spotted onto a 2.4 cm diameter Whatman DE-81 filter paper disc. The filter was washed twice in a pan containing 50 ml per filter of 0.4 mM $NH_4HCO_3$. Each wash lasted 30 minutes. This was followed by washing in 50 ml per filter of distilled $H_2O$ for 30 minutes and drying. The dried filters were then put in PPO-POPOP scintillation fluid and counted in a Beckman Liquid Scintillation Counter.

3. Time Course of Enzyme Activity and Linearity with Varying Extract Concentration

Parallel assays of HPRT in cell extract from both V-79A and trMH-19 were run according to the Basic Assay Procedure outlined above to determine the stability of the enzyme from both lines. Over a period of 60 minutes, duplicate or triplicate assays were run for intervals of 12 minutes. In addition, the amount of extract protein added to the assay

was varied in order to determine whether enzyme activity was linear with protein concentrations.

4. Substrate Affinities

Determination of the $K_m$'s for hypoxanthine and PRPP was done by modification of the Basic Assay Procedure outlined above. HPRT was assayed as cell extract and measurements were done in parallel for V-79A and trMH-19. Assays were performed by varying the concentration of either hypoxanthine or PRPP while keeping the concentration of the nonvaried substrate at constant concentration. The values of the apparent $K_m$'s were derived from double-reciprocal plots, the curves being fit by least-squares regression analysis.

# CHAPTER III

## RESULTS AND DISCUSSION

### A. Plating Efficiencies

Plating efficiencies are an indication of the ability of cultured cells to grow under different conditions after transfer or subculture (3,4). Very low numbers of dispersed cells are plated and allowed to grow for a period of time. After this growth period, the macroscopically visible colonies (outgrowth from single cells) can be stained and counted for the calculation of the plating efficiency.

HAT medium, consisting of hypoxanthine, aminopterin, and thymidine, is a counterselective medium for HPRT mutants developed by Szybalski and Szybalski (11). Aminopterin, an antifolate drug, effectively inhibits purine de novo synthesis and thymidylate synthesis at the steps which require $^{5}N^{10}N$-methylene tetrahydrofolate. Hypoxanthine and thymidine act as exogenous sources of purine and thymidine, respectively. Cells with the ability to grow in HAT medium would have to have an active HPRT in order to use hypoxanthine as a source for purines. Cells with defective HPRT would not grown in HAT medium because they could not use exogenous hypoxanthine and would be unable to synthesize purines de novo.

IAT medium, in analogy to HAT medium, contains inosine, aminopterin, and thymidine. Hochstadt and Quinlan (12), based on work with membrane preparations, hypothesized that eukaryotic cells could have a transport function analogous to the group translocation mechanism found in bacteria. This hypothesis included HPRT as an integral part of the transport mechanism. From their hypothesis, inosine could be bound to a membrane transporter different than the one for hypoxanthine, This bound inosine, by their hypothesis, would be split at the membrane using ATP, and subsequently the hypoxanthine would be converted to IMP via a membrane-linked HPRT. Utilization of inosine as a purine source by trMH-19 cells that do not take up hypoxanthine could indicate a transport defect in trMH-19. Other work by Plagemann et al. (8) showed that transport of nucleosides is independent of ATP, and that group translocation probably does not occur in mammalian cells. As inosine is degraded inside cells to hypoxanthine and ribose, IAT requires that cells growing in it have active HPRT.

Eight-azaguanine (8-AG) is a purine analog used to select for cells deficient in HPRT activity (13,24). Cells not resistant to 8-AG incorporate the compound via HPRT and consequently die. Cells resistant to 8-AG may have defective HPRT activity (24) or could possibly have altered levels of guanine deaminase activity, as recently pointed out by van Diggelin et al.(25).

Plating efficiencies for cells under normal conditions of transfer are usually 50-80%. Table I shows values obtained for plating efficiencies in normal LAC medium, HAT medium, IAT medium, and 8-AG medium with V-79A cells and trMH-19 cells. V-79A is HAT and IAT resistant and 8-AG sensitive. The mutant trMH-19 is HAT and IAT sensitive and resistant to 8-AG. I conclude from these data that trMH-19 does not utilize exogenous purines.

TABLE I

PLATING EFFICIENCY VALUES

| | LAC | HAT | IAT | 8-AG |
|---|---|---|---|---|
| V-79A | 68% | 48% | 51% | .03% |
| trMH-19 | 71% | .03% | 0% | 66% |

## B. Determination of the Fate of $^{3}$H-Hypoxanthine in Cells

The experiments to determine the fate of tritium-labeled hypoxanthine in cells had a two-fold purpose. One reason was to see whether $^{3}$H-hypoxanthine actually gets into both V-79A and trMH-19 cells. The other reason was to monitor the subsequent metabolism of the compound. Previous work done by Irene Schauer and Paul Naylor showed <u>in vitro</u> and <u>in vivo</u> transport of hypoxanthine was practically the same for V-79A and trMH-19, but metabolism of hypoxanthine in living cells

was not tested. Assuming a functional transporter, an uptake-deficient cell would come to equilibrium with the surrounding medium in terms of hypoxanthine concentration, rather than concentrate $^{3}H$ in the form of phosphorylated metabolites.

Plagemann and Richey (5) used thin layer chromatography to analyze the phosphorylated products of nucleosides taken up by Novikoff rat hepotoma cells under varying conditions of ATP depletion. Siepel and Reichert (23), in their studies on purine transport in yeast cells, developed a methanol solvent system and a LiCl solvent system for separating purine base, nucleoside, and nucleotide compounds from cell extracts on cellulose thin layer chromatography plates. Using these procedures, the amount of intracellular hypoxanthine and its metabolites in V-79A and trMH-19 was analyzed. The LiCl solvent system (see Materials and Methods section) most clearly separated the free hypoxanthine from its products.

Figure 1 shows the profile of tritium-labeled products extracted from V-79A cells and separated on PEI cellulose using the methanol solvent system. Fractions one and two show that the bulk of $^{3}H$-hypoxanthine that was taken up by the cells was converted to ATP and GTP, with the peak for the ADP masked by the ATP-GTP peak. Very small peaks for AMP (Fraction 9) and hypoxanthine (Fraction 24) were also found.

Figure 1. Separation of $^{3}$H-Metabolites from V-79A Using the Methanol System.

Cells ($3 \times 10^{5}$) on a 60 mm plate were incubated with $^{3}$H-hypoxanthine (30 µM, 80 Ci/ml) for 20 minutes. At the end of the incubation, the cells were rinsed with PBS. After rinsing, the $^{3}$H-labeled products were extracted with 5% TCA as described in Materials and Methods. Ten µl of the extract was applied to the plate and separated with the methanol solvent system also described in Materials and Methods. Positions of the standards (ATP, GTP, ADP, IMP, AMP, and HPX (hypoxanthine) are indicated.

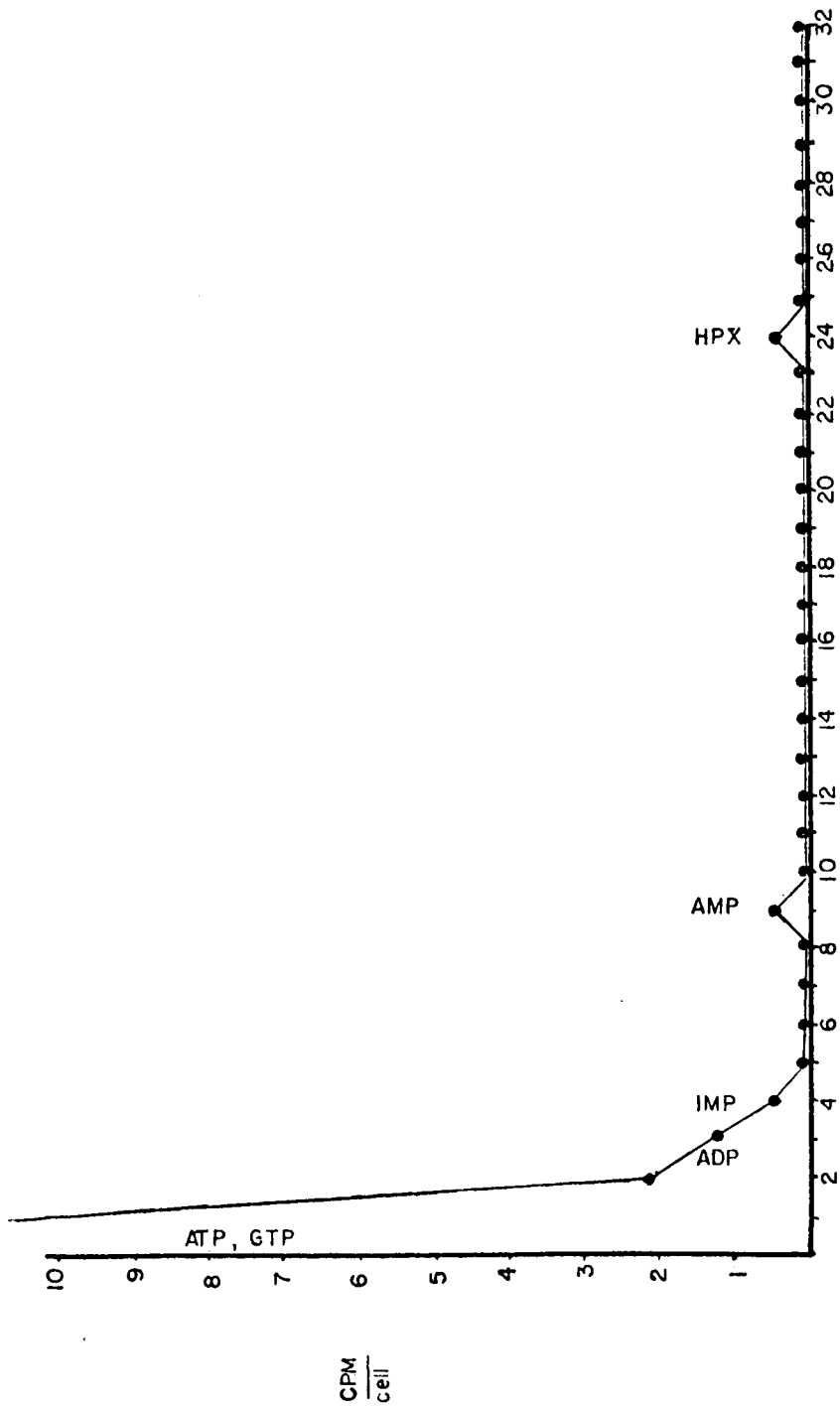

ATP, GTP
ADP
IMP
AMP
HPX
CPM/cell
fraction number
1
2
3
4
5
6
7
8
9
10
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

Figure 1

Figure 2 is the profile of tritiated products from trMH-19 cells separated on PEI-cellulose plates by the methanol system. There are no peaks for any phosphorylated products by hypoxanthine. The hypoxanthine peak (Fractions 23-25) is the amount of $^{3}$H-hypoxanthine left in the cells after washing (see Materials and Methods section). The presence of this peak indicates that $^{3}$H-hypoxanthine can enter the trMH-19 cells but cannot be further metabolized.

Figures 3 and 4 give results of separation of the tritiated products from V-79A and trMH-19 using the LiCl solvent system. Figure 3 shows that ATP (Fractions 8-14), GTP (Fractions 4-8), and ADP (Fractions 19-20) are all separable metabolites of $^{3}$H-hypoxanthine in V-79A cells. Hypoxanthine, AMP and IMP were not separated as individual components by the LiCl solvent system. Figure 4, showing the profile extracted from trMH-19, has only a small hypoxanthine peak. The presence of hypoxanthine and the absence of hypoxanthine nucleotides indicates a metabolic block in the phosphorylation of hypoxanthine (i.e., in HPRT activity) in trMH-19.

## C. Enzyme Studies

### 1. Background

HPRT catalyzes the conversion of hypoxanthine to inosine monophosphate (IMP). The enzyme has two substrates,

Figure 2. Separation of $^{3}$H-Metabolites from trMH-19 Using the Methanol System.

The experimental conditions were the same as those described in the legend to Figure 1.

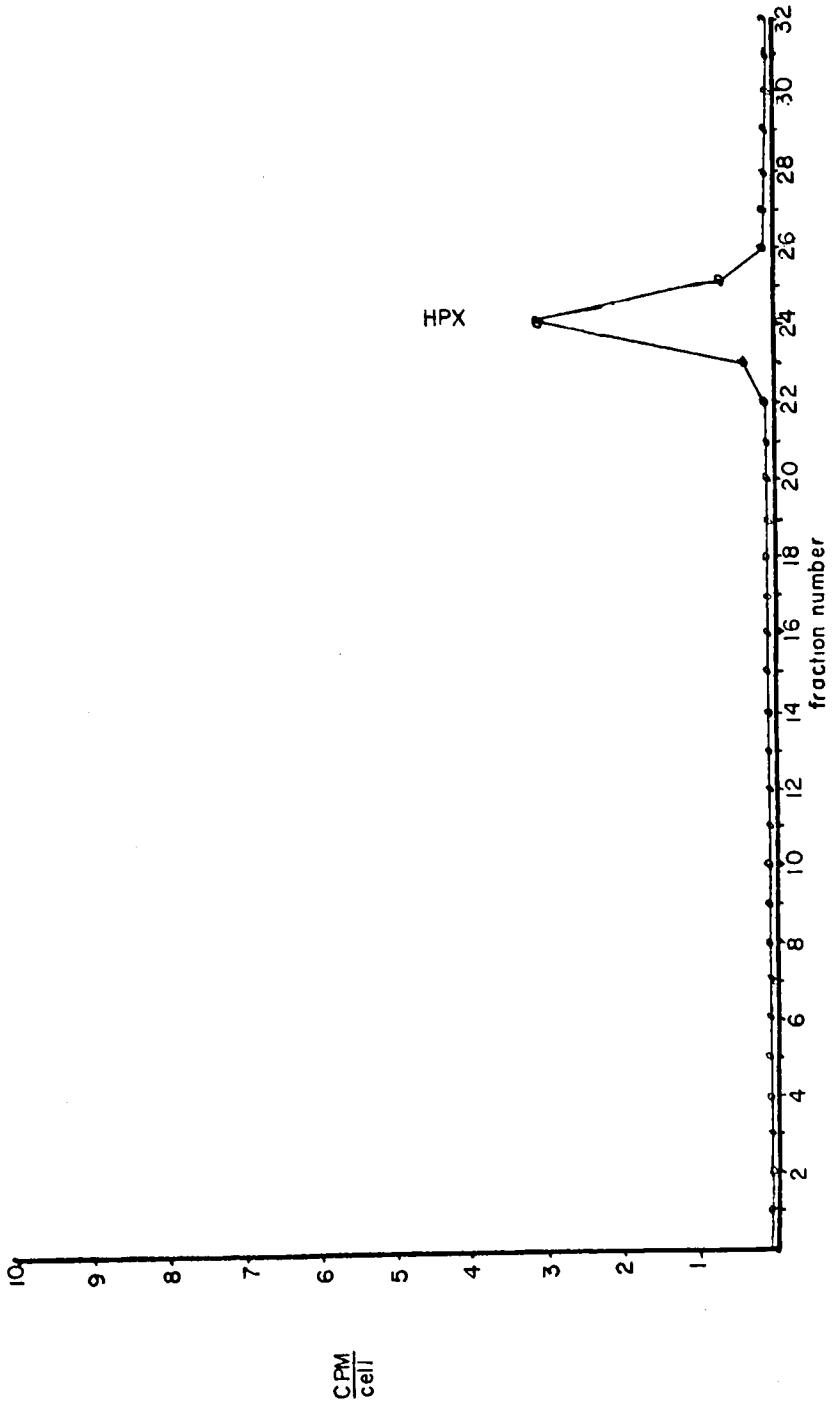

HPX
CPM/cell
fraction number
1
2
3
4
5
6
7
8
9
10
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

Figure 2

Figure 3. Separation of $^{3}$H-Metabolites from V-79A Using the LiCl System.

Cells ($3 \times 10^{5}$) on a 60 mm plate were incubated with $^{3}$H-hypoxanthine (30 µM, 80 µCi/ml) for 20 minutes. At the end of incubation, the cells were rinsed with PBS. After rinsing, the $^{3}$H-labeled products were extracted with 5% TCA as described in Materials and Methods. Ten µl of the extract was applied to the plate and separated with the LiCl solvent system described in Materials and Methods. Positions of the standards are indicated as in Figure 1.

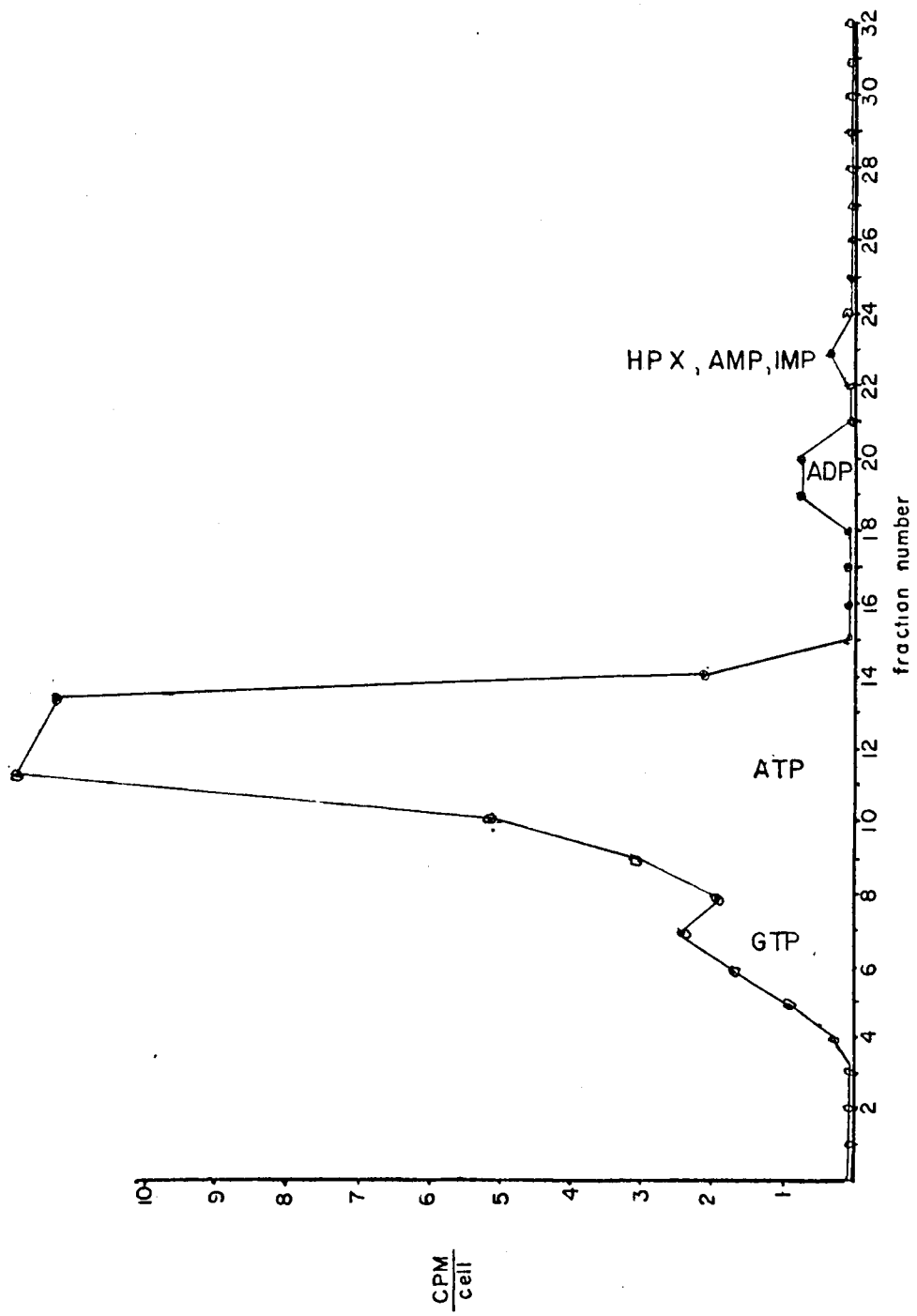



Figure 3

Figure 4. Separation of $^{3}H$-Metabolites from trMH-19 Using the LiCl System.

The experimental conditions were the same as those described in the legend to Figure 3.

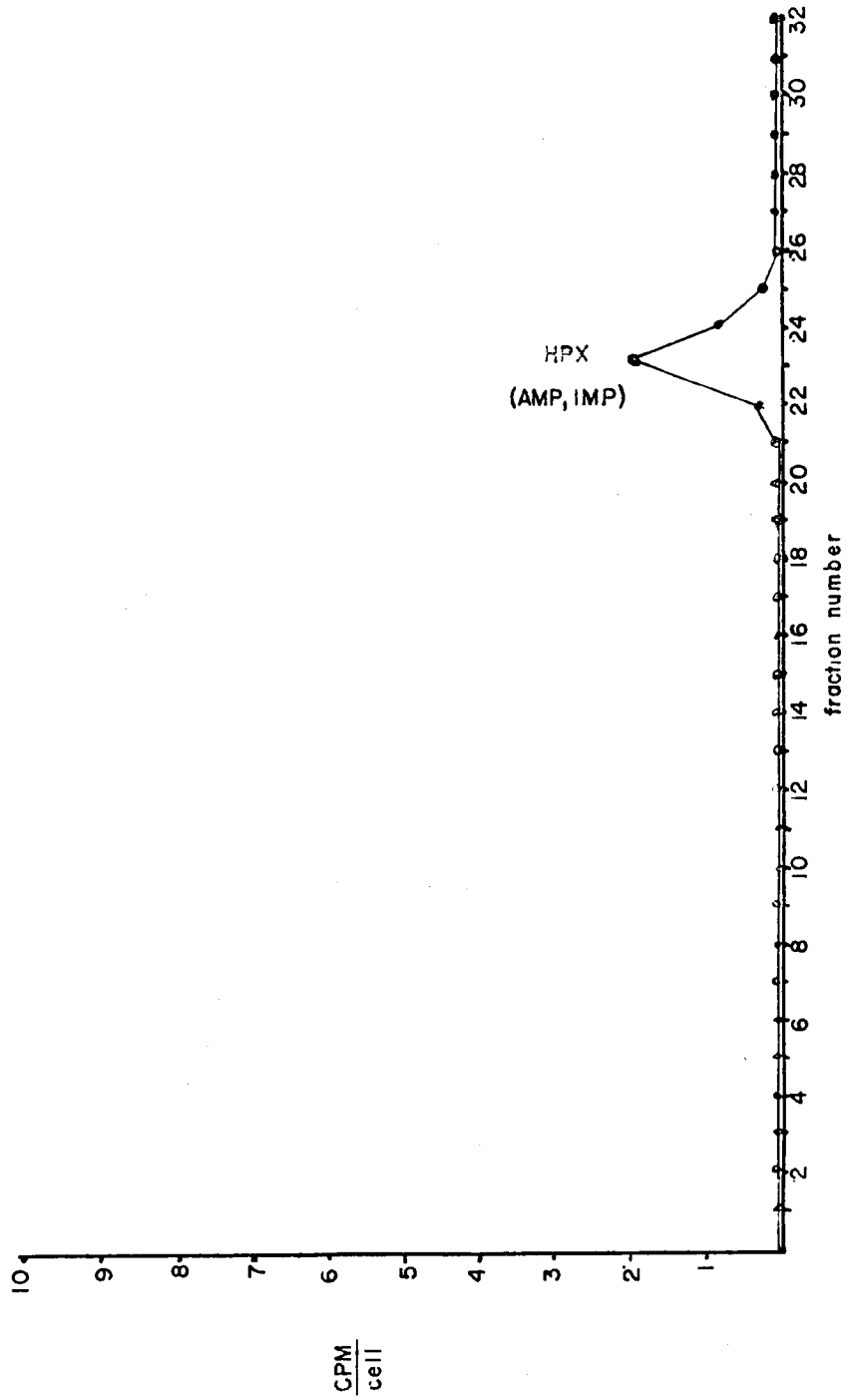

HPX
(AMP, IMP)
fraction number
CPM / cell
1
2
3
4
5
6
7
8
9
10
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

Figure 4

hypoxanthine and PRPP. It also is active with the purine analogs 6-mercaptopurine, 6-thioguanine, and 8-azaguanine (11,24).

The HPRT enzyme has been purified to homogeneity from numerous sources (1,7,16,17). Hughes (17) greatly improved the purification by using affinity column chromatography with GMP-agarose. HPRT is a multimeric enzyme consisting of identical subunits, but arguments still exist over the exact number of subunits. The subunit apparently exhibits no catalytic activity (1). Recent work by Holden and Kelley (18) gives strong evidence for a tetrameric structure.

HPRT displays Michaelis-Menton kinetics of single-substrate enzyme catalysis when one of its substrates is in limiting amounts and the other is held at constant saturating levels (7). The concentration of magnesium ion in the assay has a pronounced effect on the activity of HPRT (18). At concentrations above 80 mM, and at those below 1 mM, the activity of HPRT is adversely affected. The product of the reaction, IMP, has been found to be inhibitory when at 1 mM and higher concentrations (7). Literature values for the apparent $K_m$ for hypoxanthine range from 0.5 μM to 25 μM at fixed PRPP concentrations of 1 mM to 2 mM. Those for the apparent $K_m$ for PRPP range from 5 μM to 220 μM at fixed hypoxanthine concentrations of 60 μM to 100 μM (7,14,15,18, 19).

Many researchers have found that chemical mutagens could induce mutations at the HPRT locus (1,3,9,19). Work by Fenwick et al. (19), with HPRT mutants of Chinese hamster ovary cells, showed that alteration of the enzyme affinities for both hypoxanthine and PRPP was obtainable by mutagenesis. They produced a number of mutants with HPRT activities ranging from none, when compared with the parent line, to two- or three-fold in excess of the parent. These reduced or increased activities were reflected in changed values of the apparent $K_m$ of HPRT for hypoxanthine or for PRPP as compared to the parent line. There are certain lines of cultured cells in which $K_m$ changes of three- to ten-fold are considered evidence of a structural gene mutation (20). Fenwick et al. (19) used SDS polyacrylamide gel electrophoresis to show that the mutants with increased $K_m$'s for hypoxanthine and PRPP had altered subunits of HPRT.

The above-mentioned work of Fenwick et al. (19) provides evidence that changes in substrate affinity (apparent $K_m$) is indicative of structural changes in an enzyme (here, HPRT). The decision was made to determine the apparent $K_m$'s of HPRT from V-79A and trMH-19 for hypoxanthine and PRPP. Comparison of the obtained values of apparent $K_m$ could indicate the presence of an altered HPRT in trMH-19.

## 2. Experimental Procedures

The extraction procedure was performed using both the freeze-thaw method and Dounce homogenization (see Materials and Methods section). The freeze-thaw method yielded approximately 2 µg/µl of extract protein after the second centrifugation (see Materials and Methods section), while the Dounce procedure yielded approximately 4 µg/µl. This difference was attributed to better lysis of suspended cells in the Dounce procedure. The actual amount of HPRT activity varied somewhat from extract to extract. Generally the extracts yielded activities of 10.0-20.0 pmole IMP $min^{-1}$ $\mu g^{-1}$.

Experiments were done to determine (a) whether enzyme activity was linear with increasing activity, and (b) how long enzyme activity was linear over time for both V-79A and trMH-19. Figure 5 shows that in both V-79A and trMH-19 extracts, enzyme activity was linear with increasing amounts of enzyme concentration. Even though the amount of activity varied from extract to extract, the linearity of activity remained. Figure 6 shows a time course of activity for V-79A and trMH-19 extract. Activity was linear for at least 45 minutes of reaction time in extracts of both parental and mutant cells. A 15 minute assay time was chosen for the substrate affinity studies since 15 minutes are well within the time of linear activity. Other investigators generally use a 15 minute assay.

Figure 5. Linear Activity with Increasing Amounts of Extract Protein.

HPRT was assayed in cell extract in a mixture of tris (100 mM), $MgCl_2$ (10 mM), PRPP (2 mM), and $^3$H-hypoxanthine (0.4 mM, 25 µCi/ml) at pH 7.4. The amount of extract protein per assay was varied for each set of assays. (A) V-79A extract, (B) trMH-19 extract.

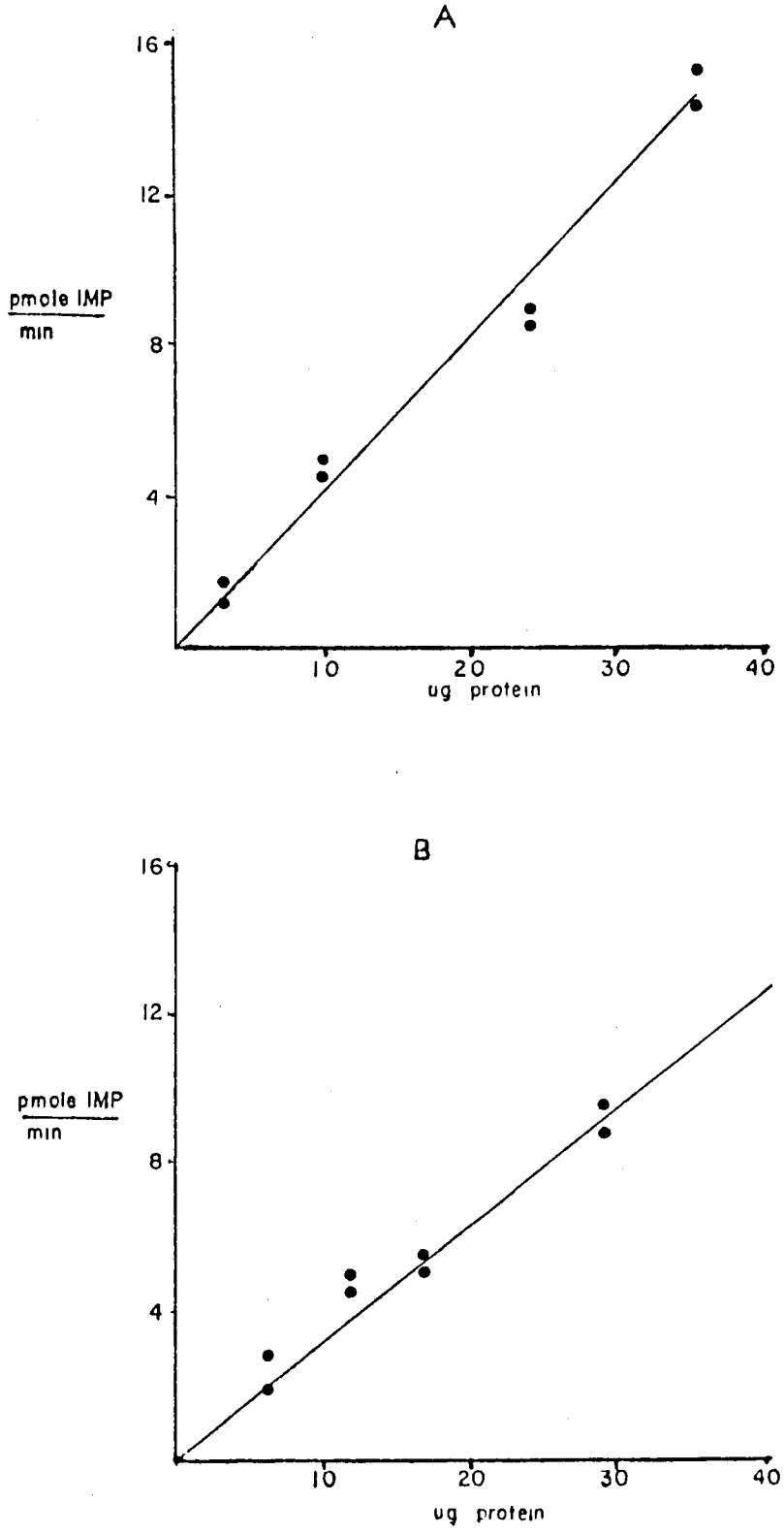
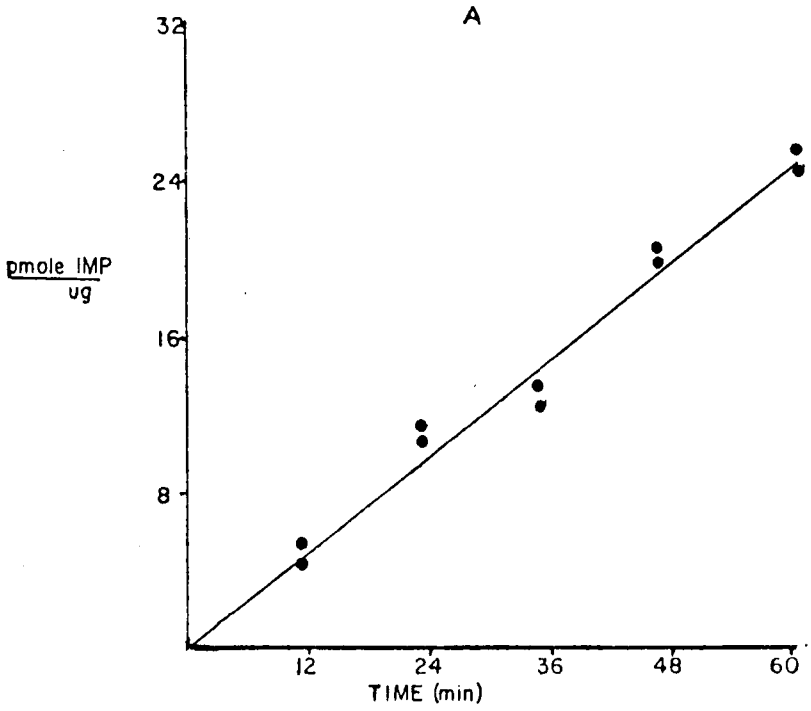

A
16
12
8
4
pmole IMP
min
10
20
30
40
ug protein
B
16
12
8
4
pmole IMP
min
10
20
30
40
ug protein

Figure 5

Figure 6. Time Course of HPRT Activity.

HPRT was assayed in cell extract in a mixture of tris (100 mM), $MgCl_2$ (10 mM), PRPP (2.0 mM), and $^3H$-hypoxanthine (0.4 mM, 25 µCi/ml) at pH 7.4. The time of reaction was varied at 12 minute intervals for each set of assays. (A) V-79A extract, (B) trMH-19 extract.


A
32
24
16
8
pmole IMP
ug
12
24
36
48
60
TIME (min)

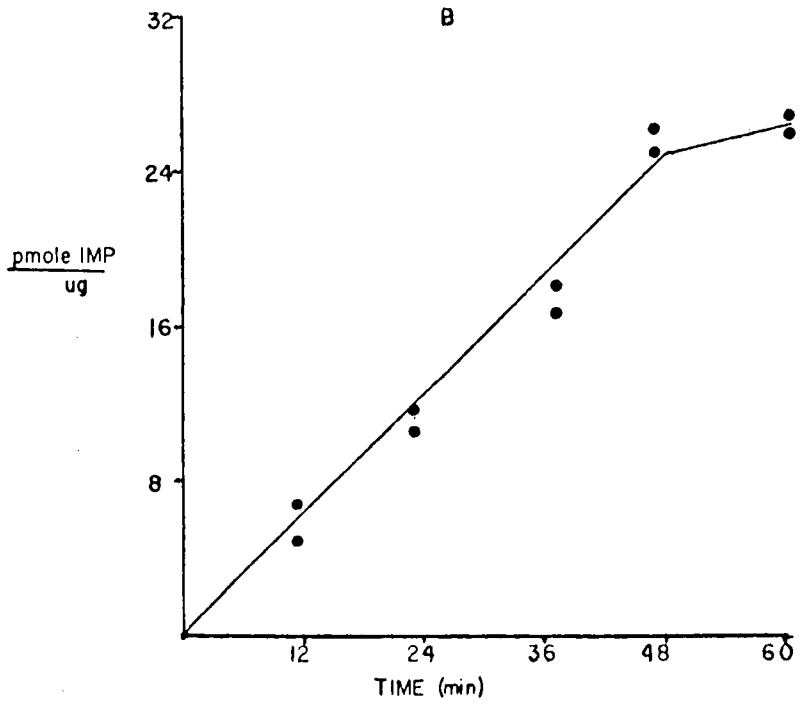

B
32
24
16
8
pmole IMP
ug
12
24
36
48
60
TIME (min)

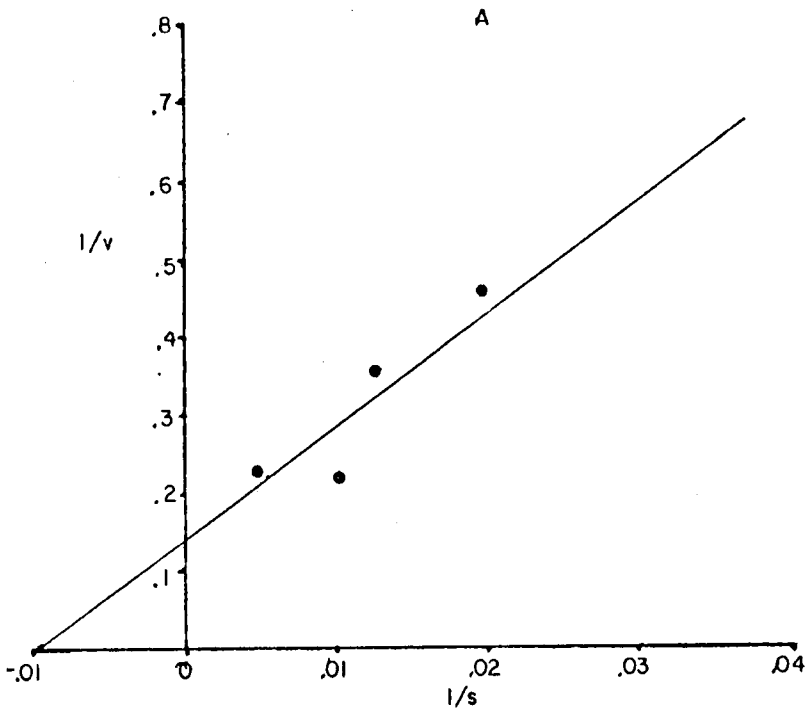
Figure 6

Preliminary experiments were done to determine whether the HPRT from V-79A and trMH-19 could be saturated at the concentrations of hypoxanthine and PRPP in the Basic Assay mixture (see Materials and Methods section). It was found that the concentration of 0.4 mM hypoxanthine and 2.0 mM PRPP were saturating amounts of these substrates for HPRT from V-79A and trMH-19 (data not shown).

On the basis of the above-mentioned saturation data, experiments were done to determine the apparent $K_m$'s for hypoxanthine and PRPP in V-79A and trMH-19. Figure 7 shows typical double-reciprocal plots from experiments in which the hypoxanthine concentrations were varied in assays of V-79A and trMH-19 extract. Apparent $K_m$'s were determined from repetitions of these experiments. Average apparent $K_m$ values of 97.72 μM for V-79A (Figure 7A) and 96.63 μM for trMH-19 (Figure 7B) were obtained. These values indicate no apparent difference in the affinity of HPRT from either cell line for hypoxanthine. Figure 8 shows double-reciprocal plots of activity from experiments in which the concentration of 5-phosphoribosyl-1-pyrophosphate (PRPP) was varied in assays of HPRT from V-79A and trMH-19. The values for $K_m$ derived from these plots, 131 μM for V-79A and 1.69 μM for trMH-19, show that the affinity for PRPP in trMH-19 is twelve-fold lower than V-79A. This greatly lowered affinity could easily cause trMH-19 to fail at hypoxanthine

Figure 7. Double Reciprocal Plots Determining Apparent $K_m$'s for Hypoxanthine.

HPRT was assayed in cell extract in a mixture of tris (100 mM), $MgCl_2$ (10 mM), PRPP (2 mM), and varying concentrations of $^3$H-hypoxanthine (50-200 µM, from 0.4 mM, 25 µCi/ml stock). The reaction was allowed to proceed for 15 minutes. Velocity is in p moles IMP minute$^{-1}$ µg protein$^{-1}$.
(A) V-79A cell extract, (B) trMH-19 cell extract.



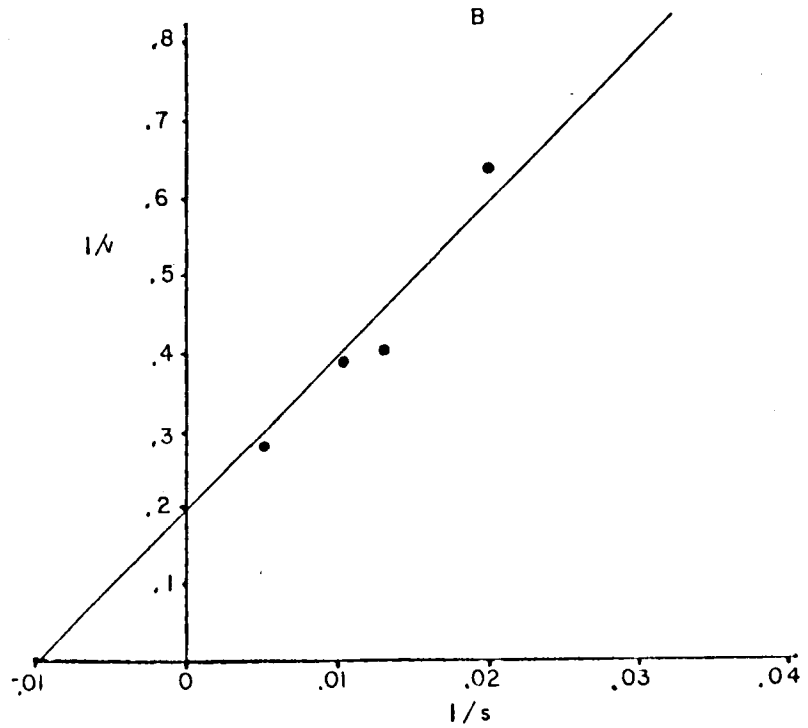
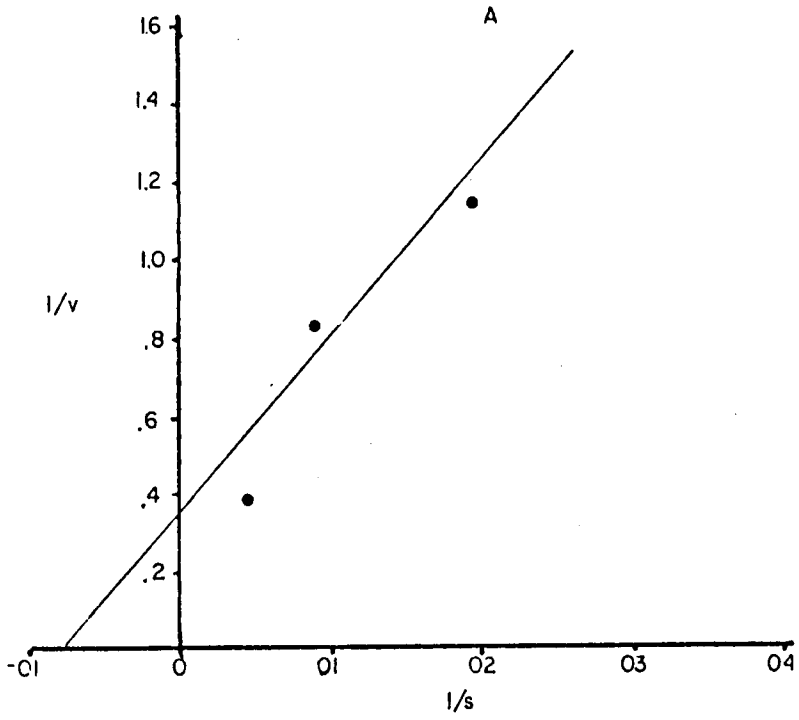




Figure 7

Figure 8. Double Reciprocal Plots Determining Apparent $K_m$'s for PRPP.

HPRT was assayed in cell extracts as described for Figure 7 except that $^3$H-hypoxanthine was at constant 0.4 mM, 25 µCi/ml. PRPP concentrations were varied (A) between 50 µM and 200 µM for V-79A, and (B) between 0.5 mM and 2.0 mM for trMH-19.



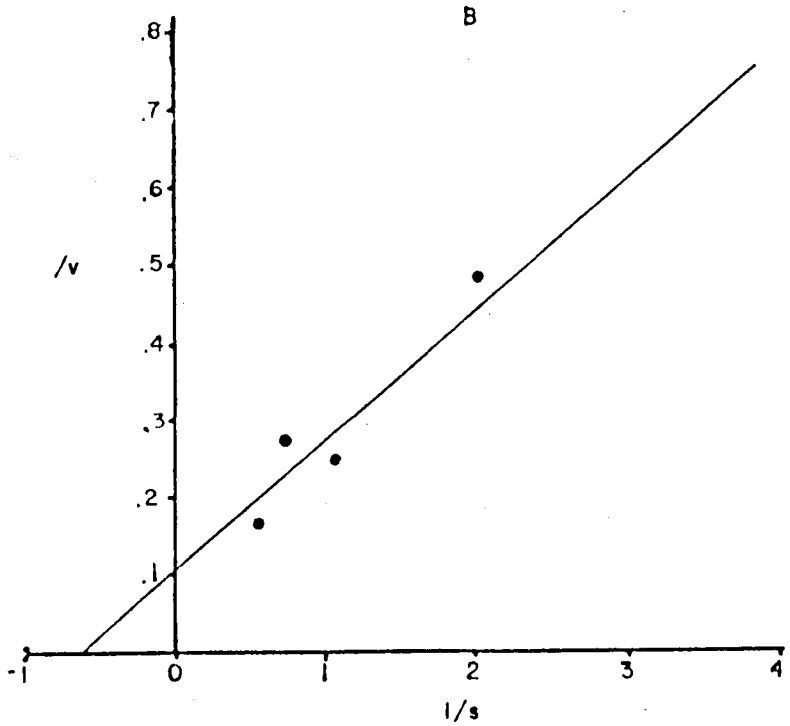




Figure 8

utilization *in vivo*. When compared with the data of Fenwick et al. (19), this twelve-fold change in apparent $K_m$ is evidence for a structural change in HPRT from trMH-19. The results of the studies on substrate affinities are summarized in Table II.

TABLE II

AVERAGE APPARENT $K_m$ VALUES

| | V-79A | | trMH-19 | |
|---|---|---|---|---|
| Substrate | $K_m$ | Standard Error | $K_m$ | Standard Error |
| Hypoxanthine | 97.72 μM | 21.6 μM | 96.63 μM | 23.8 μM |
| PRPP | 131.73 μM | 32.8 μM | 1.69 mM | .157 mM |

# CHAPTER IV

## SUMMARY

The mutant cell line trMH-19 has a 99% reduction in the uptake of hypoxanthine compared to its parental cell line, V-79A. Uptake is defined as the transport and metabolism of a specified substrate. The trMH-19 line exhibited "normal" HPRT activity *in vitro*, when assayed in a cell extract with concentrations of 0.4 *mM* hypoxanthine and 2.0 *mM* PRPP.

Plating efficiencies determine the ability of cells to grow under different conditions (3). A plating efficiency of approximately 50% is expected of normal V-79A cells under normal growing conditions. HAT medium contains hypoxanthine, aminopterin, and thymidine. Cells that survive in HAT medium must have a functional HPRT *in vivo* in order to utilize exogenous hypoxanthine as a source of purines. It can be seen, from Table I on page 14, that trMH-19 cells did not survive in HAT medium. This means that exogenous purine cannot be utilized by the mutant cells, raising the possibility that HPRT is not functional in trMH-19. IAT medium is similar except that inosine is substituted for hypoxanthine. The absence of surviving colonies of trMH-19 (Table I) in IAT medium suggested a deficient HPRT activity. V-79A cells, having normal HPRT activity, survive in both HAT and IAT media. Eight-azaguanine is a purine analog used

as a selection agent for mutants of HPRT (11). The last column in Table I (page 14) shows that V-79A had a very low survival frequency in 8-azaguanine, while trMH-19 showed the same viability as in normal LAC medium. These results strongly suggest that trMH-19 is a HPRT mutant.

The set of experiments done to investigate the fate of tritium-labeled hypoxanthine in vivo were done to answer two questions: (a) Can hypoxanthine enter trMH-19 cells? and (b) Once inside, can hypoxanthine be phosphorylated by the trMH-19 cellular machinery?

In order to answer these questions, cells were allowed to incubate in the presence of labeled hypoxanthine for a fixed time, and then were TCA-extracted. The extract was subjected to thin-layer chromatography. Two solvent systems were run. The methanol system yielded maximum separation of hypoxanthine from phosphorylated products. The LiCl system gave a better separation of the phosphorylated products (23). By comparing V-79A and trMH-19 extracts in both solvent systems, it was shown that the exogenous hypoxanthine was taken into the mutant cells, but was not converted to phosphorylated products. (See Figures 1, 2, 3, and 4, pages 16, 19, 21, and 23, respectively.) Lack of phosphorylated products of hypoxanthine is a strong indication of HPRT deficiency.

Assays of HPRT activity in cell extracts were done to determine whether there were any differences in the catalytic properties of the mutant HPRT, compared to parental HPRT. Cell extracts were assayed for a fixed time with varying amounts of either hypoxanthine or PRPP. An apparent $K_m$ was determined for each substrate in both V-79A and trMH-19 for comparison.

Double reciprocal plots fit with least-squares regression were used to determine values of apparent $K_m$'s for hypoxanthine and PRPP (Figures 7 and 8, pages 33 and 35, respectively). The data are summarized in Table II, page 37.

The apparent $K_m$ for hypoxanthine in both V-79A and trMH-19 was determined to be approximately 100 μM. This value is higher than literature values of 0.5 to 25 μM (7,14,15,18,19), but is representative of determinations by others in Dr. Bryant's laboratory using the same assay system. No good explanation of the discrepancy exists. However, the determinations yielded from my experiments clearly indicate there is no difference in the apparent $K_m$'s between V-79A and trMH-19 enzyme for hypoxanthine.

The apparent $K_m$ determined for PRPP in V-79A is approximately 131 μM, while that determined for trMH-19 is approximately 1.65 mM, twelve-fold higher than for the parent. This twelve-fold difference drastically alters the

activity of the HPRT from trMH-19. It is almost totally inactive at concentrations of PRPP less than 1 mM. This provides evidence that the HPRT is inactive *in vivo*.

HAT sensitivity and 8-AG resistance are classical indicators of HPRT mutants in general. For a cell to utilize exogenous hypoxanthine, it must have a functional HPRT. The thin-layer chromatographic data show that trMH-19 does not use exogenous hypoxanthine. The twelve-fold difference in apparent $K_m$ for PRPP is a strong indication that there is a structural change in the HPRT enzyme in trMH-19. I conclude that the twelve-fold difference in $K_m$ for PRPP is sufficient to cause a 99% reduction in uptake of hypoxanthine in the mutant cell line.

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# VITA

Daniel Grey Hatcher III was born in Wilmington, Deleware, on October 10, 1953. He attended elementary schools in Spartanburg, South Carolina, Bristol, Tennessee, and Maryville, Tennessee. He graduated from Maryville High School in June, 1971. He attended Tennessee Technological University from 1971 to 1975 and received a Bachelor of Science degree in Biology in 1975.

The author entered graduate school at The University of Tennessee in 1976. He received a teaching assistantship from 1977 to 1979 and completed his Master of Science degree in Biochemistry in June, 1980.

Mr. Hatcher is currently seeking a Master of Business Administration degree.