To the Graduate Council:

I am submitting herewith a thesis written by Richard O. Whitehouse entitled "A Characterization of Variant V79 Cells Selected by Tritium Uridine Suicide." 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

Robert E. Bryant, Major Professor

We have read this thesis
and recommend its acceptance:

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A CHARACTERIZATION OF VARIANT V79 CELLS SELECTED BY TRITIUM URIDINE SUICIDE

A Thesis

Presented for

Master of Science

Degree

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Richard O. Whitehouse

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ABSTRACT

In a previous study, mutagenized cells of the V79 hamster cell line were isolated by tritiated uridine suicide. Preliminary screening from that work indicated that some of the mutants had a decrease in whole cell incorporation of uridine and a decrease in the activity of the enzyme uridine kinase. A rigorous analysis of the uridine kinase was undertaken in this study for three of the variant clones. The results of this study indicate that the mutant cells have a maximal enzyme velocity of only 4% to 6% of that observed for the parental cell line. This decrease in velocity was accompanied by a ten-fold reduction in the Michaelis constant of this enzyme for uridine. These observations are consistent with a mutation in a structural gene for uridine kinase.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

A. Background

Well characterized mutant cell lines are important tools for the study of cellular processes. Our current knowledge of molecular genetics and biology is due in part to advances in selection and isolation of mutant phenotypes, and to the technology which has been developed for the mainipulation of mutant genes. The direct study of a single mutant phenotype allows one to compare the effects of an altered state to the normal cellular state while retaining an isogenic background (1). A mutation can be utilized as a genetic marker; once assigned to a specific location on a chromosome it can be utilized to determine linkage relationships which would aid the development of recombinant technology in eukaryotic systems, as was done in prokaryotic systems (2). Recent advances toward the understanding of regulatory mechanisms for gene expression in eukaryotic cells have been facilitated by the technology already developed to induce and manipulate specific mutations which either block or enhance the expression of another gene (3,4,5).

Early workers studied those mutations which occurred spontaneously in nature; today through the use of mutagens, such as ionizing radiation, ultraviolet light, and mutagenic chemicals, the rate at which mutations can be produced in the laboratory has been greatly enhanced. In most cases mutagens act in a random, non-discriminate manner without increasing the frequency of mutating any specific gene to a greater extent than other genes. To increase the probability of obtaining a specific class of mutation, selection schemes have

been developed by which the desired phenotype can be isolated from a mixed population of cells.

The three major classes of mutant cell lines are those resistant to chemical compounds such as drugs, nutritional auxotrophs, and conditional lethal strains. Drug resistant mutants are selected by virtue of their survival when a cell population is exposed to drugs or structural analogs of metabolites which are toxic to the "wild type" (parental) strain. Nutritional auxotrophs, cells which require a nutrient not required by the parental strain to survive, are selected by growing cells in a medium lacking an essential nutrient and containing some toxic substance. Cells which are actively growing (wild type) will incorporate the toxin, while those which require the missing nutrient will not incorporate the drug and will therefore survive the exposure. Temperature sensitive cells are the most useful of the conditional lethal mutants. They can be selected by exposing cells to a toxic substance at a "non-permissive" temperature. Cells which survive this treatment are not able to grow and incorporate the toxin at the elevated temperature.

Two major drawbacks to using drug or analog killing exist. First, structural analogs do not exist for all metabolites which might be of interest and utility for the study of intracellular interactions and regulation. Second, and perhaps more important, due to differences in affinity of enzymes for the analogs generally used for mutant selection versus the normal substrate and the methods required for selection with these substances, the underlying factors which result in the observed variant phenotype are in some cases difficult to predict and complicate a rigorous analysis of the actual mutation. This second disadvantage is best exemplified in a study by van Diggelen et al. (6) in which differences in cell resistance to 8-azaguanine (AG) and 6-thioguanine (TG) were investigated.

Cells resistant to TG always have a reduction in hypoxanthine phosphoribosyl transferase (HPRT) activity, while cells resistant to AG often contain normal levels of enzyme activity and exhibit sensitivity to TG. Kinetic evaluation of HPRT in L929 cells for the substrates hypoxanthine (hpx), AG, and TG indicated comparable Km's for hpx and TG but 1000-fold higher for AG, indicating AG is a very poor substrate for the enzyme. Additionally, evidence for two alternative modes of resistance was obtained; (1) base substitutions which would further reduce the affinity of the enzyme to AG and (2) an increase in degradation of AG by guanine deaminase (6).

In an effort to minimize problems deriving from the use of structural analogs, this laboratory's major focus has been to assess the application of tritium-labelled analogs as selecting agents for cells defective in purine, pyrimidine and nucleotide metabolism. A tritium labeled compound is an identical analog of its nonradioactive counterpart. Selecting mutant clones by "tritium suicide" is similar to selecting due to drug resistance. Cells which can incorporate the tritium labeled compounds are killed as a result of radiolytic decay, while those cells which have deficient incorporation will survive exposure to it.

To eliminate difficulties incurred due to metabolism of labeled substrates we have based our selection procedure on a study by Burki and Okada (7) in which they compared the effectiveness of tritium suicide in L5178Y cells with different tritiated substrates (thymidine, uridine, lysine or histidine). Their results indicated that freezing cells at -196°C after a pulse label with the selected tritiated compound allowed an accumulation of cell damage as a consequence of tritium decay while essentially all metabolic activity had stopped. To determine the effectiveness of tritium killing, cells were thawed

and cloned at periodic time intervals and survival was measured. Killing effeciency at different molecular sites was found to be DNA > mRNA > tRNA, rRNA > amino acids. Tritium in RNA was about 25% as effective as it was in DNA (7,8).

The successful use of tritium suicide as a selective agent for variant mammalian cell strains is documented in the literature. Breslow and Goldsby (9) were the first to exploit the technique by using tritiated thymidine to isolate clones which are defective in the uptake of thymidine. Finkelstein et al. (10,11) were successful in the isolation of two cell lines from mouse lymphocytic cells which have a reduced transport of small neutral amino acids. These investigators used tritiated aminoisobutyric acid in a selection procedure similar to the one used in this laboratory. Several aminoacyl tRNA synthetase temperature-sensitive mutant CHO cell lines have been isolated by Thompson and coworkers (12,13,14,15) by selecting clones with tritiated amino acids and allowing the accumulation of radiation damage at 4°C. A stepwise regimen of increasing concentrations of tritiated uridine plus treatment with 5-fluorouridine yielded uridine kinase-deficient 3T3 mouse cells (16); however, the stability of these mutants has recently been questioned (17). Recently, Ayusawa et al. (18) isolated a thymidine requiring auxotroph deficient in thymidylate synthetase by culturing FM3A cells with tritiated deoxyuridine. In our laboratory tritiated hypoxanthine has been used to select mutant V79 cells which have altered kinetic values for HPRT (19), while in another experiment using tritiated adenine, a cell strain has been isolated which exhibits a decreased intracellular PRPP concentration, presumably because of an altered phosphoribosyl pyrophosphate synthetase (20).

B. Purpose of Research and Plan of Experiments

The goals of this project have been twofold. The first goal was to attempt to identify specific defects in certain cell clones which had been isolated using tritium uridine suicide, that showed decreases in incorporation of uridine. The second goal was to analyze quantitatively phenotypic differences determined between the variant cells and the parental strain. The mutant cells were isolated using tritiated uridine. Preliminary screening indicated that the mutant cells were deficient for activity of uridine kinase. Mutant isolation and screening were done by Irene Schauer as part of another project in Dr. Bryant's laboratory. My thesis project consisted of an analysis of the biochemical lesions in certain mutants. A rigorous characterization of the uridine uptake defects could yield valuable information as to the nature of the effect of tritiated uridine suicide as a selecting agent for mutant cells, particularly in the case of uridine kinase deficiencies. My experimental approach was first to verify the location of the enzymatic block by chromatographic examination of the in vitro assay products, and then to determine whether a variation in kinetic properties could be documented for suspect enzyme.

CHAPTER II

MATERIALS AND METHODS

A. Maintenance of Cell Cultures

The parental cell line (wild type) used for the isolation of the mutants used in these experiments was V79, a Chinese hamster lung cell line first reported in 1958 by Ford and Yerganian (21). V79 is a stable heteroploid cell line with a modal number of 21 chromosomes (22, 23). This cell line has a doubling time of 12 to 13 hours. All cultures were grown as monolayers on plastic tissue culture plates 35, 60, 100 and 150 mm diameter (Corning Glass Works or Falcon Division of Becton Dickinson and Co.). The cells were grown in an incubator at 37° C with air supplemented with 5% CO_2. Growth medium was lactalbumin medium (LAC medium) which consists of 10.6 g/liter Hank's modified Eagle's medium (Flow Laboratories Inc.) plus 10% donor calf serum (Flow Laboratories Inc.) 1.3 g/liter sodium bicarbonate, 50 mg/liter trypsin (Grand Island Biological Company), 59 mg/liter of L-glutamine, 20 ml/liter of 10% lactalbumin hydrolysate (Grand Island Biological Company), 2 mg/liter Fungizone (Flow Laboratories Inc.), and 50 mg/liter Gentamicin (Schering Corporation). Donor calf serum was replaced by dialyzed fetal calf serum (Grand Island Biological Company) during mutagenesis and isolation of clones.

Cell growth was maintained by serial culturing the cells to new plates with fresh medium at two to three day intervals. This was accomplished by detachment of the cells from the old plates, disruption of the monolayer by a trypsinization procedure, and transfer to new plates. The trypsinization procedure consisted of the following steps:

1) The old medium was decanted

2) The monolayer was rinsed with sterile Hank's balanced salt solution

3) The monolayer was rinsed with 0.25% trypsin solution

4) The monolayer was incubated with 0.25% trypsin solution until the sheet of cells detached from the surface of the plate (about five minutes)

5) The cells were resuspended in fresh LAC medium

6) The suspension was transferred to new plates

The 0.25% trypsin solution consisted of 8.0 g/liter sodium chloride, 0.4 g/liter potassium chloride, 1.0 g/liter dextrose, 0.2 g/liter disodium ethylenediamine tetraacetate (EDTA), 2.5 g/liter trypsin, and 1.08 g/liter sodium bicarbonate.

B. Mutagenesis and Selection of Mutant Clones

The experiments to be described in Section B were done by Schauer (Schauer and Bryant, unpublished data). The procedures and results are relevant to an understanding of my results and will be summarized here. Section C continues with my own work.

Mutagenesis

Mutagenesis of V79 was performed as described by Bryant et al. (18). Briefly this procedure consisted of incubating 5×10^6 cells in LAC medium containing 10% dialyzed fetal calf serum supplemented with 400 μg/ml ethylmethanesulfonate (EMS) in a 100 mm petri dish for 19 hours. This dose level is sufficient to produce one ethylation per 10,000 bases in V79 DNA (24). The cells were then grown in fresh medium for 6 days to fix phenotypic expression. Cell survival following exposure to the mutagen was determined to be 5%.

Mutant selection by tritiated uridine suicide

Mutant selection is summarized in Table 1. Mid log phase mutagenized cell cultures were incubated with LAC medium containing dialyzed fetal calf serum plus (5,6-^{3}H)-uridine (1.35 μM, 37 Ci/mmole) (New England Nuclear) for 10 minutes. To minimize tritium loss due to leakage from the cell the trypsinization for kill cycles 2 and 3 also contained tritium uridine at the same concentrations. Following trypsinization and resuspension, dimethylsulfoxide was added to 10% of total volume, and the resulting cell suspension was distributed into vials (about 1.5 ml/vial). The cells were frozen over liquid nitrogen and stored at -70°C in an ultra low temperature freezer. Storage was maintained for a given number of days until 100-fold to 1000-fold killing of the cells had occured. The above procedure constituted one kill cycle; a total of three kill cycles were performed. After each of the first two periods of frozen storage, the cells were thawed, plated, and grown for five to seven doublings prior to the next kill cycle. After the third kill cycle, thawed cells were plated for clonal growth. Nine clones were isolated using this procedure. No gross morphological deviations from the parental lines were detected by microscopic evaluation. Clones were labeled U1 through U10 (U9 was discarded). The clones were screened by Schauer for uridine uptake and uridine kinase activity relative to wild type. Table 2 shows the results.

Table I: Tritium Suicide[a]

Kill Cycle	CPM/Cell Day 0[b]	Frozen Storage Time (days)	Cells/Vial[c]	% Survival[d]
1	0.89	4	1.6×10^6	0.1
2	0.69	6	1.2×10^6	1.0
3	0.20	7	3×10^6	3.2

[a]Schauer and Bryant unpublished data.

[b]Immediately after incorporation of ^{3}H-uridine and prior to freezing. Uridine concentration was 1.35 µM (37 Ci/mmole).

[c]Cell concentration was determined by counting a sample of cell suspension in a hemocytometer prior to freezing.

[d]Survival was determined by plating efficiency of a sample of thawed cells.

Table 2: Uridine Uptake and Uridine Kinase Activity of Clones Selected After Tritium Uridine Kill Cycle 3.[a]

Clone	Uridine Uptake (% of V79)[b]	Uridine Kinase Activity (% of V79)[c]
U1	4.0	1.5
U2	0.01	1.9
U3	3.0	2.4
U4	2.3	6.7
U5	9.5	6.0
U6	3.0	14.0
U7	93	200
U8	1.4	36.0
U10	12.0	3.0

[a]Schauer and Bryant unpublished data values are averages of duplicate assays.

[b]Perchloric acid soluble and insoluble fractions determined by "silicone sandwich" technique of Damper, D. and C.L. Patton, Pentamidine Transport in Trypanosoma brucei-Kinetics and Specificity, Biochemical Pharmacology, 25 (1976) 271-276 for whole cells incubated for two hours in 1μM uridine (6 Ci/mmole)

[c]Determined by one hour assay using the same procedure described in Materials and Methods Part C.

C. Analysis of Enzymatic Activity

Preparation of crude cell extract

Five mid log phase 150 mm petri plates were harvested to yield $5x10^7$ to $1x10^8$ cells using the trypsinization procedure. The resulting cell suspension was centrifuged for 10 minutes at 500 rpm in a Sorvall GLC-1 bench top centrifuge. The cell pellet was resuspended to 10 ml in 10 mM potassium phosphate buffer (pH 7.6) using a graduated centrifuge tube and centrifuged in the above manner to remove residual medium. The cell pellet was then suspended in 2 ml of 10 mM potassium phosphate buffer (pH 7.6) containing 1.7 mM phenolmethylsulfonylfluoride (PMFS). The cell suspension was lysed by four cycles of freezing in liquid nitrogen and thawing in a 37°C water bath. This crude cell lysate was centrifuged at 30,000xg (SS-34 Sorvall Rotor at 18,000 rpm) for 20 minutes at 4°C. The pellet was discarded and the supernatant was centrifuged at 110,000xg (50Ti Beckman rotor at 42,000 rpm) for one hour at 4°C. The resulting supernatant was divided into 100µl aliquots and stored in precooled microfuge tubes at -70°C. Protein concentration of the extract was determined using the method of Lowry et al. (25) with lysozyme as the protein standard.

Enzyme activity assays

Assays to determine enzymatic activity were performed with a total volume of 200µl. The uridine kinase (UK) assay mix consisted of the following: 25 mM Tris (pH7.4), 10 mM $MgCl_2$, 100 mM sucrose, 5 mM phosphoenolpyruvate, 0.66 units pyruvate kinase, 2 mM ATP, and 400 µM uridine (0.125Ci/mmole) (New England Nuclear or ICN Chemical and Radioisotope Division). Kinetic evaluations were performed by varying the concentration of uridine from 0.5

μM to 400 μM to determine Km for uridine or by varying the concentration of ATP 50 μM to 2 mM to determine the Km for ATP. Concentrations of ATP greater than 2mM were found to produce substrate inhibition and were not used for this study. HPRT activity was measured using a mixture consisting of 100 mM Tris (pH 7.4), 10 mM $MgCl_2$, 2 mM phosphoribosyl pyrophosphate and 400 μM hpx (New England Nuclear) (62.5mCi/mmole). The cell extract volume was 20 μl per assay for both UK and HPRT assays. Prior to the start of the reaction both the mix and the extract were warmed to 37°C. The reaction was initiated by the addition of the reaction mix to the cell extract. All assays were performed as a time course by removing 50 μl aliquots at 0, 2.5 and 5 minute intervals and stopping the reaction by the addition of 5 μl of 0.5 mM EDTA. A volume of 40 μl of this final solution was applied to 2.4 cm DEAE cellulose (Whatman DE81) filter disks (Reeve Angel and Co.). The disks were washed three times to remove unreacted substrate, thirty minutes each wash, twice in 4 mM ammonium carbonate and once in distilled water with a volume equivalent to 75 ml per disk. After draining excess water from the disks they were eluted in scintillation vials using 1 ml of 0.1 M HCl/0.2 M KCl for 15 minutes (26). The samples were counted in a Beckman LS-230 scintillation counter using triton-toulene scintillation fluid. All assays were done in duplicate. The best fit line was determined by linear least squares analysis. All assays had a correlation coefficient of 0.9 or greater.

D. Identification of Reaction Products

Polyethyleneimmine cellulose (PEI-cellulose; Polygram Cel 300 PEI, 20 x 20 cm from Macherey-Nagel, Duren, Germany; Brinkmann, USA distributors) thin layer chromotography (TLC) was used to evaluate the products produced in the UK assay. Enzymatic assays were done as in section C; however 10 µl of the inactivated reaction was applied to a PEI-TLC plate 2 cm from the bottom in a band 1.5 cm wide. Successive development with methanol followed by increasing concentrations of lithium chloride in the same direction separated uridine and each of its phosphorylated derivatives (27). The first development was with 80% methanol to 15 cm past the origin. In this stage of development, uncharged molecules, including uridine, moved along with the front. After air-drying the plate, development was continued with LiCl. First, 0.14 M LiCl was allowed to migrate to 1 cm past the origin. Immediately following without drying, 1.4 M LiCl was allowed to migrate to 15 cm past the origin. The plate was dried and then cut into 2 cm x 0.5 cm strips perpendicular to the direction of chromatography and corresponding to the lane of travel. The strips were placed in scintillation vials and radioactivity determined using toulene scintillation fluid to locate the peaks. This method separated each component in the system by charge. Uridine moved furthest, followed in order by UMP, UDP and UTP. Standards consisted of 30 µg each of uridine, UMP, UDP and UTP and were located by their absorbance of ultraviolet light.

E. Analysis for Mycoplasma Contamination

All cultures were evaluated for contamination by mycoplasma with the method described by Chen (28). This procedure used 33258 Hoechst stain, a

bibenzimidazole dye, 2(2-(4-hydroxyphenyl)-6-benzimidazole)-6-(1-methyl-4-piperazyl)-benzimidazole trihydrachloride, (gift from Dr. J. Becker) which binds specifically to adenine-thymine rich regions of DNA. Once the DNA-dye complex is formed the fluorescence of the compound is enhanced approximately four-fold (29) allowing for ease in visualization with a fluorescent microscope. Staining of whole cells to assess possible mycoplasma contamination is outlined as follows:

1) Cells were grown on a 10.5 mmx22 mm plastic coverslip (Thermanox; Lux Scientific Corp.) in a 35 mm petri dish for 36 hours.
2) Without removing the medium, about 10 drops of fixative (1 part glacial acetic acid: 3 parts absolute methanol) were added to the culture.
3) After two minutes, the solution was removed and replaced with 3 ml of fixative and allowed to stand for 10 minutes.
4) The coverslip cell culture was removed and allowed to air dry.
5) The cells were stained with sufficient amount of dye to cover the surface of the coverslip (0.05 μg 33258 Hoechst per ml distilled water).
6) After 10 minutes the cells were gently rinsed with four changes of distilled water.
7) The coverslip was then mounted face down on a microscope slide using citrate-phosphate buffer (pH 5.5).
8) The preparation was examined with a Zeiss fluorescent microscope using Zeiss attachments, excitor filter BG-12 and barrier filter combination 55/44 (or equivalent).

If present, mycoplasma will be detected as spherical or ellipsoid shapes about 0.1 to 0.3 microns in diameter which are stained uniformly, and located in, on, or around the cells (28). All cultures evaluated were negative for contamination with mycoplasma.

CHAPTER III

RESULTS

A. Clones Chosen for Further Characterization

Three of the nine clones isolated from the third kill cycle, U2, U3, and U6, were selected for a more rigorous characterization. Data obtained from the preliminary screening (see Table 2) indicated these clones incorporated uridine to 3% or less when compared to wild type for whole cell uptake and their UK activity was decreased to 1.9% to 14% of the wild type cells. The observed phenotypes remained stable throughout a six month period of study for each cell strain with continual transfers.

B. HPRT Activity Measurements

In order to monitor reproducibility among extracts, HPRT activity was measured for each extract prepared. HPRT was chosen arbitrarily since it was not related to the observed phenotype. If the HPRT activity decreased 30% or more from the average determined for the cell line in question it was discarded. Extracts were discarded on four occasions during this study. In general, U2 had near normal HPRT activity, while U3 and U6 had about two thirds the activity measured in V79. A summary of the overall average HPRT activity for each cell line is presented in Table 3.

Table 3: Average HPRT Activity of Wild Type and Mutant Cells.[a,b]

Cell line	Average HPRT Activity (pmole/min/μg prot)	% of V79
V79	4.1 ± 0.2	100
U2	4.2 ± 0.3	103
U3	2.5 ± 0.4	61
U6	2.9 ± 0.3	71

[a]Values represent a minimum of four different extracts.

[b] ± values represent standard deviations.

C. UK Activity Measurements

Control Assays

Enzymatic activity was determined to be directly proportional to protein concentration from 0.05 μg to 0.6 μg protein per μl of total assay volume. All assays in this study had a protein concentration range of 0.2 μg to 0.4 μg protein per μl of assay volume. To eliminate the possibility that the mutants might contain an inhibitor of UK activity experiments were performed in which wild type and mutant extracts were mixed in the following proportions: 0:4, 1:3, 2:2, 3:1, and 4:0 (vol:vol). In each case the enzymatic activity observed was proportional only to the volume of wild type extract present. Enzymatic activity was linear in all cases during the five minute assay period as shown in Figure 1. No enzyme activity was found to be lost during the fractionation procedure for either wild type or mutant extracts. In order to verify the nature of the material which was being retained and quantitated on the DE81 disks, the eluted solution of one set of assays from each of the cell lines was chromatographed by PEI-TLC chromatography. This was accomplished by eluting the filters with 1 ml of 0.4 M perchloric acid (PCA) two times. The 2 ml of eluted solution was pooled and applied to an activated charcoal column. The column was washed with two volumes of 0.4 M PCA followed by four volumes of distilled water. Elution was achieved with a mixture of 100% ethanol, distilled water, and ammonium hydroxide (2:2:1). The solution was lyophilized until dry, then solublized with 20 μl of the eluting solvent. The

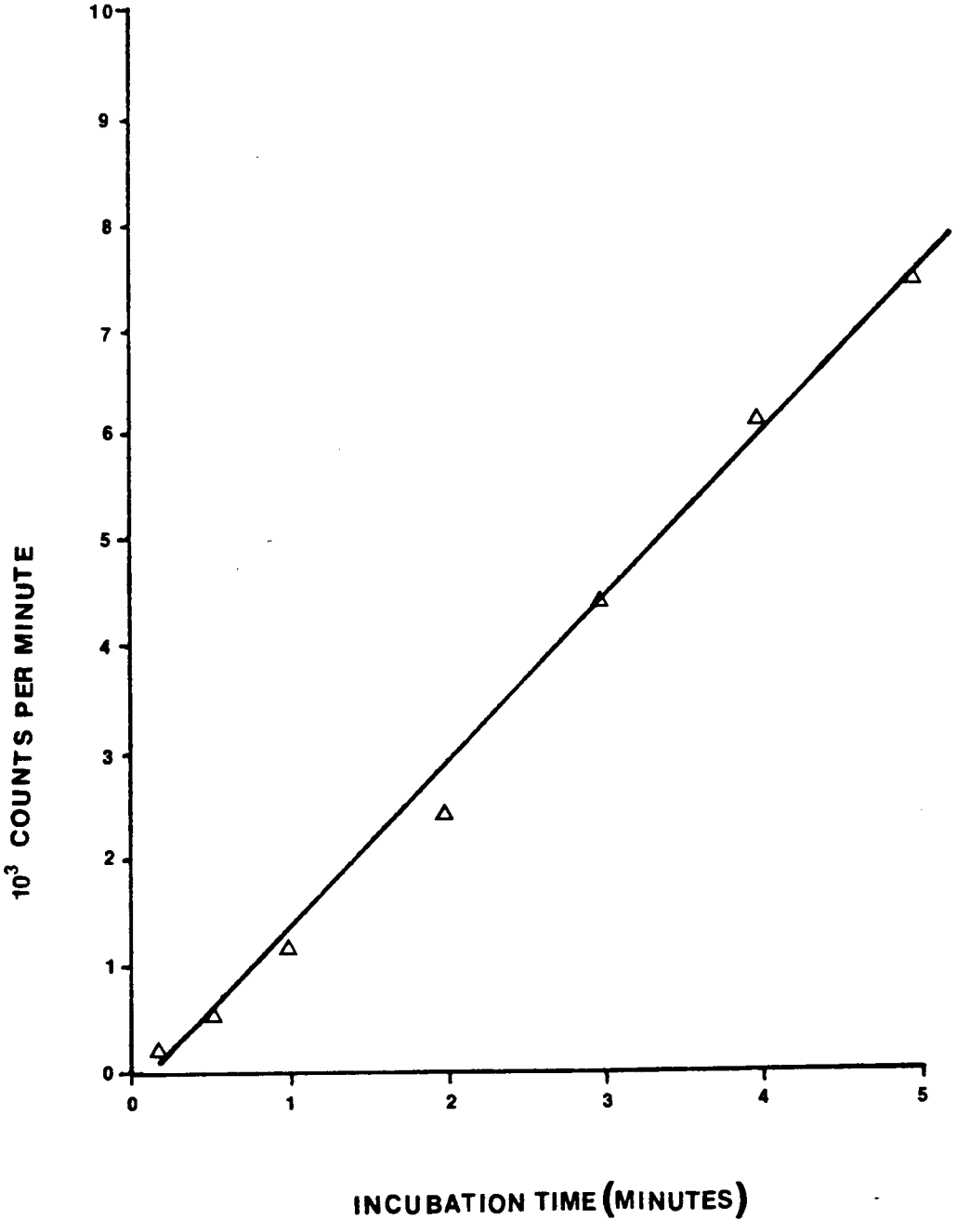

Figure 1. Five minute time course of uridine kinase activity.

material was spotted on a PEI-TLC plate and developed as described above. Elution of the DE81 filter disks followed by PEI-TLC verified that the counts being retained were due to the phosphorylated derivatives of uridine.

Chromatographic analysis of reaction products

PEI-TLC of assays using V79 extracts revealed that uridine was rapidly converted to the triphosphate form with very little accumulation of the mono- and diphosphate forms (see figure 2). The small peak migrating to 9.5 cm is an unidentified contaminant which was contained in the labeled substrate. Similar experiments with each mutant strain indicated the counts observed were due primarily to the triphosphate form (see Figures 3, 4, and 5). It was concluded from this evidence that UK was rate limiting for the kinases responsible for uridine incorporation in the extract and that the defect observed in the mutants was in the activity of UK itself.

Determination of UK mutant kinetic parameters

Kinetic values were calculated using the statistical analysis method described by Wilkinson (30). The apparent Km's for uridine and ATP for V79 extracts were determined to be 120 μM and 382 μM respectively and a Vmax of 5.3 pmole/min/ μg protein. Similar analyses for the mutant cells revealed a decrease of 10-fold in the Km for uridine, a 5-fold decrease in the Km for ATP and a decrease in Vmax to a range of 4% to 7% observed for the wild type. Double reciprocal plots of the data for V79 and the mutant strains are

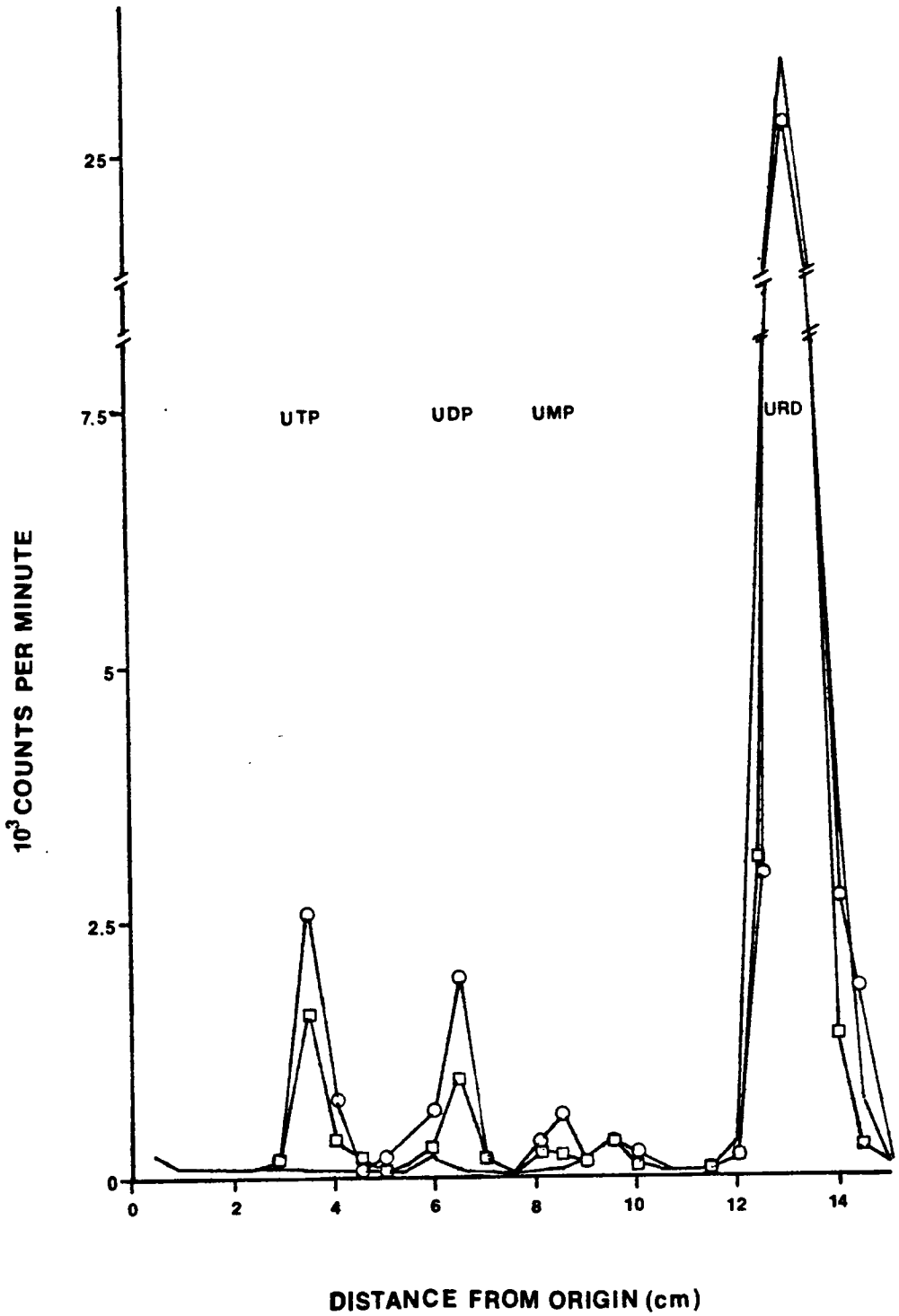

Figure 2. PEI-TLC of V79 UK assay products.

Illustration of the production of phosphorylated products with respect to time for V79 extracts. Uridine concentration was 400 μM and ATP concentration was 2 mM.
(—— 0 minutes, □—□ 2.5 minutes, ○—○ 5 minutes)

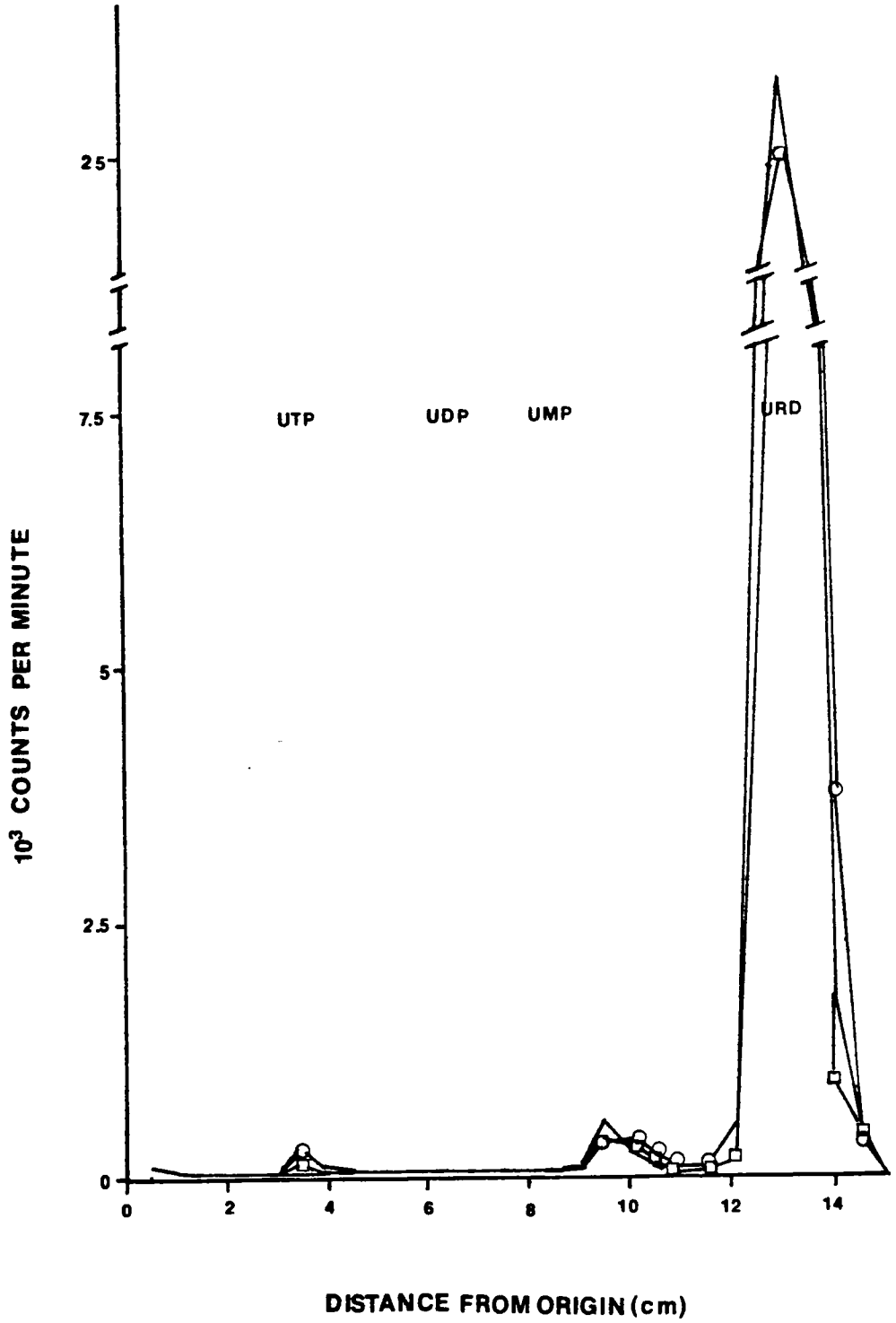

Figure 3. PEI-TLC of U2 UK assay products.

Illustration of the production of phosphorylated products with respect to time for U2 extracts. Uridine concentration was 400 μM and ATP concentration was 2 mM.
(●——● 0 minutes, □——□ 2.5 minutes, ○——○ 5 minutes)

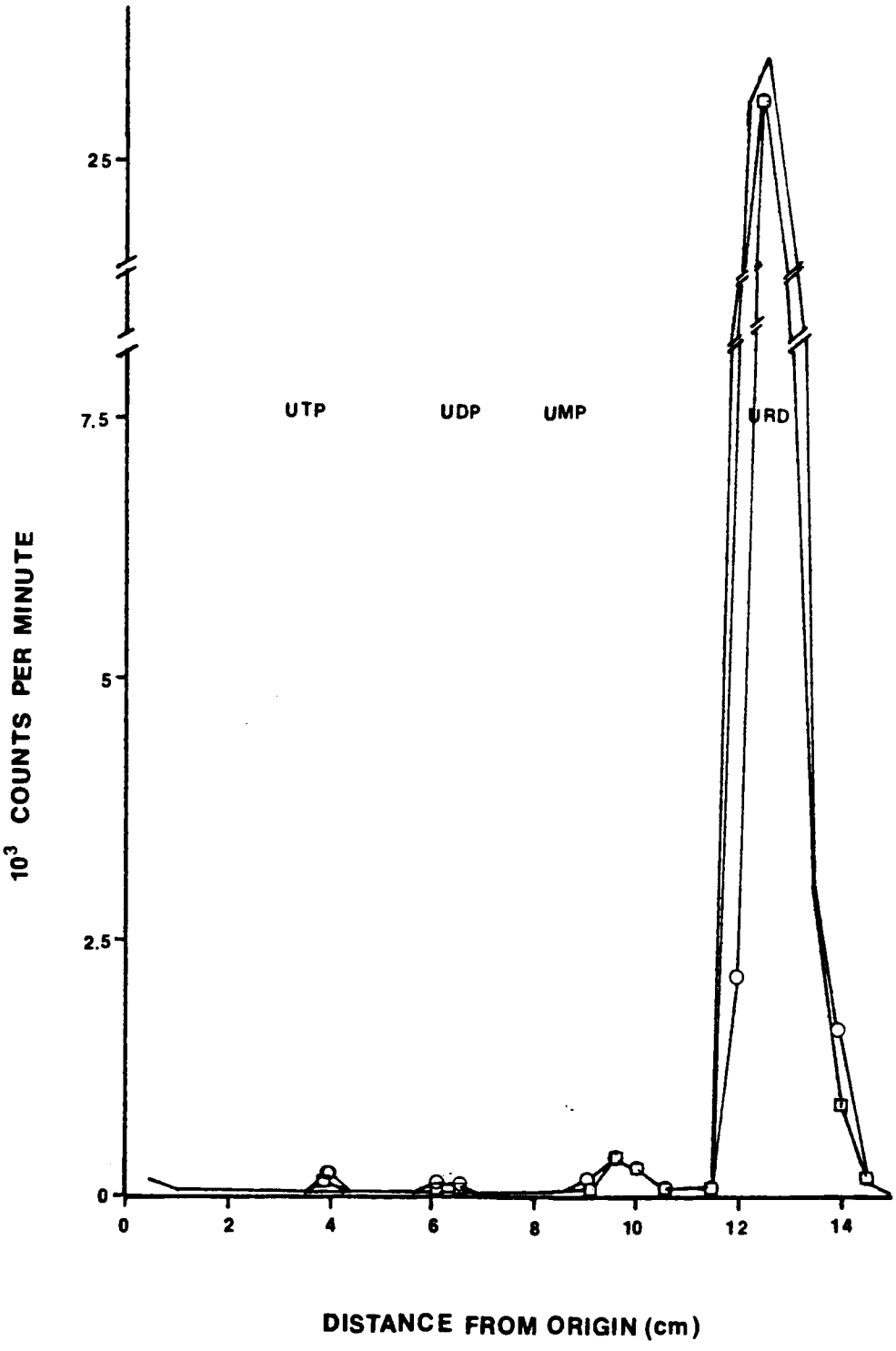

Figure 4. PEI-TLC of U3 UK assay products.

Illustration of the production of phosphorylated products with respect to time for U3 extracts. Uridine concentration was 400 μM and ATP concentration was 2 mM.
(—— 0 minutes, □——□ 2.5 minutes, O—O 5 minutes)

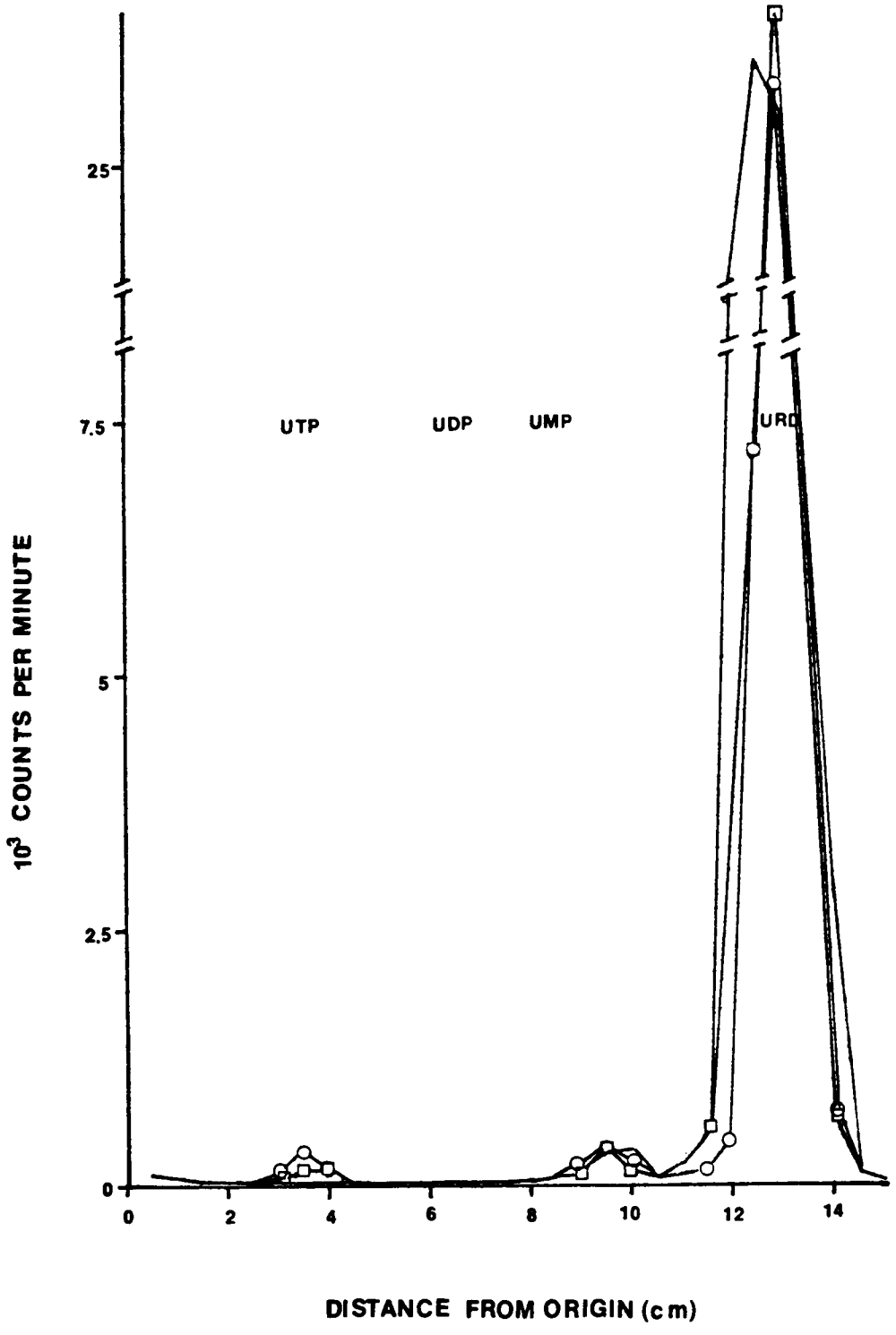

Figure 5. PEI-TLC of U6 UK assay products.

Illustration of the production of phosphorylated products with respect to time for U6 extracts. Uridine concentration was 400 μM and ATP concentration was 2 mM.
(—— 0 minutes, □—□ 2.5 minutes, ○—○ 5 minutes)

presented in Figures 6, 7, 8, and 9; the best fit line in each illustration was derived from the Wilkinson equation (31) and the vertical bars represent the range of experimental data pooled to obtain the values. Six extracts were used for V79 and two were used for each mutant. Kinetic values determined from individual experiments are presented in Table 4 and a summary of the pooled data from the averages of the time points is presented in Table 5.

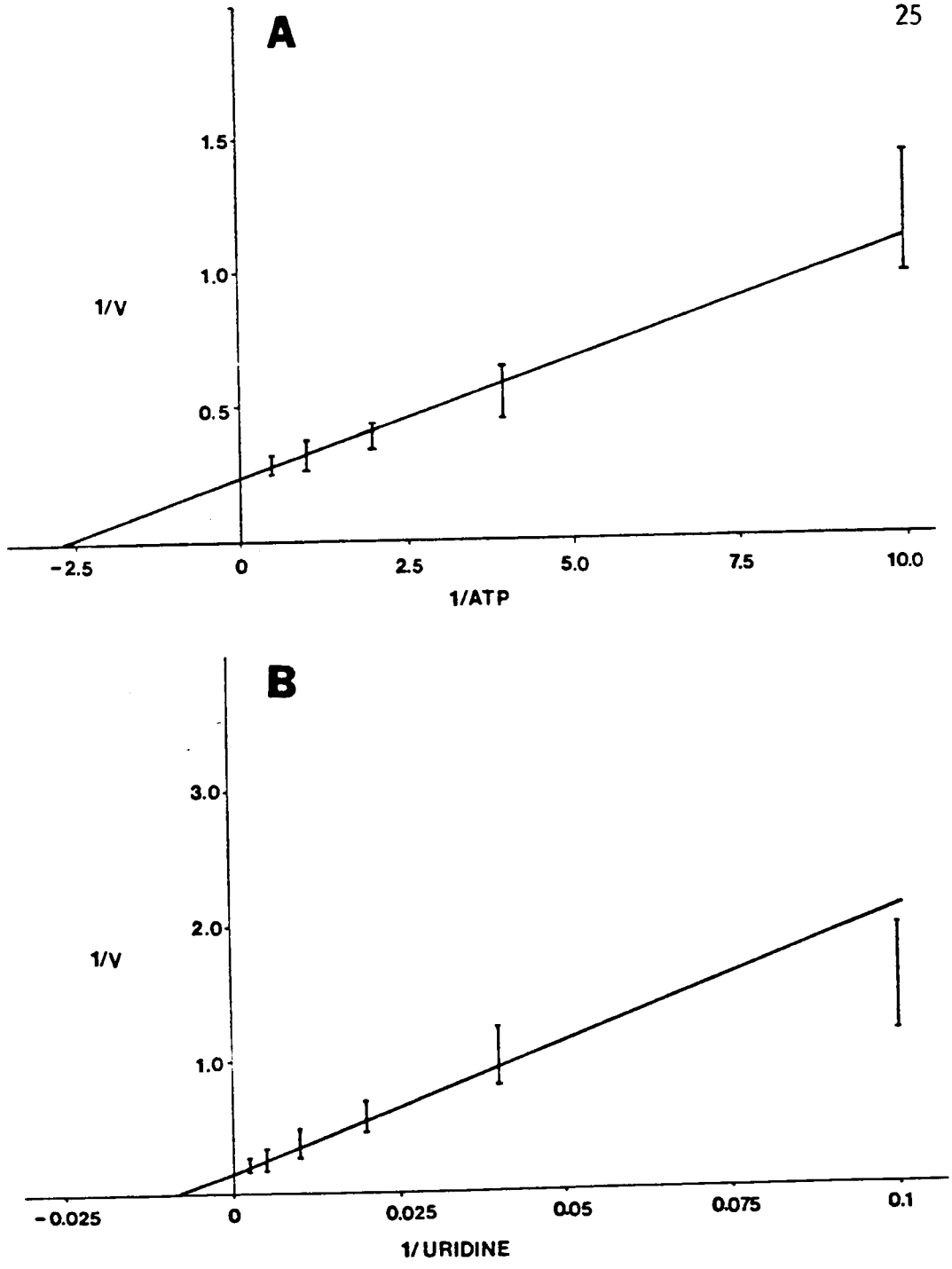

Figure 6. Double reciproocal plots illustrating the kinetic parameters for ATP and uridine as derived from the Wilkinson equation for V79.

Values were determined as described in the text under MATERIALS AND METHODS. Vertical bars represent the range of experimental data pooled to obtain values. Vertical axis values are in pmole/min/μg protein. Horizontal axis values are in μmoles/liter. (A) Kinetic determinations varying ATP at 400 μM uridine. (B) Kinetic determinations varying uridine at 2 mM ATP.

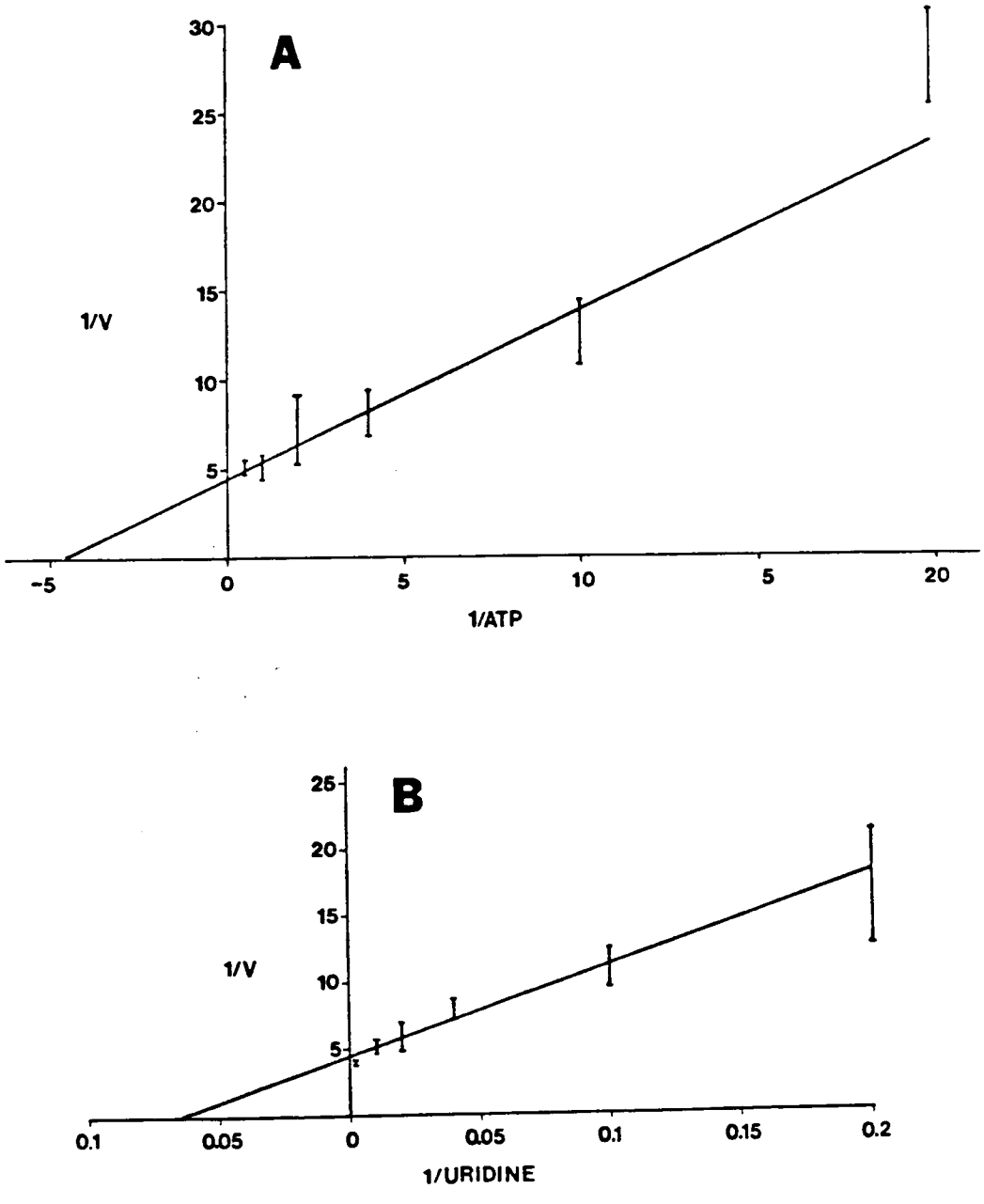

Figure 7. Double reciprocal plots illustrating the kinetic parameters for ATP and uridine as derived from the Wilkinson equation for U2.

Values were determined as described in the text under MATERIALS AND METHODS. Vertical bars represent the range of experimental data pooled to obtain values. Vertical axis values are in pmole/min/μg protein. Horizontal axis values are in μ moles/liter. (A) Kinetic determinations varying ATP at 400 μM uridine. (B) Kinetic determinations varying uridine at 2 mM ATP.

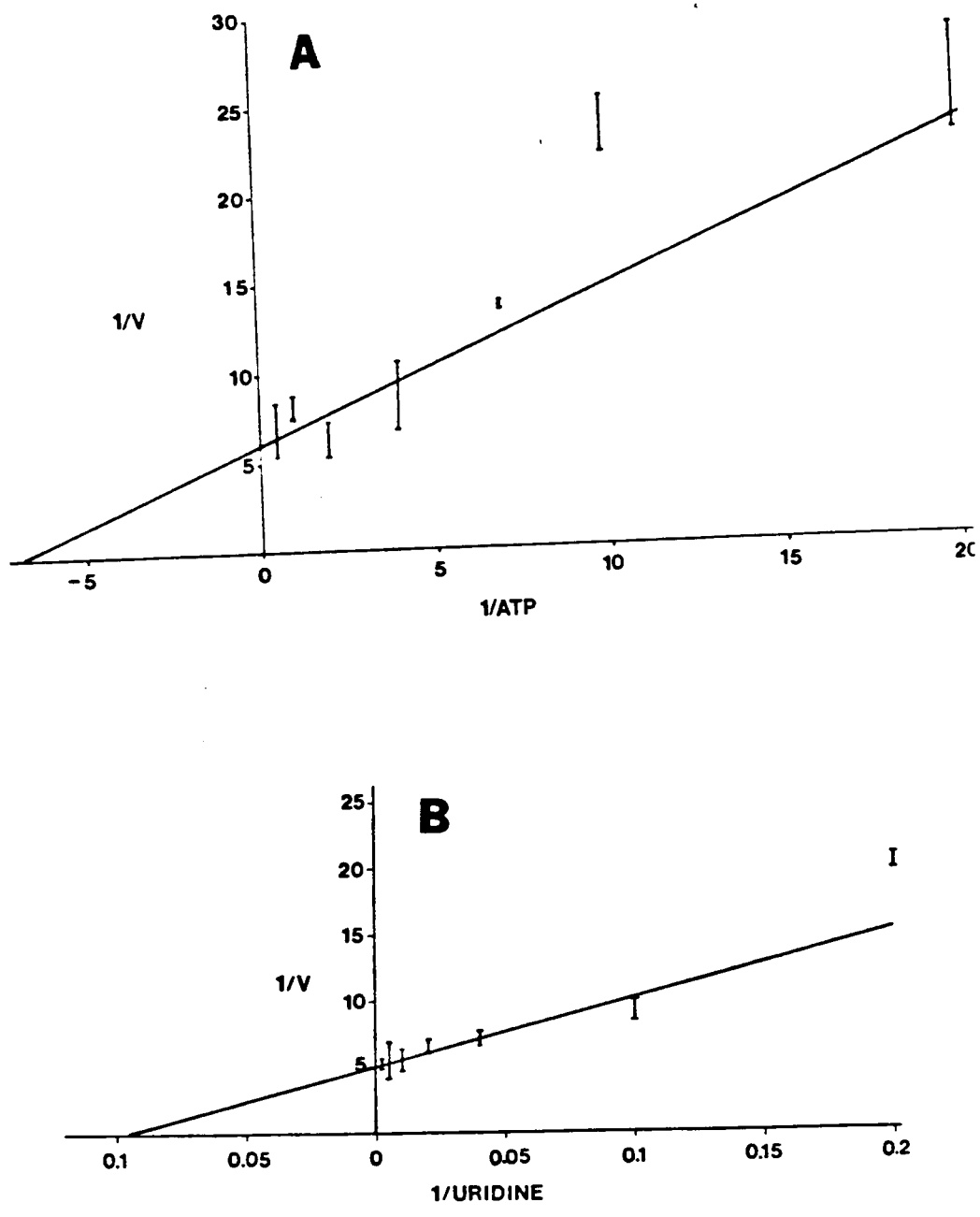

Figure 8. Double reciprocal plots illustrating the kinetic parameters for ATP and uridine as derived from the Wilkinson equation for U3.

Values were determined as described in the text under MATERIALS AND METHODS. Vertical bars represent the range of experimental data pooled to obtain values. Vertical axis values are in pmole/min/µg protein. Horizontal axis values are in µmoles/liter. (A) Kinetic determinations varying ATP at 400 µM uridine. (B) Kinetic determinations varying uridine at 2 mM ATP.

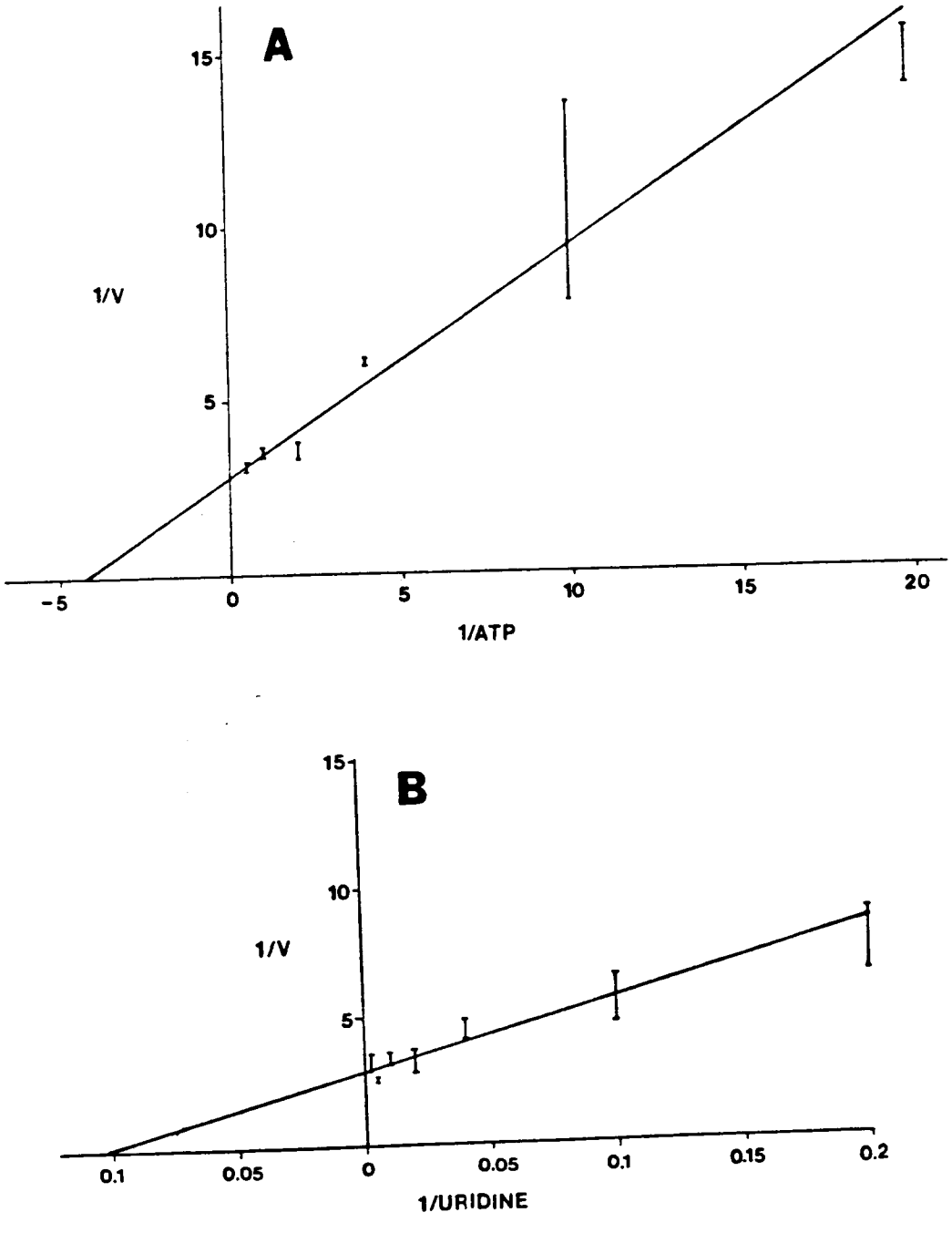

Figure 9. Double reciprocal plots illustrating the kinetic parameters for ATP and uridine as derived from the Wilkinson equation for U6.

Values were determined as described in the text under MATERIALS AND METHODS. Vertical bars represent the range of experimental data pooled to obtain values. Vertical axis values are in pmole/min/µg protein. Horizontal axis values are in µmoles/liter. (A) Kinetic determinations varying ATP at 400 µ M uridine. (B) Kinetic determinations varying uridine at 2 mM ATP.

Table 4: Apparent Km and Vmax Values From Individual Experiments for Uridine Kinase for V79 and Mutant Clones[a,b]

Cell line	Km Uridine (μM)	Km ATP (μM)	Vmax Uridine (pmole/min/μg/prot)	Vmax ATP (pmole/min/μg prot)
V79	125 ± 21	353±174	3.5 ± 0.2	2.8 ± 0.4
	132 ± 15	300±62	7.2 ± 0.4	4.6 ± 0.3
	161 ± 20	352±44	6.3 ± 0.4	3.8 ± 0.2
	101 ± 12	467±93	7.1 ± 0.3	5.3 ± 0.4
	113 ± 13	457±55	4.8 ± 0.2	4.3 ± 0.2
	139 ± 14	355±128	3.6 ± 0.2	3.0 ± 0.3
U2	6.4±4.6	192±55	0.33±0.04	0.35 ± 0.03
	14.3±4.1	301±55	0.37±0.02	0.37 ± 0.02
U3	9.7±6.2	156±53	0.22±0.02	0.20 ± 0.03
	10.2±3.8	131±50	0.18±0.01	0.14 ± 0.02
U6	13.5 ±4.7	177±41	0.25±0.03	0.25 ± 0.02
	26.7 ±9.3	290±78	0.22±0.02	0.21 ± 0.02

[a]Values determined by Wilkinson analysis of duplicate assays.

b ± values represent standard error of the estimate

Table 5: Apparent Km and Vmax Values for Uridine Kinase for V79 and Mutant Clones from Pooled Data[a,b]

Cell line	Km Uridine (μM)	Km ATP (μM)	Vmax Uridine (pmole/min/μg prot)	Vmax ATP (pmole/min/μg prot)
V79	120±20	383±56	6.3 ± 0.5	4.4 ± 0.2
U2	10±3	238±36	0.34 ±0.02	0.36 ±0.02
U3	10±4	150±45	0.20 ±0.01	0.17 ±0.02
U6	16±4	214±58	0.23 ±0.02	0.23 ±0.02

[a]Values determined by Willkinson analysis of pooled data from Table 4.

[b] ± values represent standard error of the estimate.

CHAPTER IV

DISCUSSION

The enzyme uridine-cytidine kinase (ATP: uridine 5'-phosphotransferase, EC 2.7.1.48) catalyzes the reaction:

$$\begin{matrix}\text{cytidine} \\ \text{or} \\ \text{uridine}\end{matrix} + \text{NTP} \xrightarrow{Mg^{++}} \begin{matrix}\text{cytidine 5'-monophosphate} \\ \text{or} \\ \text{uridine 5'-monophosphate}\end{matrix} + \text{NDP}$$

where NTP and NDP represent nucleotide triphosphate and diphosphate respectively. ATP is the preferred NTP; however GTP, ITP, dATP, dGTP, dTTP, dCTP and dUTP have also been observed to act as phosphate group donors (32, 33, 34, 35, 36, 37). The enzyme was first reported by Canellakis (38) in 1957 from extracts of rat liver. The only known function of UK is to phosphorylate preformed pyrimidine nucleosides from either exogenous or endogenous sources, forming the 5'-monophosphate ester.

The enzyme exhibits a rather broad specificity toward the pyrimidine moiety, phosphorylating many synthetic analogs in addition to the natural substrates (39). However, the ribofuranosyl group is the sole carbohydrate which has been observed to be acted upon (39). CTP and UTP act as feedback inihibitors to regulate the activity of UK (34, 35, 36, 37). The mechanism of this control, however, is controversial since experimental evidence from one laboratory was interpreted to indicate allosteric regulation in Paracentrotus lividus (34) and Novikoff ascites cells (35) while other laboratories observed competition for the ATP binding site in Tetrahymena pyriformis (36), P815 cells and H-Ep-2 cells (37). UK has never been purified to homogeneity for mammalian cells but gel filtration data indicates that in mammals the molecular

weight is in the 150,000 to 200,000 dalton range (35, 39, 40). Chromatography with DEAE cellulose and Sepharose 6B has revealed that the enzyme from Paracentrotus lividus (34), rat liver (40), El-4 and P815 mouse cells (41) and rat tissue (42), exists in at least two isomeric forms designated I and II with molecular weights of 120,000 and 30,000 respectively. Recently Absil et al. (43) have identified four forms in male Sherman rats by isoelectric focusing. Examinations of kinetic parameters by various laboratories for eukaryotic systems show Km values for uridine to have a range of $1x10^{-5}M$ to $4x10^{-6}M$ (34, 35, 36, 41, 42, 44, 45, 46) and Km values for ATP to have a 100 fold range of $3x10^{-5}M$ to $3x10^{-3}M$ (34, 35, 36, 40, 41, 42, 44, 45). Liacouras and Anderson (33) and Anderson (47) have presented very strong evidence that the enzyme functions via a sequential mechanism in the murine tumor cell line P815, while Orengo (35, 48) states an equally valid argument for a double displacement mechanism in the Novikoff hepatoma line.

Researchers have only in the past few years been able to isolate mammalian clones which had deficient UK activity (16, 17, 49). Medrano and Green (16) isolated a mouse line which had low activity; however, reports in the literature indicate it was not phenotypically stable (17). Plageman et al. (49) isolated a UK deficient Novikoff cell line by culturing the cells with increasing concentrations of 5-fluorouridine. Ulman et al. (17) have isolated two clones from mutagenized S49 mouse T-lymphoma cells, one by culturing in increasing concentrations of 6-azauridine and the other by using a similar procedure with 5-fluorouricil.

The observation of a decrease in Km accompanied by a simultaneous decrease in Vmax for an enzyme has occurred in other mutant strains (50). At least three possible explanations can be made for this sort of result. First,

the enzyme could be completely deleted and the activity observed could be due to another enzyme (51). Secondly, a simple kinetic model is possible in which the mutation affects the enzyme so as to decrease the rate at which product is formed by the enzyme. Provided this rate normally is significant with respect to the rate of the reverse reaction the overall result would be seen as a decrease in both the Km and Vmax. The final hypothesis, a multiple mutation event, results from the manner in which the mutant cells were selected. A multistep selection process may select for cells which contain more than one mutation producing the observed phenotype. An illustration can be demonstrated by an example with two separate mutagenic events. The first mutation could produce a net reduction in the synthesis of the enzyme or an increase in its rate of degradation, thereby decreasing the enzyme's concentration and resulting in a decrease in Vmax (52). A second mutation could alter the regulatory site for negative effectors so as to depress the release of product resulting in an observed decrease in Km. All these possibilities are equally valid since none of them have been ruled out by the information of this study. Confirmation of any of these hypotheses would require further characterization of the genetic changes and of the structural modification of the enzyme. Whether the three mutant cell lines represent three different mutations or are derived from the same clone cannot be determined from the data presented here. A study of that nature would require preparations of greater purity with which to perform a more in depth analysis and a thorough characterization of the genotypes.

Thompson's statement, "The association of an altered phenotype with an altered gene product constitutes strong evidence that a mutated gene is being expressed" (53) establishes the argument that these cells are the result of a

mutation event. Plagemann et al. (49) have determined that uridine transport is linear for less than ten seconds, therefore ruling out a possible transport defect as being responsible for the observed decrease in uridine incorporation for the mutant clones. In this study we have presented evidence verifying that variant cells selected with tritiated uridine exhibit an altered gene product (uridine kinase). Therefore we conclude that tritiated uridine suicide, using the procedure outlined in this paper, is a successful method for the isolation of mutant cells defective in uridine kinase.

CHAPTER V

SUMMARY OF RESULTS

The results presented in this investigation demonstrate that tritiated uridine suicide selection of mutagenized cells is an effective procedure for the isolation of uridine kinase deficient cell clones. The reaction products determined by PEI-TLC verified that UK was the rate limiting step for the kinases and that the observed behavior of the mutant cells was due to a decrease in the activity of that enzyme. Kinetic investigation of the enzyme revealed a decrease in both Vmax and Km for UK in the mutant cells when compared to the wild type. Evaluation of this data lead to the conclusion that these cells are mutants and that the mutations occurred in a structural gene for UK.

LIST OF REFERENCES

LIST OF REFERENCES

1. Oates, D.C., D. Vannais and D. Patterson, A Mutant of CHO-K1 Cells Deficient in Two Nonsequential Steps of de Novo Purine Biosynthesis, Cell, 20 (1980) 797-805.

2. Caskey, C.T. and G.D. Kruh, The HPRT Locus, Cell, 16, (1979) 1-9.

3. Calos, M.P. and J.H. Miller, Transposable Elements, Cell, 20 (1980) 519-528.

4. Haber, J.E., D.W. Mascioli and D.T. Rogers, Illegal Transposition of Mating-Type Genes in Yeast, Cell, 20 (1980) 519-528.

5. Hicks, J.B., J.N. Strathern and I. Herskowitz, The Cassette Model of Mating-Type Inconversion, DNA Insertion Elements, Plasmids and Episomes, Cold Spring Harbor Laboratory (1977) 457-462.

6. van Diggelen, O.P., T.F. Donahue and S.I. Shin, Basis for Differential Cellular Sensitivity to 8-Azaguanine and 6-Thioguanine, Journal of Cell Physiology, 98 (1979) 59-72.

7. Burki, H.J. and Okada, S., A Comparison of the Killing of Cultured Mammalian Cells Induced by Decay of Incorporated Tritiated Molecules at -196°C, Biophysical Journal, 8 (1966) 445-456.

8. Burki, H.J., S. Bunker, M. Ritter and J.E. Cleaver, DNA Damage from Incorporated Influence of the ^{3}H Location in the Cell, Radiation Research, 62 (1975) 299-312.

9. Breslow, R.E. and R.A. Goldsby, Isolation and Characterization of Thymidine Transport Mutants of Chinese Hamster Cells, Experimental Cell Research, 55 (1969) 339-346.

10. Finkelstein, M.C., C.W. Slayman and E.A. Adelberg, Tritium Suicide Selection of Mammalian Cell Mutants Defective in the Transport of Neutral Amino Acids, Proceedings of the National Academy of Science, USA, 74, (1977) 4549-4551.

11. Dantzig, A.H., E.A. Adelberg and C.W. Slayman, Properties of Two Mouse Lymphocyte Cell Lines Genetically Defective in Amino Acid Transport, Journal of Biological Chemistry, 254 (1979) 8988-8993.

12. Thompson, L.H., C.P. Stasnners and L. Siminovitch, Selection by ^{3}H Amino Acids of CHO-Cell Mutants with Altered Leucyl- and Asparagyl-transfer RNA Synthetases, Somatic Cell Genetics, 1 (1975) 187-208.

13. Adair, G.M., L.H. Thompson and P.A. Lind, Six Complementation Classes of Conditionally Lethal Protein Synthesis Mutants of CHO Cells Selected by ^{3}H-Amino Acid, Somatic Cell Genetics, 4 (1978) 27-44.

14. Thompson, L.H., D.J. Lofgen and G.M. Adair, Evidence for Structural Gene Alterations Affecting Aminoacyl-tRNA Synthetases in CHO Cell Mutants and Revertants, Somatic Cell Genetics, 4 (1978) 423-435.

15. Thompson, L.H., Mutant Isolation, Methods in Enzymology, 58 (1979) 308-332.

16. Medrano, L. and H. Green, A Uridine Kinase-Deficient Mutant of 3T3 and a Selective Method for Cells Containing the Enzyme, Cell, 1 (1974) 23-26.

17. Ullman, B., B.B. Levinson, D.H. Ullman and D.W. Martin, Jr., Isolation and Characterization of Cultured Mouse T-Lymphoma Cells Deficient in Uridine-Cytidine Kinase, The Journal of Biological Chemistry, 254 (1979) 8736-8739.

18. Ayusawa, D., H. Koyama, K. Iwata and T. Seno, Single-Step Selection of Mouse FM3A Cell Mutants Defective in Thymidylate Synthetase, Somatic Cell Genetics, 6 (1980) 261-270.

19. Bryant, R.E., I.E. Schauer and D.G.. Hatcher, Isolation of Hypoxanthine Phosphoribosyl Transferase Defective Mutants in Chinese Hamster V79 Cells by Tritium Suicide, Mutation Research, In Press.

20. Crawford, C.R. The Isolation and Characterization of a Variant Cell Line Deficient in Adenine Uptake, (1980) Thesis, The University of Tennessee, Knoxville, Tennessee.

21. Ford, D.K. and G. Yerganian, Observations on the Chromosomes of Chinese Hamster Cells in Tissue Culture, Journal of the National Cancer Institute, 21 (1958) 393-425.

22. Yu, C.K., Evaluation and Nature of the Near-Diploid Cells of Chinese Hamster (Cricetulus Griseus) In Vitro, Canadian Journal of Genetics and Cytology, 5 (1963) 307-317.

23. Yu, C.K. and W.K. Sinclair, Homogeneity and Stability of Chromosomes of Chinese Hamster Cells In Vitro, Canadian Journal of Genetics and Cytology, 6 (1964) 109-116.

24. Aaron, C.S., A.A. Van Zeeland, G.R. Mohn, A.T. Natarajan, ADA G.A.C. Knaap, A.D. Tates and B.W. Glickman, Molecular Dosimetry of the Chemical Mutagen Ethyl Methanesulfonate: Quantitative Comparison of Mutation Induction in Escherichia coli, V79 Chinese Hamster Cells and L5278Y Mouse Lymphoma Cells, and Some Cytological Results In Vitro and In Vivo, Mutation Research, 69 (1980) 201-216.

25. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, Protein Measurement with the Folin Phenol Reagent, Journal of Biological Chemistry, 193 (1951) 265-275.

26. Ives, D.H., J.P. Durham and V.S. Tucker, Rapid Determination of Nucleoside Kinase and Nucleotidase Activities with Tritium-Labeled Substrates, Analytical Biochemistry, 28 (1969) 192-205.

27. Randerath, K. and E. Randerath, Ion-Exchange Chromatogrpahy of Nucleotides on Poly-(Ethyleneimine)-Cellulose Thin Layers, Journal of Chromatography, 16 (1964) 111-125.

28. Chen, T.R., Microscopic Demonstration of Mycoplasma Contamination in Cell Cultures and Cell Culture Media, Tissue Culture Association Handbook, 2 (1976) 229-232.

29. Cesarone, D.V., C. Bolognesi and L. Santi, Improved Microfluorometric DNA Determination in Biological Material Using 33258 Hoechst, Analytical Biochemistry, 100 (1979) 188-197.

30. Wilkinson, G.N., Statistical Estimations in Enzyme Kinetics, Biochemical Journal, 80 (1961) 324-332.

31. Barnes, J.E. and A.J. Waring, Pocket Programmable Calculators in Biochemistry, John Wiley and Sons, New York (1980).

32. Krystall, G. and P.G. Scholefield, The Partial Purification and Properties of Uridine Kinase from Ehrlich Ascites Tumor Cells, Canadian Journal of Biochemistry, 51 (1973) 379-389.

33. Liacouras, A.S., T.Q. Garvey, III., F.K. Millar and E.P. Anderson, Uridine-Cytidine Kinase, Kinetic Studies and Reaction Mechanism, Archives of Biochemistry and Biophysics, 168 (1975) 74-80.

34. Orengo, A., Feedback Regulation in the Ribopyrimidine "Salvage" Pathway, Experimental Cell Research, 41 (1966) 338-349.

35. Orengo, A., Regulation of Enzymic Activity by Metabolites, I. Uridine-Cytidine Kinase of Novikoff Ascites Rat Tumor, The Journal of Biological Chemistry, 244 (1969) 2204-2209.

36. Plunkett, W. and J.G. Moner, The Role of Uridine-Cytidine Kinase in the Regulation of Pyrimidine Ribonucleotide Synthesis in Tetrahymena Pyriformis GL, Biochimica et Biophysica Acta, 250 (1971) 92-102.

37. Anderson, E.P. and R.W. Brockman, Feedback Inhibition of Uridine Kinase by Cytidine Triphosphate and Uridine Triphosphate, Biochemical et Biophysica Acta, 91 (1964) 380-386.

38. Canellakis, E.S., Pyrmidine Metabolism II. Enzymatic Pathways of Uracil Anabolism, The Journal of Biological Chemistry, 227 (1957) 329-339.

39. Cihak, A. and B. Rada, Uridine Kinase: Properties, Biological Significance and Chemotherapeutic Aspects (A Review), Neoplasma, 23 (1976) 233-257.

40. Fulchignoni-Lataud, M.C., M. Tuilie and J.M. Roux, Uridine-Cytidine Kinase from Foetal and Adult Rat Liver, Purification and Study of Some Properties, European Journal of Biochemistry, 69 (1976) 217-222.

41. Greenberg, N., D.E. Schumm, P.E. Hurtubise and T.E. Webb, Uridine Kinase Activities in Normal and Neoplastic Lymphoid Cells, Cancer Research, 37 (1977) 1028-1034.

42. Herzfeld, A. and S.M. Raper, Uridine Kinase Activities in Developing, Adult and Neoplastic Rat Tissues, Biochemical Journal, 182 (1979) 771-778.

43. Absil, J., M. Tuilie and J.M. Roux, Electrophoretically Distinct Forms of Uridine Kinase in the Rat, Biochemical Journal, 185 (1980) 273-276.

44. Skold, O., Uridine Kinase from Ehrlich Ascites Tumor: Purification and Properties, Journal of Biological Chemistry, 235 (1960) 3273-3278.

45. Ahmed, N.K. and A.D. Welch, Some Properties of Uridine-Cytidine Kinase from a Human Malignant Lymphoma, Cancer Research, 39 (1979) 3102-3106.

46. Drake, J.C., R.J. Stoller and B.A. Chabner, Characteristics of the Enzyme Uridine-Cytidine Kinase Isolated from a Cultured Human Cell Line, Biochemical Pharmacology, 26 (1977) 64-66.

47. Anderson, E.P., Uridine-Cytidine Kinase from a Murine Neoplasm, Methods in Enzymology, 51 (1978) 314-321.

48. Orengo, A. and S.H. Kobayashi, Uridine-Cytidine Kinase from Novikoff Ascites Rat Tumor and Bacillus stearothermophilus, Methods in Enzymology, 51 (1978) 299-307.

49. Plagemann, P.G.W., R. Marz and R.M. Wohlhueter, Uridine Transport in Novikoff Rat Hepatoma Cells and Other Cell Lines and its Relationship to Uridine Phosphorylation and Phosphorolysis, Journal of Cellular Physiology, 97 (1978) 49-72.

50. Fenwick, R.G., Jr., T.H. Sawyer, G.D. Kruh, K.H. Astrin and C.T. Caskey, Forward and Reverse Mutations Affecting the Kinetics and Apparent Molecular Weight of Mammalian HGPRT, Cell, 12 (1977) 383-391.

51. Ahmed, N.K., G.S. Germain and A.D. Welch, Phosphorylation of Nucleosides Catalyzed by a Mammalian Enzyme Other Than Uridine-Cytidine Kinase, Biochemical and Biophysical Research Communications, 95 (1980) 440-445.

52. Spector, E.B., M.S. Hershfield and J.E. Seegmiller, Purine Reutilization and Synthesis de Novo in Long-Term Human Lymphocyte Cell Lines Deficient in Adenine Phosphoribosyltransferase Activity, Somatic Cell Genetics, 4 (1978) 253-264.

53. Thompson, L.H. and R.M. Baker, Isolation of Mutants of Cultured Mammalian Cells, Methods in Cell Biology, 6 (1973) 209-281.

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