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Uridine-Cytidine Kinase IV. Kinetics of the Competition Between 5-Azacytidine and the Two Natural Substrates*

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SUMMARY

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Dual-substrate kinetics have been used to demonstrate that the phosphorylation of 5-azacytidine by a purified preparation of uridine-cytidine kinase is competitive with that of the natural substrates. When 5-azacytidine was combined with either uridine or cytidine in the reaction, the kinetic patterns observed were consistent with the theoretical plots for a single enzyme catalyzing both phosphorylations. In experiments with uridine plus 5-azacytidine, the patterns were those for alternate substrates with significantly disparate V_{max} values ($V_{max,\ uridine}$ = 1.82 μmoles phosphorylated/min/mg protein; $V_{max,\ azacytidine}$ = 1.11 μmoles phosphorylated/min/mg protein). In the phosphorylation of cytidine plus 5-azacytidine, the pattern seen was that for alternate substrates with equivalent V_{max} values ($V_{max,\ cytidine}$ = 0.97 μmoles phosphorylated/min/mg protein). Furthermore, 5-azacytidine could be shown to be a competitive inhibitor of cytidine phosphorylation, albeit at high ratios of analogue to normal substrate. Apparent K_m's were found to be: uridine, 0.135 mM; cytidine, 0.067 mM; 5-azacytidine, 8.09 mM. From the kinetic patterns in the dual substrate experiments, it can be concluded that all three nucleosides are phosphorylated by the same enzyme, presumably at a single catalytic site.

INTRODUCTION

Uridine-cytidine kinase (ATP : uridine 5'-phosphotransferase, E.C. 2.7.1.48) catalyzes the phosphorylation of uridine and cytidine, and a number of their analogues to the respective nucleoside monophosphates (2, 3). Of these analogues, 5-azacytidine (4), a triazine derivative of cytidine, has attracted particular attention because of its cytostatic, carcinostatic, and antibacterial properties (5-7). In eukaryotes, the spectrum of metabolic irregularities produced by 5-azacytidine includes effects on RNA, DNA, pyrimidine nucleotide, and protein biosyntheses (6-9), and on the breakdown of liver polyribosomes (6).

Phosphorylation of 5-azacytidine to its monophosphate could be demonstrated (6, 7, 10-12), and resistance to the analogue has been related to decreased activity of uridine-cytidine kinase (6, 8, 9, 13). In mammalian enzyme systems, 5-azacytidine phosphorylation was inhibited by both uridine and cytidine (7, 8, 11), and a purified preparation of uridine-cytidine kinase phosphorylated 5-azacytidine as well as it

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did cytidine (12). Isozymes specific for normal and fraudulent substrates are, however, known, and might be expected in this feedback-regulated enzyme (14). To determine conclusively whether or not 5-azacytidine is phosphorylated by the enzyme active for the normal substrates, we have undertaken a dual-substrate kinetic study; this technique has been successfully used for the two natural substrates (15).

Explicit theoretical analyses for dual-substrate competitions involving interaction with a single enzyme have been presented (16–19), and this kinetic approach has been applied to a variety of systems in which one enzyme catalyzes the reaction between two alternative substrates, and particularly to studies in which one of the substrates is foreign (20–24). The procedure measures the total product of both reactions and the combined velocity under steady-state conditions, as given by the equation

$$v = \frac{\frac{V_A A}{K_A} + \frac{V_B B}{K_B}}{1 + \frac{A}{K_A} + \frac{B}{K_B}} \quad (1)$$

where A and B are the concentrations of the two competing substrates, and V and K are their respective V_{max} and K_m values. If a single enzyme catalyzes the reaction with two alternative substrates, a characteristic pattern of curves is obtained when combined velocity is plotted against the concentration of either substrate; the nature of this pattern depends upon the relationship between the two V_{max} values (18). When the two reactions are, instead catalyzed by separate enzymes, the pattern is very different, and reflects the kinetic independence of each reaction.

MATERIALS AND METHODS

Materials. Uridine was purchased from Boehringer-Mannheim and cytidine from Sigma Chemical Company; [2-^{14}C]cytidine (25 mCi/mmole) came from Schwarz/Mann and 5-azacytidine was generously provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Rabbit muscle lactate dehydrogenase (360 U/mg), pyruvate kinase (150 U/mg), and the sodium salts of ATP, NADH, and phosphoenolpyruvate were also obtained from Boehringer-Mannheim. All the reagents were used without further purification, and were freshly prepared just prior to assay. ATP and phosphoenolpyruvate were titrated to pH 6.5 with NaOH.

Enzyme source. Hepatoma 129P ascites cells (25) were carried in C3H mice by intraperitoneal transfer. Uridine-cytidine kinase was isolated according to previously described methods (12) from crude extracts of acetone powders of these cells (26). The final preparation used in these kinetic studies was approximately 330-fold purified and was essentially free of enzyme contaminants which transformed either the substrates or the products into other metabolic compounds; these included UMP kinase, nucleoside diphosphate kinase, adenosine triphosphatase, cytidine aminohydrolase and enzymes which degrade UMP to uridine or uracil.

Enzyme assays. Assay I. The activity of uridine-cytidine kinase was assayed spectrophotometrically (15, 27) by coupling the production of ADP with pyruvate kinase and lactate dehydrogenase and monitoring the formation of NAD^+ at 340 nm for up to 10 min with a Gilford recording spectrophotometer.

Enzyme assays. Assay II. The conversion of [^{14}C]cytidine to radioactive products was used to assay the enzymatic activity (12) in studying 5-azacytidine inhibition of cytidine phosphorylation. Labeled substrate and products were separated and quantitated as described previously (12).

In both assays, protein content of the final purified fraction was determined with a Beckman 120B Amino Acid Analyzer; Beckman *Stand-In* in sodium citrate buffer contained the internal reference standards.

Data processing. Two computer programs were used to analyze the experimental data. From the velocity and concentration data for the experiments with each nucleoside alone, the linear slopes of the double reciprocal plots, and the V_{max} and K_m values were determined according to the Cleland Hyper program (28), using an

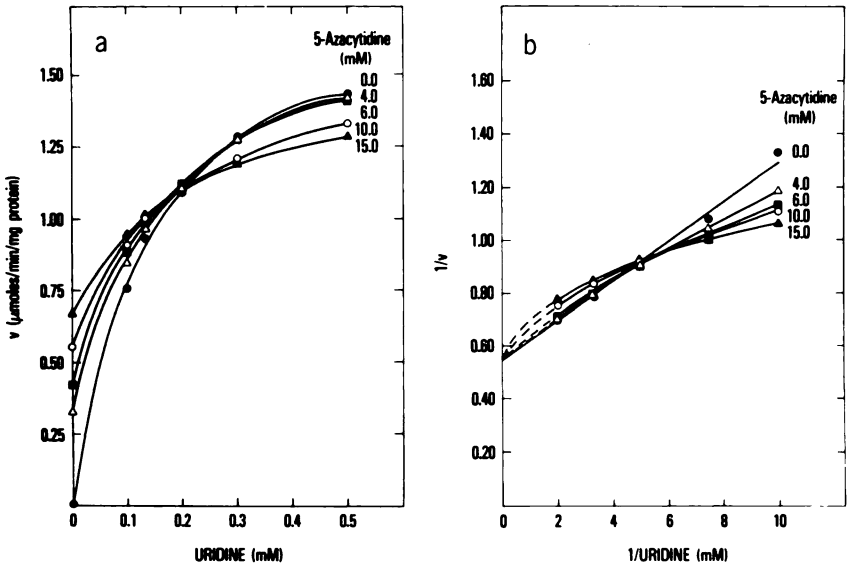

FIG. 1. *Substrate competition patterns for uridine-cytidine kinase with uridine and 5-azacytidine: 1a, combined velocity plotted against uridine concentration at different fixed concentrations of 5-azacytidine; 1b, double reciprocal plot of 1/combined velocity against 1/uridine concentration at the different concentrations of 5-azacytidine*

Each reaction cuvette contained, together with fixed and variable substrates, 5 μmol ATP, 11 μmol $MgCl_2$, 100 μmol KCl, 6 μmol phosphoenolpyruvate, 0.4 μmol NADH, 50 μg pyruvate kinase, 100 μg lactate dehydrogenase and 50 μmol imidazole-HCl buffer, pH 6.5 (Assay I). The total volume in each test was 1 ml. The fixed concentrations of 5-azacytidine were 0, ●——●; 4.0 mM, △——△; 6.0 mM, ■——■; 10.0 mM, ○——○; and 15.0 mM, ▲——▲. The reaction was initiated by the addition of 1.65 μg enzyme protein. All experiments were run in duplicate at 25°. Coordinates of the intersection point are approximately: v = 1.11 μmoles phosphorylated/min/mg protein; [S] = 0.21 mM.

IBM 360 computer. The curves for these combined substrate experiments were drawn visually (Figs. 1–4) and then compared, for correctness of fit, to a second set of computer-drawn curves. To obtain these latter, best estimates of the V_{max} and K_m values were calculated by fitting the velocity and concentration data to the mathematical function given in equation 1, using the MLAB[1] computer program (29, 30). Given these derived values for the kinetic constants, the graphics facilities of MLAB were used to plot the best-fit curve for v versus [S] and for $1/v$ versus $1/[S]$, particularly of the extrapolation to the ordinate, at each fixed concentration of the alternate substrate.

[1] MLAB, which was developed at the National Institutes of Health, is a computer program for interpretive evaluation and fitting of mathematical models. It runs on a DEC system-10 computer, and is available to the public.

RESULTS

Preliminary studies were first undertaken to determine the kinetic constants for the reaction with each substrate alone. The resulting data are shown in Table 1 (Experiments #1–3). Since the V_{max} for 5-azacytidine is nearly equal to that for cytidine and approximately half that for uridine, dual-substrate experiments combining the analogue with one of the natural substrates provide good examples of interactions between alternate substrates both with equivalent V_{max} values and with different V_{max}'s. The theoretical curves (18) predict characteristic patterns for both situations. Other preliminary experiments defined satisfactory concentration ranges for each of the substrates in combined velocity experiments.

Competition between 5-azacytidine and uridine. The kinetic patterns for combinations of 5-azacytidine and uridine, sub-

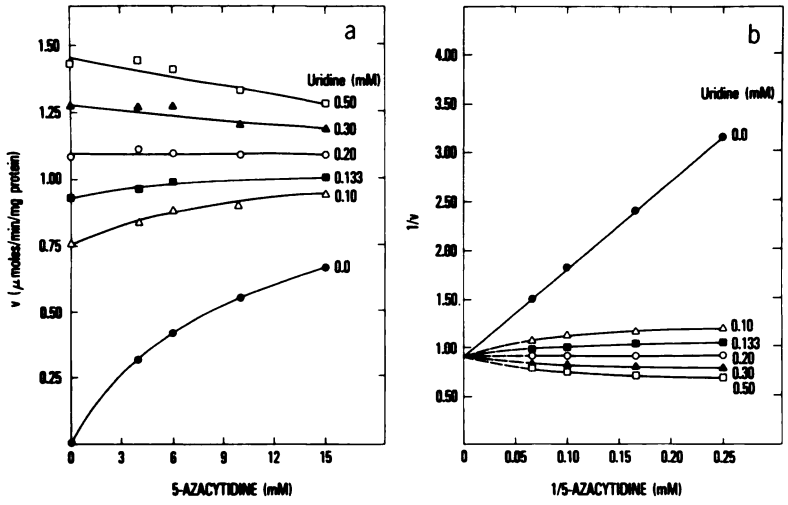

FIG. 2. *Substrate competition patterns for uridine-cytidine kinase with uridine and 5-azacytidine: 2a, combined velocity plotted against 5-azacytidine concentration at the different fixed concentrations of uridine; 2b, double reciprocal plot of 1/combined velocity against 1/5-azacytidine concentration at the several concentrations of uridine*

The data are the same as those plotted in Fig. 1; 5-azacytidine concentrations and the reaction conditions are given in the legend. The six fixed concentrations of uridine were 0, ●——●; 0.10 mM, △——△; 0.133 mM, ■——■; 0.20 mM, ○——○; 0.30 mM, ▲——▲; and 0.50 mM, □——□.

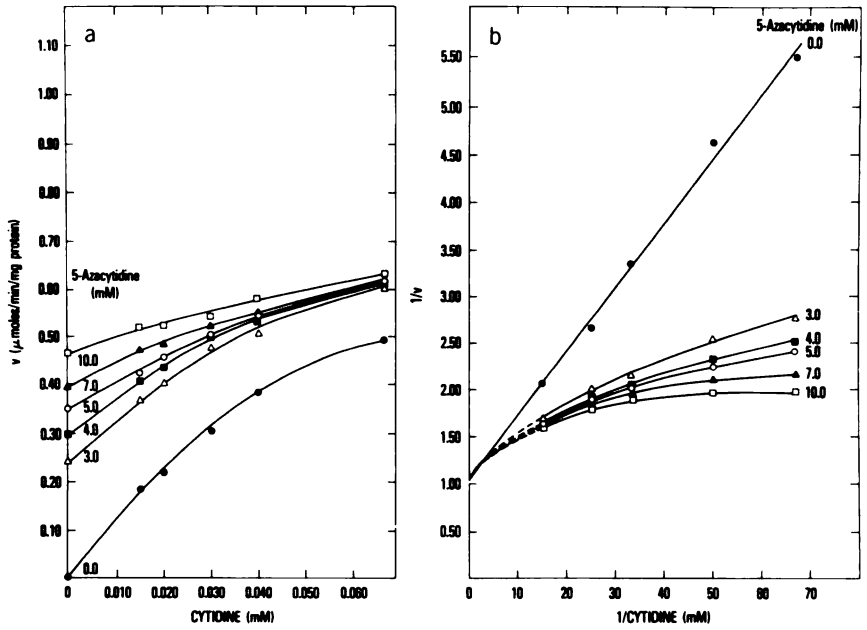

FIG. 3. *Substrate competition patterns for uridine-cytidine kinase with cytidine and 5-azacytidine: 3a, combined velocity plotted against cytidine concentration at different fixed concentrations of 5-azacytidine; 3b, double reciprocal plot of 1/combined velocity against 1/cytidine concentration at the different concentrations of 5-azacytidine*

Reaction conditions are the same as those given in Fig. 1. The six fixed concentrations of 5-azacytidine were 0, ●——●; 3.0 mM, △——△; 4.0 mM, ■——■; 5.0 mM, ○——○; 7.0 mM, ▲——▲; and 10.0 mM, □——□.

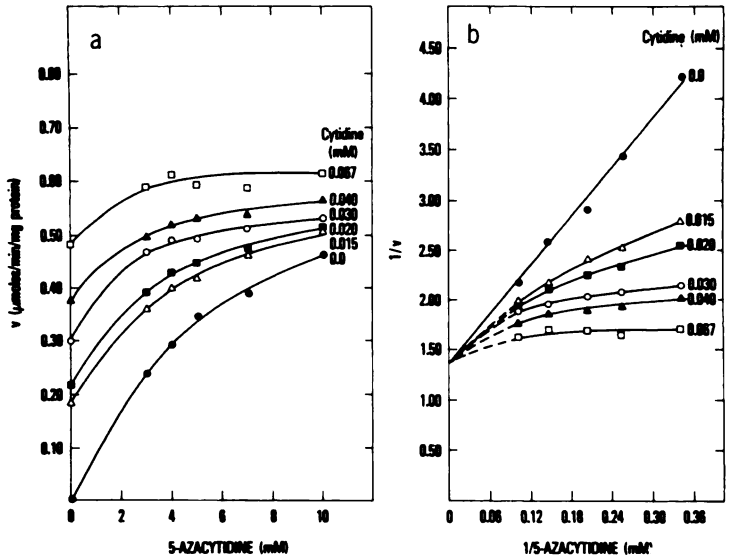

FIG. 4. *Substrate competition patterns for uridine-cytidine kinase with cytidine and 5-azacytidine: 4a, combined velocity plotted against 5-azacytidine concentration at the different fixed concentrations of cytidine; 4b, double reciprocal plot of 1/combined velocity against 1/5-azacytidine concentration at the several concentrations of cytidine*

The data are the same as those plotted in Fig. 3 and 5-azacytidine concentrations are given therein. Reaction conditions are those given in Fig. 1. The six fixed concentrations of cytidine were 0, ●——●; 0.015 mM, △——△; 0.020 mM, ■——■; 0.030 mM, ○——○; 0.040 mM, ▲——▲; and 0.067 mM, □——□.

strates with different V_{max} values, are shown in Figs. 1 and 2. In Fig. 1a, the combined velocity of the two reactions is plotted against concentration of uridine (the high V_{max} substrate) at several fixed concentrations of the presumed alternative substrate, 5-azacytidine. Uridine concentration was varied over a five-fold range from 0.1 to 0.5 mM, while 5-azacytidine concentrations ranged from 4.0 mM to 15 mM. The phosphorylating agent, ATP, was maintained at a constant 5 mM (K_m = 3.5 mM and 3.1 mM with uridine and 5-azacytidine, respectively). As can be seen, the family of curves tends to intersect near 0.21 mM uridine. The presence of the analogue increases and decreases the rate of total measured product formation at uridine concentrations below and above 0.21 mM, respectively. The double reciprocal plot of 1/combined velocity against 1/uridine concentration (Fig. 1b) again gives a family of curves that intersect the line for the reciprocal of the velocity with uridine alone. The curves were extrapolated, according to equation 1, to the reciprocal value of the V_{max} for uridine where the concentration of uridine would be infinite and hence rate determining in each case. The K_m and V_{max} values are given in Table 1 (Experiment #4). The first and second columns show the values calculated from the line for uridine alone. The last two columns show the values calculated with the MLAB program (see *Data processing*).

In Fig. 2a, the same data for combined velocity are plotted against the concentration of 5-azacytidine (the low V_{max} substrate) at various fixed concentrations of uridine. The high V_{max} substrate, uridine, makes the more significant contribution to the combined velocity, and the pattern seen is a series of curves that do not intersect, but rather tend to merge toward the V_{max} value for 5-azacytidine. Fig. 2b shows the double reciprocal plot of 1/combined velocity against 1/5-azacytidine concentration. The curves were again extrapolated to the reciprocal of the V_{max} for 5-azacytidine.

The patterns observed in Figs. 1 and 2 agree closely with the theoretical patterns derived by Cha (18) for competition, for a single enzyme, between alternate sub-

TABLE 1

Kinetic constants for 5-azacytidine, uridine and cytidine

Substrate	K_m[a] (mM)	V_{max}[a] (μmoles phosphorylated/ min/mg protein)	K_m[b] (mM)	V_{max}[b] (μmoles phosphorylated/ min/mg protein)
5-Azacytidine				
Experiment #1	7.36	0.95	—	—
Experiment #4	9.90	1.11	8.98	1.07
Experiment #5	6.28	0.74	5.71	0.74
Uridine				
Experiment #2	0.173	2.19	—	—
Experiment #4	0.135	1.82	0.124	1.79
Cytidine				
Experiment #3	0.059	1.00	—	—
Experiment #5	0.067	0.97	0.079	1.16

[a] Determined from the velocity and concentration data for the experiments with each nucleoside alone, using the Cleland Hyper computer program (28).

[b] Determined by fitting the velocity and concentration data from combined substrate experiments, to equation 1, using the MLAB computer program (29, 30).

strates with divergent V_{max} values. Similar patterns were observed in our previous study on the competition between uridine and cytidine for this enzyme (15). Thus, the results indicate that uridine and 5-azacytidine are indeed phosphorylated by a single enzyme.

Competition between 5-azacytidine and cytidine. Cytidine and 5-azacytidine have equivalent V_{max} values; this type of alternate substrate interaction is depicted in the kinetic patterns in Figs. 3 and 4. In Fig. 3a, combined velocity is plotted against cytidine concentration at several fixed concentrations of 5-azacytidine. Cytidine concentration was varied from 0.015 mM to 0.067 mM, and the concentrations of 5-azacytidine ranged from 3.0 mM to 10.0 mM. The concentration of ATP was again constant at 5 mM (K_m = 2.1 mM with cytidine, 3.1 mM with 5-azacytidine). Here the combined velocity is always greater than the reaction rate with cytidine alone, and the pattern is a series of hyperbolic curves that do not intersect, but rather approach the maximum velocity. The double reciprocal plot of 1/combined velocity against 1/cytidine concentration (Fig. 3b) shows the straight line for cytidine alone and the series of curves for the several azacytidine concentrations, each extrapolated to the reciprocal of the V_{max}. The data from these studies are shown in the Table as Experiment #5. The same data are plotted in Fig. 4a as v against [S] with 5-azacytidine as the variable substrate, at several fixed concentrations of cytidine. Figure 4b shows the comparable double reciprocal plot.

The patterns in Figs. 3 and 4 are similar, and both are in keeping with the theoretical patterns (18) for alternate substrates with equivalent V_{max} values competing for the same enzyme. This confirms the results indicated by Figs. 1 and 2, and the conclusion can be drawn that 5-azacytidine, cytidine, and uridine are all phosphorylated by the same enzyme, and presumably at a single catalytic site on the enzyme. There is no indication, therefore, of separate, closely related isozymes specific for the normal and fraudulent substrates, respectively.

Inhibition of cytidine phosphorylation by 5-azacytidine. Experiments were undertaken to explore further the capacity of 5-azacytidine to inhibit cytidine phosphorylation. These were carried out using Assay II, with [^{14}C]labeled cytidine and cold 5-azacytidine. Results of these experiments are shown in the double reciprocal plot of $1/v$ against 1/cytidine, as depicted in Fig. 5. The concentrations of cytidine were varied from 0.015 mM to 0.067 mM. The pattern with an intersection on the ordinate, indicates competitive inhibition. The K_m for cytidine, 0.05 mM, is in excellent agreement with the values determined above and from earlier studies (27). The insert shows a replot of the slopes against inhibitor concen-

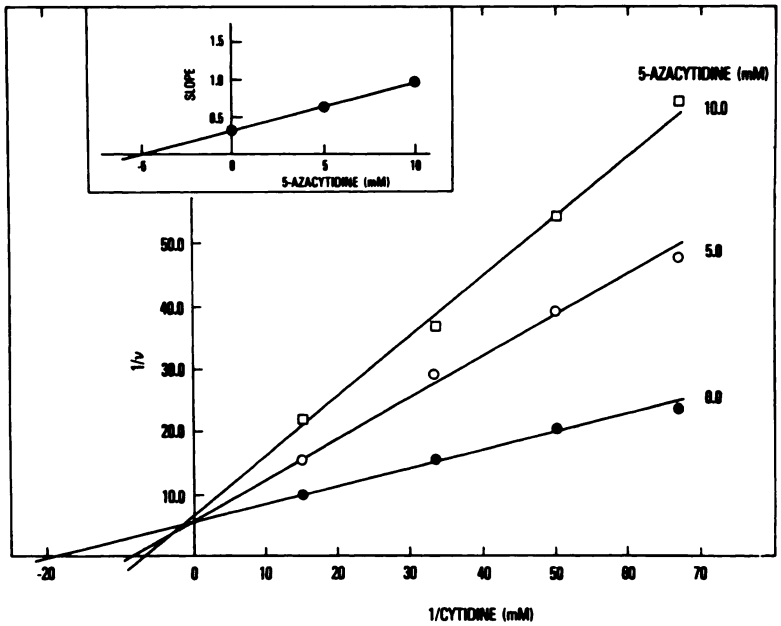

FIG. 5. *Inhibition of cytidine phosphorylation by 5-azacytidine; double reciprocal plot of 1/v against 1/cytidine concentration at fixed concentrations of 5-azacytidine*

Each reaction tube contained 0.15 μmol ATP, 0.40 μmol $MgCl_2$, 1.5 μmol imidazole-HCl buffer, pH 6.5, and various concentrations of [^{14}C]labeled cytidine and of cold 5-azacytidine, as indicated (Assay II). The total volume was 30 μl. The fixed concentrations of 5-azacytidine were 0, ●——●; 5.0 mM, ○——○; and 10.0 mM, □——□. The reaction was initiated by the addition of 1.71 μg enzyme protein (a different preparation from that used in the spectrophotometric studies), carried out at 25° for periods of up to 5 min, and stopped by adding an equal volume of absolute ethanol. Kinetic data obtained for cytidine are K_m = 0.05 mM and V_{max} = 0.183 μmol phosphorylated/min/mg enzyme protein. This V_{max} for cytidine, which is approximately one-fifth the value shown in Table 1, reflects the much lower catalytic activity of the enzyme preparation used for this one experiment. The insert shows a plot of slopes against 5-azacytidine concentrations.

tration; this is linear and intersects the abscissa to give a K_i of 5.0 mM for 5-azacytidine.

DISCUSSION

Dual-substrate kinetics have been used in the past to determine whether related compounds are metabolized by one enzyme (16–24). Feedback regulated enzymes may exhibit isoenzymic forms with different specificities for particular substrates (31, 32) or end-product inhibitors (33). Uridine-cytidine kinase is a feedback regulated enzyme (14) and isoenzymes specific for different substrates and their end-products were deemed a likely possibility, even though none were separable by the purification techniques used. There was evidence of protein heterogeneity on polyacrylamide gel electrophoresis, even of the most highly purified enzyme fraction, and some suggestion of heterogeneity of enzyme activity in gel filtration on Sephadex G-200. However, attempts to purify the enzyme to homogeneity were thwarted by the marked instability of the final preparation (12). In such a case, a dual-substrate kinetic study, which does not require a homogeneous enzyme protein, can provide a more direct answer to this question. If the two reactions examined in an experiment with combined substrates are catalyzed by distinct enzymes, even though each reaction is inhibited by the other substrate, the plot for combined velocity against concentration of the substrate with the higher V_{max} will show no common intersection (18). In other plots of v against [S], the curves will merge toward no common V_{max}. The data and the kinetic patterns resulting from the present

study thus indicate that phosphorylations of 5-azacytidine and of the natural substrates are not catalyzed by separate closely related isozymes, but rather by a single enzyme.

When alternative substrates compete for the same enzyme, the type of kinetic pattern observed depends primarily on the relationship between the two V_{max} values, and on the respective K_m's, according to equation 1 (18). When the V_{max} values for the two substrates differ significantly, as with uridine and 5-azacytidine, and combined velocity is plotted against the concentration of the high V_{max} substrate at several fixed concentrations of the low V_{max} substrate, the theory predicts a family of curves with a common intersection point where the low V_{max} substrate appears to have no effect on the velocity. The coordinates at the intersection point are $v = V_{max,\,B}$ and $[S] = V_BK_A/(V_A - V_B)$, where A is the high V_{max} substrate and B the substrate with the lower V_{max} (18–24). This is the pattern seen in Fig. 1, in good agreement with the theoretical prediction; the coordinates for the intersection point also agree with the theory, i.e., v = 1.11 μmoles phosphorylated/min/mg enzyme protein (theory 1.08 μmoles) and uridine concentration = 0.21 mM (theory 0.18 mM). When combined velocity is plotted against the concentration of the low V_{max} substrate, at the different fixed concentrations of the higher V_{max} substrate, the curves do not intersect, but instead merge toward the V_{max} value for substrate B. This is the pattern evident in Fig. 2, again in agreement with the theoretical pattern. If the difference between V_{max} values for the alternate substrates decreases until $V_A = V_B$, the pattern seen is a combined velocity which is always greater than the rate with either substrate alone, and the curves in the plot of v against $[S]$ merge toward the common V_{max} value. This is the pattern evident in Figs. 3 and 4. Interaction at the same site is further confirmed by the studies with [^{14}C]cytidine and unlabeled azacytidine (Fig. 5), in which the analogue can be seen to inhibit competitively the phosphorylation of cytidine.

The curves in Figs. 1–4, constructed visually and extrapolated to the V_{max} values, were compared to curves drawn with the MLAB computer program (see *Data processing*). The kinetic constants in Table 1 show that the values derived by MLAB agreed well with those determined from the linear plot. This strengthens the validity of the methodology and of the kinetic constants.

Our previous characterization of the purified uridine-cytidine kinase indicated a sequential reaction mechanism, based on the initial velocity pattern; kinetic parameters for uridine and cytidine were also accurately estimated by extrapolation to saturating levels of both nucleoside and ATP (27). The data in Table 1 are based, instead, on single experiments, but the agreement with previously determined values is good. It is interesting to note that the apparent K_m for 5-azacytidine is much higher than those for either of the natural substrates; a high K_m for 5-azacytidine was also reported for crude uridine-cytidine kinase from human lymphoblastic leukemia cells (9). This might have chemotherapeutic implications if phosphorylated derivatives are the active chemotherapeutic agent(s); this possibility has been suggested along with a possible role of catabolic products (8, 9). Despite the high K_m (one hundred times that for cytidine, Table 1), 5-azacytidine at sufficiently high concentrations effectively inhibited cytidine phosphorylation, as indicated in Fig. 5 (50% inhibition at a ratio of azacytidine : cytidine of approximately 250 : 1). The K_i for 5-azacytidine determined in this experiment (5.0 mM) agrees well, in fact, with the values determined for K_m (Table 1).

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