Enhanced Uridine Kinase in Rat Liver following 5-Azacytidine Administration

IN VIVO CHARACTERIZATION AND PROPERTIES OF PARTIALLY PURIFIED ENZYME

(Received for publication, July 5, 1972).

Alois Čihák and Jiří Veselý
From the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague 6, Czechoslovakia

SUMMARY

The activity of uridine kinase in rat liver is markedly enhanced following the intraperitoneal administration of 5-azacytidine by a process independent of adrenal secretion. The enhancement of enzyme activity is dose-dependent and is maximal at 24 to 28 hours following the administration of the analogue. The increase of uridine kinase in the liver is unaffected by a number of compounds interfering with DNA, RNA, and protein synthesis. Only cytidine and uridine administered in excess simultaneously with 5-azacytidine reverse the effect of the drug. Multiple doses of 5-azacytidine increase the activity of uridine kinase 4- to 5-fold followed by its subsequent decrease. The renewed enhancement of the enzyme activity is possible by further administrations of the drug. From the effect of actinomycin D, the calculated half-time of template RNA for liver uridine kinase is about 24 hours. Partially purified uridine kinase from the liver of 5-azacytidine-treated rats differs from the control enzyme preparation by its higher stability toward heating.

Uracil was utilized for nucleic acid biosynthesis to a greater extent by a chemically induced liver tumor than by normal liver. Thus, uridine kinase plays an important role in the synthesis of nucleic acid precursors by the salvage pathway (2). The enzyme is active in different rapidly proliferating tissues. The enzyme content is generally considered to reflect the relative efficiency of the system to utilize preformed pyrimidine precursors by the salvage pathway. Rutman et al. (6) reported that uracil was utilized for nucleic acid biosynthesis to a greater extent than was orotic acid. Hence, uridine kinase plays an important role in the salvage pathway of nucleic acid precursors.

In spite of its metabolic importance as the first enzyme in the salvage pathway, there are only few studies on the increase of uridine kinase activity following the administration of biologically active compounds (9-11). More attention was devoted to the effect and mechanism of action of possible feedback inhibitors (12-16) and especially to the deletion of uridine kinase during the development of resistance of various tumor cells toward different pyrimidine analogues (17-19).

5-Azacytidine is a potent cancerostatic agent (10) which affects the activity of different liver enzymes (20-22). In regenerating liver, 5-azacytidine administration results in the prolongation of the lag phase preceding the enhancement of thymidine and thymidylate kinase activity with the subsequent stimulation of RNA and DNA synthesis. The increased incorporation of orotic acid and of uridine into RNA was associated paradoxically with the diminished activity of orotidine 5'-phosphate decarboxylase (24); however, the activity of uridine kinase was increased (25). We have shown that liver regeneration is not a requisite for the increased activity of uridine kinase following 5-azacytidine administration. The present report characterizes the stimulatory effect of 5-azacytidine on uridine kinase in vivo and describes some properties of the partially purified enzyme preparation from 5-azacytidine-treated rat livers.

EXPERIMENTAL PROCEDURE

Methods

Preparation of Cell-free Extracts—Groups of Wistar male rats, including 4 to 10 animals kept under standard conditions, were used throughout the experiments. The animals were injected intraperitoneally at 8 to 9 A.M., while the controls received the same volume of 0.9% NaCl solution. Adrenalectomized animals were kept 3 days before starting the experiments and all animals were killed by cervical dislocation. Excised livers and thymuses were homogenized under cooling in a glass homogenizer with a tight-fitting Teflon pestle in 3 or 5 volumes (liver or thymus, respectively) of cold 0.25 M Tris, pH 7.5, containing 25 mM KCl and 5 mM Mg²⁺ ions. Homogenates were centrifuged (Spinco, 10,000 rpm, 20 min, 3°) and the defatted supernatants were used immediately for the estimation of enzyme activity.

Enzymes of Uridine Metabolism—The activity of uridine kinase, uridine monophosphate kinase, uridine 5'-nucleotidase, and uridine 5'-triphosphatase were assayed during a 5- to 10-min incubation period at 37° in 50 mM Tris, pH 7.5, in a total volume of 0.3 to 0.5 ml in the presence of 0.05 to 0.1 ml of the liver cell-
free extract. The reactions were linear over a 10-min period of incubation and at the amount of liver extract added (25). The assay system for uridine kinase contained 0.1 mM 6-aza-
[4,5-14C]uridine as substrate (8) and 4 mM adenosine 5'-triphosphate with equimolar Mg2+ ions. Uridine monophosphate kinase and the corresponding 5'-nucleotidase were assayed in a reaction mixture containing 0.1 mM [2-14C]uridine 5'-monophosphate and 1 mM Mg2+ ions in the presence or absence of 2 mM adenosine 5'-triphosphate. Uridine 5'-triphosphatase was followed in 50 mM potassium phosphate, pH 7.8, with 0.1 mM [2-14C]uridine 5'-triphosphate during a 5-min incubation period. Analysis of the reaction mixture was carried out by descending chromatography on Whiattman paper No. 1 in a solvent system composed of isobutyric acid-ammonium hydroxide-water (66:1.5:33) or in 1-butanol-acetic acid-water (10:1:3). The radioactive zones were cut out using standards and their radioactivity was measured. Unless otherwise stated, the activity of enzymes is expressed as micromoles of reacted substrate in cell-free extract corresponding to 1 g of tissue during 1 hour of incubation.

**Purification of Uridine Kinase**—Uridine kinase was purified by a modified method of Orenge (15). The liver supernatant fraction in 0.01 M Tris, pH 7.8, with 0.25 M sucrose obtained by the centrifugation of a liver homogenate (1 g of liver in 4 volumes of homogenization solution) in a Spinco ultracentrifuge (20,000 rpm, 3°, 20 min) was spun down at 40,000 rpm for 90 min (3°). The resulting supernatant, adjusted with 0.01 M Tris, pH 7.5, containing 0.1 mM dithiothreitol and the concentration of proteins adjusted to 3 to 4 mg of protein per ml (determined according to Lowry et al. (26)) yielded Fraction I. To 100 ml of Fraction I, 20 ml of 5% streptomycin sulfate were added, the suspension was stored overnight at 3° and centrifuged for 20 min at 20,000 rpm. The resulting supernatant (Fraction II) was brought to 27° saturation with ammonium sulfate (1 g of ammonium sulfate added at a rate of 1 g per min with continuous stirring). The suspension was stored for 1 hour (4°) and centrifuged as above. The sediment was discarded and the supernatant brought to 35° saturation with ammonium sulfate and stored for an additional hour. After centrifugation (20,000 rpm, 1 hour, 4°), the sediment was dissolved in 0.01 M Tris, pH 7.5, with 0.1 mM dithiothreitol and the concentration of proteins adjusted to 3 to 4 mg per ml (Fraction III). A portion of this fraction (20 to 30 mg of proteins) was layered on a Sephadex G-200 column (3 x 40 cm) and equilibrated with 0.01 M Tris, pH 7.5, containing 0.1 mM dithiothreitol. The elution of uridine kinase was carried out at 4° with the same buffer (flow rate 10 to 15 ml per hour; fractions of 2 ml). The optical density was recorded at 280 nm and the enzyme activity of individual fractions was assayed during a 10-min incubation period as described. The fractions with maximal enzyme activity were collected and yielded Fraction IV. The samples were maintained at -20° for at least 10 days without any appreciable loss of activity.

**Liquid Scintillation Counting**—Measurements of individual radioactive spots on paper chromatograms were obtained by out-elution in a Packard Tri-Carb liquid scintillation spectrometer after the addition of 10 ml of scintillation fluid (4 g of 2,5-diphenyloxazole, 0.15 g of p-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene).

**Materials**

Uridine 5'-monophosphate and 5'-triphosphate, adenosine 5'-triphosphate, dithiothreitol, hydroxyurea, arabinoside cytosine and cycloheximide were obtained from Calbiochem, Los Angeles. Actinomycin D was purchased from Merek Sharp and Dohme, West Point, and puromycin from Sigma, St. Louis. 5-Azacytidine, 5-fluorouridine, 6-azauridine, and 6-azauridine 5'-monophosphate were prepared at this Institute. Streptomycin was a product of Jena, Sephadex G-200 of Pharmacia, Uppsala, and ammonium sulfate was a product of Lachema, Brno (three times recrystallized). [6-14C]Orotic acid (48 Ci per pmole), 6-aza[4,5-14C]uridine (80 Ci per pmole), [2-14C]-uridine 5'-triphosphate and 5'-monophosphate (44 Ci per pmole) were delivered by the Institute for Research, Production and Uses of Radioisotopes, Prague.

**RESULTS**

**Uridine Kinase Enhancement in Liver of Rats following 5-Azacytidine Administration**—The injection of 5-azacytidine to adult rats results in the increase of liver uridine kinase (Fig. 1, A and B). The enhancement of enzyme activity is dose-dependent and repeated administration of the drug leads to further increases. At the same time it is evident that while the stimulation following 5-azacytidine treatment occurs in the liver, uridine kinase activity in the thymus is diminished. It is apparent that the activity of liver uridine kinase after a single dose of 5-azacytidine steadily rises reaching the maximum at about 24 hours following the administration of the drug. 5-Aza-2'-deoxyctydine (19) though chemically similar to 5-azacytidine does not change the activity of the enzyme.

The activity of uridine kinase has been assayed in all cases with 6-azauridine as phosphate acceptor with the exception of the experiment reported in Fig. 2 where uridine was employed as a substrate. The enhanced activity of uridine kinase in unpurified cell free liver extract is reflected by the progressively increasing formation of uridine 5'-triphosphate (25). At the same time, it is apparent that the application of 6-azauridine, as substrate of uridine kinase, is more convenient since the analogue is not degraded by the phosphorylase present in unpurified liver extracts. In addition, the 6-azauridine 5'-monophosphate formed is not further phosphorylated to higher 5'-phosphates (S).

The data given in Table I exclude the possibility that the effect of 5-azacytidine on uridine kinase is mediated through the
The animals were killed 24 hours after the last injection and uridine phosphorylation was assayed in their cell-free liver extracts as described under "Experimental Procedure." The results in brackets denote the level of uracil (U), uridine 5'-monophosphate (M), and uridine 5'-triphosphate (T). The duration of the effect of 5-azacytidine on the increase of uridine kinase activity in the liver of rats is relatively stable process. When different substances known to inhibit DNA and RNA synthesis as well as the formation of proteins are given simultaneously with 5-azacytidine, the activity of enhanced enzyme is possible when the effect of repeated administration ceases. The newly obtained effect is somewhat lower than that obtained during the first administration of 5-azacytidine and cytidine. Simultaneous administration of 5-azacytidine and cytidine did not result in an increase of uridine kinase activity. The enhancement of hepatic uridine kinase is a relatively stable process. When different substances known to inhibit DNA and RNA synthesis as well as the formation of proteins are given simultaneously with 5-azacytidine, the activity of enhanced uridine kinase is not depressed (Table III). The only exception is actinomycin D. Of the compounds administered alone only 5-Azacytidine + cytidine. Groups of five male rats (175 g) received 5-azacytidine (6 μmoles/100 g) at a zero time and then repeatedly at 24-hour intervals.
Stability of 5-azacytidine-mediated enhancement of liver uridine kinase

Compounds were administered intraperitoneally with 5-azacytidine or alone to groups of four to eight female rats 24 hours before killing. The activity of uridine kinase in cell-free liver extracts was assayed as described under “Experimental Procedure.” Control 1.08 ± 0.11 μmoles per g of liver per hour (−100%). The numbers in parentheses are percentages.

<table>
<thead>
<tr>
<th>Administered compounds</th>
<th>Dose level</th>
<th>With 5-Azacytidine</th>
<th>Without 5-Azacytidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td>μmoles/g liver/hr ± S.E.</td>
<td>μmoles/g liver/hr ± S.E.</td>
</tr>
<tr>
<td>5-Azacytidine</td>
<td>25</td>
<td>2.53 ± 0.24* (224)</td>
<td>0.86 ± 0.13 (115)</td>
</tr>
<tr>
<td>Plus actinomycin D</td>
<td>25 + 5.1</td>
<td>0.88 ± 0.13 (115)</td>
<td>0.88 ± 0.13 (115)</td>
</tr>
<tr>
<td>Plus arabinoside cytosine</td>
<td>25 + 202</td>
<td>1.15 ± 0.10 (243)</td>
<td>1.15 ± 0.10 (243)</td>
</tr>
<tr>
<td>Plus hydroxyurea</td>
<td>25 + 200</td>
<td>1.03 ± 0.16 (229)</td>
<td>1.03 ± 0.16 (229)</td>
</tr>
<tr>
<td>Plus 6-azauridine</td>
<td>25 + 100</td>
<td>1.05 ± 0.16 (225)</td>
<td>1.05 ± 0.16 (225)</td>
</tr>
<tr>
<td>Plus 5-fluorouridine</td>
<td>25 + 25</td>
<td>1.07 ± 0.10 (231)</td>
<td>1.07 ± 0.10 (231)</td>
</tr>
<tr>
<td>Plus puromycin</td>
<td>25 + 25</td>
<td>0.86 ± 0.09 (216)</td>
<td>0.86 ± 0.09 (216)</td>
</tr>
<tr>
<td>Plus cycloheximide</td>
<td>25 + 15.3</td>
<td>2.22 ± 0.16 (330)</td>
<td>2.22 ± 0.16 (330)</td>
</tr>
</tbody>
</table>

* p < 0.02.

p < 0.05.

Controls (100%) two sets of experimental groups of animals killed at the time of actinomycin D administration were used.

cycloheximide leads also to the enhancement of uridine kinase activity (27), though the mechanism of its action is completely different (28) from that of 5-azacytidine (29). The results obtained with actinomycin D administered in vivo at different time intervals following 5-azacytidine treatment and before killing are shown in Fig. 4, A and B. As control values the activity of uridine kinase at these time intervals was measured since the enzyme was still gradually rising. The calculated value of the half-time of the template RNA for liver uridine kinase is about 24 hours. The sensitivity toward actinomycin D of the enhanced uridine kinase did not differ from that of the control enzyme preparation.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>nmoles/mg</td>
<td>nmoles/mg</td>
</tr>
<tr>
<td>I. Cell-free liver extract</td>
<td>11.7</td>
<td>4,600</td>
<td>1.7</td>
</tr>
<tr>
<td>II. Streptomycin</td>
<td>10.3</td>
<td>4,500</td>
<td>2.9</td>
</tr>
<tr>
<td>III. Ammonium sulfate</td>
<td>3.3</td>
<td>2,120</td>
<td>22.6</td>
</tr>
<tr>
<td>IV. Sephade G-200</td>
<td>0.6</td>
<td>476</td>
<td>58.9</td>
</tr>
</tbody>
</table>

Characterization of Partially Purified Uridine Kinase from Liver of 5-Azacytidine-treated Rats—The effect of 5-azacytidine on liver uridine kinase seemed of sufficient significance to investigate the properties of the partially purified enzyme. Following the fractionation of crude liver extracts of control and 5-azacytidine-treated rats with streptomycin and ammonium sulfate uridine kinases were subjected to chromatography on a Sephadex G-200 column (15) resulting in a 34- to 30-fold increase of their specific activity (Table IV). The increase of the activity of both enzyme preparations during the isolation was almost the same. The activity of partially purified enzymes (Fraction IV) is illustrated in Fig. 5, A and B. The rate of 6-azauridine.
The enzyme activity was measured under the conditions of preincubation of enzyme preparations at 50°C using the rats was undertaken. As is apparent from Fig. 7, A and B, uridine kinases from the liver of control and 5-azacytidine-treated and resistant to 5-azacytidine (30), a similar investigation of uridine kinases isolated from mouse leukemic cells sensitive more resistant towards thermal inactivation than that of the enzyme was isolated from different sources (15, 16, 31) and it was pseudouridine kinase also appears to be a distinct enzyme molecule (37).

Kinetic constants for Second substrate
Uridine kinase activity was assayed as described in Fig. 7 and under “Experimental Procedure.”

<table>
<thead>
<tr>
<th>Kinetic constants for</th>
<th>Second substrate</th>
<th>Apparent K_m</th>
<th>Apparent V_max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Adenosine 5'-triphosphate</td>
<td>0.27</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>6-Azauridine</td>
<td>0.10</td>
<td>147</td>
</tr>
<tr>
<td>6-Azacytidine-treate</td>
<td>Adenosine 5'-triphosphate</td>
<td>0.27</td>
<td>125</td>
</tr>
<tr>
<td>d</td>
<td>6-Azauridine</td>
<td>0.10</td>
<td>330</td>
</tr>
</tbody>
</table>

Figure 6. Lineweaver-Burk plot for uridine kinase isolated from 5-azacytidine-treated rat liver with respect to phosphate donor and acceptor. The animals were given intraperitoneally 5-azacytidine as in Fig. 5. The enzyme activity was assayed during a 10-min incubation period at 37°C with 55 μg of Fraction IV from controls (○) and 5-azacytidine-treated liver (●). A, 6-azauridine, 0.1 mM; ATP expressed in nm, r in nanomoles of 6-azauridine 5'-phosphate formed per mg of protein during 10 min of incubation. B, adenosine 5'-triphosphate at the concentration 40 times higher than that of phosphate acceptor; 6-azauridine (6-AzUR) expressed in millimoles concentration, r in nanomoles of 6-azauridine 5'-phosphate formed.

Figure 7. Different thermostability of partially purified uridine kinases from the liver of control (○) and 5-azacytidine-treated (●) rats. Enzyme preparations (Fraction IV) were prepared as described in Fig. 5. Activity of uridine kinase was assayed as described under “Experimental Procedure” with 55 μg of purified enzyme. A, preincubation of the enzyme systems in 10 mM Tris, pH 7.5, at 50°C during different time intervals (time, min); the subsequent estimation of uridine kinase activity, expressed as nanomoles of phosphorylated 6-azauridine per mg of protein during 10 min of incubation, was carried out at 37°C. B, preincubation of enzyme in 10 mM Tris, pH 7.5, for 10 min at different temperatures (°C); the residual activity, expressed as percentage of untreated controls, was measured during 10 min of incubation at 37°C.

There is a correlation between the effect of 5-azacytidine on uridine kinase activity in rat liver and its effect on the enzyme activity as was observed during the development of resistance towards this drug. Even though in resistant tumor cells the activity of uridine kinase is decreased (19) and in 5-azacytidine-treated rat liver the activity of the enzyme increases (Fig. 3), the modified enzyme preparations are more stable toward heat inactivation in both cases.

**DISCUSSION**

Reichard and Sköld (3) provided evidence that uridine kinase was the limiting enzyme reaction influencing the synthesis of uridine 5'-monophosphate in the liver. The comparison of uridine kinase in different tissues revealed a correlation between the enzyme activity and the growth rate (4). The enzyme has been partially purified for the first time from Ehrlich ascites tumor cells (1). Out of 19 tested nucleosides as phosphate acceptors, uridine kinase reacted with uridine, cytidine, 6-azauridine, 5-fluorouridine, and 5-fluorocytidine but not with deoxyuridine, thymidine, and deoxycytidine. Later the enzyme was isolated from different sources (15, 16, 31) and it was clearly established that it differs from thymidine (32-34) and deoxycytidine kinase (35, 36). In extracts from pyrimidine auxotrophs of *Escherichia coli* it has been shown recently that pseudouridine kinase also appears to be a distinct enzyme molecule (37).

Uridine kinase belongs to a group of enzymes which are known to be under the influence of feedback inhibition (12-16). The enzyme isolated from mouse mast-cell neoplasm PS15 was subjected to feedback inhibition by uridine 5'-triphosphate and

Downloaded from http://www.jbc.org/ by guest on September 22, 2017
even more by cytidine 5'-triphosphate (12). Similar results have been obtained using uridine kinase from Novikoff ascites rat tumor (15) and from the embryos of Paracentrotus lividus (18) where feedback inhibition could be removed by guanosine 5'-triphosphate and deoxyguanosine 5'-triphosphate. On the basis of these studies it has been proposed that there exist three binding sites on the surface of the enzyme molecule; one for the phosphate donor, one for the phosphate acceptor, and the third one for the regulatory ligand (15). The molecular weight of the enzyme from Tetrahymena pyriformis has been estimated as 195,000 by gel filtration (10). In normal and neoplastic rat livers two species of uridine kinase with molecular weight of approximately 130,000 and 30,000 have been recently identified (31). The first one predominates in adult rat liver, whereas the second one is prevalent in early embryonal liver and occurs also in different tumor tissues.

The data reported in the present study suggest that the enhancement of uridine kinase following 5-azacytidine administration is tissue-specific; in the thymus the drug acts in the opposite direction (Fig. 1). None of the different analogues of nucleic acid components and drugs tested (Table III), with the exception of cycloheximide (27), was able to enhance the activity of liver uridine kinase, although drugs with different modes of biological action attacking different metabolic loci were applied. Since the administration of cytidine reverses the enhancing effect of 5-azacytidine on uridine kinase activity (Table I, and Fig. 3), it is likely that the phosphorylation and incorporation of the drug (38, 39) is required for this action.

It is noteworthy that tyrosine aminotransferase activity is also increased in the liver both by 5-azacytidine (22, 23) and cycloheximide (40-42), in addition to many other compounds (43-45). This enhancing effect has been explained by the inhibition of the enzyme degradation (20, 41, 44, 45). However, uridine kinase is not increased by many other compounds known to inhibit the degradation of liver enzymes (8-azaaguanine (44), actinomycin D (45), 5-fluoroorotic acid (23), L-tryptophan (46, 47)). Since cycloheximide, a well known inhibitor of protein synthesis (48, 49), equally increases the activity of uridine kinase in rat liver (27), it is permissible to suppose that there is no increase of de novo enzyme synthesis. Consequently it would seem that 5-azacytidine affects the enzyme degradation as was shown in the case of tyrosine aminotransferase (20, 22, 23). The mechanism of degradation of uridine kinase and its control following 5-azacytidine are apparently different from those operating in the case of the above mentioned substances (23, 43, 44, 46), which do not influence the activity of liver uridine kinase. Further evidence will be obtained after the direct measurement of the rate of uridine kinase synthesis and degradation following 5-azacytidine.

It is also possible that 5-azacytidine administration results in the formation of modified uridine kinase molecules as evidenced by their increased stability toward heating (Fig. 7, A and B). The existence of two different molecular forms of uridine kinase in rat liver and different hepatomas (31) would suggest that the administration of 5-azacytidine could lead to the changed relation of both species in favor of the more heat-stable low molecular isozone normally prevailing in embryonic rat liver.

The deletion of uridine kinase in tumor cells resistant to uridine and cytidine analogues is regarded as a requisite for the development of resistance (18, 19, 50). On the other hand, the enhanced activity of the enzyme would result in an efficient anabolic transformation of the analogues, and thus would increase their chemotherapeutic effect. The administration of 5-azacytidine in combination with selected anticancer agents could influence favorably the development of resistance to uridine and cytidine antimetabolites especially in hepatic neoplasms.

Acknowledgments—We should like to express our thanks to Miss Zdena Škachová, Mrs. Jana Müllerová, and Mrs. Dana Prášková for their outstanding technical assistance.

REFERENCES

Enhanced Uridine Kinase in Rat Liver following 5-Azacytidine Administration: IN VIVO CHARACTERIZATION AND PROPERTIES OF PARTIALLY PURIFIED ENZYME
Alois Cihák and Jirí Veselý


Access the most updated version of this article at http://www.jbc.org/content/248/4/1307

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/4/1307.full.html#ref-list-1