Pyrimidine Nucleoside Phosphorylases of Rat Liver

SEPARATION BY ION EXCHANGE CHROMATOGRAPHY AND STUDIES OF THE EFFECT OF CYTIDINE OR URIDINE ADMINISTRATION*

(Received for publication, November 17, 1967)

ESTHER W. YAMADA

From the Department of Biochemistry, University of Manitoba, Winnipeg, Canada

SUMMARY

By chromatography on diethylaminoethyl Sephadex, three enzyme fractions with pyrimidine nucleoside phosphorylase activity have been separated from extracts of normal or regenerating liver of rats. Two of these probably represent isoenzymes of uridine phosphorylase. Both are phosphate-dependent and both exhibit maximal activities toward uridine, deoxyuridine, and thymidine at pH values of 7.9 to 8.2, 6.4 to 6.8, and 5.6 to 5.8, respectively. One isoenzyme is of nuclear origin, whereas the other is predominantly of cytoplasmic origin. At the pH optimum of the respective substrates, as well as at physiological pH, both isoenzymes catalyze the phosphorylase of uridine at a more rapid rate than the phosphorylase of deoxyuridine and are much less active toward thymidine. The third enzyme, thymidine phosphorylase, is mainly of cytoplasmic origin. It is also phosphate-dependent; it is most active toward deoxyuridine at pH 5.8 and toward thymidine at pH 5.4, but shows no activity toward uridine or deoxycytidine. It has more deoxyuridine- than thymidine-cleaving activity over the pH range studied, but is probably the enzyme mainly responsible for catalyzing the phosphorylase of thymidine in liver cells.

Under the standard conditions of assay the specific activities of the nuclear and cytoplasmic uridine phosphorylases were found to be increased about 3-fold when assayed in the presence of uridine, deoxyuridine, or thymidine 6 hours after the injection of uridine. Six hours after the injection of cytidine the specific activities of both enzymes with each of the three substrates were increased about 2-fold. Concomitantly, the specific activity of thymidine phosphorylase when assayed with deoxyuridine or thymidine was not changed by uridine injection but was decreased after the injection of cytidine.

Earlier, evidence was presented for the view that the phosphorylase of uridine and deoxyuridine is catalyzed by separate enzymes in Ehrlich ascites tumor cells (1). Both enzymic activities are also found together in tissues of the rabbit (2) and in the rat liver (3) and were thought to be due to separate enzymes (1, 3). Further support for this view came from the finding that the two activities increase at different rates in regenerating liver of rats after the injection of cortisol, uridine, or cytidine (4–6). Subsequently, uridine phosphorylase and thymidine phosphorylase activities were separated by chromatography on DEAE-cellulose columns from extracts of mouse tissues (7). Much of the deoxyuridine-cleaving activity of these tissues was associated with these two enzyme fractions (7). A similar uridine phosphorylase was described for Ehrlich ascites tumor cells (7), dog tissues, and rat liver (8), but a second type of uridine-cleaving enzyme, with a lower pH optimum for uridine cleavage, was found in Escherichia coli (7, 8) and in tissues of the chicken, human, guinea pig, and frog (8).

The present report describes the separation of nuclear and cytoplasmic isoenzymes of uridine phosphorylase from rat liver. Both enzymes are nonspecific for the ribose moiety of the substrate for both show activity toward uridine, deoxyuridine, and thymidine. A third enzyme, also isolated from rat liver, is specific for the deoxyribose moiety of the substrate and catalyzes the phosphorylase of deoxyuridine and thymidine but does not act on uridine. This enzyme is designated, herein, thymidine phosphorylase.

Which of these enzymes is induced (or derepressed) in regenerating liver of rats after uridine or cytidine administration (5, 6, 9) was also investigated.

MATERIALS AND METHODS

Animals

Male Holtzman rats weighing 150 to 250 g were used. In a number of experiments the rats were partially hepatectomized, as described previously (5, 6), 48 hours prior to use. Where indicated in the text, groups of four rats were injected intraperitoneally with 1 to 2 ml of 0.9% NaCl or 1.5 mg of cytidine or uridine (Sigma) per g of body weight. The solutions were adjusted to pH 7.4 before injection. Six hours later the rats were decapitated. Their livers were perfused in situ with ice-cold 0.9% NaCl, removed, and placed in either ice-cold Buffer A

* This work was supported by the Medical Research Council of Canada and the National Cancer Institute of Canada.
Gauze (28 X 24 mesh) and then dialyzed for 16 hours in 100 volumes of Buffer A. The homogenates were filtered through four layers of cotton gauze and then dialyzed in 100 volumes of Buffer A. In the cold, in a continuous flow dialyzing apparatus (model B, Oxford Laboratories).

Preparation of Liver Homogenates

Livers were homogenized, in the cold, in 5 volumes of Buffer A in a Potter-Elvehjem apparatus equipped with a Teflon pestle. The homogenates were filtered through four layers of cotton gauze (28 X 24 mesh) and then dialyzed for 16 hours in 100 volumes of Buffer A. The homogenates were then dialyzed in Buffer A as described for whole liver.

Procedure 1—This was used to assay the activity of enzyme fractions suspended in Buffer B. It differs from Procedure 1 only in the incubation medium which contained the following (in micromoles) in a final volume of 1.5 ml: potassium phosphate buffer at pH 6.85; 30; potassium phosphate buffer at pH 7.4, 170; β-mercaptoethanol, 7.5; enzyme diluted to 1.2 ml with 0.02 M potassium phosphate buffer at pH 8.0; substrate at pH 7.0, 5. The final pH of this incubation medium was the same as that of Procedure 1.

The dialyzed homogenates of whole liver or nuclei and the dialyzed cytoplasmic fractions were centrifuged at 151,000 x g for 10 min at 2°C in a Sorvall RC-2 refrigerated centrifuge; the sediment was rehomogenized in 1 times its weight of sucrose solution and centrifuged again as above. This step was repeated once. The supernatants were combined to form the "cytoplasmic" fraction. The nuclear pellet was suspended with homogenization in 3 times its weight of Buffer A. Both nuclear and cytoplasmic fractions were then dialyzed in Buffer A as described for whole liver.

Enzyme Preparations

The dialyzed homogenates of whole liver or nuclei and the dialyzed cytoplasmic fractions were centrifuged at 151,000 x g for 1½ hours in a Spinco model L preparative ultracentrifuge at 4°C. The supernatants (SD 151) were decanted and dialyzed in 100 volumes of Buffer B (0.02 M potassium phosphate buffer (pH 8.0)-10 mM β-mercaptoethanol-1 mM EDTA, pH 8.0) or stored at -20°C until further use. At this temperature there was no appreciable loss of enzyme activity after 1 week. The dialyzed supernatants (SD 151) were analyzed immediately on ion exchange columns.

Enzyme Assays

Enzyme activity was assayed spectrophotometrically by measuring the increase in absorption resulting from the conversion of nucleoside to free base. Two methods were used.

Procedure 1—This is a modification of a method described previously (4, 6). Unless otherwise specified, the incubation medium contained the following (in micromoles) in a final volume of 1.5 ml: potassium phosphate buffer at pH 7.4, 150; β-mercaptoethanol, 7.5; enzyme diluted to 0.5 ml with 0.05 M potassium phosphate buffer at pH 7.0; uridine, deoxyuridine, or thymidine at pH 7.0, 5. The final pH of the reaction mixture was 7.34. The reaction was started by the addition of substrate and the tubes were incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.45 ml of 2.12 X HClO4 in an ice-bath. The controls were incubated without substrate, which was added just prior to the addition of HClO4. After at least 5 min in an ice bath, the tubes were centrifuged at 14,800 X g for 10 min at 2°C in a Sorvall RC-2 refrigerated centrifuge. From each tube, 1 ml of supernatant was removed and mixed with 0.07 ml of 9.6 M NaOH. All assays were done in duplicate and in all assays the test solutions were read against incubated controls. For every micromole of uracil formed from uridine the increase in absorption at 290 μμ was 4.40 and from deoxyuridine at 290 μμ was 4.10. For every micromole of thymine formed from thymidine the increase in absorption at 295 μμ was 4.30.

Procedure 2—This was used to assay the activity of enzyme fractions suspended in Buffer B. It differs from Procedure 1 only in the incubation medium which contained the following (in micromoles) in a final volume of 1.5 ml: potassium phosphate buffer at pH 6.85, 30; potassium phosphate buffer at pH 7.4, 170; β-mercaptoethanol, 7.5; enzyme diluted to 1.2 ml with 0.02 M potassium phosphate buffer at pH 8.0; substrate at pH 7.0, 5. The final pH of this incubation medium was the same as that of Procedure 1.

Under the standard conditions of both assays, the formation of free base was a linear function of time for 20 min and of protein concentration up to an optical density reading of 0.390. One unit of enzyme activity is defined as that quantity that catalyzes the formation of 1.0 μmole of free base per hour. Specific activity is defined as the number of units per mg of protein. Protein was measured by the method of Lowry et al. (10).

DEAE-Sephadex Chromatography

DEAE-Sephadex A-50 (Pharmacia) was allowed to swell in 0.02 M potassium phosphate buffer (pH 8.0) containing 1 mm EDTA (pH 8.0) in a cold room. Over a period of 24 hours the supernatant liquid and fines were decanted and replaced by fresh buffer, two to three times. The gel slurry was then poured into a glass column of 18-cm diameter to a height of 22 cm (bed volume, 56 ml). A layer of washed Sephadex G-25 (coarse) was placed on top of the DEAE-Sephadex to protect the gel surface. The column was washed with 1½ bed volumes of Buffer B to reduce the optical density of the eluents to zero at 260 and 280 μμ. The enzyme preparation (SD 151), followed by about 100 ml of Buffer B, was added to the column. Six-milliliter fractions were collected in a cold room at 2°C at a flow rate of 0.45 ml per min and washing with Buffer B was continued until the optical density of the eluents was reduced to 260 and 280 μμ. The enzyme preparation (SD 151), followed by about 144 to 150 ml of effluent had been collected, a linear gradient of KCl was started by allowing 0.5 M KCl, dissolved in 300 ml of Buffer B, to flow into a mixing chamber containing 300 ml of Buffer B and thence into the column. The protein concentration of each fraction was determined by the method of Warburg and Christian (11) from optical density readings at 260 and 280 μμ.

RESULTS

Separation of Enzymes on DEAE-Sephadex

Liver homogenates of four rats, killed 48 hours after partial hepatectomy, were pooled. SD 151 supernatants were prepared and applied to DEAE-Sephadex columns. The separation of enzyme activity into three distinct nucleoside-cleaving fractions is shown in Fig. 1. Enzyme Fractions 1 and 2 have similar substrate specificities. Under the conditions of the standard assay both catalyzed the phosphorylation of uridine at a faster rate than the phosphorylation of deoxyuridine. Both showed low uridine-cleaving activity which was reproducible from column to column. In their substrate specificity both resemble the uridine phosphorylases described for Ehrlich ascites tumor cells (7) and mouse tissues (8). Enzyme Fraction 3 appears to be specific for the deoxyribose moiety. It was more active with
deoxyuridine than thymidine, under the conditions of the standard assay, and showed no appreciable activity with uridine.

SD 151 supernatants, prepared from nuclear and cytoplasmic fractions of regenerating liver of rats, were fractionated on DEAE-Sephadex (Figs. 2 and 3). Enzyme 1 was not found in the cytoplasmic fraction and only Enzymes 2 and 3 were present (Fig. 2). In contrast, Enzyme 1, as well as significant amounts of Enzymes 2 and 3, was present in the nuclear fraction (Fig. 3). The presence of the latter two enzymes may be due, to some extent, to contamination of the nuclei by cytoplasm.

In other experiments SD 151 supernatants of four livers from normal rats were found to contain three separate enzyme activities which, by their positions in the chromatogram (Fig. 4), resemble those of regenerating liver. Less Enzyme 1 was found in normal than in regenerating liver.
Fig. 5. Rate of phosphorolysis of uridine (--), deoxyuridine (---), and thymidine (----) by Enzyme 1 of regenerating liver as a function of pH. In all assays phosphate buffer (●) was present in the reaction mixture in the concentrations outlined for Procedure 1 of the standard assay. In some assays acetate buffer (□) or glycylglycine buffer (△) was also included.

Fig. 6. Rate of phosphorolysis of uridine (--), deoxyuridine (---), and thymidine (----) by Enzyme 2 of regenerating liver as a function of pH. In all assays phosphate buffer (●) was present in the reaction mixture in the concentrations outlined for Procedure 1 of the standard assay. In some assays acetate buffer (□) or glycylglycine buffer (△) was also present.

Properties of Pyrimidine Nucleoside Phosphorylases

Substrates and pH—Effluents from each enzyme peak, within the limits indicated on the chromatograms of Figs. 1, 2, and 4, were pooled and concentrated by the addition of solid ammonium sulfate to 70% saturation. The precipitates were dissolved in 0.05 M potassium phosphate buffer at pH 7.0 containing 5 mM β-mercaptoethanol and could be stored frozen at -20° for several weeks with negligible loss of activity.

The pH optima of enzyme Fractions 1 and 2 (Figs. 5 and 6) for uridine and deoxyuridine (7.9 to 8.2 and 6.4 to 6.8, respectively) were similar to those reported for uridine phosphorylase of Ehrlich ascites tumor cells (1, 7) and rat, mouse, and dog tissues (8). At the pH optimum of each substrate, ratios of 1.2 (uridine to deoxyuridine) for Enzyme 1 and of 1.4 for Enzyme 2 were obtained. The pH optima of Enzymes 1 and 2 for thymidine cleavage (5.6 to 5.8) were lower than that reported for uridine phosphorylase of Ehrlich ascites tumor cells (7). Over the pH range studied Enzymes 1 and 2 showed much less activity toward thymidine than toward the other two substrates.

The pH optimum of Enzyme 3 for thymidine cleavage was 5.4

Fig. 7. Rate of phosphorolysis of deoxyuridine (---) and thymidine (----) by Enzyme 3 of regenerating liver as a function of pH. In all assays phosphate buffer (●) was present in the reaction mixture in the concentrations outlined for Procedure 1 of the standard assay. Where indicated acetate buffer (□) or glycylglycine buffer (△) was also included.

TABLE I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity (increase in absorbance/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uridine</td>
</tr>
<tr>
<td>Enzyme 1</td>
<td></td>
</tr>
<tr>
<td>Medium A</td>
<td>Complete</td>
</tr>
<tr>
<td></td>
<td>Minus phosphate plus glycylglycine. 0.024</td>
</tr>
<tr>
<td>Medium B</td>
<td>Complete</td>
</tr>
<tr>
<td></td>
<td>Minus phosphate plus glycylglycine. 0</td>
</tr>
<tr>
<td>Enzyme 2</td>
<td></td>
</tr>
<tr>
<td>Medium A</td>
<td>Complete</td>
</tr>
<tr>
<td></td>
<td>Minus phosphate plus glycylglycine. 0.011</td>
</tr>
<tr>
<td>Medium B</td>
<td>Complete</td>
</tr>
<tr>
<td></td>
<td>Minus phosphate plus glycylglycine. -0.002</td>
</tr>
<tr>
<td>Enzyme 3</td>
<td></td>
</tr>
<tr>
<td>Medium A</td>
<td>Complete</td>
</tr>
<tr>
<td></td>
<td>Minus phosphate plus glycylglycine. 0</td>
</tr>
<tr>
<td>Medium B</td>
<td>Complete</td>
</tr>
<tr>
<td></td>
<td>Minus phosphate plus glycylglycine. 0</td>
</tr>
<tr>
<td></td>
<td>Minus phosphate plus arsenate. 0</td>
</tr>
</tbody>
</table>
TABLE II
Pyrimidine nucleoside phosphorylase activity of regenerating rat liver fractions

Enzymic activities of the homogenates were assayed by Procedure 1. Procedure 2 was used to assay the enzymic activities of the SD 151 supernatants. The numbers of rats used per sample are given in parentheses.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Specific activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uridine</td>
<td>Deoxyuridine</td>
</tr>
<tr>
<td>1. 0.9% NaCl-injected (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.101</td>
<td>0.149</td>
</tr>
<tr>
<td>SD 151</td>
<td>0.232</td>
<td>0.330</td>
</tr>
<tr>
<td>Recovery of total activity (%)</td>
<td>86.1</td>
<td>81.0</td>
</tr>
<tr>
<td>2. Uridine-injected (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.204</td>
<td>0.246</td>
</tr>
<tr>
<td>SD 151</td>
<td>0.528</td>
<td>0.541</td>
</tr>
<tr>
<td>Recovery of total activity (%)</td>
<td>82.3</td>
<td>82.0</td>
</tr>
<tr>
<td>3. Cytidine-injected (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.191</td>
<td>0.156</td>
</tr>
<tr>
<td>SD 151</td>
<td>0.341</td>
<td>0.294</td>
</tr>
<tr>
<td>Recovery of total activity (%)</td>
<td>81.5</td>
<td>89.7</td>
</tr>
</tbody>
</table>

(Fig. 7), which agrees with the finding of others for thymidine phosphorylase of rat liver (12). The pH optimum of Enzyme 3 for deoxyuridine cleavage was 5.8. At the pH optimum for each substrate deoxyuridine was cleaved at 2.2 times the rate of thymidine. This preference for deoxyuridine is also apparent at pH 7.34, the pH of the standard assay medium, at which the ratio (deoxyuridine to thymidine) was 1.4.

Similar results were obtained with enzyme fractions from normal and regenerating liver. Under the standard conditions of assay the three enzymes showed no activity toward cytidine or deoxycytidine.

Phosphate Dependence—The concentrated enzyme Fractions 1, 2, and 3, described above, were dialyzed for 2 hours in 100 volumes of 0.05 M glycylglycine buffer at pH 7.0, 5 mM β-mercaptoethanol, and 1 mM EDTA (pH 7.0), in a cold room at 2°. The activities of the fractions were assayed in the presence and absence of inorganic phosphate. Results are given in Table I. When phosphate was omitted from the reaction mixture, the activities of Enzymes 1, 2, and 3 were negligible. Arsenate almost completely replaced phosphate in the reaction medium of Enzyme 3. These findings are in agreement with those reported earlier (4) in which the phosphate dependence of pyrimidine nucleoside phosphorylase activity was studied in homogenates of regenerating liver.

Effect of Uridine and Cytidine Administration

Groups of rats were injected with 0.9% NaCl, uridine, or cytidine 48 hours after partial hepatectomy. The rats were killed 6 hours later, the time at which maximal increases in the uridine-cleaving activity of whole liver occur(1). The livers were homogenized and dialyzed and their pyrimidine nucleoside phosphorylase activity was determined. The dialyzed homogenates from each group of rats were then pooled; the specific activities of the pooled homogenates are given in Table II. In agreement with previous findings(5, 6), the specific activity of liver homogenates, when determined with each of the three substrates, is increased after uridine administration. After cytidine injection, the uridine-cleaving activity is also increased but the deoxyuridine- and deoxythymidine-cleaving activities are affected but little. Over 80% of the activities of the homogenates
Enzyme 2, although Enzyme 1 contributes to the phosphorolysis of both uridine and deoxynucleosides. Additional phosphorolysis of deoxyuridine is catalyzed in almost equal proportions by Enzymes 2 and 3 in livers of 0.9% NaCl-injected rats but this proportion varies after the injection of the nucleosides. The phosphorolysis of thymidine is catalyzed mainly by Enzyme 3.

The activities of the effluents from each of the enzyme peaks were increased after uridine injection, whereas the activity of enzyme Fraction 3 is not apparent from a comparison of Figs. 1 and 8. A comparison of Figs. 1 and 9 shows that the activities of Enzymes 1 and 2 are also increased after cytidine injection but the activity of Enzyme 3 is decreased. These differences are not due to variations in the recovery of enzyme activity from the three columns, since the recoveries are all of the same order, nor are these changes in activity apparent when actinomycin D, chloramphenicol, or puromycin is injected 1 hour prior to the injection of uridine. However, since Enzyme 2 has been purified more than 300-fold from extracts of rat liver cytoplasm, the conclusion that Enzyme 2 is a uridine phosphorolysis of the high pH optimum type. In addition, Enzyme 2 retains its thymidine-cleaving activity throughout the purification.

Similar studies are planned for Enzyme 1.

Rat liver also contains a third enzyme which is more active in catalyzing the phosphorolysis of deoxyuridine than thymidine, both at the pH optimum of each substrate as well as at physiological pH. It shows no activity toward uridine. In these respects, it differs considerably from the thymidine phosphorolysis described for mouse liver (7), which shows a preference for thymidine over deoxyuridine, when its activity is assayed at pH 7.4, and has some activity toward uridine. It resembles thymidine phosphorolysis of horse liver (14) and pyrimidine deoxyribosyltransferase of E. coli (15) in its specificity for the deoxyribose moiety. However, the latter two enzymes were not tested for deoxycytidine-cleaving activity, although in the direction of synthesis of pyrimidine deoxyribonucleosides both were found to be active with uracil as with thymine (at pH 7.4) (14, 15). Since the enzyme of E. coli does not act on deoxycytidine, it was later designated thymidine phosphorolysase (16). Enzyme 3 of rat liver has not been tested for deoxyribosyltransfer activity (12, 17) or sensitivity to deoxycytidine (18), so it is not possible, at this time, to correlate its properties with the properties of the thymidine phosphorolysases isolated by Zimmerman (17, 18) and Zimmerman and Seidling (19). There are suggestions, however, that the latter preparations are not free from uridine phosphorolysis activity (7, 8). Thus, since Enzyme 3 appears to be the enzyme mainly responsible for the thymidine-cleaving activity of rat liver, whereas both isoenzymes of uridine phosphorolysis as well as this enzyme contribute to the phosphorolysis of deoxyuridine, the name thymidine phosphorolysase appears appropriate for Enzyme 3 at this time.

The different rates at which uridine- and deoxycytidine-cleaving activities of rat liver are found to increase at various times after the injection of uridine or cytidine (5, 6, 9) are explained by the finding that rat liver contains two phosphorolyses which act on uridine but three phosphorolyses which act on deoxycytidine. The activities of the isoenzymes of uridine phosphorolysase which increase together after uridine or cytidine administration would determine the overall rate of phosphorolysis of uridine by rat liver. On the other hand, the activities of both these isoenzymes and of thymidine phosphorolysase would determine
the over-all rate of phosphorolysis of deoxyuridine by rat liver. In contrast to findings for the uridine phosphorylases, the specific activity of thymidine phosphorylase remains at control levels after uridine injection but decreases after cytidine injection.

Present findings, taken together with those of previous studies (6, 9), indicate that both isoenzymes of uridine phosphorylase are probably induced (or derepressed) in regenerating liver of rats after uridine or cytidine administration. After cytidine administration thymidine phosphorylase is probably repressed. Whether the nucleosides are the effectors per se is not known at this time. Pertinent to our studies are the reports that thymidine phosphorylase of E. coli is induced, in growing cultures, by deoxyribonucleosides but not by uridine (16) and the activity of uridine phosphorylase of livers of tadpoles is increased in normal and thyroxine-induced metamorphosis (19).

Acknowledgments—The author is indebted to N. Grift, J. Yea, S. Silver, and S. Grant for their skillful technical assistance.

REFERENCES
Pyrimidine Nucleoside Phosphorylases of Rat Liver: SEPARATION BY ION EXCHANGE CHROMATOGRAPHY AND STUDIES OF THE EFFECT OF CYTIDINE OR URIDINE ADMINISTRATION
Esther W. Yamada


Access the most updated version of this article at http://www.jbc.org/content/243/7/1649

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/243/7/1649.full.html#ref-list-1