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Studies of Fluorinated Pyrimidines. XVIII. The Degradation of 5-Fluoro-2′-deoxyuridine and Related Compounds by Nucleoside Phosphorylase*

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The degradation of 5-fluoro-2′-deoxyuridine and a variety of other nucleosides to the corresponding pyrimidine bases by the high-speed (105,000 × *g*) supernatant fraction of Ehrlich ascites cells has been investigated. Both 5-fluoro-2′-deoxyuridine and 5-fluorouridine were degraded rapidly by this preparation at *p*H optima of 6.4 and 7.4, respectively. A number of other pyrimidine nucleosides, in particular 2′-deoxyuridine and the 5-halogen-substituted derivatives of 2′-deoxyuridine, were also degraded to the free bases at varying rates under the same conditions. 3′-Monoacetyl-5-fluoro-2′-deoxyuridine and 3′,5′-diacetyl-5-fluoro-2′-deoxyuridine were not degraded by the high-speed supernatant fraction of Ehrlich ascites cells or of human, rat, or mouse liver. However, powerful deacetylase activity was found in a particulate fraction from liver; only very weak deacetylase activity was present in Ehrlich ascites cells. The phosphorylase activity of Ehrlich ascites cells towards 5-fluoro-2′-deoxyuridine was inhibited by several compounds, in particular by 5-fluorouridine and uridine. Uridine was found to be a competitive inhibitor of the reaction.

Previous work from this laboratory has clearly demonstrated that the inhibition of thymidylate synthetase and, hence, of DNA biosynthesis, is the mechanism by which 5-fluorouracil (FU) inhibits the growth of neoplastic tissue (Bosch *et al.*, 1958; Harbers *et al.*, 1959; Hartmann and Heidelberger, 1961). The actual inhibitor of thymidylate synthetase has been shown to be 5 - fluoro - 2′ - deoxyuridine - 5′ - monophosphate (FUDRP) (Cohen *et al.*, 1958; Hartmann and Heidelberger, 1961) and, further, the formation of this inhibitory nucleotide from FU has been found to follow the same pathway as that of deoxyuridylic acid from uracil (Chaudhuri *et al.*, 1958; Harbers *et al.*, 1959; Sköld, 1960a,b). Thus, the first steps in the formation of FUDRP are the formation of 5-fluoro-2′-deoxyuridine (FUDR) or 5-fluorouridine (FUR) from FU and deoxyribose-1-phosphate or ribose-1-phosphate, respectively, reactions which are catalyzed by deoxyuridine phosphorylase or uridine phosphorylase (Sköld, 1960b). In view of this, FUDR should be a better precursor of FUDRP than FU, particularly if the main pathway of synthesis of the deoxyribonucleotide is *via* FUR and 5-fluorouridine-5′-monophosphate (FURP) (Chaudhuri *et al.*, 1958). The hypothesis was confirmed when it was found, first, that FUDR was a more effective carcinostatic agent than FU against the Ehrlich ascites carcinoma and sarcoma-180 *in vivo* (Heidelberger *et al.*, 1958); second, that the deoxyribonucleoside inhibited the incorporation of formate-C^{14} into the DNA-thymine of Ehrlich ascites cells *in vitro* to a greater extent than did FU (Bosch *et al.*, 1958); and, third, that FUDR, in the presence of ATP, was phosphorylated to FUDRP by a high-speed (105,000 × *g*) supernatant fraction from Ehrlich ascites cells, whereas FU did not give rise to FUDRP under the same conditions (Hartmann and Heidelberger, 1961). However, it was found that, *in vivo*, the effectiveness of FUDR was not as much greater than that of FU as might be

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expected (Heidelberger *et al.*, 1958). The reason for this is the reversibility of the reaction catalyzed by nucleoside phosphorylase and the consequent rapid degradation to FU of a large proportion of FUDR administered *in vivo* (Chaudhuri *et al.*, 1959). Thus, it appears that one of the main limitations to cancer chemotherapy with FUDR is the rapid degradation of the drug by nucleoside phosphorylase.

In view of these observations, the present study of the properties and specificity of the nucleoside phosphorylase activity of the Ehrlich ascites carcinoma was undertaken. In addition, the possibility of inhibiting the degradation of FUDR was investigated, first, by chemical modification of the drug and, second, by inhibition of the nucleoside phosphorylase activity with a variety of compounds.

Experimental

(*i*) *Preparation of Tissue Extracts.*—Ascites cells were harvested from Swiss mice 7 days after intraperitoneal transplantation of the Ehrlich ascites carcinoma and were washed twice with ice-cold 0.9% NaCl. The cells were suspended in two volumes of cold 0.15 M phosphate buffer (*p*H 6.4) and were disrupted for 2 minutes at 10 kc/second in a Raytheon sonic oscillator. The high-speed supernatant (S_3) fraction was obtained by centrifuging the disrupted cells for one hour at 105,000 × *g*. The particulate fraction of the cells, containing nuclei, mitochondria, and microsomes, was obtained from the pellet of material remaining after removal of the S_3 fraction. This pellet was washed with 0.15 M phosphate buffer (*p*H 6.4) and recentrifuged at 105,000 × *g* for one hour. The washed pellet was then resuspended in 0.15 M phosphate buffer (*p*H 6.4) and centrifuged for 5 minutes at 200 × *g* to remove cell debris. The resulting opaque suspension was taken as the particulate fraction.

Human tissues were obtained from patients at surgery. All solid tissues used were minced with scissors and homogenized with four to five volumes of 0.15 M phosphate buffer (*p*H 6.4) in a Potter-Elvehjem homogenizer. The homogenates were filtered through glass wool and centrifuged for 5 minutes at 200 × *g* to remove cell debris. Both the S_3 fractions and particulate fractions of these tissues were obtained from the filtered homogenates by the procedure described for sonically disrupted Ehrlich ascites cells.

The concentration of protein present in the tissue preparations was estimated by the quantitative biuret method of Gornall *et al.* (1949), with casein used as standard.

(*ii*) *Materials.*—FU,[1] FUDR, α-FUDR, FUR, 3'-monoacetyl - FUDR, 3',5' - diacetyl - FUDR, 3',5' - dipropionyl - FUDR, 5 - fluoro - 6 - methoxy - 5,6-dihydro - 2' - deoxyuridine, and 5 - bromo - 5 - fluoro-6 - methoxy - 5,6 - dihydro - 2 - deoxyuridine were generously provided by Dr. Robert Duschinsky of Hoffmann-LaRoche, Inc. FUDRP, diethyl-FUDRP, *N,N* - diethylaminoethyl - FUDRP, α,β - DNMP, and β,β-DNMP were synthesized in this laboratory

[1] Abbreviations used in this paper are: FU, 5-fluorouracil; UDR, 2'-deoxyuridine; FUDR, 5-fluoro-2'-deoxyuridine; ClUDR, BrUDR and IUDR, 5-chloro-, 5-bromo- and 5-iodo-2'-deoxyuridine, respectively; TDR, thymidine-UR, uridine; FUR, 5-fluorouridine; FUDRP, 5-fluoro; 2'-deoxyuridine-5'-monophosphate; α,β-DNMP, β-5-fluoro-2'-deoxyuridylyl(5' → 3')-α-5-fluoro-2'-deoxyuridine; β,β-DNMP, β-5-fluoro-2'-deoxyuridylyl(5' → 3')-β-5-fluoro-2'-deoxyuridine; diethyl-FUDRP, 5-fluoro-2'-deoxyuridine-(5')-diethylphosphate; N,N-diethylaminoethyl-FUDRP, 5-fluoro-2'-deoxyuridine-5'-monophosphate-N,N-diethylaminoethyl ester.

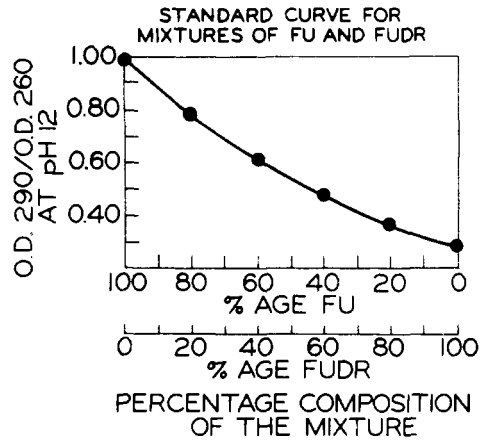

Fig. 1.—Standard curve for mixtures of FU and FUDR; plot of OD 290/260 ratios of mixtures of FU and FUDR at *p*H 12 against percentage composition.

(Remy *et al.*, 1962). 6-Azathymine, 6-azauracil, 6-azauridine, UR, UDR, ClUDR, BrUDR, and IUDR were supplied by the California Corporation for Biochemical Research. 6-Azathymidine was a gift of Dr. William Prusoff, and β-D-arabinosyl-5-fluorouracil was donated by Dr. J. J. Fox (Yung *et al.*, 1961). We are indebted to Dr. K. C. Murdock and Dr. R. Angier of Lederle Laboratories for a generous gift of *dl* - 1 - [*trans* - 3 - hydroxy - *cis* - 4 - (hydroxymethyl) cyclopentyl] thymine (Murdock and Angier, 1962) and for full details of the properties of this compound prior to publication. FUDR-2-C^{14} was synthesized from FU-2-C^{14} (purchased from the California Corporation for Biochemical Research) by Dr. D. B. Koechlin of Hoffmann-LaRoche, Inc.

(*iii*) *Enzyme Assays.*—Two methods of assaying the activity of the enzyme extracts were used.

(*a*) The Optical Method.—Incubation mixtures were prepared so that each milliliter contained 75 μmoles of phosphate buffer, 0.4 μmole of substrate, and 1.0 mg of tissue extract protein. Mixtures were incubated with continuous shaking under air at 37° and, at various times, 1.0-ml samples were transferred to tubes containing 1.0 ml of ice-cold 10% perchloric acid. After the precipitated protein had coagulated, it was centrifuged, and 1.0 ml of the supernatant fluid was withdrawn and taken to *p*H 12 by the addition of 1.0 ml of 1 N NaOH. The solution was diluted to 4.0 ml with water, and the extinctions at 260 mμ and 290 mμ were determined in a Beckman Model DU spectrophotometer. The extinctions, at the same wave lengths, of the appropriate blank (a sample taken after the same time interval from an incubation mixture complete except for the substrate) were subtracted and the ratio of the extinction at 290 mμ to that at 260 mμ was calculated. By means of a standard curve, such as the one shown in Figure 1, the percentage of free base present in the mixture was estimated. Standard curves for a number of pyrimidine bases and their nucleosides, for example, FU and FUDR (Fig. 1), were constructed by determining the OD 290/260 ratios at *p*H 12 of various known mixtures of the free base and the nucleoside.

(*b*) The Isotopic Method.—Into a conical centrifuge tube was pipetted 37.5 μmoles of phosphate buffer, 0.2 μmole of FUDR containing 0.05 μc of FUDR-2-C^{14}, and 0.5 mg of S_3 fraction protein, in a total volume of 0.50 ml. The mixture was incubated with continuous shaking at 37° for 20 minutes, and the reaction was stopped by heating at 100° for 5 minutes. The precipitated protein was centrifuged,

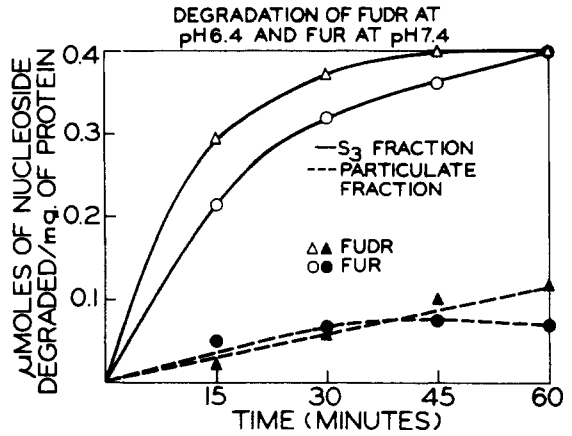

FIG. 2.—The degradation of FUDR at *p*H 6.4 and FUR at *p*H 7.4 with time. The enzyme activity was assayed by the standard optical method, described in the Experimental Section.

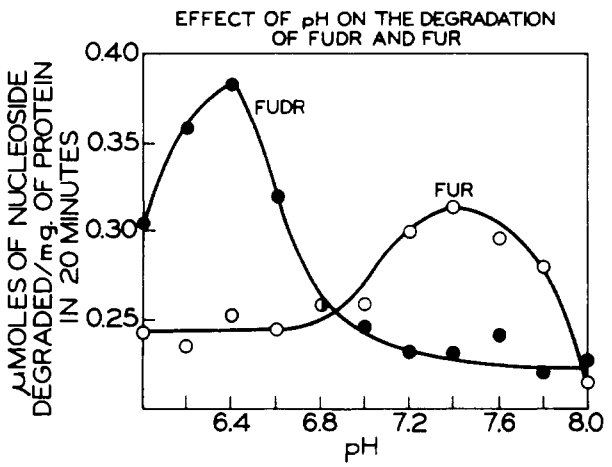

FIG. 3.—The effect of *p*H on the rate of degradation of FUDR and FUR. The enzyme activity of the S_3 fraction of Ehrlich ascites cells was assayed by the standard optical method, described in the Experimental Section. Phosphate buffers of *p*H 6.0 to 8.0 were used, and the incubation time was 20 minutes.

and 0.2 ml of the supernatant fluid was streaked onto a strip (2.5 × 51 cm) of Whatman No. 1 chromatography paper. FU and FUDR were separated by electrophoresis for 2 hours at 2,250 v and 30–35 ma in 0.05 M sodium tetraborate, *p*H 9.2. The proportion of FUDR degraded to FU was determined by passing the strips through a windowless gas-flow strip-counter equipped with an integrator (Ludwig *et al.*, 1960).

The optical method was used to study the degradation of FUDR and other nucleosides and also to measure the rate of formation of free base from nucleoside derivatives such as FUDRP, 3′-monoacetyl-FUDR, and 3′,5′-diacetyl-FUDR, which had ultraviolet spectra not significantly different from that of FUDR at *p*H 12. Semiquantitative estimates of the degradation of other derivatives of FUDR, such as α,β-DNMP, were also obtained with this method. The isotopic method was used in studies of the effects of various substrates, which themselves were metabolized in the assay system, on the cleavage of FUDR to FU.

In all experiments, excellent correspondence between the optical and the isotopic methods was obtained. Over the complete range of FU and FUDR mixtures, agreement of within 5% between the two methods was observed. In each experiment incubations were done in duplicate and each experiment repeated at least once. Appropriate blanks and zero-time controls were incubated in parallel.

RESULTS

(*a*) *The Phosphorolysis of FUDR and FUR by Ehrlich Ascites Cells.*—That FUDR and FUR were rapidly degraded to FU by the 105,000 × *g* supernatant (S_3) fraction of Ehrlich ascites cells at *p*H 6.4 and 7.4, respectively, is clearly shown in Figure 2; the particulate fraction of the same cells, containing nuclei, mitochondria, and microsomes, cleaved FUDR and FUR at a much slower rate. At *p*H 6.4 in 0.25 M Tris buffer, in the absence of added inorganic phosphate, the cleavage of FUDR was only 21% of that observed in 0.25 M phosphate buffer or 0.10 M Tris buffer plus 0.15 M phosphate buffer. Thus, as has been found recently by Jacquez (1962), the S_3 fraction contained the FUDR and FUR phosphorylase activity of Ehrlich ascites cells and so was used as the source of the enzyme without further purification.

It can be seen from figures 2, 7, and 8 that the rates at which the nucleosides were degraded by the S_3 fraction were not strictly linear and tended to reach a plateau at around 30 minutes. Although there was some deviation from linearity at 20 minutes, this time was arbitrarily chosen for most of the experiments as a means of achieving the greatest experimental accuracy, since the changes in optical density were considerably greater than those obtained at shorter time intervals. Thus, although the rates reported for degradation over a 20-minute interval may not represent maximum rates, nevertheless they are self-consistent throughout the study, and we believe the comparisons reported here to be valid.

The maximum rate of degradation to FU occurred at *p*H 6.4 for FUDR and *p*H 7.4 for FUR (Fig. 3). These *p*H optima correspond quite closely with those found by Pontis *et al.* (1961), using a purified nucleoside phosphorylase preparation, for UDR (*p*H 6.5) and UR (*p*H 8.1). The dependence of the rate at which FUDR was cleaved by a constant level of S_3 fraction protein on the substrate concentration is shown in Figure 4; the results of the complementary study, in which the rates of degradation of a constant concentration of FUDR by different concentrations of S_3 fraction protein were measured, are summarized in Figure 5. In Figure 4 it can be seen that the maximum rate of FUDR cleavage took place at 0.4 μmoles of FUDR per ml before there was a significant deviation from linearity, and this concentration was used throughout the study. In Figure 5 it is shown that a slight saturation occurred at 1.0 mg of protein per ml, and so the concentration of protein was fixed at this level throughout.

The stability of the nucleoside phosphorylase activity of the S_3 fraction is shown in Figure 6. When the preparation was kept frozen at −10°, even after 16 days 96% of the original activity was still present, although the extract rapidly lost activity if stored at 0°. In practice, fresh enzyme preparations were made each week.

(*b*) *The Degradation of Some Pyrimidine Nucleoside Derivatives by the S_3 Fraction of Ehrlich Ascites Cells.*—The specificity of the crude enzyme preparation from Ehrlich ascites cells was investigated by comparing the rates at which the S_3 fraction cleaved various nucleosides to the corresponding free bases. As is shown by Figure 7, halogenation of the 5-position of UDR did not markedly affect the rate at which the nucleoside was cleaved by the S_3 fraction. In contrast, TDR was cleaved at half the rate of UDR.

TABLE I

THE DEGRADATION OF NUCLEOSIDES TO FREE BASE BY THE S_3 FRACTION OF EHRLICH ASCITES CELLS

The Enzyme activity was assayed by the standard optical method, described in the Experimental section. The incubation time was 20 minutes.

Substrate	pH	Amt. Substrate Degraded to Free Base (μmoles/mg protein/20 min.)
FUDR	6.4	0.340
FUDR	7.4	0.216
UDR	6.4	0.328
UDR	7.4	0.210
FUR	6.4	0.238
FUR	7.4	0.287
UR	6.4	0.303
UR	7.4	0.385
β-D-Arabinosyl-5-fluorouracil	7.4	0.072
3'-Monoacetyl-FUDR	6.4	0.00
3',5'-Diacetyl-FUDR	6.4	0.00
3',5'-Diacetyl-FUDR	7.4	0.00
3',5'-Dipropionyl-FUDR	6.4	0.00
5-Fluoro-6-methoxy-5,6-dihydro-2'-deoxyuridine[a]	6.4	0.00
5-Bromo-5-fluoro-6-methoxy-5,6-dihydro-2'-deoxyuridine[a]	6.4	0.00
TDR	6.4	0.158
1-[*trans*-3-hydroxy-4-*cis*-(hydroxymethyl)cyclopentyl]thymine	6.4	0.00
6-Azathymidine	6.4	0.00
6-Azauridine	6.4	0.00

[a] Before determination of the OD 290/260 ratios for these compounds, after being adjusted to pH 12 the samples taken were incubated overnight at 37°. Under these conditions, the compounds are degraded to FUDR (personal communication from Dr. Robert Duschinsky). Standard curves for mixture of the compounds and FU were determined in parallel.

The results from experiments with other nucleosides are summarized in Table I. It can be seen that FUDR was degraded at a slightly faster rate than UDR at both pH 6.4, and 7.4 whereas UR was cleaved much more rapidly than FUR at both pH levels. β-D-Arabinosyl-5-fluorouracil was degraded by the S_3 fraction, but at a much slower rate than FUR. 3'-Monoacetyl - 3',5' - diacetyl- and 3',5' - dipropionyl-FUDR, 5 - fluoro - 6 - methoxy - 5,6 - dihydro - 2'-deoxyuridine, and 5 - bromo - 5 - fluoro - 6 - methoxy-5,6 - dihydro - 2' - deoxyuridine were not cleaved to FU by the S_3 fraction to a detectable extent. Also, neither 1 - [*trans* - 3 - hydroxy - *cis* - 4 - (hydroxymethyl) cyclopentyl] thymine nor 6-azathymidine was degraded, although TDR was readily cleaved by the S_3 fraction. Similarly, 6-azauridine was not cleaved, although UR was degraded very rapidly by the S_3 fraction.

Finally, the stereospecificity of the nucleoside phosphorylase activity of the S_3 fraction was clearly demonstrated when the S_3 fraction was found to be completely inactive toward α-FUDR (Figure 7).

The action of the S_3 fraction of Ehrlich ascites cells on various derivatives of the 5'-mononucleotide, FUDRP, is shown in Table II. As the degradation of these compounds results in more than one ultraviolet-absorbing product, assay of the rates of degradation to the various products was not possible with the optical method. However, since the ultraviolet spectra of these nucleotides at pH 12 were not significantly different from that of FUDR (Remy *et al.*, 1962), the

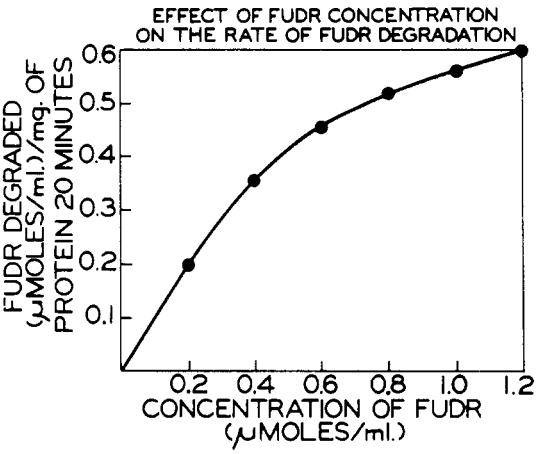

FIG. 4.—The effect of FUDR concentration on the rate of degradation of FUDR. The enzyme activity of the S_3 fraction was assayed by the standard optical method, described in the Experimental Section, except that various concentrations of FUDR were used. The incubation time was 20 minutes.

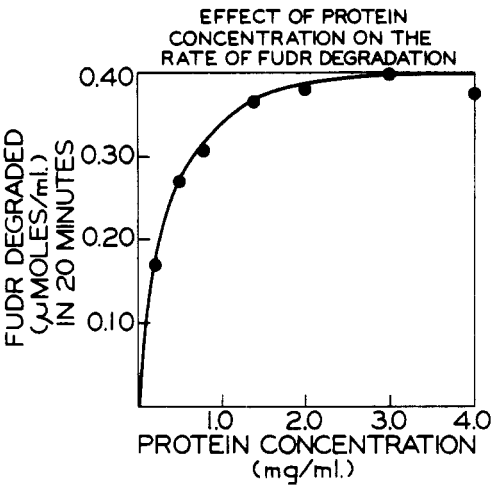

FIG. 5.—The effect of protein concentration on the rate of degradation of FUDR. The enzyme activity of the S_3 fraction was assayed by the standard optical method, described in the Experimental Section, except that various amounts of the S_3 fraction were added. The incubation time was 20 minutes.

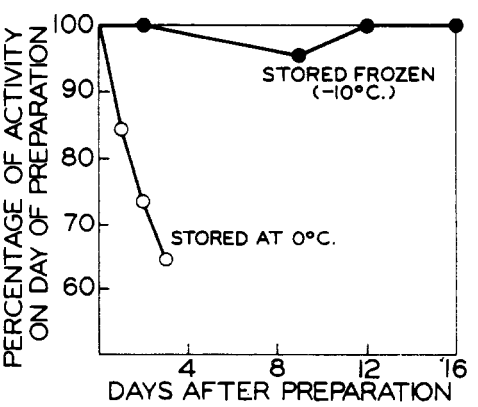

FIG. 6.—The stability of the FUDR phosphorylase activity in the S_3 fraction of Ehrlich ascites cells. The enzyme activity was assayed by the standard optical method, as described in the Experimental Section. The incubation time was 20 minutes.

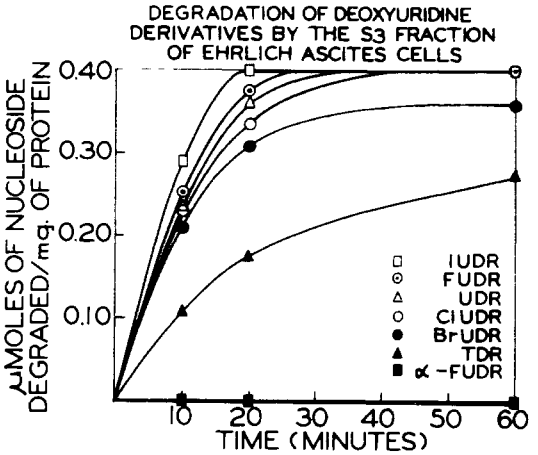

FIG. 7.—The degradation of UDR and 5-substituted derivatives of UDR by the S_3 fraction of Ehrlich ascites cells with time. The enzyme activity was assayed by the standard optical method, described in the Experimental Section.

optical method enabled estimates to be made of the rates at which the S_3 fraction degraded the nucleotides to FU as compared with FUDR. Table II shows that FUDRP was degraded to FU very much more slowly than FUDR, indicating that dephosphorylation is probably required as the initial step in the degradation of the nucleotide. Esterification of the 5′-phosphate with diethyl or *N,N*-diethylaminoethyl groups inhibited the dephosphorylation. The nucleotide was not cleaved when the 5′-phosphate was esterified with the 3′-hydroxyl of α-FUDR (α,β-DNMP), although the β,β-isomer of this compound was cleaved at the same rate as FUDRP.

(*c*) *The Degradation of FUDR, 3′-Monoacetyl-FUDR, and 3′,5′-Diacetyl-FUDR.*—A survey of the FUDR phosphorylase content of a number of tissues, the results of which are summarized in Table III, showed that FUDR is rapidly cleaved by the S_3 fractions of a variety of tissues. Considerably more FUDR phosphorylase activity per mg of S_3 fraction protein was obtained from Ehrlich ascites cells than from mouse or rat liver. The S_3 fractions from normal human gastric mucosa, spleen, and liver cleaved FUDR rapidly, as did all the human tumors studied; on the other hand, human serum appeared to be totally lacking in FUDR phosphorylase activity.

Table III also shows that the S_3 fractions of these tissues degraded the acetylated derivatives of FUDR only very slowly, if at all. However, 3′,5′-diacetyl-FUDR was degraded slowly to FU by a homogenate

TABLE II

THE DEGRADATION OF SOME NUCLEOTIDE DERIVATIVES OF FUDR BY THE S_3 FRACTION OF EHRLICH ASCITES CELLS

The enzyme activity was assayed by the standard optical method, described in the Experimental section. The incubation time was 20 minutes.

Substrate	Amt. Substrate Degraded to FU in 20 min.[a]
FUDR	100
FUDRP	10
Diethyl-FUDRP	0
N,N-diethylaminoethyl-FUDRP	0
β,β-DNMP	10
α,β-DNMP	0

[a] Estimated as a percentage of the amount of FUDR degraded under the same conditions (FUDR = 100).

TABLE III

THE DEGRADATION OF FUDR, 3′-MONOACETYL-FUDR, AND 3′,5′-DIACETYL-FUDR BY THE S_3 FRACTION OF VARIOUS TISSUES

The enzyme activity was assayed by the standard optical method, as described in the Experimental section. The incubation time was 20 minutes.

Tissue	Pre-incubation[a]	Amt. Substrate Degraded to FU (μmoles/mg protein/20 min.) FUDR	3′-Mono-acetyl-FUDR	3′,5′-Di-acetyl-FUDR
Ehrlich ascites cells	−	0.340	0.00	0.00
Ehrlich ascites cells	+	0.400	0.00	0.010
Mouse liver	−	0.166	0.00	0.00
Mouse liver	+	0.183	0.119	0.083
Rat liver	−	0.072	0.00	0.00
Rat liver	+	0.080	0.070	0.024
Human Serum	−	0.00	—	0.00
Human gastric mucosa	−	0.260	—	0.00
Human spleen	−	0.370	—	0.00
Human liver	−	0.280	0.010	0.00
Human liver	+	0.280	0.220	0.060
Human adenocarcinoma of stomach	−	0.220	—	0.008
Human squamous cell carcinoma[b]	−	0.229	—	—
Human rectal polyp[b]	−	0.220	—	—
Human reticulosarcoma	−	0.100	—	0.00

[a] Preincubation: Substrate and buffer were incubated with the particulate fraction (1.0 mg of protein) at 37° for one hour; the S_3 fraction was then added and incubation continued for 20 minutes. [b] In these cases, homogenized tissue was used in place of the S_3 fraction.

of mouse liver, which suggests that the deacetylase activity was associated with a particulate fraction rather than the S_3 fraction. When 3′-monoacetyl-FUDR and 3′,5′-diacetyl-FUDR were incubated with the S_3 fraction of mouse liver after preincubation for 1 hour with the particulate fraction of the liver, both derivatives were degraded rapidly to FU (Table III). The particulate fraction alone did not degrade the acetylated derivatives to FU to any significant extent. Similar results were obtained with both rat and human liver (Table III). Under the same conditions, Ehrlich ascites cells degraded acetylated FUDR at an extremely low, although significant, rate.

A detailed study of the rates at which human liver preparations degraded FUDR, 3′-monoacetyl-, and 3′,5′-diacetyl-FUDR confirmed that the deacetylase activity of human liver was in the particulate fraction (Fig. 8). Figure 8 also shows that 3′-monoacetyl-FUDR was cleaved much more rapidly than 3′,5′-diacetyl-FUDR, which suggests that the 5′-acetyl group is the more resistant to hydrolysis. In addition, it can be seen that, even after preincubation with the particulate fraction, 3′-monoacetyl-FUDR was cleaved to FU at a slower rate than FUDR; this indicates that the deacetylation is rate limiting.

Entirely similar results were obtained with mouse and rat liver.

(*d*) *Inhibition of the FUDR Phosphorylase Activity of Ehrlich Ascites Cells.*—A variety of compounds was screened for their ability to inhibit the degradation of FUDR by the nucleoside phosphorylase of the S_3 fraction of Ehrlich ascites cells. The results are summarized in Table IV. It can be seen that an equimolar concentration of a number of compounds inhibited

TABLE IV

INHIBITION OF THE DEGRADATION OF FUDR BY THE S_3 FRACTION OF EHRLICH ASCITES CELLS

The FUDR phosphorylase activity, in the presence of the inhibitors, was assayed by the isotopic method described in the Experimental section. The concentration of FUDR was 0.4 μmole/ml.

Inhibitor	Percentage Inhibition by 0.4 μmole/ml.	0.8 μmole/ml.
UR	39	50
UDR	7	14
6-Azauracil	0	10
6-Azathymine	15	23
5-Hydroxymethyluracil	11	—
6-Azauridine	6	—
1-[*trans*-3-hydroxy-*cis*-4-(hydroxymethyl)cyclopentyl]thymine	4	12
α-FUDR	0	0
β-D-Arabinosyl-5-fluorouracil	9	—
3′-Monoacetyl-FUDR	0	—
3′,5′-Diacetyl-FUDR	0	14
3′,5′-Dipropionyl-FUDR	—	17
5-Fluoro-6-methoxy-5,6-dihydro-2′-deoxyuridine	0	0
5-Bromo-5-fluoro-6-methoxy-5,6-dihydro-2′-deoxyuridine	0	7
FUDRP	0	—
N,N-Diethylaminoethyl-FUDRP	0	—
Diethyl-FUDRP	15	—

the degradation of FUDR to a small extent. A very small effect only was observed with 6-azauracil, although significant inhibition was obtained with 6-azathymine. 6-Azauridine did not inhibit the degradation of either FUDR or FUR. 3′-Monoacetyl-FUDR did not inhibit the cleavage of FUDR, but both 3′,5′-diacetyl-FUDR and 3′,5′-dipropionyl-FUDR inhibited to a small extent. The stereoisomer, α-FUDR, was totally inactive. Of the nucleotide derivatives, only diethyl-FUDRP inhibited the phosphorolysis of FUDR, and that only slightly. On the other hand, UR inhibited the degradation of FUDR very strongly, although UDR showed only weak inhibitory activity. This result is in agreement with the observation of Pontis *et al.* (1961), who showed that UR is a competitive inhibitor of UDR phosphorylase.

The inhibitory effects of several compounds at various concentrations were investigated, and the results are shown in Figure 9. It can be seen that, compared to the other compounds, both FUR and UR strongly inhibited the degradation of FUDR to FU. 6-Azathymidine proved to be a poor inhibitor of the degradation, as did UDR except, as was expected, at high concentrations. The end-product of the reaction, FU, was found to be a relatively potent inhibitor, particularly at high concentrations; a 10-fold molar excess of FU inhibited the degradation of FUDR to the extent of 65%.

The kinetics of the inhibition of FUDR phosphorolysis by UR were studied and the Lineweaver-Burk plot (Lineweaver and Burk, 1934), shown in Figure 10, clearly demonstrated that the inhibition is competitive. From these data, it was calculated that the Michaelis constant (K_m) for FUDR phosphorylase in the crude extract was 1.77×10^{-3} M; similarly, the inhibitor constant (K_i) for UR was found to be 7.0×10^{-4} M.

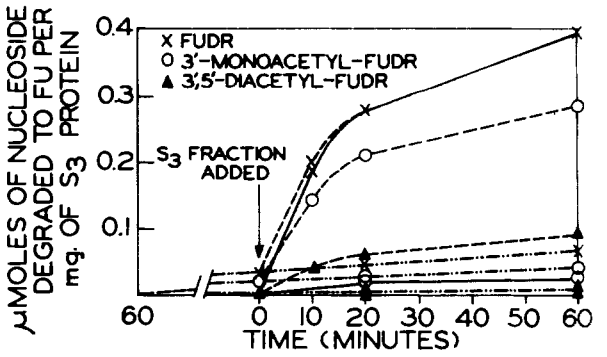

FIG. 8.—The degradation of FUDR, 3′-monoacetyl-FUDR, and 3′,5′-diacetyl-FUDR by human liver. The substrate (1.6 μmoles) and phosphate buffer (300 μmoles, *p*H 6.4), either alone (———) or in the presence of the particulate fraction (1.0 mg of protein) from human liver (– – – –), were incubated for one hour at 37°. Then the S_3 fraction (1.0 mg of protein) of the liver was added and incubation continued at 37°. A control, in which buffer was added in place of the S_3 fraction (·—·), was incubated in parallel. Samples were removed at zero, 10, 20, and 60 minutes, and the activity of the enzyme preparation was measured by the optical method, described in the Experimental Section.

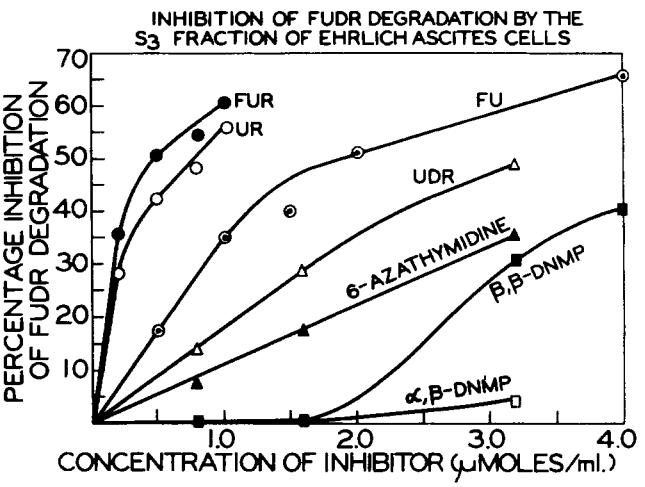

FIG. 9.—Inhibition of the FUDR phosphorylase activity of the S_3 fraction of Ehrlich ascites cells. The enzyme activity, in the presence of various concentrations (from 0 to 4.0 μmoles/ml) of the inhibitors, was assayed by the isotopic method described in the Experimental Section. The incubation time was 20 minutes.

DISCUSSION

In the present study, the S_3 fraction of Ehrlich ascites carcinoma cells was used as the source of nucleoside phosphorylase without further purification, since our primary interest in FUDR is as a clinical carcinostatic agent. Thus we felt that it was desirable to study the phosphorolysis of FUDR in a cell-free system under conditions closer to those pertaining *in vivo* than could be realized with a purified enzyme preparation, such as was used by Sköld (1960c) and Pontis *et al.* (1961). Furthermore, the use of the S_3 fraction rather than whole cells obviated the possibility that the significance of the observations might have been obscured by cell membrane permeability effects such as those observed recently by Jacquez (1962).

FUDR is rapidly degraded to FU by a large variety of human tissues, including tumors. Thus, inhibition of this degradation would be of prime importance in attempts to improve the chemotherapeutic effectiveness

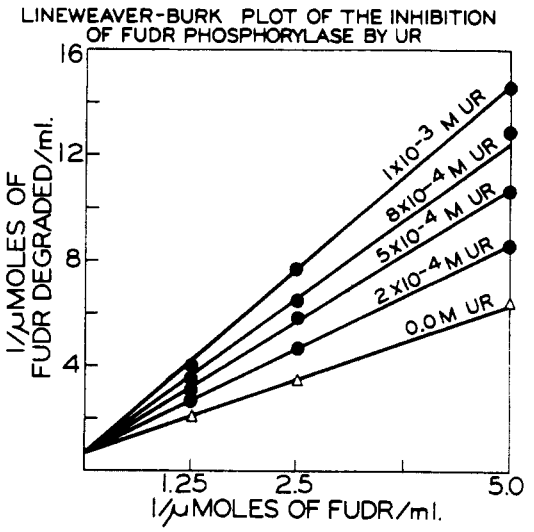

FIG. 10.—The Lineweaver-Burk plot of the inhibition of the FUDR phosphorylase activity of Ehrlich ascites cells by UR.

of FUDR. One method of inhibiting the degradation of FUDR is by modifying the molecule chemically such as was done by Remy *et al.* (1962). Variations in the structure of either the pyrimidine ring or the sugar moiety of the nucleosides markedly decreased their rates of degradation to the free base by nucleoside phosphorylase. Similarly, many of the derivatives of FUDR synthesized by Remy *et al.* (1962) resisted degradation to FU by the S_3 fraction of Ehrlich ascites cells. However, Mukherjee and Heidelberger (1962) found that none of these compounds was as effective as FUDR in inhibiting the incorporation of formate-C^{14} into the DNA-thymine of Ehrlich ascites cells, which is an indicator of chemotherapeutic efficacy.

Although both 3′-monoacetyl-FUDR and 3′,5′-diacetyl-FUDR are very slowly degraded to FU by Ehrlich ascites cells, Mukherjee and Heidelberger (1962) found that, compared with FUDR, both of these derivatives are extremely weak inhibitors of the *in vitro* incorporation of formate-C^{14} into the DNA-thymine of these tumor cells. This apparent anomaly is explained by the observation that Ehrlich ascites cells contain a very low level of deacetylase activity. Thus, the rate-limiting step in the degradation of the acetyl derivatives of FUDR is the deacetylation reaction(s), with, consequently, immediate cleavage of the resulting FUDR to FU. However, *in vivo*, the acetyl derivatives of FUDR are probably deacetylated in the liver, since human, rat and mouse liver were found to hydrolyze 3′-monoacetyl-FUDR and 3′,5′-diacetyl-FUDR to the nucleoside.

A second method of inhibiting the degradation of FUDR would be the inhibition of FUDR phosphorylase by another substrate. 6-Azauracil and 6-azathymine have some affinity for nucleoside phosphorylase, since, under the influence of a purified nucleoside phosphorylase preparation, 6-azauracil reacts with ribose-1-phosphate to form 6-azauridine and 6-azathymine with deoxyribose-1-phosphate to form 6-azathymidine (Pontis *et al.*, 1961). 6-Azauridine and 6-azathymidine were also of interest, since neither was cleaved by nucleoside phosphorylase. Similarly, the effect on the phosphorolysis of FUDR was studied with a number of compounds (Remy *et al.*, 1962) not rapidly degraded by the S_3 fraction of Ehrlich ascites cells, but possibly having some affinity for nucleoside phosphorylase by virtue of their relationship to FUDR. However, none of these compounds inhibited the degradation of FUDR to a significant extent.

The effect of UDR, UR, FU and FUR on the cleavage of FUDR to FU was studied, since UDR phosphorylase is inhibited by FU, FUDR (Sköld, 1960c) and UR (Pontis *et al.*, 1961). Although FUR inhibited the phosphorolysis of FUDR to a significant extent, the compound was not investigated further, since the use of FUR in chemotherapy is impossible because of the extreme toxicity of the drug. Since the ratio of K_m:K_i for the inhibition by UR was found to be 2.5:1, UR, although the most active of the compounds studied, excepting FUR, is not a potent inhibitor of the reaction. However, UR is not toxic to mammals and, hence, it seemed possible that administration of UR in large excess simultaneously with FUDR might increase the chemotherapeutic effectiveness of FUDR *in vivo*. However, when administered to mice, large doses of UR caused a marked increase in the toxicity of FUDR. This results, presumably, from the saturation of the degradative pathway by uracil, formed from UR by UR phosphorylase, and the consequent inability to degrade and thus detoxify FU formed from the FUDR.

The present study has enabled the characteristics of the nucleoside phosphorylase activity of Ehrlich ascites cells toward FUDR to be elucidated. However, all attempts to improve the carcinostatic effectiveness of FUDR by inhibiting the degradation of the drug have, thus far, proved unsuccessful.

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