Biochemical Pharmacology, Vol. 21, pp. 1063–1073. Pergamon Press, 1972. Printed in Great Britain.

STUDIES ON THE MODE OF ACTION OF 3-DEAZAPYRIMIDINES—1

METABOLISM OF 3-DEAZAURIDINE AND 3-DEAZACYTIDINE IN MICROBIAL AND TUMOR CELLS*

M. C. WANG and A. BLOCH

Department of Experimental Therapeutics, Roswell Park Memorial Institute, New York State Department of Health, 666 Elm Street, Buffalo, N.Y. 14203, U.S.A.

(*Received* 27 *September* 1971; *accepted* 19 *November* 1971)

Abstract—The pyrimidine analogs 3-deazauridine and 3-deazacytidine are effective inhibitors of the growth of various microbial and tumor cell lines in culture, and of some experimental tumors *in vivo*. As part of a study on their modes of action, the metabolic conversion of the analogs by intact *Escherichia coli*, leukemia L-1210 and Ehrlich ascites carcinoma cells, or by cell-free extracts derived from these or from *Streptococcus faecium* cells, was examined. Neither analog underwent cleavage of its glycosidic bond in the bacterial or tumor cell extracts, which catalyzed the cleavage of uridine but not of cytidine. Deazacytidine was not deaminated by extracts of *E. coli*, which effected the rapid deamination of cytidine. Both analogs were phosphorylated to the nucleoside triphosphate stage, as determined with Ehrlich ascites extracts, and Ehrlich ascites, L-1210 and *E. coli* cells. The possible formation of 2′-deoxyribonucleoside phosphate derivatives was examined with 3-deazacytidine, and a 2′-deoxyribonucleotide derivative of this analog was found in the acid-soluble fraction of *E. coli* cells. Neither 3-deazauridine nor 3-deazacytidine was incorporated into the RNA or DNA of Ehrlich ascites cells *in vivo*.

DEAZACYTIDINE (deaza-CR) and deazauridine (deaza-UR; Fig. 1), synthesized by Robins and Currie,[1] were found, in our laboratory, to be markedly active against *Escherichia coli*, *Streptococcus faecium*, leukemia L-1210, and Ehrlich ascites carcinoma (EAC) cells.[2] Since the metabolic conversion which nucleoside analogs undergo in the cell determines to a large extent the site at which they will interfere with the cell metabolism, the biotransformation of deaza-CR and deaza-UR was examined preliminary to further studies on their modes of action.

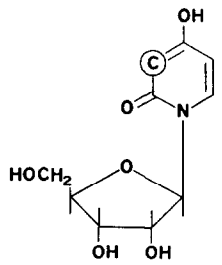

FIG. 1. Structural diagram of 3-deazauridine.

* This study was aided by Grant CA-12585 from the National Cancer Institute, U.S.-P.H.S.

MATERIALS AND METHODS

Materials

Deaza-CR, deaza-UR and 2′-deoxy-deaza-CR were generously provided by Dr. M. J. Robins of the Department of Chemistry, University of Alberta, Canada. Tritiated deaza-CR and deaza-UR were prepared according to the Wilzbach technique by New England Nuclear Corp., Boston, Mass. These tritiated compounds were purified by successive paper chromatography in the solvents A (or B), C, E and H (see below), and were used for experiments within 2 weeks after purification. This extensive purification was undertaken not only to insure the purity of the compounds, but also to remove, with the solvents employed throughout the study, any labile tritium which may have remained after initial removal. Uniformly labeled [^{14}C]-cytidine, [2-^{14}C]-uracil, [2-^{14}C]-uridine, and [γ-^{32}P]-ATP were obtained from the ICN Corp. Creatine phosphokinase was purchased from Sigma Chemical Company and snake venom (*Crotalus adamanteus*) was obtained from the Ross Allen Reptile Institute.

Cells

E. coli K12 cells, grown in the medium of Gray and Tatum,[3] and *S. faecium var. durans*, grown in the medium of Flynn *et al.*,[4] were cultured as previously described.[5]

L-1210 cells were maintained at 37° in 100-ml spinner flasks containing 60 ml RPMI No. 1630 medium[6] supplemented with 10 per cent calf serum, 62·4 mg penicillin and 66·0 mg streptomycin/liter of medium.

EAC cells were maintained by weekly intraperitoneal implantation of 5×10^6 cells into male Ha/ICR mice.

Cell-free extracts

The preparation of cell-free extracts was carried out at 4° as follows:

EAC extract. Tumor cells were collected 9–12 days after implantation. They were washed twice with cold 0·154 M saline, centrifuged at approximately 600 *g* for 1 min, and the supernatant was carefully removed by pipette. The tumor cells were then packed at 1500 *g* for 10 min, and the supernatant was discarded. To each gram of packed cells, 3 ml of 0·05 M tris–HCl buffer, pH 7·4, was added and the cells were suspended by mixing. They were then disrupted by exposure, for 20 sec, to a Bronson sonifier set at 5·8 amp, followed by centrifugation of the disrupted material at 105,000 *g* for 90 min. The supernatant solution beneath the floating lipid layer constituted the crude cell extract.

S. faecium extract. Cells grown for 18 hr at 37° were harvested by centrifugation and were washed once with 0·154 M saline. The cells were recentrifuged and the packed cells were frozen in dry ice and disrupted with a Hughes press. To each gram of disrupted cells, 3 ml tris–HCl buffer, pH 7·4, was added and, after mixing, the preparation was centrifuged at 29,500 *g* for 1 hr. The resulting precipitate was discarded and the supernatant (about 30 ml) was dialyzed overnight against 6 l. of 0·05 M tris–HCl buffer, pH 7·4.

E. coli extract. Cells grown with shaking at 37° were collected and processed as described for *S. faecium*. The disrupted cell preparation was centrifuged at 12,000 *g* and the supernatant fraction was mixed gently with one-tenth its volume of 5 per cent

streptomycin sulfate solution. After standing for 15 min in ice, the mixture was centrifuged at 29,500 *g* for 20 min. The precipitate was discarded and the supernatant was brought to 40 per cent saturation with ammonium sulfate. After gentle stirring for 15 min, the mixture was recentrifuged at 29,500 *g* and the supernatant thus obtained (20–25 ml) was dialyzed overnight against 4·1 of 0·05 M tris–HCl buffer, pH 7·4.

All extracts thus obtained were stored in 1-ml aliquots at −20°, and were used within 1 month after preparation. The protein content of the extracts was determined by the method of Lowry *et al.*[7]

Extraction of cells with perchloric acid

The cells were centrifuged at 4° (L-1210 and EAC cells at approximately 1500 *g* for 10 min; bacterial cells at 2000 *g* for 20 min) and, after discarding the medium, the cells were mixed with 2 vol. of cold 0·4 M perchloric acid and extracted for 15 min with occasional stirring. The mixture was centrifuged, and the precipitate re-extracted for 5 min with 1 vol. of cold 0·2 M perchloric acid, followed by centrifugation. Both extracts were pooled, neutralized with 4 M potassium hydroxide and centrifuged. The supernatant was then analyzed.

Termination of enzyme reactions

All enzyme reactions were terminated by heating the incubation mixtures in boiling water for 3 min, followed by removal of the precipitates by centrifugation at approximately 2000 *g* for 10 min.

Paper chromatography

For the chromatographic separation of reaction mixtures, Whatman 3 MM paper and the following solvent systems were used: system (A) *n*-butanol–glacial acetic acid–water (65:15:20, by vol.); (B) *n*-butanol–glacial acetic acid–water (50:20:30, by vol.); (C) upper phase of the mixture of ethyl acetate–*n*-propanol–water (57·2:14·3:28·5, by vol.); (D) 1 per cent sodium borate; (E) upper phase of the mixture *sec*-butanol–*tert*-butanol–water (43:8·6:48·4, by vol.); (F) 60 g ammonium sulfate and 2 ml *n*-propanol added to 100 ml of 0·1 M primary sodium phosphate, pH 6·8; (G) 95 per cent ethanol–1 M ammonium acetate containing 0·1 % EDTA (70:30, by vol.); (H) *n*-propanol–water (60:40, by vol.); (I) 70 g ammonium sulfate added to 100 ml water and 79 ml of this solution mixed with 19 ml water and 2 ml isopropanol. Descending chromatography was carried out where the solvents C, D and H were used; ascending chromatography was used with the other solvents.

Where available, appropriate carriers aiding the identification and localization of the reaction products were added to the mixtures prior to analysis.

Localization and elution of radioactive materials from paper chromatograms

After chromatography, 3-cm wide strips extending from the origin to the solvent front were cut from the chromatograms. These strips were divided into 1-cm segments and each segment was placed into a scintillation vial to which 5 ml toluene, containing 0·4 per cent 2,5-diphenyloxazole and 0·01 per cent 1,4-bis-2-(4-methyl 5-phenyl-oxazolyl)benzene,[8] was added. Radioactivity was measured in a Packard Tri-Carb model 3000 liquid scintillation counter. Those chromatographic bands corresponding

to segments containing radioactivity were eluted by cutting them into small pieces, and stirring them with 40-ml portions of distilled water for 15 min. The resulting pulp was filtered under suction, and the elution was repeated two more times. The combined eluates were evaporated to dryness at 30° in a rotary flash evaporator. The dried materials were taken up in 1–3 ml of water, and were filtered through Millipore filters to remove any remaining paper fibers.

Cleavage of the glycosidic bond

To test whether deaza-UR or deaza-CR are susceptible to cleavage of their glycosidic bond, 4·4 mM or 0·11 mM [^{3}H]-deaza-UR ($6{\cdot}2 \times 10^4$ counts/min/μmole) or 4·7 mM or 2·2 mM [^{3}H]-deaza-CR ($4{\cdot}0 \times 10^5$ counts/min/μmole) were incubated for 1 hr at 37° with 0·1 M phosphate buffer, pH 7·0, and 0·2 ml of either EAC (0·50 mg protein), *E. coli* (0·34 mg protein) or *S. faecium* (0·3 mg protein) extract in a total volume of 0·5 ml. After termination of the reaction, 0·05-ml aliquots of the mixtures were chromatographed after addition of the carriers. Solvent A was used for separation of the mixtures containing deaza-UR and solvent D for those containing deaza-CR. The respective R_f values in solvent A were: deaza-UR, 0·58; deaza-U, 0·83; in solvent D: deaza-CR, 0·80; deaza-C, 0·62. The susceptibility of the analogs to cleavage of their glycosidic bond was also evaluated by examining their ability to act as ribosyl donors. Uracil-2-^{14}C (1·2 mM; $2{\cdot}96 \times 10^4$ counts/min/μmole) was incubated together with EAC extract (0·50 mg protein) and with 5 mM each of UR or of one of the deaza-analogs, in a total volume of 0·5 ml of 0·04 M phosphate buffer, pH 7·0. After removal of heat-precipitated protein, 0·05-ml aliquots were chromatographed in solvent C, where the following R_f values apply: U = 0·38, UR = 0·22, deaza-UR = 0·26, and deaza-CR = 0·13.

Deamination

Two methods were used to examine whether deaza-CR is enzymatically deaminated. One method utilized the spectral difference between deaza-CR and deaza-UR at an acidic pH. At pH 4·0, deaza-CR and deaza-UR show a maximum difference in absorption at 257 nm. If deamination occurs, it is reflected in a decrease of absorbance at this wavelength. This method applies only at an acidic pH, for at pH 7·0 or above, the spectra of deaza-CR and deaza-UR are quite similar. Accordingly, after incubation of 1 mM deaza-CR for 2 hr at 37° with 0·01 ml *E. coli* extract (0·017 mg protein) in 0·05 M tris buffer, pH 7·4, in a total volume of 0·5 ml and removal of the protein, the incubation mixture was acidified to pH 4·0 and the spectra were determined.

The possible deamination of deaza-CR was also examined by the use of tritiated deaza-CR. The labeled compound (1 or 0·1 μmole; 6×10^4 counts/min/μmole) was incubated with *E. coli* extract under the same conditions as described above, and 0·05-ml aliquots of the incubation mixtures were chromatographed together with 0·1 μmole of the appropriate carriers in solvents A and C. Inhibition of cytidine deaminase activity by deaza-CR was measured by incubating 0·1 mM CR or 0·12 mM arabinosyl-cytosine together with 0·02 ml *E. coli* extract (0·034 mg protein) in 0·05 M tris–HCl buffer, pH 7·4, in a total volume of 1·2 ml. The rate of deamination was determined from the initial (straight line) decrease in absorbance at 282 nm[9] as measured with a Gilford recording spectrophotometer.

Phosphorylation

The phosphorylation of the 3-deazapyrimidine nucleosides was examined both *in vitro* and *in vivo*. The analysis *in vitro* proceeded as follows: 8 μmoles each of [^{3}H]-deaza-CR (6×10^4 counts/min/μmole) or [^{3}H]-deaza-UR ($1{\cdot}5 \times 10^4$ counts/min/μ-mole), [γ-^{32}P]-ATP ($5{\cdot}6 \times 10^4$ counts/min/μmole), and $MgCl_2$, and 50 μmoles of tris–HCl buffer, pH 7·4, were incubated for 1 hr at 37° together with 0·7 ml EAC extract (1·8 mg protein) in a total volume of 2 ml. After termination of the reaction, the supernatant solution was applied to the paper in a 5-in. wide band and was chromatographed in solvent F. The doubly labeled materials, which were centered around an R_f of 0·53 when deaza-UR was the precursor, and an R_f of 0·61 when deaza-CR was used, were eluted. (In solvent F, deaza-CR has an R_f of 0·42, deaza-UR of 0·46, and ATP of 0·38.) The eluates were rechromatographed in solvent G, and the distribution of radioactivity after chromatography is shown in Fig. 2. Differential counting of the two labels was carried out according to the method described by Okita *et al.*[10]

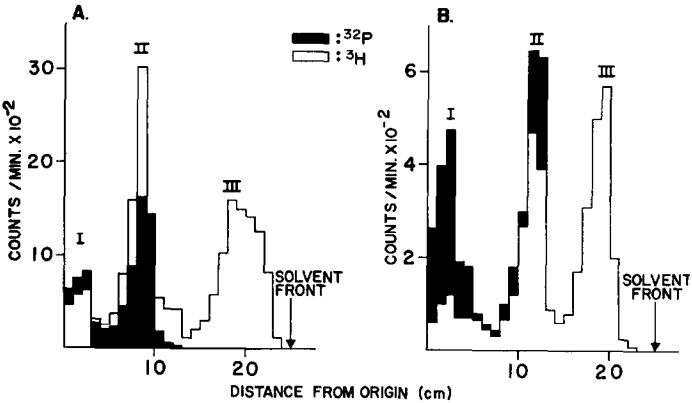

FIG. 2. Chromatographic separation and extent of labeling of derivatives of [^{3}H]-deazacytidine (A) and [^{3}H]-deazauridine (B) after their incubation together with [γ-^{32}P)]-ATP and Ehrlich ascites cell extract. Solvent: 95 per cent ethanol–1M ammonium acetate, containing 0·1% EDTA (70:30, by vol), ascending. Peaks I and II correspond to the nucleoside tri- and monophosphate derivatives respectively; peaks III, to the nucleoside analogs.

To confirm the assumption that the peaks I and II in Fig. 2A and B correspond to nucleotide derivatives of deaza-CR and deaza-UR, respectively, a larger amount of these materials was required, prepared as follows: Eight tubes, each containing 20 μmoles [^{3}H]-deaza-UR or [^{3}H]-deaza-CR, 10 μmoles ATP, 10 μmoles $MgCl_2$, 120 μmoles creatine phosphate, 390 μg phosphocreatine kinase (50 units/mg), 0·25 mole tris–HCl buffer, pH 7·4, and EAC extract (5·0 mg protein) in a total volume of 5 ml were incubated at 37° for 2 hr. After termination of the reactions, the mixtures were centrifuged at 2000 *g* for 5 min. The supernatant solutions were applied in bands to Whatman 3 MM paper, and were chromatographed in solvent G. The materials to be identified were eluted from the appropriate areas and were rechromatographed in solvent I to separate adenine nucleotides ($R_f \leqq 0{\cdot}50$) from the deazapyrimidine derivatives ($R_f \geqq 0{\cdot}60$). The latter were eluted from the paper with water, removed

from the residual salt by adsorption on charcoal, and released with a mixture of ethanol–NH_4OH–H_2O (5:1:4).[11] After filtration and evaporation, the material was placed on a 1 × 10 cm Dowex 50-H^+ column to remove any ammonium ions remaining, which appear to interact particularly with 3-deazauridine. The column was washed with 50 ml water, the effluent evaporated to dryness, and the residue taken up in 3 ml water. The deaza-CR or deaza-UR content of the compounds thus purified was determined by ultraviolet spectrometry, and their phosphorus content was determined by the method of Dryer *et al.*[12] The ultraviolet spectra thus obtained also served to confirm the identity of the compounds as deazapyrimidines. Aliquots of the eluted materials were treated with snake venom as follows: Approximately 12 n-moles of the labeled compounds were incubated for 12 hr at 37° with 0·6 mg snake venom in 0·05 M tris buffer, pH 7·4, in a total volume of 0·6 ml. After termination of the reaction by heating, 0·1 ml aliquots of the supernatant solution, together with deaza-CR, deaza-UR and 2′-deoxy-deaza-UR as carriers, were chromatographed in solvents A and E. In solvent A the untreated peak IA, IB, IIA, and IIB materials remained close to the origin ($R_f \leqq 0{\cdot}20$), whereas deaza-CR, 2′-deoxy-deaza-CR, deaza-UR and 2′-deoxy-deaza-UR had R_f values of 0·35, 0·49, 0·58 and 0·68 respectively.

The phosphorylation of 3-deazapyrimidine nucleosides in intact cells was examined with EAC, leukemia L-1210, and *E. coli* cells. EAC cells carried in mice or L-1210 cells in culture were harvested, washed twice with 0·154 M saline, and were centrifuged at 1500 *g* for 10 min. Then, 1-ml aliquots of the packed cells were mixed with 9 ml of RPMI No. 1630 medium containing 7 μmoles [^{3}H]-1deaza-UR and were incubated at 37° for 2 hr. The cells were collected by centrifugation, extracted with perchloric acid and neutralized as described above and, after removal of the salt, the extracts were evaporated to dryness *in vacuo*. The residues were taken up in 1·5-ml amounts of water, and 0·05 ml aliquots were chromatographed in solvent G. The remainder of the extracts was chromatographed in solvent A, and the material corresponding to the nucleotide analogs was eluted, treated with snake venom as described earlier, and the products were rechromatographed in solvent A. The identity of the 3-deazapyrimidine nucleosides after enzymatic digestion was further confirmed by rechromatography in solvents D and E. The respective R_f values in solvent D are: deaza-CR, 0·80; deaza-UR, 0·89; in solvent E: deaza-CR, 0·49; deaza-UR, 0·68.

To determine the phosphorylation of deaza-CR in *E. coli*, 8 ml of packed cells suspended in 200 ml medium containing 7 μmoles [^{3}H]-deaza-CR was incubated at 37° for 2 hr. The extraction and identification of the nucleotide derivatives present in the acid-soluble fraction proceeded as described for the mammalian cells.

Incorporation into nucleic acids

The possible incorporation of the deazanucleosides into the RNA and DNA of EAC cells was examined as follows: Mice were inoculated i.p. with 5 × 10^6 EAC cells. Seven days later, [^{3}H]-deaza-CR or [^{3}H]-deaza-UR, at the doses shown in Table 3, were injected i.p. into groups of two mice. The animals were killed 4 hr after administration of the drugs. The tumor cells were collected and were washed three times with 0·154 M NaCl, centrifugation being carried out at 600 *g* for 1-min periods. The cells were then packed at 2000 *g* for 10 min, and the acid-soluble and the RNA and DNA fractions were isolated from a total cell volume of 2 ml by the method of Caldwell and

Henderson.[13] Aliquots (0·5 ml) of each fraction were mixed with 10 ml Bray's scintillation fluid[14] and were counted. The concentrations of DNA and RNA were determined by the diphenylamine[15] and orcinol[16] reactions.

To examine the possible incorporation of 3-deaza-CR into the nucleic acids of *E. coli*, the *E. coli* cells were harvested in log phase by centrifugation, and enough cells to give a Klett reading of 100 were resuspended in 1 l. of medium to which 0·5 ml of 13 mM [^{3}H]-deaza-CR ($3{\cdot}6 \times 10^5$ counts/min/μmole) was added. The cell suspension was shaken for 2 hr, after which a Klett reading of 250 was recorded. After centrifugation, the cells were washed twice with saline, centrifuged at 2000 *g* for 20 min and the acid-soluble, and the RNA and DNA fractions were isolated as described above.

RESULTS

Cleavage of the glycosidic bond

The enzymatic cleavage of the glycosidic bond of CR by *L. pentosus* has been reported;[17] however, in the extracts which we prepared from *E. coli*, *S. faecium* and EAC, cleavage of CR was not detectable by the use of [^{14}C]-labeled CR. Nevertheless, the possibility that the CR analog, deaza-CR, might be subject to cleavage of the glycosidic bond was considered. After incubation of [^{3}H]-deaza-CR with extracts of the three cell types, only deaza-CR was detectable by paper chromatography. Thus, the glycosidic bond of deaza-CR, like that of CR, was not cleaved by these cell extracts. In contrast, after 1 hr of incubation with either EAC, *E. coli* or *S. faecium* extract, [2-^{14}C]-uridine was found cleaved to the extent of 80, 50 and 10 per cent respectively. Under these conditions, no cleavage of [^{3}H]-deaza-UR was detectable.

To support the finding that the deazanucleosides are not subject to cleavage of the glycosidic bond, the analogs were also examined for their potential ability to donate their ribosyl group to [2-^{14}C]-uracil for the synthesis of uridine.[18] When uracil-2-^{14}C

TABLE 1. FAILURE OF DEAZACYTIDINE AND DEAZAURIDINE TO SUPPORT THE SYNTHESIS OF URIDINE FROM URACIL

Incubation mixture*	Concentration of [2-^{14}C]-uridine formed (mM)
Ehrlich ascites + [2-^{14}C] uracil cell extract	0·02
Ehrlich ascites + [2-^{14}C] uracil cell extract + uridine	0·40
Ehrlich ascites + [2-^{14}C] uracil cell extract + deazacytidine	0·03
Ehrlich ascites + [2-^{14}C] uracil cell extract + deazauridine	0·02
Ehrlich ascites + [2-^{14}C] uracil cell extract + uridine + deazacytidine	0·40
Ehrlich ascites + [2-^{14}C] uracil cell extract + uridine + deazauridine	0·39

* The incubation mixture contained Ehrlich ascites extract (0·5 mg protein), 1·2 mM [2-^{14}C]-uracil ($2{\cdot}96 \times 10^4$ counts/min/μmole) and 5 mM uridine and/or the deaza analog, in 0·04 M phosphate buffer, pH 7·0, in a total volume of 0·5 ml. The mixture was incubated for 1 hr at 37°.

at a concentration of 1·2 mM was incubated for 1 hr at 37° in the presence of EAC extract and 5 mM UR or either of the deaza analogs, the ribosyl moiety was found transferred from UR to approximately 0·4 mM (33 per cent) of the labeled base supplied (Table 1). No transfer took place from deaza-UR or deaza-CR. The transfer of the ribose from UR to [^{14}C]-U was not interfered with when either analog was present at 5 mM. It should be emphasized, however, that in the systems used, CR itself did not undergo cleavage of its glycosidic bond or transfer of its ribosyl moiety to uracil, and the findings with deaza-CR do not, therefore, establish unequivocally that the analog cannot serve as a substrate in these reactions.

Deamination

Since *E. coli* extract catalyzes the rapid deamination of cytidine, the susceptibility of deaza-CR to deamination was examined in this system, both spectrophotometrically and by the use of labeled CR and deaza-CR. Incubation of deaza-CR with the bacterial extract for 2 hr did not lead to any detectable deamination of 1 or 0·1 μmole of the analog. Under identical conditions, CR was completely converted to UR.

Although not a substrate of CR deaminase, deaza-CR does, apparently, bind to the enzyme to some extent, as shown by its interference with the deamination of both CR and arabinosyl cytosine. At concentrations equal to or twice the concentration of these substrates (0·10 and 0·12 mM respectively), deaza-CR inhibited their deamination from 21 to 34 per cent (Table 2).

Phosphorylation

As shown in Fig. 2A and B, incubation of [^{3}H]-labeled deaza-UR or deaza-CR with EAC extract in the presence of [γ-^{32}P]-ATP resulted, in both instances, in the formation of two intermediates (peaks I and II) containing both [^{3}H] and [^{32}P] activity. The third peak (III) contained only [^{3}H] activity and, as shown chromatographically and spectrophotometrically, corresponds to deaza-CR or deaza-UR respectively. The ratio of ^{32}P/^{3}H of peak I to that of peak II was 2·8 when [^{3}H]-deaza-CR was the substrate and 3·1 when [^{3}H]-deaza-UR was used. The magnitude

TABLE 2. INHIBITION OF CYTIDINE DEAMINASE BY DEAZACYTIDINE*

Concentration of			Rate of deamination (nmoles/min)	Per cent inhibition
Cytidine (mM)	Arabinosylcytosine (mM) in incubation material	Deazacytidine (mM)		
0·10			16·8	
0·10		0·10	13·3	21
0·10		0·20	11·9	29
	0·12		1·5	
	0·12	0·12	1·1	21
	0·12	0·24	0·9	34

* The compounds were incubated at 37° together with 0·02 ml of *E. coli* extract (0·034 mg protein) in 0·05 M tris–HCl buffer, pH 7·4, in a total volume of 0·5 ml. Progress of the reactions was monitored at 282 nm with a Gilford model 2000 recording spectrophotometer, and the rates of deamination were calculated from initial slopes. The values given are the averages of triplicate determinations.

of the counts used for this comparison is indicated in Fig. 2. The purified peak I and peak II materials showed the characteristic spectra of 3-deaza-CR or 3-deaza-UR. Colorimetric assay[12] of the moles of phosphate present per mole of analog in peak I gave an average value of 2·9 for deaza-CR and 2·8 for deaza-UR. In peak II, the corresponding average values were 1·0 for deaza-CR and deaza-UR. Incubation of the phosphate-containing compounds with snake venom gave [^{3}H]-labeled 3-deazauridine or 3-deazacytidine, respectively, as confirmed by co-chromatography with authentic material in solvents A and E. Taken together, these data clearly indicate that mono- and triphosphate derivatives of the nucleoside analogs were formed. The slightly lower than theoretical values obtained for the ratio of phosphate to base of the compounds in the peaks IA and IB could be due to the presence of small amounts of diphosphate derivatives of the analogs. Such a presence, close to peak I, is also suggested by the radioactive profiles in Fig. 2. However, their small concentration prevented separate analysis.

Under the restricted conditions used in the experiments *in vitro*, the formation of deoxyribonucleotide derivatives was not expected, and no deoxyribonucleosides were encountered in the snake venom digests of eluates from the chromatographic area comprising all nucleotides and ranging from the origin to peak III.

To examine the phosphorylation of deaza-UR in intact cells, L-1210 and Ehrlich ascites cells were chosen, because the analog is more active in these systems than is deaza-CR. In contrast, the phosphorylation of deaza-CR was followed in *E. coli*, where deaza-CR is a more potent inhibitor than is deaza-UR. Chromatographic analysis of the acid-soluble extracts obtained from all these cell lines after incubation with either [^{3}H]-deaza-UR or [^{3}H]-deaza-CR showed that the labeled derivatives formed from the analogs by the intact cells had the same R_f values as the corresponding nucleoside mono- and triphosphate derivatives analyzed in detail following their isolation from cell-free extracts (see above). Enzymatic digestion of the derivatives furnished the nucleoside analogs, further confirming the identity of the compounds as the nucleotide derivatives of the 3-deazapyrimidines.

In snake venom digests of the radioactive nucleotides present in the acid-soluble fraction of *E. coli* cells treated with labeled 3-deaza-CR, an additional nucleoside was found containing approximately 15 per cent of the total radioactivity applied. In solvents A, D and E, this compound had the same R_f values (0·49, 0·74 and 0·57) as did synthetic 2′-deoxy-3-deaza-CR (the corresponding R_f values for deaza-CR are 0·35, 0·80 and 0·49), indicating that enzymatic reduction of the analog, presumably at the nucleotide stage, had occurred. The extent of phosphorylation of this deoxyribonucleoside derivative has not as yet been determined.

Incorporation into nucleic acids

In view of the metabolic formation of nucleotides of both 3-deaza-UR and 3-deaza-CR, their possible incorporation into the nucleic acids was examined. The tritiated analogs were injected i.p. into mice bearing EAC cells, and were also supplied to growing cultures of *E. coli*. No radioactivity was found in either the RNA or DNA of these cells, although under the conditions of incubation used approximately 70 and 55 per cent, respectively, of the labeled material in the acid-soluble fraction of EAC and *E. coli* appeared as the nucleoside triphosphates (Table 3).

TABLE 3. METABOLIC DISPOSITION OF DEAZAPYRIMIDINES IN EHRLICH ASCITES AND *E. coli* CELLS*

Cell type	Nucleoside	Amount of radioactivity in: Acid-soluble fraction (counts/min/g cells)	RNA (counts/min/mg)	DNA (counts/min/mg)
Ehrlich ascites	[^{14}C]-cytidine	3624	5210	3932
	[^{3}H]-deazacytidine	25,328	0	0
	[^{3}H]-deazauridine	3256	0	0
E. coli	[^{14}C]-cytidine	870	651	646
	[^{3}H]-deazacytidine	4071	0	0

* Seven days after i.p. inoculation of 5×10^6 Ehrlich ascites cells, each of two mice was injected with either 7·6 μmoles of uniformly labeled cytidine (6×10^4 counts/min/μmole), 8·3 μmoles [^{3}H]-deazacytidine ($1{\cdot}1 \times 10^6$ counts/min/μmole) or 5·4 μmoles [^{3}H]-deazauridine ($7{\cdot}4 \times 10^4$ counts/min/μmole). The mice were sacrificed after 4 hr, and the tumor cells were collected and washed twice with saline. The acid-soluble fraction, RNA and DNA were isolated by the method of Caldwell and Henderson,[13] and the radioactivity in each fraction was determined on 0·5-ml aliquots. The concentration of DNA was determined with diphenylamine[15] and that of RNA by the orcinol reaction.[16] The metabolic conversion of the analogs in *E. coli* was determined in the same way after incubation of 1 l. of a growing culture, for 2 hr, with 6·5 μmoles [^{3}H]-deazacytidine ($3{\cdot}6 \times 10^5$ counts/min/μmole).

DISCUSSION

Analogs of pyrimidines modified in their heterocycle by the replacement of a carbon by a nitrogen atom, have proved to be potent inhibitors of a variety of experimental tumors. Among such active triazines are 5-azacytidine and 6-azauridine.[19–23] The reverse replacement, that of a nitrogen by a carbon atom, can also give rise to inhibitors, as shown by the pronounced interference of the 3-deazapyrimidines with microbial and tumor cell growth.[2] This inhibitory effect is prevented, competitively, by the natural pyrimidines,[2] demonstrating the antimetabolite nature of the analogs.

The significant antitumor activity of the compounds made it desirable to obtain information concerning their modes of action, and the present investigation, dealing with the biotransformation of the analogs, provides a foundation for the further studies.

Like many other nucleoside analogs,[24] the 3-deazapyrimidines undergo phosphorylation and their inhibitory activity is, likely, exerted at the nucleotide stage, at which stage the analogs accumulate in the cell. From the point of view of structure as it relates to activity, it is noteworthy that, upon replacement of the nitrogen atom at position 3 of the pyrimidine ring with a carbon, cleavage of the glycosidic bond does not occur. Similarly, the nitrogen in that position apparently participates in determining substrate specificity for cytidine deaminase, as shown by the fact that 3-deaza-CR was not deaminated by the enzyme. Nitrogen-3 may constitute an essential binding site for these enzymes, or its ability to accept a proton from the 4-hydroxy or 4-amino groups of uridine or cytidine, respectively, may be required for catalysis. On the other hand, the absence of N-3 does not abolish the capacity of deaza-UR or deaza-CR to act as substrates for the appropriate nucleoside and nucleotide kinases. Furthermore, as shown by the formation of a 2′-deoxy derivative of 3-deaza-CR in *E. coli*, replacement of N-3 by carbon does not preclude substrate activity for, at least, the bacterial nucleotide reductase. The conversion of 3-deaza-UR to its 2′-deoxy derivative in the

bacterial system, and the reduction of both 3-deaza-UR and 3-deaza-CR in the tumor cells remain to be demonstrated. The low deoxyribonucleotide levels encountered in the mammalian cells make this demonstration difficult.

The lack of incorporation of both 3-deaza-UR and 3-deaza-CR into RNA, in spite of their extensive conversion to the triphosphate, indicates that the absence of N-3 precludes catalysis by RNA-polymerase. The finding of a deoxyribonucleotide derivative of 3-deaza-CR from *E. coli*, without concomitant incorporation of the analog into DNA, similarly suggests that the lack of N-3 precludes catalysis by DNA-polymerase.

REFERENCES

1. M. J. Robins and B. L. Currie, *Chem. Commun.* **2,** 1547 (1968).
2. M. J. Robins, B. L. Currie, R. K. Robins and A. Bloch, *Proc. Am Ass. Cancer Res.* **10,** 73 (1969).
3. C. H. Gray and E. L. Tatum, *Proc. natn. Acad. Sci. U.S.A.* **30,** 404 (1944).
4. L. N. Flynn, V. B. Williams, B. L. O'Dell and A. G. Hogan, *Analyt. Chem.* **23,** 180 (1951).
5. A. Bloch, *Biochim. biophys. Acta* **201,** 323 (1970).
6. G. E. Moore, A. A. Sandberg and K. J. Ulrich, *J. natn. Cancer Inst.* **36,** 405 (1966).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
8. R. J. Mans and G. D. Novelli, *Archs. Biochem. Biophys.* **94,** 48 (1961).
9. T. P. Wang, H. Z. Sable and J. O. Lampen, *J. biol. Chem.* **184,** 17 (1950).
10. G. T. Okita, J. J. Kabara, F. Richardson and G. V. Leroy, *Nucleonics* **15,** 111 (1957).
11. J. Mager and B. Magasanik, *J. biol. Chem.* **235,** 1474 (1960).
12. R. L. Dryer, A. R. Tammes and J. I. Routh, *J. biol. Chem.* **225,** 177 (1957).
13. I. C. Caldwell and J. F. Henderson, *Analyt. Biochem.* **34,** 303 (1970).
14. G. A. Bray, *Analyt. Biochem.* **1,** 279 (1970).
15. F. B. Seibert, *J. biol. Chem.* **133,** 593 (1940).
16. Z. Dische and K. Schwarz, *Mikrochim. Acta* **2,** 13 (1937).
17. T. P. Wang, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 2, p. 456. Academic Press, New York (1955).
18. A. R. P. Paterson, *Can. J. Biochem. Physiol.* **43,** 257 (1964).
19. F. Sorm, A. Piskala, A. Cihak and J. Vesely, *Experientia* **20,** 202 (1964).
20. F. Sorm and J. Vesely, *Neoplasma* **11,** 123 (1964).
21. J. J. Jaffe, R. E. Handschumacher and A. D. Welch, *Yale J. Biol. Med.* **30,** 168 (1957).
22. F. Sorm and H. Keilova, *Experientia* **14,** 215 (1958).
23. W. H. Prusoff, *Biochem. Pharmac.* **2,** 221 (1959).
24. J. J. Fox, K. A. Watanabe and A. Bloch, *Prog. nucleic Acid Res.* **5,** 251 (1966).