Fluorinated Pyrimidine Aldehydes. Inhibition of Nucleic Acid and Protein Biosynthesis in Ehrlich Ascites Carcinoma Cells *in Vitro*[1]

CHI-BOM CHAE,[2a] J. LOGAN IRVIN,[2b] AND CLAUDE PIANTADOSI

Departments of Biochemistry and Medicinal Chemistry, University of North Carolina, Chapel Hill, North Carolina 27514

Received December 8, 1967

A study was made of the possible mechanisms of inhibition of the growth of Ehrlich ascites carcinoma by the following pyrimidine analogs: 5-fluoroorotic aldehyde (III), 2-thio-5-fluoroorotic aldehyde (IV), 2-methylmercapto-5-fluoroorotic aldehyde (V), 2-ethylmercapto-5-fluoroorotic aldehyde (VI), and 6-(2-thio-4-oxy-6-pyrimidylmethylidene)-5′-oxo-2′-phenyloxazoline (VII). Among the pyrimidine analogs tested only VI and VII inhibited incorporation of orotic acid-6-^{14}C into RNA and DNA *in vitro*. The inhibition of the conversion of orotic acid to orotidylic acid was the main cause of the inhibition of RNA synthesis. The incorporation of formate-^{14}C into purines of RNA was inhibited by the compounds III, IV, V, and VI in contrast to the lack of effect of 5-fluorouracil (FU). These analogs, III–VI, also inhibited incorporation of formate-^{14}C and thymidine-^{3}H into DNA–thymine, but FU inhibited formate incorporation only. The incorporation of formate-^{14}C, glycine-1-^{14}C, and phenylalanine-1-^{14}C into proteins was inhibited markedly by the analogs III–VI and 2-thioorotic aldehyde (II).

We have previously reported on the synthesis of antitumor derivatives of 6-pyrimidinecarboxaldehydes: Schiff bases, hydrazones,[3] methylidenerhodanines, α-keto acids, α-thioketo acids, and α-oximino acids.[4]

Introduction of a fluorine atom at C-5 of the pyrimidine ring of 6-pyrimidinecarboxaldehyde markedly increased the antitumor activity.[5] Walker[6] reported that 6-(2-thio-4-oxy-6-pyrimidylmethylidene)-5′-oxo-2′-phenyloxazoline is another derivative of the pyrimidine aldehyde with potent antitumor activity against Ehrlich ascites carcinoma.

Heidelberger, *et al.*,[7] found that 5-fluorouracil, its nucleoside (FUR),[8] and its deoxynucleoside (FdUR) inhibit the synthesis of RNA and DNA. The principal cause of the inhibition of DNA synthesis is the block of the conversion (by thymidylate synthetase) of dUMP to TMP by FdUMP.[9] The possible site involved in the inhibition of RNA synthesis is uridinephosphorylase.[10] However, FU and its derivatives do not inhibit incorporation of formate into purines of nucleic acids[7] and of amino acids into proteins.[11]

The present paper is concerned with a study of the effects of the derivatives of 4-oxy-5-fluoro-6-pyrimidinecarboxaldehyde (5-fluoroorotic aldehyde) on the synthesis of nucleic acids and proteins in Ehrlich ascites carcinoma cells *in vitro*. Data are presented on the inhibition of the synthesis of RNA-purines and -pyrimidines, DNA-thymine, and proteins, and comparisons are made between these derivatives of 5-fluoro-6-pyrimidinecarboxaldehyde and FU in the inhibition of nucleic acid and protein synthesis. A preliminary account of this work has appeared elsewhere.[12]

Experimental Section

Ehrlich ascites carcinoma cells were harvested from female Swiss-Webster mice 8 days after transplantation. The ascitic fluid was centrifuged in an International refrigerated centrifuge, Model PR-2, for 10 min at 2000 rpm. The supernatant fluid was discarded, and the precipitated cells were washed three times with KRP buffer, pH 7.1–7.25.

Into 50-ml erlenmeyer flasks were added 5 ml of a 40% (v/v) suspension of the washed cells in KRP buffer and 1 ml of KRP buffer containing 3 mg of glucose, and the flasks were equilibrated for 5 min at 37° in a Dubnoff shaker at a rate of 90 cycles/min. Then 0.1 ml of DMSO and a pyrimidine analog dissolved in 0.1 ml of DMSO were added to control and experimental flasks, respectively. After shaking for an additional period of 5 min, 1.0 ml of a labeled precursor in KRP buffer was added to each flask, and the flasks were shaken in air for 1 hr. All groups of controls and experimentals were run at least in duplicate. After incubation, the cells were centrifuged and washed twice with cold 0.9% NaCl as quickly as possible. The acid-soluble fraction (ASF) was extracted by homogenization of the cells in 10 ml of cold 1 *N* PCA after removal of the acid-insoluble fraction by centrifugation. The ASF was neutralized with KOH. Sodium nucleates were prepared from the acid-insoluble fraction according to the procedure of Danneberg, *et al.*,[7a] and hydrolyzed with 1.5 ml of 0.3 *N* KOH at 37° for 16–18 hr.[13]

DNA and $KClO_4$ were precipitated by addition of 0.2 ml of cold 6 *N* PCA, and DNA was purified by three cycles of solution in dilute NaOH and precipitation with PCA. The purified DNA was dissolved in 1.5 ml of 0.3 *N* NaOH, and the concentration of DNA was determined spectrophotometrically at 268 mμ after

(1) This investigation was supported by grants from the National Institutes of Health, Public Health Service (CA-06346-06 and GM08318-07). Some of the equipment used in this research was obtained by means of a departmental equipment grant from the National Science Foundation (GB-4577).

(2) (a) A portion of this work was taken from a dissertation submitted by C.-B. Chae to the University of North Carolina in partial fulfillment of the requirements of the Ph.D. degree. (b) To whom inquiries should be sent.

(3) C. Piantadosi, V. G. Skulason, J. L. Irvin, J. M. Powell, and L. Hall, *J. Med. Chem.*, **7**, 337 (1964).

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(5) C. S. Kim, Ph.D. Thesis, University of North Carolina, Chapel Hill, N. C., 1967.

(6) E. Walker, M.S. Thesis, University of North Carolina, Chapel Hill, N. C., 1967.

(7) (a) P. B. Danneberg, B. J. Montag, and C. Heidelberger, *Cancer Res.*, **18**, 329 (1958); (b) L. Bosch, E. Harbers, and C. Heidelberger, *ibid.*, **18**, 335 (1958).

(8) Abbreviations: AICAr = 5-amino-4-imidazolecarboxamide riboside; AICAR = 5-amino-4-imidazolecarboxamide ribotide; ASF = acid-soluble fraction, AzU = 6-azauracil; AzUR = 6-azauridine; CMP, CDP, CTP = cytidine mono-, di-, and triphosphates; DNA = deoxyribonucleic acid; DMSO = dimethyl sulfoxide; FAICAR = 5-formamido-4-imidazolecarboxamide ribotide; FU = 5-fluorouracil; FUR = 5-fluorouridine; FdUR = 5-fluorodeoxyuridine; FdUMP = 5-fluorodeoxyuridylic acid; GAR = glycinamide ribotide; KRP = Krebs-Ringer phosphate; OA = orotic acid; OMP = orotidylic acid; OR = orotidine; PCA = perchloric acid; PEI = polyethylenimine; RNA = ribonucleic acid; TCA = trichloroacetic acid; tlc = thin layer chromatography; TdR, TMP, TDP, TTP = thymidine and thymidine mono-, di-, and triphosphates, respectively; UMP, UDP, UTP = uridine mono-, di-, and triphosphates, respectively.

(9) J. G. Flaks and S. S. Cohen, *J. Biol. Chem.*, **234**, 2981 (1959).

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(13) J. N. Davidson and R. M. S. Smellie, *Biochem. J.*, **52**, 594 (1952).

hydrolysis in 1 N PCA.[14] A portion (less than 0.5 ml) of the DNA solution in 0.3 N NaOH and an equal volume of Hyamine Hydroxide 10-X[15] were added to a glass scintillation vial, and 16 ml of POPOP–PPO Triton X-100[16] scintillation fluid was added for determination of radioactivity in a Packard liquid scintillation spectrometer, Model 3003.

Separation of DNA Bases.—Two-thirds of the total quantity of DNA was hydrolyzed with 2 ml of 70% PCA at 100° for 1.5 hr,[17] and the solution was diluted to 7 ml with H_2O. KOH (10 N) was added in a volume which neutralized one-half of the PCA, and the suspension of $KClO_4$ was clarified by centrifugation. The purine and pyrimdine bases were adsorbed on 100 mg of acid-washed Norit A[18] at 25° and eluted with 10 ml of EtOH–NH_4OH–H_2O (2:1:2)[19] overnight at 37°. The mixture was dried and separated by one-dimensional cellulose thin layer chromatography (tlc)[20] with i-PrOH–concentrated HCl–H_2O (170:41:39, v/v),[21] and the total development time for the distance of 10 cm was 2.5 hr. The R_f values were the same as those obtained with Whatman No. 1 filter paper:[21] orthophosphate, 0.92; thymine, 0.82; cytosine, 0.49; adenine 0.38; guanine, 0.26. The spots (or bands) of interest were located with a uv lamp, and the thin layer material at the site of the spots was scraped into glass centrifuge tubes and eluted with 0.1 N HCl at room temperature for 2–4 hr. The concentrations, identity, and purity of the bases were established by the ratio of the absorbance at 250/260 and 280/260 mμ and by comparison with R_f values of known compounds. The radioactivity of the bases was determined in a liquid scintillation spectrometer with a scintillation medium consisting of POPOP–PPO–Triton X-100. The specific activities of the bases were computed as cpm/μmole.

Separation of the Monophosphates of RNA Hydrolysates.—The supernatnat fluid obtained after the acidification of the 0.3 N KOH hydrolysates of nucleates was neutralized (pH 7–8) with 10 N KOH, and $KClO_4$ was removed by centrifugation. The 2′ + 3′ purine and pyrimidine monophosphates were separated from this solution by PEI-cellulose tlc according to Randerath and Randerath.[22] The spots (or bands) of interest were scraped into glass centrifuge tubes and eluted with 1 M LiCl at room temperature for 4–6 hr. The concentration of each 2′ + 3′ monophosphate was computed from the absorbancy at its absorption maximum. The concentration of RNA–P was calculated from the absorbancy at 260 mμ.[14]

Protein.—For the determination of protein the residue left from the hot NaCl extract was suspended in 5 ml of 1 N PCA and heated for 1 hr at 95°. Then the protein was washed as follows: once with 95% EtOH, twice with 0.5 N PCA, and once with 0.1 N PCA. The washed protein was dissolved in 4 ml of 0.3 N NaOH. The quantity of protein was determined by biuret reaction.[23] The radioactivity of protein was detrmined by a procedure similar to that described for DNA.

Double-Isotope Labeling Experiment.—The incubation flasks containing an equivalent of 0.5 ml packed-volume of cells in KRP buffer containing glucose were divided into two groups. To the "control" flasks were added 0.1 ml of DMSO and the ^{3}H-labeled precursor in KRP buffer. Also, in each "control" series one or two flasks received the ^{14}C-labeled precursor, and these ^{14}C-labeled controls subsequently were paired with ^{3}H labeled controls to obtain the control ^{14}C/^{3}H ratios for the nucleic acids and the various acid-soluble nucleotides. The "experimental" flasks received 0.1 ml of the pyrimidine analog in DMSO and ^{14}C-labeled precursor in KRP buffer. These ^{14}C-labeled experimentals subsequently were paired with ^{3}H-labeled controls. At the end of incubation, the contents of ^{14}C-flasks and ^{3}H-flasks were combined and centrifuged immediately. The ^{14}C,^{3}H-cells were washed twice with cold 0.9% NaCl and homogenized in 10 ml of cold 5% TCA as described previously. The ^{14}C,^{3}H-ASF was extracted repeatedly with ether to remove TCA, and the TCA-free aqueous phase was lyophilized. The lyophilized residue was dissolved in 0.4 ml of distilled H_2O (ASF′). PEI-cellulose plates (20 × 20 cm; thickness, 0.5 mm) were prepared and washed with 5% NaCl according to Randerath and Randerath.[24] Samples of ASF′ were applied once to a starting line as closely adjacent spots (diameter, 3 mm), and marker solutions containing known nucleotides were applied to the same spots previously loaded with ASF′ and to vacant positions on the starting line (see Figures 2 and 3). Ascending chromatography was carried out in closed tanks filled with solvent to a height of 1 cm.

A. Separation of UTP, CTP, UDP, and OMP.—A solvent mixture consisting of 2 N HCO_2H–1.6 M LiCl (1:1, v/v) was used. The total development time was about 40 min (Figure 2).

B. Separation of CDP, OA + UMP, OR, and CMP.—The loaded plates were treated with 500 ml of anhydrous MeOH[24] for 5 min in a flat dish, and the dried plates were transferred to the tanks containing a mixture of 2 N HCO_2H–0.2 M LiCl (1:1, v/v). The total development time was about 30 min (Figure 3). The treatment with MeOH removed uridine from the plates, but it gave better separations from the thick band following behind the bands for OA + UMP and OR.

After the solvent front had reached 10–12 cm above the starting line, the plates were dried immediately in a stream of warm air. The spots (or bands) of interest were scraped into glass scintillation vials directly, and the nucleotides were eluted with 1–1.5 ml of 1 M LiCl for 2–4 hr at room temperature. Then 18 ml of POPOP–PPO–Triton X-100 medium was added. The radioactivities of ^{14}C and ^{3}H were determined simultaneously according to Karbara, *et al.*,[25] in a Packard liquid scintillation spectrometer Model 3003. After a cycle of counting, the internal standard (^{14}C-toluene or ^{3}H-toluene) of known radioactivity was added to the vial which had been counted, and the radioactivity of the standard + sample was determined. This procedure gave accurate channel ratios for ^{14}C and ^{3}H and permitted calculation of the counting efficiency of the ^{14}C and ^{3}H of a sample. The counting efficiency of ^{14}C was not affected by the presence of the thin layer particles but that of ^{3}H was reduced by 1–2%.

^{3}H,^{14}C-RNA was obtained from the acid-insoluble fraction obtained after the homogenization of the mixed ^{14}C and ^{3}H cells.

Results and Discussion

Inhibition of Pyrimidine Incorporation into Nucleic Acids.—Among the pyrimidine analogs tested (Table I) only VI and VII showed appreciable inhibition of incorporation of orotic acid-6-^{14}C into RNA and DNA as shown in Table II. Compound VI inhibited the conversion of orotic acid into RNA by approximately 45% and into DNA by 40% at a concentration of 0.23–0.46 mM. The 2-ethylmercapto group of VI seems to be important in the inhibitory activity of VI, since compounds III, IV, and V showed no inhibitory activity at comparable concentrations. On the other hand, compound II which has the 2-ethylmercapto group but lacks the 5-fluoro group also was ineffective in inhibiting incorporation of orotic acid at 0.46 mM concentration. Consequently, it appears that both of these groups are important in enhancing inhibitory activity.

Compound VII showed about the same degree of inhibition of RNA and DNA synthesis as VI at 0.31 mM, but RNA synthesis was inhibited to a greater extent than DNA at 0.51 mM. The phenyl group on the azlactone ring enhances inhibitory activity while substitution of a methyl group at position 5 decreases the inhibitory effect against orotic acid incorporation.

(14) J. E. Logan, W. A. Mannell, and R. J. Rossister, *Biochem. J.*, **51**, 470 (1952).

(15) J. M. Passmann, N. S. Radin, and J. A. D. Cooper, *Anal. Chem.*, **28**, 484 (1956).

(16) In 1 l. of toluene 5 g of 2,5-diphenyloxazole (PPO), 0.3 g of 1,4-bis-(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP) was dissolved. Two liters of this POPOP–PPO solution and 1 l. of Triton X-100 were mixed.

(17) A. Marshak and H. G. Vogel, *J. Biol. Chem.*, **189**, 597 (1951).

(18) K. K. Tsuboi and T. D. Price, *Arch. Biochem. Biophys.*, **81**, 223 (1959).

(19) K. Kusama and E. Roberts, *Biochemistry*, **2**, 573 (1963).

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(24) K. Randerath and E. Randerath, *J. Chromatog.*, **16**, 111 (1964).

(25) J. J. Karbara, N. R. Spafford, M. A. Mekendry, and N. L. Freeman, *Advan. Tracer Methodol.*, **1**, 76 (1963).

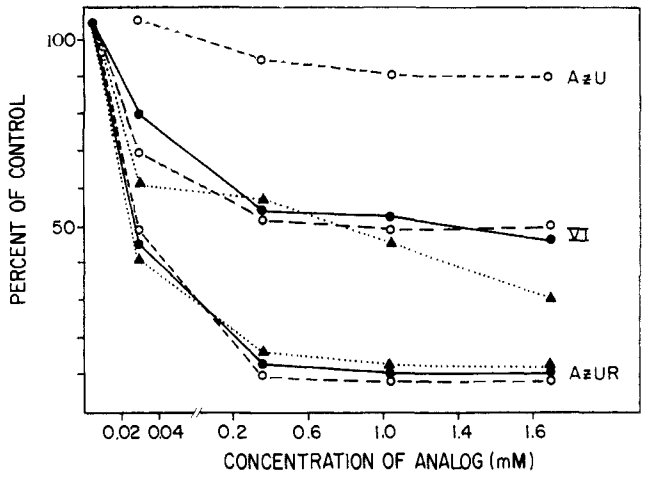


Figure 1.—Effects of analogs on the incorporation of orotic acid-5-^{3}H into RNA of Ehrlich ascites carcinoma cells *in vitro*. Incubation proceeded for 1 hr at 37°. ○ = RNA, ● = 2′ + 3′ UMP, ▲ = 2′ + 3′ CMP.

TABLE I

CHEMICAL STRUCTURES OF DERIVATIVES OF PYRIMIDINE-6-CARBOXALDEHYDE

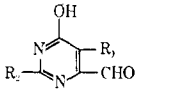
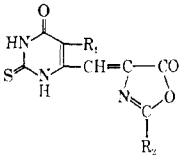
Structure	No.	R_1	R_2
	I	H	SH
	II	H	SCH_2CH_3
	III	F	OH
	IV	F	SH
	V	F	SCH_3
	VI	F	SCH_2CH_3
	VII	H	C_6H_5
	VIII	H	p-$C_6H_4C_6H_5$
	IX	H	p-C_6H_4Cl
	X	H	CH_3
	XI	CH_3	C_6H_5
	XII	CH_3	p-C_6H_4F

TABLE II

EFFECTS OF VARIOUS PYRIMIDINE ANALOGS ON INCORPORATION OF OROTIC ACID-6-^{14}C[a] INTO RNA AND DNA OF EHRLICH ASCITES CARCINOMA CELLS

Analog	Concn of analog, mM	Av exptl values (% of controls) RNA	DNA	ASF
None	0	100[b]	100[c]	100[d]
VI	0.23	57	64	78
	0.46	54	58	70
VII	0.31	57	70	71
	0.51	39	62	74
FU	0.28	92	23	94
	0.57	90	22	86

[a] Orotic acid-6-^{14}C: 0.028 mM, 2.5 μCi. [b] 1.68×10^3 cpm/mg of RNA-P. [c] 59 cpm/mg of DNA-P. [d] 3.12×10^4 cpm.

In Figure 1 a comparison is made between compound VI and AzUR and AzU. The incorporation of orotic acid-5-^{3}H into uracil and cytosine of RNA was inhibited to the same degree by compound VI up to a concentration of 1 mM, but at higher concentrations the inhibition of incorporation into RNA-cytosine was somewhat greater than the inhibition of incorporation into RNA-uracil. AzU showed no appreciable inhibition as reported previously,[26] but AzUR was more effective than compound VI with concentrations for 50% inhibition of 0.03 and 0.4 mM, respectively.

(26) (a) R. Schindler and A. D. Welch, *Science*, **125**, 548 (1957); (b) R. Schindler, *Biochem. Pharmacol.*, **1**, 132 (1958).

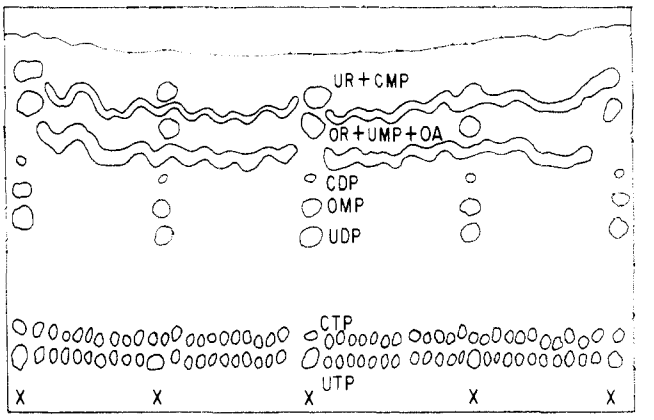


Figure 2.—Anion-exchange thin layer chromatogram of acid-soluble nucleotides of Ehrlich ascites carcinoma cells (0.5-mm thick PEI-cellulose layer). A lyophilized TCA extract of Ehrlich ascites cells in 0.4 ml of water (1 ml packed cells) was applied once. The diameter of the spots was 3 mm. To the starting spots, X, were applied the standard nucleotide solutions. Solvent: 2 N HCO_2H–1.6 M LiCl (1:1, v/v).

When uracil-2-^{14}C was used as a precursor, compounds VI and VII produced no inhibition of incorporation into RNA and DNA at 0.5 mM. Thus, the inhibition of the conversion of orotic acid into RNA and DNA obtained by 0.5 mM VI and VII seems to be attributable to the inhibition of the conversion of orotic acid to UMP. However, VI began to show some inhibition of incorporation of thymidine-^{3}H into DNA at a higher concentration, 0.8 mM (Table VI).

The inhibited biochemical step between orotic acid and UMP was determined by the analysis of the doubly labeled ribopyrimidine nucleotides on PEI-cellulose thin layer plates. However, before presenting the results of the experiments it seems desirable to discuss the limitation of the methods used. The major radioactive components in the ASF are OA, UMP, UDP, UTP, CMP, CDP, and CTP when orotic acid-6-^{14}C is used as a precursor.[27] The radioactivity of the deoxypyrimidine nucleotides is expected to be approximately 1% of the corresponding ribonucleotides since the specific activity of DNA was about $^1/_{100}$ that of RNA when orotic acid-6-^{14}C and uracil-2-^{14}C were used in our system. OMP is known not to be present in cells,[28] but it is expected to be accumulated in cells when the conversion of OMP to UMP is blocked (*e.g.*, by AzUR[29]). Orotidine accumulated in the cells treated with AzUR, and it was suggested by Pasternak and Handschumacher[29] that orotidine was derived from OMP by a phosphatase action but not directly from orotic acid.

The behavior of ribo- and deoxyribonucleotides on PEI-cellulose thin layer chromatography with various solvent systems was studied by Randerath and Randerath,[24,30] but orotic acid, orotidine, and OMP were not included in their study. Thus, various solvents used by Randerath and Randerath[24] were tested for the separation of the mixtures of the standard orotic acid derivatives, and uridine and cytidine ribonucleotides. The system 2 N HCO_2H–1.6 M LiCl (1:1, v/v) (Figure

(27) (a) R. B. Hurlbert, H. Smitz, A. F. Brumm, and V. R. Potter, *J. Biol. Chem.*, **209**, 23 (1954); (b) H. Schmitz, R. B. Hurlbert, and V. R. Potter, *ibid.*, **209**, 41 (1954); (c) I. Lieberman, A. Kornberg, and E. S. Simms, *ibid.*, **215**, 403 (1955).

(28) D. G. R. Blair, J. E. Stone, and V. R. Potter, *ibid.*, **235**, 2379 (1960).

(29) C. A. Pasternak and R. E. Handschumacher, *ibid.*, **234**, 2992 (1959).

(30) E. Randerath and K. Randerath, *J. Chromatog.*, **16**, 126 (1964).

TABLE III

EFFECTS OF VARIOUS PYRIMIDINE ANALOGS ON OROTIC ACID-6-^{14}C INCORPORATION INTO ACID-SOLUBLE NUCLEOTIDES OF EHRLICH ASCITES CARCINOMA CELLS *in Vitro*[a]

Nucleotide	Ratio $^{14}C/^{3}H$ of controls, dpm	Av exptl values (% of controls)					
		AzUR		VI		VII	
		0.8 m*M*	1.6 m*M*	1.6 m*M*	3.2 m*M*	1.6 m*M*	3.2 m*M*
OA + UMP	0.25	190	220	150	300	120	150
OR	0.18	35X[b]	35X[b]	70	20	25	6
OMP		220 dpm[c]	200 dpm[c]	0 dpm[c]	0 dpm[c]	0 dpm[c]	0 dpm[c]
UDP	0.20	30	20	70	30	45	30
UTP	0.20	10	5	60	2	20	3
CMP	0.22	30	15	100	30	120	40
CDP	0.24	4	10	100	40	60	40
CTP	0.21	10	10	60	10	35	5

[a] Orotic acid-6-^{14}C (experimental), 0.16 m*M*, 2 μCi; orotic acid-5-^{3}H (control), 0.16 m*M*, 10 μCi; incubation, 30 min at 37°; glucose, 22 m*M*. [b] 35-fold accumulation of ^{14}C. [c] ^{14}C accumulated.

2) gave satisfactory spacings of UTP, CTP, UDP, OMP, and CDP. A decreasing concentration of LiCl reduces the mobility of triphosphates and diphosphates. The system 2 *N* HCO_2H–0.2 *M* LiCl (1:1, v/v) (Figure 3) can separate orotidine, CMP, and uridine, but the spots of UMP and orotic acid are very close, and they often overlap in a single spot.

The lyophilized ASF (equivalent to 1 ml of packed cells) in 0.4 ml of distilled H_2O was applied once to the starting line to give a spot with a diameter of 3 mm. The chromatogram obtained with 2 *N* HCO_2H–1.6 *M* LiCl (Figure 2) gave visible spots corresponding to UTP and CTP. The positions of other pyrimidine nucleotides were determined from the R_f values of the authentic reference compounds. UTP, CTP, UDP, OMP, and CDP were obtained from chromatograms developed with this solvent system. The spots for these pyrimidine nucleotides may be mixtures of purine and pyrimidine nucleotides and deoxynucleotides[24] in the ASF, but the double-isotope method obviates the necessity to determine the absolute amounts of each pyrimidine nucleotide. Purine nucleotides would not be appreciably labeled with the precursors used in these experiments. Orotidine was well separated from UMP + orotic acid when the loaded plates were pretreated with anhydrous MeOH[24] before the plates were developed with 2 *N* HCO_2H–0.2 *M* LiCl (Figure 3). The MeOH treatment removes uridine from the plates. In Figure 3 reference uridine was applied after the MeOH treatment.

Table III shows the results of the double-isotope labeling experiment with AzUR and compounds VI and VII. A relatively high concentration of the analogs was chosen so that the effects of the analogs were distinctive enough to draw a definite conclusion on the probable step of inhibition. AzUR at a concentration of 0.8 m*M* resulted in the accumulation of orotic acid + UMP ($^{14}C/^{3}H$ ratio was 190% of control). This accumulation probably is attributable solely to the accumulation of orotic acid, not UMP. The accumulation of orotidine with AzUR treatment was so large that $^{14}C/^{3}H$ ratios are not given in Table III. ^{14}C-OMP was accumulated with AzUR treatment in contrast to the absence of any radioactivity in the OMP position of controls. The data for the experiment with AzUR also show a marked inhibition of incorporation of orotic acid into UDP, UTP, CDP, and CTP. All of the data for AzUR inhibition are consistent with the conclusion of Pasternak and Handschumacher[29] that AzUR inhibits OMP decarboxylase. AzUR showed an additional effect: the conversion of UDP to UTP was inhibited by 30% at 0.8 and 1.6 m*M* but not the conversion of CDP to CTP (or CTP to CDP). The per cent of control values of UTP and CTP obtained with 0.8 and 1.6 m*M* AzUR from 30-min incubations were the same as those of RNA–uracil and –cytosine (Figure 1) obtained from a 1-hr incubation at the same concentration. These data seem to exclude the possibility of the inhibition of RNA polymerase by AzUR, but a more direct evaluation would be desirable.

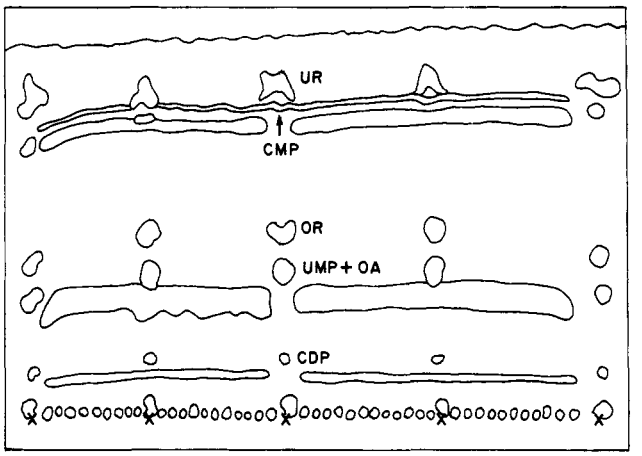


Figure 3.—Anion-exchange thin layer chromatograph of acid-soluble nucleotides of Ehrlich ascites carcinoma cells (0.5-mm thick PEI-cellulose layer). A lyophilized TCA extract of Ehrlich ascites carcinoma cells in 0.4 ml of water (1 ml packed cells) was applied once. The diameter of the spots was 3 mm. To the starting spots, X, were applied the standard nucleotide solutions. The plate was then treated with 500 ml of anhydrous methanol for 5 min, and standard UR was applied to X after the plates had dried. Solvent: 2 *N* HCO_2H–0.2 *M* LiCl (1:1, v/v).

In contrast to AzUR, 1.6 m*M* VI caused the accumulation of radioactivity in the fraction of orotic acid + UMP but inhibited incorporation of orotic acid-6-^{14}C into orotidine, UDP, UTP, and CTP (Table III). These results lead to the conclusion that compound VI inhibits the conversion of orotic acid to OMP. At this concentration of the inhibitor the $^{14}C/^{3}H$ ratios for CMP and CDP were 100% of controls.

At 3.2 m*M* concentration of VI the accumulation of the radioactivity in orotic acid + UMP fraction was doubled, and the incorporation of orotic acid-6-^{14}C into orotidine, UTP, and CTP was inhibited more strongly. Compound VII showed essentially the same type of in-

hibition as VI. The incorporation of orotic acid-6-^{14}C into orotidine (derived principally from OMP by phosphatase action) was inhibited by VII more strongly than VI (Table III).

Compounds VI (3.2 mM) and VII also inhibited the conversion of UDP to UTP. The results for CDP and CTP, however, cannot be interpreted on the basis of the present knowledge of the sequence of the synthesis of cytidine nucleotides from uridine nucleotides, *viz.*, the formation of CTP from UTP. Lieberman reported the amination of UTP to yield CTP in *Escherichia coli*.[31] UDP could replace UTP as acceptor of the amino group with approximately half the reaction rate. UMP, uridine, or uracil could not serve as substrates.[31] These pathways have been accepted to be the same in mammalian systems,[32] but no direct evidence has been reported as far as we can determine. The data for CDP and CTP (Table III) obtained with 3.2 mM VII can only support the conclusion that CTP comes from CDP. This can be seen from the observation (Table III) that the per cent of control value for CDP was higher than that of CTP. If CDP is derived from CTP the per cent of control value for CDP must be at least equal to the value for CTP. The results for CDP and CTP obtained with 3.2 mM VII are consistent with the inhibition of the conversion of CDP to CTP. The amination step cannot be established at the present time because the data for UMP are not available, but the present data would be consistent with the conversion of UDP to CDP. A planned double-isotope labeling experiment with uridine-^{3}H and uridine-^{14}C should eliminate the contamination of UMP by orotic acid and should contribute to knowledge of the pathway to the cytosine nucleotides in Ehrlich carcinoma cells.

Inhibition of Purine Biosynthesis.—Inasmuch as pyrimidine analogs III–V, which showed as strong inhibitory activity as VI for the growth of the Ehrlich ascites carcinoma,[5] showed no significant effect on the incorporation of orotic acid into nucleic acids up to a 0.5 mM concentration of the analogs, a study was made of the effects of these analogs on incorporation of formate-^{14}C into purines of nucleic acids.

The major radioactive components in RNA and DNA when 8 μCi (0.064 mM) of formate-^{14}C was used as a precursor were adenine and guanine of RNA and thymine of DNA. Table IV shows that a 0.6 mM concentration of compounds III–VI inhibits incorporation of formate-^{14}C into adenine and guanine of RNA by 50% in contrast to a small stimulatory effect of FU. Compound II showed only weak inhibitory activity. In comparing compounds II–VI, the incorporation of formate-^{14}C into DNA–thymine was inhibited to the greatest extent (45% inhibition at 0.64 mM) by compound III, but FU was a more potent inhibitor (at least tenfold) of formate utilization for DNA synthesis than compound III. At a concentration of 1.6 mM compounds III–VI inhibited formate incorporation into purines of RNA, into DNA–thymine, and into proteins almost completely. The radioactivity in the ASF was reduced by the presence of 1.6 mM III–VI. An

TABLE IV

EFFECTS OF VARIOUS PYRIMIDINE ANALOGS ON INCORPORATION OF FORMATE-^{14}C INTO NUCLEIC ACIDS AND PROTEINS OF EHRLICH ASCITES CARCINOMA CELLS

Compd	Concn of analog, mM	Av expt values (% of controls) RNA–2′ + 3′ AMP	RNA–2′ + 3′ GMP	DNA–(by-mine	Proteins	ASF
None	0	100[a]	100[c]	100[d]	100[e]	100[f]
II	0.66	80	82	101	...	108
III	0.64	44	31	55	73	101
	1.70	RNA = 14.8		4	38	28
IV	0.60	58	52	87	...	89
	1.62	22	20	15	34	72
V	0.60	46	48	79	...	88
	1.86	10	10	4	12	55
VI	0.64	37	40	77	...	...
	1.60	8	8	3	12	46
FU	0.064	113	139	55	...	100
	0.64	126	126	7	...	100

[a] Formate-^{14}C, 0.064 mM, 8 μCi. [b] 5.15×10^{5} cpm/μmole. [c] 1.13×10^{5} cpm/μmole. [d] 2.50×10^{4} cpm/μmole. [e] 1.60×10^{4} cpm/mg. [f] 1.14×10^{6} cpm.

essentially equal inhibition of formate-^{14}C incorporation into adenine and guanine of RNA by III–VI suggests that the steps after the formation of IMP are not affected by these analogs. The 5-fluoro and 6-aldehyde group of the analogs seem to be involved in the inhibitory effects *vs.* formate incorporation into RNA–purines and proteins considering the structural difference between III–VI, II, and FU. Formate is utilized for purine biosynthesis at two steps: formylation of GAR[33] and AICAR.[34] For this reason the possibility of the inhibition of the conversion of AICAR to IMP was studied. Table V shows the effects of various pyrimidine analogs on incorporation of formate-^{14}C into nucleic acids, proteins, and ASF in the presence of AICAr.

The addition of AICAr at a concentration of 0.2 mM stimulated formate incorporation into purines of RNA about 600% and into the ASF 230% but did not affect formate incorporation into DNA–thymine and into proteins. Further increase of the concentration of AICAr to 0.55 mM inhibited incorporation of formate-^{14}C into proteins and DNA–thymine. This inhibition apparently is due to the reduced size of the "one-carbon" pool available for the synthesis of serine,[35] methionine,[36] and TMP[37] as a result of the "trapping" of formate by conversion of AICAR to FAICAR. Since 0.2 mM AICAr did not affect the rate of incorporation of formate-^{14}C into DNA–thymine and into proteins, the effect of compounds III–VI on the formate incorporation into various fractions was studied in the presence of 0.2 mM AICAr. The per cent of control (0.2 mM AICAr) values of RNA–purines, proteins, DNA–thymine, and ASF obtained by the addition of 0.2 mM AICAr + 1.7 mM III were approximately the same as those obtained by 1.7 mM III alone relative to the control without added AICAr (compare data of Tables IV and V). Similar results were obtained with IV–VI. The persistence of the same degree of inhibition even with the increased concentration of AICAR and FAI-

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TABLE V

EFFECTS OF AICAr ON THE INHIBITION OF INCORPORATION OF ^{14}C-FORMATE[a] INTO NUCLEIC ACIDS AND PROTEINS BY VARIOUS PYRIMIDINE ANALOGS (III–VI)

Compd	Concn, mM	Av exptl values (% of controls) RNA-2′ + 3′ AMP	RNA-2′ + 3′ GMP	DNA-thymine	Protein	ASF
None	0	100[b]	100[b]	100[b]	100[b]	100[b]
AICAr	0.20	572	600	99	94	230
	0.55	1230	1400	61	40	550
0.2 mM AICAr						
+ III	1.70	97 (17)[c]	79 (13)[c]	0 (0)[c]	32 (32)[c]	81 (35)[c]
+ IV	1.62	204 (36)[c]	158 (26)[c]	14 (14)[c]	29 (31)[c]	160 (70)[c]
+ V	1.86	140 (25)[c]	67 (11)[c]	5 (5)[c]	13 (14)[c]	140 (60)[c]
+ VI	1.60	120 (20)[c]	43 (7)[c]	3 (3)[c]	12 (13)[c]	120 (52)[c]

[a] 0.0064 mM, 8 μCi. [b] The absolute values of specific activities and cpm for these controls are identical with those given in footnotes to Table IV. [c] Average experimental values as per cent of the corresponding data of line 2.

CAR in cells by the addition of AICAr seems to imply that there is no competition between either AICAR or FAICAR and the analogs.

The somewhat lower per cent of control values of the 2′ + 3′ GMP fraction in comparison with the 2′ + 3′ AMP fraction obtained with III–VI in the presence of AICAr suggests the presence of another block. However, this conclusion is contradictory to the one made before on the basis of the results listed in Table IV. The same situation, however, was encountered in the experiments with 4-aminopyrazolo[3,4-*d*]pyrimidine,[38] and it was suggested that a metabolic bypass may exist from AICAR to AMP without passing through IMP.[39]

Inhibition of incorporation of formate into proteins seems not to be due to the inhibition of total RNA synthesis. The specific activities of RNA obtained in the presence of 0.2 mM AICAr + 1.6–1.8 mM concentrations of III–VI were somewhat higher than that of cells untreated with either AICAr or analogs, and the radioactivities of treated cells (AICAr + III–VI) were above 100% untreated cells, but the per cent of inhibitions of formate-^{14}C incorporation into proteins obtained with AICAr + III–VI were the same as the value obtained with the analogs only (Table IV).

The possibility of the inhibition of the formylation of THFA[40] by the analogs was tested with an extract of an acetone powder of Ehrlich ascites cells. N^{10}-Formyl-THFA synthetase plus cyclohydrolase activity was assayed according to the procedure of Slaviková, *et al.*,[41] with some modifications. The amount of $N^{5,10}$-methenyl-THFA, produced by either cyclohydrolase[42] or by treatment of N^{10}-formyl-THFA with acid[43] at the end of reaction, was measured.[44] Compound III showed no effect on the enzyme activity. Compounds IV–VI were not investigated, but it is expected that these three compounds would behave similarly to compound III. The effects of these analogs on folate reductase activity are under investigation.

Inhibition of DNA Synthesis.—Compounds III–VI were much weaker than FU as inhibitors of the utilization of formate for the synthesis of DNA–thymine (Table IV). FU inhibits thymidylate synthetase specifically after its conversion to FdUMP, and FU, FUR, and FdUR do not inhibit incorporation of thymidine into DNA–thymine.[45] The inhibitory effects of III–VI also may be due to the inhibition of thymidylate synthetase, but there is an alternative possibility that, unlike FU, these pyrimidine analogs may inhibit at some stage beyond TMP. To test this possibility the effects of III–VI on the incorporation of thymidine-^{3}H into DNA–thymine were studied (Table VI). Compounds III–VI produced only weak

TABLE VI

EFFECT OF VARIOUS PYRIMIDINE ANALOGS ON INCORPORATION OF THYMIDINE-^{3}H[a] INTO DNA–THYMINE OF EHRLICH ASCITES CARCINOMA CELLS

Compd	Concn of analog, mM	Av exptl values (% of control) DNA-thymine	ASF
None	0	100[b]	100[c]
III	0.80	87	110
	1.72	7	94
IV	0.94	70	115
	1.62	9	136
V	0.92	65	113
	1.86	6	97
VI	0.80	77	128
	1.60	10	165
FU	1.60	330	143

[a] Thymidine-CH_3-^{3}H, 0.036 mM, 27 μCi. [b] 4.47 × 10^4 cpm/μmole. [c] 3.13 × 10^5 cpm.

inhibitory activity at a concentration of 0.8–0.9 mM, but a twofold increase of the concentration (1.6–1.8 mM) of the analogs showed 90% inhibition of thymidine-^{3}H incorporation into DNA–thymine. FU at the same concentration showed a stimulatory effect. Thus, the introduction of an aldehyde group at C-6 of FU drastically reduced its inhibitory effect on incorporation of formate into DNA but yielded strong inhibition of incorporation of thymidine into nucleic acid.

The results of double-isotope labeling experiments[46] with ^{14}C- and ^{3}H-labeled thymidine have shown that III–VI inhibit incorporation of thymidine into DNA by inhibiting the phosphorylation of TdR to form TMP, the subsequent formation of TTP, and by direct or indirect inhibition of the DNA polymerase reaction. These experiments also suggest the possibility that the

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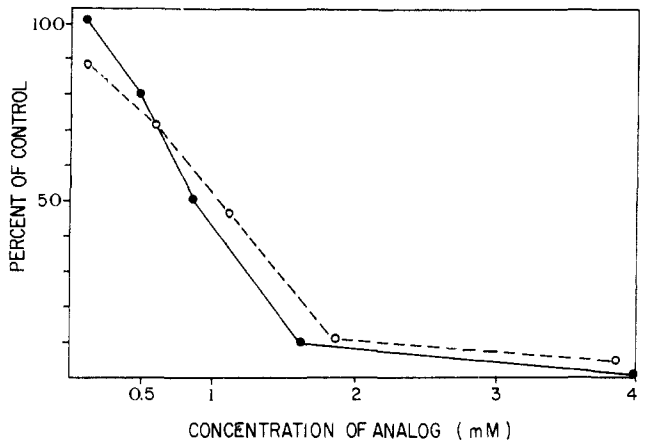

Figure 4.—Effects of pyrimidine analogs (III and V) on incorporation of glycine-1-^{14}C into proteins of Ehrlich ascites carcinoma cells *in vitro*. Incubation proceeded for 1 hr at 37°; glycine-1-^{14}C, 0.02 m*M*, 0.2 μCi. ● = III, ○ = V.

inhibition of incorporation of formate into DNA-thymine by III–VI is attributable principally to inhibition of these phosphorylation and DNA polymerase reactions. Further studies on these biochemical sites of inhibition are now in progress.

Inhibition of Protein Biosynthesis.—Compounds III–VI inhibited the incorporation of formate-^{14}C into proteins (Table IV), and these analogs also inhibited incorporation of phenylalanine-1-^{14}C (Table VII) and

TABLE VII

EFFECTS OF VARIOUS PYRIMIDINE ANALOGS ON INCORPORATION OF PHENYLALANINE-1-^{14}C[a] INTO PROTEINS OF EHRLICH ASCITES CELLS *in Vitro*

Compd	Concn of analog, m*M*	Av expt values (% of controls) Protein	ASF
None	0	100[b]	100[c]
I	0.65	100	110
	1.96	31	170
II	0.62	83	119
	1.86	96	112
III	0.56	82	113
	1.66	26	50
IV	0.55	107	98
	1.65	30	124
V	0.62	58	137
	1.87	13	130
VI	0.60	94	108
	1.80	15	153
FU	0.68	87	83
	2.60	81	85

[a] Phenylalanine-1-^{14}C, 0.02 m*M*, 0.2 μCi. [b] 7.35×10^2 cpm/mg. [c] 4.54×10^4 cpm.

glycine-1-^{14}C (Figure 4). Up to a concentration of 0.55–0.68 m*M* (Table VII) incorporation of phenylalanine-1-^{14}C into proteins was not inhibited appreciably by the analogs listed in Table VII with the exception of V which gave 40% inhibition, but at a concentration of 1.65–1.96 m*M* V and VI produced 85% inhibition and I, III, and IV gave 70% inhibition. FU and compound II showed only a slight inhibition. The radioactivity was accumulated in the ASF by I and IV–VI at this high concentration, but III showed quite the opposite result (50% of control).

Generally the same trend was observed with glycine-1-^{14}C. Compounds III and V (Figure 4) inhibited incorporation of glycine-1-^{14}C into proteins by 50 and 90% at a concentration of 1 and 1.6–1.9 m*M*, respectively. The same per cent inhibition was obtained with IV and VI at comparable concentrations (not shown here). Compound II was less active than III–VI: 70% inhibition at 2.0 m*M*. The radioactivity of the ASF was about 40–50% of controls at 1.6–1.86 m*M* concentrations of III–VI in contrast to the accumulation of radioactivity in the ASF in the case of phenylalanine. FU at the concentration tested showed no effect on incorporation of glycine into proteins. The results with FU are in agreement with previous studies[41] which have found that FU has little or no inhibitory effect on incorporation of amino acids into proteins in both mammalian and bacterial cells.

It is of interest to determine whether the inhibition of protein synthesis by III–VI is related to the inhibition of RNA synthesis by these analogs in view of the important roles of various types of RNA in protein synthesis. The data of Table V show that the addition of AICAr to a concentration of 0.2 m*M* removes the inhibition of total RNA synthesis by 1.6–1.8 m*M* concentrations of III–VI, but this concentration of AICAr failed to reverse the inhibition of incorporation of phenylalanine-1-^{14}C into proteins (Table VIII). This

TABLE VIII

EFFECTS OF AICAr ON THE INHIBITION OF PHENYLALANINE-1-^{14}C[a] INCORPORATION INTO PROTEINS BY VARIOUS PYRIMIDINE ANALOGS

Compd	Concn, m*M*	Av expt values (% of control) Proteins	ASF
None	0	100[b]	100[c]
AICAr	0.2	105	96
0.2 m*M* AICAr			
+ III	1.66	19	46
+ IV	1.62	19	73
+ V	1.88	9	68
+ VI	1.80	15	110

[a] Phenylalanine-1-^{14}C, 0.02 m*M*, 0.2 μCi. [b] 7.35×10^2 cpm/mg. [c] 4.54×10^4 cpm.

observation seems to suggest that the inhibition of protein synthesis by III–VI is not due to inhibition of RNA synthesis. However, Caffery and Irvin[47] in this laboratory found that VI inhibits the hydrocortisone induction of tryptophan pyrrolase in mouse liver, but this compound does not inhibit the substrate, tryptophan, induction of this enzyme. This result favors the conclusion that the inhibition of protein synthesis by VI occurs at the messenger RNA level. This conclusion is based on the current hypothesis that tryptophan stimulation of tryptophan pyrrolase activity depends upon an inhibition of enzyme degradation[48] while the hydrocortisone induction depends upon an increase in messenger RNA synthesis with a concomitant increase in enzyme protein synthesis.[49] Therefore it seems likely that there are two different mechanisms for the inhibition of protein biosynthesis by these pyrimidine

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analogs—a direct effect and an indirect effect mediated through inhibition of messenger RNA synthesis.

General Discussion

The experimental results presented in this paper show that the derivatives of 5-fluoroorotic aldehyde have biochemical inhibitory properties which are quite different from FU in Ehrlich ascites carcinoma cells.

Compound VI inhibits incorporation of orotic acid into RNA by blocking the conversion of orotic acid to OMP in contrast to the weak inhibitory effect of FU on incorporation of orotic acid into RNA, but the inhibition of DNA synthesis was greater with FU than VI. On the basis of the results obtained with the structural analogs of VI it is concluded that, in addition to the 6-carboxaldehyde, the 2-ethylmercapto group and the 5-fluoro group of VI are important for the inhibition of orotic acid incorporation. Compound VII shows even stronger inhibition of incorporation of orotic acid into RNA and DNA, and again the primary site of inhibition seems to be the conversion of orotic acid to OMP. The unsubstituted phenyl group on the azlactone ring and the intact hydrogen atom at C-5 of VII are necessary for the inhibition. Although VI and VII are much inferior to AzUR as inhibitors of orotic acid metabolism, they are much superior to AzU.

The analogs having a 5-fluoro and 6-carboxaldehyde group, III–VI, inhibit incorporation of formate-^{14}C into purines of RNA and thymine of DNA in contrast to the lack of inhibition of purine biosynthesis by FU. The structural requirements for the inhibition of purine biosynthesis are a C-6 carboxaldehyde and a C-5 fluorine. The inhibition seems to occur at a step preceding IMP. Compound III (and possibly IV–VI) do not inhibit N^{10}-formyl-THFA synthetase and cyclohydrolase activity.

The incorporation of formate-^{14}C and thymidine-^{3}H into DNA-thymine is inhibited by the 5-fluoroorotic aldehydes III–VI, but FU inhibits formate incorporation only. By comparison of III with FU, the aldehyde group of III seems to play an important role in the inhibition of thymidine incorporation into DNA-thymine. This inhibition is obtained at a concentration somewhat higher than that used for the experiments with orotic acid-^{14}C and uracil-^{14}C. The double-isotope labeling experiments with ^{14}C- and ^{3}H-thymidine have shown[46] that the kinase reaction for conversion of TdR to TMP and the DNA polymerase reaction are the primary sites of inhibition by these analogs. The introduction of an aldehyde group into position C-6 of FU, however, seems to reduce markedly the activity as an inhibitor of thymidylate synthetase.

Protein biosynthesis also is affected by the derivatives of 5-fluoroorotic aldehydes as shown by inhibition of formate-^{14}C, glycine-1-^{14}C, and phenylalanine-1-^{14}C into proteins. By contrast, FU showed no inhibitory activity for protein synthesis at the concentrations tested. The inhibition of RNA–purine biosynthesis by these analogs seems not to be responsible for the inhibition of protein synthesis since the restoration of purine synthesis by the addition of AICAr did not reverse the inhibition of phenylalanine incorporation into proteins. However, Caffery and Irvin[47] have made observations which support the conclusion that the inhibition of protein synthesis by VI may occur at the messenger RNA level: VI inhibits the hydrocortisone induction but not the substrate induction of tryptophan pyrrolase in liver of the adrenalectomized mouse.

Acknowledgment.—We thank Dr. R. E. Handschumacher of the Department of Pharmacology, Yale University School of Medicine, for a sample of orotidylic acid.

Synthesis and Biological Activity of Some 8-Aza-9-cyclopentylpurines[1,2]

PAULINE K. CHANG, LOUIS J. SCIARINI, ALAN C. SARTORELLI, AND MORRIS S. ZEDECK

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

Received December 28, 1967

The preparation of a number of 6-substituted 8-aza-9-cyclopentylpurines is described. Using ethyl malonate-1-^{14}C, a mixture of 4-^{14}C- and 6-^{14}C-labeled 6-chloro-8-aza-9-cyclopentylpurine has been synthesized. The results of the biological evaluation of these compounds as antineoplastic agents and as inhibitors of T2osr bacteriophage induced lysis of *Escherichia coli* B are reported.

It has been shown that 2′-deoxyadenosine inhibits the steroid-induced synthesis of Δ^5-3-keto steroid isomerase in *Pseudomonas testosteroni*, whereas adenosine was completely inactive.[3] A subsequent study of structure–activity relationships, in which analogs of 2′-deoxyadenosine were tested as inhibitors of the formation of induced enzyme, indicated that inhibitory activity was enhanced when (a) the 6-amino group of adenine was substituted by chlorine, and (b) the 8-carbon in the purine ring was replaced by nitrogen. The presence of a 2′-hydroxy substituent on the pentose moiety or a deviation from the natural β,D configuration of 2′-deoxyribose caused the loss of activity as an inhibitor of the synthesis of induced enzyme, whereas analogs deficient in 2′-hydroxy-containing substituents, such as 2′,3′,5′-trideoxyadenosine and 9-cyclopentyladenine, retained activity. These observations led to the design and synthesis of 6-chloro-8-aza-9-cyclopentylpurine (3-cyclopentyl-7-chloro-*v*-triazolo[4,5-*d*]pyrimidine, **1**). This compound, as predicted from structure–activity considerations, proved to

(1) This work was supported by a grant (CA-02817) from the National Cancer Institute, U. S. Public Health Service, and by the Connecticut Division of the American Cancer Society.

(2) Presented in part before the Division of Medicinal Chemistry at the 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1967.

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