Synthesis and Biological Activity of Dinucleoside Phosphates Containing 5-Fluorouracil Residues

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5-Fluoropyrimidines are potent inhibitors of cell growth and have found useful application in the chemotherapy of certain neoplasms of man. These compounds exert their activiy primarily as the 5'-monophosphate of 5-fluoro-2'-deoxyuridine. Since the direct administration of the mononucleotides of the fluoropyrimidines is precluded, due to a permeability barrier, the possibility was considered that dinucleoside phosphates containing 5-fluorouridine or 5-fluorodeoxyuridine as component residues might be able to penetrate the cell membrane. Accordingly, the following dinucleoside phosphates were synthesized: 5-fluoro-2'-deoxyuridylyl-(5'→5')-thymidine (I), thymidylyl-(3'→5')-2'-deoxy-5-fluorouridine (II), 5-fluoro-2'-deoxyuridylyl-(3'→5')-thymidine (III), 5-fluorouridylyl-(5'→5')-5-fluorouridine (IV), and 5-fluorouridylyl-(5'→5')-uridine (V). The biological activity of this series of compounds was evaluated, using *Streptococcus faecalis* 8043. While 5-fluorouracil and its nucleosides inhibit the growth of this organism by 50% at 1–5 $\times$ 10^{-10} M, the activity of the dinucleoside phosphates is substantially less, extending from 1 $\times$ 10^{-8} M to 2 $\times$ 10^{-7} M. Permeability studies show that the cell membrane limits the uptake of these compounds into the cell. Reversal patterns obtained from inhibition analyses indicate that the dinucleoside phosphates exert their inhibitory action at the same metabolic site at which the component nucleoside analogs act. A similar conclusion was reached from cross-resistance studies. These data suggest that the dinucleoside phosphates exert their inhibitory activity following cleavage of the ester bond. Chromatographic and microbial determinations show that the compounds undergo spontaneous hydrolysis, and it appears that this cleavage, possibly supplanted by some enzymatic hydrolysis prior to their entry into the cell, is responsible for their biological activity. Since the dinucleoside phosphates containing fluoropyrimidines are capable of slowly releasing the nucleoside components, they may possess desirable chemotherapeutic properties not found in the component nucleoside analogs alone.

The 5-fluoro analogs of uracil, cytosine, and their nucleosides are potent inhibitors of cell growth.[1] They are effective against several transplantable tumors[2,3] and have found useful application in the chemotherapy of certain neoplasms of man.[4] However, the susceptibility of these agents to metabolic degradation and excretion[5,6] requires their prolonged administration in comparatively high doses. As a result, the selective action of these drugs is decreased, and the chance for the development of drug resistance is markedly enhanced.

The 5-fluoropyrimidines inhibit cell growth primarily by interfering with thymidylate synthetase activity, and to exert this inhibition their prior conversion to FdUMP[7] is required.[4,8–10] The direct administration of nucleotides, such as FdUMP, is hindered by permeability barriers attributable to the presence of the charged phosphate group.[11] Preparation of dinucleoside phosphates containing fluoropyrimidines as residues was initiated because it was held possible that esterification of the phosphate group with another nucleoside moiety may permit the transport of the resulting dinucleoside monophosphate into the cell, followed by the intracellular liberation of the nucleotide by the action of esterases. It was also thought that these diesters may be more resistant to metabolic degradation and elimination than are the component nucleosides, resulting in the increased chemotherapeutic effectiveness of the compounds. It was further considered possible that cells resistant to FUR or FdUR may be susceptible to inhibition by the dinucleoside phosphates.

This paper describes the synthesis of a number of dinucleoside monophosphates containing FUR or FdUR residues and compares their biological effectiveness in the *Streptococcus faecalis* 8043 test system. On the basis of uptake, reversal, and cross-resistance studies, the likely mode of action of these compounds is discussed. Part of this work has been presented previously.[12]

Experimental Section

Paper Chromatography.—To determine the R_f values of the compounds synthesized, Whatman No. 1 paper and the solvent systems: A, 2-propanol-1% aqueous $(NH_4)_2SO_4$ (2:1); and B, 2-propanol-water-concentrated NH_4OH (7:2:1) were used.

Electrophoresis.—Electrophoresis was carried out in a Gilson electrophorator for 1 hr at 4500 (100 v/cm) in 0.05 M ammonium formate solution, pH 3.5, using Whatman No. 3 MM paper.

Synthesis of Dinucleoside Phosphates. 2',3'-O-Isopropylidene-5-fluorouridine.—5-Fluorouridine[13] (0.97 g, 3.7 mmoles), di(*p*-nitrophenyl) phosphate (0.126 g, 0.37 mmole) and 2,2-dimethoxypropane (3.35 g, 32 mmoles) were dissolved in 40 ml

(1) *Cancer Chemotherapy Rept.*, **6**, 64, 1960.

(2) C. Heidelberger, N. K. Chaudhuri, P. Danneberg, D. Mooren, L. Griesbach, R. Duschinsky, R. J. Schnitzer, E. Pleven, and J. Scheiner, *Nature*, **179**, 663 (1957).

(3) C. Heidelberger, L. Griesbach, B. J. Montag, D. Mooren, O. Cruz, R. J. Schnitzer, and E. Grunberg, *Cancer Res.*, **18**, 305 (1958).

(4) C. Heidelberger and F. J. Ansfield, *ibid.*, **23**, 1226 (1963).

(5) N. K. Chaudhuri, K. L. Mukherjee, and C. Heidelberger, *Biochem. Pharmacol.*, **1**, 328 (1959).

(6) K. L. Mukherjee, J. Boohar, D. Wentland, F. J. Ansfield, and C. Heidelberger, *Cancer Res.*, **23**, 49 (1963).

(7) The following abbreviations are used in this paper: FU = 5-fluorouracil, FUR = 5-fluorouridine, FdUR = 5-fluoro-2'-deoxyuridine, FUMP = 5-fluorouridine 5'-phosphate, FdUMP = 5-fluoro-2'-deoxyuridine 5'-phosphate, FUMP-FUR = 5-fluorouridylyl-(5'→5')-fluorouridine, FUMP-UR = 5-fluorouridylyl-(5'→5')-uridine, FdUMP-dTR(3'→5') = 5-fluoro-2'-deoxyuridylyl-(3'→5')-thymidine, FdUMP-dTR(5'→3') = 5-fluoro-2'-deoxyuridylyl-(5'→3')-thymidine, FdUMP-dTR(5'→5') = 5-Fluoro-2'-deoxyuridylyl-(5'→5')-thymidine.

(8) (a) S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichenstein, *Proc. Natl. Acad. Sci. U. S.*, **44**, 1004 (1958); (b) E. Harbers, N. K. Chaudhuri, and C. Heidelberger, *J. Biol. Chem.*, **234**, 1255 (1959).

(9) C. Heidelberger, G. Kaldor, K. L. Mukherjee, and P. B. Danneberg, *Cancer Res.*, **20**, 903 (1960).

(10) K-U. Hartmann and C. Heidelberger, *J. Biol. Chem.*, **236**, 3006 (1961).

(11) K. C. Leibman and C. Heidelberger, *ibid.*, **216**, 823 (1955).

(12) M. H. Fleysher, R. Thedford, and R. H. Hall, Abstracts, 149th National Meeting of the American Chemical Society, Detroit, Mich., 1965, p 9N.

(13) N. C. Yung, J. H. Burchenal, R. Fecher, R. Duschinsky, and J. J. Fox, *J. Am. Chem. Soc.*, **83**, 4060 (1961).

of absolute acetone. The solution was stirred 2.25 hr after which sufficient Amberlite IR-400 resin (OH^- form) was added to neutralize the acid. The resin was filtered off and washed with ethyl acetate. The solvents were evaporated to dryness and the residue was crystallized from ethyl acetate and petroleum ether (bp 60°). The yield was 0.85 g (76%), mp 198.5°.

Anal. Calcd for $C_{12}H_{15}FN_2O_6$: C, 47.68; H, 4.97; N, 9.27. Found: C, 47.73; H, 5.10; N, 9.20.

2',3'-O-Isopropylidene-5-fluorouridine 5'-Phosphate Pyridinium Salt.—2',3'-O-Isopropylidene-5-fluorouridine (0.72 g, 2.4 mmoles) and 2-cyanoethyl phosphate reagent[14] (5 mmoles) were dissolved in 10 ml of pyridine. The solution was evaporated to dryness and the residue was dissolved in 10 ml of anhydrous pyridine. This step was repeated three more times. To the solution of the residue in 20 ml of pyridine was added 5 g (24.1 mmoles) of dicyclohexylcarbodiimide (DCC). The clear solution was incubated at 25° for 18 hr after which 4 ml of water was added. The solution was allowed to stand for 24 hr. The cyclohexylurea was filtered off and washed with pyridine. The filtrate and washings were combined and were evaporated to dryness. The residue was dissolved in 45 ml of 9 N NH_4OH and 40 ml of pyridine, and the solution was heated for 3 hr at 100°. It was evaporated to dryness and the residue was dissolved in 20 ml of water. Barium acetate (1.65 g, 6 mmoles) was added and the solution was kept at 4° for 20 hr. The precipitate of barium phosphate was filtered off. Two volumes of ethanol were added to the filtrate and the precipitate was collected by filtration. The product was dissolved in water and the solution was passed through a short column of Dowex 50 (50–100 mesh), pyridinium form. The column was washed with water and the total effluent was lyophilized. The yield was 0.83 g, 75%.

Anal. Calcd for $C_{17}H_{21}FN_3O_9P \cdot 2H_2O$: C, 41.04; H, 5.03; N, 8.45. Found: C, 40.36; H, 4.88; N, 9.07.

5'-O-Trityl-2'-deoxy-5-fluorouridine 3'-Phosphate Pyridinium Salt.—5'-O-Trityl-2'-deoxy-5-fluorouridine[15] (2 mmoles) was phosphorylated by the method described for the preparation of 2',3'-O-isopropylidene-5-fluorouridine 5'-phosphate. The yield was 800 mg (60%).

Anal. Calcd for $C_{33}H_{31}FN_3O_8P \cdot H_2O$: C, 59.38; H, 4.96; N, 6.31. Found: C, 59.06; H, 5.02; N, 6.13.

Dinucleoside Phosphates (General Procedure).—The quantities of solvents and reagents are based on 0.5 mmole of nucleotide. The actual quantity used varied with the amounts of starting materials. The size of the column and the volume of eluting buffer was constant for all five preparations. The pyridinium salt of the blocked nucleotide (0.5 mmole) and the blocked nucleoside (1 mmole) were dissolved in 10 ml of anhydrous pyridine and the solution was concentrated to dryness. Evaporation with pyridine was repeated several times and finally the residue was dissolved in 25 ml of anhydrous pyridine and DCC (4.1 g) was added. The reaction flask was flushed with dry nitrogen, tightly stoppered, and allowed to stand at room temperature for 6 days. On day 3, more DCC (2 g) was added. Water (5 ml) was added and the mixture was left at room temperature overnight. After filtering, the mixture was evaporated to dryness *in vacuo* and traces of pyridine were removed by evaporating with water, then ethanol. The residue was treated with 80% acetic acid for 30 min at 100° in order to remove blocking groups and the product was purified on a column (1.9 × 36 cm) of DEAE-cellulose (carbonate). The column was developed with a linear gradient of triethylammonium carbonate solution (0.01 M → 0.1 M, pH 8.6, total vol 2 l.) according to the general procedure described previously.[16] The order of elution of the three principal constituents of each of the mixture was nucleoside, dinucleoside phosphate, and mononucleotide. In the case of the preparation of 5-fluorouridylyl-(5'→5')-5-fluorouridine the order of elution was changed in that the mononucleotide preceded the dinucleoside phosphate. The fraction containing the product was concentrated to dryness *in vacuo* and the excess triethylammonium carbonate was removed by repeated suspension of the residue in absolute ethanol and followed by evaporation of the solvent. The residue was dissolved in a minimum amount of absolute ethanol (2 ml) and, on addition of anhydrous ether, the product precipitated out of solution. The product (triethylammonium salt) was collected by centrifugation, washed several times with anhydrous ether, and dried over P_2O_5 for 24 hr. The compounds prepared by this procedure are described below.

5-Fluoro-2'-deoxyuridylyl-(5'→5')-thymidine (I) was prepared from 3'-O-acetylthymidine 5'-phosphate (2.4 mmoles)[17] and 3'-O-acetyl-2'-deoxy-5-fluorouridine (3 mmoles). The compound was isolated as its triethylammonium salt, yield 900 mg (60%).

Anal. Calcd. for $C_{25}H_{39}FN_5O_{12}P \cdot 3H_2O$: C, 42.62; H, 6.38; N, 9.94. Found: C, 42.92; H, 6.06; N, 9.91.

Thymidyl-(3'→5)'-2'-deoxy-5-fluorouridine (III) was prepared from 5'-O-tritylthymidine 3'-phosphate[18] (1 mmole) and 3'-O-acetyl-2'-deoxy-5-fluorouridine[15] (3 mmoles). The product isolated as the triethylammonium salt weighed 900 mg (60%). This product moved as a single ultraviolet absorbing spot when chromatographed on paper in four solvent systems and its electrophoretic mobility is characteristic of that of a dinucleoside phosphate (Table I).

TABLE I

PAPER CHROMATOGRAPHY AND ELECTROPHORESIS

Compd	R_f value Solvent A	R_f value Solvent B	Distance moved toward anode, cm
5-Fluoro-2'-deoxyuridylyl-(3'→5')-thymidine	0.50	0.224	15.8
Thymidylyl-(3'→5')-2'-deoxy-5-fluorouridine	0.499	0.224	15.9
Thymidylyl-(5'→5')-2'-deoxy-5-fluorouridine	0.47	0.194	16.4
Thymidine	0.73	0.67	
2-Deoxy-5-fluorouridine	0.726	0.51	
Thymidine 3'-phosphate	0.432	0.123	19.2
2-Deoxy-5-fluorouridine 3'-phosphate	0.372	0.049	18.8
5-Fluorouridine	0.71	0.48	
2',3'-Isopropylidene-5-fluorouridine	0.86	0.77	
5-Fluorouridine 5'-phosphate	0.53	0.27	19.1
5-Fluorouridylyl-(5'→5')-uridine	0.44	0.06	16.6
5-Fluorouridylyl-(5'→5')-5-fluorouridine	0.46	0.04	15.9
2',3'-Isopropylidine-5-fluorouridine 5'-phosphate	0.56	0.03	21.7
Uridine	0.65	0.50	
Uridine 2'(3')-phosphate			20.3

Anal. Calcd for $C_{25}H_{39}FN_5O_{12}P \cdot 2H_2O$: C, 43.66; H, 6.25; N, 10.19; P, 4.51. Found: C, 43.93; H, 6.59; N, 9.94; P, 4.24.

5-Fluoro-2'-deoxyuridylyl-(3'→5')-thymidine (III) was prepared from 5'-O-trityl-2'-deoxy-5-fluorouridine 3'-phosphate (0.5 mmole) and 3'-acetylthymidine[19] (1 mmole). The yield was 150 mg (44%).

Anal. Calcd for $C_{25}H_{39}FN_5O_{12}P \cdot H_2O$: C, 44.84; H, 6.12; N, 10.46. Found: C, 44.78; H, 6.55; N, 9.97.

5-Fluorouridylyl-(5'→5')-5-fluorouridine (IV) was prepared from 2',3'-O-isopropylidene-5-fluorouridine 5'-phosphate (0.76 mmole) and 2',3'-O-isopropylidene-5-fluorouridine (1.5 mmoles). The product was obtained as the triethylammonium salt (yield 71%). It moved as a single ultraviolet-absorbing spot on paper chromatography, and its electrophoretic mobility corresponded to that of a diribonucleoside phosphate (Table I). Upon conversion to the sodium salt *via* Dowex 50, neutralization, and lyophilization, the product was analyzed as the sodium salt.

Anal. Calcd for $C_{18}H_{20}FN_4O_{14}NaP \cdot 3.5H_2O$: C, 32.19; H, 4.02; N, 8.35. Found: C, 32.15; H, 3.76; N, 8.32.

5-Fluorouridylyl-(5'→5')-uridine (V) was prepared from 2',-3'-O-isopropylidene-5-fluorouridine 5'-phosphate (1.69 mmoles) and 2',3'-O-isopropylideneuridine (3.38 mmoles). The product as obtained from the DEAE-cellulose column was contaminated

(14) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).

(15) H. J. Thomas and J. A. Montgomery, *J. Med. Pharm. Chem.*, **5**, 24 (1962).

(16) R. H. Hall and R. Thedford, *J. Org. Chem.*, **28**, 1506 (1963).

(17) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958).

(18) G. Weiman and H. G. Khorana, *ibid.*, **84**, 419 (1962).

(19) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 951 (1953).

with 10% of uridine; therefore, an additional purification step was required. The reaction product was neutralized with Dowex 50 resin (H^+) and the solution after filtering was evaporated to dryness. The residue was dissolved in water and this solution was titrated with 0.1 N $Ba(OH)_2$ solution to pH 7.35 (14 ml consumed). The product was precipitated by the addition of 5 vol of ethanol, and the precipitate was collected by centrifugation and washed with a few milliliters of ethanol-water (3:1). The product was dried over P_2O_5 in a desiccator for 5 days. The yield was 0.89 g (83%).

Anal. Calcd for $C_{18}H_{24}FN_4O_{14}P \cdot 0.5Ba \cdot 7H_2O$: C, 28.49; H, 4.59; N, 7.35. Found: C, 28.49; H, 3.71; N, 7.15.

A more satisfactory analysis was obtained with the sodium salt which was prepared as described above.

Anal. Calcd for $C_{18}H_{24}FN_4NaO_{14}P \cdot 2H_2O$: C, 34.41; H, 3.98; N, 8.94. Found: C, 34.78; H, 4.14; N, 8.60.

The hyperchromic effect of FUMP-FUR and FUMP-UR is 12 and 8.3%, respectively. These values were obtained according to the procedure reported previously.[20]

Microbial Assay Procedures.—All growth experiments were carried out in the basal medium of Flynn, *et al.*,[21] from which uracil and the purines had been omitted, and to which 1 mμg/ml of folic acid was added. Portions (1 ml) of this double-strength medium were placed into 13 × 100 mm Pyrex culture tubes and 1 ml of water or 1 ml of the solution containing the test compound was then added to each tube. Sterilization of the medium was carried out by autoclaving for 6 min at 116–121°. The fluorinated pyrimidines and the dinucleoside phosphates containing them were sterilized by filtration and added to the autoclaved medium. The inocula were prepared from cultures of *S. faecalis* grown in 5 ml of the basal medium for 20 hr at 37°. Following centrifugation and washing twice with isotonic saline, the cells were resuspended in enough saline to give an optical density of 0.30 as measured in a Beckman Model B spectrophotometer at 470 mμ. A 1-ml portion of this suspension containing approximately 1.5×10^7 cells was diluted 1:10 in saline, and 1 drop of this final dilution was placed in each assay tube. Incubation proceeded for 20 hr at 37°, at which time the control culture is still in log phase. The extent of growth was determined by means of a Klett-Summerson photoelectric colorimeter, using a red filter (640–700 mμ). To determine their potency as inhibitors of growth, the analogs were added to the basal medium in concentrations ranging from 10^{-3} to 10^{-12} M.

The ability of various pyrimidines to prevent the inhibition of growth exerted by the fluoropyrimidines and their derivatives was evaluated by adding the metabolites to the medium at concentrations ranging from 10^{-3} to 10^{-6} M, and assaying them against concentrations of the analog extending from 10^{-3} to 10^{-12} M.

Selection of Mutant Strains.—Mutants resistant to FU, FUR, FUMP, and FUMP-FUR were obtained from the parent strain by serial transfer in increasing concentrations of the drug. The basal medium served as the selection medium.

Uptake of FU and FUMP-FUR by *S. faecalis*.—The spectrophotometric procedure of Jacquez[22] was used to determine the uptake of the analogs. The cells were grown in the basal medium containing 2 mμg/ml of folic acid. After 20 hr of incubation at 37°, the cultures were washed twice with 0.2 M phosphate buffer, pH 7.0, and were resuspended in a sufficient amount of buffer to yield a cytocrit between 0.1 and 0.2. The suspensions were preincubated for 6 min at 37° and were then incubated for 5 min in the presence of various concentrations of the analogs. The cells were separated from the extracellular fluid by centrifugation, and the compounds were extracted by boiling the cells with 95% ethanol and 0.45% NaCl (4:1). The concentration of analog in this solution was determined with a Cary Model 14 recording spectrophotometer. Allowance was made for the amount of compound contained in the intercellular space, the volume of which was determined by the use of inulin.[23] The concentration of compounds in the extracellular fluid was estimated spectrophotometrically.

Purification of the Compounds and Determination of Their Stability.—The biological test system used is highly sensitive to inhibition by the fluorinated pyrimidine bases and nucleosides (10^{-11} M). To exclude accumulated breakdown products, the dinucleoside phosphates were chromatographed immediately before their use. Chromatography was carried out overnight in solvent A on Whatman No. 3 paper, descending, at 25°. The dinucleoside phosphates were eluted from the paper by shaking in water, pH 7.0, for 1 hr.

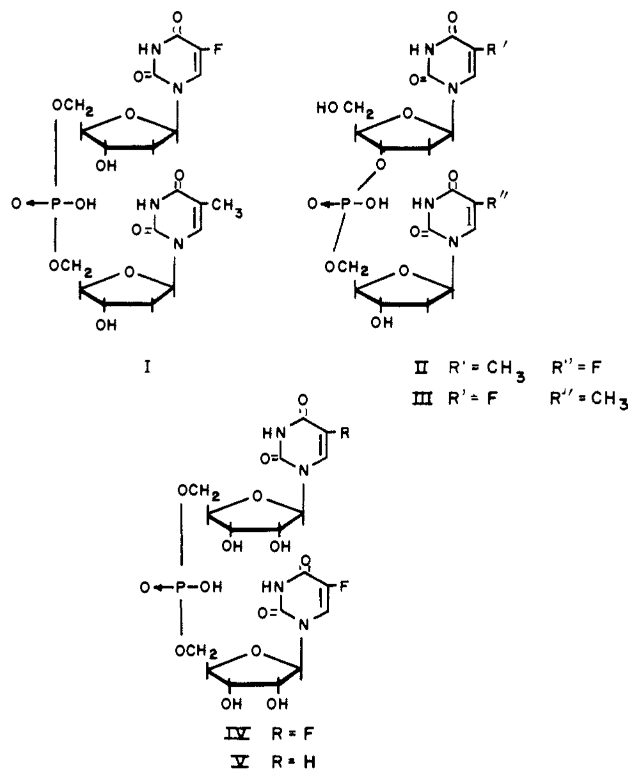
To determine the possible hydrolysis of even small portions of the compounds during chromatography, bioautography was employed. A 2.5-cm strip, cut along the length of the dried chromatogram, was placed on the solidified basal nutrient medium (1.8% agar), which had been seeded with *S. faecalis* (1 ml of standard inoculum/100 ml of medium). Following incubation for 20 hr at 37°, the location of zones of inhibition was compared with the position of zones produced by the corresponding base, nucleoside, and nucleotide analogs.

To determine the extent of hydrolysis which the dinucleoside phosphates undergo upon standing in solution, the compounds were dissolved in 0.001 M phosphate buffer, pH 7.0. One part of the solution was frozen at once, and the remaining portion was incubated at 37° for 2–5 days. All fractions were then assayed simultaneously for inhibition of the growth of *S. faecalis*.

Results and Discussion

Dinucleoside monophosphates containing one or two residues of an antimetabolite are of fundamental interest in chemotherapy. If capable of entering the cells intact, not only their potency, but also their mode of action may differ from that of the individual residues. They may be less prone to catabolic inactivation and may be active against cell strains resistant to the free analog residues. The synthesis of dinucleoside phosphates containing FUR or FdUR as part of their structure was carried out to permit an investigation of these possibilities. Chart I shows the structural formulas of the dinucleoside phosphates which we have prepared. FdUMP was combined with thymidine through a 5'→5' (I), 5'→3' (II), and 3'→5' (III) linkage in order to permit an evaluation of the effect of a given

CHART I



(20) R. Thedford, M. H. Fleysher, and R. H. Hall, *J. Med. Chem.*, **8**, 486 (1965).

(21) L. M. Flynn, V. B. Williams, B. L. O'Dell, and A. G. Hogan, *Anal. Chem.*, **23**, 180 (1951).

(22) J. A. Jacquez, *Cancer Res.*, **17**, 890 (1957).

(23) A. Bloch and D. J. Hutchison, *ibid.*, **24**, 433 (1964).

bond arrangement upon biological activity. FUMP was attached in 5'→5' linkage with a fluorouridine (IV) and with a uridine (V) residue.

The growth inhibitory activity of these compounds in the *S. faecalis* 8043 system is shown in Table II. While FU, FUR, and FdUR inhibit the growth of this organism by 50% at 1-5 × 10^{-11} *M*, the activity of the dinucleoside phosphates containing these analogs is less, extending from 2 × 10^{-8} to 5 × 10^{-7} *M*. A comparative decrease in activity is shown by FUMP. These dinucleoside phosphates are also inhibitors of Sarcoma 180 cells grown in tissue culture.[24]

TABLE II

GROWTH INHIBITION OF *S. faecalis*[a] BY FLUOROPYRIMIDINES

Analog	Amt of analog (*M*) for 50% growth inhib
FU	5 × 10^{-11}
FUR	3 × 10^{-11}
FdUR	1 × 10^{-11}
FdUMP-dTR-(3'→5')	1 × 10^{-8}
FdUMP-dTR-(5'→3')	1 × 10^{-8}
FdUMP-dTR-(5'→5')	2 × 10^{-8}
FUMP	4 × 10^{-8}
FUMP-FUR	5 × 10^{-8}
FUMP-UR	5 × 10^{-7}

[a] Grown in a defined medium for 20 hr at 37°.

The marked difference in the potency of the free nucleoside analogs as compared to that of the dinucleoside phosphates, or the nucleoside monophosphate, may be explained in a number of ways. A permeability barrier could impede the facile entrance of the dinucleoside phosphates, while permitting the simple diffusion of the nucleosides. On the other hand, the dinucleoside phosphates might enter the cells freely, but may act in a manner different from that of the free nucleosides. Alternately, cleavage to the nucleoside or the nucleotide stage may have to occur either outside or inside the cell before the dinucleoside phosphates can exert their inhibitory activity.

To establish which of these possibilities is most likely responsible for the action of these compounds, a comparative inhibition analysis was carried out.[25] Central to such an analysis is the observation that in *S. faecalis*, as in other biological systems, interference with the biosynthesis of thymidylic acid represents the primary mechanism by which the fluoropyrimidines in the form of FdUMP exert their growth inhibitory effect.[23] The reversal capacity of a given pyrimidine depends thus on the extent of its conversion to dUMP (Figure 1). Table III shows that the reversal patterns obtained with the dinucleoside phosphates coincide closely with those of FUR and FdUR. There exists, however, an approximately 1000-fold difference in the magnitude of the inhibition indices, which parallels the difference in potency of the compounds. Thymidine and thymine are the best reversing agents, and although they give rise to the product of thymidylate synthetase activity (Figure 1), their reversal effect is competitive, indicating some interference of the fluoropyrimidines with the anabolism of endogenously supplied thymine and thymidine.[23,26] Deoxyuridine,

(24) Dr. M. T. Hakala, personal communication.

(25) W. Shive, *Ann. N. Y. Acad. Sci.*, **52**, 1212 (1950).

(26) P. S. Kokolis and A. J. Weiss, *Proc. Am. Assoc. Cancer Res.*, **6**, 36 (1965).

Figure 1.—Scheme of pyrimidine interconversions.

the immediate precursor of dUMP, is a somewhat less effective reversing agent, but is more active in preventing the inhibition than are the remaining pyrimidines.

The reversal patterns obtained with the dinucleoside phosphates coincide not only with those of FUR and FdUR, but also with that of FUMP. This indicates that all of these fluoropyrimidines exert their primary inhibition at the same metabolic site in the form of the identical intermediate which, most likely, is FdUMP.

That the dinucleoside phosphates act in the same manner as do their component analog residues is also suggested by the data in Table IV. Mutant strains of *S. faecalis* which require 4 × 10^{-8} *M* FU (SF/FU) or FUR (SF/FUR) for 50% growth inhibition are cross-resistant to 3 × 10^{-5} *M* FUMP-FUR. This is a ratio of approximately 1:1000. Alternately the strain resistant to FUMP-FUR (SF/FUMP-FUR) is cross-resistant to FU and FUR at approximately the same ratio. In comparison the wild strain (SF/0) is inhibited by 3 × 10^{-11} *M* FUR and by 5 × 10^{-8} *M* FUMP-FUR which is also a ratio of about 1:1000. This observation indicates that the mechanism of resistance is directed against a site which is acted upon by a common intermediate of these analogs. If the dinucleosides were to inhibit growth in a manner different from that of FUR, then this constant ratio of inhibition could not be expected. The same observation applies to FUMP. As determined with SF/0, the ratio between the inhibitory activity of FUMP and FUR is approximately 1000:1 (Table IV). In turn, the mutant resistant to FUMP is cross-resistant to FU or FUR to approximately the same extent. This coincidence would not be likely to occur if the mode of action of these compounds were to differ.

The weaker inhibitory activity of the dinucleoside phosphates as compared to that of the free fluoropyrimidines must then result from a difference in the rate of their conversion to the active intermediate. Figure 2 demonstrates that FUR enters *S. faecalis* cells readily, the facile entry paralleling the unhindered uptake of FU.[23,27] The entrance of the dinucleoside phosphate FUMP-UR is, however, markedly retarded,

(27) J. A. Jacquez, *Proc. Soc. Exptl. Biol. Med.*, **109**, 132 (1962).

TABLE III

REVERSAL OF FLUOROPYRIMIDINE INHIBITION BY PYRIMIDINES

Pyrimidine	Inhibition index[a] obtained with—							
	FU × 10³	FUR × 10³	FdUR	FUMP	FUMP-FUR	FUMP-UR	FdUMP-dTR-(3'→5')	FdUMP-dTR-(5'→5')
Thymidine	10	10	7	10	10	20	7	7
Thymine	1	2	1	2	2	5	0.6	0.8
Deoxyuridine	1	1	1	1	0.8	1	0.4	0.6
Uridine	0.2	0.1	0.1	0.1	0.2	0.3	0.1	0.1
Deoxycytidine	0.03	0.1	0.08	0.04	0.03	0.08	0.02	0.03
Uracil	0.03	0.01	0.04	0.02	0.02	0.08	0.03	0.03
Cytidine	0.01	0.01	0.01	0.02	0.01	0.04	0.01	0.02
Cytosine	0.008	0.001	0.004	0.002	0.001	0.006	0.006	0.007

[a] Average inhibition index = [I]/[S] for 50% growth inhibition, determined at four concentrations ranging from 10^{-3} to 10^{-6} *M*.

TABLE IV

CROSS-RESISTANCE OF STRAINS OF *S. faecalis*[a] RESISTANT TO VARIOUS FLUOROPYRIMIDINES

Analog	Amt of analog required (*M*) for 50% growth inhibition of—				
	SF/0	SF/FU	SF/FUR	SF/FUMP	SF/FUMP-FUR
FU	5×10^{-10}	4×10^{-8}	4×10^{-8}	5×10^{-8}	1×10^{-9}
FUR	3×10^{-10}	4×10^{-5}	4×10^{-8}	4×10^{-8}	1×10^{-9}
FUMP	4×10^{-8}	5×10^{-6}	5×10^{-6}	5×10^{-5}	2×10^{-7}
FUMP-FUR	5×10^{-8}	3×10^{-5}	3×10^{-5}	5×10^{-5}	5×10^{-5}

[a] Grown in a defined medium for 20 hr at 37°.

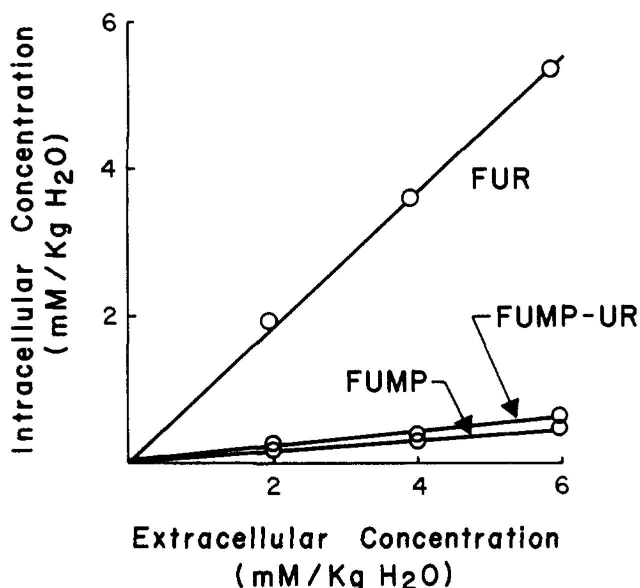


Figure 2.—Entry of 5-fluorouracil derivatives into *S. faecalis* cells. Incubation proceeded for 5 min at 37°.

indicating a permeability barrier. Figure 2 also shows that a similar barrier exists for FUMP, and since this compound cannot enter the cells in its intact form,[11] the small amount of intracellular fluoropyrimidine recovered must have resulted from cleavage prior to entrance. On the other hand the demonstration that the cell membrane limits the uptake of the dinucleoside phosphates does not constitute proof for the extracellular cleavage of these compounds. Indications for extracellular cleavage is obtained, however, from chromatographic analysis of the dinucleoside phosphates. When these compounds are removed from cold storage in the lyophilized state and chromatographed in solvent A (pH 6.5) or B (pH 11.5) at 25°, zones of inhibition at positions corresponding to dinucleoside phosphate, nucleoside monophosphate, and nucleoside can be demonstrated by means of bioautography. If the material corresponding to dinucleoside phosphate is eluted with water, and rechromatographed overnight, the same breakdown products can again be demonstrated. If relatively large amounts (50–100 mg) of the dinucleoside phosphates are applied to the paper, the products formed during chromatography can be detected by inspection under ultraviolet light. Thus, this group of compounds is apparently subject to some hydrolysis even under mild conditions and during the restricted period of time (20 hr) required for biological evaluation.

This hydrolysis is also confirmed by the information presented in Figure 3, which demonstrates the increase in potency which results when the dinucleoside phosphates are stored in phosphate buffer, pH 7.0, at 37° for various lengths of time prior to biological evaluation. The relative increase in activity of the compounds following their standing in solution would indicate that the breakdown products obtained during the period required for biological assay, together with the hydrolysis products invariably present in the starting solution, can account for much of the biological effects seen. A 5×10^{-8} *M* concentration of FUMP-FUR would have to yield less than $^1/_{10000}$ th its amount of FUR (3×10^{-8} *M*) to produce 50% growth inhibition of SF/0. Supplementation of this nonenzymic hydrolysis with enzymatic cleavage at the cell membrane can, however, not be excluded.

As shown by Figure 3 the nucleotide analog FUMP also undergoes nonenzymatic hydrolysis. That cleavage of this compound is required before it can enter the cells has been demonstrated by Leibman and Heidelberger.[11] This compound appears to be cleaved to the same extent as are the dinucleoside phosphates, and its enzymatic cleavage at the cell membrane may supplant the nonenzymatic hydrolysis.

When combined, the data obtained from the inhibition analysis and from the studies on cross-resistance, uptake, and *in vitro* hydrolysis lead to the conclusion that the dinucleoside phosphates exert their inhibitory activity following their conversion to the nucleosides, cleavage occurring most likely outside the cell. Thus,

it is questionable whether these dinucleoside phosphates *per se* possess any biological activity. Despite this fact, the compounds may be of real value in chemotherapy. The relatively slow release of the biologically active component may beneficially affect its toxicity, and may retard its rapid inactivation. Compounds such as FUMP-UR and FdUMP-dTR which contain built-in reversing agents may possess selectivity for tissues which do not use exogenous uracil or thymidine.

As shown by the data in Table IV no advantage is gained from the application of the dinucleoside phosphates to strains of *S. faecalis* resistant to the component base or nucleoside analogs. This result parallels the recent observation by Parsons and Heidelberger[28] who reported that 5-fluoro-2′-deoxyuridylyl-(5′→5′)-5-fluoro-2′-deoxyuridine did not inhibit cells resistant to FdUR. This lack of differential activity of the fluoropyrimidine-containing dinucleoside phosphates in the bacterial system differs from the marked activity exerted in tissue culture by a dinucleoside phosphate containing two 6-mercaptopurine residues. As shown by Montgomery, *et al.*,[29] this compound inhibits the growth of 6-mercaptopurine-resistant H.Ep. No. 2 cells at about $^1/_{25}$ the concentration required for equivalent inhibition by the free nucleoside or nucleotide. This observation was held to demonstrate that the molecule penetrates the cells intact.

Since the dinucleoside phosphates containing 5-fluorouracil residues do apparently represent reservoirs capable of slowly releasing the nucleoside components, they may, particularly in tissue culture, produce the effect of a continuous treatment lasting over many days and resulting in a cell response not seen with a single high dosage of the free nucleoside or nucleotide. Under such circumstances the cells in all their phases of growth are subject to the action of the drug, a fact which may result in increased inhibitory activity.

Although small, from a chemical point of view, the instability of this group of compounds is nevertheless

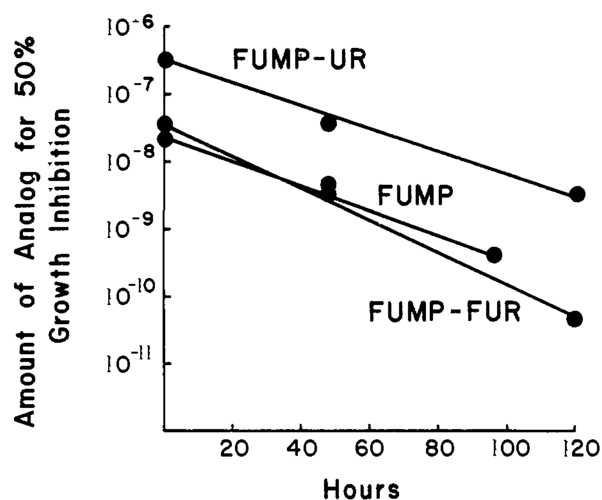


Figure 3.—Increase in biological activity of dinucleoside phosphates containing 5-fluorouracil residues following standing in neutral solution at 37° for various periods of time.

of theoretical interest. It is unlikely that the lability is due to the fluoropyrimidine residues. Indeed, compounds such as UMP-UR-(3′→5′) and 5-methyl-UMP-5-methyl-UR-(3′→5′) have also been observed in this laboratory to undergo slow cleavage upon prolonged storage in the frozen state. While, in the ribonucleoside series, the presence of a neighboring hydroxy group can be held responsible for the lability of the (3′→5′) link, no such explanation can be advanced for the relative instability of the dinucleoside phosphates combined in a (5′→5′) link, or containing two deoxyribonucleoside residues. It may well be that the limited degradation seen with the dinucleoside phosphates containing fluoro analogs is a property of dinucleoside phosphate esters in general, independent of the nature of their component residues.

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