of chloroacetic acid was added 5 ml. of water and 1.5 ml. of concentrated ammonium hydroxide. The mixture was heated in a boiling water bath for 5 min. At the end of that time there was a clear solution and the ultraviolet absorption spectrum (λ_{max} = 290 mμ at pH 1) indicated that the 6-mercapto group had been converted to a 6-carboxyethylthio group to give XLV. The solution was chilled, 50 ml. of concentrated ammonium hydroxide was added to the resultant suspension, and the mixture was heated in a sealed tube at 140° for 18 hr. After cooling, the colorless crystalline precipitate was collected, washed with water, and dried at 120° (1.1 g., 67%), m.p. 320° dec. Upon recrystallization from 40 ml. of water and treatment with Darco, the m.p. was raised to 323° dec. (sealed tube m.p. = 336° dec.) (351° [46]). Sublimation did not raise the m.p.

Anal. Calcd. for $C_6H_7N_5$: C, 48.3; H, 4.70; N, 47.0. Found: C, 47.9; H, 4.67; N, 46.6.

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Studies on Fluorinated Pyrimidines. XIV. The Synthesis of Derivatives of 5-Fluoro-2′-deoxyuridine 5′-Phosphate and Related Compounds[1]

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Phosphate derivatives of 5-fluoro-2′-deoxyuridine (I), a tumor inhibitory compound, have been chemically prepared. These derivatives represent a wide variation in (1) the amount of charge on the phosphate moiety [—O—$PO_3^=$, —O—PO_2^-(OR)$^-$, —O—$PO_2O(CH_2)_2NH(C_2H_5)_2^+$, —O—PO(OR)$_2$], (2) in the nature of the functionally substituted phosphate (steroid, amino alcohol, alcohol, amino acid, di- and triphosphates), and (3) in the stereochemical configuration at the nucleoside moiety of 5-fluoro-2′-deoxyuridylyl(5′ → 3′)5-fluoro-2′-deoxyuridine (VIII and XIII).

5-Fluorouracil[3] (5-FU), and its nucleosides, 5-fluoro-2′-deoxyuridine[4,5] (5-FUDR, I) and 5-fluorouridine[4,6] (5-FUR), have been shown to possess significant tumor inhibitory activity in certain solid human tumors[7] and in transplanted[8] mouse and rat tumors. One of the mechanisms by which these fluorinated pyrimidines exert their carcinostatic effect is the inhibition of the *de novo* biochemical pathway of thymidylic acid biosynthesis.[9] The recent work of Cohen, *et al.*,[10] with bacterial systems, and of Harbers and Heidelberger[11] and of Hartmann and Heidelberger[12] with Ehrlich ascites carcinoma systems, has shown that 5-fluoro-2′-deoxyuridine 5′-monophosphate (FUDRP, IX) is the specific, competitive inhibitor of the enzyme *thymidylate synthetase*, which is responsible for the metabolic conversion of 2′-deoxyuridylic acid to thymidylic acid. In order to inhibit *thymidylate synthetase*, 5-FU, as well as its nucleosides, 5-FUDR and 5-FUR must be metabolized *in vivo* to the nucleotide FUDRP (IX).

Various catabolic processes, however, decrease the efficiency with which the cell can incorporate and metabolize 5-FUDR and 5-FUR to FUDRP. In particular, cleavage of the glycosyl bonds of 5-FUDR and 5-FUR by *nucleoside phosphorylase* gives 5-FU, which undergoes further metabolic degradation.[13]

The therapeutic use of the (enzymically[14] or chemically[15]) preformed metabolite, FUDRP, for the inhibition of *thymidylate synthetase* appears to

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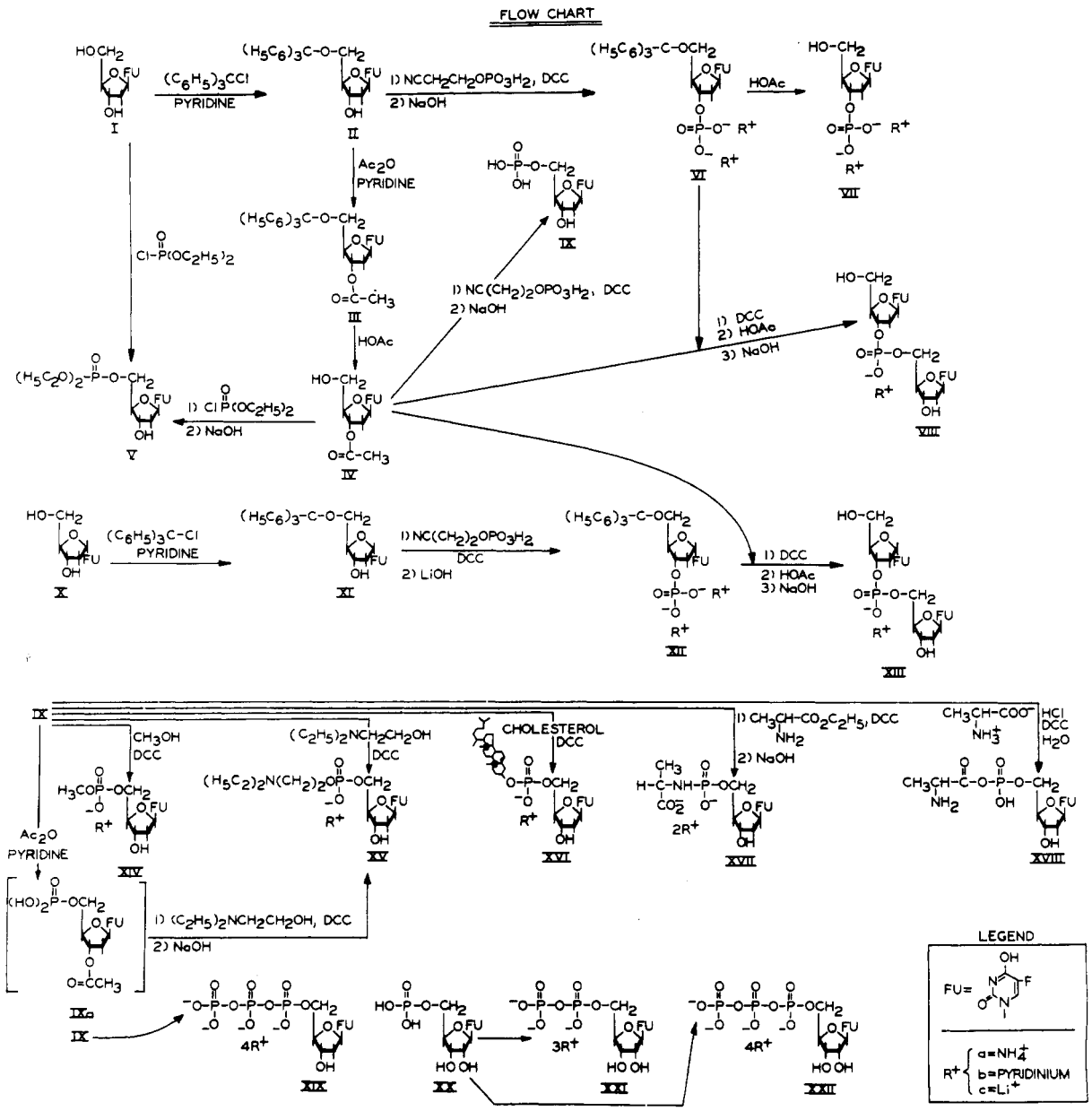

be excluded since Leibman and Heidelberger[16] have shown that cell membranes are impermeable to the passage of nucleotides. There appeared to be, however, the possibility of masking the phosphate moiety of FUDRP with a wide variety of functional substituents, one type of which,[17] it was hoped, might penetrate the complex cell membrane and be metabolized by intracellular processes (phosphodiesterases, etc.) to the active antimetabolite FUDRP.

In order to test this hypothesis, syntheses of derivatives of FUDRP were undertaken that represent (1) variation of the amount of charge on the phosphate moiety [—$OPO_3^=$—, —OPO_2OR^-, —O—PO_2-$O(CH_2)_2NH(C_2H_5)_2^+$, —O—$PO(OR)_2$], (2) variation of the nature of the functional substituent (steroid, alcohol, amino alcohol, amino acid, di-, and triphosphates), and (3) variation of stereochemical configuration in the nucleoside moiety of 5-fluoro-2′-deoxyuridylyl(5′ → 3′)-5-fluoro-2′-deoxyuridine (VIII and XIII).

Discussion

Reaction of β-5-fluoro-2′-deoxyuridine[4,5] (FUDR, I) with exactly one mole of freshly distilled diethylchlorophosphate[18] in pyridine solution gave a complex reaction mixture from which the diethyl ester of 5-fluoro-2′-deoxyuridylic acid (V) was isolated (29%) by paper chromatography. That the nucleotide derivative obtained was indeed the 5′

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rather than the isomeric 3′ derivative was shown by reaction of the protected nucleoside, 3′-acetyl-FUDR (IV), with diethyl chlorophosphate. Removal of the 3′-acetyl blocking group with dilute base after the phosphorylation reaction gave a product that was spectroscopically and chromatographically identical to that obtained by the direct reaction (I → V). Some concomitant saponification of the 5′-diethyl phosphate moiety occurred during alkaline removal of the 3′-acetyl blocking group in reaction IV → V. For this reason, the more direct reaction is preferred.

Treatment of 5′-trityl-β-FUDR (II) with β-cyanoethyl dihydrogen phosphate, patterned after Tener's[19] procedure, afforded the 5′-trityl-3′-nucleotide derivative VIa. Inasmuch as the 5′-trityl blocking group is acid labile, the free nucleotide VI, R^+ = H, cannot be isolated since the 3′-phosphate moiety causes a "self" detritylation.[20] The intermediate VI is best kept as the diammonium salt (VIa) prior to its conversion to a pyridinium salt (VIb) for use in condensation reactions. Dicyclohexylcarbodiimide (DCC) mediated condensation of VIb with the 3′-protected nucleoside IV, followed by stepwise removal of both the trityl and acetyl protecting groups according to the methods of Khorana and co-workers,[21] gave β-5-fluoro-2′-deoxyuridylyl(5′ → 3′)-β-5-fluoro-2′-deoxyuridine (VIIIa). This dinucleoside monophosphate (β,β-DNMP) was obtained in over-all yields of 15% after preparative scale paper chromatography in isopropyl alcohol–ammonia–water (7:1:2) and in *n*-butyl alcohol–acetic acid–water (5:2:3).

In a similar manner, 5′-trityl-α-5-fluoro-2′-deoxyuridine 3′-monophosphate (XIIa) was prepared from α-FUDR (X) *via* the tritylation and cyanoethyl phosphate reactions. DCC mediated condensation of XIIb with IV gave, after removal of the protecting groups, a crude reaction mixture from which the α,β-dinucleoside monophosphate XIIIa (α,β-DNMP) was isolated in 39% yield by preparative scale paper chromatography. The trityl nucleosides XIIb and VIb exhibit striking differences in solubility. The β anomer VIb is quite water-soluble, but is only moderately soluble in pyridine. The α anomer XIIb is fairly insoluble in water, but is quite soluble in pyridine.

Prolonged incubation of the α,β-DNMP (XIIIa) with crude snake venom phosphodiesterase resulted in degradation of the compound to the nucleoside components, whereas incubation of XIIIa with a purified venom phosphodiesterase[22] (*Crotalus adamanteus*) gave β-5-fluoro-2′-deoxyuridine 5′-monophosphate (IX) and α-FUDR (X). The α,β-DNMP was unaffected by a purified prostatic phosphomonoesterase. Similarly, the β,β-DNMP was degraded by the purified venom phosphodiesterase (free of 5′-phosphomonoesterase activity) to its respective 5′-nucleotide (IX) and nucleoside (I) components.

Since the mixed anhydrides of amino acids and adenylic acid (aminoacyladenylate derivatives) are involved in important metabolic processes within cells,[23] it appeared of interest to prepare an aminoacyl-5-fluorouridylate derivative for investigation as to its permeability characteristics. 5-Fluoro-2′-deoxyuridine 5′-L-alanylphosphate (XVIII) was prepared essentially by the method of Berg.[24] This alanyldeoxyfluorouridylate is far less stable than some of the aminoacyladenylates recently reported. For example, whereas Harris and MacWilliam[25] were able to purify adenine 5′-L-leucylglycylphosphate by paper chromatography in a butanol–acetic acid–water (4:1:1) system, attempted chromatography of XVIII in this system resulted in complete hydrolysis to alanine and FUDRP. Isolation and purification of XVIII was accomplished, however, by high potential electrophoresis[26] (150 v./cm., 6–8 ma.) in sodium acetate buffer, pH 3.1. At this pH, XVIII travels slowly (1–2 cm./hr.) towards the cathode, thus showing that the electrophoretic migration characteristic of the compound is consistent with the assumption that it bears a net positive charge. Because of its inherent instability, the compound was isolated only as an aqueous solution of known concentration. Analysis of the electrophoretically pure sample showed the compound to have a ratio of FUDR (spectrophotometrically determined[27]): total phosphorus[28]: number of anhydride linkages[24,29] = 1.0:1.02:0.94. (Theory requires the ratio FUDR: P:anhydride linkages = 1.0:1.0:1.0). Although it was kept in a frozen solution, rapid hydrolysis of XVIII to alanine and FUDRP occurred. Thus, after standing for four days from the time of its initial preparation, 55% of XVIII had decomposed as determined by spectrophotometric analysis of the electrophoretically separated hydrolysis products.

The preparation of the FUDRP esters XIV, XV, and XVI, was based on the observations by Khorana[30] that, in the presence of dicyclohexylcarbodi-

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imide (DCC) and a large excess of alcohol, phospho monoesters are converted to phospho diester derivatives. As based on an arbitrarily selected example, in the preparation of the *N,N*-diethylaminoethyl ester XV, there appeared to be no relative advantage to protect the 3′-hydroxyl group in FUDRP (IX → IXa) prior to the DCC condensation reaction with the alcohol. Since alkaline hydrolysis of the 3′-acetyl protecting group in reaction IXa → XV (step 2) can conceivably result in concomitant saponification at the 5′-phospho diester moiety to give FUDRP, the more direct reaction (IX → XV) is again preferred.

In the preparation of XIV and XV, the excess alcohol used in the condensation reaction was easily removed after the reaction since both methanol and diethylaminoethanol are volatile. In the case of the cholesteryl ester XVI, separation of the ester and excess cholesterol was effected by column chromatography on Florisil. Further purification of the moderately water-insoluble ester was carried out by preparative scale paper chromatography, a method that was also used for the purification of the esters XIV and XV.

Mukherjee and Heidelberger[31] have recently studied the ability of the 5-fluoropyrimidine derivatives whose syntheses are reported here to inhibit the incorporation of C^{14}-formate into DNA-thymine in Ehrlich ascites carcinoma cells *in vitro*. None of the compounds was more effective than FUDRP.

It has recently been shown that tumor cells have a high pinocytotic activity.[32] It was, therefore, of interest to prepare the polymers of the fluorinated pyrimidine nucleotides IX and XX (poly-FUDRP and poly-FURP, respectively) to see if they could be taken up by Ehrlich ascites cells.

5-Fluorouridine 5′-diphosphate (FURPP, XXI) and 5-fluorouridine 5′-triphosphate (FURPPP, XXII) were prepared from the nucleotide XX by procedures similar to those recently outlined by Khorana and co-workers.[33] Lengyel and Ochoa[34] successfully converted the diphosphate XXI into poly-FURP with *polynucleotide phosphorylase*. A ribonucleic acid (RNA) containing fluorouracil has been prepared by Weiss[35] using the triphosphate XXII, the triphosphate derivatives of guanosine, adenosine, cytidine, and *RNA-polymerase*.

5-Fluoro-2′-deoxyuridine 5′-triphosphate (XIX) was synthesized from the corresponding nucleotide IX. Using *E. coli DNA-polymerase*, Kornberg and Aposhian[36] found that the triphosphate XIX was very poorly incorporated into deoxyribonucleic acid (DNA), with fluorouracil replacing thymine, and it did not support extensive net synthesis of DNA. Accordingly, no DNA containing fluorouracil could be obtained from XIX to carry out other studies.

The compounds reported in this paper have also been studied with respect to their susceptibility to cleavage by *nucleoside phosphorylase*.[37] Preliminary studies of these compounds in transplanted mouse tumors are in progress and will be reported elsewhere.

Experimental

Methods.—Descending paper chromatography was used throughout this work, employing the following solvent systems: *A*, isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2)[38]; *B*, *n*-butyl alcohol–acetic acid–water (5:2:3)[39]; *C*, 1% ammonium sulfate in 0.0005 *M* sodium borate–isopropyl alcohol (1:2) on paper previously soaked in 1% ammonium sulfate in 0.0005 *M* sodium borate; *D*, ethyl alcohol–1 *M* ammonium acetate, pH 7.5, (7.5:3.0).[40] The type of paper used in each experiment is mentioned in the text. The compounds were detected on the papers by visual observation under an ultraviolet lamp; phosphates were located on separate corresponding chromatograms (guide strips) by an ammonium molybdate–perchloric acid spray.[41]

Ultraviolet absorption measurements were made with a Beckman spectrophotometer, Model DU or DB. Where indicated, ϵ (P) refers to the molar extinction coefficient based on 1 g.-atom of phosphorus per liter.[42] Analyses were performed by Huffman Microanalytical Laboratories, Spang Microanalytical Laboratory, and through the courtesy of Hoffmann-LaRoche, Inc. Total phosphorus was determined by the method of Fiske and Subbarow.[28] Melting points were taken on a hot stage and are uncorrected. Pyridine was distilled from and stored over calcium hydride.

We are greatly indebted to Dr. Robert Duschinsky of Hoffmann-LaRoche, Inc. for generous gifts of FUR and FUDR.

5-Fluoro-2′-deoxyuridine (5′)-Diethylphosphate (V). Method A.—A solution of 1.00 g. (0.00406 mole) of β-5-fluoro-2′-deoxyuridine (I) in 10 ml. of dry pyridine was placed in a dry, nitrogen flushed, three-necked flask equipped with condenser, dropping funnel, and magnetic stirrer. Into the dropping funnel had been weighed previously 0.7025 g. (0.00407 mole) of freshly redistilled diethyl chlorophosphate.[18] After cooling the reaction flask to −5°, the diethylchlorophosphate was added dropwise to the stirred solution over a period of 5 hr. After the addition was complete, the solution was allowed to warm to room temperature and was stirred an additional 4 hr.

The dark straw-colored solution was taken to dryness *in vacuo* on a rotary evaporator, and the residual sirup obtained was coevaporated *in vacuo* with water ten times to remove completely the pyridine and pyridinium hydrochloride. The residue was applied to 22 sheets of 18-in. wide Whatman No. 40 filter paper. Chromatography in solvent system *A* gave five bands detectable under the ultraviolet lamp of which the "heaviest" (darkest), and the only band to show a positive Hanes-Isherwood[41] test for phosphate, had an average R_f of 0.68 to 0.80. The corresponding bands were cut

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out from all papers, eluted with water, and the eluate was evaporated to dryness *in vacuo*. The resulting tan oil was dissolved in a small volume of water, filtered, and the filtrate was freeze dried to give 0.527 g. of a very viscous oil. On standing under vacuum for several days, this oil partially crystallized in large star shaped masses. Repeated trituration of the partially crystallized material with cold ether removed a colored, soluble material and left 0.425 g. (29%) of 5-fluoro-2′-deoxyuridine (5′)-diethylphosphate (V) as an extremely hydroscopic off-white powder, $\lambda_{max}^{H_2O}$ 268 mμ, ε (P) 8540; $(OD_{260}/OD_{260})_{0.01\ N\ HCl} = 0.86$; $(OD_{280}/OD_{260})_{0.01\ N\ NaOH} = 0.80$. The material was chromatographically homogenous in solvent system A (R_f 0.77), B (R_f 0.88), and in butanol–water (86:14) (R_f 0.71).

Anal. Calcd. for $C_{13}H_{20}N_2O_8FP$ (382.29): C, 40.84; H, 5.28; P, 8.10. Found: C, 40.88; H, 5.24; P, 8.56.

Method B.—A solution of 0.300 g. (0.00104 mole) of 3′-acetyl-β-5-fluoro-2′-deoxyuridine[15] (IV) in 45 ml. of dry pyridine was placed in a dry, nitrogen flushed, three-necked flask equipped with a dropping funnel and magnetic stirrer. The solution was cooled in ice and to it was added dropwise 0.6 ml. of diethylchlorophosphate over a 1.5-hr. period. The reaction mixture was stirred an additional 6 hr. at 0° and then was allowed to stand at room temperature overnight. After evaporation to dryness *in vacuo*, the residue was co-evaporated several times with water to remove pyridine and pyridinium hydrochloride. The residue was dissolved in 5 ml. of water and made alkaline (pH 13) by the addition of 6 *N* sodium hydroxide. After standing for 30 min., the solution was passed through a (1.3 × 30 cm.) column of Dowex-50 (H^+) ion exchange resin to remove sodium ions. The eluate was concentrated to a small volume and passed through a column (1.3 × 27 cm.) of Dowex-1-chloride to remove acidic components. The clear, colorless eluate was concentrated to a small volume and applied to six sheets of 18-in. wide Whatman No. 40 filter paper. Chromatography in solvent system *A* showed a very heavy band at R_f 0.67 to 0.79. The corresponding bands were cut out from all papers, eluted with water, and the eluate was freeze dried after filtration. The material obtained was chromatographically homogeneous and identical to the product obtained above (method A) when chromatographed either singly or when mixed with product prepared by method A; solvent system *A* (R_f 0.74, mixed R_f 0.75); solvent system *B* (R_f 0.87, mixed R_f 0.86); *n*-butanol–water (86:14) (R_f 0.71, mixed R_f 0.69). (All chromatograms on Whatman No. 1 filter paper); $(OD_{280}/OD_{260})_{0.01\ N\ HCl} = 0.84$; $(OD_{280}/OD_{260})_{0.01\ N\ NaOH} = 0.80$.

Preparation of the *N,N*-Diethylaminoethyl Ester of 5-Fluoro-2′-deoxyuridine (5′)-Monophosphate (XV). Method A:—To a mixture of 3 ml. of anhydrous pyridine and 3 ml. of freshly distilled acetic anhydride was added 100 mg. (0.306 mmole) of β-FUDRP[15] (IX). The solution was stirred magnetically in the absence of light for 30 hr. After evaporation of the solution to dryness *in vacuo* at 30°, the pale yellow residue was co-evaporated ten times with 5-ml. portions of water to remove pyridinium acetate. The residue was dissolved in approximately 2 ml. of water and freeze dried to give a pale tan powder. To this product was added 4 ml. of dry pyridine, 4 ml. of freshly distilled diethylaminoethanol, and 300 mg. of redistilled DCC. The flask was stoppered, and the mixture was shaken at room temperature for 14 days. During this time, dicyclohexylurea precipitated from the originally homogeneous solution. The mixture was evaporated to dryness *in vacuo* at 30°, and the residue was co-evaporated *in vacuo* several times with water to assist complete removal of the pyridine and diethylaminoethanol. The remaining product was dissolved in 10 ml. of 0.1 *N* sodium hydroxide, and the solution was filtered and extracted with 20 ml. of ether. After standing for 30 min., the aqueous phase was concentrated *in vacuo* at 30° to a small volume and passed through an Amberlite IR-120 (NH_4^+) ion exchange column. The eluate was concentrated *in vacuo* to a small volume and then was applied to two 18-in. wide sheets of Whatman No. 40 filter paper. Chromatography in solvent system *A* gave two principal bands: (1) unchanged FUDRP (R_f 0.03 to 0.09) and (2) the ammonium salt of the *N,N*-diethylaminoethyl ester of β-FUDRP (XVa) (R_f 0.41 to 0.49). This latter band was cut, eluted with water, the water concentrated *in vacuo* to 1–2 ml., and the concentrate freeze-dried. The resultant pale tan product was dissolved in 3 ml. of warm absolute ethanol, centrifuged to remove a small amount of insoluble, colored material, and the product precipitated by addition of 12 ml. of dry ether. After drying at 56° for 6 hr. *in vacuo*, there was obtained 44.7 mg. (32%) of **ammonium 5-fluoro-2′-deoxyuridine (5′)-diethylaminoethylphosphate** (XVa) as the monohydrate salt. The white powdery product had λ_{max} 268 mμ, ε (P) 8590 (in water); R_f (solvent system *A*) 0.45; R_f (solvent system *B*) 0.53 (Whatman No. 1 filter paper).

Anal. Calcd. for $C_{15}H_{24}N_3O_8FP \cdot NH_4 \cdot H_2O$ (460.41): C, 39.13; H, 6.56; P, 6.72. Found: C, 39.22; H, 6.08; P, 6.32.

Electrophoresis of the product on Whatman No. 4 paper in 0.1 *M* ammonium acetate buffer, pH 4.05, (135 v./cm., 34 ma., 0°)[26] produced a single homogeneous band migrating very slowly to the cathode thus indicating that the molecule bears a net positive charge at this pH.

Method B.—β-5-Fluoro-2′-deoxyuridine (5′)-monophosphate (IX) (100 mg., 0.306 mmole) was passed through an Amberlite IR-120 (pyridinium) ion exchange column. The eluate was evaporated to dryness *in vacuo* at 30°, and the pyridinium salt obtained was made anhydrous by co-evaporation with pyridine *in vacuo*. To the dry salt was added 4 ml. of freshly distilled diethylaminoethanol, 6 ml. of anhydrous pyridine, and 300 mg. of DCC. The initially homogeneous solution was shaken gently for 8 days during which time dicyclohexylurea precipitated. The mixture was evaporated *in vacuo* at 30°, and the residue was coevaporated five times with water to remove the pyridine and diethylaminoethanol. The residue was dissolved in 10 ml. of water and filtered. The filtrate, after being extracted with ether, was passed through an Amberlite IR-120 (NH_4^+) ion exchange column. The eluate was concentrated *in vacuo* at 30° to a small volume, and the solution was applied to two 18-in. wide sheets of Whatman No. 40 filter paper. Chromatography in solvent system *A* gave two principal bands: (1) unchanged starting nucleotide (R_f 0.05 to 0.18) and (2) the ammonium salt of the *N,N*-diethylaminoethyl ester of β-FUDRP XVa (R_f 0.42 to 0.50). The latter band was cut out and eluted with water. After concentration of this eluant *in vacuo*, the pale tan solution was freeze-dried. The residue was dissolved in 3 ml. of warm absolute ethanol, centrifuged to remove a small amount of insoluble, colored material, and the product precipitated by addition of 12 ml. of ether. The "off-white" powder, so obtained, was dried at 56° for 2 hr. *in vacuo;* λ_{max} 268 mμ, ε (P) 9300 (in water) R_f (solvent system *A*) 0.48; R_f (solvent system *B*) 0.53.

Anal. Calcd. for $C_{15}H_{24}N_3O_8FP \cdot NH_4 \cdot H_2O$: P, 6.72. Found: P, 6.24.

5-Fluoro-2′-deoxyuridine (5′)-Cholesterylphosphate (XVI).—5-Fluoro-2′-deoxyuridine (5′)-monophosphate (120 mg., 0.368 mmole) was converted to its pyridinium salt by the method previously described. Cholesterol[43] (1.300 g., 3.36 mmoles) and 2 ml. of anhydrous pyridine were added to the dry pyridinium salt, and the mixture was shaken until a homogeneous solution was obtained. To this solution was added 0.388 g. (1.88 mmoles) of DCC and 2 ml. of pyridine. The flask was sealed and placed in a mechanical shaker. Dicyclohexylurea began to precipitate from the original homogeneous mixture within an hour. After shaking for 7 days at room temperature, the precipitate was removed by filtration and the clear, light brown filtrate was evaporated to dryness *in vacuo*. Repeated co-evaporation of the yellow,

(43) Cholesterol was purified by conversion to the dibromide and then subsequent regeneration; R. J. Anderson, *J. Biol. Chem.*, **71**, 407 (1927).

gummy residue with petroleum ether helped to remove residual pyridine.

The residue was placed on a Florisil column (2.1 × 36 cm.) packed in petroleum ether (b.p. 60–80°). Excess, unchanged cholesterol was eluted from the column with 100% benzene, and a crystalline, non-ultraviolet absorbing material (presumably dicyclohexylurea) was eluted from the column with 100% ethyl acetate. Finally, washing the column with absolute methanol gave 258 mg. of a gummy white solid that absorbed in the ultraviolet at 270 mμ. This residue was dissolved in 10 ml. of methanol containing 5 ml. of concentrated ammonium hydroxide, and the resulting solution was evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml. of absolute methanol and filtered to remove an insoluble (but water-soluble) white powder. The methanolic filtrate was concentrated to a small volume and applied to 4 sheets of Whatman No. 40 filter paper (previously extracted with methanol). Chromatography in solvent system *A* gave a single band (R_f 0.85). The bands, after being cut out, were eluted with warm methanol. The methanol eluate was evaporated to dryness, and the crystalline residue was washed with 1 to 2 ml. of ice cold methanol to remove a slight amount of colored material. The white material remaining was dissolved in warm methanol, filtered, and to the clear filtrate was added 200 ml. of ether. After cooling, the precipitate was collected by centrifugation to give 41.6 mg. (16%) of ammonium 5-fluoro-2′-deoxyuridine (5′)-cholesterylphosphate (XVIa) as a fine white powder; $\lambda_{max}^{CH_3OH}$ 269 mμ, ε (P) 8420 (in methanol); R_f 0.90 (solvent system *A*, Whatman No. 1 filter paper); R_f 0.55 (in *n*-butanol–water, 86:14).

Anal. Calcd. for $C_{36}H_{59}N_3O_8FP$(711.83): C, 60.74; H, 8.36; P, 4.35. Found: C, 60.22; H, 8.06; P, 4.37.

The compound underwent indefinite decomposition at about 190–196°.

5-Fluoro-2′-deoxyuridine (5′)-Methylphosphate (XIV).—A mixture of 25 mg. (0.0765 mmole) of β-FUDRP (as the pyridinium salt), 2 ml. of anhydrous pyridine, 25 ml. of methanol (dried over calcium oxide), and 100 mg. of DCC was shaken at room temperature for 6 days. The solvents were removed *in vacuo* at 30°, and the semiliquid compound so obtained was dissolved in a small amount of water. After filtration, the aqueous solution was extracted with 3 to 10-ml. portions of ether prior to freeze-drying. The gummy residue was dissolved in absolute ethanol and precipitated with ether. The resultant white material was dissolved in a minimum quantity of water and applied to an 18-in. wide sheet of Whatman 3 MM filter paper. Chromatography in solvent system *A* gave three bands (R_f's 0.42, 0.45, and 0.70) of which the principal band was that having an R_f of 0.45. This band was cut out, eluted with water, and the eluate was freeze-dried. The resulting white powder was dissolved in absolute ethanol, centrifuged to remove a small amount of insoluble material, and the product was precipitated by addition of ether. After drying, there was obtained 12 mg. (46%) of ammonium 5-fluoro-2′-deoxyuridine (5′)-methylphosphate (XIVa) λ_{max} 271 mμ, ε (P) 8400 (in water); R_f (solvent system *C*) 0.91.

Anal. Calcd. for $C_{10}H_{13}N_2O_8FP{\cdot}NH_4$(357.24): P, 8.71. Found: P, 8.40.

β-5-Fluoro-2′-deoxyuridylyl (5′ → 3′)-β-5-fluoro-2′-deoxyuridine (VIII).—A mixture of 1.194 g. (2.45 mmoles) of 5′-trityl-β-FUDR (II), 5 ml. of a stock solution of β-cyanoethyl phosphate[19] (1 mmole/ml.), and 20 ml. of dry pyridine was evaporated *in vacuo* to a thick sirup. Additional anhydrous pyridine was added and the solution was evaporated again. After dissolving the thick, clear residue in 15 ml. of dry pyridine, 1.83 g. of DCC was added. The flask was sealed and shaken gently at room temperature for 4 days. Dicyclohexylurea began precipitating from the initially homogeneous solution within a few minutes. The mixture was transferred to a separatory funnel with the aid of 20 ml. of pyridine and 20 ml. of water. After extracting the excess, unchanged DCC with two 25-ml. aliquots of petroleum ether, the aqueous phase was allowed to stand for 3 hr. at room temperature to cleave the metaphosphates. Dicyclohexylurea was removed by filtration, and to the clear filtrate (plus washings) was added 5 ml. of 1 *M* sodium hydroxide. The basic aqueous phase was evaporated to dryness *in vacuo*. (During this evaporation, additional sodium hydroxide was added to maintain alkalinity.) To the residue was added 20 ml. of 1 *M* sodium hydroxide, and the resulting solution was refluxed for 2 hr. A small amount of precipitate was removed by filtration and washed with dilute sodium hydroxide solution. To the total aqueous filtrate was added approximately 50 ml. of Amberlite IR-120 (NH_4^+) ion exchange resin. The solution was then passed through an Amberlite IR-120 (NH_4^+) column (3.3 × 42 cm.). The eluate was concentrated *in vacuo* to a small volume (~ 15 ml.). During this concentration, ammonium hydroxide was added periodically to maintain alkalinity. The concentrate was filtered and then freeze dried to give an almost quantitative yield of 5′-trityl-5-fluoro-2′-deoxyuridine (3′)-monophosphate (VIa) as the diammonium salt. Chromatography in solvent system *A* showed a single band (R_f 0.56).

The ammonium salt of 5′-trityl (3′)-phosphate (VIa) (see above) was converted to its pyridinium salt by passage through an Amberlite IR-120 (pyridinium) column. To 305 mg. of the dry pyridinium salt was added 266 mg. (0.925 mmole) of 3′-*O*-acetyl-β-FUDR and 420 mg. of DCC. The mixture was dissolved in 1 to 2 ml. of dry pyridine, and the flask was sealed and vigorously shaken for a few minutes. The solution turned slightly brown and within 10 min., dicyclohexylurea began to precipitate from solution. The reaction was allowed to stand at room temperature for 3–4 days. The mixture was evaporated to dryness *in vacuo* at 30° and the trityl compounds were extracted with four 7-ml. portions of chloroform. The chloroform extracts were filtered. Evaporation of the chloroform gave a gummy brown solid to which was added 10 ml. of 80% acetic acid. Detritylation was effected by refluxing this solution for 10 min. Water (20 ml.) was added to the solution after cooling in ice, and the crystalline triphenylcarbinol was removed by filtration. The aqueous filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml. of water and the pH of the solution was adjusted to 13 by addition of aqueous sodium hydroxide solution. After standing 30 min., the solution was passed through a Dowex-50 (H^+) ion exchange column to remove sodium ions. The eluate was evaporated to a small volume *in vacuo*, and the material obtained was applied to four 18-in. wide pieces of Whatman No. 40 filter paper. Chromatography in solvent system *A* gave three principal bands: (1) a nucleotide band (R_f 0.04 to 0.13); (2) the dinucleoside monophosphate (VIIIa) (R_f 0.15 to 0.28); and (3) a nucleoside band (R_f 0.43 to 0.58). The second bands (R_f 0.15 to 0.28) were cut out, eluted with water, and the eluate was freeze-dried to give 116.5 mg. of a pale tan solid.

This material was dissolved in a small amount of water and applied to three 18-in. wide pieces of Whatman No. 40 filter paper. Chromatography in solvent system *B* gave single principal bands at R_f 0.18 to 0.30; these bands were cut out and eluted with water. Ammonium hydroxide (5 ml.) was added to the eluate prior to its evaporation *in vacuo*. The residue was dissolved in 20 ml. of hot absolute ethanol, filtered, and 75 ml. of ether was added to the filtrate. After cooling, the precipitate was collected by filtration and dried to give 45.2 mg. (15%) of ammonium β-5-fluoro-2′-deoxyuridylyl(5′ → 3′)-β-5-fluoro-2′-deoxyuridine (VIIIa) as a pure white powder; λ_{max} 268 mμ, ε (P) 16,700 (in water); R_f (solvent system A) 0.31 (Whatman No. 1 filter paper); R_f (solvent system B) 0.33 (Whatman No. 1 filter paper). The compound was electrophoretically homogeneous (Whatman No. 4 paper, 140 v./cm., 34 ma.) in 0.1 *M* acetate buffer, pH 4.05.

Anal. Calcd. for $C_{18}H_{20}N_4O_{12}F_2P{\cdot}NH_4{\cdot}2H_2O$ (607.44):

C, 35.59; H, 4.65; P, 5.10. Found: C, 35.26; H, 4.35; P, 5.06.

This dinucleoside monophosphate (NH_4^+ salt, 1.2 mg.) was incubated with 0.02 ml. of a purified snake venom phosphodiesterase solution[22] (*Crotalus adamanteus*) in 0.08 ml. of 0.2 *M* tris buffer, pH 8.0, for 3 hr. at 37°. Chromatography of the total mixture in solvent system *A* showed a nucleotide band (R_f 0.13), a nucleoside band (R_f 0.62), and some starting dinucleoside monophosphate (R_f 31).

5-Fluoro-2′-deoxyuridine (3′)-Monophosphate (VII).—A mixture of 215 mg. (0.357 mmole) of the ammonium salt of 5′-trityl-5-fluoro-2′-deoxyuridylic-(3′) acid (VIa) and 10 ml. of 80% acetic acid was refluxed gently for 10 min. After addition of 50 ml. of water to the ice-cooled mixture, it was filtered to remove crystalline triphenylcarbinol. The filtrate was evaporated to dryness. Several coevaporations with water completely removed the acetic acid. The residue was dissolved in 25 ml. of water, and the pH of the solution was adjusted to 7.5 by addition of an aqueous barium hydroxide solution. The solution was filtered and to the clear filtrate was added three times its volume of absolute ethanol. The mixture was cooled overnight to precipitate the barium salt of 5-fluoro-2′-deoxyuridine (3′)-monophosphate. The precipitate was collected by centrifugation and washed with absolute ethanol, acetone, ether, and dried. The white precipitate was then re-dissolved in water and passed through a column of Dowex-50 (H^+) to completely remove barium ions. The eluate was concentrated *in vacuo* to a very small volume and applied to an 18-in. wide sheet of Whatman 3 MM (acid-washed) filter paper. Chromatography in solvent system *A* gave a single major band (R_f 0.06). The band was cut out, eluted with water, and the eluate was freeze-dried to give 30 mg. (26%) of an off-white powder; λ_{max} 267 mμ, ε (P) 8350 (in water); R_f (in solvent system *B*, Whatman No. 1 paper) 0.35.

Anal. Calcd. for $C_9H_{10}N_2O_8FP \cdot 2NH_4$ (360.24): P, 8.59. Found: P, 8.80.

5′-*O*-Trityl-α-5-fluoro-2′-deoxyuridine (XI).—Into a round-bottom flask equipped with magnetic stirrer and calcium chloride drying tube was placed a solution of 5.00 g. (0.02 mole) of α-5-fluoro-2′-deoxyuridine[44] (X) in 20 ml. of anhydrous pyridine. Freshly recrystallized triphenylchloromethane[45] (6.00 g.) was added to the solution. After heating for 3 hr. on a steam bath, the solution was allowed to cool slowly overnight. The solution was poured into 400 ml. of ice water. The resulting gummy precipitate was taken up in and extracted with three 50-ml. portions of chloroform. The combined chloroform extracts were dried over sodium sulfate. After removing the sodium sulfate by filtration and the chloroform by evaporation, the residue obtained was put on a silicic acid column (3.2 × 35 cm.) packed in petroleum ether. Triphenylcarbinol was eluted from the column with benzene, after which the trityl nucleoside was eluted with methanol–ether (1:5). After evaporation of the solvent, the residue was crystallized from aqueous ethanol to give 8.04 g. (81%) of 5′-*O*-trityl-α-5-fluoro-2′-deoxyuridine (XI) as small white needles. Three recrystallizations from aqueous ethanol gave analytically pure material that contained 1 mole of water of crystallization, m.p. 170–173° (previous sweating 150–160°).

Anal. Calcd. for $C_{28}H_{25}N_2O_5F \cdot H_2O$ (506.52): C, 66.39; H, 5.37. Found C, 66.75; H, 5.30.

β-5-Fluoro-2′-deoxyuridylyl(5′ → 3′)-α-5-fluoro-2′-deoxyuridine (XIII).—To a solution of 1.194 g. (2.36 mmoles) of 5′-*O*-trityl-α-FUDR (monohydrate) in 5 ml. of anhydrous pyridine was added 5 ml. of a stock solution of β-cyanoethyl phosphate (1 mmole/ml.). The solution was evaporated to dryness *in vacuo*. Additional dry pyridine was added and the solution was evaporated again. To the thick, clear sirup was added 15 ml. of dry pyridine and 1.83 g. of DCC. The flask was sealed and shaken mechanically at room temperature for 4 days. The mixture was then transferred to a separatory funnel with the aid of 20 ml. of water and 20 ml. of pyridine. After extracting with two 25-ml. portions of petroleum ether, the aqueous pyridine solution was allowed to stand for 3 hr. Dicyclohexylurea was removed by filtration and to the clear filtrate was added 15 ml. of 1 *M* lithium hydroxide. The solution was evaporated to dryness *in vacuo* and 20 ml. of 1 *M* lithium hydroxide was added to the residue. The solution was refluxed for 2 hr., cooled, and filtered to remove an insoluble, colored gum. To the filtrate was added 100 ml. of Amberlite IR-120 (NH_4^+) ion exchange resin. The total aqueous solution then was passed through an Amberlite IR-120 (NH_4^+) ion exchange column (3.2 × 42 cm.). The eluate was concentrated *in vacuo* at 30° to a small volume (~ 50 ml.). During this concentration, ammonium hydroxide was added periodically to the solution to maintain alkalinity. On further concentration of the solution (~ 15 to 20 ml.), the diammonium salt of 5′-trityl-α-5-fluoro-2′-deoxyuridine (3′)-monophosphate (XIIa) precipitated. The precipitate was collected by centrifugation and dried in high vacuum at room temperature to give 0.8638 g. (59%) of an almost white powder. Chromatography in solvent system *A* showed a single ultraviolet absorbing band (R_f 0.77). When the supernatant from the above centrifugation was freeze-dried, there was obtained an additional 0.536 g. (36%) of the trityl-(3′) nucleotide (XIIa) as a brown, gummy solid. Trituration of this residue with ice water removed most of the color and gave additional material suitable for succeeding condensation as described below.

A 305-mg. sample of the diammonium salt of the trityl nucleotide was converted to its pyridinium salt by passage through an Amberlite IR-120 (pyridinium) ion exchange column (2.5 × 40 cm.). To the dry pyridinium salt was added 266 mg. (0.925 mmole) of 3′-*O*-acetyl-β-FUDR (IV) and 420 mg. of DCC. After the mixture was dissolved in 2 ml. of anhydrous pyridine, the flask was sealed and mechanically agitated at room temperature for 4 days. Dicyclohexylurea began to precipitate from solution within a few minutes after shaking. Pyridine was removed *in vacuo* and the trityl compounds were extracted with three 10-ml. portions of chloroform. Filtration of the combined chloroform extracts removed dicyclohexylurea and unchanged 3′-*O*-acetyl-β-FUDR. The chloroform was removed *in vacuo* and to the residual brown gum was added 10 ml. of 80% acetic acid. After refluxing gently for 10 min., the acetic acid solution was cooled in ice. Addition of water (20 ml.) precipitated triphenylcarbinol which then was removed by filtration. The clear, light brown solution was evaporated to dryness *in vacuo*. Most of the acetic acid was removed by coevaporating the residue with water. The residue was dissolved in 5 ml. of water, and sodium hydroxide was added to adjust the solution to pH 13. After standing for 30 min., the solution was passed through a Dowex-50 (H^+) ion exchange column (2.2 × 30 cm.). The eluate was concentrated *in vacuo* to a small volume and the concentrated solution was applied to four 18-in. wide sheets of Whatman No. 40 filter paper. Chromatography in solvent system *A* gave three bands: (1) a nucleotide band (R_f 0.03 to 0.12), (2) a dinucleoside monophosphate band (R_f 0.14 to 0.29), and (3) a nucleoside band (R_f 0.44 to 0.57). The second bands (R_f 0.14 to 0.29) were cut out, eluted with water, and the eluate was freeze-dried to give a pale tan solid. This solid was dissolved in 30 ml. of hot absolute ethanol, centrifuged to remove a small amount of insoluble, colored material, and to the supernatant was added 90 ml. of anhydrous ether. After cooling, the white precipitate was collected by centrifugation. Purification was effected by dissolving the material in absolute ethanol, filtering, and re-precipitating it by addition of ether. After drying *in vacuo* at 56° there was obtained 113 mg. (39%) of **ammonium β-5-fluoro-2′-de-**

(44) M.p. 174–175°; $[\alpha]^{25}D$ −20.2° (2% in water). This α-FUDR contained less than 0.02% of the β-anomer as determined by inhibition of *Sarcina lutea*. We are indebted to Dr. R. Duschinsky for these data.

(45) Aldrich Chemical Co., Milwaukee 10, Wisconsin.

oxyuridylyl(5′ → 3′)-α-5-fluoro-2′-deoxyuridine (XIIIa) as a white powder; λ_{max} 269 mμ, $\epsilon(P)_{max}$ 17,800 (in water); R_f (solvent system *A*) 0.33; R_f (solvent system *B*) 0.24 (Whatman No. 1 filter paper).

Anal. Calcd. for $C_{18}H_{20}N_4O_{12}F_2P\cdot NH_4\cdot H_2O$ (589.42): C, 36.68; H, 4.45; P, 5.26. Found: C; 36.42; H, 4.60; P, 5.03.

To a pre-incubated (37°) solution of the ammonium salt of the above dinucleoside monophosphate (1.1 mg.) in 0.08 ml. of 0.2 *M* tris buffer, pH 8.0, was added 0.02 ml. of a purified snake venom phosphodiesterase[22] (*Crotalus adamanteus*) solution. The solution was incubated for 3 hr. at 37°. The total mixture was chromatographed in solvent system *A* and gave a nucleotide band (R_f 0.13), a nucleoside band (R_f 0.63), and some starting dinucleoside monophosphate (R_f 0.28).

The above dinucleoside monophosphate was unaffected after treatment with a purified prostatic phosphomonoesterase in an acetate buffer solution (pH 5.6).

5-Fluoro-2′-deoxyuridine 5′-Alanylphosphate (XVIII).—To a magnetically stirred solution of 28.5 mg. (0.32 mmole) of L-alanine in 0.35 ml. of 1.008 *N* hydrochloric acid was added a solution of 100 mg. (0.306 mmole) of β-FUDRP in 2.4 ml. of pyridine and 0.52 ml. of water. The solution was cooled in an ice–salt mixture (−10°) and 3.2 g. (15.55 mmoles) of DCC in 6 ml. of pyridine was added. The mixture was stirred at −10° to 0° for 3.5 hr. and 150 ml. of acetone (−15°) was added. The precipitate was immediately removed by filtration, and the white residue was washed with two 25-ml. portions of cold acetone and one 25-ml. portion of cold ether. The precipitate was dried in high vacuum for 1 hr. The dry filter cake was extracted with one 5-ml. and two 3-ml. portions of 0.01 *N* hydrochloric acid; insoluble residues were removed by centrifugation and filtration. The clear, colorless filtrate was lyophilized.

The residue from this lyophilization was dissolved in a minimal amount of water and placed on two sheets of Whatman No. 4 filter paper, each 15 cm. wide, for high voltage electrophoresis (150 v./cm., 6–8 ma.) in 0.02 *M* sodium acetate buffer, pH 3.1, at 0° for 2 hr. Three ultraviolet absorbing bands were visible in each sheet after electrophoresis: (1) a band moving to the anode at the same rate as FUDRP (IX), (2) a diffuse band of unknown composition moving very rapidly to the cathode, and (3) a sharp band moving very slowly (about 1–2 cm./hr.) to the cathode (XVIII). This latter band was cut from each sheet and extracted repeatedly with small amounts of ice water. The extracts were combined, filtered, the volume of the filtrate was adjusted to 10.0 ml. Aliquots of this solution were used immediately for the following analyses.

Based on optical density measurements at 267 mμ, the solution contained 0.045 mmole (17.8 mg., 14% yield based on FUDRP) of the aminoacylfluorouridylate derivative XVIII. Analytical electrophoresis (0.02 *M* sodium acetate buffer, pH 3.1, 150 v./cm., 0°) of the solution revealed a single ultraviolet absorbing spot migrating slowly (1–2 cm./hr.) to the cathode. With a ninhydrin spray, a red-violet color developed at the corresponding site of this ultraviolet absorbing spot.[25,26] The ratio of FUDR (as determined spectrophotometrically[27]): total phosphorus[28]: number of anhydride linkages[29,46] = 1.0:1.02:0.94. Calculated FUDR:total phosphorus:anhydride linkages = 1.0:1.0:1.0. Electrophoretic analysis of the mixture obtained by treating an aliquot of the solution with 0.1 *N* potassium hydroxide for 5 min. at room temperature,[24] showed a single ultraviolet absorbing spot migrating to the anode that was indistinguishable from FUDRP.

After standing for 4 days in frozen solution, 55% of the aminoacylfluorouridylate XVIII had decomposed as estimated from spectrophotometric examination of the electrophoretically separated aminoacyl derivative XVIII and FUDRP.

***N*-(5-Fluoro-2′-deoxyuridine-5′-phosphoro)-L-alanine (XVII).**—Alanine ethyl ester was prepared by the method of Fischer.[47] The product was distilled at 50° (10–12 mm.) to give a clear, colorless liquid that was used immediately.

To the dry pyridinium salt of β-FUDRP, prepared from 100 mg. (0.306 mmole) of FUDRP, was added a solution of 107 mg. (0.918 mmole) of alanine ethyl ester in 1 ml. of dry pyridine. DCC (316 mg., 1.53 mmoles) in 2 ml. of dry pyridine was added, and the solution was shaken mechanically for 3 days. Chromatographic analysis of the crude mixture in solvent system *A* showed the presence of some unchanged FUDRP (R_f 0.02–0.08) and a large ultraviolet absorbing spot at R_f 0.43–0.50. The mixture, therefore, was allowed to shake an additional 4 days at room temperature. Dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness in high vacuum (40°) to remove pyridine and the excess alanine ethyl ester. The dry residue was extracted with 2 to 5-ml. portions of petroleum ether and 2 to 5-ml. portions of water. The combined aqueous extracts were kept at pH 13 for 30 min. by addition of 1 *N* sodium hydroxide, after which time the solution was passed through an Amberlite IR-120 (NH_4^+) column (1.7 × 25 cm.). The eluate, after evaporation (*in vacuo*) to a small volume, was applied to four sheets of Whatman No. 40 filter paper. Chromatography in solvent system *A* revealed three bands: (1) FUDRP (R_f 0.03–0.10); (2) the condensed, but unsaponified, phosphoro-alanine ester (R_f 0.45–0.50); and (3) a large band at R_f 0.13–0.24 (XVIIa). The corresponding bands of R_f 0.13–0.24 were cut out from each sheet of paper, combined, and eluted with water. This eluate was concentrated *in vacuo* to a small volume, filtered, and the filtrate was freeze-dried. The residue, a pale tan powder, was dried at 56° *in vacuo* to give 81.7 mg. (57% yield) of *N*-(5-fluoro-2′-deoxyuridine-5′-phosphoro)-L-alanine (XVIIa). This product was chromatographically homogeneous in solvent system *A* (R_f 0.22) as well as electrophoretically homogeneous in a sodium phosphate buffer (0.05 *M*, pH 8.48, 75 v./cm, 14 ma., 0°, 1.5 hr., Whatman No. 4 paper, 15 cm. wide). The product, which gave no reaction with ninhydrin reagent, had λ_{max} 267.5 mμ (H_2O), $\epsilon(P)$ 9130.

Anal. Calcd. for $C_{12}H_{15}N_3O_9FP\cdot 2NH_4\cdot 2H_2O$: N, 15.0; P, 6.63; (N/P), 5.0. Found: N, 14.78; P, 6.59; (N/P), 4.95.

A small sample of the product was dissolved in 2 *N* hydrochloric acid. This solution was heated on a steam bath for 45 min., after which time, it was allowed to stand for 6 hr. Chromatographic analysis of the hydrolysis products in solvent system *A* revealed two ultraviolet absorbing spots (R_f 0.13) and (R_f 0.54), as well as a large ninhydrin positive spot migrating with the same R_f (0.49) as authentic L-alanine (R_f 0.47).

5-Fluorouridine-5′-diphosphate (XXI).—To 400 mg. (1.06 mmoles) of 5-fluorouridine (5′)-monophosphate (XX), dissolved in 15 ml. of water was added 0.6 ml. of morpholine and 15 ml. of *t*-butyl alcohol. The solution was brought to reflux, and to the hot solution was added, very slowly and dropwise, 1.10 g. of DCC in 20 ml. of *t*-butyl alcohol. The addition took about 1.5 hr. The solution was refluxed for 36 hr. *t*-Butyl alcohol was removed *in vacuo* and the aqueous solution was extracted with three 5-ml. portions of ether. The aqueous phase was concentrated to dryness *in vacuo*, and the last traces of water were removed in high vacuum.

(46) L-Alanine hydroxamic acid was prepared by treating L-alanine ethyl ester with salt-free hydroxylamine.[23] After recrystallization from aqueous methanol, the product had a m.p. of 147–149° and gave a single, ferric chloride sensitive spot (R_f 0.29) after chromatography in *sec*-butyl alcohol–formic acid–water (75:15:10). The pure L-alanine hydroxamic acid was used for setting up a standard curve for this determination.

(47) E. Fischer, *Ber.*, **34**, 433 (1901).

The gummy product was dissolved in 8 ml. of methanol, and and the morpholidate was precipitated by addition of ether. Trituration of the sticky solid precipitate with ether gave a fine powder. After washing with additional ether, and drying *in vacuo*, there was obtained 810 mg. of the 4-morpholine-*N*,*N'*-dicyclohexylcarboxamidinium salt[33] of 5-fluorouridine (5')-monophosphate.

To an anhydrous pyridine solution of the mono(tri-*n*-butylammonium) salt of orthophosphoric acid (3.0 mmoles) (prepared by treating 345 mg. of orthophosphoric acid with 0.7 ml. of freshly distilled tri-*n*-butylamine in anhydrous pyridine solution) was added the morpholidate salt (see above) dissolved in 10 ml. of anhydrous pyridine. The solvent was removed *in vacuo*, and the dry residue was dissolved in 10 ml. of anhydrous pyridine. The solution was mechanically shaken for 1 hr. and then allowed to stand at room temperature for 2 days. Pyridine was removed *in vacuo;* trace amounts of pyridine were removed by co-evaporation with water *in vacuo*. The residue was dissolved in 15 ml. of water containing 450 mg. of lithium acetate. After extracting the solution with ether, the solution was brought to pH 12 by addition of lithium hydroxide. The solution was cooled at 0° for 30 min., and the precipitate (mostly lithium phosphate) was removed by filtration. The precipitate was washed with 0.01 *M* lithium hydroxide, and the total aqueous phase, which contains both the mono- and diphosphates of 5-fluorouridine, was brought to pH 8 by the addition of Dowex-50 (H^+). The solution was then put on a Dowex-1-chloride ion exchange column (3 × 12 cm.). After washing with water to remove nucleosides the column was eluted with a salt gradient using 0.003 *N* hydrochloric acid in the mixing chamber and 0.075 *M* lithium chloride in 0.003 *N* hydrochloric acid in the reservoir. A flow rate of 5 ml./min. was maintained. The elution pattern was followed by optical density measurements at 280 mμ. The first peak obtained contained 15% of the total material put on the column. The second peak obtained represented 60% of the material. This latter eluate was adjusted to pH 7.5 by addition of aqueous lithium hydroxide. The solution was evaporated to dryness *in vacuo* and the last traces of water were removed in high vacuum. The residual white material was dissolved in absolute methanol, centrifuged to remove a small amount of insoluble material, and precipitated by addition of acetone.

The precipitate was collected by centrifugation, again dissolved in absolute methanol, centrifuged, and reprecipitated by addition of acetone. There was obtained 205 mg. (39%) of the trilithium salt of 5-fluorouridine (5')-diphosphate (XXIc) as a hygroscopic fluffy white powder. λ_{max} 271 mμ (in water). R_f (in solvent system *C*) 0.35; R_f (in solvent system *D*) 0.16.

Anal. Calcd. for $C_9H_{10}N_2O_{12}FP_2Li_3 \cdot 8H_2O$: P, 10.6. Found: P, 10.8.

5-Fluorouridine (5')-Triphosphate (XXII).—To a mixture of 0.3782 g. (1 mmole) of 5-fluorouridine (5')-monophosphate (dihydrate), 1.16 g. (10 mmoles) of 85% orthophosphoric acid, 5 ml. of freshly distilled tri-*n*-butylamine, and 20 ml. of dry pyridine was added 10.3 g. (50 mmoles) of DCC. The flask was sealed and then was shaken vigorously to obtain a homogeneous solution. Within a few minutes, a heavy precipitate of dicyclohexylurea formed. The flask was protected from light and allowed to stand 48 hr. Dicyclohexylurea was removed by filtration, and the filter cake was washed thoroughly with water. The combined filtrate and water wash were extracted with three 70-ml. portions of ether. The combined ether fractions were washed with 15 ml. of water, and this water was added to the original aqueous phase. The total aqueous solution was concentrated to approximately 20 ml. by lyophilization. The concentrated solution was adjusted to pH 8.5 by addition of dilute sodium hydroxide, and this solution was adsorbed on a Dowex-1-chloride ion exchange column (1.6 × 14 cm.).

After washing with water, elution of the column was carried out with the following systems: I, 0.003 *N* hydrochloric acid and 0.02 *M* lithium chloride; II, 0.003 *N* hydrochloric acid and 0.05 *M* lithium chloride; III, 0.003 *N* hydrochloric acid and 0.075 *M* lithium chloride; IV, 0.003 *N* hydrochloric acid and 0.10 *M* lithium chloride; V, 0.003 *N* hydrochloric acid and 0.15 *M* lithium chloride; and VI, 2 *N* hydrochloric acid.

Fractions I, II, and III contained a total of 1000 optical density units (270 mμ). Fraction IV (2379 ml.) contained 3093 optical density units (270 mμ).

Fraction IV was neutralized (pH 7) with 1 *M* lithium hydroxide, and the solution was concentrated *in vacuo* at 34° to approximately 50 ml. Addition of an equal volume of methanol (50 ml.) followed by addition of 400 ml. of acetone resulted in the precipitation of the tetralithium salt of 5-fluorouridine (5')-triphosphate. The precipitate was collected by centrifugation and dried *in vacuo* at room temperature. Purification of the triphosphate was effected by dissolving it in 10 ml. of water, filtering, and reprecipitating it by addition of 10 ml. of methanol and 250 ml. of acetone. The product was collected by centrifugation, washed once with acetone–methanol (3:1), twice with absolute acetone, and once with ether. After drying *in vacuo* at room temperature, there was obtained 239.8 mg. (46.5%) of the tetralithium salt of 5-fluorouridine (5')-triphosphate (XXIIc) as a snow-white powder; λ_{max} 268 mμ; R_f (in solvent system *C*) 0.34, R_f (solvent system *D*) 0.13.

Anal. Calcd. for $C_9H_{10}N_2O_{15}FP_3Li_4$: P (total), 17.67; P (labile), 11.78; $\frac{P\ (total)}{P\ (labile)} = 1.50$. Found: P (total), 18.0; P (labile),[48] 11.8; $\frac{P\ (total)}{P\ (labile)} = 1.53$.

Chloride ion could not be detected in the final product by a silver nitrate test.

5-Fluoro-2'-deoxyuridine 5'-Triphosphate (XIX).—To a solution of 25 mg. (0.0765 mmole) of 5-fluoro-2'-deoxyuridine (5')-monophosphate (IX) in 2 ml. of anhydrous pyridine was added 90 mg. of 85% orthophosphoric acid and 1 ml. of tri-*n*-butylamine. The mixture was shaken vigorously until a homogeneous solution was obtained. To the clear solution was added 0.81 g. of DCC; the flask was well stoppered and allowed to stand at room temperature for 2 days. Dicyclohexylurea was removed by filtration. The precipitate was washed with 5 ml. of water, and this water was added to the filtrate. The aqueous solution, after extraction with two 5-ml. portions of ether, was freeze-dried. The gummy residue so obtained was dissolved in 5 ml. of water and put on a Dowex-1-chloride ion exchange column (3 × 12 cm.).

The column was washed with water to remove pyridine and any nucleosidic material, and then with the following solvent systems: *I*, 0.003 *N* hydrochloric acid and 0.02 *M* lithium chloride; II, 0.003 *N* hydrochloric acid and 0.05 *M* lithium chloride; III, 0.003 *N* hydrochloric acid and 0.075 *M* lithium chloride; IV, 0.003 *N* hydrochloric acid and 0.15 *M* lithium chloride; V, 0.003 *N* hydrochloric acid and 0.2 *M* lithium chloride; and VI, 2 *N* hydrochloric acid. The total optical density units of product eluted with solvent system IV was 220 (42%). This fraction (IV) was neutralized with 2 *M* lithium hydroxide to pH 7, and the solution was concentrated *in vacuo* to a sirup. This residue was diluted with 25 ml. of methanol and then 50 ml. of acetone was added to precipitate the tetralithium salt of 5-fluoro-2'-deoxyuridine 5'-triphosphate. The precipitate was collected by centrifugation and washed with a mixture of methanol and acetone. Purification of the triphosphate was effected by dissolving it in a minimum quantity of water, filtering, and reprecipitating it by addition of acetone to the aqueous filtrate. The

(48) Hydrolysis in 1 *N* hydrochloric acid in boiling water for exactly 7 min.

precipitate was finally washed with acetone, then ether, and dried at room temperature *in vacuo* to give 12 mg. (26%) of the triphosphate XIXc as a white powder, λ_{max} 268 mμ.; R_f (in solvent system *C*) 0.31.

Anal. Calcd. for $C_9H_{10}N_2O_{14}FP_3Li_4 \cdot 3H_2O$: P, 16.5. Found: P, 16.3.

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Synthesis of 2,4,7-Trichloroimidazo[4,5-*d*]pyridazine and Certain of Its Derivatives[1]

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Diethyl Δ^3- (or Δ^4)-imidazolone-2-dicarboxylate-4,5, m.p. 279°, prepared by Fenton has been shown to be identical to diethyl Δ^3- (or Δ^4)-imidazolone-2-dicarboxylate-4,5, m.p. 200°, characterized by Geisenheimer and Anschutz. The structure of this ester has been established as diethyl 2-imidazolone-4,5-dicarboxylate (diethyl Δ^4-imidazolone-2-dicarboxylate-4,5). Several esters of 2-imidazolone-4,5-dicarboxylate have been prepared. The dibutyl ester has been found to be the most satisfactory intermediate for cyclization purposes to 2,4,7-trihydroxyimidazo[4,5-*d*]pyridazine. Both 2,4,7-trihydroxyimidazo[4,5-*d*]pyridazine and 2,4,7-trichloroimidazo[4,5-*d*]pyridazine have been synthesized. Several new derivatives, both diaminated and triaminated, of 2,4,7-trichloroimidazo[4,5-*d*]pyridazine have been prepared. The chloro substituents in the 4,7-positions have been found to be the most reactive leading to 2-chloro-4,7-disubstituted derivatives. The position of the substituents of the monochlorodibenzylaminoimidazo[4,5-*d*]pyridazine was established by removing the chlorine atom with sodium in a liquid ammonia medium. The resulting dibenzylaminoimidazo[4,5-*d*]pyridazine was found to be identical to the 4,7-dibenzylaminoimidazo[4,5-*d*]pyridazine previously prepared by Carbon. It was assumed that the other derivatives were also diaminated in the 4- and 7-positions. Structures were assigned to the two new ring systems resulting from the treatment of 2-chloro-4,7-dihydrazinoimidazo[4,5-*d*]pyridazine with formic acid and with nitrous acid.

The change in the chemical activity of the chloro substituents in relation to their positions in several of the nitrogen heterocycles has been of interest to this laboratory for a number of years.[2] Recently Kuraishi[3] reported the chloro substituent on the pyridazine ring to be relatively inactive which confirmed our own observations while attempting to diaminate the trichloro derivative; Castle and Seese[4] had been unsuccessful in their efforts to diaminate the dichloropyridazine. For these reasons, it seemed worthwhile to expand the study to include both the chloropyridazines and the chloro-substituted imidazopyridazines.

Although 4,7-dichloroimidazo[4,5-*d*]pyridazine has been prepared in 17% yield by Castle and Seese,[4] both 2,4,7-trichloroimidazo[4,5-*d*]pyridazine and 2,4,7-trihydroxyimidazo[4,5-*d*]pyridazine are unknown. This latter compound, an analog of uric acid, should be preparable from the reactions of a 2-imidazolone-4,5-dicarboxylate ester with hydrazine.

The preparation of the starting material, diethyl 2-imidazolone-4,5-dicarboxylate for such a synthesis is not to be found in the more recent literature. However, Beilstein describes two different procedures for the preparation of the diethyl ester of Δ^3- (or Δ^4)-imidazolone-2-dicarboxylate-4,5, one with a melting point of 200° and the other melting at 258–259°.

The ester (m.p. 200°) had been prepared by Geisenheimer and Anschutz[5] by condensing diethyl diketosuccinate with urea to form the monoureide; this monoureide on treatment with phosphorus trichloride yielded the ester.

Fenton and Wilks[6] used an entirely different procedure to prepare the higher melting ester. These workers cyclized the so-called dihydroxymaleic acid (which in its solid state was shown by Hardtree[7] and Gupta[8] to be dihydroxyfumaric acid) with urea in an ethyl alcoholic solvent in the presence of dry hydrogen chloride.

Attempts by the authors to repeat this work, however, gave only small inconsistent yields of material melting at 200°. On the other hand, the compound prepared in this laboratory by the method of Geisenheimer and Anschutz[5] also melted at 200° as reported. Both products reacted with hydrazine to form a dihydrazide which, on treatment with dilute hydrochloric acid, gave 2,4,7(1*H*,3*H*,5*H*,6*H*)-imidazo[4,5-*d*]pyridazinetrione. It must be concluded that the two esters, m.p. 200° and 279°, are identical and that the melting point of the Fenton compound is in error.

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