Enzymatic and Chemical Synthesis of 1β-D-Arabinosyl-5-fluoropyrimidine 5′-Phosphates. A Comparative Study[1]

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The preparation of 5′-monophosphates derived from nucleoside antagonists of possible interest in neoplastic and viral disease by both enzymatic and chemical methods has been carried out for the sake of comparison. The enzymatic procedure used the well-known phosphotransferase of Chargaff and coworkers, an enzyme which was further purified (510-fold). Incubation of 1β-D-arabinosyl-5-fluorouracil with the enzyme in the presence of *p*-nitrophenyl phosphate as a donor gave the desired 5′-phosphate in 24% yield; the corresponding 5-fluorocytosine derivative was similarly phosphorylated (30%) and the two phosphates were interrelated *via* nitrous acid deamination. An alternate, purely chemical synthesis proceeded *via* conversion to the triphenylmethyl ether of the primary hydroxyl group; acylation and detritylation gave a properly protected substrate which was phosphorylated by Tener's method and deblocked (over-all yield 10%). Factors which must be considered in a comparison of the two methods include availability of substrate, availability of the enzyme, and separation problems.

1β-D-Arabinofuranosylcytosine (*ara*-C) is a potent antimetabolite in a number of systems.[2] Its mode of action may involve blockade of ribonucleotide diphosphate reductase,[3,4] although the work of Moore and Cohen[5] with purified enzyme from rat tumor cells casts some doubt on this point, or it may be involved at other loci, such as low-level incorporation into nucleic acids.[6,7] The analog, however, is rapidly deaminated to the corresponding inactive[4] uracil derivative in a variety of systems.[8,9] Accordingly, the effect of related antimetabolites was of interest, and the pronounced activity of 1β-D-arabinofuranosyl-5-fluorocytosine (*ara*-FC) has been duly noted.[10] The uracil analog (*ara*-FU) had also been prepared and its activity against transplanted mouse leukemia described.[11] Since it seems probable that the activity of these analogs is effected *via* the corresponding nucleotide analogs and their derivatives, it was desirable to make phosphates available for biological studies. The present paper compares the preparation of the 5′-phosphates of these fluorinated analogs by both enzymatic and chemical methods.

The general problem of arabinosyl nucleotide synthesis has been reviewed by Cohen.[12] Various phosphotransferases of plant origin have the useful property of abstracting a phosphate group from such substrates as phenyl phosphate and of transferring it specifically to the 5′ position of nucleosides.[13] Chargaff and coworkers have characterized and purified this activity, especially the carrot enzyme,[14] and Pizer and Cohen[5] have shown that arabinosides also serve as substrates for this phosphorylation. We are, in this laboratory, making a systematic survey of carrot nucleases and phosphatases,[15] and have been able to purify the Chargaff phosphotransferase to 510-fold enhancement over the activity of the crude extract. This preparation can now 5′-phosphorylate thymidine to an extent of 90%. The phosphotransferase has been utilized to phosphorylate fluorinated pyrimidine arabinosides, and an alternate chemical synthesis has also been carried out to permit comparison of the two methods with respect to yield and large-scale convenience. These transformations and some related reactions are outlined Chart I.

1β-D-Arabinofuranosyl-5-fluorocytosine[16] (*ara*-FU, **1**) was converted to 5′-phosphate (**2**) on a millimolar scale with 75 units[17] of an enzyme fraction of intermediate purity and a ten-fold excess of *p*-nitrophenyl phosphate. After 50 hr at 30°, some 60% of the phosphate donor had disappeared, and the reaction mixture was chromatographed on DEAE-Sephadex A-25. After eluting unreacted nucleoside analog with 0.02 *M* glycine (pH 3.2 with HCl), a solution of 0.06 *M* KCl in the glycine buffer was used to elute the phosphorylated analog **2** in 30% yield. Recovery from buffer by way of charcoal absorption gave 24% of the pure phosphate,[18] homogeneous by paper chromatography and containing the required amount of phosphate. A similar conversion of 1β-D-arabinofuranosyl-5-fluorouracil (*ara*-FU, **3**), using a 20 molar excess of phenyl phosphate, gave some 30% of phosphate **4**[19] which was separated by preparative paper chromatography. Precipitation as the barium salt and reconversion to the

(1) Presented at the 154th National Meeting of the American Chemical Society, Division of Medicinal Chemistry, Chicago, Ill., Sept 11-15, 1967. Abstracts of Papers 28.

(2) See D. A. Karnofsky and C. R. Lacon, *Biochem. Pharmacol.*, **15**, 1435 (1966), for a recent review of the literature.

(3) J. S. Evans, E. A. Musser, G. D. Mengel, R. K. Forsblad, and J. H. Hunter, *Proc. Soc. Exptl. Biol. Med.*, **106**, 350 (1961).

(4) M. Y. Chu and G. A. Fisher, *Biochem. Pharmacol.*, **11**, 423 (1962).

(5) Quoted in A. Doering, J. Keller, and S. S. Cohen, *Cancer Res.*, **26** (1), 2444 (1966).

(6) M. Y. Chu and G. A. Fisher, *Biochem. Pharmacol.*, **14**, 333 (1965).

(7) W. A. Creasey, R. J. Papac, M. E. Markiw, P. Calabresi, and A. D. Welch, *ibid.*, **15**, 1417 (1966); see, however, ref 4.

(8) L. Pizer and S. S. Cohen, *J. Biol. Chem.*, **235**, 2387 (1960).

(9) C. G. Smith, H. H. Buskirk, and W. L. Lummis, *Proc. Am. Ass. Cancer Res.*, **6**, 60 (1965); see also ref 6.

(10) J. H. Kim, M. L. Eidinoff, and J. J. Fox, *Cancer Res.*, **26** (1), 1661 (1966).

(11) N. C. Yung, J. H. Burchenal, R. Fecher, R. Duschinsky, and J. J. Fox, *J. Am. Chem. Soc.*, **83**, 4060 (1961); see also E. J. Reist, J. H. Osiecki, L. Goodman, and B. R. Baker, *ibid.*, **83**, 2208 (1961).

(12) S. S. Cohen, *Progr. Nucleic Acid Res. Mol. Biol.*, **5**, 1 (1966).

(13) G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.*, **75**, 2020, 4113 (1953).

(14) M. Tunis and E. Chargaff, *Biochim. Biophys. Acta*, **37**, 267 (1960).

(15) In addition to the phosphotransferase here discussed, and several nonspecific phosphatases, carrot extract also contains a phosphodiesterase which has been purified 300-fold: see C. Harvey, L. Malsman, and A. L. Nussbaum, *Biochemistry*, **6**, 3689 (1967).

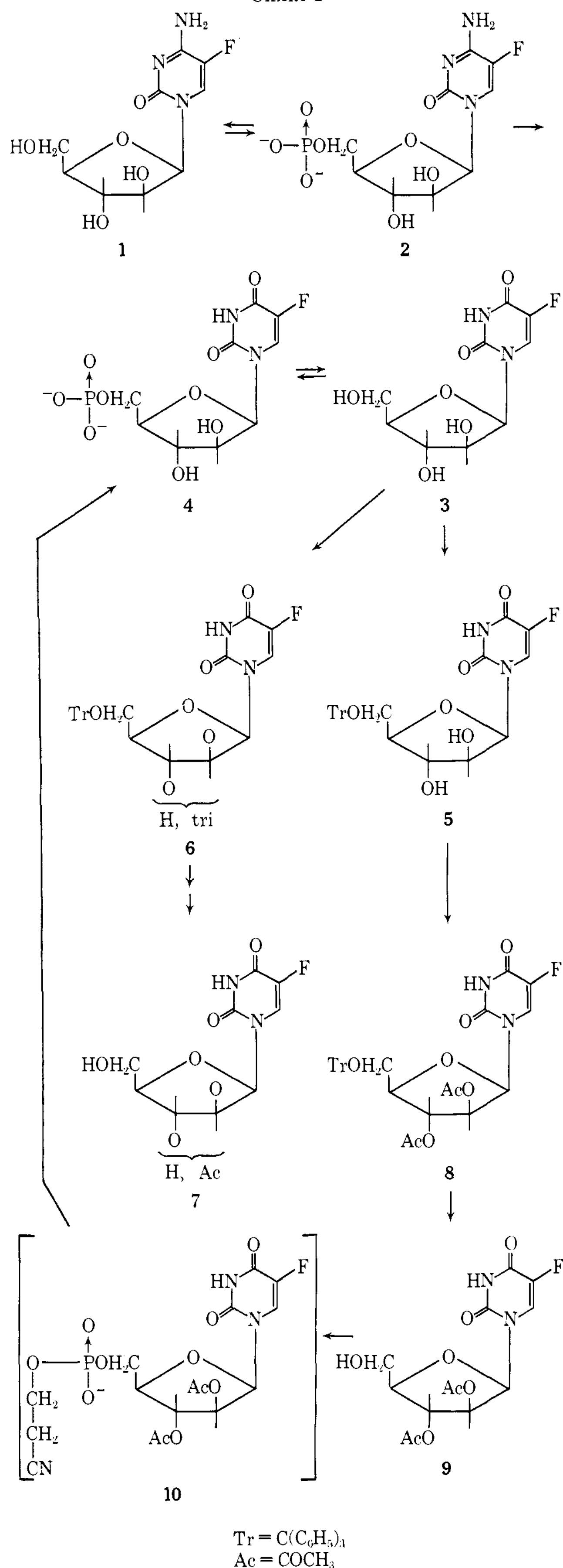
(16) J. J. Fox, N. C. Miller, and I. Wempen, *J. Med. Chem.*, **9**, 101 (1966).

(17) A unit of enzyme is defined as the amount of activity which transfers 1 μmol of phosphate from *p*-nitrophenyl phosphate to thymidine/hr under assay conditions.

(18) A chemical synthesis for this compound has been claimed in the patent literature: Upjohn Co., Netherlands Patent 66,08939 (1966) and U. S. Patent 3,338,882 (1967).

(19) This compound has been previously prepared although no preparative details were disclosed: H. D. Barner and S. S. Cohen, *Progr. Nucleic Acid Res. Mol. Biol.*, **5**, 41, 74 (1966).

Chart I



free acid with Dowex-50 (H^+) lowered the yield to 20% of paper chromatographically homogeneous material which contained the calculated amount of phosphate upon digestion with 5′-nucleotidase. The two phosphates were interrelated by conversion of **2** to **4** with nitrous acid.[20]

An alternative chemical synthesis was carried out by conventional methods.[21] Tritylation of **3** at elevated temperature[22] with an excess of triphenylmethyl chloride gave a mixture of oily 2′,5′- and 3′,5′-ditrityl derivatives (**6**), with the latter predominating. These derivatives were separated by chromatography on silica gel, acetylated, and detritylated to the 3′- and 2′-monoacetates (**7**), respectively, the latter emerging crystalline. A room-temperature tritylation, however, gave the desired crystalline monotrityl derivative (**5**), characterized as the 5′ isomer by nmr. This in turn permitted selective acetylation of the secondary hydroxyl groups to give the crystalline diacetate (**9**) after detritylation of the intermediate trityl diacetate **8** (not isolated). Phosphorylation by the method of Tener[23] gave an intermediate cyanoethyl phosphate (**10**) which was treated with sodium methoxide in methanol[24] to remove protecting groups, again giving the desired 5′-phosphate (**4**) identical with the product from enzymatic transphosphorylation. The over-all yield from these several steps was 10%.

Although the enzymatic synthesis undoubtedly gives higher yields than the chemical multistep sequence, the latter is preferred if larger quantities of material are desired. This conclusion rests mainly on the fact that it is difficult to separate the products from the surviving phosphate donor. Nevertheless, it is hoped that technical advances will make such simple one-step enzymological conversion practical for the organic chemist.

Biological Activity.—The nucleotide analogs here discussed were tested *in vivo* against the transplanted Sarcoma 180 tumor in the mouse.[25] As seen in Table I,

Table I: Activity against Sarcoma 180 in Mice[a]

Compound	Index, C/T
ara-FC	16.7
ara-FC 5′-phosphate	4.6
ara-FU	3.0
ara-FU 5′-phosphate	3.7

[a] Dosage, 50 mg of nucleoside or its molar equivalent of phosphate/kg ip. The index C/T denotes ratio of tumor weights, controls/treated animals.[25]

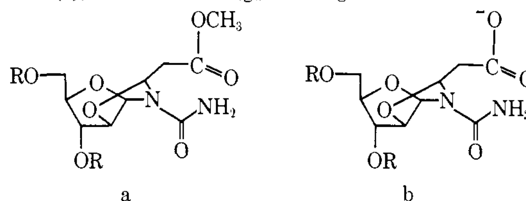
(20) A. Kleinzeller, *Biochem. J.*, **36**, 729 (1942).

(21) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic Press Inc., New York, N. Y., 1963, p 126 ff.

(22) After J. P. Horwitz, J. A. Urbanski, and J. Chua, *J. Org. Chem.*, **27**, 3300 (1962).

(23) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 165 (1961).

(24) These conditions, rather than use of the conventional aqueous alkali, were chosen in order to prevent the well-known sensitivity of such arabinosides toward ring opening [J. J. Fox, N. C. Miller, and R. J. Cushley, *Tetrahedron Letters*, 4927 (1966)]. No such opening was observed when a model (1β-D-arabinosyl-5-fluorouracil tribenzoate) was subjected to methanolysis, presumably because the postulated intermediate of this potentially destructive pathway is not prevented from reclosure (a), in contrast to the hydrolysis product (b), which carries a negative charge.



(25) Procedure as in E. Grunberg, H. N. Prince, E. Titsworth, G. Beskid, and M. D. Tendler, *Chemotherapia*, **11**, 249 (1966). We wish to thank Dr. Grunberg and his staff for making these results available to us.

Table II

Enzyme Purification[a]

	Steps	Vol, ml	Concn, units/ml	Total units	Protein, mg/ml	Spec act., units/mg	Yield, %
1.	Crude extract	10,000	0.73	7300	4.43	0.165	100
2.	pH 4 precipitation	10,000	0.42	4200	1.10	0.380	57.5
3.	80% ammonium sulfate	300	1.24	3720	5.95	2.03	51.0
4.	30–70% acetone precipitation	100	31.6	3160	9.35	3.38	42.4
5.	CM-cellulose	55	10.5	2890	0.37	28.4	39.5
6.	Sephadex G-200	42	9.6	2020	0.25	38.4	27.7
7.	ECTEOLA	72	3.45	1240	0.04	86.3	17.0

[a] From step 4, purification was carried out on 20% aliquots. Figures in the table have been prorated.

phosphorylation does not abolish the activity observed for the precursor nucleoside analogs. However, while there appears to be little effect upon phosphorylation of *ara*-FU, the more interesting *ara*-FC seems to become less active as the nucleotide, at least at the dosage studied.

Experimental Section[26]

General.—Solvents were purified by distillation and dried by standing over Molecular Sieve (Linde 10X). Melting points are uncorrected (Thomas–Hoover apparatus). DEAE-cellulose (Whatman DE-23), ECTEOLA-cellulose (H. Reeve Angel & Co., Clifton, N. J.), and DEAE-Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) were prepared as recommended by the manufacturer. Paper chromatography (descending) employed the following systems: (A) EtOH–1 *M* NH_4OAc pH 7.5, 7:3 (v/v); (B) isobutyric acid–0.5 *M* NH_4OH, 5:3 (v/v). Thin layer chromatography employed plates prepared from Camag silica gel with indicator. Concentrations *in vacuo* were carried out below 35°. Susceptibility to 5′-nucleotidase was determined as previously described.[15] The optical integrity of the nucleotides follows from their susceptibility to 5′-nucleotidsase, an enzyme which is inactive toward the "unnatural" anomer. Charcoal for nucleotide separation was prepared as described.[27]

Purification of Phosphotransferase.[28]—Purification of the enzyme is summarized in Table II. The procedure used for the first six steps was the same as that described for carrot diesterase[15] except that 0.04% Triton X-100 was added to all solutions. Also, column eluates were concentrated with Carbowax instead of by lyophilization.

The carboxymethylcellulose column separated the desired phosphotransferase from a nonspecific acid phosphatase (a phosphatase which split *p*-nitrophenyl phosphate but did not form thymidylic acid from thymidine). The nonspecific phosphatase was eluted first, followed by a phosphodiesterase and finally the phosphotransferase. When a G-200 column was used, the phosphotransferase came off in a peak following and overlapping the remaining phosphodiesterase. An ECTEOLA column was substituted for DEAE in the last step of the purification. The ECTEOLA was cleaned; a 1 × 5 cm column was poured and equilibrated with 0.01 *M* Tris·HCl (pH 7.5) as described for the DEAE column.[15] The mixing chamber contained 100 ml of the above buffer, while the reservoir contained 100 ml of this buffer also 0.3 *M* in NaCl; 1-ml fractions were taken. The phosphotransferase was collected in a broad peak and concentrated with Carbowax. The enzyme was stored at −20°.

Enzyme Assays.—The phosphotransferase was assayed in the following reaction mixture which was brought up to 0.2 ml with water: 0.5 μg of Triton X-100, 20 μmol of NaOAc (pH 5.2), 20 μmol of *p*-nitrophenyl phosphate, 2 μmol of thymidine containing 2.3 × 10^{5} cpm as thymidine-C^3H_3, and 0.04–0.2 unit of enzyme. After incubation at 37° for 1 hr, a 50-μl aliquot was spotted on Whatman No. 1 and developed overnight in EtOH–1 *M* NH_4OAc, pH 7.5 (70:30). After drying, the strips were run through a Packard Model 7201 radiochromatogram scanner system. The peaks on the recorder tracing corresponding to thymidine and thymidylic acid were cut out and weighed. A unit was defined as 1 μmol of thymidylic acid produced/hr. Assays for contaminating activities are described elsewhere.[15]

Enzymatic Phosphorylation. 1β-D-Arabinofuranosyl-5-fluorocytosine 5′-Phosphate (2).—The following reaction mixture was incubated in a stoppered flask at 30° for 50 hr: 261 mg (1 mmol) of *ara*-FC (**1**), 10 ml (10 mmol) of 1 *M* *p*-nitrophenyl phosphate, 15 ml of 1 *M* NaOAc pH 5.2, and 5 ml of carrot phosphotransferase (74.5 units, step 6): total volume was 50 ml.

At the end of incubation, the reaction was stopped by heating for 2 min at 100°. *p*-Nitrophenol was extracted with three 50-ml portions of ether and the aqueous phase was applied to a column of DEAE-Sephadex A-25, 5 × 30 cm, previously equilibrated with 0.02 *M* glycine hydrochloride buffer of pH 3.2. Elution was carried out with increasing concentrations of KCl in the glycine buffer, 20-ml fractions being collected at a rate of 200 ml/hr. The neat buffer removed the surviving nucleoside analog. The phosphate of *ara*-FC was eluted with 0.06 *M* KCl which was followed closely by *p*-nitrophenyl phosphate.

Approximate fractions were pooled and measured (2500 OD units at 281 mμ, or 0.31 m*M*), and the pH was adjusted to 2.5 with HCl and evaporated to dryness *in vacuo*. The residue was dissolved in water (5.0 ml) and 12 ml of a 30% aqueous suspension of Norit A was added. The adsorbed phosphate was separated from the supernatant by filtration through a bed of Celite and desorbed by elution with five 50-ml portions of a 50% aqueous EtOH solution 2% in concentrated NH_4OH; recovery from charcoal: 1900 OD at 281 mμ, measured at pH 7. This represents 0.24 mmol of the 5′-phosphate of *ara*-FC (**2**), homogeneous by paper chromatography (R_f 0.23 in system B).

1β-D-Arabinofuranosyl-5-fluorouracil 5′-Phosphate (4).—The following reaction mixture was incubated in a stoppered flask at 30° for 24 hr: 5 ml (1 mmol) of 0.2 *M* *ara*-FU (**3**) in H_2O; 10 ml of 2 *M* phenyl phosphate in H_2O, pH 5.2; 4 ml of 1 *M* NaOAc buffer, pH 5.2; 1 ml of 0.1 *M* $CuSO_4$; 5 ml of carrot phosphotransferase solution (73.5 units, step 4); 15 ml of distilled water; total volume was 40 ml.

At the end of the incubation period, the enzymatic action was stopped by heating for 2 min at 100°. Phenol was extracted with three portions (20-ml) of ether, and the aqueous portion was streaked on large sheets of Whatman No. 1 filter paper (2.5 ml/55-cm band). Development in system A separated surviving nucleoside analog, phosphate donor, and the desired phosphate (R_f 0.12 system A). Extraction of the appropriate zone with water gave 2900 OD units at 268 mμ (pH 7), or 0.36 mmol, of material not entirely free of phenyl phosphate. The material was redissolved in 4.0 ml of distilled H_2O and rechromatographed as above. The material, now homogeneous, represented 2300 OD units at 268 mμ (pH 7), that is, 0.27 mmol.

The paper extract was evaporated to dryness *in vacuo* (temperature being kept below 35°), dissolved in 1 ml of H_2O, and the pH adjusted to 7. $BaBr_2$ (1 *M*, 2.0 ml) was added and the resulting barium phosphate was removed by centrifugation. The precipitate was washed with H_2O (2 ml) and the combined aqueous solution was treated with 8 ml of EtOH. After overnight storage in the refrigerator, the desired barium salt was collected by centrifugation. The free acid was regenerated by passage over 3.0 ml of Dowex-50 (hydrogen cycle) ion-exchange resin and eluted with 10 ml of H_2O. The elutate contained 1570 OD units (268 mμ, pH 7.0), that is, 0.2 mmol, 20% yield.

The material was identical with that obtained from chemical phosphorylation (*vide infra*) by the following criteria: paper chromatography, susceptibility to 5′-nucleotidase (releasing

(26) Where analyses are indicated only by symbols of the elements or functional groups, analyses obtained for those elements of functional groups were within ±0.4% of the calculated values. Statements of chromatographic homogeneity are adjudged to be sensitive to ±5%.

(27) G. B. Petersen, *Biochem. J.*, **87**, 495 (1963).

(28) After submission of our manuscript, a phosphotransferase purification procedure was published [E. F. Brunngraber and E. Chargaff, *J. Biol. Chem.*, **242**, 4834 (1967)].

nucleoside analog), nmr spectrum, and uv spectrum ($100\epsilon_{268}$ = 8600, pH 7).

Interconversion.—The hydrolytic deamination followed the procedure of Kleinzeller.[20] The 5′-phosphate of *ara*-FC (**2**, 5 μmol) was dissolved in 64 μl of H_2O. The following was added, in the order specified: NaOAc (5.5 mg), AcOH (20 μl), H_2O (40 μl), and $NaNO_2$ (60 mg). The resulting solution (pH 3.7) was allowed to stand at room temperature. After various time intervals, 12.5-μl aliquots were spotted on Whatman No. 1 filter paper and subjected to descending paper chromatography in system B. After 1 hr, 85% of the starting material **2** had been converted to the analogous fluorouracil derivative **4** as indicated by migration against standards.

Chemical Synthesis. 5′-Triphenylmethyl-1β-D-arabinofuranosyl-5-fluorouracil (5).—*ara*-FU (**3**, 2.72 g, 10.4 mmol) was dissolved in 31 ml of anhydrous pyridine, triphenylmethyl chloride (3.62 g, 13.0 mmol) was added, and the mixture was stirred at room temperature for 65 hr. At the end of that period an equal volume of ice water was added, and the mixture was concentrated to a syrup *in vacuo*. The residue was partitioned between 100 ml of H_2O and an equal volume of $CHCl_3$. The organic layer was dried (Na_2SO_4) and concentrated to a small volume to give 3.39 g (6.73 mmol) of **5**, mp 124–126°. *Anal.* ($C_{28}H_{25}FN_2O_6$) C, H, N. Absorption peaks of spectra (uv, ir, nmr) were as expected.

Ditritylation of 1β-D-Arabinofuranosyl-5-fluorouracil.—Triphenylmethyl chloride (16.4 g, 59 mmol) was dissolved in 25 ml of anhydrous pyridine and *ara*-FU (**3**, 1.28 g 4.9 mmol) was added to the solution previously heated to 95°. Heating was continued for 1 hr, resulting in slight browning of the initially clear yellow solution. Pyridine was removed *in vacuo* and the residue was partitioned between water (25 ml) and $CHCl_3$ (50 ml). The aqueous portion was back-extracted with $CHCl_3$ (25 ml each portion), and the combined organic layers were concentrated to a dry residue.

Chromatography on silica gel gave, after removal of reagent with benzene, two oily materials which were eluted with C_6H_6–Et_2O: the faster moving material (1.68 g) appeared with 10% ether; this was followed by unresolved mixtures (0.80 g) eluted with 20% ether. The more polar component (0.3 g) free from admixture was obtained with 30% ether. Neither material was crystalline. Examination of the nmr spectrum indicated that both species were di(triphenylmethyl) ethers on the basis of relative abundance and polarity; the faster moving diether is tentatively assigned the 3′,5′-ditrityl structure of *ara*-FU (**6**).

1β-D-Arabinofuranosyl-5-fluorouracil 2′(?)-Acetate (7).—The material to which the 3′,5′-ditrityl structure had been assigned (the less polar species **6**, 1.52 g) was dissolved in 5.0 ml of dry pyridine; Ac_2O (2.5 ml) was added, and the solution was allowed to react overnight at room temperature with protection against moisture. An equal volume of crushed ice was added, and all volatile components were stripped *in vacuo*. The residue was dissolved in 25 ml of 80% aqueous AcOH and heated for 1 hr at 95° (oil bath). Again, the reaction mixture was concentrated to dryness, the residue was taken up in H_2O (25 ml), and insoluble trityl alcohol was removed by filtration. The precipitate was washed with 25 ml of H_2O and the combined aqueous filtrate was concentrated to dryness. The residue was crystallized from acetone–petroleum ether (bp 30–60°) to give 0.39 g of **7** (63%), mp 202–205°. An analytical sample had mp 206–208°. *Anal.* ($C_{11}H_{13}FN_2O_7$) C, H, N. Absorption peaks of spectra (uv, ir, nmr) were as expected.

Similar treatment of the more polar (minor) ditrityl derivative did not give rise to a crystalline material, although a homogeneous oil (tlc) with an nmr spectrum compatible with a monoacetate was obtained.

1β-D-Arabinofuranosyl-5-fluorouracil 2′,3′-Diacetate (9).—5′-Monotrityl derivative **5** (5.38 g, 10.7 mmol) was dissolved in 25 ml of anhydrous pyridine and 12.5 ml of Ac_2O was added. After standing overnight at room temperature with the exclusion of moisture, an equal volume of crushed ice was added, and the resulting solution was concentrated *in vacuo* to an oil. The latter was dissolved in 50 ml of 80% aqueous AcOH and heated in a stoppered flask for 1 hr at 95° (oil bath). The solution was again concentrated to a small volume *in vacuo;* H_2O was added several times, and again the solution was subjected to vacuum concentration to remove most of the acetic acid. The residue was suspended in 50 ml of H_2O, the insoluble trityl alcohol was removed by filtration, and the solid was washed twice with 50 ml of H_2O. The combined aqueous filtrates were concentrated to dryness, and the residue crystallized from MeOH to give 0.95 g of **9** (26%),[29] mp 163–165°. An analytical sample had mp 164–166°; absorption peaks of spectra (uv, ir, nmr) were as expected. *Anal.* ($C_{13}H_{15}FN_2O_8$) C, H, N.

1β-D-Arabinofuranosyl-5-fluorouracil 5′-Phosphate (4).—Diacetate **9** (0.95 g, 2.74 mmol) and 13.7 ml of a 1 *M* solution of pyridinum cyanoethyl phosphate in pyridine (prepared according to Tener[23]) were dried, by concentrating to dryness several times from dry pyridine, and dissolved in 25 ml of dry pyridine. N,N′-Dicyclohexylcarbodiimide (5.65 g, 27.4 mmol) was added, and the reaction mixture was stirred at room temperature for 24 hr, moisture being excluded. At the end of that period the reaction was stopped by addition of 10 ml of H_2O. After a further period of 18 hr in the refrigerator, the precipitated dicyclohexylurea was removed by filtration and washed with 100 ml of a 50% aqueous pyridine solution. The combined filtrate was extracted with four 100-ml portions of ether to remove excess reagent. The aqueous phase was concentrated *in vacuo*, with two additions of EtOH to remove most of the pyridine, and dissolved in 27.4 ml of a methanolic solution of 1 *M* NaOMe and the resulting suspension was stirred at 60–65° (oil bath), under N_2 in a vessel fitted with a reflux condenser. After 1 hr, the reaction mixture was cooled in an ice bath and neutralized to pH 7 with anhydrous HCl in ethanol and concentrated to dryness *in vacuo*.

The residue as taken up in 50 ml 0.005 *M* triethylammonium bicarbonate, pH 7.5, and charged on a column of DEAE-cellulose, 35 × 5 cm, equilibrated with the same buffer. A gradient dilution system was applied: mixing chamber, 4 l. of 0.005 *M* buffer as above; reservoir, 4 l. of the same, 0.3 *M*. Fractions of 20 ml were collected at the rate of 7.5/hr. The desired phosphate **4** was obtained from fractions 190–250, yield 5750 OD units at λ_{max} 268 mμ (pH 7.5), or 0.72 mmol (26% from **8**). The material was homogeneous by paper chromatography (R_f 0.19 in system A) and identical with that obtained from enzymatic phosphorylation by spectra (nmr, uv), paper mobility, and enzymatic susceptibility to 5′-nucleotidase.

(29) In another experiment the yield was 32%.