a column of Dowex 50-W (acid form, 70 ml) by washing initially with water until free of uv-absorbing material, then eluting stepwise with 0.1 *M* and 0.3 *M* pyridinum formate buffer (pH 4.5). The initial eluates (2.8 l.) were discarded; 3.1 l. of the successive eluates was evaporated to dryness. The residue was dissolved in 25 ml of water and passed through the column of Diaion SA-11B (chloride form, 10 ml), followed by washing with water. The combined effluent and washings were concentrated to dryness. The residue was triturated with EtOH and crystallization took place. Colorless needles were obtained (0.61 g, 54.55%): mp over 260°; λ_{max} ($\epsilon \times 10^{-3}$) H_2O, 230 (9.1), 269 nm (11.2); 1 *N* HCl, 230 (10.1), 269 nm (12.0); nmr (δ) 8.37 (d, 1 H, C_6-H), 5.62 (d, 1 H, $C_{1'}$-H). *Anal.* ($C_9H_{11}N_3O_4FCl$) C, H, N.

5-Iodo-ara-C (VIII). IIb (100 mg) was dissolved in 15 ml of 1 *M* triethylammonium bicarbonate (pH 7.5) and heated at 50° for 40 min. The reaction mixture was evaporated and the residue was dissolved in 10 ml of water to adjust the pH to 2.0 with 0.1 *N* HCl. The product was isolated on a column of Dowex 50-W (ammonium form, 4 ml) by washing with water until free of uv-absorbing material. The effluent was concentrated to dryness, and the residue was crystallized from EtOH: yield, 57 mg (60.5%); mp 205° dec,[13] λ_{max} H_2O, 223, 295 nm; 1 *N* HCl, 310 nm.

2',6-Anhydro-6-hydroxy-ara-C (IX). VIII (3.0 g) was dissolved in a mixture of 70 ml of DMSO and 25 ml of *tert*-BuOH. To this solution was added a mixture of 20 ml of 1 *N* sodium *tert*-butoxide and 40 ml of DMSO under stirring at 60° and heating was continued for 2 hr. The pH of the reaction mixture was adjusted to 9.0 with Dowex 50-W (ammonium form, 40 ml), and the resin was filtered off. The filtrate was concentrated to 100 ml and passed through the column of Dowex 50-W (acid form, 140 ml) to absorb a cyclized product. Isolation was achieved by washing initially with water until free of uv-absorbing material, then eluting with 5% ammonium hydroxide (1.2 l.). The eluates were concentrated to a small volume to give pure crystals. Recrystallization from minimum amount of water afforded an analytical sample: yield, 0.986 g (50.30%); mp 276° dec; λ_{max} ($\epsilon \times 10^{-3}$) pH 7, 222 (11.14), 261.5 nm (14.1); pH 1, 266 (23.3); R_f (A) 0.12, (B) 0.32. *Anal.* ($C_9H_{11}N_3O_5$) C, H, N.

The combined effluent was concentrated to dryness, and the residue was repeatedly washed with ether and dissolved in a small amount of water to give white prisms: yield, 730 mg; mp 235-237° dec; λ_{max} H_2O, H^+, OH^-, 275 nm. This compound is not yet characterized.

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Indigogenic Phosphodiesters as Potential Chromogenic Cancer Chemotherapeutic Agents†

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Several indigogenic phosphodiesters of 5-iodo- (or nitro-) indol-3-yl 5'-nucleosides were prepared by coupling 5-substituted indol-3-yl phosphodichloridate with a suitably protected nucleoside. A pyrophosphate diester of 5-iodoindol-3-yl-5'-(5-fluorodeoxyuridine) was prepared by the reaction of *N*-acetyl-5-iodo-indol-3-yl phosphate and 3'-*O*-acetyl-5-fluorodeoxyuridine 5'-phosphodichloridate. These compounds were substrates for snake venom 5'-nucleotide phosphodiesterase, and the K_m and v_{max} values were measured by following the rate of indigo formation. Cytotoxicities of the phosphodiesters were tested on a rat mammary tumor (AC 33) and HeLa cell lines.

The synthesis of indigogenic nucleoside phosphodiesters as chromogenic substrates for the localization of nucleotide 5'-phosphodiesterase (E.C. 3.1.4.1.) activity in tissue has been reported from this laboratory.[1,2] The extension of this histochemical approach to the synthesis of chromogenic cancer chemotherapeutic agents has been illustrated recently.[3] The design principle is depicted in Scheme I.

The enzymatic activity can be measured by the liberated substituted indigo dye, and the anticancer activity assessed in a tissue culture study. While the use of simple nucleoside analogs depends on thymidine kinase for their actions, this approach depends on phosphodiesterase to deliver an antimetablite as the possible chemotherapeutic agent. These phosphodiesters therefore should be effective in tumors that

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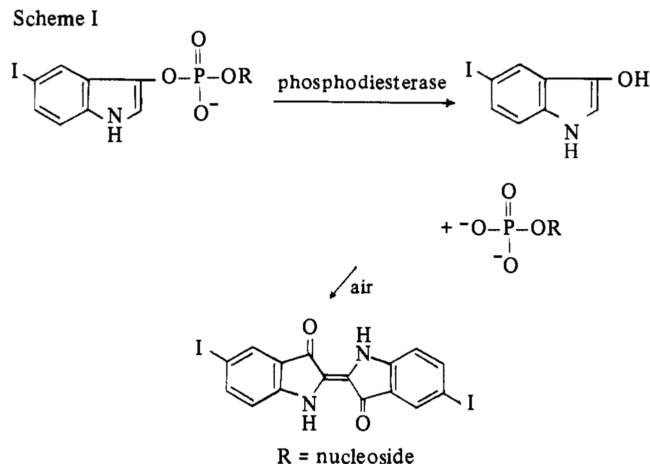
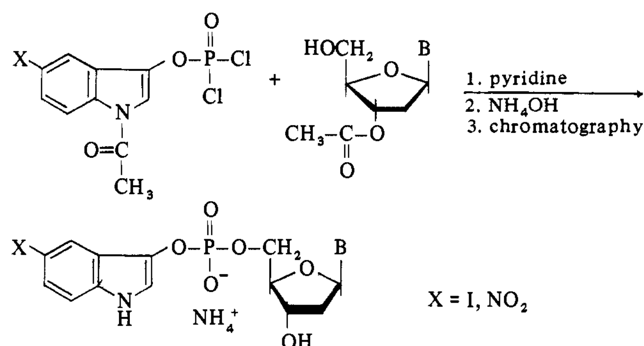
Scheme I



are resistant to the simple nucleoside analogs, because of thymidine kinase deficiency. Preliminary study of the incorporation of 5-fluorodeoxyuridine (FUdR) by this principle showed that this approach could indeed lead to useful chemotherapeutic agents.[3] The present paper reports further syntheses of other phosphodiesters containing antimetabolite nucleosides and their potential use in cancer chemotherapy based on enzyme rationale.[4,5] The synthetic procedure for the nucleoside 5′-phosphodiesters was essentially similar to that previously used for the FUdR derivative[3] and is shown in Scheme II. *N*-Acetyl-5-nitro-3-indolyl phosphodichlori-

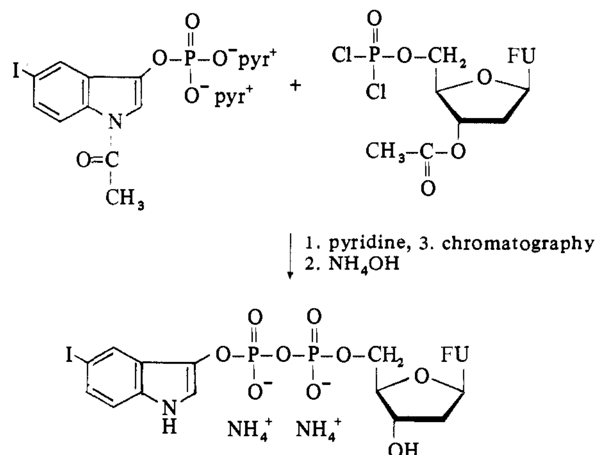
Scheme II



date was prepared by the same method as *N*-acetyl-5-iodo-3-indolyl phosphodichloridate.[6] The reaction times of the protected nucleosides and the phosphodichloridates varied from 20 to 48 hr.‡ The *N*-acetyl group was removed in 7 *M* ammonia, and the product was purified by DEAE-Sephadex A-25 chromatography, using a linear gradient of NH_4HCO_3.§

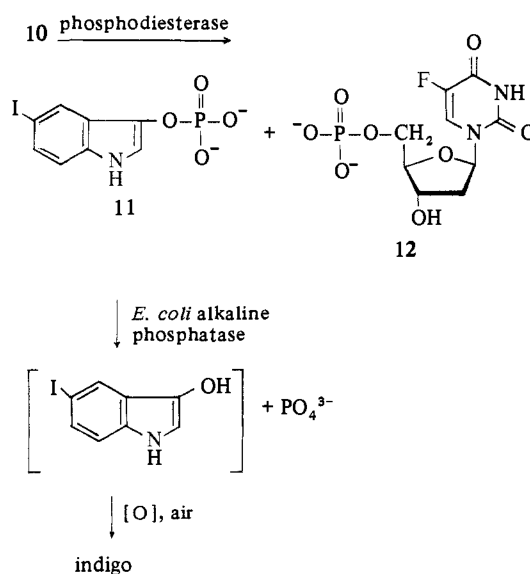
The preparation of pyrophosphoric ester of FUdR and 5-iodoindoxyl was carried out with the pyridinium salt of *N*-acetyl-5-iodo-3-indolyl phosphate and the phosphodichloridate of 3′-*O*-acetyl-5-fluorodeoxyuridine, and the product was purified by column chromatography as in the case of phosphodiesters. This procedure appears to be generally useful for such pyrophosphate derivatives. This synthetic method requires fewer steps than the usual method of coupling a phosphate monoester with a nucleoside 5′-phosphoromorpholidate.[7]

The indigo dye formed by the air oxidation of the indoxyl radical upon enzyme hydrolysis can be stabilized by 0.2% polyvinyl alcohol (PVA).[8] These solutions have an absorp-



tion maximum at 540 nm and are stable for at least 24 hr. Thus, the enzymatic reactions can be easily followed. A comparative study of the hydrolysis of compounds **1-10** (Table I) by snake venom nucleotide 5′-phosphodiesterase was made. If the biochemical results could be used to correlate the activities of these new drugs, one would then be able to probe further into synthesizing other analogous new drugs using the biochemical parameters. As shown in Table I, snake venom nucleotide 5′-phosphodiesterase shows a broad specificity toward the various nucleoside phosphodiesters.

When the enzymatic hydrolysis of **10** was followed by the formation of indigo, the apparent rate of hydrolysis was very slow. This rate was found to be almost the same as the chemical hydrolysis rate of 5-iodoindoxyl phosphate, **11**. Indeed, the products were shown to be **11** and FUdR 5′-phosphate, **12**, by paper chromatography. Therefore, the rate of hydrolysis of **10** by venom phosphodiesterase was measured again in the presence of excess *Escherichia coli* alkaline phosphatase (E.C. 3.1.3.1.) so that the pyrophosphate cleavage step is rate limiting. Commercial *E. coli* alkaline phosphatase is free of phosphodiesterase activity.



Phosphodiesterases from various sources have been shown to possess weak to moderate activities (compared to *p*-nitrophenyl 5′-thymidylate) toward compounds containing the pyrophosphate linkage such as thymidylyl (5′ → 5′) thymidylate,[9-11] NAD,[11] ADP,[9,11] ATP,[9,11] and certain nucleoside tri- and tetraphosphates.[11-14] At present, it is not certain whether the pyrophospholytic activity is inherent to phosphodiesterase or due to contamination of a pyrophospha-

‡Thin-layer chromatography was used to monitor the extent of the reaction.

§Several attempts were made to prepare the 5-iodoindoxyl phosphodiester of 6-mercaptopurine riboside, but the product was found to be too labile to be isolated.

Table I. Kinetic Parameters of Nucleotide 5'-Phosphodiesterase Catalyzed Hydrolysis of Indoxyl Nucleoside Phosphodiesters[a]

	R	R'	X	Y	B	$K_m \times 10^4$, *M*	$v_{max} \times 10^{-4}$ µmoles/hr per mg of protein
1	H	OH	I	–	5-Fluorouracil	2.50	1.05
2	OH	OH	I	–	Uracil	2.86	0.69
3	H	OH	I	–	Thymine	5.86	2.33
4	H	OH	I	–	Adenine	4.00	0.79
5	H	OH	I	–	5-Bromouracil	5.00	0.87
6	H	OH	I	–	5-Iodouracil	2.25	0.57
7	H	H	I	–	Adenine	4.90	1.25
8	H	OH	NO_2	–	5-Fluorouracil	5.00	0.83
9	OH	OH	I	–	Adenine	5.05	1.11
10	H	OH	I	PO_3^-	5-Fluorouracil	6.25	1.04[b]

[a]Reaction medium: 50 m*M* Tris-HCl buffer (pH 8.40) containing 0.2% PVA, temperature 37°. [b]*E. coli* alkaline phosphatase was included in the assay.

Table II. Cytotoxic Effect of Indigogenic Phosphodiesters in Tissue Culture at 1.0×10^{-4} *M*

	Per cent of control growth	
Compd	HeLa	Mammary tumor
1	12.6	26.1
5	76.0	30.0
6	63.0	55.5
7[a]	73.5	66.5
8	6.0	21.6
10	11.0	7.6
IUdR	65.9	61.2
BUdR	61.9	134.6
FUdR	9.6	28.0

[a]This compound was assayed also in KB cell through the cooperation of Dr. Florence White at the National Cancer Institute and was not active at 100 µg/ml.

tase. Nevertheless, compound **10**, when used in conjunction with *E. coli* alkaline phosphatase, should be a valuable substrate for the assay of pyrophosphatase.

Cytotoxicity in the tissue culture system was tested in two cell lines, the HeLa cell system and a rat mammary tumor line (AC 33). The results are shown in Table II. Compound **7** has very low cytotoxicity in both cell lines. This is a little surprising in view of the fact that 2',3'-dideoxyadenosine can act as a chain terminator in the growing DNA chain.[15] There was no significant difference in cytotoxicity between the 5-nitroindoxyl and 5-iodoindoxyl moieties when they were incorporated into the phosphodiesters. Pyrophosphate **10** is similar in cytotoxicity in HeLa cell to **1**, but it is distinctly more toxic in AC 33 cells. The reason for this difference in activity may be attributed to the difference in pyrophosphodiesterase activity. Additional compounds in this series should therefore be synthesized and evaluated in the future.

Experimental Section

Material. Lyophilized Russel's viper venom phosphodiesterase was obtained from Calbiochem. *E. coli* alkaline phosphatase was purchased from Miles Laboratory as a suspension in $(NH_4)_2SO_4$. Polyvinyl alcohol (Elvanol 72-60) was a product of Du Pont. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), mp 170-171°, was purchased from Matheson and used without further purification. Sodium *p*-nitrophenyl 5'-thymidylate was obtained from Raylo Chemicals, Ltd., Edmonton, Canada. Ammonium 5-iodoindol-3-yl 5'-uridylate (**2**) and ammonium 5-iodoindol-3-yl 5'-adenylate (**9**) were prepared by previously published methods.[1,3] Phosphodiesters **3**, **4**, and **6** were prepared by Dr. Aoyagi.#

The following is a general procedure for making nucleoside phosphodiesters.

Ammonium 5-Iodoindol-3-yl 5'-(5-Bromodeoxyuridylate) (5). To a pyridine solution of *N*-acetyl-5-iodoindol-3-yl phosphodichloridate prepared from 390 mg (1.30 mmoles) of *N*-acetyl-5-iodoindoxyl and 0.13 ml (1.30 mmoles) of $POCl_3$ was added 400 mg (1.15 mmoles) of 3'-*O*-acetyl-5-bromodeoxyuridine[16] in 10 ml of anhydrous pyridine. The mixture was stirred for 24 hr and treated with 20 ml of 7 *M* NH_4OH, and stirring was continued for another 24 hr. The reaction mixture was evaporated to dryness, diluted with 500 ml of water, and filtered. The filtrate was applied to a DEAE-Sephadex A-25 column (HCO_3 form) and eluted with a linear gradient of 4 l. each of 0.01 *M* NH_4HCO_3 and 0.40 *M* NH_4HCO_3. After the gradient, the column was eluted with 0.5 *M* NH_4HCO_3. The fractions (20 ml each) showing the desired uv were pooled and lyophilized to give a white powder.

Calcium *N*-Acetyl-5-iodoindol-3-yl Phosphate. This intermediate was prepared by a modification of the procedure of Rabiger, *et al.*,[17] in order to retain the *N*-acetyl group. A mixture of 4.4 g (14.8 mmoles) of *N*-acetyl-5-iodoindoxyl in 30 ml of dry pyridine was treated with 1.6 ml (16 mmoles) of $POCl_3$. The reaction mixture was stirred overnight and poured slowly into a filtered solution of 7.5 g of $Ca(OH)_2$ and 5.3 g of NH_4Cl in 100 ml of water with rapid stirring. The insoluble product was collected by filtration and washed with two small portions of water. The product was air-dried and weighed 3.4 g (54.7%) as a lavender powder, uv λ_{max} (H_2O) 252, 303, 312 nm.

Dipyridinium *N*-Acetyl-5-iodoindol-3-yl Phosphate. A 1.5-g portion of the above calcium salt was stirred for 1 hr with 20 ml of Dowex 50W-X8 (pyridinium form) resin and 20 ml of water followed by filtration. The dark red filtrate was decolorized by treating with activated charcoal at 80° for 10 min. The light yellow filtrate was lyophilized to give the dipyridinium salt as a hygroscopic yellow oil.

Ammonium 5-Iodoindol-3-yl-5-fluorodeoxyuridine 5'-Pyrophosphate (10). A solution of 376 mg (1.30 mmoles) of 3'-*O*-acetyl-FUdR[18] in 15 ml of pyridine was treated with molecular sieves and cooled in ice. To the solution was added 0.13 ml (1.30 mmoles) of $POCl_3$; the mixture was allowed to stand for 45 min at 0° and then was treated with a solution of 700 mg (1.30 mmoles) of dipyridinium *N*-acetyl-5-iodoindol-3-yl phosphate in 15 ml of pyridine. After stirring for 5 min at 0° and 60 min at room temperature, the mixture was treated with 20 ml of 7 *M* NH_4OH and stirred overnight. Column chromatography by the usual procedure gave **10** as a white powder.

Enzyme Assay. Kinetic measurements were made on a Beckman DB-G grating spectrophotometer equipped with a Beckman 10-in. recorder. The cell compartment of the spectrophotometer was thermostated to 37 ± 0.5° by a Haake Model FE constant temperature circulator.

In a typical run, 2.0 ml of 50 m*M* Tris-HCl buffer (pH 8.40) containing 0.2% (w/v) PVA was pipetted into a 3.5-ml cuvette (optical path, 10 mm). To this was added 20 µl of phosphodiesterase

#S. Aoyagi, unpublished results.

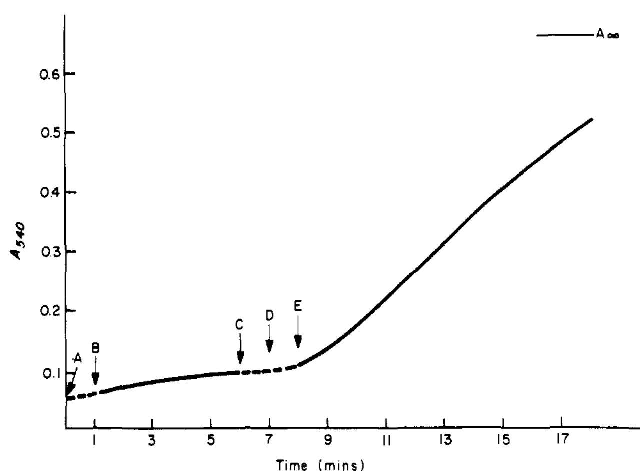


Figure 1. Reaction curve of **10**; 100 μl of 2.83 × 10^{-4} *M* of **10** was incubated with 2.0 ml of 50 m*M* Tris-HCl buffer (pH 8.40) containing 0.2% PVA. At point A (time zero), 20 μl of *E. coli* alkaline phosphatase was added. The instrument was started at point B and stopped at point C. At point D, 20 μl of snake venom phosphodiesterase was added. At point E, the instrument was started again. ——, actual recorder trace of absorbance; - - - -, extrapolated absorbance; A_{∞}, absorbance at infinity time.

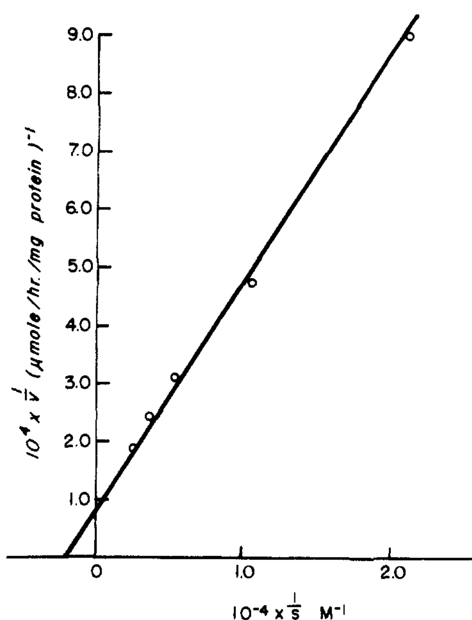


Figure 2. Lineweaver-Burk plot of Russel's viper venom nucleotide 5'-phosphodiesterase catalyzed hydrolysis of **7**.

Table III. Physical Properties and Yields of Phosphodiesters[a]

Compd	Formula	Yield, %	Uv_{max} (H_2O), nm (ε)	R_f[b]
5	$C_{17}H_{19}N_4O_8BrIP \cdot 2H_2O$	13.6	232 (25,700) 280 (10,600)	0.66[c]
7	$C_{18}H_{21}N_7O_5IP \cdot 2H_2O$	2.4	234 (27,800) 261 (13,150)	0.64[d]
8[e]	$C_{17}H_{19}N_5O_{10}FP \cdot 2H_2O$	7.4	272 (19,300)	0.41[d]
10	$C_{17}H_{23}N_5O_{11}FIP \cdot 2H_2O$	8.6	232 (31,200) 272 (10,800)	0.60[c]

[a]Satisfactory C, H analyses were obtained in all new compounds. [b]Obtained on Whatman No. 1 paper by the ascending method. [c]Solvent system: 1 *M* ammonium acetate–EtOH, 3:7 (v/v). [d]Solvent system: *i*-PrOH–concd NH_4OH–H_2O, 7:1:2 (v/v). [e]C: calcd, 37.95; found, 38.37.

stock solution (200 units/ml). The base line was set to zero at 540 nm. After allowing time for incubation, reaction was initiated by adding 100 μl of an indigogenic substrate solution. The initial rate of indigo formation was calculated. This corresponds to the rate of formation of indoxyl because the air oxidation of haloindoxyls is fast[19] and, therefore, not rate limiting.

In the case of **10** the substrate was incubated with 1.98 ml of the buffer first. After 20 μl of *E. coli* alkaline phosphatase (330 units/ml) was added, the reaction was followed for 6 min. At 7 min, 20 μl of phosphodiesterase stock solution was added, and the reaction was allowed to proceed as usual (Figure 1). For *p*-nitrophenyl 5'-thymidylate, the reaction was followed at 400 nm.

Product of Hydrolysis of 10 Catalyzed by Snake Venom 5'-Nucleotide Phosphodiesterase. A mixture of 1.0 ml of 50 m*M* Tris-HCl buffer (pH 8.40) containing 0.2% PVA, 50 μl (7.5 units) of Russel's viper venom phosphodiesterase, and 400 μl of 8.66 m*M* **10** in the same buffer was incubated at 37° for 1.5 hr. The mixture was then analyzed by paper chromatography on Whatman No. 1 paper (ascending) and the R_f values (1 *M* ammonium acetate-EtOH) are given below, together with those of the related markers: sodium 5-iodoindoxyl phosphate, R_f 0.43; **10**, R_f 0.60; 5-fluorodeoxyuridine, R_f 0.82; 5,5'-diiodoindigo, R_f 0.00; reaction mixture, R_f 0.41, 0.20; 5'-(5-fluoro)deoxyuridylate, R_f 0.20. Thus, the primary products of hydrolysis of **10** catalyzed by venom nucleotide 5'-phosphodiesterase are 5'-iodoindoxyl phosphate and 5'-(5-fluoro)-deoxyuridylate (Table III).

Determination of K_m and v_{max}. The initial rates of hydrolysis of the phosphodiesters by Russel's viper venom phosphodiesterase were measured at a fixed enzyme concentration and varying concentration of substrate. Values of K_m and v_{max} obtained from $1/v$ *vs.* $1/(S)$ plots are summarized in Table I. A typical Lineweaver-Burk plot[20] is shown in Figure 2. The protein content of the commercial enzyme preparation is too small to allow an accurate determination by the Lowry method.[21] Therefore, all the v_{max} values listed in Table I were obtained from calibration with *p*-nitrophenyl 5'-thymidylate using an extrapolated v_{max} value of 26,500 μmoles/hr per mg of protein for this substrate.[22]

Cytotoxicity Studies. Using Eagle's medium (MEM) with 10% calf serum (heat inactivated at 56° for 24 hr to remove endogenous phosphodiesterase), the cytotoxicity of the phosphodiesters were tested on rat mammary tumor (AC 33) and HeLa cell tissue culture lines using the technique described by Umeda and Heidelberger.[23] The cells were grown in Leighton tubes, and the compounds were added after 24-hr incubation at 37°. Their effect was determined by removing the cells from the glass surface of the tubes with 0.025% Pronase solution[24] in MEM without serum and counting them in a hemocytometer. Counts were made on day 4, and all activities are expressed in terms of control cell growth on day 4.

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Novel Phosphate Anthelmintics. 1. Alkyl 2,2-Dichlorovinyl Methyl Phosphates and Related Alkoxyalkyl and Cycloalkyl Analogs of Dichlorvos

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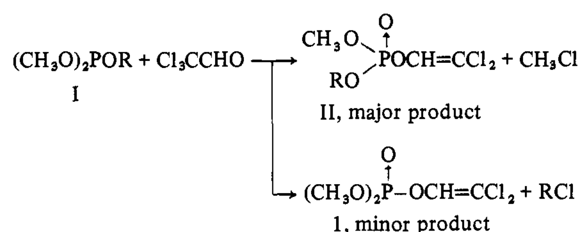
A series of new alkyl 2,2-dichlorovinyl methyl phosphates and related alkoxyalkyl and cycloalkyl analogs of dichlorvos, 2,2-dichlorovinyl dimethyl phosphate (**1**), having a high order of anthelmintic activity, has been synthesized. Novel vinyl pyrophosphates, obtained by treatment of vinyl phosphoric acids with $SOCl_2$ or diphenylcarbodiimide, facilitated the preparation of the asymmetric esters. The activity of these compounds has been assessed in rats and mice against the roundworm *Nippostrongylus brazilensis*, the tapeworm *Hymenolepis nana*, and the pinworm *Syphacia obvelata.*

The broad anthelmintic and insecticidal properties of dichlorvos,[1,2] 2,2-dichlorovinyl dimethyl phosphate (**1**), prompted investigation of possible ways of producing related compounds having improved therapeutic values either by increasing their toxicity toward target organisms, reducing their mammalian toxicity or both. Spontaneous reactivation of phosphorylated cholinesterase has been shown to depend on the structure of the phosphoryl residue and has led to the concept that the rate constant for reactivation decreases with increasing size of the alkoxyl radicals.[3] This concept is only partly correct considering the work of Berry and Davis[4] who have shown that the spontaneous reactivation of alkyl methylphosphonyl acetylcholinesterases shows the expected decrease when the alkyl group changes from Me to Pr, but an increase in spontaneous reactivation above that of the Me homolog, as the alkyl group increases from butyl through hexyl. Since increase in the rate of spontaneous reactivation should decrease toxicity, ways are then suggested for reduction of mammalian toxicity in other phosphates. The same structural modifications that may lead to reduction of mammalian toxicity may also serve to increase toxicity toward target organisms. Enzyme-substrate affinity studies of Hofstee,[5] Lewis,[6] and Bracha and O'Brien[7,8] have shown that the affinity of various esterases toward certain substrates increases to a maximum with increasing length of *n*-alkyl chains in *n*-fatty acid esters, *n*-acyl methylcarbamates, alkyl phosphates, and *S*-alkyl phosphorothiolates. Indeed, the compilation of Dixon and Webb[9] indicates that this is a common property of various substrates toward a variety of bifunctional esterases. In these examples the rate of reaction depends greatly on the presence and length of *n*-hydrocarbon chains in the substrates and often shows a definite maximum for a particular chain length for a particular esterase. Thus, replacement of one of the methyl groups in **1** by larger alkyl groups or other radicals was considered a likely route toward attainment of the desired goals. Several asymmetric esters of vinyl phosphates have been described,[10-12] but their pharmacological utility remained unappreciated until recently, perhaps due to the complexity of the products arising from conventional synthesis routes that were not fully appreciated when the early preparations were evaluated biologically. This paper describes methods for the preparation of asymmetric ester (II) analogs of dichlorvos,[13] their anthelmintic properties,[14] and their structure-activity correlations, and a new class of vinyl phosphates, vinyl pyrophosphates,[15] that greatly facilitated the synthesis work. After completion of this project four patents[16-19] were issued claiming synthesis processes (Schemes III and IV) and/or insecticidal and fungicidal properties for some of the compounds described in this paper. These compounds are referred to in the tables. A fifth patent claims compound **14** as an anthelmintic.[20]

Chemistry. The synthesis of the asymmetric esters II was carried out initially by the reaction discovered by Whetstone[1] and Perkow,[21] Scheme I.

Scheme I



Alkyl dimethyl phosphites (I)[22] were allowed to react with chloral in a usual manner.[23] The product contained from 5 to 15% of **1** and required purification by distillation and column chromatography. Because products obtained by Scheme I required lengthy purification to remove related phosphates, an alternate procedure *via* III was sought. Attempts to prepare III by treatment of **41** or **42**[24] with a 10 *M* excess of thionyl chloride at reflux gave instead **50**. This pyrophosphate may arise through intermediacy of a mixed anhydride IV, as reported for similar reactions of