(b) **Cytidine-3′,5′ Cyclic Phosphate.**—The nucleotide (0.05 mmole) and barium hydroxide (0.5 mmole) in water (2.5 ml.) were heated at 100° and the product adsorbed onto a column (10 cm. × 1.0 cm. dia.) of Dowex 1 resin (8% cross-linked, 200–400 mesh) in the formate form, as in the hydrolysis of uridine-3′,5′ cyclic phosphate. Cytidine phosphates were eluted with 0.01 *M* formic acid[51] (1.1 l.), the nucleotides being estimated at 280 mμ. Subsequently, the uridine phosphates were eluted as in the preceding experiment. The column was then calibrated with a mixture of cytidine-5′, cytidine-2′, cytidine-3′ and the corresponding uridine nucleotides. The products thus were shown to be cytidine-5′ phosphate (8%), cytidine-3′ phosphate (41%), uridine-5′ phosphate (7%) and uridine-3′ phosphate (37%). In addition, an unidentified product with a cytidine spectrum (3%) was eluted immediately after cytidine-5′ phosphate. No cytidine-2′ or uridine-2′ phosphate was detected.

(c) **Adenosine-3′,5′ Cyclic Phosphate.**—The nucleotide (0.05 mmole) was hydrolyzed with barium hydroxide (0.5 mmole) in water (2.5 ml.) at 100° as above and the product analyzed on a column (10 cm. × 1.0 cm. dia.) of Dowex 1 resin (8% cross-linked, 200–400 mesh) in the chloride form. Elution was carried out with 0.002 *M* hydrochloric acid,[51] the eluate being examined at 257 mμ. The products were adenosine-3′ phosphate (84%) and adenosine-5′ phosphate (16%). No adenosine-2′ phosphate was detected. Continued washing with a solution 0.02 *N* in sodium chloride and 0.01 *N* in lithium acetate, *p*H 5.5,[52] showed the absence of any inosine nucleotides. The hydrolysis products were also characterized paper chromatographically in solvents B and G.

(d) **Guanosine-3′,5′ Cyclic Phosphate.**—Hydrolysis was as for uridine-3′,5′ cyclic phosphate and chromatography on the standard ion exchange column in the chloride form. Elution was carried out using 0.005 *M* hydrochloric acid. The products were guanosine-3′ phosphate (80%) and guanosine-5′ phosphate (20%). No xanthosine phosphates were detected on continued elution with 0.025 *M* hydrochloric acid and 0.05 *M* sodium chloride. The products were also characterized paper chromatographically in the same way as described for the products of hydrolysis of adenosine-3′,5′ cyclic phosphate.

Hydrolysis of Uridine-3′,5′ Cyclic Phosphate by Sodium Hydroxide.—The nucleotide (0.1 mmole) in *M* sodium hydroxide (1.0 ml.) was heated under nitrogen at 100° in a polyethylene tube. The solution turned brown during the course of reaction and some gummy material separated. Aliquots were removed at suitable intervals, treated with Amberlite IR-120 resin (ammonium form) and examined chromatographically in solvent A. Ultraviolet adsorbing spots were eluted with 0.1 *M* hydrochloric acid (2 ml.) and estimated spectrophotometrically at 261 mμ; $t_{1/2}$ for the conversion of uridine-3′,5′ cyclic phosphate into other ultraviolet adsorbing substances was 1.5 hr. There was concomitant destruction of the chromophore. After 2 hr. the products were uridine-5′ phosphate (trace), uridine-3′ phosphate (major product), uracil (about 1/4 the amount of uridine-3′ phosphate), and an unidentified substance, R_f 0.77 (trace), some unreacted uridine-3′,5′ cyclic phosphate being also present.

Under the same conditions (2 hr. at 100°) uridine-5′ phosphate was completely unaffected by 1 *M* sodium hydroxide. Adenosine-5′ phosphate was partially (about 10%) degraded to adenine (identified chromatographically and spectrophotometrically) and two minor unidentified substances (R_f's 0.05 and 0.55 in solvent A).

When uridine-3′5′ cyclic phosphate (0.01 mmole) in water (0.1 ml.) containing freshly precipitated cadmium hydroxide (10 mg.) at *p*H 7.5 was heated at 100°, only a faint trace of hydrolysis could be detected after 24 hr.

Enzymic Hydrolysis of Uridine-3′,5′ Cyclic Phosphate. (a) **With Crude Snake Venom.** Crude *Crotalus adamanteus* venom (10 mg.) was added to a solution of the nucleotide (0.02 mmole) in water (1.8 ml.) containing *M* tris-(hydroxymethyl)-aminomethane buffer *p*H 8.9 (0.2 ml.). A drop of toluene was added and the mixture incubated at 37° for 24 hr. At this stage, 51% of the uridine-3′,5′ cyclic phosphate was unreacted, the products being uridine (41%) and uridine-3′ phosphate (8%). After 108 hr., 11% of the uridine-3′,5′ cyclic phosphate remained, the products being uridine (53%), uracil (15%) and uridine-3′ phosphate (11%). In another experiment, a mixture of uridine-3′,5′ cyclic phosphate (3.0 μmoles of ammonium salt) and the crude venom (3.3 mg.) in 0.05 *M* tris-(hydroxymethyl)-aminomethane, *p*H 8.2 (0.4 ml.), was kept at 35° for 13 hr. The products were uridine-3′,5′ cyclic phosphate (40%), uridine (15%) and uridine-3′ phosphate (45%).

(b) **With Purified Spleen Diesterase.**[46] The nucleotide (2.5 μmoles of ammonium salt) in *M* ammonium acetate, *p*H 6.1 (0.1 ml.), and the spleen diesterase preparation (36 μg. protein) in 0.01 *M* pyrophosphate buffer, *p*H 6.1 (0.1 ml.), were incubated together at 37° for 24 hr. No hydrolysis of the uridine-3′,5′ cyclic phosphate was detectable. The activity of the spleen phosphodiesterase used was such that 0.02 ml. of the preparation hydrolyzed completely 1 μmole of thymidylyl-(3′→5′)-thymidine in 1 hr. under the above conditions.

Hydrolysis of Adenosine-3′,5′ Cyclic Phosphate with Crude Snake Venom.[7]—The nucleotide (20 μmoles) and the crude venom (10 mg.) in water (1.8 ml.) containing *M* tris-(hydroxymethyl)-aminomethane, *p*H 8.9 (0.2 ml.), were incubated at 37° for 24 hr. in the presence of a drop of toluene. Chromatography in solvent A showed that 27% of adenosine-3′,5′ cyclic phosphate remained, the products being adenosine (36%) and adenosine-3′ phosphate (37%). After 108 hr., reaction was complete, the products being adenosine (51%) and adenosine-3′ phosphate (49%).

(52) W. E. Cohn in "The Nucleic Acids," eds. E. Chargaff and J. N. Davidson, Academic Press, Inc., New York, N. Y., 1955, Vol. I, p. 228.

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Organic Phosphorus Compounds. VI.[1] Effects of Structural Variations in Systox and Isosystox Analogs on their Reactions with Cholinesterase

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A series of Systox and Isosystox analogs of the general formulas $RP(S)(OR')OCH_2CH_2SC_2H_5$ (I) and $RP(O)(OR')SCH_2CH_2SC_2H_5$ (II), respectively, was prepared; R represents CH_3 and C_2H_5 and R′ is an alkyl group with 2–5 carbon atoms. The effect of the variation of R′ on the rate of the reaction of these compounds with eel cholinesterase was studied.

Introduction.—The higher rate of inactivation of eel cholinesterase by isopropyl methylphosphonofluoridate (Sarin), as compared to diisopropyl phosphorofluoridate (DFP),[3] and the increase in anticholinesterase activity realized by the replacement of one ethoxy group of S-(2-ethylthioethyl) phosphorothioate (Isosystox) by an ethyl[4,5] or propyl[5] radical demonstrate a pronounced effect of

(1) Paper V of this series, *J. Am. Chem. Soc.*, **81**, 148 (1959).
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(3) H. O. Michel, *Federation Proc.*, **14**, 255 (1955).
(4) H. S. Aaron, H. O. Michel, B. Witten and J. I. Miller, *J. Am. Chem. Soc.*, **80**, 456 (1958).
(5) T. R. Fukuto and R. L. Metcalf, *ibid.*, **81**, 372 (1959).

TABLE I

PHYSICAL CONSTANTS AND ANALYSES OF O-ALKYL O-(2-ETHYLTHIOETHYL) ALKYLPHOSPHONOTHIOATES, $RP(S)(OR')OCH_2CH_2SC_2H_5$ (I)

R'	Yield, %	B.p. °C.	B.p. Mm.	$n^{25}D$	d^{25}_4	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Phosphorus, % Calcd.	Phosphorus, % Found	Sulfur, % Calcd.	Sulfur, % Found
R = CH_3													
C_2H_5 (IV)	50	66–70	0.005–0.015	1.5014	....	36.82	36.4	7.51	7.5	13.57	14.4	28.09	28.1
C_3H_7 (V)	51	78–80	0.020	1.4981	1.0733	39.65	39.8	7.90	8.1	12.78	13.2	26.46	26.5
i-C_3H_7 (VI)	33	75–79	0.015	1.4930	1.0710	39.65	39.8	7.90	8.0	12.78	12.8	26.46	26.4
C_4H_9 (VII)	68	80–90	0.010–0.015	1.4941	1.0616	42.16	42.2	8.26	8.4	12.08	12.4	25.02	24.7
C_5H_{11} (VIII)	27	83–86	0.002–0.003	1.4918	1.045	44.42	44.4	8.57	8.5	11.46	11.6	23.72	23.6
R = C_2H_5													
C_2H_5 (IX)	33	86–91	0.070–0.080	1.4962	1.086	39.65	39.5	7.90	7.8	12.78	12.8	26.46	25.9
C_3H_7 (X)	56	82–87	.015– .065	1.4950	1.0632	42.16	42.5	8.26	8.1	12.08	12.2	25.02	25.1
C_4H_9 (XI)	46	88.5–92	.040– .050	1.4932	1.0478	44.42	44.4	8.57	8.6	11.46	11.7	23.72	24.0
C_5H_{11} (XII)	42	96–98	.005– .010	1.4919	1.0361	46.45	46.1	8.86	8.7	10.89	11.2	22.55	23.1

the structure on the activity of phosphorus-anticholinesterases. Fukuto and Metcalf[5] investigated recently the effect of a variety of alkyl groups replacing one of the ethoxy groups of diethyl *p*-nitrophenyl phosphate (Paraoxon), another highly effective cholinesterase inhibitor, on the activity of these compounds and elucidated a number of structural requirements for the alkyl substituent on the phosphorus atom necessary to result in high inhibitory activity. No systematic study seems to have been reported for the structural effects of different alkoxy groups on the activity of compounds of the Systox–Isosystox type. The two series of cholinesterase inhibitors represented by the general formulas $RP(S)(OR')OCH_2CH_2SC_2H_5$ (I) and $RP(O)(OR')SCH_2CH_2SC_2H_5$ (II), in which R is methyl or ethyl and R' is an alkyl group with 2–5 carbon atoms, were selected for studying the effect of changes in the OR' grouping on the activity of these two types of organic phosphorus compounds.

Experimental

All O-alkyl O-(2-ethylthioethyl) alkylphosphonothioates (I) were prepared by reaction of the appropriate O-alkyl alkylphosphonochloridothioate, $RP(S)(OR')Cl$ (III), with the sodium derivative of 2-ethylthioethanol in dry benzene in analogy to the method reported for the synthesis of Systox from diethyl phosphorochloridothioate.[6-8] Thermal isomerization of I yielded the corresponding O-alkyl S-(2-ethylthioethyl) alkylphosphonothioates (II).[9] The III required for the preparation of I was obtained in the manner described previously.[10]

General Method for the Preparation of O-Alkyl O-(2-Ethylthioethyl) Alkylphosphonothioates (I).—To a stirred suspension of 0.263 mole of powdered sodium hydride (5% excess) in 250–400 ml. of benzene was added 0.275 mole of 2-ethylthioethanol (10% excess). The mixture was stirred and heated at reflux temperature for a period of 1–2 hr. After cooling to room temperature, the resulting suspension of sodium 2-ethylthioethoxide was treated with 0.250 mole of the appropriate III which was added dropwise over a period of 10–25 min. while maintaining the temperature of the reaction mixture at 15–30° by external cooling. In order to assure complete reaction, stirring was continued for 2–4 hr. (or overnight) at room temperature. The precipitated sodium chloride was removed by washing the mixture once with 250 ml. of water and twice with 100-ml. portions of water. The aqueous washings were combined and extracted once with 200 ml. and once with 100 ml. of benzene. The combined benzene layer and benzene extracts were dried with anhydrous magnesium sulfate, filtered, and evaporated on a rotary evaporator at room temperature under reduced pressure. The oily residue of the crude I was finally distilled *in vacuo* below 0.10 mm. of pressure. It is imperative to perform the distillations at low pressures in order to avoid the thermal isomerization of the I to II.

The I prepared in this manner are listed with their physical constants and analyses in Table I. The phosphorus nuclear magnetic resonance (n.m.r.) spectra indicated that all I contained at least 95% of their phosphorus in the form of a P → S grouping. In agreement with these findings were the infrared absorption spectra. On the basis of their (n.m.r.) spectra, compounds IV and V contained 4–5% and compound XII 2–4% phosphoryl impurity, while the phosphoryl contaminations of all other I were less than 2%.

Isomerization of I to O-Alkyl S-(2-Ethylthioethyl) Alkylphosphonothioates (II).—The I was heated for a period of 3 hr. under a slow stream of dry nitrogen in a round-bottom flask of suitable size in an oil-bath maintained at 150 ± 10.° The n.m.r. spectra of the various I treated in this manner showed that only compound IV had undergone isomerization with the formation of a phosphoryl group to an extent of more than 95%. The percentage of isomerization based on the n.m.r. spectra is given for the various I in the second column of Table II. The third and fourth column of the table give the percentage of phosphorus present in the form of phosphoryl compounds after 5 and 7 hours, respectively, of heating at 150°.

TABLE II

ISOMERIZATION OF O-ALKYL O-(2-ETHYLTHIOETHYL) ALKYLPHOSPHONOTHIOATES (I) TO O-ALKYL S-(2-ETHYLTHIOETHYL) ALKYLPHOSPHONOTHIOATES, $RP(O)(OR')SCH_2CH_2SC_2H_5$ (II)

R'	Isomerization, % after 3 hr.	Isomerization, % after 5 hr.	Isomerization, % after 7 hr.
R = CH_3			
C_2H_5 (IV)	>95	..	..
C_3H_7 (V)	61	85[a]	95[b]
i-C_3H_7 (VI)	48	81[c]	95
C_4H_9 (VII)	71	86	91[d]
C_5H_{11} (VIII)	59	88	93
R = C_2H_5			
C_2H_5 (IX)	67[e,f]	..	..
C_3H_7 (X)	87	91	95[c]
C_4H_9 (XI)	36	72[c]	92[g]
C_5H_{11} (XII)	80	88	>95

[a] 6% decomposition. [b] 5% decomposition. [c] Heated 6 hr. [d] 4% decomposition. [e] 9% decomposition. [f] A heating period of 3.5 hr. resulted in 83% isomerization and 7% decomposition. [g] Heated 9 hr.

For further isomerizations beyond the initial 3-hr. heating period, 1-cc. aliquots of the partially isomerized I were sealed under dry nitrogen in narrow Pyrex glass n.m.r.

(6) G. Schrader, U. S. Patent 2,571,989 (1951).

(7) Farbenfabriken Bayer, German Patent 850,677 (1952).

(8) Ya. A. Mandelbaum, N. M. Mel'nikow and V. J. Lomakina, *Zhur. Obshchei Khim.*, **26**, 2581 (1956); *C. A.*, **51**, 1825*e* (1957).

(9) F. W. Hoffmann and T. R. Moore, *J. Am. Chem. Soc.*, **80**, 1150 (1958).

(10) F. W. Hoffmann, D. H. Wadsworth and H. D. Weiss, *ibid.*, **80**, 3945 (1958).

tubes. The n.m.r. spectra of these samples were measured after every hour of heating at 150°. Under these conditions, compound XI showed the slowest rate of isomerization, complete isomerization in this case required a heating period of 9 hours. The results of these isomerization runs are given in Table II. A second set of 1-cc. samples of the various I was then isomerized in sealed tubes for the periods of time listed in the first column of Table III. These isomerized samples were analyzed by means of their n.m.r. spectra and used for the determination of their cholinesterase inhibition.

TABLE III

PREPARATION OF O-ALKYL S-(ETHYLTHIOETHYL) ALKYLPHOSPHONOTHIOATES, $RP(O)(OR')SCH_2CH_2SC_2H_5$ (II)

R′	Total heating period, hr.	Yield, of % (II)[a]	Yield, of % Phosphorus-containing[a] decomp. prodn.	$n^{25}D$
		R = CH_3		
C_2H_5 (XIII)	3	95	..	1.5106
C_3H_7 (XIV)	7	95	5	1.5038
i-C_3H_7 (XV)	8	90	10	1.5018
C_4H_9 (XVI)	7	100	..	1.5029
C_5H_{11} (XVII)	7	100	..	1.4996
		R = C_2H_5		
C_2H_5 (XVIII)	6	90	10	1.5032
C_3H_7 (XIX)	6	100	..	1.5024
C_4H_9 (XX)	9	100	..	1.5009
C_5H_{11} (XXI)	7	100	..	1.4985

[a] Determined by n.m.r.

Kinetics of the Reactions of O-Alkyl O- and S-(2-Ethylthioethyl) Alkylphosphonothioates with Cholinesterase.—The kinetic measurements were made by the method reported previously.[4] The I or II was allowed to react with highly purified eel cholinesterase in 0.357 *M* potassium chloride, 0.1% gelatin and 0.0018 *M* tris-(hydroxymethyl)-aminomethane at *p*H 7.4 and 25°. The concentrations of the inhibitors were at least 500 times the minimum required to inactivate the enzyme. The bimolecular velocity constants for the enzyme–inhibitor reaction are given in Table IV.

Results and Discussion

The rates of inactivation of eel cholinesterase by the organophosphorus compounds I and II were all first-order with respect to enzyme concentration. Bimolecular velocity constants were computed from the rate data and the results are given in Table IV. The values of the I are all much smaller than the corresponding values for the II. Velocity constants for the II show a progressive increase with increasing chain length of the alkoxy group, the isopropoxy compound XV excepted, and in each case, a greater value for the methyl- than for the corresponding ethylphosphonate.

The difference found between the thiono (I) and thiolo (II) derivatives is in line with earlier work on Systox and Isosystox[11] which showed that the iso compound was the more active cholinesterase. There does not appear to be any systematic pattern to the velocity constants of the thionates. Because of probable spontaneous isomerization, it is possible that much of the observed reactivity of the thionates is due to trace amounts of the thiolate analogs.

TABLE IV

VELOCITY CONSTANTS FOR INACTIVATION OF EEL CHOLINESTERASE BY COMPOUNDS I AND II AT *p*H 7.4 AND 25°

R	Compound, I	k_2(±SD) × 10^{-4}, l./mole min.	Compound, II	k_2(±SD) × 10^{-4}, l./mole min.
		R′ = methyl		
Ethyl	IV	0.60 ± 0.08	XIII	17.6 ± 1.6
Propyl	V	0.51 ± .13	XIV	123.0 ± 26
Iropropyl	VI	1.39 ± .01	XV	4.9 ± 0.4
Butyl	VII	1.48 ± .30	XVI	320.0 ± 12
Amyl	VIII	0.76 ± .11	XVII	381.0 ± 23
		R′ = ethyl		
Ethyl	IX	.82 ± .22	XVIII	(13.9 ± 0.3)[a]
Propyl	X	.077 ± .001	XIV	29.2 ± 0.4
Butyl	XI	.36 ± .04	XX	85 ± 4
Amyl	XII	.32 ± .016	XXI	74 ± 4

[a] The value obtained by Aaron, *et al.*,[4] was 4.6 × 10^{-4}.

The consistently higher velocity constants of the methyl- as compared to the analogous ethyl-phosphonothiolates may be the result of a smaller steric hindrance to the approach of a nucleophilic group in the enzyme to the phosphorus atom of the phosphonate, and also could be related to providing a more favorable approach of the phosphonate to the enzyme surface.

The increased rates of reaction due to increasing chain length of the alkoxy groups in the thiolates may indicate that the latter groups can lie along the enzyme surface to increase binding through van der Waals forces, in the formation of an intermediary complex. Branching in the alkoxy group decreases the rate of reaction, again possibly because of steric hindrance. In Sarin, which shows a high rate of reaction with eel cholinesterase, but has a branched alkoxy group, the size of this group is compensated by the relatively small space requirement of the fluorine atom.

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(11) T. R. Fukuto and R. L. Metcalf, *J. Am. Chem. Soc.*, **76**, 5103 (1954).