slightly active while the 9-methyl derivative (calcd. log P, 7.48) is active. Equation 34 avoids this difficulty. From Table VII it is seen that the cut off in activity is to be expected at about log $P = 8$. This is the point where the carcinogen is 100 million times more soluble in the lipophilic phase than in the aqueous—an enormous differential. For aromatics with six rings, the differential would be over 600 million. Considering the great insolubility of such compounds in either phase, it seems quite natural to expect a small aqueous barrier to isolate the cellular site of action from these insoluble substances.

While the results from the ρ–σ–π analysis with carcinogenic hydrocarbons are not as sharp as those we have found for the other systems, they are convincing enough to show that the lipo–hydrophilic character of these molecules must be considered in attempts to rationalize structure–activity relationships.

From the results obtained with the ρ–σ–π analysis for the eight examples reported herein, plus the two previously considered cases,[3] the view emerges that, although eq. 8 was developed to rationalize structure–activity relationships of the plant growth regulators, it now appears to have a general use in a wide variety of pharmacological systems. Our basic hypothesis that, as a first approximation, biologically active compounds appear to exert a rate-controlling effect on one chemical or physical process and that biological effects resulting from structural changes can be correlated by means of regression analyses with two parameters, σ and π, seems well worth further study.

If ρ in eq. 6 is indeed the same as that in the Hammett equation as we have assumed, then eq. 6 (or its simpler forms) should be useful in estimating electronic and steric demands of enzymes without the necessity of isolating and studying them *in vitro*.

The constants associated with π provide insight into the nature of the cellular material through which a molecule must make its way before reaching the site of action. The additive character of π or log P should be of considerable help in making gross modifications in the structure of biologically active compounds in the quest for more active or more selective drugs. For example, in the synthesis of the so-called nonclassical antimetabolites,[30] where the attachment of a very large moiety to a basic structure is desired, one must, in addition to holding electron distribution constant, achieve an isolipotropic character in the derivative if one expects to find equivalent biological response. Work in progress indicates that when strong electronic interaction is absent, π is constant and additive in character. This should make the construction of isolipotropic molecules relatively easy.

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Deoxyribonucleoside-3′,5′ Cyclic Phosphates. Synthesis and Acid-Catalyzed and Enzymic Hydrolysis[1]

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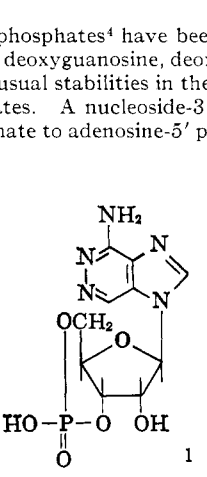
Methods previously developed for the synthesis of ribonucleoside-3′,5′ cyclic phosphates[4] have been utilized for the synthesis of cyclic phosphates derived from deoxyadenosine, deoxycytidine, deoxyguanosine, deoxyinosine, deoxyuridine, and thymidine. The glycosidic bonds in these nucleotides have unusual stabilities in the presence of acid, analogous to the stabilities found in ribonucleoside-3′,5′ cyclic phosphates. A nucleoside-3′,5′ cyclic phosphate diesterase from beef brain, which converts adenosine-3′,5′ cyclic phosphate to adenosine-5′ phosphate, hydrolyzes purine deoxynucleoside-3′,5′ cyclic phosphates.

The discovery[5] and characterization[6] of adenosine-3′,5′ cyclic phosphate (**1**) as a factor in the activation of hepatic glycogen phosphorylase has been followed by a rapidly increasing appreciation of the widespread occurrence[7,8] of the nucleotide and the multiplicity of its



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biological functions, especially as an intermediate in the action of hormones.[9] Consequently, it is of interest to

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study the chemistry and biochemical activity of a variety of analogs of adenosine-3′,5′ cyclic phosphate.[10] Thus, the synthesis of various nucleoside-3′,5′ cyclic phosphates[4] preceded and facilitated the characterization of naturally occurring guanosine-3′,5′ cyclic phosphate.[11] In the present paper, syntheses of deoxynucleoside-3′,5′ cyclic phosphates derived from adenine, cytosine, guanine, hypoxanthine, thymine, and uracil are described. Adenosine-3′,5′ cyclic phosphate diesterase is probably of considerable importance to the biological actions of the nucleotide.[7,12] Consequently, it was of some interest to determine the action of the enzyme on the deoxynucleoside-3′,5′ cyclic phosphates. The diesterase from beef brain appears to be purine specific. The unusual stability to acid of glycosidic bonds in nucleoside-3′,5′ cyclic phosphates[4-6,13] has been further investigated in the present study.

Preparation of Deoxynucleoside-3′,5′ Cyclic Phosphates.—Problems associated with the synthesis of cyclic phosphates have been discussed in detail previously.[4,13-15] The preparation of nucleoside-3′,5′ cyclic phosphates requires the relatively difficult formation of a six-membered ring fused *trans* to a five-membered ring. The most effective procedure for synthesis of ribonucleoside-3′,5′ cyclic phosphates involves the reaction of ribonucleoside-5′ phosphates, in the presence of a strong organic base (solvating agent), with dicyclohexylcarbodiimide in boiling pyridine under conditions of high dilution, followed by ion-exchange chromatography on diethylaminoethyl cellulose.[4] With only slight modifications in experimental procedure,[16] this approach has been applied successfully to the syntheses of deoxynucleoside-3′,5′ cyclic phosphates in satisfactory yield.[17,20,21] Deoxyadenosine-3′,5′, deoxycytidine-3′,5′, and thymidine-3′,5′ cyclic phosphates have been characterized previously.[17,22,23] Structures of new nucleotides are assigned by analogy and by their chromatographic and electrophoretic properties.

Hydrolysis of Deoxynucleoside-3′,5′ Cyclic Phosphates by Acid.—A striking feature of the chemistry of ribonucleoside-3′,5′ cyclic phosphates is the unusual stability of their glycosidic bonds in acid.[4-6] Adenosine-3′,5′ and guanosine-3′,5′ cyclic phosphates are converted to adenine and guanine much less readily than their parent 5′-phosphates, whereas uridine-3′,5′ cyclic phosphate is much less stable than uridine-5′ phosphate under the same conditions. Studies on thymidine-3′,5′ cyclic phosphate showed that thymine was released by acid more readily than from thymidine-5′ phosphate.[13] Consequently, it was of interest to investigate the acid-catalyzed hydrolysis of a number of other deoxynucleoside-3′,5′ cyclic phosphates. In *M* hydrochloric acid at 50°, there was no detectable change in deoxyadenosine-3′,5′ and deoxyguanosine-3′,5′ cyclic phosphates after 2 hr., whereas the corresponding 5′-phosphates were converted completely to adenine and guanine in less than 5 min. Thus the stability of the purine deoxynucleoside-3′,5′ cyclic phosphates relative to the 5′-nucleotides is much greater than in the ribonucleotide series.[4] At 100°, the half-life for conversion of deoxyadenosine-3′,5′ cyclic phosphate to adenine was 3 min. The corresponding figure for adenosine-3′,5′ cyclic phosphate is 30 min.

At 50°, thymidine-3′,5′ cyclic phosphate in *M* hydrochloric acid was half converted to thymine in 13 min., thymidine-5′ phosphate being unchanged under these conditions after 2 hr. Both deoxycytidine-3′,5′ cyclic phosphate and deoxycytidine-5′ phosphate were stable in *M* hydrochloric acid at 50°. At 100°, the cyclic phosphate and the 5′-phosphate had half-lives of 22 and 110 min. (approximately), the sole ultraviolet light-adsorbing product in each case being cytosine. Thus, the tendency for purine glycosidic linkages to be stable and for pyrimidine glycosidic bonds to be labile, relative to parent nucleoside-5′ phosphates, is a characteristic of both ribonucleoside-3′,5′ and deoxynucleoside-3′,5′ cyclic phosphates.[25]

Enzymic Hydrolysis of Deoxynucleoside-3′,5′ Cyclic Phosphates.—Earlier studies have demonstrated the widespread occurrence of an enzymic activity which hydrolyzes adenosine-3′,5′ cyclic phosphate to adenosine-5′ phosphate.[7,12] This phosphodiesterase, which does not hydrolyze diesters of adenosine other than the 3′,5′-cyclic phosphate, has been purified from rabbit brain,[12] dog heart,[26] and beef heart.[7] In the present study, the rates of hydrolysis of the deoxyribonucleoside-3′,5′ cyclic phosphates by adenosine-3′,5′ cyclic phosphate diesterase, obtained from beef brain by a modified procedure, were followed electrophoretically. Results are recorded in Table I.

From the results recorded in Table I, it can be seen that the beef brain diesterase preparation reacts specifically with purine nucleoside-3′,5′ cyclic phosphates.

"Ciba Foundation Symposium on Adrenergic Mechanisms," J. R. Vane, G. E. W. Wolstenholme, and M. O'Connor, Ed., J. and A. Churchill Ltd., London, 1960, pp. 295–304; T. W. Rall and E. W. Sutherland, *Cold Spring Harbor Symp. Quant. Biol.*, **26**, 347 (1961); P. R. Davoren, and E. W. Sutherland, *J. Biol. Chem.*, **238**, 3016 (1963); S. Tarui, K. Nonaka, Y. Ikura, and K. Shima, *Biochem. Biophys. Res. Comm.*, **13**, 329 (1963).

(10) Acyl derivatives of adenosine-3′,5′ cyclic phosphate are known to duplicate the effects, qualitatively, of the parent nucleotide in a number of biological systems [T. Posternak, E. W. Sutherland, and W. F. Henion, *Biochim. Biophys. Acta*, **65**, 558 (1962)].

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(13) G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Am. Chem. Soc.*, **80**, 6223 (1958).

(14) H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffatt, *ibid.*, **79**, 430 (1957).

(15) M. Smith, J. G. Moffatt, and H. G. Khorana, *ibid.*, **80**, 6204 (1958).

(16) These include shorter reaction times, an increase in the amount of dicyclohexylcarbodiimide used, and chromatography on DEAE-Sephadex (Pharmacia). The anion exchanger was more effective in separation of thymidine-3′,5′ cyclic phosphate from a minor, uncharacterized, reaction product (see Experimental section).

(17) There is evidence that deoxyribonucleoside-3′ phosphates or their derivatives, or ribonucleoside-3′ phosphates in which the 2′-hydroxyl function is blocked, are most effective precursors of nucleoside-3′,5′ cyclic phosphates.[4,18,19] However, these types of compounds are, at present, less accessible than nucleoside-5′ phosphates.

(18) A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4651 (1959).

(19) M. Smith and H. G. Khorana, unpublished results [see D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963), footnote 21].

(20) Thymidine-3′,5′ cyclic phosphate can be obtained readily by reaction of thymidine-5′ phosphate with dicyclohexylcarbodiimide in the absence of a strong organic base under conditions of high dilution.[13] The solubility characteristics of other nucleotides demand the modified procedure used in the present study.[4]

(21) Deoxyadenosine-3′,5′, deoxycytidine-3′,5′, and thymidine-3′,5′ cyclic phosphates previously have been described as minor products obtained during polymerization of nucleotides.[13,18,22-24]

(22) R. K. Ralph and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 2926 (1961).

(23) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961).

(24) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962).

(25) The effect on glycosidic bond stability is least in the cytosine series. This, coupled with the greater resistance to acid of the glycosidic bond in ribonucleosides, prevented any observations on the relative stability of cytidine-3′,5′ cyclic phosphate and cytidine-5′ phosphate.[4]

(26) K. G. Nair, *Federation Proc.*, **21**, 84 (1962).

TABLE I

RELATIVE RATES OF HYDROLYSIS OF NUCLEOSIDE-3′,5′ CYCLIC PHOSPHATE BY BEEF BRAIN DIESTERASE AT 37°, pH 7.5

Nucleoside-3′,5′ cyclic phosphate[a]	Rate (μmoles/mg. protein/hr.)	Relative rate
Adenosine	7.5[b]	100
Deoxyadenosine	3.75[b]	50
Deoxyinosine	3.6[b]	48
Deoxyguanosine	3.3[b]	44
Thymidine	0.09[c]	1.2
Deoxyuridine	0.08[c]	1.1
Deoxycytidine	0.06[c]	0.8

[a] Each substrate was present at a concentration of 1.5 μmoles in a final volume of 0.2 ml. [b] Protein (200 μg.) incubated 20 min. [c] Protein (1 mg.) incubated 2 hr.

By anology with adenosine-3′,5′ cyclic phosphate, the products obtained by hydrolysis of the deoxynucleoside-3′,5′ cyclic phosphates are believed to be the 5′-phosphates. The product from the diesterase hydrolysis of deoxyadenosine-3′,5′ cyclic phosphate, when treated with a specific adenosine-5′ phosphate deaminase,[27] was completely converted to deoxyinosinic acid, indicating that deoxyadenosine-5′ phosphate resulted from diesterase treatment.

Comparison of the relative rates of hydrolysis of nucleoside-3′,5′ cyclic phosphates, examined in the present study, with earlier results is of interest (see Table II). This might indicate that the adenosine-3′,5′ cyclic phosphate diesterase from different tissues can have different specificities, or that there are a number of different diesterases of this type. It may be noted that the enzyme which produces adenosine-3′,5′ cyclic phosphate from adenosine-5′ triphosphate[28] and the activating system for hepatic[9] and skeletal[29] muscle phosphorylase have much greater substrate specificities than any of the diesterase preparations so far described.

TABLE II

RELATIVE RATES OF HYDROLYSIS OF NUCLEOSIDE-3′,5′ CYCLIC PHOSPHATE BY DIFFERENT DIESTERASE PREPARATIONS

Diesterase prepn.	Nucleoside-3′,5′ cyclic phosphate	Relative rate
Rabbit brain[12]	Adenosine	100
	Guanosine	33
	Uridine	11
	Cytidine	0
Dog heart[26]	Guanosine	33
	Inosine	60
	Deoxyadenosine	100
	Uridine	15
	Cytidine	0
Beef heart[7]	Inosine	100
	Uridine	60

Experimental

General Methods.—Reagent grade pyridine was dried over calcium hydride. All evaporations were carried out at approximately 10 mm. pressure, the bath temperature being less than 40°. Paper chromatography was performed by the descending technique using Whatman No. 40 paper. Nucleotides and related compounds were detected by viewing under ultraviolet light. The R_f values of various compounds in 2-propanol–concentrated ammonia–water (7:1:2) are listed in Table III. Paper electrophoresis was carried out on Whatman No. 3MM paper in 0.05 *M* potassium phosphate, pH 7.5, for 90 min. at a field strength of 25 v./cm. Phosphate was determined by the method of King.[30]

TABLE III

R_f VALUES OF NUCLEOTIDES AND RELATED COMPOUNDS IN 2-PROPANOL–CONCENTRATED AMMONIA–WATER (7:1:2)

Compound	R_f
Deoxyadenosine-5′ phosphate	0.17
Deoxyadenosine-3′,5′ phosphate	0.43
Deoxyadenosine	0.59
Adenine	0.49
Deoxycytidine-5′ phosphate	0.14
Deoxycytidine-3′,5′ phosphate	0.43
Deoxycytidine	0.61
Cytosine	0.52
Deoxyguanosine-5′ phosphate	0.06
Deoxyguanosine-3′,5′ phosphate	0.25
Deoxyguanosine	0.40
Guanine	0.27
Thymidine-5′ phosphate	0.19
Thymidine-3′,5′ phosphate	0.52
Thymidine	0.71
Thymine	0.69

Thymidine-3′,5′ Cyclic Phosphate.—Thymidine-5′ phosphate (dihydrate of diammonium salt, 392 mg., 1.0 mmole) was converted to the pyridinium salt by passage, in water, through a column (10 × 1.5-cm. diameter) of the freshly prepared pyridinium form of Amberlite IR-120[31] cation exchanger. After removal of water by evaporation under reduced pressure, the nucleotide and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine[32] (293 mg., 1.0 mmole) were dissolved in dry pyridine (25 ml.), and the last traces of water were removed by coevaporation under reduced pressure (this step was repeated three times). The nucleotide was dissolved in dry pyridine (100 ml.), and the solution was run dropwise (1 hr.), *via* the reflux condenser, into a boiling solution of dicyclohexylcarbodiimide (1.03 g., 5.0 mmoles) in dry pyridine (100 ml.). The apparatus was protected from moisture. After removal of pyridine by evaporation, the product was extracted with water (50 ml.) and ether (50 ml.). The water–ether mixture was filtered to remove dicyclohexylurea, and the aqueous layer in the filtrate, which contained the nucleotide, was applied to a column (20 × 2.5-cm. diameter) of DEAE-Sephadex[33] in the carbonate form. After a water wash (200 ml.), elution was carried out using a linear salt gradient, with 0.002 *M* triethylammonium bicarbonate,[34] pH 7.5 (2 l.), in the mixing chamber and 0.1 *M* triethylammonium bicarbonate (2 l.) in the reservoir. Fractions (20 ml.) were collected at 10-min. intervals, the elution of nucleotides being followed spectrophotometrically at 261 mμ. The fractions (60–72) containing the major nucleotide component were combined, concentrated to dryness, redissolved in water, and lyophilized to yield the triethylammonium salt of thymidine-3′,5′ cyclic phosphate as a hygroscopic white powder (270 mg., 70%), chromatographically homogeneous in Solvent 1 and electrophoretically homogeneous at pH 7.5. Ultraviolet adsorption[13] showed λ_{max} 265 mμ (ε 9900) at pH 7.0 and λ_{max} 265 mμ (ε 7600) at pH 12.0.

Anal. Calcd. for $C_{10}H_{13}N_2O_7P \cdot C_6H_{15}N$: P, 7.55. Found: P, 7.32.

In an experiment where thymidine-3′,5′ cyclic phosphate was isolated *via* a column of diethylaminoethyl cellulose, the nucleotide was contaminated with a trace amount of an acid-resistant ultraviolet light-adsorbing substance with the same R_f in Solvent 1.

Deoxyuridine-3′,5′ Cyclic Phosphate.—Deoxyuridine-5′ phosphate was reacted with dicyclohexylcarbodiimide as above. The cyclic phosphate was isolated by preparative chromatography in Solvent 1 and was electrophoretically homogeneous at pH 7.0.

Deoxyadenosine-3′,5′ Cyclic Phosphate.—Deoxyadenosine-5′ phosphate (hemihydrate of the diammonium salt, 374 mg., 1.0 mmole) was converted to the 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium salt and treated with dicyclohexylcarbodiimide

(27) Y. Lee, *J. Biol. Chem.*, **227**, 987 (1957); *ibid.*, **227**, 999 (1957).

(28) E. W. Sutherland, T. W. Rall, and T. Menon, *ibid.*, **237**, 1220 (1962); T. W. Rall and E. W. Sutherland, *ibid.*, **237**, 1228 (1962).

(29) E. G. Krebs, personal communication; *cf.* E. G. Krebs, D. J. Graves, and E. H. Fisher, *ibid.*, **234**, 2867 (1959).

(30) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(31) Rohm and Haas Co., Philadelphia, Pa.

(32) Aldrich Chemical Co., Milwaukee, Wis.

(33) Pharmacia Fine Chemicals, Inc., New York, N. Y.

(34) Ref. 24, footnote 34.

(1.03 g., 5.0 mmoles) in boiling pyridine using the conditions described for the preparation of thymidine-3′,5′ cyclic phosphate. Work-up followed by chromatography on DEAE-Sephadex yielded deoxyadenosine-3′,5′ cyclic phosphate in fractions 70–100. The latter were combined and concentrated to dryness, and the residue was taken up in water and lyophilized to yield triethylammonium deoxyadenosine-3′,5′ cyclic phosphate (280 mg., 68%) as a hygroscopic white powder, chromatographically homogeneous in Solvent 1 and homogeneous on electrophoresis at pH 7.5. Ultraviolet adsorption showed λ_{max} 259 mμ (ε 14,500) at pH 7.0 and λ_{max} 257 mμ (ε 14,000) at pH 2.0.

Anal. Calcd. for $C_{10}H_{12}N_5O_5P \cdot C_6H_{15}N$: P, 7.48. Found: P, 7.34.

Deoxyinosine-5′ Phosphate.—The nucleotide was prepared from deoxyadenosine-5′ phosphate by nitrous acid deamination.[35]

Deoxyinosine-3′,5′ Cyclic Phosphate.—Deoxyinosine-5′ phosphate was treated with dicyclohexylcarbodiimide as described in the preparation of thymidine-3′,5′ cyclic phosphate. Preparative chromatography in Solvent 1 yielded deoxyinosine-3′,5′ cyclic phosphate in 65% yield. The nucleotide was electrophoretically homogeneous at pH 7.0.

Deoxycytidine-3′,5′ Cyclic Phosphate.—Deoxycytidine-5′ phosphate (acid, 307.2 mg., 1.0 mmole) was converted to N-benzoyldeoxycytidine-5′ phosphate[4,23] by treatment with benzoyl chloride in pyridine as in the preparation of N-benzoylcytidine-5′ phosphate.[4] The nucleotide, after conversion to the 4-morpholine-N,N′-dicyclohexylcarboxamidinium salt, was treated with dicyclohexylcarbodiimide (1.03 g., 5.0 mmoles) in boiling pyridine as in the synthesis of thymidine-3′,5′ cyclic phosphate. After removal of pyridine by evaporation, the residue was extracted with water (50 ml.) and ether (50 ml.) and filtered, and the water layer was isolated. The latter was concentrated to dryness, and the residual gum was taken up in 95% ethanol (5 ml.) and concentrated ammonia, sp. gr. 0.9 (10 ml.) and kept at 25° overnight. After removal of excess ammonia under reduced pressure, the nucleotide was adsorbed onto DEAE-Sephedex and chromatographed using a gradient of 0.005 to 0.075 *M* triethylammonium bicarbonate. The major ultraviolet light adsorbing component was eluted in fractions 59–69. These were combined and concentrated to dryness, the residue was dissolved in a small amount of water, and the nucleotide was precipitated with acetone. The latter was dissolved in water and lyophilized to yield deoxycytidine-3′,5′ cyclic phosphate dihydrate (150 mg., 47%) as a white powder, chromatographically homogeneous in Solvent 1 and electrophoretically homogeneous at pH 7.5. Ultraviolet adsorption showed λ_{max} 271 mμ (ε 9700) at pH 7.0 and λ_{max} 278 (ε 13,700) at pH 2.0.

Anal. Calcd. for $C_9H_{12}N_3O_6P \cdot 2H_2O$: P, 9.54. Found: P, 9.66.

Deoxyguanosine-3′,5′ Cyclic Phosphate.—Deoxyguanosine-5′ phosphate (diammonium salt, 382 mg., 1 mmole) was converted to the pyridinium salt of N-benzoyldeoxyguanosine-5′ phosphate by reaction with benzoyl chloride as has previously been described.[36] The cyclization and removal of the protecting group were carried out as in the synthesis of guanosine-3′,5′ cyclic phosphate,[4] followed by chromatography on DEAE-Sephadex as described in the present paper for thymidine-3′,5′ cyclic phosphate except that 0.15 *M* triethylammonium bicarbonate was used in the reservoir of the salt gradient system. The 3′,5′-cyclic phosphate was eluted in fractions 78–93. Isolation by the usual procedure yielded the triethylammonium salt of deoxyguanosine-3′,5′ cyclic phosphate (150 mg., 35%),[37] chromatographically and electrophoretically homogeneous. Ultraviolet adsorption showed λ_{max} 262 (ε 11,200) at pH 12.0, λ_{max} 253 (ε 13,000) at pH 7.0, and λ_{max} 256 (ε 12,600) at pH 2.0.

Anal. Calcd. for $C_{10}H_{12}N_5O_6P \cdot C_6H_{15}N$: P, 7.21. Found: P, 7.10.

In experiments where 4-morpholine-N,N′-dicyclohexylcarboxamidinium deoxyguanosine-5′ phosphate, suspended in pyridine, was treated with dicyclohexylcarbodiimide, yields of deoxyguanosine-3′,5′ cyclic phosphate were 10–15%.

Acid Hydrolysis of Deoxynucleoside-3′,5′ Cyclic Phosphates.—Aliquots (10–20 μl.) of a solution of nucleoside-3′,5′ cyclic phosphate (10 μmoles) in cold (0°) *M* hydrochloric acid (0.5 ml.) were sealed into capillary tubes. The tubes were heated at 50° in a water bath; at suitable intervals tubes were removed from the water bath, and the contents were examined chromatographically in Solvent 1 after the reaction had been stopped by exposure to ammonia fumes. The same procedure was employed in studying the acid hydrolysis of deoxynucleoside-5′ phosphates. Hydrolyses, at 100°, were carried out in a boiling water bath.

There was no detectable change in deoxyadenosine-3′,5′ cyclic phosphate after 2 hr. at 50°. Under identical conditions deoxyadenosine-5′ phosphate was converted completely to adenine in less than 5 min. At 100°, the half-life of deoxyadenosine-3′,5′ cyclic phosphate in *M* hydrochloric acid was 3 min., conversion to adenine being complete in 30 min. No intermediates were detectable at any stage of the hydrolysis, unlike the acid hydrolysis of adenosine-3′,5′ cyclic phosphate.[4]

Deoxyguanosine-3′,5′ cyclic phosphate was unchanged after 2 hr. at 50°, a temperature at which deoxyguanosine-5′ phosphate was converted completely to guanine in less than 5 min.

Deoxycytidine-3′,5′ cyclic phosphate and deoxycytidine-5′ phosphate were both unchanged after 2 hr. at 50°. At 100°, the half-life for the conversion (no detectable intermediates) of the cyclic nucleotide to cytosine was 22 min. The half-life for the 5′-phosphate was approximately 110 min.

Thymidine-3′,5′ cyclic phosphate was converted to thymine (with no detectable intermediates) with a half-life of 13 min., reaction being complete in approximately 2 hr. Thymidine-5′ phosphate was completely unchanged under identical conditions.

Adenosine-3′,5′ Cyclic Phosphate Diesterase.—Frozen beef brain (100 g.) was homogenized in a Serval Omnimixer with 400 ml. of acetone at −15°, and the suspension was centrifuged at 6000*g* for 10 min. at −15°. The precipitate was homogenized with an additional 400 ml. of acetone at −15° and centrifuged as before. This process was repeated once, and the final residue was dried under vacuum. The resulting dry powder was extracted twice with four volumes of 0.01 *M* tris(hydroxymethyl)aminomethane hydrochloride (tris·HCl), pH 7.5, by homogenization in a Serval Omnimixer at 0° followed by centrifugation at 80,000·*g*. To the combined supernatant solutions, saturated ammonium sulfate (pH 7.4) was added to bring the solution to 30% saturation. After 20 min. stirring at 0°, precipitated material was removed by centrifugation. The supernatant solution was brought to 40% saturation by addition of saturated ammonium sulfate. The mixture was centrifuged and the precipitate was dissolved in a small volume of 0.01 *M* tris·HCl, pH 7.0, containing 10^{-3} *M* magnesium acetate. This solution was dialyzed against 2 l. of the same buffer.

Rates of Hydrolysis of Deoxynucleoside-3′,5′ Cyclic Phosphates by Beef Brain Diesterase.—Each nucleotide, as its triethylammonium salt (1.5 μmoles), was added to tubes containing tris·HCl, pH 7.5 (50 μmoles), magnesium acetate (0.2 μmole), and water, the total volume being 0.2 ml. Brain extract (see Table I) was added and the tubes were incubated at 30°. Tubes containing the nucleotide and buffer, but no enzyme, served as controls. At appropriate times, the reaction was stopped by addition of glacial acetic acid (20 μl.), and the tubes were chilled in ice. An aliquot (25 μl.) was subjected to electrophoresis at pH 7.5 for 90 min. Nucleotides were detected by viewing under ultraviolet light, and the spots were cut out and eluted with water to a final volume of 1.5 ml. The absorbancy of the eluates were determined at the appropriate wave length for each nucleotide using cuvettes with 0.5 cm. light path. Under these conditions, the rate of hydrolysis of the purine nucleoside-3′,5′ cyclic phosphates was proportional to protein concentration provided hydrolysis did not exceed 60%. This was ascertained from separate incubations in which 100, 200, and 400 μg. of protein were used. The reaction rates are recorded in Table I.

Products of Diesterase Hydrolysis.—In the case of the purine deoxynucleoside-3′,5′ cyclic phosphates, the sole product of hydrolysis was a nucleotide with the electrophoretic mobility of appropriate deoxynucleoside-5′ phosphate. That the product of hydrolysis was the 5′-phosphate rather than the 3′-isomer in the hydrolysis of deoxyadenosine-3′,5′ cyclic phosphate was confirmed by incubation of the eluate from electrophoresis with highly purified adenosine-5′ phosphate deaminase,[12,27] the hydrolysate being converted completely to a nucleotide with the ultraviolet characteristics of deoxyinosine. The products of hydrolysis of deoxyinosine-3′,5′ and deoxyguanosine-3′,5′ cyclic phosphates are assigned nucleoside-5′ phosphate structure by analogy.[12]

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(37) As in the case of deoxycytidine-3′,5′ cyclic phosphate the over-all yields are lower than in the synthesis of thymidine-3′,5′ cyclic phosphate, presumably because of the additional handling required by the protected derivatives (*cf.* ref. 4).

When the purine nucleoside-3′,5′ cyclic phosphates were incubated for protracted periods or with excessive amounts of protein, traces of ultraviolet adsorbing products appeared with no electrophoretic mobility at pH 7.5. Presumably, these are nucleosides (or bases) resulting from the actions of contaminating traces of phosphatase (or other degradative enzymes). Analogous products were obtained in the experiments with pyrimidine deoxynucleoside-3′,5′ cyclic phosphates, which required large concentrations of enzyme and prolonged reaction before any degradation was evident.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN]

Studies on Polynucleotides. XXX.[1] A Comparative Study of Reagents for the Synthesis of the $C_{3'}$–$C_{5'}$ Internucleotidic Linkage[2]

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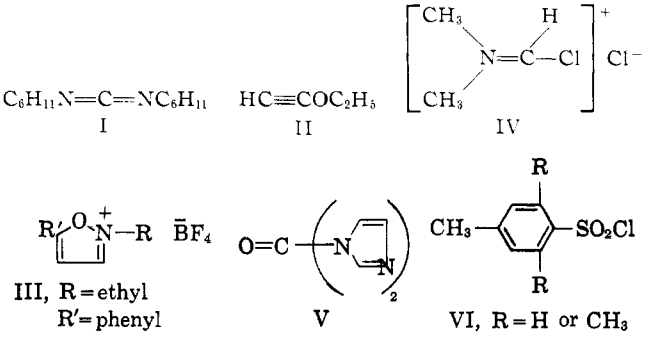
The formation of thymidylyl(3′→5′)thymidine by the condensation of (a) pyridinium 3′-O-acetylthymidine-5′ phosphate with 5′-O-tritylthymidine and (b) pyridinium 5′-O-acetylthymidine-3′ phosphate with 3′-O-acetylthymidine has been studied under identical conditions using the following reagents: dicyclohexylcarbodiimide, ethoxyacetylene, N-ethyl-5-phenylisoxazolium fluoroborate, the reagent prepared by the reaction of phosgene with dimethylformamide, ethyl metaphosphate, *p*-toluenesulfonyl chloride, and mesitylenesulfonyl chloride. Dicyclohexylcarbodiimide and the aromatic sulfonyl chlorides gave the highest (90% or better, using stoichiometric amounts of the protected nucleotide and the nucleoside) yields of the desired product. The rate of internucleotide bond synthesis using the aromatic sulfonyl chlorides was much higher than that obtained with dicyclohexylcarbodiimide. The mechanism of internucleotide bond synthesis using the aromatic sulfonyl chlorides is discussed in relation to the previous findings with dicyclohexylcarbodiimide.

A comparative study of several reagents for the purpose of polymerization of mononucleotides recently was reported.[3] Dicyclohexylcarbodiimide (I, DCC) gives the best results,[3] and this reagent has, in fact, been used in essentially all of the synthetic work in the polynucleotide field reported from this laboratory.[1,4,5] However, during the past few years a number of new reagents have been proposed for the activation of carboxylic and phosphoric acid groups. The reagents proposed include ethoxyacetylene[6] (II), substituted



isoxazolium salts[7] (III), the product (IV) from the reaction of phosgene with dimethylformamide,[8] ethyl metaphosphoric acid prepared by the reaction of ether with phosphorus pentoxide,[9] trichloroacetonitrile,[10] and carbonylbis(imidazole)[11] (V). The present paper reports on a comparative study of several of the reagents[12] for the synthesis of the $C_{3'}$–$C_{5'}$ internucleotidic linkage using relatively simple nucleotide and nucleoside derivatives. The results show that DCC and the aromatic sulfonyl chlorides[3,13] (VI) are the most efficient reagents. A characteristic feature of the latter class of reagents, however, is the very much higher rate of reaction than that obtained using DCC. A brief report of part of these results has been made previously.[14]

Dicyclohexylcarbodiimide.—The formation of thymidylyl(3′→5′)thymidine (VII) from (a) pyridinium 3′-O-acetylthymidine-5′ phosphate (VIII) and 5′-O-tritylthymidine (IX, System A), and from (b) 5′-O-acetylthymidine-3′ phosphate (X) and 3′-O-acetylthymidine (System B), using 0.1 *M* solutions in dry pyridine has been studied. Both of these condensations have been given close study in this laboratory using DCC as the reagent.[5,15,16] Two new observations were made using the latter reagent in the present work. When the duration of the aqueous pyridine treatment was reduced from 16 hr.[15] to about 1 hr., the presence of labile intermediates was noted. These

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(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, the National Science Foundation, Washington, D. C., and The Life Insurance Medical Research Fund, New York, N. Y.

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(12) An experiment using trichloroacetonitrile, carried out under conditions identical with those used in the present work, was reported previously [G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 419 (1962), and footnote 14]. Up to a period of 64 hr. at room temp., none of the desired product, thymidylyl(3′→5′)thymidine, was formed. The use of the reagent carbonylbis(imidazole) in internucleotide bond synthesis was tested recently under identical conditions in this laboratory. None of the desired thymidylyl(3′→5′)thymidine was formed (H. Schaller and H. G. Khorana, unpublished work).

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