Syntheses and Properties of Adenine and Thymine Nucleoside Alkyl Phosphotriesters, the Neutral Analogs of Dinucleoside Monophosphates^{1a-d}

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Abstract: The methyl and ethyl phosphotriester derivatives of TpT and dApdA were synthesized and were obtained as a pair of diastereoisomers in each case. As shown by pmr, CD, and uv hypochromicity measurements, the conformations of the triesters in solution are quite similar to those of the parent diesters, although there is less base stacking in the triesters. The phosphate-alkyl groups serve as monitors for the interaction between the backbone and the bases. The pmr resonances of these groups provide valuable information about the dynamics of the dimer conformations. Thermal perturbation or denaturation by DMSO causes a loss of the stacked conformation and an overall rotation of the base planes about the C-O-P bonds of the pentose-phosphate backbone. The triesters of dApdA form complexes with poly(uridylic acid) in 0.01 M Mg²⁺ with a stoichiometry of 2U:1A. The parent diester, dApdA, forms a complex with identical stoichiometry and the secondary structures of all three complexes appear to be the same. The thermal stabilities of the 2 poly(U)-triester complexes are greater than that of the 2 poly(U)-dApdA complex due to decreased repulsion between the negatively charged phosphates of the poly-(U) and the neutral phosphotriester backbone. The phosphotriesters are stable in neutral aqueous solutions and are resistant to hydrolysis by snake venom and spleen phosphodiesterase, and by micrococcal nuclease.

Several laboratories have studied the conformations of dinucleoside monophosphates in solution using the methods of pmr^{2,3} and ORD and CD spectroscopy.4-7 The importance of intramolecular basebase interactions as a determinant of the overall conformation of nucleic acid⁸⁻¹⁰ and of dinucleotides^{2a} and dinucleotide analogs¹¹ in solution has been previously emphasized.

The contribution of the sugar-phosphate backbone to nucleic acid conformation has received less attention. To further understand the influence of the sugar-phosphate backbone on dinucleotide conformation, Ts'o and coworkers have investigated and compared the conformations of ribose dinucleoside monophosphates with 3'-5', 2'-5', and 5'-5' ribose-phosphate internucleotide linkages,¹² and adenine dimers containing

(2) (a) P. O. P. Ts'o, N. S. Kondo, M. P. Schweizer, and D. P. Hollis, Biochemistry, 8, 997 (1969); (b) B. W. Bangerter and S. I. Chan, J. Amer. Chem. Soc., 91, 3910 (1969).

(3) S. I. Chan and J. H. Nelson, ibid., 91, 168 (1969).

(4) J. Brahms, J. C. Maurizot, and A. M. Michelson, J. Mol. Biol., 25, 481 (1967).

(5) C. A. Bush and I. Tinoco, *ibid.*, 23, 601 (1967).
(6) M. M. Warshaw and I. Tinoco, *ibid.*, 20, 29 (1966).

(7) C. R. Cantor, M. M. Warshaw, and H. Shapiro, Biopolymers, 9,

1059 (1970).

(8) A. M. Michelson "The Chemistry of Nucleosides and Nucleotides," Academic Press, New York, N. Y., 1963, Chapter 8.
(9) G. Felsenfeld and H. T. Miles, Annu. Rev. Biochem., 36, 407

(1967)

(10) P. O. P. Ts'o in "Molecular Associations in Biology," B. Pullman, Ed., Academic Press, New York, N. Y., 1968, p 39.
(11) D. T. Browne, J. Eisinger, and N. J. Leonard, J. Amer. Chem.

Soc., 90, 7302 (1968).

L-ribose.¹³ The methods of uv hypochromicity measurement, CD, and pmr spectroscopy were used concomitantly in these studies. The interaction of these "unnatural" dimers with homopolynucleotides was also investigated.

Tsuboi, et al.,¹⁴ studied the phosphate backbone of ApA by ³¹P nmr spectroscopy. Warshaw and Cantor¹⁵ have studied the differences in conformation between deoxyribo- and ribodinucleoside monophosphates by CD spectroscopic techniques. Our laboratory has been very interested in this subject¹⁶ and our results on the conformational differences between the ribosyl and deoxyribosyl dimers of adenine will be reported in a subsequent paper in this series.¹⁷ The conformation of the pentosyl ring in the ribosyl dimer (rAprA) and that in the deoxyribosyl dimer (dApdA) was reported and discussed in the preceding paper of this series.¹⁸ In addition, several workers have calculated the allowed conformations of the sugar-phosphate backbone using data from X-ray crystallographic studies of nucleic acids. 19-21

To further study the contribution of the sugar-phosphate backbone to dinucleotide conformation, we have prepared methyl and ethyl phosphotriester derivatives

(12) N. S. Kondo, H. M. Holmes, L. M. Stempel, and P. O. P. Ts'o, Biochemistry, 9, 3479 (1970).

(13) I. Tazawa, S. Tazawa, L. M. Stempel, and P. O. P. Ts'o, ibid., 9, 3499 (1970).

(14) M. Tsuboi, S. Takahashi, Y. Kyogoku, H. Hayatsu, T. Ukita, and M. Kainosho, Science, 166, 1504 (1969).

(15) M. M. Warshaw and C. R. Cantor, Biopolymers, 9, 1079, (1970). (16) P. O. P. Ts'o, S. A. Rapaport, and F. J. Bollum, Biochemistry, 5, 4153 (1966).

(17) N. S. Kondo, K. N. Fang, P. S. Miller, and P. O. P. Ts'o, 160th National Meeting of the American Chemical Society, Chicago, Ill., 1970, Abstract BIOL 15.
(18) K. N. Fang, N. S. Kondo, P. S. Miller, and P. O. P. Ts'o, J. Amer.

(19) M. Sundaralingam, Biopolymers, 7, 821 (1969).
(20) A. V. Lakshiminarayanam and V. Sasisekharan, *ibid.*, 8, 475

(1969).

(21) S. Arnott and D. W. L. Hukins, Nature (London), 224, 886 (1969).

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Figure 1. The diastereomeric dinucleoside alkyl phosphotriesters.

of thymidylyl-(3'-5')-thymidine and of deoxyadenylyl-(3'-5')-deoxyadenosine. The phosphate-alkyl groups were used as pmr probes to investigate the sugar-phosphate backbone conformation. The influence of the phosphate-alkyl group on the conformation of the dimers was examined. The interaction of the neutral phosphotriesters of dApdA with poly(uridylic acid) allowed us to study the effect of the negative charge of the phosphate group on nucleic acid interactions. Furthermore, the stability of these analogs in the presence of various nuclease enzymes was investigated.

Results and Discussion

Syntheses of the Phosphotriesters. The syntheses of the methyl and ethyl phosphotriester derivatives of TpT and dApdA are outlined in Scheme I. The fully

Scheme I. Syntheses of Dinucleoside

Monophosphates and Their Alkyl Phosphotriester Derivatives



protected cyanoethyl dinucleoside phosphotriesters (1) were prepared by the method of Letsinger and coworkers.²² Brief treatment of 1 with concentrated ammonium hydroxide in pyridine quantitatively removed the β -cyanoethyl group²³ to give the protected diester 2. Prolonged hydrolysis of 2 with base followed

(22) R. L. Letsinger, K. K. Ogilvie, and P. S. Miller, J. Amer. Chem. Soc., 91, 3360 (1969).



Figure 2. Pmr spectra of dApdA and dAp(CH₃)dA (0.02 M, pD 7.0, D₂O at 100 MHz, 28°).

by acid hydrolysis and DEAE-cellulose column chromatography gave the dinucleoside monophosphate **3**.

Treatment of 2 with anhydrous methanol or ethanol in the presence of *p*-toluenesulfonyl chloride followed by silica gel column chromatography gave the fully protected methyl and ethyl phosphotriester derivatives 4. The 3'-O- β -benzoylpropionyl group of the thymine dimers²⁴ and the *N*-benzoyl groups of the adenine dimers²⁵ were removed by reaction with hydrazine hydrate under neutral conditions. The remaining trityl groups were hydrolyzed with 80% acetic acid and the resulting dinucleoside alkyl phosphotriesters 5 were isolated by silica gel column chromatography.

For pmr studies $dApdA^{17,18}$ and $dAp(CH_3)dA$ were prepared with a deuterium atom in the C-8 position of the 5' portion of the dimer. These deuterated dimers allowed unambiguous assignment of the C-8 proton resonances to be made.

The synthetic method yields each phosphotriester derivative as a pair of diastereoisomers (see Figure 1). The diastereoisomers were not separated physically, but could be observed and distinguished by pmr spectroscopy as reported in detail in the following section. All the physical measurements on the dinucleoside alkyl phosphotriesters reported in this paper were performed on the pair of diastereoisomers obtained by the above synthetic route.

Pmr Spectroscopy The chemical shifts of the methyl protons of the phosphate alkyl groups are presented in Table I. The base protons of dApdA and the base and methyl protons of dAp(CH₃)dA are illustrated in Figure 2. The pentose protons (H_1' , H_2' , H_2'' , etc.) of dApdA have been discussed in the preceding paper.¹⁸

(24) R. L. Letsinger, M. H. Caruthers, P. S. Miller, and K. K. Ogilvie, *ibid.*, 89, 7146 (1967).

(25) R. L. Letsinger, P. S. Miller, and G. W. Grams, Tetrahedron Lett., 2621 (1968).

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⁽²³⁾ R. L. Letsinger and K. K. Ogilvie, ibid., 91, 3350 (1969).



Figure 3. The methyl proton resonances of $dAp(C_2H_{\delta})dA$ (0.02 M, pD 7.0, D₂O at 220 MHz) at various temperatures.

The methyl proton resonances of the methyl phosphotriesters occur as two sets of doublets, one set for each diastereoisomer, with a coupling constant J_{P-H} of 11 Hz.²⁶ The ratio of line intensities (4:6) indicates that the phosphate methyl groups of the diastereomeric $dAp(CH_3)dA$'s must be in quite different magnetic environments, since their chemical shifts are not the same. This may result in part from a difference in the

		Methyl ester			Ethyl ester		
	Temp, °C	δ_{I}	δ_2	$\Delta(\delta_1 - \delta_2)$	δ_1	δ_2	$\Delta(\delta_1 - \delta_2)$
$TpT dApdA \Delta(T - A)$	10	4.237 2.252 -0.015	4.237 4.216 0.021	0 0.036	1.742 1.761 -0.019	1.742 1.730 0.012	~0 0.031
$TpT dApdA \Delta(T - A)$	28	4.316 4.300 0.016	4.305 4.275 0.030	0.01 0.025	1.798 1.793 0.005	1.798 1.772 0.026	~0 0.021
$TpT dApdA \Delta(T - A)$	63	4.402 4.362 0.040	4.393 4.345 0.048	0.009 0.017	1.875 1.815 0.060	1.875 1.815 0.060	~ 0 ~ 0
$TpT dApdA \Delta(T - A)$	28 ^b	4.084 4.045 0.039	4.084 4.031 0.053	0 0.014	1.678 1.563 0.115	1.678 1.563 0.115	~ 0 ~ 0

Table I. Chemical Shifts of the Methyl Protons in the Alkyl Phosphotriesters of dTpdT and dApdA (TMS Capillary)^a

^a Measured at 100 MHz, pD 7.0, in D₂O, and infinite dilution. ^b Measured at 100 MHz in DMSO-d₅ and concentration was 0.04 M.

one of the diastereoisomers predominates in the mixture. The difference in chemical shifts, $\Delta(\delta_1 - \delta_2)$, of the diastereomeric phosphate methyl groups of Tp(CH₃)T are quite small, indicating that these groups are in very similar magnetic environments.²⁷ On the other hand,

(27) One would expect to observe an intrinsic difference in the chemical shifts of the methyl protons since they are anisochronis. See K.

degree of shielding from the adjacent adenine rings experienced by the two methyl groups. Upon thermal perturbation, $\Delta(\delta_1 - \delta_2)$ decreases by 1.9 Hz (measured at 100 MHz) as the temperature is increased from 10° to 63°. At 28° $\Delta(\delta_1 - \delta_2)$ in DMSO- d_6 , a solvent which

Mislow and M. Raban in "Topics in Stereochemistry," N.L. Allinger and E. L. Eliel, Ed., Vol. 1, New York, N. Y., 1967, Interscience, p 1. This intrinsic chemical shift difference of the methyl moieties of the phosphate groups of $Tp(C_2H_5)T$ was too small to be detected by present instrumentation.

⁽²⁶⁾ A similar coupling constant is observed for $P(OCH_3)_3$, $J_{P-H} = 10.8$ Hz. See J. Feeney in "Nuclear Magnetic Resonance for Organic Chemists," D. W. Mathieson, Ed., Academic Press, New York, N. Y., 1967, Chapter 9.

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Figure 4. The chemical shift positions of the base protons of dApdA and its methyl and ethyl phosphotriester derivatives at infinite dilution. Measured from an external tetramethylsilane capillary. The line intensities are drawn to scale in each case.

disrupts intramolecular base stacking,^{2, 28, 29} is 1.4 Hz, while in D_2O the observed difference is 2.5 Hz. Thus thermal perturbation or reduction of base stacking by DMSO causes the methyl protons of $dAp(CH_3)dA$ to become similar in magnetic environment.

The methyl-proton resonances of the phosphate ethyl groups of $Tp(C_2H_5)T$ occur as a triplet, $J_{H-H} =$ 6.8 Hz, which is further split by long-range coupling with the phosphorus atom, $J_{P-H} = 1.0$ Hz.³⁰ One would expect to see two such sets of resonances from the diastereomeric alkyl groups, but these were not observed.²⁷ The diastereomeric methyl groups of $dAp(C_2H_5)dA$ were clearly distinguishable, as shown in Figure 3. At 10°, the methyl proton resonances occur as a pseudoquartet which separates to two sets of triplets at 35° and finally to one triplet at 55°.

The chemical shifts of the phosphate methyl groups of $Tp(CH_3)T$ and $dAp(CH_3)dA$ increase as the temperature of the solvent increases when measured by an external standard technique (TMS capillary). This results from a change in the bulk susceptibility of the solvent.³¹ However, the increase in the chemical shifts of the methyl groups of $dAp(CH_3)dA$ is less than that observed for the methyl groups of Tp- $(CH_3)T$. This is shown by the increase in the chemical shift differences, $\Delta(T - A)$, for these two compounds upon increasing temperature. Thus the methyl groups of $dAp(CH_3)dA$ are *shielded* relative to the phosphate methyl groups of $Tp(CH_3)T$ as the temperature of the dimers is raised.³² The same shielding of the methyl groups in dAp(CH₃)dA is observed as well in DMSO (Table I).

The situation for the methyl protons in the ethyl triesters is even more striking. The difference between the methyl protons of $Tp(C_2H_5)T$ and $dAp(C_2H_5)dA$ at 10° and at 28° is small and barely significant. At 63°

(28) G. K. Helmkamp and P. O. P. Ts'o, J. Amer. Chem. Soc., 83, 138 (1961).

(29) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *ibid.*, **86**, 4182 (1964).

the difference, $\Delta(T - A)$ in Table I, becomes substantial; and in DMSO the difference is quite large (0.115 ppm). This difference again reflects the shielding effect of the ring current magnetic anisotropy of the adenine bases on the methyl protons of the phosphate ethyl group.

In Table I, Figure 3, and the above discussion, two important phenomena are noted for the phosphate alkyl groups, which serve as monitors for the interaction between the backbone and the bases in the dimer. The first is the difference in the $\Delta(\delta_1 - \delta_2)$ values of the diastereomeric phosphate alkyl groups. This difference in shielding vanishes at high temperature or in a destacking solvent such as DMSO. The second phenomenon is the increased shielding $\Delta(T - A)$ experienced by the phosphate alkyl group from the adenine ring upon elevation of temperature or solution in DMSO.

The simplest explanation for these phenomena, which is also completely consistent with the molecular model. is that there is a continuous, full rotation (360°) of the adenine base planes relative to each other in the dimer. At low temperature and in aqueous solution the rotation rate is slow and the base planes tend to spend a long time in a stacked conformation with the alkyl groups located outside the stack. Therefore shielding of the phosphate alkyl group by the adenine bases is very small as indicated by the low value of $\Delta(T - A)$. Under this semistationary condition, the alkyl group of one diastereoisomer is closer to the adenine base planes than is the alkyl group of the other isomer as shown by the value of $\Delta(\delta_1 - \delta_2)$. As the temperature is elevated, the rotation rate of the base planes increases. During this rotational process, the alkyl groups pass over the adenine planes and experience the diamagnetic effect of the ring current of the bases. Therefore, at elevated temperature the alkyl groups are relatively more shielded than at low temperature as reflected by the increase in the value of $\Delta(T - A)$. In addition, the magnetic environments experienced by the diastereomeric alkyl groups become more equivalent during this rotational process, as shown by the diminution of the $\Delta(\delta_1 - \delta_2)$ values. The same situation arises when the dimer is transferred from an aqueous environment to a destacking solvent (DMSO). Rotation in the dimer occurs in the organic solvent and the same effects ensue under this condition.

It should be noted that in aqueous solution, the temperature has to reach 55° before the rotation rate of the base planes of $dAp(CH_2CH_3)dA$ becomes sufficiently fast, so that the difference in the shielding of the methyl group of the two diastereoisomers can no longer be detectable (Figure 3). In the DMSO solution, the temperature required for such a rotational rate is below 28° (Table I). In other words, at 28° $dAp(CH_2CH_3)$ dA rotates at a faster rate in DMSO than in D_2O . This result suggests that a considerable barrier to the rotation arises from nonbonded, intramolecular base-base, hydrophobic stacking interaction in aqueous solution. Also these results confirm our previous conclusion¹² that the physicochemical properties of the dinucleoside monophosphates should not be described by a two-state model, but can be more appropriately described by a "rotating-dimer" model. This "rotating-dimer" model is similar (but not identical) to the "oscillatingdimer" model previously proposed by Tinoco and coworkers.33

⁽³⁰⁾ For examples of long range P-H coupling see ref 27, Chapter 5, Table 5.3.

⁽³¹⁾ J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill, New York, N. Y., 1959.

⁽³²⁾ We assume that the change in bulk susceptibility of the solvent, D_2O , affects the chemical shifts of the phosphate-methyl groups of $TP(CH_3)T$ and $dAp(CH_3)dA$ in the same manner.

The base-proton resonances of dApdA and its methyl and ethyl triester derivatives are illustrated schematically in Figure 4. For dApdA four resonances are observed corresponding to the four adenine base protons. The assignments were made in the preceding paper.¹⁸

One would expect to observe a total of eight lines in the spectra of the base protons of the diastereomeric alkyl triester derivatives, i.e., four lines from each diastereoisomer. A total of six lines are observed for $dAp(CH_3)dA$ while $dAp(C_2H_5)dA$ shows the theoretical eight lines. Thus the two diastereomeric C-8 protons (dpA portion) of $dAp(CH_3)dA$ occur at the same resonance (8.78 ppm), while one of the diastereomeric C-2 protons (pdA portion) and one of the diastereomeric C-8 protons (dAp portion) also occur at the same resonance position (8.58 ppm). This conclusion is supported by the distribution of the intensities of the proton signals shown in Figure 4.

The chemical shift positions of the base protons of the triesters indicate that the phosphate alkyl groups perturb the base stacking of the dimer. Thus all the proton resonances except the C-8 protons on the pdA portion of the dimers are located at a lower field than the resonances of the corresponding protons of the parent dimer, dApdA. In the triesters the deshielding effect of the phosphate group is canceled due to loss of the negative charge. Thus the C-8 proton of the dpA portion of the dimer occurs at higher field than the corresponding proton in the negatively charged diester. This observation is additional evidence that the pdA portion of dApdA is in the anti conformation.¹⁸ The reduced shielding experienced by the other base protons in $dAp(CH_3)dA$ and $dAp(C_2H_5)dA$ as compared to dApdA(Figure 4) indicates that the phosphate alkyl group has lessened the intramolecular stacking interaction between the adenine bases in the dimer.

Uv Hypochromicity and CD spectra The uv spectra of the alkyl phosphotriesters and their parent diesters are qualitatively identical. The thymine dimers show maxima at 267 nm and minima at 235 nm while the adenine dimers show maxima at 259 nm and minima at 225 nm. The extinction coefficients of each of the diesters and triesters are tabulated in Table II, together

Table II. Extinction Coefficients of Deoxyribose Dimers

Compound	$\epsilon_{\rm max} imes 10^{-3}$	% hypochromicity ^a
pT	9.6	
ΤαΤ	9.2	4.2
Tp(CH ₃)T	9.1	5.2
$Tp(C_2H_5)T$	9.0	6.3
pdA	15.3	
dApdA	12.7	17
dAp(CH ₃)dA	14.3	6.5
$dAp(C_2H_5)dA$	13,6	11

^a Relative to the monomer, at 28° in water, pH 7.

with the per cent hypochromicity relative to the corresponding monomer.

The thymine dimers each have very similar extinction coefficients. The extinction coefficients of the adenine dimers are quite different, however. Thus dApdA is



1.5

1.0

0.0 (B) × 10.4

Figure 5. Circular dichroic spectra of deoxyadenosine (---), dApdA (O-O), $dAp(CH_3)dA$ (\bullet - \bullet), and $dAp(C_2H_5)dA$ (\Box - \Box) in 0.01 M Tris (pH 7.5)-0.01 M MgCl₂ at 1.5°. Nucleotide concentration, $1 \times 10^{-4} M$.

mμ

17% hypochromic relative to the monomer, pdA, while its methyl and ethyl phosphotriester derivatives are 6.5 and 11% hypochromic. The decreased hypochromicity of the triesters is a result of decreased intramolecular base-base interaction in these dimers.³⁴ Thus the methyl and ethyl triesters of dApdA are less stacked than the parent diester, a conclusion previously reached from the pmr data.

The CD spectra of dApdA and its triester derivatives are shown in Figure 5. The maximum and minimum spectral positions of the adenine dimer and its methyl and ethyl phosphotriesters are identical. However, the magnitude of the molecular elipticities of the triesters at 250 nm and 270 nm are approximately 50%less than that observed for dApdA. The decrease in rotational strength of the triesters is consistent with decreased interaction between the electric dipoles of the adjacent adenine bases, resulting from less base-base overlap in these dimers.34

The effect of temperature on the CD spectra of the adenine dimers is shown in Figure 6. The magnitudes of the molecular elipticities at 270 nm and at 250 nm of the three dimers decrease linearly as the temperature increases. The decreased rotational strengths observed at high temperature result from decreased stacking in the dimers. The differences between the slopes of the lines in Figure 6 are small (the triester derivatives appear to give a smaller slope). Thus, the effect of thermal perturbation upon stacking appears to be similar in this temperature range for the diester and triester derivatives. These observations support the conclusion based on the pmr data that thermal perturbation leads to disruption of intramolecular base stacking and to overall rotation in the dimer.

(34) See ref 12 for a discussion of the relation of base-base interaction to uv hypochromism and circular dichroism.

⁽³³⁾ R. C. Davis and I. Tinoco, Biopolymers, 6, 223 (1968); D. Glaubiger, D. A. Lloyd, and I. Tinoco, ibid., 6, 409 (1968).



Figure 6. Temperature dependence of $[\theta]$, molecular ellipticity, at 250 nm and 270 nm of dApdA (O–O), dAp(CH₃)dA (\bullet – \bullet), and dAp(C₂H₃)dA (\bullet – \bullet) in 0.01 *M* Tris (pH 7.5)–0.01 *M* MgCl₂. Nucleotide concentration 1 × 10⁻⁴ *M*.



Figure 7. Mixing curves for poly(U) with dApdA at 260 nm $(\bigcirc -\bigcirc)$, at 275 nm $(\bigcirc -\bigcirc)$, and at 280.2 nm $(\bigcirc -\bigcirc)$ in 0.01 *M* Tris (pH 7.5)-0.01 *M* MgCl₂ at -1.0° . The total nucleotide concentration was $1 \times 10^{-4} M$.

Hydrolysis of Nucleases. Both TpT and dApdA are readily hydrolyzed to their monomeric units by snake venom and by spleen phosphodiesterase. The methyl and ethyl triester derivatives were found to be resistant to hydrolysis by these enzymes under conditions in which the parent diesters, TpT and dApdA, were com-



Figure 8. Mixing curves for poly(U) with dAp(CH₃)dA at 258 nm $(\Box - \Box)$ and 270 nm $(\blacksquare - \blacksquare)$ at 0.0°; poly(U) with dAp(C₂H₃)dA at 260 nm (O-O), 275 nm $(\bullet - \bullet)$, 281.1 nm $(\bullet - \bullet)$ at -1.5°. Each experiment was carried out in 0.01 *M* Tris (pH 7.5)-0.01 *M* MgCl₂ at a total nucleotide concentration of $1 \times 10^{-4} M$.

pletely cleaved. Furthermore, $Tp(C_2H_5)T$ and dAp-(C₂H₅)dA were found to be resistant to hydrolysis by micrococcal nuclease.³⁵ Thus it would appear that the negatively charged phosphate in the dimer is an important recognition site for these nuclease enzymes.

Interaction with Poly(U). Interaction of the dinucleoside alkyl phosphotriesters with their complementary polynucleotides is of considerable physicochemical and biological interest. Neutralization of the negatively charged phosphate should increase the stability of the triester-polymer complex by decreasing electrostatic repulsion. On the other hand, the phosphate alkyl substituent may sterically hinder the formation of the complex.

As a model system, the interaction of poly(uridylic acid) with dApdA and with the alkyl phosphotriesters was studied. Poly(U) and dApdA form a complex in 0.01 M Mg²⁺ with a stoichiometry of 2U:1A (see Figure 7). Likewise the phosphotriesters, dAp(CH₃)dA and dAp(C₂H₅)dA, form complexes with a 2U:1A stoichiometry, as illustrated by the mixing curves in Figure 8.

The conformations of these complexes were investigated by CD spectroscopy. As shown in Figure 9, the 2U:1A complexes formed by poly(U) and the adenine dimers have identical CD spectra, within experimental error. Thus the overall conformation of each complex appears to be the same.

The thermal stabilities of the complexes are quite different as shown by the melting experiments illustrated in Figure 10. The 2 poly(U)-dApdA complex melts at 7.6° in 0.01 M Mg²⁺. Under the same conditions the 2 poly(U) dAp(C₂H₃)dA complex melts at 12.0°,

(35) Dr. Eugene Sulkowski, private communication.



Figure 9. Circular dichroic spectra of the 2 poly(U)-dApdA complex (\bigcirc - \bigcirc), the 2 poly-(U)-dAp(CH₃)dA complex (\bigcirc - \bigcirc), and the 2 poly(U)-dAp(C₂H₃)dA complex (\Box - \Box) in 0.01 *M* Tris (pH 7.5)-0.01 *M* MgCl₂ at 1.5° The total nucleotide concentration was 1.5 × 10⁻⁴ *M*.

while the 2 poly(U) $\cdot dAp(CH_3)dA$ complex melts at 13.2°.

It should be noted that these experiments were conducted in 0.01 M Mg²⁺. Under this condition all the binding sites of the phosphate groups are fully occupied by the Mg²⁺ ions.³⁶ Apparently under this condition, charge repulsion between the phosphate groups still takes place. The increase in the T_m of the 2 poly(U)– dAp(R)dA complex evidently reflects a decrease in electrostatic repulsion. It is interesting to note that the stability of the 2 poly(U)·dAp(C₂H₅)dA complex is slightly lower than that of the 2 poly(U)·dAp(CH₃)dA complex. This small difference may represent the slight increase in the steric hindrance by the ethyl group of the triester for complex formation.

The present findings encourage further studies on the oligonucleotide alkyl triesters. Such compounds are likely to form complexes of considerable stability with complementary polynucleotides. Thus these compounds may be useful as probes for the single-stranded regions of nucleic acids such as tRNA's. In addition, these compounds may be permeable to cellular membranes, thus enabling their entrance into living systems. The biological effects of these compounds may prove to be of considerable interest.

Experimental Section³⁷

Reagents for Nucleotide Syntheses; Polynucleotides and Enzymes. 2'-Deoxyadenosine and thymidine were purchased from Sigma Chemical Co., St. Louis, Mo., and were checked for purity by paper chromatography before use. The dipyridinium salt of β -cyanoethyl phosphate was prepared by passage of an aqueous solution of the barium salt through a Dowex 50-X cation-exchange resin in the pyridinium form, followed by lyophilization and storage of the resulting gum in dry pyridine. Anhydrous pyridine was



Figure 10. Melting profiles of 2 poly(U)-dApdA complex (O-O), 2 poly(U)-dAp(CH₃)dA complex (\bullet - \bullet), and 2 poly U·dAp-(C₂H₃)dA complex (\bullet - \bullet) at 258 nm in 0.01 *M* Tris (pH 7.5)-0.01 *M* MgCl₂. The total nucleotide concentration was 5 × 10⁻⁵ *M* in each case. The relative absorbance is the ratio of the absorbance at a given temperature to the absorbance plateau of the melted complex at high temperature.

prepared by distillation of commercial pyridine from *p*-toluenesulfonyl chloride onto solid potassium hydroxide, followed by distillation onto Linde Type 4A Molecular Sieves. Dry N,N'dimethylformamide was prepared by distillation of commercial material from phthalic anhydride onto Linde Type 4A Molecular Sieves.

N-Benzoyldeoxyadenosine and *N*-benzoyl-5'-*O*-di-*p*-methoxytrityldeoxyadenosine were synthesized by the procedure of Schaller, *et al.*³⁸ The β -cyanoethyl ester of 5'-*O*-mono-*p*-methoxytritylthymidylyl-(3'-5')-3'-*O*-(β -benzoylpropionyl)thymidine [MTrTp-(CE)T β B] was synthesized by the method of Letsinger, *et al.*²⁴

Poly(uridylic acid) was a product of Miles Laboratories, Elkhart, Ind., and was used without further purification. A maximum molar extinction coefficient of 9.2×10^3 was used for poly(U). Spleen phosphodiesterase and snake venom phosphodiesterase (EC 3.1.4.1) were purchased from Worthington Biochemical Corp., Freehold, N. J.

Chromatography and Electrophoresis. Descending paper chromatography was performed on Whatmann 3MM paper using the following solvent systems: solvent A, 2-propanol-concd ammonium hydroxide-water (7:1:2, v/v); solvent I, 2-propanol-water (7:3, v/v). Ascending thin-layer chromatography was carried out on Eastman silica gel sheets No. 6060. Paper electrophoresis was performed on a Savant flat plate apparatus using Whatman 3MM paper and 0.05 *M* triethylammonium bicarbonate (pH 7.8) for 30 min at a potential of 40 V/cm.

Ultraviolet Absorption Spectra, Circular Dichroic Spectra, and Proton Magnetic Resonance Spectra. Ultraviolet spectra were recorded on a Cary 15 spectrophotometer; circular dichroic spectra were recorded on a Cary 60 spectropolarimeter equipped with a 6001 CD attachment and a thermojacketed cell; most of the pmr spectra were recorded on a Varian HA-100 spectrometer equipped with a Varian C-1024 computer of average transients. Temperature dependence studies on the ethyl phosphotriesters were carried out on a Varian HR-220 spectrometer equipped with a variable temperature probe. Temperature readings were calibrated with ethylene glycol and methanol standards from Varian. The hypochromicity measurements, the mixing curve experiments,

⁽³⁶⁾ C. Sander and P. O. P. Ts'o, J. Mol. Biol., in press.

⁽³⁷⁾ Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Elemental analyses were performed by Micro-Tech, Inc., Skokie, Ill.

⁽³⁸⁾ H. Schaller, G. Weiman, B. Lerch, and H. G. Khorana, J. Amer. Chem. Soc., 85, 3821 (1963).

the melting temperature measurements, as well as the CD and pmr measurements were conducted as described previously.^{12,13}

Enzyme Digestion. Snake Venom Phosphodiesterase. Approximately 0.4 μ mole of the dinucleotide was incubated with 40 μ l of enzyme (5 mg of enzyme/ml of water) in 20 μ l of 1 *M* ammonium bicarbonate (pH 9.0) for 8 hr at 37°.

Spleen Phosphodiesterase. Approximately 0.4 μ mole of the dinucleotide was incubated with 20 μ l of enzyme (16 units of enzyme/ml of water) in 20 μ l of 0.25 *M* sodium succinate buffer (pH 6.5) for 8 hr at 37°.

Syntheses of Phosphotriesters. Methyl Ester of Thymidylyl-(3'-5')-thymidine [Tp(CH₃)T]. The β -cyanoethyl ester of 5'-Omono-*p*-methoxytritylthymidylyl-(3'-5')- 3'- O- $(\beta$ -benzoylpropionyl)thymidine [MTrTp(CE)T β B] (521 mg; 0.5 mmole) was treated with 12 ml of a 50% concentrated ammonium hydroxidepyridine solution for 30 min at 28°. The solvents were evaporated and the residue was dried by repeated evaporation with anhydrous pyridine (three 5-ml portions). The resulting gum was dissolved in a solution containing dry N,N-dimethylformamide (32 ml), dry methanol (16 ml), and dry 2,6-lutidine (13 ml). The solution was treated with p-toluenesulfonyl chloride (5.8 g) for 45 min at 28° and then with 6.5 ml of water for 15 min at 0° . The solvents were then evaporated with the aid of ethanol. The residue was dissolved in 150 ml of chloroform and the solution was washed with water (two 150-ml portions). After drying over anhydrous sodium sulfate, the chloroform solution was concentrated and applied to a silica gel column (2.5 \times 35 cm). The column was eluted with diethyl ether (250 ml) followed by tetrahydrofuran (400 ml). Fractions containing material with R_{f}^{EtOAc} 0.19 and $R_{\rm f}^{\rm THF}$ 0.60 (silica gel tlc) were collected, and the solvents were evaporated. The residue was precipitated from tetrahydrofuran by addition of hexane to yield 392 mg (78%) of MTrTp(CH₃)-T β B, mp 110-113°. Anal. Calcd for $C_{50}H_{51}O_{15}N_4P$ 2H₂O: C, 59.16; H, 5.46; N, 5.52. Found: C, 58.35; H, 4.61; N, 5.90.

The fully protected dinucleoside phosphotriester (370 mg, 0.37 mmole) was dissolved in a solution containing 2.3 ml of pyridine and 0.5 ml of glacial acetic acid. The solution was treated with 89 μ l of 85% hydrazine hydrate for 5 hr at 28°. After evaporation of solvents, the residue was treated with 5 ml of 80% aqueous acetic acid for 15 min at 100°. The acetic acid was evaporated, the residue was dissolved in a minimum volume of 50% methanol-chloroform, and the solution was applied to a silica gel column (2.5 imes 40 cm) which had previously been washed with 50% methanol-chloroform. The column was eluted with 500 ml of chloroform and with 250 ml of 50% methanol-chloroform. Material with $R_{\rm f}^{\rm MeOH-CHCl_3, 1:1}$ 0.64 (silica gel tlc) was collected, the solvents were evaporated, and the residue was precipitated from methanol by addition of diethyl ether to yield 176 mg (94%) of Tp(CH₃)T: mp 200° dec; $R_{PT_m} 0.0$ (paper electrophoresis), uv, λ_{max} 267 nm, λ_{min} 235 nm.

Samples for optical measurements were further purified as follows. A 15-mg portion of the above material was chromatographed on four sheets (15×45 cm) of Whatmann 3MM paper in solvent I. A trace of an unidentified, uv-absorbing compound, R_f^{I} 0.61, was observed along with Tp(CH₃)T, R_i^{I} 0.73. The latter material was eluted from the paper with water-2-propanol (3:7, v/v) and after concentration was applied to a DEAE-cellulose column (2×30 cm) in the bicarbonate form. The column was eluted with water. The triester was collected and lyophilized from water.

Ethyl Ester of Thymidylyl-(3'-5')-thymidine $Tp(C_2H_{\circ})T$. The ethyl ester, MTrTp(C_2H_{\circ})T βB , was prepared from MTrTp(CE)T βB (521 mg, 0.5 mmol) by the same procedure described for the methyl triester above, except anhydrous ethanol was used instead of anhydrous methanol. The resulting MTrTp(C_2H_{\circ})T βB weighed 301 mg, mp 102–105°. *Anal.* Calcd for $C_{51}H_{33}O_{15}N_4P \cdot 2H_2O$: C, 59.52; H, 5.58; N, 5.44. Found: C, 59.22; H, 4.05; N, 5.51.

The protecting groups were removed from the fully blocked triester (270 mg, 0.25 mmole) in the same manner described above for the methyl ester to yield 113 mg (57%) of $\text{Tp}(C_2H_3)\text{T}$, mp 146–166 dec, $R_m p^T$ 0.0 (paper electrophoresis). Samples for optical measurements were prepared as described for the methyl ester. The resulting $\text{Tp}(C_2H_3)\text{T}$, R_i^T 0.78 (paper chromatography), was lyophilized from water.

The β -Cyanoethyl Ester of *N*-Benzoyl-5'-*O*-di-*p*-methoxytrityldeoxyadenylyl-(3'-5')-*N*-benzoyl-3'-*O*'-di-*p*-methoxytrityldeoxyadenosine [DMTrdA^{Bz}p(CE)dA^{Bz}DMTr]. *N*-Benzoyl-5'-*O*-di-*p*methoxytrityldeoxyadenosine (6.73 g, 10 mmol) and dipyridinium β -cyanoethyl phosphate (20 mmol) were dried by repeated evaporation with pyridine (4 \times 10 ml) and the resulting gum was treated with p-toluenesulfonyl chloride (5.73 g, 30 mmol) in 20 ml of dry pyridine for 7 hr at room temperature. The reaction solution was treated with 20 ml of ice-water, stirred at room temperature overnight, and then diluted with saturated sodium chloride (40 ml). The aqueous solution was extracted with chloroform (three 40-ml portions). The combined chloroform extracts were evaporated. N-Benzoyldeoxyadenosine (5.33 g, 15 mmol) was added to the resulting gum and the reagents were dried by repeated evaporation with pyridine (four 10-ml portions). The gum was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (4.25 g, 14 mmol) in 20 ml of dry pyridine for 24 hr at room temperature. Water (200 ml) was then added, and the aqueous mixture was extracted with ethyl acetate (three 200-ml portions); the ethyl acetate solution was evaporated, and the residue was evaporated with several portions of ethanol. The resulting gum was dissolved in a minimum volume of chloroform and applied to a silica gel column $(4 \times 50 \text{ cm})$. The column was eluted with 1 l. of diethyl ether, 3 l. of ethyl acetate-tetrahydrofuran (1:1), and 2.5 l. of 5% methanol in tetrahydrofuran. Material with R_{f}^{THF} 0.16 (silica gel tlc) was collected.

To increase the mobility of the protected triester, the material obtained above was evaporated with dry pyridine (two 10-ml portions), and the resulting gum was treated with di-*p*-methoxy-trityl chloride (3.4 g, 10 mmol) in 20 ml of dry pyridine for 48 hr at room temperature. Ethanol was then added, and the solvents were evaporated. The residue was dissolved in a minimum volume of chloroform and applied to a silica gel column (4×60 cm). The column was eluted with ethyl acetate followed by ethyl acetate tetrahydrofuran (1:1, v/v). Material with $R_f^{\text{EtOAe-THF}, 1:1}$ 0.34 (silica gel tlc) was collected, the solvents were evaporated, and the residue was precipitated from tetrahydrofuran by addition of hexane to yield 2.45 g (17.5%) of DMTrdA^{B2}p(CE)dA^{B2}DMTr, mp 128-130°. Anal. Calcd for $C_{77}H_{74}O_{14}N_{11}P \cdot H_2O$: C, 64.83; H, 5.37; N, 10.80. Found: C, 64.48; H, 5.07; N, 10.84.

The protecting groups were removed from a 5-mg sample of the fully protected dinucleoside phosphotriester by treatment with 1 ml of 50% concentrated ammonium hydroxide-pyridine solution for 2.5 days at room temperature, followed by treatment with 1 ml of 80% aqueous acetic acid for 2 hr at 28° and 15 sec at 100°. The dinucleotide, dApdA, was purified by paper chromatography in solvent A, R_f^A 0.33. Samples of the dinucleotide (5-OD units) were subjected to enzyme digestion with snake venom phosphodiesterase and with spleen phosphodiesterase, followed by paper chromatography in solvent A. Snake venom gave two products: R_f^A 0.60 (dA) and R_f^A 0.07 (pdA). Spleen gave two products: R_f^A 0.39 (d1)³⁹ and R_f^A 0.05 (dAp).

Methyl Ester of Deoxyadenylyl-(3'-5')-deoxyadenosine [dAp-(CH3)dA]. The fully protected dinucleoside phosphotriester, DMTrdA^{B2}p(CE)dA^{B2}DMTr (1.15 g, 0.8 mmol), was treated with 20 ml of 50% concentrated ammonium hydroxide-pyridine solution for 30 min at room temperature. After evaporation of solvents, the residue was evaporated repeatedly with dry pyridine (three 10-ml portions) and then dissolved in a solution containing dry N,N-dimethylformamide (50 ml), dry methanol (25 ml), and dry 2,6-lutidine (20 ml). The solution was treated with 9 g of *p*-toluenesulfonyl chloride at 0° for 5 min and at room temperature for 45 min. The solution was then treated with ice-water for 15 min, after which the solvents were evaporated with the aid of ethanol, and the residue was dissolved in 250 ml of chloroform. The chloroform solution was washed with water (two 250-ml portions), dried over anhydrous sodium sulfate, concentrated, and applied to a silica gel column (2.5 \times 35 cm). The column was eluted with 1 l. of ethyl acetate and 500 ml of ethyl acetate-tetrahydrofuran (1:1). Material with $R_{f}^{EtOAc-THF, 1:1}$ 0.38 (silica gel tlc) was collected, the solvents were evaporated, and the residue was precipitated from tetrahydrofuran by addition of hexane to give 368 mg (33%) of DMTrdA^{B2}p(CH₃)dA^{B2}DMTr, mp 123-127° Anal. Calcd for $C_{75}H_{73}O_{14}N_{10}P \cdot H_2O$: C, 64.92; Н, 5.44; N, 10.09. Found: C, 64.74; H, 4.77; N, 10.94.

The fully protected dinucleotide (0.25 mmol) was dissolved in 3.3 ml of pyridine. After cooling to 0°, glacial acetic acid (0.79 ml), hydrazine hydrate (0.13 ml), and water (0.04 ml) were added, and the solution was stirred at room temperature for 24 hr. The solvents were evaporated and the residue was treated with 5 ml of 80% aqueous acetic acid for 4 hr at 28° and for 15 sec at 100° .

(39) K. K. Ogilvie and R. L. Letsinger, Biochem. Biophys. Res. Commun., 30, 237 (1968).

After cooling, the solvents were evaporated, the resulting gum was extracted with 50 ml of diethyl ether, and the residue was dissolved in a minimum volume of methanol. The solution was applied to a silica gel column (2 \times 16 cm) which had previously been washed with a 50% chloroform-methanol solution; and the column was eluted with chloroform and then with 50% chloroform-methanol. Material with $R_f^{CHCl_3-MeOH, 1:1}$ 0.45 (silica gel tlc) was collected, the solvents were evaporated, and the residue was precipitated from methanol by addition of diethyl ether to give 40 mg (22%) of dAp(CH₃)dA, mp 183° dec.

Samples for optical measurements were further purified by chromatography on a Sephadex G-10 column using water as eluent. The triester eluted immediately after the void volume and was lyophilized from water.

Ethyl Ester of Deoxyadenylyl-(3'-5')-deoxyadenosine [dAp- $(C_2H_5)dA$]. The fully protected dinucleotide DMTrdA^{B2}p-(CE)dA^{B2}DMTr (1.05 g, 0.78 mmol) was converted to the ethyl ester, DMTrdA^{Bz}p(C₂H₅)dA^{Bz}DMTr, by the procedure described above for the methyl ester, except dry ethanol was used instead of methanol. The resulting $DMTrdA^{Bz}p(C_2H_5)dA^{Bz}DMTr$ weighed 647 mg (63%), mp 126-130°. Anal. Calcd for $C_{76}H_{75}O_{14}N_{10}P$. 1H2O: C, 65.13; H, 5.53; N, 9.99. Found: C, 65.06; H, 5.12; N, 10.43.

The protecting groups were removed from the fully blocked triester (650 mg, 0.49 mmol) in the same manner described above for the methyl ester to yield 232 mg (86%) of dAp(C₂H₆)dA, $R_t^{MeOH-CHCl_2, 1:1}$ 0.51 (silica gel tlc), mp 146° dec. Samples for optical measurements were further purified as described for the methyl ester. The resulting triester was lyophilized from water.

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A Spin Label Investigation of the Active Site of an Enzyme. Bovine Carbonic Anhydrase¹

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Abstract: A spin-labeled sulfonamide inhibitor bound to the active site of the enzyme carbonic anhydrase has been studied by electron paramagnetic resonance. It is demonstrated that a 1:1 complex between the label and enzyme exists and that rapid anisotropic motion of the label about one of its bonds is taking place. The data are consistent with the aromatic nucleus of the inhibitor being held rather firmly to the hydrophobic portion of the active site cleft. The value of a_N , the isotropic coupling constant of the nitrogen atom of the paramagnetic nitroxide group, indicates that this portion of the sulfonamide is in a highly polar region.

arbonic anhydrase (carbonate hydrolyase, EC \checkmark 4.2.1.1), a metalloenzyme which catalyzes the reversible hydration of CO₂, has been extensively investigated since its discovery in 1932. It is of interest not only for the elucidation of its particular catalytic mechanism but also because it is felt that the mechanism involved may serve as a useful model for the study of enzyme action in general. It is a nearly spherical molecule consisting of a single polypeptide chain of approximately 260 amino acid residues.^{2a,b} In its active form the enzyme contains a zinc(II) atom found near the center of the molecule at the bottom of a crevice. Although the presence of the zinc atom (or cobalt) is necessary in order for the enzyme catalytic activity to be present, both the crevice and the zinc atom constitute the active site for the enzyme.³ It is thought that an OH- ion attached to the zinc attacks the CO₂ molecule which is loosely bound to a hydrophobic surface or cavity near the zinc.⁴ It is also felt that proton transfer plays a role in the mechanism although the proton transfer path is not known in detail.⁵ Pocker and coworkers⁶ have suggested facilitation by a strategically located basic imidazole group of the protein.

The enzyme is inactivated by complexation with aromatic sulfonamides.⁷ It is thought that the SO₂NH₂ group (probably as the SO₂NH⁻ anion)⁸ complexes with the zinc, thus competing with the OH- ion. X-Ray studies^{2a,9} indicate that a number of anionic inhibitors occupy the same site in the enzyme, namely, the cavity or crevice at the bottom of which is the zinc atom. Sulfonamide binding is stabilized by hydrophobic interaction involving the sulfonamide ring with the enzyme cleft.⁵ From consideration of the interactions between inhibitors and the enzyme it is concluded that the cleft in the region of the zinc provides a good fit for the inhibitor.^{2a,8}

The present study concerns a spin-label investigation of the active site of the bovine carbonic anhydrase (BCA) enzyme. The need for such "site specific"

⁽¹⁾ Supported in part by National Science Foundation Grant No. GP-8298 and the NIH Biomedical Support Grant (Duke University).

^{(2) (}a) K. Fridborg, K. Kannan, A. Liljas, J. Lundin, B. Strandberg,
R. Strandberg, B. Tilander, and G. Wiren, J. Mol. Biol., 25, 505 (1967);
(b) P. Nyman and S. Lindskog, Biochim. Biophys. Acta, 85, 141 (1964).

⁽³⁾ J. E. Coleman, Nature (London), 214, 193 (1967).
(4) M. E. Riepe and J. H. Wang, J. Amer. Chem. Soc., 89, 4229 (1967).

⁽⁵⁾ P. W. Taylor, R. W. King, and R. S. V. Burgen, Biochemistry, 9, 2638 (1970).

⁽⁶⁾ Y. Pocker and J. E. Meany, ibid., 4, 2535 (1965); Y. Pocker and J. T. Stone, ibid., 7, 4139 (1968).

⁽⁷⁾ T. Mann and D. Keilin, Nature (London), 146, 164 (1940). A recent and excellent review on this subject is presented by T. H. Maren,

<sup>Physiol. Rev., 47, 595 (1967).
(8) R. W. King and A. S. V. Burgen, Biochim. Biophys. Acta, 207, 278</sup> (1970).

⁽⁹⁾ A. Liljas, K. Kannan, P. Bergstin, K. Fridborg, L. Särup, S. Lövgren, H. Paradies, B. Strandberg, and I. Waara, NASA Spec. Publ., 188, 89 (1969).