constituents in 1:2 proportion, undergo a single-step thermal transition observed at 260 and 284 mμ. The latter wavelength is specific for the triple strand melting.[7] Values of $1/T_{1/2}$, when m = 6–9, for an oligonucleotide residue concentration of 10 μM do follow a linear relationship, but A = 3.05 and B = 2.20 (Figure 1). The 1:1 complex for equimolar mixtures of $d(pA)_m$ and $d(pT)_n$ is completely formed only when $m > 16$ in SSC, and these double-stranded complexes have $T_{1/2}$ similar (within the range of our experimental error, ±0.5°) to those formed by $d(pA)_n$ and $d(pT)_m$ at equal values of m.[8] $1/T_{1/2}$ values now fit on the same line (Figure 1). The anomalous stoichiometry described above for a Na^+ concentration of 0.19 M (SSC) is also exhibited in 40 mM phosphate buffer, pH 7.0, with or without the presence of 8 mM $MgCl_2$ (data not presented here).[9]

The over-all reactions that may be postulated to occur in our studies are given in eq 1–3 (major complex at equilibrium is underlined) when equimolar amounts of

$$d(pA)_n + d(pT)_n \rightleftharpoons \underline{d(pA)_n\text{-}d(pT)_n} \rightleftharpoons d(pA)_n\text{-}2d(pT)_n + d(pA)_n \quad (1)$$

$$d(pA)_n + d(pT)_m \rightleftharpoons \underline{d(pA)_n\text{-}d(pT)_m} \rightleftharpoons d(pA)_n\text{-}2d(pT)_m + d(pA)_n \quad (2)$$

$$d(pA)_m + d(pT)_n \rightleftharpoons \underline{d(pA)_m\text{-}d(pT)_n} \rightleftharpoons \underline{d(pA)_m\text{-}2d(pT)_n} + d(pA)_m \quad (3)$$

reactant residues are mixed. Reaction 1 is analogous to the interaction of rA with rU because stable 1:1 and 1:2 complexes are formed at equilibrium when the two polymers are mixed in the 1:1 and 1:2 proportion. The kinetics of the approach to the equilibrium in the equimolar mixture of the two polymers are also similar because the complex $d(pA)_n$-$2d(pT)_n$, detected by differential spectroscopy at 284 mμ, is a transient form as in the case of rA–rU formation.[10] In reaction 2 the triple-stranded complex is not formed in equimolar mixture at $m < 11$ (probably because the conditions are not permissive), and we find that equilibrium is reached a few seconds after mixing. We anticipate that as m increases 1A–2T complexes will be observed in 1:1 and 1:2 mixtures. In reaction 3 we notice that the ability of $d(pA)_m$ to compete with $d(pT)_n$ held in the three-stranded configuration increases with increasing value of m. The equilibrium point for this reaction at $m < 16$ (SSC) is shifted toward the triple-stranded helix.

All of this discussion can be summed up by noting that an extrapolation of the $T_{1/2}$ line for triple-stranded complex to the $T_{1/2}$ axis would cross the line for two-stranded complexes (Figure 1). This means that there will be a region of m where three-stranded complexes are more stable than two-stranded complexes. The melting transitions here will be three → one. There is also a region where three-stranded complexes are less stable than two-stranded complexes. Here melting transitions will be three → two → one.

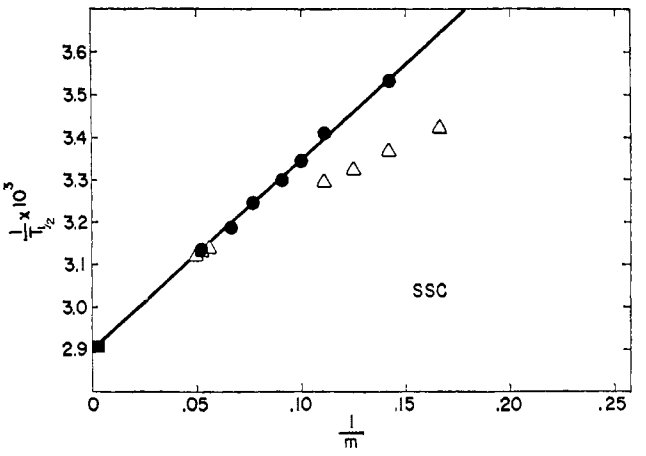

Figure 1. Melting temperature *vs.* chain length for oligodeoxynucleotide–polydeoxynucleotide complexes. Plot of the reciprocal midpoint of the thermal transition (in degrees Kelvin) of complexes formed by mixing oligodeoxynucleotides with complementary deoxypolymers as a function of the reciprocal chain length of the oligodeoxynucleotide. The total nucleotide residue concentration of the mixtures was 30 μM. The line was determined by least squares: ●, $d(pA)_n$-$d(pT)_m$ mixtures; △, $d(pA)_m$-$d(pT)_n$ mixtures; ■, $d(pA)_n$-$d(pT)_n$ mixture.

The value of m at the cross point is also related to the solvent used. We anticipate that there should be a low salt concentration where all $d(pA)_m$ form a double-stranded complex with $d(pT)_n$ in equimolar mixture, and a salt concentration (*ca.* >1.0 M) where all the $d(pA_m)$ or $d(pA)_n$ form a triple-stranded complex with $d(dT)_n$.

The complexities observed in the present case require that the contribution of the chain length to the stability of oligo–polymer interactions can be evaluated only after careful determination of the stoichiometry of the complexes formed. In the "critical region," where stoichiometry values are changing, the exact molecular stoichiometry will be indeterminate.

(11) Department of Genetics, University of Pavia, Pavia, Italy.

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(7) M. Riley, B. Maling, and M. J. Chamberlin, *J. Mol. Biol.*, **20**, 359 (1966); F. J. Bollum, unpublished data, 1965.

(8) A. M. Michelson, *Boll. Soc. Chem. Biol.*, **47**, 1553 (1965), has shown that complexes of different stability are formed when $r(A)_m$ or $r(U)_m$ of the same chain length are mixed with the complementary ribopolymer, but the stoichiometry of the complexes formed was not discussed.

(9) The choice of solvents may be considered arbitrary, but in fact the Mg^{2+} phosphate buffer is used for the enzymatic replication of homopolydeoxynucleotides. This investigation was a result of problems encountered in the replication study.

(10) R. D. Blake and J. R. Fresco, *J. Mol. Biol.*, **19**, 145 (1966).

Di-*t*-butyl Trioxide and Di-*t*-butyl Tetroxide

Sir:

Kinetic, tracer, and product studies of autoxidation[1–4] support the sequence of reactions 1–4 as controlling chain termination. Experimental observations

$$2RO_2\cdot \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RO_4R \quad (1)$$

$$RO_4R \xrightarrow{k_2} [RO\cdot O_2\cdot OR] \text{ (in solvent cage)} \quad (2)$$

$$[RO\cdot O_2\cdot OR] \xrightarrow{k_3} O_2 + ROOR \quad (3)$$

$$[RO\cdot O_2\cdot OR] \xrightarrow{k_{diff}} O_2 + 2RO\cdot \quad (4)$$

(1) H. S. Blanchard, *J. Am. Chem. Soc.*, **81**, 4548 (1959).
(2) P. D. Bartlett and T. G. Traylor, *ibid.*, **85**, 2407 (1963).
(3) J. R. Thomas, *ibid.*, **87**, 3935 (1965).
(4) G. A. Russell, *ibid.*, **79**, 3871 (1957).

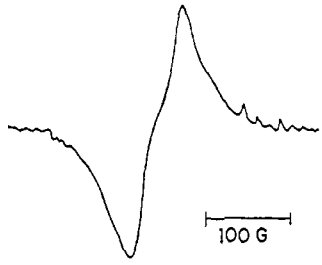

Figure 1. Esr signal observed at −196°.

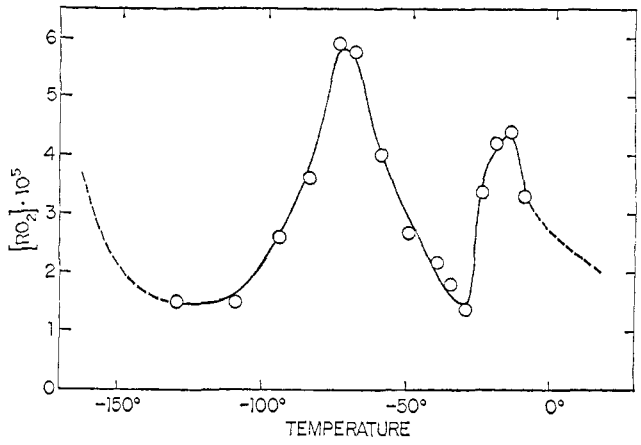

Figure 2. Change of esr signal (calibrated against DPPH) with temperature. Below −75° changes are reversible.

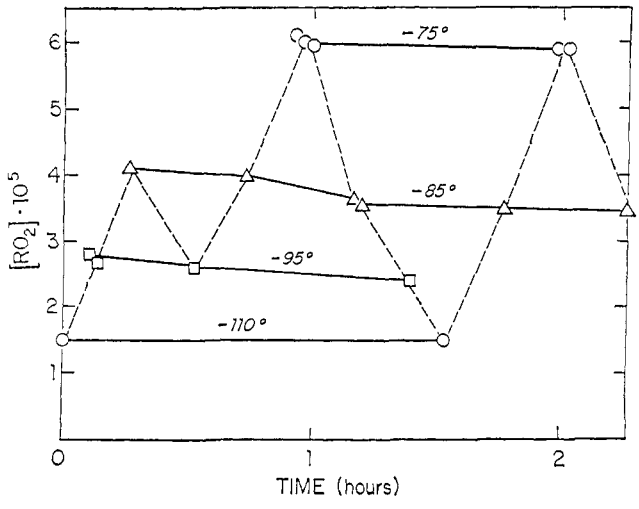

Figure 3. Reversible dependence of esr signal on temperature below −75°.

on the oxidation of alkyl hydroperoxides[5] showed that (a) t-Bu_2O_4 is not stable at −70°, but (b) a compound believed to be t-Bu_2O_3 is formed at −70° and is stable up to about −35°.[6]

Di-t-butyl diperoxycarbonate,[7] irradiated with 2540-A light in frozen methylene chloride at −196° for 20 min, gave the esr signal shown in Figure 1. In contrast to the case of 2-azobis(2-phenyl-3-methylbutane),[8] no

(5) P. D. Bartlett and P. Günther, *J. Am. Chem. Soc.*, **88**, 3288 (1966).
(6) S. W. Benson, *ibid.*, **86**, 3922 (1964), has predited greater stability for RO_3R than for RO_2R.
(7) M. M. Martin, *J. Am. Chem. Soc.*, **83**, 2869 (1961).
(8) P. D. Bartlett and J. M. McBride, paper presented at the Gomberg Symposium, Ann Arbor, Mich., Aug. 23, 1966; *Pure Appl. Chem.*, in press; see also *Chem. Eng. News*, **44**, No. 40, 106, and No. 44, 6 (1966).

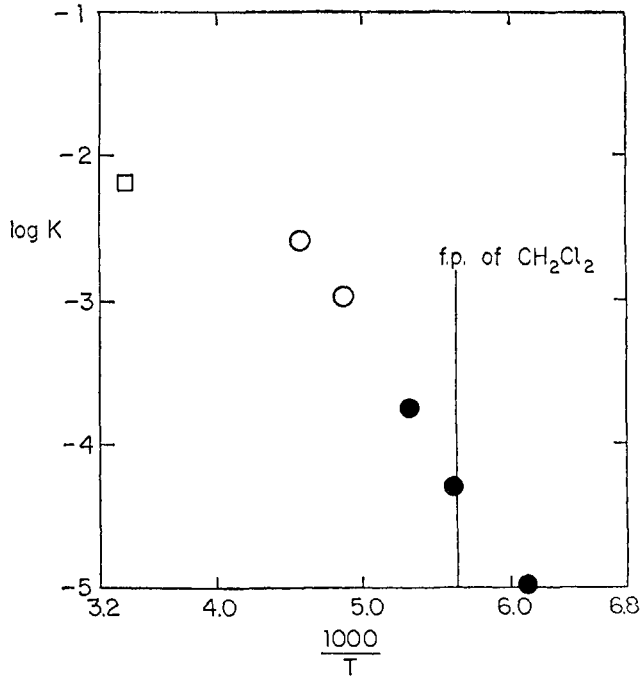

Figure 4. Dissociation constant, K, = $(RO_2\cdot)^2/(R_2O_4)$ in CH_2Cl_2: ○, from rate measurements, shortest possible extrapolation; ●, equilibrium esr, assuming complete dissociation at −75° and 6 × 10^{-5} M; □, value of Thomas,[3] from rate measurements in methanol.[10a]

triplet signal at half-field was observed. Calibrated by the radical DPPH (g = 2.0036), the signal was shown to result from overlapping of two single lines of approximate g = 2.009 and 2.016, appropriate for t-$BuO\cdot$ and t-$BuO_2\cdot$. A control irradiation of pure solvent produced no esr signal.

On removal of the light and gradual warming, the esr signal passed through two minima and two maxima as shown in Figure 2. Above −120° the line was sharp, g = 2.016, the known value for the t-butylperoxy radical.[9]

The behavior of the signal from −30° up in temperature was that expected from the previous work on di-t-butyl trioxide.[5] At any constant temperature on the falling portions of the curve of Figure 2, the intensity of the signal declined with approximate second-order kinetics, according to the equations

$$\frac{-d(RO_2\cdot)}{dt} = k_{esr}(RO_2\cdot)^2 \qquad (5)$$

$$\log k_{esr} = \left[6.02 - \frac{1100}{T}\right] \text{l./mole sec} \qquad (6)$$

However, *in the range from −110 to −85° the signal intensity remained nearly constant at any steady temperature*, and could be increased and decreased reversibly by raising and lowering the temperature. This behavior, shown in Figure 3, indicates that the equilibrium of eq 1 adjusts itself rapidly as the temperature is altered. These observations imply that di-t-butyl tetroxide is stable only a little below the temperature (−70°) of the previous[5] oxidation of t-butyl hydroperoxide.

t-Butyl hydroperoxide, about 0.25 M, was now mixed at −90° in methylene chloride with a fourfold excess

(9) M. Bersohn and J. R. Thomas, *J. Am. Chem. Soc.*, **86**, 959 (1964).

of lead tetraacetate and allowed to stand at −90° for 1.5 hr, while the lead diacetate precipitated without any oxygen evolution. The solution was then brought to constant temperatures over the range from −82 to −68° and the oxygen evolution was followed kinetically. Good *first-order* lines were obtained under these conditions according to the equations

$$\frac{d(O_2)}{dt} = \frac{-d(R_2O_4)}{dt} =$$

$$k_{O_2}(R_2O_4) = k_{O_2}([O_2]_\infty - [O_2]_t) \quad (7)$$

$$\log k_{O_2} = \left[9.27 - \frac{2440}{T}\right] \text{sec}^{-1};\ E_a = 11 \text{ kcal/mole} \quad (8)$$

The concentrated solutions prepared at −90° could be frozen and cooled to temperatures as low as −160° before they ceased to exhibit an esr signal. The fact that these signal levels were rapidly established and reasonably constant at each temperature below the freezing point (−96°) of methylene chloride indicates considerable mobility of the tetroxide and peroxy radicals, either in a polycrystalline mixture or in pockets of concentrated solution.[10]

An estimate of the dissociation equilibrium constant $K = k_{-1}/k_1$ could be made by assuming that at 0.25 M, where k_{O_2} was measured, the tetroxide was essentially undissociated, so that k_{O_2} equals the k_2 of eq 2, and that at concentrations below 10^{-4} M, as in the measurement of k_{esr}, the dissociation is nearly complete. These assumptions, together with $k_{-1} \gg k_2$, lead to the equation

$$K = \frac{2k_{O_2}}{k_{esr}} \quad (9)$$

Combination of eq 6 and 8 then yields

$$\log K = 3.55 - \frac{1340}{T};\ \Delta H = 6 \text{ kcal/mole} \quad (10)$$

The two temperatures requiring the least extrapolation, −55 and −68°, then give values for K of 2.57×10^{-3} and 1.05×10^{-3}, respectively. The assumption that the $RO_2\cdot$ at 6.0×10^{-5} M at −75° was totally unassociated affords values of $K^{-85°} = 1.8 \times 10^{-4}$ and $K^{-95°} = 4.9 \times 10^{-5}$. These values are shown plotted in Figure 4, along with the estimate made in methanol by Thomas[3] by fitting his rate measurements at 22° to the equation for decomposition of the partly dissociated t-Bu_2O_4 under nonlimiting conditions. Although we might have expected the dissociation in methanol to be higher, not lower, than in methylene chloride, this value may be compatible with ours within the uncertainties of both methods.[10a]

From these values of K we calculate that in the measurement of k_{O_2} at −68° and 0.25 M the dissociation was about 4.5%, while in the measurement of k_{esr} at −55° and 10^{-5} M the dissociation was 99%. Thus the approximations used are adequate and yield self-consistent results.

A compound formerly thought[11] to be di-t-butyl tetroxide must be, according to its decomposition temperature, the trioxide instead.[6] A stable substance reported[12] as the trioxide has been shown to be 2,2-bis-(t-butylperoxy)propane.[13,14]

Acknowledgment. This work was supported by the B. F. Goodrich Co.

(10) R. E. Pincock and T. E. Iovsky, *J. Am. Chem. Soc.*, **87**, 2072, 4100 (1965).

(10a) NOTE ADDED IN PROOF. Dr. J. R. Thomas has kindly informed us that, on the basis of new evidence, his value of K shown in Figure 4 should be regarded only as a lower limit.

(11) N. A. Milas and S. M. Djokic, *Chem. Ind.* (London), 405 (1962).

(12) N. A. Milas and G. G. Arzoumanidis, *ibid.*, 66 (1966).

(13) Bartlett and Günther, ref 5, footnote 9a.

(14) R. D. Youssefyeh and R. W. Murray, *Chem. Ind.* (London), 1531 (1966).

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A Convenient Method for Stepwise Synthesis of Oligothymidylate Derivatives in Large-Scale Quantities[1,2]

Sir:

For the synthesis of high molecular weight polydeoxyribonucleotides with arbitrary, defined sequences of the four nucleotide units, methods are needed which enable one (a) to prepare and purify readily on a relatively large scale oligonucleotides or suitable oligonucleotide derivatives and (b) to utilize efficiently the oligonucleotides (or derivatives) as building blocks for construction of high molecular weight material.[3] In this communication we describe a synthetic method which satisfies stipulation a for oligothymidylate derivatives. Work is in progress to adapt the procedure to the synthesis of mixed oligonucleotide derivatives and to the construction of polynucleotides from these units.

The basic feature of the method is that chains are built with phosphotriester rather than phosphodiester links. In a final step blocking groups are removed hydrolytically to give the desired phosphodiester chains. Initial work on this approach was carried out in experiments with polymer support reactions.[4] We now find that the method can be adapted readily to work in solution, in which case the phosphotriesters can be isolated, characterized, and utilized as intermediates in subsequent synthetic steps. There are two principal advantages to the new procedure, both of which stem from the fact that the phosphotriesters obtained are uncharged, neutral molecules. (1) The products can be handled by conventional organic techniques; for example, they can be separated by chromatography with organic solvents on silica gel, which has a much higher capacity and greater flow rate than DEAE cellulose. (2) Yields do not fall off significantly as the chain length of the oligonucleotide derivative increases. As a consequence it is not necessary to employ increasingly large excesses of nucleoside reagent as the stepwise synthesis progresses.[5]

The chemistry is illustrated by the synthesis of the β-cyanoethyl-TpT derivative I, indicated in Scheme I.

(1) Part VIII in our series on nucleotide chemistry. Part VII: K. K. Ogilvie and R. L. Letsinger, *J. Org. Chem.*, 32, 2365 (1967).

(2) This research was supported by the Division of General Medical Sciences, National Institutes of Health, Grant GM-10265.

(3) For a study of the condensation of small blocks of oligonucleotides see E. Ohtsuka and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2195 (1967).

(4) R. L. Letsinger and V. Mahadevan, *ibid.*, 87, 3526 (1965); **88**, 5319 (1966).

(5) See T. M. Jacob and H. G. Khorana, *ibid.*, 87, 368 (1965); S. A. Narang and H. G. Khorana, *ibid.*, 87, 2981 (1965).