(17) J. Dimicoli and C. Helene, *Biochimie*, **53**, 331 (1971).
(18) S. J. Gill, M. Downing, and G. F. Sheats, *Biochemistry*, **6**, 272 (1967).
(19) G. McDonald, B. Brown, D. Hollis, and C. Walter, *Biochemistry*, **11**, 1920–1930 (1972).
(20) W. Kauzmann, *Adv. Protein Chem.*, **14**, 1 (1959).
(21) G. Nemethy, I. Z. Steinberg, and H. A. Scheraga, *Biopolymers*, **1**, 43 (1963).
(22) H. S. Frank and M. W. Evans, *J. Chem. Phys.*, **13**, 507 (1945).
(23) D. M. Crothers and D. I. Ratner, *Biochemistry*, **7**, 1823 (1968).
(24) S. Hanlon, *Biochem. Biophys. Res. Commun.*, **23**, 682 (1966).
(25) H. DeVoe and I. Tinoco, Jr., *J. Mol. Biol.*, **4**, 500 (1962).
(26) B. Pullman, *J. Chem. Phys.*, **43**, S233 (1965).
(27) P. Claverie, B. Pullman, and J. Caillet, *J. Theor. Biol.*, **12**, 419 (1966).
(28) R. Foster and C. A. Fyfe, *Prog. Nucl. Magn. Reson. Spectrosc.*, **4**, 1 (1969).
(29) M. A. Slifkin, "Charge Transfer Interactions of Biomolecules", Academic Press, London, 1971.
(30) See ref 29, p 2.
(31) See ref 29, p 214.
(32) G. Nemethy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).
(33) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **76**, 6305 (1954).
(34) R. A. Bernheim, T. H. Brown, H. S. Gutowsky, and D. E. Woessner, *J. Chem. Phys.*, **30**, 950 (1959).
(35) Z. Luz and S. Meiboom, *J. Chem. Phys.*, **40**, 1058 (1964).
(36) Z. Luz and S. Meiboom, *J. Chem. Phys.*, **40**, 2686 (1964).
(37) H. M. McConnell and R. E. Robertson, *J. Chem. Phys.*, **29**, 1361 (1958).
(38) R. A. Dwek, R. J. P. Williams, and A. V. Xavier, in "Metal Ions in Biological Systems", H. Siegel, Ed., Marcel Dekker, New York, N.Y., 1974, pp 167–169.

Communications to the Editor

Isolation and Identification of Thymine Products from DNA γ Irradiated in Oxygen-Free Aqueous Solutions

Sir:

It has been suggested[1-3] that the response of living cells to ionizing radiation involves different classes of DNA damages, in aerated and deaerated conditions.[4] In deaerated solutions the radiation-induced degradation of free pyrimidine bases has been well studied. However, the actual isolation and identification of bases damaged *in the DNA chain* in oxygen-free solutions has not been accomplished. It should be noted that the radiation chemistry of the free bases may be quite different from that of bases covalently linked to the phosphate-sugar backbone of the DNA chain.[5] We report the successful characterization of thymine fragment derivatives formed in the polymer chain by γ irradiation of oxygen-free DNA solutions.

In a first step, *Escherichia coli* DNA $^{14}CH_3$ labeled in the thymine moiety was obtained from the mutant 15 $T^-A^-U^-$.[6] The DNA solution (500 μg/mL) in 10^{-3} M phosphate buffer deaerated under vacuum by repeated cycles of freezing and pumping was irradiated in sealed vials by ^{60}Co γ rays with a dose rate of 70 Gy/min at 1000 Gy (gy = gray). After irradiation, the polymeric material was separated from the low-molecular-weight material by dialysis.

The low-molecular-weight ^{14}C material was fractionated by silica gel thin layer chromatography[6] and shown to be free thymine and its radiolysis products.[7] It may be postulated that the release of the thymine fragment[8] involves OH radical attack at C-4′ and C-1′. These compounds of low molecular weight will not be discussed further because they are of lesser biological interest than the modified DNA chain.

In a second step, the high-molecular-weight material, i.e., modified DNA chain, was treated at 90 °C with 95% formic acid for 16 h.[9] The ^{14}C labeled products liberated from the DNA chain by this acid hydrolysis have been shown to be thymine (I), 5,6-dihydrothymine (II), *cis*- (III) and *trans*-5,6-dihydroxy-5,6-dihydrothymine (IV), and 5-hydroxy-5,6-dihydrothymine (V) (Figure 1).

The structure of compound II was supported by its transformation into 2-methyl-3-ureidopropionic acid in alkaline solution.[10] The oxidation of products III and IV[11] by potassium periodate gave N^1-formyl-5-hydroxy-5-methylhydantoin,[12] which suggested the presence of a glycol group, in agreement with the expected structure. 2-Hydroxy-2-methyl-3-ureidopropionic acid, which gave a colored spot with *p*-dimethylaminobenzaldehyde,[13] was obtained by alkaline solvolysis of derivative V which is consistent with the properties of 5-hydroxy-5,6-dihydrothymine.[14a] The drastic conditions of formic acid hydrolysis did not allow the isolation of 6-hydroxy "hydrate";[14b] however, isolation of VI and VII could be obtained using an enzymatic method. The polymeric material resulting from the irradiation of DNA incubated with crude enzymatic extracts of *E. coli*[15] for 2 h at 37 °C at pH 7.2 released the products I to V and in addition *cis*- (VI) and *trans*-6-hydroxy-5,6-dihydrothymine (VII).[16] The products I to VII were separated from the reaction mixture by dialysis against water and then chromatographed in the usual manner.[6,9] As expected VI and VII gave rise to thymine on warming for 10 min at 100 °C in aqueous acid solution at pH 1. Treatment of VI and VII with H_2O_2 as described previously causes their transformation to *cis*- and *trans*-6-hydroperoxy-5,6-dihydrothymine, through nucleophilic substitution of 6-OH by OOH group.[16] These peroxides were stereospecifically reduced to VI and VII. In this way VI and VII were interconverted. Finally, the structure of the products II–VII was confirmed by chemical synthesis of reference samples which were characterized by UV, IR, ^1H NMR, and mass spectrometry.[16-18]

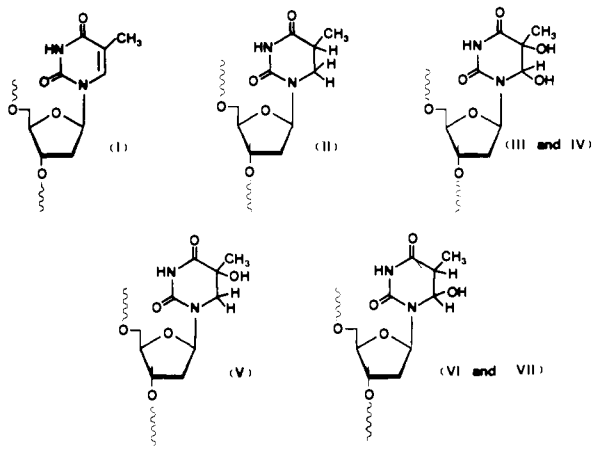

Figure 1. Unaltered thymine fragment (I) and modified thymine fragments (II–VII) in DNA chain produced by γ irradiation of DNA in deaerated aqueous solutions.

On the basis of studies performed on model compounds,[19] a mechanism for the formation of these radiation products on the DNA chain may be postulated. When water absorbs γ rays, it decomposes to OH radicals, H atoms, and e^-_{aq} which

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are short-lived species and react chemically with DNA. ESR methods clearly indicate that OH radicals can attack at both the C-5 and C-6 sites of the thymine ring. Hydrated electrons react with pyrimidine to form ions which may then undergo protonation. H atoms seem to add preferentially at C-6 and there is a difference between the H atom adduct and the electron adduct spectra indicating a different site of attack for the two short-lived species.[19] The pyrimidine radicals obtained are then able to undergo electron transfer through a dismutation reaction. Solvolytic substitution of the pyrimidic ions and H atom exchange give rise to the final radiation products II to VII.

We believe that the method described herein may be extended to the other DNA bases.[19,20]

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References and Notes

(1) T. Alper in "Biophysical Aspects of Radiation Quality", IAEA, Vienna, 1971, p 171; T. Alper, *Mutat. Res.*, **4**, 15 (1967).
(2) C. D. Town, K. C. Smith, and H. S. Kaplan, *Radiat. Res.*, **55**, 334 (1973).
(3) D. Ewing and E. L. Powers, *Science*, **194**, 1049 (1976).
(4) The terms N and O types of DNA damage should not in the future be used because, as shown below for deaerated solutions and previously for aerated solutions (R. Teoule, C. Bert, and A. Bonicel, *Radiat. Res.*, **72**, 190 (1977)), some products are identical in the presence and absence of oxygen.
(5) For example, in aerated aqueous solutions, the radiolysis of thymine fragment in the DNA chain gave rise to an *N*-formamidodeoxyribose derivative as the *major* component. A formamide derivative *is not* formed in the radiolysis of free thymine under the same experimental conditions. It is concluded that the simple extrapolation, frequently admitted, of the results obtained with free thymine to thymine in the DNA chain is thus incorrect (see ref 6 and 7).
(6) R. Teoule, A. Bonicel, C. Bert, J. Cadet, and M. Polverelli, *Radiat. Res.*, **57**, 46 (1974).
(7) (a) R. Teoule and J. Cadet, *J. Chem. Soc., Chem. Commun.*, **20**, 1269 (1971); (b) J. Cadet and R. Teoule, *Z. Naturforsch. C*, **29**, 645 (1974); (c) B. Ekert and R. Monier, *Nature (London)*, **184**, BA 58 (1959).
(8) (a) M. Dizdaroglu, C. von Sonntag, and D. Schulte-Frohlinde, *J. Am. Chem. Soc.*, **97**, 2277 (1975); (b) M. Dizdaroglu, D. Schulte-Frohlinde, and C. von Sonntag, *Int. J. Radiat. Biol.*, **32**, 481 (1977).
(9) The solution was evaporated to dryness and the residue extracted with methanol. The combined extracts were applied to TLC silica gel plates and the products separated as described in ref 6. The spots were eluted and the products submitted to microreactions.
(10) I. Blagoeva, D. J. Kurtev, and I. G. Pojarlieff, *J. Chem. Soc. B*, 232 (1970).
(11) R. Latarjet, B. Ekert, S. Apelgot, and N. Rebeyrotte, *J. Chim. Phys.*, **58** 1046 (1961).
(12) The pyrimidine ring of thymine glycols III and IV was opened between C-5 and C-6 to give N^1-formyl-N^2-pyruvylurea. However, ^{13}C NMR spectra and ^1H NMR spectra in Me_2SO show that the cyclic formula should be preferred to the linear structure. This observation does not rule out the possibility of a chain-cycle tautomerism. (R. Ducolomb et al., unpublished work.) Mass spectrometric analysis is in favor of the linear structure. (A. Cornu et al., unpublished work.)
(13) R. M. Fink, R. E. Cline, C. MacGhaughey, and F. Fink, *Anal. Chem.*, **28**, 4 (1956).
(14) (a) C. Nofre, A. Cier, R. Chapurlat, and J. P. Mareschi, *Bull. Soc. Chim. Fr.*, 332 (1965); (b) C. Nofre and M. H. Ogier, *C. R. Hebd. Seances Acad. Sci.*, **263**, 1401 (1966).
(15) *Escherichia coli* cells in logarithmic phase of growth are harvested and washed with a KCl solution (0.15 M). They are then suspended again in KCl (0.15 M) before being sonicated (30 s, 20 KHz). The cellular concentration is then 40–50 times greater than the departure cellular concentration. This solution is centrifuged (5000 rpm, 1 h, 4 °C) and the supernatant is the crude enzymatic extract.
(16) J. Cadet and R. Teoule, *Int. J. Appl. Radiat. Isot.*, **22**, 273 (1971).
(17) J. Ulrich and R. Teoule, *Org. Mass Spectrom.*, **2**, 1183 (1969).
(18) J. Cadet, J. Ulrich, and R. Teoule, *Tetrahedron*, **31**, 2057 (1975).
(19) "Effects of Ionizing Radiation on DNA. Physical, Chemical and Biological Aspects", Bertinchamps, Hütterman, Köhnlein, and Téoule, Ed., Springer-Verlag, Berlin Heidelberg, New York, 1978.
(20) Other types of DNA defects induced by various agents have been determined. E.g., (a) the cross links produced by nitrous acid, R. Shapiro, S. Dubelman, A. M. Feinberg, P. F. Crain, and J. MacCloskey, *J. Am. Chem. Soc.*, **99**, 302 (1977); (b) the thymine dimers formed in DNA by UV irradiation, S. Y. Wang, *Nature (London)*, **188**, 843 (1960).

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On the Configuration of the *tert*-Butyl Radical[1]

Sir:

The configuration[2] of the *tert*-butyl radical has been the subject of considerable debate.[3-15] The controversy has focused on the magnitude of the EPR ^{13}C hyperfine splitting of the central atom (a^{13C}),[2-8,10] the interpretation of photoelectron spectroscopic data,[9,15] and the results of ab initio calculations.[4,13,16]

Most recently the debate has rested on the temperature dependence of a^{13C}.[3,4,7,11,12,14] This parameter provides the best available measure of the C_α hybridization and hence of the configuration of the radical. If *tert*-butyl has $\theta_{min} = 0°$, where θ[18] is the angle between the plane of the methyl carbon atoms and a C-C bond, then a^{13C} should increase monotonically with increasing temperature. However, a^{13C} has been found to decrease with increasing temperature, possibly reaching a minimum at ~275 K.[3,4,7] Two independent theoretical studies[11,12] accounted for these results in terms of rapid pyramidal inversion of the radical, governed by a double-minimum potential. It was concluded that $\theta_{min} \approx 19°$ and that there is a small barrier (~600 cal/mol) to inversion. Since this theoretical work was made to fit EPR data in matrices and since it was suggested that medium effects could have caused the observed anomalies,[6,13,14] we decided to measure a^{13C} in solution over the widest possible range of temperatures. Under these conditions the spectral lines are sharp, isotropic, and free from influence by a crystalline lattice.

The radical was generated from $(CH_3)_3{}^{13}CBr$ (90 atom % ^{13}C) by standard methods[19] in either propane or isooctane as solvent. The spectral parameters were the same in both solvents (at the same temperature) and were not influenced by the method of radical generation. At each temperature the field positions and microwave frequency were recorded for 10 to 15 of the "second-order" [20] lines. The spectral parameters were computed using an exact solution of the isotropic Hamiltonian. An iterative least-squares procedure[21] was then applied which adjusted the parameters so as to obtain the best fit to all of the measured lines. Standard deviations were approximately ±0.04 G (a^{13C}), ±0.02 G (a^H), and ±0.00001 (g).[22] The ^{13}C splittings are plotted in Figure 1 and all of the data are available as supplementary material.

Our data are substantially different from those obtained in the solid state,[3,4,7] although we confirm the basic trend observed. Most notably the change in a^{13C} is almost an order of magnitude smaller in solution and there is a *well-defined* minimum at 220 K. The latter suggests that there is a small energy barrier (V_0) for the inversion of the radical. A "classical" analysis[12] gives $V_0 = 2.330$ $T_{min} = 510$ cal/mol.

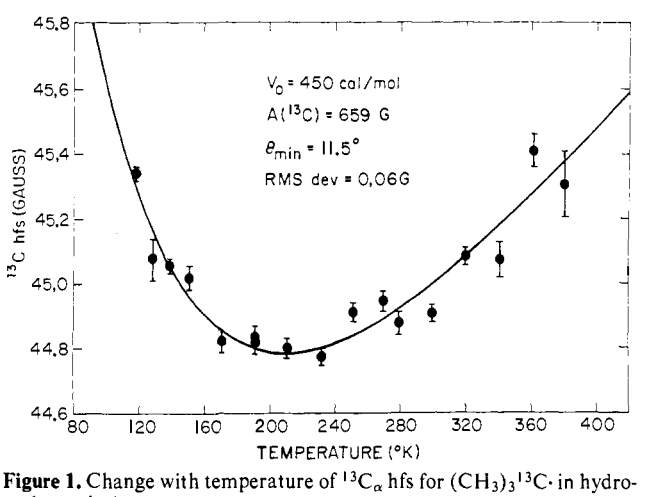

Figure 1. Change with temperature of $^{13}C_\alpha$ hfs for $(CH_3)_3{}^{13}C\cdot$ in hydrocarbon solution.

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