5-THYMINYL-5,6-DIHYDROTHYMINE FROM DNA IRRADIATED WITH ULTRAVIOLET LIGHT

A. J. Varghese

Division of Biology
The University of Texas at Dallas
P. O. Box 30365, Dallas, Texas 75230

Received December 8, 1969

SUMMARY

The major thymine-derived product (P_3) has been isolated and characterized from DNA irradiated with ultraviolet light at low temperature (-78°C). P_3 is chromatographically indistinguishable from the major photoproduct of UV-irradiated bacterial spores - generally referred to as spore-photoproduct ("sp") and of dry DNA. On the basis of UV, IR, NMR and mass spectra studies, 5-thyminyl-5,6-dihydrothymine has been assigned as the most probable structure of P_3. The mechanism proposed for the formation of P_3 in DNA involves the addition of a thyminyl radical to the 5-position of a thymyl radical.

Ultraviolet (UV) irradiation of DNA results in the formation of different types of photoproducts depending on the environment and physical state of the DNA during irradiation [for review see Smith (1)]. The major photoproduct of DNA irradiated in solution has been isolated and identified as thymine cis-syn dimer (P_2A)* (3). Two minor products, identified as uracil-thymine dimer (P_1) and 6-4'-(pyrimidin-2'-one)-thymine (P_2B) both derived from cytosine and thymine residues, have also been isolated from DNA irradiated in solution (4,5). On the other hand, UV irradiation of bacterial spores (6) and DNA at low temperature (7), or in the dry state (8), or of frozen bacteria labeled with ^3H or ^{14}C thymine, does not produce substantial amounts of P_1, P_2A or P_2B. Instead a significant amount of another thymine-derived product (P_3) is formed. Smaller amounts of P_3 have been reported to be formed when DNA is irradiated in solution (9).

* P_1, P_2A, P_2B and P_3 denote the radioactive peaks with R_f values 0.19, 0.29, 0.29 and 0.37 in n-butanol/acetic acid/water (80/12/30) detected on radiochromatograms of acid hydrolysates of thymine-2-^{14}C-labelled bacterial DNA irradiated with UV (2).

P_3 was first detected as a major product in UV-irradiated bacterial spores and is often referred to as spore-photoproduct ("sp"). The biological significance of "sp" has been studied in detail by Donnellan and co-workers (6,11). These investigators have also shown that it is formed from adjacent thymine residues in DNA. We have isolated P_3, in quantities large enough for chemical characterization, from DNA irradiated at low temperature. The UV, IR, NMR and mass spectra suggest that 5-thyminyl-5,6-dihydrothymine (V) is the most probable structure.

MATERIALS AND METHODS

Calf thymus DNA (Sigma Chemical Corp.) was dissolved in 0.075 M phosphate buffer (0.5 mg/ml). To 100 ml of the solution in an enamel pan (40 x 25 cm), 15 ml of glycerol was added. The resulting solution formed an optically clear glass at -78°C (dry ice-ethyl alcohol) which was irradiated for 25 minutes at a distance of 20 cm from 6 germicidal lamps. The irradiated solution was thawed, dialyzed against distilled water, and the dialysate evaporated to dryness. The residue was hydrolyzed in CF_3COOH at 170°C for 90 minutes and the hydrolysate was streaked on Whatman 3MM paper and developed in n-butanol:acetic acid:water (80:12:30) (10). To locate the desired area on the chromatogram, bacterial DNA labelled with thymine-2-C^{14} was used in parallel runs under identical conditions. From the chromatograms, strips (R_f 0.33-0.42) corresponding to the radioactive peak P_3, were cut out and extracted thoroughly with water. The extract was concentrated and chromatographed in the following solvent systems: (i) n-butanol:water (86:14), (ii) 2-propanol, NH_4OH:H_2O (7:1:2), (iii) n-butanol:methanol:NH_4OH:H_2O (60:20:1:20) in which P_3 had R_f values of 0.08, 0.55 and 0.43, respectively. In each case strips corresponding to P_3 were cut out from the chromatograms and were extracted with water. The UV absorption spectrum of the sample in acid and alkali indicated the relative purity of the product.

For final purification, a solution of P_3 was applied to a Dowex 50W-X12

(H^+; 100-200 mesh) column. The first and only major product eluted with water was pure P_3. This eluent was evaporated to dryness and the residue recrystallized from water. From 5 g of calf thymus DNA, 7 mg of P_3 (mp>300°) was obtained.

RESULTS AND DISCUSSION

The UV absorption spectrum of P_3 (Fig. 1) has a maximum at 265 nm (ε = 8,200) in aqueous solution at pH 2 and pH 6, shifting to 290 nm (ε = 7,500) at pH 12, which is characteristic of 5-substituted uracil derivatives, such as 5-hydroxymethyl uracil and thymine itself (12). The molar extinction coefficients of P_3 are based on the molecular weight of 252 obtained by mass spectroscopy. The infrared spectrum of P_3 shows a band at 1745 cm^{-1}, usually observed in saturated thymine derivatives, and another band at 1695 cm^{-1}, generally observed in thymine (13).

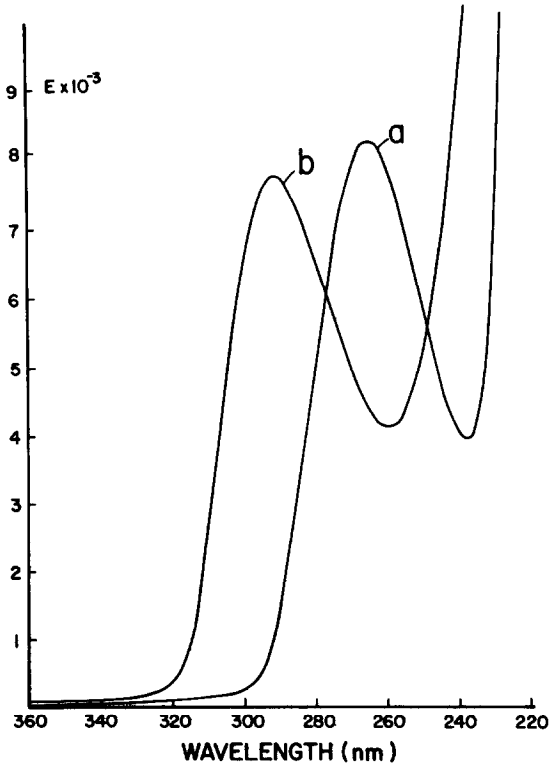


Fig. 1. Ultraviolet absorption spectra of P_3 in aqueous solution: a) at pH 5 and at pH 2; b) at pH 12.

The NMR spectrum of P_3 (Fig. 2) in CF_3COOD at 100 MHz/sec further supports the proposed structure. It shows a singlet at δ1.35 (3H) indicating the presence of the 5-methyl group. The 6-methylene protons give rise to an AB quartet with doublets centered at δ2.70 (1H) and δ3.06 (1H) with J_{AB}=14 cps; this coupling constant is consistent with geminal proton-proton coupling across an sp^3 hybridized carbon atom (14, and references cited therein). The 5-methylene protons give rise to a singlet at δ3.34 (2H) and a sharp singlet at δ7.58 (1H) indicates the 6'-vinyl proton. The two broad peaks at δ7.17 and δ9.15 are probably due to NH protons. In DMSO-D_6 two additional broad peaks at δ10.70 and δ10.94 occur, most likely attributable to protons of the other two NH groups.

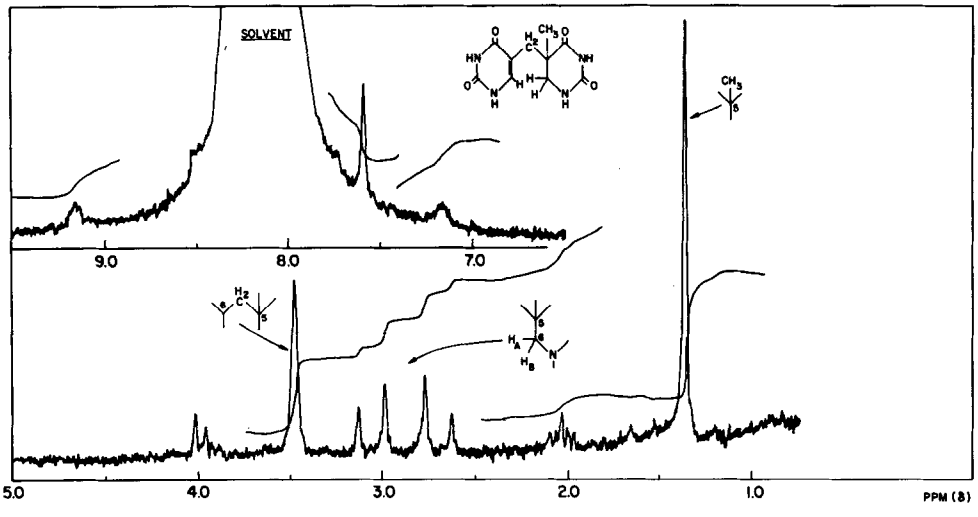


Fig. 2. NMR spectrum of P_3. The sample was examined on a Varian HA-100D, 100 MHz, proton magnetic resonance spectrometer as solution in CF_3COOD containing tetramethylsilane as an internal reference and lock signal. The observed proton absorbance bands were electronically integrated. (Examined by Sadtler Research Laboratories, Inc., Philadelphia, Pa.)

The mass spectrum of P_3 (Fig. 3) supports the proposed structure V. It shows a low intensity molecular ion peak at m/e 252 corresponding to the molecular formula of $C_{10}H_{12}N_4O_4$. The fragmentation pattern is typical of 2,4-dioxy-

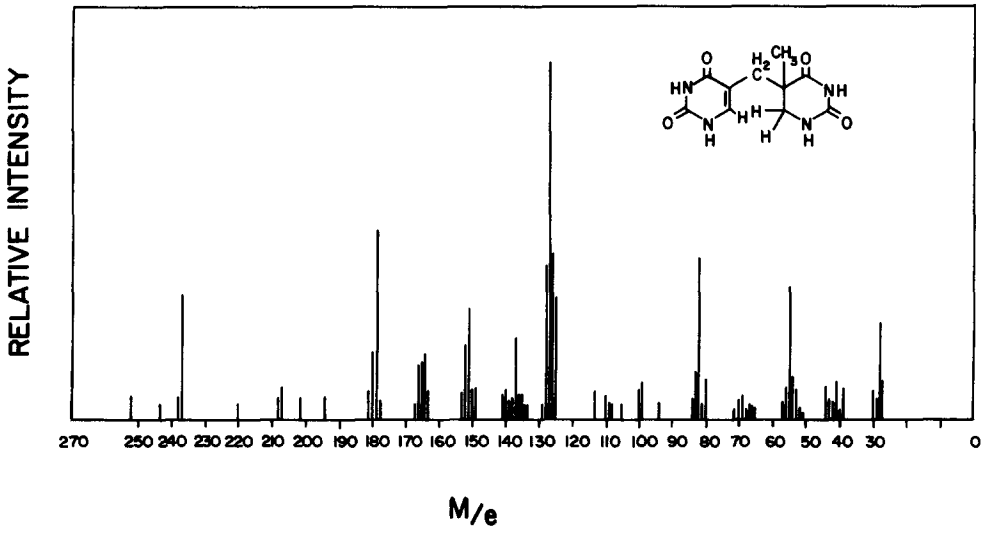
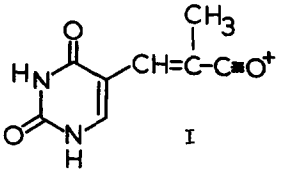


Fig. 3. Mass spectrum of P_3 at 70 e.v. (Determined by Morgan Schaffer Corp., Montreal, Quebec, Canada.)

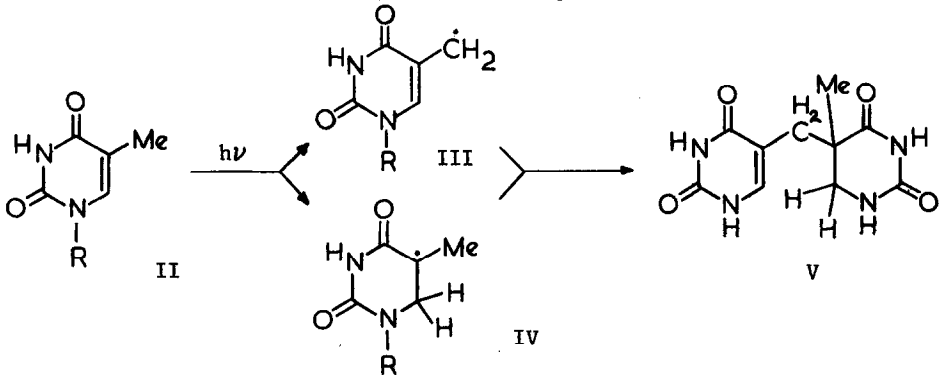
5,6-dihydropyrimidines which easily lose neutral fragments other than HNCO from the molecular ion (15); the peak at m/e 237 indicates loss of a fragment of mass 15 (tertiary methyl), as observed for dihydrothymine. A number of prominent peaks in the spectrum cannot be explained by the fragmentation pathways of known pyrimidine derivatives, notably the series of ions of mass 179, 151, and 123. Such peaks are not observed in the mass spectra of other dimeric products of thymine (16). However, from the proposed structure, formation of a fragment I



I

and subsequent loss of CO and $CH_3 \cdot CH{=}C{=}O$ from I, could produce the series of peaks mentioned above. Such eliminations have been observed in cyclic saturated ketones.

While the evidence presented above suggests that 5-thyminyl-5,6-dihydrothymine is the most probable structure of P_3, the mechanism for its formation in DNA is not clear. However, the following reaction mechanism can be proposed:



There is some evidence for the formation of radicals III and IV as a result of UV irradiation. Thus the oxidation of the 5-methyl group of thymine to give 5-hydroxymethyluracil, 5-formyluracil, and 5-carboxyuracil, has been observed when thymine is irradiated with UV (λ~254 nm) in solution (17), the first step for such a reaction sequence is probably the formation of the thyminyl radical III. The formation of the thymyl radical IV is suggested from electron-spin-resonance studies of DNA and thymine irradiated with UV or ionizing radiations (18-20). It is therefore not unreasonable to assume that UV can induce radicals III and IV under suitable conditions. To explain the differences in the E.S.R. spectra of wet and dry DNA, Pershan et al (18) have postulated the necessity of two sources of hydrogen, one of which predominates in dry DNA and the other in moist DNA. Recently Rahn and Hosszu (8) have shown that the absence of water is necessary for the formation of spore photoproducts (mainly P_3) in dry DNA. From the above considerations it can be postulated that the nature of the hydrogen source determines the type of thymine-derived product in UV-irradiated DNA. In the presence of water, hydrate formation and dimerization may take place concurrently and, the hydration product of thymine being very unstable, the isolable product will be mainly the dimer. In the dry state the methyl group of one thymine residue may be the hydrogen source for the other, thus leading to III and IV, and finally the product V.

ACKNOWLEDGEMENT

The author is indebted to Dr. David Creed for helpful discussion throughout the course of this work, to Dr. M. H. Patrick for supplying the tritiated DNA, to Drs.J. Jagger and C. S. Rupert for their critical review of the manuscript, and to Elizabeth Browning for technical assistance. This work was supported by U.S. Public Health Service Grants GM 13234, FRO 5646, and GM 16547.

REFERENCES

1) Smith, K. C. in Radiation Research (Ed. G. Silini), p. 756, North-Holland, Amsterdam (1967).
2) Varghese, A. J. and Wang, S.Y., Science 156, 955 (1967).
3) Varghese, A. J. and Wang, S.Y., Nature 213, 909 (1967).
4) Weinblum, D., Biochem. Biophys, Res. Comm. 27, 384 (1967).
5) Varghese, A. J. and Wang, S. Y., Biochem. Biophys. Res. Comm. 29, 543 (1968).
6) Donnellan, J.E. and Setlow, R.B., Science 149, 308 (1965).
7) Rahn, R. O. and Hosszu, J. L., Photochem. Photobiol., 8, 53 (1968).
8) Rahn, R. O. and Hosszu, J. L., Biochem. Biophys. Acta 190, 126 (1969).
9) Setlow, R. B., Photochem. Photobiol. 7, 637 (1968).
10) Smith, K. C., Photochem. Photobiol. 2, 503 (1963).
11) Stafford, R. S. and Donnellan, Jr., J. E., Proc. Natl. Acad. Sci., U. S. 59, 822 (1968).
12) Cline, R. C., Fink, M. R. and Fink, K., J. Am. Chem. Soc., 81, 2521 (1959).
13) Lacher, J. R., Bitner, J. L., Emery, D. J., Sefel, M. E. and Park, J. D., J. Phys. Chem., 59, 615 (1955).
14) Sternhell, S., Quart. Revs., 23, 236 (1969).
15) Rice, J. M., Dudek, G. O. and Barber, M., J. Am. Chem. Soc., 87, 4569 (1965).
16) Fenselau, C., Varghese, A. J. and Wang. S. Y., 16th Annual Conference on Mass Spectrometry and Allied Topics, Pittsburgh, Pennsylvania, May 12-17, 1968.
17) Alcantara, R. and Wang, S. Y., Photochem. Photobiol. 4, 475 (1965).
18) Pershan, P. S., Shulman, R. G., Wyluda, B. J. and Eisinger, J., Science 148, 378 (1965).
19) Salovey, P., Shulman, R. G. and Walsh, Jr.,W. M., J. Chem. Phys. 939, 839 (1963).
20) Pruden, B., Snipes, W. and Gordy, W., Proc. Natl. Acad. Sci., U. S., 53, 917 (1965).