DIHYDROTHYMINE FROM UV-IRRADIATED DNA

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Pyrimidine dimers have been isolated as stable photoproducts of UV-irradiated DNA.[1-3] Recently, another photoproduct of thymine in DNA has been isolated[4] but not yet identified. In this note we report the observation and identification of an additional photoproduct of irradiated DNA, i.e., 5,6-dihydrothymine. Wacker[2] and his co-workers have previously reported that six radioactive species are observed after Dowex-column separation of hydrolyzed DNA labeled with C^{14}-thymine. In addition to thymine (T) and the thymine dimer ($\widehat{TT}$), there was a peak which appeared first (peak I) and three others (called IV, V, and VI), much less prominent, which appeared after T and $\widehat{TT}$. By chromatographic analysis we have been able to show that peak I is 5,6-dihydrothymine (diHT).

Methods and Materials.—Labeled E. coli DNA: *E. coli* 15 T^- was grown in M-9 medium containing 4 μg/ml of radioactive thymidine (Calbiochem, Los Angeles, Calif.), enriched with 2.5 mg/ml of Difco casamino acid. DNA was extracted by the Marmur procedure.[5] The calf thymus DNA was from Worthington Biochemical Corporation, Freehold, New Jersey, and was used without further purification.

High concentrations of DNA in tritiated water were irradiated and then dialyzed against 500 vol distilled water at 5°C, changed 5 times. The DNA was then reduced to dryness by lyophilization.

Hydrolysis: Dried DNA samples were hydrolyzed in 90% formic acid at 175°C for 30 min.

Chromatography: Essentially, Wacker's procedure[2] was used for the Dowex ion-exchange chromatography. Hydrolyzed DNA solution was evaporated to dryness and dissolved in a few milliliters of the starting buffer. Elution was carried out with ammonium formate gradient from pH 10 (0.03 *M*) to pH 5 (0.36 *M*). Fractions of 2 ml were collected and the absorbancy at 260 mμ was measured. Radioactivity was measured by an Ansitron liquid scintillation counter. For the identification of the radioactive peaks, *n*-butanol–acetic acid–water (80:12:30), *n*-butanol–water (86:14), and saturated ammonium sulfate–1 *M* sodium acetate–isopropanol (40:9:1) descending paper chromatographies were used at room temperature. The dried chromatograms were analyzed for distribution of radioactivity.

Irradiation: Light from an Osram 500-watt high-pressure Hg lamp was focused by a front surface mirror onto the samples which were contained in 5-mm OD quartz ESR tubes (obtained from Varian Associates). Usually, a 3.0-cm thick aqueous solution of 0.4 *M* $NiSO_4$ was used as a filter. This passed 2200 Å $< \lambda <$ 3600 Å, although, as reported previously,[6] the spectral output of this lamp is negligible between 2200 and 2400 Å. It has previously been reported[6] that this irradiation gave $\sim 10^6$ erg/sec as the energy of UV light absorbed by the samples which had areas of $\sim$100 mm^2 for a resulting UV flux of $\sim 10^4$ erg/mm^2/sec. The largest dosages used were irradiations for 300 sec or a total of $\sim 300 \times 10^4$ erg/mm^2. These were approximately four times larger than the largest employed by Wacker.

Experimental Results.—The first experiments were an attempt to discover stable photoproducts which came from the thymine-free radical, which we had previously[6] observed in DNA at low temperatures after UV irradiation. This free radical had been shown to consist of an unpaired spin at C_5, while a hydrogen had been added at C_6. Furthermore, it had been shown that when the DNA was equilibrated before irradiation with D_2O, instead of H_2O, a deuteron was added. Hence, we reasoned that equilibration with T_2O would result in some tritium addition and any stable product formed from the free radical could be detected by tritium radio-

assay. Because we were looking for new thymine products, we used Wacker's Dowex chromatographic analysis since unidentified products had been observed in that way. Calf thymus DNA dissolved in tritiated water, frozen to 77°K, irradiated, brought to room temperature, and hydrolyzed gave two products on Dowex with approximately equal tritium counts. The same products were observed after irradiation in aqueous solution at 274°K (see Fig. 1*a*). One peak was very close to thymine. The other, which came at the same position as Wacker's peak I, we have shown to be chromatographically similar to dihydrothymine. The controls

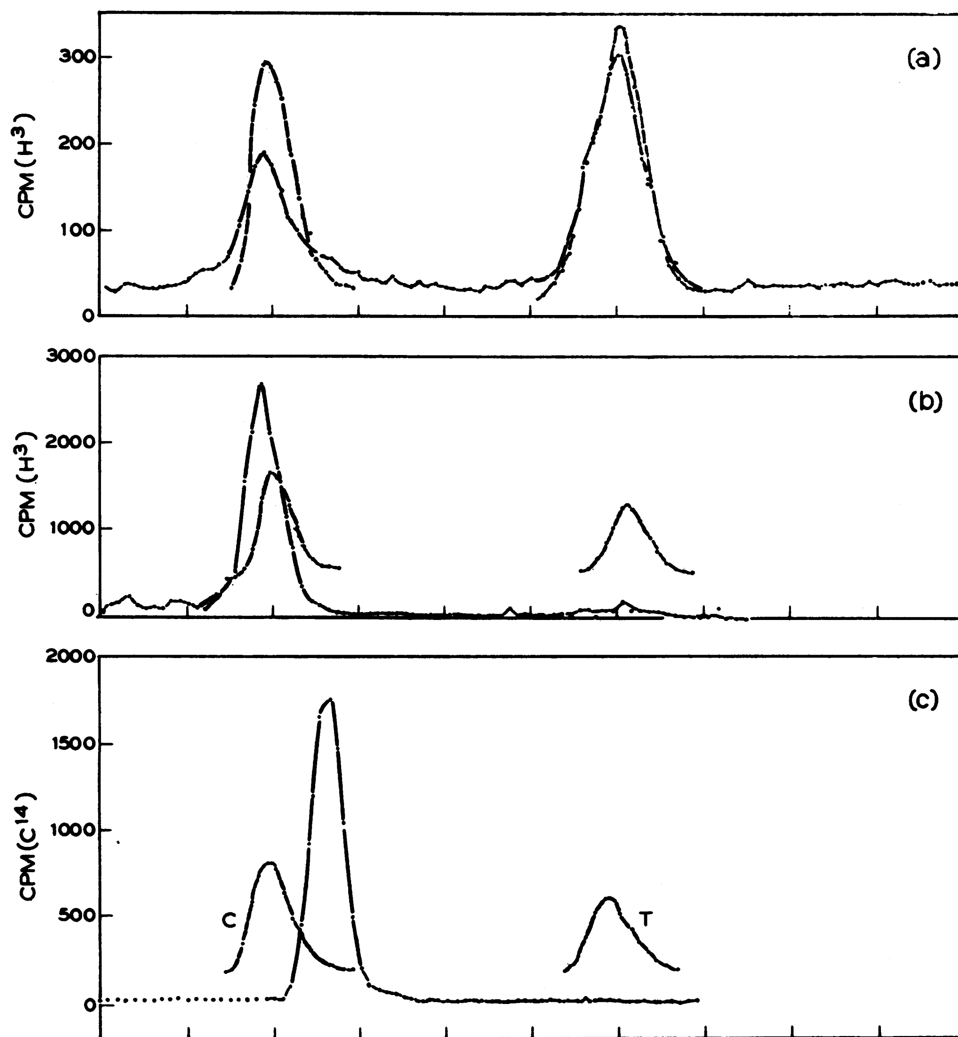

FIG. 1.—The dashed lines are the OD profiles of cytosine (C) and thymine (T) used to calibrate the Dowex column. In (*a*) calf thymus DNA was irradiated in tritiated water at ~1°C, then hydrolyzed, and the H^3 counts are indicated by the solid line. In (*b*) H^3-diHT chromatographed on the Dowex column with cytosine and thymine controls. In (*c*) the C^{14}-thymine dimer on Dowex was counted and compared with cytosine and thymine controls. Samples used in (*b*) and (*c*) were adjusted to pH 10 and ammonium formate 0.03 *M* which corresponds to the eluents at the beginning of the gradient.

used on the Dowex column were cytosine, thymine, C^{14}-thymine dimer type I as described by Weinblum and Johns,[7] which was kindly prepared and supplied by A. A. Lamola, and H^3-dihydrothymine purchased from the New England Nuclear Corporation. The results of irradiating calf thymus DNA in T_2O shown in Figure 1*a* are compared with the tritium-labeled diHT in Figure 1*b* and with the C^{14}-labeled type I thymine dimer in Figure 1*c*. C^{14}-labeled DNA showed the $\widehat{TT}$ peak in addition to the diHT and T peaks, as has been observed previously.[2]

We also froze C^{14}-thymine in H_2O and irradiated it at 77°K. It gave thymine plus two other C^{14} peaks, one at the position of dihydrothymine, the other at the $\widehat{TT}$ position. Furthermore, irradiating nonlabeled thymine in T_2O under these same conditions gave three H^3 peaks covering the region normally spanned by diHT and $\widehat{TT}$ which look like diHT, $\widehat{TT}$, and an intermediate peak, although these assignments are not confirmed. The possibility of tritium exchange between thymine and water during dimerization is not excluded in the irradiated thymine experiments but it is excluded in the irradiated DNA experiments. It is hard, but not impossible, to reconcile these results with those of Wang *et al.*,[8] who observed a loss of tritium from the thymine methyl group upon dimerization from DNA, while they did not observe a loss of tritium in the case of thymine dimerization from frozen aqueous solutions.

In addition to demonstrating the similar behavior on the Dowex column of peak I and diHT, we also compared their R_f values with three different solvents on paper chromatography. The Dowex peak I material was compared with known constituents in the following solvents: *n*-butanol, acetic acid, water (80:12:30), *n*-butanol, water (86:14), and saturated ammonium sulfate, 1 *M* sodium acetate, and isopropanol (40:9:1). The observed R_f values and the tube number of the peaks in the Dowex column are given in Table 1. For comparison the same data are presented for cytosine, thymine, the $\widehat{TT}$ (type I dimer) as well as for diHT, and the peak I of Dowex. Since no differences were observed for this Dowex peak when we irradiated thymine or DNA or when we used C^{14} or H^3-labeling, their chromatographic parameters are not distinguished.

The first striking feature shown in Table 1 is that the peak I behaves like dihydrothymine in all four chromatographic separations. On this basis we conclude peak I is diHT.

The second noteworthy feature is that the commonly used *n*-BuOH-HAc-H_2O and *n*-BuOH-H_2O solvents do not separate dihydrothymine from thymine which may explain why dihydrothymine has often not been separated as a distinct product.

TABLE 1

	Dowex Tube	R_f (*n*-BuOH, HAc, H_2O)	R_f (AmS, NaAc, iPrOH)	R_f (*n*-BuOH, H_2O)
Thymine	60	0.64	0.46	0.48
Cytosine	20	0.35	0.66	0.19
$\widehat{TT}$	26	0.25	0.66	0.1
Dihydrothymine	17	0.64	0.55	0.45
Dowex peak I	17	0.64	0.55	0.45

Chromatographic data showing the comparison of peak I from irradiated DNA and thymine with various known species. Note, first of all, its similarity to diHT under all conditions, and second, the similarity of both peak I and diHT to thymine in the (*n*-BuOH, HAc, H_2O) and (*n*-BuOH, H_2O) paper chromatography.

The yield of dihydrothymine did not change by very much when DNA was irradiated frozen at 77°K or in solution at 275°K. To date our experiments cannot be used to obtain the relative yield of diHT and $\widehat{TT}$ since they were done at high dosages of $\sim 2 \times 10^6$ erg/mm^2, and at these dosages the amount of dihydrothymine was approximately the same as $\widehat{TT}$, as judged from the C^{14}-labeled DNA. Wacker *et al.*[2] have shown that at low doses the $\widehat{TT}$ yield rises faster than does that of their peak I, so that at lower doses the $\widehat{TT}$ quantum yield should be considerably higher than the peak I or diHT yield. In this respect, and only qualitatively at this time, the diHT yield seems to follow the thymine-free radical yield,[6] and quantitative experiments to correlate these two species are under way. As an additional control, our standard irradiation of $\widehat{TT}$ labeled with C^{14} gave no diHT, indicating it is not a photoproduct of the $\widehat{TT}$.

It is interesting to note that diHT has been shown to hydrogen bond less strongly to adenine than uracil[9] and also to lower the melting temperature of poly A-poly U copolymers.[10] If diHT is shown to induce biological changes, it might be by this lowered chemical affinity for hydrogen bonding with adenine.

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[1] Beukers, R., W. Berends, *Biochim. Biophys. Acta.*, **41**, 550 (1960).
[2] Wacker, A., *Progr. Nucleic Acid Res.*, **1**, 369 (1963).
[3] Setlow, R. B., and W. L. Carrier, *J. Mol. Biol.*, **17**, 237 (1966).
[4] Varghese, A. J., and S. Y. Wang, *Science*, **156**, 955 (1967).
[5] Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961).
[6] Pershan, P. S., R. G. Shulman, B. J. Wyluda, and J. Eisinger, *Physics*, **1**, 163 (1964).
[7] Weinblum, D., and H. E. Johns, *Biochim. Biophys. Acta*, **114**, 450 (1966).
[8] Wang, S., M. H. Patrick, A. J. Varghese, and C. S. Rupert, these PROCEEDINGS, **57**, 465 (1967).
[9] Kyogoku, Y., R. C. Lord, and A. Rich, *J. Am. Chem. Soc.*, **89**, 496 (1967).
[10] Cerutti, P., H. T. Miles, and J. Frazier, *Biochem. Biophys. Res. Commun.*, **22**, 466 (1966).