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Publication Date

1965-09-01

UCRL-16426

University of California

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Berkeley, California

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UCRL-16426

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
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AEC Contract No. W-7405-eng-48

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P. E. Brown, M. Calvin, and J. F. Newmark

September 1965

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Abstract. When thymine, in dilute, deaerated, aqueous solution was irradiated with γ-rays in the presence of ethanol a high yield of products containing both the thymine and ethanol moieties was obtained. These were shown to be isomers formed by the addition of H· and $CH_3\dot{C}HOH$ radicals to the 5,6 double bond of thymine. A similar reaction was observed with N,N'-dimethylthymine, but O,O'-dimethylthymine did not react. The reaction may be relevant to the resistance of certain cells to ionizing radiation.

During the course of a search for the sensitized photodimerisation of thymine and the sensitized photodissociation of the thymine dimer, ethanol was used as cosolvent for some of the materials (1). Although as yet no photosensitization reactions have been seen a particularly efficient reaction leading to the disappearance of thymine was observed and was traced to the presence of small amounts of ethanol rather than any of the other constituents. We wish to report here the nature of that reaction, and to indicate its possible role in biological systems.

Solutions of thymine-2-C^{14} (1 mg/ml, 1μc/ml) were deoxygenated in a stream of nitrogen, treated with ethanol (5 μl/ml) and irradiated with γ-rays from a Co^{60} source. The total doses varied from 0.066 to 9.6 megarads at a dose rate of 0.40 Mrad/hr. The resultant solutions were subjected to two-way paper chromatography on Ederol paper. The solvent in the first direction was n-butanol: formic acid: water (77:13:10 v/v) and in the second direction was propanol: concentrated ammonia: water (6:3:1 v/v). Spots were detected

by radioautography and after elution their radioactivity was determined by liquid scintillation counting using a dioxane-naphthalene scintillator. The results of this experiment showed that under these conditions thymine was converted into three new compounds (A,B, and C), as shown in Figure 1. The appropriate R_f values are given in Table 1. The nature of these compounds was investigated after large scale irradiations.

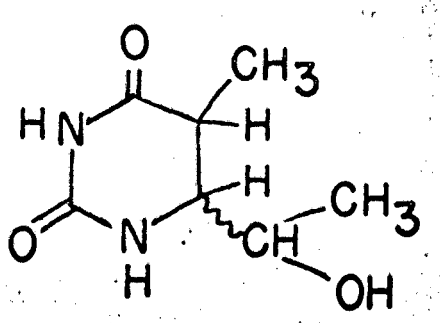
For the large scale experiment a solution of thymine (1.35 g.) in water (450 ml) was deoxygenated in a stream of nitrogen for at least one hour. Ethanol (13.5 ml) and ethanol-1-C^{14} (135 µl, 0.102 µc/µl) were added and the solution irradiated (13.2 Mrad.) Solvents were removed by rotary evaporation and the residual white solid dried as far as possible under vacuum. The product was treated with hot methanol and a small insoluble portion discarded. The solvent was removed and replaced with chloroform-methanol (9:1 v/v; 50 ml) and the solution chromatographed on silica-gel (280 g.) Only one significant fraction was eluted (821 mg, 55%) and this could not be resolved by thin-layer chromatography. However, even after several recrystallizations from methanol, this material had a wide melting change of 20°C, indicating a mixture of compounds. Two-way paper chromatography as before revealed the presence of two compounds whose R_f values corresponded to those of compounds A and B in the initial experiment.

The spots were detected both by radioautography and by spraying with 0.5 M sodium hydroxide solution followed by an acidic solution of p-dimethylaminobenzaldehyde. The latter reagent detects dihydropyrimidines and the characterization of compounds A and B as dihydropyrimidines was confirmed by the absence of a band at about 265 mµ in the ultraviolet spectrum. The similarity of the physical properties of A and B suggested that they were isomers and this received some support from elemental analysis which agreed

with that required for a 1:1 addition product between thymine and ethanol (Found: C, 49.05; H, 6.94; N, 16.31. $C_7H_{12}N_2O_3$ requires C, 48.83; H, 6.97; N, 16.27%). The infrared spectrum revealed the presence of a hydroxyl group (3450 cm^{-1}), two carbonyl groups (1700 and 1724 cm^{-1}) and amino groups (3280 cm^{-1}) whilst the n.m.r. spectrum (in D_2O) showed the presence of several $C\underline{CH}_3$ groups (overlapping bands between τ= 8.75 and 8.98) as well as several unresolved $C\underline{H}$ groups (between τ=5.5 and 6.9).

The p-nitrobenzoyl derivatives of this mixture were prepared using p-nitrobenzoyl chloride and pyridine. The product was recrystallized from ethanol and gave colorless crystals (141 mg) whose m.p. (207-230°C.) indicated a mixture of compounds. Elemental analysis agreed with this mixture being p-nitrobenzoyl derivatives of the above alcohols (Found: C, 51.61; H,4.66; N, 12.92. $C_{14}H_{15}N_3O_6$ requires C, 52.3; H, 4.67; N, 13.1%), and the infrared spectrum now lacked the 3450 cm^{-1} hydroxyl band. Preparative thin-layer chromatography of this material on silica-gel in chloroform-methanol (95:5 v/v) yielded two compounds. The first separated from ethanol as colorless prisms (1.6 mg), m.p. 212-213°C. The second compound separated from ethanol as colorless needles (3.6 mg), m.p. 235-237°C whose n.m.r. spectrum (in D_2O) showed two doublets due to $C\underline{CH}_3$ groups, each split by an adjacent CH group, confirmed that the two compounds, A and B are <u>cis</u> and <u>trans</u> isomers of the addition product of thymine and ethanol (I).

(I)



By using a computer of average transients (CAT) further resolution of the [redacted] n.m.r. spectrum was achieved, the signals confirming this structure. Bands were observed at τ=0.4 (CONHCO, singlet), 2.1 (aromatic CH, split), 5.1, 6.75 and 7.5 (three CH, all split), 9.0 and 9.3 (two CCH_3, both doublets).

Because of the small amounts of material available further confirmation of the structures was not possible. However, by using N,N'-dimethylthymine, separation and identification of the reaction products was made easier and information concerning the structural requirements for reaction was obtained. The reaction with N,N'-dimethylthymine also supported the allocation of structure I to the products A and B.

A solution of N,N'-dimethylthymine (2.25 g) in water (450 ml) was deoxygenated in a stream of nitrogen. Ethanol (22.5 ml) was added and the solution irradiated (24 Mrad.) after which solvents were removed by rotary evaporation. The resultant colorless oil was dissolved in chloroform, dried, and chromatographed on silicic acid (200 g) on which separation into three components was achieved.

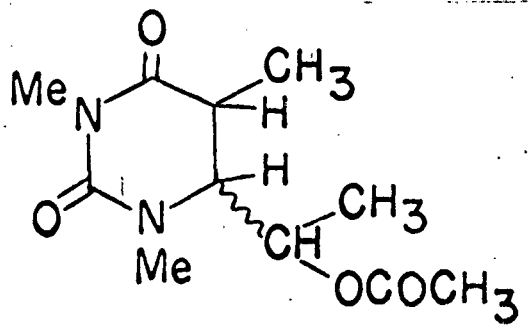
Component I (1.11 g, 31%) was recrystallized from carbon tetrachloride to give colorless needles, m.p. 153-155°C, and mixed m.p. with starting material 153-154°C. Component III (550 mg, 16%) was a colorless gum which could not be caused to crystallize and was not investigated further.

Component II (1.85 g, 53%) was shown to contain hydroxyl groups by infrared (band at 3500 cm^{-1}) and n.m.r. spectroscopy (band at τ=6.2 removed by shaking with deuterium oxide). The presence of at least two compounds was suspected from the complex CCH_3 signals in the n.m.r. spectrum and this was confirmed by thin-layer chromatography. This material was acetylated using acetic anhydride and pyridine and the product rechromatographed on silicic acid in diethyl ether when three fractions were obtained.

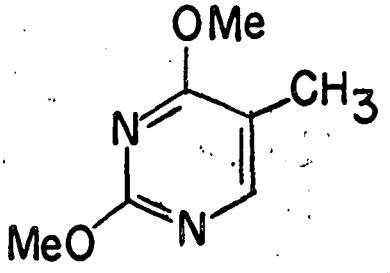
The first fraction (110 mg, 21%) was recrystallized twice from petroleum ether-ethanol to give cis or trans 6-α-acetoxyethyl- 5,6-dihydro-1,3-dimethylthymine (II) as colorless needles, m.p. 101-102°C (Found: C, 54.93; H, 7.44; N, 11.59%; MW 248 (Rast), 242 (mass spec.) $C_{11}H_{18}N_2O_4$ requires C, 54.50; H, 7.30: N, 11.45%; MW 242), λ_{max} 222mμ (log ε 3.64), ν_{max} 1740, 1720 and 1680 cm^{-1}, τ values 4.9 ($C_7\underline{H}$, quartet), 6.4 ($C_6\underline{H}$, quartet), 6.9 two N$\underline{Me}$, singlet), 7.13 $C_5\underline{H}$, doublet), 8.0 (OCOC$\underline{H}$, singlet), 8.2 and 8.92 (two CC$\underline{H}_3$, both doublets, J=7 c.p.s.).

The second fraction (225 mg, 43%) separated from petroleum ether - ether as colorless needles of the isomer, m.p. 118°C (Found: C, 54.49; H, 7.31: N, 11.54%, MW 240 (Rast), 242 (mass spec)), λ_{max}. 222 mμ (log ε 3.26), ν_{max}. 1750, 1718 and 1675 cm^{-1}, τ values 4.93, 6.66, 6.86, 6.92, 7.2, 8.75 and 8.85.

(II)



(III)



The third fraction (185 mg, 36%) could not be caused to crystallize. No further resolution was achieved by thin-layer chromatography and the n.m.r. spectrum showed peaks at τ=8.0 (OOOC$\underline{H}_3$) and 8.8 (CC$\underline{H}_3$) whose relative intensities (1:2) indicated that one mole of ethanol had reacted with one mole of thymine.

When O,O'-dimethylthymine (III) was treated in the same way thin-layer chromatography revealed that no reaction had taken place.

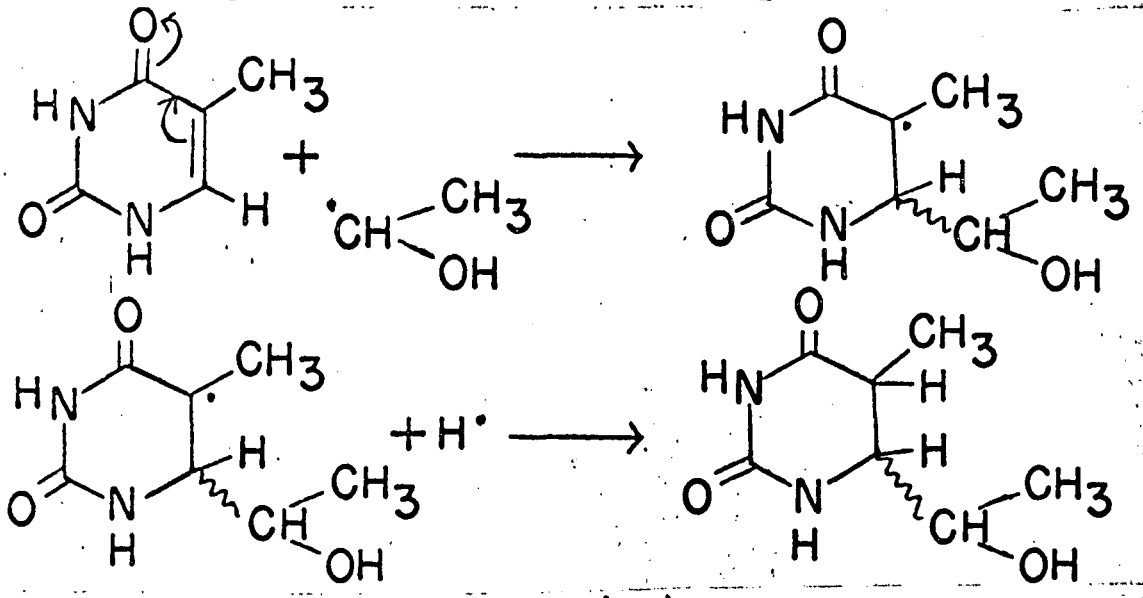
The above results show that the radiation induced addition of ethanol to certain pyrimidines is a surprizingly efficient reaction, G values being in the range normally found for radiation chemistry (2.3 for dimethylthymine, 0.9 for thymine). The reaction appears to proceed by attack of the hydroxyethyl radical on the electronegative C_6 of the pyrimidine deivative, as shown in the scheme below:

i) $H_2O \xrightarrow{\gamma} H^{\bullet} + OH^{\bullet}$

ii) $CH_3CH_2OH + H^{\bullet} \longrightarrow CH_3\dot{C}HOH + H_2$

$CH_3CH_2OH + OH^{\bullet} \longrightarrow CH_3\dot{C}HOH + H_2O$

iii)



The inability of O,O'-dimethylthymine (III) to react is evidently caused by the absence of the C_4 carbonyl group. Such a group is required to activate the C_6 position to radical attack by electron withdrawal.

Donnellan and Setlow have recently reported the formation of thymine photoproducts other than thymine dimers in ultraviolet-irradiated bacterial spores (2). Since bacterial spores are resistant to the deleterious effects of both ultraviolet light and ionizing radiation the formation of large amounts of such photoproducts implies either that these products do not interfere with DNA synthesis or that the cells have a very efficient repair mechanism for dealing with them. It follows that those cells which are resistant to ultraviolet light and ionizing radiation should form non-harmful products in preference to lethal thymine dimers. Such a preferential reaction implies an efficient chemical act between substances present in relatively high amounts within the cell.

The reaction described above is such an efficient reaction and requires only the presence of hydroxyl groups. Since these are present in large amounts within cells (e.g. sugars) it is possible that this type of reaction plays a part in the high dose radiation chemistry of cells.

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References

1. J.F. Newmark, Ph. D. thesis, University of California, Berkeley (1965).
2. J.E. Donnellan and R.B. Setlow, Science, 149, 308 (1965).

Table 1

R_f values of compounds A, B, C, and thymine on Ederol

Compound	R_f in bu-formic	R_f in prop-NH_3
A	0.54	0.71
B	0.45	0.67
C	0.71	0.75
Thymine	0.51	0.59

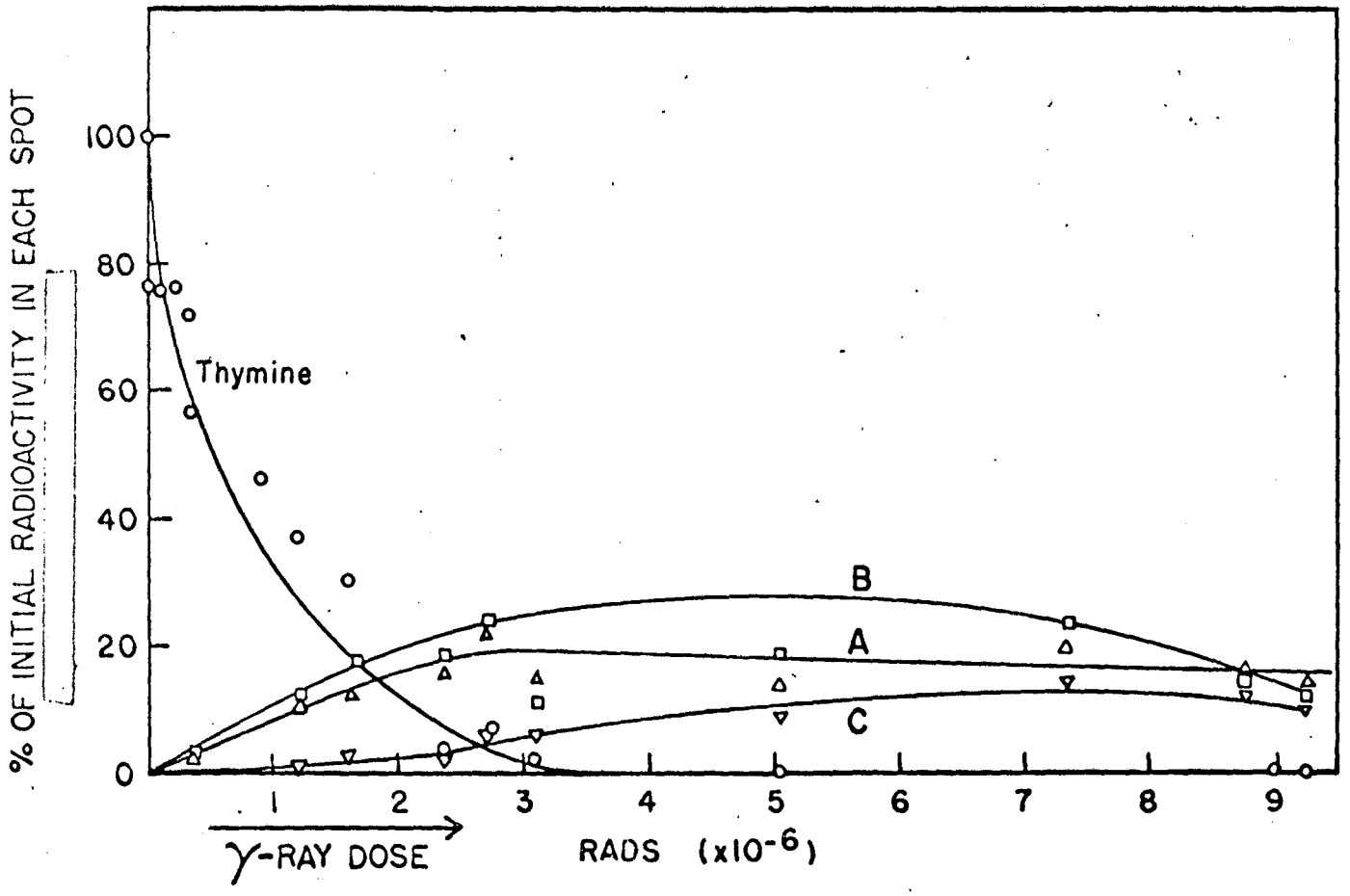

Fig. 1. Disappearance of thymine and appearance of irradiation products A, B, and C as a function of dose.

Legend: o, thymine

Δ, A

▼, B

□, C