

FORMATION OF RELATIVELY PERSISTENT O^2 -ETHYLTHYMIDINE BY DIETHYLNITROSAMINE IN RAT LIVER DNA

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1. Introduction

O-Alkylation of DNA by chemical carcinogens or mutagens has been considered as a relevant event in carcinogenesis and mutagenesis [1]. Besides O^6 of guanine and the phosphate groups, the *O* of pyrimidines are sites of modification [2–6]. O^4 -Alkylthymidine was detected after *in vitro* and *in vivo* alkylation of DNA [7–9] and O^2 -alkylthymidine after reaction of DNA *in vitro* or in cultured cells [9,10]. We now report the identification of O^2 -ethylthymidine in liver DNA of rats given the hepatocarcinogen DEN. Despite the apparent lack of mis-coding *in vitro* [1], the relative persistence of this lesion *in vivo* may indicate a relevant role in hepatocarcinogenesis.

2. Experimental

Liver DNA of female Sprague-Dawley rats given 10 mg [$1\text{-}^{14}\text{C}$]DEN/kg (0.4–2 mCi) was prepared as in [12]. [3H]Thymidine-labelled DNA, isolated from rats given labelled precursor during regeneration after partial hepatectomy [13], was ethylated (2 mg/ml 0.05 M phosphate buffer (pH 7.2), 37°C, 1 h) with ENU (Hoechst, Frankfurt/M) at 3 mg/mg DNA. After precipitation by addition of 2 vol. ice-cold ethanol

(in the presence of 1 M NaCl), DNA was washed repeatedly and dried *in vacuo*. Before enzymic hydrolysis with DNase I, venom phosphodiesterase and alkaline phosphatase [14], DNA (3–4 mg/ml 0.1 × SCC, pH 6.5–7) was heated in a sealed tube at 100°C for 1 h, precipitated as above, and washed twice in 0.01 M Tris/HCl (pH 7)–ethanol (1:3). The enzyme digest was adjusted to pH 8 and chromatographed on a 30 × 1 cm Dowex 50W-X4 column (Bio-Rad, Richmond, CA; –400 mesh, NH_4^+ form), using 0.1 M ammonium formate (pH 8) [14] as elution buffer. Fractions of 1.4 ml were collected.

For rechromatography appropriate fractions were pooled, ammonium formate removed by sublimation *in vacuo* and the residue redissolved in a small volume of water. This was chromatographed on precoated TLC plates (20 × 20 cm, with fluorescent indicator, all from Merck, Darmstadt) in the three systems detailed in the legend of table 1. Before running the chromatogram, the plates were dried for 2 min in an oven at 100°C. Radioactivity was measured by liquid scintillation counting using a scintillation cocktail containing Triton X-100 [12]. From the extinction of thymidine and deoxycytidine at 270 μM , using the molar extinction coefficients 9.6×10^3 and 8.9×10^3 , respectively, the DNA phosphate was calculated. Authentic O^2 -, O^4 - and 3-ethylthymidine were a generous gift from Dr B. Singer.

3. Results and discussion

A typical elution pattern of an enzymic digest of preheated DNA is shown in fig.1. After an initial

Abbreviations: DEN, diethylnitrosamine; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; ENU, *N*-ethyl-*N*-nitrosourea; SSC, standard saline citrate (0.15 M NaCl in 0.015 M sodium citrate); TLC, thin-layer chromatography

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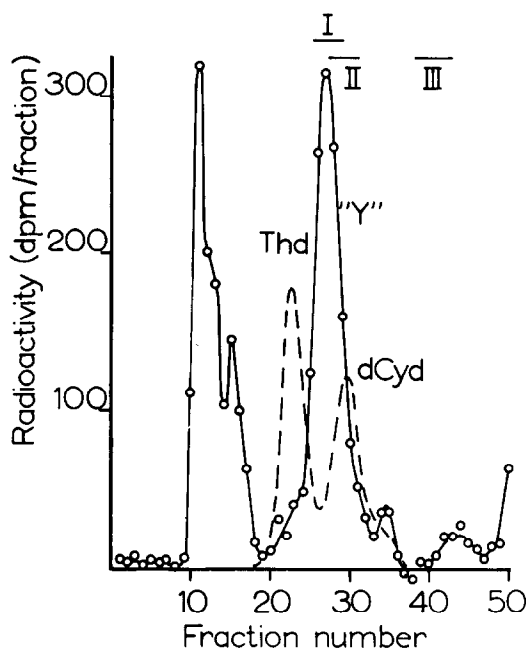


Fig.1. First 50 fractions (1.4 ml) of a Dowex-50 chromatogram of 6 mg rat liver DNA isolated 3 h after the application of 10 mg $[1\text{-}^{14}\text{C}]\text{DEN/kg}$ body wt (2 mCi/kg). The horizontal bars indicate the elution positions of authentic markers: I, O^2 -ethylthymidine; II, 3-ethylthymidine; III, O^4 -ethylthymidine. Dashed line: ultraviolet extinction of thymidine (Thd) and deoxycytidine (dCyd).

radioactive peak consisting mainly of oligonucleotides which were relatively resistant to enzymic hydrolysis (most probably because of modification at the phosphate groups), a sharp peak of radioactivity (termed 'Y') eluted between thymidine and deoxycytidine, followed (not shown in fig.1) by the purine deoxynucleosides, O^6 -ethyldeoxyguanosine, and ethylphosphotriesters [14]. A peak similar to 'Y' was observed [14] after methylation of DNA in vitro, and tentatively identified as 3-methylthymidine.

Peak 'Y', however, did not contain radioactive 3-ethylthymidine as shown by the lack of cochromatography with 3-ethylthymidine marker in all three TLC systems (table 1). Nevertheless, it was indicated that 'Y' contained a modified thymidine since $[^3\text{H}]\text{thymidine}$ -labelled DNA ethylated in vitro with ENU yielded radioactive material in the appropriate fractions of a Dowex chromatogram which behaved as 'Y' in the TLC systems (table 1). Com-

Table 1
 R_F values of 'Y' and authentic markers

Compound	TLC system ^a		
	I	II	III
$[1\text{-}^{14}\text{C}]\text{'Y'}$ ^b	0.75	0.04	0.24
$[^3\text{H}]\text{thymidine-}'\text{Y}'$ ^c	0.79	0.02	0.24
O^2 -ethylthymidine	0.73	0.06	0.26
3-ethylthymidine	0.9	0.4	0.63
O^4 -ethylthymidine	0.89	0.16	0.42
O^2 -ethylthymine ^d	e	e	0.6

^a System I, cellulose/ethanol-*n*-butanol-water (10:80:25, v/v/v); system II, silicagel/acetone-benzene (2:1, v/v); system III, silylated silicagel/acetone-benzene (2:1, v/v)

^b Radioactivity eluting from Dowex-50 between thymidine and deoxycytidine. Enzyme digest of rat liver DNA ethylated in vivo by $[1\text{-}^{14}\text{C}]\text{DEN}$ (see fig.1)

^c Same as ^b except enzyme digest of $[^3\text{H}]\text{thymidine}$ -labelled DNA ethylated in vitro by ENU. For chromatography in systems II and III, radioactivity of R_F 0.79, eluted from the cellulose plate (system I), has been used

^d Obtained by short acidic treatment (0.02 N HCl) of O^2 -ethylthymidine [16]

^e Not done

parison of authentic O^2 - or O^4 -ethylthymidine with 'Y' with respect to their chromatographic behaviour on the Dowex column (fig.1), showed that only O^2 -ethylthymidine eluted in the appropriate fractions (3-ethylthymidine eluted partially in the same fractions). O^4 -Ethylthymidine eluted later, as was expected from the behaviour of the methyl-analog [14]. The ^{14}C radioactivity obtained from peak 'Y' cochromatographed with O^2 -ethylthymidine in all three TLC systems used (table 1, fig.2), indicating that O^2 -ethylthymidine was formed in vivo after DEN application.

Conclusive evidence came from the acidic conversion of O^2 -ethylthymidine into O^2 -ethylthymine. O^2 -Ethylthymidine is very unstable in acid, the *N*-glycosyl linkage being broken within minutes at pH 1.5 [15]. Short treatment of O^2 -ethylthymidine marker and the cochromatographing radioactivity eluted from the TLC plate after chromatography in system III (fig.2, peak I), with 0.02 N HCl, and rechromatography in system III resulted in complete conversion of the marker and the radioactivity into a new product, and cochromatography of their extinction and radioactivity (fig.3).

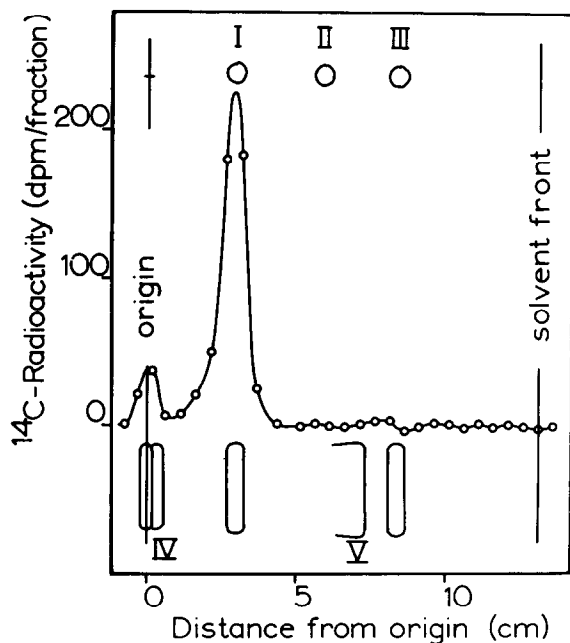


Fig. 2. Rechromatography (with internal markers O^2 - and 3-ethylthymidine) of part of 'Y' (see fig. 1) in TLC system III (silylated silicagel/acetone-benzene, 2:1). On top of the figure the positions of external markers: I, O^2 -ethylthymidine; II, O^4 -ethylthymidine; III, 3-ethylthymidine. Parent deoxynucleosides: IV, deoxycytidine; V, thymidine.

Quantitatively, 3 h after the application of 10 mg DEN/kg, rat liver DNA contained about 3 thymidine groups ethylated at the O^2 -position per 10^6 deoxynucleotides. This represented 6% of the total binding of ethyl groups to DNA.

Under the conditions of mild acid hydrolysis (0.1 N HCl, 70°C, 1 h or 37°C overnight) used to depurinate DNA completely before Sephadex G-10 chromatography, O^2 -ethylthym(id)ine is dealkylated [15]. Under such conditions of hydrolysis, O^2 -[1- 14 C]-ethylthymidine-containing DNA is expected to yield radioactive ethanol. A peak of radioactive ethanol, representing about 10% of the total ethylation, is indeed regularly detected in Sephadex G-10 chromatograms ([12], termed 'X') of rat liver DNA ethylated in vivo by [1- 14 C]DEN. It is concluded that part of peak 'X' is due to dealkylation of O^2 -ethylthym(id)ine during mild acid hydrolysis of DNA. Determination of O^2 -ethylthymidine in rat liver DNA at different times after the application of DEN

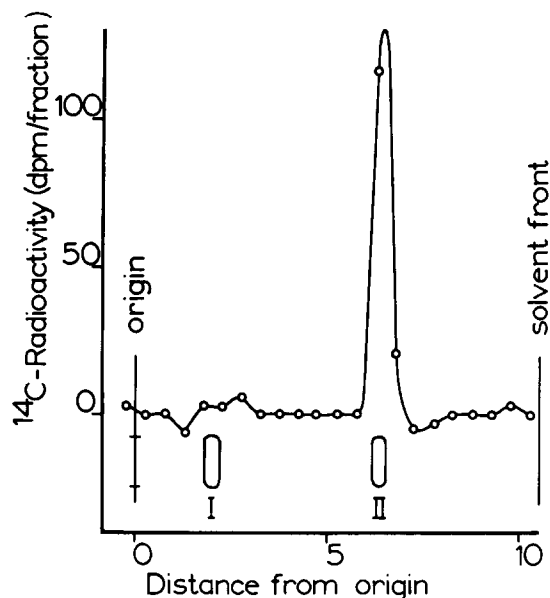


Fig. 3. TLC in system III of a short acid incubate (0.02 N HCl, 25°C, 5 min) of O^2 -ethylthymidine and part of the radioactive peak from TLC of 'Y' in system III (see fig. 2). O^2 -Ethylthymidine (I) has been added after incubation as internal marker.

revealed that O^2 -ethylthymidine is quite persistent in vivo: 2.2 μ mol/mol DNA-P were measured 2 weeks, and 1.2 μ mol/mol DNA-P 4 weeks after the application of DEN. These figures yield a half-life of about 20 days for O^2 -ethylthymidine in rat liver DNA.

From the known stability of 3-ethyl- and O^4 -ethyl-thymidine [15] under the conditions of hydrolysis used in the present investigation, it would have been expected that, if present, both these DNA adducts could have been detected in enzymic digests of rat liver DNA. However, neither 3-ethylthymidine (fig. 2) nor O^4 -ethylthymidine (fig. 1) were detected 3 h after the application of DEN. This is in partial agreement with published results. Studying salmon sperm DNA ethylated in vitro with ENU or ENNG, the former adduct was not detected [9], whereas the latter was only a minor product. In DNA of cultured cells ethylated with ENU, again no 3-ethylthymidine was detected. O^4 -Ethylthymidine, however, represented 2.1–4.3% of the total ethylation [10]. In the present study radioactivity eluting from the Dowex column in the vicinity of marker O^4 -

ethylthymidine amounted to $>1\%$ of the total radioactivity (fig.1). Together with the higher figures for O^4 -ethylthymidine in DNA ethylated in vitro or in cultured cells [10], this may indicate efficient removal of O^4 -ethylthymidine from rat liver DNA in vivo.

It is not known whether O^2 -ethylthymidine is a promutagenic lesion or not. From the in vitro RNA synthesis with synthetic polynucleotides containing O^2 -ethyluridine as template, it was concluded [6] that the presence of O^2 -ethyluridine increases the amount of misincorporation of GMP and CMP into RNA. However, based on experiments using poly-(dA-dT)methylated with methylnitrosourea or dimethylsulfate as template [11], it was argued that O^2 -methylthymidine does not miscode during DNA synthesis. They argued further that base pairing between O^2 -methylthymine and guanine is not indicated because of steric hindrance between the deoxyriboside moiety and the *O*-methyl group of O^2 -methylthymidine. However, even regular base pairing between O^2 -alkylthymine and adenine is not possible if the hydrogen on the *N*-3 position is lost to yield an unchanged base. Therefore, DNA synthesis may be hampered at the site of O^2 -alkylthymine, resulting in an increased error frequency during replication of O^2 -alkylthymine-containing DNA. The induction of mutations in vivo would be favoured by the persistence of this lesion. Since O^2 -ethylthymidine was quite persistent in rat liver DNA, its formation could be relevant for hepatocarcinogenesis.

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