O^2-ALKYLCYTIDINE - - A NEW MAJOR PRODUCT OF NEUTRAL, AQUEOUS REACTION OF CYTIDINE WITH CARCINOGENS

B. SINGER

Department of Molecular Biology and Virus Laboratory, and Space Sciences Laboratory, University of California, Berkeley, California 94720 USA

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1. Introduction

All of the positions available for nucleophilic displacements and addition reactions in purine bases of nucleic acids have been found to be reactive, to varying extents, when treated at or near neutrality with directly acting alkylating agents (N-1, N^2, N-3, N-7 and O^6 of G and N-1, N-3, N-7 and N^6 of A) [1]. In the case of thymidine, O-alkylation has generally been found only upon diazomethane reaction, except for a very small amount of O^4-methylthymidine arising upon N-methyl-N-nitrosourea treatment of salmon sperm DNA (0.1–0.6% of total methylation) [2,3]. Uridine in nucleic acids has been believed to be virtually unreactive toward alkyl sulfates and alkyl sulfonates [1]. However it has recently been shown that the N-3 of both uridine and thymidine is alkylated by methylating agents at neutrality [4]. Regarding O-alkylation, both O^2- and O^4-alkyluridines have been prepared and described [5,6]. Cytidine, on the other hand, reacts at the N-3 and N^4 under a variety of conditions [7] but the O^2 position has never been described as reacting with alkylating agents nor has O^2-alkylcytidine been synthesized to my knowledge. And indeed O^2 substitution has been considered to be impossible since the resulting compound was presumed to be very unstable. I now report that O^2-methylcytidine and O^2-ethylcytidine are major products of neutral, aqueous reaction of cytidine with the potent carcinogens, N-methyl-N-nitrosourea and N-ethyl-N-nitrosourea, respectively. Ethyl methanesulfonate and diethyl sulfate also react at this site but dimethyl sulfate which is not appreciably carcinogenic, does not alkylate cytidine on the O^2.

Abbreviations: MeNU, N-methyl-N-nitrosourea; EtNU, N-ethyl-N-nitrosourea; EtMS, ethyl methanesulfonate; Me_2SO_4, dimethyl sulfate; Et_2SO_4, diethyl sulfate; Me, methyl; Et, ethyl; Cyd, cytidine.

2. Materials and methods

Cytidine, 25 mg/ml, was treated with alkyl sulfates and EtMS* in a Radiometer pH-stat at the desired pH (± 0.1 unit). The pH of the cytidine-alkyl nitrosourea reaction was maintained by 0.2 M cacodylate buffer (pH 6.1 or pH 7.3). All reactions were performed at 20–25°C for 7 h with an excess of reagent present at all times. Electrophoresis of 5–20 mg of cytidine in pH 5.7 formate buffer was used to first separate the 'high pK' products (N-3 and O^2) from 'low pK' products (N^4 and unreacted cytidine) [7]. Further separation of the products was achieved by chromatography of each fraction in 80 butanol–25 ethanol–10 water on Whatman 3 MM, descending (20 h for ethylated samples; the methylated samples separate better in 48 h). The R_F of each is as follows: 3-Me Cyd 0.11, O^2-Me Cyd 0.22, N^4-Me Cyd 0.33; 3-Et Cyd 0.24, O^2-Et Cyd 0.37, N^4-Et Cyd 0.49; Cyd 0.15.

The amount of each derivative was based on the optical density using the following E_{max} ($\times 10^{-3}$): 3-alkylcytidine (pH 1), 11.8; N^4-alkylcytidine (pH 1), 14.3; O^2-alkylcytidine was de-alkylated, then determined as cytidine (pH 1), 13.4.

Reactions were also performed using [^{14}C]EtNU (2 μCi/mg) and [^{14}C]Et_2SO_4 (0.2 mCi/mmol). The labeled products were purified by repeated electrophoresis and chromatography until constant specific activity was achieved. These labeled derivatives were used to study the stability of the ethyl group. 3-Et Cyd and N^4-Et Cyd were recovered unchanged after treatment with 0.01 N NaOH (37°C, 2 h) or with 1 N HCl (100°C, 1 h) while O^2-Et Cyd was completely converted to cytidine upon such treatment. Derivatives isolated from alkylated cytidine were subjected to such NaOH and HCl treatment as one test for O^2 substitution.

3. Results

The extent of reaction of cytidine with the alkylating agents used varied greatly as did the proportion of alkyl groups bound to the N-3, N^4 and O^2 of the ring (table 1). In the case of EtNU reaction, at both pH 6.1 and 7.3, about half of the ethyl groups were bound to the ring oxygen, a third to the exocyclic amino group and the remainder ($\sim$ 15%) to the N-3. Conversely, under the same conditions Me_2SO_4 reacted almost completely with the N-3 position.

The identification of the new O-alkylcytidines was based on the following data. (1) The spectra at pH 1 and pH 7 (fig.1) were virtually identical to those of an analogous compound, O^2,2′-O-cyclic cytidine 3′5′

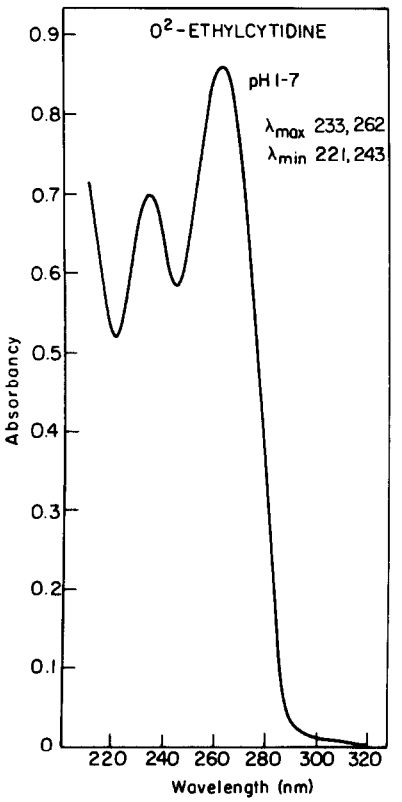


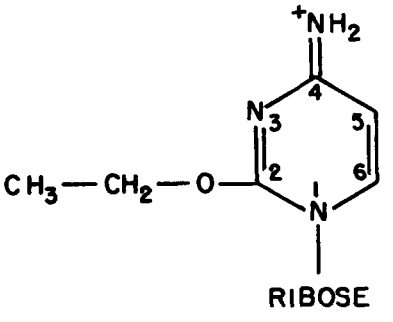
Fig.1. Ultraviolet spectrum of O^2-ethylcytidine in water. The λ_{max} and λ_{min} are given in the figure and are the same at pH 1 and pH 7. Above pH 9 the derivative begins to de-ethylate and the spectrum slowly changes to that of the basic form of cytidine. At pH 14 the de-ethylation is complete within 5 min at 20°C and the λ_{max} is 272 at pH 14 and 279 at pH 1.

Table 1
Amount and proportion of products of cytidine alkylation[a]

Reagent	pH	% Reaction	% of Total alkylation O^2-Alkyl	3-Alkyl	N^4-Alkyl
EtNU[b]	7.3	0.7	50	19	31
	6.1	7	52	13	36
EtMS	6.3	2.3	19	64	17
Et_2SO_4[b]	6.9	0.6	38	51	11
	6.3	1.2	26	63	11
MeNU	7.3	21	20	75	5
	6.1	29	24	69	4
Me_2SO_4	6.3	85	<0.05	99	1

[a]See Materials and methods for reaction conditions.
[b]Includes data obtained with ^{14}C labeled reagents.

diphosphate (a generous gift of C. A. Dekker) [8]. (2) At pH 12 (20°C), the compound was de-alkylated completely in 40 min to cytidine, conditions under which O^2,2′-O-cyclocytidine is also hydrolyzed, but in this case to arabinocytosine [9]. (3) 1 N HCl at 100°C for 60 min de-alkylates O^2 alkylcytidine. These conditions are also sufficient to de-alkylate other O-alkyl nucleosides such as O^6-alkylguanosine, O^2-alkyluridine and O^4-alkyluridine; (4) both ^{14}C reagents used (Et_2SO_4 and EtNU) gave a derivative with a specific activity indicating one alkyl group bound; and (5) the pK_a of O^2-alkylcytidine is greater than 9 since it migrates further in pH 9.2 ammonium carbonate electrophoresis than do 3-methylcytidine (pK_a 8.3–8.9) or 1-methyladenosine (pK_a 8.3–8.8). The high apparent pK would indicate that the derivative is a quaternary base, a necessity for any presumed structure such as:



4. Discussion

In a previous paper from this laboratory [7] we described an unknown derivative of high pK resulting from the neutral aqueous reaction of cytidine and poly C with Et_2SO_4 and EtMS which we suggested could be O^2-alkylated. The chromatographic behavior, spectrum and de-ethylation with acid or alkali of the purified compound now identify this derivative as O^2-ethylcytidine. While O^2-ethylcytidine is formed in poly C we have as yet no data on its presence in alkylated nucleic acids, since it is labile under the usual conditions of acid hydrolysis and can only be detected in enzyme digests when the digestion is under neutral conditions. In our recent study of ethylation of RNA and DNA in vitro and in vivo [10,11] enzyme digests were chromatographed in the same system described in Methods in order to detect O^6-ethylguanosine. However the area of the chromatogram which would contain any O^2-ethylcytidine was subsequently hydrolyzed in strong acid, a procedure which de-ethylates all O-alkyl nucleosides.

It is pertinent to note that EtNU primarily ethylates oxygens. In nucleic acids about 60–70% of the ethylation is on phosphodiesters, and another 10–12% is O^6-ethylguanosine [10,11]. In the present work about 50% of the ethylation of cytidine is on the oxygen. The correlation, suggested by us [10], that carcinogenesis by alkylating agents is related to affinity for oxygen alkylation, appears supported by the data in this paper.

In the last few years both 3-alkylcytosine and O^6-alkylguanine have been shown to mispair when incorporated into polynucleotides [12–15], in contrast to 7-alkylguanine which pairs normally [16]. In addition we have found a strong correlation between the extent of methylation of the N-3 of cytosine and mutation of TMV [17]. Alkylation of the O^2 of cytosine would be expected to also cause mispairing and thus be likely to be a mutagenic event. However any relationship between mutagenic and carcinogenic reactions is yet to be proven.

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