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Biochemistry

Nucleoside adducts are formed by cooperative reaction of acetaldehyde and alcohols: Possible mechanism for the role of ethanol in carcinogenesis

(mixed acetals/hydroxyethylation/exocyclic amino groups/mammary cancer)

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ABSTRACT **The exocyclic amino groups of ribonucleosides and deoxyribonucleosides react rapidly at ambient temperature with acetaldehyde and alcohols to yield mixed acetals [—NH—CH(CH$_3$)OR]. Nucleotides and nucleoside di- and triphosphates also react. Depending on the nucleoside used and on the relative amounts of aldehyde, alcohol, and water, preparative reactions reach equilibrium with yields up to 75% in a few hours. The structures have been confirmed by fast atom bombardment MS and proton NMR. Half-lives at 37°C have been determined, and maximum stability is in the pH range of 7.5–9.5. In the absence of alcohol, acetaldehyde-nucleoside adducts could be isolated at 4°C, but these were too unstable to characterize except for their UV spectra, also at 4°C. Ethanol is often present in human blood and tissues, and acetaldehyde is its initial metabolic product, as well as being formed by many other metabolic processes. Both chemicals have separately been implicated in carcinogenic and other cytopathologic processes, but no cooperative mechanism has been proposed. The reactions reported here are of biological concern because they also occur in dilute aqueous solution. These findings supply a mechanism by which ethanol can be covalently bound to nucleic acids under physiological conditions.**

The reactions of aldehydes with amino groups in aqueous solution are well known to form mono- or bishydroxyalkyl compounds [—NH—CHROH and —N—(CHROH)$_2$, where R = alkyl groups]. Schiff's bases (N=CHR) represent a minor product but have the advantage that they can be stabilized by reduction. The hydroxyalkyl products readily undergo reversal. However, conditions for isolation of hydroxymethyl nucleosides, resulting from formaldehyde treatment, have been studied by McGhee and von Hippel (1, 2) and, under their conditions using acetaldehyde, we have now made and studied the corresponding hydroxyethyl derivatives.

Since acetaldehyde has been classified as carcinogenic in animals (3) and is produced *in vivo*, and since a particular alcohol, ethanol, is present in many people at high levels and first is metabolized to acetaldehyde (4, 5), it appeared of interest to study the reactions of the amino groups of nucleosides with acetaldehyde in the presence of alcohols. We then observed that products, considerably more stable than the aldehyde addition products, are formed at room temperature and neutral pH by very rapid condensation of the initial labile addition product, the N-hydroxyethyl compound, with the alcohol: R—NH—CH(CH$_3$)OH + HOR′ → R—NH—CH(CH$_3$)OR′ + H$_2$O (in which R represents the purine or pyrimidine ring, and HOR′, the alcohol used).

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This type of nucleoside derivative, which can be termed a mixed acetal, has to our knowledge been described only once. Bridson *et al.* (6) refluxed protected nucleosides for 5 hr with formaldehyde and ethanol and reported analogous structures. We now find the acetaldehyde reaction with alcohols to proceed rapidly without heating. We report the isolation and characterization of reaction products from cytidine, deoxycytidine, adenosine, deoxyadenosine, 3-methylcytidine, and N^6-methyladenosine on the one hand; and with methanol, ethanol, propanol, and isopropanol on the other. The yields of the reactions, in the absence of added water, can be as high as 75% within a few hours under the gentle conditions mentioned above.

No DNA or nucleoside reactions with ethanol have been reported and none would be expected to occur under physiological conditions in the absence of an activating agent such as free radicals and/or exposure to UV or visible light (7–9). The reactions now described represent a new mechanism for ethanol to form nucleic acid derivatives.

METHODS AND MATERIALS

Reactions for preparative purposes were performed at 22–25°C with 5–50 μmol of nucleoside, usually in 1- to 3-ml total volume containing the alcohol, 0–10% H$_2$O depending on the solubility of the nucleoside, and 33–50% acetaldehyde. Aliquots were taken at various times (2–144 hr) to measure the extent of reaction. These were chromatographed on silica thin-layer plates (Eastman 1318) or Whatman 3MM paper, depending on the amount of reaction mixture to be applied. The same solvent, butanol/ethanol/H$_2$O, 80:10:25 (vol/vol) was used. UV-absorbing areas were eluted with water, 0.01 M ammonium carbonate (pH 8.5), or methanol. UV spectra were obtained on a Varian-Cary 219 spectrophotometer. This instrument, equipped with temperature control and a programmable repetitive scanner, was used for stability measurements. pK_a values were also determined spectrophotometrically (10).

High performance liquid chromatographic separations of eluates or total reaction mixtures were performed with a Bio-Rad programmable system, a Bio-Rad 250 × 4 mm Aminex HP-C cation column (NH_4^+ form) and 0.4 M ammonium formate (pH 9) at 25°C.

Proton NMR spectra in ^2H_2O were recorded on a Brucker AM 500-mHz Fourier transform spectrophotometer with dioxane as an internal reference.

Fast atom bombardment (FAB) MS was conducted on a Kratos MS-50 mass spectrometer equipped with a Kratos FAB ion source operating at 7-kV accelerating voltage (Kratos Analytical Instrument, Ramsey, NJ).

Acetaldehyde may have to be redistilled from aqueous H$_2$SO$_4$. [^{3}H]Deoxycytidine and [^{3}H]deoxyadenosine were obtained from New England Nuclear.

RESULTS

Isolation and Characterization of Mixed Acetals. The chromatograms of reaction mixtures showed only two strong 254-nm UV-absorbing bands representing the original nucleosides and their mixed acetals. The latter moved from 1.2 to 2.4 times further on thin-layer plates and up to 4.2 times further on paper than did the starting materials, depending on the alcohol and nucleoside used (Fig. 1). An example of the rates and extent of product formation is illustrated in Table 1. In addition to the nucleosides listed on this table, 3-methylcytidine was also studied. In 50% acetaldehyde/50% alcohol in the absence of water, it reacted 52% with methanol and 32% with ethanol after 144 hr. For mechanistic studies, diethylacetal [$CH_3CH(OC_2H_5)_2$] was used as the only reagent. The same products were formed at a very slow rate, even at 37°C.

When nucleoside diphosphates (ADP, CDP) were treated or triphosphate (dCTP) was treated with 1:1:1 (vol/vol) ethanol/acetaldehyde/ 0.05 M Tris, pH 7.8, and the reaction mixture was chromatographed on cellulose thin-layer plates (Eastman 12354) with 1 M ammonium acetate, pH 7/ethanol, 30:75 (vol/vol), the products moved considerably further than the starting material.

The UV spectra of the mixed acetals were not affected by the alcohol moiety. Reaction products of adenosine (Fig. 2) and deoxyadenosine showed an E_{max} shift of 4–6 nm to the higher wavelength (265 vs. 259) (Fig. 2). A similar shift was observed with the mixed acetals of N^6-methyladenosine. The cytidine products (Fig. 3), including CDP, showed smaller shifts (271 vs. 269 at neutrality). These compounds had, however, an additional maximum with a λ_{max} at 235 nm, which is not shown by the starting material (Fig. 3). The E_{max} of the cytidine products at pH 1 was 285 nm, compared to 278 nm for cytidine, with a similar red shift as well as a decrease in the λ_{min}. Such red shifts were reported for the hydroxymethyl derivatives by McGhee and von Hippel (1, 2). The 3-methylcytidine reaction product differed in not having a second λ_{max} but also showed the red shift. At low wavelengths (below 220 nm), additional differences were noted.

The molar extinction coefficients of all products were about 18% higher than those of the original nucleosides (Figs. 2 and 3). To calculate product yield from UV absorbancy, we corrected the total recovered absorbancy for the extinction coefficients.

HPLC was used to further characterize the products and monitor their purity. Retention times for cytidine and its methyl, ethyl, and propyl acetals were 7.4, 6.4, 6.8, and 8.4 min; the values for adenosine and its methyl, ethyl, and propyl acetals were 13.6, 16.2, 22.9, and 36 min.

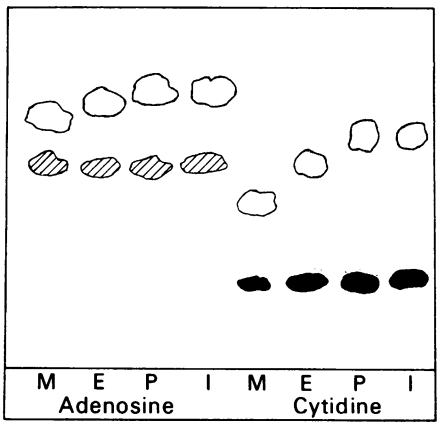

FIG. 1. TLC separation of unmodified adenosine (black areas) and cytidine (hatched areas) from mixed acetals formed by acetaldehyde and methanol (M), ethanol (E), 1-propanol (P), or 2-propanol (I). Detection was at 254 nm λ_{min}.

Table 1. Formation of nucleoside mixed acetals

Nucleoside	Mixed acetals formed, %					
	Methanol		Ethanol		2-Propanol	
	2 hr	20 hr	2 hr	20 hr	2 hr	20 hr
Cytidine	26	54	41	54	18	32
2′-Deoxycytidine	31	60	18	54	7	27
Adenosine	49	60	46	51	23	39
2′-Deoxyadenosine	45	55	49	54	11	47
N^6-Methyladenosine				11*		

Data are percentage yields of mixed acetals in a typical experiment with 2 mg of nucleoside, 0.1 ml of H_2O, 0.4 ml of alcohol, and 0.5 ml of acetaldehyde at 24°C for 2 hr and 20 hr.
*Twenty-two percent formed after 72 hr.

Fast atom bombardment MS confirmed the calculated molecular weights of the various products: N^4-propoxyethylcytidine, 329 (M + 1); N^4-methoxyethylcytidine, 301 (M + 1); and N^4-methoxyethyldeoxycytidine, 285 (M + 1); an apparent dimer of N^4-methoxyethyldeoxycytidine was also seen; N^6-ethoxyethyladenosine, 339 (M + 1); and N^4-methoxyethyl-3-methylcytidine, 316 (M + 1).

Proton NMR spectra of N^4-methoxyethyl deoxycytidine [δ1.41 (3H, d, J = 5.8 Hz, CH_3); 3.35 (3H, s = OCH_3); and 5.52 (1H, q, J = 5.8 Hz, αCH)] confirmed the assigned structure.

The alkoxyethyl group is by orders of magnitude more stable than the alkoxy group. Most $t_{1/2}$ values were determined at 37°C, and in general, stability was greatest in weak alkali (≈pH 9). The cytidine mixed acetals were several-fold more stable than the adenosine products (Table 2). In acid, reversal was rapid. Bridson *et al.* (6) reported similar acid

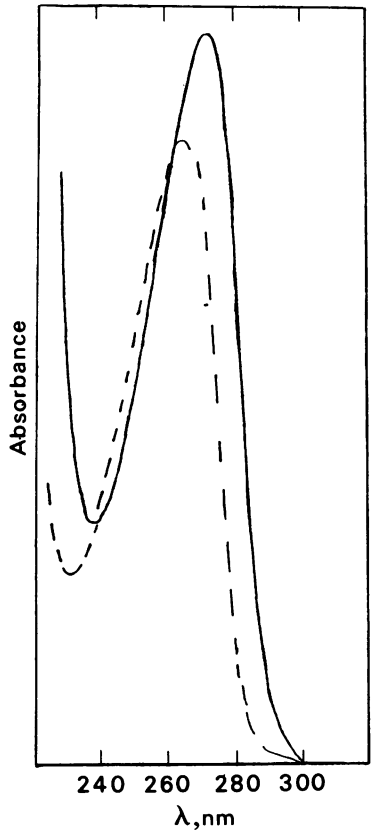

FIG. 2. UV spectra at pH 7 of N^4-ethoxyethyladenosine (—) and of adenosine (– –) formed after heating at 37°C. The relative extinction coefficients can be determined from these data.

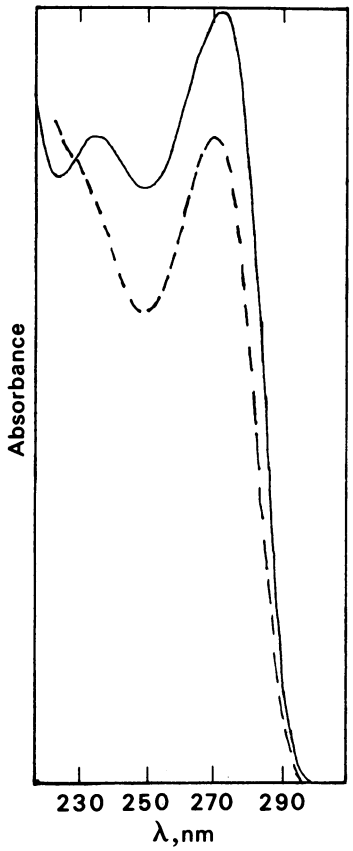

FIG. 3. UV spectra at pH 7 of N^6-ethoxyethyladenosine (—) and of cytidine (--) formed after heating at 37°C. The relative extinction coefficients can be determined from these data.

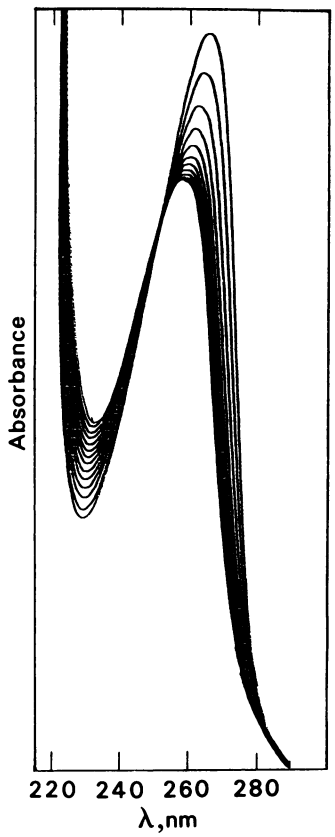

FIG. 4. Rate of loss of the ethoxyethyl group from the N^6 of N^6-ethylethoxyadenosine at pH 7 and 37°C determined by repetitive UV scans. The highest absorbancy is 0 time. Each scan represents 15 min. The total time shown is 3.5 hr.

sensitivity and alkali resistance for the ethoxymethyl group. An example of the rate of reversal of a mixed acetal is shown in Fig. 4. The initial kinetics appeared to be first order.

The determination of the pK_a values was made difficult by the acid-catalyzed reversal to unmodified nucleosides in the region of the pK_a values of cytidine and adenosine. The value obtained for N^4-ethoxyethyldeoxycytidine is ≈3.7, compared to 4.3 for deoxycytidine. Other related N^4 substituents also have a slightly lower pK_a than that of the parent nucleoside (11).

Reaction of Acetaldehyde with Nucleosides. Adenosine and cytidine treated with acetaldehyde alone (in the absence of added water and alcohol) produced within minutes UV-absorbing products. These could be isolated by rapid P2 gel filtration with 0.01 M ammonium carbonate (pH 8.5) at 4°C (1). Their spectra were identical to those of the corresponding mixed acetals, which indicates that the spectral shifts in Figs. 2 and 3 can be attributed to the aldehyde addition part of the reaction. These hydroxyethyl products also show similar properties to the hydroxymethyl products described by McGhee and von Hippel (1). The ethoxy group was released only slowly when stored at −70°C. During chromatography, both on paper or on silica plates, the cytidine derivative reversed completely, and the adenosine product partly.

Reactions in Aqueous Solution. Since water is formed during the reaction [R—NH_2 + $(CH_3)CHO$ + R′OH → R—NH—$CH(CH_3)OR'$ + H_2O], the reaction conditions are never anhydrous. Experiments using dilute aqueous solutions of deoxy[^{3}H]cytidine containing various proportions of ethanol and acetaldehyde (1–10%) have shown that the expected mixed acetals are formed, rapidly reaching equilibrium concentrations (Table 3). Although water would not

Table 2. Stability of nucleoside mixed acetals

pH	Nucleoside ethylacetal $t_{1/2}$ at 37°C	
	Deoxycytidine	Deoxyadenosine
1.0	7 min*	2 min†
3.6	≈0 min	≈1.3 min†
6.0	43 min	≈10 min
7.2	2.2 hr	30 min
7.5	24 hr	2.5 hr
7.8	24 hr	2.5 hr
9.4	>48 hr	>24 hr
11.0	>48 hr	10 hr
12.5	30 min	10 min
13.0	9 min	1.5 min

*At 18°C: pH 1, 40 min.
†At 18°C: pH 1, 4 min; pH 3.5, 6 min.

Table 3. Rate and extent of formation of N^4-ethoxyethyldeoxy[^{3}H]cytidine in dilute aqueous solution or pH 7.5 SSC at 22–24°C.

Reaction component, %				Product at time in minutes, %			
C_2H_5OH	CH_3CHO	H_2O	SSC	0	10	30	120
10	10	80	—	0.15	2.5	2.5	2.6
10	10	—	80	—	—	—	4.3
10	1	89	—	ND	0.35	0.43	0.47
10	1	—	89	1.1	1.1	1.2	1.0
1	1	98	—	ND	0.20	0.22	0.23
1	1	—	98	1.6	—	—	1.2

Products were separated from unreacted deoxycytidine using TLC as shown in Fig. 1. Radioactivity coinciding with authentic marker was used to determine the yield. Reaction mixtures contained 1 mg of deoxy[^{3}H]cytidine per ml. SSC, 15 mM NaCl/1.5 mM Na citrate; ND, not detected.

be expected to favor reaction, the lowest reagent concentrations used (1%, ≈200 mM), which more closely approximate physiological levels (4), do indicate that significant reaction occurs. Reaction in pH 7.5 standard sodium citrate solution markedly increased the rate and extent of product formation (Table 3). It appears that acetaldehyde concentration is the limiting factor.

DISCUSSION

We have synthesized a series of nucleoside derivatives that are modified on the exocyclic amino group by rapid reaction with acetaldehyde in the presence of various alcohols. The structures of the modifying group have the general formula —$CH(CH_3)OR$ where R represents an alkyl group. Such structures are optically active but diastereoisomers have not yet been resolved. The orientation of the modifying group may be *syn* or *anti*. However, N^6-methyladenosine has the methyl group syn to the N-1 (12), yet is able to form a mixed acetal, which must for reasons of steric hindrance be anti on the disubstituted N^6. This may generally not be the favored conformation because reaction of N^6-methyladenosine is markedly slower than that of adenosine (Table 1).

Bridson *et al.* (6) had reported a similar series of nucleoside derivatives by utilizing aqueous formaldehyde but found it necessary to reflux the reaction mixture for several hours. Under our conditions (22–25°C), formaldehyde does not form acetals even after 10 days of reaction, while subsequent addition of acetaldehyde to such a reaction mixture led to the formation of the expected mixed acetal. The difference in the ability of the two aldehydes must be attributed to the methyl side chain of acetaldehyde favoring the rapid condensation with alcohols at the lower temperature.

In another approach to the mechanism of this reaction, diethylacetal [$CH_3CH(OC_2H_5)_2$] was used instead of acetaldehyde and ethanol. That it gave by group transfer the same product with cytidine, but very much more slowly even at 37°C, also supports the sequence of initial binding of acetaldehyde to the amino group followed by alcohol condensation.

In general, reversal of aldehyde reactions is favored by water. We, however, find that mixed acetals are formed rapidly in aqueous solution containing only 200 mM each acetaldehyde and ethanol (Table 3). These conditions begin to approach those present in people consuming ethanol. Lieber *et al.* (4) report that alcoholics have blood ethanol concentrations up to 50 mM. *In vivo*, the two chemicals necessary for this nucleoside modification are always present as a result of drinking alcoholic beverages.

This newly described reaction may represent a unique situation in that the ingested chemical requires its first metabolite for modification of nucleic acids. These reactions could play a role in the recently documented association between moderate alcohol use and increased incidence of breast cancer. Both Willett *et al.* (13) and Schatzkin *et al.* (14) in long-term epidemiological studies of cohort groups of women concluded that any alcohol drinking increases risk 50–100%.

Of the derivatives described in this paper, N^4-ethoxyethyl deoxycytidine is similar in structure to other N^4-substituted cytidines that have been found to mispair and, thus, represent mutagenic events (15, 16).

Note Added in Proof. Guanosine was found to react in pH 7.8 buffered solution to form multiple unidentified products. Poly(rC) and poly(rA) also react with acetaldehyde and ethanol, in 80% water. The polymeric products are considerably more stable than are the isolated nucleoside mixed acetals.

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