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ALKALINE OPENING OF IMIDAZOLE RING OF 7-METHYLGUANOSINE. 2. FURTHER STUDIES ON REACTION MECHANISMS AND PRODUCTS*

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SUMMARY

High performance liquid chromatography (HPLC) was used to follow the kinetics of the alkaline induced opening of the imidazole ring of 7-methylguanosine (7-meGuo). The kinetics show an initial rapid formation of a major transient intermediate and some minor products that were chromatographically separable into seven peaks. This phase of the reaction is followed by the formation of a dominant pyrimidine derivative whose liquid chromatography retention time in a 6% methanol, 0.01 M $NH_4H_2PO_4$ (pH 5.1) solvent is 6 min; during the rest of the reaction time this dominant species was progressively converted to a co-dominant species that has a 4.5-min column retention. Mass spectroscopy confirmed the existence of two species of ring opened 7-methylguanine (7-meGua), one formylated and another deformylated. Schiff's reaction demonstrated that the species in the second HPLC peak is the formylated one. The ring opened 7-methylguanine (rom^{7}Gua) released by formamidopyrimidine (FAPy)-DNA glycosylase was shown to coelute with the formylated species. These results demonstrate that the enzyme excises formylated rom^{7}Gua from DNA Analysis of rom^{7}Guo by NMR showed that there are two signals assignable to methyl protons and two to formyl protons. These chemical shifts were interpreted as being due to the opening of the imidazole ring at two sites and to the formation of formylated and deformylated rom^{7}Gua.

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Abbreviations: NMR, nuclear magnetic resonance; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; FAPy, formamidopyrimidine is used synonymously with formylated rom^{7}Gua; roAde, ring opened adenine; r_T = retention time; AFB_1-Gua, aflatoxin B_1-guanine.

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INTRODUCTION

The opening of the imidazole ring of purines by alkali has been studied by a number of investigators [1--5]. In the case of guanine it is now known that its ring fission by alkali is expedited by the presence of a ribose and a 7-alkyl group. The N-7 alkylation of guanosine generates a quaternary N-7 site resulting in electron deficiency in the imidazole ring [6]. In nucleic acid polymers this alkylation labilizes the glycosyl bond and leads to spontaneous chemcial depurination of guanine adducts [7]. On the other hand, rom^{7}Gua residues in nucleic acids become very stable and less prone to spontaneous release [8].

A full understanding of the basic mechanisms of alkaline ring fission remains enigmatic. Hecht et al. [9] have proposed a scheme for alkaline ring opening of 7-meGua involving a nucleophilic attack of the C-8 site by a hydroxyl ion. The resulting intermediate is a carbinolamine in which the presence of a hydroxyl group and a proton at C-8 changes the double bond between N-7 and C-8 to a single bond. The resulting rearrangement of electrons leads to a ring strain relievable by fission of the imidazole ring.

The position assigned to the formyl group in the resulting pyrimidine derivatives is N^5 [3,10]. The fact that no evidence has been presented for this assignment of the position of the formyl group leaves unanswered the question as to whether this group is on the N^5 or N^6 or both positions of rom^{7}Gua. The assignment of the formyl group to the N^5 position of ring opened 7,9-di(2-hydroxyethyl)guanine [2] where the presence of hydroxyethyl groups at both N-7 and N-9 positions would be assumed to provide equivalent electron environments at these two nitrogen sites is a case in point. Smith et al. [11] demonstrated the formation of two structural isomers upon ring opening of 1,2,3,5-tetramethylbenzimidazole, a compound rather similar to 7-meGua.

We have examined the kinetics of the reaction of the alkaline opening of the imidazole ring of 7-meGuo and have further analyzed the major products by proton NMR, mass spectroscopy and the Schiff reaction for aldehydes. Our results indicate that the ring opening reaction leads to the formation of both formylated and deformylated rom^{7}Gua as well as leading to deribosylation of rom^{7}Guo. Evidence suggesting the opening of the imidazole ring at two different sites is discussed.

MATERIALS AND METHODS

HPLC. The details of our HPLC conditions are given in the accompanying paper [8]. In most separations a 3% or 6% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1) solvent system was used isocratically. In other separations, elution with the above solvent was followed by further elution with 90% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1). All HPLC separations were performed at 24°C.

Kinetics of alkaline imidazole ring opening in 7-meGuo. A 2 mg/ml solution of 7-meGuo in 0.2 N NaOH was incubated at 37°C. At selected

time intervals 10 μl aliquots of reacted material were collected and analyzed by HPLC using a 6% methanol, 0.01 M $NH_4H_2PO_4$ (pH 5.1) solvent system. By observing the changing array of chromatographic peaks and retention times exhibited by the reacting 7-meGua at progressive stages of incubation, we were able to follow the kinetics of the imidazole ring opening reaction.

Mass Spectrometry. A Finnigan automated gas chromatograph/E_1-C_1 model 4023 mass spectrometer interfaced with a Tektronix model 4010 data system was used. The output from the elementary analysis of rom^{7}Gua preparations was recorded on a Printronix model P 150 printer. We used rom^{7}Guo which had been treated with 85% formic acid to remove ribose residues [10] in this analysis; some formic acid treated material is poorly resolved due to extensive fragmentation by electron impact.

For obtaining mass spectra the solid sample was placed in a small cup and introduced into the source through a vacuum lock. The sample cup was heated according to a programmed sequential rise in temperature. Mass spectra were recorded at a source temperature of 290°C.

Proton NMR spectroscopy. A Bruker 360 and a Varian 60 MHz NMR spectrometer were used in analyzing rom^{7}Gua. Spectra from the 360 MHz spectrometer showed more details. As a standard we have used the compound 2,6-diamino-3-methyl-4-hydroxy-5-formamidopyrimidine (Vega Biochemicals, Tucson, AZ), a structural isomer of rom^{7}Gua. This is the only compound we have been able to obtain whose structure is closest to that of rom^{7}Gua. Samples in D_2O were placed into 5 mm tubes and the spectra taken at a frequency of either 60 MHz or 360 MHz at 24°C. The chemical shift (δ) of protons are expressed in parts per million (ppm) and in terms of Hz from tetramethylsilane (TMS).

Schiff's reaction with rom^{7}Gua. For the calibration of our reaction system, we used benzaldehyde as a standard. Varying amounts of benzaldehyde (0—200 μmol) were taken up in 1 ml of 90% acetic acid and incubated for 45 min at 55°C. After allowing the material to cool to 24°C, 1 ml of Schiff's reagent was added and incubation carried out for 15 min. Two milliliters of *t*-butanol saturated with SO_2 were added and the system allowed to react for 2 min at 24°C. The reaction products were quantitated by measuring the A_{570} of each sample [12].

The reaction of rom^{7}Gua with Schiff's reagent was carried out in a similar manner and the amount of formylated rom^{7}Gua estimated from the A_{570} of the reaction products. We also found it convenient to react rom^{7}Gua with Schiff's reagent and then analyze and quantitate the reaction products by HPLC. The HPLC column was first eluted with 3% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1); further elution was carried out with 90% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1) to remove both unreacted Schiff's reagent and rom^{7}Gua-Schiff's reagent complexes from the column.

For simple qualitative determination of the presence of formyl groups in rom^{7}Gua, a given species of acidified rom^{7}Gua was reacted with Schiff's reagent at 24°C. The development of a pink or purple color is a positive test for the formation of a Schiff base in this reaction.

Reaction of enzyme released rom^{7}Gua with Schiff's reagent. A 0.12-ml

reaction mixture consisting of 0.05 M Hepes/KOH (pH 7.4), 0.1 M KCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, [^{3}H] FAPy-DNA (2200 cpm) and 10 microunits of FAPy-DNA glycosylase was incubated for 30 min at 37°C [13]. The material was filtered through a Sep-pak to remove protein and DNA from the enzyme released product which was recovered in the filtrate.

The filtrate was reacted with Schiff's reagent. During subsequent analysis of the product by HPLC, fractions were collected at 1 min time intervals. The first set of fractions was eluted with 3% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1); the last set of fractions was eluted with 90% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1). For measuring radioactivity each fraction was mixed with 10 ml of aqueous counting solution and then counted in a Beckman LS-8100 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Kinetics of conversion of 7-meGua to pyrimidine derivatives by alkali. We have attempted to follow the time course of the alkaline induced conversion of 7-meGuo to its imidazole ring opened derivatives at 37°C. When a 2 mg/ml solution of 7-meGuo in 0.2 N NaOH was incubated at 37°C, HPLC analysis of 10-μl aliquots collected at selected time intervals over a 45-h time period made possible the identification of putative reaction products as well as pathways followed by the process of ring opening of 7-meGuo. We shall refer to these reaction products by the number of peaks in which they elute.

The results in Fig. 1 indicate that the conversion of 7-meGuo to rom^{7}Guo goes through a number of intermediate products. The HPLC profiles of the sample collected from the reaction mixture immediately following the addition of alkali (approx. t = 0 min) indicates that 7-meGuo is rapidly converted to a number of transient intermediates (Fig. 1a), the major one being product 5 (peak 5). Thus most of 7-meGuo is initially converted to product 5 (r_T = 5 min) with product 6 (7 min) being next in amount, while products 1 (3.5 min) and 3 (4.5 min) are the lowest in amount. As product 5 disappears rapidly at 37°C, we undertook to determine its half-life by treating 7-meGuo with 0.2 N NaOH at 24°C and collecting 10-μl aliquots every minute for HPLC analysis. The rate of decrease in size of peak 5 showed that product 5 has a half-life of 2 min. Peak 8 (8.3 min) contains a species presumed to be unreacted 7-meGuo as deduced from the retention time. Formate ions elute in peak 1. A minor peak eluting with r_T = 12.5 min corresponding to that of 7-meGua was observed (data not shown). Our monitoring of the column effluents at 265 nm made it impossible for us to detect the elution position of ribose as it does not absorb UV light; thus ribose can not account for any one of the peaks in Fig. 1.

After 10 min peak 3 increases in size to surpass both peaks 2 (3.8 min) and 4 (4.5 min) of (a) while peak 8 (8.3 min) of (a) has disappeared (Fig. 1b); presumably all of the 7-meGuo has now been converted to rom^{7}Guo. Most

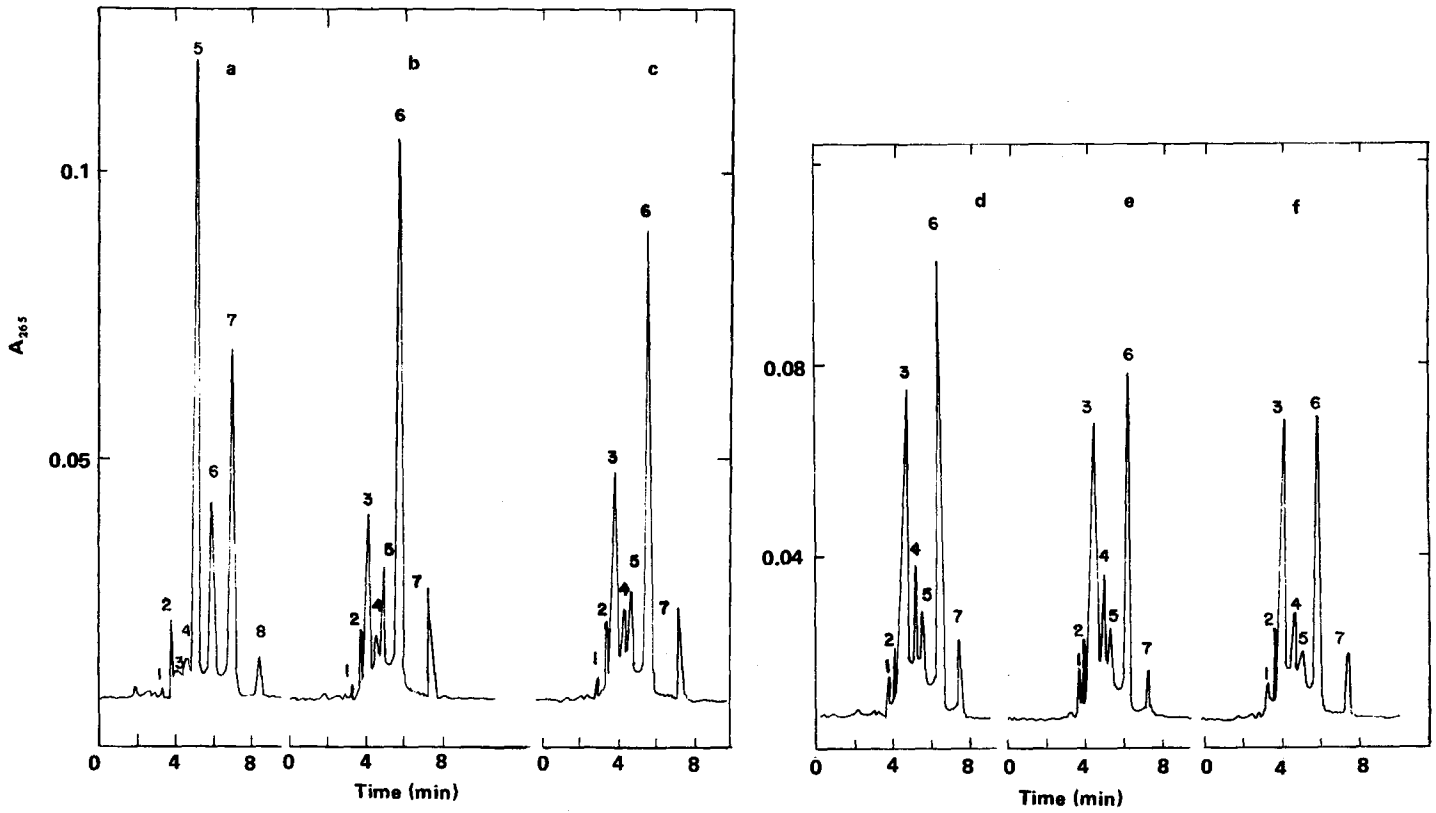

Fig. 1. Kinetics of alkaline ring fission of 7-meGuo as revealed by HPLC. About 2 mg of 7-meGuo in 1 ml of 0.2 N NaOH was incubated at 37°C. At selected time intervals 10-μl samples were collected and resolved by HPLC. The incubation time for the sample with HPLC profile in (a) = 0 min, (b) = 10 min, (c) = 20 min, (d) = 40 min, (e) = 120 min and (f) = 4.5 h. The solvent system was 0.01 M $NH_4H_2PO_4$ (pH 5.1), 6% methanol.

of the material that had been in peak 5 (5 min) of (a) seems to be now converted to product 6 (6 min) of (b); next in amount is product 3 in the newly emerged peak 3 (4.3 min). (c)—(e) show a progressive decline in the amount of product 6 (6 min) accompanied by an increase in the size of peak 3 (4.3 min). It can also be observed that peak 5 which was the largest one in Fig. 1a becomes greatly reduced in height in (e). Thus the major proportion of the reaction going on beyond the first 10 min, and on up to the first 2 h of treatment with alkali (Fig. 1e) is the conversion of product 6 to the species steadily accumulating in peak 3. After 4.5 h peaks 3 and 6 become equal in size; this equal distribution of material between these two peaks remained the same after 45 h. These results suggest that the reactions for preparing these two forms of rom^{7}Gua in 0.2 N NaOH require up to 4.5 h of incubation to reach completion. It appears that products 3 and 6 represent the final products from alkaline treatment of 7-meGuo; thus peaks 3 and 6 seem to contain two species of rom^{7}Gua which are in equilibrium. Attempts at collecting the material from peak 5 (Fig. 1b) were unsuccessful. One reason for this failure is that the material in the adjacent peaks could not be sufficiently eliminated. Secondly, the half-life of peak 4 material (approx. 2 min at 24°C) precluded further processing of this material as it quickly became converted to product 6. Products 3 and 6 correspond to products 1 and 2 below.

At the present time we have not yet studied the products in the minor peaks of (a) and those in peaks 1, 2, 4, 5 and 7 of (b)—(f) (Fig. 1). When alkaline treated 7-meGuo is subsequently treated with 85% formic acid in order to remove the ribose residues, peaks 1, 2, 4, 5 and 7 in (b)—(f) as such are all practically eliminated from the preparation; the HPLC profiles of such samples will show the presence of some residual background material. It should be pointed out that the HPLC profile of rom^{7}Guo in Fig. 1a is more readily obtained if the reaction is carried out at 24°C or at an even lower temperature.

Mass spectra. Figure 2 depicts a typical electron impact mass spectrum of rom^{7}Gua. The relative intensities of the species corresponding to the various *m/e* ratios are normalized to that of the species giving the highest peak (*m/e* = 96) which is assigned an intensity of 100%. It will be observed that there is no peak corresponding to 7-meGuo (mol. wt. 298), apparently due to its fragmentation, to give rise to smaller products. There is also no peak attributable to rom^{7}Guo (mol. wt. 315). Alkaline ring opening is known to hydrolyze the ribose residues (mol. wt. 133) from the base [4,5]. The peak at *m/e* = 183 is attributed to formylated rom^{7}Gua. The peak at *m/e* = 155 is attributed to deformylated rom^{7}Gua and the *m/e* = 154 attributed to deformylated rom^{7}Gua from which a proton, perhaps the N-1 proton, has been lost. The presence of *m/e* = 183 and *m/e* = 155 peaks in the mass spectrum is consistent with the presence of both formylated and deformylated species of rom^{7}Gua in our sample of rom^{7}Gua [8].

There are several other fragments whose *m/e* ratio peaks are rather prominent. One of these fragments has a peak at *m/e* = 148 which may result from

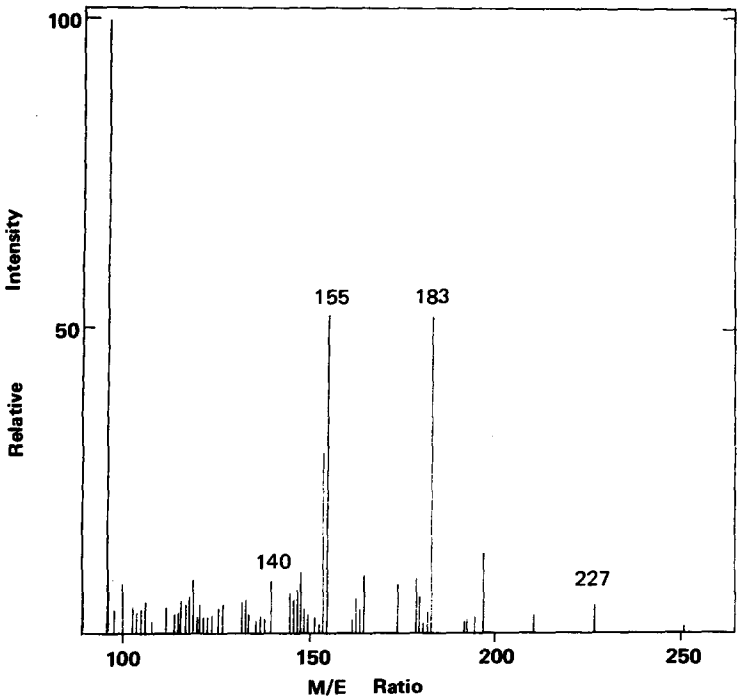

Fig. 2. Electron impact mass spectrum of rom^{7}Gua. A sample of 7-meGuo was treated for 4 h with 0.2 N NaOH at 37°C. The mass spectrum was taken after the sample had been treated with acid and then lyophilized.

a combined loss of both an amino group and the C-4 hydroxyl group from rom^{7}Gua [4]. The peak at *m/e* = 140 is attributed to loss of a formamido radical from rom^{7}Gua. Formation of fragments has been observed by other investigators [14,15] under comparable conditions.

Schiff's reaction for the detection of aldehydes. We further attempted to determine which of products 1 and 2 (Fig. 3a) is formylated rom^{7}Gua by using the well known reaction between aldehydes and Schiff's reagent. We reacted 10 μg of rom^{7}Gua with Schiff's reagent to determine which of the two HPLC peaks contains the rom^{7}Gua species which participates in the formation of a Schiff base.

Figure 3b shows that it is the species of rom^{7}Gua retained for 8 min (see peak 2 of Fig. 3a) which disappears after the reaction with Schiff's reagent. A 3% methanol solvent system was used to obtain the elution profiles in (a) and (b). During calibration procedures we established that the material retained for 4—5 min is largely acidic components derived from Schiff's reagent. The profile in (c) was obtained when a 90% methanol solvent system was subsequently used in the column from which profile (b) had been obtained. The region under peak 1 of (c) consists of unreacted Schiff's reagent. The Schiff base consisting of rom^{7}Gua-Schiff's reagent complexes

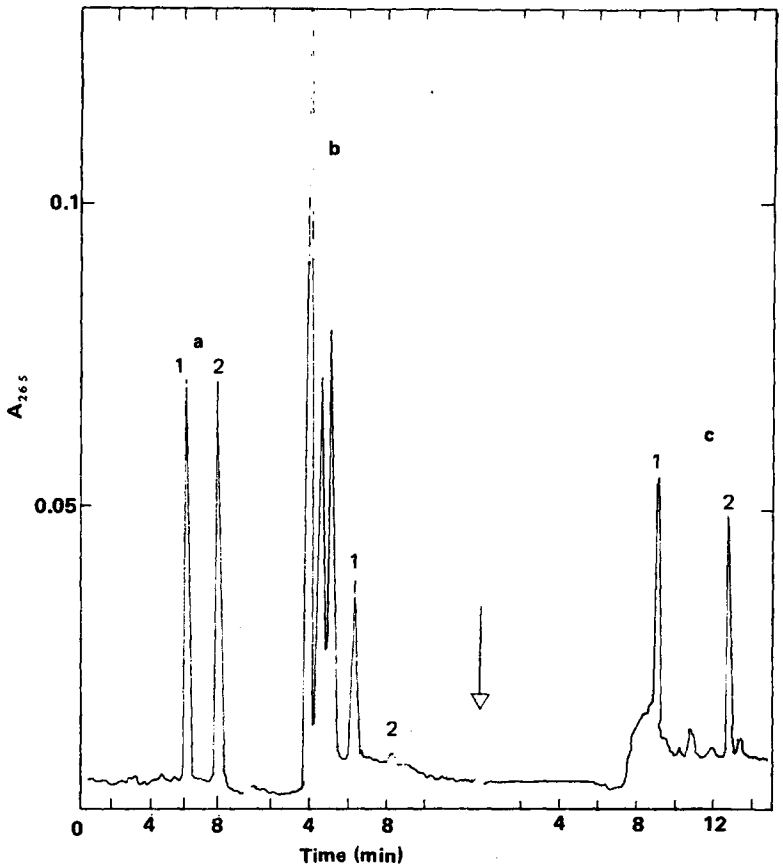

Fig .3. Schiff base formation. A sample of rom^{7}Gua with HPLC profile shown in (a), was reacted with Schiff's reagent and the reaction products analyzed by HPLC using a 3% methanol, 0.01 M $NH_4H_2PO_4$ (pH 5.1) system (b). Then a 90% methanol, 0.01 M $NH_4H_2PO_4$ (pH 5.1) solvent system was used to elute the Schiff base complex (c).

was recovered in material under peak 2 (r_T = 12.5 min). The spectroscopic properties of this complex (λ_{max} 570) are characteristic of the Schiff base [12].

These results demonstrate that it is the material retained for 8 min that contains formylated rom^{7}Gua. The material retained for 6.3 min thus represents deformylated rom^{7}Gua. Schiff's reaction perturbs the equilibrium between the two species. The peak size for the deformylated species is smaller in (b) than it is in (a); this reduction may occur during a compensating reformylation to reestablish equilibrium. The reformylated rom^{7}Gua could simultaneously react with Schiff's reagent to form a Schiff base (r_T = 12.5 min).

Schiff's reaction with enzyme released rom^{7}Gua. As a further test for the form of rom^{7}Gua that is released from [^{3}H]rom^{7}Gua-DNA by FAPy-DNA glycosylase, we reacted enzyme released rom^{7}Gua with Schiff's reagent.

The reaction products were analyzed by HPLC. Figure 4a shows that the elution positions of deformylated rom^{7}Gua (r_T = 6.3 min) and formylated rom^{7}Gua (r_T = 8 min) contained small amounts of [^{3}H] rom^7 Gua. More than 80% of the rom^{7}Gua reacted with Schiff's reagents to form a Schiff base which is elutable in peak 2 with 90% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1). The resulting Schiff base has r_T = 12.5 min.

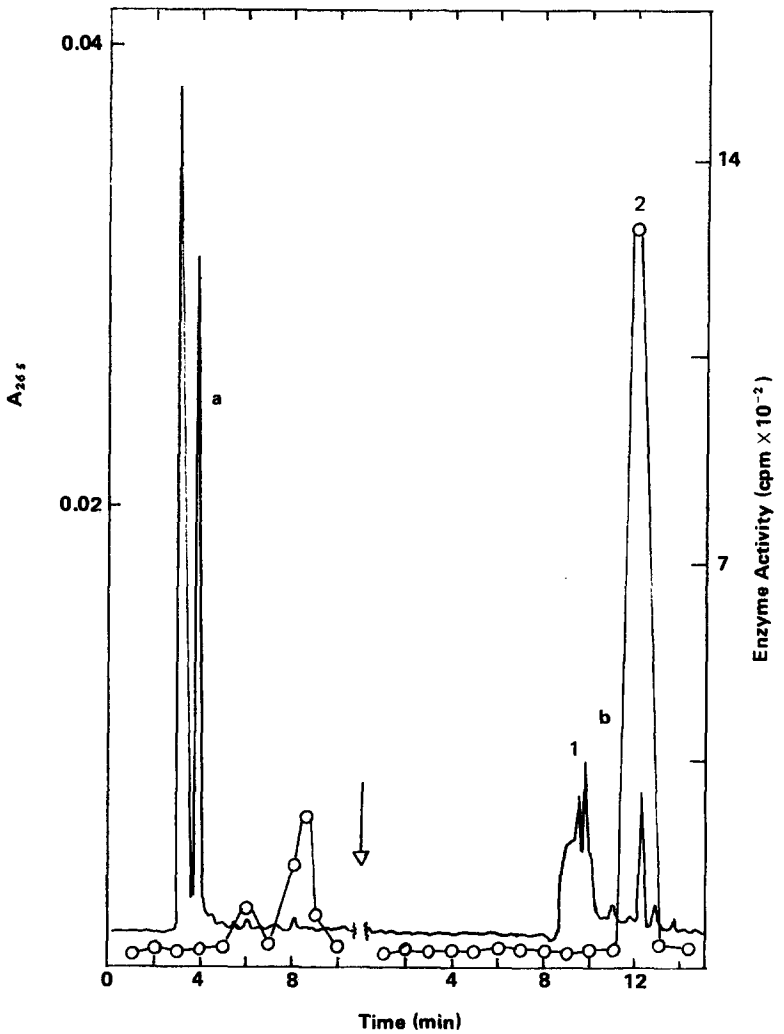

Fig. 4. Schiff's reaction for enzyme released aldehydes. The products released from [^{3}H]FAP_Y-DNA (2200 cpm) by 10 microunits of enzyme were filtered through a Sep-pack. The filtrate was reacted with Schiff's reagent and the products analyzed by HPLC. Each fraction was collected during a 1 min interval. Fractions in (a) were eluted with 3% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1) and those in (b) with 90% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1). Arrow indicates point at which the 90% methanol solvent was applied.

These results clearly demonstrate that it is the formylated form of rom^{7}Gua that is released by FAPy-DNA glycosylase [16]. Our preliminary results indicate that formylated rom^{7}Gua is the main product formed when DNA containing 7-meGua is treated with 0.2 N NaOH. Kohn and Spears [17] showed that DNA containing alkylated [8-^{14}C]guanine, when treated with alkali, does not lose the ^{14}C-labeled formyl groups. It was the subsequent treatment of the DNA with $HClO_4$ and Hg^{2+} that they found to remove the formyl groups and to liberate the ^{14}C in the form of $^{14}CO_2$.

Figure 5 presents a scheme of a possible series of steps in the pathway for the imidazole ring opening reaction. The initial phase involves a hydroxide ion attack on C-8 to produce transient carbinolamine isomer IIA or IIB. In kinetic studies this transient intermediate would elute in the major peak (r_T = 5 min) observed immediately after adding alkali to 7-meGuo (Fig. 1a). The half-life of the carbinolamine intermediate in 0.2 N NaOH is 2 min.

The formation of a carbinolamine intermediate is the mechanism generally postulated to lead to the scission of the imidazole ring of purines like guanine adducts and adenine. In aflatoxin B_1 (AFB_1)-guanine adducts, Miller et al. [18] have proposed the formation of a carbinolamine intermediate precedent to the generation of ring opened pyrimidine derivatives. The AFB_1-Gua adducts resemble 7-meGua in that AFB_1 is attached to the N-7 position of guanine. Imidazole ring fission at the C-8 to N-9 bond thus leads to formation of AFB_1-FAPy. Garrett and Mehta [5] have studied the conditions for opening the imidazole ring of adenosine and found that efficient ring scission in this case was achieved by using 1.0 N NaOH at 80°C. This fission was also proposed to go through a carbinolamine intermediate. In the case of 7-meGuo, we are proposing that subsequent to the formation of the carbinolamine, ring fission leads to the production of structure IIIA or IIIB (Fig. 5) depending on whether the ring opens between N-7 and C-8, or between C-8 and N-9 [11].

We further propose the formation of another transitional structure IVA or IVB before the loss of formate ions to give rise to deformylated rom^{7}Gua (V). Isomers IVA and IVB could be responsible for some peaks that appear in kinetic studies (Fig. 1 (a—f)). A similar intermediate has been proposed to precede the generation of deformylated ring opened adenine (roAde) [5].

It appears that it is structures III and V that exist in equilibrium and are responsible for the twin HPLC peaks observed with most preparations of rom^{7}Gua. The conversion of structure III to V in 0.2 N NaOH takes 4 h to reach equilibrium; such a slow rate of deformylation of structure III suggests that the conversion pathway goes through some intermediate(s) or that the alkalinity of the medium slows the conversion. The formate ions released during the deformylation of III are held in reserve and can later reformylate V under conditions which skew the equilibrium in favor of III [8].

Ion exchange chromatography using ammonium formate buffer (pH 8.9) (Dowex 50 NH_4^+ form) provides rom^{7}Gua which consists of 90% structure III [8]. It appears that these chromatography conditions provide formate ions

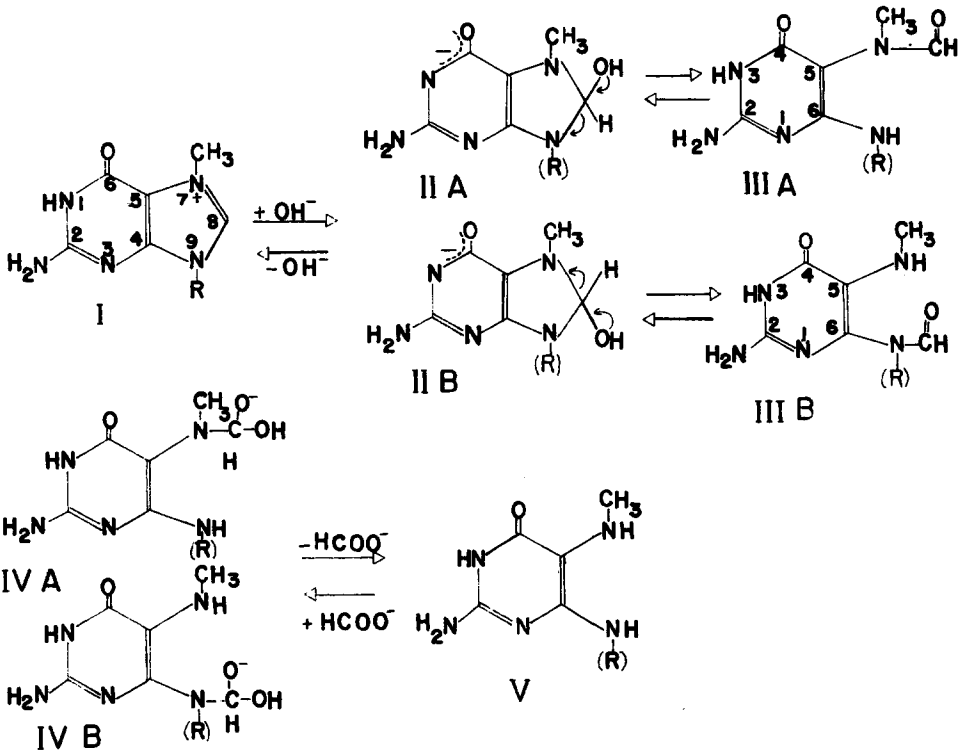

Fig. 5. A scheme proposed to explain the reaction pathway through which 7-meGuo (I) goes during alkaline induced opening of the imidazole ring. R = ribose; the brackets around R are meant to denote that some of structures II—V are deribosylated.

that promote the reverse reaction from deformylated rom^{7}Gua (V) through structure IV and back to the formylated species (III). The rom^{7}Gua released by the enzyme from FAPy-DNA substrates coelutes with structure III when analyzed by HPLC.

Proton NMR spectra. We have been unable to completely separate the material in each of the two HPLC peaks of rom^{7}Gua. Some success was had in recovering material containing 90% formylated rom^{7}Gua (r_T = 8 min) and 10% deformylated rom^{7}Gua (r_T = 6.3 min) as estimated by the peak heights. This material as well as rom^{7}Gua was analyzed by proton NMR with similar results.

Among the questions we attempted to resolve by NMR was whether one can distinguish between the chemical shifts due to the protons of methyl groups in formylated and those in deformylated rom^{7}Gua, and whether formylated rom^{7}Gua consists of two isomers, one with N^5 formyl groups and another with N^6 formyl groups, as in structures IIIA and IIIB (Fig. 5). The formation of such structural isomers from alkaline imidazole ring opened heterocyclic compounds is not without precedent. Smith et al. [11] showed that two structural isomers are produced when the imidazole

ring of 1,2,3,5-tetramethylbenzimidazole is opened by treatment with alkali.

The NMR spectrum of 2,6-diamino-3-methyl-4-hydroxy-5-formamidopyrimidine is depicted in Fig. 6. The spectrum shows a singlet at $\delta = 3.4$ attributed to the protons of the N-3 methyl group. The singlet at $\delta = 8.7$ represents the chemical shift of the N^5-formyl proton. In the spectrum obtained with our rom^{7}Gua preparation (Fig. 7), the chemical shifts assigned to the protons of the formyl groups are observed as a downfield quartet centered around $\delta = 7.9$ and a triplet centered around $\delta = 8.38$. *N*-Formyl protons are generally known to give signals in the 8.03—8.70 ppm region [19,20]. Townsend and Robins (3) assigned a $\delta = 8.15$ signal to the formyl proton to rom^{7}Gua. In the NMR spectrum of rom^{7}Gua-AFB$_1$, Lin et al. [8] assigned the singlet at $\delta = 8.36$ to the *N*-formyl proton.

The upfield region of Fig. 7 consists of two sets of quartets, one centered at $\delta = 2.95$ and the other at $\delta = 3.3$. The signals in the 2.8—3.5 ppm region

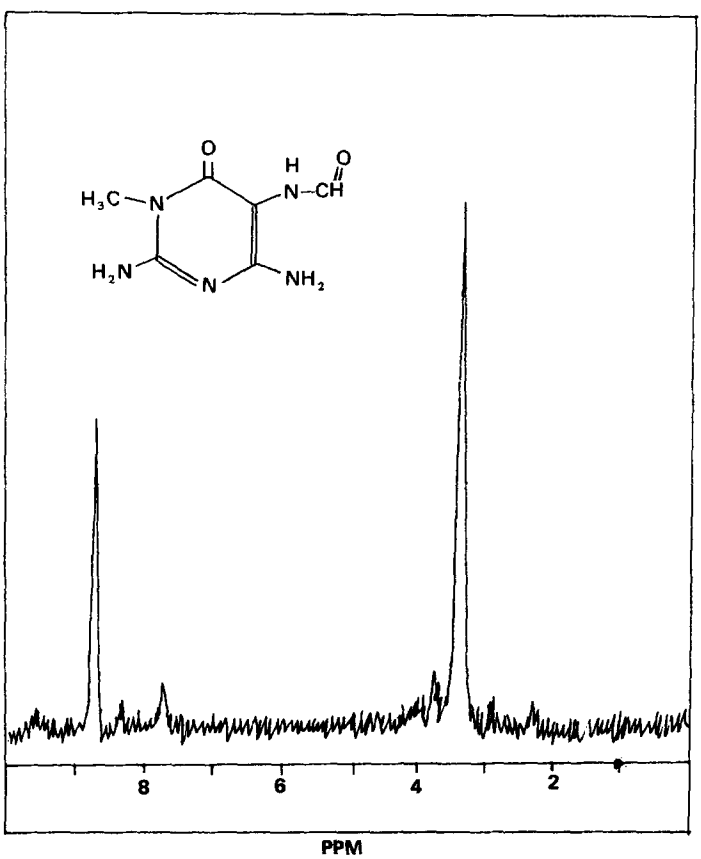

Fig. 6. Proton nuclear magnetic resonance spectrum of 2,6-diamino-3-methyl-4-hydroxy-5-formamidopyrimidine in D_2O. The spectrum was taken with a 60 MHz spectrometer.

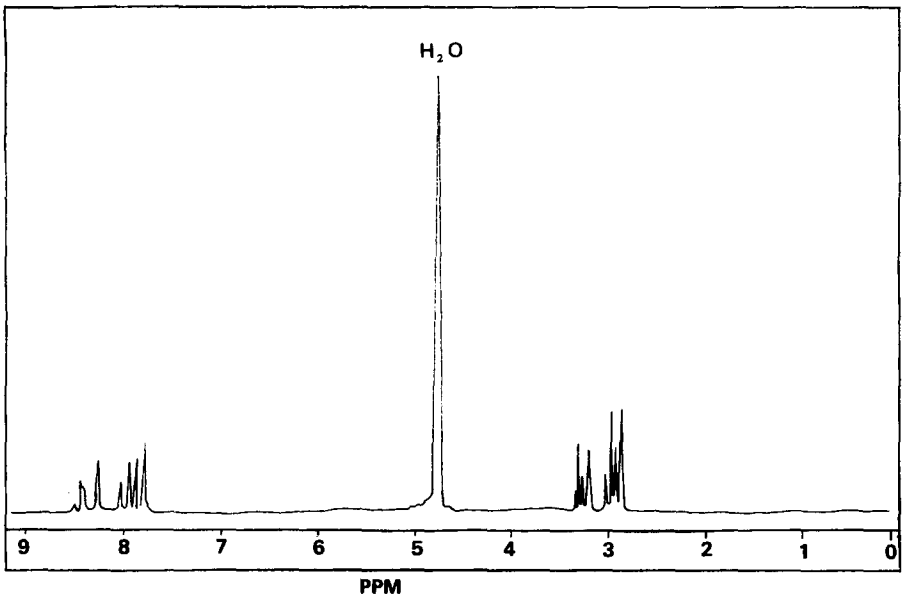

Fig. 7. Proton nuclear magnetic resonance spectrum of rom^{7}Gua in D_2O. The ring scission in 0.2 N NaOH had been allowed to take place for 4 h. This was followed by acid treatment to remove the ribose residues. The spectrum was taken with a 360 MHz spectrometer.

are usually attributed to protons on methyl groups that are attached to either the ring nitrogens or those belonging to methyl constituents of exocyclic methylamines [20,21]. In the present study we have assigned the peaks centered at δ = 2.95 and 3.3 to the protons of the N-3 methyl groups and the exocyclic methylamines. Although chemical shifts of methyl protons are generally in the 2 ppm region, the insertion of a nitrogen atom between the methyl groups and the ring is known to slightly deshield the protons, thus effecting a downfield shift in the resonance of methyl protons [19,21].

Box et al. [22] using ^{13}C-NMR spectroscopy also obtained two chemical shifts for the methyl carbon atoms of rom^{7}Gua. They proposed that the two signals are due to the existence of two conformers of rom^{7}Gua; the formation of the presumptive conformers may result from a rotation about the C-5 to N^5 bond. Thus an alternative explanation for the two signals that we have assigned to methyl protons could be that they are due to chemical shifts of methyl protons in two rom^{7}Gua conformers. When our spectra of rom^{7}Gua and those obtained by Box et al. [22] are compared with the spectrum of 2,6-diamino-3-methyl-4-hydroxy-5-formamidopyrimidine, it appears that the presence of the methyl at the N-3 position of this isomer, as compared to the N^5 position methylation of rom^{7}Gua (Fig. 5), makes the isomer to give one resonance signal for the formyl proton and one for the methyl protons; rom^{7}Gua on the other hand gave two signals for the formyl proton and two for the methyl protons.

The spectrum in Fig. 6 was taken with a Varian 60 MHz NMR spectrometer

while that in Fig. 7 was taken with a Bruker 360 MHz NMR spectrometer. The splitting observed with the signals of the formyl and methyl protons in Fig. 7 may be partly attributed to the greater sensitivity of the 360 MHz spectrometer. No splitting of these signals was observed when spectra of our rom^{7}Gua sample were taken with the 60 MHz spectrometer (data not shown). The presence of the two downfield signals is attributed to the fact that the N^5-formyl proton (IIIA) exists in a magnetic environment different from that of the N^6-formyl proton (IIIB). The two methyl signals also suggest that the protons in one methyl group exist in a magnetic environment different from that of the protons in the other methyl group. The notion of dissimilar magnetic environments around the methyl protons of rom^{7}Gua would be consistent with structures IIIA and IIIB, as well as structure V. From the spectrum of 2,6-diamino-3-methyl-4-hydroxy-5-formamidopyrimidine in Fig. 6 and other structures with methyl groups on ring nitrogens or on exocyclic nitrogens [19], it appears that the chemical shift at $\delta = 3.3$ (Fig. 7) might be assigned to the methyl protons in structures IIIB and V. The signal at $\delta = 2.95$ could be due to the protons of the methyl group in IIIA; this is the position of the peak expected for protons of methyl groups bound to the nitrogen of an amide [23]. If this interpretation is correct, these results can be taken to indicate that the imidazole ring of 7-meGua opens either between the N-7 and C-8 bond or between the C-8 and C-9 bond with a frequency that may not necessarily be equal.

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