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ALKALINE OPENING OF IMIDAZOLE RING OF 7-METHYLGUANOSINE. 1. ANALYSIS OF THE RESULTING PYRIMIDINE DERIVATIVES*

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SUMMARY

Column chromatography and spectroscopy have been employed in analyzing pyrimidine derivatives obtained from alkaline-treated 7-methylguanosine (7-meGuo). High performance liquid chromatography (HPLC) revealed that the alkaline generated products consist predominantly of two forms of ring opened 7-methylguanine (rom^{7}Gua) in equal amounts. Material from both Dowex 50 and Sephadex LH-20 columns was readily resolvable into two HPLC peaks. The species in one peak appears to be composed of formylated and that in the other of deformylated rom^{7}Gua. The presence of a deformylated species is supported by the absence of radioactivity in one of the two peaks obtained when ring opened [8-^{14}C]-guanosine was analyzed by HPLC. The formylated species was retained on the liquid chromatography column for 8 min with a 3% methanol, 0.01 M $NH_4H_2PO_4$ (pH 5.1) solvent and for 6 min with a 6% methanol, 0.01 N $NH_4H_2PO_4$ (pH 5.1) solvent system; the deformylated species was retained for 6.3 min with the first solvent and 4.5 min with the second solvent. Subsequent to Dowex 50 chromatography in an ammonium formate solvent, about 90% of the material was formylated. When stored at 24°C for 72 h in a solvent without formate ions, the material was shown by

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Abbreviations: AFB_1, aflatoxin B_1; AP, apurinic-apyrimidinic; DMS, dimethylsulfate; FAPy, formamidopyrimidine (a shortened form of formylated rom^{7}Gua called 2,6-diamino-4-hydroxy-5-*N*-methyl formamidopyrimidine); HPLC, high performance liquid chromatography; 7-meGua, 7-methylguanine; 7-meGuo, 7-methylguanosine; MMS, methylmethanesulfonate; MNU, *N*-methyl-*N*-nitrosourea; rom^{7}Gua, ring opened 7-methylguanine; rom^{7}Guo, ring opened 7-methylguanosine; deformylated rom^{7}Gua, 2,6-diamino-4-hydroxy-5-methylaminopyrimidine.

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HPLC to consist of equal amounts of the formylated and deformylated species. These results indicate that the two species of rom^{7}Gua are in equilibrium. The rom^{7}Gua excised from DNA by formamidopyrimidine (FAPy)-DNA glycosylase was shown to coelute with the formylated species.

INTRODUCTION

Chemical agents like *N*-methyl-*N*-nitrosourea (MNU), dimethylsulfate (DMS) and methylmethanesulfonate (MMS) exert their carcinogenic or mutagenic effects by methylating the nucleic acids in the target tissues [1,2]. They largely alkylate the nucleophilic N-7 site of guanine moieties to form 7-meGua adducts in nucleic acids. DMS and MMS are considered to be weak carcinogens; they have been shown to induce local sarcomas when injected into rats subcutaneously [3].

The fate of the 7-meGua adducts in DNA in vivo has been a matter of some uncertainty until the recent discovery of an enzymatic mechanism for their removal from DNA [4–7]. The quarternization of ring nitrogen resulting from alkylation reactions destabilizes the glycosyl bond of 7-meGua in DNA leading to depurination and apurinic-apyrimidinic (AP) site production [8]. In addition, this N-7 alkylation makes facile the imidazole ring opening in 7-meGua by alkali [9,10]. Ionizing radiation has been shown to cause comparable ring fission in non-alkylated guanine and adenine nucleosides [11–14].

Recently we discovered in *Escherichia coli* cell extracts a DNA glycosylase that removes a species of rom^{7}Gua (2,6-diamino-4-hydroxy-5-*N*-methylformamidopyridine, shortened to formamidopyrimidine (FAPy)) from DNA [15]. This enzyme, FAPy-DNA glycosylase, has now been purified to near homogeneity and further characterized [16]. A similar activity has been isolated from hamster and rat liver cell extracts [6].

The chemical nature of rom^{7}Gua derivatives has been a subject of some disagreement. Haines et al. [9] using paper chromatography to analyze products from alkaline-treated 7-meGuo reported the formation of homogeneous FAPy while Lawley and Shah [10] resolved these products into four components on a Dowex 50 (NH_4^+ form) column. It is not clear whether the data from these two laboratories are at variance simply because they used different chromatographic methods of analysis or because of some post-alkaline treatment modification of the products.

In an effort to resolve this disagreement as well as to better define the substrate specificity of FAPy-DNA glycosylase, we have undertaken a detailed investigation of the products from alkaline rom^{7}Gua using HPLC. We report here that the alkaline ring opening in 7-meGuo results in the formation of two major products, namely FAPy and 2,6-diamino-4-hydroxy-5-methylaminopyrimidine, a deformylated FAPy derivative. The possible basis for the several products reported by Lawley and Shah [10] and the

likely biological impact of a prolonged retention of rom^{7}Gua in cellular DNA are considered.

MATERIALS AND METHODS

Materials

Escherichia coli B cells were obtained as frozen pastes from Grain Processing Corp. (Muscatine, IA). Dowex 50 was obtained from Bio-Rad Laboratories while Sephadex LH-20, 7-meGuo, 7-meGua and Hepes buffer were obtained from Sigma. [8-^{14}C]Guanosine was obtained from ICN (Irvine, CA). Solvents used for HPLC were analytical grade and were filtered before use. Aqueous counting scintillant was obtained from Amersham Corp.

Methods

Preparation of pyrimidine derivatives from 7-meGuo. To open the imidazole ring of 7-meGuo, a reaction mixture consisting of 100 mg of 7-meGuo in 2 ml of 0.2 N NaOH was incubated at 37°C for 4 h. After neutralizing with 2 N HCl the reaction mixture was made 85% in formic acid and incubated for 72 h at 24°C to remove the ribose residues. The material was neutralized with NH_4OH, lyophilized and finally dissolved in 1 ml of water.

The material was applied to a Dowex 50 (H^+ form) column (1.5 × 14 cm) equilibrated with water. After washing the column with water and then 1.0 N HCl, elution was carried out with 2.0 N HCl. The rom^{7}Gua was recovered as a 2.0 N HCl eluate and was subsequently fractionated on a Sephadex LH-20 column (2.5 × 36 cm) in a 25% ethanol solvent. The column was washed with 50% ethanol and elution carried out with a 50—80% ethanol linear gradient. The material recovered in the different peaks was analyzed by UV spectroscopy as well as reversed-phase HPLC. The rom^{7}Gua residues have a λ_{max} 265 nm and a λ_{min} 240 nm (vide infra).

In some experiments a Dowex 50 (NH_4^+ form) column (1.5 × 20 cm) was used in place of the Dowex 50 (H^+ form) column described above. The column was equilibrated with 0.3 M ammonium formate (pH 8.9). The column was washed with 0.3 M followed by 1.0 M ammonium formate (pH 8.9). The rom^{7}Gua material was recovered in the void volume.

Methylation and ring opening of [8-^{14}C]guanosine. The reaction mixture consisted of 1 mg unlabeled guanosine, 25 μCi [8-^{14}C]guanosine (42 mCi/mmol), 2 μl DMS in 0.5 ml of 0.02 M cacodylic acid (pH 7.0). Incubation at 37°C was carried out in the dark for 24 h. The sample was made 0.2 N in NaOH and incubated 4 h at 37°C to induce imidazole ring fission. To ensure alkalinity of the medium during treatment with 0.2 N NaOH, it is essential to carry out the above methylation reaction in not more than 0.02 M cacodylic acid (pH 7.0). Following alkaline treatment the sample was neutralized with HCl and subsequently heated in 0.2 N HCl to hydrolyze the ribose residues. Following a final neutralization the sample was analyzed by HPLC. The optical activity of the column effluents was moni-

tored at 265 nm and 0.02-ml fractions collected for radioactivity measurements.

HPLC. A Waters Associates HPLC system was used in both preparative and analytical separations. The system consisted of a Waters Model 450 variable wavelength detector, two model 6000A pumps, a Model 660 solvent programmer, and a C_{18}-μBondapak column (4 mm × 25 cm); in addition an omniscribe recorder from Houston Instruments was used. A guard column containing LC-18 pellicular packing (Supelco Inc.) preceded the C_{18}-μBondapak column in the chromatography system setup.

The 3% (or 6%) methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1) solvent was filtered through a 0.45 μm pore filter mounted on a Millipore filtration apparatus. Samples were filtered in a Sep-pak C_{18} cartridge prior to application to the C_{18}-μBondapak column. During column development the retention times of the species resolved into different peaks were monitored at 265 nm. All HPLC separations reported in this communication were performed isocratically at 24°C. A flow rate of 1 ml/min was used in most HPLC analyses. Both rom^{7}Gua and rom^{7}Guo were resolved into products 1 and 2 by HPLC. In collecting each product separately for lyophilization and further characterization, better resolution was obtained (6.3 min and 8 min) with a 3% methanol, 0.01 M $NH_4H_2PO_4$ (pH 5.1) solvent system. The rate and degree of interconversion of products 1 and 2 was determined by incubating each one in 0.3 M ammonium formate (pH 8.9) or in 0.2 N NaOH at 24°C and collecting 10-μl aliquots at selected time intervals for HPLC analysis.

Separation of 7-meGua, 7-meGuo and pyrimidine derivatives by HPLC. This experiment was carried out in two stages. The solvent system used was either 3% or 6% methanol in 0.01 M $NH_4H_2PO_4$ (pH 5.1). During the first stage solutions of 7-meGua, 7-meGuo and rom^{7}Gua were each separately resolved by reversed phase HPLC to establish the retention time for each individual compound. The three samples were then mixed and jointly chromatographed under the same conditions as those used for each one individually.

Preparation of DNA substrate. Calf thymus DNA methylated with [*methyl*-^{3}H]DMS (New England Nuclear Corp., spec. act., 4.3 Ci/mol) was dialyzed against 0.2 N NaOH at 25°C for 24 h to effect imidazole ring opening in 7-meGua residues of DNA. After dialyzing against 2000 ml of 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) the DNA was allowed to reanneal at 65°C for 72 h. The DNA now with 85% rom^{7}Gua was dialyzed against two changes of 2000 ml of 0.05 M Tris–HCl (pH 7.4), 1 mM EDTA and used as substrate for FAPy-DNA glycosylase.

Release of 7-meGua and rom^{7}Gua from DNA. A 0.25 ml solution containing 85 μg of 7-[^{3}H]meGua-DNA (1800 cpm/μg) and another containing 85 μg of [^{3}H]rom^{7}Gua-DNA (1600 cpm/μg) in 0.1 M KCl, 0.05 M Hepes/KOH (pH 7.4), 0.01 M $MgCl_2$, 1 mM EDTA were incubated at 37°C. At selected time intervals 10-μl aliquots were collected and chromatographed on Whatman 3 MM paper for 18 h together with marker 7-meGua and

rom^{7}Gua in a methanol/ethanol/conc. HCl/H_2O (50 : 25 : 6 : 19) solvent system. The UV-absorbing spots containing the markers and released bases were cut out and the radioactivity in them measured.

Enzyme purification and assay. The enzyme was prepared from *E. coli* B. The cells were lysed in an X-press (Tekmar Co., Cincinnati, OH) at −25°C. The detailed procedure for enzyme purification including the properties of this enzyme have been described elsewhere [16].

Enzyme activity was measured in a 50-μl reaction mixture consisting of 0.05 M Hepes/KOH (pH 7.4), 0.1 M KCl, 1 mM dithiothreitol, 1 mM EDTA 5% glycerol, 2 μg [^{3}H]FAPy-DNA (4000 cpm) and 6 microunits of enzyme unless specified otherwise. After incubating for 20 min at 37°C and chilling with 150 μl of ethanol, the ethanol-soluble enzyme released material was recovered in the supernatant (12 000 rev./min, 15 min). The radioactivity in this released material was measured by cutting the appropriate marker spots after paper chromatography and counting them. Alternatively, the ethanol soluble material was mixed with appropriate markers and resolved by reversed phase HPLC. The HPLC fractions were counted in a Beckman LS-8100 liquid scintillation counter.

RESULTS

Absorption spectra of 7-meGuo and its pyrimidine derivatives. The UV absorption spectrum of alkaline-treated 7-meGuo differs considerably from that of untreated 7-meGuo. Figure 1 shows that alkaline rom^{7}Guo (peak *b*) has λ_{max} 265 nm and λ_{min} 240 nm. Figure 1 also shows the absorption spectrum of 7-meGuo (λ_{max} 252 nm, shoulder at 272 nm) in 0.02 M $NaPO_4$ buffer (pH 7.2). Because 7-meGua is practically insoluble at neutral pH, it was dissolved in 0.01 N NaOH in order for its spectrum to be taken; its absorption spectrum was found to be similar to that of 7-meGuo (peak *a*). The UV absorption spectrum of rom^7 Guo in Fig. 1 was used as a reference profile [17] for this pyrimidine derivative in the data presented hereafter. The UV spectrum of rom^{7}Guo treated with 85% formic acid to remove the ribose residues is similar to that of rom^{7}Guo.

Ion exchange chromatography of rom^{7}Gua derivatives. After rom^{7}Guo had been treated with acid, it was fractionated on a Dowex 50 (NH_4^+ form or H^+ form) column. Figure 2 shows that Dowex 50 (H^+ form) resolves rom^{7}Gua into three peaks. The first peak contains largely non-ring opened 7-meGua (λ_{max} 250 nm at pH 1) as well as some other products not yet identified; the second peak contains orcinol reacting material which accounts for the ribose residues removed from rom^{7}Guo during earlier treatment with 85% formic acid. The third peak contained material having λ_{max} 265 nm and the general UV absorption profile characteristic of rom^{7}Gua. HPLC analysis resolved this material into products 1 and 2 (Fig. 3, peaks 1 and 2).

Figure 3 also shows the elution positions of 7-meGua and 7-meGuo. The HPLC column had been calibrated to establish the retention times

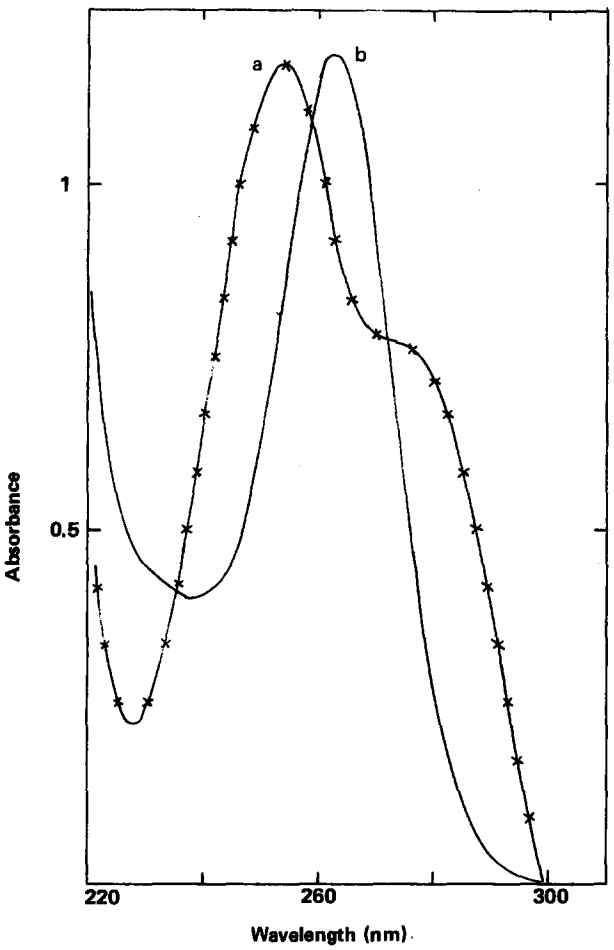

Fig. 1. Absorption spectra of (a) 7-meGuo (×——×) and (b) rom^{7}Guo (——) in 0.02 M sodium phosphate (pH 7.2). Other pyrimidine derivatives of 7-meGuo with UV spectra similar to that of rom^{7}Gua are specified in the text.

of rom^{7}Gua, 7-meGua and 7-meGuo using a 6% methanol, 0.01 M $NH_4H_2PO_4$ (pH 5.1) solvent. It can be observed from this analysis that HPLC resolves rom^{7}Gua into product 1 retained for 4.5 min and product 2 retained for 6 min; peak 3 contains 7-meGuo (8.5 min) while peak 4 contains 7-meGua (13 min). As this solvent system gives lower retention times for rom^{7}Gua species, we used it in carrying out fast analyses of reaction products from routine experiments with alkaline rom^{7}Gua.

In an effort to separately recover products 1 and 2 from the HPLC column (Fig. 3), the rom^{7}Gua from Dowex 50 (H^+ form) was applied to a Sephadex LH-20 column in 25% ethanol. Trace amounts of rom^{7}Gua were recovered together with some unidentified material in the first peak of Fig. 4. No material was recovered upon washing the column with 50%

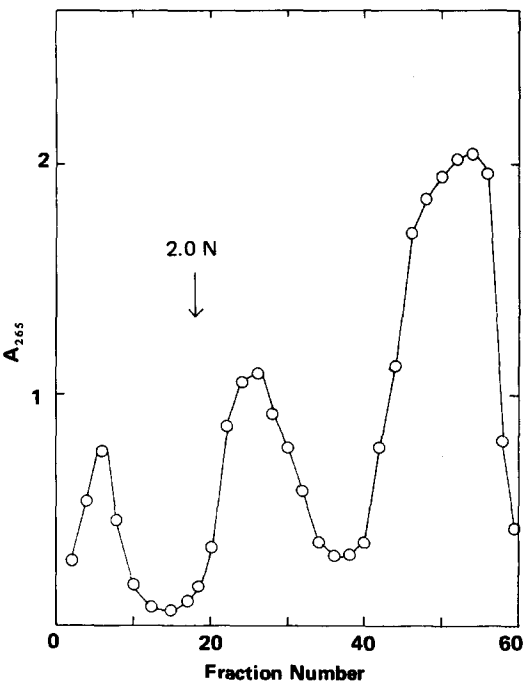

Fig. 2. Chromatography of rom^{7}Gua on Dowex 50 (H^+ form). After alkaline ring fission and acid removal of ribose groups, rom^{7}Gua was applied to the column in water. Washing with water removed peak 1 material (λ_{max} 250 nm) while 2 N HCl eluted the material in peaks 2(λ_{max} 272 nm) and 3(λ_{max} 265 nm). Fractions collected were 10.4 ml in volume.

ethanol. When a linear gradient of 50—80% ethanol was applied to the column, rom^{7}Gua (λ_{max} 265 nm) was recovered in fractions 18—24 (60% ethanol). The third peak (fractions 52—56) contained material whose absorption spectra showed a peak at 230 nm and a shoulder at 270 nm; the species in this peak has not been further characterized. The rom^{7}Gua recovered from the Sephadex LH-20 column had an HPLC elution profile identical to that of the rom^{7}Gua recovered from the Dowex 50 (H^+ form) as depicted in Fig. 3 (peaks 1 and 2). Thus the adsorption chromatography on Sephadex LH-20 could not separate the two species of rom^{7}Gua.

As an alternative to fractionation of acid-treated rom^{7}Guo on Dowex 50 (H^+ form) we next used Dowex 50 (NH_4^+ form) in another attempt to separate the species of rom^{7}Gua that are eluted from HPLC in two peaks. The column was equilibrated with 0.3 ammonium formate (pH 8.9). The material showing the UV absorption spectrum characteristic of rom^{7}Gua came off the column in the void volume (Fig. 5). The loading buffer also removes material with λ_{max} 220 nm in the second peak. Using 1.0 M ammonium formate (pH 8.9), a third species of material (λ_{max}, 240 nm) was eluted from the column; the material in the last two peaks has not yet been identified. The disadvantage of using Dowex 50 (NH_4^+ form) is that the

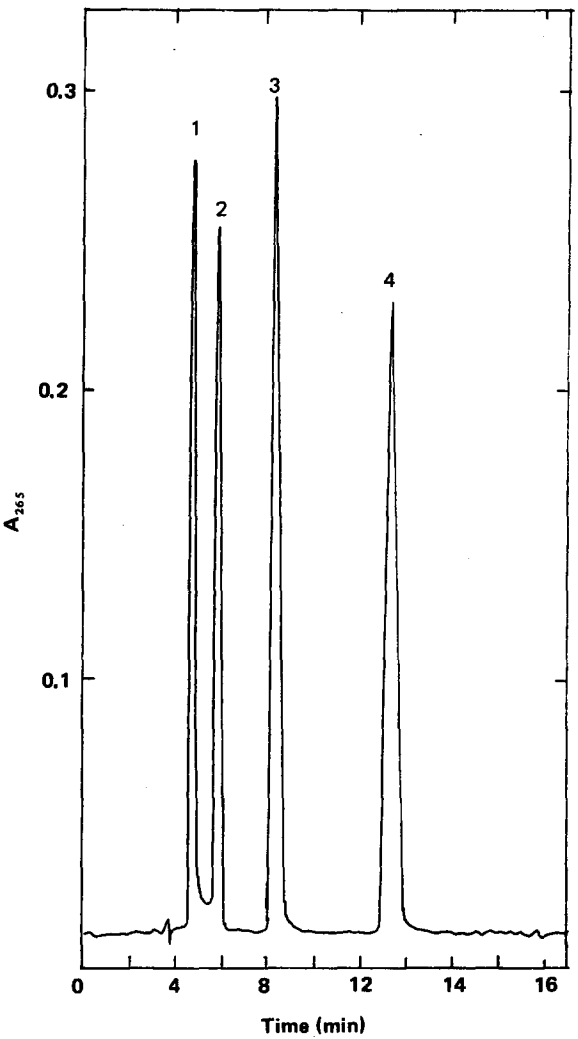

Fig. 3. HPLC separation of rom^{7}Gua (peaks 1 and 2), 7-meGuo (peak 3) and 7-meGua (peak 4). A 0.01 M $NH_4H_2PO_4$ (pH 5.1), 6% methanol solvent system was used. Separations were monitored by measuring the A_{265} of the column effluents.

brownish ribose residues and the rom^{7}Gua are not separated, but are both recovered in the first peak.

The samples of rom^{7}Gua from Dowex 50 (H^+ form) and from Dowex 50 (NH_4^+ form) were each resolved into two peaks by HPLC giving the profiles shown in Figs. 6a and 6b, respectively. A 72-h incubation of the rom^{7}Gua from Dowex 50 (NH_4^+ form) at 24°C did not change the predominance of product 2 shown in Fig. 6b. When the material from Dowex 50 (NH_4^+ form) is lyophilized, dissolved in water, desalted by gel filtration (Bio-Gel P-2), and finally lyophilized again, HPLC showed that the pro-

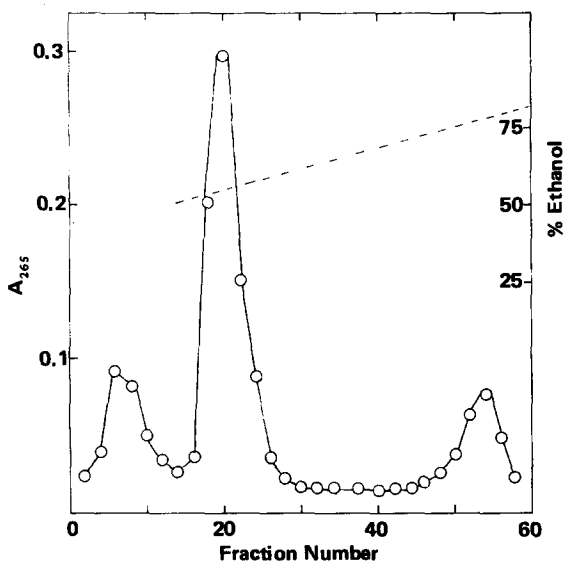

Fig. 4. Sephadex LH-20 chromatography of rom^{7}Gua recovered from Dowex 50 (H^+ form). Material loaded in 25% ethanol and 9.0-ml fractions collected. After washing with 25% and 50% ethanol, a linear gradient (50—80% ethanol) was used to elute the rom^{7}Gua (fractions 18—24) and an unidentified species in fractions 52—56.

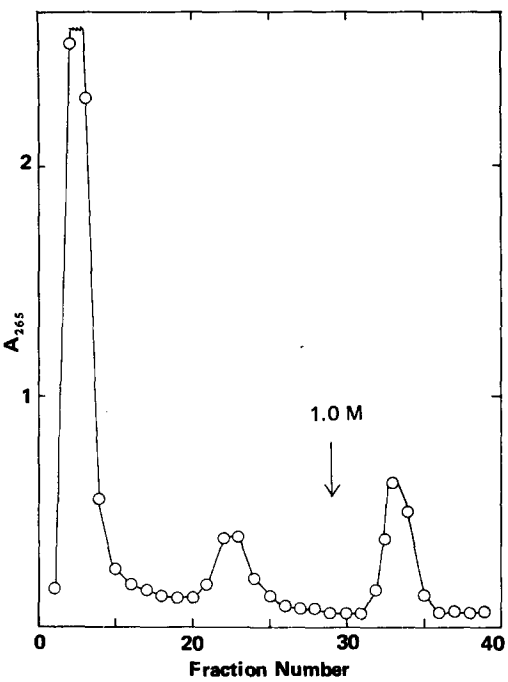

Fig. 5. Dowex 50 (NH_4^+ form) chromatography of rom^{7}Gua following acid treatment to hydrolyze the ribose residues. The rom^{7}Gua having the typical λ_{max} 265 was recovered in the first peak. Material in peak 2 had λ_{max} 220 nm and that in peak 3 had λ_{max} 240 nm. The species in peaks 1 and 2 were removed with 0.3 M ammonium formate (pH 8.9), and that in peak 3 with 1.0 M ammonium formate (pH 8.9). 6-ml fractions were collected.

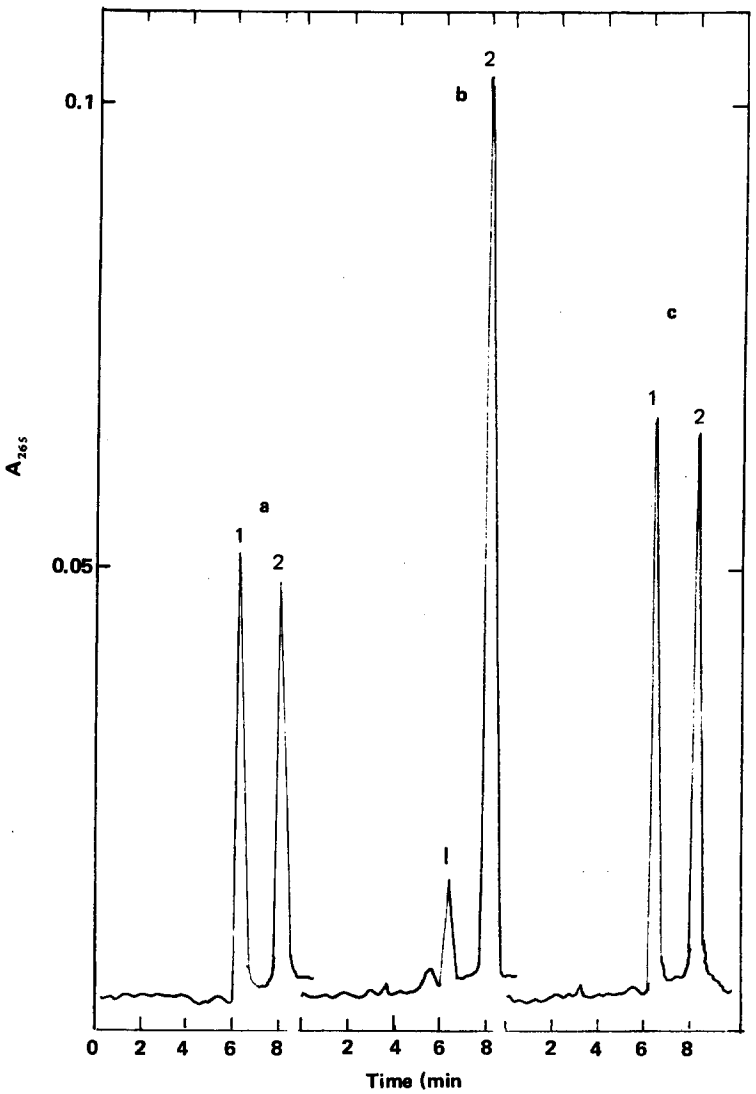

Fig. 6. HPLC of rom^{7}Gua after different conditions of processing. (a) HPLC profile of rom^{7}Gua from a Dowex 50 (H^+ form) column. (b) HPLC profile of unprocessed material from Dowex 50 (NH_4^+ form) or that of similar material that had been lyophilized, and dissolved in water just before HPLC. (c) HPLC profile resulting after material in (b) had been processed and stored at 24°C for 72 h. The solvent system was 3% methanol, 0.01 M $NH_4H_2PO_4$ (pH 5.1). Product 1 had r_T = 6.3 min and product 2 r_T = 8.0 min.

portion of the heights of peaks 1 and 2 was initially similar to that in Fig. 6b. Upon prolonged storage at 24°C, part of product 2 was converted to product 1; the process continued until peaks 1 and 2 became equal in size (Fig. 6c). Thus the post-Dowex processing does not appear to have removed all formate ions from the rom^{7}Gua sample.

These observations suggest that the two products obtained when rom^{7}Gua is resolved by HPLC (Fig. 3, peaks 1 and 2; Fig. 6a) are in fact, two pyrimidine derivatives of rom^{7}Gua which are in equilibrium. The processing associated with Dowex 50 (NH_4^+ form) chromatography would appear to provide conditions that shift the equilibrium in favor of product 2.

To test whether the conversion of product 1 to product 2 was due to factors other than the presence of formate ions in the solvent used for Dowex 50 (NH_4^+ form) chromatography, we treated 7-meGuo with 0.2 N NaOH and analyzed the resulting rom^{7}Guo by HPLC; this resolved the material into products 1 and 2 as in Fig. 6a. When product 1 (peak 1) was stored in 0.2 N NaOH for 72 h at 24°C and analyzed by HPLC (3% methanol, 0.01 M $NH_4H_2PO_4$), Fig. 7a shows the presence of a large amount

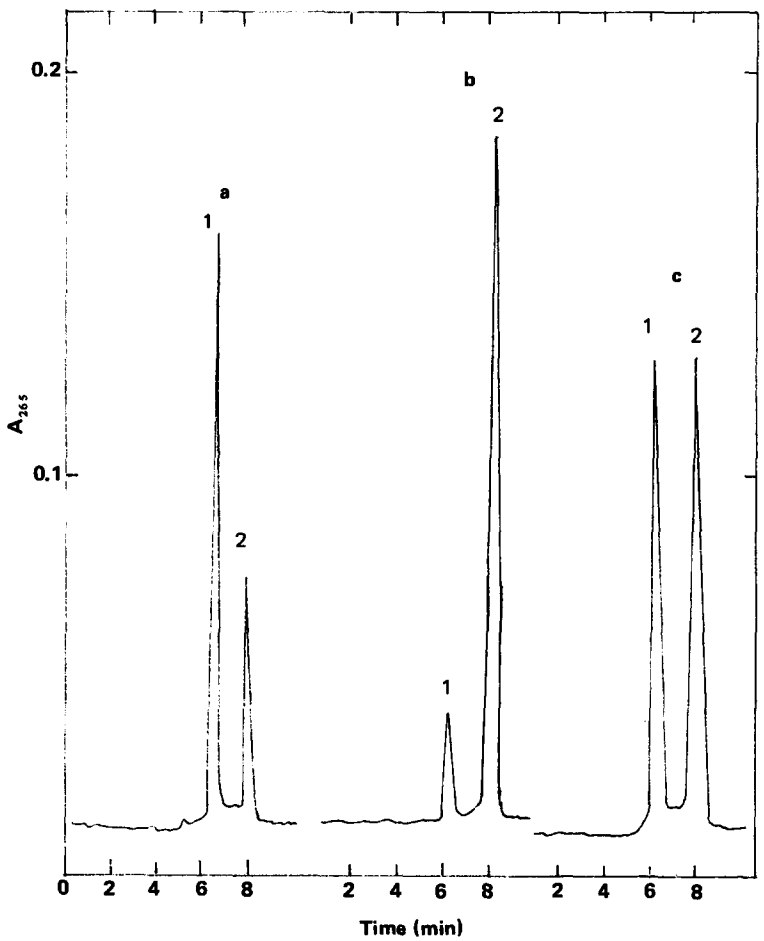

Fig. 7. Conditions influencing the establishment of equilibrium between products 1 and 2. (a) HPLC profile of product 1 incubated in 0.2 N NaOH at 24°C for 72 h. (b) HPLC profile of product 1 similarly incubated in 0.3 M ammonium formate (pH 8.9). (c) HPLC profile of product 2 similarly incubated in 0.2 N NaOH.

of product 1 and a small amount of product 2; this amount of product 2 was present before the 72-h storage and seems to represent product 2 material not successfully eliminated during the collection of product 1 from the HPLC column. When similarly stored in 0.3 M ammonium formate, most of product 1 was converted to product 2 (Fig. 7b). On the other hand

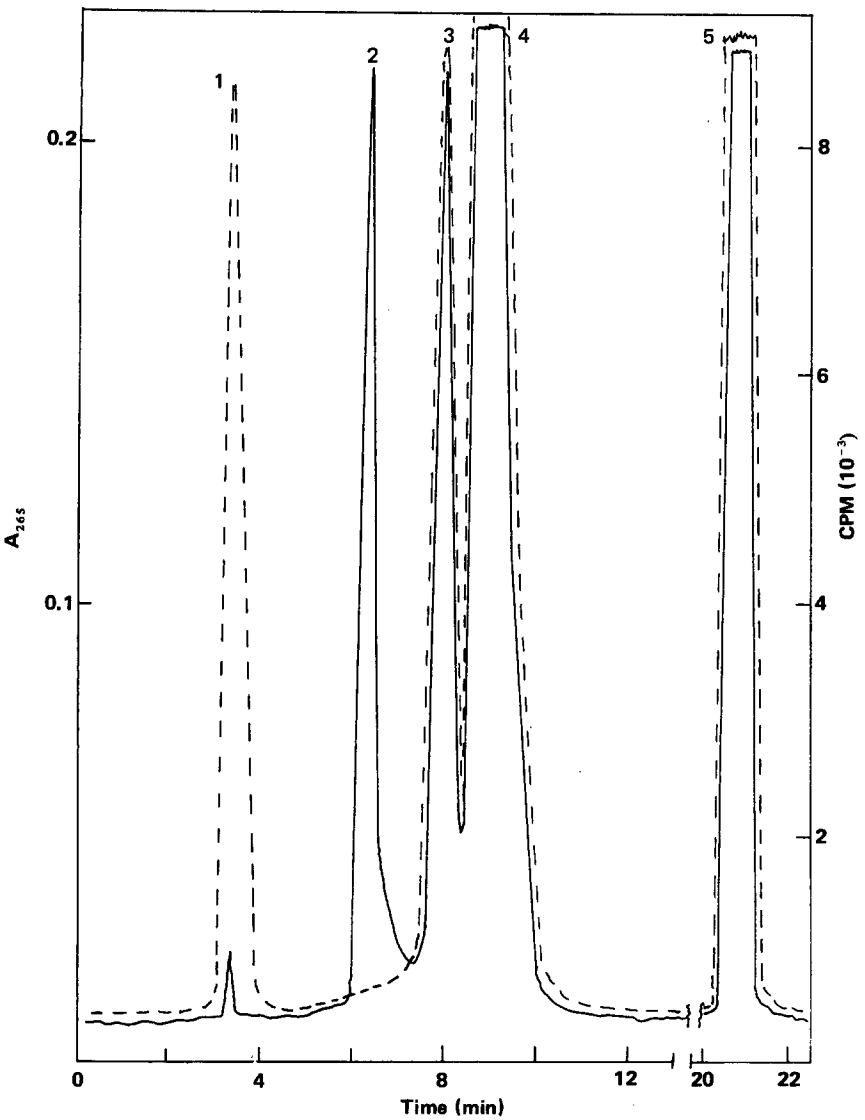

Fig. 8. Determination of fate of formyl groups in ring opened 7-[8-^{14}C]meGua. HPLC analysis of adduct derivatives after treatment with 0.2 N NaOH and 0.2 N HCl. For measuring radioactivity 0.02-ml fractions were collected during column development. A_{265} (——); cpm (----).

a similar processing of product 2 in 0.2 N NaOH allowed part of it to be converted to product 1 until equilibrium was established (Fig. 7c); a faster conversion occurred when product 2 was stored in a buffer at pH 7.4. The UV absorption spectra of products 1 and 2 were both similar to that of rom^{7}Guo (Fig. 1).

One interpretation is that product 2 (Fig. 7) is formylated rom^{7}Guo which becomes deformylated to product 1. Another possible interpretation is that product 1 is a carbinolamine intermediate (see Fig. 5, IIA and IIB in accompanying paper [18]) which is in equilibrium with product 2. As we could not investigate the latter possibility for want of a carbinolamine standard, we sought to determine whether product 1 represents deformylated rom^{7}Gua.

In investigating this possibility we treated 7-[8-^{14}C]meGuo with 0.2 N NaOH to open the imidazole ring of guanine, followed by acid treatment to deribosylate the rom^{7}Guo. The resulting material was fractionated by HPLC during which process 0.02-ml fractions were collected. The radioactivity in each fraction was measured. Fractions from each A_{265} peak were pooled and their UV absorption spectra taken. From the HPLC profile obtained in this experiment (Fig. 8) peaks 2 (r_T = 6.3 min) and 3 (r_T = 8 min) correspond to the elution positions of products 1 and 2, respectively, while peak 4 is in the elution position of guanine. Pooled material from each of peaks 2 and 3 had a UV spectrum similar to that of rom^{7}Gua (Fig. 1); material from peaks 4 and 5 had a UV spectrum characteristic of unmethylated guanine. Peak 5 material coeluted with marker guanosine.

An examination of the distribution of the radioactivity throughout the HPLC profile shows radioactivity in peak 1 that represents the elution position of formate ions. There is no radioactivity in peak 2 (product 1), while there is considerable radioactivity in peak 3 (product 2). As the UV spectrum of material from each of these two peaks indicates that the two peaks consist of rom^{7}Gua, the absence of radioactivity in peak 2 suggests that the [^{14}C]formyl group has been released from product 1. This radioactivity presumably elutes as formate ions in peak 1. The material in peak 4 represents deribosylated [8-^{14}C]guanine. These results are consistent with the notion that the equilibrium observed between the heights of peaks 1 and 2 (Fig. 6 and 7) is, in fact, an equilibrium between deformylated and formylated rom^{7}Gua, respectively. As product 2 (peak 3) is the radioactive species of rom^{7}Gua, it is presumed to be the formylated derivative.

HPLC analysis of product released by enzyme from DNA. The rom^{7}Gua released from DNA by FAPy-DNA glycosylase was cochromatographed with rom^{7}Gua standards. Figure 9 shows that the majority of enzyme released rom^{7}Gua coelutes with product 2 while a small amount of it coeluted with the species of marker rom^{7}Gua that is retained for 6.3 min. It is conceivable that if the enzyme released rom^{7}Gua were subsequently incubated for an appropriate length of time, it would eventually be equally distributed between the two peaks upon attainment of equilibrium.

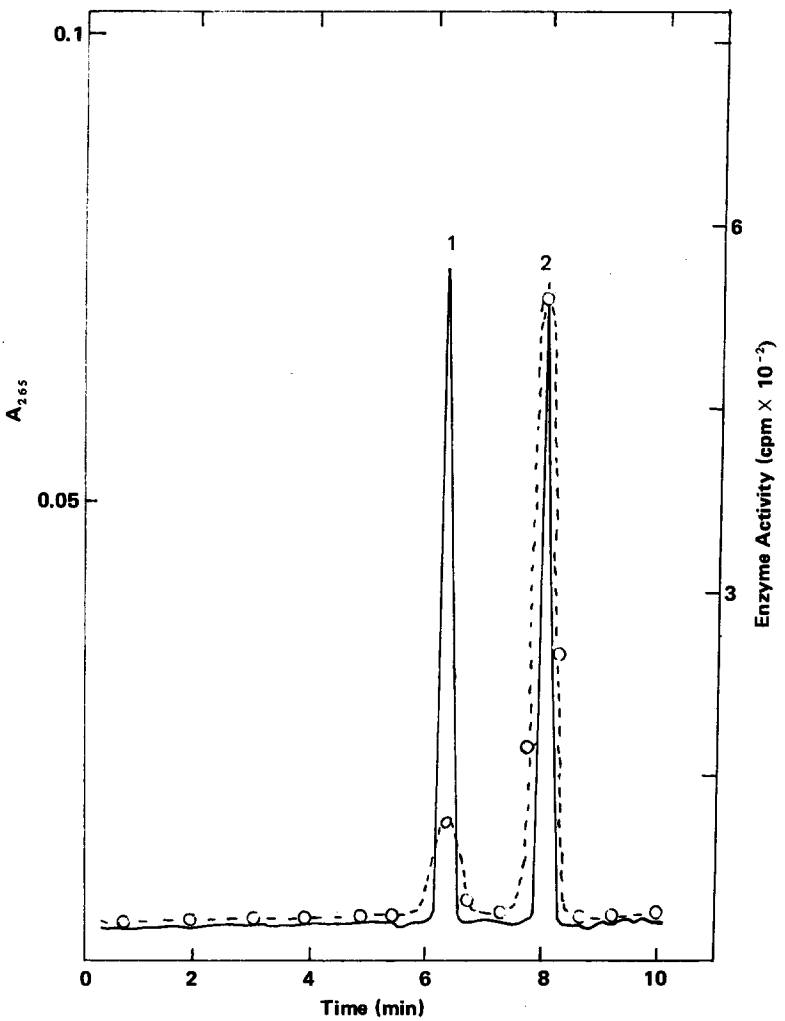

Fig. 9. HPLC of [^{3}H]rom^{7}Gua released from DNA by FAPy-DNA glycosylase mixed with unlabeled rom^{7}Gua marker. Separations were followed by measuring the radioactivity in the drops from the column as well as A_{265} nm of the column effluents. A_{265} (——); cpm (-----).

Chemical hydrolysis of 7-meGua and rom^{7}Gua under physiological conditions. The recently discovered enzymatic mechanism for the excision of 7-meGua from DNA [5,7] does not remove these 7-meGua residues with opened imidazole rings. Table I shows that rom^{7}Gua residues are hardly released by chemical hydrolysis under physiological conditions in vitro, while 7-meGua is released with $t_{1/2}$ = 144 h. These results indicate how long the alkylated ring opened guanines are retained in DNA under physiological conditions, and further point to the considerable odds for mutagenesis in the biological systems exposed to these alkylating agents.

TABLE I

SPONTANEOUS RELEASE OF 7-meGua AND rom^{7}Gua FROM DNA

Each reaction mixture consisted of 7-[^{3}H]meGua-DNA or [^{3}H]rom^{7}Gua-DNA in 0.1 M KCl, 0.05 M Hepes/KOH (pH 7.4), 0.01 M $MgCl_2$, 1 mM EDTA. Incubation was at 37°C. 10-μl aliquots (approx. 5500 cpm) were collected after the indicated time intervals and processed for counting.

Time (h)	cpm in released base	
	7-[^{3}H]meGua	[^{3}H]rom^{7}Gua
0	82	95
1	180	—
2	415	84
6	566	96
18	667	102
24	881	87
48	1241	81
72	1700	116
96	2186	90
120	2411	141
144	2746	142
168	3153	160
192	3494	150
240	5018	195

DISCUSSION

The experiments carried out in this investigation show that there are two major products generated by alkaline treatment of 7-meGuo. Lawley and Shah [10] resolved alkaline rom^{7}Gua into several peaks by Dowex 50 (NH_4^+ form) ion exchange chromatography. Haines et al. [9] had earlier reported the production of one form of rom^{7}Gua by alkaline treatment of 7-meGuo. The imidazole ring fission kinetic studies in the accompanying paper show that rom^{7}Gua consists of several species [18]. In the present study, Dowex 50 (NH_4^+ form) chromatography was found to fractionate rom^{7}Gua into three peaks, two of which contained material with optical properties not characteristic of rom^{7}Gua. The fact that our Dowex 50 (NH_4^+ form) chromatography of acid treated rom^{7}Guo did not fractionate this material into the several peaks reported by Lawley and Shah [10] suggest an elimination of the minor products by this acid treatment.

After desalting the post-Dowex 50 (NH_4^+ form) material by gel filtration and subsequent lyophilization, more than 90% of rom^{7}Gua was resolved into one major peak by HPLC; the formation of this major species in a medium containing formate ions suggests that product 2 is formylated rom^{7}Gua. It appears that the ammonium formate (pH 8.9) buffer used in the Dowex 50 (NH_4^+ form) column chromatography step provided formyl groups that shifted the equilibrium between the two species of rom^{7}Gua

in favor of formylated rom^{7}Gua. An analysis of ring opened 7-[8-^{14}C]-meGua by HPLC showed that product 1 is deformylated rom^{7}Gua. Storage of product 2 for 72 h at pH 7.4 resulted in its separation by HPLC into products 1 and 2. These results are consistent with the notion that the two peaks contain two different species of rom^{7}Gua which have a tendency for gravitating towards equilibrium. Margison and Pegg [6] reported the separation of rom^{7}Gua by HPLC into two peaks using 0.02 M ammonium formate (pH 4.0) as solvent; under these conditions the retention times for the material in the two peaks were 3 and 8 min.

The existence of two major species of rom^{7}Gua in equilibrium raises the question as to which species is the substrate for FAPy-DNA glycosylase. When FAPy-DNA substrate is incubated with FAPy-DNA glycosylase, the species of rom^{7}Gua released by the enzyme comigrates with formylated rom^{7}Gua whose HPLC retention time is 8 min; only a small amount of enzyme released rom^{7}Gua coelutes with the deformylated species which is retained for 6.3 min. This indicates that the enzyme releases formylated rom^{7}Gua.

The fact that the alkaline treatment of 7-meGuo gives two forms of rom^{7}Gua which are in equilibrium does not necessarily indicate that two forms of rom^{7}Gua exist in FAPy-DNA substrates. The electron system of rom^{7}Gua in DNA is unlikely to allow as facile an interconversion of the two pyrimidine derivatives as is the case with the rom^{7}Gua residues derived from free 7-meGuo. As our results show that it is the formylated rom^{7}Gua that is excised from DNA by FAPy-DNA glycosylase [18] it remains to be resolved whether alkaline treated DNA also contains deformylated rom^{7}Gua.

We found that rom^{7}Gua is stably bound to DNA and is not as easily spontaneously released from DNA as 7-meGua. Kohn and Spears [19] observed a similar stabilization of N-7 alkylated guanine in DNA subsequent to ring fission. The stabilization of rom^{7}Gua in DNA allows the damaged adduct time for expression and may be the mechanism by which alkylating agents exert their mutagenic or carcinogenic effects. In this regard it is interesting to note that the hepatocarcinogen aflatoxin B_1 (AFB_1) [20,21] has been reported to cause ring fission in guanine adducts under physiological conditions. There is evidence that the AFB_1-FAPy adducts are retained in DNA much longer than the intact AFB_1-guanine adducts [21]. The existence of these ring opened pyrimidine adducts in cellular DNA over a protracted time period is likely to register a mutagenic effect on the genome during the ensuing rounds of DNA replication; the carrier cells may also be rendered susceptible to neoplastic transformation.

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