¹H and ¹³C NMR Study of the Interaction of Formaldehyde on Adenine and its Derivatives

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It has previously been shown that formaldehyde causes the formation of hydroxymethylated derivatives with nucleobases, but these were identified after separation of the reaction products; such handling might destroy labile derivatives or remove products present in small proportions. This work is based on a study of the reactivity of formaldehyde on adenine, adenosine and adenosine monophosphate in the reaction medium. It demonstrates the high reactivity of the N-9 site of adenine, the reactivity of the hydroxymethyl at the C-5' of the ribose and brings to light such new derivatives as N-9-CH₂OCH₂OH and N-6-CH₂OCH₂OH. Further, the order of appearance of different derivatives and their approximate determination were achieved.

KEY WORDS Formaldehyde Adenine Adenosine Adenosine monophosphate

INTRODUCTION

From experiments conducted on the rat,¹ it is known that formaldehyde can cause cancer in the rat's nasal cavity and have a genotoxic effect.^{2,3} One study using ¹⁴C-labelled formaldehyde showed activity from RNA, DNA and proteins,⁴ but it is known that formaldehyde, with tetrahydrofolic acid acting as an intermediary, can enter into the C1 pool of intermediary metabolism and be incorporated into macromolecules, including nucleic acids.⁵

However, contrary to statements by Craft *et al.*⁶ formaldehyde not only reacts on the exocyclic amino group of deoxyadenosine, deoxyguanosine and deoxy-cytidine, but also, as we have previously shown, produces N-3-hydroxymethylated derivatives with uridine, thymidine⁷ and inosine.⁸ ¹H NMR studies have already shown that formalde-

hyde reacts with adenosine (Ado) to yield N-6hydroxymethyladenosine^{9,10} and, in the presence of a strong concentration of formalin, yields N-6dihydroxymethyl-Ado. However, structural determinations were carried out after chromatographic separation of the reaction products and, as the authors concede, the separation process may have destroyed labile compounds or may have removed products which were present in small proportions. The results presented here are based on the reaction of formaldehyde with adenine, adenosine and adenosine monophosphate (AMP), and new derivatives such as N-9-CH₂OCH₂OH and N-6-CH₂OCH₂OH have been identified, which facilitates the interpretation of the NMR spectra of the solutions. This study is also based on the identification and the order of appearance of the products actually in the reaction medium and not, as in previous work, on the products after separation. It is also the first stage in the study of the long-term reactions of formaldehyde.

EXPERIMENTAL

Adenine and adenosine were used as solutions in hexadeuteriated dimethyl sulphoxide (CEA) (DMSO- d_6) at approximately 0.15 m; the residual proton signal served as the internal reference at 2.5 ppm and the central methyl signal at 39.5 ppm for the ¹³C NMR spectra. A 31% by weight solution of formaldehyde, containing no methanol, was used. It is known that at higher concentrations commercial solutions are stabilized with methanol, yielding hemiacetals, CH₃(OCH₂)_nOH.¹¹ Keeping in mind that formaldehyde exists in solution as oligomers, but that only formaldehyde (CH₂O) or methylene glycol [CH₂(OH)₂] is the reacting agent, the concentration of the --CH₂O- groups must be much greater than those of the nucleobases otherwise the kinetics might be controlled by the depolymerization of the poly(oxymethylene) glycols. The disappearance of the monomer brings about the depolymerization of other oligomers and, in addition, the added formalin was approximately 0.15 M of the methylene glycol; the monomer concentration of a 31% formalin solution is approximately 3.1 м and 11.3 м of the total concentration in oxymethylene groups.¹²

NMR Spectra were obtained at 40 ± 1 °C on a Bruker WM 250 spectrometer; the temperature was measured by using the variation in the chemical shift of the two ethylene glycol signals. The conditions used for the ¹H NMR spectra were sweep width 2500 Hz, magnetizing angle *ca* 40° and number of scans 40. For the ¹³C spectra the sweep width was 11 000 Hz, the magnetizing angle *ca* 30°, the acquisition time 0.737 s and the memory size 16K.

In order to simplify the ¹H NMR spectra for adenosine and AMP, which in DMSO are in a crowded region with the hydroxymethyl signals from poly(oxymethylene) glycols and from ribose, a formalde-



Figure 1. ¹H NMR spectrum of an adenine (0.15 m)-formalin ([monomer] = 0.15 m) solution in DMSO-d₆. Reaction time = 14.5 h.

hyde solution in D_2O was used to obtain certain spectra; the preparation of such solutions has been described previously.¹²

Action of formaldehyde on adenine

After several hours at 40 °C the ¹H NMR spectrum from an adenine-formaldehyde solution (Fig. 1) becomes complex and difficult to analyse; the reaction products can be determined only from the order of appearance of the signals.

After 15 min the first product, 1, appears (about 25%), which is identified by new signals for H-2, H-8 and the NH₂ group, and by the appearance of a doublet and a triplet at 5.50 and 6.76 ppm, respectively. The integration of the two signals corresponding to the NH₂ group does not vary throughout the first phase of the reaction. A ¹³C NMR study shows the largest change in shifts to be for C-8 (+1.9 ppm) and C-4 (-1.1 ppm) (Table 1). Moreover, the chemical shift of the hydroxymethyl signal at 65.7 ppm is identical with that obtained with dimethylaminopurine at 65.8 ppm.⁸

At 45 min a second product, 2, appeared in the ¹H NMR spectra, characterized by a new doublet in the immediate vicinity of the N-9-CH₂OH signal (+0.03 ppm) associated with a triplet at 6.88 ppm. This derivative is therefore the result of hydroxymethylation of derivative 1. The ¹³C NMR study confirmed these results; a new signal is observed at 65.86 ppm (+0.16 ppm) for the N-9-CH₂OH signal, and there is a broad signal at 64.22 ppm. However, by considering the values found for the model compounds in the study of urea-

formaldehyde resins¹³ (NHCH₂OH, $\delta^{13}C = 62-66$ ppm), this signal is attributed to hydroxymethylation of the nitrogen atom in position 6; hindered rotation around the C-6—N bond would explain the width of this methylene absorption. Hence, the second reaction product is N-6,N-9-di(hydroxymethyl)adenine (2) (Table 2).

Simultaneously, although more slowly, there is an increase in two other signals in the H-2,H-8 proton group at 7.130 and 8.232 ppm. In contrast, the remainder of the spectrum is not altered (Fig. 1). These chemical shifts suggest that the third reaction product could be N-6-hydroxymethyladenine (3).

The signals of the hydroxymethyl groups in the N-6 position are broad in both the ¹H and ¹³C NMR spectra. Hydroxymethylation at N-6 has only a small effect on the chemical shift of N-9-CH₂OH (+0.03 and +0.15 ppm); conversely, in view of the width, hydroxymethylation at N-9 has an unobservable effect on the chemical shift of N-6-CH₂OH. The ¹H and ¹³C NMR signals of N-6-CH₂OH will be the same for 2 and 3, and by the same reasoning we can assume the existence of 5.

At 41 °C more than half of the initial adenine had disappeared after 1 h, which explains the more rapid formation of N-6,N-9-di(hydroxymethyl)adenine than N-6-hydroxymethyladenine.

Compound 4 is characterized by a broad signal at 5.36 ppm, at +0.34 ppm for the N-6-CH₂OH signal, a variation already observed between the CH₂ signal of monomethylolurea and N,N-dimethylolurea,¹⁴ and a doublet at 5.59 ppm for the N-9-CH₂OH methylene group (Fig. 1). A signal at 70.22 ppm is observed in the ¹³C NMR spectrum, located in the region for a double

 Table 1. ¹³C chemical shifts of adenine (Ade), its derivatives and their reaction products with formaldehyde

Compound	C-2	C-4	C-5	C-6	C-8	сн₂он
Adenine	152.5	150.3	118.5	156.0	138.9	
1	152.7	149.2	118.7	155.9	140.8	65.7
N-6-Dimethyl-Ade	151.6	151.1	119.6	154.1	138.0	
N-9-Hydroxymethyl-N-6-dimethyl-Ade	152.0	150.0	119.3	153.9	139.7	65.8
Adenosine	152.4	149.1	119.4	156.2	140.0	
6	152.5	149.5	119.8	154.6	140.3	64.2
AMP	152.2	148.4	118.1	154.8	139.9	
9	152.3	148.7	118.7	153.7	140.1	64.44

,	Ade	1	2	3	4	5
N-6	н	н	н	н	СН2ОН	СН₂ОН
1	н	н	CH2OH	СН,ОН	сн,он	сн,он
<i>δ</i> 'Η	6.99	7.11	5.02	5.02	5.36	5.36
$\delta^{13}C$			64.22	64.22	70.22	70.22
N-9-	н	CH₂OH	CH₂OH	н	СН₂ОН	н
$\delta^{1}H$	12.85	5.50	5.53	а	5.59	а
$\delta^{13}C$	_	65.7	65.86		65.86	
ť	—	3 min	45 min	4 h	9 h	14 h
%1 h ^c	48	48	4	3	3	-
%7 h ^d	25	53	15		7	

Table 2. Chemical shifts of reaction products of formaldehyde with adenine (Ade)

^a These signals are no longer visible after the addition of formalin.

^bAppearance time of reaction products at 41 °C; concentrations, 84 mmol of

Ade + 15 μ l of formalin (30% w/w) in 0.5 ml of DMSO- d_6 .

^c Percentage of compounds after 1 h.

^d Percentage of compounds after 7 h.

hydroxymethylation of urea-N-(CH₂OH)₂ (68–70 ppm).¹⁵ Although this chemical shift range is quoted for solid urea-formaldehyde resins, these values are nevertheless exploitable if one compares the chemical shifts for $-NHCH_2OH$ in DMSO- d_6^{-13} and in the solid state (64.5–65.2 ppm).¹⁵ Therefore, the fourth reaction product is N-6,N-6,N-9-tri(hydroxymethyl)adenine (4).

When free formaldehyde is removed on bubbling with nitrogen, the equilibrium is broken down and the derivatives decompose (54% of 1 and 46% of 2 after 5 days). In addition, 3 and 5 for which the N-9 site is available can never be quantitatively detected. This may provide an explanation for the lack of a mutagenic effect by adenosine which has been treated with formaldehyde, as opposed to that of adenosine itself.¹⁶

Action of formaldehyde on adenosine

The ¹³C spectra of an adenosine–formaldehyde solution show the absence of a signal at 65.7 ppm, which was attributed above to the hydroxymethyl in position 9. This therefore confirms the initial conclusion since the N-9 site is no longer available owing to its link with ribose.

In contrast, two broad signals can be observed, as before, at 64.20 and at 70.32 ppm (Fig. 2). This spectrum was obtained with mixed ¹³C-labelled and normal formaldehyde solution, in order to identify the new signals appearing on formaldehyde attack. Moreover, a decrease in temperature from 50 to 30 °C caused this last signal to broaden from 15 to 46 Hz, confirming the hypothesis that this phenomenon is due to hindered rotation around the C-6—N bond. The broadened N-6hydroxymethyl signal at 5.02 ppm is found again in the ¹H NMR spectrum. Irradiation of this signal caused the coalescence of signals in the H-2,H-8 group at 8.2 ppm, which are otherwise not visible when formaldehyde in D₂O solution is used. Hence the N-6-<u>H</u> proton is coupled with the hydroxymethyl.

The time of appearance of these derivatives at $39.5 \,^{\circ}$ C is 30 min for **6** and 2 h for **7**. Their amounts after 1 and 4 h are Ado 84% and 50% and **6** 16% and 28%.

After 24 h at 40 °C three major compounds are found in almost identical proportions: the adenosine and 6 and 7. However, the proportion of the derivative (8) due to a reaction at position 5' develops very slowly (C-5'H₂-OCH₂OH = 88.78 ppm).

Action of formaldehyde on adenosine monophosphate

The first traces of the initial product 9 are detected by ${}^{1}H$ NMR at 40.6 °C as early as the second minute of the



Figure 2. Partial ¹³C NMR spectrum of adenosine (0.15 M)-¹³C-labelled formalin in DMSO- d_6 at an average time of 24 h; cf. Experimental. F = signals of formaldehyde oligomers.

reaction, reaching ca 20% in 10 min; a second compound (10) appears after 13 min and a third (11) after 40 min. After 1 h, the composition is AMP 21%, 9 55%, 10 17%, 11 6%. A broadened ¹³C NMR signal is again seen at 64.44 and 70.30 ppm corresponding to the N-6hydroxymethylated 9 and the N-6, N-6-dihydroxymethylated derivative 10, but no signal is observed in the vicinity of 65.7 ppm (previously attributed to N-9hydroxymethylation) or in the vicinity of 88.8 ppm (corresponding to C-5'-hydroxymethylation) (Table 4). On the contrary, a ³¹P NMR study showed a slight shift in the phosphorus signal in terms of time, from +0.28 ppm (in relation to H₃PO₄) to -0.036 ppm 14 days later, indicating the reaction of formaldehyde on the distant phosphate site. In AMP, the reactive site is, again, the NH_2 of adenosine.

The presence of new signals similar to those of formaldehyde oligomers could indicate the creation of polyoxymethylene chains on N-6 (N-6-CH₂OCH₂OH; ¹H 5.13 and 4.72 ppm, ¹³C 68.06 and 88.65 ppm); we have previously shown the existence of this type of reaction on N-9 of dimethylaminopurine.⁸

CONCLUSION

The N-9 site of adenine is highly reactive with formaldehyde and almost half the starting adenine disappeared within 1 h. There are also a large number of reaction products owing to the formation of compounds of the

 $^{6}N \xrightarrow{(CH_{2}O)_{n}H} (CH_{2}O)_{n''}H$ and $^{9}N(CH_{2}O)_{n''}H$ types, with all pos-

sible combinations between n, n' and n'' in adenine and n and n' in AMP. It is also obvious that formaldehyde reacts at C-5' of adenosine; no reaction takes place in the vicinity of the phosphate group, even after 14 days.

In addition, a reaction might take place on N-7, often a major position of electrophilic attack,¹⁷ but no signal confirms the presence of such a reaction during the first stage of the reaction studied here. Further, the reactivity



of the hydroxyl groups of the ribose moiety should not be excluded, and this will be studied in the near future.

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