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STUDIES ON CHEMICAL ALTERATIONS OF NUCLEIC ACIDS AND THEIR COMPONENTS—VII[a]

C-ALKYLATION OF PURINE BASES THROUGH FREE RADICAL PROCESS CATALYZED BY FERROUS ION[b]

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Abstract—Guanine, guanosine, and 5'-guanylic acid were methylated with t-butylhydroperoxide in the presence of ferrous ion to give the corresponding 8-Me derivatives in fairly good yields. Adenine, hypoxanthine, and their ribosides also underwent the methylation to give the corresponding mono- and di-methyl derivatives where Me groups were substituted in position-2, -8, or both. N,N-Dimethylcarbamoyl group was introduced to position-8 of guanosine under a similar condition using dimethylformamide as the reaction solvent.

INTRODUCTION

Extensive investigation has been done on the chemical modifications of nucleic acid bases in relation to the lesion of the genetic principle of DNA because of its biological importance. The chemical process in such modifications involves ordinary ionic, photochemical, and/or radiochemical mechanisms but no information is available of free radical reactions induced by metal-ion catalysis, especially with purine bases. This paper describes C-methylation of guanine, adenine, hypoxanthine, and their ribosides and in addition, 5'-guanylic acid as a model of the nucleotides, with the Me radical in acidic media. Preliminary results of general application of this free radical process with radicals other than Me will also be described. As the Me radical source, the one homolytically derived from t-butylhydroperoxide catalyzed by ferrous ion was used. The latter reaction system was extensively studied by Minisci *et al.* with the protonated species of N-heteroaromatic compounds such as quinolines, as illustrated in Chart 1.[1,2]

All the reaction conditions chosen in the present study were analogus to those described by Minisci *et al.*; with excess amounts of the peroxide and catalyst in acidic media (0·1 or 1·0 N H_2SO_4 in almost all cases).

RESULTS

Guanine, guanosine, and 5'-guanylic acid were readily methylated with excess of t-butylhydroperoxide and ferrous sulfate in aqueous 1N H_2SO_4 to afford the corresponding 8-Me derivatives in fairly good yield. The results are summarized in Chart 2 and Table 1. The UV and R_f data of the products are shown in Table 2.

8-Methylguanine thus obtained was identified with an authentic specimen[3]† by cochromatography and by UV and NMR spectroscopy. The spectroscopic data of the product of guanosine suggested that it might be 8-methylguanosine and when a sample of this was heated at 100° for 1 h in 1N HCl, 8-methylguanine was obtained in quantitative yield. In order to identify the structure of the 8-Me derivative of 5'-guanylic acid, the sample was hydrolyzed by incubating with *E. coli* alkaline phosphomonoesterase to give 8-methylguanosine in quantitative yield.

In the reaction of guanine, the total UV intensity recovered from the mixture corresponded to that of the starting material originally used, indicating that the guanine nucleus was almost quantitatively recovered, not accompanied by any great extent of degradation of the guanine nucleus under the reaction conditions used. However, in other cases of guanine derivatives and also of other bases and their nucleosides, the UV recoveries of the crude products were somewhat less than those expected from the starting amount of the materials, indicating that degradation of the aromatic nucleus might take place to some extent during the reaction. It may be worth mentioning that one of the factors influencing the UV recovery was acid-

[a] Part VI: G.-F. Huang, T. Okamoto, M. Maeda and Y. Kawazoe, *Tetrahedron Letters* 4541 (1973)

[b] A part of this work was preliminarily reported as a communication to the editor. Y. Kawazoe, M. Maeda and K. Nushi, *Chem. Pharm. Bull. Tokyo* **20**, 1341 (1972).

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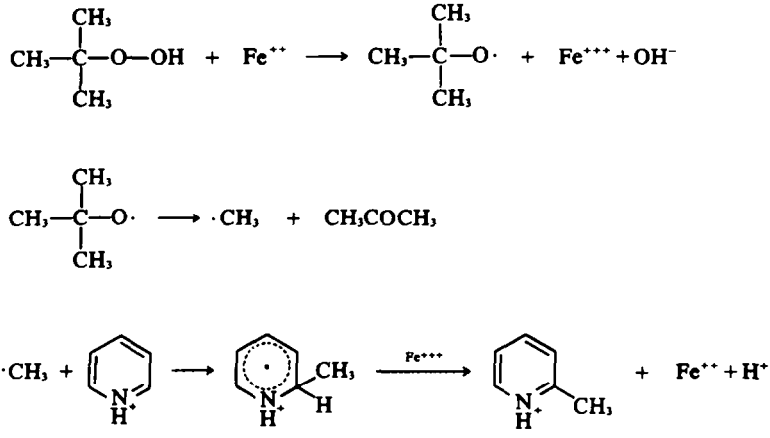
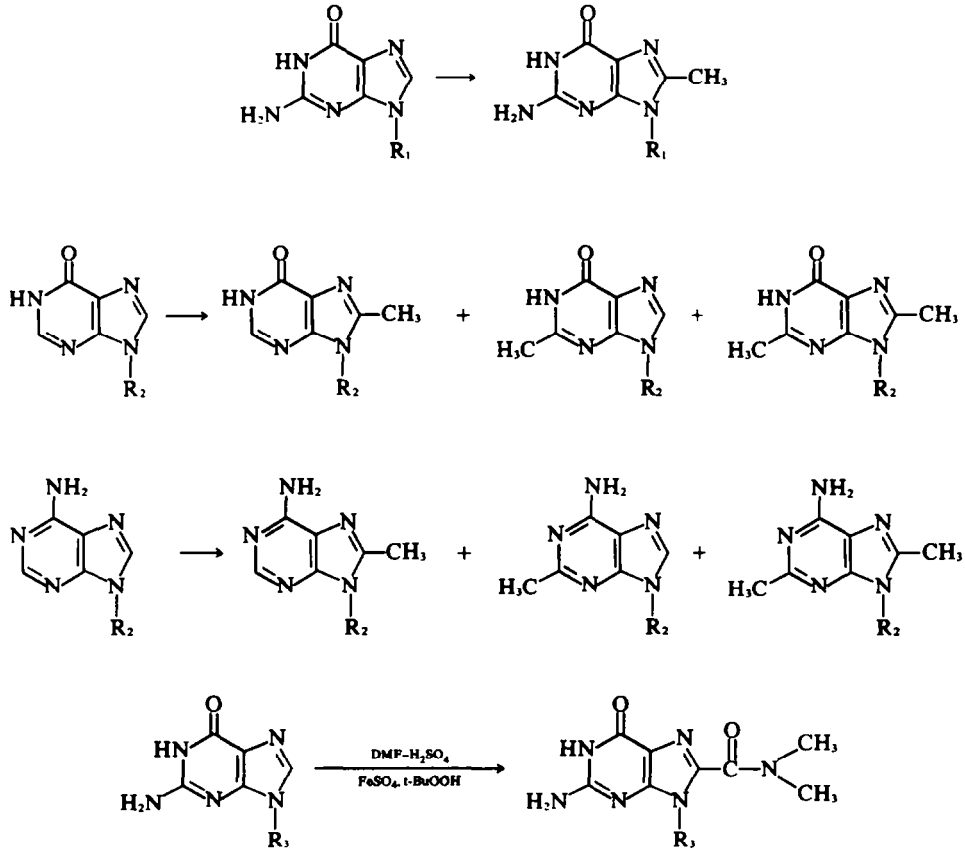


CHART 1.



R_1: H,β-D-ribofuranosyl, or 5-Phosphoryl-β-D-ribofuranosyl
R_2: H,β-D-ribofuranosyl
R_3: β-D-ribofuranosyl

CHART 2.

Table 1. Methylation of nucleic acid bases and their derivatives by treatment with t-butylhydroperoxide and ferrous sulfate in aqueous sulfuric acid at 0°C or room temperature

Compound	Conc. sulfuric acid	Product yield (%) 2-Me	8-Me	2,8-diMe	Recovery (%) of starting material
Guanine	0·2N		45		50
	1·0		57		40
Guanosine	1·0		68		7
5′-Guanylic acid	(H_2O)		1·4		31
	0·2		7·4		13
	0·5		30		52
	1·0		43		53
	1·0		59		30
	2·0		35		36
Hypoxanthine	1·0	2·1	19·0	3·6	19·5
Inosine	1·0	1·0	19·4	2·6	20·9
Adenine	1·0	5·1	13·8	9·9	38·1
Adenosine	0·2	8·3	8·3	6·3	65·1
	1·0	5·4	7·2	8·3	49·4

Table 2. UV and R_f data of alkylated guanine derivatives

Compound	UV Absorption maximum (nm) (ε) pH 1		pH 7		pH 12	R_f (Avicel-SF) solvent system A[a]	B[b]	D[c]
Guanine	248	276 (sh)	246	276 (sh)	274	0·10	0·14	
8-Me-guanine	249 (12300)		247 (10700)	276 (sh)	275 (9100)	0·26	0·27	
Guanosine	256	277 (sh)	253	271 (sh)	256–266	0·21	0·18	0·22
8-Me-guanosine	257 (11700)	275 (sh)	253 (12800)	275 (sh)	258 (10800)	0·35	0·24	
8-(DiMe-carbamoyl) guanosine	268 (14910)		268 (15060)		281 (10710)	0·30	0·29	
8-Hydroxymethyl-guanosine	259	277 (sh)	257	272 (sh)	262–270			0·17
5′-Guanylic acid	256	277 (sh)	252	271 (sh)	258	0·45		
8-Me-5′-guanylic acid	258	275 (sh)	257	275 (sh)	258	0·61		

[a] MeOH : conc. HCl : H_2O = 7 : 2 : 1.
[b] Isopropanol : conc. NH_4OH : H_2O = 7 : 1 : 2.
[c] n-Butanol : AcOH : H_2O = 3 : 1 : 1.

concentration of the medium, as seen in Table 1, although details are still left unclear.

Hypoxanthine, inosine, adenine, and adenosine underwent the methylation reaction under the same conditions described for guanine derivatives to give the corresponding methyl derivatives. The results are summarized in Chart 2 and Table 1. The UV and R_f data of the products are shown in Table 3.

The products from hypoxanthine and adenine were identical to those which were synthesized by the authentic preparative methods, respectively.[4,5] Those from inosine and adenosine were converted to the corresponding bases by acid-hydrolysis, which were identical to the authentic Me derivatives of hypoxanthine and adenine, respectively.

In order to extend applicability of this kind of homolytic C—C formation reaction catalyzed by ferrous ion, guanosine was treated with t-butylhydroperoxide and ferrous sulfate in dimethylformamide (DMF) containing 1% sulfuric acid, when 8-(dimethylcarbamoyl)guanosine was produced in 24% yield. When the reaction solvent was replaced by methanol containing 1% sulfuric acid, the products were 8-methyl and 8-hydroxymethyl derivatives of guanosine, structures of which were suggested by UV and NMR spectroscopy. The

Table 3. UV and R_f data of methylated hypoxanthine and adenine derivatives

Compound	UV Absorption maximum (nm) pH 1	pH 7	pH 12	R_f (Avicel-SF) solvent system A[a]
Hypoxanthine	246	248	261	0·22
2-Me-hypoxanthine	249	250	261	0·32
8-Me-hypoxanthine	247	250	262	0·45
2,8-DiMe-hypoxanthine	249	250	264	0·71
Inosine	248	246	253	0·26
2-Me-inosine	250	250	255	0·42
8-Me-inosine	250	250	255	0·36
2,8-DiMe-inosine	250	250	254	0·63
Adenine	261	262	268	0·13
2-Me-adenine	264	262	269	0·41
8-Me-adenine	265	263	269	0·49
2,8-DiMe-adenine	265	268	270	0·70

[a] MeOH:conc HCl:H_2O = 7:2:1.

yields were 22% and 50%, respectively. It is worth noting that 8-hydroxymethylguanosine was the only product when the reaction was carried out with hydrogen peroxide (instead of t-butylhydroperoxide) and ferrous sulfate in acidic methanol. The details are now under investigation.

DISCUSSION

Recent investigations have enabled us to introduce an alkyl group directly to the ring carbons of the purine nucleus through photo- or radiochemical processes involving production of alkyl free radicals as the reactant. In general, the purine bases are relatively stable under the influence of UV light and γ-ray, compared with the pyrimidines which readily undergo photohydration, photodimerization, homolytic addition of hydroxyl radical, etc.[6,7] Purine itself undergoes photochemical hydroxyalkylation and aminoalkylation in the presence of alcohols and alkylamines, respectively, to give 6-substituted 1,6-dihydropurines.[8-10] Caffeine and some purine nucleosides are converted to 8-hydroxy- and amino-alkyl derivatives by treatment with alcohols and alkylamines, respectively, under UV or γ-ray irradiation.[11-19] The present study dealt with homolytic alkylation of purine nucleus with alkyl radicals initiated by ferrous ion catalysis. Taking into account the metal-catalyzed redox system in the living organisms, it may be suggested that the latter type of homolytic alkylation may play a role in modifications of genetic substances such as DNA by carcinogenic peroxides.[20,21] Discussion along this line will be made elsewhere in the near future. It became evident that methylation occurred in purine bases probably through nucleophilic attack of a Me radical as Minisci *et al.* evidenced with other classes of heteroaromatic compounds.[1,2] The possibility also occurred that Me radical or probably OH radical, produced from t-butylhydroperoxide or hydrogen peroxide by metal-catalysis, abstracts a hydrogen from solvent molecules to generate other types of carbon radicals such as ·$CONMe_2$ and ·CH_2OH, which, in turn, react with the substrate of purines. Details are now under investigation.

EXPERIMENTAL

The solvent systems for TLC on Avicel-SF cellulose plates used for separation and identification of the products were as follows: solvent system A [MeOH:conc HCl:water = 7:2:1]; solvent system B [i-PrOH:conc ammonia:water = 7:1:2]; solvent system C [n-BuOH:formic acid:water = 45:5:5]; solvent system D [n-BuOH:AcOH:water = 3:1:1]. The nucleic acid bases, nucleosides, and nucleotide used were purchased from Sigma Chem. Co. (Missouri) or Takeda Pharm. Co. (Osaka). t-Butylhydroperoxide was a product of Nippon Oil and Fat Co. (Tokyo) and all other reagents were those of Tokyo Kasei Kogyo (Tokyo). The papers for chromatography were No. 51A of Toyo Roshi Kaisha or Whatman 3MM. Avicel-SF thin-layer plates were purchased from Funakoshi Pharm. Co. (Tokyo). NMR spectra were taken with JNM-3H-60 spectrometer operating at 60 MHz and UV spectra were recorded with a Cary-14 spectrometer.

8-Methylguanine. To a soln of guanine (100 mg; 0·67 mmoles) and $FeSO_4 7H_2O$ (750 mg; 2·7 mmoles) in 25 ml of 1N H_2SO_4, t-butylhydroperoxide (180 mg; 2·0 mmoles) in 5 ml water was added in drops at room temp during 5 min with stirring. After 30 min standing at this temp, $Ba(OH)_2$ aq was added to adjust the pH of the mixture to above 10. After centrifugation of the brown slushy mixture, the supernatant was separated and saturated with CO_2. The ppt was collected on a funnel and separated into 8-methylguanine and guanine by paper chromatography (solvent system A or B). The yield of 8-methylguanine was 57%. When 70 ml of 0·2N H_2SO_4 was used instead of 1·0N H_2SO_4, the yield lowered to 45%. The 8-methylguanine thus obtained was identical with an authentic specimen[3] by UV and NMR spectroscopy and by co-chromatography with solvent system A and B.

8-Methylguanosine. To a soln of guanosine (100 mg; 0·35 mmoles) and $FeSO_4 7H_2O$ (400 mg; 1·4 mmoles) in 20 ml 1N H_2SO_4, t-butylhydroperoxide (100 mg; 1·1 mmoles) in 5 ml water was added in drops at room

temp during 5 min with stirring. After 30 min standing at this temp, KOH aq was added to adjust the pH of the mixture to neutral. After centrifugation of the brown slush, the supernatant was separated. The brown slush was washed with hot water. The washings were combined with the supernatant and concentrated under reduced pressure. The concentrated soln was applied on a charcoal column (2·2 cm × 16 cm). After the column was washed with 1 l of water, the product was eluted with 2 l of a mixture of MeOH and conc ammonia (3 : 1 in volume). The eluate was evaporated to dryness to give a crude product consisting of 90% of 8-methylguanosine and 10% of the starting material. The crude product was recrystallized twice from H_2O to give 8-methylguanosine as white needles which melted at 185° and decomposed at 230°. The yield was 68%. (Found: C, 41·79; H, 5·33. Calcd. for $C_{11}H_{15}N_5O_5H_2O$: C, 41·90; H, 5·43%). A sample of this was heated in 1 N HCl at 100° for 1 h. The hydrolyzate was identical with 8-methylguanine by TLC on Avicel plate (solvent system A and B). The UV and NMR data supported this structural identity.

8-Methylguanosine 5'-monophosphate. To a soln of disodium guanosine 5'-monophosphate (360 mg; 0·90 mmoles) and $FeSo_4$ $7H_2O$ (1·12 g; 4·0 mmoles) in 21 ml 1·0N H_2SO_4, t-butylhydroperoxide (270 mg; 3·0 mmoles) in 10 ml water was added in drops at room temp during 5 min with stirring. The mixture was made alkaline with NaOH aq. After centrifugation, the pH of the supernatant was adjusted to 7·5 by addition of conc HCl. The aqueous soln thus obtained was concentrated to a volume of 50 ml under reduced pressure. The concentrated soln was applied on a charcoal column (1·7 cm × 7 cm). The column was washed with about 1 l of water and then, the product was eluted with a mixture of MeOH and conc ammonia (3 : 1 in volume). The residue was separated into 8-methylguanosine 5'-monophosphate and the starting material by thin-layer and paper chromatography (solvent system D). The yield of the product was 59% and 30% of the starting material was recovered. The 8-methylguanosine 5'-monophosphate thus obtained was confirmed as the phosphoric monoester by electrophoresis (pH 8·0 in triethylammonium bicarbonate buffer). The product was hydrolyzed into 8-methylguanosine by incubating with *E. coli* alkaline phosphomonoesterase (Boehringer Mannheim) in 0·05 M triethylammonium bicarbonate at 37° for one day.

Methylation of hypoxanthine. To a soln of hypoxanthine (272 mg; 2 mmoles) and $FeSO_4$ $7H_2O$ (2·22 g; 8 mmoles) in 40 ml 1N H_2SO_4, t-butylhydroperoxide (540 mg; 6 mmoles) in 10 ml of water was added in drops at room temp during 5 min with stirring. After 30 min standing at this temp, NaOH aq was added to adjust the pH to above 10. After centrifugation of the brown slushy mixture, the supernatant was neutralized with conc HCl and evaporated to dryness *in vacuo*. The residue was applied to a charcoal column (2·2 cm × 15·5 cm). After the column was washed with about 1 l of water, the bases were eluted with about 1 l of a mixture of MeOH and conc ammonia (3 : 1 in volume). The eluate was evaporated to dryness to afford a mixture of the bases in 44% yield. The mixture was separated by paper chromatography into the starting material, 2-methyl-, 8-methyl-, and 2,8-dimethylhypoxanthine in 19·5, 2·1, 19·0, and 3·6% yield, respectively. The products were identified with the respective authentic specimens[3,22-27] by co-chromatography (solvent systems A and B) and by UV and NMR spectroscopy.

Methylation of inosine. To a soln of inosine (268 mg; 1 mmole) and $FeSO_4$ $7H_2O$ (1·11 g; 4 mmoles) in 20 ml 1N H_2SO_4, t-butylhydroperoxide (270 mg; 3 mmoles) in 10 ml water was added in drops at room temp during 5 min with stirring. After 30 min at this temp, NaOH aq was added to adjust the pH to above 10. After centrifugation of the brown slushy mixture, the supernatant was neutralized with conc HCl and evaporated to dryness *in vacuo*. The residue was applied to a charcoal column (2·2 cm × 20 cm). After the column was washed with about 2 l of water, the nucleosides were eluted with about 2 l of a mixture of MeOH and conc ammonia (3 : 1 in volume). The eluate was evaporated to dryness to afford a mixture of the nucleosides in 44% yield. The mixture was separated by paper chromatography (solvent system A) into the starting material, 2-methyl-, 8-methyl-, and 2,8-dimethylinosine in 20·9, 1·0, 19·4, and 2·6% yields, respectively, the structures of these products being suggested by UV and NMR spectroscopy. By treatment with 1N HCl at 100° for 1 h, the products were hydrolyzed into the respective methylated hypoxanthines which were identified with the authentic specimens by co-chromatography (solvent systems A and B).

Methylation of adenine. To a soln of adenine (270 mg; 2 mmoles) and $FeSO_4$ $7H_2O$ (2·22 g; 8 mmoles) in 30 ml 1N H_2SO_4, t-butylhydroperoxide (540 mg; 6 mmoles) in 10 ml water was added in drops at room temp during 5 min with stirring. After 30 min at this temp, NaOH aq was added to adjust the pH to above 10. After centrifugation of the brown slushy mixture, the supernatant was neutralized with conc HCl and evaporated to dryness *in vacuo*. The residue was applied on a charcoal column (2·2 cm × 13 cm). After the column was washed with about 1 l of water, the bases were eluted with about 1·5 l of a mixture of MeOH and conc ammonia (3 : 1 in volume). The eluate was evaporated to dryness to afford a mixture of the bases in 67% yield. The mixture was separated by paper chromatography (solvent system A) into the starting material, 2-methyl-, 8-methyl-, and 2,8-dimethyl-adenine in 38·1, 5·1, 13·8, and 9·9% yields, respectively. The products were identical to the authentic specimens, respectively,[22,24,25,27,28] by co-chromatography (solvent systems A and B) and by UV and NMR spectroscopy.

Methylation of adenosine. To a soln of adenosine (534 mg; 2 mmoles) and $FeSO_4$ $7H_2O$ (2·22 g; 8 mmoles) in 40 ml 1N H_2SO_4, t-butylhydroperoxide (540 mg; 6 mmoles) in 10 ml water was added in drops under ice-cooling during 5 min with stirring. After 30 min at 0°, NaOH aq was added to adjust the pH to above 10. After centrifugation of the brown slushy mixture, the supernatant was neutralized with conc HCl and evaporated to dryness *in vacuo*. The residue was applied to a charcoal column (2·2 cm × 13 cm). After the column was washed with about 1 l of water, the nucleosides were eluted with 2·5 l of a mixture of MeOH and conc ammonia (3 : 1 in volume). The eluate was evaporated to dryness to afford a mixture of the nucleosides in 70% yield. All were the nucleosides and the hydrolyzed adenine derivatives were not detected by thin-layer chromatography. The yield was estimated by the recovered UV intensity. As this mixture could not be separated into each of the nucleoside components, they were hydrolyzed to the corresponding bases by heating at 100° for 1 h in 1N HCl. The hydrolyzate was separated by TLC (solvent system C on Avicel-SF plate) into adenine, 2-methyl-, 8-methyl-, and 2,8-dimethyl-adenine which were identical to the authentic specimens, respectively, by co-chromatography (solvent systems A and B) and by UV and NMR spectroscopy. The yields of

the methylated adenosines were estimated on the basis of the product ratio of the hydrolyzate to be 49·4, 5·4, 7·2, and 8·3% for the recovered adenosine, 2-methyl-, and 8-methyl-, and 2,8-dimethyl-adenosine, respectively.[29-31] When the methylation reaction was carried out in 0·2N H_2SO_4 instead of 1N, the yields of the products slightly changed; 65·1, 8·3, 8·3, and 6·3% for adenosine, 2-methyl-, 8-methyl-, and 2,8-dimethyl-adenosine, respectively.

8-(Dimethylcarbamoyl)guanosine. To a soln of guanosine (3 g; 10·5 mmoles) in 30 ml dimethylformamide containing 2·1 g conc H_2SO_4, $FeSO_4$ $7H_2O$ (11 g; 40 mmoles) in 30 ml water and t-butylhydroperoxide (3 g; 33 mmoles) in 30 ml water were individually and simultaneously added in drops under ice-cooling with vigorous stirring during 30 min. After another 30 min stirring, the mixture was diluted with 100 ml water under ice-cooling and make alkaline (pH 10–11) by addition of 30% NaOH. After centrifugation of the brown slush thus produced, the supernatant was neutralized with HCl aq and applied on a charcoal column (2 cm × 15 cm). The column was washed thoroughly with water and the product was eluted with a mixture of MeOH and conc NH_4OH (3:1 in volume). The eluate was evaporated into dryness *in vacuo* and crystallized from water to afford 1·5 g of guanosine. The mother liquid was evaporated and the residue was crystallized from a mixture of EtOAc and acetone. The crystalline material (900 mg) was recrystallized from EtOH containing ether to give 8-(dimethylcarbamoyl)guanosine in 24% yield. (Found: C, 40·83;, H, 4·97; N, 21·82 (hygroscopic). Calcd. for $C_{13}H_{18}N_6O_6 \cdot 3/2H_2O$: C, 40·94; H, 5·55; N, 22·04%). NMR (δ in dimethylsulfoxide-d_6): 3·00 and 3·04 for two N-Me, 5·84 for 1'-H, 6·67 for NH_2, $J_{1'2'}$ 6·1 Hz.

8-(Hydroxymethyl)guanosine. To an ice-chilled soln of guanosine (2·83 g, 10 mmoles), $FeSO_4$ $7H_2O$ (11·1 g; 40 mmoles) and 10 g of conc H_2SO_4 in 200 ml MeOH, 4·55 g of *ca* 33% H_2O_2 was added in drops during 10 min with stirring. After 1·5 h standing under the same conditions the mixture was applied to a charcoal column (20 ml) and washed with water and then, eluted with a mixture of methanol and conc ammonia (3:1 in volume). The eluate was evaporated to dryness under reduced pressure. The reaction was repeated at least once more under the same conditions to increase the yield of the product. The evaporated residue finally obtained was recrystallized from an appropriate volume of water to give pure guanosine. The mother liquid contained about more than 70% of 8-(hydroxymethyl)guanosine.

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