of its conversion to a conjugated imino enol which cannot undergo photoionization. It is unlikely that **2** and **3** are formed in concerted sigmatropic rearrangements.

Experimental Section

Commercial ethyl *N*-phenylcarbamate was decolorized by activated carbon in refluxing ethanol and purified by sublimation, mp 52 °C (lit.[10] 52 °C). Aniline and ethyl *o*-aminobenzoate was distilled before use and ethyl *p*-aminobenzoate was purified by crystallization in ethanol. A mixture of *meso*- and *dl*-2,3-butanediol was prepared by the reduction of 2,3-butanedione with lithium aluminum hydride in refluxing ether.

Photochemical Apparatus. A 100-ml photocell was employed which was provided with a 24/40 joint for inserting a Hanovia low-pressure mercury lamp (2.5 W) emitting 90% of radiations of 2537 Å. The cell was provided with an air-tight condenser and flushed with nitrogen before and during photolysis. The solution in the cell was stirred magnetically. The cell was wrapped in aluminum foil.

After photolysis, the solution was concentrated[24] on a rotary evaporator and was directly analyzed in a gas chromatograph using a 6-ft 20% Carbowax (20M) column (0.125 in. diameter) operating between 170 and 210 °C. Sulfolane was used as an internal standard whenever necessary and the response ratios were evaluated from pure authentic samples. The experimental results are summarized in Table II. When photolyzed in the presence of an acid (usually HCl, 10^{-1} M) the photoproducts were basified and extracted with ether. The ether solution was dried ($MgSO_4$), concentrated, and analyzed as before.

Kinetic Runs. The photocell was similar to the one described above. A 5×10^{-5} solution of **1** in ethanol was taken in the flask and photolyzed over a period of 24 h. Samples (5 ml) were withdrawn periodically by means of a syringe and the uv spectrum recorded for each sample. From the change in the absorbance at 2340–2350 Å, the rate of disappearance of I was evaluated to be $4.587 \pm 0.178 \times 10^{-3}$ min^{-1} corresponding to a half-life of 2.5 h.

The output of the lamp was determined with an uranyl oxalate actinometry according to the method of McLaren and Schugar.[22] The quantum yield was of the order of 0.45.

Acknowledgment. The authors wish to express their thanks to the Aero Materials Laboratory, Naval Air Development Center, Warminster, Pa., for support of this research.

Registry No.—**1**, 101-99-5; **2**, 87-25-2; **3**, 94-09-7; **4**, 62-53-3.

References and Notes

(1) J. C. Anderson and C. B. Reese, *Proc. Chem. Soc., London*, 217 (1960).
(2) A large body of literature including two review articles listed below are available. (a) D. Bellus, *Adv. Photochem.*, **8**, 109 (1971); (b) V. I. Stenberg, *Org. Photochem.*, **1**, 127 (1967); (c) R. A. Finnegan and J. J. Mattice, *Tetrahedron*, **21**, 1015 (1965).
(3) C. E. Kalmus and D. M. Hercules, *J. Am. Chem. Soc.*, **96**, 449 (1974).
(4) H. Kobsa, Abstracts, 148th National Meeting of the American Chemical Society, Chicago, Ill., August 1964, No. 12E.
(5) M. R. Sandner, E. Hedaya, and D. J. Trecker, *J. Am. Chem. Soc.*, **90**, 7249 (1968).
(6) M. R. Sandner and D. J. Trecker, *J. Am. Chem. Soc.*, **89**, 5725 (1967).
(7) G. M. Coppinger and E. R. Bell, *J. Phys. Chem.*, **70**, 3479 (1966).
(8) D. J. Trecker, R. S. Foote, and C. L. Osborn, *Chem. Commun.*, 1034 (1968).
(9) D. Bellus and K. Schaffner, *Helv. Chim. Acta*, **51**, 221 (1968).
(10) (a) H. C. Beachell and I. L. Chang, *J. Polym. Sci.*, **10**, 503 (1972). (b) The photoproducts **2**, **3**, and **4** react with oxygen during photolysis.
(11) K. Schwetlick, R. Noack, and G. Schmieder *Z. Chem.*, **12**, 107 (1972).
(12) H. J. Hageman, *Recl. Trav. Chim. Pays-Bas.*, **91**, 362 (1972).
(13) R. Noack and K. Schwetlick, *Z. Chem.*, **12**, 108 (1972); **12**, 140 (1972); **12**, 143 (1972); **14**, 99 (1973).
(14) K. Schwetlick and R. Noack, *Z. Chem.*, **12**, 109 (1972).
(15) (a) M. V. Alfimov and I-G. Batekha, *Khim. Vys. Energ.*, **2**, 123 (1968); (b) M. V. Alfimov, V. A. Smirnov, *ibid.*, **2**, 186, (1968); (c) M. V. Alfimov, V. A. Smirnov, N. V. Seregin and V. Ya, *Agroskin, Dokl. Nauk SSSR*, **191**, 103 (1970).
(16) D. M. Findlay and M. F. Tchir, *J. Chem. Soc., Chem. Commun.*, 514 (1974).
(17) W. G. Dauben, L. Salem, and N. J. Turro, *Acc. Chem. Res.*, **8**, 41 (1975).
(18) F. Kraus, E. Gregorek, and H. Von Weyssenhoff, *Z. Phys. Chem.*, **82**, 139 (1972).
(19) R. W. Yip, R. O. Loutfy, Y. L. Chow, and L. K. Magdzinski, *Can. J. Chem.*, **50**, 3426 (1972).
(20) Y. Shigemitsu, T. Tominaga, T. Shimodair, Y. Odaira, and S. Tsutsumi, *Bull. Chem. Soc. Jpn.*, **39**, 2463 (1966).
(21) V. V. Zharkov and N. K. Rudnevskii, *Tr. Khim. Khim. Tekhnol.*, 175 (1965).
(22) A. D. McLaren and D. Shugar, "Photochemistry of Protein and Nucleic Acids", MacMillan, New York, N.Y., 1964, p 378.
(23) A referee suggested that a larger photoyield is to be expected at lower concentrations of **1** since more quanta of light are available for each molecule. However photolysis of **1** at the higher concentrations for extended periods of time (1 week) did not increase the photoyields over those obtained in 3 days.
(24) Care was taken to avoid loss of photoproducts during concentration.

Methylation of Nucleic Acid-Bases with Trimethyl Phosphate

Kiyoshi Yamauchi,* Toshizumi Tanabe, and Masayoshi Kinoshita

Department of Applied Chemistry, Osaka City University, Sumiyoshi-ku, Osaka, Japan

Received April 30, 1976

The action of trimethyl phosphate (TMP) on cytosine, thymine, uracil, adenine, and guanine has been examined in a homogeneous aqueous phase at 25–60 °C, pH 9–12. All these nucleic acid–bases underwent methylation reactions, showing the following reactivity order on the methylating site in each base: cytosine, N-1 > N-3; thymine and uracil, N-1 ~ N-3; adenine, N-9 ~ N-3 > N-7, N-1; guanine, N-1 ≳ N-7 > N-3 > N-9, O-6. These results and reactions of monomethylated bases with TMP suggested TMP as an useful modifying agent for nucleic acids. The use of TMP as additives for commercial products was also considered briefly.

The direct N-alkylation of basic moieties of nucleic acids has been the subject of considerable chemical and biological interest in recent years. Such reactions may be not only useful in a synthetic point of view but also relevant to the study of mutagenic and carcinogenic effects which occur in living systems caused by alkylating agent.

Various alkylating agents have been employed in such investigations such as nitrogen[1,2] and sulfur[3,4] mustards as well as diazoalkanes,[5,6] alkyl esters of sulfurous oxy acids,[7-13] alkyl halides,[14-16] and others.[17-20]

However, there have been no alkylation studies with trimethyl phosphate (TMP), although TMP has been shown recently to cause mutagenic effects in male mice[21] as well as in Neurospora.[22] In vivo, TMP has been reported to function as alkylating agent, degrading in the rat to dimethyl hydrogen phosphate with formation of *S*-methylcysteine as an urinary metabolite.[23] It would, therefore, be interesting to study the reactivity of TMP toward nucleic acids, their components, and other natural products.

With these aspects in mind, we have studied the reactivity of TMP, showing previously N-methylation of imidazoles, purines, and pyrimidines upon fusing these bases in TMP at 160–220 °C.[24,25] Aside from its synthetic utility, however, this procedure would be too vigorous for the study of the action

Table I. Product Distribution in the Reactions of Pyrimidines and Purines with Trimethyl Phosphate (TMP)[a]

Base	Product[b]	pH	R_f[c]	λ_{max} (log ϵ)[d] at pH 7	Spectroscopic yield, %[e] 25 °C 24 h	25 °C 48 h	37 °C 10 h	37 °C 24 h	60 °C 10 h	60 °C 24 h	Isolated[f] yield, %	Registry no.
Cytosine (1)	1-Methyl-C (4)	11–12	0.41	275.0 (4.02)	34	48	33	40	36	41	28	1122-47-0
	1,3-Dimethyl-C (5)		0.08	281.5 (3.98)	0	0	0	0	0	0	9	6749-87-7
	1,3-Dimethyl-U (11)		0.80	267.0 (3.92)	0	0	0	0	5	10	12	874-14-6
	(Cytosine)		0.19	267.0 (3.79)	61	46	66	57	55	40		
Thymine (2)	1-Methyl-T (6)	9–10	0.62	273.0 (3.99)	13	16	14	21	18	23	12	4160-72-9
	3-Methyl-T (8)		0.82	266.0 (3.85)	12	14	14	15	16	18	17	4160-77-4
	1,3-Dimethyl-T (10)		0.88	272.0 (3.97)	7	17	6	18	31	36	52	4401-71-2
	(Thymine)		0.20	265.0 (3.90)	55	41	63	42	32	20		
Uracil (3)	1-Methyl-U (7)	9–10	0.58	267.5 (3.93)	13	20	16	21	20	21	12	615-77-0
	3-Methyl-U (9)[g]		0.74	260.0 (3.87)	12	17	15	17	18	18	13	608-34-4
	1,3-Dimethyl-U (11)		0.88	267.0 (3.92)	7	17	6	18	31	36	5	
	(Uracil)		0.26	260.0 (3.91)	60	33	57	36	25	17		
Adenine (12)	3-Methyl-A (13)	9–10	0.51 0.28	274.0 (4.01)	27	34	20	25	29	23	6	5142-23-4
	7-Methyl-A (15)		0.18 0.09	269.0[h,n]	0	6	5	5	3	2		935-69-3
	9-Methyl-A (14)		0.74 0.46	263.0 (4.09)	28	34	24	30	32	28	27	700-00-5
	N^6,9-Dimethyl-A (16)		0.84 0.58	268.0 (4.10)	0	4	0	2	10	10	10	2009-52-1
	3,7-Dimethyl-A (17)		0.02 0.01	278.0[i,n]	0	0	0	0	6	14		60065-12-5
	Imidazole ring-opened N^6,7,9-trimethyl-A (18)		0.18 0.04	263.5[j]	0	3	4	5	4	6	1	49581-54-6
	(Adenine)		0.55 0.31	260.0 (4.13)	32	13	34	19	3	2		
Guanine (22)	1-Methyl-G (23)	11–12	0.08 0.33	250.0 (3.87) 274.5 (3.77)			27	30		20	1	938-85-2
	7-Methyl-G (24)		0.15 0.47	250.0[s] 283.5[k,n]			0	10		0		578-76-7
	1,7-Dimethyl-G (25)		0.23 0.65	253.0 (3.76) 284.0 (3.81)			10	8		15	2	26758-00-9
	3,7-Dimethyl-G (26)		0.10 0.53	270.0 (3.76)			11	12		16	10	19143-67-0
	Imidazole ring-opened 1,7,9-trimethyl-G (27)		0.23	269.0 (4.22)[l]			Tr	Tr		7	20	60065-13-6
	O^6,3,7-Trimethyl-G (28)		0.34	(3.82)[m] 269.0			Tr	Tr		5	4	60065-14-7
	(Guanine)			245.0 (4.04) 274.0 (3.92)			17	14		6		

[a] Reaction size: a base (0.90 mmol) + TMP (10.80 mmol) + H_2O (5 ml) at 25 °C; a base (1.80 mmol) + TMP (5.40 mmol) + H_2O (5 ml) at 37 and 60 °C. [b] C, T, U, A, and G refer to cytosine, thymine, uracil, adenine, and guanine rings, respectively. [c] Aluminum oxide TLC, solvent C for reaction mixtures of thymine and uracil; silica gel TLC, solvent D for the reaction mixture of cytosine; solvent D (left R_f) and solvent C (right R_f) for the reaction mixture of adenine; solvent B (left R_f) and solvent E (right R_f) for the reaction mixture of guanine. [d] Ultraviolet spectra in acidic (pH 1) and basic (pH 13) conditions were identical with those reported in literatures cited in the Experimental Section. [e] Tr refers to a trace yield. [f] Yields based on isolated amounts of products. Reactions were carried out using a large excess of TMP; see Experimental Section for details. [g] Mp 189.5–191 °C (EtOH–ether) (lit.[40] 174–175 °C). [h] λ_{max} 274.0 nm (pH 1), 269.0 (pH 13) [G. B. Elion, *J. Org. Chem.*, **27**, 2478 (1962): λ_{max} 274 nm (pH 1), 270 (pH 13)]. [i] λ_{max} 281.0 nm (pH 13) [lit.[27] λ_{max} 277 nm (pH 7) and 281 (pH 13)]. [j] λ_{max} 269.0 nm (pH 1), 261.0 (pH 13) [ref 12 reports λ_{max} 271 nm (pH 1) and 260 (pH 13) for the imidazole ring-opened N^6,7-dimethyladenosine]. [k] λ_{max} 251.5 and 272.0[s] nm (pH 1) [W. Pfleider, *Justus Liebigs Ann. Chem.*, **647**, 167 (1961), reports λ_{max} 250 and 270[s] nm (pH 1); 248,[s] 283 (pH 7)]. R_f of the authentic sample: 0.15 (solvent B) and 0.47 (solvent E). [l] λ_{max} 269.0 nm (4.33) (pH 1) and 269.0 (4.21) (pH 13). [m] λ_{max} 267.0 nm (3.90) (pH 1) and 291.0 (3.72) (pH 13). [n] Compound was not isolated. [s]Shoulder.

of TMP on many natural products, where milder conditions are required.

This paper presents reactions of TMP with nitrogen heterocycles of nucleic acids in an aqueous solution of pH 9–12 at 25–60 °C, revealing facile methylation of these bases. Whereas other alkylating agents such as mentioned above are little soluble in water, TMP is miscible freely with water and allowed alkylation reactions to be run in a homogeneous aqueous phase for the first time.

Following are the characterization of products from reactions of cytosine (1), thymine (2), uracil (3), adenine (12), and guanine (22). The reactivity of these nucleic acid–bases will be also compared qualitatively in the succeeding section and discussed in terms of selective alkylation by TMP.

Results and Discussion

Reactions were generally carried out at 25, 37, and 60 °C by mixing a base and TMP in water at an appropriate pH (pH 9–11 for **2**, **3**, and **12** and pH 11–12 for **1** and **22**). Although the relatively high pH was used for **1** and **22** to overcome the low solubility of these bases, hydrolysis of TMP was slow even under this condition. For example, when the same molar mixture of TMP and water as in the alkylation reaction was kept at 37 and 60 °C for 24 h at pH 11, the extent of hydrolysis of the ester was about 3 and 10%, respectively. The decomposition of the ester was virtually negligible between pH 9 and 10.

Products were separated by a combination of extraction and column chromatography. Alkylation sites were determined most conveniently by ultraviolet, NMR, and mass spectra. Other physical constants (R_f, melting point, elemental analysis, etc.) were also employed for the identification of products.

Cytosine (1), Thymine (2), and Uracil (3). Alkyl halides in dimethyl sulfoxide in the presence of potassium hydroxide[26] and dimethyl sulfate in dimethylformamide or dimethylacetamide[11] have been most frequently utilized for the direct alkylation of these pyrimidines. These reagents convert **1** to

Table II. Reactions of Monomethylpyrimidines and -purines with Trimethyl Phosphate (TMP)[a]

Reactant	Product	Yield, %[b] 7 h	14 h	24 h
1-Methylcytosine (4)	1,3-Dimethyluracil (11)	Tr	Tr	4 (95)
1-Methyluracil (7)	11	15 (83)	23 (73)	37 (60)
3-Methyluracil (9)	11	12 (84)	24 (73)	40 (55)
3-Methyladenine (13)	3,7-Dimethyladenine (17)	8 (77)	11 (74)	14 (70)
9-Methyladenine (14)	N^1,9-Dimethyladenine (21)	Tr	Tr	2 (94)
	N^6,9-Dimethyladenine (16)	Tr	Tr	3

[a] Reaction size: 4, 7, 9, and 13 (0.127 mmol) + TMP (0.371 mmol) + H_2O (0.35 ml); 14 (0.127 mmol) + TMP (0.74 mmol) + H_2O (0.70 ml). pH used: 9.5–10.0 (NaOH). Reaction temperature: 37 °C for reactions of 4, 7, 9, and 14; and 60 °C for the reaction of 13. [b] Tr refers to a trace yield. The percentage of the starting monomethyl bases unreacted are shown in parentheses.

a mixture of 3-alkyl (major) and 1,3-dialkyl derivatives[10] and 2 and 3 generally to a mixture of 1-alkyl and 1,3-dialkyl derivatives.

The present method with TMP in a water phase gave results somewhat different from these reactions. Table I summarizes the distribution of products at various reaction times and temperature. Thus, cytosine (1) was substituted at the N-1 position mainly to produce 1-methylcytosine (4) with a small amount of 1,3-dimethyluracil (11). Upon using a large excess of TMP, 1,3-dimethylcytosine (5) was isolated in addition to these products. 3-Methylcytosine was not formed in the present reaction. Methylation of the exocyclic 4-NH_2 and C^2O groups was not observed, either. The occurrence of a substantial amount of 11 may be attributed to the hydrolysis of 5. Indeed, an authentic sample of 5 was transformed to 11 under comparable conditions. The alternative formation of 11, e.g., the deamination of 1 or 4 and the subsequent methylation at the N-3 position of the resulting uracil derivatives (2 or 7), would be less likely as the source of 11 since both 1 and 4 are fairly stable under the present reaction condition, undergoing deamination to the extent of only 1–2%.

Thymine (2) and uracil (3), on the other hand, were substituted at both the N-1 and N-3 positions to give a mixture of 1-methyl (6 and 7), 3-methyl (8 and 9), and 1,3-dimethyl (10 and 11) derivatives. Methylation of the C^2O and C^4O groups was not observed at all. As depicted in Table I, the distribution of these products is similar in both 2 and 3.

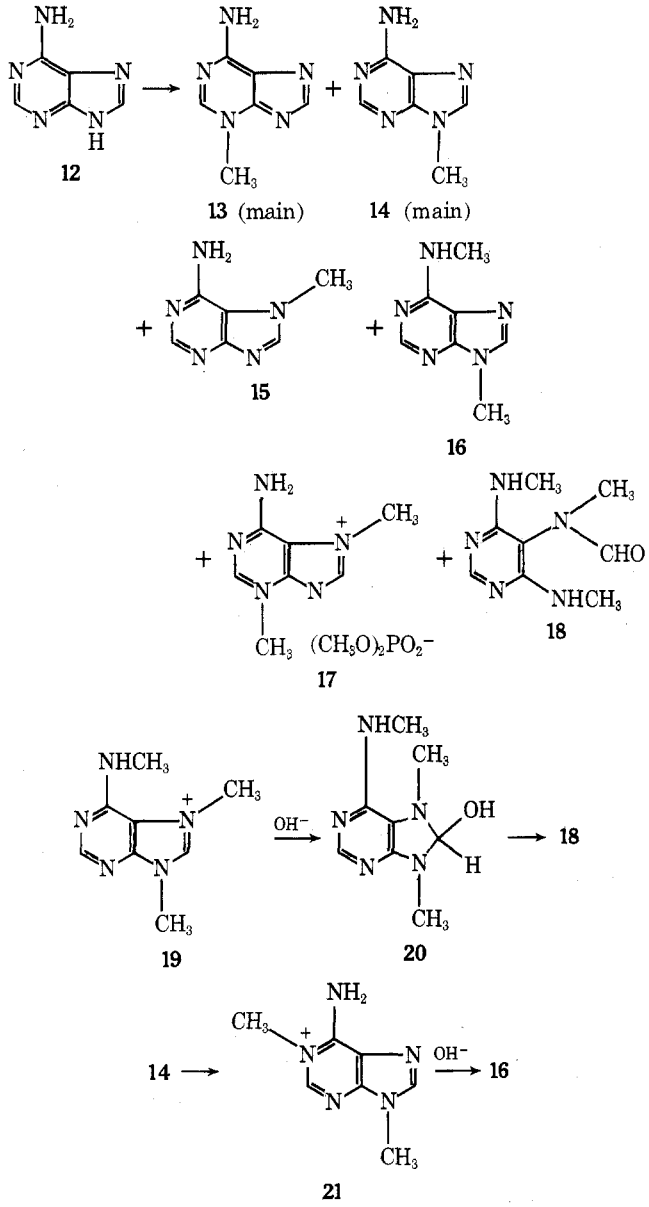
The controlled experiments revealed that the yield of 11 from methylation of 7 is approximately equal to that from 9 (see Table II). TMP, thus, appears to be attacked at the N-1 and N-3 positions of 2 and 3 equally to produce monomethyl derivatives (7 and 9), both of which are then remethylated to afford 1,3-dimethyl derivatives at similar rates. Upon using a large excess of TMP, 10 and 11 were isolated in semiquantitative yields. The synthetic utility of the facile methylation of 6 and 7 will be discussed later in General Remarks.

Adenine (12). Alkylation of 12 has been conducted with various alkylating agents such as dimethyl sulfate, diazomethane, alkyl halides, etc. Accumulating data show that the alkylation sites are influenced a great deal by solvent, pH, steric factors, temperature, etc. It, however, has been generally observed that the N-3 position is alkylated preferentially to the N-9 position under neutral conditions,[7,8,17,27] whereas under basic conditions the N-9-substituted product is formed mainly with the conformation of N-3-substituted adenine.[15,20,28]

The present reaction of adenine (12) with TMP took place smoothly to generate about six ultraviolet-absorbing products (13–18, see Table I). Compounds 13 and 14, which were major products obtained in comparable yields, and 16 were identified as 3-methyl-, 9-methyl-, and N^6,9-dimethyladenines, respectively, through their known physical constants. Products 15 and 17 could not be isolated but were identified tentatively as 7-methyl- and 3,7-dimethyladenines, respectively, based on their ultraviolet spectra which had close resemblances to those of the assigned compounds. At low temperatures the yields of these products were negligible as shown in Table I, but the formation of 17 increased considerably at 60 °C and appeared to accompany the consumption of 13 and 15. The reaction of 13 and TMP also afforded 17 as the chief product under comparable conditions. These results coincide with Leonard's observation that N-3-substituted adenine is alkylated mainly at the N-7 position and vice versa.[27]

Product (18) was generated in a small yield; it had an ultraviolet spectrum similar to that of imidazole ring-opened N^6-methyl-7-ethyladenosine.[12] The mass spectrum showed ion peaks at *m/e* 195 ($M\cdot^+$), 166 ($M - NHCH_2$), 137 [$M - N(CH_3)CHO$], etc. From these, 18 was identified as 4,6-di(methylamino)-5-methylformamidopyrimidine. Its precursor may be N^6,7,9-trimethyladenine (19), in which the electrophilic C-8 position would be attacked by hydroxyl ions to undergo imidazole ring-opening reaction via 20.

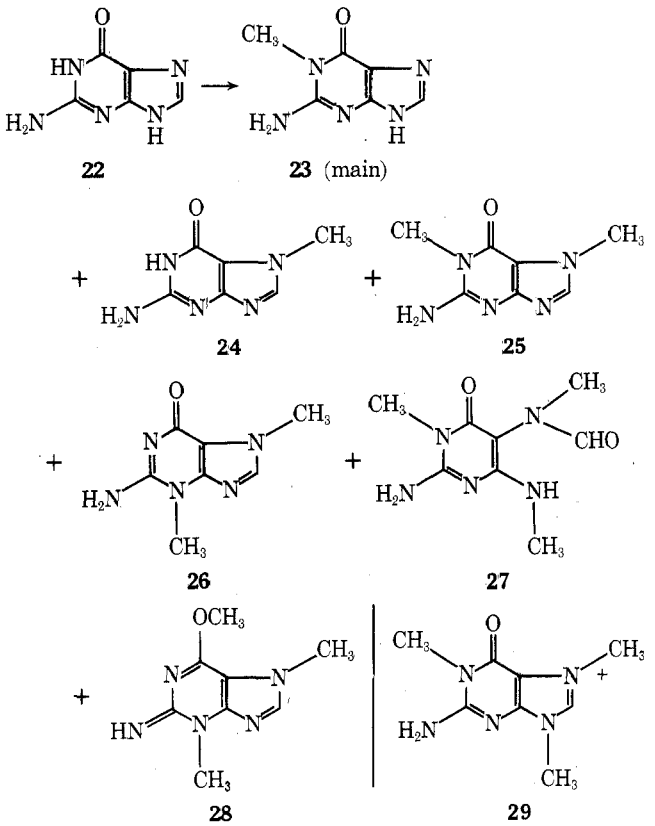
Although 1,9-dimethyladenine (21) was not detected in the reaction mixture, its formation via 14 would be expected from the very basic and nucleophilic nature of the N-1 position in 9-alkyladenine.[14,29,30] Actually, the reaction of 14 and TMP produced 21 and 16 in comparable yields (see Table II). The coproduct (16) may be derived from the Dimroth rearrangement of alkali-labile 21 rather than the direct methylation at the 6-NH_2 group.[31]



The above results thus show that adenine (**12**) is methylated with TMP at the N-3 and N-9 positions mainly. Methylation of the N-1 and N-7 positions provide relatively small contribution to the overall reactions but tend to increase with a rise of reaction temperature, generating di- and trimethylated derivatives.

Guanine (22). The guanine moiety has been established as a prime target in the alkylation of DNA by alkylating agents. However, the present knowledge of alkylation of **22** is limited. Pal reported four products in the reaction with ethyl methanesulfonate at pH 12 but identified only 7-ethylguanine.[7] Reiner and Zamenhof used dimethyl and diethyl sulfates at pH 10.8 and assigned one of the products as 7-methylguanine (**24**) on a spectrometric basis, but several other products were not identified.[9] Robins et al., on the other hand, found a quantitative formation of 7,9-dimethylguanine with dimethyl sulfate in dimethylacetamide.[8]

In the present reaction of **22** with TMP at pH 11–12, six ultraviolet-absorbing products were observed by thin layer chromatography of the reaction mixture (**23–28**, see Table I). One of the spots (compound **24**), which was seen when the reaction was carried out at 37 °C for 24 h as shown in Table I, was assigned as 7-methylguanine (**24**) based on the ultraviolet spectrum of the aqueous extract. 7-Methylguanine (**24**) is rather reactive and appears to undergo further methylation



reaction (vide infra). The other products were all isolated to give 1-methylguanine (**23**, a major product) and 1,7-dimethyl- and 3,7-dimethylguanines (**25** and **26**) both in substantial yields. Here, the structure of **26** was determined from its NMR spectrum and conversion to 3,7-dimethylxanthine (theobromine) through nitrous acid treatment. The structure of a minor product (**27**) was established as 2-amino-1,6-dihydro-1-methyl-4-methylamino-5-*N*-methylformamido-6-oxopyrimidine from the NMR spectrum and its resemblance in the ultraviolet spectrum to that of imidazole ring-opened 1,7-dimethylguanosine.[13] 1,7,9-Trimethylguanine (**29**) may be considered as the precursor of **27**, being attacked by hydroxyl ion at the C-8 position in a similar way as the formation of **18**. Another minor product (**28**) was brightly fluorescent under ultraviolet light and was stable under alkaline conditions, suggesting a nonpolar O^6-methyl derivative. The NMR spectrum in deuteriochloroform showed three singlets each integrating as three protons at 2.40, 2.55, and 3.60 ppm, and a singlet integrating as one protone at 7.55 ppm, indicating attachment of two methyl groups on nitrogen atoms and one methyl group on an oxygen atom. The mass spectrum showed *m/e* 193 ($M^{\cdot+}$) and 136 ($M - CH_3OCN$), which may suggest the absence of a methyl group on the N-1 position. These considerations leave O^6,3,7-trimethylguanine as the most possible structure for **28**. The formation of O^6,3,9-trimethylguanine would be hindered sterically since the 3-methyl and 9-methyl groups are situated in peri position to each other.

The yield distributions of products mentioned above indicate that the N-1 position of guanine (**22**) is comparative to or more reactive than the N-7 position followed by the N-7 and N-3 positions in methylation with TMP, although the N-7 position was believed to be the most susceptible site with other alkylating agents even under alkaline medium.

General Remarks

The present procedure allowed us to carry out methylation reactions of nucleic acid–bases in homogeneous aqueous phase owing to water-soluble and stable properties of TMP. Most

other alkylating agents are relatively short lived under an aqueous environment or insoluble in water, and reactions are generally performed in an organic solvent or a mixture of water and an organic solvent. These characteristics of TMP would favor its use for the alkylation reactions of water-soluble compounds.

In the present reactions, TMP was found to alkylate the major heterocyclic moieties of nucleic acids. Inspection of Table I shows the reactivity of heterocycles in the following qualitative order based on the consumption of the starting materials: adenine (**12**) > guanine (**22**) > uracil (**3**) ~ thymine (**2**) > cytosine (**1**). In each of these bases, the first methylation occurs in the following order: adenine, N-9 ~ N-3 > N-7, N-1; guanine, N-1 ≳ N-7 > N-3 > N-9, O-6; uracil, N-1 ~ N-3; thymine, N-1 ~ N-3; cytosine, N-1 > N-3.

These results and distribution of products may indicate that (1) the successive methylation of 1-methyluracil and -thymine (**6** and **7**) to 1,3-dimethyl derivatives (**10** and **11**) takes place easily, but (2) that conversion of 1-methylcytosine (**4**) and of 9-methyladenine (**14**) to dimethyl derivatives occur very slowly; (3) guanine (**22**), on the other hand, appears to undergo a fast methylation to produce mono- and dimethyl derivatives. Aspects 1 and 2 were ascertained in control experiments (see Table II). Facile methylation of 9-alkylguanine with TMP was confirmed by the rapid formation of 1-methyl- and 1,7-dimethylguanosines from guanosine.[32] The above differences in the methylation of bases might be useful for the modification of nucleic acids and their components, allowing especially selective methylation of thymidine and uridine both at the N-3 position.

In addition to N-methylation, O-6-methylation of guanine (**22**), which is seen in the formation of **28**, might be noteworthy from the physiological point of view despite its low yield, because O-6-alkylation of guanine residues in nucleic acids has been considered to be the cause of powerful mutagenic effects through an atypical base pairing.[33] The present results of above-mentioned N- and O-methylations may, thus, emphasize the careful consideration for the employment of TMP as food additives and for other purposes.[34]

The kinetics and mechanisms of the present reactions have not been examined, but methylation may take place most likely in a bimolecular fashion between the anionic forms of the bases and TMP under the present conditions. Here, TMP reacts with the bases efficiently in an alkaline pH range and uses one of its three methyl groups for methylation, being converted to dimethyl hydrogen phosphate, which no longer exhibits alkylating properties.

Experimental Section

Melting points were uncorrected. Ultraviolet spectra were measured with a Hitachi 3T spectrometer. ^1H NMR spectra were recorded on a Hitachi Perkin-Elmer R-20 spectrometer with a dilute solution in deuteriochloroform, deuterioxide, or dimethyl sulfoxide-d_6 and tetramethylsilane as an internal or an external standard. Mass spectra were obtained using Atlas CH-4B and JEOL 01SG-2 spectrometers.

Thin layer chromatography was performed on silica gel [GF_{254} (type 60), Merck] or aluminum oxide [PF_{254} (type 150), Merck] using a mixture of chloroform and methanol in the following volume ratio: solvent A, 9:1; B, 7:1; C, 5:1; and D, 5:2, or solvent E, 1-propanol–concentrated ammonium hydroxide, 5:1. Column chromatography was carried out using silica gel (Merck, art. 7734, 70–230 mesh) or aluminum oxide (Merck, art. 1097).

Commercially available cytosine, thymine, uracil, adenine, and guanine were used without further purification. Trimethyl phosphate (TMP) was distilled prior to use.

Determination of Product Distribution in the Reaction Mixture of a Base and TMP. The base (1.80 or 0.90 mmol) was dissolved in aqueous sodium hydroxide solution of pH 9–12 (5.0 ml) and the solution was treated with TMP (5.40 or 10.80 mmol) at a specified reaction temperature as shown in Table I. The pH of the solution was maintained in the same level with occasional addition of 1 N aqueous sodium hydroxide. Two or three aliquots of 3 μl of each of the reaction mixtures were spotted on a thin layer chromatogram after 10, 24, and 48 h. The plate was developed immediately after spotting, using solvent D for **1**, C for **2, 3,** and **12,** and E for **22.** The product spots were then scraped individually from the plate in order to extract the substance from the spot with water (3 ml). Absorbancy of the solution was recorded at the respective λ_{max}. The yield of the product at each reaction time was calculated from the equation $100\epsilon_a A/\epsilon_p A^0$, where ϵ_a and ϵ_p are molecular absorbancies of the starting base and that of the product, and A^0 and A are the absorbancies of the starting base at zero time and that of the product at a suitable reaction time.

The products and their distributions in the reaction mixtures are summarized in Table I. Control experiments were performed for bases (**4, 7, 9, 13,** and **14**) in a similar way as mentioned above. The reaction size, conditions, and results are described in Table II.

Isolation of Products. The reactions of bases and TMP were carried out in a manner similar to that mentioned above. Following are reaction sizes and isolation procedures. The mobilities (R_f) in thin layer chromatography are shown in Table I with references on the ultraviolet spectral peak at pH 7 used for identification. Ultraviolet spectra at pH 1 and 13 as well as the melting points of all known compounds agreed in most cases with literature values. The NMR spectra were obtained in all compounds and coincided with the assigned structures. Yields are calculated after recrystallization and are based on the isolated amounts of products.

Cytosine (1). The base (1.00 g, 9.01 mmol), TMP (3.75 g, 26.79 mmol), and water (15 ml) were mixed at pH 9–10, 60 °C for 24 h. The reaction mixture was extracted with chloroform (100 ml). The organic extract was concentrated and mixed with *n*-hexane (20 ml) to afford 1,3-dimethyluracil (**10**) as crystals: 0.15 g (12%); mp 123–125 °C (EtOH–water) (lit.[35] mp 120–121 °C). The water layer was concentrated and the residue was applied to a silica gel column (1.5 × 60 cm). Elution with chloroform–methanol (5:2 v/v) provided 1-methylcytosine and then 1,3-dimethylcytosine as the dimethyl hydrogen phosphate salts (0.55 and 0.17 g, respectively). The salts were subsequently treated with an anionic exchange resin (Dowex 1 × 8, 200–400 mesh, OH^- form). Elution with water gave the free form of 1-methylcytosine (**4,** 0.31 g, 28%); mp 298–300 °C (water) (lit.[36] 299–300 °C). 1,3-Dimethylcytosine (**5**) was obtained subsequently, 0.11 g (9%), mp 149–152 °C (sublimed) (lit.[11] 145 °C).

Thymine (2). The base (2.20 g, 17.46 mmol), TMP (7.50 g, 53.57 mmol) and water (30 ml) were mixed at pH 9.5–10.5, 60 °C for 24 h. The reaction mixture was neutralized with concentrated hydrochloric acid and the resulting solution was kept at 3 °C for 24 h to give 1-methylthymine (**6,** 0.12 g, 5%) as crystals, mp 282 °C (water) (lit.[37] 280–282 °C). The mother liquor was concentrated under reduced pressure to give the residue, which was then separated by aluminum oxide chromatography (2 × 40 cm), using chloroform–methanol (10:1 v/v) as a solvent. Twenty-five milliliters of the eluate was collected in each tube to give the following products. Fractions 1–2: 1,3-dimethylthymine (**10,** 1.39 g, 52%), mp 157–159 °C (EtOH–water) (lit.[37] 155 °C). Fractions 3–7: 3-methylthymine (**8,** 0.42 g, 17%), mp 211–212 °C (EtOH) (lit.[38] 209–210 °C). Fractions 10–19: 1-methylthymine (**6,** 0.17 g, 7%).

Uracil (3). After a mixture of uracil (1.00 g, 8.93 mmol) and TMP (7.50 g, 53.57 mmol) in water (11 ml) was warmed at pH 9–11, 60 °C for 48 h, the following products were obtained after treating the reaction mixture in a manner similar to that mentioned in thymine: 1-methyluracil (**7**), 0.13 g (12%), mp 232–234 °C (EtOH–water) (lit.[39] 233–234 °C); 3-methyluracil (**9**), 0.15 g (13%), mp 189.5–191 °C (EtOH–ether) (lit.[40] 174–175 °C); 1,3-dimethyluracil (**11**), 0.06 g (5%).

Adenine (12). The base (1.22 g, 9.04 mmol), TMP (3.75 g, 26.79 mmol), and water (10 ml) were mixed at pH 10–11, 60 °C for 24 h. The reaction mixture was adjusted to pH 14 by the addition of 4 N sodium hydroxide and allowed to stand at room temperature for 24 h, precipitating 9-methyladenine (**14**) as crystals, 0.31 g (23%), mp 307–313 °C dec (EtOH–water) (lit.[41] 310 °C). The mother liquor was concentrated to give a residue which was subsequently mixed with ethanol (50 ml) and separated from undissolved substances. The residue which was obtained after concentrating the alcoholic solution was then divided into a chloroform-soluble part (A) and -insoluble part (B).

Part A was treated by a silica gel column (1 × 60 cm). Elution with chloroform–methanol (7:1 v/v) provided first N^6,9-dimethyladenine (**16,** 0.15 g, 10%); mp 193–195 °C (benzene–EtOH) (lit.[41] 190–191 °C), then **14** (0.06 g, 4%). The subsequent elution with the same solvent afforded the imidazole ring-opened N^6,7,9-trimethyladenine (**18,** 0.02 g, 1%): mp 224–226 °C (chloroform); mass spectrum (75 eV) *m/e* 195 (molecular ion, 32), 181 (71), 163 (37), 153 (100), 137 (46), 123 (25),

109 (40), 95 (42), 82 (20), and 67 (19). The ultraviolet spectrum of 18 was almost identical with that of the imidazole ring-opened N^6,7-dimethyladenosine as footnoted in Table I.

Part B was treated similarly by a silica gel column (1 × 60 cm) using the same developing solvent to give 14 (0.02 g, 1%) followed by 3-methyladenine (**13**, 0.08 g, 6%), mp 295–301 °C dec (water) (lit.[42] 309–312 °C).

Guanine (22). The base (2.62 g, 17.35 mmol) was suspended in a solution of TMP (15.00 g, 107.14 mmol) and water (50 ml, pH 13). During stirring at 60 °C, a homogeneous solution was obtained, showing a pH of 11.5–12.0. After 24 h, the reaction mixture was neutralized with concentrated hydrochloric acid to give a precipitate. The mother liquor, after extracting out unreacted TMP with chloroform, was combined with the aqueous washings of the above precipitate with hot water. The solution was then concentrated as much as possible. The resulting residue was applied to a silica gel chromatography column (3 × 50 cm) and eluted using chloroform–methanol (10:1 v/v) as a developing solvent. Twenty-five milliliters of the eluate was collected in each tube to provide the following products.

Fraction 12–16: O^6,3,7-trimethylguanine (**28**); 0.14 g (4%); mp 201 °C (EtOH); mass spectrum (75 eV) *m/e* 193 (molecular ion, 100), 178 (9), 164 (28), 149 (13), 138 (23), 136 (18), 124 (19), 109 (31), 96 (15), 82 (29), and 67 (34); NMR δ ($CDCl_3$) 2.40 (s, N^3- or N^7-CH_3, 3), 2.55 (s, N^7- or N^3-CH_3, 3), 3.60 (s, OCH_3, 3), 7.53 (s, H^8, 1), 7.55 (broad s, NH, 1).

Anal. Calcd for $C_8H_{11}H_5O_1 \cdot 0.8H_2O$: C, 46.29; H, 6.08; N, 33.75. Found: C, 46.31; H, 5.54; N, 33.38.

Fractions 18–48: 1,7-dimethylguanine (**25**), 0.06 g (2%), mp 312–315 °C (EtOH–water) (lit.[36] 330–331 °C).

Fractions 54–60: imidazole ring-opened 1,7,9-trimethylguanine (**27**); 0.74 g (20%); mp 254.5–256.5 °C (EtOH–water); NMR (Me_2SO-d_6) δ 2.70 (d, 6-N-CH_3, J = 5 Hz), 3.11 (s, 1-N-CH_3, 3), 6.35 (q, 6-NH, 1, J = 5 Hz), 6.93 (broad s, NH_2, 2), 7.65 (s, CHO, 1).

Anal. Calcd for $C_8H_{13}N_5O_1 \cdot H_2O$: C, 41.91; H, 6.60; N, 30.55. Found: C, 41.70; H, 6.26; N, 30.52.

Fractions 63–84: 3,7-dimethylguanine (**26**), 0.33 g (10%), mp 327–333 °C dec (EtOH–water) (lit.[43] 328–330 °C).

Fractions 98–140: 1-methylguanine (**23**), 0.02 g (1%), mp 370 °C (water) (lit.[41] 350 °C).

The identification of **26** was also performed by converting it into the xanthine derivative; e.g., a mixture of **26** (0.01 g) and sodium nitrite (0.01 g in 3 ml of 4 N hydrochloric acid) was warmed at 60 °C for 30 min. The silica gel thin layer chromatogram of the reaction mixture showed a single spot which had the same mobility as that of 3,7-dimethylxanthine [0.54 with solvent A and 0.70 with a mixture of acetone and methanol (5:1)]. The ultraviolet spectra of the extracted aqueous solution of the spot were identical with those of the authentic sample at pH 1, 7, and 13.

Registry No.—**1**, 71-30-7; **2**, 65-71-4; **3**, 66-22-8; **12**, 73-24-5; **22**, 73-40-5.

References and Notes

(1) C. C. Price et al., *Biochem. Biophys. Acta*, **166**, 327 (1968).
(2) L. Fishbein, W. G. Flamm, and H. L. Falk, "Chemical Mutagens", Academic Press, New York, N.Y., 1970, p 142.
(3) P. D. Lawley and P. Brookes, *Biochem. J.*, **77**, 478 (1960).
(4) P. Brookes and P. D. Lawley, *Biochem. J.*, **80**, 496 (1961).
(5) M. Asai, M. Miyaki and B. Shimizu, *Chem. Pharm. Bull.*, **15**, 1856 (1967).
(6) J. A. Hains, C. B. Reese, and L. Todd, *J. Chem. Soc.*, 5281 (1962).
(7) C. B. Pal, *Biochemistry*, **1**, 558 (1962).
(8) J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, **84**, 1914 (1962).
(9) B. Reiner and S. Zamenhof, *J. Biochem.*, **228**, 475 (1957).
(10) P. D. Lawley and P. Brookes, *Biochem. J.*, **92**, 19c (1964).
(11) P. Brookes and P. D. Lawley, *J. Chem. Soc.*, 1348 (1962).
(12) B. Singer, L. Sun and H. Frankel-Conrat, *Biochemistry*, **13**, 1913 (1974).
(13) B. Singer, *Biochemistry*, **11**, 3939 (1972).
(14) J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, **85**, 193 (1963).
(15) M. Rasmussen and J. H.-S. Chan, *Aust. J. Chem.*, **28**, 1031 (1975).
(16) J. A. Montgomery and H. J. Thomas, *J. Heterocycl. Chem.*, **1**, 115 (1964).
(17) A. Vincze, R. E. A. Henderson, J. J. McDonard, and N. J. Leonard, *J. Am. Chem. Soc.*, **95**, 2677 (1973).
(18) R. Roe, Jr., J. S. Paul, and P. O'B Montgomery, *J. Heterocycl. Chem.*, **10**, 859 (1973).
(19) J. Zemlicka, *Collect. Czech. Chem. Commun.*, **35**, 3572 (1970).
(20) A. Loveless, *Nature (London)*, **223**, 206 (1969).
(21) S. S. Epstein, W. Bass, E. Arnord, and Y. Bishop, *Science*, **168**, 584 (1970).
(22) G. Kolmark, *C. R. Trav. Lab. Carlsberg, Ser. Physiol.*, **26**, 205 (1956).
(23) H. Jackson and A. R. Jones, *Nature (London)*, **220**, 591 (1968).
(24) K. Yamauchi and M. Kinoshita, *J. Chem. Soc., Perkin Trans. 1*, 391, 2506 (1973).
(25) K. Yamauchi, M. Hayashi, and M. Kinoshita, *J. Org. Chem.*, **40**, 385 (1975).
(26) P. Brookes and P. D. Lawley, *J. Mol. Biol.*, **25**, 143 (1967); P. D. Lawley, *Prog. Nucleic Acid Res. Mol. Biol.*, **5**, 89 (1966).
(27) N. J. Leonard and R. A. Laursen, *J. Am. Chem. Soc.*, **85**, 2026 (1963); N. J. Leonard and T. Fujii, *ibid.*, **85**, 3719 (1963).
(28) E. P. Lira and C. W. Huffman, *J. Org. Chem.*, **31**, 2188 (1966).
(29) C. L. Angell, *J. Chem. Soc.*, 504 (1961).
(30) N. J. Leonard and T. Fujii, *Proc. Natl. Acad. Sci. U.S.A.*, **51**, 73 (1964).
(31) D. J. Brown, "Mechanisms of Molecular Migrations", B. S. Thyagarajan, Ed., Interscience, New York, N.Y., 1968, p 209. The direct methylation at the external amino group did not occur in cytosine (**1**) and guanine (**22**), either.
(32) K. Yamauchi and M. Kinoshita, to be published.
(33) The miss pairing may be attributed to the lack of the N-1 proton on a guanine ring after O-6-alkylation.
(34) TMP is currently used as a solvent for paints, polymers, gasoline additives, etc. Recently, triethyl phosphate has been proposed as an additive for the stabilization of egg white: M. R. Gumbmann, W. E. Gagne, and S. E. Williums, *Toxicol. Appl. Pharmacol.*, **12**, 360 (1968).
(35) J. H. Burckhalter and H. C. Scarborough, *J. Am. Pharm. Assoc.*, **44**, 545 (1955).
(36) A. R. Katritzky and A. J. Waring, *J. Chem. Soc.*, 3046 (1963).
(37) J. B. Johnson and S. H. Clapp, *J. Biol. Chem.*, **5**, 49 (1908).
(38) E. Wittenburg, *Chem. Ber.*, **99**, 2391 (1966).
(39) R. K. Ralph, G. Shaw, and R. N. Naylor, *J. Chem. Soc.* 1169 (1959).
(40) C. W. Whitehead, *J. Am. Chem. Soc.*, **74**, 4267 (1952).
(41) R. K. Robins and H. H. Lin, *J. Am. Chem. Soc.*, **79**, 490 (1957).
(42) R. Denayer, *Bull. Soc. Chim. Fr.*, 1358 (1962).
(43) I. M. Orcharova et al., *Khim.-Farm. Zh.*, **2**, 18 (1968).