alkaline methanol solution (Plates I and II), In acidic aqueous solution, however, hydrolysis of the pyridoxal Schiff bases was found to increase to a considerable extent,[10] notwithstanding the application of a 10^4 molar excess of the amino acid over pyridoxal in order to retard such hydrolysis. Thus, the observation of cationic pyridoxal species analogous to I_A, I_B and V has not been possible.

The initial dissociation constants for salicylidenevaline, and for 3-hydroxy-4-pyridylidenevaline, are somewhat larger than one might predict. The ease of removal of the first proton seems to be an indication of the strength of hydrogen bond formed in II_B–III_B and II_A–III_A mixtures.

The large difference in the last dissociation constants (in methanol) of the pyridinoid and benzenoid Schiff bases is considered significant. The fact that the dissociation of the pyridine derivative is nearly one hundred times (two *pK* units) greater than that of salicylidenevaline shows that the equilibrium concentration of IV_B would be much greater than that of IV_A under the same solution conditions in the range where species II and III are the more stable forms. Thus the formation of IV, and its metal chelates, would be greatly favored when an electron-withdrawing group such as the pyridine nitrogen atom is present in the Schiff base. It is seen, therefore, that the requirement of an electron-withdrawing substituent on the aromatic ring, pointed out earlier by Snell,[11] has a direct bearing on the equilibrium formation of the reactive Schiff base species in solution.

The acid dissociation constants used in this paper are only apparent constants, since ionization of the acetic acid added to the system certainly cannot be complete. Since the dielectric constants of methanol and especially dioxane, are considerably lower than that of water, one can assume that the acetic acid molecules (or a mixture of acetic acid molecules and ion pairs formed between acetate ion and the protonated solvent) comprise the acidic reactant. Thus the constants given are actually acid–base equilibrium constants with acetic acid as one of the reacting species. The dissociation constants would therefore be similar to aqueous dissociation constants, in which acetic acid molecules (or ion pairs) replace the hydronium ion.

(11) E. E. Snell, *Physiol. Revs.*, **33**, 509 (1953).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ARIZONA STATE UNIVERSITY, TEMPE, ARIZ.]

Purine Nucleosides. III. Methylation Studies of Certain Naturally Occurring Purine Nucleosides[1]

BY JESSE W. JONES AND ROLAND K. ROBINS

RECEIVED JUNE 29, 1962

1-Methyladenosine (V) and 2′-deoxy-1-methyladenosine (VI) have been isolated and characterized as products from the methylation of adenosine and 2′-deoxyadenosine, respectively. Guanosine and 2′-deoxyguanosine provided 7-methylguanosine and 2′-deoxy-7-methylguanosine upon methylation in neutral media. These latter compounds were isolated and characterized in the form of an unusual internal nucleoside zwitterion (XIV, XV). Xanthosine and inosine under similar conditions gave 7-methylxanthosine (XVI) and 7-methylinosine (XXI), which were also isolated in the betaine form. At *p*H 8.5 inosine was methylated to give 1-methylinosine (XIV). 1-Methyladenosine and 2′-deoxy-1-methyladenosine rearranged in aqueous sodium hydroxide to give N^6-methyladenosine (VIII) and 2′-deoxy-N^6-methyladenosine (IX), respectively. These studies are discussed in view of previously reported alkylations of purine nucleosides. The possible biochemical significance of the present work is considered.

A substantial number of N-methylpurines have been isolated from various biological sources and identified in recent years.[2] A significant increase in 1-methylhypoxanthine, 8-hydroxy-7-methylguanine and 7-methylguanine has been noted in the urine of patients with leukemia.[3] It seems quite possible that the simple N-methylpurines might arise as degradation products of methylated purine nucleosides or nucleotides hydrolyzed enzymatically *in vivo* or cleaved during isolation procedures.[4] Several of these N-methylpurines have been isolated directly from nucleic acid.[5–9] Littlefield and Dunn[10] identified N^6-methyladenosine (6-N-methylamino-9-β-D-ribofuranosylpurine) and N^6,-N^6-dimethyladenosine (6-N,N-dimethylamino-9-β-D-ribofuranosylpurine) as degradation products of ribonucleic acid from various microbial, plant and mammalian sources. This identification was based on chromatographic and ultraviolet absorption data and was greatly aided by the fact that both 6-N-methylamino-9-β-D-ribofuranosylpurine and 6-N,N-dimethylamino-9-β-D-ribofuranosylpurine were readily available from previous syntheses.[11,12] 2′-Deoxy-N^6-methyladenosine (6-N-methylamino-9-β-D-2′-deoxyribofuranosylpurine) has been isolated[13] from the DNA of a strain of *Escherichia coli*. At least three different N-methylguanosine derivatives have been detected from various sources of ribonucleic acid.[14] Identification of these purine nucleosides was based on chromatographic and spectroscopic comparison with samples prepared by enzymatic means. It is quite clear that various N-methylpurine ribosides and deoxyribosides of established structure, available in pure crystalline form, are needed for present biochemical studies and would be of considerable assistance in the isolation and identification of various purine nucleosides as minor constituents of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The preparation of such N-

(1) Supported by research grants CY-4008(C3) and CY-4008(C4) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

(2) See J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, **84**, 1914 (1962), for a list of pertinent references.

(3) R. W. Park, J. F. Holland and A. Jenkins, *Cancer Research*, **22**, 469 (1962).

(4) D. B. Dunn and J. D. Smith, in "Proc. Intern. Congr. Biochem., 4th Congr.," Vol. VII, Vienna, 1958, p. 72, Pergamon Press, Inc., New York, N. Y., 1959.

(5) D. B. Dunn and J. D. Smith, *Nature*, **175**, 336 (1955).

(6) J. W. Littlefield and D. B. Dunn, *ibid.*, **181**, 254 (1958).

(7) M. Adler, B. Weissmann and A. B. Gutman, *J. Biol. Chem.*, **230**, 717 (1958).

(8) D. B. Dunn, *Biochim. et Biophys. Acta*, **46**, 198 (1961).

(9) F. F. Davis, A. F. Carlucci and I. F. Roubein, *J. Biol. Chem.*, **234**, 1525 (1959).

(10) J. W. Littlefield and D. B. Dunn, *Biochem. J.*, **70**, 642 (1958).

(11) H. M. Kissman, C. Pidacks and B. R. Baker, *J. Am. Chem. Soc.*, **77**, 18 (1955).

(12) J. A. Johnson, Jr., H. J. Thomas and H. J. Schaeffer, *ibid.*, **80**, 699 (1958).

(13) D. B. Dunn and J. D. Smith, *Biochem. J.*, **68**, 627 (1958).

(14) J. D. Smith and D. B. Dunn, *ibid.*, **72**, 294 (1959).

methylpurine nucleosides was the main objective of the present work.

A possible route to the preparation of such derivatives would appear to be by the direct methylation of the readily available naturally occurring common purine ribosides and their 2′-deoxyribosides. This approach is especially attractive since methylation of such purine nucleosides in the laboratory under approximate physiological conditions (near neutral *p*H) might well provide alkylation on the purine moiety at the same site as *in vivo* enzymatic methylation. The fact that methionine has recently been shown to serve as a methyl donor for the methylated purines found in soluble RNA of mammalian cells[15] is strong support for such an *in vivo* methylation mechanism. Borek, Mandel and Fleissner[16] have recently shown that methionine-starved *E. coli* lacked the methylated purine bases which are normally found in the soluble RNA. Another reason for studying the direct methylation of the purine nucleosides was to attempt to learn more about the various sites of purine alkylation. Presumably, methylation will occur first on the nitrogen of highest electron density. This position should also be the most probable site of protonation in the naturally occurring purine nucleoside and perhaps a probable site of attachment of an enzyme system concerned with biological transformations. This information is of considerable importance in studying such phenomena as hydrogen bonding and base pairing in DNA and RNA.

Of major current interest is the action of the various cytotoxic alkylating agents on DNA. There is substantial evidence that the purine bases provide important sites of alkylation by these agents acting on nucleic acid.[17]

It is noteworthy that although several alkylated purines have been isolated and identified from studies of nucleic acid and various antitumor alkylating agents, *no alkylated purine nucleosides* resulting from such a study have yet been adequately characterized or identified. In the present work, since the main objective was to prepare and characterize the N-methylated purine nucleosides, the conditions for methylation were carefully selected to prevent loss of D-ribose or 2′-deoxy-D-ribose. Preliminary studies in our laboratory indicated that the purine 2′-deoxynucleosides were particularly unstable to methylation conditions at temperatures near 100°. The loss of 2′-deoxy-D-ribose from the purine was noted at 100° in solvents such as dimethylformamide.[18] Methylation studies were therefore executed at room temperature. The methylating agents employed were methyl iodide, dimethyl sulfate and methyl *p*-toluenesulfonate. Dimethylacetamide, dimethylformamide or dimethyl sulfoxide was selected as a reaction solvent. These solvents were necessary to gain sufficient solubility of the purine nucleoside at the temperature employed. These solvents also acted as good buffering solutions which allowed the methylation studies to proceed at approximately *p*H 5–6, thus preventing loss of the sugar by acid hydrolysis. The use of dimethylacetamide has been successfully employed in similar methylation studies of the simple purines.[2]

Methylation of Adenosine.—The first methylation studies of adenosine were made by Levene and Tipson.[19] These workers treated adenosine with dimethyl sulfate and aqueous sodium hydroxide in the presence of acetone and isolated a crystalline tetramethyladenosine as the hydrochloride. Degradation studies gave 2,3,5-trimethyl-D-ribose and a monomethyl adenine derivative whose structure was not determined. Bredereck and co-workers[20a,b] studied the methylation of adenosine in strongly alkaline solution and at *p*H 8–10 and *p*H 6 with dimethyl sulfate. These investigators[20] reported N^6-methyladenosine in strongly alkaline solution, 1,N^6-dimethyladenosine at *p*H 8–10 and 1-methyladenosine at *p*H 6. In each instance, however, the products were inadequately characterized. A nitrogen analysis of the crude reaction product or a nitrogen analysis of the picrate of the purine base obtained after hydrolysis was the only analytical datum given. The identification of the methylated adenosine derivative by this means was far from certain since the structure of the various possible methyladenines had not been previously established. Indeed, Wacker and Ebert[21] repeated the work of Bredereck, *et al.*,[20a] and found that Bredereck could not possibly have had the N^6-methyladenosine and 1,N^6-dimethyladenosine claimed, since under the strongly alkaline conditions employed, considerable methylation of the ribose portion of the molecule took place. Methylation of the ribose moiety under similar conditions has also been observed by earlier workers.[19,22] Izatt and Christensen[23] have recently shown that adenosine dissociates to yield a proton at a *p*H of approximately 12, presumably from the ribose portion of the molecule. Wacker and Ebert further examined the methylation products of adenosine described by Bredereck, *et al.*,[20a] and showed by paper chromatography that at *p*H 6–8 in aqueous solution at least four products were obtained. At *p*H 8.8–9 Wacker and Ebert[21] report two additional spots, presumably due to alkylation on the sugar. Brookes and Lawley[24] recently studied the methylation of adenosine with dimethyl sulfate in dimethylformamide. These workers make no reference to previous methylation studies of adenosine by other investigators[19–21] who had employed the same methylating agent. They used three equivalents of dimethyl sulfate in dimethylformamide at 100°. No methylated purine nucleoside derivatives were isolated or characterized, but the crude reaction mixture was hydrolyzed and the various methyladenines identified.

In the present work adenosine (I) was methylated in N,N-dimethylacetamide at room temperature with excess methyl *p*-toluenesulfonate. 1-Methyladenosine was isolated as the tosylate salt III. No adenosine or other methylated purine nucleoside derivative could be detected by a chromatographic study of the filtrates. This compound was fully characterized by treatment with aqueous sodium hydroxide which readily converted it to the known N^6-methyladenosine (VIII) which has previously been prepared[12] from 6-chloro-9-β-D-ribofuranosylpurine. An excess of methyl iodide and adenosine in dimethylacetamide at room temperature similarly gave 1-methyladenosine which was isolated as the iodide and converted to the free crystalline 1-methyladenosine with dilute aqueous ammonia at *p*H 9. Under these conditions no rearrangement to N^6-methyladenosine was noted. Acid hydrolysis of V gave D-ribose and 1-methyladenine (VII),[24] obtained

(15) B. B. Biswas, M. Edmonds and R. Abrams, *Biochem. Biophys. Research Comm.*, **6**, 146 (1961).

(16) E. Borek, L. R. Mandel and E. Fleissner, *Federation Proc.*, **21**, 379 (1962).

(17) For a review of this subject see G. P. Wheeler, *Cancer Research*, **22**, 651 (1962).

(18) S. Greer and S. Zamenhof, *J. Mol. Biol.*, **4**, 123 (1962), have studied the effect of heating DNA in solutions of various ionic strengths. These investigations show considerable release of purines at temperatures of 75–100°.

(19) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **94**, 809 (1932).

(20) (a) H. Bredereck, H. Haas and A. Martini, *Ber.*, **81**, 307 (1948); (b) H. Bredereck, G. Müller and E. Berger, *ibid.*, **73**, 1059 (1940).

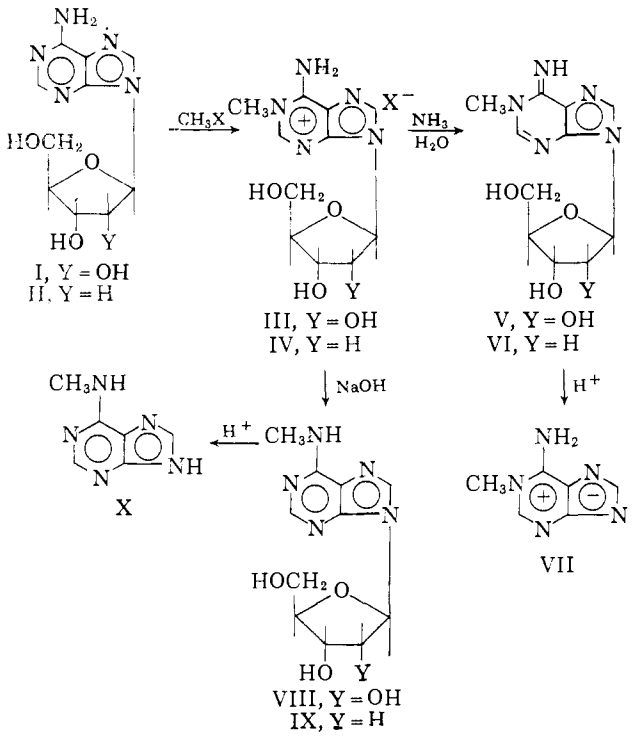
(21) A. Wacker and M. Ebert, *Z. Naturforsch.*, **14b**, 709 (1959).

(22) A. S. Anderson, G. R. Barker, J. M. Gulland and M. V. Lock, *J. Chem. Soc.*, 369 (1952).

(23) R. M. Izatt and J. J. Christensen, *J. Phys. Chem.*, **66**, 359 (1962).

(24) P. Brookes and P. D. Lawley, *J. Chem. Soc.*, 539 (1960).

REACTION SCHEME I



for the first time as the free base. From these studies it is apparent that adenosine in neutral or weakly acidic media alkylates most readily at position 1. In view of these results it would appear that the 3-methyladenine, isolated by Brookes and Lawley[24] from methylation of adenosine, probably arose from direct methylation of adenine present in the reaction mixture at the temperature employed. Adenosine heated two hours in dimethylformamide at 100° in the presence of dimethyl sulfate showed considerable adenine when the solution cooled and was chromatogrammed. Jones and Robins[2] have shown that adenine under similar methylating conditions gives rise to 3-methyladenine. The 1,3-dimethyladenine noted by Brookes and Lawley[24] could well be the result of methylation of 3-methyladenine rather than a direct methylation of adenosine or 1-methyladenosine. Our results are consistent with the recent work of Windmueller and Kaplan[25] who showed that adenosine and ethylene oxide in aqueous solution at room temperature alkylated only at position 1.

Methylation of 2′-Deoxyadenosine.—Methylation of 2′-deoxyadenosine (II) under conditions similar to those employed for adenosine with methyl iodide gave 2′-deoxy-1-methyladenosine isolated as the iodide salt IV. No other product could be detected in the alkylation mixture. The assignment of the methyl group to position 1 was verified by acid hydrolysis to 1-methyladenine[24] and treatment of VI with aqueous sodium hydroxide to give 2′-deoxy-N^6-methyladenosine[25a] (IX). The structure of IX was established since mild acid hydrolysis gave 6-methylaminopurine (X)[26] and 2′-deoxy-D-ribose. It is noteworthy that 2′-deoxy-N^6-methyladenosine, thus prepared, possessed properties, *i.e.*, ultraviolet absorption spectra, R_f values, etc., identical to those reported by Dunn and Smith[13] for 6-methylaminopurine deoxyriboside isolated from *E. coli*. 2′-Deoxyadenosine was also methylated with methyl *p*-toluenesulfonate to give 2′-deoxy-1-methyladenosine isolated as the tosylate salt.

Methylation of Guanosine.—Bredereck and Martini[27] treated triacetylguanosine with an excess of diazomethane and isolated a crystalline monomethyl derivative of guanosine which melted at 163°. This compound was assigned the structure 1-methylguanosine on the basis of a nitrogen analysis and the supposition that the hydrolysis product was 1-methylguanine as judged by the preparation of a picrate of the purine base. Later Bredereck, Haas and Martini[20a] prepared a monomethylguanosine by the action of dimethyl sulfate on guanosine at a *p*H of 4. This material was also assigned the structure 1-methylguanosine by comparison with the product prepared with diazomethane. Both reported preparations of 1-methylguanosine were repeated in our laboratory following the directions of Bredereck.[20a,27] In each case a product was isolated with the properties described. The products were found to be homogeneous, analyzed for a monomethyl derivative of guanosine, and were identical as judged by chromatography and infrared spectra. However, when the monomethylated guanosine was treated with hydrochloric acid, the purine base isolated was shown to be 7-methylguanine[28,29] instead of 1-methylguanine[29] as reported by Bredereck. Since the methylation of guanine under neutral conditions has recently been shown to give 7,9-dimethylguanine,[2] a similar internal betaine structure is now postulated for 7-methylguanosine (XIV). The alkylation of guanosine under neutral conditions appeared to be quite general. Methyl iodide in N,N-dimethylformamide readily gave the iodide which was converted with methanolic ammonia into 7-methylguanosine in good yield. These results are consistent with the work of Brookes and Lawley[30] who recently studied the alkylation of guanosine with diethyl sulfate at 100° in a sealed tube and noted 7-ethylguanine among the hydrolysis products of the reaction. These investigators, however, did not isolate or characterize an alkylated derivative of guanosine, nor did they make any reference to the earlier work of Bredereck. Under the reaction conditions employed it is extremely doubtful that 7-ethylguanosine would have survived since it has now been found that 7-methylguanosine readily loses D-ribose under the influence of acid and/or heat to give 7-methylguanine. Bredereck, Haas and Martini[20a] report that methylation of guanosine at *p*H 13–14 with dimethyl sulfate in the presence of aqueous sodium hydroxide gave N^2-methylguanosine. The product was not analyzed, purified, or further characterized, and from other studies[19–22] it seems rather likely that methylation also occurred on ribose under these conditions.

Methylation of 2′-Deoxyguanosine.—2′-Deoxyguanosine (XII) was treated with methyl iodide in dimethyl sulfoxide to give 2′-deoxy-7-methylguanosine isolated as the iodide salt and converted to the internal betaine XV with aqueous methylamine. The structure of XV was verified by hydrolysis to 7-methylguanine (XVII) and 2′-deoxy-D-ribose.

Methylation of Inosine.—Bredereck and Martini[27] reported that diazomethane and triacetylinosine gave a solid which was assigned the structure 1-methylinosine. The product was not further characterized. Miles[31] repeated the methylation according to Bredereck[27] and showed that the methylation product contained about 20% 6-methoxy-9-β-D-ribofuranosylpu-

(25) H. G. Windmueller and N. O. Kaplan, *J. Biol. Chem.*, **236**, 2716 (1961).

(25a) NOTE ADDED IN PROOF.—A. Codington [*Biochim. Biophys. Acta*, **59**, 472 (1962)] has recently studied the methylation of 2′-deoxyadenosine with essentially the same results based on ultraviolet and chromatographic data. However, no crystalline nucleoside derivatives were isolated.

(26) A. Albert and D. J. Brown, *J. Chem. Soc.*, 2060 (1954).

(27) H. Bredereck and A. Martini, *Ber.*, **80**, 401 (1948).

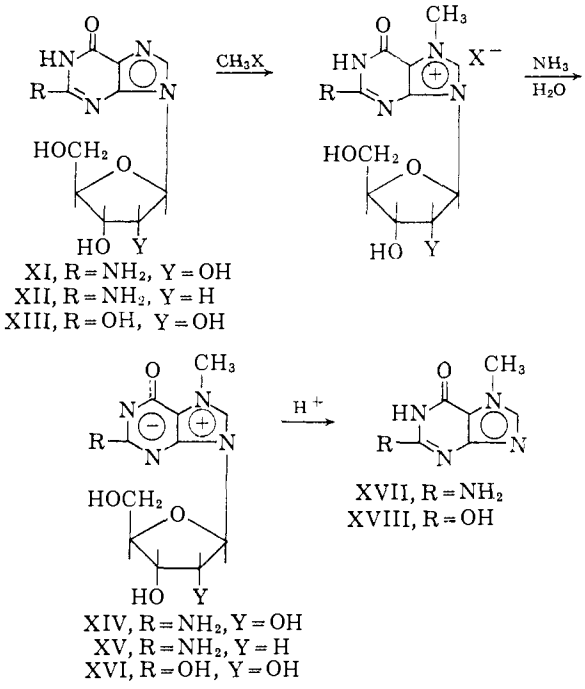
(28) E. Fischer, *Ber.* **31**, 542 (1898).

(29) W. Traube and H. W. Dudley, *ibid.*, **46**, 3839 (1913).

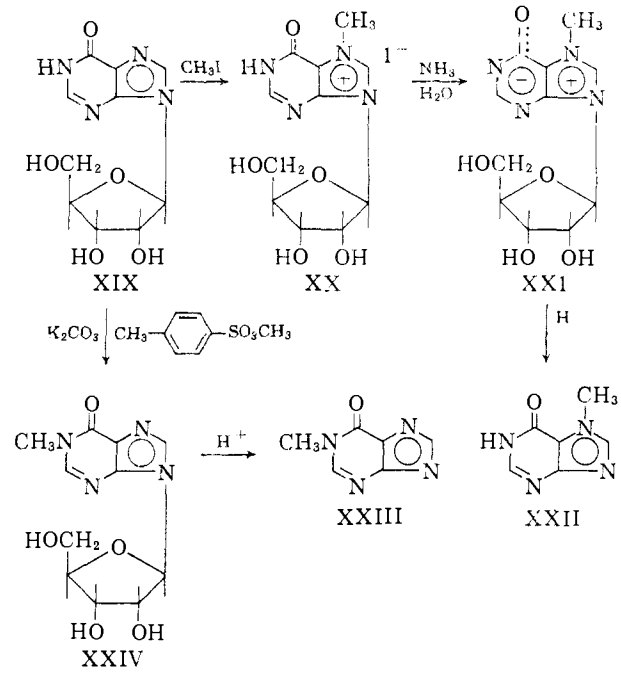
(30) P. Brookes and P. D. Lawley, *J. Chem. Soc.*, 3923 (1961).

(31) H. T. Miles, *J. Org. Chem.*, **26**, 4761 (1961).

REACTION SCHEME II



REACTION SCHEME III



rine as well as 1-methylinosine and at least two other unidentified components. The methylation of triacetylinosine with diazomethane according to Bredereck[27] was also repeated in our laboratory, and an additional nucleoside component which gave a highly fluorescent spot when separated by paper chromatography was noted. By means of ultraviolet spectra and R_f values this product was judged to be identical with 7-methylinosine (XXI), which was prepared in our laboratory by direct methylation of inosine with methyl iodide in dimethyl sulfoxide. 7-Methylinosine (XXI) was isolated as a crystalline solid and characterized by hydrolysis to D-ribose and 7-methylhypoxanthine (XXII).[32] The synthesis of XXI by this procedure is not unexpected since 9-methylhypoxanthine under similar conditions yields 7,9-dimethylhypoxanthine[2] with dimethyl sulfate. A betaine structure (XXI) is proposed for 7-methylinosine similar to that proposed for 7,9-dimethylhypoxanthine.[2] Shaw[33] has shown that benzyl chloride and inosine in N,N-dimethylformamide in the presence of sodium bicarbonate results in a 50% yield of 1-benzylinosine. Methyl chloromethyl ether under similar conditions[33] alkylates inosine presumably at positions 1 and 5′. In view of these results methylation of inosine in N,N-dimethylacetamide with methyl *p*-toluenesulfonate in the presence of potassium carbonate was studied in our laboratory. Under these conditions 1-methylinosine (XXIV) was obtained. The crystalline product which was isolated directly without recourse to column chromatography proved to be identical to the product described by Miles.[31] Further structural confirmation was obtained by hydrolysis of XXIV to 1-methylhypoxanthine (XXIII)[34] and D-ribose. It is interesting that in the presence of potassium carbonate a proton is presumably removed from nitrogen 1, and under these conditions methylation occurs at the anion in preference to position 7.

Methylation of Xanthosine.—Levene,[35] in 1923, reported the preparation of 1,3-dimethylxanthosine. Gulland and co-workers[36] repeated Levene's early work and showed his 1,3-dimethylxanthosine to be a mixture of methylated xanthines and methylated derivatives of ribose; no trace of theophylline was detected in the acid hydrolysates. Attempts to methylate xanthosine with methyl iodide and silver oxide or dimethyl sulfate and alkali were reported by Gulland and co-workers[36] to yield only caffeine and methylated derivatives of D-ribose.

In the present work xanthosine was treated with dimethyl sulfate in N,N-dimethylacetamide to give 7-methylxanthosine (XVI) isolated as the methyl sulfate salt. The crystalline betaine XVI was isolated by neutralization of an aqueous solution of the salt with concentrated aqueous ammonia. Hydrolysis of XVI gave 7-methylxanthine (XVIII)[32] and D-ribose. 7-Methylxanthosine (XVI) was the expected product since under similar conditions 9-methylxanthine yields 7,9-dimethylxanthine.[2]

General Discussion

1-Methyladenosine and 2′-deoxy-1-methyladenosine appear to resemble adenosine and 2′-deoxyadenosine, respectively, with regard to stability toward loss of ribose or 2′-deoxyribose. The 7-methylpurine nucleosides, on the other hand, tend to lose the sugar rather easily.

Although 7-methylguanosine (anhydrous) is stable indefinitely when stored at room temperature, 7-methylxanthosine, 7-methylinosine and 2′-deoxy-7-methylguanosine decompose to give the corresponding 7-methylpurines within a 3-month period. No decomposition was noted, however, when these nucleosides were stored for extended periods of time near 0°. Heating of the 7-methylpurine nucleosides in dilute acid, alcohol or water on the steam-bath resulted in the quantitative isolation of the 7-methylpurine within 30 minutes. The 7-methylpurine nucleosides decomposed rapidly in 1 *N* aqueous sodium hydroxide. The ultraviolet absorption spectra of the resulting products indicated probable opening of the imidazole ring similar to that observed by Brookes and Lawley[30] for 7,9-bis-(β-hydroxyethyl)-guanine. Bredereck, Kupsch and Wieland[37] have observed a similar ring

(32) R. N. Prasad and R. K. Robins, *J. Am. Chem. Soc.*, **79**, 6401 (1957).
(33) E. Shaw, *ibid.*, **80**, 3899 (1958); **83**, 4770 (1961).
(34) L. B. Townsend and R. K. Robins, *J. Org. Chem.*, **27**, 990 (1962).
(35) P. A. Levene, *J. Biol. Chem.*, **55**, 437 (1923).
(36) J. M. Gulland, E. R. Holiday and T. F. Macrae, *J. Chem. Soc.*, 1639 (1934); J. M. Gulland and T. F. Macrae, *ibid.*, 662 (1933).

opening of 9-methylcaffeine in base. When 7-methylinosine, 7-methylxanthosine and 7-methylguanosine were allowed to stand in aqueous solution for one to three days, opening of the imidazole ring was likewise observed. The isolation and characterization of certain of these ring opened products is presently under further investigation.

Inspection of the ultraviolet absorption spectra of inosine and 7-methylinosine in acid solution reveals that these two spectra are remarkably alike. This evidence suggests that inosine is protonated at position 7 in acid solution.

On the basis of infrared studies in deuterium oxide, Tsuboi and co-workers[38] concluded that guanosine undergoes protonation in aqueous solution at either the 3- or 7-position. A study of the ultraviolet absorption of 7-methylguanosine and guanosine in aqueous acidic solution (*p*H 1) reveals that the spectra are virtually identical. Although 3-methylguanosine is not available for comparison, it is noteworthy that 3-methylguanine has recently been prepared[39] and has been shown to possess an ultraviolet absorption spectrum considerably different from that of guanine or other mono-N-methylated guanine derivatives. If one assumes that both protonation and alkylation will each occur at the nitrogen atom of highest electron density in the guanosine molecule, the present study offers additional evidence that position 7 is the preferred site. Pfleiderer[40] has concluded from ultraviolet absorption studies that 9-methylguanine is protonated most readily at position 7. A study of the ultraviolet absorption spectra of 1-methyladenosine and adenosine at *p*H 1 reveals that the spectra are strikingly similar. Since a methyl group at the 3- or 7-position is known to change the spectrum of adenine to a considerable extent,[2] it can be stated from the present work that position 1 is the most basic center of adenosine and is the first position to accept a proton. This conclusion is in accord with the study of Windmueller and Kaplan[25] and Tsuboi and co-workers[38] who studied the infrared spectrum of adenosine in acidic deuterium oxide and definitely showed that protonation did not occur on the nitrogen of the amino group. Angell[41] has recently concluded on the basis of infrared studies that adenylic acid exists in the zwitterion form with a proton at nitrogen 1 of the adenosine moiety.

Alexander, Lett and Parkins[42] have advanced the hypothesis that the initial site of attack of an alkylating agent on DNA is the phosphate group, and the phosphate ester so formed alkylates the ring nitrogens of the purines. Ross[43] has postulated that alkylation of position 7 of the guanine moiety in DNA is due to alkyl-group transfer from the primary phosphate which is sterically in a favorable position for such a reaction. Ross[43] further states that alkylation occurs at position 7 under these conditions as N_7 is favored since it is not involved in the hydrogen bonding in the Watson–Crick model for DNA. From our present work on the alkylation of guanosine it is unnecessary to invoke either of these theorems to explain alkylation at N_7. Ross[43] further suggests that the work of Lawley, *et al.*,[44] supports a more rapid alkylation of guanine in the form of DNA or guanylic acid possibly due to alkyl transfer from the phosphate group. Our present methylation studies of guanosine indicate that alkylation on N_7 occurred as readily as under similar conditions employed by Lawley and Wallick[44] for alkylation of DNA or methylation of guanylic acid.

The alkylation of adenosine at position 1 is of considerable recent interest since in bacterial systems it has been shown[45–47] that 5-phosphoribosyl-1-pyrophosphate (PRPP) alkylates adenosine triphosphate at position 1 to give N-1-(5′-phosphoribosyl)-adenosine triphosphate which loses N_1 and C_2 toward the biosynthesis of histidine.

The occurrence of 1-methyladenine (VII) (spongopurine) in a siliceous sponge[48] and in the acid hydrolysates of a variety of sources[49,50] of RNA strongly suggests the possible occurrence of 1-methyladenosine (V) and 2′-deoxy-1-methyladenosine (VI) in biological materials.

Camargo[51] noted that the berries of the coffee tree *arabia* contain large quantities of guanosine, which he postulated was the precursor of the caffeine molecule. Anderson and Gibbs[52] have recently shown that methionine is the source of the methyl groups in caffeine and that caffeine arises by methylation of a preformed purine derivative which accumulates in the coffee plant. The present studies show that guanosine and xanthosine (at neutral *p*H) are readily methylated at position 7 and that such methylation aids cleavage of the ribosyl linkage. Wiardi and Jansen[53] isolated 7-methylxanthine (heteroxanthine) by extracting yeast with a large volume of dilute acid (*p*H 4.5) at room temperature. Such conditions will not hydrolyze the more common purine ribosides but readily cleave 7-methylxanthosine to 7-methylxanthine.

It is quite possible that more carefully controlled enzymatic hydrolysis of various sources of RNA and DNA will reveal the presence of such nucleosides as 7-methylguanosine, 7-methylxanthosine, 1-methyladenosine, 1-methylinosine and the similar methylated purine 2′-deoxyribosides. The present synthesis of these and related derivatives should be of great assistance in the future identification of such products from biological sources. It would also be of considerable interest to explore by various means the possible incorporation of such nucleosides as 1-methyladenosine (V) and 2′-deoxy-1-methyladenosine (VI) into RNA and DNA. Since the 1-position is blocked by the methyl group, hydrogen bonding with thymine in the usual Watson–Crick fashion would be prevented. Littlefield and Dunn[10] have speculated that perhaps the methylated purines present in nucleic acid are important for the specific coding of an unusual amino-acid, or in some way produce the correct termination of a polypeptide. The true biochemical role of the N-methylated purines and purine ribosides, however, is yet to be elucidated.

The greatly increased water solubility of the 7-methylpurine nucleosides over that of the parent compounds is strong support for a betaine-type structure (XIV, XV, XVI, XXI). In each case the 7-

(37) H. Bredereck, G. Kupsch and H. Wieland, *Chem. Ber.*, **92**, 583 (1959).

(38) M. Tsuboi, Y. Kyogoku and T. Shimanouchi, *Biochim. et Biophys. Acta*, **55**, 1 (1962).

(39) R. K. Robins and L. B. Townsend, *J. Am. Chem. Soc.*, **84**, 3008 (1962).

(40) W. Pfleiderer, *Liebigs Ann. Chem.*, **647**, 167 (1961).

(41) C. L. Angell, *J. Chem. Soc.*, 504 (1961).

(42) P. Alexander, J. T. Lett and G. Parkins, *Biochim. et Biophys. Acta*, **48**, 423 (1961).

(43) W. C. J. Ross, "Biological Alkylating Agents," Butterworths, London, 1962, pp. 45, 79–80.

(44) P. D. Lawley, *Ann. Rept. Brit. Emp. Cancer Camp.*, **36**, 16 (1958); P. D. Lawley and C. A. Wallick, *Chemistry and Industry*, 633 (1957).

(45) H. S. Moyed and B. Magasanik, *J. Biol. Chem.*, **235**, 149 (1960).

(46) B. N. Ames, R. G. Martin and B. J. Garry, *ibid.*, **236**, 2019 (1961).

(47) B. Magasanik and D. Karibian, *ibid.*, **235**, 2672 (1960).

(48) D. Ackermann and P. H. List, *Naturwiss.*, **48**, 74 (1961); D. Ackermann and P. H. List, *Z. physiol. Chem.*, **323**, 192 (1961).

(49) D. B. Dunn, *Biochim. et Biophys. Acta*, **46**, 198 (1961).

(50) D. B. Dunn, J. D. Smith and P. F. Spahr, *J. Mol. Biol.*, **2**, 113 (1960).

(51) T. de A. Camargo, *J. Biol. Chem.*, **58**, 831 (1924).

(52) L. Anderson and M. Gibbs, *ibid.*, **237**, 1941 (1962).

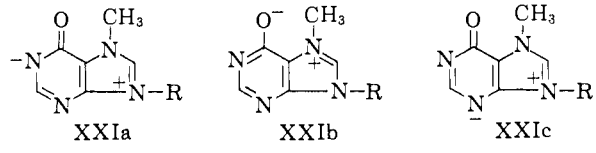
(53) P. W. Wiardi and B. C. P. Jansen, *Rec. trav. chim.*, **53**, 205 (1934).

TABLE I

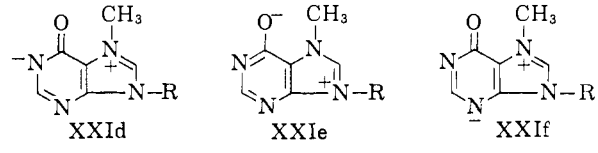
ULTRAVIOLET ABSORPTION SPECTRA OF THE N-METHYLPURINE NUCLEOSIDES

Compound	pH 1				Water				pH 11			
	λ_{max}, mμ	ε	λ_{min}, mμ	ε	λ_{max}, mμ	ε	λ_{min}, mμ	ε	λ_{max}, mμ	ε	λ_{min}, mμ	ε
N^6-Methyladenosine	261	16,300	231	2,900	265	16,300	229	2,030	265	15,000	223	0
7-Methylinosine	252	10,800	229	5,400	261	8,400	238	0	256	6,600	238	5,400
7-Methylguanosine	256	13,300	230	6,150	275	9,650	243	5,500	265	12,200	243	6,350
	270–280(s)	9,650	0									
2′-Deoxy-7-methylguanosine	256	10,800	229	1,500	275	9,000	243	5,400	266	9,000	243	5,400
	270–280(s)	7,800										
2′-Deoxy-1-methyladenosine·HI	256.5	15,050	239	7,350	257.5	15,050	241	10,600	257.5	15,050	242	8,750
2′-Deoxy-N^6-methyladenosine	261	15,100	230	0	265	15,400	229	2,785	265	15,400	226	0
7-Methylxanthosine	262	9,800			253	9,100	230	5,890	268.5	15,400	239	6,250
					280	8,800						
1-Methyladenosine	256.5	13,650	231	2,250	257	14,600			257	14,600	223	3,200
					260–265(s)				260–265(s)			

methylpurine nucleoside betaine was isolated as a hydrate. The amount of hydration was determined by Karl Fischer titration since attempts to remove the water by heating *in vacuo* resulted in partial loss of the sugar. This tendency to hydrate is also typical of the betaine salts. The existence of a molecule of this zwitterion nature is aided by the fact that various resonance structures can be drawn which contribute to a rather wide distribution of charges. Some of the more probable resonance forms of 7-methylinosine (XXI) are illustrated (R = β-D-ribofuranosyl). This



XXIa XXIb XXIc



XXId XXIe XXIf

compound exhibits the type of properties which are indicative of a general deficiency of electrons throughout the imidazole ring and a general excess of electrons in the pyrimidine ring. Thus, the opening of the imidazole ring after long exposure to aqueous solution is probably due to attack of a nucleophilic water molecule or a hydroxyl ion at the electron-deficient 8-carbon. It is of interest that a similar kind of polar purine structure has recently been postulated to account for certain unusual chemical properties of some 1-N-methyl-[54] and 3-N-methylpurines.[39] The stabilizing effect of the 2-amino group of 7-methylguanosine (XIV) can be interpreted as the indirect increase of electron density to the deficient nitrogen atoms in the imidazole ring. The increased lability of the 7-methylpurine nucleosides to loss of the sugar moiety over that of the parent nucleosides strongly suggests that the acid hydrolysis of such natural purine nucleosides as guanosine and inosine is probably preceded by protonation at N_7.

The ultraviolet absorption spectra of the new purine nucleosides are listed in Table I. The specific rotation in aqueous solution has been recorded in Table II for each of the N-methylpurine nucleosides. It is of interest that all the 7-methylpurine betaine nucleosides exhibit a characteristic light-blue fluorescence when irradiated with ultraviolet light (254 mμ). This fluorescence allows for ready detection of these derivatives on paper chromatograms. The R_f values of the methylated purine nucleosides are listed in Table III. It is of interest that the 7-methylpurine betaine nucleosides possess a smaller R_f value than the corresponding naturally occurring parent purine nucleoside. This is additional support for an ionic type structure for these compounds since the more polar derivatives are likely to be retarded in the stationary phase (water). Usually methylation of purine derivatives gives compounds possessing a greater R_f value[55,56] (more soluble

TABLE II

SPECIFIC ROTATIONS OF SOME METHYLATED PURINE NUCLEOSIDES

Compound	Concn., g./100 ml.	Temp., °C.	Solvent	$[\alpha]_D$
7-Methylguanosine (XIV)	0.40	27	Water	−33.5°
7-Methylxanthosine (XVI)	.924	27	Water	−17.04
7-Methylinosine (XXI)	.58	27	Water	−35.4
1-Methylinosine (XXIV)	.50	28	Water	−49.2
2′-Deoxy-1-methyladenosine hydroiodide (IV)	1.0	28	0.2% NaAc	− 8.3
1-Methyladenosine	2.00	26	Water	−58.95
N^6-Methyl-2′-deoxyadenosine IX	1.00	25.5	Water	−23.45

in organic solvents). The only exception noted is with certain 3-methylpurines[57] which have recently been assigned a polar structure.[39] In this regard 1-methyladenosine (V), N^6-methyladenosine (VIII), 2′-deoxy-1-methyladenosine (VI), 2′-deoxy-N^6-methyladenosine (IX) and 1-methylinosine all possess R_f values greater than the unmethylated parent nucleoside. This would normally be expected since these purine nucleosides exhibit a higher solubility in organic solvents and do not possess a zwitterion structure. The small R_f values, coupled with the characteristic fluorescence of the 7-methylpurine nucleosides, provide a good diagnostic tool for the future detection of these derivatives in biological materials.

(54) L. B. Townsend and R. K. Robins, *J. Org. Chem.*, **27**, 990 (1962).

(55) F. Bergmann, H. Kwietny, G. Levin and D. J. Brown, *J. Am. Chem. Soc.*, **82**, 598 (1960).

(56) S. Dikstein, F. Bergmann and M. Chaimovitz, *J. Biol. Chem.*, **221**, 239 (1956).

(57) F. Bergmann, G. Levin, A. Kalmus and H. Kwietny-Govrin, *J. Org. Chem.*, **26**, 1504 (1961).

TABLE III

R_f VALUES OF METHYLPURINES AND PURINE NUCLEOSIDES[a]

Compound	Solvent[b] A	B	C	D	E	F	G	H
Adenosine	0.42	0.11	0.51	0.77	0.53			0.16
1-Methyladenosine	.47	.52	.75	.83	.57			
N^6-Methyladenosine	.67	.39						
2′-Deoxyadenosine	.44	.18	.54	.60	.56		.68	
2′-Deoxy-1-methyladenosine·HI			.74	.83	.62			
2′-Deoxy-N^6-methyladenosine	.71				.69		.66	
N^6-Methyladenine	.56	.61						
D-Ribose	.71	.18	.86	.85	.56			
2′-Deoxy-D-ribose	.74	.34	.86	.89	.64			
Inosine					.51	.63	.64	.37
7-Methylinosine					.36	.59	.53	.32
1-Methylinosine					.58	.72	.67	.50
7-Methylhypoxanthine		.19	.63		.28			
Xanthosine					.46		.55	
7-Methylxanthosine	.36				.44		.41	
7-Methylxanthine			.64		.51		.55	
Guanosine				.73	.47	.58	.58	.32
7-Methylguanosine				.79	.34	.41	.35	.28
7-Methylguanine		.22	.53		.53			
1-Methyladenine	.22	.34			.56			
2′-Deoxyguanosine						.59	.68	.40
2′-Deoxy-7-methylguanosine						.39	.29	.40

Chromatograms developed on Whatman No. 1 paper. [b] *Solvents systems* (descending method): A, concd. aqueous ammonia–N,N-dimethylformamide–isopropyl alcohol, 10:25:65 (vol:vol); B, 1-butanol–water (saturated solution); C, 5% aqueous ammonium bicarbonate; D, ethyl acetate–pyridine–water, 75:23:165 (vol:vol). *Solvent systems* (ascending method): E, 1-butanol–acetic acid–water, 5:1:4 (vol:vol); F, methanol–water, 7:3 (vol:vol); G, isopropyl alcohol–water, 6:4 (vol:vol); H, ethanol–water, 7:3 (vol:vol).

Experimental

7-Methylxanthosine (XVI).—Xanthosine, 5 g., was added to 50 ml. of N,N-dimethylacetamide and the suspension cooled to 20° and treated all at once with 5 ml. of dimethyl sulfate. The mixture was then stirred for 1 hr. and 45 min. at 20–23°, during which time the xanthosine gradually dissolved. The resulting solution was treated with Celite and filtered. The filtrate was added to 500 ml. of chloroform stirring at 7°. The resulting suspension was allowed to stand at 7–10° for 20 min. and was then filtered. The white product was washed first with 100 ml. of chloroform and finally with 100 ml. of ethyl ether. The resulting product was dried under reduced pressure in a rotary evaporator at room temperature to yield 5.8 g. of the methyl sulfuric acid salt of 7-methylxanthosine. The compound was homogeneous as judged by paper chromatography. The methyl sulfate salt of 7-methylxanthosine softened at 63–66° and gradually decomposed on further heating.

Anal. Calcd. for $C_{12}H_{18}N_4O_{10}S\cdot{}^1/_2H_2O$: C, 34.4; H, 4.5; N, 13.4; H_2O, 2.2. Found: C, 34.4; H, 4.9; N, 13.2; H_2O, 2.3.

A small sample of the above salt (2.5 g.) was dissolved in 25 ml. of water and chilled immediately to 5°. The solution was then carefully adjusted to *p*H 8 with concentrated aqueous ammonia and finally diluted to 250 ml. with acetone. The solution was then readjusted to *p*H 8 at 5°, and a product separated gradually from the solution. The solid was separated by decanting the supernatant liquid, and the crude product was washed with three 50-ml. portions of acetone followed by two 75-ml. portions of ethyl ether. The resulting 7-methylxanthosine was dried at room temperature under reduced pressure in a rotary evaporator to yield 1.3 g. of a chromatographically pure product. 7-Methylxanthosine melted with decomposition at 140–150° when placed on a melting point block preheated to 130°.

Anal. Calcd. for $C_{11}H_{14}N_4O_6\cdot H_2O$: C, 42.0; H, 5.1; N, 17.8; H_2O, 5.7. Found: C, 41.6; H, 5.3; N, 17.7; H_2O, 4.6.

Hydrolysis of 7-Methylxanthosine (XVI).—The methyl sulfate salt of XVI (1.0 g.) was dissolved in 25 ml. of water and the solution heated on the steam-bath for 1 hr. The solution was then cooled and filtered, and the white solid, 7-methylxanthine (0.4 g.), was identified by its ultraviolet absorption spectra[32] and its identity confirmed by comparison by means of paper chromatography with an authentic sample.[32] The presence of D-ribose was confirmed in the filtrate by paper chromatography.[58]

7-Methylguanosine (XIV). Method 1.—Guanosine dihydrate (10 g.) was stirred for 30 hr. in 100 ml. of N,N-dimethylacetamide containing 10 g. of methyl iodide. The temperature of the reaction mixture was maintained at 30°. The solution that resulted was treated with 2 g. of Celite and filtered. To the filtrate was added 500 ml. of ethanol, and the resulting solution was diluted to 1200 ml. with petroleum ether (b.p. 65–110°). The supernatant liquid was decanted from the oily residue that separated. The oily residue was treated with 300 ml. of acetone, and a white solid separated immediately. The suspension was stirred for 5 min. and the precipitate filtered and washed with excess acetone (300 ml.) followed by 400 ml. of ether. The resulting white precipitate was then dried at room temperature under reduced pressure in a rotary evaporator to yield 8 g. of product. The iodide salt of 7-methylguanosine was found to be chromatographically pure and was analyzed without further purification.

Anal. Calcd. for $C_{11}H_{15}N_5O_5\cdot HI$: C, 31.1; H, 3.7; N, 16.5; I, 29.9. Found: C, 31.5; H, 3.8; N, 16.2; I, 29.3.

The iodide salt of 7-methylguanosine (3 g.) was dissolved in 25 ml. of water at room temperature and chilled to 10°. The solution was adjusted to *p*H 9.5 with concentrated aqueous ammonia and diluted immediately to 250 ml. with acetone. Additional concentrated aqueous ammonia was added to the acetone solution until the solution was readjusted to *p*H 9. The precipitate that immediately separated was filtered and slurried first in 100 ml. of acetone and finally in 100 ml. of ether. The product was dried at room temperature under reduced pressure in a rotary evaporator and found to be chromatographically and analytically pure. 7-Methylguanosine melted at 160–161°.

Anal. Calcd. for $C_{11}H_{15}N_5O_5\cdot 2H_2O$: C, 39.6; H, 5.7; N, 21.0; H_2O, 10.9. Found: C, 39.8; H, 5.8; N, 21.0; H_2O, 11.2.

Method 2.—Guanosine dihydrate (20 g.) was stirred at 25–28° for 6 hr. in 200 ml. of N,N-dimethylacetamide containing 15 g. of dimethyl sulfate. The mixture was treated with 3 g. of Celite and filtered. The colorless filtrate was chilled at 15–20° and carefully adjusted to *p*H 8 with concentrated aqueous ammonia. The solution was then diluted to 600 ml. with acetone, and the precipitate that separated at 15–20° for 2 hr. was filtered and washed with 200 ml. of absolute ethanol. The white product was stirred in 200 ml. of ether, filtered, and finally washed with 75 ml. of ether. The chromatographically pure product was dried under a stream of air to yield 18.5 g., m.p. 159–160°.

Anal. Calcd. for $C_{11}H_{15}N_5O_5\cdot 2H_2O$: C, 39.6; H, 5.7; N, 21.0. Found: C, 39.5; H, 5.7; N, 21.0.

Method 3.—The procedures of Bredereck and Martini[27] and Bredereck, Haas and Martini[20a] for the preparation of "1-methylguanosine" were repeated in our laboratory. In each case a product which possessed the properties described by the above investigators was isolated and proved to be identical to 7-methylguanosine prepared by methods 1 and 2. Mixed melting points showed no depression, and the products obtained by methods 1, 2 and 3 possessed identical ultraviolet and infrared spectra and the same R_f values in three different solvents.

Hydrolysis of 7-Methylguanosine. Method 1 (Water).—7-Methylguanosine (1 g.), in 25 ml. of water, was heated for 1 hr. on a steam-bath. The reaction mixture was chilled at 5–10° for 10

(58) S. M. Partridge, *Nature*, **158**, 270 (1946).

hr., and the product that separated was filtered, washed with cold water and air-dried. The isolated product was identified as 7-methylguanine by ultraviolet spectra and paper chromatographic comparison with an authentic sample of 7-methylguanine.[28,29] D-Ribose was shown to be present in the filtrate by chromatographic procedures.[58]

Method 2 (Methanol).—7-Methylguanosine was suspended in 50 ml. of methanol and heated for 1 hr. on a steam-bath. The insoluble product was filtered, washed with cold water, and dried. The isolated product was established as 7-methylguanine[28,29] by the procedures outlined in method 1.

Method 3 (Acid).—7-Methylguanosine (1 g.) was added to a solution of 0.5 ml. of concentrated sulfuric acid in 10 ml. of water. The mixture was heated on a steam-bath for 2 hr. The solution was cooled to room temperature and allowed to stand for 24 hr. The sulfate salt of 7-methylguanine was filtered and washed with cold water (2 ml.), and the filtrate was chromatogrammed in solvents B, C and E to give R_f values identical to those of D-ribose. The isolated product was shown to be 7-methylguanine[28,29] by the procedures described in method 1.

2'-Deoxy-7-methylguanosine (XV).—2'-Deoxyguanosine (monohydrate) (10 g.) was carefully dissolved in 75 ml. of dimethyl sulfoxide (50°) and the solution cooled to 20°. To the reaction mixture was added methyl iodide (10 ml.), and the resulting solution was stirred at 20° for 1 hr. in a tightly sealed flask. The temperature of the solution was then allowed to increase gradually to 28° over a period of 3.5 hr. The excess methyl iodide was allowed to evaporate in an open flask at room temperature. The dark red solution was then diluted with 600 ml. of chloroform previously chilled to 15°. The reaction mixture was allowed to stand at 15° for 3 hr., and the precipitate that separated was filtered and washed first with ethanol (50 ml.) and then with ethyl ether (30 ml.). The tan product weighed 10 g. after drying in a rotary evaporator at room temperature and was analyzed without further purification. The hydroiodide salt of 2'-deoxy-7-methylguanosine begins to turn brown at 135° and gradually darkens over a wide range without melting.

Anal. Calcd. for $C_{11}H_{15}O_4 \cdot HI$: C, 32.3; H, 3.9; N, 17.1; I, 31.1. Found: C, 32.6; H, 4.2; N, 17.2; I, 31.1.

Two grams of the hydroiodide salt of XV was suspended in 100 ml. of ethanol at room temperature and the resulting suspension treated with 40% aqueous methylamine (20 ml.). The reaction mixture was stirred at 28° for 1 hr., and the resulting white precipitate was filtered and washed with ethanol (50 ml.) followed by ethyl ether (50 ml.). The analytically pure product was dried at room temperature under reduced pressure in a rotary evaporator to yield 1.2 g.

Anal. Calcd. for $C_{11}H_{15}N_5O_4 \cdot {}^5/_4H_2O$: C, 43.5; H, 5.8; N, 23.1; H_2O, 7.4. Found: C, 43.6; H, 6.0; N, 23.2; H_2O, 7.6.

Hydrolysis of 2'-Deoxy-7-methylguanosine Hydroiodide (XV).—2'-Deoxy-7-methylguanosine hydroidide (20 mg.), in 0.1 *N* hydrochloric acid (20 ml.), was heated on a steam-bath for 20 min. The solution was cooled to room temperature and adjusted to *p*H 8 with concentrated aqueous ammonia. The ultraviolet spectrum of the solution was identical to that of 7-methylguanine. The solution was subjected to paper chromatography and showed 7-methylguanine and 2'-deoxy-D-ribose as the only detectable spots.

7-Methylinosine (XXI).—Inosine monohydrate (2.5 g.) was suspended in 25 ml. of dimethyl sulfoxide containing 2.5 ml. of methyl iodide. The mixture was stirred at 26–28° for 6 hr. and diluted with acetone (200 ml.). The solution was treated with Celite, filtered, and allowed to stand at 0° for 48 hr. The very small amount of precipitate that separated was then filtered and discarded. The filtrate at 0° was carefully adjusted to *p*H 9 with methanol saturated with ammonia at 0°. The white precipitate that separated was filtered and washed with excess acetone (400 ml.) followed by ethyl ether (200 ml.). The remaining solid was dried under reduced pressure in a rotary evaporator to yield 2.1 g. of homogeneous product as judged by paper chromatography. 7-Methylinosine softened at 138–140° and decomposed over a wide range on further heating.

Anal. Calcd. for $C_{11}H_{14}N_4O_5 \cdot H_2O$: C, 44.0; H, 5.3; N, 18.7; H_2O, 6.0. Found: C, 43.5; H, 5.3; N, 18.5; H_2O, 5.7.

Hydrolysis of 7-Methylinosine. Method 1.—7-Methylinosine was stored in a tightly sealed container at room temperature for 3 months. A dark, gummy residue resulted, and a small sample of this product was chromatogrammed in solvents B, C and E to give R_f values identical to those of 7-methylhypoxanthine.[32] The ultraviolet spectrum of an authentic sample of 7-methylhypoxanthine and that of the isolated product were identical.

Method 2.—7-Methylinosine (0.5 g.), in 10 ml. of 0.2 *N* hydrochloric acid, was heated on a steam-bath for 1 hr. The reaction mixture was chilled to room temperature and adjusted to *p*H 7 with concentrated aqueous ammonia. The solution was chromatogrammed directly and gave evidence of the presence of 7-methylhypoxanthine and D-ribose. The ultraviolet spectrum of the solution was identical to that of 7-methylhypoxanthine.[32]

1-Methyladenosine (V). Method 1.—Adenosine (5 g.) was added to a mixture of 10 g. of methyl *p*-toluenesulfonate in 500 ml. of N,N-dimethylformamide. The mixture was stirred for 24 hr. at room temperature, treated with 1 g. of Celite, and filtered. The colorless filtrate was diluted to 500 ml. with acetone. The precipitate that separated at 15° for 48 hr. was filtered and air-dried under a heat ray lamp. The white product was dissolved in 250 ml. of methanol, routinely treated with 3 g. of Celite, and filtered. The filtrate was allowed to stand at 10–15° for 24 hr. The precipitate (4 g.) was collected and washed with a small quantity of cold ethanol. An analytical sample of the tosylate salt III was dried at 85° for 12 hr., m.p. 197–199°.

Anal. Calcd. for $C_{18}H_{22}N_5O_7S$: C, 47.7; H, 5.1; N, 15.5. Found: C, 47.5; H, 5.0; N, 15.5.

Method 2.—A mixture of adenosine (20 g.) and 20 ml. of methyl iodide in 250 ml. of N,N-dimethylacetamide was stirred at 28° for 18 hr. The resulting solution was treated with 2 g. of Celite, filtered, and added to 1 l. of acetone. The mixture was allowed to stand at 5–10° for 14 hr. during which time a white precipitate separated. This product was filtered, washed with 200 ml. of acetone followed by 75 ml. of ethyl ether, and dried under a heat ray lamp. The product (25 g.) was chromatographically pure and was analyzed without further purification.

Anal. Calcd. for $C_{11}H_{15}O_4N_5 \cdot HI$: C, 32.3; H, 3.9; N, 17.1, Found: C, 32.6; H, 3.9; N, 16.8.

The hydroiodide salt of 1-methyladenosine (25 g.) was dissolved in 100 ml. of water and adjusted to *p*H 8 with concentrated aqueous ammonia. The solution was then diluted to a total volume of 500 ml. with acetone. The resulting mixture was allowed to stand at 10–15° for 48 hr., during which time a white product (14 g.) separated as fine needles. The product was filtered, washed with 300 ml. of acetone, and dried under a heat ray lamp. The chromatographically pure product softened at 210° and melted with decomposition at 214–217°.

Anal. Calcd. for $C_{11}H_{15}N_5O_4$: C, 47.0; H, 5.3; N, 24.8. Found: C, 47.1; H, 5.7; N, 24.7.

Hydrolysis of 1-Methyladenosine.—1-Methyladenosine (1 g.), in 20 ml. of 0.5 *N* hydrochloric acid, was heated on a steam-bath for 45 min. and cooled to room temperature. The resulting solution was adjusted to *p*H 7.5 with concentrated aqueous ammonia. The solution was subjected to paper chromatography in three different solvent systems and gave evidence of two components: 1-methyladenine and D-ribose. The neutralized solution was allowed to stand at room temperature for 4 hr., during which time a white precipitate separated. The product was filtered, washed with water, and dried at 110° for 18 hr. The ultraviolet spectrum of this sample proved to be identical to that of 1-methyladenine.[24]

N^6-Methyladenosine[12] (6-Methylamino-9-β-D-ribofuranosylpurine) (VIII).—1-Methyladenosine (V, 5 g.), in 25 ml. of 0.25 *N* sodium hydroxide, was heated for 75 min. on a steam-bath. The solution was cooled to 5–10° and adjusted to *p*H 7.5 with a 10% solution of *p*-toluenesulfonic acid. The resulting solution was evaporated to dryness under reduced pressure on a steam-bath. The residue was treated with 50 ml. of methanol and evaporated as before. The resulting solid was continuously extracted for 10 hr. with ethyl acetate in a soxhlet extractor, and the ethyl acetate was removed under reduced pressure on a steam-bath to give 3.2 g. of crude product. A sample was prepared for analysis by recrystallization from ethyl acetate. The compound was dried at 110° for 10 hr. to give an anhydrous product that softened at 206–208° and melted at 219–221°. An authentic sample of VIII prepared by the method of Johnson, *et al.*,[12] showed no depression when mixed and melted with the above product.

Anal. Calcd. for $C_{11}H_{15}N_5O_4$: C, 47.0; H, 5.4; N, 24.9. Found: C, 46.9; H, 5.3; N, 24.5.

2'-Deoxy-1-methyladenosine (VI). Method 1.—2'-Deoxyadenosine monohydrate (2 g.) was added to a mixture of 6 g. of methyl *p*-toluenesulfonate in 35 ml. of N,N-dimethylformamide and stirred for 15 hr. The resulting solution was treated with 1.5 g. of Celite and filtered. The filtrate was added to 300 ml. of acetone, and the mixture was stirred vigorously for 20 min. The white precipitate that separated was collected, washed with acetone, and air-dried to yield 2.8 g. of pure product. The tosylate salt softened at 153–155° and decomposed over a wide range on further heating.

Anal. Calcd. for $C_{18}H_{22}N_5O_6S \cdot {}^1/_2H_2O$: C, 48.5; H, 5.2; N, 15.7; H_2O, 2.0. Found: C, 48.3; H, 5.4; N, 15.8; H_2O, 1.9.

Method 2.—2'-Deoxyadenosine monohydrate (15 g.) was stirred for 18 hr. at 25–28° in a tightly sealed flask containing 50 ml. of N,N-dimethylacetamide and 15 ml. of methyl iodide. The 2'-deoxyadenosine gradually dissolved, and later a new product precipitated. Acetone (250 ml.) was added to the suspension, and stirring was continued for 30 min. The white precipitate was filtered, washed with 250 ml. of acetone, and dried immediately under reduced pressure (rotary evaporator) at room temperature to yield 20 g.

Anal. Calcd. for $C_{11}H_{16}N_5O_3 \cdot HI \cdot H_2O$: C, 32.2; H, 4.5; N, 17.1. Found: C, 32.2; H, 4.4; N, 17.3.

Hydrolysis of 2'-Deoxy-1-methyladenosine (Water).—A dilute solution of 2'-deoxy-1-methyladenosine hydroiodide (20 mg. in 0.5 ml. of water) was heated for 20 min. on a steam-bath. The solution was then cooled to room temperature and adjusted to *p*H 7.5 with concentrated aqueous ammonia. This solution was subjected to paper chromatography and gave evidence of the presence of 1-methyladenine and 2-deoxy-D-ribose.

1-Methyladenine.[24]—2'-Deoxy-1-methyladenosine hydroiodide (IV, 10 g.), in 100 ml. of methanol, was heated on a steam-bath for 30 min. The mixture was allowed to cool at room temperature for 10 hr. The precipitate that separated was filtered and dissolved in 20 ml. of water, and the resulting solution was treated with Celite and filtered. The filtrate was adjusted to *p*H 8.5 with concentrated aqueous ammonia. The white precipitate that separated was filtered, washed with cold water, and dried under a heat ray lamp to yield 2.5 g. of crude product. For analysis a small sample was recrystallized twice from water. The purified product softened at 290–295° and melted with decomposition at 296–299°; ultraviolet data: *p*H 1, λ_{max} 257.5 mμ, ε 11,800; λ_{min} 227 mμ, ε 1,400; *p*H 11, λ_{max} 268.5 mμ, ε 12,100; λ_{min} 241.5 mμ, ε 1,400.

Anal. Calcd. for $C_7H_7N_5$: C, 48.3; H, 4.7; N, 47.0. Found: C, 48.5; H, 5.1; N, 46.5.

2'-Deoxy-N^6-methyladenosine (2'-Deoxy-6-methylamino-9-β-D-ribofuranosylpurine).—The iodide salt of 2'-deoxy-1-methyladenosine monohydrate (IV, 2 g.), in 50 ml. of 0.2 *N* sodium hydroxide, was heated on a steam-bath for 30 min. and then chilled to 28°. The resulting solution was carefully neutralized to *p*H 7 with a 10% aqueous solution of *p*-toluenesulfonic acid. The mixture was evaporated to dryness under reduced pressure on a rotary evaporator, and the residue was extracted with 50 ml. of cold methanol in 2 portions. The insoluble residue was recrystallized from 35 ml. of hot methanol to yield 0.6 g. (dried at 80° for 2 hr.), m.p. 206–208° (block preheated to 180°). This product chromatogrammed as a single homogeneous spot in three different solvents identical to the previous reported R_f values for the same compound prepared by enzymatic means.[13]

Hydrolysis of 2'-Deoxy-N^6-methyladenosine (IX).—A small sample of IX (0.2 g.) in 5 ml. of 0.1 *N* hydrochloric acid was heated for 10 min. on a steam-bath. The resulting solution was neutralized with concentrated aqueous ammonia and chromatogrammed in three different solvents. The chromatograms indicated a single ultraviolet absorbing spot identical to that of an authentic sample of 6-methylaminopurine.[26] A dark, homogeneous spot identical to that obtained from 2'-deoxyribose resulted after developing the chromatograms with silver nitrate in acetone followed by alcoholic potassium hydroxide. When the neutralized hydrolysate was allowed to stand at room temperature, a white precipitate separated. The product proved to be identical to 6-methylaminopurine as judged by ultraviolet and infrared spectra.

1-Methylinosine.—Inosine (5 g.) was added to 50 ml. of N,N-dimethylacetamide, containing 3 g. of potassium carbonate, heated at 80°. The mixture was treated with 3.5 g. of methyl *p*-toluenesulfonate (added dropwise) and the solution stirred at 100° for 2 hr. The resulting solution was treated with Celite and filtered and the filtrate diluted to 250 ml. with acetone. The precipitate that separated was collected, washed with 75 ml. of acetone, dissolved in 300 ml. of methanol, and neutralized to *p*H 7 with *p*-toluenesulfonic acid in methanol. The solution was allowed to stand at room temperature for 15 hr., and the crystalline potassium tosylate was filtered. The solution was then refrigerated at 15° for 48 hr. and filtered and the filtrate evaporated in a rotary evaporator at 80°. The residue was suspended in 75 ml. of isopropyl alcohol and dissolved by addition of methanol. The filtered solution was allowed to crystallize for 24 hr. at 15°, and the white product (2 g.) that separated was filtered and finally recrystallized from ethyl alcohol. The pure product melted at 210–212° (lit.[31] 211–212°).

Anal. Calcd. for $C_{11}H_{14}N_5O_4$: C, 46.8; H, 5.0; N, 19.9. Found: C, 46.9; H, 5.4; N, 19.6.

[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO., PEARL RIVER, N. Y.]

The Tertiary Butyl Group as a Blocking Agent for Hydroxyl, Sulfhydryl and Amido Functions in Peptide Synthesis

BY FRANCIS M. CALLAHAN, GEORGE W. ANDERSON, ROLF PAUL AND JOAN E. ZIMMERMAN

RECEIVED JULY 23, 1962

The *tert*-butyl group has been used to protect hydroxyl groups during peptide synthesis and conditions for its subsequent removal have been determined. Its removal from a sulfhydryl or amido group was too difficult for practical application to peptide synthesis. Several intermediates are described.

The reaction to form a peptide bond between the ester of an amino acid and an acylated amino acid is usually a straightforward procedure if the starting amino acids are the simple ones such as glycine, alanine or valine, which have no additional reactive centers. However, with amino acids bearing hydroxyl or sulfhydryl groups, *O*- or *S*-acylation is a common and an expected side reaction. Where a simple amido group is exposed, as in asparagine, many peptide-forming reagents lead to some nitrile formation[1,2] as well as other anomalies.[3] In addition to intermolecular side reactions, compounds containing glutamine or asparagine have a tendency to form undesirable cyclic structures.[4–6] To avoid such side reactions a number of groups have been used to protect amido, hydroxyl and sulfhydryl functions during peptide synthesis. A widely used means of O-protection has been acylation by the tosyl,[7–11] acetyl,[12–15] carbobenzoxy[16–18] and the *p*-nitrocarbobenzoxy groups.[19] Less used are the benzylsulfonyl,[20] β-naphthalenesulfonyl[21] and benzoyl derivatives.[22]

Cysteine has been dicarbobenzoxylated and conditions for removal of the two acyl groups were determined.[23]

Among the ether blocking groups for the hydroxyl function the benzyl[24–26] group is outstanding because of its easy removal by catalytic hydrogenation. The

(1) C. Ressler, *J. Am. Chem. Soc.*, **78**, 5956 (1956).
(2) M. Zaoral and J. Rudinger, *Proc. Chem. Soc.*, 176 (1957).
(3) S. J. Leach and H. Lindley, *Austral. J. Chem.*, **7**, 173 (1954).
(4) A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955).
(5) E. Sondheimer and R. W. Holley, *J. Am. Chem. Soc.*, **79**, 3767 (1957).
(6) J. Rudinger, *Angew. Chem.*, **71**, 742 (1959).
(7) E. Fischer, *Ber.*, **48**, 93 (1915).
(8) T. Oseki, *J. Tokyo Chem. Soc.*, **41**, 8 (1920); *C. A.*, **14**, 2780 (1920).
(9) A. A. Patchett and B. Witkop, *J. Am. Chem. Soc.*, **79**, 185 (1957).
(10) P. G. Katsoyannis, D. T. Gish and V. du Vigneaud, *ibid.*, **79**, 4516 (1957).
(11) J. Kurtz, G. D. Fasman, A. Berger and E. Katchalski, *ibid.*, **80**, 393 (1958).
(12) M. Bergmann, L. Zervas, L. Salzmann and H. Schleich, *Z. physiol. Chem.*, **224**, 17 (1934).
(13) A. E. Barkdoll and W. F. Ross, *J. Am. Chem. Soc.*, **66**, 951 (1944).
(14) M. Frankel and M. Halmann, *J. Chem. Soc.*, 2735 (1952).
(15) S. G. Waley and J. Watson, *Biochem. J.*, **57**, 529 (1954).
(16) E. Abderhalden and A. Bahn, *Z. physiol. Chem.*, **219**, 72 (1933).
(17) P. A. Levene and A. Schormüller, *J. Biol. Chem.*, **105**, 547 (1934).
(18) E. Katchalski and M. Sela, *J. Am. Chem. Soc.*, **75**, 5284 (1953).
(19) D. T. Gish and F. H. Carpenter, *ibid.*, **75**, 950 (1953).
(20) H. B. Milne and Chi-Hiesh Peng, *ibid.*, **79**, 639 (1957).
(21) E. Fischer and P. Bergell, *Chem. Ber.*, **36**, 2592 (1903).
(22) C. R. Harington and R. V. Pitt Rivers, *Biochem. J.*, **38**, 417 (1944).
(23) A. Berger, J. Noguchi and E. Katchalski, *J. Am. Chem. Soc.*, **78**, 4483 (1956).
(24) L. Velluz, G. Amiard and R. Heymes, *Bull. soc. chim.*, 201 (1955).
(25) K. Okawa, *Bull. Chem. Soc. Japan*, **29**, 488 (1956).
(26) W. Grassman, E. Wünsch, P. Deufel and A. Zwick, *Chem. Ber.*, **91**, 538 (1958).