112. *The Methylation of Adenosine and Adenylic Acid.*

By P. Brookes and P. D. Lawley.

Methylation of adenosine with dimethyl sulphate in dimethylformamide, followed by acid hydrolysis, gave as main products 1- and 3-methyladenine, 5-aminoimidazole-4-*N'*-methylcarboxamidine, and (probably) 1,3-dimethyladenine, which were separated by ion-exchange chromatography. The amidine results from the action of acid on 1-methyladenine. The ultraviolet spectra of these compounds are given and the action of alkali on them is discussed.

Methylation of adenylic acid in aqueous solution at pH 7 results in reaction at $N_{(1)}$ and $N_{(3)}$.

The action of alkali on 1-methyladenylic acid was shown, as with 1-methyladenine, to result in net migration of the methyl group from $N_{(1)}$ to the extranuclear amino-group.

In preliminary studies of the alkylation of nucleic acids and their constituent nucleotides [1] it was found that reaction occurred on the bases guanine, adenine, and cytosine. Whereas it has been shown [2,3] that in the case of the guanine nucleotides alkylation occurs at $N_{(7)}$, alkylation of adenine nucleotides yielded two products, the structures of which were not established. The object of the present work was to determine the position of methylation of adenosine and adenylic acid.

Of the possible *N*-methyladenines, 3-methyladenine, 7-methyladenine, and 6-methylaminopurine are known.[4] The synthesis of 1-methyladenine has not so far been reported and Elion [4] failed to obtain it by the action of ammonia on 6-mercapto-1-methylpurine.

Since some methyladenines are known to be unstable in alkaline conditions,[4,5] such conditions were avoided in our work. Methylation of adenosine with dimethyl sulphate in dimethylformamide was followed by acid hydrolysis of the methylated nucleosides. The resulting bases were separated by chromatography on a cation-exchange resin. The total acid-hydrolysate was thus found to contain five major components in addition to adenine (Fig. 1a). However, as the principal product could not be adequately separated

[1] Lawley, *Biochim. Biophys. Acta*, 1957, **26**, 450.
[2] Lawley and Wallick, *Chem. and Ind.*, 1957, 633.
[3] Lawley, *Proc. Chem. Soc.*, 1957, 290.
[4] Cf. Elion, "Ciba Foundation Symposium on the Chemistry and Biology of Purines," J. and A. Churchill Ltd., London, 1957, p. 39.
[5] Leese and Timmis, Abs. of 7th Internat. Cancer Congress, London, 1958, p. 144.

from adenine in this way, preliminary removal of adenine was necessary. This was done on the assumption that the methyladenines would yield uncharged bases in weakly alkaline solution, in contrast to adenine itself which forms an anion. The mixture of bases in aqueous ammonia at pH 10 was passed through an anion-exchange column: adenine was retained by the resin. When the filtrate from this column was chromatographed on a cation-exchange resin the pattern of elution of the remaining bases was substantially as before (Fig. 1*b*), except that the product (*A*) eluted first was missing (attempts to isolate

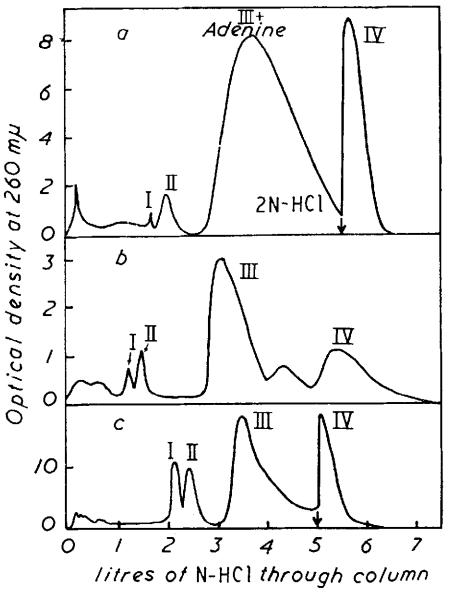


FIG. 1. *Elution from Dowex-50 (H^+-form) of bases contained in the acid-hydrolysates from methylated adenosine.*

Me_2SO_4: *(a, b)* 1·5 *mols., (c)* 3 *mols. In (a), adenine was not removed; in (b) and (c) adenine had been removed.*

this product were unsuccessful). Evaporation of the solvent resulted in loss of the specific ultraviolet absorption due to material *A* ($\lambda_{max.}$ 277 mμ). The four major products which were isolated and purified are numbered (I)—(IV).

Two series of experiments were carried out, with molar ratios of dimethyl sulphate to adenosine of 1·5 and 3. In neither case was complete conversion of adenosine observed, suggesting that side reactions had developed which inhibited methylation. The principal

TABLE 1. *Properties of products from the methylation of adenosine.*

Compound	pH	$\lambda_{max.}$ (mμ)	$10^{-3}\,\varepsilon$	$\lambda_{min.}$ (mμ)	$\varepsilon_{280}/\varepsilon_{260}$	pK_a'*	R_F † in solvent: 1	2	3
(III)	4	259	11·7	228	0·23	7·2	1·3	1·6	0·8
	13	270	14·4	239	0·85	11·0			
(II)	2	274	15·9	235	1·26		1·5	1·2	1·1
	13	273	12·8	244	1·48				
(IV)	4	281	12·8	242	1·8	9·5	1·3	1·8	0·5
	12	290	15·4	251	2·6				
(I)	4	276	15·7	239	1·5	11·0	1·8	2·1	0·3
	13	279	14·1	240	2·0				

* *I* 0·05 at 20°. † Relative to adenine; for solvents see p. 543.

effect of the larger proportion of dimethyl sulphate was to increase the relative yield of product (I) (Fig. 1*b* and *c*), suggesting that this might be a dimethyladenine: at the same time the relative amounts of products (III) and (IV) remained constant, suggesting that one might be a transformation product of the other.

Ultraviolet absorption spectra of these products, in aqueous solution at various pH, are shown in Table 1 and Fig. 2. Comparison with the data given by Elion [4] for the known

methyladenines shows only one correspondence, namely, of compound (II) with 3-methyladenine: detailed comparison confirmed this identity.

Compound (III), purified as sulphate, gave analyses of a monomethyladenine and the molecular weight of the picrate, determined spectrophotometrically,[6] was consistent with this. The pK_a values were determined spectrophotometrically (Fig. 2 and Table 1): absence of a dissociation at pH ~4 suggests that the compound forms an imino-, rather than an amino-base. For a monomethyladenine this would be the case only if the methyl group were attached to a pyrimidine-ring nitrogen atom. Since compound (II) is 3-methyladenine, compound (III) should then be 1-methyladenine. The values of pK_a observed, 7·2 and 11·0, would then be due to the dissociation of protons from the amino-group and from the imidazole ring, probably in that order. Confirmation was provided when compound (III) was converted by alkali into 6-methylaminopurine (V) in over 80% yield, this being analogous to the conversion [7] of 1,2-dihydro-2-imino-1-methylpyrimidine into 2-methylaminopyrimidine. Further, Elion [4] found that 6-mercapto-1-methylpurine with

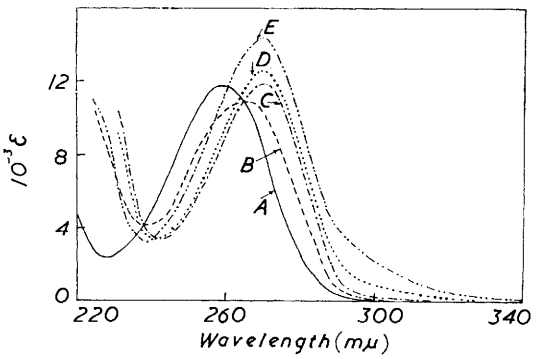
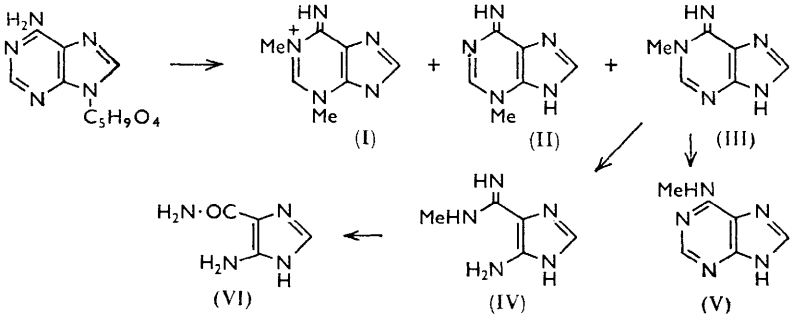


FIG. 2. *Absorption spectra of* 1-*methyladenine*, (*A*) pH 4, (*B*) pH 7·2, (*C*) pH 9, (*D*) pH 10·7, (*E*) pH 13.

hot aqueous ammonia yielded 6-methylaminopurine; and Leese and Timmis [5] reported that methylation of 9-phenethyladenine yielded a quaternary salt which was not identified but gave 6-methylamino-9-phenethylpurine on treatment with alkali (the evidence now available suggests that the quaternary salt was 1-methyl-9-phenethyladenine).

Compound (I) was purified as sulphate and gave analyses for a dimethyladenine, with



which the molecular weight of the picrate was in agreement. The absorption spectrum in acid solution was very similar to that of 3-methyladenine (Table 1). For an alkaline solution the absorption curve, although similar in shape to that for 3-methyladenine, showed a bathochromic shift, as found with 1-methyladenine and 6-methylaminopurine.

[6] Cunningham, Dawson, and Spring, *J.*, 1951, 2305.
[7] Brown, Hoerger, and Mason, *J.*, 1955, 4035.

Heating compound (I) in alkaline solution greatly reduced the ultraviolet absorption above 225 mμ, the remaining absorption having no maximum in this region. By analogy with the behaviour of other dialkyladenines, it would be expected that compounds having one substituent on the pyrimidine-ring nitrogen and the other on the imidazole-ring nitrogen would, under similar alkaline conditions, give, in high yield, stable products having specific ultraviolet absorption with maxima in the region of 260—280 mμ. These considerations suggest that compound (I) has both methyl groups attached to the pyrimidine moiety and, in view of the structures of compounds (II) and (III), it is proposed that compound (I) is a 1,3-dimethyladenine salt, although the 3-methyl-6-methylaminopurine cannot be excluded.

The analysis of compound (IV) as a dihydrochloride and the molecular weight of the dipicrate were consistent with a formula $C_5H_9N_5$ for the base. With alkali this compound

TABLE 2.

Time (min.)	0	20	40	70	140	240	1400
Conversion (%)	0	8·4	16	26·4	45·8	65	100
D_{280}/D_{260} (mμ) (obs.) ...	0·23	0·33	0·38	0·49	0·72	0·925	1·3
D_{280}/D_{260} (mμ) (calc.)...	—	0·31	0·38	0·49	0·72	1·01	1·66
D_{290} (mμ) (obs.)	0·024	0·092	0·138	0·204	0·275	0·363	0·275
D_{290} (mμ) (calc.)	—	0·085	0·140	0·216	0·356	0·496	0·75

yielded 5-aminoimidazole-4-carboxamide (VI) as major product and is thus probably 5-aminoimidazole-4-*N'*-methylcarboxamidine. This compound could have been derived

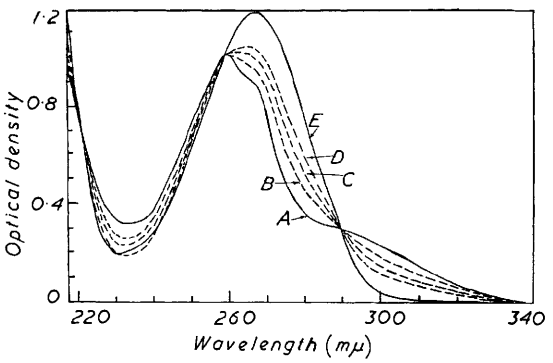


FIG. 3. *Absorption spectra showing the conversion of* 1-*methyladenylic acid at* pH 11·7 *and* 37°; (*A*), *initial*; (*B*), *after* 35 *min.*; (*C*), *after* 70 *min.*; (*D*), *after* 105 *min.*; (*E*), *after* 18 *or* 27 *hr.*

from 1-methyladenosine or 1-methyladenine by fission of the pyrimidine ring with loss of $C_{(2)}$. Such a ring opening, but without loss of $C_{(2)}$, was proposed by Taylor [8] as a possible intermediate step in the rearrangement of 1-methyladenine to 6-methylaminopurine: since then our experiments had involved acid-treatment of the bases from methylated adenosine, the action of acid on 1-methyladenine was studied. We found that refluxing 1-methyladenine with 6N-hydrochloric acid did afford compound (IV). This reaction was followed by ultraviolet spectroscopy, by using the known extinction coefficients of 1-methyladenine and 5-aminoimidazole-4-*N'*-methylcarboxamidine. The initial stages were in line with this assumption, indicating a first-order reaction with a half-life of 158 min. (Table 2), but in the latter stages further degradation, into material with lower ultraviolet absorption, was indicated.

The position of methylation of adenosine in an organic solvent having been determined, methylation of adenylic acid in aqueous solution was investigated. The acid was methylated in neutral phosphate buffer. Paper chromatography showed that unchanged adenylic acid accounted for ~80% of the products having absorption in the ultraviolet

[8] Taylor, "Ciba Foundation Symposium on the Chemistry and Biology of Purines," J. and A. Churchill Ltd., London, 1957, p. 57.

region. Two other products were observed and these were isolated from the paper and hydrolysed in acid. The major product (approx. 15% yield) gave 1-methyladenine; the other (approx. 5% yield) gave 3-methyladenine.

1-Methyladenine was shown, by the change in its ultraviolet spectrum (Fig. 3), to be converted by aqueous alkali quantitatively into a product with spectra in acid and alkali almost identical with those of 6-methylaminopurine riboside.[9] Acid hydrolysis of this product yielded 6-methylaminopurine identified by comparison with an authentic specimen (Table 3).

TABLE 3. *Ultraviolet absorption spectra of the principal product obtained by methylation of adenylic acid at* pH 7 *and* 37°, *and of products from its subsequent conversion in aqueous alkali.*

	pH	$\lambda_{max.}$ (mμ)	$\varepsilon_{280}/\varepsilon_{260}$
Initial product	7	258·5	0·26
	12	259	0·39
After irreversible change in alkali	12	266	0·66
	2	262	0·45
After subsequent acid-hydrolysis	0	267	0·80
	12	273	1·17
6-Methylaminopurine riboside	12	266	0·66
	2	262	0·43
6-Methylaminopurine	0	267	0·73
	12	273	1·20

It is therefore shown that the methylation of adenylic acid in aqueous solution and of adenosine in dimethylformamide are analogous in that alkylation occurs principally at $N_{(1)}$, but also at $N_{(3)}$. Treatment of 1-methyladenylic acid with alkali results, as with 1-methyladenine, in net migration of the methyl group from $N_{(1)}$ to extranuclear nitrogen.

It is of interest that the adenine nucleotides are alkylated on the pyrimidine ring, but guanine nucleotides on the imidazole ring.[2,3] These findings are in agreement with theoretical estimates of the relative reactivities of ring-nitrogen atoms of the two purines by Nakajima and Pullman.[10]

EXPERIMENTAL

M. p.s were observed on a microscope hot stage. Absorption spectra were measured with a Unicam SP.500 spectrophotometer for aqueous solutions.

Paper chromatography was carried out on Whatman No. 1 filter paper, the following solvents being used: (1) methanol–concentrated hydrochloric acid–water (7 : 2 : 1); (2) propan-2-ol–5% aqueous ammonium sulphate (1 : 19); (3) butan-1-ol saturated with water–aqueous ammonia (*d* 0·88) (100 : 1). Descending chromatography was used for solvent system (1) and (3), and ascending for solvent (2).

Methylation of Adenosine.—Adenosine (1·2 g.; dried *in vacuo*) was heated in dry, redistilled dimethylformamide (15 c.c.) and dimethyl sulphate [1·7 g., 3 equiv.; dried (K_2CO_3) and redistilled] at 100° for 2 hr. The solvent was removed *in vacuo* and the residue refluxed in N-hydrochloric acid (20 c.c.) for 1 hr. Evaporation gave a residue, paper chromatography of which disclosed a number of products including much adenine.

Removal of adenine. The above aqueous solution was adjusted to pH 10 with concentrated ammonia solution and applied to a column (15 × 1·8 cm.) of Dowex-1 (Cl^- form) which had been washed until neutral with water. The column was washed with ammonia solution of pH 10 until the filtrate had no significant absorption at 260 mμ. A paper chromatogram of the combined column filtrate was identical with that of the input to the column, except that adenine was no longer present.

Separation of the products. The filtrate from the anion-exchange column was made acid by adding one-tenth of its volume of concentrated hydrochloric acid and applied to a column (17·2 × 2 cm.) of Dowex-50 (H^+ form, equilibrated with N-hydrochloric acid). The column was developed with N-acid, and 25 c.c. fractions were collected. The optical density of the fractions at 260 and 280 mμ was measured, with dilution where necessary with N-hydrochloric

[9] Littlefield and Dunn, *Biochem. J.*, 1958, **70**, 642.
[10] Nakajima and Pullman, *Bull. Soc. chim. France*, 1958, 1502; Pullman, *J.*, 1959, 1621.

acid. The value for $\lambda_{max.}$ of any product found in the fractions was also noted. The first 200 fractions contained three major products. The strength of the eluting acid was then increased to 2N, and a fourth product obtained. No further products were obtained when elution was continued with 4N-hydrochloric acid.

The yield of each product was estimated by summation of the optical densities at 260 mμ of appropriate fractions. Subsequent purification and analysis of these products enabled their molar extinction coefficients to be determined, and hence the total amounts in the column fractions were calculated. The molar proportion of adenine in the mixture of bases, estimated from the loss in ultraviolet absorption resulting when the mixed bases were passed through the anion-exchange resin, amounted to 28% of the initial adenosine. The yields were (I) 6%, (II) 7%, (III) 31%, and (IV) 20%.

1,3-*Dimethyladenine salts.* Fractions 82—88 contained a product (I), $\lambda_{max.}$ 276 mμ, and on evaporation gave a solid chloride, which could not be crystallised. It was converted into the *sulphate* which crystallised from methanol as needles, m. p. 302—303°(Found: C, 31·8; H, 4·0; N, 26·2. $C_7H_{11}O_4N_5S$ requires C, 32·2; H, 4·2; N, 26·8%). A derived *picrate* crystallised from water as plates, m. p. 256—257° [Found: *M* (spectroscopic method of Cunningham, Dawson, and Spring [6]), 392. $C_{13}H_{12}O_7N_8$ requires *M*, 392].

3-*Methyladenine.* Fractions 96—102, containing product (II), $\lambda_{max.}$ 274 mμ, gave a hydrochloride which could not be crystallised. The sulphate crystallised from methanol as needles, m. p. 268—270°, identical with the sulphate prepared from 3-methyladenine (kindly supplied by Dr. G. H. Hitchings of the Burroughs Wellcome Laboratories, New York) as shown by ultraviolet spectra, m. p., and R_F in 3 solvent systems. The picrate crystallised as needles (from water), and sublimed above 270° (Found: *M*, 376. Calc for $C_{12}H_{10}O_7N_8$: *M*, 378).

1-*Methyladenine.* Fractions 135—170° containing product (III), $\lambda_{max.}$ 259 mμ, yielded a hydrochloride which failed to crystallise. The *sulphate* separated from methanol as prisms, m. p. 276—278° (Found: C, 29·5; H, 3·7; N, 27·6. $C_6H_9O_4N_5S$ requires C, 29·2; H, 3·6; N, 28·4%). A *picrate* recrystallised from water as yellow prisms, m. p. 253—255° (Found: *M*, 380. $C_{12}H_{10}O_7N_8$ requires *M*, 378).

5-*Aminoimidazole*-4-N′-*methylcarboxamidine.* Fractions 203—220 contained a product (IV), $\lambda_{max.}$ 279 mμ, and on evaporation to dryness yielded a crude hydrochloride which did not crystallise. A *dipicrate* recrystallised from water as yellow needles, m. p. 200—201° (Found: *M*, 598. $C_{17}H_{15}O_{14}N_{11}$ requires *M*, 597). Analysis of this dipicrate failed to give satisfactory results so the *dihydrochloride* was regenerated with hydrogen chloride in dry ether. It then recrystallised from methanol–ethyl acetate as needles, m. p. 233° (Found: C, 28·0; H, 5·6; N, 33·3. $C_5H_{11}N_5Cl_2$ requires C, 28·3; H, 5·2; N, 33·0%).

Action of Alkali on Methylation Products.—0·1—0·5 mg. of products (I), (III), and (IV), separated on the ion-exchange column, were separately heated in 0·1 c.c. of concentrated aqueous ammonia (sealed tubes) at 100° for 18 hr. The tubes were then opened and the solvent was distilled off.

Substance (I) (1,3-dimethyladenine sulphate) gave a mixture which was shown by paper chromatography to contain 4 compounds. The major product behaved chromatographically like the starting material; the others were not identified.

Substance (II) (1-methyladenine sulphate) gave only one product, whose ultraviolet absorption at pH 1 and 13 and the R_F values in 3 solvent systems were identical with those of 6-methylaminopurine (kindly supplied by Mr. G. M. Timmis and Dr. C. L. Leese, of this Institute).

Substance (IV) gave a product with an ultraviolet absorption spectrum closely resembling that of 5-aminoimidazole-4-carboxamide. Paper chromatography showed 5 substances to be present, but only one occurred in significant amount (40% yield). This compound was eluted from the paper and its ultraviolet spectra at pH 1 and 13 and its R_F in 3 solvent systems confirmed its identity with 5-aminoimidazole-4-carboxamide.

0·01% solutions of the compounds (I) and (II) in 0·1N-sodium hydroxide were prepared, and the ultraviolet absorption spectra of the solutions measured before and after 18 hours' heating at 100°. For compound (II) the spectrum of the product obtained was identical with that of 6-methylaminopurine. With compound (I) this treatment resulted in 92% loss of ultraviolet absorption at the original maximum of 280 mμ.

Methylation of Adenylic Acid.—Neutralised adenosine-5′ phosphate (35 mg.) was treated in 0·4N-phosphate buffer (pH 7·2; 1 c.c.) with dimethyl sulphate (13 mg.) and kept at 37° for

1 hr. The solution was then chromatographed on Whatman No. 4 paper, with saturated aqueous ammonium sulphate–propan-2-ol–0·1N-phosphate (79 : 2 : 19), at pH 7·2, as solvent. When examined in ultraviolet light (λ 253·7 mμ) three components were observed, one of R_F 0·3 being unchanged adenosine-5′ phosphate, one of R_F 0·65 showing light blue fluorescence, and a third of R_F 0·8. The relative yields were estimated by elution into 0·04N-phosphate buffer (pH 7) and measurement of the optical density at 260 mμ. This indicated that ~80% of the adenosine-5′ phosphate was unchanged, there being 5% of the product of R_F 0·65 and 15% of the product of R_F 0·8.

When the products were eluted with N-hydrochloric acid and hydrolysed at 100° for 1 hr., the compound of R_F 0·8 yielded 1-methyladenine, while that of R_F 0·65 gave 3-methyladenine.

In a further experiment the major product was eluted from paper with water and the solution adjusted to pH 11·7 and kept at 37°. The ultraviolet absorption spectrum was taken immediately and again after 35, 70, 105 min., 18 and 27 hr., on a Cary automatic recording spectrophotometer. The results (Fig. 3 and Table 3) show quantitative conversion into a product with ultraviolet absorption spectrum almost identical with that of 6-methylaminopurine riboside. To the final solution obtained was added 0·1 volume of concentrated hydrochloric acid, and the solution was kept at 100° for 1 hr. The ultraviolet absorption was then identical with that of 6-methylaminopurine.

Action of Acid on 1-Methyladenine.—1-Methyladenine (~4 mg.) was refluxed in 6N-hydrochloric acid (3 c.c.) 2 hr. The solution was evaporated, and the residue dissolved in methanol (1 c.c.) and treated with ethyl acetate until a cloud was obtained. A seed of 5-aminoimidazole-4-*N*′-methylcarboxamidine dihydrochloride was added. After 3 hr. at 0° the crystals were collected and shown to be 5-aminoimidazole-4-*N*′-methylcarboxamidine dihydrochloride.

In a similar experiment the acid solution was kept at 100°. Samples were removed at intervals and after dilution with water the ultraviolet absorption was determined (see Table 2).

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THE CHESTER BEATTY RESEARCH INSTITUTE,
THE INSTITUTE OF CANCER RESEARCH: ROYAL CANCER HOSPITAL,
FULHAM ROAD, LONDON, S.W.3. [*Received, July 24th,* 1959.]