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Reaction of Adenosine with Ethylating Agents†

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ABSTRACT: The products of ethylation of adenosine with diethyl sulfate and ethyl methanesulfonate in neutral aqueous solution are 1-ethyladenosine, N^6-ethyladenosine, and 7-ethyladenosine. In addition, lesser amounts of unidentified compounds are found which may be degradation products of 3-ethyladenosine. Reaction with anhydrous ethyl iodide or with methylating agents alkylates the 1 and 7 positions but not the exocyclic amino group. The new finding that up to half of the total ethylation was direct substitution of the N^6 position is paralleled by the recent finding that cytidine is also directly ethylated at the N^4 position (Sun, L., and Singer, B. (1974), *Biochemistry 13*, 1905). 7-Ethyladenosine and N^6,7-dialkyladenosine (obtained from the alkylation of N^6-methyladenosine) were isolated and characterized for the first time. They are both brightly fluorescent under ultraviolet light and the imidazole ring is rapidly opened in neutral or alkaline solution. The relative amount of 7-alkylation by both ethylating and methylating agents is higher than previously reported and it is suggested that the great lability of 7-alkyladenosine, like that of 3-alkyladenosine, has led to erroneously low values for alkylation at these sites. The extent of ethylation of poly(A) and poly(A)·poly(U) was extremely low and even the use of ^{14}C-labeled reagents did not permit detection of the products of their reaction with poly(A)·poly(U). N^6-Ethyladenine and a lesser amount of 3-ethyladenine were identified as products of the reaction of [^{14}C]diethyl sulfate and [^{14}C]-ethyl methanesulfate with poly(A), although the presence of other products was not excluded.

Adenosine, guanosine, and cytidine are the three nucleosides generally alkylated by mutagenic or carcinogenic alkylating agents. In order to understand the mechanism of the biological effect of alkylation on RNA and DNA, and particularly the differing effects of their methylation and ethylation, we have first isolated and characterized the products of ethylation of nucleosides by reagents such as ethyl iodide, diethyl sulfate, methyl methanesulfonate, and ethyl methanesulfonate. The products of ethylation of guanosine and cytidine have been discussed in the previous two papers (Singer, 1972; Sun and Singer, 1974). This paper deals with the isolation and characterization of various methyl- and ethyladenosines after reacting adenosine with ethyl iodide, diethyl sulfate (Et_2SO_4), dimethyl sulfate (Me_2SO_4), methyl methanesulfonate ($MeMeSO_3$), and ethyl methanesulfonate ($EtMeSO_3$).

No ethyladenosines have thus far been characterized, although some ethyladenines have been described, either obtained directly from the reaction of $EtMeSO_3$ with adenine (Pal, 1962), or from neutral hydrolysis of diethyl sulfate treated DNA (Lawley and Brookes, 1964), or ethyl methanesulfonate treated deoxyadenosine, yeast RNA, salmon sperm DNA, T2 phage DNA (Lawley and Brookes, 1963), and poly(A) (Ludlum, 1969). These include 1-ethyl-, 3-ethyl-, 7-ethyl-, and 9-ethyladenine. In addition N^6-ethyl- and N^6,N^6-diethyladenine have been synthesized by Elion *et al.* (1952).

The literature on methylation of adenine, adenosine and nucleic acids is extensive and many mono-, di-, and trimethyl derivatives have been either isolated from direct methylation or synthesized. There are two expected derivatives which have been described only as the base, not as the nucleoside. These are the 3- and 7-substituted adenines, predicted to be labile as the nucleoside (Leonard *et al.*, 1965).

The present paper presents data on the isolation and characterization of 1-ethyladenosine, N^6-ethyladenosine, 7-ethyladenosine, and N^6,7-dialkyladenosine and makes some comparisons of the relative reactivities of the 1, 3, N^6, and 7 positions of adenosine toward dimethyl sulfate, diethyl sulfate, and ethyl methanesulfonate.

Experimental Section

Reaction of Adenosine with Dimethyl Sulfate, Diethyl Sulfate, Methyl Methanesulfonate, and Ethyl Methanesulfonate. Several sets of conditions were used, depending on the purpose of the experiment. To obtain markers for experiments using ^{14}C-labeled reagents, 500 mg of adenosine in 6 ml of 0.4 M ammonium bicarbonate or pH 7.2 cacodylate buffer were reacted with 0.2 ml of Et_2SO_4 or $EtMeSO_3$ at 30°. After 8-hr stirring, 2.5 ml of ammonium bicarbonate or pH 7.2 cacodylate buffer and 0.1 ml of Et_2SO_4 or $EtMeSO_3$ were added and stirring was continued 18 hr. The final pH of these reaction mixtures was pH 3–4.

To compare the reaction products of the neutral reaction of Me_2SO_4 and Et_2SO_4 with adenosine at room temperature, 50 mg of adenosine in 2 ml of H_2O was adjusted to pH 7.0 in a Radiometer pH-Stat; 20 μl of Me_2SO_4 or Et_2SO_4 was added and the pH was maintained at 7.0 with 1 N NaOH. After 5 hr

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TABLE I: Chromatographic Behavior of Alkylated Adenosines and Adenines.

Derivative	R_F[a] in Solvent A Nucleoside	R_{Ado}[b] in Solvent B Nucleoside	Base
1-Ethyl-	0.64[c]	0.5[c]	1.1[c]
3-Ethyl-			1.8[c]
7-Ethyl-	0.57[c]	0.4[c]	1.4
Imidazole ring-opened 7-ethyl-	0.63	0.5 streak	
N^6-Ethyl-	0.85	2.2	2.6
X	0.45	0.1	
YI	0.76	1.9	
YII	0.82	1.9	
1-Methyl-		0.35[c]	0.8[c]
3-Methyl-			1.3[c]
7-Methyl-		0.25[c]	1.0
Imidazole ring-opened 7-methyl-		0.3 streak	1.0
N^6-Methyl-		1.8	2.2
1,N^6-Dimethyl-		0.60[c]	
N^6,7-Dimethyl-		0.80[c]	
Adenosine	0.59	1.0 (R_F 0.30)	
Adenine			1.6

[a] See Experimental Section for details. Solvent A is isopropyl alcohol–H_2O (70:30, v/v). [b] See Experimental Section for details. Solvent B is butanol–H_2O–ethanol (80:25:10, v/v). Movement is given relative to adenosine since it is advantageous to allow solvent to run off the end of the paper to separate derivatives with lower or similar R_F values than adenosine or adenine. [c] Designates the derivatives with pK values above 6 which can be separated from the others by prior electrophoresis at pH 5.7.

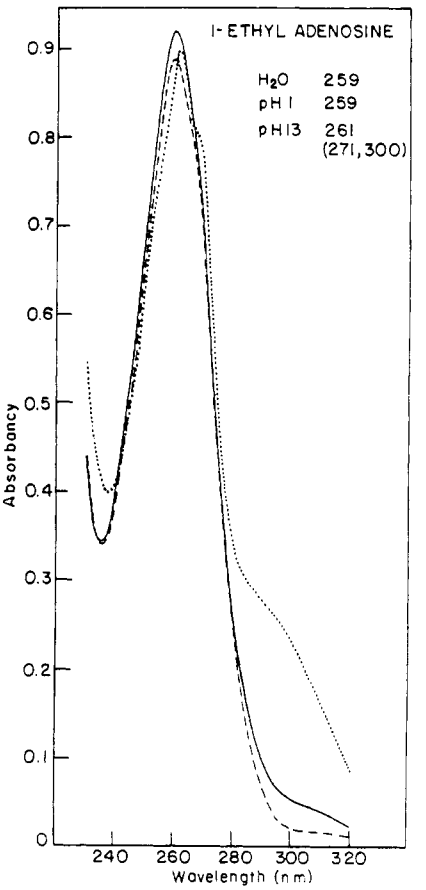

FIGURE 1: Ultraviolet absorption spectra for 1-ethyladenosine in H_2O (—), 0.1 N HCl (— —), and 0.1 N KOH (· · · · ·). The absorption maxima at each pH are given in the figure. A characteristic of adenosine or adenine alkylated on the 1 position is a high absorption at 300 nm when in the anionic form. This is also shown in Figures 2 and 5A.

and 2 more additions of 20 μl of reagent the Me_2SO_4 reaction was stopped. At the same time an aliquot was taken of the Et_2SO_4 reaction which was then continued for 24 hr with several additions of reagent as the Et_2SO_4 hydrolyzed.

Experiments with ^{14}C-labeled Me_2SO_4 and Et_2SO_4 were performed as follows. Adenosine (5 mg) in 2 ml of H_2O was adjusted to either pH 4 or 7 in a Radiometer pH-Stat. Reagent (5 μl; 0.2 Ci/mol) in 25 μl of ethanol was added and the desired pH was maintained for 5 hr at room temperature. Excess reagent was removed by repeated ether washing.

Alternatively, 2 mg of adenosine was reacted with 10 μl of labeled or unlabeled Et_2SO_4 and $EtMeSO_3$, or unlabeled Me_2SO_4 and $MeMeSO_3$ in 0.4 ml of pH 7.2 cacodylate buffer for 5–24 hr at room temperature. The final pH was 6.

Reaction of Adenosine with Ethyl Iodide. Adenosine (100 mg) dissolved in dimethyl sulfoxide (1 ml) was reacted with ethyl iodide (0.1 ml) at 37° for 7 hr. Aliquots of the reaction mixture were electrophoresed or chromatographed without prior removal of the reagent.

Reaction of 1-Methyladenosine and N^6-Methyladenosine with Me_2SO_4 and Et_2SO_4. Two milligrams of 1-methyladenosine or N^6-methyladenosine in 0.3 ml of pH 7.3 1 M cacodylate buffer was reacted with 10 μl of Me_2SO_4 or Et_2SO_4 for 18 hr at 37°. The final pH was 6.

Reaction of Poly(A) and Poly(A)·Poly(U) with [^{14}C]Et_2SO_4 and [^{14}C]$EtMeSO_3$. Polymer (1 mg; Miles Laboratory) was dissolved in 1 ml of 0.2 M pH 4 ammonium formate or pH 7 ammonium acetate. Labeled reagent (2 μl; Et_2SO_4 0.9 Ci/mol; $EtMeSO_3$ 5.3 Ci/mol) was added and after 2 hr at room temperature an additional 2 μl of reagent was added. The pH remained constant. The polymers were dialyzed against 10^{-4} M HCl at 0° to remove reagent and prevent any possible alkali-catalyzed reactions, then hydrolyzed in 1 N HCl at 100° for 1 hr.

Methods for the Separation of Alkylated Adenosines and Adenines. (1) Paper electrophoresis on Whatman No. 3MM in 0.05 M pH 5.7 ammonium formate (1000 V, 3 hr/30 cm) separates the products with quaternary nitrogens (1-, 3-, 7-, 1,N^6-, and N^6,7-alkyladenosines and 1-, 3-, and 1,N^6-alkyladenines) from all other products (such as unreacted adenosine or adenine, N^6- and N^6,N^6-dialkyladenosines, ring-opened 7-alkyladenosine, and N^6-, N^6,N^6-, and 7-alkyladenines). After elution of the two areas of the electropherogram (to be termed high and low pK areas), further separation was accomplished by paper chromatography. (2) Descending paper chromatography on Whatman No. 3MM was in either or both of two solvent systems, either on the entire sample or after prior electrophoretic separation. Solvent A was isopropyl alcohol–H_2O (7:3, v/v). Solvent B was butanol–ethanol–H_2O (8:1:2.5, v/v). Both solvents separated 1-, N^6- and 7-ethyladenosines from each other with differing effectiveness but only solvent B resolved 1-ethyladenosine from unreacted adenosine. Two of the three unidentified derivatives, of low pK, were resolved only in solvent A. Chromatographic movement for all derivatives is given in Table I.

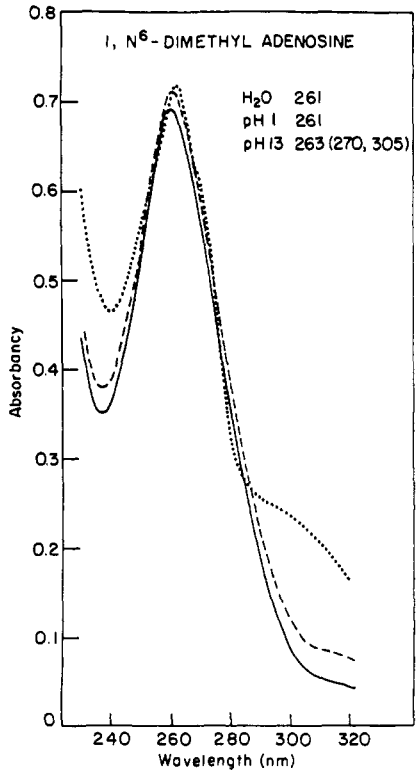

FIGURE 2: Ultraviolet absorption spectra for 1,N^6-dimethyladenosine in H_2O (—), 0.1 N HCl (- - - -), and 0.1 N KOH (· · · ·). The absorption maxima at each pH are given in the figure.

Identification of Alkylated Adenosines. Paper chromatograms or electropherograms were observed under ultraviolet light to detect various derivatives. Note was made of those which were brightly fluorescent. These were found to correspond to 7-alkyladenosine and to N^6,7-dialkyladenosine. After elution with water by capillarity, the spectra of the various ethyl- and methyladenosines were plotted using a Cary 15 recording spectrophotometer. HCl (6 N) was added to the same solution to a final concentration of 0.1 N and the spectrum was replotted. KOH (6 N) was added to a final concentration of 0.1 N and the spectrum was immediately replotted. Figures 1–5 show spectra of several alkyl nucleosides and bases. In the case of 7-ethyladenosine or N^6,7-dialkyladenosine, where imidazole ring opening is alkali catalyzed, the spectra in 0.1 N KOH were observed until there was no further change. HCl was then added to a final concentration of 0.1 N to determine the acid spectra of the ring-opened derivatives (Figures 3B and 4). The spectrum of imidazole ring-opened 7-methyladenine obtained after heating the open nucleoside in 1 N HCl 100° 1 hr is shown in Figure 6.

Since authentic samples of 1-methyladenosine and N^6-methyladenosine (Terra-Marine Bioresearch) were available, the analogous structures were assigned the ethyl derivatives based on their almost identical spectra. When hydrolyzed to the corresponding bases, the spectra corresponded to those of 1-methyladenine and N^6-methyladenine (Table IIB). It should be noted that all derivatives alkylated on the 1 position exhibit a high absorbancy at 300 nm when in the neutral form. This characteristic, shown in Figures 1, 2, and 5A, is useful in assigning structures. 7-Alkylpurine nucleosides are all fluorescent and exhibit alkali-catalyzed imidazole ring opening (Jones and Robins, 1963). Therefore we assigned the derivative with these properties the structure of 7-ethyladenosine. N^6-methyladenosine when alkylated gave a product which also fluoresced and ring opened in alkaline solution. It was therefore assigned the structure N^6,7-dialkyladenosine, the spectrum of which is shown in Figure 4. Both these nucleosides (7-ethyladenosine and N^6,7-dialkyladenosine) were further

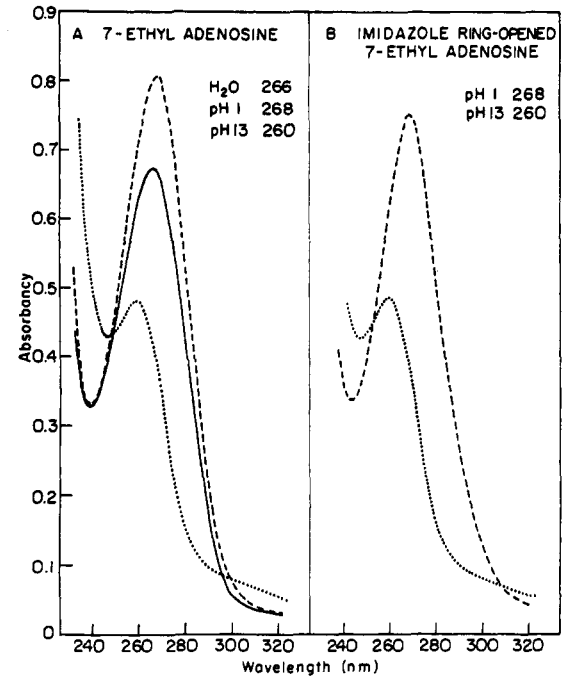

FIGURE 3: Ultraviolet absorption spectra for 7-ethyladenosine (A) and imidazole ring-opened 7-ethyladenosine (B) in H_2O (—), 0.1 N HCl (— —), and 0.1 N KOH (· · · ·). The absorption maxima at each pH are given in the figure.

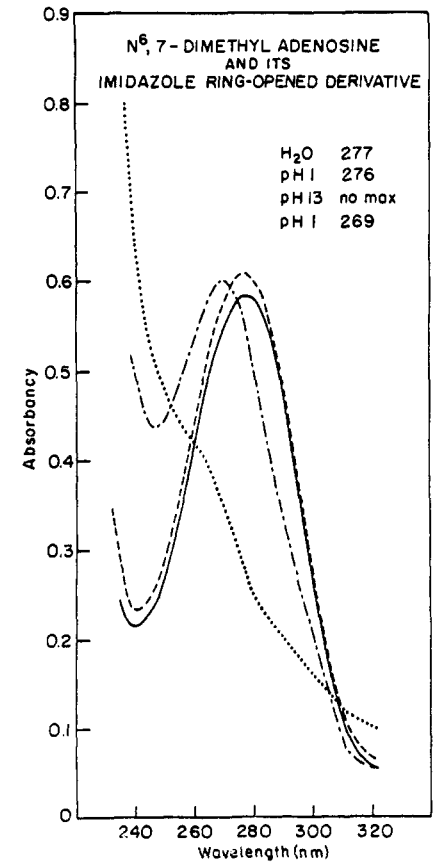

FIGURE 4: Ultraviolet absorption spectra for N^6,7-dimethyladenosine in H_2O (—) and 0.1 N HCl (— —). In 0.1 N KOH (· · · ·) the imidazole ring opens. The acid form of the ring-opened compound is also shown (— · — · —). The absorption maxima are given in the figure.

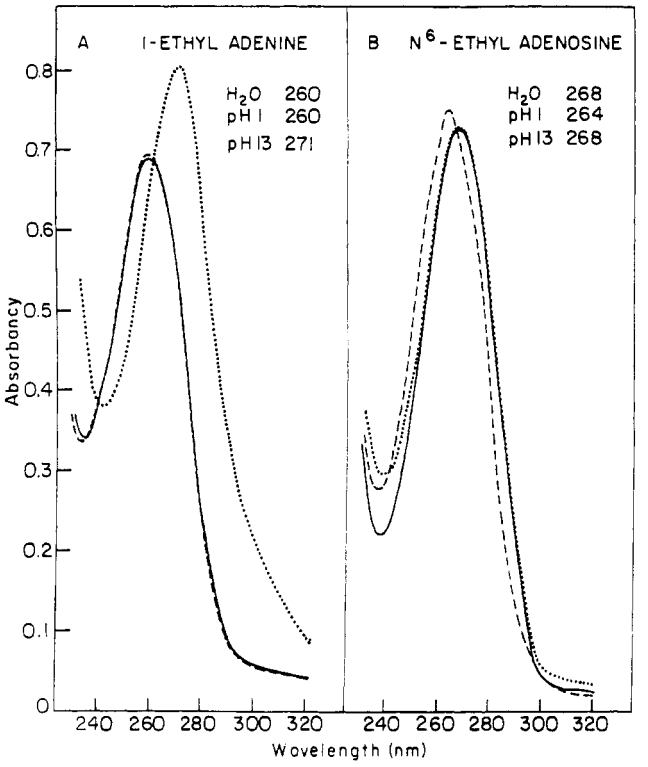

FIGURE 5: Ultraviolet absorption spectra of 1-ethyladenine (A) and N^6-ethyladenosine (B) in H_2O (—), 0.1 N HCl (— —), and 0.1 N KOH (· · · ·). These are the products of acid depurination and alkaline rearrangement respectively, of 1-ethyladenosine. The absorption maxima at each pH are given in the figure.

characterized by hydrolyzing them in 1 N HCl at 100° 1 hr to convert them to bases. The spectra then corresponded to

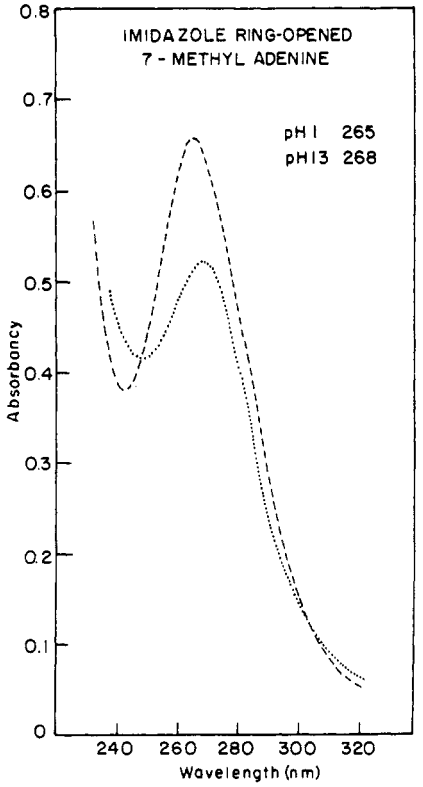

FIGURE 6: Ultraviolet absorption spectra of imidazole ring-opened 7-methyladenine, derived from 1 N HCl hydrolysis at 100° 1 hr of imidazole ring-opened 7-methyladenosine, in 0.1 N HCl (- - -) and 0.1 N KOH (· · · ·). The absorption maxima are given in the figure.

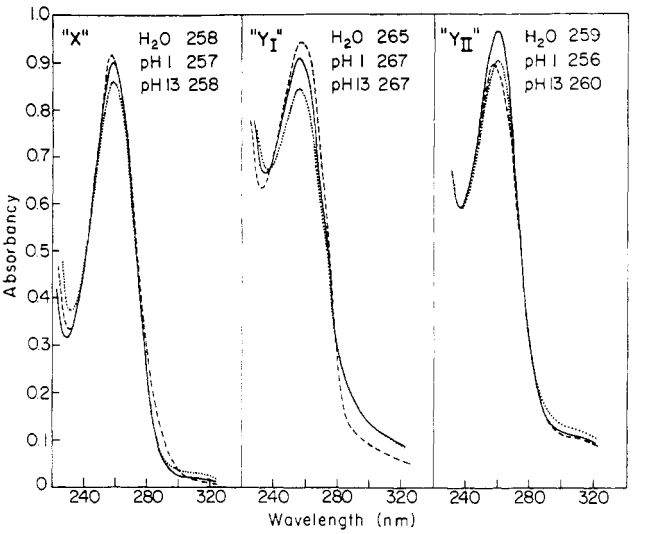

FIGURE 7: Ultraviolet absorption spectra of unidentified ethyl derivatives of adenosine, discussed in the text, in H_2O (—), 0.1 N HCl (— —), and 0.1 N KOH (· · · ·). The absorption maxima at each pH are given in the figure.

the spectra of 7-methyladenine (Cyclo Chemical) and N^6,7-dimethyladenine (Prasad and Robins, 1957; Taylor and Loeffler, 1960) (Table IIB). 1,N^6-Dimethyladenosine was obtained as a product of methylation of N^6-methyladenosine and its spectrum (Figure 2) was almost identical with that reported by Wacker and Ebert (1959) and Broom *et al.* (1964) (Table IIA).

Three ethyl derivatives, termed X, YI, and YII, were characterized by their spectra and chromatographic and electrophoretic movements (Table I and Figure 7), but their structure is not known.

Spectral data for isolated alkyl nucleosides and bases are summarized in Table IIA,B.

Materials. [^{14}C]Ethyl methanesulfonate (5.3 Ci/mol) was obtained from Amersham/Searle. [^{14}C]Diethyl sulfate (0.9 Ci/mol) was obtained from ICN.

Results

Reaction of Adenosine with Diethyl Sulfate, Dimethyl Sulfate, Ethyl Methanesulfonate, and Methyl Methanesulfonate. The extent and products of the reaction of adenosine with Et_2SO_4 or $EtMeSO_3$ at pH 6–7 were similar. The extent of reaction up to 24 hr did not exceed about 5%. The major product was 1-ethyladenosine (50%), as expected. However, new findings were that about half as much N^6-ethyladenosine (25%) was found regardless of the extent of reaction and that 7-ethyladenosine, amounting to about 10% of the products, was isolated and identified for the first time. Although 3-ethyladenosine is not known, 3-ethyladenine is. We failed to identify any product which, as the base, was ethylated on the 3 position. Three unidentified products with pK's of about 4, termed X, YI, and YII, amounted to about 15% of the total, and it is possible that one or more of these could represent ring-opened compounds derived from 3-ethyladenosine. Their spectra as isolated (Figure 7) or after HCl hydrolysis to bases did not correspond to any known mono-, di-, or trialkyladenines.[1]

[1] In addition to comparing the spectra of these derivatives with the ethyladenines discussed in this paper, their spectral characteristics were also compared to published data for 3,N^6-dimethyladenine, 3,7-dimethyladenine, 1,7-dimethyladenine, 9-methyl- and N^6,9-dimethyladenine, 1,N^6,N^6-trimethyladenine, 3,N^6,N^6-trimethyladenine, N^6,N^6,7-trimethyladenine, N^6,N^6,9-trimethyladenine, 3,N^6,7-tribenzyladenine, and a similar series of alkylhypoxanthines.

TABLE II: Spectral Identification of Alkyladenosines (Part A) and of Alkyladenines (Part B).[a]

Compound	Neutral λ_{max}	Neutral λ_{min}	Acidic λ_{max}	Acidic λ_{min}	Basic λ_{max}	Basic λ_{min}	Source[b]
Part A							
Adenosine	259	227	257	231	260	233	P-L Biochemicals
1-Ethyladenosine[c]	259	235	259	235	261 (268)	237	Experimental
1-Ethyldeoxyadenosine	259	234	259	231	260 (268)	236	Experimental
1-Methyladenosine	257	233	257	233	259 (265)	236	Experimental
1-Methyladenosine	257	236	257	236	258 (266)	238	Terra-Marine
1-Methyladenosine	257 (263)[e]		257	231	257 (263)	223	Jones and Robins (1963)
1-Methyldeoxyadenosine	257	241	257	239	257	242	Jones and Robins (1963)
N^6-Ethyladenosine[c,d]	268	242	264	239	268	243	Experimental
N^6-Ethyldeoxyadenosine	267	241	263	237	268	240	Experimental
N^6-Methyladenosine	265	233	262	233	265	241	Experimental
N^6-Methyladenosine	266	232	263	234	267	236	Terra-Marine
N^6-Methyladenosine	265	229	261	231	265	223	Jones and Robins (1963)
N^6-Methyldeoxyadenosine	265	229	261	230	265	226	Jones and Robins (1963)
7-Ethyladenosine[c]	266	239	268	239			Experimental
Imidazole ring-opened 7-ethyladenosine[c]	260	247	268	243	260	248	Experimental
Imidazole ring-opened 7-methyladenosine	258	247	267	243	253–257		Experimental
1,N^6-Dimethyladenosine[c]	261	237	261	237	263 (270)	240	Experimental
1,N^6-Dimethyladenosine			261	234	262	234	Broom *et al.* (1964)
N^6,7-Dimethyladenosine	277	240	276	241			Experimental
N^6-Methyl-7-ethyladenosine[c]	277	240	276	242			Experimental
Imidazole ring-opened N^6-methyl-7-ethyladenosine			271	247	260	253	Experimental
N^6,N^6-Dimethyladenosine	275	236	268	234	276	237	Townsend *et al.* (1964)
Unidentified ethyl-X	258	228	257	231	258	232	Experimental
-Y_I	265	242	267	242	267	243	Experimental
-Y_{II}	259	237	256	237	260	238	Experimental
Part B							
Adenine	261	225	263	229	269	238	P-L Biochemicals
1-Ethyladenine	260	234	260	233	271	242	Experimental
1-Ethyladenine			260		271		Ludlum (1969)
1-Methyladenine	263	238	259	229	271	241	Cyclo Chemical
N^6-Ethyladenine[d]	267	231	268	231	274	241	Experimental
N^6-Ethyladenine			270		273		Elion *et al.* (1952)
N^6-Methyladenine	266	229	267	231	273	240	Sigma
7-Ethyladenine			272	239	270 (280)	234	Experimental
7-Ethyladenine			273		270		Lawley and Brookes (1964)
7-Methyladenine	272 (280)	232	273	237	270 (280)	238	Cyclo Chemical
3-Ethyladenine	273	246	274	240	273	247	Experimental
3-Ethyladenine			273	238	271	245	Pal (1962)
3-Methyladenine	273	243	274	236	273	244	Cyclo Chemical
1,N^6-Dimethyladenine			261	235	273	249	Experimental
1,N^6-Dimethyladenine			260	230	274	245	Broom *et al.* (1964)
N^6,N^6-Diethyladenine			276		282		Elion *et al.* (1952)
N^6,N^6-Dimethyladenine			277	236	281	245	Elion *et al.* (1952)
N^6-Methyl-7-ethyladenine			277 (285)	241	276	244	Experimental
N^6,7-Dimethyladenine			280		275		Prasad and Robins (1957)
N^6,7-Dimethyladenine			278				Taylor and Loeffler (1960)
Imidazole ring-opened 7-ethyladenine			265	238	268	247	Experimental

[a] The spectra of the experimental and commercial samples were plotted in H_2O, 0.1 N HCl, and 0.1 N KOH. The corresponding literature data were obtained under a variety of neutral, acidic and basic conditions. [b] Part A: experimental samples were isolated from paper chromatograms after prior electrophoretic separation; part B: all experimental compounds, except 3-ethyladenine, were products of acid hydrolysis of alkyl nucleosides. [c] Converted to the corresponding adenine derivatives by heating in 1 N HCl 100° 1 hr. Spectral characteristics of the alkyl bases are given in part B. [d] Also obtained by heating 1-ethyladenosine in 1 N KOH 37° 18 hr. [e] Numbers in parentheses indicate shoulders or inflections. λ, nm.

TABLE III: Relative Alkylation of 1 and N^6 Position of Adenosine by Ethylation and Methylation.[a]

Reagent	Time (hr)	1-Alkyl (%)	N^6-Alkyl (%)	N^6:1-Alkyl
Me_2SO_4	5	15	0.6	0.04
Et_2SO_4	5	1	0.9	0.9
	24	3	1.5	0.5
$MeMeSO_3$	1	32	nd	~0
$EtMeSO_3$	5	2	1.3	0.65
	8	6	3	0.5

[a] Reactions were performed with similar results, either in a Radiometer pH-Stat at pH 7.0 or in pH 7.2 cacodylate buffer (the final pH being 6.5) at room temperature with stirring. See Experimental Section for details.

TABLE IV: Rate of Alkali-Catalyzed Rearrangement of 1-Alkyladenosine to N^6-Alkyladenosine.

Adenosine Derivative	Half-Life (hr)[a] pH 12.5 22–25°	pH 12.5 37°	pH 7 37°
1-Methyl-	4.4	1.7	32
1-Ethyl-	4.5	2.5	
1-Ethyldeoxy-	5.2	2.4	

[a] Purified derivatives of approximately 0.9 absorbancy unit of each derivative were prepared in 0.75 M K_2CO_3 (pH 12.5) or in 0.01 M phosphate buffer (pH 7). The stoppered cuvets were maintained at the desired temperature. The spectra were plotted in a Cary 15 recording spectrophotometer over a period of 6–48 hr, depending on the rate of rearrangement. The change of $A_{280:260}$ was calculated as per cent of 1-alkyladenosine remaining and plotted against time. A straight line can be drawn for approximately the first 40% of the conversion and the half-life can be extrapolated to 50% conversion. The quantitative significance of such a calculation was confirmed by isolation, using paper chromatography, of the products of the reaction at pH 12.5, 22–25°, after 4–7 hr. The proportions of 1-alkyladenosine and N^6-alkyladenosine were almost identical with those calculated from the change of $A_{280:260}$.

Similar reactions with Me_2SO_4 yielded, with 30% reaction in 4 hr at room temperature, 80% 1-methyladenosine, 16% imidazole ring-opened 7-methyladenosine, and 2% N^6-methyladenosine. We also found approximately 2% of an unknown nucleoside of $pK > 6$ which when hydrolyzed to the base did not correspond to any of the known alkyladenines.

The direct substitution of the exocyclic amino group by ethylating, and not methylating, agents was demonstrated in two ways. A comparison of the ratio of N^6:1 alkylation at the early times of reaction with Et_2SO_4 and Me_2SO_4 or $EtMeSO_3$ and $MeMeSO_3$ at room temperature indicated that methylation at N^6 never exceeded 3% of that at 1 while ethylation at N^6 was at least 50% of that at 1 (Table III). The rate of alkali-catalyzed rearrangement of 1-alkyl- to N^6-alkyladenosine was determined at the same pH as the alkylation reaction (pH 6.5–7.2 cacodylate buffer) and found to account only for the small amount of N^6-methyladenosine found (Table IV). The rate of conversion of 1-methyl- and 1-ethyladenosine to the N^6 derivatives was similar (Table IV) (Lawley and Brookes, 1963). At pH 7 and 37° the half-life for conversion of 1- to N^6-methyladenosine was 32 hr. Therefore, since our reactions were at 22–25° and the reaction time was 5–24 hr, the N^6-ethyladenosine represents a product of direct substitution, occurring at about pH 6–7, both in buffered solution and when maintained by pH-Stat.

Both methylating and ethylating reagents alkylate the 7 position. However 7-methyladenosine is more alkali labile than 7-ethyladenosine and is mainly found as the imidazole ring-opened derivative when the reaction is performed at neutrality. The amount found greatly exceeds that reported by Lawley and Brookes (1964). These authors found a small amount of 7-methyladenine after neutral hydrolysis of Me_2SO_4 treated deoxyadenosine but since their reaction was performed at pH 7 it appears likely that much more alkylation of the 7 position occurred but was not detected due to subsequent ring opening.

Reaction of Adenosine with [^{14}C]Et_2SO_4 *and* [^{14}C]$EtMeSO_3$. The use of labeled reagents did not lead to additional information since it proved difficult to remove all excess reagent by the techniques used. Since we particularly wished to detect 3-ethyladenosine, as well as N^6-ethyladenosine as a direct alkylation product, the pH was maintained below pH 7 and the temperature below 25°. Reacted adenosine was washed repeatedly with ether, then chromatographed in solvent B. Nevertheless considerable radioactivity attributable only to the reagent and its decomposition products appeared largely in a single region of the chromatograms. Since this was near the area where 1- and 7-ethyladenosines should be, these derivatives could not be quantitated. After alkali treatment to rearrange 1-ethyladenosine to N^6-ethyladenosine, we could calculate the amount of 1-ethyladenosine from the increase in the amount of N^6-ethyladenosine found on the chromatograms. The amount of 1-ethyladenosine after reaction with ethylating agents at pH 4 or 7 was about twice that of N^6-ethyladenosine, agreeing with the relative amounts of these derivatives found by absorbancy measurement. Additional reproducible areas of radioactivity could not be correlated with the R_F values of known derivatives. Acid hydrolysates were also chromatographed with 1-ethyl-, 3-ethyl-, N^6-ethyl-, and 7-ethyladenines as external markers (3-ethyladenine was isolated from ethylated DNA, 1-ethyl- and N^6-ethyladenines were obtained from 1-ethyladenosine isolated from experiments with unlabeled reagent. 7-Ethyladenine was obtained from 7-ethyladenosine, similarly isolated). The results were dubious in terms of identification of derivatives, again partially due to contaminating radioactivity.

Reaction of Adenosine with Ethyl Iodide. Adenosine was reacted with ethyl iodide in neutral dimethyl sulfoxide, as we have previously reacted guanosine (Singer, 1972) and cytidine (Sun and Singer, 1974). The reaction proceeded much further than with Et_2SO_4 or $EtMeSO_3$ (20% reaction) and the major product was 1-ethyladenosine. 7-Ethyladenosine was a minor product. No N^6-ethyladenosine or 3-ethyladenosine was detected. The three unidentified derivatives X, YI, and YII were also present in small amounts. Although N^6-ethyladenosine was not a product, a small amount of 1,N^6-diethyladenosine (analogous spectra Figure 2) was found after the "high pK" fraction from electrophoresis was chromatographed. This could arise either from rearrangement of 1- to N^6-ethyladenosine and subsequent ethylation of the 1 position or from direct N^6-ethylation of 1-ethyladenosine.

Another strongly fluorescent product (amounting to about 1% of the total), found only after reaction with ethyl iodide, had a pK_a estimated at 5–5.5 and an unusual spectrum [λ_{max}(pH 1) 272 nm; λ_{max}(H_2O and pH 13) 265, 274 nm, shoulders at 258, 290 nm]. At pH 13 it changed rapidly to a product with a broad and much higher maximum at 263 nm. The acid spectrum of this alkali-treated compound had a maximum at 267 nm. Heating of the fluorescent derivative in 1 N HCl to convert it to a base gave a compound with λ_{max}(pH 1) 273 nm and λ_{max}(pH 13) 274 nm. Although these absorption maxima correspond to those for 3-ethyladenine, the spectra differ markedly. Neither the acid- nor alkali-treated compound could be recovered from paper chromatograms as a single uv-absorbing spot; it is possible that the compound undergoes degradation leading to multiple products.

Reaction of 1-Methyladenosine and N⁶-Methyladenosine with Et_2SO_4 and Me_2SO_4. 1-Methyladenosine and N^6-methyladenosine were both reacted with an excess of Me_2SO_4 and Et_2SO_4 at pH 6–7.3. Methylation of N^6-methyladenosine led to the formation of 1,N^6-dimethyladenosine and N^6,7-dimethyladenosine in small but similar amounts. N^6,7-Dimethyladenosine, the spectrum of which is shown as the nucleoside and after imidazole ring opening (Figure 4), has not previously been found as a direct product of alkylation. Methylation of 1-methyladenosine yielded a barely detectable amount of 1,N^6-dimethyladenosine. Ethylation of both 1-methyladenosine and N^6-methyladenosine yielded derivatives alkylated on the same positions as after methylation. No N^6,N^6-dialkyladenosine was found in either of these model experiments nor in reactions with adenosine. It is concluded that N^6-methyladenosine is more readily alkylated at the 1 and 7 positions than is 1-methyladenosine alkylated at the 6 or 7 positions.

Reaction of Poly(A) and Poly(A)·Poly(U) with [^{14}C]Et_2SO_4 and [^{14}C]$EtMeSO_3$. The extent of reaction with polymers at either pH 4 or 7 was very much lower than the reaction with adenosine which, in turn, reacts to only about 2% under comparable conditions. Poly(A) was more reactive than poly(A)·poly(U) but was less than 0.1% reacted. Furthermore, analysis of the products is complicated by the difficulty in removing all excess reagent, even after extensive dialysis.

After HCl hydrolysis followed by chromatography in solvent B, which separates 1-, 3-, N^6- and 7-ethyladenines from each other (Table I), N^6-ethyladenine was found in ethylated poly(A) both at pH 4 and 7. The radioactivity found in the area of 3-ethyladenine amounted to about one-fourth of that of N^6-ethyladenine. No other derivatives could be clearly quantitated due to the contaminating radioactivity in the portion of the chromatogram corresponding to 1- and 7-ethyladenine.

The amount of radioactivity in the poly(A)·poly(U) samples was not sufficient to detect any derivative with assurance. There were indications, however, of a small amount of radioactivity coinciding with N^6-ethyladenine.

Discussion

Adenosine reacted with Et_2SO_4 or $EtMeSO_3$ in neutral aqueous solution was directly alkylated on the exocyclic amino group to an extent amounting to 25–40% of the total reaction. Under comparable conditions of reaction, neither of the corresponding methylating agents (Me_2SO_4 or $MeMeSO_3$) caused directly alkylation of the N^6 position. The small extent of N^6-methylation was shown to be due to rearrangement of 1-methyladenosine, while the N^6-ethylation was almost entirely due to direct substitution. Wacker and Ebert (1959) have reported N^6-methylation of adenosine, after reaction at pH 6–8 with Me_2SO_4, but their pH was not controlled. Jones and Robins (1963) methylated adenosine with methyl *p*-toluenesulfonate in *N,N*-dimethylacetamide and found no evidence of N^6 substitution. Haines *et al.* (1964) found a small amount of N^6-methyladenosine only after extensive reaction of adenosine with diazomethane. Ethylation of the N^6 position is a striking illustration of the qualitative difference between closely related alkylating agents (ethyl *vs.* methyl) and is paralleled by the previous finding from this laboratory that cytidine is ethylated, but not methylated, on the exocyclic amino group (Sun and Singer, 1974). Poly(A), like poly(C), is also ethylated on the amino group. The biological implication of N^6-alkylation of adenosine is not clear. Both N^6-MeADP and N^6,N^6Me_2ADP are polymerized by polynucleotide phosphorylase (Griffin *et al.*, 1964) but only poly(N^6-MeA) forms a 1:1 complex with poly(U). N^6,9-Dimethyladenine also binds to poly(U) stoichiometrically (Pörschke *et al.*, 1973) and thus there is little evidence from these studies that monoalkylation of an exocyclic amino group would cause mispairing in nucleic acids.

We find no evidence that N^6-methyl- or N^6-ethyladenosine can be additionally alkylated to form N^6,N^6-dialkyl derivatives, but we do find that 1,N^6-dialkyladenosine and N^6,7-dialkyladenosine are the products.

7-Ethyladenosine was isolated as a major derivative from ethylated adenosine. It is a brightly fluorescent compound which is unstable in alkali, forming the imidazole ring-opened derivative, but more stable than 7-methyladenosine which was isolated almost only as the ring-opened derivative. This is paralleled by the relatively higher alkali stability of 7-ethylguanosine compared to 7-methylguanosine (Singer, 1972). Although 7-alkyladenine has been found in acid-hydrolyzed alkylated RNA, DNA, or poly(A) (Lawley *et al.*, 1973; Lawley and Shah, 1972; Pegg, 1973; Lawley and Brookes, 1963, 1964; Singer and Fraenkel-Conrat, 1969) those investigators who previously alkylated adenosine with Me_2SO_4 (Wacker and Ebert, 1959; Brookes and Lawley, 1960), benzyl bromide (Brookes *et al.*, 1968), or methyl *p*-toluenesulfonate (Jones and Robins, 1963) either did not find or look for 7-alkyladenosine. Since all these reactions were at pH 7 or higher it is likely that 7-alkyladenosine was formed but ring opened during the reaction.

3-Ethyladenosine has not yet been described but since it is predicted to be unstable, as are 7-alkyladenosines, one or more of the minor unidentified ethylation products may represent its degradation products.

Et_2SO_4 or $EtMeSO_3$ ethylated poly(A) slowly as also found by Ludlum (1969), but using ^{14}C-labeled reagents N^6-ethyladenine was identified as well as a lesser amount of 3-ethyladenine. 3-Methyladenine has generally been reported as a quite minor product of methylation of poly(A) or RNA (Lawley and Brookes, 1963; Ludlum, 1966; Singer and Fraenkel-Conrat, 1969; Lawley and Shah, 1972; Pegg, 1973) but to what extent this is due to the presumed instability of 3-alkyladenosines remains to be established. The even lower extent of reaction of ethylating agents with poly(A)·poly(U) did not permit positive identification of any derivatives.

The relative reactivities of the various nitrogens in adenosine toward Me_2SO_4 and Et_2SO_4 are similar except for the exocyclic amino group which is substituted only by Et_2SO_4 or $EtMeSO_3$. The decreasing order of reactivity toward Et_2SO_4 or $EtMeSO_3$ in neutral solution is 1 > 6 > 7 > 3; toward Me_2SO_4 or $MeMeSO_3$ 1 > 7 > 3.

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Regulation of Adenylyl Cyclase from Isolated Pancreatic Islets by Prostaglandins and Guanosine 5′-Triphosphate†

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ABSTRACT: Adenylyl cyclase activity of homogenates or membrane preparations from isolated rat pancreatic islets was slightly activated by 10 μM prostaglandin E_1 (PGE_1) or GTP. However, when both PGE_1 and GTP were used in combination, adenylyl cyclase activity was increased twofold to a level that was 70% of that obtained with 10 mM sodium fluoride. In the presence of GTP (20 μM) PGE_1 stimulation was evident at 0.2 μM and maximal at 10 μM. Prostaglandins E_2, F_2, and A_1 (0.1 or 10 μM) had no effect on basal enzyme activity, but PGE_2 or PGA_1 (10 μM) increased activity slightly in the presence of GTP (20 μM). Kinetic analysis indicated that PGE_1 plus GTP increased both the apparent Michaelis constant for ATP and the maximum velocity of adenylyl cyclase. Neither compound had any effect on the activity of cyclic nucleotide phosphodiesterase in pancreatic islet homogenates.

Considerable evidence has accumulated in recent years to suggest that release of insulin from the β cells of the pancreas involves cAMP[1] (Turtle and Kipnis, 1967; Montague and Cook, 1971; Atkins and Matty, 1971). Substances that activate adenylyl cyclase in other tissues, such as glucagon (Sutherland and Robison, 1966), elevate cAMP levels in pancreatic islet tissue and release insulin (Turtle and Kipnis, 1967). Other agents that are thought to act by inhibiting cAMP catabolism by cyclic nucleotide phosphodiesterase, like theophylline (Turtle *et al.*, 1967) or tolbutamide (Lacy *et al.*, 1968), similarly augment insulin release.

Previous studies in our laboratory (Johnson *et al.*, 1973) indicated that prostaglandins, particularly PGE_1, increased glucose-stimulated release of insulin. PGE_1 also increased the accumulation of cAMP formed from ^{14}C-labeled precursor by incubated pancreatic islets. These results suggested that PGE_1 might affect insulin secretion by stimulating adenylyl cyclase activity or by inhibiting cyclic nucleotide phosphodiesterase. Therefore, we have investigated the effects of PGE_1 on adenylyl cyclase and cyclic nucleotide phosphodiesterase activities of pancreatic islet tissue from the rat. Since GTP is required for the prostaglandin activation of adenylyl cyclase in platelet membranes (Krishna *et al.*, 1972) and thyroid plasma membranes (Wolff and Cook, 1973), we also studied the effects of GTP on the pancreatic islet enzymes either alone or in combination with PGE_1.

Experimental Section

Materials. Male Wistar rats (300–400 g) were purchased from Simonson Laboratories, Gilroy, Calif. Chemicals were

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[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; PGE_1, prostaglandin E_1.