Reaction of Guanosine with Ethylating Agents†

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ABSTRACT: The products of the reaction of guanosine with ethyl iodide and ethyl methanesulfonate at various pH's were isolated and characterized. These included 7-ethylguanosine, 1-ethylguanosine, 1,7-diethylguanosine, 6-*O*-ethylguanosine, and a diethyl derivative related to 6-*O*-ethylguanosine, as well as the imidazole ring-opened derivatives of 7-ethylguanosine and 1,7-diethylguanosine. Data are presented for the ultraviolet absorption spectra, fluorescence excitation and emission spectra, acid dissociation constants, rate of imidazole ring opening, and rate of glycosidic bond cleavage of these and the corresponding methylguanosines. Treatment of guanosine with ethyl iodide in dimethyl sulfoxide containing K_2CO_3 or with ethyl methanesulfonate in aqueous solution at pH 9 yields, as a major product, 6-*O*-ethylguanosine. Much less 6-*O*-methylguanosine is found after similar reactions with methyl iodide or methyl methanesulfonate. Little or no 6-*O*-alkylguanosine can be detected after reaction with diethyl sulfate or dimethyl sulfate in aqueous solution at various pH's. The amounts of the other derivatives also varied with the reagent and conditions of pH and temperature.

Alkylation of guanosine has been the subject of many biological and chemical studies in recent years and has been reviewed by Lawley (1966) and Shapiro (1968). However attention has been primarily on methylation and there are few reports on ethylation of guanosine although in some biological systems only ethylation, not methylation, is mutagenic (Loveless, 1959; Tessman *et al.*, 1964; Bautz and Freese, 1960; Green and Krieg, 1961). In our own studies of chemical mutagenesis of TMV-RNA we found that while alkylation was poorly mutagenic in general, and diethyl sulfate was not mutagenic, ethyl methanesulfonate and ethyl ethanesulfonate were as good mutagens as dimethyl sulfate (Singer and Fraenkel-Conrat, 1969). It therefore was of interest to study the reaction of nucleosides with these and another ethylating agent, ethyl iodide, in an attempt to correlate specific chemical events with mutation.

This paper deals with the isolation and characterization of various methyl- and ethylguanosines and succeeding papers will discuss ethylation of other nucleosides and the chemical and biological effects of these reagents on viral nucleic acids.

The present knowledge of the chemistry of ethylation of guanosine or guanine is as follows. Reiner and Zamenhof (1957) found four or five ethylated guanines when guanine was reacted with diethyl sulfate at alkaline pH but did not identify them. Pal (1962), using ethyl methanesulfonate at pH 14, identified 7-ethylguanine but also found appreciable amounts of three other unidentified derivatives. Rhaese and Freese (1969) reacted guanosine, dGMP, and DNA with ethyl methanesulfonate and, using depurination as an analytical tool, could only identify 7-ethylguanine. Lawley and Brookes (1963) prepared 7-ethylguanosine using ethereal diazoethane and determined the pK_a, rate of alkaline ring fission, and rate of hydrolysis of the glycosidic bond. After reacting yeast RNA with ethyl methanesulfonate the yisolated 7-ethylguanine. Loveless (1969) described the preparation of 6-*O*-ethyldeoxyguanosine by reacting deoxyguanosine with ethyl methanesulfonate or *N*-ethyl-*N*-nitrosourea, but did not find this derivative after reaction with diethyl sulfate. Kriek and Emmelot (1963) agree with other investigators that ethylation proceeds more slowly than methylation. They were able to isolate from aqueous diazoethane treated RNA 7-ethylguanine and a small amount of 1-ethylguanine (spectrally identified by analogy to authentic 1-methylguanine).

By contrast, the following methylated guanines or guanosines have been either directly synthesized or identified after isolation from reaction mixtures or natural sources: 1; 2-N; 2,2-N; 3; 6-O; 7; 1,7; 2′-O; and 3′-O. This extensive literature has been summarized by Shapiro (1968) and Hall (1971).

The present paper presents data on the preparation and characterization of 7-ethylguanosine, 1-ethylguanosine, 1,7-diethylguanosine, and 6-*O*-ethylguanosine and makes some comparisons to the corresponding methyl derivatives. The kinetics of the formation of these derivatives will be discussed. as well as the secondary reactions occurring upon 7 ethylation,

Experimental Section

Methods for the Separation of Alkylated Guanosines. (1) Paper electrophoresis on Whatman No. 3MM in 0.05 M pH 3.5 NH_4COOH separates 7- or 1,7-alkylguanosines from all other products. After elution of the two areas of the electropherogram, 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 (7:2, v/v). Solvent B was butanol–ethanol–H_2O (8:1:2.5, v/v). Both solvents resolved 1,7-dialkylguanosine from 7-alkylguanosine; and 1-alkylguanosine, 6-*O*-alkylguanosine, guanosine, and imidazole ring-opened 1,7-dialkylguanosine from each other, although with differing effectiveness. R_F values are given in Table I.

Identification of Alkylated Guanosines. Paper electropherograms or chromatograms were observed under ultraviolet light to detect various derivatives. Note was made of those areas which were brightly fluorescent. These corresponded to the 7-, 1,7- and 6-*O*-alkylguanosines. After elution with water by capillarity, the spectra were plotted using a Cary 15 recording spectrophotometer. 6 N HCl was added to the same solution to a final concentration of 0.1 N and the spectra

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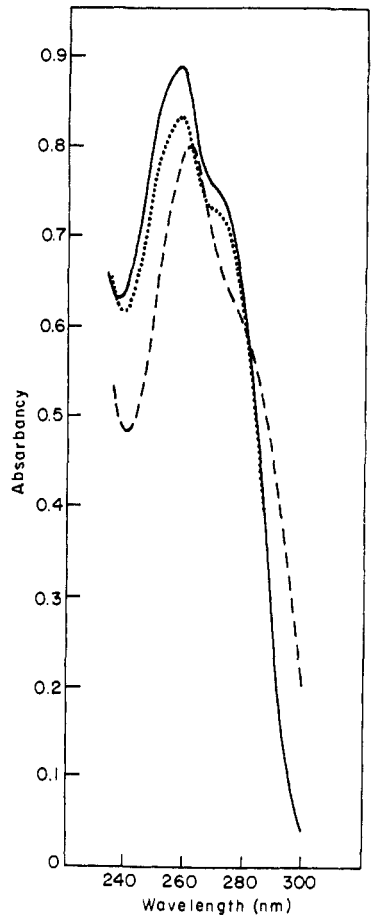

FIGURE 1: Ultraviolet absorption spectra of 1-ethylguanosine in water (——), 0.1 N HCl (– –), and 0.1 N KOH (. . . .).

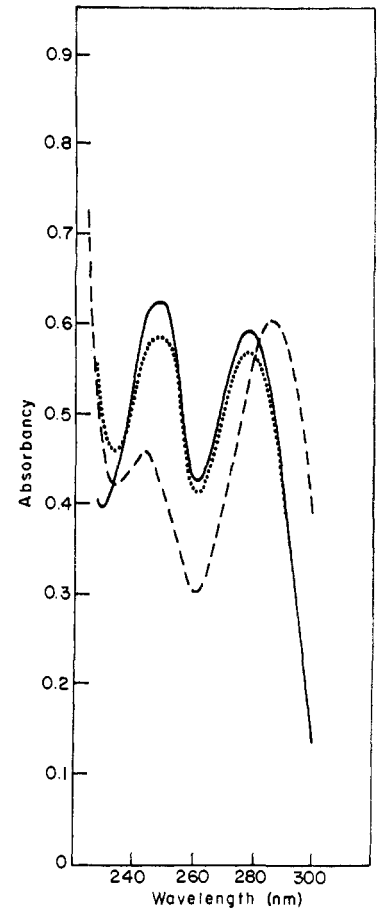

FIGURE 2: Ultraviolet absorption spectra of 6-*O*-ethylguanosine in water (——), 0.1 N HCl (– –), and 0.1 N KOH (. . . .).

TABLE I: R_F Values of Alkylated Guanosines.

Guanosine Derivative[a]	R_F[b] Solvent A	Solvent B
1-Methyl-	0.58	0.10
6-*O*-Methyl-	0.68	0.43
6-*O*,*X*-Dimethyl-	0.79	0.65
7-Methyl-	0.36	0.07
1,7-Dimethyl-	0.56	0.13
Imidazole ring-opened 7-methyl-	0.36	0.08
Imidazole ring-opened 1,7-dimethyl-	0.61	0.16
1-Ethyl-	0.68	0.34
6-*O*-Ethyl-	0.80	0.65
6-*O*,*X*-Diethyl-	0.88	0.80
7-Ethyl-	0.52	0.15
1,7-Diethyl-	0.71	0.31
Imidazole ring-opened 7-ethyl-	0.55	0.15
Imidazole ring-opened 1,7-diethyl-	0.70	0.30
Guanosine	0.45	0.10

[a] All compounds except 1-methylguanosine and 7-methylguanosine were obtained only from reaction mixtures and identified as discussed in the text. [b] Solvent A: isopropyl alcohol–H_2O (70:20, v/v); solvent B: butanol–ethanol–H_2O (80:10:25, v/v). Samples were applied to Whatman No. 3MM paper and run descending for approximately 18 hr.

were replotted. KOH (3 N) was added to a final concentration of 0.1 N and the solution immediately replotted (Figures 1–5). In the case of the 7, or 1,7 derivatives, 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 4 and 5).

Since authentic samples of 1-methylguanosine and 7-methylguanosine were available (Sigma Chemical Co.) and 1,7-dimethylguanosine could be prepared from either of these, it was possible to assign analogous structures to the ethyl derivatives based on their almost identical spectra. 6-*O*-Methyl- and 6-*O*-ethyldeoxyguanosine have been described by Friedman *et al.* (1965) and Loveless (1969) and their unique spectral characteristics were also found in the derivatives assigned the structures of 6-*O*-methyl- and 6-*O*-ethylguanosine (Figure 2). Another fluorescent derivative of guanosine, with an R_F in both solvents A and B greater than that of 6-*O*-ethylguanosine and found in lesser amounts than 6-*O*-ethylguanosine, had a spectrum which was somewhat different (Figure 3) and on the basis of mass spectra data (obtained by Dr. D. Daves of the Oregon Graduate Center) is believed to be a dialkyl derivative with one ethyl group on the 6-*O* and the other ethyl not yet assigned. This will be referred to as 6-*O*,*X*-diethylguanosine. Spectral data for ethylated and methylated guanosines are given in Table II.

Action of Alkali and Acid on 7-Substituted Guanosines. Both 7- and 1,7-alkylguanosines exhibit imidazole ring opening in alkaline solution. The approximate rate of ring opening

TABLE II: Ultraviolet Absorption Data for Alkylated Guanosines.

Guanosine Derivative	H_2O		pH 1		pH 13	
	λ_{max} (nm)	λ_{min} (nm)	λ_{max} (nm)	λ_{min} (nm)	λ_{max} (nm)	λ_{min} (nm)
1-Ethyl-	257		261		258	
	270 (s)[a]	237	272 (s)	240	270 (s)	239
7-Ethyl-	257		257			
	277 (s)	235	277 (s)	244		
1,7-Diethyl-	263		263			
	270 (s)	238	270 (s)	237		
6-*O*-Ethyl-	248	229	244	233	247	233
	277	261	286	260	278	261
6-*O*,*X*-Diethyl-	253	233	246	239	252	237
	282	268	292	267	281	268
Imidazole ring-opened 7-ethyl-			272	243	265	247
Imidazole ring-opened 1,7-diethyl-			272	243	273	248
1,7-Dimethyl-	259		260			
	270 (s)	235	270 (s)	236		
6-*O*-Methyl-	247	236	243 (s)		243	239
	277	261	284	259	277	261
Imidazole ring-opened 7-methyl-			271	248	265	247
Imidazole ring-opened 1,7-methyl-			269	238	270	243
1-Methyl-7-ethyl-	259		259			
	277 (s)	233	277 (s)	233		
Imidazole ring-opened 1-methyl-7-ethyl-			271	246	272	248
1-Ethyl-7-methyl-	261		261			
	275 (s)	235	275 (s)	235		
Imidazole ring-opened 1-ethyl-7-methyl-			270	246	272	250

[a] (s) = shoulder.

in 0.1 M pH 8.9 phosphate buffer was determined from the spectral change at 290 nm. The half-life was calculated from a plot of the log of the difference between the absorbancy at a given time and the absorbancy of the completely ring-opened compound, against time. The length of time these plots were found to be linear varied with the derivative. Figure 5 illustrates the spectra of partially and completely ring-opened 7-ethylguanosine.

The approximate rate of glycosidic bond breakage in acid solution was determined by the change in the ratio of absorbancies at 280 nm/260 nm at neutral pH reflecting the change in spectrum from nucleoside to base. Samples in 1 N HCl were heated at 37°. After various times, aliquots were diluted 20-fold with 0.2 M pH 7 phosphate buffer and the spectra were plotted. (As an example of the spectral difference between the nucleoside and base, Figure 6 shows the spectrum of 1,7-diethylguanine in 0.2 M pH 7 phosphate which can be compared to Figure 4A which shows the neutral spectrum of 1,7-diethylguanosine.) A plot of the difference between the 280 nm/260 nm ratio at a given time and the 280 nm/260 nm ratio of the base, against time, was linear. The half-life of glycosidic bond cleavage could thus be calculated.

Acid Dissociation Constants. A spectrophotometric method was used, similar to that of Cohn (1951). Samples containing 1 absorbancy unit were adjusted to various pH's from 1 to 9 with 0.1 N HCl and 0.1 N NaOH. In the case of the 1,7-dialkylguanosines which are easily ring-opened, 0.1 M pH 5 acetate buffer was added to minimize the possibility that the addition of NaOH would cause the pH to rise above 7. The spectra were plotted using a Cary 15 recording spectrophotometer and the ratio of absorbancy at the several wavelengths (*e.g.*, 300 nm/250 nm, 280 nm/260 nm, etc.) which showed the greatest change with pH was plotted against pH. The apparent pK_a was taken as the midpoint of the transition from cation to neutral species or neutral species to cation. A calculation based on two to four separate absorbancy ratios for each determination had a variation of no more than 0.1 p*K* unit from the values in Table VI.

Results and Discussion

Reaction of Guanosine and Alkylguanosines with Ethyl Iodide and Methyl Iodide. Broom *et al.* (1964) have described a method of preparing 1-methylguanosine by reacting guanosine in anhydrous solution, containing K_2CO_3, with methyl iodide. Jones and Robins (1963) prepared 7-methylguanosine by long reaction of guanosine with methyl iodide in neutral anhydrous medium. Both 1-methylguanosine and 7-methylguanosine can be additionally methylated in neutral anhydrous media to yield 1,7-dimethylguanosine. It seemed likely that these types of reactions could also be used to prepare the corresponding ethylguanosines by reaction with ethyl iodide.

Using Broom's conditions with ethyl iodide to prepare 1-ethylguanosine, it was first found that much of the reaction products were soluble in methylene chloride, used by Broom *et al.* (1964) to precipitate 1-methylguanosine, and that this methylene chloride supernatant contained several ethyl derivatives of guanosine as judged by the variety of products

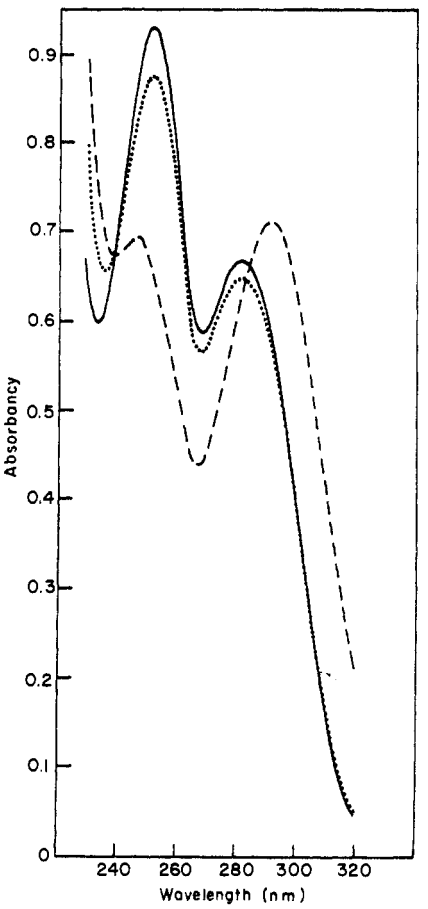

FIGURE 3: Ultraviolet absorption spectra of 6-*O*,*X*-diethylguanosine in water (——), 0.1 N HCl (– –), and 0.1 N KOH (. . . . . .).

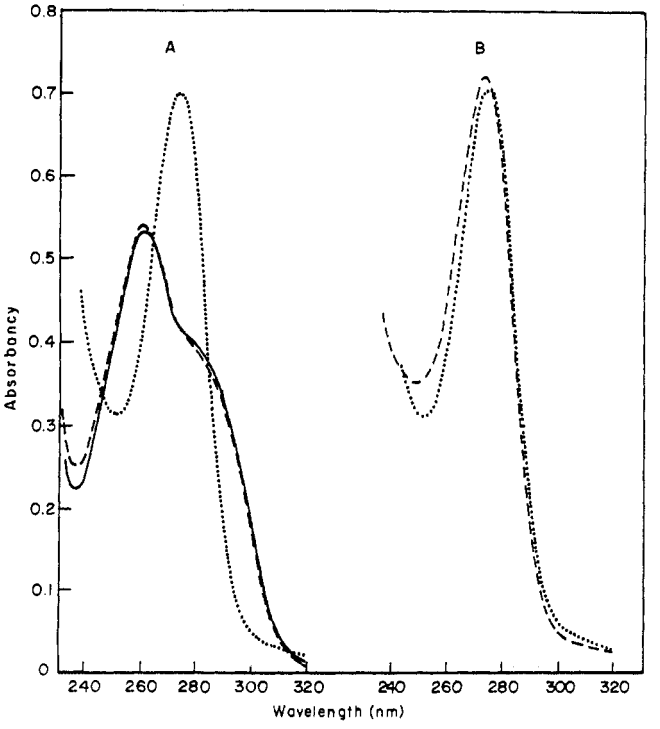

FIGURE 4: Ultraviolet absorption spectra. (A) Of 1,7-diethylguanosine in water (——), 0.1 N HCl (– –), and 0.1 N KOH (. . . . .). In 0.1 N KOH the imidazole ring opens almost instantaneously and the spectrum shown is that of the ring-opened compound. (B) Of imidazole ring-opened 1,7-diethylguanosine in 0.1 N KOH (. . . . .) and 0.1 N HCl (– –).

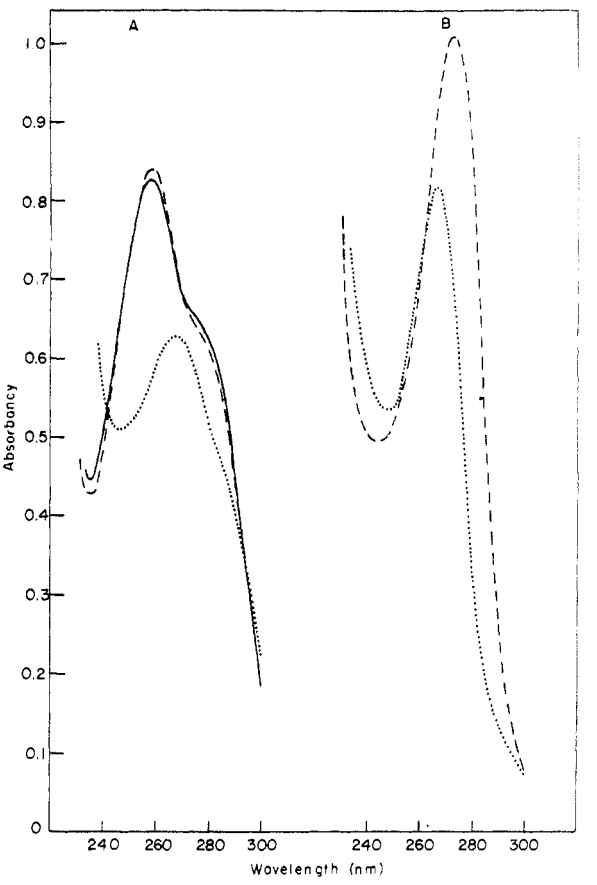

FIGURE 5: Ultraviolet absorption spectra. (A) Of 7-ethylguanosine in water (——), 0.1 N HCl (– –), and 0.1 N KOH (. . . . . .). The spectrum shown in 0.1 N KOH is that of the partially ring-opened compound. (B) Of completely imidazole ring-opened 7-ethylguanosine in 0.1 N KOH (. . . . .) and 0.1 N HCl (– –).

visible under ultraviolet light on paper chromatograms. The methylene chloride precipitate was primarily 1-ethylguanosine but the other products were present. If the entire reaction mixture was chromatographed in either solvent A or B it was possible to isolate, by further chromatography and/or electrophoresis, identify and quantitate 1-ethylguanosine, 7-ethylguanosine, 1,7-diethylguanosine, and 6-*O*-ethylguanosine, as well as another compound tentatively identified as a 6-*O*-ethylguanosine with an additional ethyl group, the position of which has not been established. There was also present, under certain conditions, the imidazole ring-opened 1,7-diethylguanosine (1-ethyl-2-amino-6-hydroxy-5-ethylformamido-4-ribosylaminopyrimidine), but not imidazole ring-opened 7-ethylguanosine. The kinetics of ring opening will be discussed later as well as the acid catalyzed glycosidic bond cleavage of these derivatives.

The analogous methyl iodide reaction mixture, when chromatographed without methylene chloride precipitation, gave in general the same methyl derivatives but the distribution was different. Methylene chloride precipitation gave better separation of 1-methylguanosine from the other methyl derivatives indicating that these methylguanosines are more soluble in methylene chloride than the ethyl derivatives, although much less material was found in the supernatant than with the ethylated guanosine.

It is not surprising that Broom *et al.* (1964) did not find these methylguanosines since they are present in low amounts in the methylene chloride precipitate, but very easy to detect

TABLE III: Distribution of Alkylguanosines Found after Reaction with Ethyl Iodide or Methyl Iodide in the Presence of K_2CO_3.[a]

Guanosine Derivative Found	% Recovd Absorbancy[b]			
	Ethyl Iodide		Methyl Iodide	
	22–25°, 2 hr	25–35°, 3 hr	22–25°, 2 hr	25–35°, 3 hr
1-Alkyl-	30	44	15	51
7-Alkyl-	7	3	2	nd
6-*O*-Alkyl-	13	22	2	6
6-*O*,*X*-Alkyl-	1	1	nd	<1
1,7-Dialkyl-	1	26	8	3
Ring-opened 1,7-dialkyl-	nd	nd	nd	40
Guanosine	48	4	73	nd

[a] The reaction conditions were essentially those of Broom *et al.* (1964) except that only 10–100 mg of guanosine were reacted with approximately stoichometric amounts of the alkyl iodide in dimethyl sulfoxide containing K_2CO_3 (100 mg of guanosine, 1 ml of dimethyl sulfoxide, 60 mg of anhydrous K_2CO_3, and 25 μl of alkyl iodide, followed by a later addition of 20 mg of K_2CO_3 and 10 μl of alkyl iodide). When samples were stirred on a magnetic stirrer the temperature rose to 35° over a 3-hr period (experiments designated as 25–35°). In other experiments, occasional manual shaking was used and the temperature remained at 22–25°. All samples were filtered through a Pasteur pipet with about 2-cm Celite supported by glass wool. Pressure was applied to increase the speed of filtration since some reaction products were unstable. A portion of the filtrate was precipitated with eight volumes of methylene chloride. The resulting precipitate was kept at 0° for a few minutes, then centrifuged in the cold. After methylation, the precipitate contained over 90% of the absorbancy while the precipitate from ethylated guanosine contained only about 60%, 40% being in the supernatant. The precipitates were dissolved in a small amount of dimethylformamide. At various times, any or all of the three samples derived from an experiment (whole filtrate, precipitate, and supernatant) were analyzed in terms of reaction products. [b] Since molar extinction coefficients were not determined for the various compounds, these values can only be regarded as approximations to actual yields.

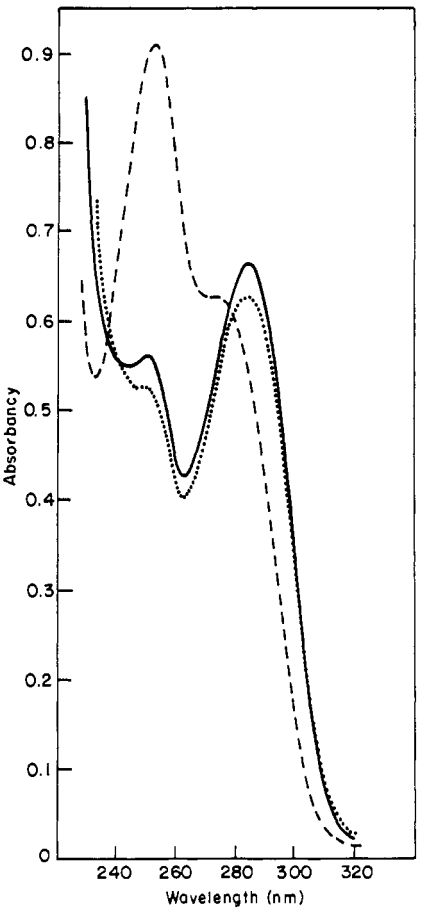

FIGURE 6: Ultraviolet adsorption spectra of 1,7-diethylguanine in 0.2 M pH 7 phosphate (——), 0.1 N HCl (– –), and 0.1 N KOH (. . . . . .).

on paper since 7-, 1,7-, and 6-*O*-alkylguanosines fluoresce brightly under ultraviolet light.

Comparisons of the amounts of ethyl- and methylguanosines formed under various conditions of time and temperature are given in Table III. The unexpected finding that much dialkylguanosine is produced under these conditions and especially the readiness with which 1,7-diethylguanosine is produced led to experiments in which it was attempted to determine whether 1-ethyl- or 7-ethylguanosine was the preferred intermediate. It was therefore surprising to find that neither 1-ethylguanosine nor 7-ethylguanosine (after isolation from paper chromatograms) reacted with additional ethyl iodide in dimethyl sulfoxide (room temperature for 18 hr) or in dimethylformamide containing K_2CO_3 (3 hr at 37°) and that formation of 1,7-diethylguanosine from 1-ethylguanosine was incomplete even upon treatment with a great excess of ethyl iodide in dimethyl sulfoxide for 24 hr at 37°.

1-Methyl- and 7-methylguanosine, on the other hand, were quantitatively converted to 1,7-dimethylguanosine in dimethyl sulfoxide containing an excess of methyl iodide. However the same reaction with 7-methylguanosine in the presence of K_2CO_3, dimethylformamide and excess methyl iodide yielded mostly the imidazole ring-opened 1,7-dimethylguanosine and a small amount of 1,7-dimethylguanosine. This indicated that under alkaline conditions the second methyl group can be added but the resulting derivative is ring opened at a rapid rate.

Since much 1,7-diethylguanosine is produced from the reaction at 35° of guanosine with ethyl iodide in dimethyl sulfoxide containing K_2CO_3, but little if any starting with 1- or 7-ethylguanosine, the reaction mechanism remained in doubt. Since 1-methyl- and 7-methylguanosine were commercial samples but 1-ethyl- and 7-ethylguanosine were isolated from paper chromatograms, it appeared possible that a contaminant from the paper might interfere with the alkylation of the ethylguanosines. To test this in two ways, 1-methylguanosine was reacted with ethyl iodide and 1-ethylguanosine was reacted with methyl iodide. Little 1,7-dialkylguanosine (<2%) was found when 1-methylguanosine was reacted with ethyl iodide, but all of the 1-ethylguanosine reacted with methyl iodide to form 1-ethyl-7-methylguanosine. Thus the ease of dialkylating 1-alkylguanosine seems to be a function of the alkylating reagent, methyl iodide being much

TABLE IV: Products of the Reaction of Guanosine, 1-Alkyl- and 7-Alkylguanosines with Ethyl Iodide and Methyl Iodide.

Reaction Mixture[a]	Products[b,c]
Guanosine, ethyl iodide	7-Ethylguanosine (very little reaction)
Guanosine, methyl iodide	6% 1,7-dimethylguanosine, 94% 7-methylguanosine
1-Methylguanosine, ethyl iodide	25% 1,7-dialkylguanosine, 75% 1-methylguanosine
1-Methylguanosine, methyl iodide	1,7-Dimethylguanosine
1-Ethylguanosine, ethyl iodide	<2% 1,7-diethylguanosine, >98% 1-ethylguanosine
1-Ethylguanosine, ethyl iodide (37°, 24 hr)	50% 1,7-diethylguanosine, 25% ring-opened 1,7-diethylguanosine, 25% 1-ethylguanosine
1-Ethylguanosine, ethyl iodide, K_2CO_3	1-Ethylguanosine
1-Ethylguanosine, methyl iodide	1,7-Dialkylguanosine
7-Methylguanosine, methyl iodide	1,7-Dimethylguanosine
7-Methylguanosine, methyl iodide, K_2CO_3	10% 1,7-Dimethylguanosine, 90% ring-opened 1,7-dimethylguanosine
7-Ethylguanosine, ethyl iodide	7-Ethylguanosine
7-Ethylguanosine, ethyl iodide, K_2CO_3	7-Ethylguanosine

[a] Reaction mixtures lacking K_2CO_3 (otherwise the same) were held for 18 hr at room temperature. Those in the presence of K_2CO_3 (see Table III, footnote *a*) were at 37° for 3 hr, unless otherwise noted. Five milligrams of 1-methylguanosine or 7-methylguanosine was reacted with 10 μl of methyl iodide in 50 μl of dimethylformamide to prepare the 1,7-dimethyl derivative (Broom *et al.*, 1964). About 10 absorbancy units of 1-ethylguanosine or 7-ethylguanosine (eluted from paper chromatograms and repurified by additional chromatography) were reacted with 20 μl of ethyl iodide or with 10 μl of dimethyl sulfoxide containing 80 μg of K_2CO_3 and 0.35 μl of ethyl iodide in attempts to prepare 1,7-diethylguanosine. These conditions are similar to those of the reaction of guanosine with alkyl iodide in the presence of K_2CO_3 (Table III, footnote *a*) or in neutral solution (Broom *et al.*, 1964). About 10 absorbancy units of 1-ethylguanosine in 50 μl of dimethylformamide was reacted with 1 μl of methyl iodide, or 1 mg of 1-methylguanosine in 50 μl of dimethylformamide was reacted with 2 μl of ethyl iodide to prepare 1-ethyl-7-methylguanosine or 1-methyl-7-ethylguanosine. [b] When a single product is listed, no other product, other than a small amount of the starting material, was found. [c] See Table III, footnote *b*.

more efficient under these conditions than ethyl iodide. Table IV presents a summary of the products isolated after various ways of alkylating 1- or 7-alkylguanosine with methyl or ethyl iodide.

Reaction of Guanosine with Ethyl Methanesulfonate and Diethyl Sulfate. The reaction of guanosine with ethyl methanesulfonate differed in several respects from that with ethyl iodide. The reaction with the former is in aqueous solution, the extent and nature of the products being pH dependent. The reaction is very slow compared to that with ethyl iodide, which will almost quantitatively alkylate guanosine in a few hours at 25–35°.

When 10 mg of guanosine was treated at 37° in 1 ml of 1 M pH 5 ammonium formate, in 1 M potassium phosphate adjusted to various pH's from 6 to 8, or in saturated ammonium carbonate (pH 9) with 0.1 ml of ethyl methanesulfonate, the two major products of the reaction are 7-ethylguanosine and 6-*O*-ethylguanosine. Since 7-ethylguanosine is unstable on both the acid and alkaline side of neutrality (glycosidic bond cleavage and imidazole ring opening, respectively), it is not possible to extend this reaction for long periods of time, without incurring such secondary reactions. Therefore the data presented are for reactions with less than 5% alkylation of the original guanosine (2–8 hr at 37°).

The amount of 6-*O*-ethylguanosine is five to ten times higher at pH 9 than at pH 7. Slightly less 7-ethylguanosine is found at pH 9 than at pH 5 or 7. 6-*O*-Ethylguanosine can be detected on paper chromatograms after long reaction at pH 5 but the amount is too low for an accurate determination. At pH 9, 6-*O*-ethylguanosine amounts to about 25% of the total ethylation.

When diethyl sulfate was used as an ethylating agent under the same conditions, 6-*O*-ethylguanosine is only barely detectable at pH 9 and not al all at pH 7. The ratio of 6-O:7-alkylation with diethyl sulfate at pH 9 was about one-tenth of that found with ethyl methanesulfonate at the same pH, 6-*O*-ethylguanosine amounting to about 3% of the total ethylation. Thus, while 6-*O*-ethylguanosine can be detected as a reaction product after diethyl sulfate reaction with guanosine, it is probably not of biological interest since it is not found near neutrality. No 6-*O*-methylguanosine can be detected after dimethyl sulfate reaction. Very little 6-*O*-methylguanosine is found after reaction with methyl methanesulfonate, the ratio of 6-O:7-alkylation being less than 0.04.

1-Ethylguanosine is also a product of ethylation with ethyl methanesulfonate at pH 9, but in contrast to its predominance when guanosine was reacted with ethyl iodide in a nonaqueous medium containing K_2CO_3, it was found only in lesser amounts than 6-*O*-ethylguanosine.

1,7-Diethylguanosine could not be detected after reaction with ethyl methanesulfonate, indicating again that 7-ethylguanosine is not readily alkylated on the 1 position.

Characterization of Alkylguanosines. The various ethylated and methylated guanosines were characterized in several ways: (1) ultraviolet spectra, (2) fluorescence excitation and emission spectra, (3) acid dissociation constants, (4) rate of glycosidic bond cleavage, and (5) rate of imidazole ring opening.

Table II presents data on the ultraviolet spectra of the neutral, anionic and cationic species of all the compounds previously discussed. In general, the ethyl and methyl substituents on the same position show similar spectra. However the small differences in maxima and minima reported are characteristic of each derivative.

Fluorescence spectra of the 7-, 1,7- and 6-*O*- derivatives differed markedly. The spectra of the ethyl and methyl series are shown in Figures 7–10. Table V gives the uncorrected excitation maxima and the ratios of the intensity of the two excitation peaks found with all the derivatives used. The

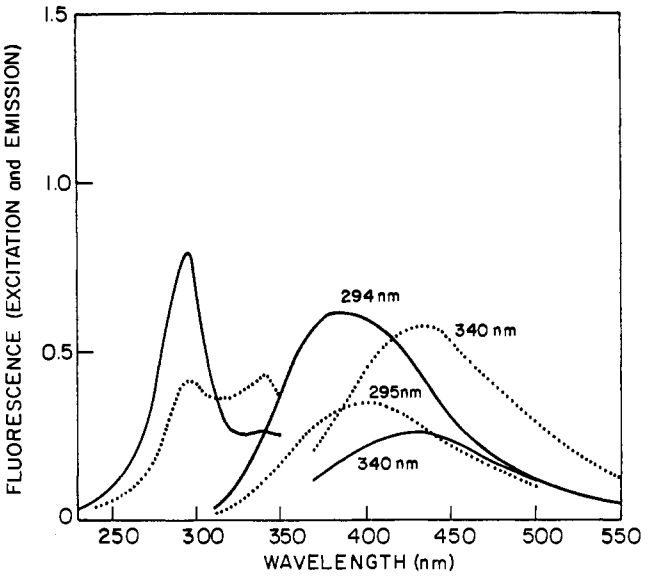

FIGURE 7: Fluorescence excitation and emission spectra of 6-*O*-ethylguanosine (——) and 6-*O*-methylguanosine (. . . . .) in 0.01 M NaCl–0.005 M cacodylate buffer, pH 7, 20°. Fluroescence intensity is in arbitrary units. Spectra are corrected to uniform slit widths and a concentration of 1 absorbancy unit. Emission is at 400 nm for excitation spectra. Nanometers of excitation for emission spectra are shown on the figure.

second excitation peak at 340–355 nm has not been previously found. This peak found with a commerical sample of 7-methylguanosine and other derivatives isolated from paper chromatograms could have been overlooked in the case of 7-methylguanosine since the intensity becomes very low at 320 nm where most fluorescence spectra are discontinued. Its validity is shown by a second emission peak at 460 nm when excited at 350 nm (Figure 9). 7-Ethylguanosine, with a strong second excitation peak at 340 nm, has a second emission peak at 440 nm. All other λ_{max} of emission differ from each other (Table V). Therefore it seems unlikely that this second excitation maximum is due to an artifact.

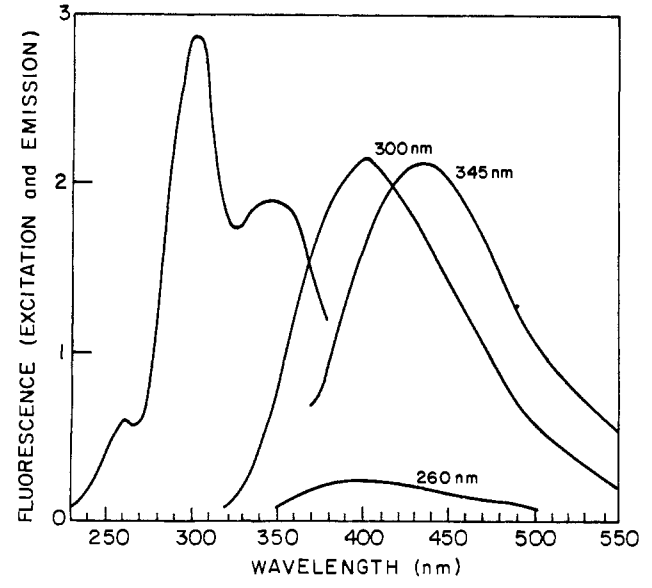

FIGURE 8: Fluorescence excitation and emission spectra of 6-*O*,*X*-diethylguanosine. See legend for Figure 7 for conditions.

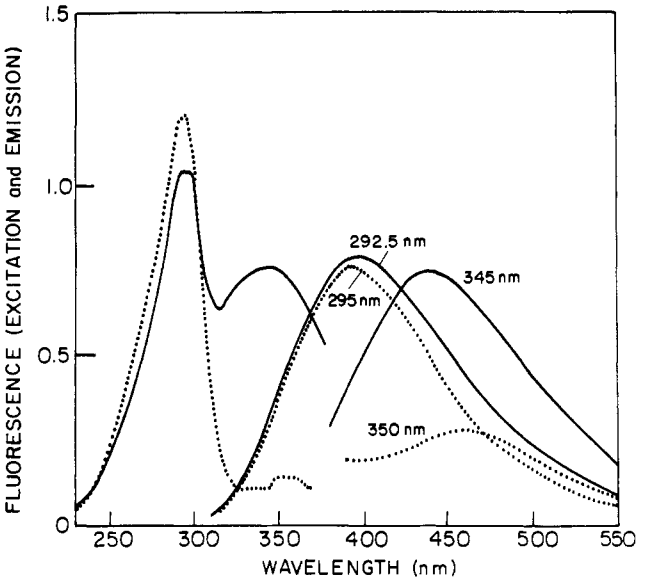

FIGURE 9: Fluorescence excitation and emission spectra of 7-ethylguanosine (——) and 7-methylguanosine (. . . . .). See legend for Figure 7 for conditions.

The approximate acid dissociation constants, determined spectrophotometrically, are given in Table VI. The value for guanosine is higher than the usual figure of 2.1–2.2 (Shapiro, 1968) and approximates that of 2.4 reported by Simpson (1964) who used a method similar to ours. Simpson usually adjusted the pH with $HClO_4$ or NaOH, sometimes in the presence of weak buffers, such as acetate. The only other comparable data, that of Lawley and Brookes (1963) for 7-methylguanosine and 7-ethylguanosine, who report a pK of 7.3 for both compounds, agree with the present value of 7.3 and 7.4, respectively. In general, the pK_a of the ethyl derivative is slightly higher than that of the methyl compound. Neither alkylation of the 1 nor the 6 position would be expected to

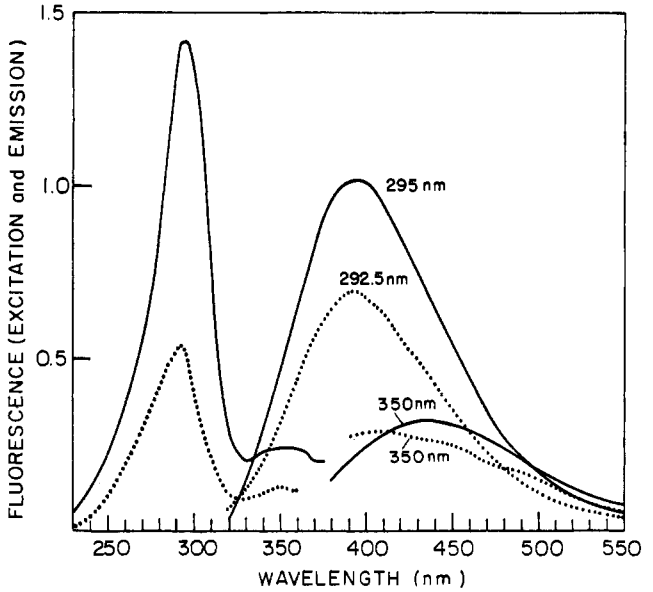

FIGURE 10: Fluorescence excitation and emission spectra of 1,7-diethylguanosine (——) and 1,7-dimethylguanosine (. . . . .). See legend for Figure 7 for conditions.

TABLE V: λ_{max} Fluorescence Excitation of Alkylguanosines.[a]

Guanosine Derivative	λ_{max} (nm) I	λ_{max} (nm) II	Fluorescence Intensity λ_{max} II: λ_{max} I
6-*O*-Ethyl-	295 (380)	340 (425)	0.35
6-*O*-Ethyl-, pH 0[b]	305		
6-*O*,*X*-Diethyl-	302 (400)	345 (435)	0.62
6-*O*,*X*-Diethyl-, pH 0[b]	312	~360–380[c]	~0.5
7-Ethyl-	294 (400)	340 (440)	0.73
1,7-Diethyl-	295 (395)	355 (435)	0.17
6-*O*-Methyl-	295 (395)	341 (435)	0.93
7-Methyl-	295 (395)	355 (460)	0.12
1,7-Dimethyl-	293 (392)	350 (410)	0.24

[a] Fluorescence excitation and emission spectra were determined using a double-monochrometer Zeiss spectrofluorimeter. Samples were diluted (except where otherwise noted) in 0.01 M NaCl–0.005 M cacodylate (pH 7) at 20°; usually 1 absorbancy unit/ml was the concentration of alkylguanosine. The author is indebted to Dr. A. M. Michelson, Institute de Biologie Physico-Chimique, Paris, for the use of the spectrofluorimeter and discussions on the interpretation of fluorescence spectra. The maxima are uncorrected for lamp intensity. The figures in parentheses are the λ_{max} of emission when excited at the λ_{max} of excitation. See Figures 7–10 for actual spectra. All compounds except 7-methylguanosine were obtained from reaction mixture. [b] After fluorescence excitation spectra were plotted in 1 N HCl, these samples were heated at 100° for 2 hr and the spectra were again plotted. 6-*O*-Ethylguanosine lost about 85% of its fluorescence as would be expected since this compound is converted to guanine in strong acid. After similar treatment of 6-*O*,*X*-diethylguanosine, the fluorescence spectrum was of the same intensity but with λ_{max} I at 325 nm and λ_{max} II at 360 nm, the ratio of fluorescence intensity of λ_{max} II:λ_{max} I being dramatically increased to 1.4. [c] Shoulder only.

TABLE VI: Acid Dissociation Constants of Alkylguanosines.[a]

Guanosine Derivative	pK_a
Guanosine	2.5
1-Ethyl-	2.8
7-Ethyl-	7.4
6-*O*-Ethyl-	2.5
6-*O*,*X*-Diethyl-	3.0
1-Methyl-	2.6
7-Methyl-	7.3
6-*O*-Methyl-	2.4

[a] See Experimental Section for experimental procedure. 1,7-Diethylguanosine and 1,7-dimethylguanosine gave apparent pK_a's at 5.7 and 5.5, respectively. However the electrophoretic movement in relation to 7-ethyl- and 7-methylguanosines indicated a pK_a above 7.4, as would be expected on a theoretical basis.

TABLE VII: Rate of Alkali-Catalyzed Imidazole Ring Opening and Acid Hydrolysis of Alkylguanosines.

Guanosine Derivative	Ring-Opening[a] Half-Life (hr)	Acid Hydrolysis[b] Half-Life (hr)
7-Ethyl-	15	15
1,7-Diethyl-	1.1	17
7-Methyl-	4	15
1,7-Dimethyl-	0.2	17

[a] Samples were diluted to an absorbancy of 1 in 0.1 M phosphate (pH 8.9). The stoppered cuvets were maintained at 37°. See Experimental Section for experimental details. [b] Samples with an absorbancy of 20 in 1 N HCl were maintained at 37°. Aliquots were diluted 20-fold with 0.2 M phosphate (pH 7) after various time periods and the spectra plotted. See Experimental Section for experimental details.

cause a large change in the acid pK. The high pK_a for 7 alkylation is the result of the quaternization of N-7. The 1,7-dialkylguanosines move appreciably further than the 7-alkylguanosines when electrophoresed at pH 5.7. This indicates that the pK_a of the 1,7 derivatives is higher than 7.4 and is in the pH range where ring opening is observed. The finding of apparent pK_a's of 5.5 for 1,7-dimethylguanosine and 5.7 for 1,7-diethylguanosine remains unexplained.

The rate of acid-catalyzed glycosidic bond cleavage of 7- and 1,7-alkylguanosines was similar for both ethyl and methyl substituents and little difference was found between 7-alkyl- and 1,7-dialkylguanosine (Table VII), indicating that the stability of the glycosyl bond of ribonucleosides is a function of the quaternization of the N-7 nitrogen and is not affected by the 1-alkyl substituent fixing the purine ring in the C=O (keto) form. The possibility exists that in 7-alkylguanosines this form is not as predominant as it may be in guanosine (Jones and Robins, 1963).

The rate of alkali-catalyzed imidazole ring opening of 7- and 1,7-alkylguanosines varies greatly, both with the nature of the alkyl group and the addition of a 1-alkyl group to 7-alkylguanosine (Table VII). At pH 8.9 at 37°, the ethyl derivatives are 4–6 times as stable as the methyl derivatives, and 7-alkylguanosines are 8–14 times as stable as the corresponding 1,7-dialkylguanosine. Since the sequence of events leading to ring opening is not known it is not possible to do more than speculate on the reasons for the differences between ethyl and methyl, and between 7 and 1,7 derivatives. It might be assumed that the stability of the formamido intermediate, which can reclose, is decreased by the presence of an alkyl group on the 1 position. However Tomasz (1970) observed that the rapid labilization of the C-8 proton of 7-methylguanosine determined by deuterium exchange was identical with that of 1,7-dimethylguanosine and 7-methylinosine, concluding that the state of the six-membered ring of the purine has no influence on the acidity of the C-8 proton.

Concerning previous studies of this problem, Lawley and Brookes (1963) did not examine in any detail the difference in the rate of the ring opening of 7-methylguanosine and 7-ethylguanosine but found 7-methylguanosine, at pH 10.2 at 20°, to have a half-life of 1.5 hr and on the basis of one point estimated that 7-ethylguanosine was about three times as

stable. In a later paper Brookes *et al.* (1968) compared the rate of ring opening of 7-methylguanosine and 7-benzylguanosine at pH 9.5 at 37° and found them to have an identical half-life of 0.5 hr. It appears that the rate of ring opening is greatly affected by small changes in pH and temperature and thus comparisons are valid only under identical conditions.

Attempts to use high-resolution mass spectrometry proved disappointing. Dr. D. Daves examined all of the ethylguanosines obtained with ethyl iodide, with and without acetylation of the ribose moiety. In most cases the base was cleaved and dealkylation occurred. A similar observation was reported by Lawley and Jarman (1972) who used mass spectrometry to identify the products of reaction of adenine and guanine with propylene oxide. This was not the case for the methylguanosines. However the data obtained for the ethylguanosines were sufficient to assign mono- or dialkyl structures. This was particularly important in the case of 6-*O*,-*X*-diethylguanosine which showed, among others, ions at *m/e* 207 corresponding to diethylguanine whereas 6-*O*-ethylguanosine lacked this peak.

Acknowledgment

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Isolation and Characterization of Pancreatic Procarboxypeptidase B and Carboxypeptidase B of the African Lungfish†

Gerald R. Reeck‡ and Hans Neurath*

ABSTRACT: Procarboxypeptidase B of the African lungfish, *Protopterus aethiopicus*, has been purified by a combination of ion-exchange chromatography, affinity chromatography, and gel filtration and characterized. The monomeric zymogen has a molecular weight of 45,000 and its amino acid composition is similar to procarboxypeptidases B of other species. The amino-terminal sequence of 15 residues has been determined. The zymogen displays intrinsic peptidase and esterase activities which increase markedly upon activation with trypsin. The activation reaction proceeds *via* an intermediate and ultimately is accompanied by the release of a large peptide (approximately 10,000 daltons). The enzyme, carboxypeptidase B, has also been characterized by chemical and enzymatic methods which have revealed extensive similarities to the corresponding bovine, porcine, dogfish, and rat enzymes. The amino-terminal sequence of lungfish carboxypeptidase is homologous to those of bovine carboxypeptidases A and B.

As part of the study of the phylogenetic variations of the structure and function of certain proteases, we have undertaken an analysis of the pancreatic zymogens and enzymes of the African lungfish, *Protopterus aethiopicus* (Reeck *et al.*, 1970). A detailed study of trypsinogen and trypsin (Reeck and Neurath, 1972) revealed considerable basic similarity to the corresponding bovine and dogfish proteins but also

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