Studies on the Alkylation of Purines and Pyrimidines

BIMAL C. PAL

From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, and Department of Chemistry, Tuskegee Institute, Alabama*

Received January 15, 1962

Adenine, 5-hydroxymethylcytosine, and thymine were treated with ethyl methanesulfonate at 40° and at *p*H 7.0, while guanine was similarly treated at *p*H 12.0. The products, separated and isolated by paper chromatography and identified spectrophotometrically, included 1-ethyladenine, 3-ethyladenine, 9-ethyladenine, and 7-ethylguanine. Three additional derivatives of guanine and one of 5-hydroxymethylcytosine were formed but were not identified. Thymine did not form any derivative. The ultraviolet absorption spectra and *pK* values of some of these derivatives were obtained. 7-Methyladenine is not a product of alkylation of adenine with dimethyl sulfate, and no correlation between alkylation sites and theoretically predicted relative basicities of the ring nitrogen atoms of adenine was observed.

Ethyl methanesulfonate causes mutations in bacteriophage (Loveless, 1958, 1959; Green and Krieg, 1961), and its action on the phage DNA is the most probable cause of this mutation. This assumption finds additional support in the work of Bautz and Freese (1960), who found that a related chemical mutagen, diethyl sulfate, will ethylate the guanine residues in herring sperm DNA. It has also been shown that ethyl methanesulfonate reacts with extracellular T4 bacteriophage to cause mutations during subsequent reproduction of the treated phage in untreated bacteria (Green and Krieg, 1961). Since T4 phage DNA contains adenine, guanine, 5-hydroxymethylcytosine, and thymine, the action of ethyl methanesulfonate on these bases was investigated. The results, presented in this paper, are compared with the actions of other alkylating agents, such as dimethyl sulfate and diethyl sulfate, on the DNA bases, on nucleotides, and on DNA itself (Reiner and Zamenhof, 1957; Lawley, 1957; Brookes and Lawley, 1960).

Experimental Procedure

The bases and the ethyl methanesulfonate used in these experiments were commercial products. Samples of the bases were run on paper in different solvent systems such as *n*-butanol–H_2O, isopropanol-NH_3, isopropanol-HCl, and isobutyric acid–NH_3, and found to be chromatographically pure. The ethylations were carried out under conditions similar to those that are mutagenic for bacteriophage (Loveless, 1958, 1959; Green and Krieg, 1961).

Synthetic samples of 1-methylguanine, 7-methylguanine, 6-hydroxy-2-methylaminopurine, 7-methyladenine, 9-methyladenine, 3-methyladenine, and 6-methylaminopurine were obtained through the courtesy of Dr. G. H. Hitchings of the Wellcome Research Laboratories, Tuckahoe, N. Y. Each of these samples was found to be chromatographically pure by running in three different solvent systems such as *n*-butanol–H_2O, isopropanol-HCl, and isopropanol-NH_3. Their spectra agreed well with the spectral data published in the literature for 1-methylguanine, 6-hydroxy-2-methylaminopurine (Smith and Dunn, 1959), 7-methylguanine (Reiner and Zamenhof, 1957), 7-methyladenine, 9-methyladenine, 3-methyladenine, and 6-methylaminopurine (Elion, 1957). A sample of synthetic 8-methylguanine was obtained from Ronald K. Robins of Arizona State University, Tempe, Ariz. This was synthesized by the method of Koppel and Robins (1958a,b), and the spectrum of the sample agreed with the published data.

Alkylation of the Bases.—The bases were dissolved in 0.1 M phosphate buffer (*p*H 7.0 except where stated otherwise) to give concentrations of 1 mg per ml. Guanine was dissolved by addition of NaOH to bring the solution to *p*H 12. Six ml of the solution was treated with 0.15 ml (1.6 mmoles) of ethyl methanesulfonate in a *p*H-stat Radiometer Type TTTla, Copenhagen, N NaOH solution being added from the syringe buret to maintain a constant *p*H. The reaction was continued at 40° until the whole of the alkylating agent was consumed as indicated by cessation of addition of alkali. Samples (0.1 ml) were then withdrawn for paper chromatography. In each case different solvent systems such as *n*-butanol–H_2O (Markham and Smith, 1949), isopropanol-NH_3 (Hershey *et al.*, 1953), isopropanol-HCl (Wyatt, 1951), and isobutyric acid-NH_3 (Löfgreen, 1952) were tried and the one that gave the best separation was chosen.

Spectrophotometry.—Substances, located on the paper by scanning with ultraviolet light, were eluted with water and observed in a Cary Recording Spectrophotometer Model 14 at concentrations giving absorbancies of about 1.0 at λ_{max} at *p*H 2.0. The spectra were determined at *p*H 2.0, 7.0, and 12.0 on the same solution after adjustment of the *p*H by addition of very small

* Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

TABLE I

ALKYLATED PRODUCTS OF ADENINE, GUANINE, 5-HYDROXYMETHYLCYTOSINE, AND THYMINE[a]

For each sample the spectra at different *p*H are for solutions of equal concentration. To avoid confusion A1M, A2M, A3M, and A4M refer to the same derivatives called by Reiner and Zamenhof (1957) by the same designation. Identity was established by comparing λ_{max} in 0.1 N HCl and 0.1 N NaOH.

Reagent	Parent Compound	Derivatives	R_M	Yield in Percentage of Parent Compound Used	Identification and/or Spectrum	*pK*
Ethyl methanesulfonate	Adenine	A1E	1.3[b]	8.4	1-Ethyladenine	6.9, 11.4
		A2E	1.59[b]	8.8	9-Ethyladenine (Fig. 1, *left*)	4.1[e]
		A3E	1.87[b]	25.4	3-Ethyladenine (Fig. 1, *right*)	6.5
Dimethyl sulfate	Adenine	A2M		[d]	9-Methyladenine (Fig. 1, *left*)	3.9[e]
		A4M		[d]	3-Methyladenine (Fig. 1, *right*; 2, *left*)	6.1[f]
		A3M		[d]	1-Methyladenine	
		A1M		[d]	Unidentified	
Ethyl methanesulfonate	Guanine	G1E	1.53[b]	17.2	7-Ethylguanine (Fig. 2, *right*)	3.7, 9.5
		G2E	1.94[b]	13.7	Fig. 3	4.5, 9.4
		G3E	2.32[b]	(weak band)		
		G4E	2.68[b]	(weak band)		
Ethyl methanesulfonate	5-Hydroxymethylcytosine	5-HMClE	1.6[c]	21.3		3.8, 11.2
	Thymine	None				

[a] Abbreviations: $R_M = \frac{R_F \text{ of the derivative}}{R_F \text{ of the parent compound}}$; 5-HMC, 5-hydroxymethylcytosine; A, adenine; G, guanine; E, ethyl derivative; M, methyl derivative. [b] Isopropanol-HCl. [c] Isopropanol-NH_3. [d] Reiner and Zamenhof (1957). [e] *pK* of synthetic 9-methyladenine was found to be 3.9. [f] *pK* of synthetic 3-methyladewas found to be 6.1; *pK* of synthetic 7-methyladenine was found to be 4.2.

amounts of concentrated HCl or NaOH. The ultraviolet spectra of the authentic compounds were obtained in the same way. Absorption values at different wave lengths chosen arbitrarily from the Cary recordings of the spectra of the alkylated products were normalized to the E_{max} of the spectra of authentic specimens at *p*H 2 and plotted as points in Figures 1 and 2. The *pK* values of those ionizations determinable spectrophotometrically were obtained by plotting the ratios A_{250}/A_{260}, A_{280}/A_{260}, and A_{290}/A_{250} as a function of *p*H and noting the *p*H at the midpoints of the absorbance shifts (Cohn, 1951). The yields of different ethyl derivatives of adenine were estimated on the assumption that their spectra and molar extinction coefficients at *p*H 2.0 at λ_{max} are the same as those of the corresponding methyl derivatives (Brookes and Lawley, 1960; Friedrich and Bernhauer, 1956, 1957; Gulland *et al.*, 1934; Gulland and Story, 1938). The yields of guanine and 5-hydroxymethylcytosine derivatives were calculated assuming molar extinction coefficients at *p*H 2.0 at λ_{max} to be the same as those of the parent compounds. The ethylated purines were identified spectrophotometrically.

RESULTS

Three ultraviolet-absorbing spots were isolated from the reaction mixture of adenine with ethyl methanesulfonate (A1E, A2E, and A3E in Table I). The spectrum of A1E is very similar to the spectrum of 1-methyladenine (Brookes and Lawley, 1960). For both substances, the positions of the absorption maxima at *p*H 4, 7, and 13 are almost the same, and the extinction coefficient at λ_{max} decreases between *p*H 4 and 7 and then increases between *p*H 7 and 13, reaching its highest value at *p*H 13. It shows two *pK*'s (6.9, 11.4) similar to those of 1-methyladenine (7.2, 11.0). These indicate that A1E is probably 1-ethyladenine.

A2E was identified spectrophotometrically as 9-ethyladenine (Fig. 1, *left*). Since the formation of 9-methyladenine from adenine and dimethyl sulfate was not reported by Reiner and Zamenhof (1957), their experiment was repeated. One of the resulting derivatives (the one designated by Reiner and Zamenhof as A2M) was spectrophotometrically identical with synthetic 9-methyladenine (Fig. 1, *left*) and had R_F values identical with it in isopropanol-HCl, isopropanol-NH_3, and isobutyric acid–NH_3.

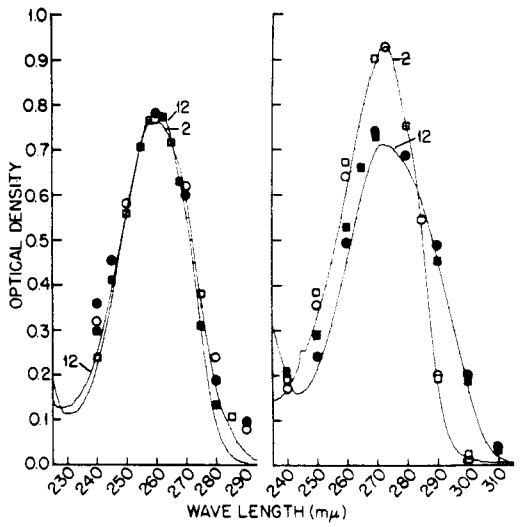


FIG. 1.—Ultraviolet-absorption spectra of 9-alkyladenines (*left*) and 3-alkyladenines (*right*) at *p*H 2 and 12. The continuous lines represent the spectra of the authentic 9-methyladenine and 3-methyladenine. Points (○, ●) are for the ethyl derivative of adenine (A2E, *left;* A3E, *right*) isolated from the reaction mixture of adenine and ethyl methanesulfonate at *p*H 2 and 12, respectively. Points (□, ■) are for the methyl derivative of adenine (A2M, *left;* A4M, *right*) isolated from the reaction mixture of adenine and dimethyl sulfate at *p*H 2 and 12, respectively. All points are taken from Cary recordings of the entire spectra.

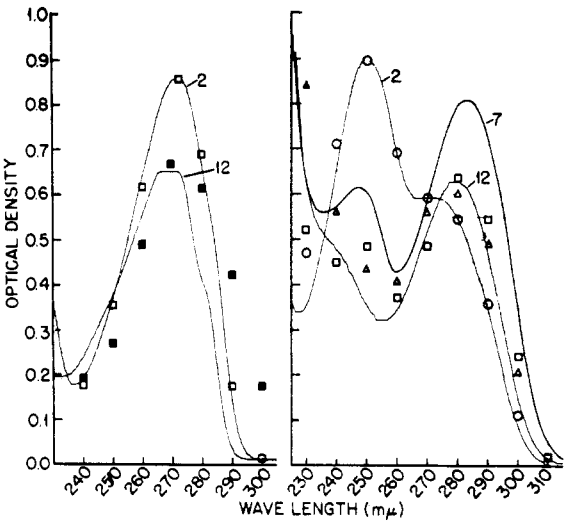


FIG. 2.—*Left,* ultraviolet-absorption spectra of synthetic 7-methyladenine at *p*H 2 and 12 (represented by continuous lines). Points (□, ■) are for the methyl derivative of adenine, A4M, isolated from the reaction mixture of adenine and dimethyl sulfate at *p*H 2 and 12, respectively, and are taken from Cary readings of the entire spectra. *Right,* ultraviolet-absorption spectra of 7-alkylguanines at *p*H 2, 7, and 12. Full lines represent the spectra of authentic 7-methylguanine. Points (○, □, △) are for the ethyl derivative of guanine, G1E, isolated from the reaction mixture of guanine and ethyl methanesulfonate at *p*H 2, 7, and 12, respectively, taken from Cary recordings.

A3E was identified spectrophotometrically as 3-ethyladenine (Fig. 1, *right*). It was reported earlier that 7-methyladenine is produced on methylation of adenine with dimethyl sulfate (Reiner and Zamenhof, 1957). We repeated the experiment under identical conditions with dimethyl sulfate; the spectrum of one of the resulting derivatives (A4M) was found to be identical with that of 3-methyladenine (Fig. 1, *right*) and not with that of synthetic 7-methyladenine (Fig. 2, *left*). Although A4M and 7-methyladenine have comparable λ_{max} values in acid and alkali, their spectra are entirely different. The isosbestic point at 283 mμ, characteristic of the spectrum of A4M and of authentic 3-methyladenine, is absent in the spectrum of 7-methyladenine. A4M and synthetic 3-methyladenine have identical R_F values in isopropanol-HCl, isopropanol-NH_3, and isobutyric acid-NH_3. The *pK* values of A4M and 7-methyladenine also differ (6.1 and 4.2 respectively). The fraction A4M and the authentic specimen of 3-methyladenine have the same isosbestic point and the same *pK* value of 6.1. These indicate that 7-methyladenine, even if it is formed during methylation of adenine with dimethyl sulfate, is not present in any significant amount in fraction A4M. Bautz and Freese (1960) were unable to detect 7-ethyladenine in the dialysate of DNA alkylated with diethyl sulfate. Rutman *et al.* (1961) were also unable to find any positive evidence for the formation of 7-substituted adenine on alkylation of DNA with nitrogen mustard (HN2). From these observations, it appears that the previous identification (Reiner and Zamenhof, 1957) of the derivative A4M as 7-methyladenine is in error.

Reiner and Zamenhof (1957) reported altogether four methylated derivatives of adenine: A1M, A2M, A3M, and A4M. Of these A2M, A3M, and A4M have now been identified spectrophotometrically as 9-methyladenine, 1-methyladenine, and 3-methyladenine respectively. A3M shows the same λ_{max} values at *p*H 4 and 13 and similar *pK* values as reported for 1-methyladenine (Brookes and Lawley, 1960). A1M is formed only in traces and has not been characterized.

One of the derivatives of guanine, G1E, was similarly identified as 7-ethylguanine (Fig. 2, *right*). The spectra of 7-methylguanine and 7-ethylguanine at *p*H 2 and 12 are very similar. The spectra at *p*H 7 show considerable difference in extinction values but they show clearly the same λ_{max} values at 248 and 282 mμ and λ_{min} values at 236 and 260 mμ. The spectra of 7-methylguanine and 7-ethylguanine are thus qualitatively similar and quantitatively different. Differences of this kind in the extinction values of methyl and ethyl analogs have been observed before. Koppel and Robins (1958a,b) report molar

extinction coefficients of 14,200 and 12,400 at 252 mμ at *p*H 1 for 9-methylguanine and 9-ethylguanine respectively.

The nature of the alkylated product may depend on the nature of the substance undergoing alkylation, the alkylating agent, and the *p*H. For instance, methylation of imidazoles substituted at the 4(5) position with NO_2, Br, or C_6H_5 yields mixtures of 1,4- and 1,5-isomers in proportions that depend on the substituent, on the methylating agent employed, and, notably, on the presence or absence of alkali in the reaction (Forsyth and Pyman, 1925). A variety of products from the methylation of nucleosides at different *p*H values has also been reported (Bredereck *et al.*, 1948). Since adenine forms 9-ethyladenine at *p*H 7 and guanine forms 7-ethylguanine at *p*H 12, the products from adenine after treatment with ethyl methanesulfonate at *p*H 4.0 and *p*H 12.0 were examined. The same products were found after treatment at *p*H 12.0 as were obtained at *p*H 7.0, while at *p*H 4.0 there was no alkylation of adenine.

The spectra of the three unidentified derivatives of guanine were compared with the spectra of 1-methylguanine, 7-methylguanine, deoxyguanylic acid, 8-methylguanine, and 6-hydroxy-2-methylaminopurine but were different from all of these. Deoxyguanylic acid, 9-methylguanine, and 9-ethylguanine should have similar spectra. The three spectra (Fig. 3) of the xanthine derivative obtained by nitrous acid treatment of G2E (Weissman *et al.*, 1957), the unidentified major derivative of guanine, is not similar to the spectra of 3-methylxanthine, 1,7-dimethylxanthine, 3,7-dimethylxanthine, or 1,3-dimethylxanthine (Cavalieri *et al.*, 1954). Both G2E and its xanthine derivative give a blue color with Folin phenol reagent (Hitchings, 1941).

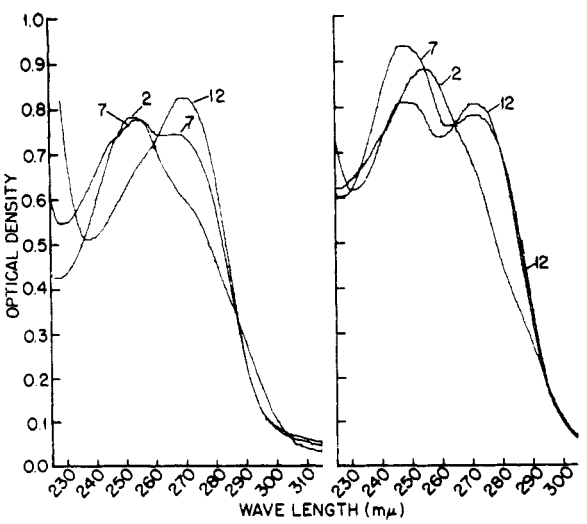
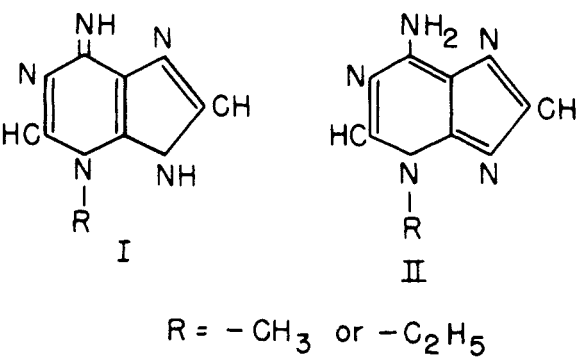


FIG. 3.—Ultraviolet-absorption spectra (Cary recording) of the ethyl derivative of guanine, G2E, isolated from the reaction mixture of guanine and ethyl methanesulfonate, at *p*H 2, 7, and 12, without (*left*) and with (*right*) nitrous acid treatment.

DISCUSSION

Although 1-ethyladenine shows a shift of 11 mμ in the position of λ_{max} between *p*H 2 and 12, 3-ethyladenine shows very little shift. 1-Ethyladenine has two *pK* values in this interval, whereas 3-ethyladenine has only one. The *pK* of 6.9 of 1-ethyladenine probably arises from the protonation of N-1 and the second *pK* of 11.4 from the dissociation of the imidazole —NH—. 1-Ethyladenine shows a higher λ_{max} at *p*H 12 than at *p*H 2, whereas 3-ethyladenine shows exactly the opposite behavior. Since 3-ethyladenine does not show an anionic *pK*, one might suspect the most likely structure is that represented in formula (1) as II rather than I, but we have been unable to find any more direct chemical evidence in support of the structure of II. If, as in structure II, the



NH_2 group of 3-ethyladenine remains intact, then it should react readily with nitrous acid and form 3-ethylhypoxanthine, but no change in the ultraviolet spectrum of 3-ethyladenine is detectable after nitrous acid treatment. 3-Methyladenine is converted into 3-methylhypoxanthine by boiling in aqueous alkali (Elion, 1957), whereas adenine under similar conditions remains unaffected (Kruger, 1892, 1894; Kossel, 1882, 1888). These considerations, although not very conclusive, suggest that the 3-alkyladenines have an imino group at the 6 position, as in structure I.

In agreement with earlier findings (Reiner and Zamenhof, 1957), N-7 of guanine is more reactive than N-9 whereas in adenine N-9 is more reactive than N-7. This is of interest in two respects. The influence of the 6-NH_2 group in adenine and the 2-NH_2 and 6-OH groups in guanine on the reactions of N-7 and N-9 in the imidazole ring with alkylating agents shows one kind of interaction between the two component rings of purines. There is also evidence for a reverse interaction as regards substitution in the imidazole ring and the reactivity of the N-1 and N-3 in the pyrimidine ring. However, before discussing this evidence, let us consider another implication of the difference in reactivities of the imidazole nitrogens of

adenine and guanine. In the known biosynthetic pathways the purine ring is formed after the sugar-phosphate moiety is attached to the potential 9 nitrogen atoms of the nucleotide. Hence, it is not surprising that the normal guanine nucleotide has the glycosidic linkage at the 9 position rather than the more reactive 7 position. If the sugar-phosphate moiety were attached after formation of the purine ring, guanine would tend to form a 7-glycosyl. This argument ignores the role of enzymes in altering chemical reactivities. The enzymatic synthesis of guanylic acid from guanine and a ribose compound has been reported by Kornberg *et al.* (1955), but the location of the ribosyl moiety has not been specified. 7-Purineglycosides are not unknown in nature. In contrast with the 9 linkage found in other natural adenine nucleotides, the presence of 7-ribosyl-adenine in pseudovitamin B12 has been reported by Friedrich and Bernhauer (1956, 1957).

The relative yields found for alkylation derivatives of adenine and adenylic acid can be compared with theoretical predictions of the reactivity of the various positions of adenine. Such predictions have been made for a number of purines by use of the calculated basicities of the various atoms in the rings (Pullman and Nakajima, 1958; Pullman, 1959). In addition, the reactivity of the nitrogen atoms was predicted to be highest for N-1, less for N-3, and very little for N-7. The observation that methylation of adenylic acid produced the 1-methyl derivative in highest yield has been taken as confirmation of the prediction (Brookes and Lawley, 1960; Pullman, 1959). However, it now appears that ethylation of adenine gives the 3-ethyl derivative in the highest yield and 1- and 9-ethyl derivatives both in considerably lower yield. While these results support the theoretical prediction of little or no reactivity for the 7 position, the discrepancies pointed out as to maximum yields indicate the limited applicability of the correlation of reactivity with calculations of basicity. Moreover, we do not see any correlation of the reactivity of adenine in solution with the observations, from x-ray crystallographic data on adenine hydrochloride hemihydrate, that protonation occurs on the N-1 and hydrogen bonding occurs on the N-1, N-6, and N-7 positions (Cochran, 1951; Pauling and Corey, 1956). Similar objections have been raised previously; Cochran's (1951) correlation of bond lengths obtained from crystallographic data with other properties of adenine as measured in solution has been criticized by Jordan (1955).

The comparison of our results for ethylation of adenine and the results for methylation of adenylic acid (Brookes and Lawley, 1960) do afford another indication of the important effect of substituents in one of the rings of a purine on reactivities at various positions within the other ring. Whereas, in adenine, N-3 on the pyrimidine ring is more reactive than the N-1, a 9-ribosyl on the imidazole ring causes N-1 to become more reactive than N-3. This fact, together with the previously noted differences in reactivity at the 7 and 9 positions of the imidazole rings arising from different substituents on the pyrimidine rings of guanine and adenine, demonstrates the two directions in which the interaction may occur between the two rings of a purine molecule. Similar cases of the influence of substituents on the imidazole ring on the reactivity of the substituents on the pyrimidine moiety of the purine ring system have been noticed before. For instance, 9-methylisoguanine was prepared from 2,6-dichloro-9-methylpurine through the stages of 2-chloro-6-amino-9-methylpurine and 2-ethoxy-6-amino - 9 - methylpurine. 7 - Methylisoguanine could not be obtained from 2,6-dichloro-7-methylpurine by the same route (Falconer *et al.*, 1939). Isoguanine does not readily react with nitrous acid (Bendich, 1955); on the other hand 9-ribosylisoguanine is deaminated by nitrous acid (Falconer *et al.*, 1939).

Acknowledgment

Thanks are due to Dr. David R. Krieg for suggesting this problem and to Dr. Waldo E. Cohn for his interest and many helpful suggestions during the course of this work.

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Uridine, Cytidine, and Deoxyuridine Derivatives*

T. K. Fukuhara and D. W. Visser

From the Department of Biochemistry, School of Medicine, University of Southern California, Los Angeles, California

Received August 30, 1961

5-(*p*-Chlorobenzylidene)-aminouridine, 5-(2′,4′-dichlorobenzylidene)-aminodeoxyuridine, 5-(3′,4′-dichlorobenzylidene)-aminouridine, 5-(3′,4′-dichlorobenzylidene)-aminodeoxyuridine, 5-bromoacetamidouracil, and 5-hydroxycytidine were synthesized, and the inhibitory effects of these compounds on growth of wild-type Neurospora and *Escherichia coli* K-12 were determined. All of the Schiff's base derivatives inhibited growth of Neurospora. Bromoacetamidouracil and hydroxycytidine were inactive. All the new compounds completely inhibited growth of *E. coli*. Inhibition reversal studies in *E. coli* indicate that amino acid metabolism, rather than nucleic acid metabolism, is involved in the inhibitory effects of the aminouridine Schiff's bases. The corresponding aminodeoxyuridine derivatives interfere with nucleic acid metabolism in *E. coli*.

The hydrogen at the 5 position of uracil and cytosine may be displaced readily by other substituents with intact pyrimidine or pyrimidine nucleosides used as starting materials. The 5 position is also the locus of numerous substitutions which take place biologically, such as the formation of thymine (Friedkin and Kornberg, 1957), hydroxymethylcytosine (Flaks and Cohen, 1959), methylcytosine (Johnson and Coghill, 1925), and pseudouridine (Cohen, 1959; Davis and Allen, 1957). This combination of circumstances has resulted in the synthesis of a number of 5-substituted derivatives which have been shown to produce a variety of interesting biological effects involving the formation or function of naturally occurring pyrimidine compounds (Handschumacher and Welch, 1960). This communication described the synthesis and biological activity of additional 5-substituted pyrimidine nucleosides.

* Aided by a research grant from the National Institutes of Health (CY-2372) United States Public Health Service.

The choice of chemical groups to be substituted at the 5 position was based on a desire to prepare compounds which might be useful for correlating the chemical nature of the 5 substituents with biological activity. Some substituents were selected because of their similarity to compounds previously shown to be interesting antimetabolites. Others were prepared as potential alkylating agents which retain structural characteristics of nucleic acid precursors. The derivatives, 5-aminouridine and 5-aminodeoxyuridine, which have been shown to inhibit growth of Neurospora (Roberts and Visser, 1952b) and bacteria (Beltz and Visser, 1957), were used as convenient starting materials for the preparations of Schiff's bases.

The 5-benzylidineaminouracil derivatives were of interest because of their structural similarity to 5-(3′,4′-dichlorophenyl)-6-(ethyl)-2,4-diaminopyrimidine, an antitumor drug (Sugiura, 1955), and to 5-(*p*-chlorophenyl)-6-ethyl-2,4-diaminopyridmidine (Falco *et al.*, 1951), an antimalarial drug; the latter has been shown to interfere with folic acid metabolism (Hitchings, 1952). These