Nucleosides. III. Studies on 5-Methylamino-2′-deoxyuridine as a Specific Antiherpes Agent

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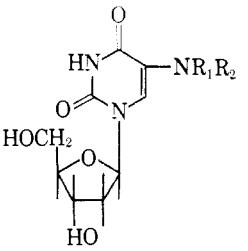
5-Methylamino-2′-deoxyuridine (I) was shown to be a specific antiherpes agent with very low toxicity in cell cultures and chick embryo. It has no activity against other DNA viruses like vaccinia and adeno 2. Replacement of the 5-methylamino group in I with 5-ethylamino and 5-dimethylamino resulted in reduction of potency and selectivity. The 5-amino, 5-hydroxy, 5-diazo, and 5-(N-acetylmethylamino) analogs are inactive. Some physical and biochemical properties of I are described. A tritium-labeled I was prepared for incorporation study.

The effectiveness of using nucleoside antimetabolites as selective antiviral agents was well demonstrated by the clinical efficacy of 5-iodo-2′-deoxyuridine (idoxuridine) in the treatment of herpes keratitis.[1-4] While the mechanism of antiviral action of idoxuridine, and of the more recently described 5-trifluoromethyl-2′-deoxyuridine,[5,6] remains to be clarified, it is well known that these nucleosides are readily incorporated into DNA *in vivo* and *in vitro*.[4,7-9] Inhibition of the synthesis and utilization of thymidylate, particularly at the viral kinase or polymerase stage, was also suggested as a possible mode of action.[10] Obviously, an inhibitor of viral-specific enzymes would be a safe and selective antiviral agent.

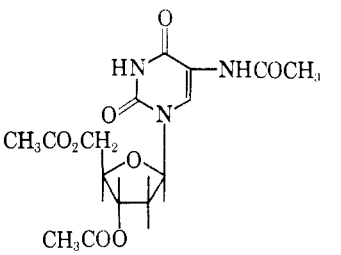
In order to explore the possibility of dissociating the property of DNA incorporation from viral kinase or polymerase inhibition, we have studied a number of 5-substituted pyrimidine-2′-deoxyribosides, assuming that the structural requirement at the 5 position would be different for these two modes of action. Our first compound was selected in the light of recent work by Visser and co-workers[11,12] in which 5-methylamino-2′-deoxyuridine was shown to be a new thymidine antimetabolite in several bacteria systems. It was noted that the van der Waals radius for a methylamino group is about 3.5 A, being much greater than the radii of 2–2.5 A for methyl, halogen, and trifluoromethyl groups. The basic nature of this nucleoside is also uniquely different from other thymidine antagonists. In contrast to idoxuridine, both the free base and its hydrochloride are very soluble in water and stable on standing.

In both tissue culture and rabbit eye assays, 5-methylamino-2′-deoxyuridine (MADU) was shown to have a potent and highly specific antiherpes activity comparable to that of idoxuridine.[13] On the other hand, it has an extremely low cytotoxicity. In grivet monkey kidney (GMK) cells the cytotoxic level is greater than 5 mg/ml, more than 2000 times that of the antiviral concentration. It also was not active against the human adenocarcinoma (HAd) no. 1 grown in the embryonated egg; a dose as high as 10 mg/egg was not toxic to the chick embryo.[14] The highly specific antiherpes activity of MADU was further emphasized by its apparent lack of activity against the several DNA and RNA viruses tested. It is well known that thymidine analogs, such as 5-bromo-,[15] 5-iodo-,[15] and 5-trifluoromethyl-2′-deoxyuridines[10] are active against several DNA viruses, for example, herpes, adeno, pseudo-rabies, polyoma, and vaccinia. For MADU, no activity against adeno 2 and vaccinia was found.[16] It was also inactive against two RNA viruses, Coxsackie B2, and parainfluenza in tissue culture.[16]

Encouraged by this discovery, several 5-alkylamino and 5-acylamino derivatives were synthesized and evaluated. The 5-amino analog was a more potent antimetabolite than MADU in the *Escherichia coli* system,[11] but was surprisingly ineffective against herpes (see Table I). The dimethylamino analog had little effect on *E. coli;* it showed slight antiviral activity with a therapeutic index of 16. Modest antiviral activity was found with the 5-ethylamino analog (IV), but the therapeutic index was only 8. No activity was found



I, $R_1 = H$; $R_2 = CH_3$
II, $R_1 = R_2 = H$
III, $R_1 = R_2 = CH_3$
IV, $R_1 = H$; $R_2 = C_2H_5$



V

(1) H. E. Kaufman, E. L. Martola, and C. Dohlman, *Arch. Ophthalmol.*, **68**, 235 (1962).
(2) H. E. Kaufman and E. D. Maloney, *Proc. Soc. Exptl. Biol. Med.*, **112**, 4 (1963).
(3) H. E. Kaufman, *Chemotherapia*, **7**, 1 (1963).
(4) H. E. Kaufman, *Postgrad. Med.*, **35**, 518 (1964).
(5) C. Heidelberger, D. G. Parsons, and D. C. Remy, *J. Am. Chem. Soc.*, **84**, 3597 (1962).
(6) C. Heidelberger, D. G. Parsons, and D. C. Remy, *J. Med. Chem.*, **7**, 1 (1964).
(7) A. D. Welch, P. Calabresi, W. H. Prusoff, W. A. Creasy, and R. W. McCollum, *Exptl. Cell. Res.*, **9**, 479 (1963).
(8) B. Djordjevic and W. J. Szybalski, *J. Exptl. Med.*, **112**, 509 (1960).
(9) E. D. Maloney and H. E. Kaufman, *Invest. Ophthalmol.*, **2**, 55 (1963).
(10) H. E. Kaufman and C. Heidelberger, *Science*, **145**, 585 (1964).
(11) D. W. Visser, S. Kabat, and M. Lieb, *Biochim. Biophys. Acta*, **76**, 463 (1963).
(12) S. Kabat and D. W. Visser, *Biochim. Biophys. Acta*, **80**, 680 (1964).
(13) M. M. Nemes and M. R. Hilleman, *Proc. Soc. Exptl. Biol. Med.*, **119**, 515 (1965).
(14) This experiment was carried out by Dr. Charles Gitterman of these laboratories, following the procedure published previously: C. O. Gitterman, E. L. Dulaney, E. A. Kaczka, D. Hendlin, and H. B. Woodruff, *Proc. Soc. Exptl. Biol. Med.*, **109**, 852 (1962).
(15) For a summary see A. S. Kaplan, T. Ben-Porat, and T. Kamiya, *Ann. N. Y. Acad. Sci.*, **130**, 226 (1965).
(16) M. M. Nemes and M. R. Hilleman of these laboratories, unpublished results.

TABLE I

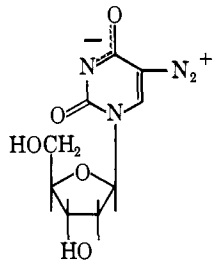
ANTIVIRAL ACTIVITIES OF 5-AMINO ANALOGS OF THYMIDINE[a]

Compd	5-Substituent	Cytotoxicity to GMK cells, μg/ml	Antiherpes, concn, μg/ml	Therapeutic index
II	NH_2	1250	>1250	—
I	$NHCH_3$	>5000	2.5	>2000
IV	NHC_2H_5	>400	50	8
III	$N(CH_3)_2$	>2500	150–300	>8–16
V[b]	$NHCOCH_3$	400	>400	
VI[c]	$NHCH_3$	400	>400	

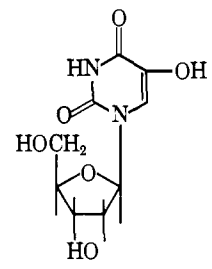
[a] Compounds tested by Drs. M. M. Nemes and M. R. Hilleman by methods previously reported.[13] [b] As 3′,5′-di-O-acetyl derivative. [c] Pyrimidine base, 5-methylaminouracil.

with the acetylated derivative (V), or with the pyrimidine base, 5-methylaminouracil (VI), itself.

Two more thymidine analogs, the zwitterion 5-diazo-2′-deoxyuridine (VII)[17] and 5-hydroxy-2′-deoxyuridine (VIII),[18] were also found to be inactive. Compound VIII was prepared by mild hydrolysis of 5-



VII



VIII

bromo-2′-deoxyuridine in aqueous sodium bicarbonate, a procedure adapted from the preparation of 5-hydroxyuracil.[19] Chemically, the ease of hydrolysis of the 5-bromo group is remarkable; other substitution reactions usually require much more strenuous conditions.[11,20] A comparison of these analogs is summarized in Table II. It should be noted that while inhibition of

TABLE II

COMPARISON OF THYMIDINE ANALOGS

5-Substituent	Antiviral activities: Herpes	Antiviral activities: Adeno 2 and vaccinia
F	−	−
Cl	−	−
Br	+	+
I	+	+
CF_3	+	+
$NHCH_3$	+	−
NHC_2H_5	+	−
N_2	−	−
OH	−	−

thymidylate synthetase may be contributary to the antiviral activity of idoxuridine and 5-trifluoromethyl-2′-deoxyuridine, 5-fluoro-2′-deoxyuridine (FUdR) does not possess similar therapeutic antiviral activities.

In an attempt to correlate the unique biological activity of MADU with its physical–chemical properties, a spectrophotometric study[21] was undertaken. Other pyrimidine nucleosides, such as the weakly acidic 2′-deoxyuridine and the weakly basic 2′-deoxycytidine show two limiting ultraviolet curves (neglecting the ionization of the sugar moiety at pH 13–14), corresponding to a neutral species and a dissociated species. However, ultraviolet measurements of MADU as a function of pH show three limiting curves in the pH 1–12 range (Figure 1). Isosbestic points common to the three curves are noted at 248 and 281 mμ. pK_{a_1} and pK_{a_2} values of 2.55 and 9.30 determined by spectrophotometric titration show MADU to be a weaker base than 2-deoxycytidine (pK_a = 4.3[21]) and to have an acid dissociation constant comparable to that reported for the 4-enolization of 2-deoxyuridine (pK_a = 9.3[21]). These data indicate a cationic, a neutral, and an anionic species which probably are resonance and tautomeric forms of structures A, B, and C. It would seem that at the physiological pH, the equilibrium composition favors the unprotonated species, B.

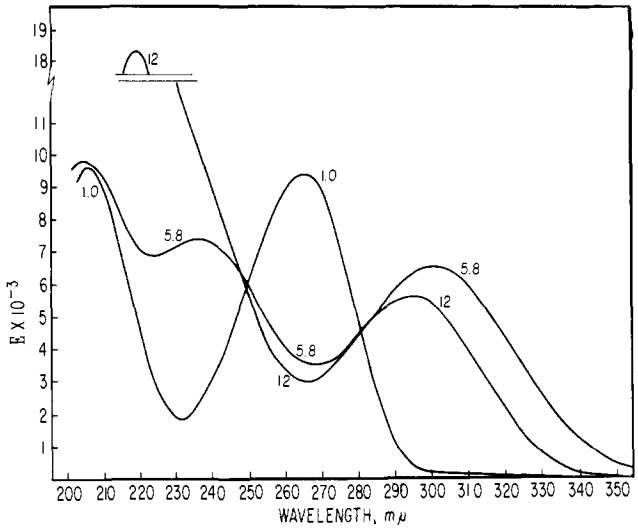
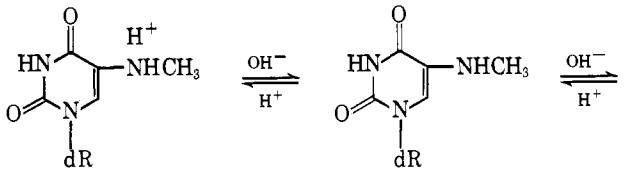
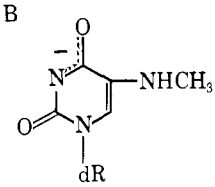


Figure 1.—Limiting ultraviolet absorption curves of the neutral and ionic species of MADU with the pH values indicated.



A B



C

The effect of change in the chromophore on the optical rotatory dispersion properties of MADU is given in Figure 2. These curves show a positive Cotton effect which recently has been shown[22] to be characteristic of the β-anomeric configuration for

(17) J. P. Paolini, R. K. Robins, and C. C. Cheng, *Biochim. Biophys. Acta*, **72**, 114 (1963).
(18) R. E. Beltz and D. W. Visser, *J. Am. Chem. Soc.*, **77**, 736 (1955).
(19) S. Y. Wang, *J. Am. Chem. Soc.*, **81**, 3786 (1959).
(20) T. Ueda, *Chem. Pharm. Bull.* (Tokyo), **10**, 788 (1962).
(21) J. J. Fox and D. Shugar, *Biochim. Biophys. Acta*, **9**, 369 (1952).
(22) T. L. V. Ulbricht, J. F. Jennings, P. M. Soopes, and W. Klyne, *Tetrahedron Letters*, **No. 13**, 695 (1964).

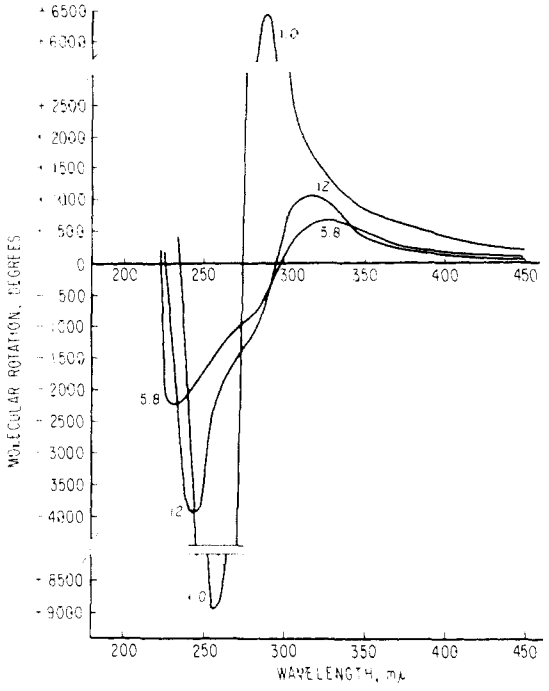
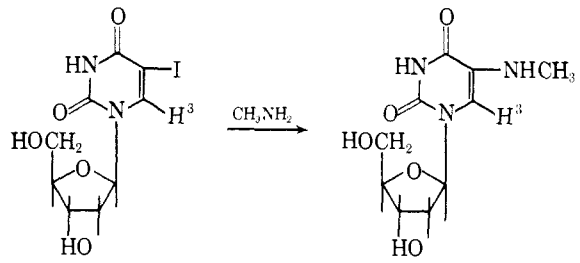


Figure 2.—Optical dispersion curves of the neutral and ionic species of MADU with the pH values indicated. The molecular rotation values are ±100° or ±10%, whichever is larger.

D-2′-deoxyribofuranosylpyrimidine nucleosides in water solutions.

The mechanism of action of 5-methylamino-2′-deoxyuridine remains to be elucidated. Its unique selective toxicity toward the herpes virus is certainly of some theoretical interest. MADU is cleaved by human spleen thymidine phosphorylase *in vitro*, but the rate is only about one-fourth that of thymidine. In an *in vitro* ascites cell system it is a very weak inhibitor of hypoxanthine and orotic acid incorporation, even at 500 μg/ml. It has no effect on protein synthesis.[23]



In order to study the incorporation of MADU, a 6-^{3}H-labeled nucleoside (IX) was prepared from labeled 5-iodo-2′-deoxyuridine-6-^{3}H in a manner similar to the amination of 5-bromo-2′-deoxyuridine. Incorporation studies using human amnion cells are being carried out by Dr. Richard Burg of these laboratories. Preliminary data have been noted.[13] Further studies on the metabolism and antiviral activity of this unique antiherpes nucleoside are in progress.

Experimental Section[24]

5-Methylamino-2′-deoxyuridine (I) was prepared by treatment of 5-bromo-2′-deoxyuridine with anhydrous liquid methylamine according to Visser.[13] An analytical sample was found to have the following physical properties: mp 178.5–179°; pK_{a_1} = 2.55, pK_{a_2} = 9.30 (±0.10 by ultraviolet spectrometric method in H_2O); $[\alpha]^{25}D$ +48° (*c* 1, 0.1 *N* HCl); $\lambda_{max}^{pH\ 1}$ 204, 266 mμ (ε 9400, 9300); at pH 5.8 $\lambda_{max}^{H_2O}$ 203, 237, 300 mμ (ε 9800, 7300, 6500); $\lambda_{max}^{pH\ 12}$ 218, 293 mμ (ε 18,600, 5800). Nmr spectra[25] at pH 12 and pH 5.8 were indistinguishable and showed singlet signals at τ 3.93 for the C-6 position and at 7.36 for the N-methyl protons. A progressive downfield shift of these signals to τ 2.18 and 7.10, respectively, was observed upon titration to pH 1 indicating a rapid equilibration of the active proton(s) in the acidic pH range with complete conversion to the protonated form, structure A, at pH 1.

5-Amino-2′-deoxyuridine hydrochloride (II) was prepared according to Friedland and Visser[26] except that 5-bromo-2′-deoxyuridine instead of the acetylated drivative was treated with anhydrous liquid NH_3. A 63% yield (500 mg) of II was obtained; mp 186–187°; $\lambda_{max}^{pH\ 1}$ 206, 265 mμ (ε 8300, 7950); $\lambda_{max}^{pH\ 12}$ 217, 289 mμ (ε 14,000, 5400).

5-Dimethylamino-2′-deoxyuridine (III) prepared by treatment of 5-bromo-2′-deoxyuridine with anhydrous liquid dimethylamine according to Visser, *et al.*,[13] has the following properties: mp 188–190°; $\lambda_{max}^{pH\ 1}$ 267 mμ (ε 9500); $\lambda_{max}^{pH\ 12}$ 220, 284 mμ (ε 13,000, 6200).

5-Ethylamino-2′-deoxyuridine (IV).—Treatment of 5-bromo-2′-deoxyuridine (1.00 g) with anhydrous liquid ethylamine (25 ml), in the same manner as for I, furnished 0.17 g (20%) of IV: mp 179–181°; $\lambda_{max}^{pH\ 1}$ 267 mμ (ε 9500); $\lambda_{max}^{pH\ 12}$ 212, 292 mμ (ε 18,100, 5600).

Anal. Calcd for $C_{11}H_{17}N_3O_5$: C, 48.70; H, 6.32; N, 15.49. Found: C, 48.82; H, 6.16; N, 15.39.

5-Amino-2′-deoxyuridine Triacetate (V).—5-Amino-2′-deoxyuridine hydrochloride (II) (106 mg) in 5 ml of dry pyridine and 5 ml of acetic anhydride was stirred at 25° overnight. The solution was concentrated to dryness *in vacuo*. Crystallization of the residue from hot water furnished 75 mg of product, colorless needles melting at 239–240°.

Anal. Calcd for $C_{15}H_{19}N_3O_8$: C, 48.78; H, 5.19; N, 11.38. Found: C, 48.98; H, 5.38; N, 11.18.

5-Methylaminouracil (VI) was prepared by modification of a previously reported method.[27] Treatment of 5-bromouracil (3.00 g) in 25 ml of anhydrous liquid methylamine at 80° for 18 hr furnished 0.79 g (36%) of VI, mp 294–296° dec, $\lambda_{max}^{pH\ 1}$ 260 mμ (ε 7600), $\lambda_{max}^{pH\ 12}$ 291 mμ (ε 4400).

5-Hydroxy-2′-deoxyuridine (VIII).—A solution of 5-bromo-2′-deoxyuridine (2.76 g, 9.0 mmoles) and sodium bicarbonate (1.13 g, 13.5 mmoles) in 90 ml of water was refluxed under nitrogen until the ultraviolet shift, $\lambda_{max}^{pH\ 12}$ 277 to 303 mμ, was complete (7 hr). The solution was diluted and treated batchwise with 30 ml of Dowex 50W-X4 (H^+ form). The acidic filtrate was neutralized by adding portions of Dowex 2-X8 (HCO_3^- form). The neutral filtrate was evaporated *in vacuo* at 50–60° in a rotary evaporator. The residue was dissolved in ethanol, and the solution evaporated. Repetition of this step gave 1.76 g of a near colorless residue. Crystallization several times from ethanol after concentration to a small volume furnished 0.63 g (28%) of VIII, mp 208–211° (lit.[18] mp 209–211°), pK_a = 7.8 (titrimetric method in H_2O), $\lambda_{max}^{pH\ 1}$ 281 mμ (ε 8300), $\lambda_{max}^{pH\ 12}$ 304 mμ (ε 6700).

5-Methylamino-2′-deoxyuridine-6-^{3}H (IX).—A 50% ethanolic solution containing 0.50 meurie of 5-iodo-2′-deoxyuridine-6-^{3}H (obtained from Schwarz BioResearch Inc.) with a reported specific activity of 0.30 curie/mmole was evaporated to dryness in a thick-walled glass tube (3 mm i.d. × 9.0 cm) under a stream of nitrogen. After the residue was dried *in vacuo* over P_2O_5, 9.0 mg of unlabeled iododeoxyuridine (IDU) and 0.15 ml of 10%

(23) This experiment was carried out by Dr. H. T. Shigeura of these laboratories, following the procedure published previously: H. T. Shigeura and C. N. Gordon, *J. Biol. Chem.*, **237**, 1932 (1962).

(24) Melting points were taken on a Kofler micro hot stage equipped with a stage-calibrated thermometer. Ultraviolet data were obtained using 0.1 *N* HCl (pH 1) and 0.01 *N* NaOH (pH 12) solution, a Bausch and Lomb Spectronic 505 recording spectrophotometer for qualitative measurements, and a Cary, *Model 11, recording spectrophotometer* for precise measurements. Counting measurements were determined using a toluene–ethanol (70:30) phosphor and a Packard Tri-Carb scintillation spectrometer, Model 3003.

(25) Spectra were obtained using a 7% concentration of MADU in D_2O solutions (adjustments of pH were made with DCl and NaOD) and a 60-Mc/sec high-resolution spectrometer. Benzene protons were used as an external standard and assigned a value of 3.50. Nmr analysis was performed by courtesy of Dr. Nelson R. Trenner and Mr. Byron Arison of these laboratories.

(26) M. Friedland and D. W. Visser, *Biochim. Biophys. Acta*, **51**, 148 (1961).

(27) T. B. Johnson and I. Marsico, *J. Am. Chem. Soc.*, **41**, 782 (1919).

anhydrous methanolic methylamine were added. The tube was sealed and heated at 50° for 65 hr in a rocking bomb.[28] The solvent was evaporated under a stream of nitrogen, and the residue was dried *in vacuo* over P_2O_5. The residue (15.9 mg) was taken up in water and run into a column (5 mm i.d. × 15 cm) containing 3 ml of Dowex 50W-X4 (H^+ form). Neutral, ultraviolet-absorbing materials were removed by washing with water, and then the product was eluted with 1.0 *N* NH_4OH. Evaporation *in vacuo* in a rotary evaporator in a water bath (25–50°) followed by drying *in vacuo* over P_2O_5 furnished 6.6 mg of crude product (ultraviolet spectra were qualitatively indistinguishable from the standard). The product was further purified on a column (5 mm i.d. × 22 cm) of silica gel (J. T. Baker, 3405) (2.5 g), packed in methylene chloride–methanol (97:3). The column was developed with the same solvent. Upon increasing the methanol concentration to 6%, the product was eluted in six 10-ml cuts. The four center cuts were combined on the basis of ultraviolet and counting data and also by radiopapergram analysis. Concentration *in vacuo* furnished 2.8 mg (0.12 mcurie, 11 mcuries/mmole) of amorphous product IX which was indistinguishable from MADU by ultraviolet analysis (optical density ratio comparisons) and homogeneous by radiopapergram analysis using unlabeled MADU as standard.

Radiopapergram Analysis.—Whatman No. 1 filter paper strips and 2-propanol–concentrated (38%) HCl–water (75:8:17) solution were used for descending-type papergram analysis. Sections of the paper were placed in phosphor solution and read directly in a liquid-scintillation counter.[24] MADU-6-H^3 was differentiated from possible interfering contaminants (see Table III).

TABLE III

Standards	R_f values
5-Methylaminouracil	0.22
MADU	0.32
Uracil	0.63
2-Deoxyuridine	0.73
5-Iodouracil	0.75
IDU	0.80

In Vitro **Cleavage Studies of Substituted 5-Amino-2′-deoxyuridines.**—The assay for determining the cleavage of nucleosides by human spleen thymidine phosphorylase was carried out by Dr. Morris Zimmerman of these laboratories essentially as described[29] for measuring the formation of 2′-deoxyribose 1-phosphate. The nucleoside was incubated with the enzyme in phosphate buffer. The cleavage rate of thymidine was taken as 100%. The rates with the 5-substituted 2′-deoxyuridines are as follows: 5-amino (II), 36%; 5-methylamino (I), 27%; 5-dimethylamino (III), 0%.

Acknowledgment.—The authors wish to thank Mr. Richard N. Boos for microanalytical data, Mr. Alec Kalowsky for ultraviolet measurements, Mr. Edward A. MacMullan for spectrophotometric analysis (p*K* and isosbestic point determinations), Dr. Charles Rosenblum for assistance with radioactivity measurements, and Dr. James J. Wittick for ORD analysis.

(28) IDU was completely destroyed using the same conditions, 80° for 18 hr, found successful for amination of BrDU. A series of 10-mg runs with unlabeled IDU was performed to determine optimum conditions.

(29) G. E. Boxer and C. E. Shonk, *J. Biol. Chem.*, **233**, 535 (1958).

Anticancer and Potential Antiviral Activity of Complex Inorganic Compounds

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Recent studies indicate that viruses are associated with many types of cancerous tumors. The authors propose that complex inorganic compounds might be successfully used in the alteration of these viruses with the consequent destruction or diminution of their activity. Twenty-six complex inorganic compounds have been prepared in this study, and among them are five which are active against certain cancers, including complexes containing coordinating agents which are themselves carcinostatic, such as 6-mercaptopurine. Both the platinum(IV) and palladium(II) complexes of 6-mercaptopurine have been found to be extremely active against Adenocarcinoma 755 and Sarcoma 180.

Despite considerable controversy in recent years regarding the possible role of viruses as cancer-producing agents, there is no longer any question that certain types of cancer are virus caused and some scientists[1] feel that most, if not all cancers, have one or more viruses associated with them. Since viruses contain proteins and nucleic acid units, some atoms of which are excellent coordinating agents for metal ions, it was decided to study the possibility of altering the virus *via* its coordination and/or chelation to metal ions with the expectation that this alteration will result in the elimination or diminution of its viral activity.

Discussion

Perhaps the most important aspect of this problem involves "appropriate" introduction of a metal ion into the vicinity of a virus so that complexation or coordination may occur. Mere introduction of simple or hydrated metal ions into living organisms will not suffice because of their almost certain coordination to one or more of the many nonviral proteins, amino acids, and other coordinating groups found in most parts of the animal system. Consequently, this investigation is concerned with the introduction of metal ions in the form of moderately stable complex inorganic compounds (coordination compounds), the ligands of which might possibly be displaced by viruses, thus forming new complexes with the metal ion, for example

$$m(\mathrm{Vi}) + [\mathrm{M}^{+x}(\mathrm{L}^{-y})_n]^{x-ny} = [\mathrm{M}^{+x}(\mathrm{Vi})_m]^{+x} + n\mathrm{L}^{-y} \quad (1)$$

where Vi represents a virus; M, a metal ion; L, a ligand; and where m and n represent the number of molecules of virus and ligand, and x and y represent the charges on the metal ion and ligand, respectively.

Another important aspect of this problem is finding and capitalizing upon some difference between viral systems which may cause abnormal growth and the many benign coordinating systems in animals, because

(1) S. E. Luria, *Cancer Res.*, **20**, 669 (1960).