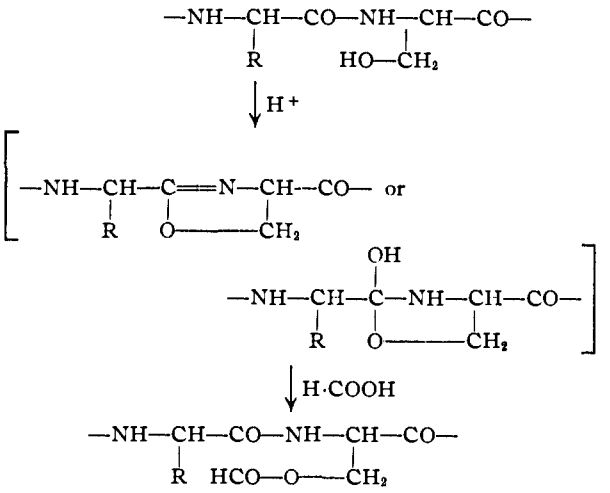
in the proteins. The possibility that contaminating traces of formaldehyde in the radioactive formic acid were bound to the protein was ruled out by the finding of negative results on formaldehyde estimation.[25] Since all radioactivity in the 25% sulfuric acid hydrolysate of the radioactive protein sample were recovered in the distillate in alkaline solution, the protein bound radioactive material must be formic acid. The amounts of radioactive carbon bound by various proteins seemed to be in parallel with their total hydroxyamino acid content (serine + threonine) as summarized in Table III. Therefore the main reaction of formic acid with proteins must be O-formylation of their hydroxyamino acid residues. O-Formylation of the tyrosine residue appears not to occur in anhydrous formic acid, since the paper chromatographic behavior and ultraviolet absorption spectra in both acid and alkali solution of the formic acid treated N-acetyl-L-tyrosine were the same as for the untreated compound.

Since serine did not give O-formylserine by reaction with anhydrous formic acid, O-formylation of the hydroxyl group of serine (and threonine) probably takes place with the assumed intermediate oxazoline or hydroxyoxazoline compound

Owing to the great differences in the stability of the O-formyl groups of proteins observed in the present experiments, it was not possible to estimate the exact number of formyl groups introduced into the protein by the reaction between formic acid and protein. Formic acid may nevertheless prove to be useful as a specific reagent for the hydroxyl group of serine (and threonine) residues in protein, since it appears to be milder and possibly more specific than concentrated sulfuric acid.[22]



Acknowledgments.—The author is grateful to Dr. H. Fraenkel-Conrat for suggestions and discussion throughout this investigation and to Mr. H. Shichi for technical assistance.

BERKELEY, CAL.

(25) H. Fraenkel-Conrat, M. Cooper and H. S. Olcott, THIS JOURNAL, 67, 950 (1945).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, SCHOOL OF MEDICINE, UNIVERSITY OF SOUTHERN CALIFORNIA]

5-Bromodeoxycytidine and 5-Chlorodeoxycytidine[1]

BY DAVID M. FRISCH AND DONALD W. VISSER

RECEIVED DECEMBER 11, 1958

Two new deoxynucleoside derivatives, 5-chlorodeoxycytidine and 5-bromodeoxycytidine, have been prepared by a photocatalytic process. The inhibitory effect of these derivatives on the turbidity and viability of a thymine requiring mutant of *Escherichia coli* and a pyrimidine-requiring mutant of Neurospora are described.

Introduction

Several 5-halopyrimidines, their ribosides and deoxyribosides, have interesting inhibitory effects upon growth of bacteria, bacteriophage, molds and tumors. Some of these antimetabolites inhibit thymine synthesis, others prevent thymidine utilization, and depending to some extent on the size of the halogen,[2,3] the abnormal pyrimidine may be incorporated into either RNA or DNA. Shive and Skinner[4] have summarized these activities in a recent review. The unusual activities of the above derivatives suggested the synthesis of two new derivatives, 5-chlorodeoxycytidine and 5-bromodeoxycytidine, by a photocatalytic method similar to that reported for the synthesis of 5-halocytidine.[5] In addition to the possible usefulness of these derivatives in studies of nucleic acid metabolism, genetics and as chemotherapeutic agents, the new compounds are potential replacements for 5-methyldeoxycytidine found in some plant and animal DNA and 5-hydroxymethyldeoxycytidine in the coliphage DNA of the even varieties.

Experimental

5-Bromodeoxycytidine.—Deoxycytidine (2.15 g., 0.0095 mole), was dissolved in 155 ml. of anhydrous acetic acid, 110 ml. of anhydrous pyridine was added and the solution chilled to −10°. The cold solution was exposed to ultraviolet light at a distance of 9–10 cm. and 1.53 g. (0.0097 mole) of bromine in 10 ml. of cold anhydrous carbon tetrachloride was added with constant stirring. The ultraviolet source was a Lamp Projector, model 93LS, manufactured by the Keese Engineering Co. of Hollywood, Calif. The amber-colored solution was removed from the ultraviolet light and allowed to stand at room temperature overnight. The solvents were removed at temperatures not exceeding 40° using reduced pressure until a thick sirup was obtained. This material was dissolved in 30 ml. of absolute methanol and again reduced to a sirup. This process was repeated five more times or until a semi-crystalline white solid was

(1) This investigation was supported by the National Institutes of Health, Grant No. C2372.

(2) S. Zamenhof, B. Reiner, R. De Giovanni and K. Rich, *J. Biol. Chem.*, 219, 165 (1956).

(3) D. B. Dunn and J. B. Smith, *Nature*, 174, 305 (1954).

(4) Shive and Skinner in *Ann. Rev. Biochem.*, 27, 643 (1958).

(5) T. K. Fukuhara and D. W. Visser, THIS JOURNAL, 77, 2393 (1955).

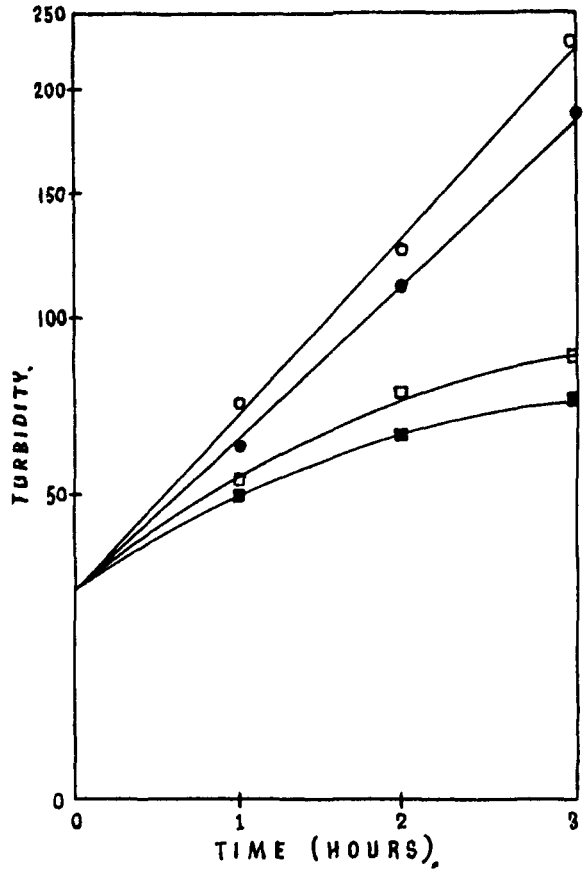


Fig. 1.—Effect of 5-chlorodeoxycytidine on turbidity of *Escherichia coli* 15T⁻ cells, plotted on logarithmic scale. Each curve represents a different concentration of inhibitor: O, control; ●, 3.3×10^{-5} *M*; □, 1.6×10^{-4} *M*; ■, 3.3×10^{-4} *M*.

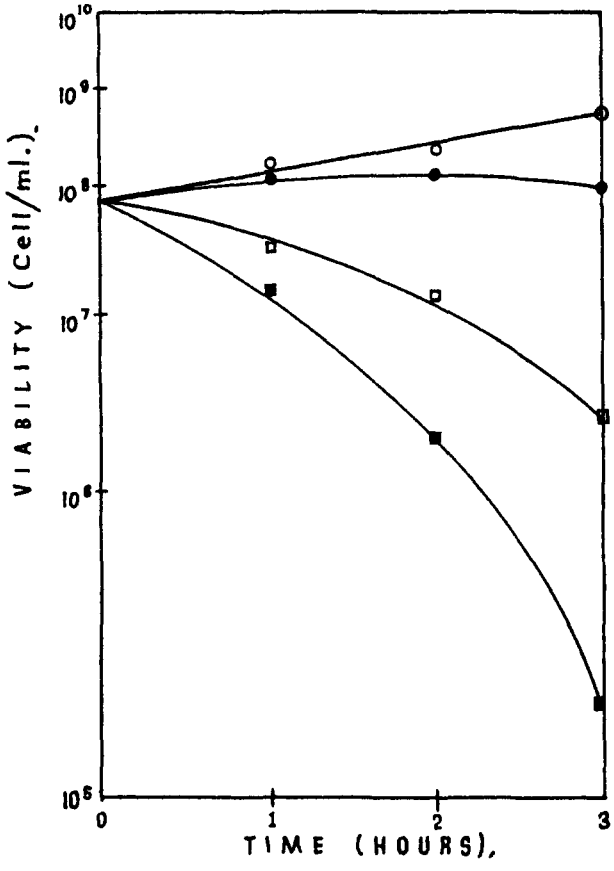


Fig. 2.—The effect of 5-chlorodeoxycytidine on viability of *Escherichia coli* 15T⁻ cells, plotted on a logarithmic scale. Each curve represents a different concentration of inhibitor: O, control; ●, 3.3×10^{-5} *M*; □, 1.65×10^{-4} *M*; ■, 3.3×10^{-4} *M*.

obtained. To this substance was added 200 ml. of cold absolute methanol solution containing 5–6% anhydrous ammonia. The mixture was shaken until the solid dissolved and then allowed to stand at room temperature for three days. Methanol and ammonia were removed at room temperature and reduced pressure. The amber-colored sirupy material remaining was dissolved in a minimum amount of water and passed through an Amberlite IR-120 resin (chloride form) column (2.2 × 25 cm.). The column was washed thoroughly with water to remove deaminated material. Ammonium hydroxide (0.4 *N*) was passed through the column and the eluate, containing 5-bromodeoxycytidine, was lyophilized leaving 2.8 g. of an amorphous, tan material. This material was dissolved in a minimum volume of hot absolute methanol, treated with Norite and filtered. Ethyl acetate was added to the filtrate until the solution became cloudy. The solution was then allowed to remain uncovered at room temperature until crystallization occurred. Successive crystallizations yielded 1.9 g. (62%) of 5-bromodeoxycytidine, m.p. 175–179°, $\lambda_{max}^{pH\ 2}$ 300 mμ (ε 9,600). *Anal.* Calcd. for $C_9H_{12}O_4N_3Br$: C, 35.31; H, 3.95; N, 13.73. Found: C, 35.39; H, 3.89; N, 13.76.

5-Chlorodeoxycytidine was prepared in an analogous manner to the 5-bromodeoxycytidine, yield 38%, m.p. 184–186°, $\lambda_{max}^{pH\ 2}$ 295 mμ (ε 9,980). *Anal.* Calcd. for $C_9H_{12}O_4N_3Cl$: C, 41.31; H, 4.62; N, 16.06. Found: C, 41.36; H, 4.71; N, 16.50.

Microbiological.—The deoxycytidine derivatives were tested for their effect on the growth of Neurospora using methods described previously.[6] Both compounds competitively inhibit growth of the pyrimidine-requiring mutant, Neurospora 1298, when either uridine or cytidine are added as the growth requirement. As observed with the halo derivatives of uridine and cytidine,[5] the chloro derivative is more effective than the bromo derivative and uridine is a more effective reversing agent than cytidine. In the presence of sufficient cytidine to satisfy the pyrimidine requirement of the mutant and a concentration of chloro- or bromodeoxycytidine which inhibits growth completely, deoxyuridine or deoxycytidine reverses the inhibition, whereas thymidine is ineffective. Neither of the derivatives had an apparent effect on the growth of wild type Neurospora.

Escherichia coli cells (thymine-requiring mutant 15T⁻) were grown aerobically in the synthetic medium of Cohen and Abrogast[7] supplemented with 1 γ/ml. of thymine. When about 70% of maximal growth was reached, aliquots of the culture were transferred to fresh medium so that the final cell count was 7–9 × 10^7 cells/ml. The fresh medium contained thymine to give a final concentration of 1 γ/ml. and varying amounts of inhibitor to give final molar concentrations of 3.3×10^{-5}, 1.65×10^{-4} and 3.3×10^{-4}. Samples were taken at 0, 1, 2 and 3-hour intervals. Turbidity was measured in a Klett-Summerson colorimeter using a 420 mμ filter. Viability was determined by spreading small aliquots of diluted cultures on broth agar plates.[8] The highest concentration of 5-chlorodeoxycytidine produced a 40% decrease in turbidity as compared to the control at 3 hours; see Fig. 1. The viability dropped rapidly during the 3-hour period until less than 1% of the cells remained viable; see Fig. 2. The curves for similar concentrations of 5-bromodeoxycytidine were substantially the same as for the 5-chloro derivative. Microscopic examination showed that the inhibited cells were about 5 times the size of the controls.

(6) M. Roberts and D. W. Visser, *J. Biol. Chem.*, **194**, 695 (1952).

(7) S. S. Cohen and R. Abrogast, *J. Exptl. Med.*, **91**, 619 (1950).

(8) S. S. Cohen, *J. Biol. Chem.*, **168**, 511 (1947).

The bromodeoxycytidine derivative produces a high frequency of plaque-type mutations in T-2 bacteriophage.[9] Screening results at Sloan-Kettering Institute[10] have shown that 5-chlorodeoxycytidine has an inhibitory effect on tumor growth (Ca 755) in rodents, whereas the 5-bromo derivative is inactive.

Discussion

All of the 5-chloro- and 5-bromopyrimidine ribosides and deoxyribosides previously tested competitively inhibit utilization of uridine by the pyrimidine-requiring mutant, Neurospora 1298. The competitive nature of these inhibitions,[5,6] and the fact that the wild-type Neurospora is not inhibited by comparatively high concentrations of these compounds indicate that the primary site of inhibition in the nutant probably involves the conversion of uridine or cytidine to the nucleotide by nucleoside kinase.

In *E. coli*, 15T$^-$ the deoxycytidine derivatives inhibit growth and decrease viability in a manner similar to that described for 5-bromodeoxyuridine.[11] Thus, it is possible that the deoxycytidine derivative exerts its effect by a prior deamination to bromodeoxyuridine and subsequent incorporation into DNA in place of thymidine.[12] However, other interesting changes in DNA structure may result from the presence of the deoxycytidine derivative, such as incorporation into DNA in lieu of deoxycytidine, or formation of an abnormal base as with 5-aminouracil.[13] These possibilities are under investigation.

The high frequency of plaque-type mutations in T-2 bacteriophage produced by bromodeoxycytidine also suggests the possibility that the analog may be incorporated into phage DNA either as the halogenated deoxyuridine, in place of thymidine, if deamination takes place, or as the halogenated deoxycytidine in place of viral 5-hydroxymethyldeoxycytidine. If the latter occurs, it would be an interesting model for chemotherapy application.

(9) J. Gregory, private communication.
(10) Data of D. Clarke, K. Sugiura and C. C. Stock.
(11) S. S. Cohen and H. D. Barner, *J. Bacteriol.*, **71**, 588 (1956).
(12) S. S. Cohen and H. D. Barner, *J. Biol. Chem.*, **226**, 631 (1957).
(13) D. B. Dunn and J. B. Smith, *Nature*, **175**, 336 (1955).

LOS ANGELES, CALIF.

[CONTRIBUTION FROM THE SUBDEPARTMENT OF SYNTHETIC CHEMISTRY IN RELATION TO MEDICAL RESEARCH, BANTING AND BEST DEPARTMENT OF MEDICAL RESEARCH, UNIVERSITY OF TORONTO]

Synthesis of L-α-(Dioleoyl)-cephalin; with a Comment on the Stereochemical Designation of Glycerolphosphatides[1]

BY ERICH BAER AND DMYTRO BUCHNEA

RECEIVED JUNE 30, 1958

A synthesis of L-α-(dioleoyl)-cephalin is reported. The unsaturated cephalin is obtained by phosphorylation of D-α,β-diolein with phosphorus oxychloride and quinoline, immediate esterification of the resulting L-α-dioleoylglycerylphosphoryl dichloride with 2'-hydroxyethylphthalimide in the presence of pyridine, and removal of the protective phthaloyl group by hydrazinolysis. The configurational and structural purity of L-α-(dioleoyl)-cephalin was confirmed by catalytic reduction to L-α-(distearoyl)-cephalin, and comparison of its specific rotation with that of authentic material. The infrared spectrum of L-α-(dioleoyl)-cephalin, and the solubility of the substance in various solvents are reported.

The synthesis of structurally and configurationally pure glycerolphosphatides containing unsaturated fatty acid residues has lagged behind that of the saturated analogs, although unsaturated phosphatides are known to occur more widely in nature. It is only in the last few years that improvements in the techniques for the separation of lipid mixtures have made possible the synthesis of such unsaturated compounds *via* less protected intermediates than those used in the saturated series. Two years ago we reported the synthesis of L-α-(dioleoyl)-lecithin.[2] The syntheses of the nitrogen-free phosphatides, dioleoyl L-α-glycerylphosphoric acid,[3a] tetraoleoylbis-(L-α-glyceryl)-phosphoric acid[3a] and (dioleoyl-L-α-glycerylphosphoryl)-L-α-glycerol[3b] are reported by us in two recent papers. The purpose of the present paper is to describe the synthesis of L-α-(dioleoyl)-cephalin. It is interesting to note that oleic acid appears to be the principal unsaturated fatty acid in naturally occurring cephalins.[4,5]

As mentioned in an earlier publication[2] the procedures developed in this Laboratory for the synthesis of saturated α-phosphatides, employing phenylphosphoryl dichloride as phosphorylating agent, are not suitable for the synthesis of unsaturated phosphatides, since the removal of the phenyl group by catalytic hydrogenolysis would lead to a simultaneous reduction of the fatty acid double bonds. At the time it was, however, felt desirable to retain the use of phenylphosphoryl dichloride for the synthesis of dioleoyllecithin and a method was devised which made this possible.[2] The L-α-(dioleoyl)-lecithin was obtained *via* the following sequence of intermediates: D-acetoneglycerol → acetone-L-α-glycerylphenylphosphoryl chloride → acetone-L-α-glycerylphenylphosphoryl ethylene chlorohydrin → L-α-glycerylphosphoryl ethylene chlorohydrin → L-α-dioleoylglycerylphosphoryl ethylene chlorohydrin → L-α-dioleoylglycerylphosphoryl-choline. It was hoped that L-α-(dioleoyl)-cephalin could be obtained by using the same procedure,

(1) The synthesis of L-α-(dioleoyl)-cephalin was reported in a lecture presented at a "Symposium on Phosphoric Esters and Related Compounds" held by the Chemical Society at Cambridge, England, April 9-12, 1957.
(2) E. Baer, D. Buchnea and A. G. Newcombe, THIS JOURNAL, **78**, 232 (1956).
(3) (a) E. Baer and D. Buchnea, *Arch. Biochem. and Biophys.*, **78**, 294 (1958); (b) *J. Biol. Chem.*, **232**, 895 (1958).
(4) J. Parnas, *Biochem. Z.*, **22**, 411 (1909).
(5) C. G. MacArthur and L. V. Burton, THIS JOURNAL, **38**, 1375 (1916).