a reflection of the general lability of purine desoxyribosyl compounds rather than an indication of the position of esterification of phosphate on the desoxyribose residue for, as shown in Fig. 4, the phosphoester linkage of desoxyadenylic acid is more labile in 1 *N* sodium hydroxide than yeast or muscle adenylic acids.

CLEVELAND 6, OHIO RECEIVED AUGUST 28, 1950

[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

The Isolation and Identification of Desoxy-5-methylcytidylic Acid from Thymus Nucleic Acid[1]

BY WALDO E. COHN

Ion-exchange fractionation of enzymatic digests of DNA from thymus has resulted in the isolation of a desoxynucleotide of 5-methylcytosine in amounts small compared to the amount of cytidylic acid present. This confirms the earlier reports on the isolation of 5-methylcytosine from acid digests of DNA and suggests that this base occurs in desoxyribonucleic acids as a nucleotide similar to the others, being released upon enzymatic digestion.

Introduction

Since the time that Johnson and Coghill reported[2a] the isolation, from an acid hydrolysate of tuberculinic acid, of a substance crystallographically identical with synthetic 5-methylcytosine,[2b] it has been an open question as to whether this substance was a *bona fide* constituent of nucleic acids in the usual combination with a pentose phosphate. Johnson and Harkins[3] demonstrated that none of this material could be isolated from "yeast nucleic acid." Aside from this observation, there was no follow-up of the original finding; it remained an uncorrelated oddity until very recently when Hotchkiss observed,[4] in the base mixtures derived from desoxyribonucleic acid preparations, a small amount of a cytosine-like base which he termed "epicytosine." Although it seemed likely to him that this substance was 5-methylcytosine, the amounts available for study were too small to permit identification.

In the course of isolating gram amounts of the desoxyribonucleotides from enzymatically degraded DNA,[5] the presence of a hitherto unobserved substance was detected in the desoxycytidylic acid fraction.[6] From approximately 800 mg. of the crude desoxycytidylic acid, enough of this substance (*ca.* 20 mg.) was isolated to permit identification of it as the desoxypentosyl phosphate of 5-methylcytosine. The isolation and identification of this nucleotide are described in this communication.

Experimental

The source of 5-methylcytidylic acid was the desoxycytidylic acid fraction prepared from thymus DNA by Volkin, Khym and Cohn.[5] The separation was performed on the same ion-exchange column (33 sq. cm. × 12 cm. 200–500 mesh strong-base anion exchanger, in formate form) with 0.003 *M* formic acid. The 5-methylcytidylic acid fraction was reabsorbed on a smaller column and refractionated to remove the last traces of desoxycytidylic acid; this was necessary because of the very low amount (*ca.* 3%) of the new nucleotide present in the original desoxycytidylic acid. The desired fraction was concentrated by reabsorption and elution with 0.1 *M* formic acid. All subsequent tests were carried out upon this formic acid solution which contained approximately 2 mg. per ml. Phosphate[7] and desoxypentose determinations[8] upon the nucleotide and spectrophotometric comparison of the derived base with synthetic 5-methylcytosine, kindly supplied by Dr. Hitchings[9] of Wellcome Laboratories, were carried out in this Laboratory, as were all ion-exchange tests. Paper chromatographic analyses of the nucleotide, the derived base, and the enzymatically deaminated[10] derivative of the base (thymine), as well as spectrophotometric comparison of both bases with synthetic substances, were most generously made by Drs. Erwin Chargaff and Jacob Kream of Columbia University.

Results

(a) Identification of the Base.—Hydrolysis of the nucleotide was accomplished by heating for 40 minutes on a steam-bath in 72% perchloric acid.[10a] The digest was diluted tenfold with water and poured through a 1 cm. × 1 sq. cm. bed of sulfonic-acid type cation-exchanger to recover the pyrimidine base which was then eluted with ammonium hydroxide. This substance had the spectrophotometric properties, compared to synthetic 5-methylcytosine,[9] shown in Fig. 1. It could not be absorbed upon a strong base anion-exchanger except in the hydroxy form, which is characteristic of cytosine alone among other nucleic-acid bases.[11]

Hydrolysis[12] in 99% formic acid at 175° for 2 hours followed by paper chromatographic analysis[13] in butanol–water for 16 hours gave but one component detectable by ultraviolet fluorescence.[14] This component lay in the same position as 5-methylcytosine (R_F 0.28), which differs from cytosine (0.22), uracil (0.36) and thymine (0.52). When eluted from the paper, it had a spectrum identical with that of synthetic 5-methylcytosine. Another portion of the formic acid hydrolysate, containing 159 μg. of the pyrimidine, was incubated with puri-

(1) Work performed under Contract W-7405-eng-26 for the Atomic Energy Commission.

(2) (a) T. B. Johnson and R. D. Coghill, THIS JOURNAL, **47**, 2838 (1925); (b) H. L. Wheeler and T. B. Johnson, *Am. Chem. J.*, **31**, 591 (1904); *cf.* ref. 10.

(3) T. B. Johnson and H. H. Harkins, THIS JOURNAL, **51**, 1779 (1929).

(4) R. D. Hotchkiss, *J. Biol. Chem.*, **175**, 315 (1948).

(5) E. Volkin, J. X. Khym and W. E. Cohn, THIS JOURNAL, **73**, 1533 (1951).

(6) W. E. Cohn, *ibid.*, **72**, 2811 (1950).

(7) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques and Tissue Metabolism," Burgess Publishing Co., Minneapolis, Minn., 1949, p. 190.

(8) S. S. Cohen, *J. Biol. Chem.*, **156**, 691 (1944).

(9) G. H. Hitchings, *et al.*, *ibid.*, **177**, 357 (1949).

(10) E. Chargaff and J. Kream, *ibid.*, **175**, 993 (1948); *Fed. Proc.*, **9**, 192 (1950).

(10a) A. Marshak and H. J. Vogel, *ibid.*, **9**, 85 (1950).

(11) W. E. Cohn, *Science*, **109**, 377 (1949).

(12) The results reported in the remainder of this paragraph were obtained by Drs. Kream and Chargaff at Columbia University upon a sample of the nucleotide furnished by the author.

(13) E. Vischer and E. Chargaff, *J. Biol. Chem.*, **176**, 703 (1948).

(14) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

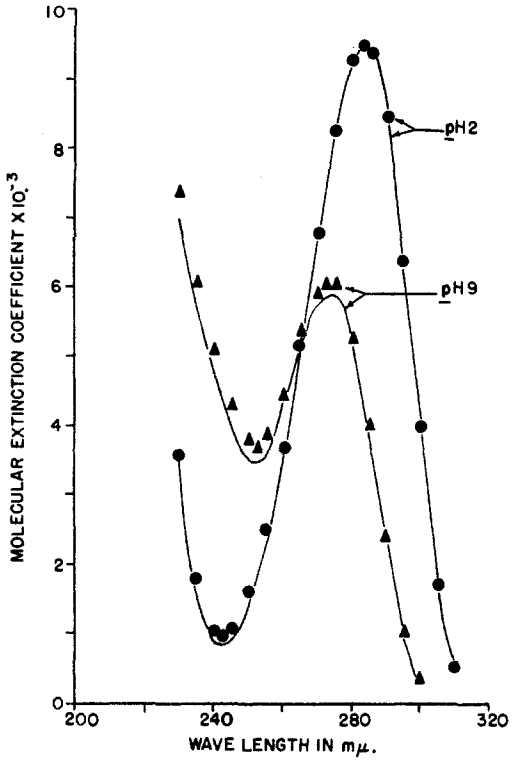

Fig. 1.—Spectra of synthetic 5-methylcytosine (solid line) and isolated substance (points: ●*p*H 2; ▲*p*H 9) normalized to 285 mμ on the *p*H 2 curve.

fied cytosine deaminase from yeast[10] (0.4 *M* phosphate buffer at *p*H 6.8, 90 minutes, 31°); the digest contained but one ultraviolet-fluorescing component, identical in R_F value and in spectrum with thymine (produced in about 80% of theoretical yield) and capable, in 3-μg. amounts, of promoting growth of a thymine-requiring *Escherichia coli* mutant which did not respond to the enzyme nor to 100 μg. of 5-methylcytosine.

(b) **Properties of the Nucleotide.**—Paper chromatographic analysis of the original nucleotide solution in an isoamyl alcohol–disodium phosphate system[14] (carried out by Dr. Carter of this Laboratory) gave but one spot in the area to which all other pyrimidine nucleotides migrate.[12] However, in the isobutyric–ammonia system,[14a] it is easily separated from adenylic, guanylic and cytidylic (ribo-) acids and gives but one spot.

The content of phosphate, desoxypentose and 5-methylcytosine, using the quoted extinction[9] of the synthetic material to assess the latter, and with desoxyadenylic acid[5] as a desoxypentose standard, was 1.6:1.0:1.8; with desoxycytidylic acid as a standard, it was 1.6:2.4:1.8. In our hands, the desoxypentose colorimetric test was not a quantitative procedure for the individual desoxynucleotides; it should be noted, however, that an average of the above values, which might result if DNA itself were used as the standard, would be 1.6:1.7:1.8. The reason for the discrepancies is not known but may lie in different rates of hydrolysis of the nucleotides used.

The ultraviolet absorption spectra of the nucleotide, which are similar to those of the other cytidylic acids, are shown in Fig. 2. As in the case of the

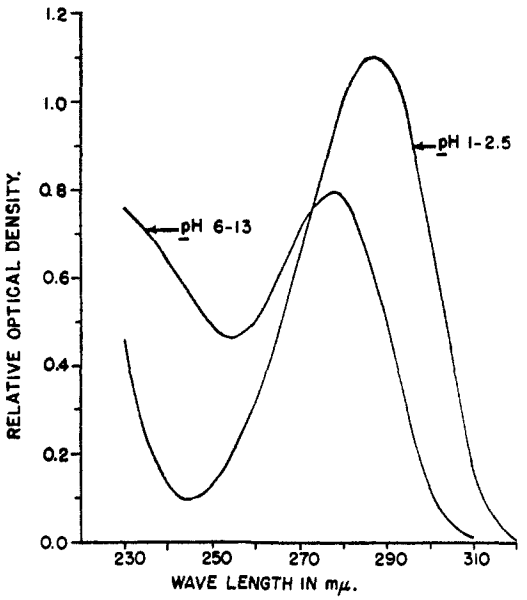

Fig. 2.—Spectra of desoxy-5-methylcytidylic acid.

three cytosine nucleotides and riboside, this compound exhibits an acid form and a neutral form only; no basic form exists below *p*H 12 at least. This is induced from the data of Fig. 3, indicating the variation with *p*H of arbitrarily chosen spectrophotometric extinction ratios.

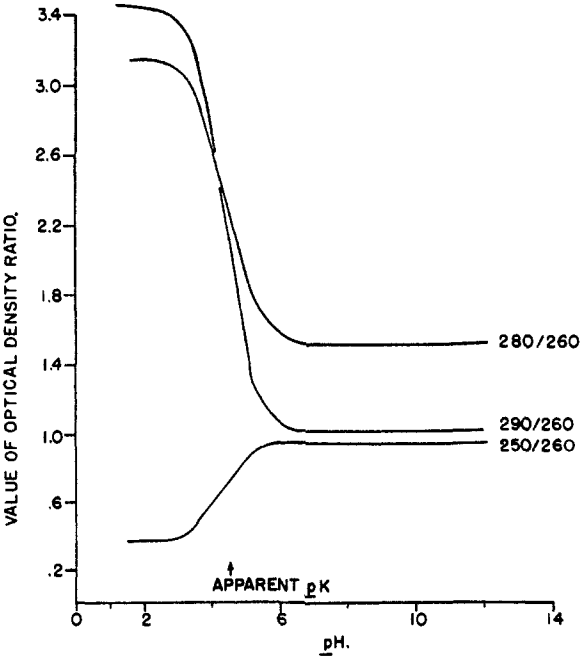

Fig. 3.—Variation in spectral constants of desoxy-5-methylcytidylic acid as functions of *p*H.

Discussion

Although lacking the nicety of a crystalline compound and elementary analyses thereof, the evidence presented above does indicate that there ex-

(14a) Magasanik, Vischer, Doniger, Elson and Chargaff, *J. Biol. Chem.*, **186**, 37 (1950).

ists, in enzymatic digests of thymus nucleic acid, a substance possessing the properties and constitution of a desoxynucleotide with 5-methylcytosine as its base component. Unless it is assumed that desoxycytidylic acid is partially methylated (or thymidylic acid partially aminated) during the enzymatic digestion period, the desoxynucleotide of 5-methylcytosine must be regarded as a constituent of thymus nucleic acid analogous to those of adenine, guanine, thymine and cytosine.

Two factors set this nucleotide apart from the others, however. One is its low molar concentration, which here approximates 3% of the amount of desoxycytidylic acid found (Wyatt[15] has reported, in preliminary form, analyses of DNA samples derived from a multitude of biological sources which indicate that 5-methylcytosine may range from zero to as high as 30% of the cytosine found). The other is that 5-methylcytosine is structurally related to both of the two pyrimidine bases of DNA, thymine and cytosine.

(15) G. R. Wyatt, *Biochem. J.* (*Proceedings*), **47**, vii (1950); *Nature*, **166**, 237 (1950).

OAK RIDGE, TENNESSEE RECEIVED OCTOBER 5, 1950

[CONTRIBUTION NO. 1439 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Acetyl- and Nicotinyl-L-tryptophanamide in Aqueous Solutions at 25° and *p*H 7.9[1]

BY H. T. HUANG AND CARL NIEMANN[2]

The kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tryptophanamide have been determined at 25° and *p*H 7.9 and it has been shown that the hydrolytic reaction is competitively inhibited by the corresponding acylated L-amino acids and by the D-antipodes of the above substrates.

The α-chymotrypsin catalyzed hydrolysis of certain acylated α-amino acid amides to the corresponding acylated α-amino acids and ammonia[3] is one of the simplest reactions that can be used for the determination of the mode of action of this enzyme. Therefore, with this latter goal in mind we have examined the kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-tryptophanamide at 25° and *p*H 7.9 in an aqueous solution 0.02 molar with respect to a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. In this study consideration was given to (a) the possibility of interaction of the enzyme with the buffer components; (b) the dependence of the activity of the enzyme upon the *p*H of the reaction system; (c) the possibility of inhibition of the hydrolytic reaction by one or both of the hydrolysis products; (d) the development of suitable rate expressions and the determination of rate constants; and (e) the possibility of inhibition of the hydrolysis of the above substrates by their D-antipodes. Since it is known[3] that α-chymotrypsin requires neither a coenzyme nor an activator it shall be assumed that the catalytically active species is identical with crystalline α-chymotrypsin.

In a previous study[4] it was found that the activity of α-chymotrypsin, when determined by its effect upon the extent of hydrolysis of nicotinyl-L-tryptophanamide, in systems buffered at *p*H 7.8 with either a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, an ethylenediamine–hydrochloric acid buffer, or a potassium phosphate buffer, was independent of the nature of the buffer. Furthermore, with the first two buffers the activity of the enzyme was found to be independent of the concentration of the buffer when it was varied from 0.005 molar to 0.05 molar with respect to the amine component.[4] Therefore, in the subsequent discussion consideration will be limited to a reaction system containing only enzyme, *i.e.*, α-chymotrypsin, substrate and reaction

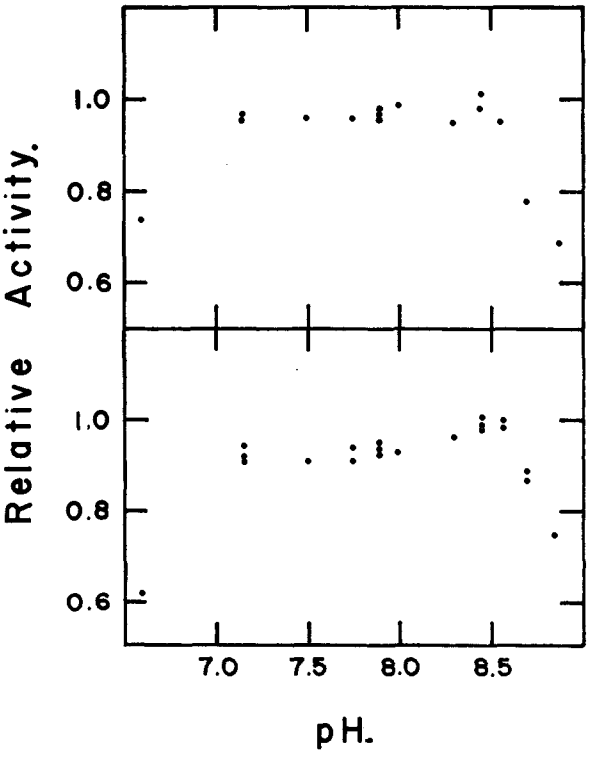

Fig. 1.—Lower half; acetyl-L-tryptophanamide, $[S]_0 = 10 \times 10^{-3}$ *M*, [E] = 0.208 mg. protein-nitrogen per ml.: upper half; nicotinyl-L-tryptophanamide, $[S]_0 = 10 \times 10^{-3}$ *M*, [E] = 0.069 mg. protein-nitrogen per ml.

(1) Supported in part by a grant from Eli Lilly and Co.

(2) To whom inquiries regarding this article should be sent.

(3) See H. Neurath and G. Schwert, *Chem. Revs.*, **46**, 69 (1950), for a resumé of previous studies on the enzymatic properties of α-chymotrypsin.

(4) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).