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

Enzymatic Evidence for the Structure of Desoxyribonucleotides

BY C. E. CARTER

Desoxyribonucleotides prepared by enzymatic degradation of thymus desoxyribonucleic acid and isolated by ion-exchange chromatography are dephosphorylated by an enzyme isolated from bull semen which hydrolyzes the phosphate group of nucleotides esterified in the 5′-position and is inactive against ribonucleotides derived from ribonucleic acids. Desoxyadenylic acid is deaminated by muscle adenylic acid deaminase. The phosphate group of desoxyadenylic acid is labile in acid and alkali, whereas the phosphate group of adenosine-5′-phosphate is relatively resistant to hydrolysis. These data are interpreted to support a 5′-nucleotide structure of desoxynucleotides.

The position of the phosphoester linkage in desoxyribonucleotides has been considered analogous to that demonstrated for several ribonucleotides derived from ribonucleic acid, namely, the third carbon of the carbohydrate moiety of the molecule.[1] Although the finding of isomeric ribonucleotides raises the question of C_2-phosphoester linkages in these compounds,[2,3] which as yet remains unsettled, similar considerations do not apply to desoxyribonucleotides because of the absence of the C_2-hydroxyl. Furthermore, there is no evidence for isomerism in the desoxyribonucleotides.[4] The position of the phosphoester linkage must then be either C_3 or C_5. Evidence for the structure of the desoxyribonucleotides isolated by ion-exchange procedures[4] has, in the present investigation, been obtained from experiments employing these compounds as substrates for a specific 5′-nucleotidase purified from bull semen by Heppel,[5a,5] (and also Mann[6]) and, in the case of desoxyadenylic acid, muscle adenylic acid deaminase. It has been found that desoxyadenylic, -guanylic, -cytidylic and -thymidylic acids as well as muscle adenylic acid are all dephosphorylated by the 5′-nucleotidase, whereas riboadenylic, -guanylic, -cytidylic and -uridylic acids are not substrates for the enzyme. Desoxyadenylic acid is deaminated by a muscle adenylic acid deaminase at a rate slower than that for adenosine-5′-phosphate but to complete deamination.

The hydrolysis of the phosphate group of the desoxynucleotides in acid and alkali has been studied and it was found that the phosphoester groups of desoxyadenylic and desoxyguanylic acid are hydrolyzed in 1 *N* hydrochloric acid at approximately the same rate as riboadenylic and riboguanylic acids. In 1 *N* sodium hydroxide inorganic phosphate is liberated more rapidly from desoxyadenylic acid than from muscle adenylic acid or yeast adenylic acid ("A" and "B").[2,3] It is probable that the chemical lability of desoxyribose[7] as contrasted with ribose accounts for the hydrolytic behavior of the phosphoester in the desoxynucleotides and makes comparative rate studies of hydrolysis of the phosphoester groups with the analogous ribonucleotides questionable evidence for structure.

Because of the well-defined specificity of the 5′-nucleotidase and muscle adenylic acid deaminase[8] the finding that desoxynucleotides are substrates for the enzymes supports a 5′-phosphoester structure for these compounds, a conclusion which must remain tentative until proof of structure by chemical degradation and synthesis is established.

Experimental

The 5′-nucleotidase employed in these studies was generously supplied by L. A. Heppel of the National Institutes of Health. Specificity studies conducted in this Laboratory confirm those published by Heppel.[5] The preparation and characterization of the desoxyribonucleotides is described by Volkin, Khym and Cohn.[4]

Muscle adenylic acid deaminase was prepared by first extracting myosin from rabbit muscle by the procedure described by Szent-Gyorgy,[9] separating this fraction from the insoluble proteins by pressing through cheese cloth and then extracting the deaminase from the latter portion by homogenizing for 30 minutes in 4 volumes of 0.1 *N* ammonium acetate. Following centrifugation in the cold, a turbid supernatant solution was obtained which contains most of the original muscle deaminase activity.

Enzymatic Assay with 5′-Nucleotidase.—Substrates were dissolved in *p*H 6.2 veronal buffer to give a concentration of 100 μg. of nucleotide phosphorus per ml. The enzyme was dissolved in distilled water to give a concentration of 0.5 mg. per ml. For enzymatic assay 0.1 ml. of enzyme was incubated at 38° with 0.25 ml. of substrate and 0.05 ml. of 0.01 *M* magnesium chloride solution. Tubes were removed at intervals and inorganic phosphorus determined by the Fiske–SubbaRow method. All desoxyribonucleotides were completely dephosphorylated by the enzyme but at different rates as shown in Fig. 1. None of the mononucleotides derived from ribonucleic acid were degraded by the enzyme. Muscle adenylic acid (adenosine-5′-phosphate) and desoxyadenylic acid were enzymatically dephosphorylated at the same rate.

Enzymatic Assay with Muscle Adenylic Acid Deaminase.—Adenosine-5′-phosphate and desoxyadenylic acid were dissolved in 0.1 *N* succinate buffer *p*H 6.0, to give a concentration of 15 μg. of nucleotide per ml. To 3 ml. of these solutions, 0.1 ml. of enzyme solution was added and the digest incubated in spectrophotometer cuvettes at 26°. The course of the enzymatic deamination was followed spectrophotometrically by Kalckar's method.[5] As shown in Fig. 2, both adenosine-5′-phosphate and desoxyadenylic acid are deaminated by the muscle enzyme, the rate for the latter substrate being slower although both reactions go to complete deamination. Yeast adenylic acids (adenylic "A" and "B")[2,3] are not substrates for the enzyme. The products of the enzymatic reactions were identified by paper chromatography.

Acid and Alkaline Hydrolysis.—The desoxynucleotides were adjusted to a concentration of 25 μg. of nucleotide phosphorus in 1 ml. of 1 *N* hydrochloric acid and placed in

(1) H. Brederek, "Fortschritte der Chemie organischer Naturstoffe," Band I, Julius Springer, Berlin, 1948, p. 121.

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

(3) W. E. Cohn, *ibid.*, **72**, 1480 (1950).

(4) E. Volkin, J. X. Khym and W. E. Cohn, *ibid.*, **73**, 1533 (1951).

(5a) In a personal communication Heppel has reported that this enzyme hydrolyzes nicotinamide ribose-5′-phosphate and synthetic uridine-5′-phosphate and cytidine-5′-phosphate as well as adenosine- and inosine-5′-phosphate.

(5) L. A. Heppel, *Federation Proc.*, **9**, 184 (1950).

(6) J. T. Mann, *Biochem. J.*, **39**, 345 (1945).

(7) W. R. Tipson, "Chemistry of Nucleic Acids in Advance in Carbohydrate Chemistry," Vol. 1, Academic Press, New York, N. Y., 1945, p. 198.

(8) H. M. Kalckar, *J. Biol. Chem.*, **167**, 445 (1947).

(9) A. Szent-Gyorgy, "Muscular Contraction," Academic Press, New York, N. Y., 1946, p. 136.

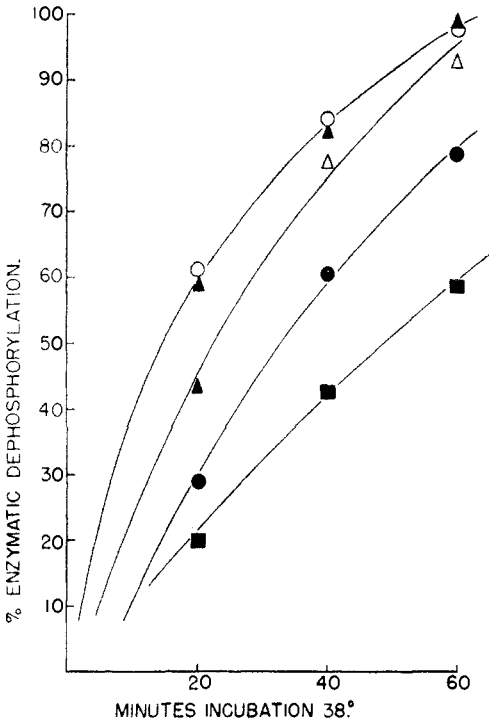

Fig. 1.—Enzymatic dephosphorylation of desoxyribonucleotides by 5′-nucleotidase. The monoribonucleotides derived from yeast ribonucleic acid were not dephosphorylated by the enzyme: O, desoxyadenylic acid; ▲, muscle adenylic acid; △, desoxyguanylic acid; ●, desoxycytidylic acid; ■, thymidylic acid.

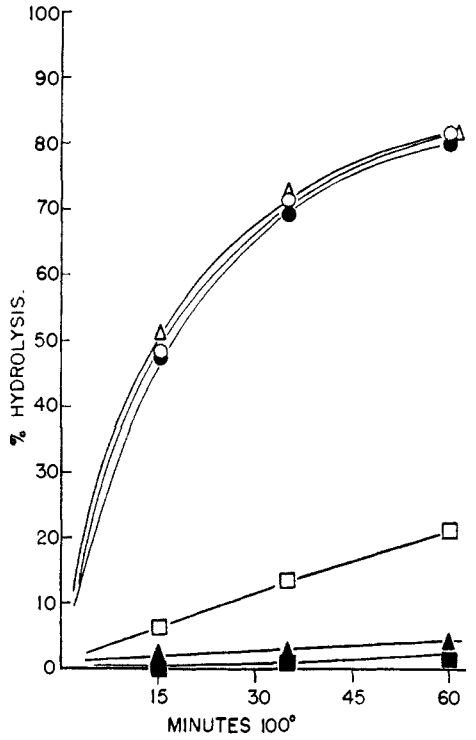

Fig. 3.—Acid hydrolysis of the phosphoester linkage in desoxyribonucleotides and yeast and muscle adenylic acids: △, desoxyguanylic acid; O, desoxyadenylic acid; ●, adenylic acids "A" and "B"; □, desoxycytidylic acid; ▲, muscle adenylic acid; ■, thymidylic acid.

a boiling water-bath in stoppered tubes. The rate of hydrolysis of the several compounds including yeast and muscle adenylic acids is shown in Fig. 3. The acid hydrolysis curves for yeast and desoxyadenylic acids are identical and in contrast with the relatively stable phosphate linkage in adenosine-5′-phosphate. This is interpreted as

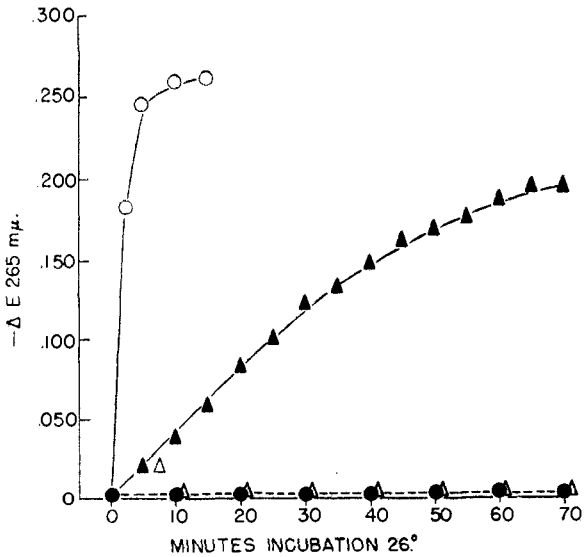

Fig. 2.—Deamination of adenosine-5′-phosphate and desoxyadenylic acid by muscle adenylic acid deaminase: O, muscle adenylic acid; ▲, desoxyadenylic acid; ●}△, yeast adenylic acids.

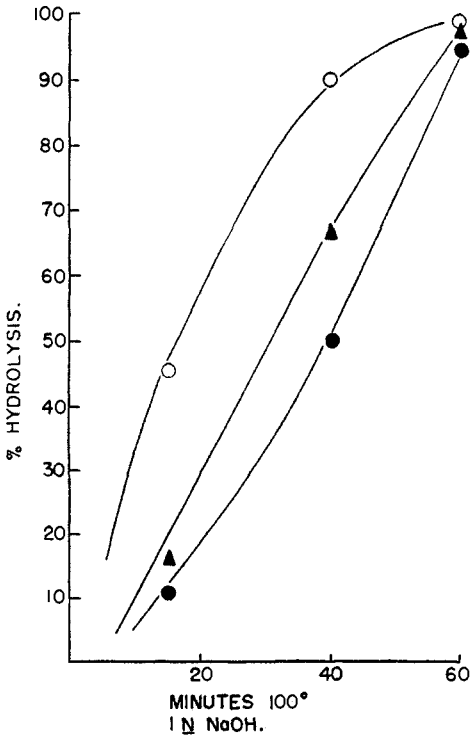

Fig. 4.—Alkaline hydrolysis of the phosphoester linkage o desoxyadenylic, yeast adenylic and muscle adenylic acids: O, desoxyadenylic acid; ▲, yeast adenylic acids; ●, muscle adenylic acid.

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).