TABLE VIII

PHYSICAL CONSTANTS AND ANALYSES

N-Acetyl derivative of	M.p.,[a] °C.	$[\alpha]_D$,[b] deg.	Calcd. for	Analyses, % Calculated C	H	N	Found C	H	N
L-Butyrine	131	−40.0(c)	$C_6H_{11}O_3N$	49.7	7.6	9.7	49.6	7.8	9.7
D-Butyrine	131	+40.0(c)					49.6	7.9	9.6
L-Norvaline	100	−35.0(c)	$C_7H_{13}O_3N$	52.8	8.1	8.8	52.8	8.2	8.9
D-Norvaline	100	+35.0(c)					52.5	8.6	8.8
L-Norleucine	112	−20.0(c)	$C_8H_{15}O_3N$	55.5	8.7	8.1	55.6	9.0	8.2
D-Norleucine	112	+20.0(c)					55.3	8.9	8.1
L-Heptyline	108	− 8.0(d)	$C_9H_{17}O_3N$	57.7	9.1	7.5	57.9	9.3	7.6
D-Heptyline	108	+ 8.0(d)					57.9	9.2	7.6
L-Capryline	105	+ 7.5(e)	$C_{10}H_{19}O_3N$	59.7	9.5	7.0	60.1	9.7	6.9
D-Capryline	105	− 7.5(e)					60.1	9.7	7.0
L-Valine	168	+ 7.5(c)	$C_7H_{13}O_3N$	52.8	8.1	8.8	52.5	8.4	8.7
D-Valine	168	− 7.5(e)					52.8	8.5	8.8
L-Aspartic acid	142	+57.0(e)	$C_6H_9O_5N$	41.1	5.1	8.0	41.1	5.2	8.1
D-Aspartic acid	142	−57.0(e)					41.3	5.5	8.0

[a] All melting points corrected. [b] All solutions at 1–2% at 25°. [c] In water. [d] In 50% acetic acid. [e] In glacial acetic acid.

solved in this solution and the total volume brought to 38 l. Two hundred mg. of acylase I powder was dissolved in the solution which was then incubated at 38°. The initial rate of hydrolysis under these conditions was 6600 μmoles of substrate hydrolyzed per hour per mg. N. Less than one-third the amount of enzyme was employed than customary in the absence of added cobalt. The reaction was complete in 24 hr., but the solution was allowed to stand for another 24 hr. In working up each of the optical antipodes[18] no difficulty due to the presence of Co^{++} was encountered, and the isomers were obtained in a state of high optical and chemical purity, in yields of 50–60% of the theoretical.

(18) J. P. Greenstein, L. Levintow, C. G. Baker and J. White, *J. Biol. Chem.*, **188**, 647 (1951); J. P. Greenstein, S. M. Birnbaum and L. Levintow, *Biochem. Preparations*, **3**, 84 (1953).

Product of Hydrolysis of Acetyl-D-alanine by Acylase I in the Presence of Co^{++}.—The action of acylase I on acetyl-D-methionine led to a product which was isolated in good yield and identified as D-methionine.[8] The experiment was repeated with acylase I using acetyl-D-alanine as substrate and in the presence of 4×10^{-2} Co^{++}. At the end of the reaction D-alanine was isolated in 62% of the theoretical yield, $[\alpha]^{25}D$ −30.4 (*c* 0.6, glacial acetic acid). The rotation of L-alanine under these conditions has been reported as +29.4°.[8]

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BETHESDA, MARYLAND

[CONTRIBUTION FROM THE DEPTS. OF ANATOMY, MICROBIOLOGY AND IMMUNOLOGY, AND BIOCHEMISTRY, MARQUETTE UNIVERSITY SCHOOL OF MEDICINE]

A Deoxyribonuclease of *Micrococcus pyogenes*[1]

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Micrococcus pyogenes var. *aureus* was found to release large quantities of a calcium-activated deoxyribonuclease (DNase) into the culture medium when grown with vigorous aeration. This enzyme was unusually stable and could be purified by boiling, followed by precipitation from the culture fluid with $(NH_4)_2SO_4$ and a series of washings, first with saturated NH_4Cl containing 2.5% trichloroacetic acid, and then with 83% ethanol. The *p*H optimum was 8.6. After completion of the action of the *M. pyogenes* DNase on deoxyribonucleic acid, studies with the aid of ion-exchange chromatography indicated that the number of bonds in the substrate which were attacked was greater than that for pancreatic DNase and less than that for snake venom phosphodiesterase. Mononucleotides liberated by the action of the bacterial enzyme were not dephosphorylated by snake venom 5′-nucleotidase, indicating that *M. pyogenes* DNase, unlike pancreatic DNase and various phosphodiesterases, could split the 5′-phosphodiester bond in deoxyribonucleic acid.

In the course of a survey of deoxyribonucleases (DNases) from various sources, it was found that an enzyme present in the supernatant fluid of centrifuged cultures of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) had several unusual properties. It required calcium, instead of magnesium, as cationic activator. Prior to separation from the original culture medium, it could be boiled with little or no loss of activity. The enzyme passed through a dialyzing membrane in easily detectable quantities, providing further evidence that it was a rather small molecule.

The unusual stability of the enzyme gave promise that it might be obtainable sufficiently free of interfering enzymes, so that a study of the mechanism of action on deoxyribonucleic acid (DNA) could be undertaken. After prolonged digestion of the substrate with various preparations of the enzyme, mononucleotides and other small molecules were obtained in rather large yield, without the production of inorganic phosphate in detectable quantity. The patterns produced by chromatog-

(1) A preliminary report was presented at the meeting of the American Chemical Society in Minneapolis, September, 1955.

raphy of these digests indicated that the extent of degradation was intermediate between that brought about by the action of pancreatic DNase alone, and that obtained by the action of pancreatic DNase followed by that of venom phosphodiesterase.

In the following, the term "micrococcal DNase" will be applied to thermostabile calcium-activated enzyme obtained from *M. pyogenes* var. *aureus*, without necessarily implying that these bacteria produce only one DNase.

Materials and Methods

Materials.—DNA was prepared by the method of Kay, *et al.*,[2] either unmodified ("old" DNA) or using a Spinco Model L ultracentrifuge for throwing down impurities in steps five, eight and nine ("new" DNA) instead of using the Servall SS-1.[3] After complete digestion with micrococcal DNase, much less insoluble material was produced, using the "new" DNA, then using the "old." This precipitate contained no detectable inorganic phosphorus and less than 1% of the total phosphorus of the DNA preparations, but was strongly biuret positive.

Dowex 1, 2% cross-linked, was washed with acid and alkali as recommended by Sinsheimer.[4] Deoxycytidylic acid (diammonium salt), thymidylic acid (calcium salt), deoxyadenylic acid and a relatively impure sample of deoxyguanylic acid—all prepared by the method of Hurst, *et al.*,[5] were purchased from the California Foundation for Biochemical Research, 3408 Fowler St., Los Angeles 63, California, and served as standard deoxyribomononucleotides. Venom of *Crotalus adamanteus* was purchased from Ross Allen Reptile Institute, Silver Springs, Florida, and a one mg./ml. solution of the crude venom served as a stock solution of 5'-nucleotidase.

Viscometric Assay of DNase.—The method of Laskowski and Seidel[6] was used, with certain modifications. The volumes of all reagents were reduced tenfold, but activities of enzyme solutions, as units per ml., were calculated as if one ml. of the enzyme solution had been used, as in the original method. The reagents were mixed in an 18 by 37 mm. test-tube and 0.7 ml. was drawn into a microviscometer[7] which was then placed in a 37° water-bath for determination of the flow time of the mixture at intervals. All flow times were corrected by subtracting the flow time of water as determined in the same viscometer under the same conditions. After an initial lag period of about ten minutes, inconstantly observed, the plot of the corrected flow times on semilogarithmic paper fell as a straight line for 15–20 minutes, and this line was used for calculating units of enzyme activity. With one unit of activity, there was a reduction of 0.001 per minute in the logarithm of the corrected flow time. Units calculated using corrected flow times were about one-half as large as those calculated by the original method. For some reason which was not understood, a series of dilutions had to be made with each sample of enzyme which was to be tested, till a dilution was found to give ten units or less.

Bacterial Cultures.—All typical strains of *M. pyogenes* var. *aureus* (as judged by production of golden pigment and coagulase, and by growth and acid production on mannitol salt agar) which were tested were found to produce micrococcal DNase. The majority of strains freshly isolated from clinical sources produced 2,000 to 10,000 units per ml. under optimum culture conditions; one (designated as Strain SA-B)[8] routinely gave over 100,000 units per ml. Stocks were stored in the refrigerator at 2–5° on slants of Bacto nutrient agar (Difco) containing 0.5% yeast extract. Before inoculation, the strain to be used was serially subcultured several times on nutrient yeast agar plates incubated at 37°. Typical 20-hour colonies were emulsified in broth and 10^8 to 10^9 cells were inoculated into liter quantities of sterile Bacto brain-heart infusion (Difco) in 4-liter flasks. The flasks were placed on an Eberbach variable speed shaking apparatus and incubated at room temperature for 48–72 hours. Maximum enzyme production required strong aeration, which was furnished by constant vigorous shaking, accompanied by considerable frothing.

Preparation of Enzyme.—Before opening, each culture flask was placed in a large 95° water-bath for 15 minutes. All subsequent procedures were carried out at room temperature. The dead cells (containing relatively very little enzyme) were thrown down in a Servall SS-1 centrifuge at 14,000 r.p.m. and discarded. The supernatant fluid was saturated with $(NH_4)_2SO_4$ and stored for five days, after which it was centrifuged again at 14,000 r.p.m. for 15 minutes and the supernatant fluid discarded. The sediment from each culture flask contained from two million to one hundred million units, depending on the strain used.

If desired, the enzyme could be purified further, eliminating gross contamination with $(NH_4)_2SO_4$. In a preparation starting with forty million units (from Strain SA-B) the precipitate (7 g., from one-half of one culture) was suspended in 15 ml. of distilled water, to which NH_4Cl was added to saturation, and sufficient 50% trichloroacetic acid solution to give 2.5% concentration. After centrifugation for ten minutes at 14,000 r.p.m. the supernatant fluid was discarded and the sediment was suspended in 15 ml. of a saturated solution of NH_4Cl, containing 2.5% trichloroacetic acid, and centrifuged as before. The sediment was then taken up in 5 ml. of water to which was added 37 ml. of 95% ethanol. The suspension was centrifuged as before (in two stainless steel centrifuge tubes), thus extracting salt and trichloroacetic acid. The sediment was washed once (in one tube) with 20 ml. of 83% ethanol, and finally suspended in 5 ml. of water and lyophilized, producing 76 mg. of powder. Solutions of the powder, one mg. per ml., were stored at 5° for 24–48 hours for reactivation, the final activity attained being thirty million units per ml. The very high yield in this particular preparation suggested that an inhibitor was removed or inactivated. (However, with other strains the yield was lower.) For comparison, crystalline pancreatic DNase (Worthington) had four billion units per mg. when tested under conditions which have been found to be optimal—$MgCl_2$ at a final concentration of 0.025 *M*, imidazole hydrochloride buffer at a final concentration of 0.1 *M*, *p*H 7.4, gelatin[9] used as for micrococcal DNase (see below).

Requirements for Enzyme Activity.—With a given enzyme sample "old" DNA gave much higher values than "new" DNA. However, this could be corrected by making dilutions of enzyme in 0.1% U.S.P. gelatin[9] and adding the enzyme in 0.1 ml. of solution, so that the final concentration of gelatin was 0.014%. Boric acid–sodium borate buffer was used at a final concentration of 0.01 *M*. With $CaCl_2$ at 0.01 *M*, the *p*H optimum was found to be about 8.6 (using enzyme treated with trichloroacetic acid and ethanol), as shown in Table I.

TABLE I

EFFECT OF *p*H ON RATE OF DEPOLYMERIZATION OF DNA BY MICROCOCCAL DNase

*p*H	8.2	8.6	8.9	9.7
Units	19	24	16	9

The effect of certain cations at *p*H 8.6 was as shown in Table II.

Preparation and Analysis of Digests

Prolonged Digestion of DNA.—DNA fibers were suspended, 20 mg./ml., in a solution containing 0.01 *M* $CaCl_2$,

(2) R. M. Kay, N. S. Simmons and A. L. Dounce, THIS JOURNAL, **74**, 1724 (1952).

(3) Suggested by Dr. A. L. Dounce, oral communication.

(4) R. L. Sinsheimer, *J. Biol. Chem.*, **208**, 445 (1954).

(5) R. O. Hurst, A. W. Marko and G. C. Butler, *ibid.*, **204**, 807 (1953).

(6) M. Laskowski and M. K. Seidel, *Arch. Biochem. Biophys.*, **7**, 465 (1945).

(7) Bore 0.45 mm., length 14 cm., flow time 13–20 seconds, single bulb. Obtained from Otto R. Greiner Co., 221–223 High St., Newark 2, N. J.

(8) Collected by Miss Barbara Weckman

(9) M. McCarty, *J. Gen. Physiol.*, **29**, 123 (1946).

TABLE II

EFFECT OF CATIONS ON ACTIVITY OF MICROCOCCAL DNase

Cation,[a] final concn., *M*	Units
Ca, 0.001	7.5
Ca, 0.01	24.0
Ca, 0.1	4.0
Ca, 0.01, Na, 0.1	14.0
Mg, 0.01	0.0

[a] (These cations were introduced as the chlorides. Calcium acetate and calcium chloride were equally effective.)

and the *p*H adjusted to 8.6 with NaOH, at 37°. The substrate was brought into solution and digested by addition of enzyme at intervals (for example, 5,000 viscometric units every three hours for 15 hours, for 200 mg. of substrate), with frequent additions of alkali to maintain *p*H 8–9. Liberation of acid groups gave a measure[4] of the progress of the digestion, 50–60%[10] of the secondary phosphoryl groups being released at the time digestion was terminated. Completion of digestion was assumed, when addition of enzyme in excess failed to result in significant further production of acid. At this point, and previously during the digestion, it was found that addition of more calcium, even to 0.04 *M*, did not increase the rate of digestion. Similar results were obtained, whether the untreated $(NH_4)_2SO_4$ precipitate was used (see exp. A, Table III) or the ethanol–trichloroacetic acid preparation was used (exp. B, C, Table III), except that ammonia release interfered with the acid titration in the former case. At 45°, relatively smaller quantities of enzyme were required for the digestion. No detectable inorganic phosphorus was formed.

The larger polynucleotides of the digest of DNA by pancreatic DNase,[11] which required 2 molar formate buffer for elution from ion-exchange columns ("2M fraction") were also used as substrate for micrococcal DNase. NH_4Cl–trichloroacetic acid–ethanol treated micrococcal DNase, 0.1 ml. of a solution containing 80,000 units per ml., was added to two ml. of a 5 mg./ml. solution of the "2M fraction" containing 0.01 *M* $CaCl_2$ and the mixture was made alkaline (*p*H about 8.5, thymol blue indicator) with NH_4OH.

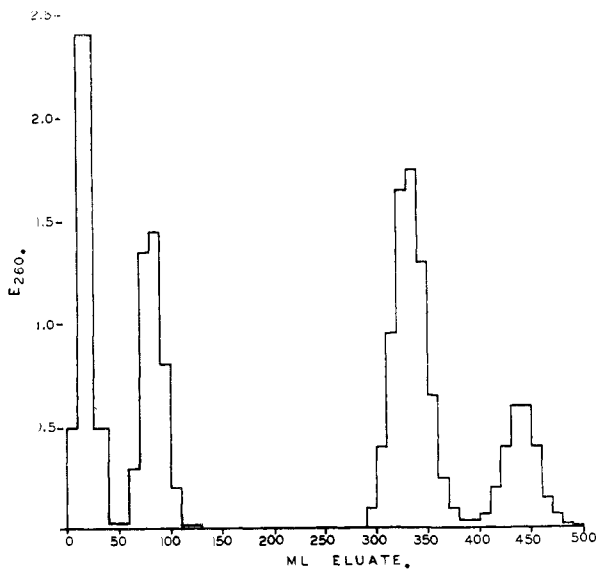

Fig. 1.—Elution diagram of a digest of 50 mg. of DNA by micrococcal DNase, 0.1 *M* ammonium formate buffer, *p*H 4.5. The first peak was identified as deoxycytidylic acid, and the others as thymidylic acid, deoxyadenylic acid and deoxyguanylic acid, in that order.

(10) M. Seraidarian, Thesis, Science Faculty, Tufts College, 1952. Described by G. Schmidt, pp. 567–568 in E. Chargaff and J. N. Davidson "The Nucleic Acids," Vol. I, Academic Press, New York, N. Y., 1955.

(11) M. Privat de Garilhe and M. Laskowski, *J. Biol. Chem.*, **215**, 269 (1955).

Two hours incubation at 37° sufficed to produce the maximum increase in optical density of the digest at 260 millimicrons.

Analysis of Digests.—Digests were chromatographed on anion exchange resin according to the method of Sinsheimer[4] as modified,[11] eluting with ammonium formate buffer at *p*H 4.5, the ionic strength being increased stepwise.

In 0.1 *M* buffer, four peaks were obtained (Fig. 1), whose spectra indicated that they were, in order of emergence, deoxycytidylic acid, thymidylic acid, deoxyadenylic acid and deoxyguanylic acid. These mononucleotides had been obtained in the same order in the same buffer in previous experiments.[11] After the materials were lyophilized, the molecular extinction coefficients[4] based on phosphorus content[12] were the same as those of the corresponding standard deoxyribomononucleotides. Each of the four compounds was then compared, by means of paper chromatography, with the appropriate standard mononucleotide, using the isopropyl alcohol–ammonia–water system of Markham and Smith.[13] The spots for each pair of mononucleotides were found to occupy parallel positions, indicating that all the compounds were indeed mononucleotides, with two dissociable hydroxyls on the phosphoryl group. The last experiment served to exclude the possibility that the materials were dinucleoside monophosphates, nucleoside diphosphates or dinucleotides, all of which are readily separable from mononucleotides in this system.[14]

The yields of these four mononucleotides were calculated from the total absorbtion at 260 mμ and expressed as per cent. of the ultraviolet absorbing material put on the column (Table III). The molar ratios in Table III were calculated from the extinction coefficients.[4]

TABLE III

MONONUCLEOTIDES RELEASED FROM DNA BY MICROCOCCAL DNase

	Exp. A		Exp. B		Exp. C	
	%	Moles/mole deoxyadenylic	%	Moles/mole deoxyadenylic	%	Moles/mole deoxyadenylic
Deoxycytidylic	5	0.7	7	1.0	5	0.8
Thymidylic	7	0.8	7	1.0	6	0.9
Deoxyadenylic	15	1.0	13	1.0	12	1.0
Deoxyguanylic	1	0.1	4	0.4	4	0.4
Total	28		31		27	

Figure 2 shows a typical elution diagram obtained in higher ionic strength buffers. It indicates the presence of several different dinucleotides and larger polynucleotides.

Further Studies of the Mononucleotides.—An attempt was made to remove the terminal phosphate of the mononucleotides with snake venom 5′-nucleotidase, with the result that no detectable inorganic phosphate was produced (less than 5%). The following conditions were used: substrate 20–30 μg. expressed as mononucleotide phosphorus (except in the case of thymidylic acid from the micrococcal DNase digest, of which only six micrograms of mononucleotide phosphorus were available); 0.5 ml. of buffer, 0.15 *M* glycine, *p*H 9.0, containing 0.03 *M* $MgCl_2$; enzyme 0.1 ml. of $^1/_{100}$ dilution of the stock solution in 0.1 *M* NaCl; incubation 45 minutes at 37°. Under these conditions, the yield of inorganic phosphorus from commercial 5′-mononucleotides was 15% for deoxycytidylic, 75% for thymidylic and 43% for deoxyadenylic (no satisfactory commercial deoxyguanylic acid was available for this comparison).

In another experiment, the deoxycytidylic acid from a larger digest was lyophilized as usual and then reprecipitated from a mixture of acetic acid, ethanol and diethyl ether as small rectangular crystals mixed with amorphous material. Digestions of this material, and of commercial deoxycytidylic acid, were carried out as before, except that the quantity of 5′-nucleotidase was increased tenfold. The yield of inorganic phosphorus from the 5′-isomer was calculated as 74% at six minutes incubation, and 106% at ten minutes. At 13 minutes, too little phosphorus had been liberated from the deoxycytidylic acid liberated by micrococcal DNase to be determined (less than 5%).

(12) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(13) R. Markham and J. D. Smith, *ibid.*, **49**, 401 (1951).

(14) L. A. Heppel, P. R. Whitefeld and R. Markham, *ibid.*, **60**, 8 (1955).

Thymidylic acid (identified as such by ionophoresis[15] as well as by its spectrum and chromatographic behavior), obtained from a digest of "2M fraction," was exposed to the action of 5′-nucleotidase as described above, except that paper ionophoresis,[15] rather than determination of inorganic phosphorus, was used to observe any dephosphorylation that might have occurred. Thymidine was not produced in detectable quantities.

Discussion

Micrococcal DNase was shown to differ from other DNases by virtue of its calcium requirement and thermostability. Furthermore, the studies of the split products, though incomplete, indicated that this enzyme was also peculiar in its specificity.

The experiments with 5′-nucleotidase indicated that mononucleotides released by micrococcal DNase differed in some way from 5′-isomers containing the same bases. On the usual assumption[16] of 3′,5′-linking of the nucleoside residues in DNA, it was concluded that micrococcal DNase was able to split the 5′-bond, producing fragments having terminal phosphate attached at the 3′-position.

The property of hydrolyzing the 5′-phosphodiester bond served to distinguish micrococcal DNase from pancreatic DNase and the phosphodiesterases of intestinal mucosa and snake venoms. These latter enzymes had been shown to be specific for the 3′-phosphodiester bond in DNA by a number of investigations of materials interchanged between different laboratories. The phosphodiesterases of intestinal mucosa[17] and of the venoms of *Crotalus adamanteus*[18] and Russel's viper[18,19] released the same deoxyribomononucleotides, which were dephosphorylated readily by 5′-nucleotidases of snake venom and bull semen.[20] The specificity of pancreatic DNase was shown to be the same as that of the above phosphodiesterases, by the results obtained when various small molecules, released by pancreatic DNase, were digested with phosphodiesterases[5,18,19,21,22] or with 5′-nucleotidase.[23] Finally, various inferences from enzymatic studies were in agreement with results of studies of 3′- and 5′-isomers of thymidylic acid and deoxycytidylic acid obtained by unambiguous syntheses starting with thymidine[24] and deoxycytidine.[25] The synthetic 3′-isomers were not dephosphorylated by the 5′-nucleotidase of the crude venom of *Crotalus atrox*,[24,25] and it appeared that crude *Crotalus* venom was a satisfactory reagent for identification of 5′-deoxyribomononucleotides.

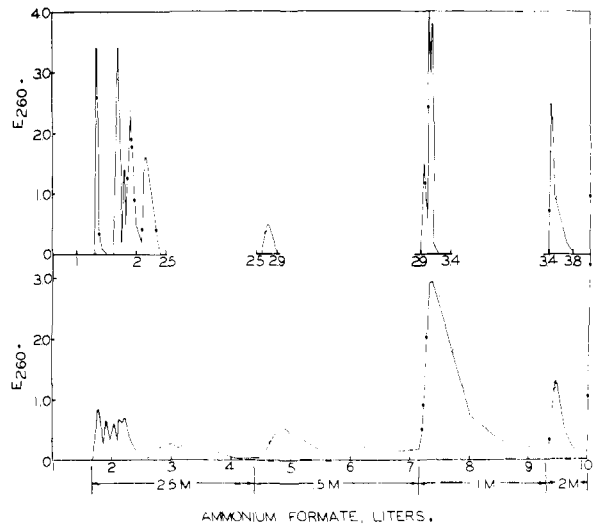

Fig. 2.—Comparison of elution diagrams of digests of DNA (200 mg.) by micrococcal DNase (upper) and pancreatic DNase[11] (lower), with concentrations of eluting buffer (ammonium formate, *p*H 4.5) shown beneath the pattern for the pancreatic DNase digest. The earlier peaks in the pattern for the pancreatic DNase were shown to be dinucleotides.[4,11] Only the eluates with buffer of ionic strength higher than 0.1 *M* were depicted, because the mononucleotide peaks for the micrococcal DNase digest would have been inconveniently large if plotted on this scale. The pattern for the micrococcal DNase digest was broken so that peaks obtained in the same buffer concentration could be compared.

Certain observations indicated that micrococcal DNase could attack more of the bonds in DNA than could pancreatic DNase. Pancreatic DNase released only about one-fourth of the secondary phosphoryl groups[4,26] of the substrate and liberated very small quantities of mononucleotides.[4,27] Some of the polynucleotides remaining after action of pancreatic DNase were attacked by micrococcal DNase. On the other hand, micrococcal DNase did not degrade DNA entirely to mononucleotides, and apparently was not a non-specific phosphodiesterase.

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