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Studies of the Specificity of Deoxyribonuclease I*

III. HYDROLYSIS OF CHAINS CARRYING A MONOESTERIFIED PHOSPHATE ON CARBON 5'

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Kunitz (3) crystallized pancreatic deoxyribonuclease[1] and studied its kinetics and the products of its action on deoxyribonucleic acid. With a ratio of enzyme to substrate not higher than 1:500, the products obtained after exhaustive digestion had an average length of a tetranucleotide. Studies from this and Sinsheimer's laboratory (for details see the recent review (4)), showed that the digest thus obtained represented a complex mixture of fragments varying in length from mononucleotides to at least octa-, possibly dodecanucleotides. The attempts to redigest larger oligonucleotides (by the use of a low enzyme-substrate ratio) into smaller fragments failed (5). It was therefore assumed that the digest thus obtained is a "limit digest" and that it is composed of fragments containing only internucleotide linkages which are totally resistant to the action of DNase I.

Hurst (6) and Hurst and Findlay (7) observed that with a very large amount of enzyme (ratio of enzyme to substrate of the order 1:10), at pH 8.0, in the presence of ethylenediaminetetraacetate and Mn^{++}, the hydrolysis proceeds beyond the "limit digest" stage. To explain their findings, Hurst and Findlay (7) postulated that crystalline DNase I contains still another enzyme, an oligonucleotidase, which is capable of hydrolyzing oligonucleotides present in the "limit digest" into smaller fragments, as measured by solubility in a uranyl acetate reagent.

Independently, in the previous paper of this series (2), it was observed that when oligonucleotides carrying monoesterified phosphate on carbon 3' were digested with very high amounts of DNase I, the predominant products were derivatives of dinucleotides. The hypothesis was advanced (2) that dinucleotides represent an ultimate product of the action of DNase I on deoxyribonucleic acid and that an apparent end point ("limit digest") is caused by an insufficient amount of enzyme and by product inhibition. Thus, an alternative explanation was suggested, without postulating a contamination of DNase I by an oligonucleotidase.

The unusual complexity of kinetics as well as heterogeneity of the fragments present in the "limit digest" of deoxyribonucleic acid by DNase I would be expected if one postulates that during the course of the reaction the substrate mixture becomes more resistant (4), until finally, at the stage of the so-called "limit digest," the reaction becomes too slow to be measurable with a low enzyme-substrate ratio. The consequence of such a hypothesis is that the "limit digest" is not a final stage of the reaction and that under a favorable set of conditions (very high concentration of enzyme and absence of product inhibition) the reaction should proceed further, until the majority of products reach the size of dinucleotides and trinucleotides, the ultimate products of the reaction. The present paper describes experiments that support this hypothesis.

EXPERIMENTAL PROCEDURE

Enzymes—Crystalline pancreatic DNase I was purchased from the Worthington Biochemical Corporation. Crude pancreatic DNase I was obtained as a by-product in the preparation of chymotrypsinogen B (8). After crystallization of chymotrypsinogen, the mother liquors were lyophilized and stored in the refrigerator.

Previously described methods were used for preparing DNase II[1] (9), venom phosphodiesterase (10), and prostatic phosphatase (11, 12). The last two were gifts of Dr. S. C. Sung and Dr. L. Cunningham, respectively.

Substrates—Native thymus DNA was prepared according to Kay *et al.* (13), and was a gift of Dr. J. G. Georgatsos.

A **1-minute digest** of DNA was prepared by exposing the native DNA to 1 minute of digestion by DNase I under such conditions that approximately 1 to 2% of the internucleotide bonds were hydrolyzed, as determined by the pH-Stat (see Methods). DNA, 100 mg, was dissolved in 50 ml of 0.05 M Tris buffer, pH 8.0, containing 0.0075 M $MnCl_2$ and 0.0045 M EDTA, and warmed in a water bath to 37°. DNase I, 100 μg, was added to the DNA solution, and the mixture was allowed to incubate for 1 minute. The reaction was stopped by the addition of 2 volumes (100 ml) of cold 95% ethanol. The precipitate which formed was centrifuged and dissolved in cold citrate buffer, 0.015 M, pH 7.1, containing 0.14 M NaCl. Duponol, recrystallized sodium dodecyl sulfate, was added to denature the possible remaining DNase I; the NaCl concentration was increased to 5%; and the solution was warmed to room temperature. After removal of the excess Duponol by crystallization in the cold, followed by centrifugation, 9 volumes of 95% ethanol were added to the supernatant, and the mixture was centrifuged. The

* This study was supported by grant AT(11-1)-293 from the United States Atomic Energy Commission and grant E-57C from the American Cancer Society. Parts I and II of this study have been published (1, 2).

[1] The abbreviations used are: DNase I, pancreatic deoxyribonuclease, and DNase II, splenic deoxyribonuclease. The abbreviations of the derivatives of nucleic acids are those used by *The Journal of Biological Chemistry*. When dealing with fragments of unknown composition, nucleosides are represented by capital letters *X*, *Y*, *Z*.

TABLE I

Characterization of substrates

	$\epsilon_{260\ m\mu}$ (P)	Specific viscosity at 25°	
		2.8 μmoles of P/ml	17.4 μmoles of P/ml
DNA	7000	7.91	
1-minute digest	7400	0.06	0.35
10-minute digest	8600	0.00	0.00
DNase I, oligonucleotide fraction	8900		

precipitate was dissolved in citrate buffer (as above) and reprecipitated with 4 volumes of 95% ethanol. It was then dissolved in water and dialyzed overnight against water, at 4°. Characterization of substrates is given in Table I.

A **10-minute digest** of DNA was prepared in the same manner as the **1-minute digest,** except that digestion was carried out for 10 minutes and resulted in cleaving approximately 10% of the internucleotide bonds.

DNase I, oligonucleotide fraction, was prepared by digesting 200 mg of thymus DNA in 100 ml of 0.1 M Tris buffer, pH 8.0, containing 3×10^{-3} M $MnCl_2$, with 400 μg of DNase I at 37° for 5 hours. The digest was chromatographed on Dowex 1-X2 columns and eluted with ammonium acetate, pH 4.5. Fractions eluted with 2 M buffer were discarded, and the remainder of the digest, eluted with 4 M buffer, was collected, lyophilized free of buffer, and used as substrate. Fragments composing this fraction are heterogeneous with respect to length and composition, but all were terminated in 5′-phosphate.

DNase II, oligonucleotide fraction was obtained by digesting DNA with DNase II under conditions previously described (14). The fraction eluted from a Dowex 1 column between 2 and 4 M acetate was collected. Fragments constituting this fraction differed from the **DNase I, oligonucleotide fraction** by being terminated in 3′-phosphate.

Methods—Hydrolysis of substrates was followed by titration of liberated secondary phosphoryl groups with a pH-Stat as described by Williams *et al.* (10).

Chromatography on Dowex 1-X2 columns was performed by standard procedures (15, 16). Two-dimensional paper chromatography was performed as described previously (17).

RESULTS

Fig. 1 and Table II show the results of experiments in which DNase I was allowed to hydrolyze either DNA or its degradation products, and the rates of reaction were recorded in the pH-Stat. In the experiments shown in Fig. 1, four substrates were used: *Curve A,* **native DNA**; *Curve B,* **1-minute digest**; *Curve C,* **10-minute digest**; and *Curve D,* **DNase I oligonucleotide fraction.** These substrates had the same statistical composition of nucleotides. They all were terminated in 5′-phosphate. They differed in length of the chains from about 17,000 nucleotides in the native DNA to about 5 to 10 in the **DNase I, oligonucleotide fraction.**

The amount of substrate was kept constant in respect to phosphorus, but the amount of enzyme varied from 3.6 μg (*Curve A*) to 720 μg (*Curve D*). This increase in enzyme was necessary to obtain rates which were roughly comparable and could be shown on the same graph.

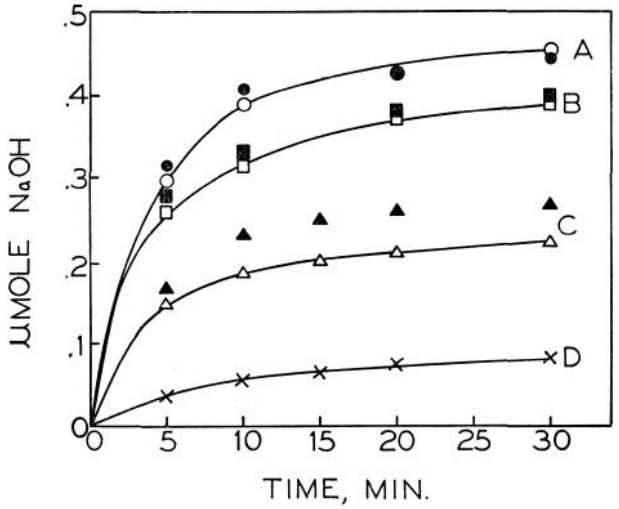

FIG. 1. Comparison of rates of hydrolysis of DNA and its fragments by two preparations of DNase I, crystalline (○, □, △) and crude (●, ■, ▲). Curves: *A,* **native DNA**; *B,* **1-minute DNase I digest**; *C,* **10-minute DNase I digest**; and *D,* **DNase I, oligonucleotide fraction**. Amount of each substrate was measured in absorbancy units, A_{260}, and was equivalent to 1 mg of native DNA. In all experiments, Mn^{++}, 7.5×10^{-3} M + 4.5×10^{-3} M EDTA was used; pH was kept at 8.0; and the total volume was 10 ml. In *Curve A,* ○——○ represents 3.6 μg of crystalline DNase; ●——●, 39 μg of crude DNase. In *Curve B,* □——□ represents 7.2 μg of crystalline DNase, ■——■, 78 μg of crude DNase. In *Curve C,* △——△ represents 360 μg of crystalline DNase, ▲——▲, 3.9 mg of crude DNase. For simplicity lines are drawn only for the experiments with crystalline DNase I. In *Curve D,* ×——× represents 720 μg of crystalline DNase. Titration curves are continuous, and symbols do not represent the experimental points, but are used to distinguish curves.

TABLE II

Dependence of activity of DNase I on concentration of divalent cations, pH 8.0

	Concentration	NaOH titrated at 15 minutes*		
		DNA, 23.0 A_{260} units†		DNase I, oligonucleotide fraction, 30.0 A_{260} units†
		DNase, 15 μg	DNase, 3 μg	DNase, 1 mg
	M	μmole	μmole	μmole
$MgCl_2$	2×10^{-2}	0.145		
	1×10^{-2}	0.180		0.035
	2.5×10^{-3}	0.215		
	1×10^{-3}	0.255		0.055
	5×10^{-4}	0.190		
	1×10^{-4}	0.030		0.020
$MnCl_2$	1×10^{-2}		0.385	0.070
	3×10^{-3}		0.380	
	1×10^{-3}		0.380	0.080
	5×10^{-4}		0.390	
	1×10^{-4}		0.035	0.050

* At this time, the reaction rate had not fallen greatly below the initial rate for any of the determinations.

† Amounts are given per sample; the total volume was 10 ml.

The results show that a continuous decrease in susceptibility occurs during the digestion of DNA by DNase I. Even a short exposure to DNase I in the **1-minute digest,** resulting in the cleavage of no more than 2% of the bonds, decreases the susceptibility of the substrate. The striking change in susceptibility occurs between the **1-minute digest** and the **10-minute digest.** It appears, therefore, that the continuously increasing resistance of the remaining substrate is not linear in respect to time, but resembles a sigmoid curve. Maximal decrease in susceptibility is observed with the **DNase I, oligonucleotide fraction.** A 200-fold excess of enzyme acting on this substrate produces only one-eighth of the rate observed with native DNA.

Similar conclusions are reached by examining the results shown in Table II. Several hundred fold more enzyme is required to hydrolyze the **DNase I, oligonucleotide fraction** than to hydrolyze DNA at a similar rate.

Experiments presented in Fig. 1 show no significant difference in the behavior of the two preparations of DNase, crystalline and crude. It was not possible to make a valid experiment with crude DNase and **DNase I, oligonucleotide fraction** as substrate, since the amount of enzyme required was too high for pH-Stat determination. With **native DNA,** 39 μg of crude preparation produced a rate almost identical to that of 3.6 μg of crystalline enzyme. With the **1-minute digest** and **10-minute digest,** the amounts of enzyme used were, respectively, 2- and 100-fold higher, but the ratio of crude (Fig. 1; ●, ■, ▲) to crystalline (Fig. 1; ○, □, △) was kept constant. As is seen from Fig. 1, the rates observed with the crude enzyme do not significantly differ from the rates expected from the activity of the crystalline DNase. These results do not substantiate the existence of a different oligonucleotidase in our preparation of crude DNase.

A second argument which has been used in favor of the existence of a separate oligonucleotidase was that requirements for the activating cation were different for the early and late stages of digestion. In the late stages (7), higher rates and more extensive hydrolysis were observed in the presence of Mn^{++} than in the presence of Mg^{++}. By the same method (pH-Stat),

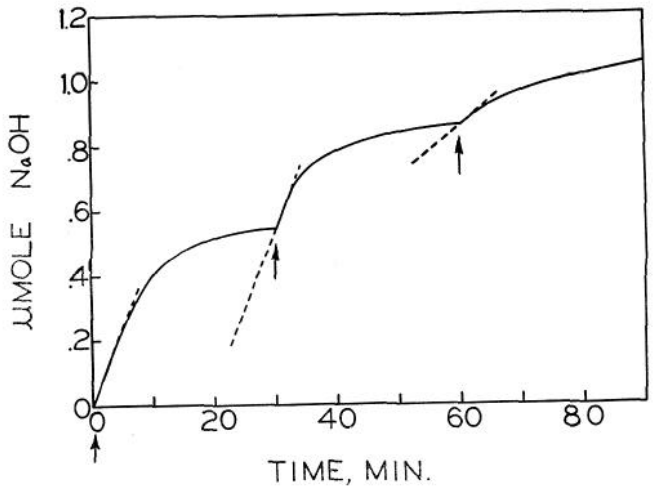

FIG. 2. Time-activity curve of crystalline DNase I. Initial conditions were: 1.07 mg of DNA, 7.5×10^{-3} M $Mn^{++} + 4.5 \times 10^{-3}$ M EDTA, pH 8.0, total volume of 10 ml, and 2 μg of DNase I. At 30 minutes 200 μg of DNase I, and at 60 **minutes** 800 μg were added to bring the total amount of enzyme to 1 mg. The rates are indicated as *tangents,* calculated for the first 2.5 minutes for each enzyme concentration.

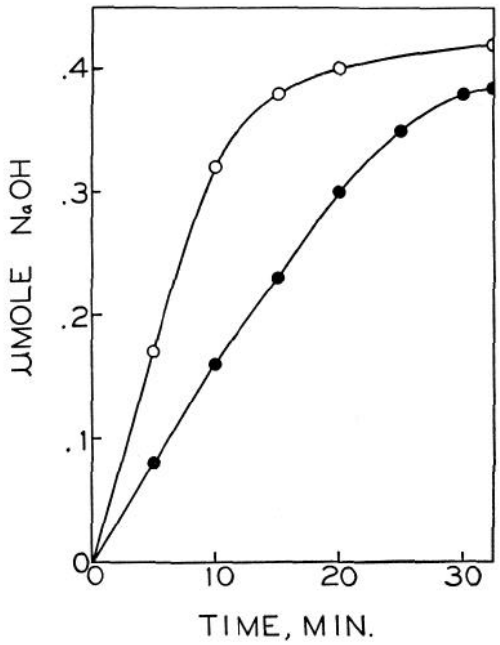

FIG. 3. Effect of the reaction products on hydrolysis of DNA by DNase I. ○——○ represent 1 mg of DNA, 2.4 μg of DNase I, 3×10^{-3} M Mn^{++}, pH 8.0, in a total volume of 10 ml. ●——● represents the same, plus 300 A_{260} units of **DNase I, oligonucleotide fraction.** As in Fig. 1, symbols are used to distinguish curves.

experiments were performed to compare the activating effect of Mg^{++} and Mn^{++} on two substrates, native DNA (mol. wt. approximately 5×10^6) and the **DNase I, oligonucleotide fraction** (mol. wt. about 2×10^3). Concentration of substrates in respect to phosphorus was the same in both cases. The experimental curves are not reproduced; only 15-minute values are compiled in Table II.

In agreement with previous workers, Mn^{++} was found to be a better activator whether DNA (initial stages of reaction (18)) or the **DNase I, oligonucleotide fraction** (terminal stages of reaction (7)) was used. It may also be noted that in agreement with several previous workers (4) the optimal concentration of a bivalent cation correlated with the concentration of the substrate expressed in terms of phosphate, but did not correlate with the molar concentrations of either substrate or enzyme. The similarity of optimal concentrations found with both substrates suggests that the requirement for bivalent cations does not change during the course of the reaction. This finding, again, does not substantiate the hypothesis that two phases of the reaction are due to two different enzymes.

The decreasing susceptibility of substrate during the course of the reaction may also be illustrated by a different type of experiment (Fig. 2). The original system contained 1 mg of DNA and 2 μg of crystalline DNase. After 30 minutes, the rapid phase of the reaction had ended.[2] At that time, 200 μg of enzyme (100 times the original amount) were added, and the original rate of the reaction was restored, as shown by the *tangents* (Fig. 2), drawn on the basis of the rate for the first $2\frac{1}{2}$ minutes for each enzyme concentration. At 60 minutes, a further addition of 800

[2] Numerous control experiments (not reproduced) established that little or no denaturation of the enzyme occurs during such exposures. Thus, the decreased reaction rate cannot be ascribed to the inactivation of the enzyme.

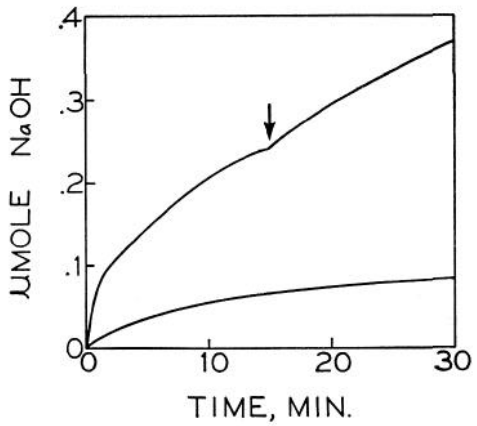

FIG. 4. Relative rates of hydrolysis of oligonucleotides terminated in 3′- (**DNase II, oligonucleotide fraction**, *upper curve*) and 5′-phosphate (**DNase I, oligonucleotide fraction,** *lower curve*). Conditions of hydrolysis were: 30 A_{260} units of substrate, 720 μg of crystalline DNase I, 3×10^{-3} M $MnCl_2$, pH 8.0, in 10 ml of total reaction volume. At the time indicated by *arrow*, 720 μg of DNase I was added to **DNase II, oligonucleotide fraction.**

μg (total amount, 1 mg of DNase present in the system) restored the rate of the reaction to only approximately 30% of the original value. At 90 minutes, 34% of the internucleotide phosphodiester bonds have been cleaved, with the reaction still proceeding at a detectable rate. In some of the similar runs, up to 40% of all internucleotide bonds have been hydrolyzed.

The major difference between the experiments illustrated in Fig. 1 and Fig. 2 is that in the latter, products of the reaction remained. However, the increased demand for enzyme at the corresponding stages of reaction was of a similar order of magnitude in both cases. This suggested that the observed phenomenon is mainly caused by newly acquired structural resistance of shortened chains and that inhibition by the reaction products plays a lesser role, if any.

Cavalieri and Hatch (19) studied the kinetics of acid liberation during the degradation of DNA by DNase I and concluded that DNase I is inhibited by the products formed. Even though the short chains are susceptible to massive doses of enzyme, one would expect that they would act as inhibitors when introduced into a system composed of highly susceptible substrate and normal amounts of enzyme. That this is indeed the case is illustrated in Fig. 3. As expected, inhibition by the products, which in this case accounts for approximately 50% is by far the smaller of the two factors contributing to the decrease in the rate of reaction.

It has been previously shown (2) that DNase I cleaves 3′,5′-mononucleoside diphosphates from oligonucleotides terminated in 3′-phosphate, but does not cleave either mononucleotides or nucleosides. This was interpreted as a labilizing influence of the 3′-phosphate group. If this interpretation is correct, one might expect that oligonucleotides terminated in 3′-phosphate should be better substrates than those terminated in 5′-phosphate. The experiment in which **DNase I, oligonucleotide fraction** and **DNase II, oligonucleotide fraction** were compared is shown in Fig. 4. As expected, the latter was found to be a better substrate. The two fractions certainly differed in the termination of the chain, DNase I fragments terminating in 5′-phosphates and DNase II fragments terminating in 3′-phosphates. The average length of the chain was presumably the same, since both fractions were eluted from a Dowex 1 column between 2 and 4 M acetate buffer. The statistical composition of nucleotides was also similar and did not differ substantially from the composition of DNA. No statement can be made concerning the sequences present. It is quite probable that sequences in DNase II products were more favorable to digestion by DNase I than were those remaining after previous exposure to DNase I. The observed difference in rate (Fig. 4) is of the same order of magnitude as that between **DNase I, oligonucleotide fraction,** and the **10-minute digest** (Fig. 1), and hardly can be attributed to preferential sequences, if they were present. The enhancing influence of the free 3′-terminal phosphate is also supported by the next experiment (Fig. 5) showing that pXpYpZ[1] is resistant to DNase I, regardless of composition, whereas XpYpZp was previously shown to be susceptible (14, 7), also regardless of composition.

It remained to prove that dinucleotides (and trinucleotides) of the type pXpY and pXpYpZ are the ultimate products of the reaction. A so-called "limit digest" of DNA was prepared in a standard manner and was chromatographed on Dowex 1-X2. The mononucleotides, dinucleotides, and most of the trinucleotides were removed by 1.25 M ammonium acetate buffer, pH 4.5. The fraction which was eluted between 1.25 and 2.5 M ammonium acetate was collected, lyophilized free of buffer, and subjected to a second digestion by DNase I. The fraction contained 623 absorbancy units (A_{270} = 623) of oligonucleotides (approximately 25 mg). The chains varied in length, probably from 4 to 6 nucleotides, but presumably were uniform in regard to termination in monoesterified phosphate, which was on carbon 5′. The fraction was dissolved in 2 ml of 0.1 M Tris buffer, pH 7.0, containing 0.02 M $MgCl_2$; enzyme was added in 2 mg portions daily, until a total of 8 mg had been added. Toluene was used as a preservative. Incubation was stopped after 5 days. The mixture was then chromatographed as before. Fig. 5 illustrates the pattern obtained. The last dinucleotide, pGpG, together with some trinucleotides, appeared in the last peak of 1 M buffer.

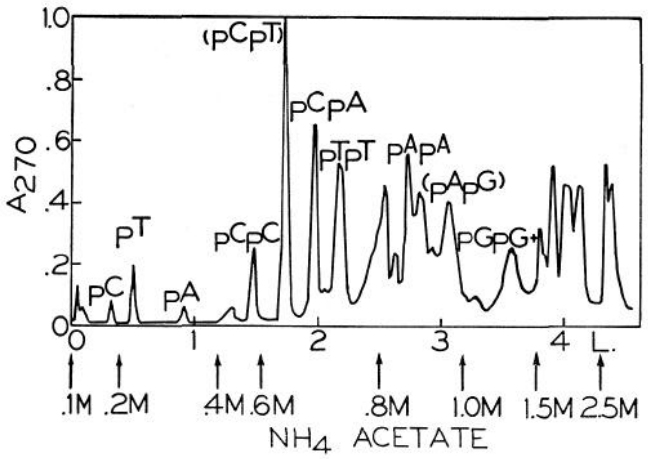

FIG. 5. Chromatographic pattern of the digest of oligonucleotides terminated in 5′-phosphate by a massive dose of DNase I. Chromatography carried out on a column (20 × 0.9 cm) of Dowex 1-X2 with ammonium acetate, pH 4.5, as eluent. Volume of effluent and changes in concentrations of buffer are indicated along the abscissa. The mononucleotides and some identified dinucleotides are indicated above the peaks. Peak marked pGpG+ is heterogeneous and contains pGpG + some trinucleotides.

It is estimated that 65% of the eluted material were mononucleotides and dinucleotides; 25%, trinucleotides; and 10% remained undigested. Since the usual amounts of mononucleotides and dinucleotides in a "limit digest" are about 1 and 17%, respectively, the presently found 1.8% of mononucleotides and 63% of dinucleotides represents an increase of mononucleotides by a factor of 2 and dinucleotides by a factor of 4. Previous reports from this and Sinsheimer's laboratory (4) showed that dinucleotides are resistant to further digestion by DNase I and cannot serve as precursors of mononucleotides. Recent results of Ralph *et al.* (20) indicate that certain trinucleotides are also resistant. This experiment showed that all dinucleotides and trinucleotides present in the digest are resistant to extremely high concentration of DNase I, and therefore do not serve as a source for mononucleotides.

The last peak of Fig. 5 (eluted with 2.5 M buffer), when subjected to further action by an equal amount of DNase I, was approximately 70% digested. The various products were present in too small amounts to identify, except pG(pApG).

DISCUSSION

Although the present results did not definitely rule out the possibility that crystalline DNase I contains an additional oligonucleotidase, no confirming evidence has been found. The alternative, for which the substantiating evidence has been presented, is that one enzyme is responsible for the observed phenomena. As the reaction proceeds, products are formed which act as inhibitors when added to the preceding stage of the reaction, but which are still substrates when massive doses of enzyme are applied. The data at hand do not allow one to speculate on a more detailed mechanism of the reaction.

The described case may not be a unique example of hydrolysis of nucleic acids. It now seems likely, that a slow second phase reaction observed by Koerner and Sinsheimer (21) with DNase II may have a similar explanation.

Since several criteria for the classification of enzymes attacking DNA have been recently reviewed (4), it seems worth mentioning that the ability to readily hydrolyze dinucleotides may also serve as a criterion. Thus, endonucleases (DNase I, micrococcal nuclease (22)), and an exonuclease, phosphodiesterase from *Escherichia coli* (23) are incapable of hydrolyzing dinucleotides regardless of their composition, whereas mung bean nuclease (24) readily attacks dinucleotides of a definite composition, and phosphodiesterase from venom, attacks all dinucleotides.

SUMMARY

During the process of hydrolysis of deoxyribonucleic acid by pancreatic deoxyribonuclease, the reaction rate progressively decreases. The process is continuous, but not linear with time. As the reaction progresses, the compounds which are formed are inhibitors when added to the system containing native deoxyribonucleic acid, but are substrates if treated with a massive dose of pancreatic deoxyribonuclease.

The optimal concentrations of activating bivalent cations remain the same throughout the course of the reaction and are not dependent on the enzyme concentration. Chains of oligonucleotides of the same average length appear to be better substrates if monoesterified phosphate is carried in position 3′ rather than in position 5′. With a very high amount of enzyme (ratio of pancreatic deoxyribonuclease to substrate of the order 1:1), dinucleotides account for over 60% and trinucleotides for approximately 25% of the digest.

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