MICROCOCCAL NUCLEASE: CONSIDERATION OF ITS MODE OF ACTION*

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Studies on the action of certain proteolytic enzymes have shown that native proteins possessing a high degree of secondary structure are more resistant to attack than the denatured molecules derived from them (Linderstrøm-Lang, 1952). This resistance is presumably due to the stereochemical restrictions placed upon the native molecule by the hydrogen bonds and the inability of the enzyme to form the associations obligatory for hydrolysis. In view of the high degree of structural regularity in the two-stranded deoxyribonucleic acid (DNA) molecule and the rigidity imposed by the unique base-pairing, it is not surprising that a similar resistance to enzymatic attack should be found.

Such indeed is the case, for studies with micrococcal nuclease, an enzyme first described by Cunningham and coworkers (1956), have revealed that it preferentially hydrolyzes the phosphodiester bonds of DNA in which the secondary structure has been disrupted (Dirksen and Dekker, 1958; Dirksen, 1959). While our initial studies showed only a four-fold rate difference between native and heat-denatured DNA, subsequent investigation demonstrated, that under conditions of high ionic strength, differences of 20- to 100-fold could be obtained. In Figure 1 are shown the rates of hydrolysis of phosphodiester bonds, as measured by constant pH titration,

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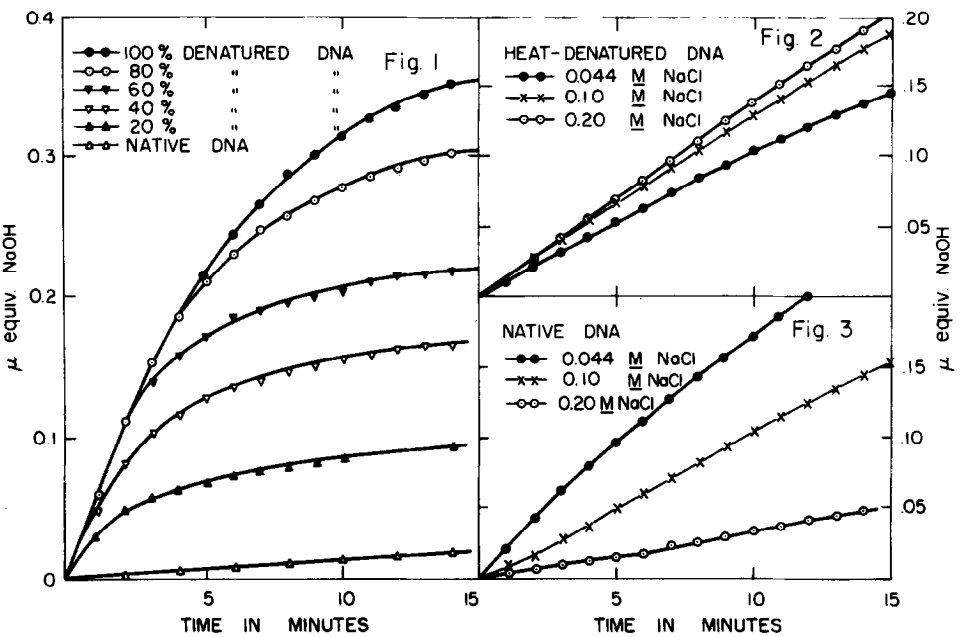
observed when synthetic mixtures of native and heat-denatured (30 minutes at 100°, pH 8.6) DNA were subjected to attack by micrococcal nuclease.



All experiments were conducted at 25° ± 0.1°C; total volume, 9 ml; ($CaCl_2$) = 0.01 M. The pH was maintained at 8.6 by automatic addition of 2×10^{-3} N NaOH.

Fig. 1. (NaCl) = 0.2 M; (DNA) = 1.05×10^{-4} gm atoms P/l; Enzyme, 5 λ of a 1 to 5 diluted solution was used for all substrates with the exception of native DNA which required 50 λ for a measurable rate of hydrolysis. Since the rate was found to be proportional to enzyme concentration, the curves were normalized to equal enzyme concentration.

Fig. 2. (DNA) = 1.37×10^{-4} gm atoms P/l; Enzyme, 25 λ of a 1 to 100 diluted solution.

Fig. 3. (DNA) = 1.37×10^{-4} gm atoms P/l; Enzyme, 25 λ of a 1 to 10 diluted solution.

Although the total DNA concentration of all samples was the same, the initial rate of hydrolysis rose with increasing proportion of denatured material in the substrate, up to about fifty percent denatured. When the fraction of denatured DNA exceeded this value the initial rates were identical indicating that the enzyme was saturated with respect to substrate.

Increasing the ionic strength of the solution led to a slightly accelerated rate of attack on heat-denatured DNA at pH 8.6 (Figure 2). In contrast, the same change decreased the rate of hydrolysis of native DNA (Figure 3), a result in accord with the known stabilizing effect of high ionic strength on the two-stranded structure. Moreover, Q_{10} (25° - 35°) determinations in 0.2 M sodium chloride gave a value of 4.9 for native DNA and 2.6 for the denatured molecule. The higher activation energy observed for the native molecule implies that attack in this case involves cooperative effects.

Since it has recently been shown that the heat stability of DNA in solution is directly related to the guanine-cytosine content of the polymer (Marmur and Doty, 1959), we sought to determine whether the more readily denatured A-T stretches of the molecule (i.e., the sequences rich in deoxyadenylic and thymidylic acid residues) were also those most readily attacked by the enzyme. A review of the published evidence relating to the products resulting from the attack of the enzyme on DNA (Cunningham, 1959) revealed a common structural feature, i.e., that almost all of the dinucleotides isolated had either an A** or a T as the residue bearing the free 5'-hydroxyl group. Moreover, among the mononucleotide products, A and T predominated while C occurred in a slightly smaller amount and G was present only as a minor component. In consideration of the action of the enzyme on native DNA these facts would appear to be consistent with the following mechanism which must

** Each 3'-nucleotide or 3'-nucleotide residue in an oligo- or poly-nucleotide will be designated by the first letter of the trivial name of the nucleotide. Whether the abbreviation refers to a DNA or an RNA derivative should be clear from the context.

be recognized as largely hypothetical at the present time: The enzyme approaches a DNA molecule and attacks at the most susceptible portion of the chain, namely that in which there are stretches consisting only or predominantly of A and T residues. The hydrolysis occurring in this region of increased flexibility is repeated at locations of similar composition along the chain. This endoesterase attack gives rise to oligonucleotides which are further degraded by a combination of endo- and exo-esterase action, the latter proceeding from the end bearing the 3'-monoesterified phosphate and terminating when the dinucleotide stage is reached (Roberts and Dekker, 1960). The rate at which the oligonucleotides first produced are further hydrolyzed will be determined by their base composition and we would suggest that the presence of guanine, the base most notorious for its ability to interact with other bases or itself, will impart the greatest resistance to further hydrolysis. While the above mechanism would appear to be adequate to explain the products arising from the digestion of the two-stranded DNA molecule, one might expect a different pattern of products for the single stranded ribonucleic acid (RNA) molecule. One does, in fact, observe a broader specificity in the hydrolysis of RNA, but even in this case the bulk of the dinucleotide products have A or U as the residue bearing the free 5'-hydroxyl group (Cunningham, 1959; Reddi, 1959a, b). Although alternative explanations can be offered, this may be another indication (see also Haschemeyer, et al., 1959; Doty, et al., 1959; Kalnitsky, et al.,1959) that RNA in solutions of high ionic strength or containing divalent metal ions is extensively hydrogen-bonded. The regions of high G-C content, being more involved in this association, would then be more resistant to the enzyme.

To explore further the A-T preference, we have compared the rates of hydrolysis of native DNA, heat-denatured DNA and the "A-T" polymer (Lehman, 1959; the authors are indebted to Dr. H. K. Schachman for this sample) by the approximate method of measuring the increase of optical density with time. The results are shown in Figure 4. The "A-T" polymer, in spite of its highly organized structure, is more readily denatured (Marmur and Doty, 1959) and

thus, as expected, more easily digested than native DNA. The initial lag indicates slower hydrolysis while all or part of the secondary structure is intact.

Fig. 4

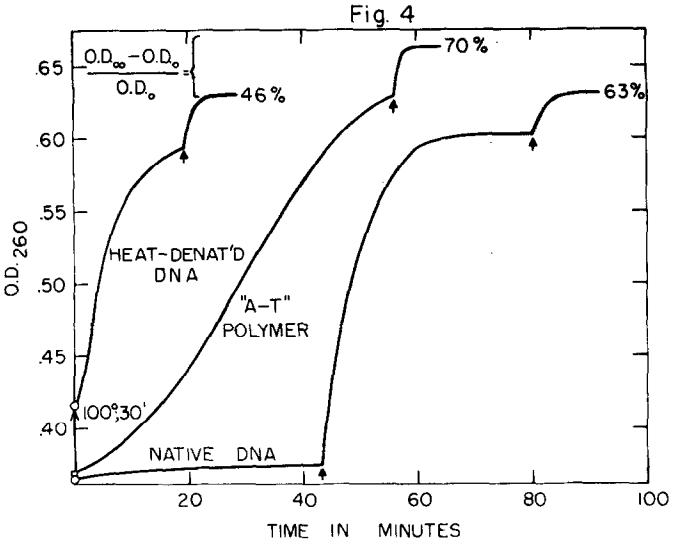


Fig. 4. All experiments were conducted at $25^\circ \pm 0.2^\circ$ in 0.05 M borate buffer, pH 8.6, 0.01 M in $CaCl_2$; total volume, 3 ml; $(DNA_{nat.}) = (DNA_{denat'd}) = 5.65 \times 10^{-5}$ gm atoms P/l; ("A-T") = ca. 6×10^{-5} gm atoms P/l; Enzyme, 5 λ of a 1 to 15 diluted solution was added at zero time in each case. To insure complete digestion, 5 λ of undiluted enzyme was added at times indicated by short arrows (↑).

Since the enzyme can hydrolyze polycytidylic acid (Reddi, 1959a) and can also release a large amount of the guanylic acid of RNA as mononucleotide (Cunningham, 1959; Reddi, 1959a,b), it does not seem that group specificity alone (e.g., towards an A or T(U) residue resulting in the liberation of its 5'-hydroxyl group) can account for the results obtained. The specificity indicated by the above data is therefore considered to be largely configuration-dependent which would suggest that the enzyme may be a valuable tool for structural investigations. Additional experimental evidence supporting this type of specificity is adduced in the following communication (Rushizky, et al., 1960).

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