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# PURIFICATION OF ACID DEOXYRIBONUCLEASE FROM HeLa CELLS

# INHIBITION KINETICS OF NATIVE AND MODIFIED FORMS OF THE HeLa AND CALF-SPLEEN ENZYMES

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SUMMARY

A procedure is described which results in a 750-fold purification of acid deoxyribonuclease (deoxyribonucleate 3'-oligonucleotidohydrolase, EC 3.1.4.6; deoxyribonuclease II) from cultured HeLa $S_3$ cells. Methods are also described for the additional purification of a commercially available, partially purified calf-spleen acid deoxyribonuclease (Worthington Biochemicals).

Treatment of either the HeLa or calf-spleen acid deoxyribonuclease with 2-mercaptoethanol resulted in the production of at least two modified, enzymatically active species of acid deoxyribonuclease which could be clearly resolved from the native enzyme molecules by chromatography on carboxymethyl-cellulose columns. The sedimentation constant of each of these mercaptoethanol-elicited species was compared with that of the native enzyme and found to be slightly lower.

All species of the enzyme (native or modified) were capable of degrading native DNA by 'single hit' kinetics.

Heat-denatured DNA was found to be an inhibitor of native DNA hydrolysis by acid deoxyribonuclease. Low amounts of heat-denatured DNA acted as a non-competitive inhibitor of native DNA degradation by acid deoxyribonuclease while higher amounts of denatured DNA acted as a competitive inhibitor of native DNA hydrolysis. In both of the mercaptoethanol-elicited modified species of acid deoxyribonuclease, low and high amounts of denatured DNA acted as competitive inhibitors of native DNA hydrolysis.

In the case of the merceptoethanol-elicited modified enzyme species, possible allosteric effects were observed inasmuch as the enzyme in combination with denatured DNA was activated in a sigmoidal fashion by the addition of increasing amounts of native DNA substrate.

A 'two active site' model is presented for acid deoxyribonuclease and discussed in terms of the present kinetic data.

## INTRODUCTION

Extracts of cultured HeLa cells catalyze the endonucleolytic degradation of both native and heat-denatured DNA at pH 4.8 (refs. 1,2). 'Acid' deoxyribonuclease, (deoxyribonucleate 3'-oligonucleotidohydrolase, EC 3.1.4.6; deoxyribonuclease II) possessing activity comparable to that observed in extracts from HeLa cells have been purified from a variety of sources, including chicken erythrocytes, hog spleen and calf spleen[3,4]. From studies of the acid deoxyribonuclease-catalyzed decrease in the molecular weight of DNA it was suggested that both 'single hit' degradation (simultaneous cleavage of both strands of native DNA at the same level) and 'double hit' degradation (introduction of single-strand breaks into the DNA double helix) can take place[5]. In order to explain the 'single hit' degradation the hypothesis was advanced that the enzyme possesses two identical active sites in the appropriate spatial configuration such that each site cleaves one of the two single strands of the DNA helix[5,6]. The finding that hog-spleen acid deoxyribonuclease was composed of two identical subunits[7,8] was consistent with the latter theory since each subunit might represent an active site. However, the production of an active monomeric subunit has not been reported nor has any direct experimental evidence supporting the 'two active sites' model been forthcoming up to the present time, to the best of our knowledge. Furthermore, there is no *a priori* reason to assume that two active sites are required for 'single hit' degradation by acid deoxyribonuclease.

In the present study kinetic experiments (using purified enzyme preparations from HeLa cells and calf spleen) were designed to test the hypothesis that there are two active sites per molecule of acid deoxyribonuclease. Evidence for the occurrence of two active sites is presented, which was derived from inhibition studies with both native and modified species of the enzyme molecule. The modified molecular forms display an allosteric type of activation by native DNA under certain conditions.

## MATERIALS AND METHODS

HeLa $S_3$ cells were cultured in suspension as previously described[9].

Radioactive DNA ($^3H$) was prepared by MARMUR's procedure[10] from *Escherichia coli* strain 15 $T^-$ grown to a limit in Tris-glucose medium containing 1 mC [$^3H$]-thymine (*plus* 2 mg carrier thymine) per l. The $^3H$-labelled DNA prepared in this manner gave about 5000 counts/min per $\mu$g DNA as measured in Bray's solution under the same conditions as were used for the estimation of enzyme activity (see below).

Acid deoxyribonuclease was assayed by using 12.5 $\mu$g of $^3H$-labelled DNA dissolved in 0.35 ml of 0.01 M sodium acetate buffer (pH 4.8) containing 50 m$\mu$-moles of $MgCl_2$. Ten $\mu$l of enzyme solution was added and the reaction mixture incubated at 37° for 15–30 min. The reaction was terminated by chilling the reaction mixture to 0° and the addition of 0.1 ml of a solution containing 2.5 mg/ml DNA and 5 mg/ml bovine serum albumin. To each tube was then added 0.5 ml of ice-cold 0.5 M $HClO_4$ and the tubes were centrifuged at 30 000 $\times$ *g* for 5 min. The supernatant fraction was added to 10 ml of Bray's solution and the radioactivity was estimated in a Packard Tricarb liquid scintillation counter (window: 50–1000; gain: 70%). A unit of enzyme activity is arbitrarily defined as the amount of enzyme catalyzing the degrada-

tion of 0.2 $\mu$g of DNA to acid soluble fragments per min (*i.e.* 1000 counts/min acid-soluble material per min with the DNA employed). The assay was linear with respect to time over a period of at least 1 h.

For purification of the acid deoxyribonuclease $1.6 \cdot 10^9$ HeLa cells were harvested by centrifugation and washed once with 0.05 M Tris–HCl (pH 7.4). The cells were then allowed to swell in hypotonic medium containing $1 \cdot 10^{-2}$ M Tris–HCl (pH 7.4) *plus* $1 \cdot 10^{-2}$ M KCl, before disruption in a Dounce homogenizer. The cell concentration was adjusted to $2.5 \cdot 10^7$ cells/ml prior to disruption. The homogenization procedure was carefully controlled so as to minimize breakage of nuclei (less than 5%) and the number of intact cells (less than 5%). The nuclear fraction was removed by centrifugation at 600 $\times$ *g* for 5 min; the cytoplasmic supernatant was then centrifuged at 30 000 $\times$ *g* for 30 min. The supernatant was assayed for traces of activity and generally discarded. The pellet was resuspended in 18 ml of 0.5 M Tris–HCl buffer (pH 7.4) containing 0.25% sodium deoxycholate. The suspension was sonicated for 3 min at 5° using a Mullard MSE sonicator. The suspension was then centrifuged (104 000 $\times$ *g* for 60 min) and the supernatant solution brought to pH 5.0 by the addition of 0.1 M acetate buffer (pH 4.8). The precipitate which formed was removed by centrifugation (30 000 $\times$ *g* for 30 min) followed by a further centrifugation (104 000 $\times$ *g* for 30 min). The clear supernatant solution was dialyzed against 100 volumes of 0.5 M Tris–HCl buffer (pH 7.4) overnight. The dialyzed solution was then added to a 1.5 cm $\times$ 15 cm column of DEAE-cellulose (Bio-Rad Cellex D) which had been previously equilibrated with 0.05 M Tris (pH 7.4). The column was developed with a linear 400-ml gradient of NaCl (0–1 M NaCl) in 0.05 M Tris–HCl buffer (pH 7.4). Ten-ml fractions were collected. The active fractions from DEAE-cellulose were combined and dialyzed overnight against the same Tris buffer as above. The dialyzed material was then added to a 1.5 cm $\times$ 15 cm column of CM-cellulose (Bio-Rad CM-Cellex) and the elution schedule was identical to that for the DEAE-cellulose column chromatography. The active fractions from CM-cellulose were dialyzed against 0.01 M Tris–HCl (pH 7.4) and used for the kinetic studies to be reported herein.

Calf-spleen acid deoxyribonuclease was purchased from Worthington Biochemicals in a partially purified state[4]. The latter preparation was further purified by chromatography on a 1.5 cm $\times$ 20 cm Sephadex G-75 column (Pharmacia) with 0.05 M Tris buffer (pH 7.4) as the eluant. The active 2-ml fractions were then pooled and subjected to CM-cellulose chromatography as described above.

Assays of acid deoxyribonuclease activity by viscometry were carried out as previously described[11].

Denatured DNA was prepared by heating a solution of highly polymerized DNA (Worthington) in 0.01 M Tris–HCl buffer (pH 7.4) (100 $\mu$g DNA/ml) at 100° for 10 min followed by rapid cooling in an ice bath. The denatured DNA was dialyzed overnight against 100 volumes of 0.01 M Tris–HCl buffer (pH 7.4).

The determination of the sedimentation constants of acid deoxyribonuclease preparations were carried out on the Spinco model E analytical ultracentrifuge by the moving-partition cell method of YPHANTIS AND WAUGH[12]. Samples were run at 59 780 rev./min at 20° for 60 min. Assays of the activity in the original solution and of the upper and lower compartments of the centrifuge cell were carried out in duplicate.

## RESULTS

### *Purification of acid deoxyribonuclease from HeLa cells*

Table I provides an outline of the purification sequence for acid deoxyribonuclease from HeLa cells. Several points of interest are summarized below:

TABLE I

PURIFICATION OF ACID DEOXYRIBONUCLEASE FROM HeLa CELLS

| *Fractions* | *Total units* | *Volume (ml)* | *Total* protein (mg)* | *Specific activity* |
|---|---|---|---|---|
| Crude homogenate | 2020 | 50 | 590 | 3 |
| Cytoplasmic fraction | 2300 | 50 | 495 | 5 |
| 28 000 × *g* supernatant | 166 | 50 | 300 | 0.5 |
| Deoxycholate extract | 4215 | 18 | 126 | 33 |
| pH 5.0 supernatant | 4178 | 20 | 21.4 | 195 |
| DEAE-cellulose | 2381 | 40 | 1.4 | 1700 |
| CM-cellulose | 930 | 50 | 0.34 | 2700 |

* Determined according to LOWRY *et al.*[13].

(1) Acid deoxyribonuclease occurs almost entirely in the cytoplasmic fraction of HeLa cells.

(2) More than 70% of the acid deoxyribonuclease activity is sedimentable by centrifugation of the cytoplasmic fraction at 30 000 × *g* for 30 min.

(3) Treatment of the particulates with sodium deoxycholate releases most of the bound enzyme into a soluble form which effects a 2-fold greater total activity of the enzyme in the total extract.

(4) Acid deoxyribonuclease is soluble at pH 5.0.

(5) Acid deoxyribonuclease binds weakly to DEAE-cellulose and is eluted as a single peak at a NaCl concentration of approx. 0.1 M.

(6) Acid deoxyribonuclease binds onto CM-cellulose columns and is eluted as a

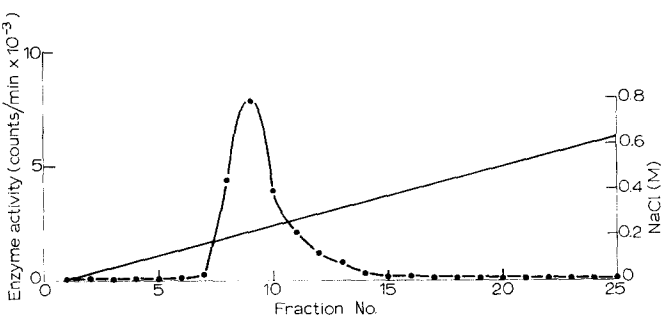


Fig. 1. Elution profile of HeLa acid deoxyribonuclease activity after column chromatography on CM-cellulose. A 400-ml NaCl gradient (0–1 M) was used as eluant. Each fraction contained 10 ml. The theoretical concentration of NaCl in each fraction is given by the straight line as indicated on the left-hand ordinate.

single peak whose midpoint corresponds to an NaCl concentration of 0.2 M (Fig. 1).

The present purification procedure generally results in a 700-fold purification of the enzyme activity with a yield of about 30–40% of the original activity present in crude extracts. However it should be pointed out that an exact estimation of the level of purification is difficult to obtain due to increase in enzyme activity by such treatments as deoxycholate or removal of nucleic acids during the purification procedure. The present purification scheme is similar to that employed by BERNARDI AND GRIFFE for hog-spleen deoxyribonuclease[3]. Important modifications we have introduced are (1) the enzyme is never exposed to pH values below 5 thus avoiding deamidation of the enzyme as observed by G. BERNARDI (personal communication) with the spleen deoxyribonuclease, (2) preliminary subcellular fractionation to effect a 4-fold increase in specific activity, and (3) the use of sodium deoxycholate to liberate the enzyme from a particulate state.

*Purification of calf spleen enzyme*

The Worthington product was chromatographed on Sephadex G-75. The major impurity was a low molecular weight protein component which eluted after the acid deoxyribonuclease activity. The low molecular weight of this impurity was confirmed by the observation that this substance failed to sediment as a peak after centrifugation for 2 h at 59 780 rev./min in the analytical ultracentrifuge. CM-cellulose chromatography of the active fractions from Sephadex G-75 resulted in a 50-fold purification of the original Worthington preparation. This 50-fold purified enzyme was used in the present studies.

The elution profile of the calf-spleen acid deoxyribonuclease from CM-cellulose closely resembled that obtained with the HeLa enzyme (Fig. 1).

*Mercaptoethanol-induced modifications of acid deoxyribonuclease*

Treatment of acid deoxyribonuclease with 8 M urea *plus* 0.1 M 2-mercaptoethanol has been reported to produce monomeric subunits of the hog-spleen enzyme[7] but no mention had been made as to their retention of enzymatic activity. Conditions were therefore investigated in our laboratory for the production of modified enzyme components which retained a certain measure of activity. In our experience, the 8 M urea treatment results in a rapid and total loss of enzymatic activity.

Treatment of the HeLa enzyme at pH 5.0 with 0.05 M 2-mercaptoethanol at 37°

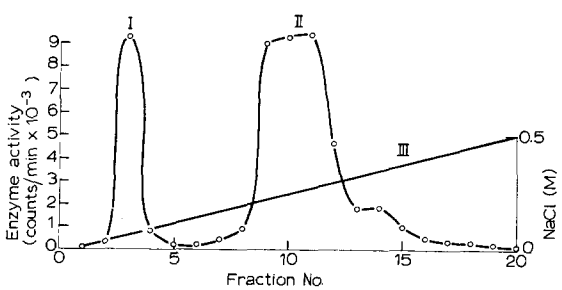


Fig. 2. Elution profile of acid deoxyribonuclease activity after CM-cellulose chromatography. The HeLa acid deoxyribonuclease was treated with 0.05 M 2-mercaptoethanol at pH 5.0 for 2 h at 37° followed by 48 h at 5° ,and then subjected to column chromatography.

for 2 h followed by storage in the refrigerator (approx. 5° for 48 h) resulted in the formation of two new peaks after chromatography on CM-cellulose (Fig. 2). The early eluting peak we shall refer to as 'Peak I', the native peak, which elutes next, as 'Peak II', and the third peak eluting after the native enzyme as 'Peak III'. Treatment of the calf-spleen enzyme preparation in the same manner with mercaptoethanol resulted in the production of very little Peak I material and most of the enzyme was converted to Peak III material. No native, Peak II material was detected (see Fig. 3). A shorter treatment of the calf-spleen enzyme at pH 5.0 (*e.g.* 60 min at 37°) with 0.05 M mercaptoethanol resulted in the conversion of 50% of Peak II material to Peak III

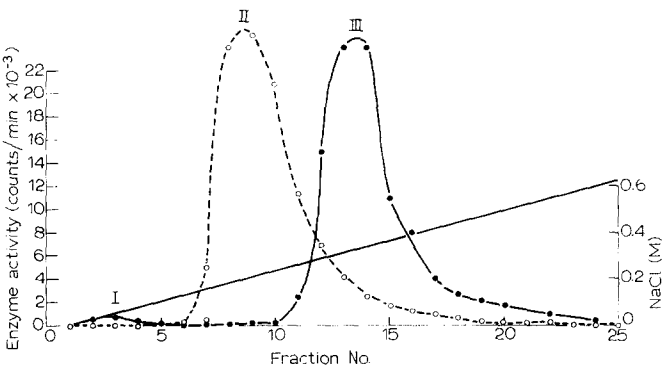


Fig. 3. Elution profile obtained after CM-cellulose chromatography of calf-spleen acid deoxyribonuclease which had been previously treated with 2-mercaptoethanol (0.05 M at pH 5.0 for 2 h at 37° followed by 48 h at 5°.) Solid line represents the mercaptoethanol-treated enzyme. Dashed line represents the elution profile usually obtained with the native calf-spleen enzyme.

material. No Peak I material was detectable. Preliminary results indicate that at pH 7.0 treatment of the calf-spleen enzyme with 0.05 M 2-mercaptoethanol (2 h at 37° followed by 24 h at 5°) produces no detectable amount of Peak III material and a small amount of Peak I material. Most of the enzyme, however, elutes in a position corresponding to that of the native enzyme.

In view of the possibility that one of the two mercaptoethanol-elicited species of acid deoxyribonuclease might represent a monomeric subunit of the enzyme, the sedimentation constant of the material in each of the CM-cellulose peaks was determined in the case of the calf-spleen enzyme. To conserve material the sedimentation constant was determined by a method dependent upon a direct measurement of enzymatic activity in each of the compartments of a partitioned ultracentrifuge cell (see MATERIALS AND METHODS). Table II lists the calculated sedimentation constants obtained by this method for each of the peaks.

Since the sedimentation constant of both Peak I and Peak III materials (approx. 2.5 S) is close to that observed for the native enzyme (2.7 S) it is unlikely that the former are monomeric subunits of Peak II acid deoxyribonuclease. The slightly lower sedimentation constant of the mercaptoethanol-modified enzyme peaks, however, does suggest that alterations in the structure of the native enzyme have occurred. Also included in Table II is the sedimentation constant of the native HeLa acid deoxyribonuclease (2.7 S) which is identical with that of the calf-spleen enzyme.

TABLE II

SEDIMENTATION CONSTANTS OF NATIVE AND MERCAPTOETHANOL-ELICITED SPECIES OF ACID DEOXYRIBONUCLEASE

| *Deoxyribonuclease* | *Sedimentation constant* |
|---|---|
| *Calf spleen* | |
| Peak II (native) | 2.7 ± 0.05 |
| Peak III | 2.5 ± 0.05 |
| Peak I | 2.5 ± 0.05 |
| *HeLa* | 2.7 ± 0.05 |

The HeLa and calf-spleen native deoxyribonuclease and the mercaptoethanol-elicited modifications all have the ability to degrade heat-denatured DNA to acid-soluble fragments at about one-fifth the rate obtained with native DNA substrate.

*Kinetic studies*

Heat-denatured DNA inhibits the degradation of native DNA by acid deoxyribonuclease. The nature of this inhibition was studied using native $^3$H-labelled DNA

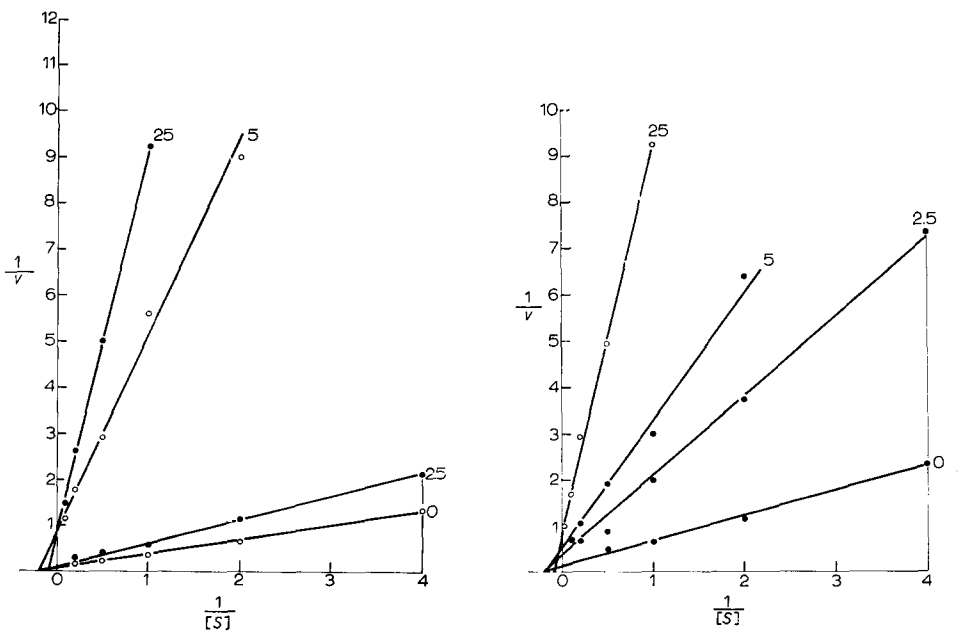


Fig. 4. Lineweaver–Burk plot of the kinetics of calf-spleen acid deoxyribonuclease degradation of $^3$H-labelled native DNA in the presence of different amounts of heat-denatured DNA. The number beside each curve represents the number of $\mu$g of heat-denatured DNA present in the reaction mixtures applying to that curve (see text for details).

Fig. 5. LINEWEAVER–BURK plot of the kinetics of degradation of $^3$H-labelled native DNA by HeLa acid deoxyribonuclease in the presence of different amounts of heat-denatured DNA (see legend of Fig. 4).

as substrate and non-radioactive heat-denatured DNA from calf thymus as inhibitor. Fig. 4 is a LINEWEAVER–BURK plot[14] of the data obtained. Amounts of inhibitor up to 5 $\mu$g (total volume of reaction mixture was always 0.4 ml) cause a non-competitive type of inhibition characterized by a lowering of the $v_{max.}$ at constant $K_m$. Higher amounts of inhibitor (*e.g.* 25 $\mu$g) however, bring about a competitive type of inhibition which is characterized by a increase in the $K_m$ at constant $v_{max.}$ The latter findings apply also to the HeLa acid deoxyribonuclease (Fig. 5). The $K_m$'s of the HeLa and calf-spleen enzymes are quite similar (4.5 and 5.0 $\mu$g of native DNA, respectively). It was also observed during the present studies that amounts of native DNA substrate greater than 5 $\mu$g caused some inhibition of acid deoxyribonuclease, which resulted in deviations from linearity in the LINEWEAVER–BURK plots in the region of high substrate concentration.

Fig. 6 illustrates the effect of increasing inhibitor concentration (denatured DNA) on the activity of acid deoxyribonuclease (spleen) at two different native DNA substrate concentrations. It can be seen that the final degree of inhibition is not total. In fact, 60 and 34% of the enzymatic activity is retained with 10 and 2 $\mu$g of substrate, respectively. Therefore the mode of inhibition of the enzyme by heat-denatured DNA is not purely competitive nor purely non-competitive as might be indicated at first sight from the LINEWEAVER–BURK plots.

The hydrolysis of heat-denatured $^3$H-labelled DNA by calf-spleen acid deoxyribonuclease is inhibited by unlabelled native DNA. Fig. 7 illustrates the LINEWEAVER–BURK plot obtained from which it can be seen that 5 $\mu$g of native DNA appears to act as a non-competitive inhibitor of heat-denatured DNA degradation. The $K_m$ for single-stranded DNA, 5 $\mu$g DNA, is the same as that observed for native DNA, but it should be noted that on a molar basis it would be about twice that observed for native DNA.

The mode of inhibition by denatured DNA of native DNA degradation was also examined using the mercaptoethanol-modified enzyme molecules. Fig. 8 illustrates the data obtained with Peak III from CM-cellulose plotted after LINEWEAVER–BURK. The inhibition even by low amounts of denatured DNA seems to be competitive. Of particular interest is that the reciprocal of enzyme activity in the presence of inhibitor decreases with substrate in a non-linear fashion until the concentration of substrate is increased to 1 $\mu$g. The latter phenomenon which appears to be due to an activation of enzyme *plus* inhibitor by native DNA is clearly illustrated by a substrate saturation plot (Fig. 9). The $K_m$ of the Peak III enzyme, 0.91 $\mu$g native DNA, is considerably lower than the $K_m$ of the native enzyme.

From the results of studies on the inhibition by denatured DNA of the degradation of native DNA by Peak I enzyme (Fig. 10) it can be seen that in this case also the inhibition appears to be of the competitive type. On the other hand, a plot of enzyme activity *versus* inhibitor concentration (Fig. 11) shows that a plateau is reached at about 80% inhibition. The latter observation applies also to the Peak III enzyme. This suggests that the inhibition in this case is not purely competitive. The $K_m$ of Peak I enzyme, 1.48 $\mu$g native DNA, is also considerably lower than the $K_m$ of the native enzyme.

The ability of the modified enzyme peaks to carry out 'single hit' degradation of native DNA was tested by viscometry. Fig. 12, Curve A illustrates the result obtained by the action of pancreatic deoxyribonuclease (Worthington deoxyribonucle-

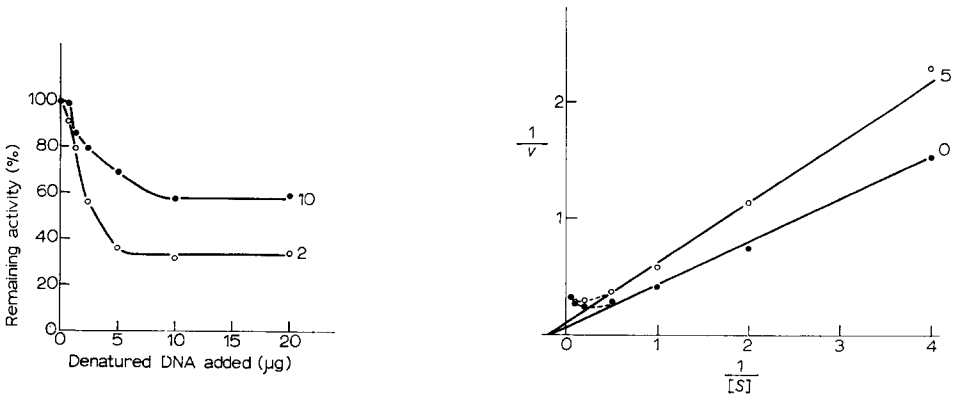


Fig. 6. Degree of inhibition by increasing amounts of heat-denatured DNA of the hydrolysis of $^3$H-labelled native DNA by calf-spleen acid deoxyribonuclease. The numbers beside the curves indicate the amount of $^3$H-labelled native DNA present in each reaction mixture applying to each of the two curves. Inhibition is expressed as a percentage of the initial activity (without inhibitor) retained after addition of various amounts of inhibitor (abscissa).

Fig. 7. LINEWEAVER–BURK plot of the saturation kinetics of hydrolysis of $^3$H-labelled heat-denatured DNA by calf-spleen acid deoxyribonuclease and its inhibition by native DNA. The number beside each curve indicates the amount of native DNA present in each reaction mixture applying to that curve. The dashed portion of the curves indicate deviations from the theoretical saturation curves caused by inhibition of the enzyme by excess substrate.

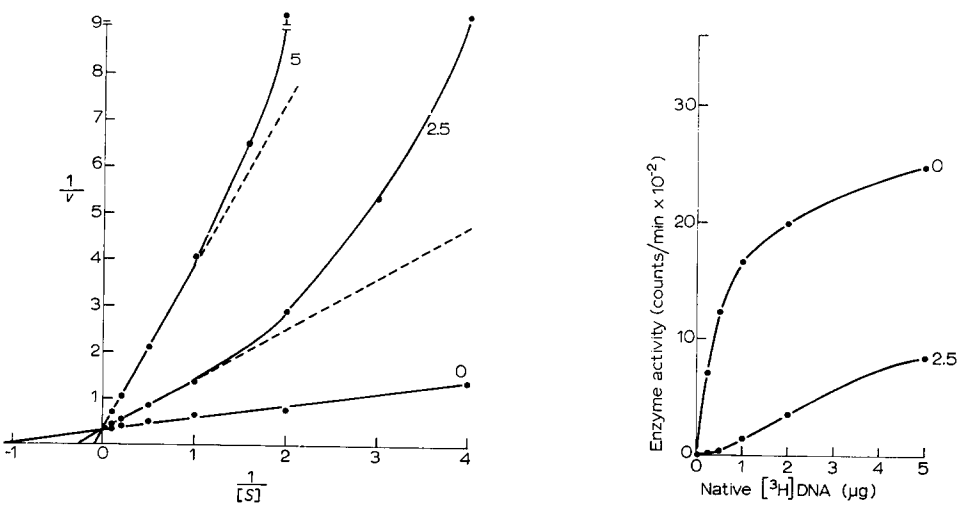


Fig. 8. LINEWEAVER–BURK plot of the kinetics of inhibition by heat-denatured DNA of the hydrolysis of $^3$H-labelled native DNA by the mercaptoethanol-elicited Peak III material from calf-spleen acid deoxyribonuclease. The dashed lines indicate the theoretically expected linear plots while the solid lines represent the experimentally observed results. The number beside each curve indicates the amount (in μg) of heat-denatured DNA present in the reaction mixtures applying to that curve.

Fig. 9. Saturation kinetics of calf-spleen acid deoxyribonuclease Peak III modified enzyme by native $^3$H-labelled DNA in the presence of 0 (upper curve) and 2.5 μg (lower curve) of heat-denatured DNA.

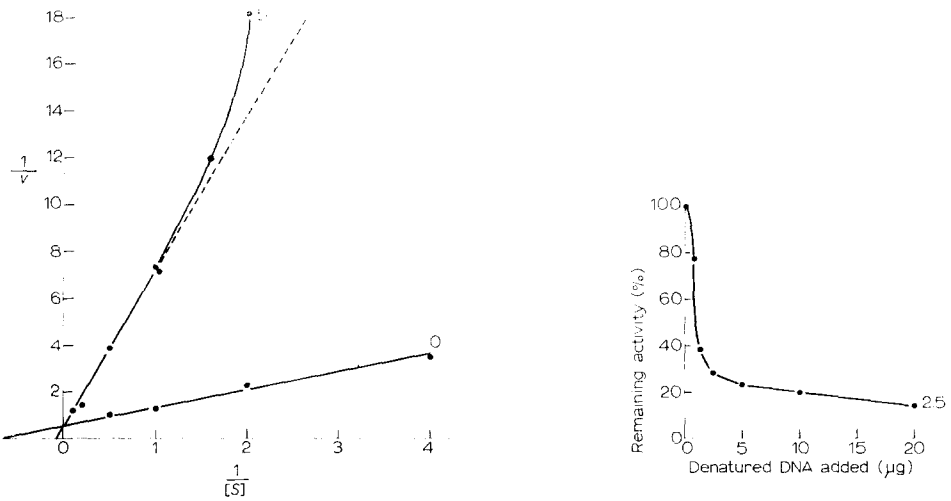


Fig. 10. LINEWEAVER-BURK plot of the saturation kinetics of calf-spleen acid deoxyribonuclease modified species Peak I. The number beside each curve indicates the number of $\mu g$ of heat-denatured DNA present in the reaction mixtures applying to that curve. The dashed line indicates the theoretical linearity expected in a LINEWEAVER-BURK plot. The solid portion represents the experimentally observed results.

Fig. 11. Inhibition by heat-denatured DNA of the hydrolysis of $^3$H-labelled native DNA by the modified calf-spleen acid deoxyribonuclease (Peak I) (see legend of Fig. 10).

ase I) which is known to degrade DNA by 'double hit' kinetics[6]. As expected, an initial lag occurs prior to a decrease in the relative viscosity. Curve B shows the results obtained with the modified form of calf-spleen acid deoxyribonuclease (Peak I). It can be seen that there is no lag in the decrease of relative viscosity. The same result applies to the native enzyme (Curve C) and the modified enzyme eluted as Peak III. Thus all forms of the enzyme can degrade native DNA by the 'single hit' mechanism.

It was noted that heat-denatured DNA inhibited the degradation of native DNA by acid deoxyribonuclease to a definite value and that further inhibition was not achieved by addition of excess inhibitor. It was reasoned that perhaps in the

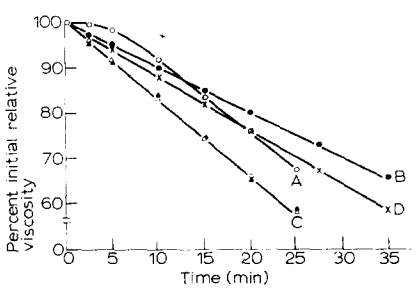


Fig. 12. Kinetics of the decrease in relative viscosity of native DNA solutions catalyzed by: Curve A, pancreatic deoxyribonuclease; Curve B, the modified form of calf-spleen acid deoxyribonuclease (Peak I); Curve C, either native calf-spleen acid deoxyribonuclease or the modified species (Peak III); Curve D, native calf-spleen acid deoxyribonuclease in the presence of excess heat-denatured DNA (see text for details).

presence of excess denatured DNA the enzyme might have one of its sites saturated by inhibitor and if both sites were required for 'single hit' degradation, the latter should not be observed by viscometry. Curve D (Fig. 12) indicates that this prediction is not tenable since again no lag was observed. Similar results were obtained with Peaks I and III in the presence of excess denatured DNA (3 mg heat-denatured DNA *plus* 1 mg native DNA in total volume of 5.8 ml). It should be pointed out that the above viscometric analysis is purely qualitative and does not rule out the possibility that denatured DNA severely inhibits 'single hit' degradation of native DNA by acid deoxyribonuclease.

## DISCUSSION

HeLa acid deoxyribonuclease was found to be similar to the calf-spleen enzyme on the basis of several criteria including $K_m$, pH optimum, ratio of activity with native *versus* denatured DNA as substrate, mode of inhibition by denatured DNA, sedimentation constant, chromatographic behavior, and the qualitative nature of mercaptoethanol-elicited molecular variants of the enzyme. A distinct difference between the two enzymes is the quantitative proportion of the two different molecular modifications of the enzyme produced by mercaptoethanol at pH 5.0. The HeLa enzyme produces more Peak I material than Peak III material while the spleen enzyme produces primarily Peak III material.

Since our primary interest in this laboratory has been the study of virus-induced deoxyribonucleases[1,2,15,16], the occurrence of modified forms of the host cell's enzymes with different physical and catalytic properties is of direct concern to us. Furthermore, they point out that extreme caution should prevail in instances where a novel virus-induced enzymatic activity appears before the conclusion is drawn that it represents a different protein from that present in the host cell.

LINEWEAVER–BURK plots of the data obtained at low levels of denatured DNA indicated that the latter acted as a non-competitive inhibitor of the hydrolysis of native DNA by acid deoxyribonuclease. At higher concentrations of inhibitor, however, an identical treatment of the data indicated that denatured DNA now acted as a competitive inhibitor of native DNA degradation. It was also found that inhibition of native DNA degradation by increasing amounts of denatured DNA was never total (Fig. 6). The latter results are not consistent with the classical model for purely non-competitive inhibition[17], but suggest instead, the occurrence of a mixed type of inhibition. This phenomenon may result from the binding of inhibitor to a second site distinct from the catalytic site for native DNA hydrolysis and a concomitant structural modification of the enzyme molecule resulting in a lower $v_{max.}$ for native DNA degradation. Upon total conversion of the available enzyme to an enzyme–inhibitor complex additional inhibitor might interact with a different site and thus be in a more competitive relationship with the substrate. However, the partially competitive phase of inhibition of native DNA degradation by high concentrations of denatured DNA cannot be explained solely on the basis of competition with native DNA for the same binding site since the latter situation would result in purely competitive inhibition. It is more likely that a second factor is involved—perhaps, a further structural modification of the enzyme resulting in a lower affinity for native DNA substrate. Heat-denatured DNA is also an acceptable substrate of acid deoxyribonuclease. Therefore

it is probable that denatured DNA acts as an inhibitor by binding to a portion of a catalytic site even at low concentrations. It thus appears necessary to postulate the existence of two active sites per deoxyribonuclease molecule in order to explain the fact that denatured DNA can act simultaneously as a substrate and as a partially non-competitive inhibitor of native DNA degradation.

The mercaptoethanol-elicited modifications of acid deoxyribonuclease appeared to be competitively inhibited by heat-denatured DNA in their ability to degrade native DNA (Figs. 9 and 11). However it was demonstrated that the inhibition was not purely competitive in that excess inhibitor did not cause total inhibition. Thus the inhibition kinetics for the mercaptoethanol-produced enzyme species (Peaks I and III) resemble those of the native enzyme in the presence of excess inhibitor. This similarity strengthens the supposition that the enzyme–inhibitor complex of the native enzyme represents a structurally modified entity distinct from the native enzyme in the absence of inhibitor. The observed activation of the modified enzyme species by native DNA in the presence of denatured DNA might then represent a partial restoration of the activity of the damaged site brought about by a change in the molecular configuration of the enzyme. The lower $K_m$ of the modified enzyme species relative to that of the native enzyme is consistent with the hypothesis that the latter has largely lost the activity of one of its substrate binding sites.

In conclusion, we tentatively favor a model for acid deoxyribonuclease which includes two active sites per enzyme molecule, each of which site binds a native DNA molecule and catalyzes its hydrolysis by both 'single hit' and 'double hit' degradation. The observation that the modified enzyme as well as the native enzyme saturated with inhibitor are still able to degrade native DNA by 'single hit' kinetics is not inconsistent with the idea concerning the ability of each theoretical site to carry out double-strand scission.

An enzyme corresponding to the model suggested above might be ideally suited for a role in genetic recombination by a breakage and reunion mechanism. Such an enzyme would be able to carry out double strand breaks in two different native DNA molecules which are in close proximity to each other.

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