Studies on Acid Deoxyribonuclease. III. Physical and Chemical Properties of Hog Spleen Acid Deoxyribonuclease*

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ABSTRACT: The enzyme acid deoxyribonuclease, recently isolated from hog spleen, has been characterized in some of its physical and chemical properties: sedimentation and diffusion coefficients, electrophoretic properties, and amino acid composition. The enzyme appears to be a basic protein having a molecular weight of about 38,000 and containing a carbohydrate moiety.

Acid deoxyribonuclease has been recently isolated from hog spleen as a chromatographically homogeneous enzyme, completely free from phosphomonoesterase, exonuclease (spleen phosphodiesterase), and nucleoside polyphosphatase, and showing only a trace contamination of ribonuclease activity (Bernardi and Griffé, 1964). This contamination may be removed by using a modified purification procedure.[1]

Work carried out in the past 4 years on acid deoxyribonuclease has revealed several highly interesting features: the enzyme is able to split native DNA according to "single-hit" kinetics[2] (Bernardi and Sadron, 1961, 1964a,b; MacHattie *et al.*, 1963); it has a "phosphodiesterase" activity on bis(*p*-nitrophenyl)phosphate (Bernardi and Griffé, 1964), this "allosteric" substrate showing a cooperative type of binding to the enzyme (Bernardi, 1965); it is competitively inhibited in both its deoxyribonuclease and phosphodiesterase activities by t-RNA and some synthetic polyribonucleotides (Bernardi, 1964); it is very widely distributed and probably present in all the cells of multicellular organisms (Cordonnier and Bernardi, 1965). Such an enzyme has not been described so far in bacteria; it has been found, however, that endonuclease I from *Escherichia coli* (Lehman *et al.*, 1962) degrades DNA like acid deoxyribonuclease (Bernardi and Cordonnier, 1965).

All the above-mentioned findings prompted a physical and chemical investigation on the enzyme. The results obtained, already reported in part in a preliminary form (Bernardi *et al.*, 1963), are presented here.

Materials

Hog spleen acid deoxyribonuclease was prepared according to the method of Bernardi and Griffé (1964) (preparations HS2 and HS3) or to a modification of it[1] (preparation HS7). All measurements were carried out on both types of preparations. These were indistinguishable in their amino acid composition and physical properties.

Methods

Sedimentation. A Spinco Model E analytical ultracentrifuge with temperature-control unit was used. Sedimentation-velocity experiments were carried out generally at 59,780 rpm using conventional 12-mm cells or synthetic-boundary cells (rubber-valve or capillary type). Schlieren or ultraviolet absorption optics were used; the boundary displacements were measured using a Gartner microcomparator or a Joyce-Loebl microdensitometer, respectively. Sedimentation coefficients were computed from plots of log x (x being the boundary distance to rotation axis) versus t (time). These were corrected to standard conditions.

Diffusion. Owing to the limited amount of material available, diffusion measurements were carried out in the analytical ultracentrifuge at 12,500 rpm, with the use of a synthetic-boundary cell. The enzyme concentrations were close to 0.5% in standard acetate buffer, which is 0.15 M acetate buffer–0.01 M EDTA, *p*H 5.0 (Bernardi and Griffé, 1964). Schlieren patterns were enlarged, areas were measured with an Amsler mechanical integrator, and the diffusion coefficient was calculated from plots of $(A/H)^2/4\pi$ versus t (A being the area under the peak, H the height of the peak). This was then corrected to standard conditions. The correction for the effect of the centrifugal field on different parts of the boundary was negligible.

In one case the ultraviolet absorption method was used, with a protein concentration of about 0.1%. The diffusion coefficient was calculated from densitometer tracings according to Jacob (1959).

Partial Specific Volume. This was calculated from the amino acid composition and the partial specific volumes

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[1] G. Bernardi and A. Bernardi, to be published.

[2] This finding has been recently confirmed by the independent work of Young and Sinsheimer (1965).

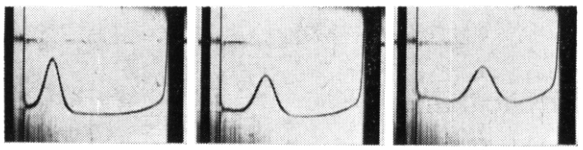

FIGURE 1: Sedimentation pattern of hog spleen acid deoxyribonuclease in standard acetate buffer. Exposures taken after 52, 84, and 122 minutes at 59,780 rpm.

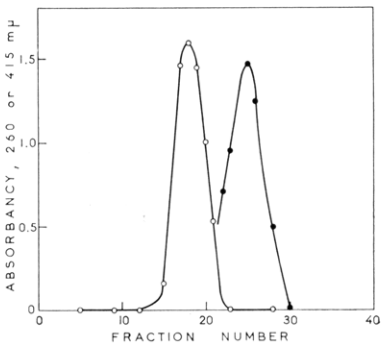

FIGURE 2: Sucrose-gradient centrifugation of hog spleen acid deoxyribonuclease; cytochrome *c* was used as a reference protein. A total of thirty-three fractions was collected. Circles indicate deoxyribonuclease activity, points, the optical density of cytochrome *c* at 415 mμ. Bottom of the cell is at the left. A linear molarity gradient was obtained by using 5% and 20% sucrose solutions in standard acetate buffer. Centrifugation was carried out for 16 hours at 4° at 38,00 rpm using a SW-39 rotor and a Spinco Model L ultracentrifuge.

of the amino acid residues (Cohn and Edsall, 1943); the partial specific volume of glucosamine was taken as 0.60 ml/g. Electrostriction effects were not taken into account.

Sucrose-gradient centrifugation was performed in a Model L Spinco preparative ultracentrifuge with a temperature setting of 4°. An SW-39 swinging-bucket rotor was used, the centrifugation time being 16 hours, the speed 38,000 rpm. Linear sucrose-concentration gradients were obtained using 5% and 20% sucrose solution in standard acetate buffer. As a reference protein (Martin and Ames, 1961), beef heart cytochrome *c* (Sigma, Type V) was added to the enzyme solution (total volume 0.1 ml) to be layered on the top of the sucrose gradient. On the average, thirty-three fractions were collected from each tube; they were assayed for optical density at 415 mμ, enzyme activity, and refractive index (using a Zeiss refractometer of the Abbé type).

Zone electrophoresis was done on cellulose acetate strips with standard acetate, potassium phosphate, or sodium borate buffers of ionic strength 0.1 as the solvents. A field strength of 6 v/cm was used. Staining was done with amido black, excess dye being removed overnight with Smithies' solution at 37° (Smithies, 1955). Alternatively, Ponceau S in trichloroacetic acid was used. *Ultraviolet spectra* were determined with a Cary Model 15 instrument.

Amino Acid Analyses. Aliquots of deoxyribonuclease solutions containing 3–5 mg protein were freeze-dried in heavy-walled special Pyrex tubes. One ml of 6 N HCl (Mallinckrodt, AR) was added to each tube. After freezing in solid CO_2-acetone bath the tubes were connected through a vacuum stopcock to a mechanical pump (Edwards 25 C 150) and evacuated to 10 μ. The stopcock was then closed and the solution was thawed, frozen, and evacuated again. The tubes were sealed and allowed to stand in an oven at 110° for the desired time. After cooling to room temperature, the tubes were opened and dried *in vacuo* over NaOH pellets. The hydrolysate was then dissolved in 1 ml of distilled water and centrifuged (30 minutes at 20,000 × *g*) to discard a minute precipitate. Then 4 ml of citrate buffer, *p*H 2.2, was added to 0.4 ml of the supernatant. This solution was chromatographed using a Spinco Model 120 amino acid analyzer. For the separation of glucosamine from phenylalanine the buffer-change timer for the long column was set at 7 hours.

Another 0.5-ml aliquot of the supernatant was vacuum dried in an aluminum foil boat, and its nitrogen content was determined using a Coleman Model 29 analyzer. The dry weights were determined on a Cahn electrobalance. The performic acid oxidation was performed at 0° (Schram *et al.*, 1954) or at room temperature (Sjöquist, 1960). The oxidized protein was recovered by freeze drying and hydrolyzed as before. The tryptophan content was determined by spectrophotometric titration with *N*-bromosuccinimide (Patchornik *et al.*, 1958). Total nitrogen and total sulfur were determined on deionized protein samples.

Results

The ultracentrifuge analysis (Figure 1) showed that the enzyme sedimented as a single boundary in acetate, *p*H 3.0 and 5.0, and phosphate buffer, *p*H 7.8; in all cases the ionic strength was 0.1. The sedimentation coefficient in the above-mentioned solvents and in standard acetate buffer was found to be 3.4 S at the low concentration (about 0.1%) used with the absorption optics. In glycine buffer, *p*H 8.9, $\mu = 0.1$, a slightly higher value ($s_{20,w} = 4.0$ S) was found, and a small amount of a faster component was present. In glycine buffer, *p*H 2.0, $\mu = 0.1$, the sedimentation analysis indicated extensive aggregation. A dependence of the sedimentation coefficient upon the concentration was found; in standard acetate buffer the sedimentation coefficient at a concentration of 1% was 2.8 S. The sedimentation coefficient of the enzymatic activity as calculated from the sucrose gradient centrifugation (Figure 2) was found

TABLE I: Physical Properties of Hog Spleen Acid Deoxyribonuclease.

$s^{\circ}_{20,w}$ (Svedbergs)	3.4
$D_{20,w}$ (10^{-7} cm^2/sec)[a]	7.8
v (ml/g)[b]	0.72
mw	3.8×10^4
f/f_0	1.34
$E^{1\%,\,1cm}_{280m\mu}$	12.1

[a] This value was obtained at concentrations of 0.5% and about 0.1%. [b] Calculated value (see text).

to be equal to 3.2 S using an $s_{20,w}$ of 1.7 S for cytochrome *c* (Ehrenberg, 1957). Over 90% of the loaded activity was recovered in the peak.

The diffusion coefficient was found to be equal to 7.8×10^{-7} cm^2/sec. The same value was obtained from several experiments and also from an experiment at very low concentration where the ultraviolet optics was used. The value given above is therefore likely to be close to the value at zero concentration. The calculated partial specific volume, molecular weight and frictional ratio are given in Table I.

The zone electrophoresis on cellulose acetate showed a single band only at four different *p*H values ranging from 4.6 to 9.2. By extrapolating the mobilities obtained at the different *p*H values to zero mobility, an isoelectric point close to 10.2 was found. The ultraviolet spectrum of the enzyme is shown in Figure 3, and its extinction coefficient at 280 mμ is given in Table I.

Finally, the results of the amino acid analyses are reported in Table II. The destruction of serine and threonine in the acid hydrolysis was moderate; valine and isoleucine were liberated slowly. The nitrogen content calculated from the amino acid analysis was in excellent agreement with the nitrogen analysis of the hydrolysate. The sulfur content was also in very good agreement with the value expected from the amounts of cysteine/cystine and methionine found in the protein.

TABLE II: Amino Acid Analysis of Hog Spleen Deoxyribonuclease.

	Grams of Amino Acid Residues per 100 g of Protein[a] in Hydrolysis Time of:				Moles of Amino Acid per Mole of Protein mw 38,000	Nearest Integral Number of Residues per Mole of Protein[c]
	22 hr	48 hr	72 hr	Corrected Values[b]		
Lys	6.94	6.80	6.95	6.89	20.44	20
His	2.13	2.17	2.31	2.20	6.08	6
(NH_3)	(23.05)	(23.65)	(24.06)	(22.5)	(49.4)	(49)
Arg	5.04	5.55	5.37	5.46	13.30	13
Asp	10.19	10.14	9.36	9.89	32.68	33
Thr	5.44	5.20	4.80	5.63	21.16	21
Ser	8.30	8.10	7.18	8.46	36.93	37
Glu	11.03	11.09	10.66	10.93	32.19	32
Pro	7.09	7.14	6.51	6.91	27.05	27
Gly	3.88	3.97	3.87	3.90	25.99	26
Ala	4.84	4.72	4.33	4.63	24.77	25
$^1/_2$ Cys	1.77	1.79	1.79	1.79	6.65[d]	8
Val	2.62	3.33	3.35	3.35	12.84	13
Met	1.63	1.65	1.30	1.53	4.45	4
Ileu	2.08	2.49	2.52	2.52	8.47	8
Leu	10.47	11.13	10.49	10.67	35.87	36
Tyr	5.23	5.17	4.83	5.28	12.31	12
Phe	6.30	6.56	6.07	6.31	16.30	16
Try					6.3[e]	6
Glucosamine	3.07	3.32	3.06	3.15	7.45	(8)
Total	98.05	100.32	94.75	(99.50)		343
N recovery %	98.9	98.7	97.4			

[a] Total N is 17.2%; total S is 1.0%. [b] In calculating the corrected values, the criteria given by Tristram and Smith (1963) have been followed. [c] The selection of the integral numbers of residues has been done taking into account also results from other analyses. [d] After performic acid oxidation, cysteic acid 8.2 residues. [e] From *N*-bromosuccinimide titration.

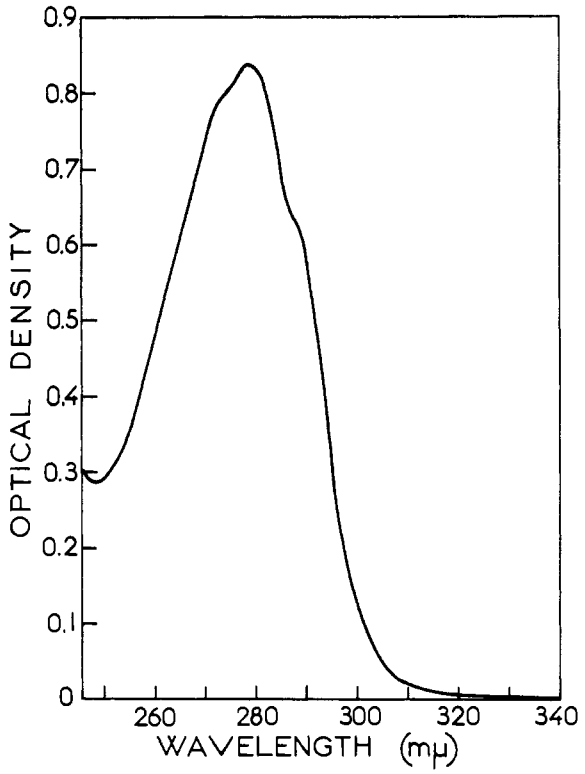

FIGURE 3: Ultraviolet spectrum of hog spleen acid deoxyribonuclease in standard acetate buffer.

Discussion

The amino acid analysis reported here shows a high level of aromatic amino acids, as expected from the high extinction coefficient at 280 mμ. The high ammonia content of the acid hydrolysate suggests that a large percentage of the dicarboxylic acids might be present in the protein as the corresponding amides. Some preliminary work on enzymic (pronase) digests of the protein confirmed its high amide level. These findings explain the alkaline isoelectric point of the enzyme.

An unexpected analytical result is the finding of glucosamine residues bound to the enzyme. Acid deoxyribonuclease is therefore one of the few enzymes which may be classified as glycoprotein. Preliminary results on the tryptic hydrolysate of the enzyme (obtained in collaboration with Dr. Baglioni) indicate that all the glucosamine residues are bound to one single peptide. A partial hydrolysis of glycosidic linkages might occur at the low *p*H used in one step of the purification procedure, as suggested by the work of Plummer and Hirs (1964) on ribonuclease B. Therefore, a complete characterization of the carbohydrate moiety of acid deoxyribonuclease and the neutral sugars it possibly contains must await further work.

To explain the simultaneous breakage of both DNA strands at the same level caused by acid deoxyribonuclease, Bernardi and Sadron (1964a,b) put forward the hypothesis that the enzyme is a dimeric protein with one active site on each (identical) subunit. In connection with this hypothesis, it seems pertinent to point out that all the amino acids which are present at a low level in the enzyme were found in an even number of residues per mole of protein. Conclusive evidence for the dimeric structure of the enzyme, based on the tryptic peptides and the dissociation of acid deoxyribonuclease in strong urea solutions, has been reached very recently.[3]

The physical results indicate that hog spleen deoxyribonuclease is a basic globular protein having a molecular weight close to 38,000. As far as its macromolecular properties are concerned, the enzyme appears to be quite stable, at least in the experimental conditions used in this work. No dissociation of the protein into subunits is caused by dilution, as indicated by the centrifugation experiments in sucrose density gradient. Although the frictional ratio reported in Table I is to be accepted with some caution, its high value suggests a certain degree of molecular asymmetry.

In conclusion, the present work gives a basic physical and chemical characterization of hog spleen deoxyribonuclease, and therefore complements the previously published work on its mechanism of action (Bernardi and Sadron, 1964b) and its purification and enzymological properties (Bernardi and Griffé, 1964). To our knowledge, a basic characterization such as this one is not available at the present time for any other of the enzymes acting on DNA, nor for the large majority of "allosteric" enzymes.

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Structural Requirements of Nucleosides for Binding by Adenosine Deaminase*

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ABSTRACT: The substrate specificity of adenosine deaminase has been studied in detail. It has been observed that a significant difference exists between the binding of those compounds altered in the 6 or 9 position of adenine. Substitutions in the 6 position (N^6-methyl, -hydrogen, or -mercapto) of adenosine result in compounds that are competitive inhibitors. Substitution of a chlorine atom for the amino group in the 6 position (6-chloropurine ribonucleoside) results in a nucleoside that is, in fact, a substrate for adenosine deaminase. Changes in the 9 substituent of adenine results in compounds that are either substrates (e.g., adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 3′-amino-3′-deoxyadenosine, xylofuranosyladenine, and arabinofuranosyladenine) or inhibitors (e.g., 9-hexyladenine, 9-pentyladenine, 9-cyclopentanoladenine, and 9-cyclohexanoladenine).

Seven of the 9 position substituent analogs studied were not bound by the enzyme (adenine, psicofuranine, fructofuranosyladenine, 2′-adenylic acid, 3′-adenylic acid, 5′-deoxyadenylic acid, and 9-cyclohexyladenine). Based on these observations, it is concluded that the "binding site" of adenosine deaminase is more specific for the substituent on position 9 than for the substituent on position 6 of adenine.

It has been shown by several workers (Kalckar, 1947; Chilson and Fisher, 1963; Coddington, 1962) that, in addition to adenosine, 2′-deoxyadenosine, 2-fluoroadenosine, 2,6-diaminopurine riboside, and 3′-deoxyadenosine (cordycepin) are substrates for adenosine deaminase. N^6-Methyldeoxyadenosine is a competitive inhibitor of adenosine deaminase, while adenine, adenylic acid, nicotinamide–adenine dinucleotide, and cytidine are neither substrates nor inhibitors of this enzyme.

A number of adenosine analogs were surveyed as substrates or inhibitors of adenosine deaminase isolated from calf serum (Weinbaum *et al.*, 1964). The activity of the calf serum deaminase was too low to determine the nature of the inhibition experimentally. To overcome this difficulty, calf intestinal adenosine deaminase was used. With this enzyme, we have evaluated experimentally the nature of the inhibition and the inhibition constants. This study reports on the structural requirements necessary for compounds to bind at the "active site" as either substrates or competitive inhibitors of calf intestinal adenosine deaminase.

Materials and Methods

Adenosine deaminase from calf intestinal mucosa (type 1, 210 units/mg) was purchased from Sigma Chemical Co. Specific activity is expressed as μmoles of adenosine deaminated per minute/mg of protein. Compounds were tested as substrates by measuring the change in absorbance at 265 mμ, using cells with 5-mm path lengths, in a Beckman DU spectrophotometer. Reactions were carried out at 24–26°. Deaminase inhibition was determined by observing the decrease in

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