Purification from Calf Spleen of Two Inhibitors of Deoxyribonuclease I. Physical and Chemical Characterization of the Inhibitor II*

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ABSTRACT: This paper contains a full account of the purification of two proteins from calf spleen, both of which inhibit deoxyribonuclease I. The protein designated inhibitor II was found to be the most homogeneous of the two and was analyzed with respect to physical and chemical properties. Its molecular weight was determined by "approach to equilibrium" and "sedimentation equilibrium" measurements and also by combination of sedimentation and diffusion data. The values obtained were 61,000, 59,000, and 57,000, respectively.

Amino acid analysis was performed on inhibitor II with a recovery of 100.2% by weight and indicated a composition of: $Asp_{49}Thr_{38}Ser_{35}Glu_{54}Pro_{27}Gly_{44}Ala_{42}Val_{37}Met_{20}Ile_{34}Leu_{42}Tyr_{19}Phe_{19}Lys_{31}His_{12}Arg_{25}$(total half-cys$_9$)$(CONH_2)_{28}$. Especially noteworthy was the relatively low content of sulfur-containing and aromatic amino acids. The inhibitor II was shown to be a labile protein which, however, could be stabilized with potassium phosphate buffer of high ionic strength or by the addition of glycine at high concentration. The effect of the inhibitor II on the activity of other enzymes was tested and found to be limited to the inhibition of deoxyribonuclease I.

A detailed description is here given of the purification procedure for the two DNase inhibitors from calf spleen, the crystallization of which was recently reported (Lindberg, 1966). Since many changes were introduced in the early stages of the procedure reported earlier (Lindberg, 1964), it was felt necessary to deal with this part in detail. The inhibitors, designated I and II, were both obtained in a high degree of purity. They were completely separated from each other on hydroxylapatite and behaved as single components on analytical disc electrophoresis.

This paper describes, in addition, a physical and chemical characterization of inhibitor II. The most consistent value for the molecular weight was found to be 59,400, which is almost double the molecular weight of DNase I (31,000) (Lindberg, 1967). The stabilization of inhibitor II by phosphate at high ionic strength and by glycine was investigated. This effect was not limited to stabilization against heat denaturation but also included the prevention of the rapid inactivation by urea and *p*-mercuribenzoate observed in 0.05 M phosphate buffer, pH 7.6, in the absence of glycine. All of the material subjected to chemical analyses could be accounted for by the amino acid recovered.

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Materials and Methods

Spleens from young calves which were less than 3 months old were obtained directly from the slaughter house and brought to the laboratory on ice, where they were frozen directly in a Dry Ice–alcohol mixture and stored at −16°. The spleens could be kept in this condition for about 1 year without detectable loss of activity.

DNase I, once crystallized from bovine pancreas, was purchased from Sigma Chemical Co., St. Louis, Mo. A stock solution of DNase containing 1 mg/ml was made in 0.05 M Tris buffer, pH 7.5, and stored frozen (0.1-ml portions). Before use in the standard DNase assay described earlier (Lindberg, 1964), a 0.1-ml portion of the DNase stock solution was mixed with 0.9 ml of 0.05 M Tris buffer, pH 7.5, containing 2 mg of serum albumin. The presence of albumin stabilized the dilute solution of enzyme, which then could be kept at 0–4° for about 1 week.

DNase II from calf spleen was extracted and subjected to ammonium sulfate fractionation as described by Fredericq and Oth (1958). The enzyme was then further purified by adsorption to and elution from Cγ-alumina hydroxide gel, followed by fitration on Sephadex G-100. The specific activity of the final preparation was 30 times that of the ammonium sulfate fraction.

Endonuclease I from *Escherichia coli* was a gift from Dr. I. R. Lehman, Stanford University, Palo Alto, Calif. Both the DNase II and the endonuclease I were assayed by spectrophotometric methods identical with that employed for DNase I. DNase II was assayed at pH 5.6 using acetate buffer (0.05 M) and a level of

40 μg of DNA/ml. Endonuclease I activity was measured under the conditions given by Lehman (1963).

RNase (bovine pancreas) was purchased from Sigma. Its activity was measured at pH 7.8 according to Shortman (1961).

Phosphodiesterase, from snake venom, was obtained from Sigma Chemical Co., and bis-*p*-nitrophenyl phosphate was used as substrate for the enzyme. The incubation mixture contained 1.0 μmole of bis-*p*-nitrophenyl phosphate, 50 μmoles of Tris buffer, pH 8.6, and 2–10 μg of the enzyme in a total volume of 1.0 ml. After incubation at 37° for 10 min the absorbance at 415 mμ was measured.

Alkaline phosphatase from *E. coli* K 12 starved for phosphate was prepared according to Garen and Levinthal (1960). The purification was subjected to chromatography on DEAE-cellulose. The enzyme was assayed by the *p*-nitrophenyl phosphate method (Garen and Levinthal, 1960).

Highly polymerized DNA (type I, Sigma Chemical Co.) was used for the DNA standard substrate solution, which was prepared as follows. DNA (48 mg) was cut into small pieces with a pair of steel scissors and mixed with 1 l. of a solution containing 120 mmoles of Tris buffer, pH 7.5, 4.8 mmoles of $MgSO_4$, and 2.1 mmoles of $CaCl_2$. The DNA dissolved on slow stirring for 48 hr at room temperature. The solution was filtered and stored at 0–4°. The assay results obtained with this substrate solution were much more reproducible than those obtained with a DNA solution prepared as described earlier (Lindberg, 1964).

RNA substrate solutions were prepared according to Shortman (1961).

Cγ-alumina hydroxide gel was prepared in large batches according to Willstätter and Kraut (1923). After the final washings with redistilled water, the gel was often used without further aging. However, the amount of gel needed for complete adsorption of a certain amount of inhibitor increased markedly during the first 3 months. For this reason the conditions of adsorption were retested at intervals of 2 months.

The gel suspension was diluted to contain 15–30 mg of dry substance/ml. Usually 0.03–0.05 ml of Cγ-alumina/mg of the dialyzed ammonium sulfate fraction was required to obtain 100% adsorption of the inhibitor activity present in the solution.

DEAE-Sephadex A 50 medium was obtained from Pharmacia, Uppsala, Sweden. The ion exchanger was washed and equilibrated as described earlier (Lindberg, 1964). The maximal amount of protein from the Cγ step which could be put onto a column of DEAE-Sephadex was found to be 8 mg/ml of packed column bed.

Sephadex G-100 (Pharmacia, Uppsala, Sweden) was allowed to swell for at least 48 hr in 0.5 M phosphate buffer, pH 7.6. The gel suspension was well evacuated and a column of suitable size was packed according to the recommendations given by the supplier. Before use the gel bed was washed with 0.5 M potassium phosphate buffer until the absorbance at 215 mμ was constant in the effluent (about 0.02 when 50 μl was diluted to 1 ml with water).

Hydroxylapatite (Hypatite-C, Clarkson Chemical Co., Inc., Williamsport, Pa.) was supplied fully equilibrated with 0.001 M potassium phosphate buffer, pH 6.7, and was not treated further before use. The capacity of the different batches was given by the supplier as between 75 and 95 mg of bovine serum albumin adsorbed/mg of hydroxylapatite. Gel of higher capacity was also tried but gave unsuitably low flow rates.

In our experiments the maximal load of protein from the Sephadex G-100 filtration step on the hydroxylapatite was 1.6 mg/ml of packed column. Larger amounts resulted in incomplete separation of the two inhibitors. The earlier described *inhibitor assay* was used but the unit of inhibitor activity was redefined as the amount of protein giving a 1% decrease in the activity of 1 μg of DNase.

Protein was determined during purification by the turbidimetric method of Bucher (1947). With the pure inhibitor II, protein could be determined using the $E^{1\%}_{1cm}$ at 280 mμ (=10.2), which was determined as described below.

Physical Analyses

Determination of $E^{1\%}_{1cm}$ at 280 mμ and dn/dc at 546 mμ. Simultaneous determinations were made of the absorbance, the refractive index increment, and the dry weight of different inhibitor II preparations. The ultracentrifuge was used as a differential refractometer at 20°.

Disk electrophoresis in polyacrylamide was performed according to Ornstein (1964) and Davis (1964) in a discontinuous buffer system. In addition, electrophoresis in polyacrylamide was run in continuous buffer systems at different pH values, as described by Hjertén *et al.* (1965).

Ultracentrifugation analyses were made in a Spinco Model E analytical ultracentrifuge equipped with a phase plate and the RTIC temperature control unit. The schlieren optical system was used in all experiments.

Before samples of the inhibitor II were used for ultracentrifugation they were equilibrated with 0.1 M potassium phosphate buffer, pH 7.6, on a column of Sephadex G-100 (1 × 50 cm). This procedure also resulted in the removal of high molecular weight aggregates which formed on storage of the inhibitor preparation (see under Results). The pooled fractions after Sephadex filtration were finally concentrated by ultrafiltration in a collodion bag, which was carefully prewashed with phosphate buffer.

Sedimentation analyses were performed at 59,780 rpm using a 12-mm, double-sector cell with a filled Epon centerpiece and calculations were made according to Schachman (1957). Diffusion coefficients were evaluated according to Van Holde (1960) from runs made in a 12-mm, capillary-type synthetic boundary cell, at 5227 rpm. Molecular weights were determined by the approach to equilibrium technique of Archibald modified as suggested by Ehrenberg (1957). The approach to equilibrium runs were performed in 12-mm,

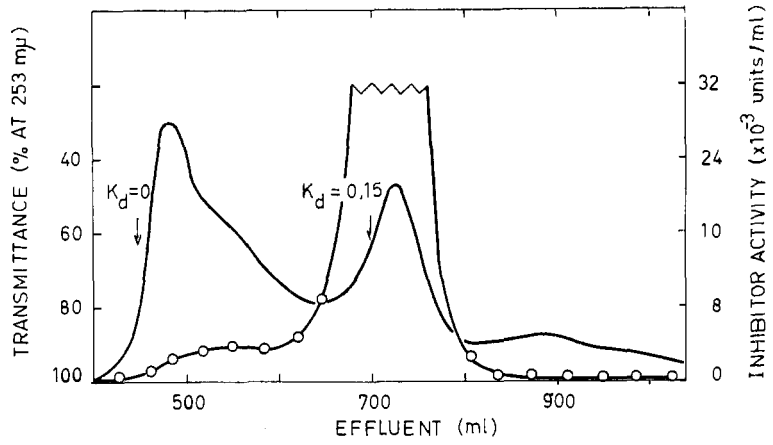

FIGURE 1: Filtration of DNase inhibitor on Sephadex G-100 after DEAE-Sephadex chromatography. The solid line (————) represents the transmittance recorded automatically on the effluent with an LKB-UVICORD ultraviolet absorptiometer. The open circles (—O—O—O—) represent inhibitor activity measured on each one-third fraction.

double-sector cells (filled Epon centerpieces) at 20,410 rpm. The concentration was determined in capillary-type synthetic boundary cells and from absorbance measurements. Molecular weights were also determined by sedimentation equilibrium runs using the multi short-column cell designed by Yphantis (1960). Distances on the photographic plates were measured in a two-dimensional microcomparator and areas were obtained by mechanical planimetry on enlarged (ten times) photographic prints.

Chemical Analyses

A desalted stock solution of inhibitor II was prepared, as described for DNase I (Lindberg, 1967), and used in the chemical analyses mentioned below. The pH of the water, used for the equilibration of the Sephadex G-25, was adjusted to about 6.5 with dilute ammonium hydroxide. This was done in order to avoid precipitation of the protein in the column, which occurred at more acid pH values.

Amino Acid Analyses. The acid hydrolyses were performed according to Mahowald *et al.* (1962) and Noltmann *et al.* (1962) with the modifications described in the chemical analysis of DNase I (Lindberg, 1967). Samples of protein (4.08 mg of dry weight) were hydrolyzed in 6 M hydrochloric acid (5 ml) at 110° for the times given below. The dried hydrolysates were dissolved in 8 ml of 0.2 M citrate buffer, pH 2.2, and 1.0 ml of these solutions was mixed with reference amino acids. The chromatographic procedure of Moore *et al.* (1958) was used in a Spinco Model 120B amino acid analyzer.

Total half-cystine was determined as cysteic acid as described by Moore (1963). After performic acid oxidation the sample (2.3 mg) was hydrolyzed in hydrochloric acid (as above) for 20 hr. Tryptophan was determined spectrophotometrically according to the method of Bencze and Schmid (1957) on about 1.0-mg amounts of protein.

Other Analyses

Amide nitrogen was determined by diffusion in Conway units, using alkaline hydrolysis as described by Stegemann (1958, 1959). Duplicate analyses were made with about 3.0 mg in each sample. Amide ammonia values were also estimated by extrapolation of the results obtained in the chromatographic analysis of the acid hydrolysates. The total nitrogen content of inhibitor II was determined by Kjeldahl procedure and related to dry weight[1] per milliliter. *Ash* and *sulfur* analyses were made by the Mikroanalyslaboratoriet, Uppsala University, Sweden.

Results

Purification of Inhibitors

The modifications in the purification procedure reported earlier (Lindberg, 1964) were introduced mainly in order to make the handling of the larger batches more convenient. As before, all operations were carried out in the cold (0–4°). Mercaptoethanol had a stabilizing effect on the inhibitor activity during the early purification steps and was included during such operations.

Preparation of Extract. Cell paste (1200 g) scraped out of the spleens was treated in a Waring blender for 0.5 min with five volumes of 0.004 M $MnCl_2$ and 0.01 M mercaptoethanol. The suspension was centrifuged in an International refrigerated centrifuge Model SR-3 (4-l. capacity) at 3000 rpm.

Ammonium Sulfate Fractionation. The bright red supernatant fluid (5700 ml) was immediately mixed with 2477 ml of a saturated ammonium sulfate solution (pH 7.0) to give a final salt concentration of 30%. The addition required about 45 min and the solution was stirred for a further 15-min period. The precipitate

[1] Samples were dried *in vacuo* over phosphopentoxide at 100–105° for 24 and 48 hr.

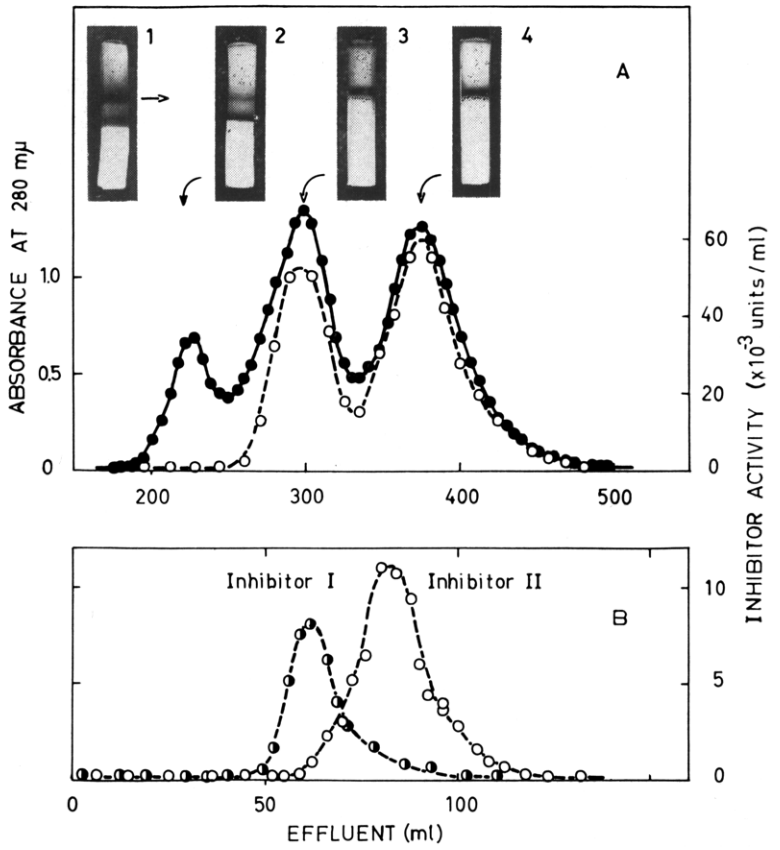

FIGURE 2: Separation of the two DNase inhibitors on hydroxylapatite and rechromatography of inhibitor I (13 mg) and inhibitor II (8 mg) on separate columns (2 × 3 cm). (A) The column used was 4 × 9 cm and the total gradient volume was 800 ml (see text). The solid circles (—●—●—●—) give the absorbance at 280 mμ, and the open circles (—O—O—O—) represent inhibitor activity. (B) The total gradient volume was 300 ml. The semisolid circles (—◑—◑—◑—) represent inhibitor I and the open circles (—O—O—O—) inhibitor II activity. Also included in A are the disc electrophoretic patterns obtained with: (1) the pooled and concentrated Sephadex fractions to be chromatographed on hydroxylapatite, (2) the first peak in the actual chromatogram, which was an impurity, (3) a second peak which was designated inhibitor I and, finally, (4) the third peak, designated inhibitor II. Samples containing 40 μg of protein were analyzed in the discontinuous buffer system (in which separation of proteins occurs at pH 10.3) of Ornstein (1964) and Davis (1964) with 100 v and 2 ma/tube for 2.5 hr.

which had formed was removed with suction on a Büchner funnel, Celite being added to ensure rapid filtration.

More saturated ammonium sulfate solution (3303 ml) was then added to the clear filtrate to obtain 50% saturation. After the addition (60 min) the solution was stirred for a further 60 min. It was then distributed in large separatory funnels and left overnight.

The next day the loosely packed sediment (about 2 l.) was removed from the funnels and centrifuged. Before the precipitate was dissolved it was washed with 60% saturated ammonium sulfate and centrifuged again at 14,000 rpm in an International refrigerated centrifuge (Model HR-1). Finally, the precipitate was dissolved in 0.05 M potassium phosphate, pH 7.6 (final volume, 390 ml). At this stage the inhibitor solution could be stored frozen for at least 6 months without loss of activity.

Adsorption with Cγ-Alumina Hydroxide Gel. Before this step the ammonium sulfate fraction was dialyzed for 2 hr against 2 × 2.5 l. of 0.005 M phosphate buffer, pH 7.6, containing 0.04 M mercaptoethanol. The solution was then diluted with water to 430 and 600 ml of the Cγ-alumina solution added (0.05 ml/absorbance unit). The suspension was stirred for 3 min and centrifuged. The precipitate was first washed with 500 ml of 0.005 M phosphate buffer, pH 7.6, containing 0.01 M mercaptoethanol. The inhibitor was then eluted with three portions of 0.07 M phosphate buffer, pH 7.6, containing 0.01 M mercaptoethanol (total volume of 900 ml). The gel suspension was stirred for 10 min before each centrifugation.

Chromatography on DEAE-Sephadex. The eluate from the Cγ step was adsorbed to a column (8 × 10 cm) of DEAE-Sephadex previously equilibrated with 0.22 M phosphate buffer, pH 7.6 (no mercaptoethanol). Elution was started with the same buffer and continued until a second inactive peak was almost eluted. When

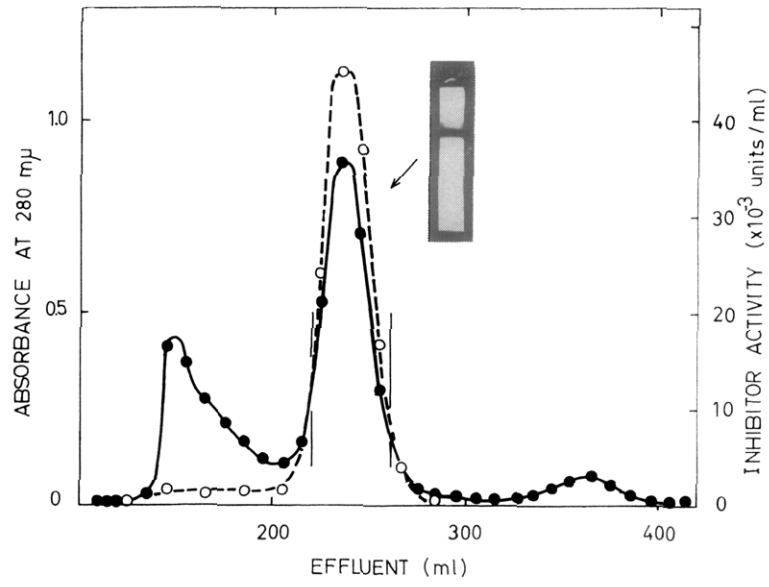

FIGURE 3: Rechromatography of DNase inhibitor II on Sephadex G-100. The inhibitor preparation (50 mg) was applied to the column (1 × 150 cm) and the latter developed with 0.5 M potassium phosphate, pH 7.6. The solid circles (—●—●—●—) represent the absorbance of the fractions at 280 mμ, and the open circles (—○—○—○—) inhibitor activity.

the absorbance at 280 mμ had dropped to below 1.5 the buffer was changed to 0.5 M phosphate, pH 7.6, containing 1.0 M glycine, and the inhibitor activity was eluted in one pool (1140 ml). Both buffers were forced through the column at the highest possible flow rate, which was achieved with a hydrostatic pressure of 30 cm.

The inhibitor was then concentrated by salt precipitation. The protein solution was dialyzed against saturated ammonium sulfate which, in addition to precipitating the protein, reduced the volume of the pool to one-third. The precipitate was collected by centrifugation. The protein could be stored (at 0–4°) as an amorphous precipitate or it could be dissolved and then stored frozen at −16°.

Filtration through Sephadex G-100. The concentrated protein solution (512 mg in 16 ml) from the previous step was filtered through a column of Sephadex G-100 (6 × 70 cm), equilibrated with 0.5 M phosphate buffer, pH 7.6. Approximately 15-ml fractions were collected each 30 min. Figure 1 shows a diagram from a typical filtration experiment. The pooled active fractions were concentrated by ultrafiltration or by precipitation with ammonium sulfate.

Chromatography on Hydroxylapatite. The material from the filtration step (178 mg in 9 ml) was dialyzed against 0.005 M phosphate buffer, pH 7.6, for 2 hr and then diluted with water to give a protein concentration of 10 mg/ml. This solution was then adsorbed to the column of hydroxylapatite (diameter, 4 cm; length, 9 cm). During this process the top one-quarter of the column was stirred with a glass rod and the hydroxylapatite in this way was well mixed with the protein solution. This precaution prevented the formation of cracks in the column during introduction of the sample. The column was eluted with a linear gradient prepared from 400 ml of 0.01 M phosphate buffer, pH 7.6, in the mixing chamber and 400 ml of 0.04 M phosphate buffer, pH 7.6, containing 1.5 M glycine in the other reservoir. The gradient was fed into the column with a peristaltic pump and 8-ml fractions/20 min were collected. The latter chromatographic step resulted in the resolution of two active protein peaks, designated inhibitor I and II, respectively (Figure 2A).

The possibility that the separation was a chromatographic artifact was eliminated by rechromatography of the two separated fractions (Figure 2B). Only the activity patterns are given but the respective protein curves were similarly displaced.

In order to obtain as complete a separation of the two inhibitors as possible the fractions of the original chromatogram were usually divided into three pools: (1) fore-portion of inhibitor I, (2) intermediary portion, and (3) after-portion of inhibitor II. Intermediary portions from several chromatograms were pooled and rechromatographed on hydroxylapatite. The purity of the different pools was checked by analytical disc electrophoresis (Figure 2). The inhibitor fractions were precipitated by dialysis against saturated ammonium sulfate and stored either as precipitates or as frozen, concentrated protein solutions.

Refiltration through Sephadex G-100. Both inhibitor proteins exhibited a high tendency to form aggregates, a property which in part explains the low over-all yield during the purification procedure. Such aggregation occurred both during the purification procedure, during storage of the protein solutions (concentrated

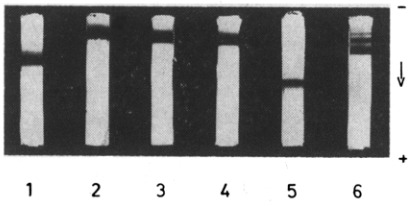

FIGURE 4: Analytical disc electrophoresis of DNase–inhibitor II. (1) A 40-μg sample of inhibitor protein was analyzed in the system of Ornstein (1964) and Davis (1964) (pH 10.3), with 100 v and 2 ma/tube for 2.5 hr. (2–4) A 40-μg sample of inhibitor protein analyzed in continuous Tris buffer systems of pH 8.9, 8.0, and 7.1, respectively (μ = 0.3). The conditions for the electrophoresis were 40 v and 4 ma/tube for 2.5 hr. (5) A 40-μg sample of inhibitor protein analyzed in Veronal buffer, pH 9.15, and μ = 0.173 (continuous system) with 60 v and 7 ma/tube for 2.5 hr. (6) A control 40-μg sample of an impure preparation of serum albumin was run in the same system as 2.

or dilute) at 0–4°, and on repeated freezing and thawing of a sample.

These aggregates were of high molecular weight and were easily removed by filtration on Sephadex G-100. In Figure 3 is shown the refiltration of inhibitor II. The methods followed in pooling the fractions and the protein patterns of this pool on disk electrophoresis are also reported. For most experiments, such as crystallization, ultracentrifugation, or studies on the complex formation with DNase, a sample of the inhibitor was refiltered through Sephadex G-100.

Crystallization. The crystallization of the two inhibitors were described earlier (Lindberg, 1966). The studies on the characteristics of inhibitor II described below were made with noncrystalline material since at that time the inhibitors had not yet been obtained in crystalline form. A summary of the purification procedure is given in Table I.

Studies on Inhibitor II

An efficient separation of the two inhibitor proteins was evident from their behavior on hydroxylapatite. Coincidence of activity and protein was especially marked in the case of inhibitor II. For this reason inhibitor II was chosen for a more detailed investigation of its homogeneity, physical characteristics, and mechanism of action.

Homogeneity

Disc Electrophoresis. As shown earlier inhibitor II behaved as a homogeneous protein when analyzed by disc electrophoresis according to Ornstein (1964) and Davis (1964) (Figure 3). An extension of this analysis was made by running the analytical electrophoresis on polyacrylamide in continuous buffer systems at varying pH.

Only a single band was obtained when inhibitor II was analyzed in Tris buffer at pH 8.9, 8.0, 7.1 (μ = 0.3), or Veronal buffer, pH 9.15 (μ = 0.17), as shown in Figure 4. An impure sample of bovine serum albumin was run in parallel at pH 8.9 in order to demonstrate the resolving power of the method.

Complex Formation. In a following paper the homogeneity of inhibitor II will also be illustrated in experiments where the specific complex formation with DNase I is studied. It was shown that essentially all

TABLE I: Summary of the Purification of the Spleen Inhibitors.

Fraction	Act. (units/ml)	Act. (total units ($\times 10^{-6}$)	Protein mg/ml	Protein Total mg	Sp Act. (units/mg)	Yield (%)
$MnCl_2$ extract	14,500	107	15.9	117,000	910	100
After $(NH_4)_2SO_4$ fractionation	146,000	57.4	32.4	12,700	4,510	54
After dialysis	135,000	58.0	26.8	11,500	5,040	54
After Cγ-alumina hydroxide gel adsorption	37,500	33.5	3.7	3,310	10,150	31
After DEAE-Sephadex chromatography	14,500	16.5	0.5	512	32,400	15
After concentration	892,000	14.3	32.0	512	28,000	13
After Sephadex G-100 chromatography	45,600	7.5	1.2	198	38,000	7
After concentration	834,000	7.5	19.8	178	42,000	7
After hydroxylapatite chromatography and concentration I-1	1215,000	1.7	30.5	42.7	40,500	1.6
I-2	1610,000	2.9	40.0	72.0	40,300	2.7

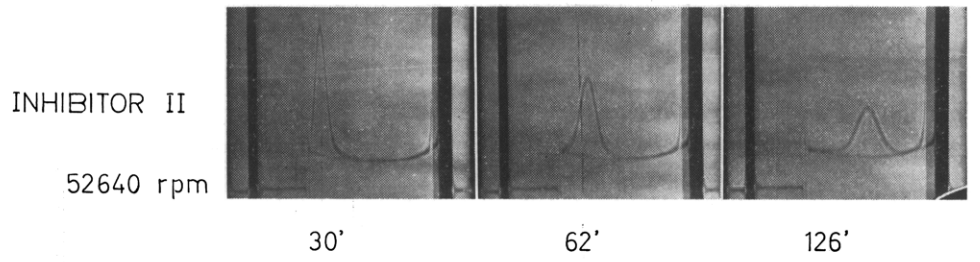

FIGURE 5: Sedimentation of DNase–inhibitor II.

of the protein present in the inhibitor II preparation could complex DNase.

Sedimentation and Diffusion Analyses. It was essential to show that all high molecular weight aggregates were removed by the Sephadex treatment, and that such aggregates were not formed under the conditions chosen for the ultracentrifugal analysis. If present they would seriously influence the outcome of molecular weight determinations. Therefore, inhibitor II was subjected to a boundary-spreading analysis.

A single symmetrical boundary was observed when the inhibitor was sedimented at 20° in 0.1 M potassium phosphate buffer, pH 7.6 (Figure 5). The Gaussian symmetry of the peak was tested (Hall and Ogston, 1956) by plotting log dn/dx *vs.* $(x_i - x_h)^2$; x_i is a position in the boundary measured from the center of rotation and x_h is the position of the maximum. This plot gave straight lines for both limbs of the boundary (Figure 6A), as it should if the boundary peak was symmetrical.

The protein concentration calculated from the area under the gradient curve was constant (9.9 mg/ml) during the experiment, if corrected for radial dilution, and was close to that of the starting material (9.7 mg/ml) (see Figure 6B). The concentration of the starting material was calculated from absorbance data using the value 10.2 as the $E^{1\%}_{1cm}$ at 280 mμ.

The concentration dependence of the sedimentation coefficient of inhibitor II (see below) was small and the spreading of the sedimenting boundary was analyzed according to Van Holde (1960). The data from this analysis are given in diagram C of Figure 6 and they

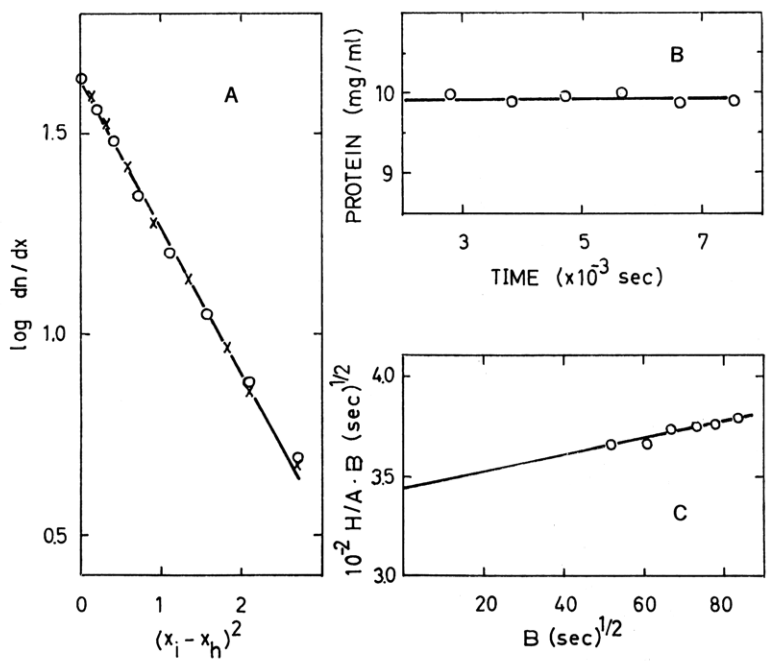

FIGURE 6: A test of the Gaussian form of the sedimenting boundary gradient obtained with DNase inhibitor II at 52,640 rpm, dependence on time of the protein concentration (corrected for radial dilution) calculated from the areas under the boundary gradient curves, and analysis of the boundary spreading according to Van Holde (1960). (A) Log dn/dx is plotted against the square of the distance from the center of the boundary $(x_i - x_h)^2$. The units chosen are arbitrary on both axes: (—O—O—O—) trailing limb; (—×—×—×—) advancing limb. (B) The protein concentration calculated with the use of the $E^{1\%}_{1cm}$ 10.2 was 9.7 mg/ml. (C) $B = (1 - 1/2\omega^2 St)^{-1} t^{1/2}$.

seem to fall on a straight line as expected for a homogeneous protein. Inhibitor II was thus shown to be homogeneous and stable under the conditions to be used in the molecular weight determinations below.

Physical Characterization

Determination of the Sedimentation Constant. The sedimentation coefficient ($s_{20,w}$) was determined as a function of protein concentration. A slight concentration dependence was observed, which is obvious from the data in Table II, and the sedimentation constant ($s^0_{20,w}$ = 3.92 S) was obtained by extrapolation of the data to zero concentration.

TABLE II: Studies on the Concentration Dependence of $s_{20,w}$ and $D_{20,w}$ of DNase Inhibitor II.

Sedimentation		Diffusion		
Protein Concn (mg/ml)	$s_{20,w}$ ($\times 10^{-13}$ sec)	Protein Concn (mg/ml)	$D_{20,w}$ ($\times 10^{-7}$ cm²/sec Low Rpm	$D_{20,w}$ ($\times 10^{-7}$ cm²/sec) High Rpm
9.85	3.58	9.55	6.1	6.4
6.57	3.70	5.72	6.3	
3.28	3.80	4.77	6.5	
0.00	3.92	2.39	6.2	
		0.00	6.5	

Determination of the Diffusion Constant. Diffusion measurements (at low angular speed) were made at different concentrations of inhibitor II. The respective diffusion coefficients were evaluated by the method of Van Holde (1960). The values are listed in Table II, together with the diffusion constant obtained by extrapolation of the experimental data to zero protein concentration. This value, 6.5 × 10^{-7} cm²/sec, is not significantly different from the $D_{20,w}$ obtained at high speed in the boundary-spreading experiments (6.4 × 10^{-7} cm²/sec).

Molecular Weight Determinations. The molecular weight of inhibitor II was determined on three different preparations by approach to equilibrium (Ehrenberg, 1957) and by sedimentation equilibrium in short columns (Yphantis, 1960). In addition, the sedimentation and diffusion data of Table II were combined in the Svedberg equation (Svedberg, 1925) to give an estimation of the molecular weight. The values obtained (see Table III) were grouped around an average of 59,400 (average deviation about the mean is ±1500). This was considered as the most consistent value for the molecular weight of inhibitor II that it was possible to obtain by these ultracentrifugation methods. The partial specific volume, which was used in all these calculations, was derived from the amino acid composition data given below.

TABLE III: Summary of Physical and Chemical Characteristics of DNase Inhibitor II.

Characteristic Analyzed		Results Obtained
$E^{1\%}_{1cm}$ at 280 mμ		
Experimental		10.2[a]
From amino acid composition		10.4[b]
		10.7[c]
Refractive index increment (ml/g)[d]		0.187 ± 0.003[e]
$s^0_{20,w}$ ($\times 10^{-13}$ sec)		3.92
$D^0_{20,w}$ ($\times 10^{-7}$ cm²/sec)		6.5
Partial specific volume (from amino acid composition)		0.737
Molecular weight		
Approach to equilibrium	60,200	
	61,900	
Sedimentation equilibrium	59,900	
	57,900	
Sedimentation diffusion	57,200	Av 59,400
Total nitrogen (%)		
Experimental		16.6 ± 0.3[e]
From amino acid composition		16.6
Total sulfur (%)		
Experimental		1.64 ± 0.04[e]
From amino acid composition		1.52

[a] In 0.1 M K_2HPO_4–KH_2PO_4 buffer, pH 7.6. [b] In 0.1 M NaOH, pH 13. [c] This value was calculated using the molar extinction coefficients for the different amino acids in 0.1 M NaOH. [d] dn/dc was determined as described under Materials and Methods. [e] The average deviation about the mean is given based on four determinations.

Determination of $E^{1\%}_{1cm}$ at 280 mμ and dn/dc. Inhibitor II showed the typical absorbance spectrum of a protein (see Figure 7), and a maximum was recorded at 278 mμ and a shoulder at 283 mμ when the protein was dissolved in 0.1 M potassium phosphate buffer, pH 7.6. As expected the spectrum was shifted markedly toward longer wavelengths when instead the protein was dissolved in 0.1 M NaOH (Tanford, 1963). The absorbance (in 0.1 M potassium phosphate buffer, pH 7.6) was related to the dry weight of the protein solution per milliliter and an $E^{1\%}_{1cm}$ at 280 mμ was determined. Two different preparations were used and the mean value obtained was 10.2, which was close to the value (10.4) obtained in 0.1 M NaOH. The latter value agreed well with that of 10.7 which was calculated according to Goodwin and Morton (1946) from the tyrosine and tryptophan content, using the molar extinction coefficients for the two amino acids in 0.1 M NaOH.

The refractive index increment of inhibitor II at 20° was also related to dry weight, and a value of 0.1866 ± 0.0032 ml/g was found (based on three determinations). This was used in the calculation of

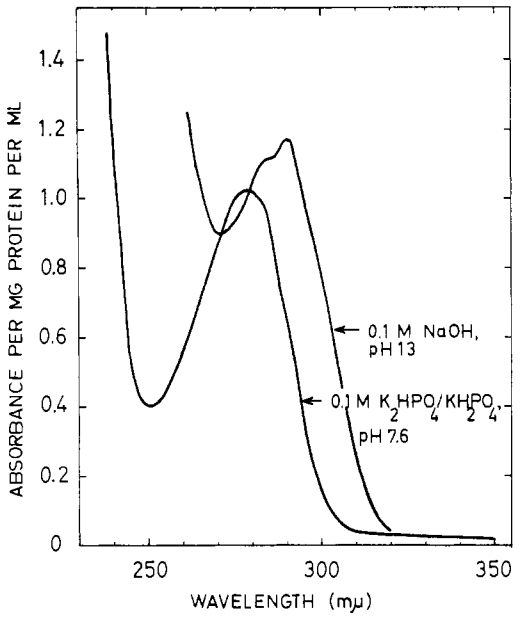

FIGURE 7: The ultraviolet absorption spectrum of DNase inhibitor II in 0.1 M potassium phosphate buffer, pH 7.6, and in 0.1 M NaOH, pH 13.

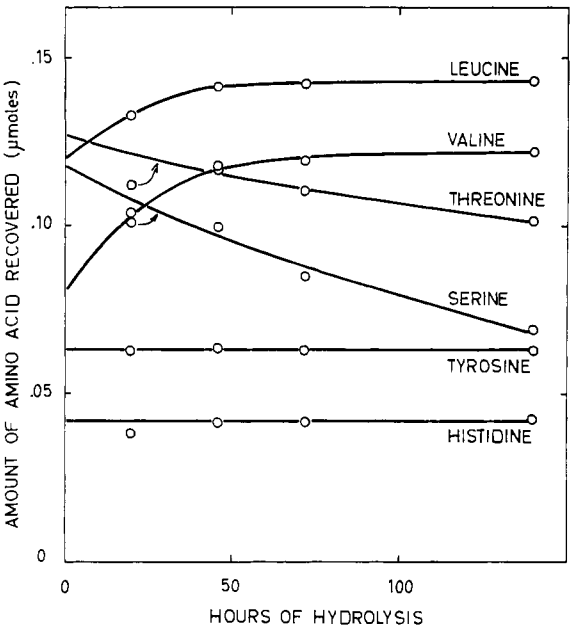

FIGURE 8: Amino acid recoveries as a function of time. Ordinate, micromoles recovered from 0.203 mg of protein.

TABLE IV: Amino Acid Recoveries after Acid Hydrolysis of the DNase Inhibitor II.[c]

Amino Acid Residue	Hours of Hydrolysis for Recovery				Amino Acid Recov Extrapolated to 0-Time Hydrolysis or at Maximal Value
	20	46	72	140	
Aspartic acid	0.1568(2)	0.1677(2)	0.1669(2)	0.1653(2)	0.1673
Threonine	0.1121(2)	0.1170(2)	0.1101(2)	0.1018(2)	0.1270
Serine	0.1012(2)	0.1000(2)	0.0852(2)	0.0696(2)	0.1180
Glutamic acid	0.1700(2)	0.1835(2)	0.1822(2)	0.1813(2)	0.1829
Proline	0.0748(2)	0.0885(2)	0.0836(2)	0.0805(2)	0.9000
Glycine	0.1390(2)	0.1501(2)	0.1496(2)	0.1506(2)	0.1501
Alanine	0.1310(2)	0.1425(2)	0.1417(2)	0.1429(2)	0.1424
Valine	0.1038(2)	0.1173(2)	0.1198(2)	0.1223(2)	0.1223
Methionine[a]	0.0628(2)	0.0712(2)	0.0624(2)	0.0682(2)	0.0673
Isoleucine[b]	0.0960(2)	0.1118(2)	0.1145(2)	0.1163(2)	0.1163
Leucine	0.1330(2)	0.1416(2)	0.1421(2)	0.1432(2)	0.1432
Tyrosine	0.0628(2)	0.0639(2)	0.0631(2)	0.0629(2)	0.0633
Phenylalanine	0.0617(2)	0.0655(2)	0.0635(2)	0.0648(2)	0.0646
Lysine	0.0910(1)	0.1053(2)	0.1026(2)	0.0934(2)	0.1040
Histidine	0.0380(1)	0.0417(2)	0.0418(2)	0.0426(2)	0.0420
Arginine	0.0780(1)	0.0874(2)	0.0835(2)	0.0820(2)	0.0843
Ammonia	0.1180(1)	0.1300(2)	0.1520(2)	0.1680(2)	0.1020
Column number: 1	2	3	4	5	6

[a] No methionine sulfoxides were detected under the standard conditions. [b] Small amounts of alloisoleucine are included in these figures. [c] The values given are micromoles recovered from 0.203 mg of protein. The numbers within parenthesis are the number of determinations performed on each hydrolyzed sample. To illustrate the precision of the analyses, the values are given to the fourth decimal. The over-all accuracy was not better than 2.5± %.

TABLE V: Amino Acid Composition of DNase Inhibitor II.

Amino Acid Residue	g of Amino Acid Residues/100 g of Protein[a]	Minimal Mol Wt[b]	Amino Acid/ 60,000 g of Protein (moles)	Nearest Integral No. of Amino Acid Residues/ 60,000 g of Protein	Nearest Integral No. Multiplied by Minimal Mol Wt	Integral No. of Residues Multiplied by Mol Wt of Respective Residue
Aspartic acid	9.49[c]	1,213	49.46[c]	49	59,437	5,640
Threonine	6.33	1,597	37.57	38	60,686	3,843
Serine	5.06	1,720	34.88	35	60,200	3,049
Glutamic acid	11.64	1,109	54.10	54	59,886	6,974
Proline	4.31	2,254	26.62	27	60,858	2,623
Glycine	4.22	1,352	44.38	44	59,488	2,511
Alanine	4.99	1,425	42.11	42	59,850	2,986
Valine	6.06	1,636	36.67	37	60,532	3,663
Methionine	4.35	3,016	19.89	20	60,320	2,624
Isoleucine	6.49	1,744	34.40	34	59,296	3,849
Leucine	7.99	1,417	42.34	42	59,514	4,754
Tyrosine	5.09	3,206	18.71	19	60,914	3,100
Phenylalanine	4.68	3,145	19.08	19	59,755	2,797
Lysine	6.57	1,951	30.75	31	60,481	3,974
Histidine	2.84	4,831	12.42	12	57,972	1,646
Arginine	6.49	2,407	24.93	25	60,175	3,905
Amide ammonia						
By chromatography	0.85[d]		30.00	30		
By diffusion	0.80 ± 0.03[d]		28.24	28		(−28)[e]
Tryptophan	2.11		6.80[f]	7		1,303
Total half-cystine	1.50		8.72[g]	9		928
Total	100.18[h]				59,960 ± 511	60,141
Column number: 1	2	3	4	5	6	7

[a] Calculated from column 6 of Table IV. [b] Calculated from the relationship amino acid residue molecular weight × 100/percentage of amino acid residue in the protein. [c] To avoid errors in the calculations owing to "rounding-off," two decimal places were retained here. [d] Omitted from total. [e] The difference in the molecular weight of OH and NH_2 was corrected for by subtracting 0.989/amide residue. [f] A molecular ratio of 2.75 was determined for tyrosine:tryptophan by the method of Bencze and Schmid (1957). When this ratio was used, 6.80 moles of tryptophan was calculated/ 60,000 g of protein, from the amount of tyrosine given in column 6 of Table IV. This corresponds to 2.11% of tryptophan in the protein. [g] Determined as cysteic acid after performic acid oxidation. [h] Total nitrogen was determined experimentally on the desalted stock solution from which the samples to the acid hydrolyses were taken. Using the independently found nitrogen content of 16.6% a recovery in the amino acid analysis of 99.2% was calculated, which is in excellent agreement with the above given value. The latter was based on absorbance measurements on the same stock solution and converted to dry weight content with the use of $E^{1\%}_{1cm}$ 10.2 at 280 mμ.

the protein concentrations in Figure 6B from the schlieren patterns obtained in the sedimentation run.

Chemical Analyses

Amino Acid Analyses. Acid hydrolyses were performed on desalted lyophilized samples of inhibitor II for 20, 46, 72, and 140 hr and two complete runs in the amino acid analyzer were made on each hydrolysate.

Table II contains figures on the recovery of the amino acid after the different times of hydrolysis. The recoveries of some of the amino acids are plotted as a function of hydrolysis time in Figure 8 in order to illustrate the trends observed. The absolute amounts in μmoles of each amino acid/0.204 mg of protein (column 6 of Table IV) were calculated after careful inspection of the hydrolyses curves for the different amino acids.

Two independent determinations on different batches by the spectrophotometric method (Bencze and Schmid,

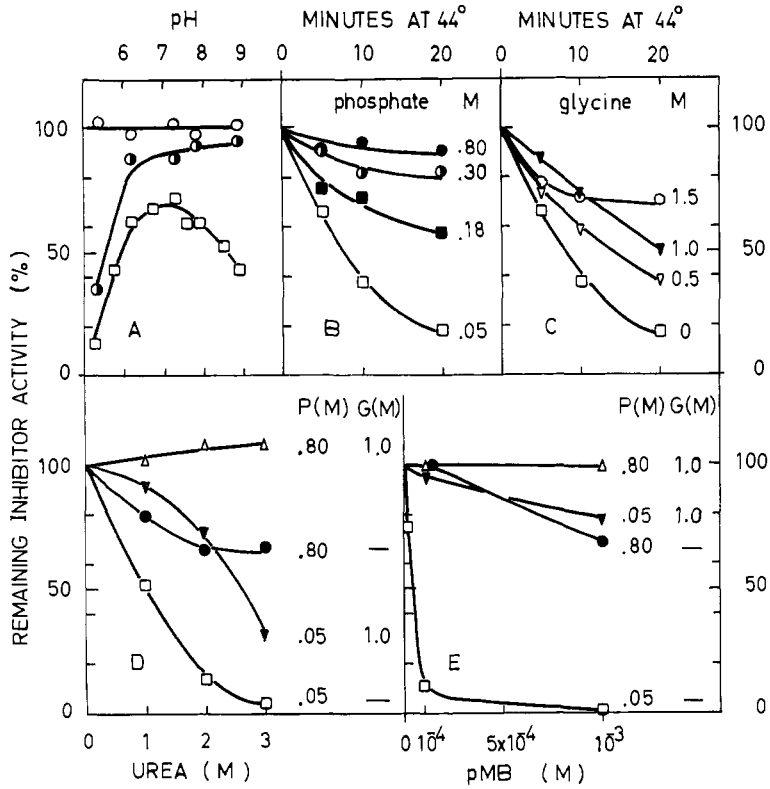

FIGURE 9: The effect of potassium phosphate and glycine on DNase inhibitor II under various conditions. (A) —○—○— represents the inhibitor activity of the control kept at 0° for 15 min in phosphate buffers (μ = 0.3). □—□—□ is the inhibitor activity of the samples after incubation at 44° for 5 min in phosphate buffers (μ = 0.3). Finally, —◑—◑—◑— is the inhibitor activity after incubation at higher ionic strength (μ = 1.0), for 5 min and at 44°. (B) The stabilizing effect of phosphate at high ionic strength. The samples were incubated at 44° for the different times and in the different phosphate concentrations given in the figure. (C) The stabilizing effect of glycine. The incubations were done as in B. The phosphate concentration was kept constant at 0.05 M. (D) The stabilization against inactivation by urea. The concentrations of phosphate and glycine in the incubation mixture are given in the figure. The inhibitor was incubated for 1 min at 44°. (E) The stabilization against inactivation by PMB. The concentrations are given in the figure. The incubations were made as in D.

1957) gave 2.19 and 1.92% for tryptophan, and 5.10 and 4.68% for tyrosine. This checks well with the figure of 5.09% for tyrosine found by chromatography after acid hydrolysis, and from these data a tryptophan content of 2.1% was calculated using the determined Tyr:Try ratio.

After performic acid oxidation 1.50% of the protein was recovered as cysteic acid. No differentiation was made between cysteine and cystine.

Additional Analyses

Amide nitrogen gave a value of 0.80% (mean value of two determinations) by diffusion and a value of 0.85 was found by extrapolation of the chromatographic results. Four analyses of *total nitrogen* content gave the values 16.7, 16.1, 17.0, 16.7 (average 16.6%), in good agreement with the figure 16.6% found from the amino acid composition. The *ash* content of the desalted inhibitor II protein was less than 1.0%. Finally the *total sulfur* was determined experimentally as 1.64 ± 0.04% (based on four determinations). This figure is in agreement with the 1.52% calculated from the methionine and total half-cystine content, which was obtained in the amino acid analyses.

The over-all accuracy obtained in the amino acid analyses was ±2.5%. This means that amino acids present in higher numbers than 40 residues/mole of inhibitor were not determined better than ±1–2 residues. The high yield obtained in the amino acid analyses (100.2%) makes it unlikely that the inhibitor should contain any other components than the amino acids reported here.

The integral numbers of the amino acids (column 5, Table V) were calculated assuming a molecular weight of 60,000, which is the value found by ultracentrifugation. From the products of these whole numbers and the minimal molecular weights listed in column 3 of the same table, an average molecular weight of 59,960 was computed. When the integral numbers were multiplied with the molecular weights

of the respective amino acid residues and the products summed up, a molecular weight of 60,141 was obtained.

From the amino acid composition a value for the partial specific volume[2] was calculated (Cohn and Edsall, 1943). This value of 0.737 ml/g was used in the calculations of the molecular weight of inhibitor II in the ultracentrifugation experiments described above.

Other Studies

Stability of Inhibitor II. It was shown earlier (Lindberg, 1964) that the inhibitor activity is sensitive to elevated temperatures and to acid and alkali. As much of the loss in inhibitor activity during the purification procedure was due to a tendency to form high molecular weight aggregates, the purified inhibitor was stabilized by phosphate at high ionic strength and by glycine, as demonstrated below. First, a study of the effect of pH was made. The inhibitor solution (0.01 ml containing 0.15 mg) was mixed with 1 ml of the buffer solution. Potassium phosphate buffers were used at all pH values; above pH 7.6 the solutions also contained 0.1 M Tris–Cl in order to increase the buffer capacity. The mixtures were incubated for 5 min at 44°, as indicated in Figure 9A. As a control a series of mixtures were kept at 0°. Figure 9A illustrates that phosphate at high ionic strength increased the stability of the inhibitor significantly, especially at alkaline pH values. The stability optimum of the crystallized inhibitor II was around pH 7.0.

The stabilization of the inhibitor by phosphate and by glycine was investigated further at pH 7.6 (Figure 9B,C). In these experiments 0.02 ml containing 0.3 mg of inhibitor protein was incubated with 0.180 ml of phosphate buffers of different concentrations (Figure 9B) or with 0.05 M phosphate buffer containing different concentrations of glycine (Figure 9C). Aliquots were withdrawn at various times and kept at 0° until they were assayed for remaining inhibitor activity.

In 0.05 M phosphate buffer almost complete inactivation of the inhibitor was obtained after incubation for 20 min, while after the same time period in 0.8 M phosphate buffer 90% of the activity was still retained. Good stabilization was also observed when 1.5 M glycine was added (low phosphate concentration), 70% of the activity of the sample remaining after 20-min incubation.

Finally, the effect of phosphate and glycine on the inactivation of inhibitor II by urea and *p*-hydroxymercuribenzoate was studied (Figure 9D,E). For this purpose 0.01 ml (0.15 mg) of the inhibitor solution was mixed with 0.09 ml of the urea or PMB[3] solutions (pH 7.6) together with the additional components present in the concentrations given in the figure. The mixtures with urea were incubated at 44° for 1 min before assay, while those with PMB were assayed immediately.

At low ionic strength and in the absence of glycine, the inhibitor was rapidly inactivated by the presence of either urea or PMB. It is apparent from the data in the figure that this inactivation could be prevented by the combined action of phosphate and glycine at high concentrations. In filtration experiments on Sephadex G-100, not reported here in detail, it was shown that the inactivation of inhibitor II (similarly exposed to urea or PMB) was accompanied by complete aggregation of the protein material present.

TABLE VI: The Effect of DNase Inhibitor II on Different Related Enzymes.[a]

Enzyme	Amount	pH	Inhibitor II	
			Amt (μg)	Inhibn (%)
DNase I from bovine pancreas	1.0 μg or 60 units	7.5	2	85
DNase II from calf spleen	22 units	5.6	100	0
Endonuclease I from *E. coli*	1.2 units	7.5	80	0
Phosphodiesterase from snake venom	5.0 μg	8.6	80	9
Alkaline phosphatase from *E. coli*	0.1 μg	7.5	80	10
RNase from bovine pancreas	0.25 μg	7.5	160	0

[a] The assay conditions for the various enzymes are given under Materials and Methods.

Specificity of the Inhibitory Effect. The effect of DNase inhibitor II on various nucleases is given in Table VI. In these experiments about 80 μg of inhibitor II (0.01 ml) was added to the incubation mixtures in the assay procedures used for the respective enzymes. Information about the different assay methods is given under Materials and Methods.

The inhibitor showed significant activity only against DNase I (bovine pancreas). A slight effect was demonstrated with alkaline phosphatase and phosphodiesterase. It was not determined if this inhibition was caused by residual phosphate ions, despite the fact that the protein preparation prior to the experiments was filtered on Sephadex G-25.

It is of particular interest that neither DNase II from calf spleen nor endonuclease I from *E. coli* were affected by the inhibitor. These two enzymes have been shown to be inhibited by RNA (Bernardi, 1964; Lehman *et al.*, 1962).

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[2] More careful and detailed amino acid analyses render this value slightly higher than that reported earlier (Lindberg, 1966).

[3] Abbreviation used: PMB, *p*-hydroxymercuribenzoate.

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Molecular Weight and Amino Acid Composition of Deoxyribonuclease I*

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ABSTRACT: The molecular weight of pancreatic deoxyribonuclease has been determined. A value of 31,000 was found by ultracentrifugation, using the approach to equilibrium technique. Sedimentation and diffusion measurements gave a molecular weight of 29,000. The amino acid composition was studied in detail. A molecular weight of 30,664 ± 322 was calculated from the integral numbers of the residues present per 31,000 g of protein.

Leucine was found as the only N-terminal amino acid. The minimal molecular weight obtained from the recovery of leucine was 31,300.

Few data have been published on the homogeneity and the physical and chemical characteristics of pancreatic deoxyribonuclease (DNase I) even though quite a long time has elapsed since the crystallization of the enzyme (Kunitz, 1950). It should be mentioned also that several modified purification procedures have been published giving better yields of material of equal purity as that of the crystallized enzyme (Polson, 1956; Baumgarten *et al.*, 1958). The separation of DNase I from contaminating RNase by chromatography on DEAE-cellulose was reported recently (Zimmerman and Sandeen, 1966).

During studies on the DNase inhibitors from calf spleen (Lindberg, 1966, 1967b), it was found that the previously reported molecular weight of DNase I (Kunitz, 1950; Smith, 1953) was probably in error. It was therefore considered necessary to reexamine the problem. The present paper shows that the enzyme obtained after filtration of the commercial product through Sephadex G-100 was pure by both electro-

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