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Molecular Weight and Amino Acid Composition of Deoxyribonuclease I*

Uno Lindberg

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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phoretic and ultracentrifugal criteria. From the ultracentrifugal data a molecular weight of about 30,000 was calculated.

A detailed examination of the amino acid composition was also undertaken. The results differed significantly from those of Gehrman and Okada (1957). Leucine was the only N-terminal amino acid found.

Materials and Methods

DNase I (once crystallized from bovine pancreas) and substrate *DNA* were the products of Sigma Chemical Co. Enzyme and substrate stock solutions were prepared and the *DNase activity* was measured as described earlier (Lindberg, 1964).

Sephadex G-100 and G-25 (Pharmacia, Uppsala, Sweden) was used for the gel filtrations. The columns were packed as recommended by the manufacturer. Before use, the gel beads were suspended in buffer and allowed to swell for at least 48 hr.

Physical Analyses

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

Before samples of DNase I were taken for ultracentrifugation they were equilibrated with 0.1 M potassium phosphate buffer, pH 7.6, by chromatography on a column of Sephadex G-25, which had been thoroughly rinsed with the buffer. Sedimentation analyses, diffusion measurements, and molecular weight determinations were performed under the conditions given in a previous paper describing the characterization of DNase inhibitor II (Lindberg, 1967a).

Disc electrophoresis was performed according to Ornstein (1964) and Davis (1964). Instead of mixing the sample with spacer gel it was introduced in a layer of 0.1 M Tris–Cl buffer containing 20% sucrose between the reservoir buffer and the concentration gel.

Chemical Analyses

DNase I (50 mg, purified on Sephadex G-100 as described below) was desalted on a column of Sephadex G-25 which had been first washed extensively with redistilled water, the pH of which was adjusted to about 3.5 with hydrochloric acid. Unless this acidic pH was used, the protein precipitated in the column. The desalted sample was used for the chemical analyses mentioned below.

Amino Acid Analyses. Aliquots (0.2 ml containing 2.06 mg of protein) were taken from the desalted stock solution and subjected to acid hydrolysis according to Mahowald *et al.* (1962) and Noltmann *et al.* (1962). The only deviation from these procedures was that the hydrolysis tubes were sealed under atmospheric pressure of argon. The tubes were then heated at 110° for the desired periods.

After hydrolysis and lyophilization of the solutions the dry residue was dissolved in 5 ml of 0.2 M sodium citrate buffer, pH 2.2. Aliquots containing 0.406 ml were mixed with 0.5 ml of a reference mixture consisting of 0.457 μmole of *n*-leucine and 1.202 μmoles of α-amino-β-guanidopropionic acid. Finally, 40% of this mixture was analyzed on each of the columns used. Chromatography was performed according to the procedure of Moore *et al.* (1958) with a Spinco Model 120 B amino acid analyzer.

Cysteine and cystine were determined separately as cysteic acid (Moore, 1963) after performic acid oxidation. The oxidized material was hydrolyzed in 6 N hydrochloric acid for 20 hr under argon under the conditions described above. Tryptophan was analyzed by the method of Bencze and Schmid (1957) using 0.7 and 1.0 mg of DNase in two separate determinations.

N-Terminal amino acid analysis was performed according to Edman's (1950) phenyl isothiocyanate method. The technical details are given by Blombäck and Yamashina (1958). The residue of leucine phenylthiohydantoin[1] was identified by thin layer chromatography using solvent systems E and D of Edman and Sjöquist (1956) and II and III of Brenner *et al.* (1962).

To differentiate between leucine and isoleucine the PTH derivative of the N-terminal amino acid was subjected to alkaline hydrolysis according to Van Orden and Carpenter (1964) following which the free amino acid was identified in the amino acid analyzer.

Additional Analyses

Amide nitrogen was determined by the method of Stegemann (1958, 1959) adapted to Conway diffusion units, using 3–5 mg of protein. Estimation of the amide content was also made by extrapolation to zero time of the amounts of ammonia produced during the acid hydrolyses. *Total nitrogen* was determined by the micro-Kjeldahl procedure and was related to the dry weight[2] content and the absorbance at 280 mμ in 0.1 M potassium phosphate buffer, pH 7.6. *Ash* and *sulfur* analyses were made by the Mikroanalyslaboratoriet, Uppsala University, Sweden.

Results and Discussion

Purification

Filtration of DNase I through Sephadex G-100. The commercial enzyme obtained from different firms always showed some degree of heterogeneity and therefore required further purification. Filtration through Sephadex G-100 was chosen for this purpose.[3] Figure 1 illustrates such a filtration experiment. The first small peak and the main peak in the chromatogram both contained DNase activity. The similarity of these two DNase activities is illustrated by the fact that both were completely inhibited by the purified DNase inhibitors (Lindberg, 1967a). It thus seems likely that this small peak contained an aggregated form of DNase I rather

[1] Abbreviation used in this work: PTH, phenylthiohydantoin.

[2] Samples were dried to constant weight at 100–105°, *in vacuo*, and over P_2O_5.

[3] Recently, Tkocz and Losse (1966) also reported the use of Sephadex G-100 in the purification of DNase I.

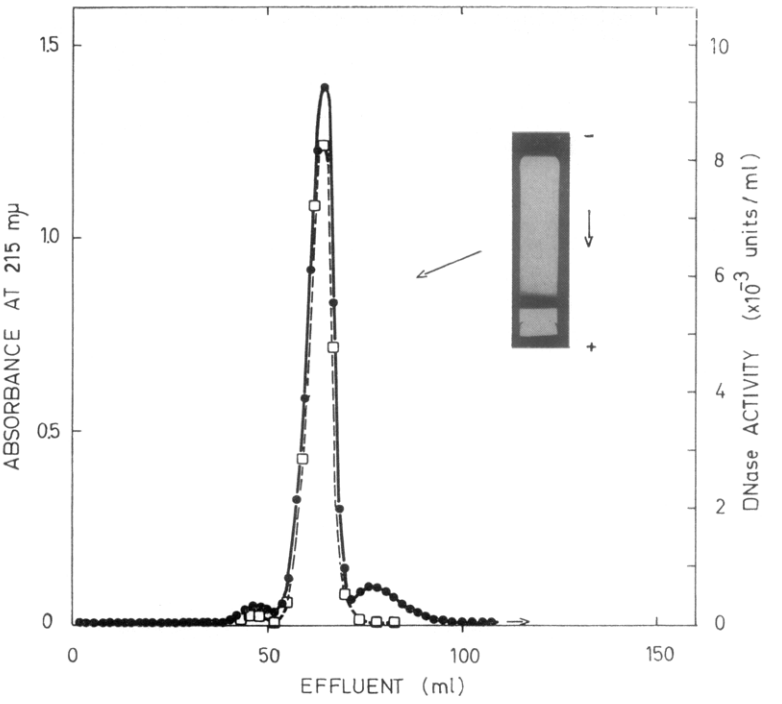

FIGURE 1: Filtration of DNase I through Sephadex G-100. A sample of 75 mg of the once-crystallized enzyme was put onto a column (1 × 170 cm) equilibrated with 0.5 M potassium phosphate buffer, pH 7.6. Filled circles (—●—●—●—) represent the absorbance at 215 mμ of the fractions after 200-fold dilution with water. Open squares (—□—□—□—) stand for enzyme activity. After the filtration experiment, the major DNase peak was analyzed by disc electrophoresis in the discontinuous buffer system of Ornstein (1964) and Davis (1964) separating proteins at pH 10.3. A photograph of a representative gel from such an experiment is included in the figure.

than a different enzyme. The small peak which emerged after the main DNase peak contained neither DNase nor RNase activity. The recovery of the total DNase activity in the filtration experiment was 80–90% and the specific activity of the enzyme was raised by 20–40%. The specific activity of the purified enzyme was 80,000–100,000 when assayed as reported earlier (Lindberg, 1964).

The specific activity of the DNase in the main peak was occasionally found to be lower in the first part than in the rest of the peak. This was interpreted as indicating heterogeneity and only the last two-thirds of the peak were used in my work.

Homogeneity of DNase I

Disc Electrophoresis. Protein (40 μg) was used for each analysis. The runs were made for 2.5 hr at 0–2° with a potential drop over the tubes of 100 v. A photograph of one stained gel is shown in Figure 1. In this type of electrophoresis experiments an advancing front is seen as a faint yellow band ahead of which no material from the sample moves. In the run shown in Figure 1 the yellow band was localized before staining with amido black. The position of the stained protein was found to be about 0.5 cm behind the advancing front. A single sharp band was obtained with the Sephadex-treated DNase, and by this criterion the protein is homogeneous.

N-Terminal Residue. DNase I (7.8 mg) was used for the determination of the N-terminal amino acid, performed as described above. After the cyclization step, 0.25 μmole of PTH-amino acid derivative was obtained as calculated from the ultraviolet absorbance of the

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

Sedimentation		Diffusion		
			$D_{20,w}$ ($\times 10^{-7}$ cm²/sec)	
Protein Concn (mg/ml)	$s_{20,w}$ ($\times 10^{-13}$ sec)	Protein Concn (mg/ml)	Low Rpm	High Rpm
6.76	2.79	7.41	8.6	8.3
4.08	2.77	5.57	8.6	
1.63	2.71	3.71	8.8	
0.00	2.78	1.85	8.7	
		0.00	8.7	

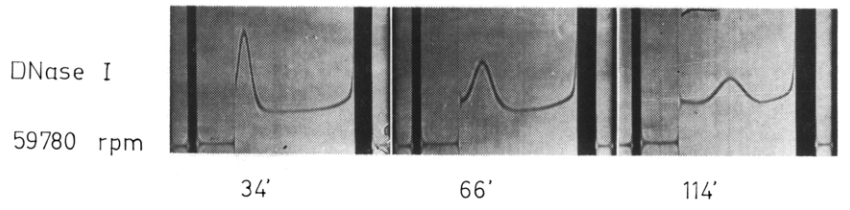

FIGURE 2: Sedimentation of DNase I in 0.1 M potassium phosphate buffer, pH 7.6, at 20°.

sample. Leucine was identified as the only N-terminal amino acid.

Assuming a 100% yield of the N-terminal amino acid, the result obtained indicated a molecular weight of 31,300 for DNase I. This is in good agreement with the value found by ultracentrifugation (average 30,300).

Complex Formation with Spleen Inhibitor II. In experiments described in a following paper (Lindberg, 1967b), it was shown that the protein in the enzyme preparation quantitatively participated during complex formation with the DNase inhibitor II. Since the spleen inhibitor was shown (Lindberg, 1967a) to be selective for DNase I, these experiments were considered to provide the most specific of the homogeneity criteria tested.

Physical Characterization

All studies in the ultracentrifuge were made at 20° in 0.1 M potassium phosphate buffer, pH 7.6.

Sedimentation Analyses. The sedimentation coefficient ($s_{20,w}$) of DNase was determined at three concentrations (see Table I), and in Figure 2, a representative sedimentation pattern is given. As is evident from the values in Table I, no dependence of $s_{20,w}$ was found and the value of $s^0_{20,w}$ was determined to be 2.78 Svedberg units (S). A sedimentation coefficient of 2.63 S was found by Polson (1956) in 0.067 M potassium phosphate buffer, pH 7.0, which is close to the value reported here.

Diffusion Studies. The diffusion of DNase I was also studied as a function of protein concentration. The experiments were performed in the ultracentrifuge at low angular speed (5227 rpm) and the values obtained are listed in Table I. The apparent concentration dependence of the diffusion coefficient which was

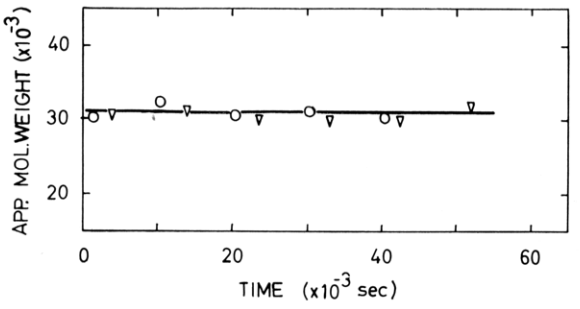

FIGURE 3: Variation with time of the apparent molecular weight of DNase I at the meniscus. The values were obtained from two experiments in 0.1 M potassium phosphate buffer, pH 7.6.

TABLE II: Summary of the Physical and Chemical Characteristics of DNase I.

Characteristic Analyzed	Results Obtained
$E^{1\%}_{1cm}$ at 280 mμ	
Experimental	12.3[a] 13.9[b]
From amino acid composition	15.3[c]
Refractive index increment (ml/g)[d]	0.196 ± 0.007[e]
$s^0_{20,w}$ (× 10^{-13} sec)	2.78
$D^0_{20,w}$ (× 10^{-7} cm²/sec)	8.7
Partial specific volume (from amino acid composition)	0.733
Molecular weight	
Approach to equilibrium	30,900 30,700
Sedimentation–diffusion	29,400
N-Terminal determination	31,300
Isoelectric point	
Experimental	4.7[f]
Total nitrogen (%)	
Experimental	16.7 ± 0.4[e]
From amino acid composition	16.2
Total sulfur (%)	
Experimental	0.75 ± 0.02[e]
From amino acid composition	0.81

[a] In 0.1 M K_2HPO_4–KH_2PO_4 buffer, pH 7.6. [b] In 0.1 N NaOH, pH 13. [c] This value was calculated using the molar extinction coefficients for the aromatic amino acids tyrosine and tryptophan given by Goodwin and Morton (1946). [d] dn/dc was determined at 20°, using the ultracentrifuge as a differential refractometer. [e] The average deviation about the mean is given based on four determinations. [f] Determined by Kunitz (1950) and by Polson (1956).

TABLE III: Amino Acid Recoveries after Acid Hydrolysis of DNase I.[c]

Amino Acid Residue	Hours of Hydrolysis for Recovery				Amino Acid Recov Extrapolated to 0-Time Hydrolysis or at Maximal Value
	20	42	70	140	
Aspartic acid	0.1756 (2)	0.1757 (2)	0.1769 (2)	0.1778 (1)	0.1770
Threonine	0.0763 (2)	0.0742 (1)	0.0715 (2)	0.0659 (1)	0.0780
Serine	0.1483 (2)	0.1362 (1)	0.1236 (2)	0.0979 (1)	0.1570
Glutamic acid	0.1045 (2)	0.1071 (2)	0.1049 (2)	0.1020 (1)	0.1055
Proline	0.0477 (2)	0.0486 (1)	0.0462 (2)	0.0492 (1)	0.0477
Glycine	0.0503 (2)	0.0475 (2)	0.0488 (2)	0.0522 (1)	0.0493
Alanine	0.1236 (2)	0.1190 (2)	0.1203 (2)	0.1249 (1)	0.1219
Valine	0.1136 (2)	0.1295 (2)	0.1352 (2)	0.1404 (1)	0.1404
Methionine[a]	0.0196 (2)	0.0182 (2)	0.0166 (2)	0.0161 (1)	0.0210
Isoleucine[b]	0.0502 (2)	0.0583 (2)	0.0632 (2)	0.0647 (1)	0.0637
Leucine	0.1227 (2)	0.1248 (2)	0.1248 (2)	0.1271 (1)	0.1260
Tyrosine	0.0829 (2)	0.0835 (2)	0.0795 (2)	0.0802 (1)	0.0840
Phenylalanine	0.0581 (1)	0.0633 (2)	0.0606 (2)	0.0621 (1)	0.0613
Lysine	0.0424 (2)	0.0440 (2)	0.0465 (2)	0.0475 (2)	0.0471
Histidine	0.0282 (2)	0.0314 (2)	0.0332 (2)	0.0321 (2)	0.0322
Arginine	0.0540 (2)	0.0648 (2)	0.0624 (2)	0.0652 (2)	0.0542
Ammonia	0.1198 (2)	0.1328 (2)	0.1392 (2)	0.1832 (2)	0.1080
Column no. 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 amounts given as micromoles were recovered from 0.162 mg of protein. The numbers given in 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. No better over-all accuracy is claimed than ±2.5%.

observed was of the order of the precision of the measurements. Therefore, instead of extrapolating the values to zero concentration, a diffusion constant ($D^0_{20,w}$) of 8.7 × 10^{-7} cm^2/sec was afforded by taking an average of the values.

When the diffusion coefficient was measured at high speed (59,780 rpm) in a sedimentation experiment, a value of 8.3 × 10^{-7} cm^2/sec was obtained at 6.76 mg/ml. At low speed and similar protein concentrations the value was 8.6 × 10^{-7} cm^2/sec. The difference in $D_{20,w}$ found was less than 5%, thus showing that the concentration and pressure dependence of $D_{20,w}$ was small. This suggests that the $D^0_{20,w}$ given is close to the true diffusion constant.

Molecular Weight Determination. The values for the molecular weight obtained in two determinations by the modified (Ehrenberg, 1957) Archibald method were 30,900 and 30,700, respectively.[4] In Figure 3, the data from these two experiments are illustrated by plotting the apparent molecular weight at the meniscus. Combination of the sedimentation and diffusion data (from Table I) in the Svedberg (1925) equation gave a molecular weight of 29,400, in reasonable agreement with the values quoted above.

The molecular weight of DNase I obtained here is about one-half the value calculated from diffusion measurements by Kunitz (1950). Kunitz' studies were conducted under conditions close to the isoelectric point of the protein, and aggregation might have occurred. In order to check this point a solution of DNase was equilibrated with 0.5 M NaCl–0.01 M acetate buffer, pH 4.5 (conditions used in Kunitz' experiments), and the molecular weight determined as above by the Archibald method. After 1- and 4-days' storage of the solution at 0–4° molecular weights of 31,300 and 31,500, respectively, were found. After 7 days the sample was also analyzed by sedimentation. Only one symmetrical peak (2.84 S) was obtained. These experiments show that in our hands no aggregation occurred under the conditions of Kunitz' experiments. A summary of the physical and chemical data found in this work is given in Table II.

Chemical Composition

Amino Acid Analyses. In the light of the new information gained about the homogeneity and physical characteristics of DNase I, it was felt warranted to reinvestigate the chemical composition of the enzyme.

[4] An error was made in the calculation of the molecular weight of DNase I published earlier (Lindberg, 1966), giving a value (33,200) which is too high.

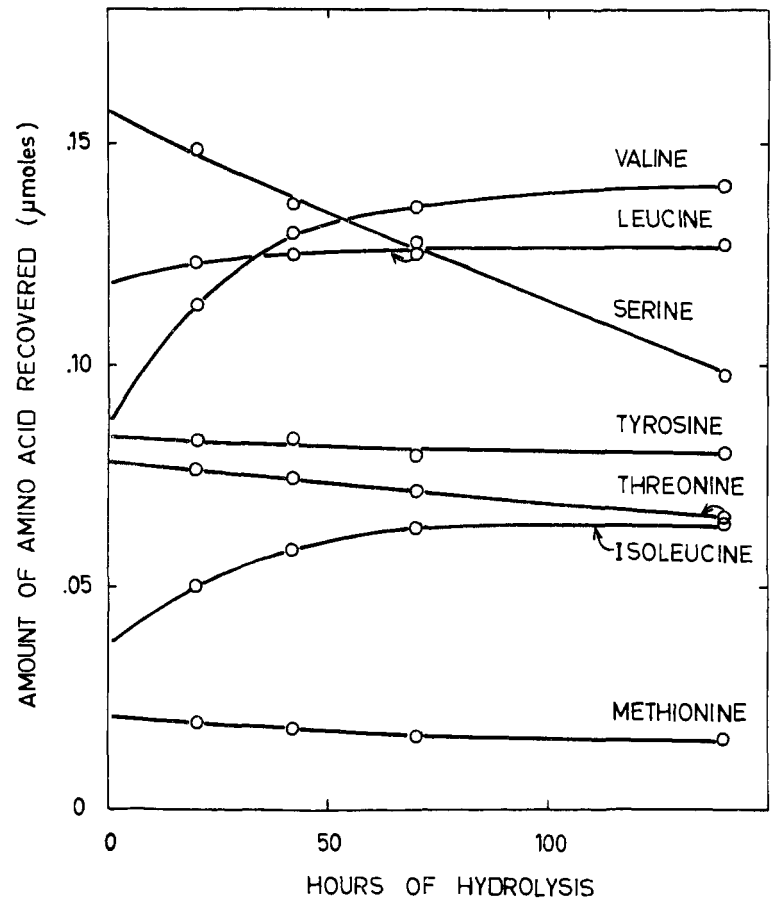

FIGURE 4: Amino acid recoveries as a function of time. Ordinate: micromoles recovered from 0.162 mg of protein.

Samples of desalted DNase I were hydrolyzed for 20, 42, 70, and 140 hr, respectively, and duplicate analyses were made on each hydrolysate (Table III). The recoveries of the different amino acids are included in Table III, the last column of which contains the absolute amounts (in micromoles) of each amino acid per 0.162 mg of protein.

When the recoveries of the different amino acids were examined closely as a function of hydrolysis time, some general trends could be observed, similar to those reported by Mahowald *et al.* (1962) and Noltmann *et al.* (1962). The values listed in the last column of Table III were chosen with respect to these trends. In order to justify the selection of data Figure 4 illustrates hydrolysis curves for some of the amino acids. The over-all accuracy of the analyses was within the limits (±3%) given for the instruments by the manufacturer.

The tyrosine and tryptophan contents were determined as 8.10 and 2.32%, respectively (Tyr:Trp = 3.98). The value obtained for tyrosine agrees well with that found by chromatographic analysis (8.48%). The latter value was used for the calculation of the tryptophan content (2.47%) using the measured Tyr:Trp ratio of 4.

After performic acid oxidation 1.24% of the protein was found as cysteic acid. No differentiation was made between cysteine and cystine.

Additional Analyses

The amount of amide nitrogen found by the diffusion technique was 1.19 ± 0.03% (three determinations). Extrapolation to zero time of the values found in the different acid hydrolysates gave a value of 1.20%. The *total nitrogen* amounted to 16.7 ± 0.4% (four determinations) when determined by the Kjeldahl procedure. A value of 16.2% was calculated from the amino acid composition. The *ash* content was less than 0.6%. Finally the *total sulfur* was determined to be 0.75 ± 0.02% (four determinations), a value which agrees reasonably well with the amount calculated from the cysteic acid and methionine recovered during the amino acid analyses (0.81%).

Out of the 18 amino acids analyzed by the routine technique, eight were determined with some assurance (numbers in boldface types in column 8, Table IV). A variation of ±2.5% in the amount recovered could not change the integers for these amino acids. The others were determined to ±1 residue. The high yield of 98.41% makes it unlikely that the DNase molecule could contain any major components except the amino

TABLE IV: Amino Acid Composition of DNase I.

Amino Acid Residue	g of Amino Acid Residues/100 g of Protein Wt[a]	Minimal Mol Wt[b]	Amino Acid/ 31,000 g of Protein (moles)	Nearest Integral No. of Amino Acid Residues/ 31,000 and 30,664 g of Protein	Nearest Integral No. Multiplied by Minimal Mol Wt	Integral No. of Residues Multiplied by Mol Wt Respective Residue	Amino Acid/ 30,664 g of Protein (moles)
Aspartic acid	12.60[c]	914	33.92[c]	34	31,069	3,914	33.56
Threonine	4.88	2,072	14.96	15	31,081	1,517	**14.80**
Serine	8.46	1,030	30.11	30	30,885	2,613	29.79
Glutamic acid	8.43	1,532	20.23	20	30,638	2,583	**20.01**
Proline	2.87	3,385	9.16	9	30,461	874	**9.06**
Glycine	1.74	3,280	9.45	9	29,518	514	9.35
Alanine	5.36	1,327	23.37	23	30,510	1,635	23.12
Valine	8.61	1,152	26.92	27	31,090	2,677	26.63
Methionine	1.70	7,718	4.02	4	30,870	525	**3.97**
Isoleucine	4.46	2,538	12.21	12	30,457	1,358	**12.08**
Leucine	8.83	1,282	24.18	24	30,768	2,717	23.92
Tyrosine	8.48	1,902	16.10	16	30,434	2,611	**16.12**
Phenylalanine	5.58	2,638	11.75	12	31,656	1,766	11.62
Lysine	3.74	3,428	9.04	9	30,850	1,154	**8.95**
Histidine	2.73	5,026	6.17	6	30,154	823	**6.10**
Arginine	6.21	2,515	12.32	12	30,184	1,874	12.19
Amide ammonia							
By chromatography	(1.20)[d]					(−22)[e]	
By diffusion	(1.19)[d]						
Tryptophan	2.47		4.09[f]	4		745	4.07
Total half-cystine	1.24		3.73[g]	4		413	3.69
Total	98.41[h]				30,664 ± 322	30,291	
Column no 1	2	3	4	5	6	7	8

[a] Calculated from column 6, Table III. [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 due 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 3.98 was determined for Tyr:Trp by the method of Bencze and Schmid (1957). When this ratio was used, 4.09 moles of tryptophan was calculated from the amount of tyrosine given in column 6, Table III. This corresponds to 2.47% of tryptophan in the protein. [g] Determined as cysteic acid after performic acid oxidation. [h] This recovery figure was based on a dry weight determination on the desalted stock solution, from which the samples to the acid hydrolyses were taken. In four independent analyses on different DNase solutions the total nitrogen was related to the dry weight and a mean value of 16.7 ± 0.4% was obtained. The nitrogen content of the above-mentioned stock solution was also determined, whereby a further check on the recovery in the amino acid analyses was gained. A value of 99.8% was then arrived at.

acids determined.

Based on the assumption of a molecular weight of 31,000, as obtained in the ultracentrifuge, the integral numbers of amino acid residues were calculated (column 5 of Table IV). Using these numbers together with the minimal molecular weights (column 3), an average molecular weight of 30,664 ± 322 could be obtained. An average molecular weight of 30,291 was found from the sum of the individual residue molecular weights multiplied by their nearest integral numbers (column 7).

Finally, a value for the partial specific volume of 0.733[5] ml/g was calculated according to the method of Cohn and Edsall (1943) from the amino acid com-

[5] This was the result of more careful and detailed amino acid analyses, and the partial specific volume reported earlier (Lindberg, 1966) must be considered too low.

position. This value was used when the molecular weights were computed from the ultracentrifugation data. Experimental measurements have not been made, but the agreement between the molecular weight calculated from the N-terminal recovery and that calculated from ultracentrifugations indicates that this figure for the partial specific volume must be very nearly correct.

The amino acid composition found here differs markedly from that reported by Gehrman and Okada (1957). The differences amounted to as much as 40% in some cases.

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