CHROM. 3956

GEL-FILTRATION BEHAVIOUR AND MOLECULAR WEIGHT OF NAD-GLYCOHYDROLASE (EC 3.2.2.5) FROM STREPTOCOCCI IN COLUMN CHROMATOGRAPHY ON SEPHADEX GELS*

FRANZ J. FEHRENBACH
Hygiene-Institut, University of Freiburg, Freiburg im Breisgau (G.F.R.)
(Received January 20th, 1969)

SUMMARY

The gel filtration of NAD-glycohydrolase from group C streptococci on Sephadex G-100 and G-200 leads to the separation of enzymatically active protein into at least two fractions. The elution behaviour of NADase was independent of the ionic strength of the buffer systems used. It could be shown, however, that there existed a positive concentration dependence of the molecular weight of NADase on the total protein concentration. Extrapolated to a protein concentration of zero, the molecular weight of the smallest enzymatically active unit amounted to 56000 ± 5000.

INTRODUCTION

Diphosphopyridine nucleosidases, NADases** (EC 3.2.2.5)[1], cleave NAD*** at the pyridine nitrogen-ribose bond[2]. They have been found in tissues of numerous mammals and in microorganisms. Animal NADases are associated with microsomal particles[3] and, hence, are primarily insoluble. They can be liberated with lipolytic and proteolytic enzymes and extracted with organic solvents[4–7].

NADases from plants and microorganisms are soluble. A few of the NADases have been characterized biochemically, including those from *Mycobacterium butyricum*[8], *Aspergillus niger*[9], *Neurospora crassa*[10,11] and *Streptococcus* AC 203 S[12].

The investigation of NADase from Group A streptococci has revealed furthermore that the enzyme functions as an antigen. Specific antibodies against NADase have been demonstrated in human sera, especially during and after streptococcal infections[13].

The antigen-antibody reaction leads to the inactivation of NADase, thus explaining the characterization Enzyme–Antienzyme System[14].

Investigation of the molecular weight, the degree of molecular integrity and

* This work was supported by the Deutsche Forschungsgemeinschaft.
** NADase—Nicotinamide–adenine dinucleotidase.
*** NAD—Nicotinamide–adenine dinucleotide. Syn.: DPNase, NAD-Glycohydrolase, NAD-nucleosidase.

the possible state of aggregation appeared to be important for an understanding of the quantitative expression of the enzyme–antienzyme reaction (anti-NADase reaction), especially considering the significance of such data in the diagnosis of streptococcal disease[15].

The aim of the present investigation has been to determine the molecular weight of NADase from *Streptococcus* H 46 A, a Lancefield Group C strain, by means of the gel-permeation technique, using dextrans of the Sephadex type[16–20].

MATERIALS AND METHODS

Lyophilized* NADase from the supernatant of a broth culture of *Streptococcus* H 46 A, Lancefield group C, was used. The specific activity, S.A., was

$$\text{S.A.} = \frac{U/\text{ml}}{E_{280}} = 3.9 \times 10^2$$

where U represents units NADase and E_{280} = optical density, O.D. (= extinction), at $\lambda = 280$ mμ.

The starting material was dissolved in a Tris–HCl or phosphate buffer (0.05 M, pH 7.00) and precipitated stepwise with $(NH_4)_2SO_4$.

Ammonium sulphate fractionation

The ammonium sulphate fractionation was carried out in two steps following the principles outlined by DIXON[21].

In a preliminary step, extraneous protein was eliminated from the solution by the addition of solid ammonium sulphate up to 50 % saturation. After 1.3 h the precipitate was separated in an MSE centrifuge (Mod. High Speed, 18, MSE Ltd., London S.W. 1) at 12000 r.p.m. for 30 min at 6° and discarded.

The pH was kept constant at the previously established optimal pH 7.00 throughout the precipitation, deviations being corrected by addition of half-concentrated NH_4OH solution. The precipitation was completed in 3 h and carried out at 10°.

In the range of 60 to 80 % $(NH_4)_2SO_4$ saturation the enzyme was harvested in 1.2- to 1.7-fold enrichment, in terms of the S.A. of the starting material, in good yield (58–70 % of the total activity).

The precipitate was collected in an MSE centrifuge at 12000 r.p.m. for 30 min at 6° and dissolved in 3–5 ml buffer. Desalting was done on a Sephadex G-25 fine column. The enzyme was either immediately processed further or stored at —20°.

Chromatography

Ascending column chromatography was carried out in Wissler System equipment (Bioplant GmbH, 318 Wolfsburg, Rieslingerstr. 12, G.F.R.).

The columns were water jacketed and held at constant temperature by means of an Ultra-Kryomat-Lauda, Mod. TK 30D (Dr. WOBSER K.G., Lauda, Pfarrstr. 16, G.F.R.). The gel was placed between two adjustable pistons, fitted with Teflon collars and filter discs. The dead volume was less than 0.1 %.

The effluent was collected in an LKB fraction collecter (LKB Producter AB,

* This material was kindly supplied by Dr. SCHWICK, Behringwerke, Marburg/Lahn, G.F.R.

Stockholm 12, Sweden) equipped with an alcohol cooling bath, thus facilitating the freezing of the enzyme at —15°.

Determination of molecular weight of NADase on Sephadex G-200

Sephadex G-200 (Pharmacia, Uppsala, Sweden; particle size, 40–120 μ; Lot No. 8987) was equilibrated for eight weeks in de-aereated phosphate buffer (0.05 *M*, pH 7.35) containing 0.05 *M* KCl, at 4°. The buffer was stirred mechanically and renewed every second day. By means of repeated resuspension and sedimentation at definite time intervals and the above-mentioned constant change of the equilibrating buffer, the smallest gel particles were eliminated by decanting, and those of essentially uniform size were retained. The gel was sedimented in a 3 × 60 cm column, treated with dichlorodimethylsilane (Bio-Rad, Richmond, Calif.) to a height of 60 cm under constant hydrostatic pressure and flow rate.

The column was washed (ascending) at a constant flow rate (Sigma-Pump). 9 ml/h, at 6°, for several days with de-aereated buffer. The column was calibrated under exactly the same conditions with Blue Dextran (bl Dextr) (Pharmacia, Uppsala, Sweden), mol. wt. $\geq 2 \times 10^6$; γ-Globulin (γ-Glob) (bovine, Op. Nr. 251154 reinst, Behringwerke, Marburg/Lahn, G.F.R.), mol. wt. 14×10^4; Bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis), mol. wt. $65–70 \times 10^3$; Ovalbumin (Ov-alb) (5 × cryst. 4969/111009, Mann Res. Lab. Inc., New York), mol. wt. 45×10^3; Myoglobin (Myo) (whale sperm, cryst., salt-free, lyph., Cat. No. 6649/53073, Mann), mol. wt. 17×10^3; Cytochrome-c (Cyt-c) (horse heart, salt-free, No. 61384551, C. F. Boehringer, Mannheim, G.F.R.), mol. wt. 13×10^3; D-Glucose (D-Gluc) (p.a. Merck, Darmstadt, G.F.R.), mol. wt. 198.2.

The column was calibrated in two separate steps:

(1) 5 ml of buffer contained: 10 mg bl Dextr, 50 mg BSA, 25 mg Cyt-c and 5 mg D-Gluc.

(2) 5 ml of buffer contained: 50 mg γ-Glob, 50 mg Ov-alb*, and 20 mg Myo*.

The calibrated column was used immediately for the molecular weight determination of NADase.

Also, afterwards, to check constancy of conditions, bl Dextr, Cyt-c and BSA were re-run on the column. Column effluent was measured on constant-flow absorption meters (Uvicord I and II, LKB Producter AB, Stockholm 12, Sweden) at 260 mμ and 280 mμ, and traced on two separate recorders (LKB).

Determination of molecular weight of NADase on Sephadex G-100

G-100 Sephadex (Pharmacia, Uppsala, Sweden; particle size, 40–120 μ; Lot No. T_0-4342) was prepared as described for G-200. A 2 × 80 cm System Wissler column was used for the molecular weight determination. The gel filtration behaviour of NADase was investigated in ascending chromatography with an applied volume of 1.5 ml at different protein concentrations (1–10 mg prot./ml), at 6° and at a flow rate of 5.0 ml/h.

Analytical

bl Dextr: The solution showed characteristic absorption bands at 200, 260,

* These substances were used for calibrating Sephadex G-100 columns only.

280, 380 and 625 mμ. For identification the optical density of the solution was measured at 280 mμ and 260 mμ ($E_{280}/E_{260} = 0.9$).

D-Gluc: The anthrone method according to SCOTT AND MELVIN[22] or MORSE[23] was used for the determination of glucose at 625 mμ. The reagent was 0.2 % anthrone in concentrated H_2SO_4 $D_{20} = 1.83$ g/ml. BSA, Ov-alb, Myo and Cyt-c were determined by measuring O.D. at 280 mμ and 260 mμ (*cf.*, $Q = 280/260$). Further for Myo and Cyt-c the O.D. was measured at 408 mμ.

NADase: Activity was measured either by the ADH* method or by the cyanide addition method of KAPLAN[24].

For NADH**, O.D. at $\lambda = 366$ mμ $= E_{366} = 3.30 \times 10^3$ (cm^2 mmole^{-1}).

For NAD-CN***, O.D. at $\lambda = 334$ mμ $= E_{334} = 5.535 \times 10^3$ (cm^2 mmole^{-1}).

One enzyme unit (U) is defined as the hydrolytic activity which cleaves 1 μmole NAD, at 37° and pH 7.35 in 0.05 M Tris-HCl buffer in 1 h at the pyridinium-N-ribose bond.

RESULTS

Elution behaviour of NADase on Sephadex G-200

Sephadex G-200 separates macromolecules in the molecular weight range of 10^3 to 2×10^5. The suggested molecular weight of 1×10^5 (ref. 25) would indicate its selection for gel filtration of NADase. The experimental data for a typical calibration chromatogram on Sephadex G-200 are given in Table I. The calibration chromatogram of NADase on G-200 (Fig. 2) showed clearly that the enzyme activity curve had several peaks and did not lead to a complete separation of individual enzyme fractions. Besides, enzymatically active material could be detected with $V_e = V_0$.

TABLE I

ELUTION PARAMETERS IN MOLECULAR WEIGHT DETERMINATION OF NADASE ON SEPHADEX G-200

Substance	*Mol. wt.*	V_e[a]	V_e/V_0[b]	*Conditions*	
bl Dextr	$2 \cdot 10^6$			column:	3 × 60 cm
γ-Glob	$14 \cdot 10^4$	286.0	1.46	buffer:	phosphate
BSA	$67–70 \cdot 10^3$	320.0	1.63	molarity:	0.05
NADase I	$63 \cdot 10^3$	329.0	1.68	KCl:	0.05
NADase II	$55 \cdot 10^3$	343.0	1.75	pH:	7.35
Cyt-c	$13 \cdot 10^3$	419.5	2.14	applied vol.:	5 ml
D-Gluc	198	470.0	2.40	l. fract. = 4.4 ± 0.1 ml = 30 min temp. = 6°	

[a] V_e = elution volume. (For abbreviations see ref. 26.)
[b] V_e/V_0 = elution volume/void volume; $V_0 = 196.0$.

In accordance with the exclusion theory[16,19] the molecular weight of this inhomogeneous enzyme fraction $\geq 2 \times 10^6$ and was therefore not separated by the gel. The principal part of the NADase—representing about 70 % of the total activity—

* ADH = alcohol-dehydrogenase (Yeast), EC 1.1.1.1.
** NADH = reduced NAD.
*** NAD-CN = cyanide addition complex of NAD (cyanide adds at the 4 position of the pyridine ring).

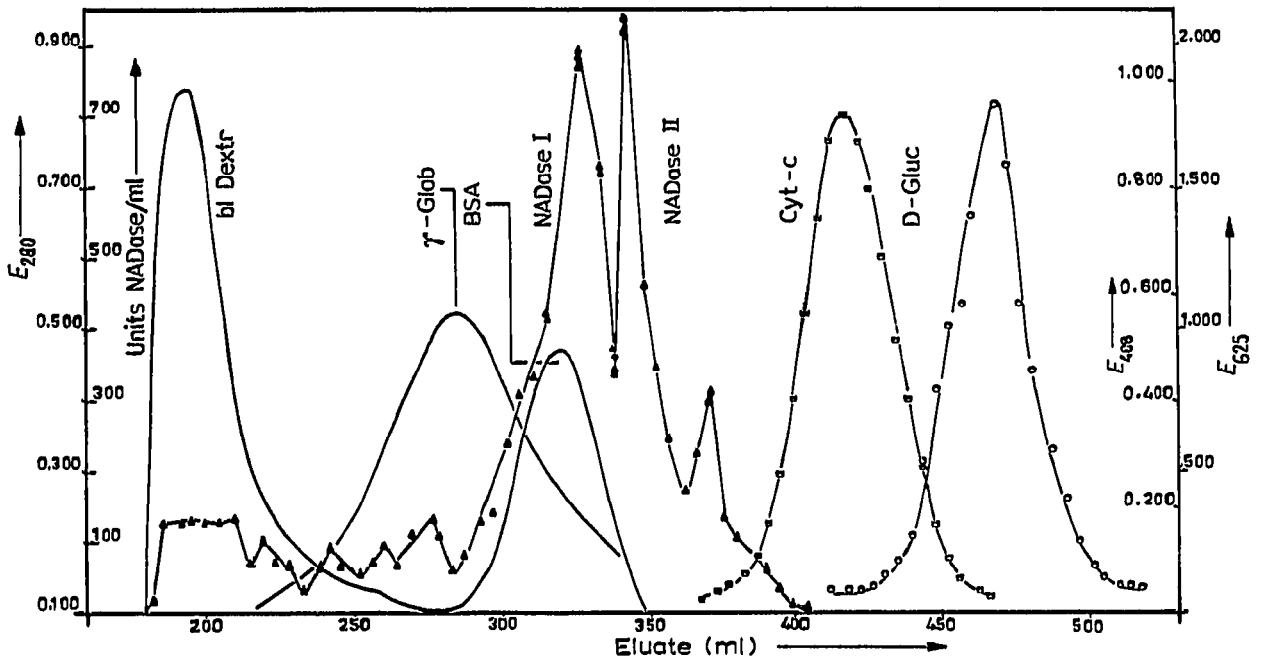

Fig. 1. Calibration chromatogram for NADase on Sephadex G-200, 0.05 M phosphate buffer, pH 7.35, containing 0.05 M KCl, 6°, column: 3 × 60 cm. (———) E_{280} = O.D. at 280 mμ (1.0 cm quartz cuvette) for bl Dextr, γ-Glob and BSA. (-▲-▲-) NADase activity. (-□-□-)E_{408} for Cyt-c. (-O-O-) E_{625} for D-Gluc (anthrone method).

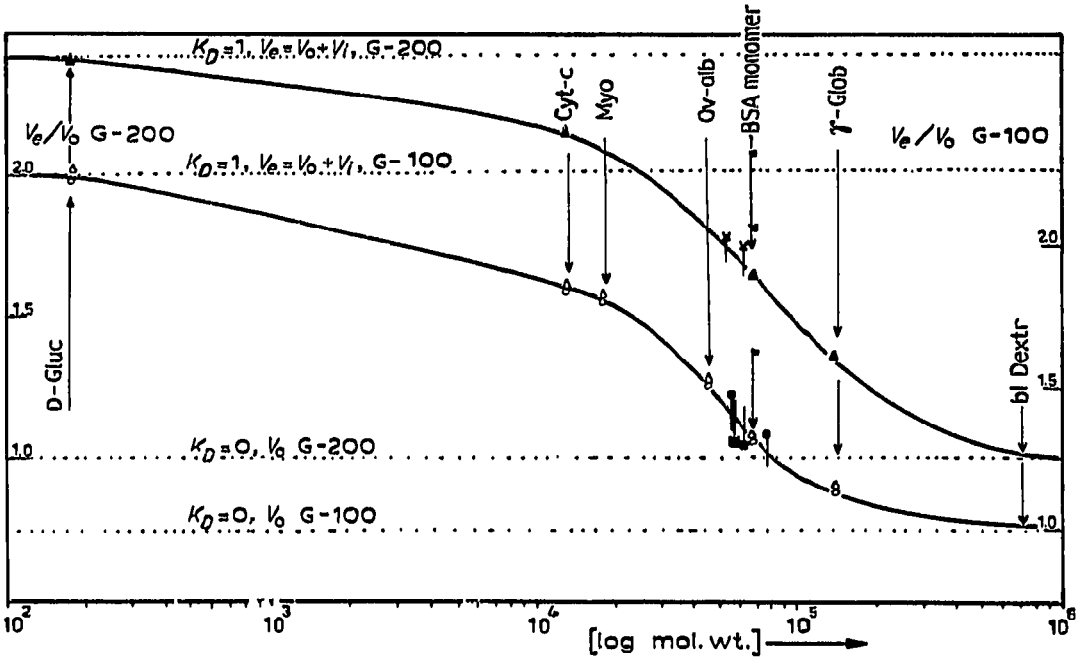

Fig. 2. Plot of V_e/V_0 as a function of log mol. wt. for calibration substances on Sephadex G-100 (lower curve) and G-200 (upper curve). O and ▽, V_e/V_0 values derived from two separate column runs on G-100. ▲, V_e/V_0 values on G-200. The symbols × × (G-200), ● ● (G-100) and ■ ■ refer to V_e/V_0 values of NADase derived, respectively, from Figs. 1, 3 and 4.

was found under peaks I and II (Fig. 1), with $V_{eI} = 329.0$ ml and $V_{eII} = 343.0$ ml (Table I).

In Fig. 2, $Q = V_e/V_0$ was plotted as a function of log mol. wt. for the calibration substances and NADase. Thus, in Fig. 2, the mol. wt. values for both NADase fractions (upper curve, $\times\times$) can be read directly from the abscissa. V_{eI}/V_0 corresponds to mol. wt. 63000 $\pm$ 6000; V_{eII}/V_0 corresponds to mol. wt. 55000 $\pm$ 5000.

Elution behaviour of NADase on Sephadex G-100

Table II summarizes the experimental data typical for a calibration run on G-100. Under these conditions the NADase elution profile (Fig. 3) shows good agree-

TABLE II

ELUTION PARAMETERS IN MOLECULAR WEIGHT DETERMINATION OF NADASE ON SEPHADEX G-100

Substance	*Mol. wt.*	V_e	V_e/V_0[a]	*Conditions*	
bl Dextr	$2 \cdot 10^6$			column:	2 × 80 cm
γ-Glob	$14 \cdot 10^4$	117.0	1.13	buffer:	phosphate
NADase I	$76 \cdot 10^3$	132.8	1.27	molarity:	0.05
BSA monomer	$67 \cdot 10^3$	134.0	1.29	KCl:	0.05
NADase II	$55 \cdot 10^3$	147.2	1.42	pH:	7.35
Ov-alb	$45 \cdot 10^3$	154.0	1.48	applied vol.:	1.5 ml
Myo	$17 \cdot 10^3$	187.5	1.81	l. fract.	= 4.6 ± 0.1 ml = 30 min
Cyt-c	$13 \cdot 10^3$	191.5	1.85		
D-Gluc	198	229.5	2.21	temp.	= 6°

[a] $V_0 = 103.7$.

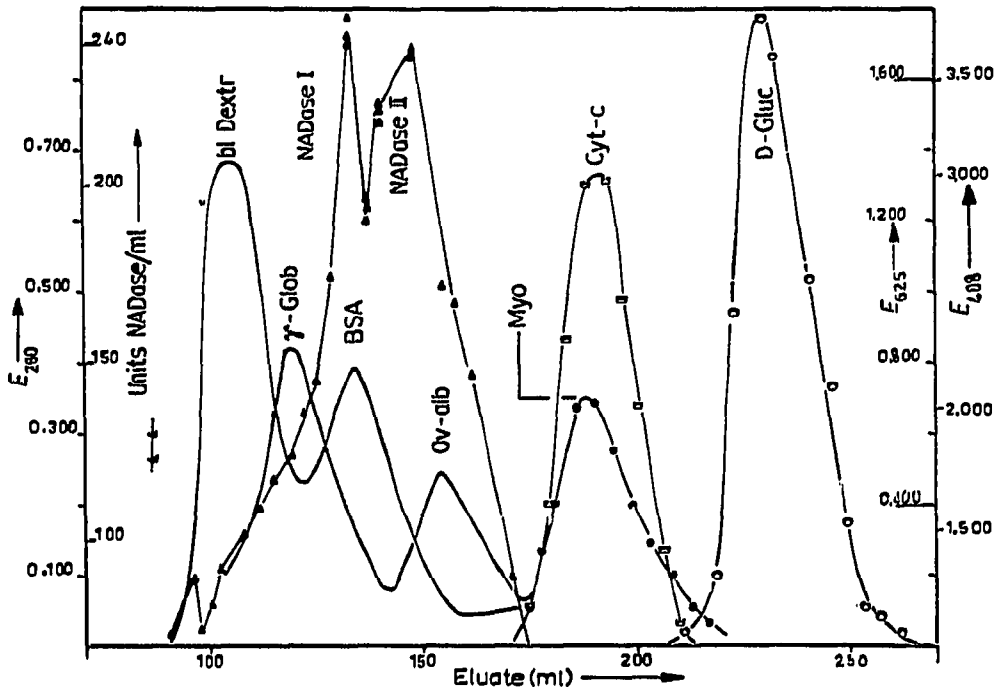

Fig. 3. Calibration chromatogram for NADase on Sephadex G-100, 0.05 *M* phosphate buffer, pH 7.35, containing 0.05 *M* KCl, 6°, column: 2 × 80 cm. (———) E_{280} = O.D. at 280 mμ (1.0 cm quartz cuvette) for bl Dextr, γ-Glob, BSA and Ov-alb. (-▲-▲-) NADase activity. (-●-●-) E_{408} for Myo. (-□-□-) E_{408} for Cyt-c. (-○-○-) E_{625} for D-Gluc (anthrone method).

ment with the curve in Fig. 1. As expected, enzymatically active protein is excluded by the gel. Fig. 1 shows further that this material was better separated on G-200.

With the aid of the elution volumes, $V_{eI} = 132.8$ ml and $V_{eII} = 147.2$ ml, taken from Table II, the corresponding molecular weights 76000 ± 7000 and 55000 ± 5000, are read directly from the abscissa of the plot of $V_e/V_0 = f$ (log mol. wt.) in Fig. 2 (lower curve, ●●).

The gel filtration chromatogram on G-100 (Fig. 3) shows 80% of the NADase activity under the two peaks; the rest, 20%, was excluded by the gel. The total yield of NADase was 50%.

Influence of ionic strength on the elution behaviour of NADase on G-100

The gel filtration of NADase in 0.05 *M* Tris–HCl buffer, containing 0.15 *M* KCl at pH 7.35 (as shown in Fig. 4) verifies the result of the chromatogram given in Fig. 3.

Two NADase fractions could be detected with $V_{eI} = 142.2$ ml and $V_{eII} = 148.5$ ml (Table III). From Fig. 2, the molecular weights of 63000 ± 6000 and 56000 ± 5000 are obtained (lower curve, ■■).

Other than a decrease in peak breadth, no change in elution behaviour of the enzyme was observed. This indicates that under the given conditions, the gel permeation behaviour of NADase on G-100 does not depend on the ionic strength. The dependence on pH and temperature of the elution parameters was not investigated.

Dependence of the molecular weight of NADase on the protein concentration

The molecular weight of NADase is shown as a function of the relative protein concentration (mg prot., Biuret[27], using Ov-alb as standard) in Fig. 5. Since each

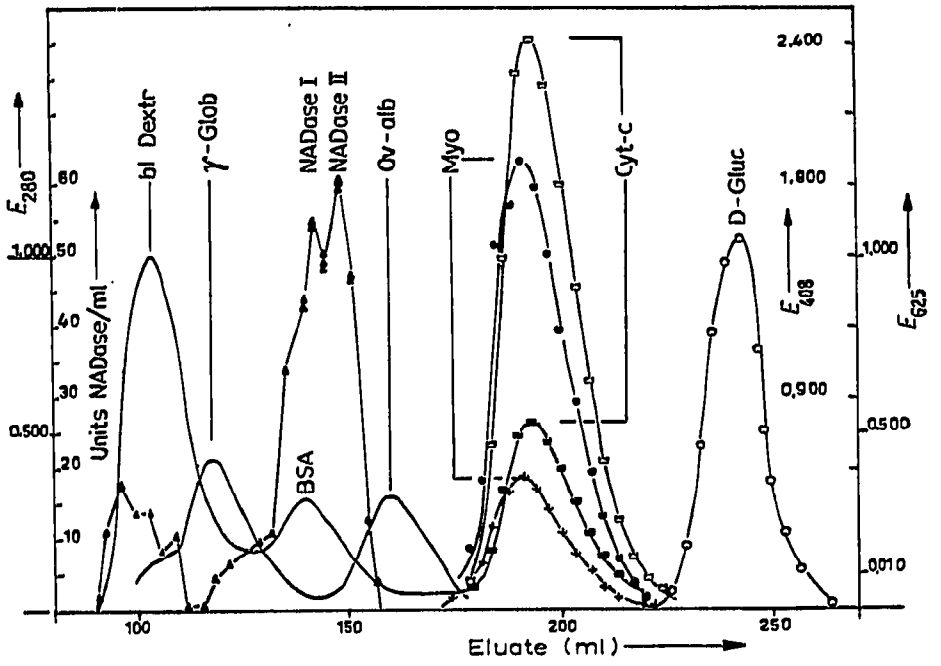

Fig. 4. Calibration chromatogram for NADase on Sephadex G-100, 0.05 *M* Tris–HCl buffer, pH 7.35, 6°, column: 2 × 80 cm. (———) E_{280} = O.D. at 280 mμ (1.0 cm quartz cuvette) for bl Dextr, γ-Glob, BSA and Ov-alb. (-▲-▲-) NADase activity. (-●-●-) E_{408} and (-×-×-) E_{280} for Myo. (-□-□-) E_{408} and (-■-■-) E_{280} for Cyt-c. (-○-○-) E_{625} for D-Gluc (anthrone method).

TABLE III

INFLUENCE OF IONIC STRENGTH ON MOLECULAR WEIGHT OF NADASE ON SEPHADEX G-100

Substance	*Mol. wt.*	V_e	V_e/V_0[a]	*Conditions*	
bl Dextr	$2 \cdot 10^6$			column:	2 × 80 cm
γ-Glob	$14 \cdot 10^4$	118.5	1.13	buffer	Tris–HCl
BSA	$67 \cdot 10^3$	140.0	1.34	molarity:	0.15
NADase I	$63 \cdot 10^3$	142.2	1.36	KCl:	0.15
NADase II	$56 \cdot 10^3$	148.5	1.42	pH:	7.35
Ov-alb	$45 \cdot 10^3$	160.0	1.53	applied vol.:	1.5 ml
Myo	$17.8 \cdot 10^3$	191.0	1.83	l. fract.	= 3.3 ± 0.1 ml = 40 min
Cyt-c	$13 \cdot 10^3$	193.5	1.85		
D-Gluc	198	241.7	2.30	temp.	= 6°

[a] $V_0 = 104.5$.

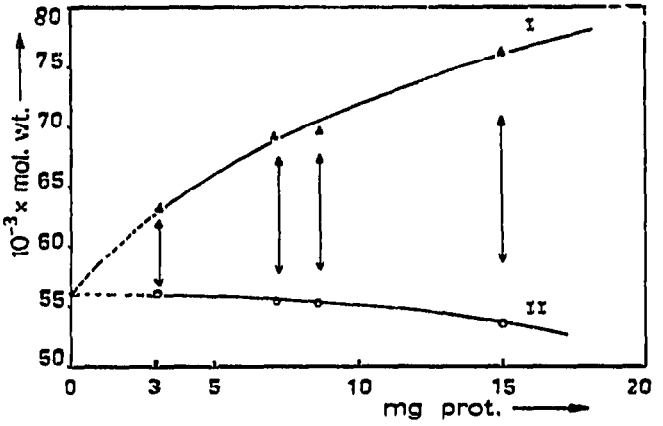

Fig. 5. Effect of total protein concentration on the molecular weight of NADase estimated by Sephadex gel filtration on G-100. Each of the corresponding pairs of values (▲–○) is derived from a single calibration chromatogram.

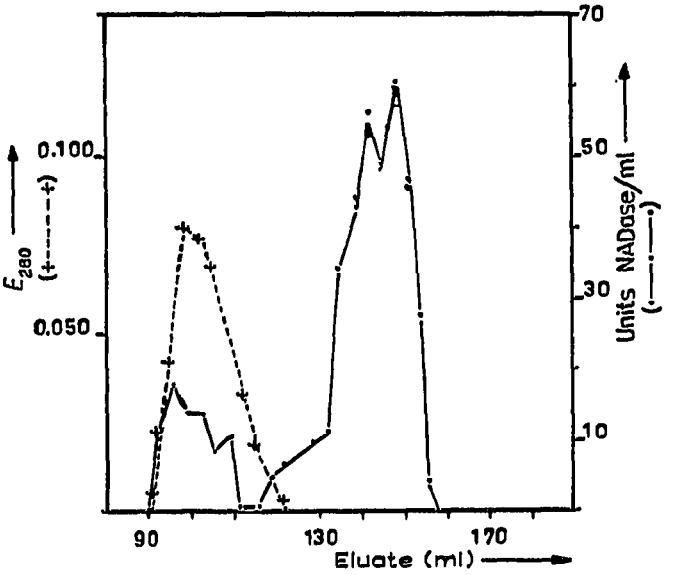

Fig. 6. Purification of NADase by gel filtration on Sephadex G-100. Column: 2 × 80 cm. Tris–HCl buffer (0.05 *M*, pH 7.35) containing 0.15 *M* KCl at 6°. (-+--+-) O.D. at 280 mμ; (-●-●-) NADase activity, *U*/ml. Measured in quartz cuvettes; 1 cm light path.

chromatogram gives two NADase peaks, the tracing of $c^* = f$ (mol. wt.) leads to two corresponding curves, I and II. Curve I represents the higher molecular weight fractions and shows positive concentration dependence. Curve II (Fig. 5) shows a weakly negative concentration dependence.

Purification of NADase by gel filtration on Sephadex G-100

The NADase activity curve, taken from the chromatogram in Fig. 4, and the optical density curve (E_{280}) are given in Fig. 6. The diagram shows a good separation of extraneous protein from NADase: only 20 % of the enzyme activity was eluted with extraneous protein.

The specific activity (S.A.) before the run was 4.4×10^2; after gel filtration S.A. $= 7.5 \times 10^3$. The enrichment in this step was 17-fold. The yield was 45–50 %.

DISCUSSION

The theoretical treatment of the gel filtration process exhibited a linear relationship between $\sqrt{\text{molecular weight}}$ and $\sqrt[3]{K_D}$ (K_D = diffusion coefficient), assuming that the form of certain macromolecules is a function of their molecular weights.

This principle is the basis of column chromatographic determination of molecular weight. Actually a number of globular proteins adhere to this general relationship in good approximation, as investigations into the gel filtration process have shown. More or less gross deviations from this general rule exist for whole series of natural substances which have been investigated[28–30]. Thus the gel filtration process is not suited for the molecular weight determination of substances which exist in a concentration-dependent association–dissociation equilibrium, as, for example, β-lactoglobin[28].

NADase which was investigated on Sephadex G-100 and G-200, displays an elution behaviour suggesting that this system does not comply with the above relationship either. The demonstration of a definite dependence of the molecular weight of NADase on the total protein concentration supports this view. It appears reasonable to assume this system to be an association–dissociation equilibrium. Whether it is a matter of enzyme–enzyme interaction (such as reversible concentration–dependent association or dissociation of subunits of NADase), or whether the system includes an enzyme–extraneous protein aggregation, cannot be elucidated by this study, since the molecular weight determination was carried out with a raw product.

It is assumed that the determination of the molecular weight of NADase in the ultracentrifuge, where concentrated protein solutions must be used, would have led to quite comparable results.

Among other factors, pH, ionic strength, presence or absence of specific metallic ions[31] and the oxidation state of central atoms in metallo–protein complexes[32,33] influence the column chromatographic behaviour of proteins to a considerable extent. Which of these factors essentially determines the molecular behaviour of NAD-glycohydrolase must be the subject of further investigations with purer enzyme.

ACKNOWLEDGEMENT

The author wishes to thank Miss CHRISTEL MOHRMANN for expert technical assistance.

* c = mg. prot.

REFERENCES

1 *Report of the Commission of Enzymes of the International Union of Enzymology*, Pergamon Press, New York, 1961, p. 112.
2 PH. HANDLER AND J. R. KLEIN, *J. Biol. Chem.*, 143 (1942) 49.
3 H. MCILWAIN AND B. RODNIGHT, *Biochem. J.*, 44 (1949) 470.
4 S. G. A. ALIWISATOS AND D. W. WOOLLEY, *J. Biol. Chem.*, 219 (1956) 823.
5 D. STATHAKOS AND K. WALLENFELS, *Biochem. Z.*, 346 (1966) 89.
6 D. STATHAKOS AND K. WALLENFELS, *Biochem. Z.*, 346 (1966) 107.
7 E. C. G. HOFMANN, *Biochem. Z.*, 329 (1957) 428.
8 M. KERN AND R. NATALE, *J. Biol. Chem.*, 231 (1958) 41.
9 D. S. SARMA, S. RAJALKSHUN AND P. S. SARMA, *Biochim. Biophys. Acta*, 81 (1946) 311.
10 N. O. KAPLAN, in S. P. COLOWICK AND N. O. KAPLAN (Editors), *Methods in Enzymology*, Vol. II, Academic Press, New York, 1957, p. 622.
11 L. GROSSMAN AND N. O. KAPLAN, *J. Biol. Chem.*, 231 (1958) 772.
12 A. S. CARLSON, A. KELLNER, A. W. BERNHEIMER AND E. B. FREEMAN, *J. Exptl. Med.*, 106 (1957) 15.
13 A. KELLNER, E. B. FREEMAN AND A. S. CARLSON, *J. Exptl. Med.*, 108 (1958) 299.
14 K. RAJEVSKY, *15. Kolloquium der Gesellschaft für Physiologische Chemie, Mosbach, Germany, 1966.*
15 K. F. PETERSEN, *Z. Hyg.*, 148 (1962) 596.
16 G. K. ACKERS, *Biochemistry*, 3 (1964) 723.
17 G. K. ACKERS AND R. L. STEERE, *Biochim. Biophys. Acta*, 59 (1962) 137.
18 P. ANDREWS, *Nature*, 196 (1962) 36.
19 P. FLODIN, *J. Chromatog.*, 5 (1961) 103.
20 R. L. STEERE AND G. K. ACKERS, *Nature*, 196 (1962) 475.
21 M. DIXON, *Biochem. J.*, 54 (1953) 457.
22 T. A. SCOTT AND E. H. MELVIN, *Anal. Chem.*, 25 (1953) 1656.
23 E. E. MORSE, *Anal. Chem.*, 19 (1947) 1012.
24 N. O. KAPLAN, *J. Biol. Chem.*, 191 (1951) 447.
25 A. W. BERNHEIMER, P. D. LAZARIDES AND A. T. WILSON, *J. Exptl. Med.*, 106 (1957) 27.
26 H. DETERMANN, in H. DETERMANN (Editor), *Gelchromatographie*, Springer Verlag, Berlin, Heidelberg, New York, 1967, p. 71.
27 T. E. WEICHSELBAUM, *J. Klin. Pathol.*, 10 (1946) 40.
28 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
29 G. K. ACKERS, *J. Biol. Chem.*, 242 (1967) 3026.
30 G. K. ACKERS, *J. Biol. Chem.*, 243 (1968) 2056.
31 K. KAKIUCHI, S. KATO, A. IMANISHI AND T. ISEMURA, *J. Biochem. (Tokyo)*, 55 (1964) 102.
32 R. BANERJEE AND C. R. CASSOLY, *Acad. Sci. (Paris)*, 262 (1966) 1375.
33 T. MERRETT, *Biochim. Biophys. Acta*, 124 (1966) 389.