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Light Scattering Determination of the Molecular Weights of the Ammodytes Viper Venom, Its Antivenin and Their Aggregates

J. Kratohvil and Dj. Ajduković

Department of Physical Biochemistry, »Andrija Štampar« School of Public Health, Faculty of Medicine, University of Zagreb, Mirogojska 4, Zagreb, Croatia, Yugoslavia

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A light scattering determination of the molecular weights of the venom of *Viperae Ammodytes*, its antivenin and their aggregates in 0.15 *M* sodium chloride solutions were performed. Venom and antivenin were extensively dialyzed against water or sodium chloride solution. The weight-average molecular weight of the venom amounted to 43,600, and of the antivenin (pepsin digested) to 83,000. The size of the venom-antivenin aggregates depended upon the time of the reaction and the venom-antivenin ratio. The corresponding molecular weights varied from 202,000 to 4,800,000. The results were compared with the investigation on similar systems, and the significance of the results was discussed.

In spite of the vast amount of papers dealing with the studies of toxic proteins, little has been done in this field of research by means of the usual macromolecular physical techniques. Such investigations were mainly confined to some bacterial toxins and, to a much less extent, to some animal venoms (for reviews see refs. 1,2). As regards to the association of toxic proteins with antibodies directed toward them, only few results obtained by the use of the macromolecular tools of experimentation could be found in the literature. So far as we know only the combination of diphtheria toxin with antitoxin has been studied in this way.[3] However, the macromolecular studies of the interaction between typical globular proteins and their antibodies are greater in number.[4–11] All these investigations have proved the usefulness of the macromolecular methods in studies of the antigen-antibody interaction.

Snake venoms are very interesting biological material because of their biochemical, toxic, immunological and therapeutic properties and possibilities. They are mainly proteinic in character,[1] and many, if not all, properties of the venoms have to be associated with the proteins present. These proteins represent also the main macromolecular components of a particular venom.

The venom of the snake *Vipera Ammodytes* has been intensively studied[12–18] and much information about its composition, properties and reactions has been collected. The present paper discusses the determination of the molecular weights of the Ammodytes viper venom and of the corresponding antivenin by means of light scattering measurements. Some molecular weights of the products of the venom-antivenin reaction are also put forward. The general feature of this reaction has been studied previously and the results partly published.[19].

EXPERIMENTAL

Materials

The *venom* (purchased from the Serovaccinal Institute, Zagreb) has been collected from living snakes, dried and homogenized by grinding in a mortar. Some of its chemical and immunological properties were described previously.[12–19] The crude venom was dissolved in 0.15 *M* sodium chloride solutions. The samples of venom solutions (in concentrations of about 0.5—1%) were dialyzed in Visking cellophane tubes against large volumes of either distilled water or saline solutions for 5—7 days at 2° C. About 14% of the venom, originally present in solution, has been precipitated during the dialysis against water. The precipitate formed was readily dissolved in 0.15 *M* sodium chloride. After the dialysis the whole solution was brought to 0.15 *M* NaCl.

The *antivenin* was supplied as an about 6% solution by the Serovaccinal Institute, Zagreb. It was a pepsin-digested purified immune horse serum concentrated by ammonium sulphate precipitations and ultrafiltrations.[20] In all experiments the antivenin of the same series of production was used. It was dialyzed against distilled water in the same manner as the venom and brought to 0.15 *M* NaCl. During the dialysis of the antivenin, the loss of the material was negligible. But for the venom about one third of the crude material was lost in the dialysis.

To study the light scattering of the *venom-antivenin aggregates,* the mixtures containing various amounts of the solutions of the venom and antivenin were prepared. We have worked at two venom-antivenin ratios: one nearly corresponding to the optimal precipitation concentrations[19] (venom-antivenin ratio 0.01667), and the other in the venom excess zone (venom-antivenin ratio 0.00333). The predetermined volumes of the solutions containing sufficient quantities of the venom and antivenin were mixed and the mixtures were kept at room temperatures (about 25° C) for 24 hours or more. After that time the appropriate dilutions of the mixture with 0.15 *M* NaCl were made and the light scattering intensity was measured.

Apparatus and procedure

Light scattering apparatus. The apparatus used was essentially similar to that of Zimm[21] and was constructed and built at the Institute »Jožef Stefan«, Ljubljana. Since the apparatus is described in a recent paper from that Institute,[22] only some special comments and a brief outline of the procedure will be given here.

The measurements were carried out using unpolarized incident light of 436 mμ wave length. Only vertically polarized component of the scattered light has been measured. The light scattering cells were of conical shape (Erlenmeyer type).[21] The angular symmetry was tested with dilute solutions of fluorescein (with yellow filter before the receiver), and the cells were aligned so that the products of the instrument readings for vertically polarized light and the factor $\sin\theta$ were constant to 2% over the range of the angles of observation, θ, from 45° to 135°. The cells were immersed in a large liquid bath containing a medium (ethyl benzene), whose refractive index was nearly the same as that of the glass.

Calibration. The instrument was calibrated with benzene carefully purified in a manner described by Edsall et al.,[23] and contained in a sealed Erlenmeyer-type glass cell. It also served as a working standard. The Rayleigh ratio of benzene was assumed to be 48.4×10^{-6} cm.$^{-1}$ for unpolarized light and 33.1×10^{-6} cm.$^{-1}$ for vertical component of scattered light of 436 mμ.[24,25,26] The calibration of the instrument and the whole procedure were verified by the measurements of light scattering in solutions of bovine serum albumin (Armour & Co., Chicago) in 0.15 *M* sodium chloride. From these measurements the molecular weight of bovine serum albumin was calculated to be 75,000, in good agreement with the results of other investigators (see, e. g., ref. 27, p. 139).

Correction factors. Owing to a suitable geometry of the instrument, the volume correction[24] was small and may be neglected. However, since the instrument has been calibrated with benzene, and the measurements were performed in aqueous solutions, the refractive index correction[24,28] had to be applied. Meyer[29] has shown that the n^2 correction is valid also for conical cells. Since the receiver did not see past the edges of the incident beam and $l \ll r$ (l is the distance between the

cell wall and the receiver, and r is the distance between the center of the incident beam and the cell wall), the refractive index correction can be set equal to $(n_w/n_b)^2$, where n_w and n_b are the refractive indices of aqueous solution and benzene, respectively. To allow for the change in the scattering volume, a factor, $\sin\theta$, was applied to the observed intensity for angles, θ, other than 90°.

Clarification. All solutions and the solvent were clarified from dust particles by nitrogen pressure filtration through Schott sintered G4 and G5 glass filters. The cells used were cleaned by the washing in butanone vapour in an apparatus described by Thurmond.[30]

Concentration determinations. Concentrations were determined after the intensity measurements by dry weight analysis (110° C), the weight of sodium chloride being subtracted from total weight.

Refractive index increments. To obtain the refractive index increment of the non-dialyzable portion of Ammodytes viper venom, the difference in refractive index between solution and solvent (0.15 *M* NaCl) was measured at 25° C in a Haber-Löwe interferometer manufactured by Zeiss.[31] The solutions approximately 0.03% to 0.07% in concentration were made and the readings converted to refractive index differences by the well-known equation:

$$n - n_0 = (\lambda / d))(\Delta N) \tag{1}$$

when n is the refractive index of a solution, n_0 of solvent, λ the wave lenght of light used (436 mμ), d thickness of liquid through which the light passes (2.00 cm.), and (ΔN) the number of fringes. The procedure applied in working with monochromatic light is described in ref. 31, p. 1231. The values of $n—n_0$ for aqueous solutions of sodium chloride were in close agreement with the values listed by Kruis.[32] The value of refractive index increment for the Ammodytes viper venom amounted to 0.194 ml./g. This value falls within the range found for other proteins (ref. 27, p. 144).

The value of refractive index increment for the antivenin was assumed to be 0.196 ml./g., *i. e.* the same as Heide[33] has measured for γ-globulin.

Owing to small difference between the refractive index increments of venom and antivenin, it is sufficient to set the mean value (0.195 ml./g.) as characteristic for their aggregates.

RESULTS

Treatment of light scattering data

The weight-average molecular weights, M_w, were calculated from the equation (cf., e. g., ref. 27):

$$M_w = [k\,(n_w/n_b)^2]/[(c/I_{90})_0 P(90)K] \tag{2}$$

where

$$K = 2\pi^2\, n^2_0\, [(n—n_0)/c]^2/N\lambda^4 \tag{3}$$

(for unpolarized incident light). In these equations the symbols have the following meanings and values:

$k = 33.1\times10^{-9}$ cm.$^{-1}$ (calibration constant; Rayleigh ratio of benzene for vertical component of scattered light multiplied by 10^{-3});

$(n_w/n_b)^2 = 0.77$ (refractive index correction factor, n_w and n_b being the refractive indices of 0.15 *M* NaCl and benzene, respectively);

c, concentration of macromolecular material (non-dialyzable portion) in g./ml.;

I_{90}, intensity of light scattered at 90° angle, corrected for the scattering of solvent (in instrument scale units);

$P(90)$, particle scattering factor, correcting the observed intensity at 90° for the loss due to internal interference;

n_0, refractive index of the solvent;

n, refractive index of the solution at the concentration c;
N, Avogadro's number;
$\lambda = 4.36 \times 10^{-5}$ cm. (wavelength of light in vacuum).

The data were treated by plotting c/I_{90} against c (Fig. 1 and 2), and the intercepts at zero concentration, $(c/I_{90})_0$, were deduced by linear extrapolation. By inserting the value of $(c/I_{90})_0$ in eq. (2), the molecular weight can be com-

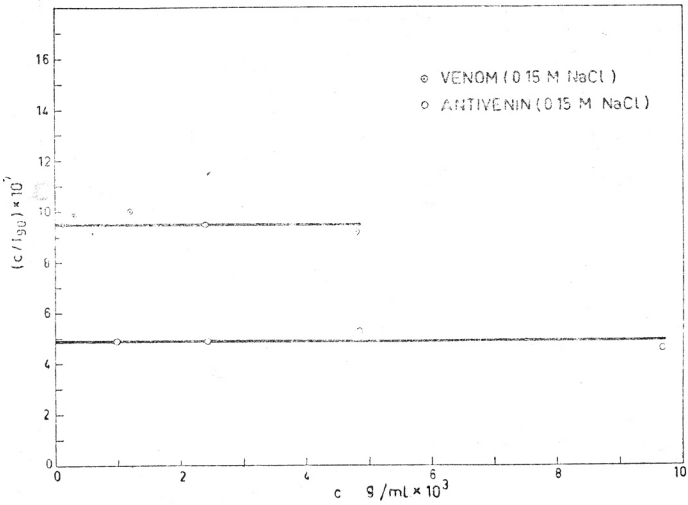

Fig. 1. Reduced intensity of scattered light at 90° versus concentration of venom and antivenin.

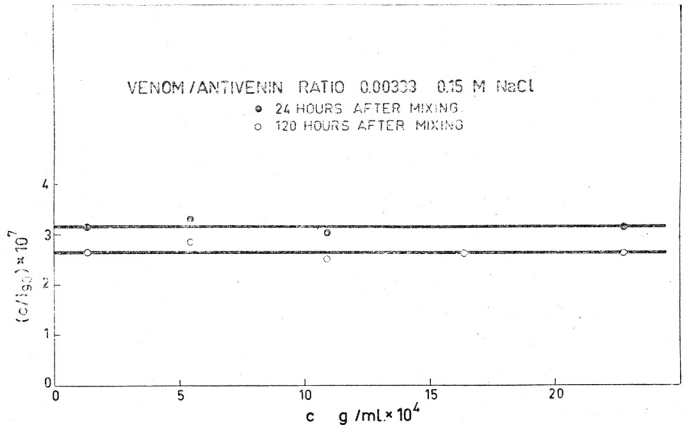

Fig. 2. Reduced intensity of scattered light at 90° versus concentration of venom-antivenin aggregates 24 and 120 hours after mixing. Antivenin excess zone.

puted if the particle scattering factor, $P(90)$, is known. It is usually evaluated from the intrinsic dissymmetry, $[z]$, making some assumptions about the shape of the particles.

In the case of venom and antivenin the dissymmetries ($z = I_{45}/I_{135}$) at all concentration were less than 1.10 (usually better than 1.05) and the particle scattering factor, $P(90)$, can be set equal to unity. However, for the venom-antivenin aggregates the particle scattering factor must be taken into account. From the instrinsic dissymmetry, [z], and by the use of tables and graphs of Doty and Steiner,[34] the corresponding value of $P(90)$ was determined assuming both a spherical and a rodlike model for the shape of aggregates.[9]

In one case (venom-antivenin ratio 0.01667) the molecular weight has seemed greater than 10^6. Consequently, the angular measurements of light scattering covering the range from 45° to 110° were performed at four concentrations. The values of c/I_θ were extrapolated to zero concentration and zero angle by the method of Zimm.[21] Since the particle scattering factor, $P(\Theta)$, is unity for $\Theta = 0$, the insertion of $(c/I_0)_0$ in eq. (3) gives the molecular weight without making assumptions about the shape of the particles.

In Fig. 3 the limiting line of c/I_θ at zero concentration is plotted against $\sin^2(\Theta/2)$ for the venom-antivenin aggregates at the ratio 0.01667.

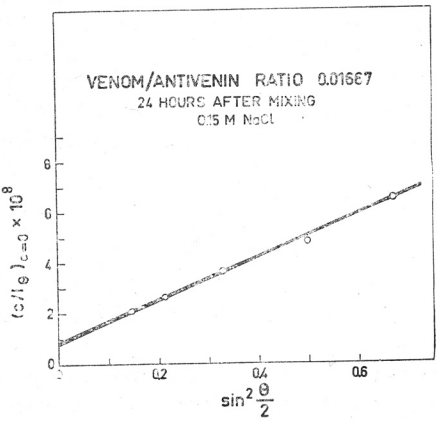

Fig. 3. Reduced intensity of scattered light for zero concentration at various angles of observation. Venom-antivenin ratio nearly at the neutralization point.

Molecular weight data

According to our measurements the weight-average molecular weights of the venom, antivenin, and their aggregates have the following values (in 0.15 *M* NaCl as solvent):

venom, $M_w = 43{,}600$;
antivenin, $M_w = 83{,}000$;
venom-antivenin aggregates,
venom/antivenin 0.00333 (antivenin excess zone),
24 hours after mixing, $M_w = 202{,}000$ (spheres),
$M_w = 228{,}000$ (rods);
120 hours after mixing, $M_w = 254{,}000$ (spheres),
$M_w = 298{,}000$ (rods);
venom/antivenin 0.01667 (nearly at the neutralization point),
24 hours after mixing, $M_w = 4{,}8000{,}000$.

The preliminary values of the molecular weights noted previously[19] were about 35% lower mainly because of the neglect of the refractive correction and a systematic error in numerical calculations.

DISCUSSION

We are fully aware of the fact that animal venoms represent a complicated mixture of various biologically active substances different in their biochemical and physicochemical properties. However, many of their properties have to be ascribed to the proteins present. For this reason, it seemed to us that the investigation of macromolecular composition, structure and properties of the venom might be warranted. The determination of the molecular weight of the whole venom (non-dialyzable portion) is the first step toward this aim.

Micheel and Jung[35] have found that the venoms of Viperidae do not pass through the cellophane membranes. According to our unpublished experiments, dialyzed Ammodytes viper venom shows all the immunological properties of the undialyzed venom, as revealed through the precipitation reaction with the corresponding antivenin under various experimental conditions (venom-antivenin ratio, *p*H, ionic strength, time). This fact indicates that the essential, particularly macromolecular, composition of the venom is maintained in the course of dialysis. Nevertheless, if one wishes to evaluate the complete macromolecular composition and structure of the Ammodytes viper venom, it would be necessary to fractionate the venom either according to a fractionating scheme described earlier[15] or by the preparative continuous paper electrophoresis. However, a first step in the fractionation is at hand: by the dialysis of the venom against solutions of low ionic strength (or against water itself) two fractions separated, one soluble at low ionic strength, and the other (about 14% of the total venom) insoluble. The determination of the molecular weights of various fractions of the Ammodytes viper venom is contemplated in the near future.

It may be of interest to compare the molecular weight of the venom investigated with the few data for other snake venoms. Gralen and Svedberg[36] found from diffusion and sedimentation measurements that the molecular weight of crotoxin (*Crotalus terrificus* neurotoxin) is 30,000, and De[37] found from diffusion constant a value of 33,200 for cobra haemolysin.

Since in the course of the preparation of Ammodytes viper antivenin a pepsin digestion was applied, a smaller molecular weight than that for normal γ-globulin had to be expected. Several authors[3,38,39] have also found that the pepsin or trypsin digested diphtheria antitoxin has much smaller molecular weight (90,000 to 100,000) than nontreated antitoxin (160,000).

As regards the size of the venom-antivenin aggregates, it may be noted that the molecular weights obtained are in the range covered by other antigen-antibody systems.[8,9,10] Our data must be considered only as provisional, because this point of investigation leaves much to be desired. It would be necessary to study the combination of the venom and antivenin, by the macromolecular physical techniques, in its dependence upon the various parameters such as venom-antivenin ratio, type and amount of added electrolyte, *p*H, temperature, concentration of hydrogen-bond breaking agents (*e. g.* urea) to obtain better insight into the mechanism of this reaction. A fuller

discussion of this point does not seem warranted by the limited experimental material now presented.

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REFERENCES

1. W. E. van Heyningen, in: *The Proteins,* edited by H. Neurath and K. Bailey, II, A. New York 1954, pp. 345—387.
2. P. Boquet, *Venins de serpents et antivenins,* Paris 1948.
3. P. Johnson and R. H. Ottewill, *Discussions Faraday Society* **18** (1954) 327.
4. D. Gitlin and H. Edelhoch, *J. Immunolog.* **66** (1951) 67.
5. R. J. Goldberg and D. H. Campbell, *J. Immunolog.* **66** (1951) 79.
6. J. Oncley, E. Ellenbogen, D. Gitlin and F. Gurd, *J. Phys. Chem.* **56** (1952) 85.
7. S. J. Singer and D. H. Campbell, *J. Am. Chem. Soc.* **74** (1952) 1794; **75** (1953) 5577; **77** (1955) 3499, 3504, 4851, 4855.
8. E. J. Becker, *J. Immunolog.* **70** (1953) 372.
9. R. F. Steiner, *Arch. Biochem. and Biophys.* **55** (1955) 235.
10. A. Winkler, Š. Hupka, C. Oravec and I. Winklerova, *Collection Czechoslow. Chem. Commun.* **21** (1956) 84.
11. S. I. Epstein, P. Doty and W. X. Boyd, *J. Am. Chem. Soc.* **78** (1956) 3306.
12. N. Muić and M. Piantanida, *Radovi jugoslav. akad. znanosti i umjetnosti* **298** (1953) 207; *Bull. intern. acad. yougoslave sci. et beaux-arts, Classe sci. math. et nat.* N. S. **9** (1953) 159.
13. M. Piantanida and N. Muić, *Arch. Biochem. and Biophys.* 46 (1953) 110.
14. N. Muić and M. Piantanida, *J. Immunolog.* **73** (1954) 115.
15. N. Muić and M. Piantanida, *Hoppe-Seyler's Z. physiol. Chem.* **299** (1955) 6.
16. N. Muić and A. Meniga, *Arhiv kem.* **27** (1955) 131.
17. N. Muić, *Bull. sci. Conceil acad. RFP Yougoslavie* **2** (1956) 105.
18. N. Muić and Dj. Ajduković, *Arhiv hig. rada* **8** (1957) 89.
19. J. Kratohvil, Dj. Ajduković and N. Muić, *J. Polymer Sci.* **30** (1958) 155.
20. A. J. Harms, *Biochem. J.* **42** (1948) 390.
21. B. H. Zimm, *J. Chem. Phys.* **16** (1948) 1099.
22. F. Krašovec, N. Vene and A. Peterlin, *Reports J. Stefan Inst.* **4** (1957) 165.
23. J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, *J. Am. Chem. Soc.* **72** (1950) 4641.
24. C. I. Carr and B. H. Zimm, *J. Chem. Phys.* **18** (1950) 1616.
25. B. A. Brice, M. Halwer and R. Speiser, *J. Opt. Soc. Am.* **40** (1950) 768.
26. A. Oth, J. Oth and V. Desreux, *J. Polymer Sci.* **10** (1953) 551.
27. K. A. Stacey, *Light Scattering in Physical Chemistry.* London 1956.
28. J. J. Hermans and S. Levinson, *J. Opt. Soc. Am.* **41** (1951) 460.
29. D. J. Meier, *J. Chem. Phys.* **21** (1953) 1892.
30. C. D. Thurmond, *J. Polymer Sci.* **8** (1952) 607.
31. N. Bauer and K. Fajans in: *Physical Methods of Organic Chemistry,* edited by A. Weissberger, 2. ed., II. New York 1949.
32. A. Kruis, *Z. physik. Chem.* **B34** (1936) 13.
33. K. Heide, *Kolloid-Z.* **146** (1956) 52.
34. P. Doty and R. F. Steiner, *J. Chem. Phys.* **18** (1950) 1211.
35. F. Micheel and F. Jung, *Z. physiol. Chem.* **239** (1936) 217.
36. N. Gralen and Th. Svedberg, *Biochem. J.* **32** (1938) 1375.
37. S. S. De, *J. Indian Chem. Soc.* **22** (1945) 10.
38. J. H. Northrop, *J. Gen. Physiol.* **25** (1941-2) 465.
39. A. Rothen, *J. Gen. Physiol.* **25** (1941-2) 487.

IZVOD

Određivanje molekularne težine otrova *Viperae Ammodytes*, pripadnog protuotrova i njihovih agregata mjerenjem rasipanja svijetla

J. Kratohvil i Dj. Ajduković

U okviru studija fizičko-kemijskih, biokemijskih i imunoloških svojstava toksičkih proteina i pripadnih antitoksina primijenjena je metoda mjerenja rasipanja svijetla za određivanje molekularnih težina otrova *Viperae Ammodytes* (poskoka) i pripadnog protuotrova. Kao otapalo upotrebljena je 0,15 *M* otopina natrijeva klorida, a otrov i protuotrov bili su podvrgnuti višednevnoj dijalizi. Nađeno je, da molekularna težina (težinski prosjek) otrova iznosi 43 600, a protuotrova (digeriranog pepsinom) 83 000. Određena je i veličina nekih agregata, koji nastaju reakcijom otrova i protuotrova. Molekularne težine agregata ovise o vremenu reakcije i o omjeru otrov/protuotrov, a nalaze se u granicama od 202 000 do 4 800 000.

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ZAGREB

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