Phosphatidase *A* Activity of Ammodytes Viper Venom

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The venom of *Vipera Ammodytes* was resolved by electrophoresis in seven proteinic components. These components were tested for phosphatidase *A* activity. The reliability of the method for haemolysis activity determination is discussed.

INTRODUCTION

It was discovered by Slotta and Fraenkel-Conrat[1] that phosphatidase *A* (crotoxin) could be prepared in crystalline form from *Crotalus t. terrificus* venom. The activity of phosphatidase *A* in biological excreta is usually tested by its haemolytic activity against horse red blood cells in isotonic buffered suspension. Following the principles of Delezenne and Fourneau[2] many investigators have used this method for characterization of various animal venoms modifying simmultaneously the experimental conditions of haemolytic process. Ghosh and De[3] expressed the haemolysin content of various fractions of Cobra venom in arbitrary units taking the haemolytic potency of one gram Cobra venom as a standard; the guinea pig erythrocytes were used as substrat, and complete haemolysis as analytical principle. Slotta and Szyska[4] using horse erythrocytes for haemolysis test recommended a similar method. These methods could not be considered as quite reliable. The use of complete lysis as an analytical endpoint does not afford as sensitive an index of haemolytic activity as an endpoint in the region of partial lysis. We proposed, therefore, in a previous communication[5] a method utilizing colorimetric estimation of the degree of partial haemolysis produced by venom enzymes. Similar methods were used by v. Euler and Swen Gard[6], Paić and Chorokoff[7], Deutsch[8], Mayer, Eaton and Heidelberger[9] for complement fixation tests in immunology.

In this communication the results of our investigations of electrophoreticaly separated *Vipera Ammodytes* proteins are presented and some characteristics of the method for phosphatidase *A* determination are discussed.

MATERIALS AND METHODS

The toxin of *Vipera Ammodytes* was obtained from living snakes, frozen, desiccated and preserved in ampullae. The DLM *(dosis letalis minima)* of each sample was tested by intravenous injection of serial dilution into mice. As dosis letalis minima was considered such an amount of venom that killed mice within 8—24 hours[10]. DLM of venom we used was approximately 18 γ for a mouse weighing 20 gr.

The electrophoresis apparatus used in the present work was that developed by Tiselius (1937) with slight modifications proposed by Wiedemann[11]. We used an optical system due to Philpot and Swensson with the cylindrical lens and the diagonal knife edge. Prior to electrophoretic analysis, each sample of venom

was dialysed 48 hours at 4° against two portions of the buffer. Immediately before electrophoresis, toxin solutions were diluted with the buffer to a protein concentration of 3% (w/v). Phosphate buffer pH 6.2 proposed by Polson, Joubert and Haig[12] (0.0224 M KH_2PO_4, 0,046 M Na_2HPO_4 and 0.44 M NaCl), silver chloride electrodes covered with saturated KCl solution, and a long analytical single cell 2×24 mm. of a 14 ml. capacity were used. The duration of electrophoresis varied from 2 to 4 hours. The experiments were discontinued when resolution of the fraction was satisfactory, $t = 2^9$, $C = 3\%$ (w/v), $I = 12.3$ mA, $U = 198$ V, $E = 5.0$ V/cm, $\varphi = 45°$.

Zone electrophoresis was carried out with an equipment constructed according to Durrum[13], Flynn and de Mayo[14], Tiselius and Per Flodin[15]. Phosphate buffer pH 7.2, ionic strength 0.1; Whatman No. 1 paper strip 3—5/30 cm., 6.5 V/cm, 2mA, 4 hr. Veronal-veronal sodium buffer pH 8.6, ionic strength = 0,05, Whatman No. 1, 5 V/cm., 0.75 mA, 2 mg. toxin, 20 hours. After electrophoresis, the paper strips were dried 10 minutes at 105°, and stained with 0.03% tetrabromophenolblue in 1% aqueous mercury chloride, and dried again. The electrophorograms were made transparent by treatment with paraffin oil and Cedax (15% Behringwerke). Thereafter each millimeter of the paper strip was measured with Fisher's electrophotometer by means of a special attachment using a 525 B green filter.

For phosphatidase A determination we have always used a 2% suspension of fresh and with 0.85% sodium chloride solution washed sheep erythrocytes in a weak hypertonic sodium shloride solution buffered to pH 7.2. Haemolytic system: 78 ml. 0,85% NaCl, 10 ml. phosphate buffer 1/15 M, pH 7.2, 2 ml. erythrocytes, 10 ml. 0.05% lecithin (Pangborn[16]). To a series of tubes each containing 3 ml. of that mixture, 0.2 ml. of haemolysin effluents were added, obtained by extraction of electrophorogram cuttings (each 5 mm. wide) in 0.5 ml. 10% sodium chloride. After standing in an incubator at 37° for 2 hours or at room temperature for 24 hours, all tubes were simultaneously centrifuged, clear haemoglobin supernatant with distilled water (1:10) diluted and the optical density of samples measured with Fisher's electrophotometer (filter 425 B). Control tubes without test substance showed no haemolysis.

RESULTS AND DISCUSSION

We separaed Ammodytes viper venom by means of free electrophoresis in the phosphate buffer at pH 6.2 in several protein components. At least seven components could be well distinguished. It is possible that the components at

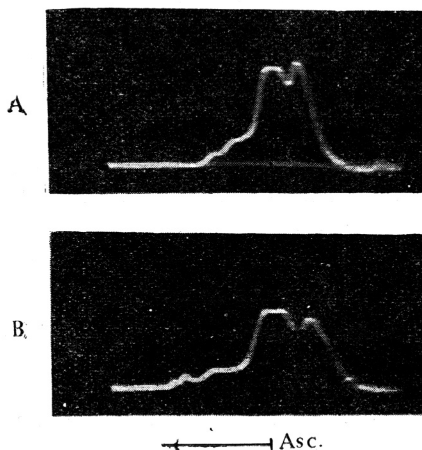

Fig. 1. Electrophoretic patterns of Ammodytes viper venom. The initial boundary positions are indicated by vertical lines and direction of migration by the arrow. Ascending limbs. Phosphate buffer pH 6.2 (Polson[13]), $I = 12.3$ mA, $U = 198$ V, $E = 5$ V/cm, $C = 3\%$ (w/v). Duration of electrophoresis $A = 120$ min., $B = 180$ min.

the starting point were associated with salt boundary (Fig. 1). These results are in good agreement with those obtained with zone electrophoresis. We have already published[5, 17] these results and they have been later on confirmed by other authors[18]. Similar resolution of venom proteins could not be obtained in acidic glycine buffer pH 3 or veronal buffer pH 8.6. We did not succeed

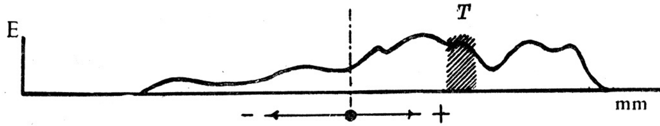

Fig. 2. — Paper strip electrophorogram of Vipera Ammodytes venom. *T* position of phosphatidase *A*. Veronal-veronal sodium buffer pH 8.6, $\mu = 0.05$, Whatman No. 1., $E = 5$ V/cm, $I = 0.75$ mA, 2 mg. of toxin, 20 hrs. Haemolysis of 2% sheep erythrocytes suspension, 2 hrs., 37°.

in improving the resolution of the components by increasing the ionic strength of the buffers used, as proposed by Gonçalves and Vieira[19]. Polson, Joubert and Haig[12] achieved the resolution of proteinic components by free electrophoresis in a series of various snake venoms. They claimed that haemolytic activity in all venoms they investigated was always associated with one proteinic component only, having a definite electrophoretic mobility. In all their haemolysis tests the readings were taken at the end of 20 minutes. It is a very significant fact, that these authors observed complete haemolysis in all haemolytic mixtures, when readings were taken after standing overnight. On the contrary, Grassmann and Hannig[18] have recently demonstrated, that

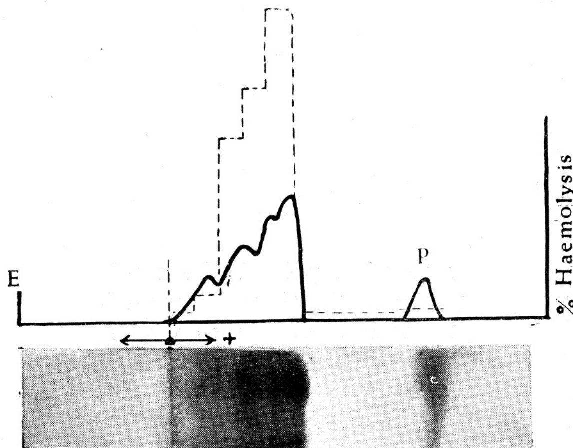

Fig. 3. — Paper strip and electrophorogram of Vipera Ammodytes venom with superposed haemolysis diagram. Phosphate buffer pH 7.2, 0.05 *M*, Whatman No. 1., $I = 2$ mA, $E = 6.5$ V/cm, 2 mg. of toxin, 4 hrs. Haemolysis of 2% sheep erythrocytes suspension 24 hrs. at 20°.

phosphatidase *A* activity can be frequently associated with two electrophoretically separated venom components. Since with boundary electrophoresis only the upper component can be thoroughly separated in one run in each limb of the cell, we preferred zone electrophoresis for our phosphatidase *A* activity investigation. The venom (2 mg.) dissolved in barbiturate buffer at pH 8.6 (0.02 ml.) was applied as a narrow line in the middle of the paper strip. After

electrophoresis the paper strip was cut along into two halves. One half was stained with tetrabromophenolblue and employed for the examination of the electrophorogram. The other half was clipped in 5 mm. narrow cuttings perpendicularly to the direction of the electrophoretic separation. The paper cuttings were extracted with 10% sodium chloride solution for 2 hours on a shaking machine. We experienced[5] that the venom proteins could be only partially eluted from the paper with 1% sodium chloride solution. The effluents were made isotonic thereafter by dilution with water and used for haemolysis tests. Fig. 2 represents the electrophorogram of Ammodytes viper venom with marked position of maximal haemolytic activity. Thus it seams that the maximal phosphatidase *A* activity is associated with component T. Even after 24 hours at room temperature no haemolysis ocurrs if no lecithin is added. Since the lecithin suspension alone does not produce haemolysis, we can assume that the Ammodytes viper venom does not contain any factor producing haemolysis directly.

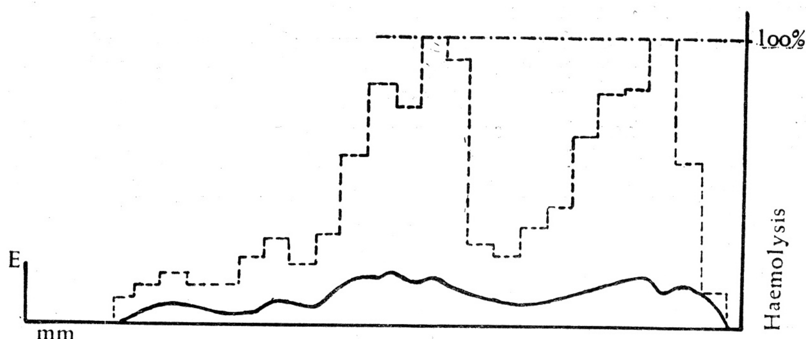

Fig. 4. — Paper strip electrophorogram of Vipera Ammodytes venom with superposed haemolysis diagram. Veronal-veronal sodium buffer pH 8.6, $\mu = 0.05$, Whatman No. 1, $I = 0.75$ mA, $E = 5$ V/cm, 2 mg. of toxin 20 hrs. Haemolysis of 2% sheep erythrocytes suspension 24 hrs. at 20°.

In phosphate buffer solution pH 7.2 the resolution of venom proteins was not as good as on paper electrophorograms in barbiturate buffer pH 8.6 However, another component *P* (Fig. 3) was well separated. This substance could be well stained with bromophenolblue, but badly fixed on paper by aqueous mercury chloride solution. It does not produce haemolysis of sheep erythrocytes even after 24 hous at 20°. The component *P* is very probably identical with the substance we found in the filtrates of aqueous venom solutions after the adsorption of other venom proteins on shredded filter paper column.

In order to elucidat the statement given by Polson, Joubert and Haig[12], that all components produced haemolytic effect if readings of haemolysis tests were taken after overnight standing, we measured the degree of haemolysis electrophotometrically (Fig. 4).

According to the results of our investigations it seems that haemolytic activity of electrophoretically separated venom proteins is in a certain way determined by the amount of proteins on the electrophorogram.

In our previous investigations we found that Ammodytes viper venom contained among other proteins at least two proteinic components showing very weak or no haemolytic activity. One component can be isolated from crude venom solution by repeated precipitation at its isoelectric point (pH 5.2)[17]. Another component with chemical properties different from that mentioned before was was separated by chromatography on shredded filter paper column[5].

Since the haemolysis test is an analytical method of great sensitivity it is probable that minute quantities (~ 2 γ/ml.) of phosphatidase *A*, adsorbed on other venom proteins, produce a significant haemolysis in reaction mixtures after a certain time. A very good separation of component *P* (Fig. 3) from other venom proteins and phosphatidase *A* gives evidence on the possibility of a perfect electrophoretical resolution of venom proteins, without any pulling of haemolysin with buffer solution during the electrophoresis. Finally, we think, that the determination of phosphatidase *A* activity by a prolonged haemolysis test (10—24 hours) should be considered as misleading.

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IZVOD

Aktivitet fosfatidaze *A* **u otrovu** *Viperae ammodytes*

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1. Elektroforezom otrova *Viperae ammodytes,* otopljenog u fosfatnom puferu pH 6.2, dokazano je, da se otrov sastoji od najmanje sedam proteinskih komponenata.

2. Upotrebom papirne elektroforeze ustanovili smo, da je fosfatidaza *A* vezana u toksinu uz jednu proteinsku komponentu *(T).*

3. Elektroforezom na papiru odvojili smo još jednu novu proteinsku komponentu *(P),* koja ne pokazuje nikakva hemolitičkog aktiviteta.

4. Priopćena je rasprava o vrijednosti metodike za određivanje aktiviteta fosfatidaze *A.*

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