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Purification of cobra venom factor from phospholipase A contaminant

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Summary. It has been demonstrated that cobra venom factor prepared by the usual combination of ion exchange chromatography and sephadex gel filtration is contaminated by substantial amounts of a 'heavy' phospholipase A. The two activities may be separated by isoelectric focusing. Cobra venom factor focuses at pH between 5·75 and 6·75 whereas the phospholipase is all found at pH below 5·75. In certain test systems, particularly *in vitro*, and particularly where albumin concentrations are low, the contaminating phospholipase may produce effects that have been attributed to complement activation.

INTRODUCTION

Cobra venom activates complement and lyses erythrocytes in the presence of serum (Flexner & Noguchi, 1903). The factor responsible (cobra venom factor (CVF)) has been isolated (Nelson, 1966; Müller-Eberhard & Fjellstrom, 1971) and shown to combine with serum factor B in the presence of Mg^{2+} (Müller-Eberhard, 1967) and of factor D (Hunsicker, Ruddy & Austen, 1972) which cleaves the factor B in the complex. The resulting complex $\overline{\text{CVF-Bb}}$, splits C3 into its active fragments of C3a and C3b, and also splits C5, leading to the activation of the terminal complement sequence and passive lysis of erythrocytes (Shin, Gewurz & Synderman, 1968; Pickering, Wolfson, Good & Gewurz, 1969; Ballow & Cochrane, 1969; Götze & Müller-Eberhard, 1970). In all these respects cobra venom behaves like an analogue of C3b (Lachmann & Nicol, 1973) and it has recently been demonstrated that the cobra venom factor may indeed be cobra C3b (Alper & Balavitch, 1976).

We report here that cobra venom of the *Naja haje* and *Naja naja* contain a 'heavy' phospholipase A (mol. wt approximately 100,000 – 150,000) which is detectable in the CVF-containing fractions after successive chromatography on DEAE and Sephadex and requires electrofocusing for its separation. CVF brings about lysis of unsensitized erythrocytes in the presence of factor B and D and C5—9 but not in the presence of lecithin alone. Conversely, the heavy phospholipase A induces lysis of unsensitized erythrocytes in the presence of egg yolk—or serum phospholipids independent of the presence of complement.

MATERIALS AND METHODS

Cobra venom factor

Cobra venom was obtained from *Naje haje* (Miami Serpentarium, Florida) or from *Naja naja* (Sigma).

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Lecithin

Purified egg yolk lecithin was generously given by Dr Rex Dawson (Institute of Animal Physiology, Babraham, Cambridge). The lecithin stock solution (4 mg/ml) was diluted before use in CFT at 56° and mixed at this temperature until the hexane evaporated.

EA

Sheep erythrocytes were incubated for 15 min at 4° with rabbit IgM anti-sheep erythrocyte serum and washed three times in CFD (Lachmann, Hobart & Aston, 1973).

Buffers

Complement fixation diluent (CFD) (Mayer, 1961); PBS–EDTA, phosphate-buffered saline containing 0·01 M EDTA, pH 7·2.

Factor B and factor D

Factor B was purified by precipitation of human serum with 20 per cent Na_2SO_4, followed by CM-cellulose chromatography of the supernatant (Lachmann *et al.*, 1973). Factor D was obtained by filtration of normal human serum through Sephadex G-75 (Martin, Lachmann, Halbwachs & Hobart, 1976).

Polyacrylamide gel disc electrophoresis

Electrophoresis was performed in 2·5 ml, 5 per cent polyacrylamide gels (5 per cent w/v acrylamide: 0·9 per cent w/v *N*, *N'*-methylene bisacrylamide, 0·165 per cent w/v tetramethylethylenediamine), polymerized by the addition of 1·65 per cent w/v ammonium persulphate after degassing in plexiglass cylinders 7·0 mm (i.d.) × 9·0 cm 0·1 M Veronal buffer, pH 8·2–8·3 was used as both gel and electrophoresis buffer. Electrophoresis was carried out at 6 mA/tube (200 V) at 24° × 4 hr or until migration of the tracking dye to the anodal pole of the gels. Then gels were longitudinally cut and stained with 0·25 per cent Coomassie blue in 0·7 per cent acetic acid or sectioned horizontally with cuts 0·14 cm apart and eluted in 1·0 ml saline for assay.

Isoelectric focusing

Analytical isoelectric focusing. It was performed on a 15 × 24 cm thin layer plate of 5 per cent polyacrylamide gel, containing 1·5 per cent ampholine, by the method of Awdeh, Williamson & Askonas (1968), modified by addition of 0·2 M Taurine to the gel (Osterman, personal communication, Alper *et al.*, 1975). A convenient pH range was obtained by using: 60 per cent ampholine of pH range 5–7; 30 per cent ampholine of pH range 4–6; 10 per cent ampholine of pH range 3·5–10. Fifty-microlitre samples (2 mg/ml proteins in 0·2 M Taurine) were applied to filter paper strips close to the cathode. Focusing was allowed to proceed overnight at 4° to a final voltage gradient of 50 V/cm. Three samples were run to test CVF activity, phospholipase activity and proteins in parallel.

Gels for detecting CVF and phospholipase activity, described below, were poured directly onto the focusing gel. The third part of the plate was cut off and stained for proteins with Coomassie brilliant blue (Salaman & Williamson, 1971).

Preparative isoelectrofocusing (Vesterberg & Svenson, 1966) was carried out in an LKB 8100–1 column containing 110 ml of 2 per cent of ampholine in 0·2 M Taurine to avoid protein precipitation. The system was stabilized by a sucrose gradient from 5 to 20 per cent and the cobra venom factor sample (7 ml of 1·2 mg proteins/ml in 0·2 M Taurine) was added to the light gradient solution before filling the column. The ampholine composition was the same as for the analytical electrofocusing. The output voltage was adjusted to give a power through the column of about 3 W, and the whole system was kept at a temperature below 10°C.

After a 24 hr run, when the resistance was in excess of 10^6 ohms the column was emptied carefully and the eluted fractions were tested for CVF and phospholipase as described below. The pH was also measured immediately after elution.

Test for CVF activity

Titration of the anti-complementary activity. Serial dilutions of 0·1 ml of the CVF samples were incubated with 0·1 ml guinea-pig complement (diluted 1/50 in CFD) at 37° for 1 h. 0·1 ml 1 per cent EA were added and the extent of lysis was measured by haemoglobin release. One unit of CVF activity is defined as giving 50 per cent inhibition of the haemolytic activity of the guinea-pig complement.

Alternatively, in monitoring columns, the activity of fraction was expressed as percentage complement inhibition. In this case the undiluted fraction (0·05 ml) was added to 0·05 ml undiluted guinea pig serum and after incubation at 37° for 1 h, the haemolytic titre of the guinea-pig complement on EA was determined. The percentage complement inhibition is given as

$$100\left(1-\frac{\text{titre of CVF-treated complement}}{\text{titre of untreated complement}}\right).$$

Passive haemolysis of guinea-pig erythrocytes (complement-induced). Dilutions of venom fractions (0·1 ml) were mixed with 0·1 ml 1 per cent guinea-pig erythrocytes and 0·1 ml 1/20 normal human serum. The reaction mixture was incubated at 37° for 30 min and lysis measured by haemoglobin release.

Overlay assay on electrofocusing polyacrylamide gel
A first gel containing about 0·3 mg/ml of factor B and 0·037 mg/ml of semi-purified factor D in agarose and CFD was poured onto the electrofocusing plate. It was incubated for 1·5 h at 37° to allow the formation of the CVF–C3 convertase. A second gel was prepared containing normal human serum at a dilution of 1/20, 1 per cent GPE and agarose in PBS–EDTA. This gel detects the presence of pre-formed CVF-C3 convertase and it was poured onto the first gel. Diffusion was allowed to occur 2 h in the cold, then the plate was warmed at 37° until bands of lysis appeared.

Single radial diffusion plate assay
Each sample to be tested for CVF was mixed with an equal volume of normal human serum and incubated 15 minutes at 37°. 7 μl was then applied to a well in a plate layered with the CVF-C3 convertase detection gel described above. Diffusion was allowed at 4° overnight, then the plate was warmed for 2 hours at 37°, enlarged and the areas of the lysis measured by planimetry.

Test for phospholipase activity

Titration of the phospholipase activity by passive lysis of guinea-pig erythrocytes (lysolecithin-induced)
Serial dilutions of the sample to be tested were made in 0·1 ml CFD. 0·1 ml of lecithin (0·4 mg/ml solution in CFT) and 1 per cent guinea-pig erythrocytes (GPE) in 1·5 per cent bovine albumin, were added and incubated 10 min at 37°. One unit of phospholipase activity was defined as giving 50 per cent haemolysis of the GPE. Albumin was used to prevent background lysis due to traces of contaminating lysolecithin present in the lecithin solution.

Overlay assay on the electrofocusing plate
A gel containing 1 per cent GPE, 1·5 per cent bovine serum albumin, 0·1 mg/ml of lecithin, and agarose 1 per cent in CFD was poured on the focusing plate. Zone of lysis developed rapidly at room temperature.

Single radial diffusion plate assay
The samples to be tested for phospholipase A were diluted 1/3 in CFD were tested on a plate containing the phospholipase detection gel described above. The plate was left at room temperature for about 2 h and the area of lysis was measured.

Egg yolk-clearing activity
For monitoring phospholipase A in column fractions a further simple assay was employed. A suspension of whole egg yolk in saline was centrifuged to remove large particles and standardized to give an absorbence of 0·6–0·7 at an optical density of 925 nM. Three millilitres of this suspension was incubated with 0·1 ml venom fractions and the percentage clearing activity measured as:

$$100\left(1-\frac{\text{optical density of treated suspension}}{\text{optical density of control suspension}}\right).$$

Thin-layer chromatography
Silicic acid (DO without binder) was obtained from Camag (Muttenz, Switzerland). Chloroform and ether were redistilled; other organic solvents of analytical grade. Phospholipid solvent contained chloroform:methanol:water:acetic acid:65:18:3:1 (v/v/v/v).

Protein determination

This was done according to the Folin method (Lowry, Rosebrough, Farr & Randall, 1951), using bovine serum albumin as a standard. To remove the ampholine which interfered with the protein determination, the pooled fractions were dialysed first against 10 times concentrated PBS. They were then dialysed against normal PBS and concentrated by ultrafiltration using an Amicon PM10 membrane.

RESULTS

(1) Ion-exchange chromatography on DEAE–cellulose

5·25 g of *Naja haje* venom in 350 ml of 5 mM phosphate, pH 7·4 and 7 mM NaCl was applied to a 5 × 53·5 cm column and eluted with a linear salt gradient according to a method of Nelson (1966) at 4°. The elution profile is shown in Fig. 1. Fractions

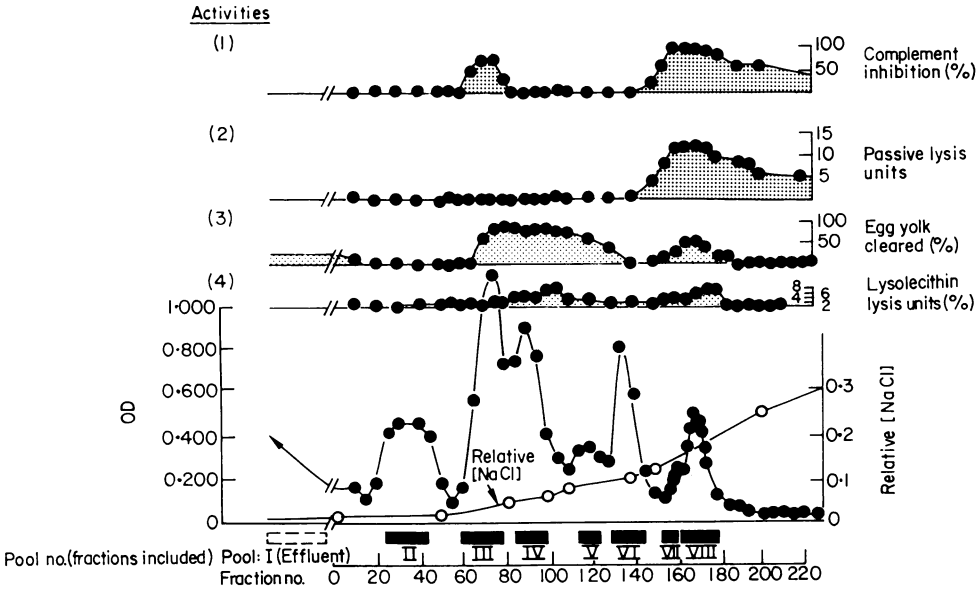

Figure 1. Analysis of DEAE–cellulose chromatogram of *Naja haje* venoms. Profile of DEAE–cellulose columns (5 × 53·5 cm) fractions (1–220) of crude venom (5·25 g), eluted with linear gradient in 0·005 M phosphate buffer with initial 1 M NaCl of 0·007, pH 7·4, at 4° at 90 ml/h, 15-ml fraction. Protein concentration was estimated by OD at 280 nm. Other techniques are described in the Materials and Methods section. (1) Anticomplementary, (2) complement-lysis inducing, (3) egg-yolk clearing, (4) lysolipid lysis-inducing.

Table 1. Correlation of activities of pools I–VIII of fractions of *Naja haje* venom

Pool	Rel. (NaCl) at elution (M)	Protein (mg/ml)	Volume (ml)	Times concentrated	Total protein (mg)	Total protein (%)	Anti-complementary activity*	Complement lysis inducing activity*	Egg-yolk clearing activity†	Lysolecithin lysis inducing activity*
I	0·007	48·0	75	20	3600·0	88·5	2	TR§	290	729
II	0·013	0·2	14	18	2·8	0·1	2	TR	169	150
III	0·035	6·5	30	7·5	195·0	4·8	64	TR	430	7000
IV	0·050	4·9	20	10	98·0	2·4	8	TR	1050	700
V	0·085	1·1	35	4	38·5	0·9	TR‡	TR	170	100
VI	0·110	3·0	20	10	60·0	1·5	4	TR	140	100
VII	0·155	1·2	13	6·5	15·6	0·4	128	1024	49	30
VIII	0·175	2·8	21	11	58·8	1·5	256	2048	140	30

* See Materials and Methods section.
† Dilution of venom fractions in 0·3 ml CFD that clears 1·0 ml egg yolk suspension after incubation at 37° for 1 h.
‡ Trace titre of < 3.
§ Trace of < 5.

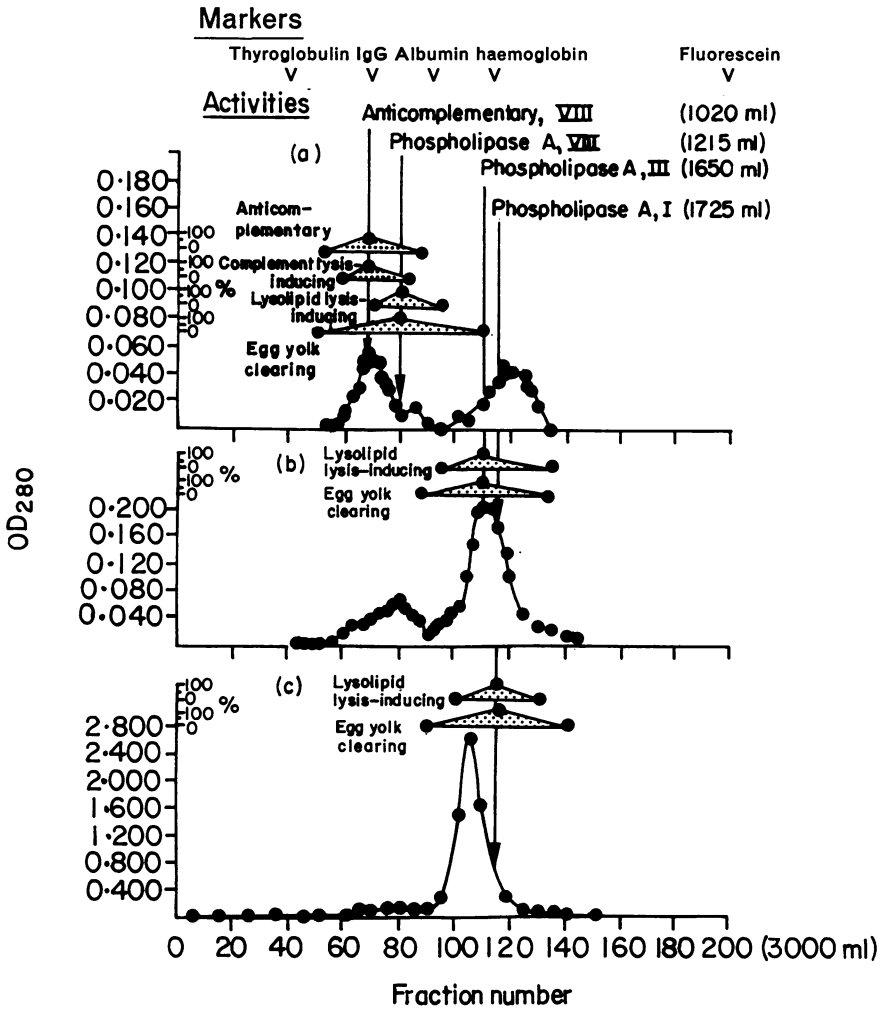

Figure 2. G-200 Sephadex gel filtration of DEAE pools of cobra venom (as Fig. 1). Columns C-II, III and IV: G200 Sephadex, 5 × 80 cm. (a) Column C-II, 29·5 mg. Pool VIII venom; (b) C-IV, 59·5 mg. Pool III venom; (c) C-III, 4850 mg. Pool I venom.

Table 2.

	Proteins mg/ml	CVF (u/ml)	Phospholipase (u/ml)	CVF/phospholipase activity ratio	CVF specific activity (u/mg protein)
Crude venom	12·2	8	50,000	0·0001	0·7
↓					
DEAE–CVF	6·2	48	32,000	0·0015	7·7
↓					
G-200–CVF	1·2	16	960	0·017	13
↓					
Electrofocusing					
Pool 1—CVF	1·38	32	1	32·0	23
Pool 2—Phospholipase	0·4	1	1280	0·0008	2·5
Heated CVF (30 min, 70°) [G-200 pool]	1·2	0	480	0	0

were pooled into eight pools as shown in Fig. 1, and in Table 1 are shown the activities of the eight pools. It can be seen that the bulk of the phospholipase activity is to be found in the excluded section and in those fractions eluted from the column by salt concentrations up to 50 mM. These fractions contain only a minor anti-complementary activity which is not associated with the ability to induce passive lysis of erythrocytes. The true anti-complementary cobra venom factor is eluted in pools 7 and 8 at salt concentrations between 150 and 200 mM. This is in accord with the published data. However, it can be clearly seen that this late eluting peak is still associated with quite considerable phospholipase activity, which can be detected both by egg-yolk clearing and by lysolecithin-mediated passive haemolysis.

(2) Gel filtration of DEAE-purified cobra venom factor on Sephadex G-200

The elution profile of Pool VIII is shown in Fig. 2. It can be seen that the anti-complementary activity appears in the same region as the IgG marker. However, this peak is still contaminated with (high molecular weight) phospholipase activity. For comparison a Sephadex G-200 fractionation of pools I and III from DEAE is shown. Here the lower mol. wt phospholipase is present in large quantity. The two activities in the cobra venom factor pool can be distinguished by their resistance to heating. Phospholipase A resists heating to 100° while CVF does not (Phillips, 1969). As can be seen in Table 2, CVF is destroyed by heating for 30 min at 70°, whereas the phospholipase A substantially resists this treatment.

(3) Polyacrylamide gel electrophoresis

DEAE-purified CVF (pool 8) was submitted to electrophoresis (Fig. 3). Phospholipase A activity (as measured by per cent egg yolk suspension clearance and lysis of sheep erythrocytes in egg yolk suspension) migrated in advance of the complement-consuming and passive lysis-inducing activity. Phospholipase A activity was present in both the faster bands.

(4) Analytical electrofocusing

CVF purified by DEAE–cellulose and Sephadex G-200 fractionation was subjected to analytical isoelectrofocusing. Several phospholipase haemolytic bands appear near the anode and very quickly it becomes impossible to distinguish discrete bands because the cells lyse on the whole anodal region. The CVF-induced haemolysis appear clearly in a different region. A broad zone of lysis is observed from the cathode to the middle of the plate (followed by another clearly distinct band at a lower pH). Protein staining shows a wide set of bands near the cathode, corresponding to the CVF activity region.

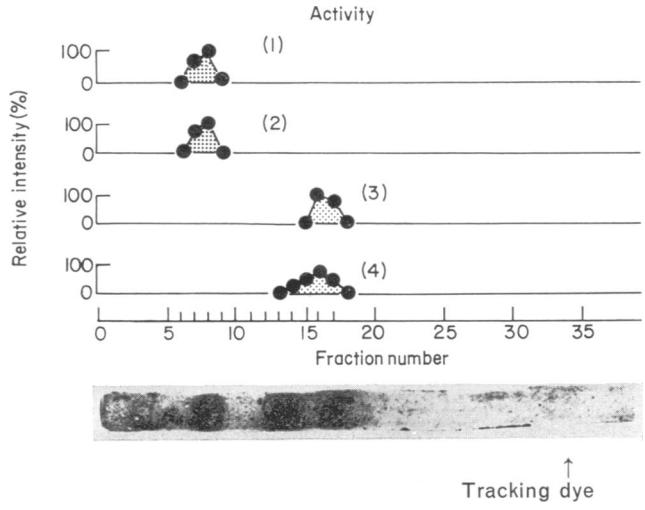

Figure 3. Polyacrylamide gel electrophoresis with DEAE purified cobra venom factor. Polyacrylamide gel, 102 × 7 mm, Pool VIII venom, 84 mg. (1) Anticomplementary, (2) complement lysis-inducing, (3) lysolecithin lysis-inducing, (4) egg-yolk clearing.

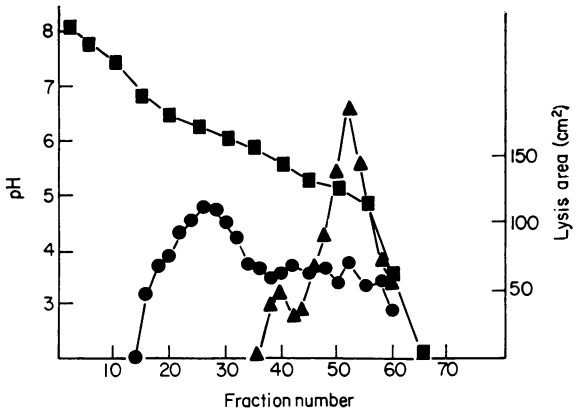

Figure 4. Elution profile of isoelectrofocusing column. CVF and phospholipase activities of the fractions are expressed as enlarged areas of lysis produced in the detecting gels, as described in the Materials and Methods section. (■——■) pH; (▲——▲) phospholipase activity; (●——●) CVF activity.

Another smaller set of protein bands focus at the other end, near the anode, corresponding to the phospholipase activity. Because of the diffusion, both from the focusing gel to the detection gel, and within the detecting gel, the haemolysis regions are much broader than the stained protein bands.

(5) Preparative isoelectric focusing

As shown on Fig. 4 during the electrofocusing, most of the cobra venom factor activity focus between pH 5·75 and 6·75, while the phospholipase activity is only found at pH below 5·75. The fractions between pH 5·75 and 6·75 do not show any phospholipase activity on the lecithin plate. The strong rings of lysis appearing on the complement-induced passive lysis plate, disappear completely if the samples are heated 30 min at 70° before reaction with normal human serum. The fractions of pH below 5·75 cause lysis in the lecithin plate and this activity is not affected by heating at 70°. On the complement plate, two sorts of ring of lysis are observed with these fractions: small rings of lysis appear at 4° and do not increase during incubation at 37°. Phospholipase, probably interacting with serum phospholipids, must be responsible for this haemolysis, since it is not affected by heating at 70°. These fractions also have some CVF activity, as shown by another larger ring of lysis, which appears only at 37° and disappears completely when the sample is heated 30 min at 70°. Thus, the preparative technique in column gives poorer resolution than the analytic technique. The CVF pool is free of phospholipase activity, but the phospholipase pool still contains traces of CVF. If the phospholipase pool is submitted to a second isoelectric focusing, some of the CVF activity focuses above pH 5·75 and can be isolated. But most of this CVF contaminant of phospholipase pool, still focus together with the phospholipase after two successive electrofocusing runs.

Table 2 gives quantitative results. Preparative electrofocusing increases the specific activity of the CVF by a little less than 2-fold but the ratio of CVF activity to phospholipase activity is increased 2000-fold.

DISCUSSION

The results presented in this paper show that, when most of the phospholipase activity is removed from CVF by ion-exchange and Sephadex chromatography, more sophisticated techniques are necessary to purify CVF from another, heavier phospholipase A.

We have shown that CVF could be obtained free of detectable phospholipase activity either by polyacrylamide gel disc electrophoresis or by using the isoelectric focusing technique, the latter being more convenient for preparative work. When purified cobra venom factor was submitted to electrofocusing, two protein peaks could be distinguished. One peak, of isoelectric point between pH 5·75 and 6·75, contained CVF and no phospholipase activity. The other peak had an isoelectric point lower than 5·75 and contained phospholipase and traces of CVF activity.

The residual phospholipase activity in usual CVF preparations, has been found to affect *in vitro* experiments where phospholipids were present in reactions involving CVF, complement and cells. For example, the passive haemolysis of guinea-pig erythrocytes in the presence of limulus serum, once thought to show a complement-like factor in this invertebrate haemolymph, we have been able to show to be caused by phospholipase A. A similar finding with lobster haemolymph has been reported by Hall, Rowlands & Nilsson (1972). The absence of albumin (which binds lysolecithin) in the haemolymph, greatly enhances the lysolecithin-induced lysis. Similarly, when CVF was used to study the alterna-

tive pathway of mouse complement activation, some lysophosphatide-induced erythrocyte lysis was observed, interfering with the complement-mediated haemolysis. This was found to a greater extent with mouse complement than with human, presumably because the mouse serum phospholipids contain more unsaturated fatty acid and thus give rise to more lytic lysophosphatides. The lysophosphatide induced lysis is not inhibited by heating the serum for 30 min at 56°, which distinguishes it from complement-induced passive lysis. However, EDTA inhibits phospholipase A as well as complement. Another example is the observation by Waldmann & Lachmann (1975), that mouse serum treated with CVF inhibited antibody production *in vitro* by mouse spleen cells. This was initially thought to be due to complement inactivation by CVF, but when it was shown that the same inhibition was observed both with mouse serum heated for 30 min at 56° and with CVF heated for 30 min at 70° and not seen with highly purified CVF, it became clear that the effect was thus due to the phospholipase A. It is probably the result of the generation of immunosuppressive, unsaturated fatty acids or of lysophosphatides containing such fatty acids (Mertin, Hughes & Stewart-Wynne, 1974).

It is therefore necessary to be aware that conventionally purified CVF shows quite substantial contamination with phospholipase A. Particularly in *in vitro* experiments and particularly where the albumin concentration in the medium is low effects may be attributed to complement activation which in fact are caused by lysophosphatides.

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