Table II. Specific activities of human arterial phosphatidyl choline palmitic, stearic and oleic acids after incubation with acetate-1-^{14}C, with and without estradiol[a]

	No estradiol	Estradiol
16:0	2,450 ± 260	4,300 ± 382
18:0	620 ± 103	560 ± 116
18:1	270 ± 54	225 ± 70

[a] DPM/mg fatty acid ± S.D.

[14] This investigation was supported by Grant No. 5ROI-HE-10172 from the National Heart Institute, U.S.P.H.S.

phate precursors into phosphatidyl choline. The determination of the exact stage of phosphatidyl choline synthesis which is affected by estradiol will require further study[14].

Zusammenfassung. Bei Bebrütung von Arterien in Gegenwart von Östradiol wurde der Einbau von ^{14}C-Azetat und ^{14}C-Cholin in Phosphatidylcholin deutlich vermehrt.

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Inhibition of Phospholipase A by a Naturally Occurring Peptide in *Bothrops* Venoms

Compared with other snake venoms, the activity of phospholipase A (EC 3.1.1.4.) in the venoms of *Bothrops* species (e.g. *B. neuwiedii* (Argentine and Brazilian), *B. jararaca, B. jararacussu, B. atrox*) is relatively weak[1]. Furthermore, with these venoms the enzyme activity shows a characteristic lag-period (15–18 min), which can be observed either with egg-yolk lipoprotein[1,2] or with pure sonicated phosphatidylcholine[3] as substrate. The results reported here show that both the apparent inactivity of the enzyme and the lag-effect are due to a peptide inhibitor, normally present in *Bothrops* venoms which, under well defined medium conditions, is highly effective on phospholipase A. It must be recalled that Habermann[4] had already postulated the role of a natural inhibitor as cause of the initially low activity of phospholipase A in the venom of *Crotalus d. terrificus*.

Venom enzyme and inhibitor can be separated by a fractionation procedure which, briefly, consists of the following steps: a) two gel-filtrations on Sephadex G-50 at pH 4.5; b) chromatography on SE-Sephadex C-25 at pH 4.5 with concave gradient elution and c) gel-filtration on Sephadex G-25 at pH 7.4. Figure 1 presents a typical elution pattern for Step 3. Peak F_e, eluted with the void volume, contained enzyme activity but did not show lag. The total phospholipase activity recovered in peak F_e was 275% of that measured in the original venom sample. The second, included peak F_i (K_{av}-value = 0.252) was enzymatically inactive but its addition to F_e provoked both a strong inhibition of the enzyme and a lag which resembled that observed[5] with the crude venom (Figure 1; upper-right corner). Further purification of F_e on DEAE-cellulose at pH 7.6, with linear or concave gradient elution (step 4 of phospholipase A purification) yielded 2 electrophoretically homogeneous fractions with specific activities 62- and 54-fold higher, respectively, than the crude venom. The pure phospholipase fractions were also strongly inhibited by F_i.

Purification of F_i by chromatography on SE-Sephadex at pH 4.5 (concave gradient elution), by paper electrophoresis or by paper chromatography yielded a ninhydrin-reacting material. F_i lost its inhibitory activity after treatment with trypsin or acid hydrolysis (10 N HCl). Paper chromatography of the hydrolysate yielded several amino acids, which is consistent with the peptide nature of F_i. After this work was completed a similar peptide inhibitor has been reported by Braganza et al.[6] in *Naja naja* venom (fam. Elapidae).

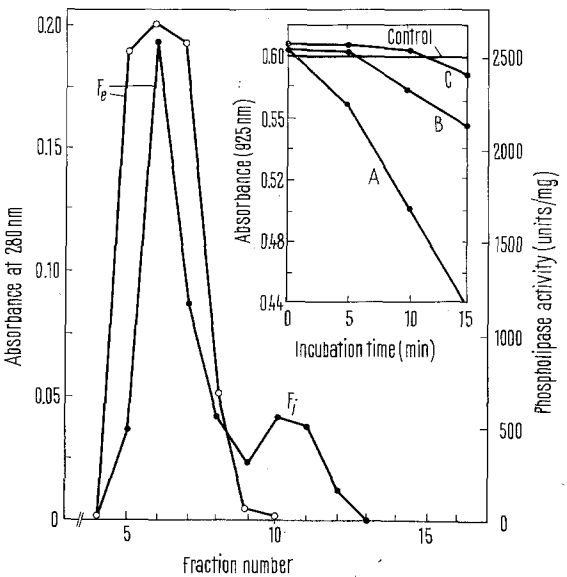

Fig. 1. Separation of phospholipase A and its inhibitor by gel-filtration on Sephadex G-25 (step 3 of the purification procedure). The column (20.5 × 1.8 cm; gel-bed volume, 53.5 ml) was equilibrated with 1 mM EDTA, 5 mM phosphate-*Tris* buffer pH 7.4, at 0–2 °C. The sample (2.2 ml, 248 m-units of A_{280} per ml) was applied to the column and eluted with the equilibration buffer. 2.7 ml fractions were collected at a flow rate of 2.3 ml/h. ●, absorbance at 280 nm; △, phospholipase specific activity measured with the turbidimetric method[1]. At the right, upper corner, inhibition of F_e by F_i. The curves represent the phospholipase activity of 0.14 mg of F_e preincubated 5 min at 0 °C with buffer (curve A) or with 0.56 (curve B) and 1.13 (curve C m-units A_{280} of F_i.

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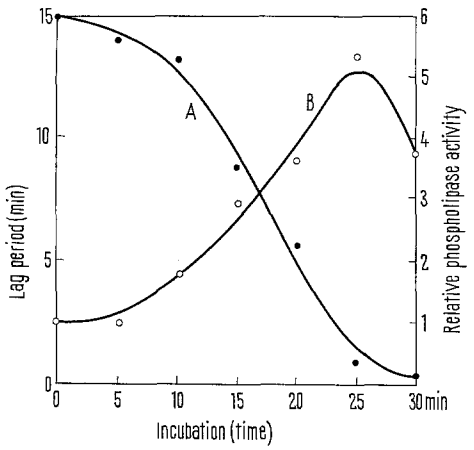

Fig. 2. Effect of the autolysis on the lag-period and phospholipase A activity of crude *B. neuwiedii diporus* venom. Crude, dried-venom was dissolved in 0.15 *M* NaCl at a final concentration of 1.0 mg/ml. The sample was adjusted to pH 6.0 and incubated at 15 °C for the time indicated in the abscissa. 0.2 ml aliquots were taken as indicated and tested for phospholipase activity with the turbidimetric method[1]. Curve A, duration of lag-period (left-ordinate) and curve B, relative phospholipase activity, which is expressed by the ratio between enzyme activity in autolyzed sample and that in the original (zero time) sample (right ordinate).

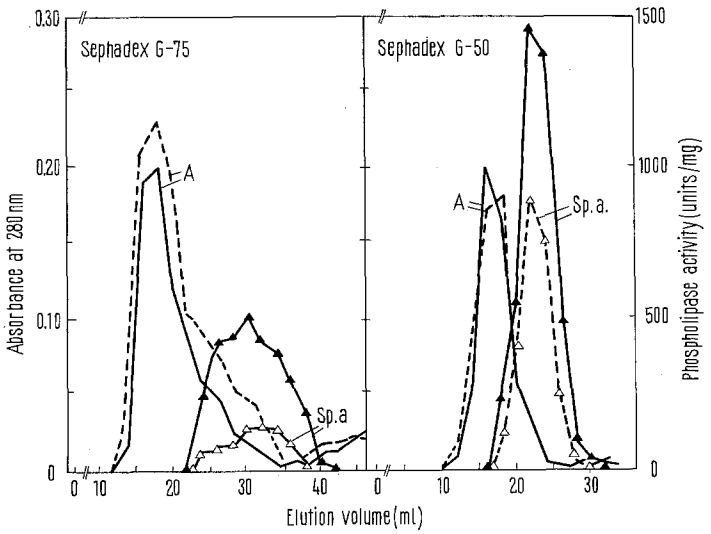

Fig. 3. Elution behavior of autolyzed (A) and crude (C) venom on Sephadex G-75 and G-50. Venom sample (A) was dissolved in 0.15 *M* NaCl adjusted to pH 4.5 and centrifuged for 10 min at 10,000 × *g*. The supernatant was adjusted to pH 6.0 and autolyzed at 15 °C to disappearance of the lag-period (about 25 min). Autolysis was then stopped by the addition of 1 m*M* EDTA, 0.1 *M* ammonium formate (final concentration, pH 4.5) and cooling at 0 °C. Venom sample (C) was directly dissolved in 0.15 *M* NaCl, 1 m*M* EDTA, 0.1 *M* ammonium formate buffer (pH 4.5) and centrifuged. Samples (A) and (C) were successively chromatographed on Sephadex G-75 (17.5 × 1.8 cm column) and G-50 (15 × 1.8 cm column) equilibrated respectively with 1 m*M* EDTA, 0.1 *M* ammonium formate buffer, pH 4.5. Elutions were performed at 0 °C with the equilibration buffer and 1.0 ml fractions were collected at a flow-rate of 3 ml/cm² per h. A, A_{280} of —— (A) and --- (C) venom samples. Sp.A., phospholipase activity of ▲ (A) and △ (C) samples, determined with the turbidimetric method[1].

Dissociation (association) of phospholipase fractions and F_i was strongly dependent on the pH of the medium. Thus, in steps 1 and 2 of the fractionation procedure (both at pH 4.5), F_i was eluted together with the enzymatically active fractions, in spite of the different molecular sizes of phospholipases and the inhibitor. On the other hand, in step 3 at pH 7.4, a clear separation occurred.

The presence of inhibitor F_i explains satisfactorily the kinetics of self-activation of phospholipase A in crude *B. neuwiedii* venom. In fact, upon autolysis of venom at pH 6.0, the lag effect decreased and simultaneously the apparent enzyme activity increased to an optimal value (Figure 2). Subsequent decrease of phospholipase activity can be attributed to the further action of venom proteases on the free enzyme. The effect of autolysis was prevented by inhibiting the venom proteases with EDTA or an acid pH (4.5). F_i could not be detected in the venom samples autolyzed to maximal activation while, on the other hand, the same degree of autolysis did not affect phospholipase fractions as evidenced by the enzymes chromatographic behavior on Sephadex G-50 and G-75 (Figure 3). Moreover, these latter fractions retained their sensitivity to the inhibitory activity of F_i.

In *B. neuwiedii* venom, both proteases and phospholipase inhibitor would constitute a self-activating physiological mechanism aimed to regulate in vivo the enzyme activity. Thus, in freshly emitted venom, the pH is about 4.0 (unpublished observation) and this would result in 2 consequences: a) the closer association of inhibitor and enzyme, and b) the inactivity of venom proteases[5,7]. When venom is injected into the victim's tissues (pH 7.3), the enzyme inhibitor inactive complex would dissociate and, in addition, the pH change would activate proteases. These latter would then hydrolyse F_i without altering the catalytic properties of phospholipase A as shown in Figure 3.

The possibility that in *Bothrops* venoms phospholipase A should exist as an inactive pre-enzyme, as occurs with the porcine-pancreas phospholipase A[8,9] is at variance with two important pieces of evidence, namely, a) maximal enzyme activation of protease-free fractions (step 2) was achieved after separation of the inhibitor by a simple physical method (Figure 1), b) venom fractions initially devoid of phospholipase activity were not converted to the active enzyme by autolysis (Figure 3)[10].

Zusammenfassung. Im Schlangengift von *Bothrops* befindet sich ein Peptid, welches als Phospholipase-Hemmer beschrieben wird.

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