33. INHIBITION OF ELECTRON TRANSPORT CHAIN BY PURIFIED PHOSPHOLIPASE A FROM *BOTHROPS NEUWIEDI* VENOM

J. C. VIDAL *, B. N. BADANO, A. O. M. STOPPANI and A. BOVERIS

Institute of Biochemistry, School of Medicine, University of Buenos Aires, Argentine

Introduction

It is known that snake venoms inhibit mitochondrial electron transfer systems (1-5). Badano and Stoppani (6, 7) studied the action of crude and heated venoms of several species of *Bothrops* on nonphosphorylating heart muscle extracts (Keilin and Hartree preparation) and found that the inhibitory power was proportional to the phospholipase A activity of those venoms. In this paper studies with purified phospholipase A (phosphatide acyl-hydrolase, E.C. 3.1.1.4) from *B. neuwiedi* venom are described.

Materials and methods

Enzyme preparations — Keilin & Hartree heart-muscle preparations were obtained from pig heart according to Slater (8). Protein concentration was measured with the biuret method (9).

Measurement of catalytic activity of heart-muscle preparation — These were carried out spectrophotometrically at 30° (6, 7, 10). Reaction mixtures (final vol. 3.0 ml) were made up as follows: a) $NADH_2$-oxidase: 0.25 mM $NADH_2$, 0.04 mM cytochrome *c*, 0.13 M phosphate buffer, pH 7.4; b) $NADH_2$-cytochrome *c* reductase: 1 mM KCN, 0.07 mM cytochrome *c* (oxidant) and other conditions as in (a); c) $NADH_2$-CoQo reductase: 0.13 mM Qo (oxidant); other conditions as in (b); d) Menadiol oxidase. Conditions as described by Colpa-Boonstra and Slater (11); e) $NADH_2$-dehydrogenase. Conditions as described by Minakami *et al.* (12); f) Succinate oxidase: 28 mM succinate, 0.02 mM cytochrome *c* and 0.13 M phosphate buffer (pH 7.4); g) Succinate dehydrogenase: conditions as described by Arrigoni and Singer (13); h) Cytochrome oxidase: conditions as described by Smith (14).

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* Fellow of Conselho Nacional de Investigaciones Cientificas y Técnicas.

Abbreviations: $NADH_2$, nicotinamide adenine dinucleotide, reduced form; Q_o, 2, 3-dimethoxy-3'-methyl-*p*-benzoquinone; EDTA, ethylenediaminetetra-acetate; DEAE-cellulose, diethylaminoethylcellulose; A, absorbancy; cyt. *c*, cytochrome *c*; K_3H_2, menadiol; PMS, phenazine methosulphate; Tris, tris (hydroxymethyl) aminomethane.

Measurement of phospholipase A activity — The following methods were used: a) The turbidimetric method of Marinetti (15). In those conditions one unit of enzymatic activity was defined as the amount of venom that, in 10 min produced a decrease of 0.010 in absorbancy at 925 mμ. *B. neuwiedi* venom presents a "lag" period before enzymatic activity is observed, and therefore the change in absorbancy at 925 mμ was considered after the end of the "lag" period. b) The method of Habermann and Neumann (16). The inhibition of coagulation of an eggyolk suspension incubated with venom is compared with a control sample incubated in the same conditions but without venom. With this method one unit of enzymatic activity was defined as the amount of venom that increases in one minute the coagulation time of the control. This method does not detect the "lag" period. c) The method of Magee and Thompson (17) was used with purified phospholipase fractions. Lecithin hydrolysis was followed by measuring the concentration of ester bonds by hydroxamate formation, according to Synder and Stephens (18). The hydrolysis required Ca^{++} ions as it has been established for phospholipase A obtained from other sources (19).

Protein concentration was measured by absorbancy at 280 mμ with the corresponding correction for nucleotide absorbancy.

Results

Purification of phospholipase A — The method described by Saito and Hanahan (20) slightly modified (21) was applied to dried venom.

Table I shows the several steps of enzyme purification. In gel filtration (step 2), all material with the phospholipase A activity was excluded and an another fraction (about 16%) without enzymatic activity remained included in the column.

TABLE I — PURIFICATION OF PHOSPHOLIPASE A FROM VENOM OF *BOTHROPS NEUWIEDI*

Experimental conditions as in Fig. 2. Enzyme activity measured by the turbidimetric method (ref. 15).

Step.	Total activity (Units)	Total activity Yield %	Specific activity units / mg of protein (a)
Crude venom	3234	100	54 (1.0)
1. Heat-treatment at pH 3.0	3168	97	115 (2.1)
2. Gel-filtration through Sephadex G-25	3798	117	168 (3.1)
3. Chromatography on DEAE-cellulose:			
F_1 (Tube No. 7-19)	380	12	73 (1.7)
F_2 (Tube No. 53-65)	2887	84	1750 (32.0)
F_3 (Tube No. 79-84)	491	15	614 (11.4)

(a) Increase in specific activity obtained at each step.

The elution diagram of DEAE cellulose chromatography (Fig. 1) shows three fractions (F_1, F_2, and F_3) having phospholipase A activity.

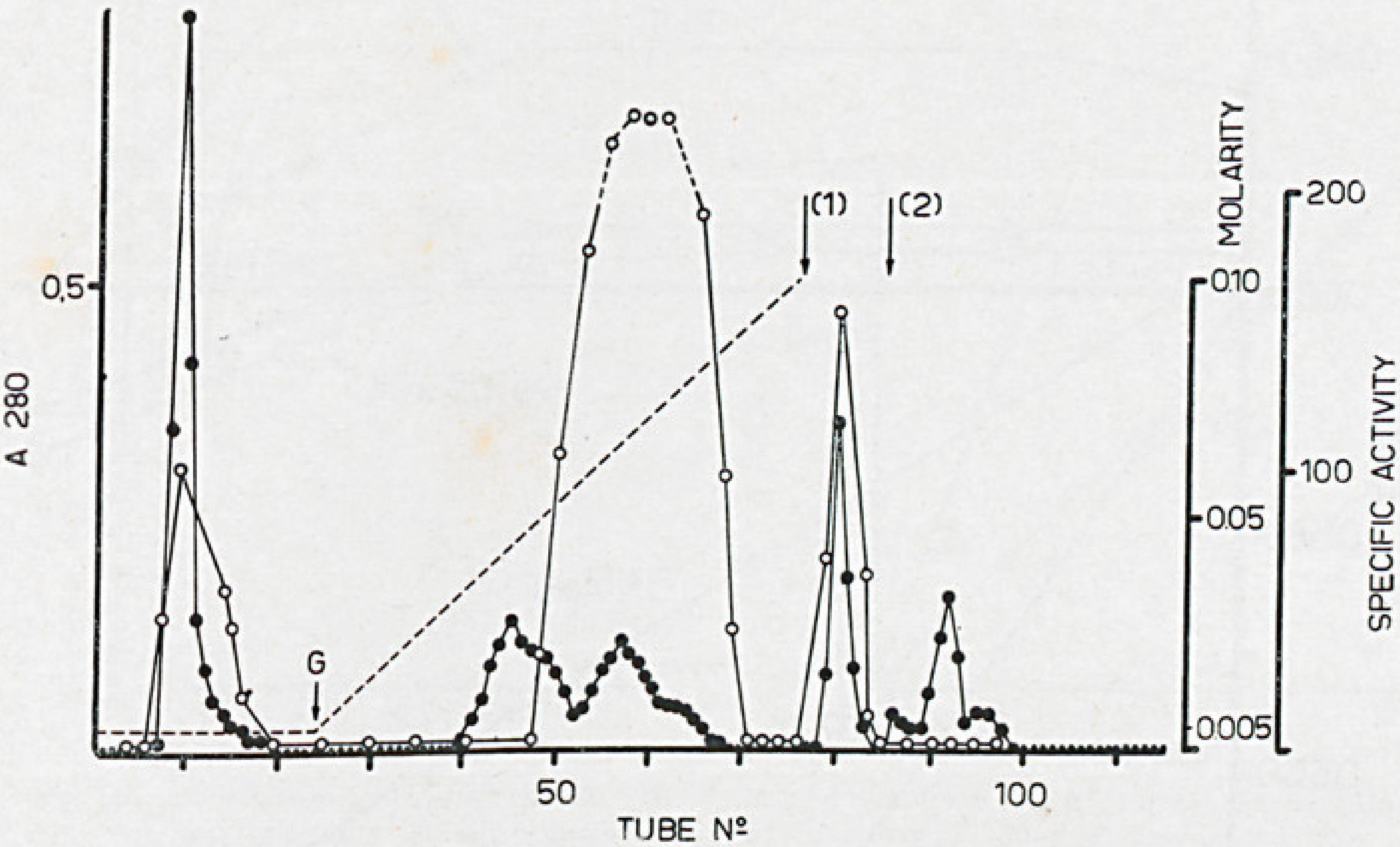

Fig. 1 — Chromatography of Step 2 fraction on DEAE-cellulose. 41 mg of protein were applied on top of a 21 × 1.2 cm DEAE-cellulose. Elution with potassium phosphate buffer (pH 7.4). The gradient was initiated at arrow G. Arrows (1) and (2) indicate the points at which 0.1 M potassium phosphate buffer pH 7.0, and 1.0 M potassium phosphate buffer, pH 7.0, were used as eluants. ● Absorbancy at 280 μm; O phospholipase specific activity (units/mg) measured by the turbidimetric method (15).

The phospholipase A activity in the crude venom samples presented an appreciable "lag" period (Fig. 2) that was not affected by the concentration of venom in the reaction mixture, by the addition of Ca^{++} ions or by increase of the temperature at which activity was measured. On the other hand, the active fraction obtained after Steps 2 and 3 of the purification procedure did not present any "lag" (Fig. 3). Similarly, autolysis of crude venom samples suspended in saline at 15°C produced a progressive disappearance of the "lag" period with simultaneous increase in total phospholipase activity (Fig. 4). The effect of autolysis could be observed only if venom proteases were active.

These facts suggest the presence in crude venoms of a low molecular weight peptide inhibitor of phospholipase A. The inhibitor would form a termostable complex with the enzyme at pH 4.5 but at pH 7.4 the complex would dissociate and then the components could be separated on Sephadex G-25. This interpretation is confirmed by the direct demonstration of an inhibitor of phospholipase A in the included fraction obtained from Sephadex G-25. In fact, in another series of experiments (21), it was found that when the included fraction was added to the active one, it provoked a "lag" with the same characteristics as that of the crude venom and simultaneously, the included fraction produced a strong inhibition of phospholipase activity. No inhibitory action was found in the included fraction obtained from samples of "autolyzed" venom (see above), nor in samples of the included fraction preincubated with trypsin.

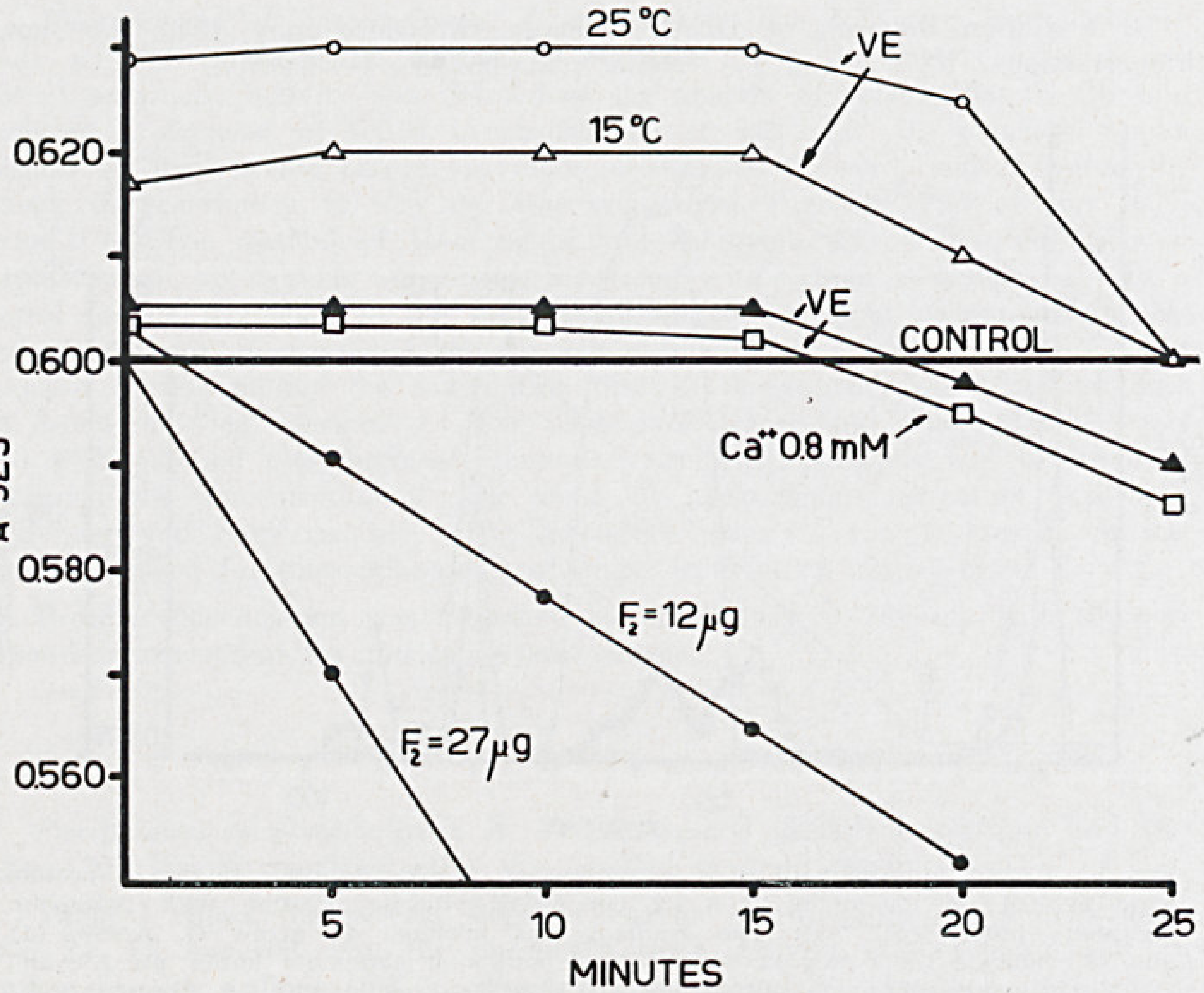

Fig. 2 — Effect of venom concentration, temperature and Ca^{2+} addition on the "lag" period. Enzyme activity measured turbidimetrically (15). The reaction mixtures were incubated at 25° with 88 μg (○) or 42 μg (▲, □) of crude venom, or at 15° with 88 μg of crude venom (△). VE: crude venom. The other curves (●) were obtained at 25° with the stated amount of F_2.

Fraction F_2 which appears homogeneous after electrophoresis on polyacrylamide gel, was tested as inhibitor of oxidizing heart muscle extracts.

According to Table II (Expts. *A* and *F*), treatment with phospholipase A produced a significant inhibition of the $NADH_2$-oxidase and succinate oxidase systems. The addition of cytochrome *c* (0.04 mM) lowered the inhibition of the $NADH_2$-oxidase system to 78%.

In order to establish the phospholipase-sensitive sites, the activity of the different components of electron transport chain was measured using adequate electron acceptors (or donors) and/or blocking cytochrome oxidase activity with KCN. Neither $NADH_2$-dehydrogenase (Expt. *E*) nor succinate dehydrogenase (Expt. *G*) were affected after phospholipase treatment. This result is very interesting because it has been demonstrated (9, 22) that digestion with phospholipase A produced the solubilization of $NADH_2$-dehydrogenase. However, it is possible that in the experiments described in Table II the concentration of enzyme used was not sufficient to produce liberation of a significant amount of $NADH_2$-dehydrogenase. It must be recalled that under similar experimental conditions Badano and Stoppani (7) found complete inhibition of succinate dehydrogenase after

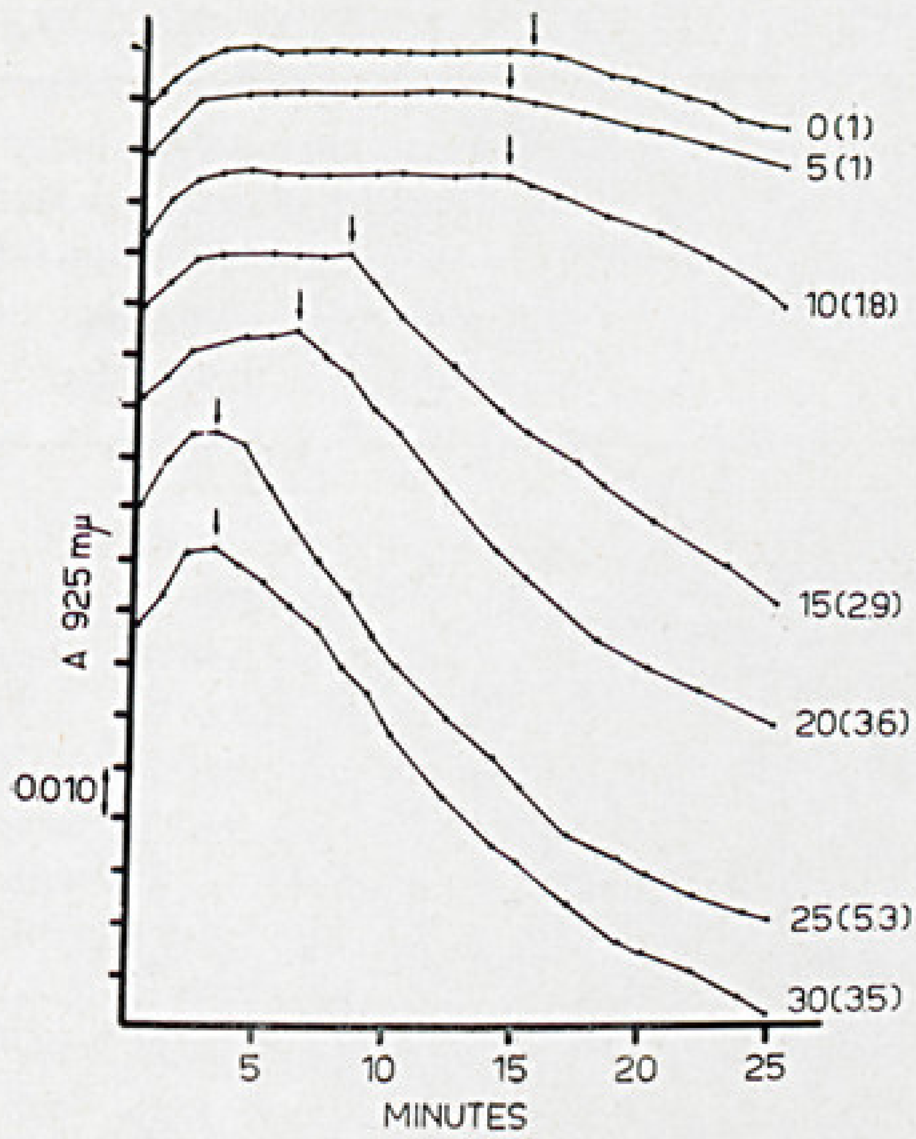

Fig. 3 — Effect of autolysis on the "lag" period. Samples containing 250 μg of crude venom were dissolved in 1 ml 0.9% (w/v) NaCl and incubated at 15° for the time (minutes) stated at the end of each plot. Phospholipase activity was measured by the turbidimetric method (15). The data in parentheses indicate the relative phospholipasic activity of the autolysed sample versus that of the original control sample.

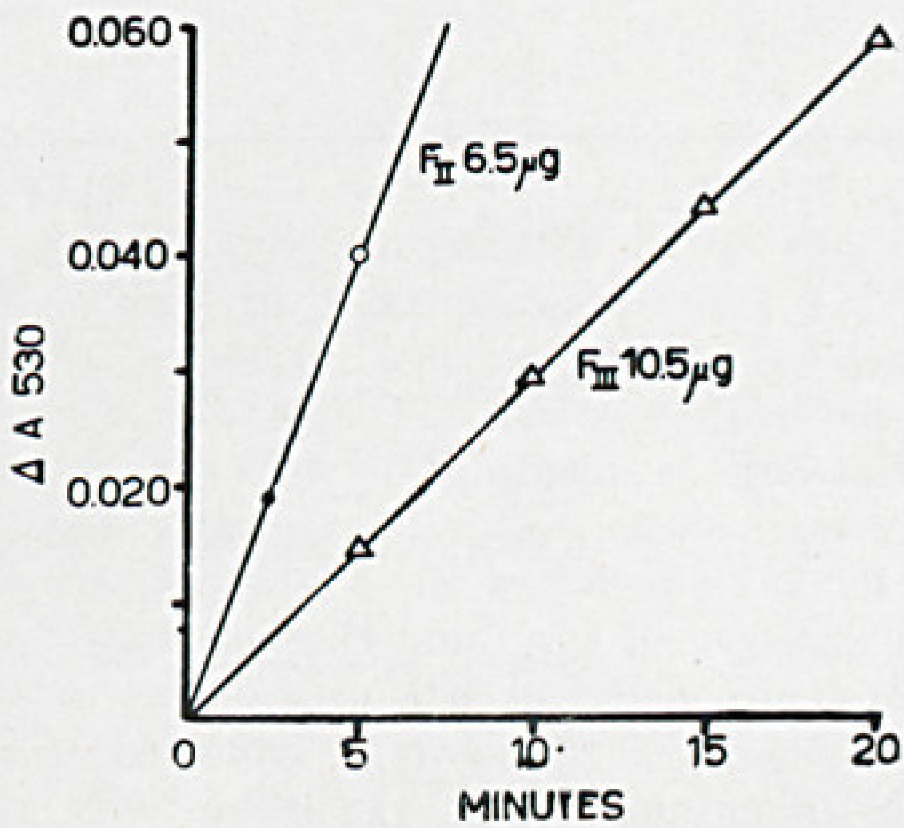

Fig. 4 — Rate of hydrolysis of lecithin by purified F_2 and F_3 measured the turbidimetric method (15). 10 mg sonicated lecithin in aqueous suspension was incubated with enzyme as stated. Reaction mixture: lecithin 5 mg/ml; 50 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA and 5 mM $CaCl_2$. Final volume 2.0 ml.

treatment of Keilin-Hartree preparation with crude venom. Since succinate dehydrogenase is inhibited by treatment with proteolytic enzymes, they interpreted (7) the enzyme inhibition as due to the venom proteases. The present results support that interpretation.

Reduction of Qo (Exp. *C*) by the phospholipase treated preparation ($NADH_2$-Qo reductase activity) was partially inhibited but the magnitude of this inhibition was not sufficient to explain the inhibition of the whole system. On the other hand, menadiol oxidase (Expt. *D*) and $NADH_2$-cytochrome *c* reductase (Expt. *B*) activities were strongly inhibited, in agreement with the inhibition of the whole system. Finally, cytochrome oxidase activity (Exp. *H*) was partially inhibited.

In order to ascertain whether the inhibitions presented in Table II were due to alteration of the lipid constituents of the electron-transport particles, phospholipase-treated and control preparations were extracted according to Fleischer *et al.* (23) and analysed by thin-layer chromatography on Silicagel G (24). After revelation, phosphorus was determined in each spot according to Doizaki and Zieve (25). Such studies showed a decrease in the concentration of lecithin, phosphatidyl-ethanolamine and a third component (probably phosphatidic acids) and a correlative increase in the respective lysoderivatives by an amount that represents about 40% of the total phospholipid content (26).

TABLE II — EFFECT OF TREATMENT WITH PHOSPHOLIPASE A ON THE CATALYTIC PROPERTIES OF THE KEILIN-HARTREE HEART-MUSCLE PREPARATION

Expt.	Reaction	Control preparation (Sp. activities)*	Inhibition of activity by phospholipase A (%)	
			10 min incubation	80 min incubation
A	$NADH_2 \longrightarrow O_2$	0.674	57	85
B	$NADH_2 \longrightarrow$ cyt. c^{3+}	0.175	67	73
C	$NADH_2 \longrightarrow Q_0$	0.483	58	61
D	$K_3H_2 \longrightarrow O_2$	10.0	71	71
E	$NADH_2 \longrightarrow Fe(CN)_6^{3-}$	8.4	0	1
F	Suc. $\longrightarrow O_2$	0.539	41	48
G	Suc. $\longrightarrow$ PMS	2.5	−2	0
H	Cyt. $c^{3+} \longrightarrow O_2$	125.0	61	65

* *Specific activities:*

Expts. *A*, *B*. *C*, and *E*, μmole of $NADH_2$ oxidized/min/mg protein;

F and *G*, μmole of succinate oxidized/min/mg protein;

D and *H*, k'/min^{-1} (mg protein/ml)$^{-1}$ (k', constant of the first order equation).

Discussion

The role of phospholipids in electron transport has been studied in detail by Brierley *et al.* (27) and Fleischer *et al.* (23). It is well known that lipids represent 26% of mitochondrial weight; that phospholipids represent 90% of total lipids and that they are indispensable for electron transport. In fact, extraction with adequate solvents or enzymatic hydrolysis by phospholipases inhibits that transference.

The present study indicates that phospholipase A produces its inhibitory effect by acting on at least three points. 1) The segment Qo-cytochrome *c* is the most sensitive to phospholipase A attack. Elective inhibition of electron transport between quinone and cytochrome *c* seems to be a rather common property of phospholipases (28). 2) The fact that the quinone (Qo) reductase activity was affected, while $NADH_2$-dehydrogenase activity did not show any decrease, indicated that another site lies between the flavoprotein ($NADH_2$-dehydrogenase) and Qo. 3). The third point is near the cytochrome oxidase.

These conclusions which are in agreement with claims by other authors (2, 3, 5), show that the inactivation is in fact due to phospholipase A action, but it remains undecided whether it is caused by the hydrolysis of phospholipid constituents of the electron transport particle ("structural damage"), by the action of the products of hydrolysis on the electron carriers, or by both. When extracted submitochondrial lipids were treated with phospholipase A and the hydrolysate was added to the Keilin and Hartree preparation there was some inhibition of $NADH_2$-CoQo reductase and menadiol oxidase activities but in a lesser extent than after direct treatment of $NADH_2$-oxidase with phospholipase A. Succinate oxidase and cytochrome oxidase activities were not inhibited by the hydrolysed lipid extract. The difference between the "direct" and "undirect" inhibition of the electron transport system must be attributed to "structural damage" by phospholipase A (26).

Summary

Phospholipase A from *Bothrops neuwiedi* venom was purified by a procedure involving heat treatment at pH 3.0, gel-filtration through Sephadex G-25 and chromatography on DEAE-cellulose. Three active fractions were isolated (F_1, F_2 and F_3) with specific activities 1.7, 32 and 11 times, respectively, higher than that of the crude venom sample. F_2 and F_3 migrated as homogeneous proteins after electrophoresis on polyacrylamide gel. The crude venom contains an inhibitor of phospholipase A activity which is responsible of the initial "lag" period in the development of enzyme activity and can be separated by filtration through Sephadex G-25 since it remains in the included fraction. When this fraction was added to the active fractions it provoked a prolonged "lag" and a strong inhibition of activity. The inhibitory effect was nullified after treatment with trypsin or by autolysis of entire venom at 15°. The incubation of submitochondrial heart muscle particles (Keilin-Hartree preparation) with F_2 intensely inhibited $NADH_2$-oxidase, succinate and $NADH_2$-cytochrome *c* reductase activities. There was a less effective inhibition of menadiol oxidase, $NADH_2$-Qo reductase and cytochrome oxidase, but $NADH_2$-dehydrogenase and succinate dehydrogenase activities were not affected after treatment with phospholipase A.

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