ketone (VII) with bp 70-72°C (2 mm), n_D^{20} 1.4340. IR spectrum (ν, cm^{-1}): 975, 3025 (trans-—HC=CH—), 1715 (—CO—). PMR spectrum (δ, ppm): 1.25 (2H, —CH_2—); 1.57 (3H, CH_3CH=); 2.0 (3H, CH_3CO—); 2.30 (4H, —CH_2CO—, CH_2CH=); 5.30 (2H, —CH=CH—); M^+ 126.

SUMMARY

A new route for the synthesis of the attractant of the honey bee *Apis mellifera* using readily accessible reactants has been developed.

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AN INVESTIGATION OF THE VENOM OF RENARD'S VIPER *Vipera ursini renardi*.

III. ISOLATION OF PHOSPHOLIPASES A_2

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Two phospholipases A_2 with molecular weights of 15,600 and 13,200 and pI values of 7.57 and 6.73, respectively, have been isolated from viper venom in the pure state with the aid of gel filtration on Sephadex G-75 followed by ion-exchange chromatography on CM-cellulose and SE-Sephadex C-25.

Phospholipase A_2 (phosphatide acylhydrolase, E. C. 3.1.1.4) is a component part of many snake venoms and plays a fundamental role in the mechanism of their action [1, 2]. The majority of the biological effects of the enzyme are probably due to the hydrolysis of membrane phospholipids, the degree of degradation of which determines the beginning of lysis of the cells [3]. The phospholipase A_2 from the venoms of different snakes differ from one another in their capacity for attacking a membrane substrate [4]. Thus, the enzyme from the venom of the Palestine viper *Vipera palestinae*, in contrast to the phospholipase A_2 from the venom of the cobra *N. naja*, does not penetrate into the structure of a substrate organized in a membrane. We have previously [5, 6] isolated pure phospholipase A_2 from the venom of Central Asian cobra *Naja oxiana* Eichwald and have studied its hemolytic action. The aim of the present investigation was to isolate pure phospholipases A_2 from the venom of the viper *V. ursini renardi* Ch.

The initial phospholipase A_2 fraction (fraction III) was obtained by the gel filtration of the whole viper venom on Sephadex G-75 [7]. It was subjected to further separation by chromatography on CM-cellulose (Fig. 1). On application to the column of ion-exchange resin, the part of fraction III denoted by the letter A and having no phospholipase activity was not adsorbed and issued from the column with the equilibrating 0.05 M ammonium acetate buffer (pH 4.7). The superimposition of a gradient of ammonium acetate buffer from 0.05 M (pH 4.7) to 0.5 M (pH 6.7) permitted separation into three components, designated in the order of their emergence from the column III-1, III-2, and III-3, and the addition of 0.01 M caustic soda solution to the column desorbed component III-4. They all possessed phospholipase A_2 activity. The initial fraction III contained mainly protein-peptide components (98%). On chromatography on CM-cellulose, the bulk of the proteins was distributed in the following way: III-1, 17.66%; III-2, 49.1%; III-3, 22.76%; and III-4, 4.4%; 6.08%

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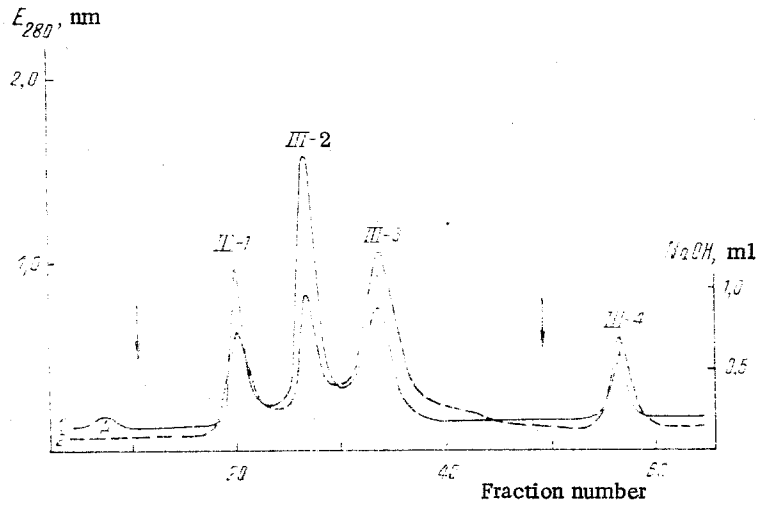

Fig. 1. Separation of the initial fraction III of the viper venom on CM-cellulose (the arrows show the moment of application of the gradient and of the addition of a 0.01 M solution of caustic soda to the column): 1) extinction at 280 nm (protein content); 2) phospholipase A_2 activity (amount of added NaOH in milliliters); 3) A, III-1, III-2, III-3, and III-4 are fractions combined according to the "protein peaks."

of the proteins was present in fraction A or formed the losses on chromatography.

For the subsequent work, we used the fractions III-2 and III-3, obtained with the highest yields. The results of their separation on the ion-exchange resin SE-Sephadex C-25 are shown in Fig. 2. Each of the fractions was separated into two components, both of which possessed phospholipase A_2 activity. Comparative electrophoretic studies showed that fractions III-2 and III-3 were each contaminated with the other, the impurities being separated by chromatography on SE-Sephadex. Consequently, we subsequently combined component III-2-1 with component III-3-1 and component III-2-2 with III-3-2 (Fig. 2). The results of disk electrophoresis and also of isoelectric focusing showed the complete individuality of phospholipases A_2 III-2 and III-3.

With the aid of gel filtration on Sephadex we established that the molecular weights of phospholipases A_2 III-2 and III-3 were 15,600 and 13,200, which corresponds to the molecular weights given in the literature [2] for monomeric forms of the enzyme. Isoelectric focusing enabled us to determine the pK values of the enzymes that we had isolated: 7.57 for III-2, and 6.73 for III-3. Analysis of the results of chromatography and comparative electrophoresis of the fractions obtained, showed the possibility of the presence of numerous forms of phospholipase A_2 in viper venom. The literature also contains information indicating the existence of a large number of molecular forms of the enzyme differing in pI value and molecular dimensions, in the venoms of viperine snakes [8].

EXPERIMENTAL

The venom of the viper *Vipera ursini renardi* Ch., dried over calcium chloride, was obtained from the Institute of Zoology and Parasitology of the Academy of Sciences of the Uzbek SSR and the Kirghiz Zonal Zoological Combine. We used CM-cellulose CM-32, Sephadexes SE C-25, G-75, and G-25, a set of reagents for electrophoresis, sets of ampholines, cytochrome c, ovalbumin, bovine serum albumin, ribonuclease, trypsin, and the neurotoxin of the venom of the Central Asian cobra [9], and also other reagents of grades KhCh ["chemically pure"] or ChDA ["pure for analysis"].

The initial fraction III was obtained from viper venom by the method described previously [7] and was chromatographed on CM-cellulose (column 300 × 20 mm) equilibrated with 0.05 M ammonium acetate buffer (pH 4.7). Elution was performed at the rate of 36 ml/h with the

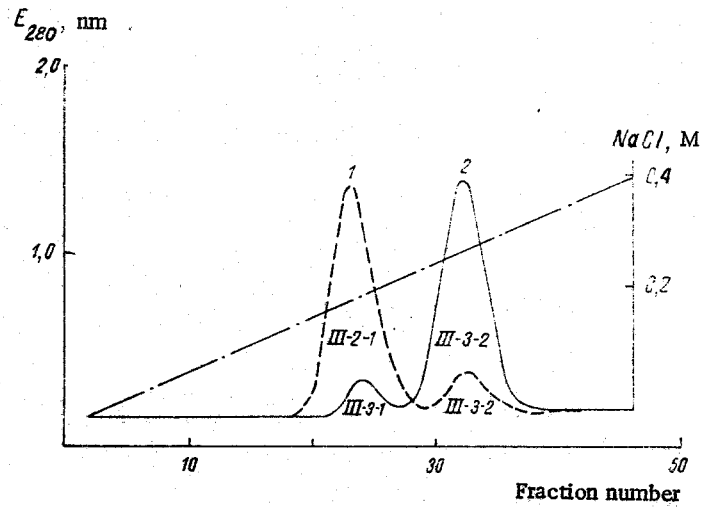

Fig. 2. Chromatography of fractions III-2 and III-3 on SE-Sephadex: 1, 2) extinction at 280 nm (protein content) in fractions III-2 (1) and III-3 (2); 3) concentration of sodium chloride in the eluate. III-2-1, III-2-2, III-3-1, and III-3-2 are fractions combined according to the "protein peaks."

equilibrating buffer in which the concentration of salt was changed by a linear gradient from 0.05 M to 0.5 M with a change in the pH from 4.7 to 6.7, and finally 0.01 M caustic soda solution was added to the column.

For chromatography on SE-Sephadex we used a 100 × 9 mm column equilibrated with 0.05 M ammonium acetate buffer (pH 4.7). Elution was carried out at the rate of 12 ml/h with equilibrating buffer in which the amount of sodium chloride was increased according to a linear gradient to 0.4 M.

Fractions with a volume of 3 ml were collected and were combined according to the protein "peaks," evaporated in a rotary evaporator, desalted on Sephadex G-25, and freeze-dried. The amounts of protein [10] and the phospholipase A_2 activities [11, 12] were determined in the combined liquid or dried fractions.

Disk electrophoresis was carried out in 7.5% PAAG in β-alanine (pH 4.3) and tris-glycine (pH 8.3) buffer systems [13], and isoelectric focusing was carried out in pH 3.5-9.0 ampholines in accordance with the recommendation of the manufacturers. Molecular weights were determined by gel filtration on a column of Sephadex G-75 [14] calibrated with proteins having molecular weights between 6000 and 67,000.

SUMMARY

1. The successive use of gel filtration on Sephadex G-75 and ion-exchange chromatography on CM-cellulose and SE-Sephadex C-25 has shown the possibility of numerous forms of phospholipase A_2 in viper venom and has enabled two forms of the enzyme to be obtained in the pure state.

2. The pure phospholipases A_2 obtained differed in molecular weight and isoelectric point. It has been established that the molecular weight and pI value of phospholipase A_2 III-2 are 15,600 and 7.57, respectively, and for phospholipase A_2 III-3 they are 13,200 and 6.73.

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METABOLITES OF THE PATHOGENIC FUNGUS *Verticillium dahliae*.

IX. PENTAKETIDES OF MUTANTS AND THEIR ROLE IN THE BIOSYNTHESIS OF MELANIN

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From mutants of the fungus *Verticillium dahliae* Kleb. having different genetic blocks in the biosynthesis of melanin we have isolated and identified scytalone, flaviolin, 4-hydroxyscytalone, and 2-hydroxyjuglone and have detected 3,4,8-trihydroxytetralone and 4,8-dihydroxytetralone, which, in combination with the results of complementation analysis, have confirmed the scheme of melaninogenesis in *V. dahliae* put forward previously. The participation of 1,3,6,8-tetrahydroxynaphthalene in the basic pathway of the biosynthesis of melanin has been shown.

Recently, mutants have been widely used in investigations on the biosynthesis of antibiotics, toxins, growth regulators, and other metabolites produced by microorganisms [1]. A number of melanin-deficient mutants has been used in the study of melaninogenesis in the fungus *Verticillium dahliae* Kleb. [2]. We have already reported on some compounds (flaviolin, scytalone) participating in this process [3, 4].

In the present paper we consider the pentaketides of various mutants of the fungus in connection with their role in the biosynthesis of melanin in *V. dahliae*.

We used melanin-deficient mutants from the collection of the Department of General Genetics of the Cotton Plant of the Academy of Sciences of the TadzhSSR, which were induced by ultraviolet irradiation (index UV), γ rays (index R), or nitrosomethylurea (index X), and also spontaneous mutants (index S). Among the 27 mutants that we studied, according to the literature [5], eight were from the group of chm mutants (UV-130, X-145, X-172, X-262, R-196, R-420, S-1, and X-272), possessing red-brown microsclerotia, 13 were from the group of alm mutants (R-177, R-503, R-550, X-146, X-127, X-222, UV-172, UV-117, UV-142, UV-160, UV-131, UV-128, and S-2), having white microsclerotia, and six were from the group of brm mutants with dark brown microsclerotia (X-171, X-269, R-841, R-83, S-3, and UV-233).

From acetone extracts of cultures of all the chm mutants apart from X-272 by column chromatography (CC) and preparative thin-layer chromatography (PTLC) on silica gel in system 1 we isolated and identified 3,6,8-trihydroxy-3,4-dihydro-1(2H)-naphthalenone (scytalone) (II) and 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin) (III) (Scheme 1) and also a substance with mp 98-103°C which on the basis of a comparison of R_f values and mass spectrum with literature information was identified as 4-hydroxyscytalone (IV) [6].

Only compounds (III) and (IV) were isolated from a culture of the mutant X-272, and no compound (II) was detected.

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