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IDENTIFICATION AND CHARACTERIZATION OF A NEW ENZYME OF THE GROUP "PHOSPHOLIPASE D" ISOLATED FROM *CORYNEBACTERIUM OVIS*

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SUMMARY

1. A new enzyme belonging to the group of phospholipases D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) was found in *Corynebacterium pseudotuberculosis (ovis)*. It splits sphingomyelins to *N*-acylsphingosylphosphates and choline, and lysophosphatidylcholine to lysophosphatidic acids and choline.

2. Methods of purification of the enzyme by $(NH_4)_2SO_4$ and methanol precipitation and elution from CM Sephadex columns are described.

3. The behaviour of the enzyme upon Sephadex columns, ion-exchangers and electrophoresis was investigated.

4. Optimal conditions of the enzymic reaction are studied and the K_m determined using sphingomyelins as a substrate ($K_m = 6.2$ mM) and lysophosphatidylcholines ($K_m = 8.3$ mM).

5. Using a new modification of paper and thin-layer chromatography the product of enzymic hydrolysis of sphingomyelins, *N*-acylsphingosylphosphates, were separated from other related compounds.

6. A new colour reaction for the characterization of *N*-acylsphingosylphosphates is described.

7. The spectrum of sphingosine bases in sphingomyelins and *N*-acylsphingosylphosphates is characterized by 4-sphingenine (as the main component) and sphinganine (present only in a trace amount).

8. The main fatty acids are in both sphingolipids $C_{24:1}$ and $C_{16:0}$ acids. The minor components are composed from $C_{24:0}$, $C_{22:0}$, $C_{18:0}$, $C_{18:1}$, $C_{22:1}$, $C_{23:0}$ and $C_{20:0}$ acids.

INTRODUCTION

During studies of the toxin of *Corynebacterium pseudotuberculosis (ovis)*, *Corynebacterium haemolyticum* and *Corynebacterium ulcerans* we proved that the toxins are adsorbed onto the surface of sheep erythrocytes and these erythrocytes are not hemolyzed by phospholipases C (phosphatidylcholine cholinephosphohydrolase, EC

3.1.4.3) produced by *Staphylococcus pyogenes* and *Clostridium perfringens*[1–4]. The comparison of phospholipids extracted from normal erythrocytes and erythrocytes after the action of the corynebacterial toxins showed the decrease of sphingomyelins on paper chromatography. In a preliminary note[5] we have shown that the toxin of *C. ovis* hydrolyses sphingomyelins and lysophosphatidylcholines and releases free choline. Determination of the concentration of phosphorus and nitrogen during the enzymic reaction proved that the phospholipids are not split to phosphorylcholine as is known for phospholipase C but the enzyme belongs to the group of phospholipases D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4). The enzyme which we have described is produced by the bacteria into the cultivation medium and does not hydrolyse phosphatidylcholines under conditions described for the plant phospholipases D (refs. 6, 7). In the present paper we give the results of the study of A, the basic characteristics of the enzyme and methods of its purification, B, the kinetic data of the hydrolytic reaction, and C the identification of the splitting product of sphingomyelins *N*-acylsphingosylphosphates, because this compound has not been isolated from the natural sources nor has it yet been synthetized.

MATERIALS AND METHODS

Preparation of enzyme

A toxigenic strain of *C. pseudotuberculosis (ovis)* NCTC 4655 from the National Type Culture Collection in London was used for the production of the enzyme. It was cultivated in Todd–Hewitt's broth without glucose at pH 7.6 for 48 to 72 h at 37° using static cultivation in Erlenmeyer flasks. The best yield of the enzyme is obtained when the ratio of surface (in cm^2) to volume (in ml) equals 0.8, under these conditions the strain grows in a surface pellicle[8].

Bacterial cells are removed by centrifugation for 45 min at 3000 × *g* at 5° and the supernatant is stored at 4°. There is no decrease in enzyme activity during storage of crude supernatants for several months.

Enzyme assay

Hydrolysis of lysophosphatidylcholines was estimated in the reaction mixture of 2 ml, the substrate (2–40 μmoles) is dissolved in 0.05 M borate buffer (pH 8.0), 1 ml of the substrate solution is incubated with 1 ml of enzyme solution in a water bath at 37°, reaction is terminated after the incubation for 5–120 min by the addition of 0.5 ml 1 M trichloroacetic acid, the mixture is made alkaline to phenolphthalein with 1 M NaOH.

Hydrolysis of sphingomyelins is determined in the reaction mixture of 2 ml, the substrate is emulsified by sonication in the M.S.E. disintegrator for 20 min at 20 kcycles. We used 1–15 μmoles of sphingomyelins in 0.05 M borate buffer (pH 8.0). 1 ml of substrate emulsion is mixed with 1 ml enzyme solution as described above.

Estimation of enzymic units

20 μmoles of lysophosphatidylcholines in 0.05 M borate buffer (pH 8.0) with 1% Neopepton (Difco) are mixed with 1 ml enzyme solution in the same buffer, incubated for 20 min at 37° in a water bath, and the reaction is terminated by the addition of 0.5 ml 1 M trichloroacetic acid. After being made alkaline to phenolphtha-

lein with 1 M NaOH, free choline is determined. 1 unit is that amount of enzyme which releases 1 μmole of choline in 1 min (ref. 9).

For the qualitative estimation of enzymic activity, 0.5 μmoles of lysophosphatidylcholines are dissolved in 0.25 ml of enzyme solution (pH 8.0) and incubated for 5 to 120 min at 37°. Free choline is determined qualitatively by the reaction with enneaiodid after BOOTH[10].

Determination of choline

Free choline in the reaction mixture is determined after the addition of 1.5 ml of 2% methanolic solution of ammonium reineckate[6], the mixture is stirred with a glass rod and kept at 5° for 120 min. The precipitate is separated by centrifugation and washed twice with 2 ml portions of *n*-propanol. The choline reineckate crystals are dissolved in 4 ml of acetone and the solution is cleared by centrifugation. The absorbance of the solution at 526 nm is read with a Zeiss Universal Spectrophotometer in 10-mm cuvettes and the amount of choline is determined by reference to a standard curve. This method determines 50–600 μg of choline in 1 ml.

Preparation of the substrates

Preparation of sphingomyelins. Sphingomyelins are prepared from the phospholipids extracted by chloroform and methanol from sheep erythrocytes according to WAYS AND HANAHAN[11]. The extracts are submitted to mild alkaline hydrolysis[12] and the sphingomyelins are obtained after fractionation in a silicic acid column in the fraction of chloroform–methanol (4:1, v/v), after the evaporation of the solvents the sphingomyelins are dissolved in chloroform and stored.

Preparation of lysophosphatidylcholines. Egg phosphatidylcholines are prepared after PANGBORN[13] and purified on a silicic acid column, eluted with a chloroform–methanol mixture (3:2, v/v) and after the evaporation of solvents, phosphatidylcholines are dissolved in chloroform and stored. Lysophosphatidylcholines are prepared by the method of LONG AND PENNY[14] which involves enzymic hydrolysis by phospholipase A from the snake venom *(Vipera ammodytes)*. Lysophosphatidylcholines are stored in chloroform.

Preparation of phosphatidic acids. Phosphatidic acids are prepared by enzymic hydrolysis of phosphatidylcholines by phospholipase D from cabbage according to KATES[15] and purified on a silicic acid column with chloroform–methanol (1:50 to 1:5, v/v) as eluate.

Chromatography on a silicic acid column

Silica gel for column chromatography (40–100 μ, Spolana Neratovice) was activated for 12 h at 120°, suspended in chloroform–methanol (1:1, v/v) and washed by chloroform–methanol (1:1, v/v) and chloroform[16]. A column of 1.8 cm × 7 cm is prepared from 3 g of silicic acid, the phospholipids in the sample reach 1 mg of phosphorus of phospholipids in 1 ml chloroform for 1 g of silicic acid. The columns are eluted by 100 ml portions of solvents: chloroform, chloroform–methanol in the ratio 4:1 to 1:4 (v/v) (ref. 17). For analytical purposes the fraction volumes were 10 ml, but for preparative purposes 100-ml fractions were collected. Phosphorus was determined in the fractions and the samples of fractions were examined by means of paper chromatography.

Paper chromatography

Paper chromatography according to the method of MARINETTI[18] was carried out using silicic acid impregnated papers with a diisobutylketone–acetic acid–water (40:20:3, v/v) system.

For the detection of the spots the usual colour reaction described in the literature[5] was used.

The fractions obtained from column chromatography containing *N*-acylsphingosylphosphates were separated on Whatman No. 3 paper impregnated with silica gel in chloroform–methanol–conc. NH_4OH (60:80:1, by vol.). The spots were detected by the usual colour reactions[5] but with some modifications[19]. A very useful test was applied for the characterization of the chemical structure of *N*-acylsphingosylphosphates and its differentiation from other related compounds. It depends upon the reaction with acid fuchsin-brilliant green and uranyl ions. *N*-Acylsphingosylphosphates give a green coloration. Any substance containing only a negative charged group, in our case PO_4^{3-}, is coloured with brilliant green and so a green colour will develope on a chromatogram where an acid substance is located. Other compounds, for instance sphingomyelins and other both positive and negative groups close together give a differently coloured shade-blue, red or red violet.

Thin-layer chromatography

Thin-layer chromatography was carried out on Silufol UV 254 sheets (Sklárny Kavalier n.p., Votice, Czechoslovakia, serie 052168) (4 cm × 7.5 cm or 7.5 cm × 7.5 cm) and developed in chloroform–methanol–conc. NH_4OH (50:15:2, by vol.).

Hydrolysis of sphingomyelins and N-acylsphingosylphosphates

The samples of original sphingomyelins (isolated from sheep erythrocytes) and the product from the enzymic reaction were hydrolyzed with 5% HCl in methanol for 18–20 h at 60–70°. After cooling, the reaction mixture was diluted with the same volume of distilled water and the methyl esters of fatty acids were extracted three times with *n*-hexane. The hexane layer was purified with distilled water and dried with Na_2SO_4. After the evaporation of the solvent the methyl esters of fatty acids were further purified by preparative thin-layer chromatography on Silufol UV 254 in *n*-hexane–diethyl ether (10:90, v/v). The fraction of esters was scraped off and extracted with *n*-hexane.

The aqueous methanolic layer was made alkaline with potassium hydroxide and sphingosine bases were then extracted with diethyl ether. The ether solution was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The dinitrophenol-derivatives were prepared according to KARLSSON[11] and separated by paper chromatography[20] or thin-layer chromatography on Silufol UV 254 sheets.

The isolated methyl esters of fatty acids were analysed by gas chromatography.

General procedures

Determination of phosphorus was done by the modified method of FISKE-SUBBAROW according to ALLEN *et al.*[22]. Protein was determined according to the procedure of LOWRY *et al.*[23].

Absorbance was measured in a Zeiss Universal Spectrophotometer using 10-mm cuvette and wave length 280 nm.

RESULTS

Purification of the enzyme

The enzymic activity of phospholipase D was detected in the extracellular products of *C. pseudotuberculosis (ovis)* so we used the supernatants of the growth medium as a starting material for the purification procedures. The concentration of the enzyme activity reaches its maximum in 48–72 h of static incubation at 37°. We tried to find a convenient synthetic medium for the production of the enzyme but in all media without peptones the production of enzyme was lower. The activity of the enzyme does not change in supernatants during 2 months of storage at 4°.

Fractionation with $(NH_4)_2SO_4$ and methanol

In the preliminary study we used the fractionation with ammonium sulfate at 10–50% saturation and it was shown that the enzyme is precipitated at 30–50% saturation, the yield reaches about 50% and the purification is 10-fold. We used $(NH_4)_2SO_4$ precipitation for samples of large volume.

Precipitation with methanol

For the precipitation of the enzyme we used COHN's[24] VIth method with methanol instead of ethanol. The enzyme is precipitated in the second and third fraction and in the fourth fraction, both these fractions together give a yield of about 50 to 60%, but the purification is higher in the second and third fraction reaching 30 instead of 10 to 20 in the fourth fraction. The effect of pH on the precipitation with methanol at constant ionic strength (approx. 0.10) was carried out according to PILLEMER[25] using a 40% concentration of methanol. The precipitation was not considerably influenced by pH. As starting material for the methanol precipitation the crude supernatant or samples after precipitation with $(NH_4)_2SO_4$ were used.

Gel filtration in Sephadex columns

Crude supernatants or samples after $(NH_4)_2SO_4$ or methanol precipitation were

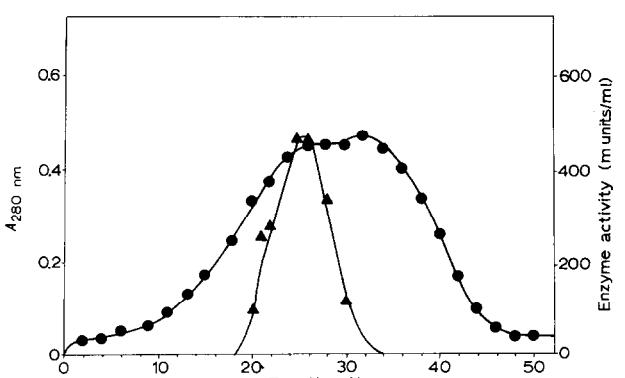

Fig. 1. Sephadex G-200 gel filtration. Sephadex G-200 (Pharmacia Uppsala) was added to 0.3 M NaCl in 0.006 M phosphate buffer (pH 7.8) and was allowed to swell for 7 days, thereafter the gel suspension was washed with the same buffer by repeated decantation. A column (2 cm × 45 cm, V_t = 205 ml) was prepared. 2 ml of enzyme solution (16.8 mg protein per ml) dissolved in 0.3 M NaCl in 0.006 M phosphate buffer (pH 7.8) was eluted with 0.3 M NaCl in 0.006 M phosphate buffer (pH 7.8), fractions of 5 ml each were collected after the void volume (V_0 = 65 ml), flow rate was 30 ml/h. Elution was performed at 20°. ●—●, $A_{280\ nm}$; ▲—▲, enzyme activity (munits/ml).

used for gel filtration. The purified samples were dissolved in the buffer solution used for the elution of the columns. Sephadex G-100, G-150 and G-200 were used for the analysis of the enzyme solutions. The enzymic activity was determined qualitatively and where possible also quantitatively. The enzyme is eluted in one peak from all the Sephadex columns we used. The effect of the concentration of salts and the pH of the eluting solutions was studied and in all cases only one peak with enzyme activity was found. Fig. 1 illustrates a typical experiment.

Purification on ion exchangers

In preliminary experiments the ion-exchanger columns of CM- and DEAE-

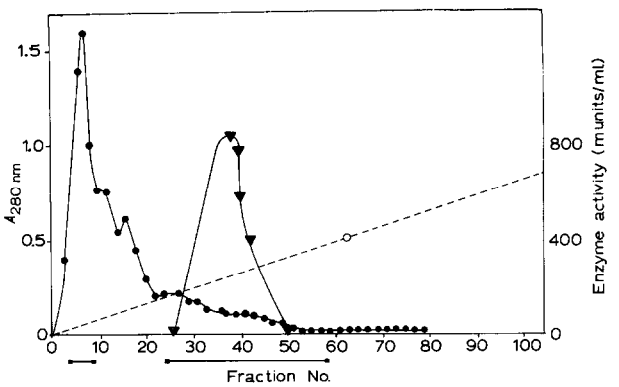

Fig. 2. CM-Sephadex chromatography. CM-Sephadex C-50 (3 g) was equilibrated in 0.0125 M phosphate buffer (pH 6.3) by repeated decantation in the same buffer. A column (2 cm × 28 cm) was prepared and the ion exchanger equilibrated by 3 volumes of 0.0125 M phosphate buffer (pH 6.3). The sample (160 mg protein) was dissolved in 8 ml 0.0125 M phosphate buffer (pH 6.3). Elution was done at 20° with a linear gradient obtained with 500 ml 0.0125 M phosphate buffer in a mixing chamber and 500 ml 2 M NaCl in buffer in a second container. The flow rate was 24 ml/h and the fraction size was 4 ml. ●—●, $A_{280\,nm}$; ▼—▼, enzyme activity (munits/ml); ■—■, release of free choline determined qualitatively.

cellulose (Whatman) and CM- and DEAE-Sephadex were used and for further studies CM-Sephadex was chosen. Fig. 2 illustrates a typical case where methanol-purified material was analysed. The sample was dissolved directly in the starting buffer solution (0.0125 M phosphate buffer pH 6.3). The enzyme is eluted in two peaks, the first one is not retained by the CM-Sephadex column, the sum of the activity in this region does not exceed approx. 1% of the original sample. The majority of the enzymic

TABLE I

PURIFICATION OF ENZYME

Purification step	*Total vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Purification*	*Recovery (%)*
Crude supernatant	250	700	40	0.015	1	100
Methanol	10	200	22.5	0.112	7.4	56.2
CM-Sephadex eluate (Fractions 38–42)	20	5.6	14.84	2.65	176.6	37.3

activity is eluted by a NaCl gradient (0.2–0.4 M) with a maximum at 0.28 M NaCl. The enzyme obtained from the ion exchanger reaches the highest specific activity in our experiments. The results of attempts to purify the enzyme are summarized in Table I.

Electrophoresis

For further analysis of the characters of the phospholipase D agarophoresis was used. In the electrophoresis the enzyme is detected by hydrolysis of lysophosphatidylcholines, the product (lysophosphatidic acids) is not dissolved in water and a precipitate is formed in the region of the enzymic activity.

The enzymic activity can be shown only in one zone, but in some samples after purification with $(NH_4)_2SO_4$ and methanol the zone is broader and moves more towards anode.

Processing of kinetic data

Lysophosphatidylcholines and sphingomyelins were used as substrate for the phospholipase D. The enzyme solutions were in most cases prepared by methanol precipitation and were dissolved in 0.05 M borate buffer (pH 8.0). The pH optimum

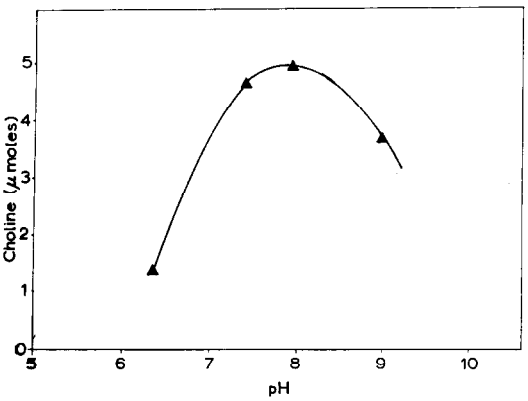

Fig. 3. Reaction rate as a function of pH. 20 μmoles of lysophosphatidylcholines were dissolved in 1 ml 0.05 M acetate buffer (pH 5.0–6.0), 0.05 M phosphate buffer (pH 6.0–7.0), 0.05 M borate buffer (pH 7.6–8.0) and incubated with 1 ml enzyme solution for 20 min at 37°.

is 7.6–8.0 (Fig. 3). Fig. 4 illustrates the reaction rate as a function of time. Using 20 μmoles of lysophosphatidylcholines the reaction rate is of zero order up to 20 min in methanol precipitates not exceeding 2 mg protein in the reaction mixture. In higher concentrations the reaction rate is of first order. The enzyme solutions were diluted for the determination of enzymic units so that the reaction velocity would be proportional to the concentration of the enzyme (Fig. 5). The velocity of the enzymic reaction is enhanced by Neopepton (Difco) which is used in 1% concentration in the buffer solutions used for the determination of the enzymic units. The effect of substrate concentration on the reaction velocity was studied. Figs. 6 and 7 illustrate the LINEWEAVER–BURK[26] plot, $K_m = 8.3$ mM lysophosphatidylcholines and $K_m = 6.2$ mM sphingomyelins, so that the affinity of the enzyme to both substrates used does not differ considerably.

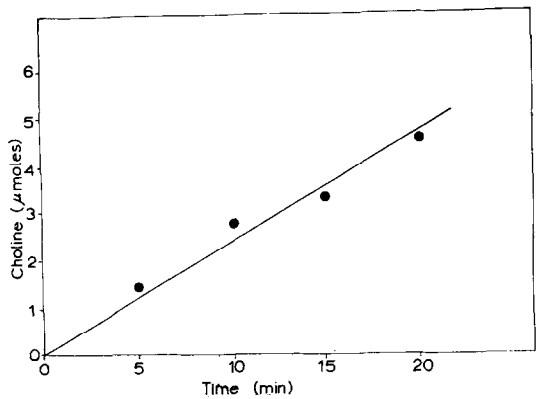

Fig. 4. Reaction rate as a function of time. 2 ml reaction mixture contained 20 μmoles of lysophosphatidylcholines with enzyme solution (0.5 mg protein) in 0.05 M borate buffer (pH 8.0) with 1% Neopepton (Difco), incubation was performed at 37°.

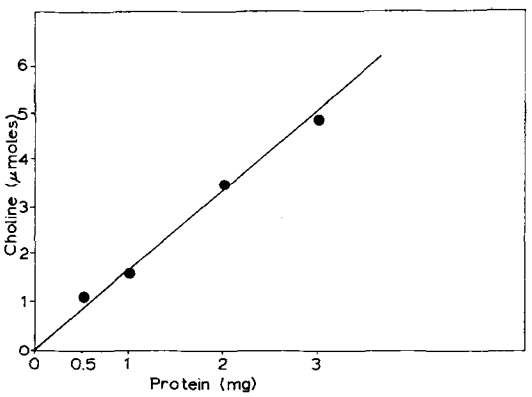

Fig. 5. Reaction rate as a function of enzyme concentration. 2 ml reaction mixtures contained 20 μmoles of lysophosphatidylcholines and enzyme solution in 0.05 M borate buffer (pH 8.0), incubation was performed at 37° for 20 min.

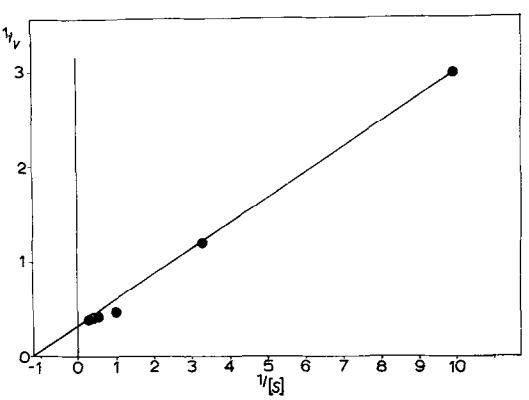

Fig. 6. Lineweaver–Burk plot of the relationship between substrate (lysophosphatidylcholines) concentration ([S] in 0.01 M) and reaction velocity (v). Reaction velocity v is given in μmoles of cholines in 1 ml of the reaction mixture. Lysophosphatidylcholines were dissolved in 1 ml 0.05 M borate buffer (pH 8.0) and incubated with 1 ml enzyme solution 20 min at 37°. K_m = 8.3 mM lysophosphatidylcholines.

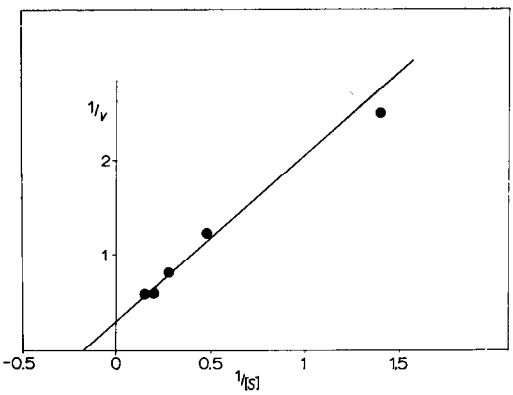

Fig. 7. Lineweaver–Burk plot of the relationship between substrate (sphingomyelins) concentration ([S] in mM) and reaction velocity (v). The reaction was carried out as in Fig. 6 using sphingomyelins emulsified by sonication. K_m = 6.2 mM sphingomyelins.

Identification of the hydrolytic products

The hydrolytic product of sphingomyelins from 70 μmoles of sphingomyelins incubated in 20 ml of 0.05 M borate buffer (pH 8.0) with 12 mg protein of enzyme solution with 0.05 M $CaCl_2$ and 20 ml diethyl ether for 120 min at 37° under permanent shaking was prepared.

During the reaction the hydrolytic product is found in the diethyl ether phase. After the incubation the diethyl ether phase is separated and another portion of diethyl ether is added to 200 ml volume. The upper phase is shaken with 0.3 M HCl until the opalescence disappears. This phase is separated and diethyl ether is evaporated in vacuum and the residue is dissolved in chloroform and used for column chromatography in a silicic acid column. The elution is illustrated in Fig. 8. All fractions were submitted to paper chromatography and their content of phosphorus

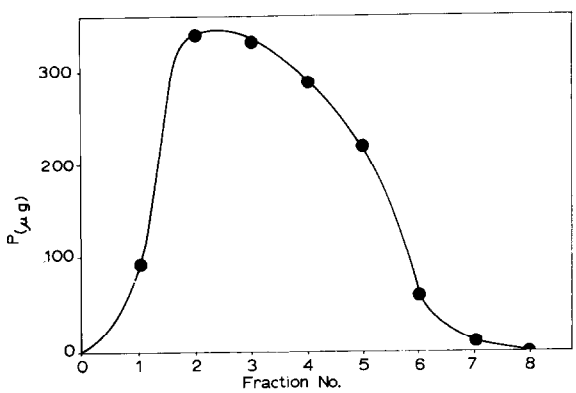

Fig. 8. Elution of *N*-acylsphingosylphosphates from the silicic acid column. Preparation of the column is described in MATERIALS AND METHODS. The sample contained 30 mg dry wt. of the hydrolysis product of sphingomyelins. Elution with 100-ml portions of chloroform–methanol in ratios 100:1, 50:1, 20:1, 10:1, 1:5, 1:1, 1:4 (v/v), fraction size 100 ml. The solvents were evaporated and phosphorus determined.

was determined. Fractions of 2–20% methanol in chloroform (v/v) were pooled and used for further identification.

The hydrolytic product of lysophosphatidylcholines was prepared in the same manner, but it was eluted in 10–50% methanol in chloroform (v/v) from the silicic acid column. The identity of lysophosphatidic acids was determined by paper and thin-layer chromatography (Figs. 9 and 10).

As we have reported in our previous paper[5] the products of the enzymic hydrolysis of sphingomyelins were separated by paper chromatography. The different mobility and some colour reactions of this hydrolytic product demonstrate that it will be a substance (or a mixture of substances) with a more non-polar character than that of the sphingomyelins (according to the chromatographic mobility), probably *N*-acylsphingosylphosphate.

Paper chromatographic separation together with a new colour reaction permits the identification of *N*-acylsphingosylphosphates from sphingomyelins and other related compounds. The combined acid fuchsin–brilliant green staining procedure differentiates between substances which contain negative groups and substances with both positive and negative groups close together. The latter are stained with acid fuchsin and uranyl ions at pH 2 and the compounds with only a negatively charged

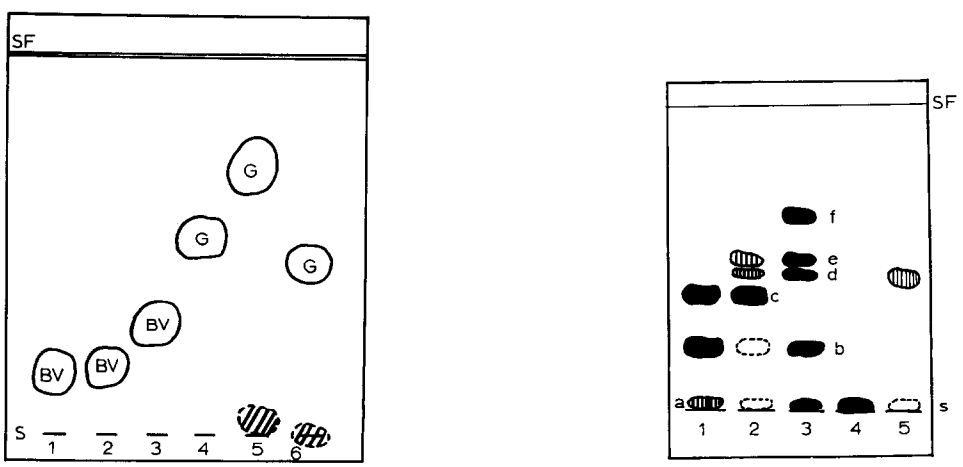

Fig. 9. Paper chromatography of sphingomyelins and related compounds. Paper: Whatman No. 3 impregnated with silica gel, solvent system, chloroform–methanol–conc. NH_4OH (60:8:1, by vol.), detection, acid fuchsin–brilliant green–uranyl nitrate. 1, sphingomyelins (isolated from sheep erythrocytes); 2, hydrogenated sphingomyelins; 3, acetylated sphingomyelins; 4, *N*-acylsphingosylphosphates (isolated from sphingomyelins after enzymic reaction); 5, phosphatidic acids; 6, lysophosphatidic acids. S, starting line; SF, solvent front; BV, blue-violet; G, green.

Fig. 10. Thin-layer chromatographic separation of sphingomyelins and related compounds on Silufol UV 254 sheets. Solvent system, chloroform–methanol–conc. NH_4OH (50:5:2, by vol.); detection, Zinzadze reagent. 1, Glycerophospholipids from sheep erythrocytes; 2, glycerophospholipids of sheep erythrocytes after enzymic hydrolysis with "phospholipase D" isolated from *C. ovis*; 3, standard samples; 4, lysophosphatidylcholins prepared from phosphatidylcholines; 5, lysophosphatidic acids originated from lysophophatidylcholines after enzymic reaction with "phospholipase D" isolated from *C. ovis*. a, lysophosphatidylcholines; b, sphingomyelins; c, phosphatidylethanolamines; d, lysophosphatidic acids; e, *N*-acylsphingosylphosphates; f, phosphatidic acids. S, starting line; SF, solvent front. Blank spots, traces; striped spots, middle strong spots; black spots, very strong spots.

group are coloured with brilliant green, for instance *N*-acylsphingosylphosphates, phosphatidic and lysophosphatidic acids. For this reason the detection with this reagent has a special importance in our experiments.

The characterization of all substances described on thin-layers (on Silufol UV 254) is very useful since the spots are very well separated and the identification is without troubles (Fig. 10).

From our results obtained by paper or thin-layer chromatography, we can conclude that the main sphingosine bases of *N*-acylsphingosylphosphates are 4-sphingenine and small amounts of sphinganine. It is necessary to note that the majority of 4-sphingenin is present in the threo-isomer because the acid hydrolysis causes the conversion of the naturally present erythro-form. The same spectrum was found in sphingomyelins. The spectra of the long-chain bases obtained after the chemical hydrolysis of "*N*-acylsphingosylphosphates" were identified as those of dinitrophenol-derivatives on thin-layer chromatography and paper chromatography. After oxidative cleavage of these derivatives, the isolated higher aliphatic aldehydes were characterized on thin-layer chromatography and paper chromatography[27].

The main component of the fatty acid spectrum in sphingomyelins and *N*-acylsphingosylphosphates is $C_{24:1}$ acid (about 46% in sphingomyelins and 49.3% in *N*-acylsphingosylphosphates). Palmitic acid is present to the extent of 23.6% (sphingomyelins) and 23.7% (*N*-acylsphingosylphosphates). The other acids are present as minor components. A remarkable difference was observed in the concentrations of $C_{18:1}$ (6.0% and 1.2%). We suppose this difference is caused by the isolation and hydrolytic conditions.

DISCUSSION

Our present paper deals with the basic characteristics of a new enzyme belonging to the group of phospholipase D (EC 3.1.4.4). It is a product of the toxin-producing strains of *C. ovis*. This enzyme differs from the other phospholipases D, *i.e.* enzymes splitting phospholipids liberating nitrogen bases, not only by the origin, but mainly by its different substrate specificity. The bacterial phospholipase D does not hydrolyse phosphatidylcholines in the pure state or bound in lipoproteins of blood serum or membranes of erythrocytes under the conditions used for plant phospholipases D. The plant phospholipases D hydrolyse phosphatidylcholines with activation by diethyl ether and Ca^{2+} (see ref. 28). Bacterial phospholipase D hydrolyses sphingomyelins and lysophosphatidylcholines without activation by Ca^{2+} and diethyl ether.

We have studied the enzyme production under various conditions, namely during the growth phases of the bacterium, using different cultivation media. Until this time we were not able to prepare a simple synthetic medium for a good production of the enzyme. Using a combination of $(NH_4)_2SO_4$ and methanol precipitation and ion exchangers we could purify the enzyme 170-fold. The loss of activity during the salting out and methanol precipitation steps is about 40–50%. A low recovery is in contradiction with the stability of the enzyme both in crude supernatants or in the partially purified state during storage.

It is noteworthy that the enzyme is homogeneous in gel filtration but in ion exchangers the enzymic activity is eluted in two separated peaks, the first component which is not bound in CM Sephadex rapidly loses its enzymic activity. The ion

exchanger celluloses are not suitable for the purification of the enzyme as they adsorb the enzyme which cannot be eluted even with 1 M NaCl.

We have studied the hydrolytic activity of the enzyme using pure phosphatidylcholines, lysophosphatidylcholines and sphingomyelins and the phospholipids bound in lipoproteins of blood serum and cell membranes. We could demonstrate only the hydrolysis of lysophosphatidylcholines and sphingomyelins. We have studied the kinetics using both these substrates and the K_m determined showed a very related affinity in the two cases. We have determined the conditions for estimation of the enzymic activity in units and we use lysophosphatidylcholines as substrates because they can be dissolved in buffer without sonication; on the other hand sphingomyelins must be emulsified by sonication. The reaction rate can be modified by the physical character both of the substrate and the hydrolytic products[29]. The reaction rate is of zero order till 20 min using low concentrations of the enzyme. The reaction velocity is enhanced by neopepton and albumin which both contribute to the stability of the enzyme in purified samples but also can activate the enzyme.

The bacterial phospholipase D can be compared with the known phospholipases D of plant origin. These enzymes exist in two forms[30–32], the first one is bound to the plastids and is activated by diethyl ether and Ca^{2+} and splits phosphatidylcholines to phosphatidic acids and free choline. It does not, however, split lysophosphatidylcholines and according to KATES[28] it has a very low hydrolytic activity to sphingomyelins. The soluble form of plant phospholipases D is able to hydrolyse lysophosphatidylcholines to lysophosphatidic acid. CASU *et al.*[33], contrary to our finding with phospholipase D of bacterial origin, could not show the hydrolysis of sphingomyelins in red cell membranes by the plant phospholipase D. So the bacterial phospholipase D is the first enzyme which is known to split sphingomyelins to *N*-acylsphingosylphosphates. This compound was isolated and we have described its chromatographic behaviour and the other analytical procedures to verify the identity of this substance.

On the other hand various bacteria are known as producers of phospholipases C, for instance Clostridia, *Staphylococcus pyogenes*[34], *Bacillus cereus*[35] *etc.* In our previous studies we described the interaction of both groups of bacterial phospholipases on the membranes of red blood cells[4]. Erythrocytes after the action of phospholipase D are not hemolyzed by phospholipases C of *Clostridium perfringens* and of *Staphylococcus pyogenes*. It is interesting that phospholipases D and C of bacterial origin have a toxic effect in experimental animals and are important factors of pathogenity of the bacteria. We succeeded in confirming the enzyme activity of phospholipase D in other species of the family Corynebacteriaceae, namely in *C. haemolyticum* and *C. ulcerans*.

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