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Received October 4, 1963. P.S.E..B.M., 1963, v114.

Influence of Fatty Acids on Appearance of Bacterial Phospholipase D in Incubated Plasma. (28804)

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In addition to clostridia, a number of bacilli are known to elaborate phospholipase D, degrading phospholipids to diglyceride (Dg) and phosphate ester(1). Since some of these bacilli are commonly present in air, water, and dust, incubations of biologic material with phospholipids under nonsterile conditions may lead to destruction of the phospholipids from phospholipase D of bacterial origin. Thus Gollub *et al*(2) found thromboplastin was destroyed by an enzyme elaborated by *B. cerus - B. megatherium.* Kushner and Feldman(3) showed that this bacillus elaborated phospholipase D.

During a study of a post heparin phospholipase A(4), phospholipase D of bacterial origin appeared in serum or plasma after incubation intervals of 8 to 10 hours. Fatty acids (FA) were required for the appearance of this enzyme. While the growth of some bacteria is enhanced by FA(5), an effect of FA on the elaboration of phospholipase D by bacteria has not previously been reported (1).

Methods. The collection of post heparin plasma, the use of Armour #2293 fraction V bovine albumin, and the preparation of egg phosphatidylethanolamine (PE) and lecithin (Le), have been described(4). Globulin was bovine fraction IV of Nutritional Biochemicals Corp. The oleic acid and palmitic acid added to incubation systems were the highest purity products of the Hormel Foundation and Nutritional Biochemicals Corp., respectively. A suitable aliquot of the FA in ethyl ether was introduced into the incubation tube and the ether removed with a current of nitrogen while the tube was warmed to 40°C. The μmoles of FA added to incubation systems are expressed as a ratio to the μmoles of phospholipid present (FA/PL). The optimal value of this ratio varies with the amount of protein present since the available fatty acid is that which exceeds the binding sites on proteins. Alteration of the albumin content of the system required a corresponding change in the amount of fatty acids required for diglyceride formation.

Bacterial contaminant. From incubation systems containing phospholipase D activity, a gram positive, aerobic, spore forming bacillus was isolated. Its morphology and colonial growth suggested *B. subtilis.* This was the organism first reported by Gollub *et al*(2) as the bacterium involved in destruction of thromboplastin. Further specification of the contaminant encountered was not attempted.

Results. Demonstration of diglyceride and phosphoethanolamine from the degradation of PE by bacterial phospholipase D. The two incubations of Fig. 1 differed in that on the left FA were derived from the degradation of PE to LyPE and FA by post heparin plasma(4), while on the right with pre hepa-

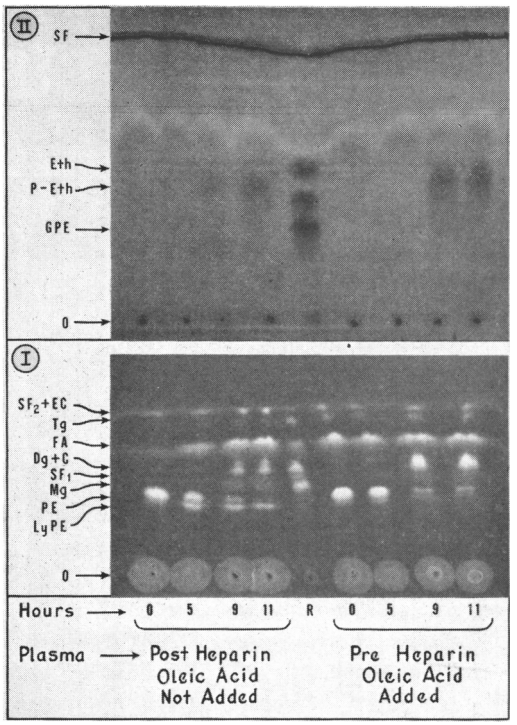

FIG. 1. Degradation of phosphatidylethanolamine to diglyceride and phosphoethanolamine.

The substrate for left and right side of chromatograms contained 12 mg glucose and 200 mg albumin adjusted to pH 8.0 at 5.0 ml with NaOH and used to dissolve 800 μg PE phosphorus. On the right, oleic acid equimolar to the PE was added. Incubations were with 1.0 ml post heparin plasma on the left and with 1.0 ml pre heparin plasma on the right for time intervals indicated. Direct application of 15 μl aliquots of incubation mixtures were made to plate I; 5 μl of a mixture of neutral lipids were applied at R. Chromatogram I was developed with chloroform : methanol : water (80:35:4) for 6½ min, dried at room temperature, then developed again for 6½ min with petroleum ether : ethyl ether : acetic acid (80:15:1). Plate was then sprayed with 0.2% dichlorofluorescein in ethanol.

For chromatogram II, 1 vol of each incubation mixture was added to 4 vol of 4% trichloroacetic acid in chloroform : methanol (2:1). After centrifuging, 10 μl of the upper phase were applied to plate. The 5 μl reference solution applied at R contained ethanolamine, o-phosphoethanolamine and glycerylphosphorylethanolamine. The chromatogram was developed for 35 min with methanol : 10% trichloroacetic acid : 1 M NaCl (90:6:6) and sprayed with ninhydrin reagent.

Abbreviations: O, Origin; LyPE, Lysophosphatidylethanolamine; PE, Phosphatidylethanolamine; Mg, Monoglyceride; SF_1, First solvent front; Dg + C, Isomeric diglycerides and cholesterol; FA, Fatty acids; Tg, Triglyceride; SF_2 + EC, Second solvent front and esterified cholesterol; GPE, Glycerylphosphorylethanolamine; P-ETh, Phosphoethanolamine; Eth, Ethanolamine.

rin plasma, oleic acid equimolar to the PE was added. Aliquots were applied to the plates immediately after addition of the plasma and after incubation intervals of 5, 9 and 11 hours, during which bacterial growth occurred. Thin layer chromatography (TLC) of the degradation of PE to Dg is shown in I; the appearance of phosphoethanolamine proportional to the Dg formed is shown in II. If oleic acid were omitted from the system containing pre heparin plasma, Dg did not appear even at intervals of 20 hours.

Small amounts of monoglyceride are visible in I above the PE area at 9 and 11 hours. At longer intervals this component and FA increase with a decrease of the Dg component. Also the phosphoethanolamine component in II decreases in intensity and ethanolamine appears. If pancreatic lipase is added to the incubation mixtures after all the phospholipid has been degraded to Dg, a rapid conversion of 40-50% of the Dg to monoglyceride and FA occurs.

Demonstration of requirement for FA in elaboration of bacterial phospholipase D. In Fig. 2 a lecithin substrate is used to show the dependence of the elaboration of phospholipase D on the amount of FA. An ali-

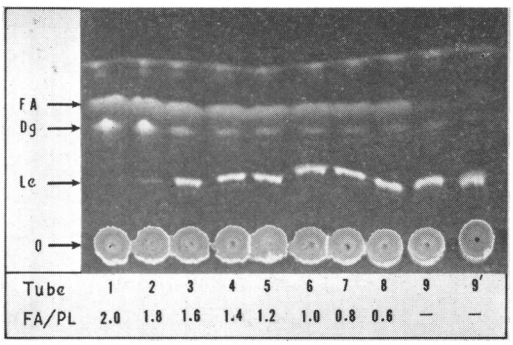

FIG. 2. Requirement for FA in production of bacterial phospholipase D.

Oleic acid was added to tubes 1-8 in an amount to give FA/PL ratios indicated. 26 μmoles of lecithin in 1.0 ml H_2O and 1.0 ml of normal plasma were then added to tubes 1-9. Then 5.0 ml of a solution containing 15 mg glucose, 300 mg albumin, 2.5 ml of 0.1 M Na_2HPO_4 and having an unadjusted pH of 7.3 was added to each tube. A 15 μl aliquot of tube 9 was applied immediately at 9'. After 14 hr of incubation at 38°C, 15 μl aliquots of tubes 1-9 were applied to plate. The chromatogram was developed as chromatogram I in Fig. 1.

Abbreviations: O, Origin; Le, Lecithin; Dg, Diglyceride; FA, Fatty acids.

TABLE I. Effect of Type of FA and Protein on Conversion of Lecithin and PE to Diglyceride.

Phospholipid	Protein	Incubation (hr)	Estimated % conversion phospholipid to diglyceride: Ratio oleic acid/PL .4	.8	1.2	1.8	3.0	No FA	Ratio palmitic acid/PL .4	.8	1.2	1.8	3.0
Lecithin	Albumin	10	5	5	5	5	0	0	0	0	5	5	0
		11½	10	15	15	90	20	0	5	5	15	15	5
	Globulin	10	40	40	90	95	95	15	15	15	25	25	20
		11½	70	80	100	100	100	15	20	40	50	40	40
PE	Albumin	10	15	15	50	60	20	0	10	15	25	25	25
		11½	15	20	60	80	40	0	10	20	30	40	40
	Globulin	10	70	85	85	70	70	60	60	60	60	60	60
		11½	95	95	95	95	95	90	90	90	90	90	90

A large volume of solution contained reagents in the following proportion in 6.0 ml: 15 mg glucose, 300 mg protein, 2.5 ml 0.1 M Na_2HPO_4 adjusted to pH 7.2 at 5.0 ml with HCl; 800 μg phospholipid phosphorus; and 1.0 ml normal plasma. The system was varied to include albumin or globulin and lecithin or PE. 6.0 ml of the solution was added to tubes containing pure oleic acid or palmitic acid at FA/PL ratios shown in Table. Aliquots of 15 μl were applied to plates for TLC of the lipids after 10 and 11½ hr of incubation at 38°C. Visual estimation of the percentage conversion of phospholipid to diglyceride was entered in Table.

quot of a mixture without added oleic acid was applied before and after a 14 hour incubation at 9′ and 9 respectively. Aliquots of similar mixtures containing various amounts of oleic acid were applied at 1-8 after 14 hour incubations. Though some Dg appears at low FA/PL ratios, the maximum conversion was at a ratio of 2.0. Above 2.0, the elaboration of the enzyme was again depressed.

The appearance of the enzyme was observed in incubations ranging between pH 6.2 and pH 9.0, with the optimum near 7.0. A very similar range of activity and optimum pH was found for the enzyme of *B. cereus* by Chu(6).

Effect of type of FA and protein on conversion of phospholipid to diglyceride by bacterial phospholipase D. Table I provides estimates from thin layer chromatograms of the degree of conversion of lecithin and PE to diglyceride in the presence of albumin or globulin and varying concentrations of oleic or palmitic acid. The degradation of both phospholipids was greater in the presence of the unsaturated fatty acid, and the optimal concentration of oleic acid was similar for both. PE was more readily acted upon than lecithin. The conversion to diglyceride was more extensive and the exact concentration of FA was less important in the presence of globulin than albumin. With PE as the substrate and globulin as the added protein, no appreciable requirement for FA was evident.

Additional findings. The enzyme was elaborated irregularly in incubations of plasma in phosphate buffer without added albumin as used in the study of Fig. 2. The optimal FA/PL ratio was much lower without added albumin, as might be expected, being about 0.6 to 0.8. Incubations which included added albumin were uniformly successful in demonstrating phospholipase D activity. The enzyme did not appear if sterile reagents and aseptic technique were used. The 8-10 hour interval for the appearance of the enzyme could be shortened to 4 hours if the system were inoculated with bacteria isolated from mixtures in which the enzyme had already appeared.

If fresh plasma were not used, additional glucose, galactose, or inositol had to be added to insure appearance of the enzyme.

Sodium deoxycholate (0.35%), sodium versenate (0.002 M) and chloromycetin or tetracycline (12 μg/ml) prevented the appearance of the enzyme. Oxalate and citrate had no effect.

Summary. The role of fatty acids in the elaboration of bacterial phospholipase D in incubations of fresh human plasma has been studied. An optimal amount of fatty acid could be specified, dependent on the amount and type of protein present. Enzyme activity

was greater in the presence of cephalin (PE) than lecithin, oleic acid than palmitic acid, and globulin than albumin.

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Received October 4, 1963. P.S.E.B.M., 1963, v114.

Strain Specific Complement-Fixation Differentiation of Influenza B Viruses Isolated in Tissue Culture.* (28805)

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The nature and relationships to each other of the two types of complement-fixing (CF) antigens (soluble and viral) of influenza viruses have been well established(1-5). With the development of strain specific and type specific antisera and antigens(5-7) it has been possible to apply the CF method in serodiagnosis and epidemiologic studies of influenza infections(8,9). In these studies, however, emphasis was placed on influenza type A strains, propagated or isolated in the developing chick embryo.

Many influenza B strains isolated during the 1961-62 epidemics in the Midwest of the United States did not propagate in the chick embryo, but were propagated in monkey kidney tissue culture (MKTC). We have been able to apply the strain-specific CF test and the hemagglutination-inhibition (HI) primarily to study antigenic variations of the isolated strains by using acute and convalescent sera from influenza B epidemics in this area. The results of the analysis are reported here.

Materials and methods. Tissue culture (TC) cells cultivated from kidneys of rhesus *(Macaca mulatta)* monkeys (MKTC) were used for virus isolation. The cells were maintained in a modified Eagle's medium with high cystine as described previously(10).

Virus strains. Influenza B virus strains isolated in MKTC from oropharyngeal secretions of patients with influenza were selected for serologic studies. Strain Illinois 61-952 came from Grand Tower in southern Illinois, strain Iowa 62-21 from Hazelton in northeast Iowa, strain Missouri 61-482 from Potosi, Mo., strain Missouri 61-967 from Jefferson City, Mo., and strain Missouri 62-36 from Marshall, Mo. Jefferson City and Marshall are located in central Missouri, separated by a distance of about 70 miles. Potosi is about 65 miles southwest of St. Louis. All strains were isolated from influenza cases during the winter of 1961-1962. Each strain has had less than 5 passages in MKTC.

Sera. Acute and convalescent sera were obtained from 8 cases of influenza: 1 from Kansas City, Mo., and 7 from Potosi in southeast Mo. Influenza B virus was isolated from the oropharyngeal secretions of the 8 cases. None of the cases had given a history of recent influenza vaccination.

Complement-fixation (CF) test. The method used has been described(11). Sera were inactivated at 56°C for 30 minutes before use.

Hemagglutination (HA) and hemaggluti-

* This investigation was supported in part by U. S. P. H. S. research grant from Nat. Cancer Inst.