Phospholipid Antithromboplastin Not Related to Sphingosine.* (23563)

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In recent papers(1,2), Hecht has reiterated a statement that the phospholipid anticoagulant studied by us(3,4) probably owes its activity to sphingosine.

The work described here tests this hypothesis by chemical reactions that destroy sphingosine or the phospholipid selectively. These reactions were brought about using mixtures made artificially from the phospholipid and sphingosine. By means of chromatography and the assay for antithromboplastic activity(4) it was possible to detect the undestroyed products. In addition, many differences between sphingosine and the phospholipid antithromboplastin, both chemical and biological are listed.

Materials and methods. The assay for anticoagulant activity has been described elsewhere(4). The clotting system contained 0.1 ml of human plasma, 0.1 ml of the solution to be tested, (or water in the control), 0.1 ml of human brain thromboplastin, 0.1 ml of 0.02 M calcium chloride solution. The test solution of the phospholipid was made using sodium desoxycholate as a solubilizing agent. The final concentrations of test substance and of sodium desoxycholate were the same. Sodium desoxycholate alone gave clotting times similar to those of the control, in the range of concentrations employed. The dl-sphingosine† was tested in aqueous solution without desoxycholate. Clotting tests were done in siliconized tubes at 37°. The meaning of the numerical activities assigned to the phospholipid fractions is described elsewhere(4). The unit of activity is the ratio of the clotting time of the test material to that of a standard at the same concentration of 1 mg/ml in a clotting system described above. The paper chromatography used Whatman No. 1 paper in a system of diethyleneglycol, n-butanol, and water (4:1:1, by volume) and the material was located by spraying with ninhydrin (0.5% in butanol saturated with water).

Results. The first experiment was based on the ready destruction of sphingosine by periodate(5-7).

Experiments were run in parallel using 200 mg of phospholipid antithromboplastin with and without the addition of 20 mg of dl-sphingosine.† As a control, 20 mg of sphingosine was treated separately. The experiments were carried out in aqueous solutions using 600 mg of sodium periodate (0.3 M) for periods from 45 minutes to 48 hours. Two different phospholipid fractions were employed of 200 and 180 units of activity per mg(4,8). In order to determine whether any sphingosine was left after the oxidation, the oxidation products were chromatographed on paper beside synthetic dl-sphingosine. Athough 5 μg of sphingosine could be detected on the paper with ninhydrin, no sphingosine could be found in the oxidation products, even when the entire product from 20 mg of sphingosine was put on the paper. In order to test for anticoagulant activity, it was necessary to remove the inorganic salts by dialysis. The 2 phospholipid preparations then had anticoagulant activity of 150 and 160 units/mg.

Preparations of the phospholipid anticoagulant were completely degraded by reduction with lithium aluminum hydride in ether, although sphingosine resists this treatment. This reaction was indeed utilized by Grob and Gadient(9) in their total synthesis of sphingosine. When a mixture of 200 mg of phospholipid and 100 μg of dl-sphingosine was reduced and the mixture was treated with just sufficient water to decompose the lithium aluminum hydride, and the ether was removed after drying over sodium sulfate, the product had no anticoagulant activity.

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† The dl-sphingosine was kindly provided by Dr. D. Shapiro and by Dr. C. A. Grob and was obtained by total synthesis.

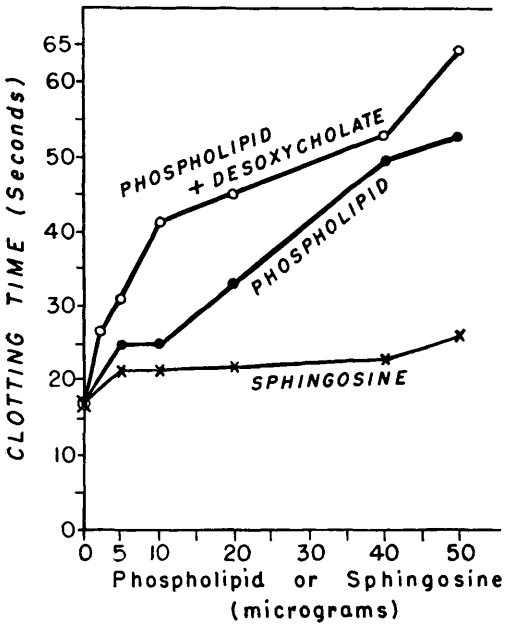

FIG. 1. Effect of phospholipid antithromboplastin fraction and dl-Sphingosine† (aqueous solution) on clotting time of activated, normal human, citrated plasma. Clotting system—see text.

Sphingosine was shown to be still present by paper chromatography of an amount corresponding to 16 micrograms of the original sphingosine. This paper chromatography was complicated by the effect noted by Marinetti's group(5), a slowing of the movement of the sphingosine in the presence of the products from the reduction of the phospholipid. This difficulty was overcome by reducing the phospholipid and then adding sphingosine to the reduction products to compare with the reduced mixture of sphingosine and phospholipid. The ethereal solution from the reduction of the phospholipid alone did not give a reaction with ninhydrin.

Comparison of anticoagulant activity. Sphingosine is relatively inactive (Fig. 1), in the activated clotting system used to assay the phospholipid, which measures the ability of the substance to neutralize the clot-accelerating effect of brain thromboplastin. This clotting test employs citrated human plasma, with which sphingosine forms a precipitate. The phospholipid antithromboplastin is, moreover, an active anticoagulant when injected into dogs(4). Unfortunately, the activity of sphingosine has not been studied *in vivo*.

Comparison of other chemical properties. Sphingosine forms a water-soluble hydrochloride, but the phospholipid antithromboplastin retains its activity when thoroughly washed with dilute hydrochloric acid(10). The phospholipid is destroyed by very mild treatment with alkali, while sphingosine is ordinarily prepared by release from its sulfate with alkali and resists alkali at 100° (5,11,12).

The purified phospholipid antithromboplastin has been studied by the paper chromatographic methods of Amelung and Böhm(13) and of Lea, Rhodes and Stoll(14). Dr. Marinetti of the University of Rochester has kindly studied its behavior for us in his solvent systems(15,16). The intact phosphatide has 2 components corresponding to phosphatidylserine and probably a lyso- or acetal-phosphatidylserine. After hydrolysis, serine was the only nitrogenous base found by paper chromatography in a variety of solvents. Cruder fractions, like the Folch fraction III (17), with anticoagulant activity of 160 units/mg. also contain phosphatidylethanolamine and some contaminating amino-acids. The amino-acids can be removed by the cellulose column method of Lea, Rhodes and Stoll(14), with complete retention of activity. It is of interest that when 100 μg of dl-sphingosine in chloroform solution is percolated through 13 g of moist cellulose, the sphingosine is completely retained. This is also true when the sphingosine solution is acidified(14).

Summary. Methods involving separate selective destruction of sphingosine and phospholipid antithromboplastin in mixtures of the two indicate that they cannot be the same. Differences in their chemical and biological properties also support the view that they are distinctly different anticoagulants.

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Inhibition of Azo Dye Carcinogenesis by Adrenalectomy and Treatment With Desoxycorticosterone Trimethylacetate.*† (23564)

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Symeonidis, Mulay and Burgoyne(1) reported that adrenalectomy or overdosage of intact rats with desoxycorticosterone acetate (DCA) prevented azo dye carcinogenesis. On the other hand Griffin, Richardson, Robertson, O'Neal and Spain(2) got just the opposite results. The former authors employed 4-dimethylaminoazobenzene (DAB) and subcutaneous implants of pellets of DCA while the latter workers used 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), and subcutaneous injections of DCA in oil. The amount of steroid given per month by either group was approximately the same, making it highly unlikely that differences in methods of administration of DCA accounted for the divergent findings. Also, it seems highly unlikely that the use of different carcinogens could account for the discrepancies since the effects produced by carcinogenic azo dyes are qualitatively the same(3); 3'-Me-DAB is a more rapidly acting hepatic carcinogen than is DAB(4). During the past 5 years, we have been attempting to determine the effects of adrenal steroids on the course of azo dye carcinogenesis and have followed the work of the above authors with considerable interest. We find, as did Symeonidis *et al.*(1), that treatment of totally adrenalectomized rats with 11-desoxycorticosterones prevents azo dye carcinogenesis, but unlike these workers, we find that overdosage of intact rats with 11-desoxycorticoids fails to prevent the carcinogenic process. Furthermore, we find that the presence of accessory or regenerated adrenal cortical tissues inhibits the protective effect of adrenalectomy and hormonal treatment. The basis of these conclusions is reported here.

Methods and materials. Male rats of the Long-Evans strain weighing approximately

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