Non-Antigen–Antibody Precipitin Reactions Observed with Dextran Sulfate, DEAE-Dextran, Antibiotics, Proteins, and Phospholipids[1] (34580)

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Routine bacteriologic agar and several culture media have been shown to contain inhibitors of the polymyxin and aminoglycoside antibiotics (1). This effect can be abolished by substitution with agarose, which is virtually free of sulfate groups, or by addition of protamine or DEAE-dextran to agar. It is postulated that the inhibitory effect observed with agar is due to formation of complexes between negatively charged sulfate groups and positively charged amino groups of the basic antibiotics.

The availability of an inhibitor-free gel, agarose, permitted further extension of these observations to identify substances in tissue which bind antibiotics (2) and the current finding of formation of visible precipitin bands between dextran sulfate and basic antibiotics of the polymyxin and aminoglycoside families. During the course of these studies, precipitin reactions also were observed between inositol phosphatide and polymyxin, DEAE-dextran and heparin, and dextran sulfate with lysozyme and human serum beta lipoproteins. All of these are unrelated to antigen–antibody interactions which are frequently studied by means of precipitin formation in gels.

Materials and Methods. Diffusion tests were conducted using 10 ml of 2 agarose (Sea-Kem, Marine Colloids, Inc.) in medium 199 (Microbiologic Associates) in 60-mm plastic cups. The pH was adjusted to desired level with 0.1 *N* HCl or NaOH. The agar was cut with a punch template to form seven wells. After solutions were added to the wells, the plates were held for 48 hr at 4° in a moist chamber. Miniature precipitin studies were conducted by placing 0.15 ml of agarose medium containing various concentrations of dextran sulfate (Sigma) in the small plastic cups used for Micro-Test determinations (Falcon Plastics Division of Bioquest). Medium 199 containing various concentrations of antibiotic was then added in a volume of 0.15 ml. Plates were read after 30-, 60-, and 90-min incubation at room temperature. Serum was studied for presence of alpha and beta lipoproteins by paper chromatography using the method of Lees and Hatch (3). Ultrafiltration was performed by the method of Toribara (4).

Antibiotics tested included polymyxin B, colistin methanesulfonate, gentamycin, neomycin, streptomycin, kanamycin, penicillin G, tetracycline, fusidic acid, and cephalexin, obtained as assay standards from the manufacturers. Other reagents were heparin, lithium salt (Calbiochem), inositol phosphatide, phosphatidyl ethanolamine (Nutritional Biochemicals Corp.) egg yolk lecithin (prepared by Dr. Fritz Henn, University of Virginia, Department of Biochemistry), egg white lysozyme or muramidase (Worthington Biochemical Corp.), human urine lysozyme and rabbit antihuman lysozyme (kindly supplied by Dr. Elliot Osserman, College of Physicians and Surgeons, Columbia University (5), and similar material from Lysozyme Products, Inc. Diethylaminoethyl dextran (mol wt 2×10^6) was obtained from Pharmacia, Uppsala, Sweden, and Triton X-144 (alkyl phenoxy polyethoxy ethanol) from Rohm and Haas.

Results. Reactions between antibiotics and dextran sulfate. Serial 2-fold dilutions in

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TABLE I. Effect of pH of Medium on Formation of Precipitin Lines in Gel Diffusion Between Dextran Sulfate (mol wt 2,000,000) and Polymyxin and Aminoglycoside Antibiotics.

	Minimum conc (μg/ml) of antibiotic producing precipitin lines			
Antibiotics pH:	3	5	7	9
Polymyxin B	64	64	64	64
Colistin methane sulfonate	125	250	>500	>500
Neomycin	64	64	64	250
Gentamycin	32	32	64	500
Kanamycin	64	125	250	>500
Streptomycin	250	250	500	>500

medium 199 of polymyxin B, kanamycin, neomycin, gentamycin and streptomycin, starting at 500 μg/ml were tested in agarose gel diffusion plates adjusted to pH 3, 5, 7, or 9 against solutions of dextran sulfate in the center well. Dextran sulfates (mol wt 5–40,000, 60,000, 200,000, 500,00 and 2 million) were tested at concentrations of 2 mg/ml. Precipitin lines were not observed with dextran sulfate of 5–40,000; light bands were seen with preparations of 60,000 and 200,000, but well defined lines were noted with both 500,000 and 2 million molecular weight polymers. Dextran sulfate 2 million was used in all further experiments at the optimal concentration of 2 mg/ml. The effect of pH on formation of precipitin lines is shown in Table I. Note that the greatest sensitivity was at low pH values.

A much more rapid and slightly more sensitive method of observing precipitin reactions was obtained by use of direct overlay of antibiotic solutions in medium 199 in Micro-Test cups containing agarose gel employing concentrations of dextran sulfate ranging from 0.25 to 2 mg/ml. Precipitin lines appeared within a few minutes and reached maximum intensity within 1 hr. They were most marked with dextran sulfate, 0.25 mg/ml. Equal results were obtained at pH 3, 4, and 5; reactions were less sensitive at higher pH. The minimum concentrations of antibiotic that could be detected were: polymyxin B, 32; colistin methane sulfonate, > 1000; neomycin, 16; gentamycin, 32; kanamycin, 64; and streptomycin, 125-μg/ml, respectively.

Precipitin lines were not observed with the following antibiotics: penicillin G, tetracycline, fusidic acid, or cephalexin.

Reactions between antibiotics and other reagents. Heparin at a concentration of 10,000 units/ml reacted with the antibiotic polymyxin B at 500 μg/ml or greater, but with none of the other antibiotics tested. Inositol phosphatide (2 mg/ml) also produced a clear precipitin line with polymyxin at concentrations up to 32 μg/ml (Fig. 1D). No lines were observed between phosphatidyl ethanolamine or egg yolk lecithin (solubilized by addition of Triton X-144) and polymyxin B under similar experimental conditions.

Precipitin lines formed with DEAE-dextran. This compound was tested in gel diffusion plates at 2 mg/ml against antibiotics as described above. No precipitin bands were noted even at antibiotic concentrations of 1000 μg/ml. Well-defined lines were noted, however, with heparin; the lowest detectable level of heparin was 5 units/ml (Fig. 1A). No reactions were observed between DEAE-dextran and 30 human sera.

Precipitin lines formed by dextran sulfate with human serum. Thirty human sera sent to the routine clinical chemistry laboratory were tested at pH 3, 5, 7, and 9 versus 2-mg/ml dextran sulfate solution placed in the center well. Single bands were observed within 24–48 hr with all sera at all pH values tested (Fig. 1E). Heating serum at 56° for 2 hr did not affect precipitin formation. Ultrafiltrates of 4 human sera contained no precipitable material in 2 and very light bands were seen only at pH 3 in the other two. No precipitin reactions were observed between dextran sulfate and urine from healthy subjects. Direct addition of 0.1 ml of the dextran sulfate solution to 0.9 ml of serum produced a marked floculation. Three substances in serum were considered as possible candidates for this reaction. These were (i) the very basic protein, lysozyme, (ii) protamine, and (iii) beta lipoproteins which are known to form complexes with dextran sulfate (6).

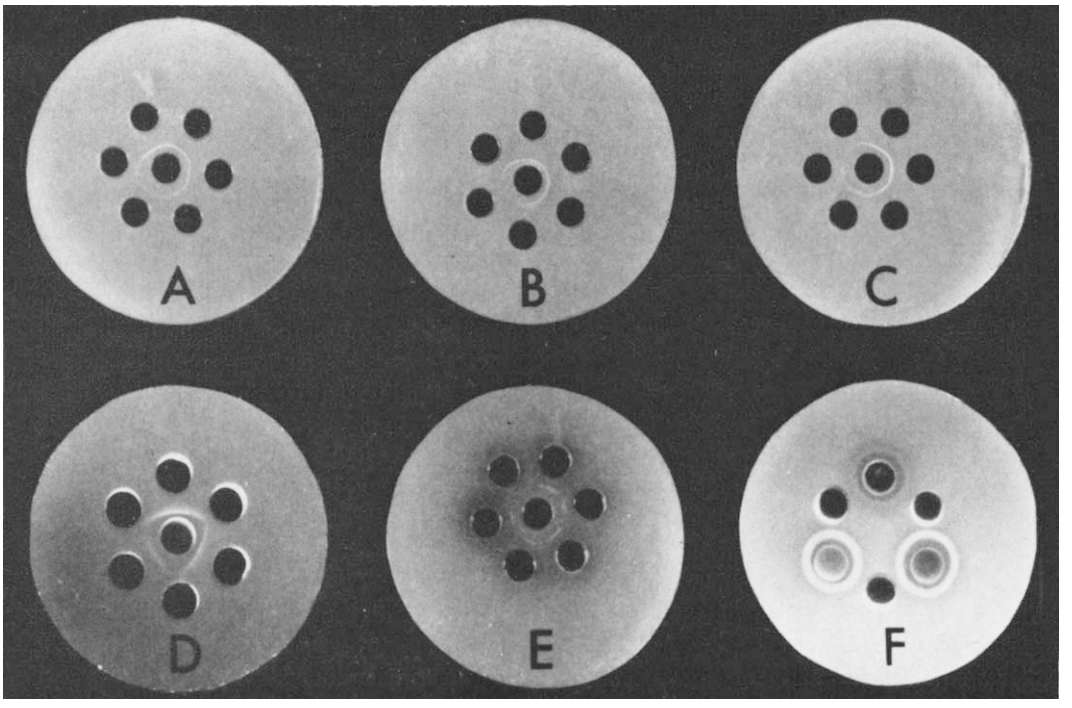

FIG. 1A–D. Precipitin lines formed between 2-fold dilutions of: heparin (beginning at 156 units/ml versus DEAE-dextran 2 mg/ml in the center (A); protamine (beginning at 1 mg/ml) versus 2 mg/ml of dextran sulfate in the center(B); egg white lysozyme (beginning at 1 mg/ml) versus 2 mg/ml of dextran sulfate in the center(C); and polymyxin B (125, 64, and 32 μg/ml) versus inositol phosphatide 2 mg/ml in the center well (D). (E) Demonstrates lines formed between 6 undiluted samples of human serum in the outer wells and dextran sulfate 2 mg/ml in the center. (F) Reveals ring precipitin reactions noted with 3 human sera placed in alternate wells in agarose gel containing 0.05 mg/ml of dextran sulfate. The serum in the upper most well is from a patient with hypo-beta lipoproteinemia. The other 2 are from normal subjects.

Dextran sulfate was tested against protamine and found to produce single bands at concentrations of 32 μg/ml (Fig. 1B). Egg white lysozyme also formed bands at 32 μg/ml (Fig. 1C). Human urinary lysozyme (5) reacted at 64 μg/ml. Rabbit antihuman lysozyme, however, was much more active since undiluted rabbit antiserum produced a single band against as little as 8 μg/ml of human lysozyme. Despite the far greater sensitivity of rabbit antiserum to detect lysozyme than dextran sulfate, no precipitin bands were observed between antilysozyme and serum from healthy humans. Thus, lysozyme appeared to be an unlikely candidate to explain the reaction between dextran sulfate and human serum. Protamine would not be expected to be present in serum. Accordingly, the known reaction between dextran sulfate and beta lipoprotein was further examined.

Evidence that precipitin lines formed between dextran sulfate and human serum are due to beta lipoproteins. Dextran sulfate and other sulfated polymers have been extensively used in the isolation and purification of beta lipoproteins (6). Fresh human serum, 0.9 ml, was treated with 0.1 ml of 2-mg/ml dextran sulfate in medium 199. The precipitate that immediately formed was removed by centrifugation and the supernate as well as untreated serum were subjected to paper electrophoresis by the method of Lees and Hatch (3), in barbital–albumin buffer in a Beckman/Spinco electrophoresis cell. Papers were developed with oil red O dye and exam-

ined. Both alpha and beta lipoproteins were readily characterized by this procedure in normal human serum. Beta lipoproteins were selectively removed from serum treated with dextran sulfate.

A ring diffusion precipitin test was developed using 0.05 mg/ml of dextran sulfate dissolved in 2 % agarose–199 medium at pH 5.0. Serum from normal subjects, added to cups produced a well defined ring on overnight incubation at 4° (Fig. 1F). Serum from a patient with known hypo-beta lipoproteinemia produced only a very faint zone, seen in the top well in Fig. 1F. Thus, the formation of a precipitin band between dextran sulfate and human serum appeared to be related to the presence of beta lipoproteins.

Discussion and Summary. A series of precipitin reactions in agarose gel are described which resemble, but are unrelated to antigen–antibody reactions commonly studied by similar methods. This appears to be due to the lack of interference in agarose by sulfate groups present in ordinary agar. The reactions appear to be due to complexes formed between highly negatively charged compounds such as dextran sulfate, heparin, and inositol phosphatide and the highly positively charged polymyxin and aminoglycoside antibiotics, lysozyme, protamine, DEAE-dextran, and presumably beta lipoproteins as well. Unfortunately, the formation of a precipitin band between human serum and dextran sulfate and sensitivity limited to antibiotic concentrations several-fold higher than achieved during antimicrobial therapy, prevents direct adaptation of these methods to a rapid procedure for detecting basic antibiotics in serum. The approach could conceivably be adapted to measurement of basic drugs in urine or to ultrafiltrates of serum.

Evidence is presented that the precipitin band formed between human serum and dextran sulfate is due to complexes formed with beta lipoproteins. A simple method for screening for hypo-beta lipoproteinemia is described. It is unlikely that protamine is present in sufficient concentrations in human serum to account for this band. Human serum contains small amounts of lysozyme, but in somewhat lower concentrations than can be measured by the precipitin reactions reported here with dextran sulfate. Osserman (5) reported lysozyme levels of about 7 μg/ml in human serum, while dextran sulfate detects about 64 μg/ml. It is possible that dextran sulfate could be useful in detecting lysozyme in urine of patients with monocytic leukemia and other diseases.

A non-antigen–antibody precipitin reaction in agarose has recently been described by Gardner and Rosenberg (7) between a lipoidal tissue extract and an IgM serum component. Several other isolated reports of similar phenomina are cited by these authors. This is the first report, to our knowledge, of precipitin reactions between relatively small molecules such as antibiotics and sulfated polysaccharides or acid phospholipids and between heparin and DEAE-dextran.

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1. Kunin, C. M., and Edmondson, W. P., Jr., Proc. Soc. Exp. Biol. Med. **129**, 118 (1968).

2. Kunin, C. M., J. Infec. Dis. **121**, 55 (1970).

3. Lees, R. S., and Hatch, F. T., J. Lab. Clin. Med. **61**, 518 (1963).

4. Toribara, T. Y., Tevepak, A. R., and Dewey, P. A., J. Clin. Invest. **36**, 738 (1957).

5. Osserman, E. F., and Lawlor, D. P., J. Exp. Med. **124**, 921 (1966).

6. Cornwell, D. G., and Kruger, F. A., J. Lipid. Res. **2**, 110 (1961).

7. Gardner, E. and Rosenberg, L. T., Immunology **17**, 71 (1969).

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