curves suggest protein-protein interaction at higher concentrations of the proteins.

The result of the alkali denaturation test is interesting. Each protein fraction gave a specific T ½ for "molecular decay reaction." Both protein waves were affected in similar fashion, indicating that decay reaction involved molecule as a whole. Activation reaction for albumin, however, revealed differences between two waves indicating that this probably concerns different places on same protein molecule. Similar observations in various Cohn fractions and plasma albumin obtained from different species have been reported in the literature(2).

Summary. The polarographic characteristics of pure protein fractions appear to be specific in nature. These techniques therefore could be utilized as useful tools for studying normal and abnormal serum proteins.

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Differentiation of Muramidase and β-Lysin. (31046)

DAVID M. DONALDSON* AND JOHN G. TEW
Department of Bacteriology, Brigham Young University, Provo, Utah

β-lysin and muramidase (lysozyme) are 2 bactericidal substances found in the sera from a variety of mammalian species. These two substances have numerous similarities which make them difficult to differentiate but which separate them from bactericidal antibody-complement systems. Both β-lysin and muramidase are lethal for Gram-positive species in contrast to the antibody-complement systems which are primarily bactericidal for Gram-negative organisms. They are also less specific than antibody in their bactericidal spectra. β-lysin and muramidase resist inactivation at boiling temperatures for short periods of time; whereas, complement is readily destroyed by heat. Furthermore, it has been demonstrated that β-lysin can be separated from both antibody and complement by Seitz filtration of serum(1). Data will be presented indicating that this is also the case with serum muramidase. Early evidence indicating that β-lysin and muramidase were probably different substances was obtained by Myrvik and Weiser(2) who demonstrated cytologic differences in the appearance of *Bacillus subtilis* exposed to egg white muramidase and to rabbit serum. Their observation that the destruction of the bacterial cell was more complete with rabbit serum than with egg white muramidase could be due to the combined effects of serum β-lysin and serum muramidase. Much of the evidence to be reported demonstrating that these substances are different was dependent on the discovery of purification techniques for β-lysin(1) and the subsequent production of neutralizing anti-β-lysin serum(3).

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Materials and methods. Rabbit β-lysin and anti-rabbit-β-lysin were prepared and as-

TABLE I. Effects of Filtration, Bentonite Treatment, and Anti-β-Lysin on Bactericidal Activity of Samples Containing β-Lysin and/or Muramidase.

Sample	*Bacillus subtilis* Control	Filtered*	Bentonite treated†	Anti-β-lysin‡	*Micrococcus lysodeikticus* Control	Filtered*	Bentonite treated†	Anti-β-lysin‡
	units/ml				units/ml			
Rabbit β-lysin	32	<1	<1	<2	8	<1	<1	<2
Egg muramidase (80 mb/ml)	8	<1	<1	8	128	<1	2	128
Rabbit spleen muramidase	<1	—	—	—	4	<1	<1	8
Alveolar macrophage extract	<1	—	—	—	16	<1	<1	16
Rabbit serum	32	<1	<1	<2	8	<1	<1	<2
Guinea pig serum	<1	—	—	—	4	<1	<1	4

* Fifteen ml samples filtered through a Hercules, type ST, cellulose-asbestos filter.
† Samples mixed with bentonite (1.5%) and shaken for 3 to 5 min before removal of bentonite by centrifugation.
‡ Samples exposed to equal volume of anti-rabbit-=-lysin at 37°C for 30 min prior to bactericidal assay.

sayed for bactericidal and neutralization activities as previously described(1,3), except that in the bactericidal tests the contact time between the *Bacillus subtilis* and the test sample was reduced from two hours to one hour. In some of the bactericidal and neutralization tests *Micrococcus lysodeikticus* was substituted for *B. subtilis* as the test organism. The *M. lysodeikticus* was grown for 48 hours at 37°C in brain heart infusion broth and diluted in 2.5% peptone saline to make a suspension containing 300,000 to 500,000 organisms/ml. One-tenth ml of this bacterial suspension was added to 1 ml of the different dilutions of the sample being assayed for bactericidal activity. These mixtures were incubated at 37°C for one hour before pour plates were prepared to determine the numbers of surviving bacteria. The 99% bactericidal unit is defined as that amount of bactericidal substance required to kill 99% of the organisms during the one hour incubation. In all neutralization tests the Seitz-filtered anti-rabbit-β-lysin was allowed to react with an equal volume of the test sample for 30 minutes at 37°C before the antiserum-sample mixtures were diluted and assayed for bactericidal activity.

The 3× crystallized egg white muramidase was supplied by Sigma Chemical Co. Rabbit spleen muramidase was prepared as outlined by Jolles(4). The collection of alveolar macrophages and the preparation of a muramidase-rich extract of these cells was as described by Myrvik *et al*(5). In addition to the bactericidal assay, muramidase was assayed using a modification of Smolesis and Hartsell(6) technique in which the decrease in turbidity of a killed *M. lysodeikticus* suspension was measured to determine enzyme concentration. The dead *M. lysodeikticus* was allowed to react with the enzymes for 10 minutes at 37°C in 0.1 M phosphate buffer at pH 6.0. The capacity of the test samples to reduce the turbidity of *B. subtilis* was measured with the same conditions used in the muramidase assay.

In the gel filtration studies 5 ml of serum was placed on a column of 15 mm diameter with a Sephadex G-75 bed volume of 70 ml. The flow rate of 0.05 M NaCl was 30 ml/hour, and the temperature was 21°C.

Results. Table I presents results on the effect of filtration through an asbestos-cellulose filter of Seitz type, and treatment with either bentonite or anti-rabbit-β-lysin on the bactericidal activity of samples containing muramidase and/or β-lysin. An examination of these results will point out some similarities and differences between β-lysin and muramidase as well as give insight into the types of bactericidal substances present in serum and alveolar macrophages. In every case where the sample was bactericidal for either

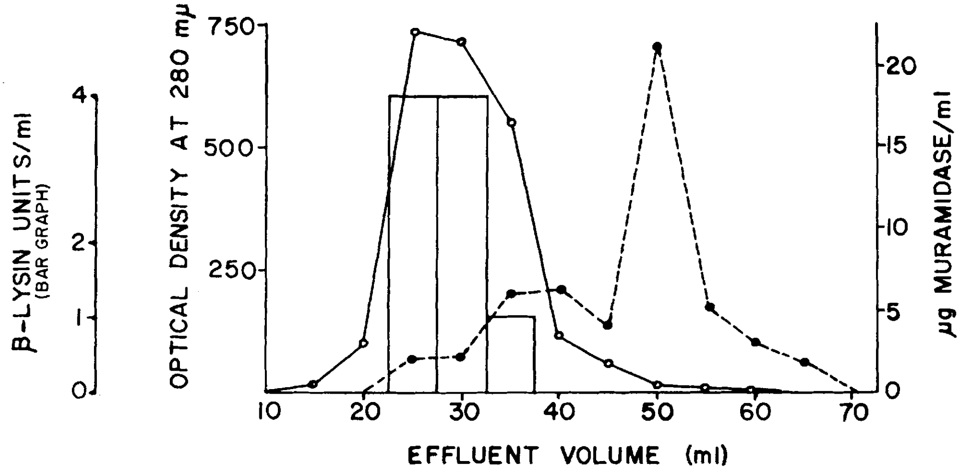

FIG. 1. Separation of serum muramidase and β-lysin by filtration through Sephadex G-75. ——— Optical density of 280 μ of sample diluted 1:15. - - - - - - mg muramidase/ml.

organism, Seitz type filtration or treatment with bentonite removed the lethal activity. The results of the first two lines of this Table reveal that although both organisms are susceptible to the lethal action of egg white muramidase and β-lysin, *B. subtilis* is about 4 times more susceptible to β-lysin than is *M. lysodeikticus*. On the other hand, *M. lysodeikticus* is about 15 times more susceptible than *B. subtilis* to muramidase. β-lysin is a primary bactericidal substance in rabbit serum for both organisms since the anti-rabbit-β-lysin, which neutralized 32 units of purified β-lysin, neutralized rabbit serum bactericidal activity for both organisms. Such antiserum did not neutralize the bactericidal activity of either egg white muramidase, rabbit spleen muramidase, aqueous extracts of rabbit macrophages, or guinea pig sera against *M. lysodeikticus*. This fact in addition to the observation that these preparations had relatively little effect on the viability of *B. subtilis* indicate the β-lysin was not present in the guinea pig serum, the aqueous extracts of rabbit macrophages, and the muramidase preparations.

The results of turbidity-reducing activity of the various preparations on both *M. lysodeikticus* and *B. subtilis* were as expected if one assumes that the reduction in turbidity is due to muramidase and not β-lysin. This assumption is based on the observations that purified β-lysin did not reduce the turbidity of a suspension of killed *M. lysodeikticus* cells; whereas, the egg white and spleen muramidase preparations reduced such turbidity. Turbidity reduction was also observed with rabbit and guinea pig sera as well as the aqueous extracts from alveolar macrophages. In no case did anti-rabbit-β-lysin of guinea pig origin differ from filtered guinea pig serum in interfering with the turbidity reducing activity of muramidase. In accordance with the discussed bactericidal results, Seitz type filtration and bentonite treatment removed the turbidity-reducing capacity of all active preparations. The reductions in turbidity of *M. lysodeikticus* suspensions catalyzed by egg and rabbit spleen muramidase were several times greater than the reductions with suspensions of *B. subtilis* with the same enzyme preparations.

Gel filtration was used to separate the serum β-lysin from serum muramidase. The Sephadex G-75 employed has an approximate exclusion limit of molecules with molecular weights greater than 50,000. Gel filtration studies in our laboratory indicate that in serum, β-lysin is associated with molecules which have molecular weights of 200,000 or greater. In contrast, muramidases are basic proteins with molecular weights near 15,000 (7). The results of the serum gel filtration are illustrated in Fig. 1. As expected, the lethal activity of serum for *B. subtilis* was found in the effluent fractions containing the majority of the proteins; whereas, the peak turbidity reducing activity for *M. lysodeikticus* was found in later fractions which contained low concentrations of protein. Anti-β-lysin neutralized the activity of all fractions that were bactericidal for *B. subtilis* indicating that the lethal factor in these fractions was β-lysin. The double muramidase peaks in Fig. 1 were observed every time this experiment was repeated suggesting that two types of muramidase may be present in rabbit serum.

Discussion. In contrast to the action of muramidase, little is known concerning the biochemical reactions involved in bactericidal effect of β-lysin. All attempts in this laboratory to substitute β-lysin for either complement or antibody in known antibody-complement bactericidal systems have been unsuccessful. Furthermore, attempts to enhance the lethal effects of antibody-complement systems with purified β-lysin have been unsuccessful. The observation that β-lysin did not reduce turbidity of non-viable suspensions of *B. subtilis* or *M. lysodeikticus* indicates that the mode of action of β-lysin differs from that of muramidase and is not related to hydrolysis or dissolution of the bacterial cell wall.

Further insight into the specificity of neutralizing antibody against β-lysin was acquired when it was shown to be incapable of neutralizing muramidase preparations of either avian or rabbit origin. Such anti-β-lysin has previously been shown to neutralize the bactericidal activity of β-lysins from different species as well as plakin, the bactericidal substance active against *B. subtilis* found in rabbit platelets(3). Like muramidase, leukin, the bactericidal substance of peritoneal leukocyte origin was not neutralized by anti-β-lysin. The failure of anti-β-lysin to neutralize the bactericidal activity of alveolar macrophage extracts coupled with the reported lack of susceptibility of *B. subtilis* of these macrophage extracts indicated an absence of β-lysin in these cells. The turbidity reductions and bactericidal test results on *M. lysodeikticus* implicating muramidase as an active constituent in these cells are consistent with results of Myrvik *et al*(5) who used *Sarcina lutea* as an indicator organism in determining lysozyme content of alveolar macrophages.

It is believed that the use and expansion of both the neutralization and gel filtration techniques will be of value in making both quantitative and qualitative measurements of the bactericidal components of various tissues and fluids suspected of containing β-lysin and muramidase.

Summary. Although β-lysin and muramidase are similar in their bacterial spectrum, heat stability, and adsorption by bentonite on an asbestos-cellulose filter pad of the Seitz type, they differ in other respects. β-lysin is relatively more effective as a bactericidal agent for *Bacillus subtilis* than for *Micrococcus lysodeikticus*. This quantitative relationship is reversed with muramidase. Unlike muramidase, purified β-lysin did not reduce the optical density of killed suspensions of bacteria. Anti-β-lysin serum did not neutralize the bactericidal activity of muramidase. Serum β-lysin and muramidase were separated by gel filtration with a Sephadex G-75 column.

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