cluded. Further studies are indicated on the comparative effects of oral *vs* parenteral administration of proteolytic enzymes and of stomach tube feeding *vs* ingestion as a component in the diet. Such studies might indicate whether the effects of the proteolytic enzymes reported above were mediated at the buccal, intestinal or systemic level.

Summary. Immature male rats were fed a natural food stock ration supplemented with graded levels of trypsin, chymotrypsin or proteolytic enzymes derived from *Carica papaya*. A marked hypertrophy of the submaxillary glands occurred in rats fed the above diets in contrast to those of rats fed the unsupplemented stock ration.

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Activity of the Antibody-Complement System and Lysozyme Against Rough Gram Negative Organisms.* (28519)

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The killing of gram negative bacteria by serum has been attributed generally to the antibody-complement system(1). Once killed by the substances of this system, these bacteria may be lysed by the action of lysozyme (2). Recent observations that purport to modify this view, at least with rough organisms, have been reported(3,4,5). In contrast to smooth strains of the gram negative enteric bacteria, rough variants are extremely sensitive to the bactericidal action of normal serum and this property constitutes one of the distinguishing characteristics of rough variants(6). The bactericidal activity of precolostral calf serum(3) and of the serum of piglets reared under sterile conditions without maternal colostrum(4) against rough strains has been attributed to non-specific substances in the apparent absence of antibody. In another approach toward determining the serum substances responsible for bactericidal action, with which this report is directly related, the presence of antibody was considered, but the interesting suggestion was made that it played no role in bactericidal action of normal serum(5). This was based upon the finding that absorption of normal serum with cells of a rough variant, *Escherichia coli* strain Lilly, resulted in a loss of lysozyme in the serum with a concomitant loss of bacteriolytic activity that was restored by addition of lysozyme to the absorbed serum. It was postulated that the killing and lysis of that organism resulted from the combined actions of complement and lysozyme without the mediation of antibody. The loss of activity resulting from the absorption was attributed to lysozyme rather than to antibody(5). This conclusion, which is incompatible with the classic concept that complement functions in the killing and lysis of bacteria sensitized by antibody, warranted further study.

Materials and methods. Egg white lysozyme, 2× crystallized, and histone, products of Worthington Biochemical Corp., were used. The cultures of *Escherichia coli* B and *Salmonella typhosa* Mrs. S were obtained from Mr. A. Abrams, Walter Reed Army Inst. of Research, and *Escherichia coli* Lilly from Dr. A. Wardlaw, Connaught Laboratories, Univ. of Toronto. These cultures satisfy most of the criteria used in defining rough variants(6). Sera were absorbed with 3× saline washed, heat killed (60°C for 1 hr) organisms using about 10^{11} cells per ml of serum. Antisera were prepared in rabbits by 2 subcutaneous injections of about 10^9 living organisms suspended in saline after 15 hours growth on meat extract. An interval of one week was allowed between injections and the

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TABLE I. Bactericidal Antibody Response in Rabbits.

	Pre-immunization control serum	Post-immunization serum vs. the homologous organism	Titer rise
E. coli Lilly	222*	6650	30×
E. coli B	116	7693	66×
S. typhosa Mrs. S	25	2500	100×

* Represents bactericidal antibody titer or reciprocal of amount of heat (56°C for 30 min.) inactivated serum required for killing 50% of a standard inoculum in conjunction with complement(8).

animals were bled 10 days after the second injection.

The bactericidal reaction was performed by a quantitative photometric assay(7) in which extent of lysis is not determined. Hemolytic complement was assayed by a standardized procedure(8).

Results. The first question raised was whether the serum of an animal injected with the cells of a rough organism would exert a greater level of bactericidal activity, in conjunction with complement, than the pre-immunization control serum. The results with the 3 organisms tested indicated a marked increase in activity (at least 25-fold) of the serum after immunization in comparison with the pre-immunization control (Table I). Also noteworthy is the inverse relationship between bactericidal antibody titer of the normal control serum and titer rise against a particular organism.

Next, the effect of addition of egg white lysozyme to different bactericidal reaction systems was determined. In all experiments, although itself inactive, lysozyme was found to enhance the action of antiserum plus complement or of fresh normal rabbit serum itself. Table II represents a summary of results obtained against *S. typhosa* Mrs. S indicating the enhanced reactivity of an immune system with added lysozyme. Similar results were obtained with the *E. coli* strains. With normal rabbit serum itself serving as a source of normal antibody and complement, an appreciable increase in bactericidal action was noted also with addition of 10-100 μg of lysozyme to the reaction mixtures with *E. coli* B and *E. coli* Lilly as test organisms, but no such enhancement was observed with a smooth organism, *Salmonella typhosa* strain 0901.

TABLE II. Enhancement of Activity of Rabbit Immune Serum and Complement by Lysozyme Against *S. typhosa* Mrs. S.

Immune serum (ml)	Absorbed rabbit complement (ml)	Lysozyme (μg)	% kill
—	.1	—	19
8×10^{-5}	.1	—	38
8×10^{-5}	.1	20	58
1.6×10^{-6}	.1	—	25
1.6×10^{-6}	.1	20	30
3.2×10^{-7}	.1	—	22
3.2×10^{-7}	.1	20	24

In accord with previous results(5), lysozyme was found capable of potentiating the action of normal rabbit or human serum in which a considerable proportion of its activity was lost as a result of absorption with cells of the test organism (Table III). Considerably larger amounts of lysozyme were required than with the immune systems studied. Comparable amounts of histone were without detectable effect.

TABLE III. Activating Effect of Lysozyme Upon Normal Rabbit serum Previously Absorbed with Cells of Test Organism, *E. coli* Lilly.

Rabbit serum abs. 4× (ml)	Lysozyme (mg)	% kill
.1	—	54
.1	4.00	80
.1	.80	63
.1	.16	53
abs. 6× (ml)		
.1	—	16
.1	4.00	34
.1	.80	30
.1	.16	30

Experiments were performed then to test the possibility that lysozyme exerted its effect by neutralizing the anticomplementary activity of acid soluble bacterial products dissolved in serum as a result of the absorption process. Hemolytic complement levels of absorbed serum declined significantly as compared to a control, untreated portion. Addition of lysozyme, however, to absorbed serum had no detectable influence on its complement titer.

Finally, when normal rabbit or human serum was absorbed 8 or 9 times so that it

TABLE IV. Bactericidal Effects of Addition of Lysozyme and of Immune Serum to Absorbed Normal Rabbit Serum, Itself Practically Lacking Bactericidal Action, Against *E. coli* Lilly.

Absorbed rabbit serum (ml)	Lysozyme	Heat inactivated rabbit antiserum (10^{-6} ml)	% kill
.1	—	—	4
.1	20 μg-20 mg	—	4
.1	—	2.60	89
.1	—	1.30	67
.1	—	.65	37
.1	—	.33	13

lost detectable activity, no amount of lysozyme was capable of restoration of activity (Table IV). Such an absorbed serum was itself inactive, or practically inactive since the 4% kill noted in Table IV is not greatly beyond the experimental error of the method. The absorbed serum was inactive despite addition of different amounts of lysozyme ranging from 20 μg to 20 mg, but functioned effectively as a complement source with as little as 3.3×10^{-6} ml of antiserum. A typical response to different amounts of antiserum was obtained(9). These results were obtained with *E. coli* Lilly, and were similar in tests with *E. coli* B and *S. typhosa* Mrs. S.

Discussion and summary. The experimental results have confirmed previous observations that lysozyme, generally without effect itself in the killing or lysing of gram negative bacteria, enhances the bactericidal action of the antibody-complement system against these organisms(10). Both so-called normal and immune systems were affected. In addition, previous observations have indicated that cells killed by the antibody-complement system may be lysed or, in stabilizing media, converted to spheroplasts by lysozyme(2).

The extreme susceptibility of rough variants to the bactericidal and bacteriolytic action of normal sera has led naturally to the postulate that specific substances or antibody may not be required for such action. The activity of precolostral calf and of newborn piglet sera against these organisms has provided some experimental basis for this concept, but further analysis of these situations is required.

Partial loss of the bacteriolytic activity of normal serum against a rough organism by absorption with the cells of the homologous organism may be restored by addition of egg white lysozyme. This finding provided a basis for the concept that complement may act independently of antibody in the bactericidal reaction(5). Experiments forming the basis of this report were performed then to consider possible alternative explanations. The first of these involving the neutralization by lysozyme of the anticomplementary activity of endotoxin or other bacterial products proved untenable. The second possibility, that small amounts of normal antibody remaining in the absorbed serum were required for enhancement of activity by lysozyme, was compatible with the observed results. Addition of lysozyme to partially absorbed serum, which retained some degree of bactericidal activity, elicited a marked increase of that activity. In contrast, more completely absorbed serum was incapable of bactericidal activity with addition of lysozyme. Portions of such absorbed serum, however, served as an excellent source of bactericidal complement. Although practically inactive itself, it was highly bactericidal in conjunction with diluted immune serum (Table IV). Finally, an easily demonstrable rise in bactericidal antibody activity, as a result of immunization, may be observed with rough organisms (Table I) as with less sensitive smooth organisms(9).

In conclusion, the results obtained are compatible with the classical concept that the action of complement and lysozyme in killing and lysing gram negative bacteria, both rough and smooth, requires the mediation of antibody. In addition, lysozyme may augment the bactericidal action of complement on bacteria sensitized by antibody.

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Macromolecular Properties of Hexadimethrine Bromide, an Antiheparin Agent. (28520)

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Hexadimethrine bromide* has been used in recent years as a phamacological agent to neutralize the anticoagulant activity of heparin(1,2). Several publications(3-5) have reported on the relationship between molecular size of this drug and its pharmacological and toxicological properties. It appeared that a critical study of its macromolecular properties would be of interest especially if the critical molecular size for the appearance of detrimental effects could be pinpointed.

Methods. Toxicity and antiheparin studies. The same methods were employed as previously reported(5).

Light scattering. Light scattering molecular weights were determined by standard methods(6) with a Brice-Speiser-Phoenix light scattering photometer using light of 4358A. The refractive index increment at this wave length was 0.154 ml/g. Determinations of fluorescence were made by employing a yellow filter (Corning No. 3384) which transmits light above 4900A in front of the photomultiplier tube.

Sedimentation. Sedimentation data were obtained on a Spinco Model E ultracentrifuge. Sedimentation velocity runs were made at 59,780 rpm and equilibrium runs at 50,740 rpm, at or near room temperature. Calculations of sedimentation coefficients and equilibrium molecular weights were made by methods described by Schachman(7).

Diffusion. Determinations were made in a Spinco Model H Electrophoresis-Diffusion apparatus at 1°C. Diffusion coefficients were calculated from Rayleigh fringe patterns by standard methods(7). No prior dialysis of the polymer was carried out since the molecule is too small to be retained by a cellophane membrane.

Partial specific volume. This value, determined by the densitometric method, was 0.72 ml/g.

Results. All of the molecular weights reported in the literature were determined by the light scattering method. In our study we became aware that all samples fluoresced at the wave length of light used for the light scattering determinations. This means that all published figures for the molecular weight of hexadimethrine bromide are in error, being much higher than actual values.

When attempts were made to correct the results for fluorescence by the method of Brice *et al*(8) it was found that the true scattering values were too low for reliable results, being only a deflection of 10 galvanometer units at full sensitivity of the instrument. Thus, it became apparent that the true molecular weights of hexadimethrine bromide are too low to be established by the light scattering method.

On the other hand, ultracentrifuge methods are more versatile and can be used even for small molecules. Sedimentation velocity runs in a synthetic boundary cell showed very low values of the sedimentation coefficient, S_{20w}. For different samples the S_{20w} ranged from 0.23 to 0.41 S and was not dependent on concentration or ionic strength. In view of the large experimental error for such a small S_{20w} these values should be considered essen-

* Polybrene®, Abbott Laboratories, North Chicago, Ill.