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Lysis of Gram-Negative Bacteria by Serum

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The antibacterial property of serum, first reported by Nuttall in 1888, has attracted many investigators and an extensive literature has accumulated. The sera of most mammals tested have been found to possess bactericidal activity, but a notable exception is the mouse, whose serum, apparently through lack of complement, is unable to kill any of a number of bacterial strains which are sensitive to other sera (Marcus, Esplin & Donaldson, 1954). Many types of bacteria are sensitive to serum killing, although definite lysis does not invariably occur. Most workers, including ourselves, have been concerned primarily with killing, as measured by failure to produce colonies on a culture plate.

Table 1. *Inactivation of the bactericidal power of human serum by inactivation of the components of complement*

Treatment of serum	Complement components inactivated
Heat at 56° for 30 min.	C'1, C'2, C'3
Absorption with antigen-antibody precipitate	C'2, C'4
Zymosan at 37°	C'3
NH ₄ OH	C'4

Several distinct bactericidal mechanisms in serum have been described. It is widely accepted that Gram-negative organisms in general are killed by mechanisms which involve the thermolabile agent complement. Complement itself is not a single entity but consists of four components designated C'1, C'2, C'3 and C'4, all of which are necessary for bactericidal activity, and all of which are believed to be proteins. None of the components of complement has been isolated in the pure state, although highly enriched preparations were obtained by Pillemer, Ecker, Oncley & Cohn (1941) and by Lepow, Ratnoff, Rosen & Pillemer (1956). Almost all the studies on complement components have hitherto been done, not with preparations of individual components, but with serum lacking one or more components as a result of various treatments (Table 1). Different opinions have been expressed about the ability of complement itself to kill bacteria or whether antibody is invariably required. Different views have also been held about the origin, specificity and role of the naturally occurring antibodies. It is not, however, our purpose to review this topic here as it has been discussed in standard texts (e.g. *Topley and Wilson's Principles*, 1955).

Investigation has shown that the complement-dependent bactericidal activity of normal human serum towards certain organisms requires magnesium ion, and also a serum euglobulin, properdin, which is distinct from antibody and complement (Pillemer, Blum, Lepow, Ross, Todd & Wardlaw, 1954; Wardlaw & Pillemer, 1956). The requirement for properdin was demonstrated by the loss of bactericidal activity following absorption of serum with the yeast cell-wall fraction 'zymosan'. The conditions of absorption were controlled so that properdin was removed without loss of component C'3. Properdin itself was eluted from the zymosan residue, and was able to restore bactericidal activity to properdin-deficient serum. Properdin by itself was non-bactericidal. The requirement for Mg ion was similarly demonstrated by the decrease in bactericidal activity following treatment of the serum with the cation exchange resin Amberlite IRC-50 (sodium cycle). Addition of MgCl_2 restored bactericidal activity to resin-treated serum. It was found with the same test organism (*Shigella shigae*) that removal of any of the four components of complement from serum also resulted in loss of bactericidal activity. Thus, altogether at least six distinct serum factors participate in the destruction of this organism. Clearly an extremely complex sequence of reactions remains to be defined.

Table 2. *The greatest dilution of various sera which produced 50 % killing of a standard inoculum in 90 min.*

Strain	Guinea pig	Rat	Human	Rabbit	Chicken
<i>Escherichia coli</i> 2206	1/2	1/2	1/20	1/32	1/32
<i>E. coli</i> K12	1/1	1/16	1/100	1/16	1/4

A possible approach to this problem is suggested by several procedures which, when applied to bacteria, alter their serum sensitivity. A well-authenticated example is that rough mutants of a given strain are usually much more sensitive to serum than the smooth parent, which suggests that the somatic (O) antigen may in some way be involved. Another example is the finding that bacteria in the logarithmic phase of growth are much more readily killed by serum than those in the lag phase (Fig. 1). Thus, in the experiment with *Escherichia coli* illustrated, bacteria in the logarithmic phase were killed in less than 5 min., whereas lag-phase organisms were still viable at 60 min. The sensitivity of bacteria can also be altered by the choice of culture medium. Thus, organisms grown in broth containing 0.005 M-phloridzin were much more sensitive than organisms grown in broth without phloridzin (Fig. 2).

Another type of variation that is frequently encountered is the variation in bactericidal activity of different sera and the sensitivity of various strains (Table 2). It is difficult to decide from these data which is the most sensitive strain and which is the most active serum. *Escherichia coli* 2206 is less sensitive to rat serum than *E. coli* K12, yet the reverse is true for chicken serum; human serum is the most active against strain K12. These variations make difficult the comparison of the results obtained by different investigators who have used a wide variety of test organisms and sera.

With regard to the mechanism of bactericidal action, we have considered the hypothesis that serum contains an enzyme system whose substrate is an essential component of the bacterial cell. The finding that *Escherichia coli* grown in the presence of phloridzin, which is a known inhibitor of phosphorylation, had a lowered resistance, suggested that a phosphate-containing structure might be attacked. The knowledge that smooth strains were more resistant than their rough mutants led to a consideration of the somatic (O) antigen as a possible substrate. On this basis, the lipopolysaccharide component of this antigen has been labelled with ^{32}P as an integral part of the macromolecule

Fig. 1

Fig. 2

Fig. 1. Changes in sensitivity of *Escherichia coli* to bactericidal action of serum at different phases in the growth cycle. The solid line shows the increase in viable count following inoculation of the organisms into heat-inactivated serum and incubation at 37° . The dotted lines are death-time curves of samples taken from this culture and immediately transferred to unheated serum.

Fig. 2. Bactericidal activity of dilutions of human serum on *Escherichia coli* grown in broth, with and without added phloridzin.

(Rowley, Howard & Jenkin, 1956). These lipopolysaccharides have molecular weights in the range of 1–10 million and are therefore non-diffusible through a cellophane membrane. The action of serum on these materials was examined by incubating mixtures of serum and ^{32}P -lipopolysaccharide in tubes for 30 min., then cooling to 0° , transferring the contents to a dialyser and dialysing overnight at 2° against saline. The diffusate was counted for radioactivity and the proportion of ^{32}P liberated from lipopolysaccharide then calculated. With this technique it was found that serum liberated up to 25 % of the total ^{32}P of the lipopolysaccharide in the form of inorganic phosphate. The reaction appeared to be enzymic in that it had temperature and pH optima of 37° and 8.5 respectively and was inhibited by EDTA. Heating the serum at 56° for 30 min. completely destroyed activity. Whole haemolytic complement was not required for the reaction, since mouse serum was quite active, as was human serum from which complement component C'3 had been removed. These data

identify the enzyme as an alkaline phosphatase, and this is confirmed by the finding that purified intestinal alkaline phosphatase liberated the same 25 % of ^{32}P at a similar rate. Other data indicate that the serum enzyme which splits phosphate from lipopolysaccharide is not identical with the serum alkaline phosphatase that is measured by the King-Armstrong method (hydrolysis of phenol phosphate). Thus, the two activities did not run parallel in different sera and serum fractions. Furthermore, phenol phosphate did not inhibit the action of serum on lipopolysaccharide, whereas lipopolysaccharide strongly inhibited the action of serum on phenol phosphate. It seems, therefore, that serum may contain two phosphatases, one of low specificity capable of acting on phenol phosphate and lipopolysaccharide the other of high

Table 3. *Effect of rat serum on various bacterial strains and the radioactive lipopolysaccharides isolated from them*

Organism	Reciprocal of serum dilution giving 50 % maximum splitting of ^{32}P -lipopolysaccharide	Reciprocal of serum dilution giving 50 % killing of parent strain in 90 min.
<i>Salmonella paratyphi</i> B11 (rough)	1000	50
<i>Escherichia coli</i> (Lilley)	200	40
<i>S. paratyphi</i> B1 (rough)	200	50
<i>E. coli</i> 2206	100	10
<i>S. paratyphi</i> B1 (smooth)	25	4
<i>S. paratyphi</i> B11 (smooth)	25	4
<i>E. coli</i> 2380	20	1
<i>E. coli</i> 145	10	> 1

specificity and capable of acting only on lipopolysaccharide. As yet there is no definite evidence that the dephosphorylating action of serum on lipopolysaccharide is in any way involved in the bactericidal action of serum on living cells. However, certain data suggest that there may be a connexion between the two phenomena (Table 3). Here a comparison is made between the sensitivities of various lipopolysaccharides to the dephosphorylating action of serum and the serum sensitivities of the parent strains. It is apparent that in general the most sensitive strains yielded the most sensitive lipopolysaccharides and vice versa.

If, in conclusion, one compares the present state of knowledge of serum bactericidal action with that existing around 1900, it is apparent that we yet know little more about the actual mechanism of the reaction. Furthermore, the discovery of new serum factors without accompanying knowledge of their modes of action greatly complicates the task. It is clear from other papers given in this Discussion that hypotheses other than the one we have held will have to be considered. But even if one adheres to the enzyme-substrate type of hypothesis, there is still a basic theoretical question to settle, and this applies equally to the lysozyme system, namely, if one takes bacterial strains of increasing resistance to serum, is their resistance due to a greater or lesser content of substrate or to an altered substrate, or what? It is apparent that theoretical arguments can be advanced in favour of any of these.

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