A COMPARISON OF THE ANTIBACTERIAL ACTIVITY OF HUMAN SERUM AND OXALATED PLASMA ON STRAINS OF ESCHERICHIA COLI AND AEROBACTER AEROGENES

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The antimicrobial action of plasma and its product serum has been investigated for the past 70 years with varying emphasis. During this time generalizations have been made only to be modified as tests for the antimicrobial action of plasma or serum were extended to different classifications of microorganisms, to different species, or even to different strains within species (Waisbren and Brown, 1962). Hence, after 70 years of investigations one generalization can be made, namely, that plasma or serum is a highly complex physical chemical system the biological properties of which vary in an unpredictable manner. It is the purpose of this paper to present evidence of variations in the antimicrobial action of serum on several strains of Escherichia coli and Aerobacter aerogenes related to the test organism used, the donor of human serum, the presence or absence of oxalate or citrate ions, and the procedure used for carrying out the tests.

Various factors in plasma or serum have been suggested as responsible for its antimicrobial action. These include heat-labile substances such as hemolytic complement (Bordet, 1895) or bactericidal complement (different?), heat-stable “beta lysins” (von Behring, 1889), “normal antibodies” (Steinhardt, 1905), immune globulins (Bordet, 1895), lysozyme (Fleming and Allison, 1922), platelet extracts (Hirsch, 1960), and histones (Miller et al, 1942). Therefore, in designing the procedures for this study it seemed wise not to accept without challenge the current standard procedures based on the hypothesis that Gram-negative bacteria are acted on by complement and antibodies present in plasma or serum.

METHODS

Source and maintenance of strains of organisms.—Strains of Escherichia coli and Aerobacter aerogenes were isolated from patients with urinary tract infections at the University of Michigan hospital. All cultures were lyophilized after 1 transfer on trypticase-soy agar (TSA) to prevent changes associated with repeated subculturing. The contents of a lyophile tube were transferred to TSA and incubated at 37 C overnight. From this plate the test organism was cultured in TS broth for 18 hours at 37 C. Dilutions of the broth culture in gelatin-saline (0.1% gelatin in 0.9% sodium chloride) were used for the bactericidal tests.

Serum and plasma.—Blood was obtained from normal volunteers by venipuncture with syringes coated with Siliclad (Clay-Adams, Inc.). The blood was divided into 2 or more samples from which were obtained the following: serum and oxalated plasma (0.2% oxalate) or ion exchange plasma (Fenwal...
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Pack, Fenwal Laboratories, Morton Grove Illinois), citrated plasma (2% citrate), and heparinized plasma.

Bactericidal tests. —To 1 ml of serum or plasma were added 0.25 ml of a gelatin-saline dilution of broth culture. The mixture was incubated at room temperature (26 to 29 C) unless otherwise stated, and three 0.1-ml samples were spread on the surface of TSA plates at 2, 6 and 24-hour intervals. All agar plates were incubated 24 hours at 37 C and the number of colonies counted. The zero hour population of the tests was based on the number of organisms present in the dilution of the broth culture added to the serum or plasma. Each serum and plasma was tested simultaneously with 2 different dilutions of culture, which resulted in a final concentration of 200 to 2000 bacteria per ml of mixture. In each test comparison was made of serum with plasma and of E. coli with A. aerogenes. All tests were set up within 2 hours of the removal of blood from the subject to minimize the inactivation of complement.

Lysozyme determination.—Approximately 50 mg of lysozyme substrate (Difeo) were suspended in 100 ml of pH 6.2 phosphate buffer which gave 10% light transmission at a wave length of 540 mwire with a Bausch and Lomb Spectronic 20 spectrophotometer. Ten mg of purified lysozyme (Worthington muramidase) were dissolved in 100 ml of buffer and dilutions giving final concentrations of 5, 2.5, 1.25, 0.625 and 0.3125 μg per ml were made in buffer. Five-ml amounts of enzyme were added to 5-ml amounts of substrate, and per cent light transmission was read in the spectrophotometer immediately and after 20 minutes incubation at room temperature. A standard curve was plotted from this data. The lysozyme content of serum and plasma was determined by adding 5 ml of dilutions of these substances to 5 ml of a standard suspension of substrate and reading per cent light transmission immediately and after 20 minutes incubation at room temperature. From these data the corresponding amounts of lysozyme were read from the standard curve.

Complement determinations.—Fifty per cent (C'50) units of serum and plasma were assayed by the method of Kabat and Mayer (1961) but with barbiturate buffer without the addition of calcium and magnesium. The final volume in the 13X125-mm tubes was 7.5 ml, and the concentration of sensitized sheep erythrocytes was 1X10⁹. The tests were incubated at 37 C for 60 minutes, and the optical density was read at 540 μm.

Hemagglutination tests. —Lyophilized cultures of E. coli and A. aerogenes were suspended and grown on brain-heart infusion agar overnight at 37 C. Roux flasks of brain-heart infusion agar were inoculated from the plate cultures and incubated at 37 C for 18 hours at which time the bacteria were harvested in 30 ml of 0.9% sodium chloride and the suspension was boiled for 2 hours. After cooling, 3 ml of ethanol were added, and the suspension was centrifuged at 5000 rcf for 15 minutes at 0 C. The supernatants were stored at 4 C until used for coating erythrocytes (Neter et al, 1952). Erythrocytes from the individual whose serum was to be tested were collected in Alsever's solution and washed 3 times in 0.9% sodium chloride. A 1:10 dilution of antigen was incubated with a 1% suspension of the erythrocytes for 1 hour in a 37 C water bath shaker. The treated erythrocytes were washed 3 times in 0.9% saline, adjusted to a final concentration of 1%, and used within 5 minutes after preparation. Mixtures containing 0.25 ml of 2-fold dilutions of the individual's serum and 0.25 ml of antigen-coated erythrocytes were incubated for 3 hours at 37 C. The re-

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ciprocal of the highest dilution of serum producing a dispersed pattern of cells was designated as the titer of the serum.

RESULTS

Reproducibility of results.—In these studies unexpected findings were obtained when a comparison was made of the antibacterial effect of human serum and oxalated plasma on selected and recently isolated strains of E. coli and A. aerogenes as measured by plate counts at 2, 6 and 24-hour intervals. Therefore, the reliability of plate counts is of high significance in interpreting the data obtained. In replicate platings the average variation in over 200 counts was 7.85%, and the range of variation was from 0 to 21%.

Selection of bacterial strains.—Several strains of E. coli and A. aerogenes were isolated from the urine of patients suffering from urinary bladder infections. These strains were then tested for their susceptibility to the antimicrobial action of serum or oxalated plasma from a healthy donor. The results (table 1) point out that there was variation among the strains of both genera of bacteria with respect to the number of hours of incubation necessary to reduce plate counts to zero or near zero. In addition, the strains of E. coli and A. aerogenes varied in their susceptibility or resistance to the antibacterial action of oxalated plasma, E. coli apparently being more susceptible to the bactericidal action of oxalated plasma than to that of serum, while oxalated plasma had definitely less antimicrobial action for some strains of A. aerogenes than did serum from the same donor.

Antimicrobial action of serum vs. oxalated plasma.—One strain of E. coli (E. coli-2) and one of A. aerogenes (A. aerogenes-4) were selected for further study of the unexpected differential action of serum and oxalated plasma on these 2 genera of Gram-negative bacteria. Samples of blood were repeatedly collected from subject A and 6 other volunteers, and the 2 bacterial strains were exposed to the action of the serum and oxalated plasma prepared from these samples. Each graph in figure 1 represents the average of from 8 to 14 tests made on the serum or oxalated plasma of 3 donors.

The oxalated plasma from all of the 6 subjects in every test reduced the number of E. coli-2 surviving after 6 hours exposure to the plasma to less than 1% of the original population, while the serum of the 3 individuals studied most extensively allowed at least a 2-fold increase in the number of the same organ-

Table 1.—The bactericidal effect of serum and oxalated plasma from donor A on strains of Escherichia coli and Aerobacter aerogenes

<table>
<thead>
<tr>
<th>Organism</th>
<th>Per cent original population* at indicated hours</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td>E. coli-1</td>
<td>8</td>
</tr>
<tr>
<td>E. coli-2</td>
<td>140</td>
</tr>
<tr>
<td>E. coli-3</td>
<td>83</td>
</tr>
<tr>
<td>E. coli-4</td>
<td>16</td>
</tr>
<tr>
<td>E. coli-5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. coli-6</td>
<td>185</td>
</tr>
<tr>
<td>A. aerogenes-3</td>
<td>138</td>
</tr>
<tr>
<td>A. aerogenes-4</td>
<td>88</td>
</tr>
<tr>
<td>A. aerogenes-5</td>
<td>181</td>
</tr>
<tr>
<td>A. aerogenes-7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>A. aerogenes-8</td>
<td>3</td>
</tr>
<tr>
<td>A. aerogenes-9</td>
<td>&lt;1</td>
</tr>
<tr>
<td>A. aerogenes-10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>A. aerogenes-11</td>
<td>27</td>
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</tbody>
</table>

* The original population (0 hours) ranged from 145 to 500 bacteria per ml of plasma or serum.
Activity of Human Serum and Plasma

Figure 1.—The bactericidal effect of human serum and oxalated plasma on *Aerobacter aerogenes*-4 (solid lines) and *Escherichia coli*-2 (broken lines) (averages of 8 to 14 tests).

isms in the same time interval. The difference in antibacterial action of serum and oxalated plasma on *E. coli*-2 was most striking in the case of donor C. Individual variation was apparent in the antibacterial activity of oxalated plasma against *A. aerogenes*-4, since the bactericidal activity shown by serum from subjects A and B was not noted in the oxalated plasma of these individuals. In contrast to this situation, the oxalated plasma of subject C had greater bactericidal activity for *A. aerogenes*-4 in the first 6 hours of incubation than did the serum from this donor, thus paralleling the findings made with *E. coli* exposed to oxalated plasma of subjects A, B and C.

The other portions of the blood samples which were treated to yield low calcium plasma (ion exchange, Fenwal Pack), heparinized plasma, citrated plasma, and plasma-serum (Hirsch, 1960) with either low or high platelet content were also compared for bactericidal action against the 2 test organisms. Heparinized plasma and both the platelet-free and high platelet content plasma-sera gave results similar to those of serum in all tests. *E. coli*-2 and *A. aerogenes*-4 showed the same pattern of resistance or susceptibility to ion ex-
change plasma that they had shown to oxalated plasma. The antibacterial activity of citrated plasma did not parallel that of oxalated plasma in all situations, and this difference is being investigated further.

Oxalate on test organisms in broth.— The question whether oxalate per se enhanced or inhibited the growth of *E. coli* 2 or *Aerobacter aerogenes* 4 was answered by inoculating the same volume of broth or oxalated broth with the same inoculum of the organisms used in the bactericidal tests (table 2). Samples were removed after 2, 6 and 24 hours of incubation at room temperature and spread on the surface of agar plates. The results proved conclusively that oxalate neither inhibits nor enhances the growth of the 2 organisms in broth.

Incidental to these tests on the effect of oxalate on the growth rate of *E. coli* 2 and *Aerobacter aerogenes* 4 in broth the effect of diluting serum with broth (1:2) was examined. No effect on the antibacterial action of serum against *E. coli* 2 by diluting the serum with broth 1:2 was noted. However, serum diluted with broth 1:2 had no antibacterial effect on *Aerobacter aerogenes* 4. A dilution of serum 1:2 with saline had marked antimicrobial action against both *E. coli* 2 and *Aerobacter aerogenes* 4. These findings add to the evidence that the mechanisms responsible for the antibacterial action of serum against *E. coli* 2 and *Aerobacter aerogenes* 4 are in part different. They also contribute to an appreciation of the complexity of the antimicrobial action of soluble blood substances.

**Oxalated serum vs. oxalated plasma.—** The effect of oxalate on the antimicrobial action of serum and plasma was compared. Results given in figure 2 indicated that oxalated plasma acted in the same manner as oxalated serum on *E. coli* 2. However, the effect of oxalated plasma on *Aerobacter aerogenes* 4 varied, depending on the donor of the blood. Oxalated serum of donor A had somewhat greater antimicrobial action against *Aerobacter aerogenes* 4 than did the corresponding oxalated plasma. No difference was noted between oxalated serum or oxalated plasma from donor B. Again by using 2 selected test organisms and blood from several donors the complexity of the antimicrobial action of the fluid constituents of blood is indicated.

**Temperature of incubation.**—The effect of temperature of incubation on the antibacterial action of serum and oxalated plasma on the 2 strains of bacteria was studied, and the results showed that the antibacterial activity was more rapid at 37 C than at 26 to 29 C. The general patterns shown in figure 1 were not altered. *E. coli* 2 remained more susceptible to the bactericidal action of oxalated plasma than to that of serum. However, this increased susceptibility was apparent after 90 minutes incubation rather than after 6 hours incubation. Thus, by lowering the incubation temperature of the test system the rate of reaction has been decreased to the point where differences in the effects of serum and oxalated serum can readily be observed.

**Hemolytic complement.**—A heat-labile

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**Table 2**—The effect of oxalate on the growth of *Escherichia coli* 2 and *Aerobacter aerogenes* 4

<table>
<thead>
<tr>
<th>Medium</th>
<th>Bacterium</th>
<th>Number of bacteria per ml at indicated hours</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Trypticase-soy broth</td>
<td><em>Aerobacter aerogenes</em> 4</td>
<td>4.40×10^4</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> 2</td>
<td>5.60×10^4</td>
</tr>
<tr>
<td>Trypticase-soy broth (0.2% oxalate)</td>
<td><em>Aerobacter aerogenes</em> 4</td>
<td>4.40×10^4</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> 2</td>
<td>5.60×10^4</td>
</tr>
</tbody>
</table>
component, now designated as complement, has been considered necessary for the bactericidal action of serum since Buchner (1889) showed that bactericidal activity was decreased by heating serum. Muschel and Treffers (1956) and Michael et al (1962) have included antibody ("normal" or immune) as a necessary reactant of the process, and Muschel et al (1959) as well as Wardlaw (1962) have added lysozyme to the list of active substances contributing to the bactericidal property of the humoral portion of the blood. Because of the observed increased antibacterial activity of serum and plasma for *E. coli*-2 when oxalate was added, determinations were made of the effect of oxalate on the amount of complement, lysozyme, and antibody present in the test.

The addition of oxalate to serum or plasma had a deleterious effect on hemolytic complement, since the presence of oxalate in serum or plasma decreased the number of 50% hemolytic units by 21% (table 3). This decrease in complement due to oxalate could not be related to a decrease in bactericidal power of oxalated plasma for *A. aerogenes*-4, since donor C's serum and oxalated
plasma contained the smallest amount of complement of the 3 donors. Nevertheless, the oxalated plasma from this donor had a more rapid bactericidal effect on *A. aerogenes*-4 than did her serum (figure 1).

If hemolytic complement is a necessary factor in the complex mechanism of antibacterial action of blood serum and plasma, it may be postulated that it has an enzymatic action or possibly a triggering effect, since assays for the amount of hemolytic complement present in oxalated serum after 2, 6 and 24 hours of incubation with *E. coli*-2 and *A. aerogenes*-4 demonstrated no loss of complement resulting from bactericidal action (table 4).

**Antibody.**—Antibody content of serum and oxalated serum from donors A, B, and C against the somatic O antigens of *E. coli*-2 and *A. aerogenes*-4 was determined by agglutination with antigen-coated erythrocytes. The same titers were obtained with oxalated serum as those listed in table 5 for serum without oxalate. Although the antibody titers of the sera from the 3 donors were greater for *E. coli*-2 than for *A. aerogenes*-4, reference to figure 1 indicates that the antibacterial effect of the sera of the 3 individuals took effect more rapidly when acting on *A. aerogenes* than when acting on *E. coli*.

**Lysozyme.**—The presence of oxalate in the serum or plasma of the 3 donors did not change the lysozyme content of the serum or plasma as measured by its lytic effect on desiccated *Micrococcus lysodeikticus* (table 6). Contrary to Wardlaw's (1962) results, we did observe a decrease in the lysozyme activity of heated serum.

**Calcium.**—Although oxalate appeared to have no enhancing effect on the lytic effect of complement and lysozyme or on the aggregating ability of antibody for antigen, the fact remains that the growth of *E. coli*-2 was more rapidly inactivated by oxalated serum or plasma than by serum alone. Since oxalate binds calcium ions which are present in blood and serum, it is possible that the increased bactericidal activity of oxalate was due to its ability to chelate calcium ions.
FIGURE 3.—The effect of calcium on the bactericidal properties of serum for *Aerobacter aerogenes*-4 and *Escherichia coli*-2. 1, serum; II, oxalated serum neutralized with an equivalent amount of calcium chloride; III, oxalated serum with an excess of 0.387 mg calcium per ml of serum.
lated plasma and oxalated serum for E. coli-2 may be associated with the low* calcium level in those materials. In order to test this hypothesis, moles of calcium chloride equivalent to the moles of oxalate used were added to oxalated serum. The effect of an excess of calcium ions was tested on other samples of oxalated serum. The results presented in the graphs of figure 3 suggest that the absence of calcium ions in oxalated plasma and serum may be responsible for the increased speed of bactericidal action on E. coli-2. The graphs also indicate that an amount of calcium chloride intermediate between the 2 concentrations used would produce results similar to those obtained with serum. The difference in the calcium requirements of A. aerogenes and E. coli is again emphasized, considering the fact that the averages given in figure 3 represent results of duplicate oxalated serum samples. The same amount of calcium which was not quite sufficient to support the bactericidal effect of serum on E. coli-2 was slightly greater than the amount needed to bring about the antibacterial pattern of serum on A. aerogenes-4.

DISCUSSION

Although it has been stated that anticoagulants such as citrate and oxalate decrease the bactericidal power of serum (Maaløe, 1946), oxalate increased the bactericidal power of human serum for the E. coli strain used in this work. That the same situation does not hold for all strains of Gram-positive bacteria is evident from the results of Jacox (1950) as well as those of Donaldson and Marcus (1958). According to Repaske (1958) and Wardlaw (1962) certain strains of E. coli, usually classified as rough, which are not susceptible to the action of lysozyme under the usual conditions become susceptible when treated with EDTA at pH 8. We feel that the increased susceptibility to the bactericidal action of oxalated serum exhibited by the E. coli strain used in our work is due to an increased susceptibility to the action of lysozyme of the oxalated plasma, brought about by the removal of calcium and the increase in pH which results from the loss of carbon dioxide when blood is removed from the body.

Even though the hemolytic complement of the serum is decreased by the addition of oxalate and the resulting decrease in calcium, obviously there is a sufficient amount of complement present to act along with antibody to produce a bactericidal effect on E. coli, if antibody and complement are involved in this activity. Although most workers in this field continue to implicate C' in the bactericidal action of serum, Muschel (1960) notes that proof is lacking that hemolytic and bactericidal complement are the same. Therefore, we suggest that it would be more accurate to designate a "heat-labile substance, as yet unidentified" to replace the term "complement."

The strain of A. aerogenes used shows a great degree of variation in susceptibility to the action of oxalated plasma and oxalated serum, depending on the donor, a situation which may be related to the fact that A. aerogenes infections in man are more resistant to treatment than those infections in which E. coli is the causative agent. This variation does not appear to be linked with calcium ions, since replacement of calcium does not restore bactericidal activity to the oxalated serum of all donors.

It seems that the substances in serum required for bactericidal activity against A. aerogenes may be different from

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* The amount of calcium per ml of oxalated plasma was 5 to 10 µg, determined by emission spectroscopy. This can be accounted for by the solubility of calcium oxalate.
those required for bactericidal activity against *E. coli* and the problem of in vivo destruction of bacteria by serum or plasma is a far more complicated one than has been indicated previously.

**SUMMARY**

The addition of oxalate to human serum or plasma increased the rate of bactericidal action of the plasma and serum against certain recently isolated strains of *Escherichia coli*. Oxalate added to the serum and plasma of certain individuals inhibited or destroyed the bactericidal activity of the serum and plasma for some strains of *Aerobacter aerogenes*. If allowed to act for 24 hours, serum from the 3 individuals tested was bactericidal for the strains of *Escherichia coli* and *Aerobacter aerogenes* used. Oxalate decreased the complement of serum and plasma by 5 to 27% but did not inactivate the bactericidal substances effective against the strain of *Escherichia coli* used.

**REFERENCES**
