# REDUCTION OF SERUM COMPLEMENT IN RABBITS AFTER INJECTION OF ENDOTOXIN

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After finding that lethal endotoxin shock requires a heat-labile factor in serum, Spink and Vick (2) suggested that complement may be essential for the production of shock by endotoxin. It has also been reported that endotoxin inactivates complement *in vitro* (3) and that normal serum contains antibody to endotoxin (4, 5). In view of these observations, it seems reasonable to consider that the injurious effects of endotoxin may be mediated by an immune reaction involving complement. This question was examined in the following study by relating the lethality of endotoxin to its effects *in vivo* on complement and antibody.

## *Materials and Methods*

*Endotoxin.*—Endotoxin was extracted from *Escherichia coli* serotype 0:113, and *Proteus mirabilis* (North) by methods previously described (6). The $LD_{50}$ of *E. coli* endotoxin in mice was 0.140 mg, while that of *P. mirabilis* was 0.102 mg. These assays were performed by methods described elsewhere (7).

*Serum.*—10 ml blood was removed by cardiac puncture for assay of antibody and complement (C′) just before the injection of endotoxin and 3 hours after injection. 5 ml blood was withdrawn 1 hour after injection for assay of C′ alone. The blood was collected in tubes immersed in an ice bath, and then refrigerated at 4°C for ½ hour. The tubes were next centrifuged twice at 2000 RPM for 10 minutes at 4°C. The sera were separated at 4°C and stored at −70°C. The sera were thawed in an ice bath just before they were assayed for C′ and antibody.

*Complement Assay.*—Fifty per cent hemolytic units of C′ were assayed in duplicate by the method of Osler, Strauss, and Mayer (8). The three sera from each animal were titrated simultaneously in tubes immersed in an ice bath. Anti-sheep hemolysin was obtained from Cappel Laboratories, West Chester, Pennsylvania.

*Animals.*—Mature albino rabbits, of mixed breed and weighing 2.6 to 3.0 kg, were housed in air-conditioned quarters and fed rabbit pellets obtained from Rockland Farms, New York. The $LD_{50}$ for *E. coli* endotoxin in rabbits was determined by intravenous injection of doses ranging from 6.4 to 0.00613 mg. Deaths were recorded through 72 hours, and the $LD_{50}$ calculated by the method of Reed and Muench (9). The marginal ear vein was used for all intravenous injections.

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‡ Presented before the American Association of Immunologists, April, 1962 (1).

*Hemagglutination Test for Antibody to Endotoxin.*—Antibody levels were measured by a modification of the hemagglutination test of Neter (10). The hemagglutination test was performed by sensitizing human group O erythrocytes with NaOH-treated *E. coli* endotoxin. 1 ml of a suspension of endotoxin (10.0 mg/ml in normal saline) was added to 4.5 ml 0.25 N NaOH and placed in a 37°C water bath for 3 hours. The solution was then neutralized by the addition of 4.5 ml of 0.25 N HCl. Human group O erythrocytes were washed 4 times with saline and 1.0 ml of packed cells were suspended in the solution of NaOH-treated endotoxin and incubated for ½ hour in a water bath at 37°C. These cells were then immediately washed 3 times in saline and resuspended to give a cell concentration of 0.25 per cent in saline. Sera were inactivated by incubating in a water bath for 30 minutes at 56°C, absorbed overnight at 22°C with group O human cells to remove red cell agglutinins, and diluted serially by adding 0.5 to 0.5 ml of saline. To each tube containing 0.5 ml of serially diluted serum was

TABLE I

*Assay for $LD_{50}$ of E. coli Endotoxin in Rabbits*

| Dose endotoxin | Deaths/Total injected | Mortality |
|---|---|---|
| *mg* | | *per cent* |
| 6.4 | 14/15 | 93 |
| 5.0* | 8/10 | 80 |
| 3.2 | 5/10 | 50 |
| 1.6 | 3/10 | 30 |
| 0.8 | 5/10 | 50 |
| 0.4 | 6/10 | 60 |
| 0.2 | 3/10 | 30 |
| 0.1 | 1/10 | 10 |
| 0.050 | 5/15 | 33 |
| 0.025 | 2/10 | 20 |
| 0.0125 | 1/10 | 10 |
| 0.00613 | 2/20 | 10 |

* Not included in the $LD_{50}$ calculations.

added 0.1 ml of the suspension of endotoxin-sensitized erythrocytes and the serum-cell mixture incubated at 37°C for 60 minutes. Agglutination was observed after overnight refrigeration at 4°C. Controls for the test consisted of sensitized O cells and saline alone, unsensitized red cells added to serum, and serially diluted hyperimmune serum to which sensitized red cells were added. The paired sera from each animal were titrated simultaneously.

*Glassware.*—All glassware and needles were made pyrogen-free by heating in a dry oven at 170°C for 2 hours.

*Radioactive Endotoxin.*—Endotoxin was labeled by direct incubation with $Na_2Cr^{51}O_4$ and radioactivity in serum measured by methods described previously (6).

*In Vitro Addition of Endotoxin to Rabbit Sera.*—The effect of endotoxin on serum complement *in vitro* was determined by mixing 0.9 ml serum with 0.1 ml of a solution of endotoxin in saline. The complement level was compared with that in a portion of the same serum to which saline alone was added. All measurements were performed in duplicate, and all manipulations carried out in an ice bath.

*Artificial Immunization.*—Animals were immunized by an intravenous injection of 0.0001 mg of *E. coli* endotoxin 1 week prior to challenge.

## RESULTS

*Lethality.*—The $LD_{50}$ of *E. coli* endotoxin was 0.468 mg. This was much higher than anticipated in light of the profound physiologic disturbances produced in rabbits by minute doses of this endotoxin (5, 7). The mortality produced by each dose of endotoxin is presented in Table I. Deaths began 2 hours after

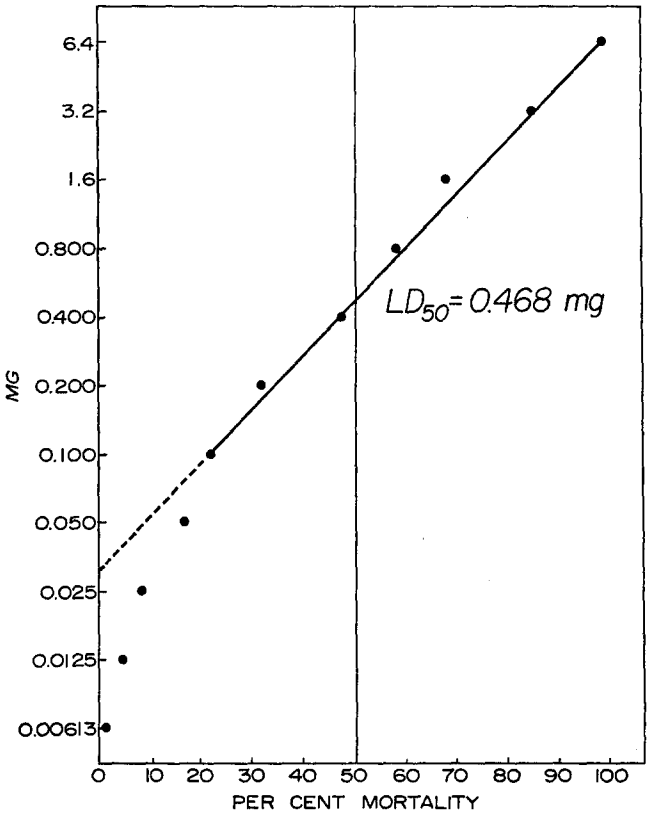


FIG. 1. Relationship of dose of *E. coli* endotoxin to cumulative mortality in rabbits

injection, were most frequent between 6 and 18 hours, and all but 2 died within 24 hours. Consistent mortality occurred with 6.4 and 5.0 mg. The lowest dose 0.00613 mg was still lethal, but for only 10 per cent of rabbits. Between these two extremes deaths appeared not to be related to dose except for a somewhat higher mortality rate above 0.100 mg. Landy and Johnson (11) have noted a similar variability in the lethality of rabbits inoculated with *Salmonella typhosa* endotoxin over a smaller dose range (0.010 to 0.5 mg).

In Fig. 1, a linear relationship was found between cumulative mortality and

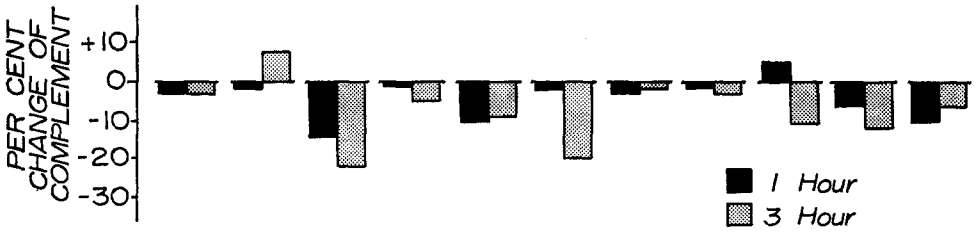


FIG. 2. Complement levels in control rabbits subjected to bleeding but not injected with endotoxin.

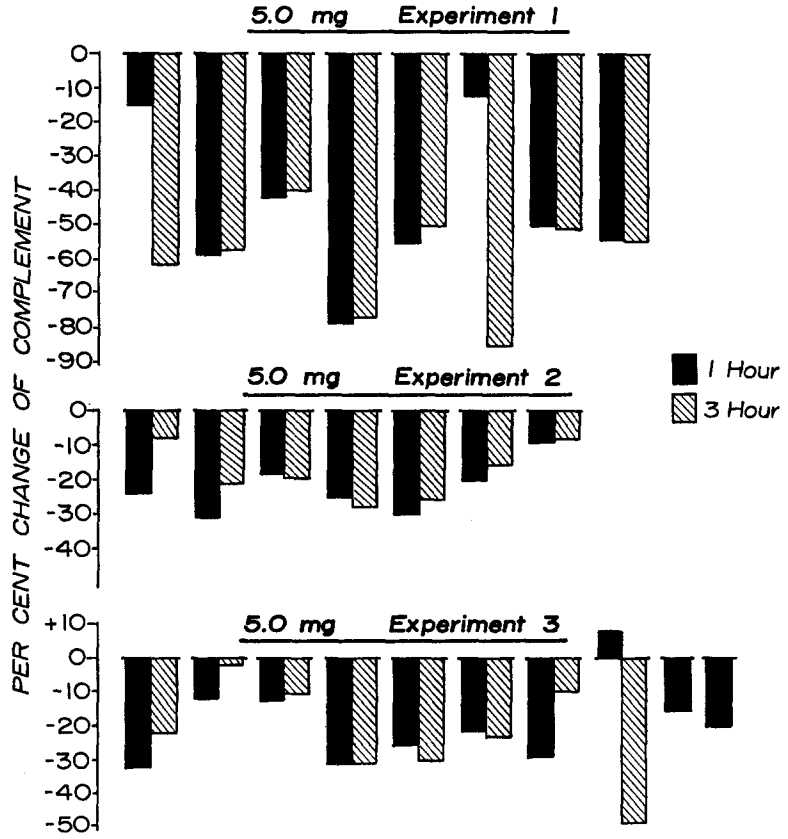


FIG. 3. Complement levels after injection of 5.0 mg of *E. coli* endotoxin into non-immunized rabbits.

dose in the range between 6.4 and 0.100 mg, but at lower doses the cumulative mortality deviated from the straight line. It is possible that these deviations may reflect a mechanism of death which differs from that of the higher doses of *E. coli* endotoxin.

*Complement Levels in Control Rabbits.*—Fig. 2 illustrates the per cent change in C′ at 1 and 3 hours in rabbits given no endotoxin. The mean fall in C′ was −4.16 per cent at 1 hour and −7.99 per cent at 3 hours. A complement fall was considered to be significant if the levels fell at or beyond the 95 per cent confidence limits of the control fluctuations.[1] These limits were −16.6 per cent at 1 hour and −28.4 per cent at 3 hours.

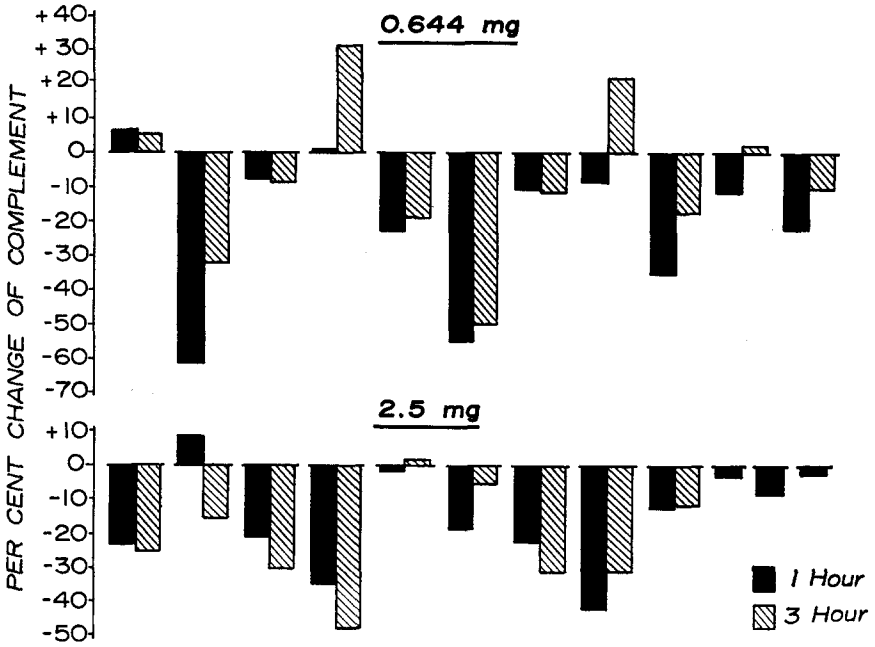


FIG. 4. Complement levels after injection of 2.5 and 0.644 mg of *E. coli* endotoxin into non-immunized rabbits.

*Complement and Antibody Levels after Injection of E. coli Endotoxin into Non-Immunized Rabbits.*—

*A. 5.0 mg:* Fig. 3 demonstrates the changes in C′ levels in three groups of rabbits after injection of 5.0 mg of *E. coli* endotoxin. In the first experiment, C′ fell sharply in 6 of 8 rabbits at 1 hour and in all 8 at 3 hours. In the other two experiments, the depression in C′ levels was not as marked as the changes in the first group. The reasons for these differences are not apparent. Antibody measured in the first two experiments fell four fold or completely disappeared from the sera (Table II A). Serum complement levels in these three experiments dropped in 18 of 25 animals (72 per cent) at 1 hour and in 12 of 23 (52.2 per cent) at 3 hours.

[1] Calculated by Student's t test: Standard deviations at 1 and 3 hours were 5.59 and 8.90.

*B. 2.5 and 0.644 mg:* These two doses above the $LD_{50}$ (but below consistent lethality) lowered C′ with less regularity. Fig. 4 illustrates that 2.5 mg produced a fall in C′ in 6 of 12 (50 per cent) rabbits at 1 hour and in 4 of 9 (44.4 per cent) at 3 hours. Antibody fell in 4 of 12 rabbits (Table II A) and could be correlated with a C′ fall in 3 animals. 0.644 mg lowered C′ in 5 of 11 (45.5 per cent) at 1 hour and in 2 of 11 (18.2 per cent) at 3 hours. In 2 rabbits C′ increased at 3 hours. At this dose a fall in antibody was noted in only 1 rabbit.

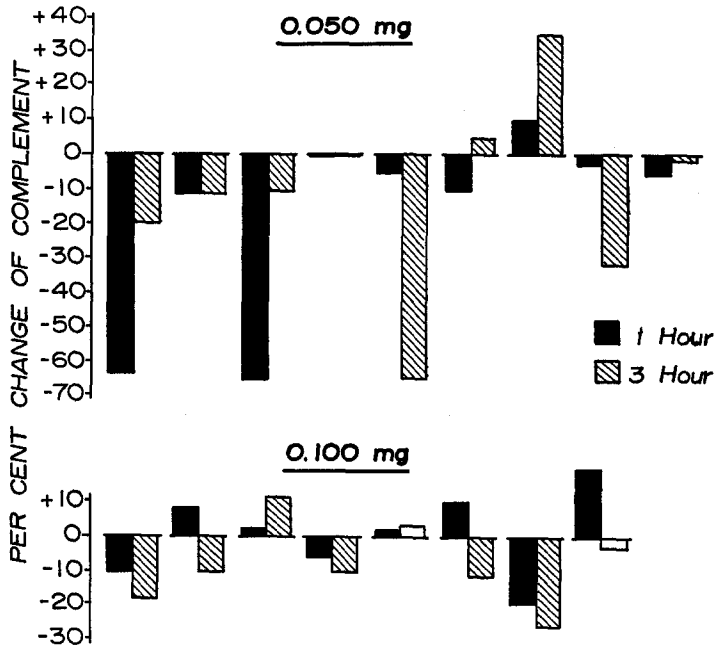


FIG. 5. Complement levels after injection of 0.100 and 0.050 mg of *E. coli* endotoxin into non-immunized rabbits.

*C. 0.100 and 0.050 mg:* These doses below the $LD_{50}$ lowered C′ sporadically (Fig. 5). At 0.100 mg only 1 rabbit displayed a fall in C′ at 1 hour and none at 3 hours. At 0.050 mg C′ fell −64.5 and −66.8 per cent in 2 rabbits at 1 hour only, and −66.6 and −32 per cent in 2 animals at 3 hours only. These two doses induced an occasional increase in C′. (Table II A illustrates that antibody never fell with these two doses of endotoxin.)

*D. 0.00613 mg:* At the other extreme of the $LD_{50}$, 0.00613 mg also caused an occasional fall in C′ (Fig. 6). In the first experiment C′ fell just beyond the 95 per cent confidence limits in 3 of 10 at 1 hour but in none at 3 hours. In the second experiment, this dose produced in 3 of 11 rabbits a fall in C′ levels at

1 and 3 hours ranging from 50 to 85 per cent. Antibody, measured in the second experiment, did not change (Table II A).

*E. Summary of the incidence of C′ fall at each dose:* Fig. 7 summarizes the incidence of C′ fall in each group of rabbits at various doses within the dimensions of the $LD_{50}$. The lethal 5.0 mg dose of endotoxin reduced C′ in 72 per cent at 1 hour and in 52.2 per cent at 3 hours. Here antibody titers almost always fell. At 2.5 mg C′ fell at 1 and 3 hours in about half the rabbits, with a fall in antibody in only 4 animals. At 0.644 mg, a dose just above the $LD_{50}$, C′ fell

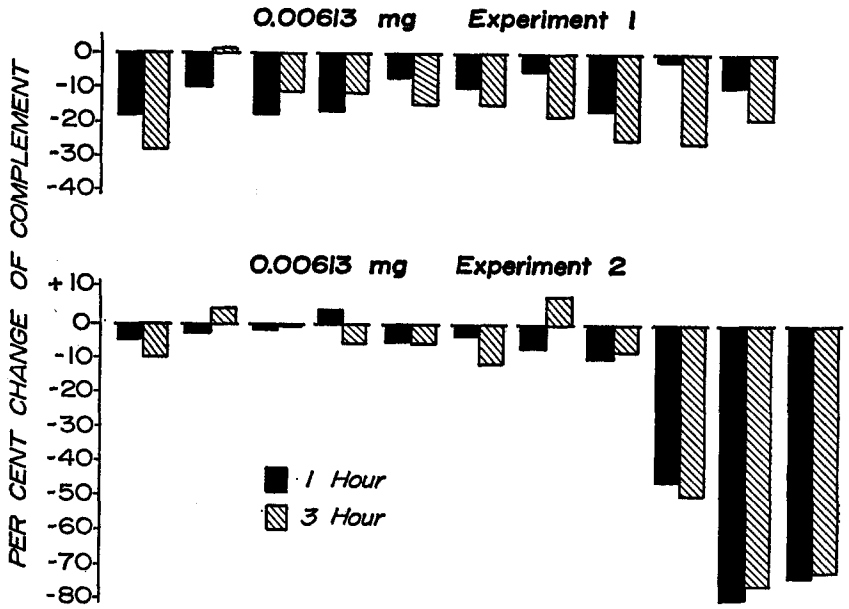


FIG. 6. Complement levels after injection of 0.00613 mg of *E. coli* endotoxin into non-immunized rabbits.

in 45.5 per cent at 1 hour and in 18.2 per cent at 3 hours, and antibody dropped in only 1. Below the $LD_{50}$ C′ fell sporadically. A dose of 0.100 mg produced a fall at 1 hour in 12 per cent, but no reduction occurred at 3 hours; 0.050 mg lowered C′ in 22 per cent at 1 and 3 hours; and 0.00613 mg reduced C′ in 28.6 per cent at 1 hour and 14.3 per cent at 3 hours. These doses below the $LD_{50}$ never lowered the antibody titers to endotoxin.

*Complement and Antibody Levels after Injection of E. coli Endotoxin into Immunized Rabbits.*—The purpose of this experiment was to examine the influence of high antibody titers on the C′ changes induced by endotoxin. Antibody titers up to 1:1024 developed in these rabbits (Table II B).

Fig. 8 demonstrates the changes in C′ in immune rabbits receiving 5.0 and

TABLE II

*Reciprocal Titer of Hemagglutinins for Endotoxin-Sensitized Erythrocytes before and after Injection of E. coli Endotoxin in Rabbits*

| Dose endotoxin, *mg* | Animal No. | Before endotoxin | 3 hrs. after endotoxin | Dose endotoxin, *mg* | Animal No. | Before endotoxin | 3 hrs. after endotoxin |
|---|---|---|---|---|---|---|---|
| *A. Non-immunized rabbits* | | | | | | | |
| 5.0 Exp. I | 1 | 4 | 0 | 0.050 | 1 | 16 | 16 |
| | 2 | 4 | 0 | | 2 | 32 | 32 |
| | 3 | 4 | 0 | | 3 | 16 | 16 |
| | 4 | 2 | 0 | | 4 | 8 | 4 |
| | 5 | 4 | 0 | | 5 | 8 | 8 |
| | 6 | 8 | 0 | | 6 | 4 | 4 |
| | 7 | 8 | 0 | | 7 | 8 | 8 |
| | 8 | 4 | 0 | | 8 | 8 | 8 |
| | | | | | 9 | 4 | 4 |
| 5.0 Exp. II | 1 | 16 | 2 | | | | |
| | 2 | 16 | 1 | 0.00613 Exp. II | 1 | 2 | 0 |
| | 3 | 8 | 0 | | 2 | 2 | 2 |
| | 4 | 8 | 2 | | 3 | 64 | 64 |
| | 5 | 4 | 0 | | 4 | 8 | 8 |
| | 6 | 4 | 1 | | 5 | 16 | 16 |
| | 7 | 64 | 0 | | 6 | 8 | 8 |
| | | | | | 7 | 8 | 8 |
| 2.5 | 1 | 4 | 0 | | 8 | 8 | 8 |
| | 2 | 64 | 4 | | 9 | 32 | 32 |
| | 3 | 8 | 4 | | 10 | 8 | 8 |
| | 4 | 64 | 2 | | 11 | 8 | 4 |
| | 5 | 16 | 8 | *B. Artificially immunized rabbits* | | | |
| | 6 | 128 | 8 | | | | |
| | 7 | 8 | 4 | 5.0 | 1 | 1024 | 32 |
| | 8 | 16 | 8 | | 2 | 1024 | 16 |
| | 9 | 8 | 8 | | 3 | 256 | 8 |
| | | | | | 4 | 32 | 2 |
| 0.644 | 1 | 16 | 4 | | 5 | 256 | 4 |
| | 2 | 8 | 8 | | 6 | 64 | 1 |
| | 3 | 4 | 4 | | | | |
| | 4 | 4 | 4 | 0.050 | 1 | 64 | 2 |
| | 5 | 8 | 8 | | 2 | 32 | 0 |
| | 6 | 32 | 32 | | 3 | 128 | 16 |
| | 7 | 4 | 2 | | 4 | 1024 | 128 |
| | 8 | 2 | 2 | | 5 | 64 | 4 |
| | 9 | 8 | 4 | | 6 | 1024 | 1024 |
| | 10 | 8 | 4 | | 7 | 512 | 128 |
| | 11 | 4 | 4 | | 8 | 512 | 128 |
| | | | | | 9 | 256 | 64 |
| 0.100 | 5 | 8 | 8 | | 10 | 256 | 16 |
| | 6 | 16 | 16 | | | | |
| | 7 | 4 | 8 | | | | |
| | 10 | 32 | 32 | | | | |

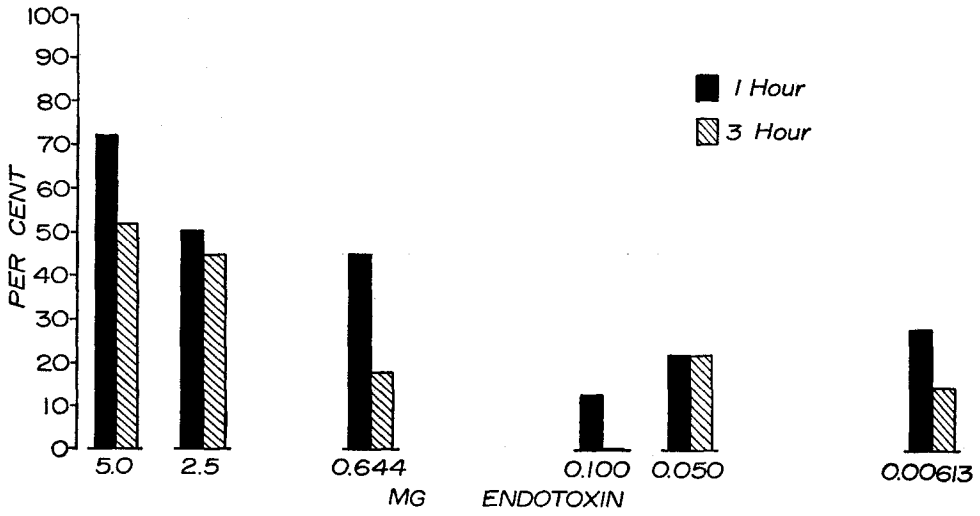


FIG. 7. Influence of the dose of *E. coli* endotoxin on the incidence of complement fall in non-immunized rabbits.

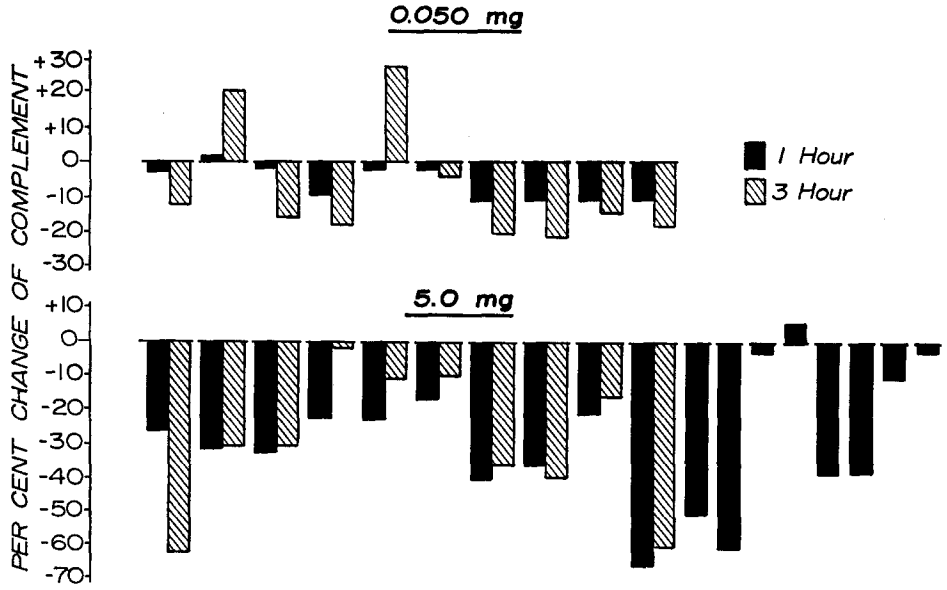


FIG. 8. Complement levels after injection of 5.0 and 0.050 mg of *E. coli* endotoxin into artificially immunized rabbits.

0.050 mg *E. coli* endotoxin. 5.0 mg lowered C′ in 14 of 18 animals (72.7 per cent) at 1 hour and in 6 of 10 (60 per cent) at 3 hours. Antibody titers fell sharply in most of the animals examined for antibody (Table II B). After injection of 0.050 mg endotoxin, antibody levels dropped in 9 of 10 immunized rabbits

(Table II B), but C′ fell in none. These findings differed from those in non-immune animals where C′ but not antibody fell in 22 per cent of rabbits after injection of 0.050 mg of endotoxin.

*Complement Levels after Injection of Proteus mirabilis Endotoxin into Non-Immunized Rabbits.*—5.0 mg of *Proteus mirabilis* endotoxin lowered C′ dras-

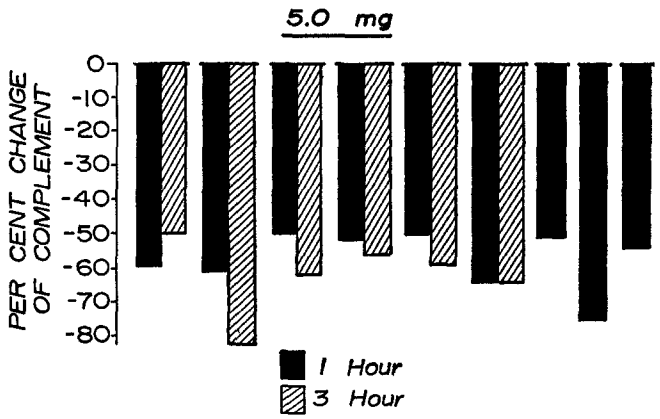


FIG. 9. Complement levels after injection of 5.0 mg of *Proteus mirabilis* endotoxin into non-immunized rabbits.

TABLE III

*Concentration of Endotoxin in Rabbit Sera 1 and 3 Hrs. after Injection of 5.0 mg Radioactive E. coli Endotoxin*

| Rabbit | 1 hr. | | 3 hrs. | |
|---|---|---|---|---|
| | CPM/ml | mg/ml | CPM/ml | mg/ml |
| 1 | 2,714 | 0.0327 | 1,657 | 0.0200 |
| 2 | 2,315 | 0.0279 | 1,294 | 0.0156 |
| 3 | 2,725 | 0.0328 | Expired | — |
| 4 | 1,601 | 0.0193 | 1,323 | 0.0120 |
| 5 | 2,634 | 0.0317 | 1,510 | 0.0183 |
| 6 | 2,339 | 0.0282 | 1,432 | 0.0173 |

tically in all 9 rabbits at 1 hour and in all 6 survivors at 3 hours (Fig. 9). Antibody to *Proteus* endotoxin was not measured in these animals. These findings indicate that the *in vivo* reduction of C′ by endotoxin is not unique to the endotoxin obtained from *E. coli.*

*Radioactive Endotoxin.*—In order to measure the amount of endotoxin in sera obtained at 1 and 3 hours, and its *in vitro* effect on C′, a separate study was performed with 5.0 mg of *E. coli* endotoxin labeled with radioactive chromium. Table III lists the radioactivity of serum and its equivalent concentra-

tion of endotoxin. In the 6 rabbits tested, the radioactive equivalent of not more than 0.033 mg/ml of endotoxin was present at 1 hour and not more than 0.020 mg/ml at 3 hours. These data on circulating levels of endotoxin were used to evaluate the *in vitro* effect of endotoxin on C′ after the serum was removed from the rabbit.

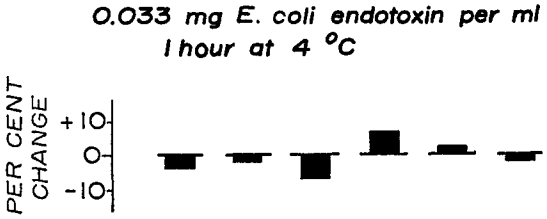


FIG. 10. Complement levels after addition of *E. coli* endotoxin to non-immune rabbit sera, *in vitro* at 4°C.

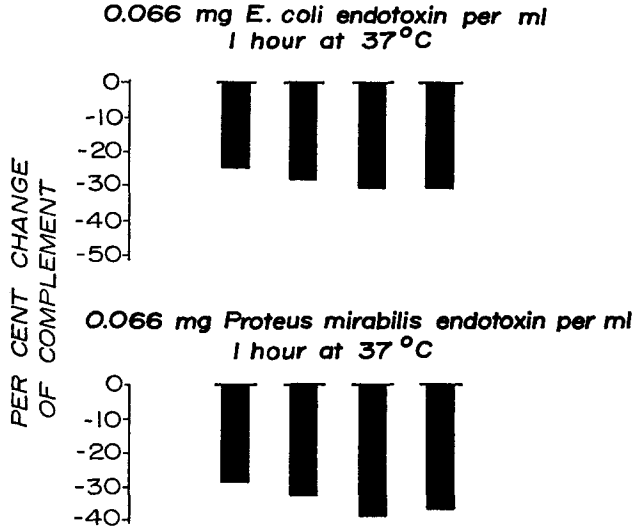


FIG. 11. Complement levels after addition of *E. coli* and *P. Mirabilis* endotoxin to non-immune rabbit sera, *in vitro* at 37°C.

*Complement Levels in Sera after Addition of Endotoxin in Vitro at 4°C.*—In order to prove that C′ was not lowered *in vitro* by the circulating endotoxin removed at the 1 and 3 hour periods, the maximum quantity of *E. coli* endotoxin calculated to be present at 1 hour, 0.033 mg/ml, was incubated *in vitro* at 4°C for 1 hour in six sera from non-immunized rabbits. Fig. 10 demonstrates that C′ was unchanged when the serum-endotoxin mixtures were manipulated under the same temporal and thermal condition as blood.

*Complement Levels in Sera after Addition of Endotoxin in Vitro at 37°C.*—The

direct interaction of endotoxin and non-immunized rabbit sera at 37°C is illustrated in Fig. 11. A concentration of 0.066 mg/ml, which could be expected to be present in circulating rabbit plasma immediately after equilibration of the 5.0 mg intravenous dose of endotoxin, lowers C′ *in vitro* at 37°C. This quantity of either *E. coli* or *Proteus mirabilis* endotoxin dropped C′ from 25 to 40 per cent in eight separate sera when incubated for 1 hour.

## DISCUSSION

The rapid fall in serum C′ that occurs after injection of an antigen into immunized animals has been construed to be the results of "complement fixation" *in vivo* (12). A similar mechanism is suggested by the rapid and sustained fall in serum complement observed in rabbits after injection of large doses of endotoxin. Unlike other antigens, however, endotoxin lowers complement in animals that have not been given artificial immunizing injections. This unique property of endotoxin might be attributed to "natural" immunization of these animals by intestinal bacteria (13). Antibodies to endotoxin were easily demonstrated in normal rabbits, and their reaction with endotoxins *in vivo* was suggested by a fall in titer 3 hours after injection.

Complement and antibody usually fell together after injection of large doses of endotoxin into either artificially immunized rabbits or those with "natural" antibody. This parallelism disappeared, however, in contrasting ways as the dose was lowered. In artificially immunized rabbits, a low dose of endotoxin reduced the antibody titers but not C′. In animals with "natural" antibody, lower doses produced a marked fall in C′ occasionally, but antibody titers remained unchanged. The first of these observations suggests that in artificially immunized animals the participation of complement in endotoxin-antibody reactions may lessen as the ratio of endotoxin to antibody diminishes. The second observation suggests that in animals not subjected to artificial immunization the sharp reduction of C′ by low doses may be the result of mechanisms which are not necessarily immune in nature. It is possible, for example, that anti-complementary substances may be released from injured tissue, or that C′ may be sequestered in organs injured by endotoxin. It has also been suggested by Pearlman, Sauers, and Talmage (14) that complement may be bound by antibody-forming cells after injection of endotoxin.

In addition to these questions on the mechanism whereby serum complement levels are reduced, the present studies raise other questions concerning the role of complement in mediating the injurious action of endotoxin. Spink and Vick (2) were the first to suggest that complement may be required for such injury after they observed that lethal endotoxin shock was prevented when a heat-labile factor was removed from dog plasma. Further evidence along this line is provided by our observation that consistent falls in complement levels occur only with a consistently lethal dose of endotoxin. Lower doses of endotoxin

also reduced C′ levels and killed animals but did so only sporadically. No direct correlations could be made, however, in individual animals between complement changes and lethality because removal of blood for C′ assay affected lethality. Instead it was necessary to measure complement levels and lethality ($LD_{50}$) separately and correlate the results between the two groups. Although these limitations, and others, call for caution in interpretation of these results, they are consistent with the theory that endotoxin produces injury, at least in part, through an antigen-antibody reaction. This theory is based on the similarities between anaphylactic and endotoxic shock (15); on the *in vivo* release of histamine and serotonin after injection of endotoxin (16–18); and on the Arthus-like reaction to endotoxin in human skin (4). While these analogies imply that endotoxin may be involved in an immediate type of hypersensitivity reaction, Stetson (19) has pointed to certain effects of endotoxin that resemble delayed hypersensitivity. The results of the present study are more consistent with the view that endotoxin has an anaphylactic effect.

## SUMMARY

Injection into rabbits of a consistently lethal dose of endotoxin regularly produced a rapid and sustained fall of complement levels and lowered the titers of antibody to endotoxin. Doses of endotoxin below the $LD_{50}$ lowered complement levels sporadically but not the titers of antibody to endotoxin. The possibility is discussed that complement may be involved in the mechanisms responsible for the lethal action of endotoxin.

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