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Sequential Metabolic Expressions of the Lethal Process in Human Serum-Treated *Escherichia coli*: Role of Lysozyme

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Several metabolic parameters indicative of *Escherichia coli* function and integrity were kinetically examined in response to treatment with normal human serum in the presence and absence of functional human lysozyme. Specific inhibition of this enzyme in bacteriolytic and bactericidal reactions was accomplished by using purified rabbit anti-human lysozyme immunoglobulin G. Initiation of the complement-mediated alterations of cytoplasmic membrane integrity, as judged by the leakage of ^{86}Rb from prelabeled cells or the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside by a cryptic strain, was found to be independent of lysozyme action. Furthermore, inhibition of macromolecular synthesis by *E. coli* in response to serum treatment occurred at the same time regardless of the functional state of lysozyme. Although the rate and extent of bacteriolysis were reduced in the absence of lysozyme, the bactericidal kinetics was unaffected. These results demonstrate that the lethal events associated with the action of antibody and complement on gram-negative bacteria are independent of lysozyme, suggeting an accessory role for this enzyme in immune reactions. A possible temporal sequence of complement-induced effects occurring at the cell surface is presented.

The bactericidal and bacteriolytic properties of mammalian sera have been recognized for nearly 100 years. An extensive literature on these subjects has accumulated during this time (reviewed by Inoue [17]). Even with the vast amount of data thus far gathered, however, much remains to be learned concerning the biochemical basis by which serum kills susceptible gram-negative bacteria. Although the involvement of antibody and complement has been well established, the temporal sequence of metabolic and structural aberrations leading to serum-induced death of these bacteria remains a mystery. Impressive advances have been made in understanding the hemolytic effect of antibody-complement, but the bactericidal reaction mechanism has not been as extensively studied. Thus, the possible role of serum-induced lysis of invading gram-negative organisms in host defense remains an open question.

We have reinvestigated the role of serum lysozyme in the human serum bactericidal and bacteriolytic reactions. Special emphasis was placed on how quantitatively measurable metabolic parameters of the test bacteria were sequentially affected by human serum in the presence and absence of functional human lysozyme. Specific inhibition of this enzyme was accomplished not by the commonly used method of bentonite absorption (12, 17, 33, 34), but by reaction with rabbit anti-human lysozyme antibody. This technique has been successfully used in studies on the bactericidal action of mammalian lysozymes and sera against *Bacillus subtilis* (3, 30).

The results presented here indicate that normal human serum lysozyme is not directly involved in the in vitro serum bactericidal reaction against *Escherichia coli*. Its role in bacteriolysis, however, is far more direct, since specific inhibition of lysozyme greatly reduces the kinetics of cellular lysis. Thus, although the presence of lysozyme severely affected the final lytic rate, the bactericidal event per se was found to be independent of cell lysis. Furthermore, our results suggest a sequence of events which may occur on the cell surface that ultimately lead to cellular death.

MATERIALS AND METHODS

Organisms and growth conditions. Three strains of *E. coli* were used. Strain W7 (kindly supplied by M. Thomashow), a K-12 derivative, is a diaminopimelic acid (DAP) and lysine auxotroph; strain A324-4 (a gift from A. Fowler and I. Zabin, Department of Biological Chemistry, University of California, Los Angeles), also derived from strain K-12, is an inducible merodiploid having the genotype *lacIZ*$^+$*Y*$^+$*A*$^+$ *pro*/F*lacI*$^+$*Z*$^+$*Y*$^+$*A*$^+$ *pro*$^+$; strain ML35 (*lacI lacY*$^+$) is constitutive for β-galactosidase and is galactoside permease negative. Strains W7 and A324-

4 were found by B. A. D. Stocker of Stanford University to be susceptible to rough-specific phages, whereas ML35 was resistant. All strains were grown at 37°C in a rotary shaking water bath in minimal medium 63 (16), using 0.4% glycerol as the carbon source, supplemented with 0.25% Casamino Acids (Difco Laboratories). For growth of strain W7, the medium was also supplemented with 0.1 mM DAP; 1 μg of thiamine per ml was added to the medium for the growth of A324-4. Overnight cultures, started from a slant inoculum, were diluted 1:100 into prewarmed medium and incubation was continued until the cell density was between 1×10^8 to 2×10^8 per ml (late logarithmic growth phase), at which time the cultures were harvested by centrifugation (12,000 × *g*, 5 min, 4°C). The sedimented cells were suspended to a density of approximately 10^9 per ml in 50 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM Mg^{2+}, 0.15 mM Ca^{2+}, and 0.4% glycerol (standard buffer solution). The cell suspensions were chilled in ice and used immediately. Control experiments with all strains indicated that storage in ice for as long as 3 h did not affect the experimental outcome.

Preparation of normal human serum. Blood was drawn from healthy adult donors by venipuncture, using a 16-gauge needle and plastic syringes. A clot was allowed to form in sterile plastic centrifuge tubes at room temperature for 2 h, at which time the tubes were rimmed and refrigerated at 4°C for 12 h. The contracted clot was sedimented at 6,000 × *g* for 30 min at 4°C, the serum supernatant was decanted, and equal portions were stored in sterile plastic tubes at −20°C. For some experiments, the serum was exhaustively dialyzed against phosphate buffer containing Mg^{2+} and Ca^{2+} ions (as above) at 4°C. The dialysis treatment had no effect on serum bactericidal or bacteriolytic properties.

Purification of human lysozyme and antilysozyme IgG. The purification of human lysozyme, preparation of rabbit antiserum, and separation of the immune immunoglobulin G (IgG) fraction were as previously described (30). Protein concentrations were determined by the method of Lowry et al. (20). In experiments where lysozyme activity was to be neutralized by its specific immune IgG, the serum was preincubated at 37°C with the IgG for 2 min before the reaction was initiated by the addition of cell suspensions.

Iodination of human lysozyme. Purified human lysozyme was iodinated according to the procedure of David (6), using solid-state lactoperoxidase (Worthington Biochemicals Corp.) and carrier-free ^{125}I (ICN). The iodinated protein was recovered by filtration through a column (0.5 by 45 cm) of Sephadex G-50, followed by dialysis against phosphate buffer. The protein product used in these experiments contained 2.5×10^9 cpm per mg of protein.

Bactericidal assay. The bactericidal assay was performed as previously described (21). In these experiments, the rate of reduction of colony-forming units was used as a measure of killing kinetics. All samples were examined in duplicate, with and without neutralization of the lysozyme with its specific immune IgG. Values for such samples agreed within 10%. It has been previously demonstrated that the immune IgG prepared from rabbit anti-human lysozyme antiserum acts as a specific inhibitor of human lysozyme (12, 30) in both the bactericidal and the bacteriolytic reactions. As in the previous investigations (12, 30), normal rabbit serum IgG neither enhanced nor suppressed the reactions studied. Human serum heated at 56°C for 30 min to inactivate the complement system was used as a control in all assays.

Bacteriolytic assay. Lysis of bacterial suspensions (1×10^8 to 2×10^8 cells per ml) was continuously monitored at 37°C by following the reduction in absorbance at 540 nm (A_{540}) in a double-beam recording spectrophotometer. For purposes of clarity, we define lysis as a reduction in A_{540}. We do not wish to imply in this definition that dissolution of the peptidoglycan or disintegration of the bacterial cell occurs. The relative activity of lysozyme in serum samples was quantitated by examining the initial rates of lysis of a standard suspension of *Micrococcus lysodeikticus* (30). With this method, 5 to 10 ng of human lysozyme could be readily detected.

Some variability in the extent of the lag period and actual A_{540} reduction was observed, especially with strain ML35. These fluctuations may be ascribed to the physiological state of the cells, although this point was not carefully examined. Meticulous control of the physiological age of the culture at the time of harvest resulted in highly reproducible data from day to day. This control was not always achieved.

Release of [^{3}H]DAP from prelabeled *E. coli* W7. Overnight cultures of strain W7 were diluted into medium 63 containing 0.01 mM DAP; 1 μCi of [^{3}H]DAP (Amersham-Searle) per ml was then added. Incubation was continued until a cell density of 1×10^8 to 2×10^8 per ml was reached, at which time the culture was harvested by centrifugation. The cells were washed once with chilled standard buffer solution supplemented with 0.25% Casamino Acids and 0.01 mM DAP and then resuspended to 10^9 cells per ml in the same medium.

The incubation mixtures for measuring the release of [^{3}H]DAP from prelabeled cell suspensions contained 2×10^8 cells per ml and 20% (vol/vol) dialyzed serum in the above medium. The kinetics of release of polymerized [^{3}H]DAP was measured by removing 0.05-ml portions of the incubation mixture at timed intervals and placing these samples onto 2.3-cm Whatman 3MM filter disks. Within 30 s, the disks were placed into cold 10% trichloroacetic acid containing 0.01% DAP. At the end of the experiment, the accumulated disks were washed three times with cold 10% trichloroacetic acid, three times with 95% ethanol, and three times with ethyl ether (4). After air drying, the disks were counted in a scintillation counter, using Spectrafluor scintillation cocktail (Amersham-Searle).

Oxygen consumption. Oxygen uptake by cell suspensions was measured at 37°C in a Gilson Oxygraph. Air-saturated standard buffer solution was used as the suspending medium for 2.5×10^7 cells per ml. After an initial slope of oxygen consumption was recorded, the desired additions were made from prewarmed solutions in a total volume of 0.1 ml. Final volume of the reaction mixture was 2.0 ml.

Membrane permeability. Exponentially growing cells of strain ML35 were harvested and resuspended

to 10^9 cells per ml in standard buffer solution. The reaction mixture, containing 10^8 cells per ml and 50% (vol/vol) dialyzed serum in standard buffer solution, was incubated at 37°C with occasional mixing. At intervals, 0.1 ml (10^7 cells) was transferred to a prewarmed assay mixture consisting of 0.5 mg of *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma Chemical Co.) in 0.9 ml of 50 mM phosphate buffer, pH 7.4. This assay mixture was immediately mixed and transferred to a prewarmed 1-cm cuvette. The initial kinetics of *o*-nitrophenol production were monitored continuously at 410 nm (A_{410}) in a double-beam recording spectrophotometer at 37°C against a reference cell containing 10^7 cells per ml in buffer. Data are presented as ΔA_{410} per minute versus incubation time in serum. This assay, which is based on that used by Rittenberg and Shilo (27), measures the unmasking of a cytoplasmic enzyme in the cryptic *E. coli* strain.

The release of ^{86}Rb, a potassium analog, from *E. coli* W7 was used to confirm aberrations in membrane permeability. Incorporation of ^{86}Rb was carried out in 50 mM sodium phosphate buffer, pH 7.4, containing Mg^{2+}, Ca^{2+}, and glycerol (as above), as well as 0.1 mM DAP, 0.4 mM lysine, and 10 μCi of ^{86}Rb per ml (specific activity, 2 to 4 mCi/mg; ICN) at 37°C. At intervals, the level of incorporation was monitored by filtering 0.1 ml of the cell suspension through a 0.45-μm membrane filter (Millipore Corp.) which had been previously saturated with 0.1 M potassium phosphate. The filters were washed five times with 1 ml of 50 mM sodium phosphate buffer, air dried, and then counted in Spectrafluor with a scintillation counter, using the ^{14}C-^{3}H window for maximum efficiency. Preliminary experiments revealed that five washes were sufficient to reduce the incorporated counts to a constant basal level.

The washed, ^{86}Rb-prelabeled cells were suspended to 1×10^9 to 2×10^9 per ml in sodium phosphate buffer containing Mg^{2+}, Ca^{2+}, glycerol, DAP, and lysine (as above). Serum and other additives, as indicated in the figure legends, were added, and incubation was carried out at 37°C. At intervals, 0.1 ml of this incubation mixture was removed and filtered through a 0.45-μm membrane filter. The filter was washed with 1 ml of 0.1 M potassium phosphate buffer, pH 7.4; the filtrate and wash were collected directly into scintillation vials and counted as above, using a 25% Triton X-100 cocktail. Under these conditions, a small but significant spontaneous release rate was observed in the absence of serum which has been subtracted from the data presented.

Induction of β-galactosidase in *E. coli* strain A324-4. Exponentially growing cells were harvested and resuspended to 10^9 cells per ml in standard buffer solution. The induction mixture contained 2×10^8 cells per ml, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma), and 0.25% Casamino Acids in standard buffer solution. Dialyzed serum was added at the prescribed times to a final concentration of 20% (vol/vol), and 0.1-ml samples were transferred at intervals to ice-cold assay medium consisting of 0.5 mg of ONPG per ml, 100 μg of chloramphenicol, 0.5 M 2-mercaptoethanol, and 1% (vol/vol) toluene in 0.9 ml of phosphate buffer, pH 7.4. The tubes were mixed and iced until the experiment was completed, at which time they were incubated at 37°C for 60 min. One milliliter of 1 M Na_2CO_3 was added to stop the reaction, and the absorbance at 410 nm due to the liberation of *o*-nitrophenol was recorded. Dialyzed serum had to be used in these experiments to prevent catabolite repression of the inducible β-galactosidase by serum glucose.

Incorporation of radioactively labeled precursors into cellular macromolecules. Most of these experiments were performed with strain W7, although confirmatory data were obtained with the other strains. All strains behaved similarly. The cells were preincubated in standard buffer solution (10^8 cells/ml) for 10 min at 37°C before the experiment was begun. At this time, either an isotopically labeled precursor (2.5 μCi of [^{14}C]leucine or 1.0 μCi of [^{14}C]uracil per ml) or serum was added, and incubation was continued for 2 to 3 min. Serum or isotope was then added, and at timed intervals 0.05-ml samples were removed, spotted onto Whatman 3MM disks, and treated as described above.

[^{3}H]thymidine incorporation was performed in a similar fashion, except that the cells were preincubated at 37°C for 20 min in standard buffer solution containing 0.1 mM DAP, 0.25% Casamino Acids, and 150 μg of deoxyadenosine (Sigma) per ml. [^{3}H]thymidine (specific activity, 1.0 mCi/mmol) was added at 25 μCi/ml and incubation was continued for an additional 10 min, at which time serum was added. Sampling and counting were performed as above. The susceptibility of the incorporated [^{3}H]thymidine counts to the action of deoxyribonuclease I (DNase I) was examined by incubating 0.1-ml samples from the reaction mixtures with 10 μg of DNase I per ml, 2.5 mM Mg^{2+}, and 1 mM thymidine for 30 min at 37°C; 0.05 ml of this mixture was then spotted onto 3 MM disks and treated as above. DNase I and Sepharose-bound DNase I were obtained from Worthington Biochemicals Corp.

RESULTS

Killing kinetics of human serum. Initial experiments were performed to examine the role of human serum lysozyme in the in vitro bactericidal reaction. Figure 1 shows the kinetics of killing of *E. coli* W7 by three concentrations of human serum. The effect of neutralizing all lysozyme activity with its immune IgG on the bactericidal capacity of serum is also presented. No significant differences in the rate or extent of reduction of colony-forming units were seen when lysozyme activity was completely neutralized, regardless of the initial serum concentration used. Virtually identical results were obtained with strains A324-4 and ML35. Thus, the presence of serum lysozyme had little effect on the observed in vitro killing kinetics due to human serum.

Bacteriolytic effects of human serum. In contrast to the lack of effect on killing by serum noted above, antilysozyme IgG markedly affected the serum bacteriolytic reaction. Figure 2 presents a recorder tracing (A_{540} reduction) of a lysis experiment using 10^8 cells of strain W7 per

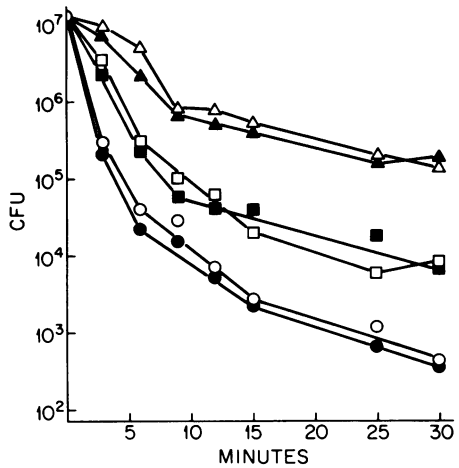

FIG. 1. *Kinetics of killing of E. coli W7 by (▲) 5% (vol/vol), (■) 10%, and (●) 20% human serum. The corresponding open symbols represent the killing kinetics by the same serum concentrations in which all lysozyme activity had been neutralized by its specific immune IgG.*

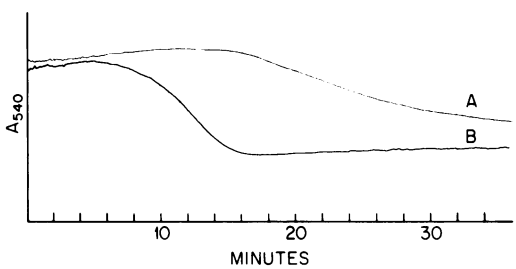

FIG. 2. *Recorder tracing of the lysis of E. coli W7 by human serum. The data presented represent the reduction A_{540} of reaction mixtures containing (A) 5% (vol/vol) serum plus 5 μg of immune IgG and (B) 5% human serum.*

ml and 5% (vol/vol) serum. A lag of 6 to 8 min was routinely observed, followed by a rapid reduction in absorbance due to lysis of the organisms. Neutralization of the lysozyme with immune IgG resulted in an extension of the lag to about 16 to 18 min. After the lag, lysis did occur but with a markedly reduced rate and total extent as compared with what was observed when lysozyme was fully active. The length of the lag period, as well as the rate of lysis, appeared to be functions of serum concentration and differed with each strain tested. At high serum concentrations, the lag period was shortened and the rate of lysis increased. Regardless of the serum concentration used, however, neutralization of serum lysozyme resulted in an extension of the lag period before the onset of lysis and in a reduction in the rate and extent of lysis.

That the slow rate of lysis observed in the presence of antilysozyme IgG was not due to dissociation of the immune IgG-lysozyme complex was confirmed by the results of several control experiments: (i) lysozyme activity against *M. lysodeikticus* was not detectable in any of the neutralized serum samples; (ii) addition of a fourfold excess of immune IgG to reaction mixtures did not decrease the slow rate of lysis observed when the antibody was at the equivalence point; and (iii) removal of the immune IgG-lysozyme complex from serum by high-speed centrifugation before analysis had no effect on the kinetics of killing or lysis compared with samples in which the complex had not been removed. This was determined using ^{125}I-lysozyme. Centrifugation of the IgG-labeled lysozyme complex sedimented greater than 95% of the neutralized enzyme.

The role of lysozyme in the serum bactericidal reaction was further investigated by adding a 30-fold excess of purified human lysozyme (2 μg/ml) to incubation mixtures containing 5% (vol/vol) serum and monitoring the lysis kinetics of strain W7 (Fig. 3). Addition of lysozyme as early as 1 min after initiating the experiment induced an almost immediate reduction in absorbance, whereas the controls (5% serum) showed a 5- to 6-min lag before lysis. Addition of excess human lysozyme at zero time to 5% serum reduced the lag period to about 2 min; a biphasic lytic re-

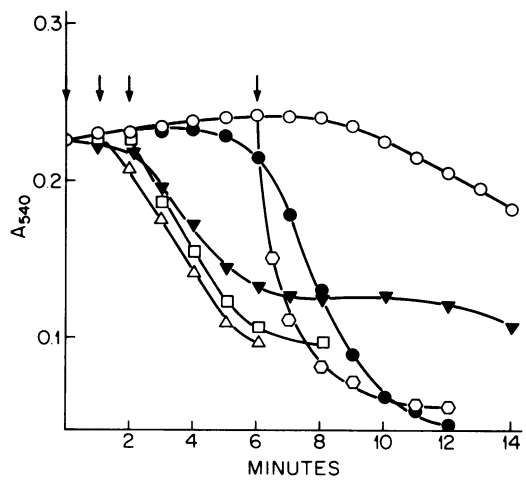

FIG. 3. *Kinetics of lysis of E. coli W7 by 5% (vol/vol) serum and the effect of addition of 2 μg of human lysozyme. Symbols: ●, 5% serum; ○, 5% serum plus 15 μg of immune IgG; ▼, 5% serum plus 2 μg of human lysozyme added at zero time; △, 5% serum plus 2 μg of human lysozyme added at 1 min; □, 5% serum plus 2 μg of human lysozyme added at 2 min; ◯, 5% serum plus 2 μg of human lysozyme added at 6 min.*

sponse was repeatedly observed under these conditions. When excess lysozyme was added to an incubation mixture containing 5% neutralized serum before initiation of the lytic phase, an immediate and dramatic reduction in absorbance was observed. Concentrations of purified human lysozyme as high as 50 μg/ml in buffer had no effect on the *E. coli* strains tested.

Cell wall effects. From the data presented above, one would predict that the *E. coli* cell wall must be undergoing extensive degradation catalyzed by lysozyme after treatment with serum. In those reactions where the enzyme was inhibited with immune IgG, and where lysis proceeded at a much reduced rate (after a prolonged lag), degradation of murein by lysozyme-independent mechanisms may or may not have occurred. To examine this possibility, strain W7 was selected for study because of its auxotrophic requirements for DAP and lysine. These properties allow specific labeling of the murein with [^{3}H]DAP, thus affording measurement of the kinetics of DAP release from the murein by analysis of counts remaining trichloroacetic acid precipitable in response to serum treatment.

Figure 4 presents the results of a typical experiment in which [^{3}H]DAP-prelabeled cells were incubated with 20% (vol/vol) serum in the presence and absence of immune IgG. Lysis kinetics were measured on the same cell suspension under identical conditions. In normal serum, approximately 90% of the polymerized cellular DAP was released within 20 min, with kinetics coincident with cellular lysis. Inhibition of serum lysozyme resulted in a much reduced rate of release, with a lag of about the same duration as that observed in the lysis process. The kinetics of DAP release was roughly 10-fold lower in the lysozyme-inhibited sample, even though the absorbance drop was only 3-fold reduced. This suggested that the absorbance drop was not due solely to murein hydrolysis, which was confirmed by phase microscopic examination. At 20 min postincubation, no intact cells were seen in the serum-treated samples, indicating extensive lysis. In the lysozyme-inhibited samples, however, cells appeared rod shaped and "intact," with very little debris seen in the microscopic field (see reference 29).

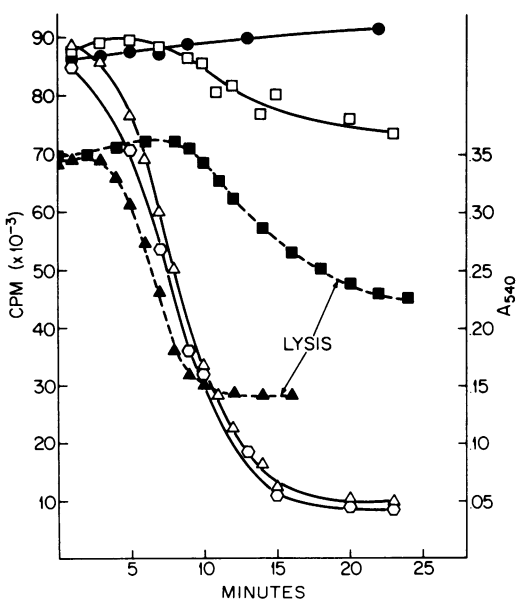

FIG. 4. *Kinetics of [^{3}H]DAP release from labeled E. coli W7 by 20% (vol/vol) serum and the effect of neutralization of lysozyme by anti-human lysozyme IgG. Symbols: ●, no serum control plus 60 μg of immune IgG; □, 20% serum plus 60 μg of immune IgG; △, 20% serum; ○, 20% serum plus 56 μg of normal rabbit serum IgG; ▲, lysis of W7 by 20% serum; ■, lysis by 20% serum plus 60 μg of immune IgG.*

Membrane effects. The lethal effect of antibody and complement in the serum bactericidal reaction is presumed to be manifested at the cytoplasmic membrane (19). Several experimental approaches were used to investigate this possibility. The rate of oxygen consumption by bacterial cell suspensions has been used routinely as an indicator of cytoplasmic membrane metabolic integrity (14). Figure 5 presents an oxygen electrode recorder tracing from a reaction in which 5×10^7 W7 cells were oxidizing glycerol at 37°C. At the arrow, serum (prewarmed to 37°C) was added, and monitoring was continued (final cell concentration, 2.5×10^7 per ml). Serum at 5% (vol/vol) had no effect on the rate of oxygen consumption for 7 to 8 min, at which point the rate decreased dramatically. With 10% (vol/vol) serum, a reduction was observed within 4 to 5 min. Neutralization of serum lysozyme by immune IgG before its introduction into the reaction vessel resulted in a 10- to 12-min lag before a reduced rate of oxygen consumption was observed. Heat-inactivated human serum had no effect on the rate of oxygen consumption. These results are very similar to the lysis patterns presented above and suggest that the cessation of respiration in these asynchronous cultures may be the result of lysis.

An alternate and perhaps more direct measure of membrane integrity involves analysis of substrate permeation into, as well as leakage of metabolites out of, the cells in response to serum treatment. The kinetics of ONPG hydrolysis were examined as a function of incubation time of the cryptic strain ML35 with serum in the

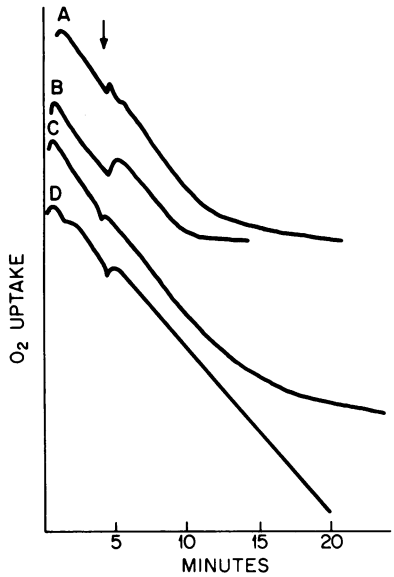

FIG. 5. *Effect of serum on the kinetics of oxygen uptake by E. coli W7. The data presented represent oxygen electrode tracings of reaction mixtures containing (A) 5% (vol/vol) serum, (B) 10% serum, (C) 5% serum plus 15 μg of immune IgG, and (D) 5% heated serum (56°C, 30 min). The arrow shows the time of additions.*

presence and absence of immune IgG. Strain ML35 is unable to hydrolyze ONPG as long as the permeability barrier remains functional. A plot of ΔA_{450} per minute (a function of ONPG hydrolysis per unit time) versus incubation time in serum is presented in Fig. 6. Significant differences in permeability to ONPG were not observed between normal and lysozyme-inhibited serum fractions. Loss of the permeability barrier induced by serum was roughly coincident with initiation of lysis (examined under the same conditions of serum and cell concentration) in normal serum. However, in the absence of lysozyme activity (by neutralization with immune IgG), the permeability barrier to ONPG was destroyed long before lysis was observed. Hydrolysis of ONPG was not induced by lysozyme alone or by heated serum.

Alterations in membrane permeability resulting from incubation with serum were also examined by analysis of low-molecular-weight solute leakage. This was done by analyzing the rate of release of ^{86}Rb from prelabeled W7 cells. ^{86}Rb is a potassium analog not incorporated into cellular macromolecules, and therefore it allows measurement of altered membrane permeability to small molecules. Prelabeled cells were incubated in the presence of 5% (vol/vol) serum with and without immune IgG, and the specific release rates were determined as described in Materials and Methods. Significant amounts of ^{86}Rb were released into the extracellular fluid starting at between 2 and 3 min postincubation (Fig. 7). Neutralization of serum lysozyme did not retard the onset of release of the isotope, but did greatly

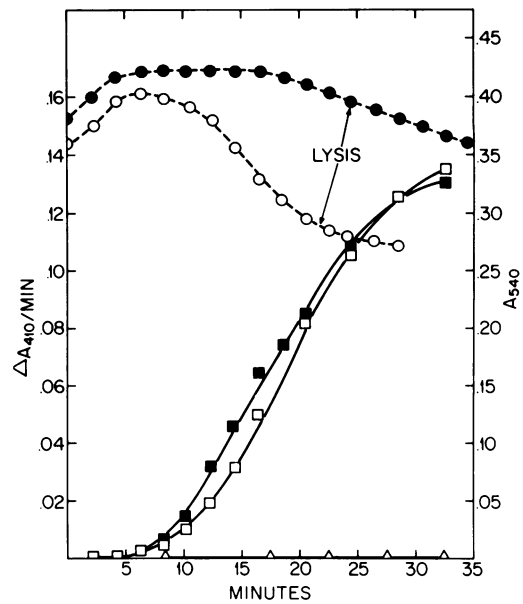

FIG. 6. *Effect of 50% (vol/vol) serum on the kinetics of ONPG hydrolysis by E. coli ML35. Symbols: ●, lysis by 50% serum plus 150 μg of immune IgG; ○, lysis by 50% serum; □, ONPG hydrolysis in 50% serum; ■, ONPG hydrolysis in 50% serum plus 150 μg of immune IgG; △, ONPG hydrolysis in 50% heated serum (56°C, 30 min).*

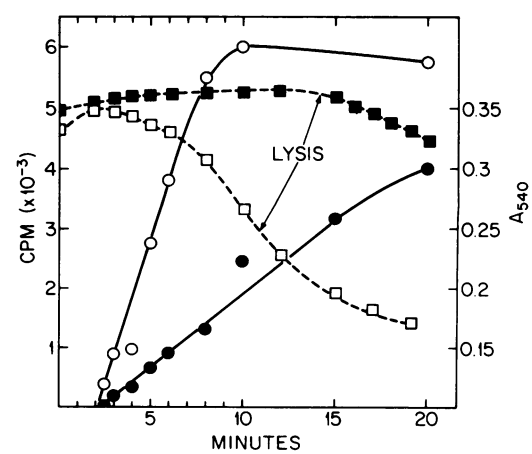

FIG. 7. *Release of ^{86}Rb by prelabeled E. coli W7 induced by 5% (vol/vol) serum. Symbols: ○, 5% serum; ●, 5% serum plus 15 μg of immune IgG; □, lysis by 5% serum; ■, lysis by 5% serum plus 15 μg of immune IgG.*

reduce the release rate. Similar results were obtained at higher serum concentrations. Much to our surprise, the kinetics of release proved linear with time of incubation in serum. We had anticipated a sigmoidal curve of ^{86}Rb efflux as observed for the elimination of crypticity in strain ML35. The results, though puzzling, were reproducible.

Effects on macromolecular synthesis. To determine whether serum treatment induces other metabolic alterations before membrane damage and lysis, the effect of human serum on the macromolecular synthetic potential of *E. coli* W7 was examined with and without neutralization of serum lysozyme. The effect of such incubations on cellular protein and ribonucleic acid (RNA) synthesis was examined by two approaches: (i) by addition of serum to cell suspensions prelabeled for 3 min to follow the fate of incorporated isotope; and (ii) by addition of the isotopes 3 min after initiating serum treatment. Temporally, both types of experimental approaches generated identical results, and therefore only data from the latter will be presented. Figure 8A presents the data for [^{14}C]leucine incorporation into trichloroacetic acid-precipitable protein and indicates a deviation from control rates within 4 to 5 min, or 7 to 8 min after serum addition. Figure 8B shows the data for [^{14}C]uracil incorporation into acid-precipitable RNA. Within 4 min (7 min after serum addition), isotope incorporation was inhibited. Neutralization of lysozyme had no observable effect in the serum-induced inhibition of protein or RNA synthesis.

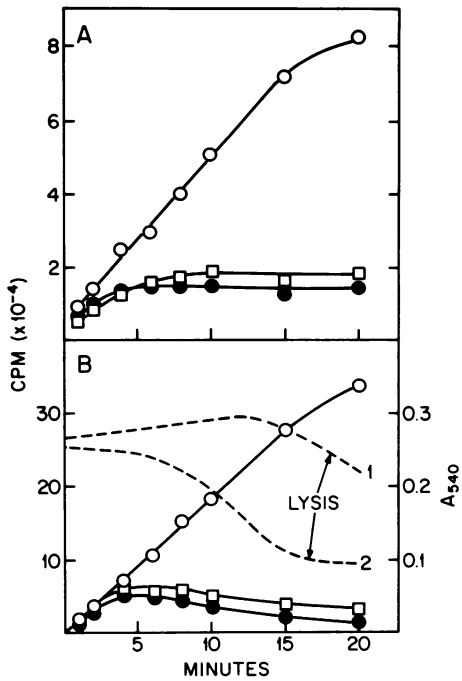

FIG. 8. *(A) Effect of 5% (vol/vol) serum on the incorporation of [^{14}C]leucine into trichloroacetic acid-precipitable protein by E. coli W7. Symbols: ○, 5% heated serum; □, 5% serum plus 15 μg of immune IgG; ●, 5% serum. (B) Effect of 5% (vol/vol) serum on the incorporation of [^{14}C]uracil into trichloroacetic acid-precipitable material by strain W7. Symbols: ○, 5% heated serum; □, 5% serum plus 15 μg of immune IgG; ●, 5% serum. Lysis curve 1, 5% serum + 15 μg of immune IgG; curve 2, 5% serum.*

The preceding data indicate that the potential for both protein and RNA synthesis in *E. coli* was eliminated within 7 min after serum addition. The question was asked whether the protein and messenger RNA made during the initial 6 to 7 min were functional, or became aberrant in structure as a result of serum treatment. Such a response would ultimately lead to death of the cell. To check the fidelity of macromolecular syntheses under these conditions, *E. coli* strain A324-4, an inducible merodiploid that makes roughly 25% of its total protein as β-galactosidase, was used. Figure 9 presents the results of a typical induction experiment. Cell suspensions were incubated in medium containing IPTG as gratuitous inducer; at the arrows, dialyzed serum was added to 10% (vol/vol). Samples were then removed at timed intervals to a cold, toluenized solution containing chloramphenicol to inhibit further protein synthesis. β-Galactosidase activity was assayed with ONPG, and is plotted as the A_{410} versus time of induction with IPTG. The insert in Fig. 9 shows the results of serum addition after 20 min of induction, sampling the incubation mixture at closely spaced intervals. It is clear that regardless of the time of serum addition, enzyme synthesis continued for about 5 min, and thereafter further increase in activity was inhibited. These data, as expected, show the inhibition of protein (β-galactosidase) synthesis within 7 min after serum addition. Messenger RNA and protein made in the presence of serum were, thus, found to be functional.

Incorporation of [^{3}H]thymidine into high-molecular-weight deoxyribonucleic acid (DNA) in strain W7 was also examined. Cell suspensions were preincubated with the labeled precursor for 10 min before the addition of serum with or without immune IgG. Thus, the kinetics of initiation of DNA synthesis, as well as the completion of on-going rounds of replication, were being randomly measured. At intervals, samples were treated with 10% cold trichloroacetic acid (or with DNase I to ascertain the specificity of incorporated counts into DNA). DNA synthesis

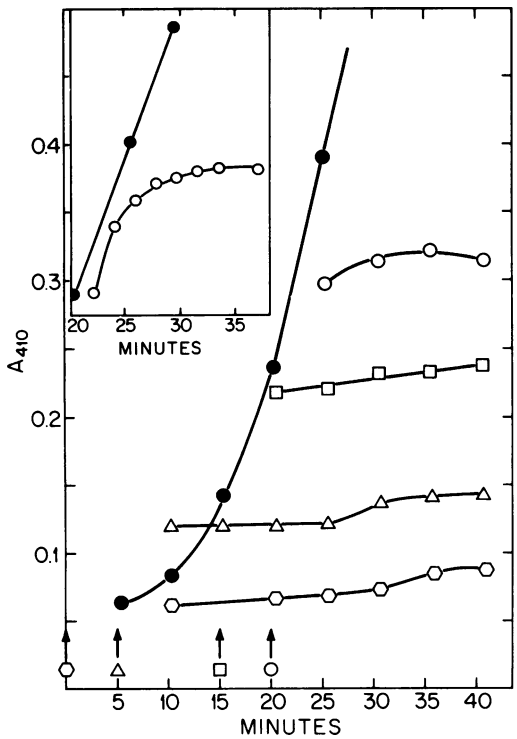

FIG. 9. *Effect of serum on β-galactosidase synthesis in E. coli A324-4. Ten percent (vol/vol) dialyzed serum was added at the times indicated by the arrows to incubation mixtures containing IPTG. The activity of the enzyme at specific times was measured on samples of these cultures by the ONPG procedure as described in the text and is expressed as A_{410}. The symbols under the arrows correspond to the respective curves. ●, 10% heated serum control.*

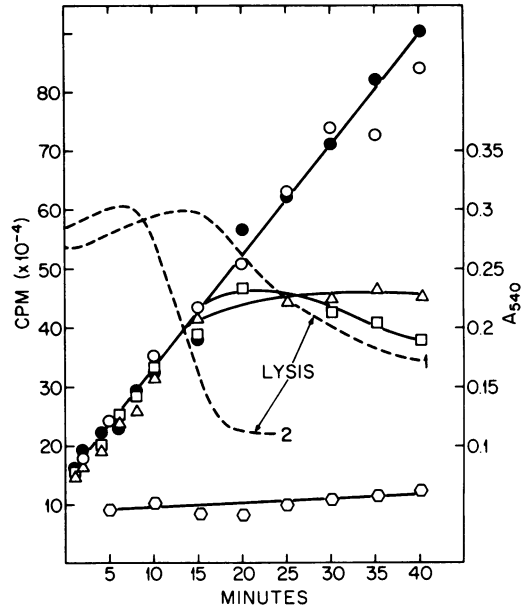

FIG. 10. *Effect of serum on the incorporation of [^{3}H]thymidine into DNA by E. coli W7. Symbols: ○, 5% heated serum; ●, 5% heated serum, DNase I-treated samples; □, 5% serum; △, 5% serum plus 15 μg of immune IgG; ◯, 5% serum plus 15 μg of immune IgG, DNase I-treated samples. Lysis curve 1, 5% serum plus 15 μg of immune IgG; curve 2, 5% serum.*

continued unabated for about 15 min after serum addition at a rate which was indistinguishable from the control rate (Fig. 10). After that point, incorporation ceased rapidly. Most of the label incorporated in the presence of serum was susceptible to DNase I hydrolysis, since the incorporated label was no longer acid precipitable after enzyme treatment. Inhibition of lysozyme activity with immune IgG did not significantly affect the prolonged synthesis of DNA or the susceptibility of incorporated counts to the action of DNase. However, if DNase I coupled to Sepharose beads was used in the experiments, most of the DNA in the lysozyme-inhibited sample was inaccessible to hydrolysis for up to 30 min. In normal serum, results analogous to those obtained with free DNase were obtained. This suggests that the accessibility of polymerized DNA to the action of DNase I most likely reflects the result of cellular lysis. In the control (heat-inactivated serum), none of the incorporated counts were accessible to DNase I.

DISCUSSION

Current theories of immune hemolysis indicate that the late-acting components of complement (C5b, C6, C7, C8, and C9) fuse to form a membrane attack complex (MAC) (1, 19) capable of altering cellular permeability to small ions and water (13). Several models have been proposed which account for this transmembrane channeling effect. The doughnut hypothesis, advanced by Mayer (22), suggests that the MAC forms a hollow, hydrophilic protein channel which allows free exchange of salts and water across the cytoplasmic membrane. On the other hand, the model proposed by Podack and Muller-Eberhard (27) suggests that the MAC combines with membrane phospholipids to form mixed protein-phospholipid micelles, thus producing hydrophilic lipid channels through which salts and water exchange. Both models gain support from experiments which indicate that: (i) certain portions of MAC polypeptide chains insert into the lipid bilayer (15, 24); (ii) fusion of complement components to form the dimeric MAC results in significant increases in detergent (27) and phospholipid (26) binding; and (iii) reaction of the MAC with the cytoplasmic membrane results in reorganization of ordered bilayer

lipids (8) and alteration of membrane fluidity (5).

The above studies define events which result in hemoglobin release and erythrocyte lysis, but additional serum reactions may be involved in the bactericidal action of antibody and complement on gram-negative microorganisms. The multilayered cell envelope of gram-negative bacteria, composed of an inner (cytoplasmic) and outer membrane separated by a peptidoglycan layer, represents a physical barrier which may apply serious constraints on complement action. Other serum components, such as lysozyme, have been examined for their involvement in the bactericidal reaction with conflicting interpretations.

The role of lysozyme in the antibody-complement-mediated bacteriolytic and bactericidal reactions has a long history replete with controversy. There have been many proponents for, as well as opponents against, its requirement in one or both reactions (for review, see reference 17). We believe that the discrepant results and conclusions arrived at by different investigators may be due to (i) the use of bentonite as a "specific" adsorbent for this enzyme from different mammalian sera, and (ii) the use of egg white lysozyme as a replacement for this enzyme in "lysozyme-depleted" sera. Both approaches are fraught with difficulties. Bentonite is far from specific for the quantitative and sole removal of lysozyme (7, 11, 32), and the specific activities of different lysozymes are highly variable in the bacteriolytic and bactericidal reactions (3, 30). To our knowledge, the only other reports using anti-human lysozyme antibody as a specific inhibitor for the human serum enzyme in these reactions were those of Glynn and Milne (11, 12). Although the experimental methodologies we used were drastically different from theirs, the results and conclusions are in reasonable agreement. Their data, however, show that inhibition of serum lysozyme with specific antiserum prevented lysis, but also reduced killing considerably.

The biochemical and physical evidence presented here clearly demonstrates that at the human serum concentrations examined, lysozyme is not directly involved in the in vitro bactericidal reaction against *E. coli*. This follows from an examination of cellular biochemical processes which indicates that serum-induced effects begin to occur at the same time (post-initiation), regardless of the functional state of lysozyme. Thus, although release of peptidoglycan components and cellular lysis are retarded and reduced in response to lysozyme neutralization, increased permeability and inhibition of macromolecular synthesis proceed as in untreated samples. Lysozyme therefore appears to play an accessory role in the serum bactericidal reaction against *E. coli* by facilitating entry of complement components to underlying sites on the cytoplasmic membrane (9, 10, 29).

Our data suggest a temporal sequence of serum-induced aberrations which result in the loss of *E. coli* viability. This hypothesis is based on examination of data obtained under standardized reaction conditions, i.e., 5% (vol/vol) human serum. It further assumes, based on the results from other laboratories (18, 26, 35) that before (or coincident with) an observable permeability effect, complement action may release outer membrane phospholipids into the incubation medium, a phenomenon we have not examined. Making this assumption, our data suggest the following sequence of events. Between 1 and 2 min after the addition of human serum, extensive outer membrane damage occurs which significantly alters the permeability properties of this structure (Fig. 3). This rapid reaction rate is consistent with complement-induced alterations of rat muscle cell membrane potential (31) as well as ion release rates in complement-treated tumor cells (Fig. 7) (see reference 2).

Once outer membrane integrity is lost, lysozyme and complement components penetrate to underlying target sites. It is clear that the complement-damaged outer membrane becomes permeable to these components quite early, since addition of excess lysozyme at zero time initiates cell lysis within 2 min (Fig. 3). This lysis does not occur in the absence of complement. In normal serum, degradation of peptidoglycan results from the action of lysozyme and possibly other factors. Induction or activation of autolytic enzymes could account for these effects, but the results of Schreiber et al. (29) tend to exclude this possibility. Working with purified complement components, they demonstrated that bactericidal activity of the alternate pathway occurred equally well in the absence of lysozyme. Additionally, without lysozyme the turbidity of cell suspensions continued to increase during the complement-dependent bactericidal reaction. The bactericidal reaction is therefore independent of bacteriolysis. The presence of other hydrolytic serum components (i.e., elastase; 25) may account for the slow release of cell wall material and reduced rate of lysis observed in lysozyme-neutralized reactions.

Within 3 to 4 min post-initiation, the permeability barrier of the cytoplasmic membrane breaks down, releasing low-molecular-weight cytoplasmic constituents into the surrounding medium. The ^{86}Rb release data (Fig. 7) further suggest that lysozyme functions primarily to facilitate access of complement components

through the extensively cross-linked peptidoglycan layer. This is apparent from a comparison of the release and lysis data obtained in the presence of lysozyme-neutralized serum. Within 15 min of incubation, and although no cell lysis was observed, the amount of ^{86}Rb released was 65% of that seen in normal serum samples. Cytoplasmic membrane damage therefore occurred long before significant alterations in cell wall structure became apparent.

At 5 to 6 min post-initiation, reductions in biosynthetic capabilities are observed. Protein and RNA syntheses are inhibited at roughly the same time after addition of serum, regardless of lysozyme action. Until this point, cytoplasmic messages and their translated products remain fully functional. The effect of complement on oxygen consumption, however, could be delayed by up to 6 min (13 min post-initiation) when cells were incubated with lysozyme-neutralized serum. This probably reflects reduced rates of respiratory substrate diffusion across the membrane, since lysozyme neutralization also results in reduced rates of ^{86}Rb release. This explanation does not hold for DNA synthesis, however, since inhibition is not observed until well after cell lysis. The significance of this extended synthesis remains unclear. No data are available concerning the fidelity of DNA replication during serum treatment.

It has been demonstrated that complement-mediated membrane depolarization (31) and ^{86}Rb release (2) are nonlethal events in nucleated cells. The results of Boyle et al. (2) further indicate that release of larger cytoplasmic molecules such as aminoisobutyric acid occurs within 3 min after serum addition and, unlike ^{86}Rb release, requires all nine complement components. If similar events occur on the bacterial cytoplasmic membrane, the above sequence suggests that serum-induced death of *E. coli* results from prolonged alterations of membrane permeability, depleting cytoplasmic pools. Similar alterations in cellular metabolism have been reported in response to treatment with hyperimmune rabbit antiserum and guinea pig complement (23). Examination of cellular energetics and precursors, particularly during the first 10 min of complement action, should provide additional information concerning serum control of microbial viability.

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