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# Properties of the Hemolytic Activities of *Escherichia coli*

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Some properties of the cell-free and cell-associated hemolysins of *Escherichia coli* were studied. Several strains of *E. coli* that were isolated from intestines of pigs with edema disease produce large quantities of cell-free hemolysin when grown in the presence of an extract of meat. The component of meat that stimulates production of cell-free hemolysin is not extracted by lipid solvents and is not dialyzable. The cell-free hemolysin is an acidic substance that occurs in two forms. It is inactivated by trypsin but not by lecithinase, lysozyme, ribonuclease, or deoxyribonuclease, shows optimum activity between *p*H 7 and 8, and requires calcium ion for activity. It does not appear to be an enzyme. The kinetics of the lytic reaction are most consistent with the hypothesis that one molecule of cell-free hemolysin is sufficient to lyse one erythrocyte and that it is inactivated in the lytic reaction. The cell-free hemolysin does not sufficiently damage the cell during the prelytic period to cause lysis after the hemolysin-calcium-erythrocyte complex has been disrupted. The cell-associated hemolysin was not separated from the cell by autolysis, freezing, sonic treatment, or treatment with trypsin or lysozyme. It appears to be closely associated with the metabolic status of the cell. Organisms that are highly hemolytic under usual conditions of assay immediately lose most of their hemolytic capability in the presence of sodium cyanide, streptomycin, nalidixic acid, and rifampin.

Certain strains of *Escherichia coli* are capable of hemolyzing red blood cells. Lovell and Rees (6) first reported the isolation of a filterable hemolysin from young cultures of *E. coli* grown in a cooked meat medium. Later Smith (9) described two hemolysins produced by strains of *E. coli*, one (alpha) that could be obtained free from bacterial cells and another (beta) that could not. According to Smith, who tested a large number of hemolytic *E. coli*, alpha hemolysin was not produced on ordinary laboratory media but only on cooked meat medium. However, Snyder and Koch (10) reported that both filterable and nonfilterable hemolysin were produced both in defined media and in heart infusion medium. Recently, a third hemolysin (gamma) has been described in mutants that are resistant to nalidixic acid (12).

The purpose of this paper is to report the production of hemolysins by strains of *E. coli* isolated from the intestine of post-weanling pigs with edema disease and to describe some properties of the cell-free and cell-associated hemolysins from a typical organism of this group. We are concerned with the hemolysins of *E. coli* because hemolysin production has been associated with strains that produce enteric diseases in some species of animals and extra-intestinal diseases in man. The role of these substances in the genesis of disease is a problem of practical importance. The problem is not likely to be solved until they can be purified and studied in animals and cell preparations.

## MATERIALS AND METHODS

**Organisms.** The organisms used in this work were hemolytic strains of *E. coli* that were isolated from the intestines of pigs with edema disease. Petri plates containing sheep erythrocyte agar medium were inoculated with intestinal contents and incubated aerobically at 37 C overnight. Hemolytic colonies were purified by subculturing on MacConkey agar. Typical lactose-fermenting colonies were also submitted to the indole, citrate utilization, hydrogen sulfide production, and carbohydrate fermentation tests. The bacteria were maintained on agar slants. Cultures were routinely incubated in a shaking water bath at about 120 cycles per min at 37 C. The concentration of bacteria in liquid culture was determined by measuring the turbidity at 600 nm. The turbidity of the culture was related to the concentration of viable cells by plate counts. All portions of this work after the initial tests of the conditions required for hemolysin production were conducted with a single strain of *E. coli*, serotype 0141: 85ac:NM (Minnesota stock culture 4331).

**Media.** Alkaline meat extract broth was prepared

as described by Smith (9). Other media used in this work were those described by Snyder and Koch (10) and by Fraser and Jerrel (4). Nutrient Broth was a product of Difco.

**Preparation of alpha-hemolysin.** A culture of *E. coli* was grown in alkaline meat broth to late log phase at which point both beta and alpha hemolysins are present at nearly maximum levels. The culture was chilled in ice, and the cells were removed by centrifugation at 25,000 × *g* for 30 min. The centrifuge was kept at 5 C. The supernatant was filtered through a sterile membrane filter with 45-μm pores (Millipore Corp., Bedford, Mass.). The filtrate, which was the crude alpha hemolysin, was stored at 4 C at *p*H 7.5. The hemolysin is stable for several weeks at 4 C. The concentration of hemolysin in this preparation was usually between 50 and 80 units per ml.

**Erythrocyte suspensions.** Blood was collected from normal sheep and mixed with Alsever's solution. The cells were used within a week after bleeding. Before use in hemolysin assays, the cells were washed three times with 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, *p*H 7.5, containing 0.135 M sodium chloride (Tris-buffered saline). After washing, the cells were suspended in the same buffer at 2% concentration. The 2% cell suspension was always used in assays on the same day it was prepared.

**Preparation of red cell fractions.** Red cells were lysed at 4 C in 10 mM Tris-chloride buffer (*p*H 7.5) at a lysate-packed cell volume ratio of 50:1. A fraction (lysed-cell suspension) of 3 ml was removed and diluted with 9 ml of the buffer containing 10 mM Tris-chloride, 20 mM calcium chloride, and 170 mM sodium chloride. The remainder of the preparation was centrifuged at 10,000 × *g* for 15 min. The supernatant was removed, and 3 ml was diluted as above (cell-free lysate). The precipitate was resuspended in Tris-buffered saline, resedimented, and resuspended in 17 ml of Tris-buffered saline containing 20 mM calcium chloride (ghosts).

**Measurement of hemolytic activity.** Hemolytic activity was measured on the basis of the amount of hemoglobin released in a solution containing hemolysin or hemolytic bacteria, erythrocytes, Tris-chloride buffer, calcium chloride, and sodium chloride. The incubation mixture (2.0 ml total volume) contained 1% sheep erythrocytes, 0.01 M Tris-chloride buffer, *p*H 7.5, 0.02 M calcium chloride, 0.115 M NaCl, and 0.01 to 0.1 units of hemolysin. The hemolysin was diluted for assay in 0.01 M Tris-chloride buffer, *p*H 7.5, containing 0.135 M sodium chloride. The assay vessels were incubated at 40 C for 60 min. At the end of the incubation period, the cells were sedimented by centrifugation at 1,000 × *g* for 10 min. The amount of hemoglobin in the supernatant solution was determined by measuring its absorbance at 540 nm in a model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The extinction coefficient of hemoglobin monomer at 540 nm was taken as $14.7 \times 10^3\ M^{-1}\ cm^{-1}$. One unit of activity is defined as the amount that causes the release of 1 μmole of hemoglobin monomer in 1 hr under the conditions used for assay. Control vessels without hemolysin were treated in the same way as the assay vessels in each experiment. The amount of hemoglobin released was shown to be approximately proportional to the amount of hemolysin in the incubation vessel up to about 0.1 units of hemolysin.

**Chromatography.** Sephadex G-200 and Sepharose 6B were products of Pharmacia. The columns of Sephadex G-200 and Sepharose 6B were 1.5 cm in diameter and 136 cm long. The eluant was Tris-buffered saline, and the elution rate was 6 ml per hr.

Diethylaminoethyl (DEAE) cellulose was Cellex-D, a product of Bio-Rad Laboratories. Samples were dialyzed against 10 mM phosphate buffer, *p*H 6, before chromatography on DEAE cellulose. A column (9 mm in diameter and 150 mm long) was prepared. Elution was carried out with a linear gradient of sodium chloride generated by two cylindrical vessels placed in series. Both vessels contained 10 mM phosphate buffer, *p*H 6, and the vessel away from the column also contained 400 mM sodium chloride.

**Other materials.** The dialysis tubing used in these experiments was boiled for 15 min in 10% sodium carbonate, then in 10 mM ethylenediaminetetraacetic acid (EDTA), *p*H 10, and finally in distilled water. The tubing was washed with distilled water after each step, and it was stored in 10 mM EDTA, *p*H 7.5. Casein hydrolysate was a product of Nutritional Biochemicals Corp., Cleveland, Ohio. Enzymes were from Worthington Biochemical Corp., Freehold, N.J. The water was distilled, passed through a mixed bed demineralizer and an organic removal cartridge (Barnstead), and redistilled from an all-glass still.

## RESULTS

**Production of hemolysins.** The production of alpha- and beta-hemolytic activities was examined with 12 different isolates of hemolytic *E. coli*. The whole cultures of all of the organisms were hemolytic when grown on any of the media used. The maximum concentration of beta hemolysin in cultures was usually between 100 and 500 units per ml. Culture supernatants from which the cells had been removed by centrifugation contained only very low levels of alpha hemolysin (generally less than 1 unit per ml) when cells were grown on any of the media except the alkaline meat extract broth. When cells were grown in alkaline meat extract broth, alpha hemolysin was produced in large quantities (between 20 and 50 units per ml). A typical pattern of cell growth and hemolysin production in alkaline meat extract medium is shown in Fig. 1.

**Conditions that affect production of alpha hemolysin.** The dialyzability of the substance present in meat broth that stimulates production of alpha hemolysin was tested. Meat extract medium (100 ml) was placed in a dialysis bag, and the dialysis bag was placed in 100 ml of Fraser's medium. The meat broth was allowed to dialyze overnight at 4 C. The dialyzed meat

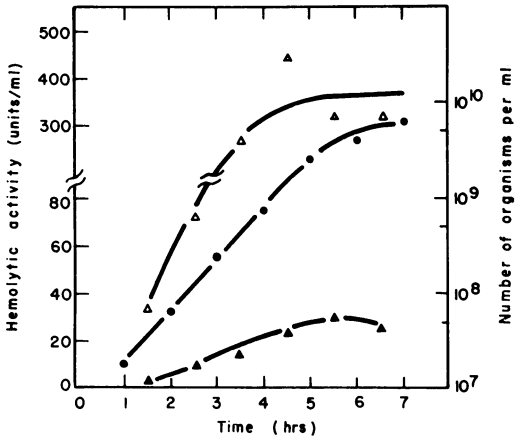


FIG. 1. *Production of hemolysins in alkaline meat extract medium. Concentration of bacterial cells, ●; alpha hemolysin, ▲; and beta hemolysin, △. Samples from the whole culture were diluted with Tris-buffered saline for assay of beta hemolysin.*

broth and the Fraser's medium (diffusate) were transferred to Erlenmeyer flasks, inoculated with hemolytic *E. coli*, and incubated on the shaker bath. Cell-free hemolysin was produced in normal quantities (40 units per ml) in the dialyzed meat broth culture, but less than 1 unit per ml was produced in Fraser's medium (diffusate). A similar experiment was carried out with uncooked meat broth, and the results were the same as with the cooked meat broth.

Lipids were extracted from muscle tissue by sequential extraction with acetone (2×), ether (3×), boiling ethanol (1×), and chloroform-methanol, 3:1 (3×). Approximately 4 ml of solvent was used per gram of tissue in each extraction. The first two solvents were used to extract cholesterol and phospholipids. Both extracted triglycerides. The last two solvents were used as general extractants to remove as much lipid as possible from the meat. No detectable lipid was obtained in the final extraction with chloroform-methanol. Each lipid fraction was pooled, evaporated to dryness, and tested for its ability to stimulate hemolysin production. Each fraction was added to a separate flask containing 50 ml of Fraser's medium which was then inoculated with hemolytic *E. coli*. The cultures were tested periodically for alpha hemolysin throughout the growth period. None of the lipid fractions stimulated production of appreciable alpha hemolysin. The meat residue from which the fat had been extracted was used to prepare meat extract broth in the usual way. This broth stimulated production of alpha hemolysin to about the same extent as meat extract broth prepared by the usual procedures.

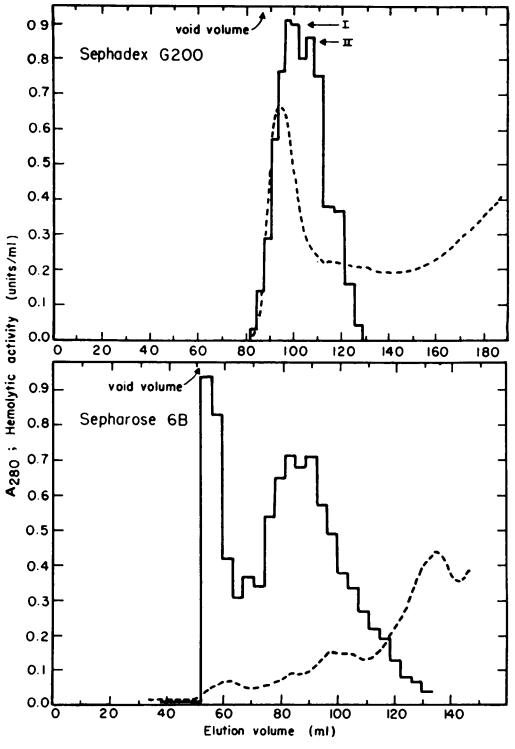


FIG. 2. *Gel filtration of alpha hemolysin. —, Hemolytic activity; ····, $A_{280}$. The supernatant solution obtained by centrifugation of a culture of hemolytic E. coli was the alpha hemolysin preparation.*

**Physical and chemical properties of alpha hemolysin.** A sample of alpha hemolysin (3 ml) containing 27 units of activity was chromatographed on a column of Sephadex G-200. Fractions of 2.97 ml were collected. The absorbance at 280 nm of each fraction was measured, and samples were tested for hemolytic activity. The hemolytic activity was recovered in two major peaks (Fig. 2). Approximately 80% of the hemolytic activity was recovered. Although the peaks are not well resolved, they consistently appeared as separate peaks in different experiments. Because all of the hemolytic activity was eluted from Sephadex G-100 in the void volume as a single sharp peak of activity, the molecular weight of the smaller hemolytic species is probably in excess of $3 \times 10^5$.

A sample of alpha hemolysin (4 ml) was also passed through a column of Sepharose 6B. In this experiment, the hemolytic activity was

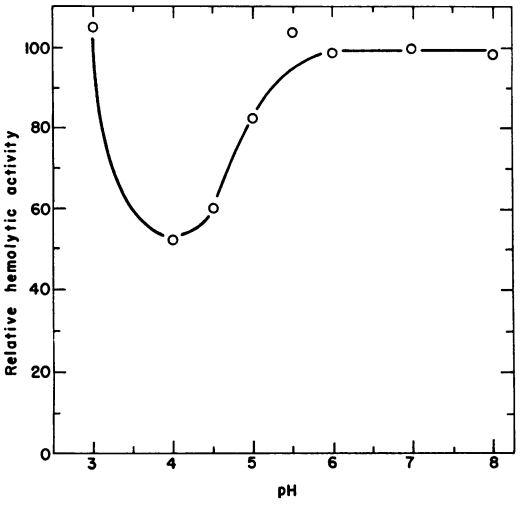


FIG. 3. *Solubility of alpha hemolysin at different pH values.*

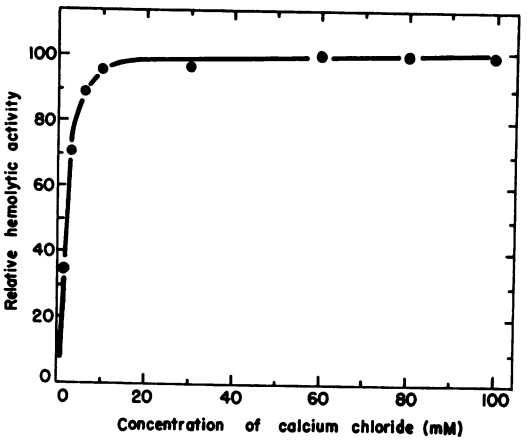


FIG. 4. *Activity of alpha hemolysin as a function of calcium ion concentration in the assay mixture.*

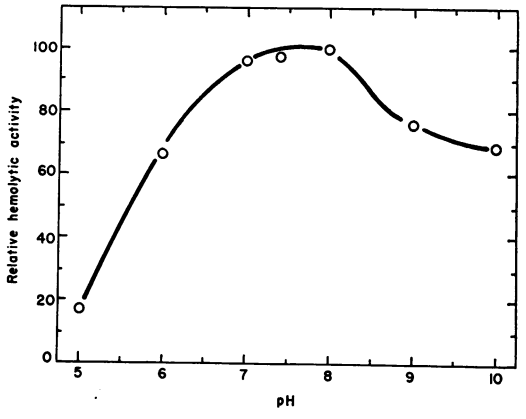


FIG. 5. *Activity of alpha hemolysin as a function of the pH of the assay mixture.*

also recovered in two major peaks (Fig. 2). The hemolysins represented by the two peaks of activity were eluted together from DEAE cellulose. They also behaved the same way under different conditions of assay.

After the hemolysin had been subjected to gel filtration and chromatography on DEAE cellulose, it was subjected to hydrolysis by lecithinase, lysozyme, deoxyribonuclease, ribonuclease, and trypsin. Of the enzymes tested, only trypsin inactivated the hemolysin. Other enzymes were without affect even at very high levels.

Tubes of hemolysin were placed in an ice bath and adjusted to *p*H values between 3 and 8 by adding hydrochloric acid or sodium hydroxide. Visible precipitate was present only at *p*H 4, 4.5, and 5.0. The tubes of hemolysin were centrifuged at 20,000 × *g* for 15 min. The supernatant solutions were collected, adjusted to the same final volume, and assayed by the standard procedure at *p*H 7.5. The results are shown in Fig. 3. Residual activity was recovered from the redissolved precipitates.

**Conditions for hemolysis by alpha hemolysin.** The effect of varying the calcium ion concentration on hemolytic activity is illustrated in Fig. 4. Changing the erythrocyte concentration in the assay vessel did not affect the magnitude of the requirement for calcium. The alpha hemolysin is inactive when incubated in the presence of 10 mM EDTA. The alpha hemolysin is active over a broad range of *p*H, and the optimum range for assay is between *p*H 7 and 8 (Fig. 5). Buffers used were ammonium acetate, *p*H 5; histidine chloride, *p*H 6 and 7; Tris-chloride, *p*H 7 through 9; sodium glycinate, *p*H 9 and 10. The relationship between temperature of incubation and hemolytic activity is shown in Fig. 6. At 60 C the erythrocytes turn brown and lyse. When the concentration of erythrocytes in the assay mixture is increased and the quantity of hemolysin is kept constant, the amount of hemoglobin released is proportional to the logarithm of the erythrocyte concentration up to a concentration of 2 to 4% red cells (Fig. 7). A concentration of 1% was arbitrarily selected for use in routine assays.

The effect of erythrocyte concentration on the extent of hemolysis is shown in Table 1. The data used to prepare Fig. 7 were recalculated to obtain the observed per cent hemolysis at each concentration of erythrocytes. The Poisson formula (1) was used to obtain the calculated

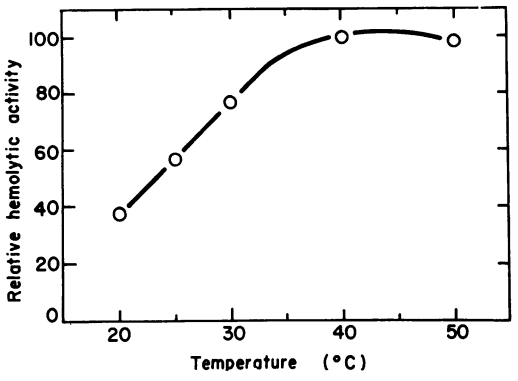


FIG. 6. *Activity of alpha hemolysin as a function of the temperature of the assay medium.*

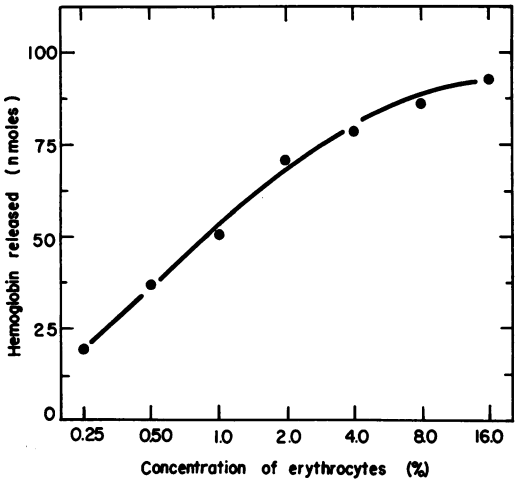


FIG. 7. *Activity of alpha hemolysin as a function of the concentration of erythrocytes in the assay mixture.*

per cent hemolysis at each concentration of erythrocytes. The assumptions were: (i) only one molecule of hemolysin is required to lyse one erythrocyte; (ii) the hemolysin is used up in the lytic reaction; and (iii) the ratio of hemolysin molecules to erythrocytes in the vessel containing 16% erythrocytes was sufficiently low that virtually none of the red cells sustained two or more hits. Under these conditions 2% hemolysis should be produced at a multiplicity of 0.0202 hemolysin molecules per red cell.

To test the possibility that alpha hemolysin might act by oxidation of some component of the cell surface and that the oxidation could be inhibited by one of the common reducing agents, assays of the hemolysin were carried out in the presence of mercaptoethanol (1 to 10 mM), ascorbic acid (1 to 10 mM), and dithiothreitol

TABLE 1. *Variation of per cent hemolysis with concentration of erythrocytes*

| Concn of erythrocytes (%) | Observed hemolysis (%) | Calculated hemolysis (%) |
|---|---|---|
| 16 | 2 | 2 |
| 8 | 3 | 4 |
| 4 | 6 | 7 |
| 2 | 10 | 13 |
| 1 | 15 | 25 |
| 0.5 | 23 | 44 |
| 0.25 | 23 | 69 |

(10 to 100 μM). Control vessels contained no reducing agent. There was no detectable inhibition of hemolytic activity by any of the reducing agents. Assays were also carried out in the presence of cholesterol and lecithin (1 to 10 mg/ml), and no inhibition was observed.

**Direct observation of hemolysis.** The course of hemolysis of erythrocytes by alpha hemolysin was observed with a phase-contrast microscope in order to determine whether partial lysis of a cell population represented complete lysis of some of the cells or leakage from all of the cells. For this purpose the assay was performed with sufficient hemolysin to release between 10 and 30% of the hemoglobin in a suspension of erythrocytes. The preparation was observed through a phase-contrast microscope throughout the period of hemolysis. Shortly after the start of incubation, spherocyte formation was observed. The fraction of red cells that underwent sphering increased as the incubation period continued. The lysing of spherocytes was always observed as an instantaneous bleaching. A large portion of the red cell population appeared normal throughout the incubation period.

**Time course of hemolysis by alpha hemolysin.** The relationship between the rate of release of hemoglobin and the time of incubation of red cells with alpha hemolysin is shown for different red cell concentrations in Fig. 8. Each vessel contained 20 ml total volume of incubation mixture. Cell-free hemolysin (0.8 units) was added to each incubation vessel after it was preincubated at 40 C for 5 min. A control vessel, without hemolysin, was prepared for each cell concentration. The concentrations of the other substances in the incubation vessels were the same as described for routine assays. The vessels were incubated in a shaker bath, shaking just sufficiently fast to keep the erythrocytes in suspension. Fractions of 2.0 ml were withdrawn from the assay and control vessels at different times, transferred to prechilled tubes, and centrifuged at 2,000 × *g* for 3 min at 4 C. The amount

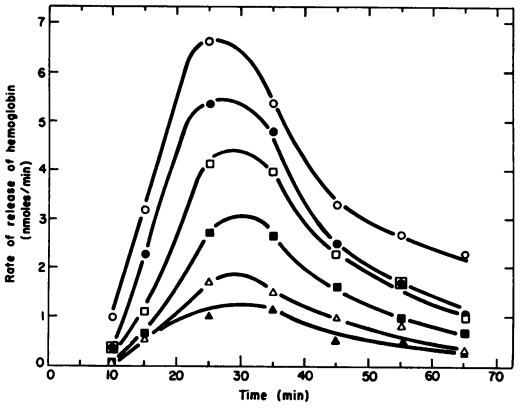


FIG. 8. *Time course of hemolysis at various concentrations of erythrocytes. The initial concentrations of erythrocytes in the incubation mixtures were:* ○, *8%;* ●, *4%;* □, *2%;* ■, *1%;* △, *0.5%;* ▲, *0.25%.*

of hemoglobin released was calculated from the $A_{540}$ of the supernatant solutions. Hemoglobin is released at an increasing rate between 10 and 25 min. After about 35 min the rate of release of hemoglobin decreases. Additional experiments were designed to explore the following possibilities for the rapid decrease in the rate of hemolysis just as the peak rate was attained. (i) The lysed cells or their contents reduce the calcium ion concentration below effective levels by providing additional binding sites. (ii) An inhibitor of hemolysin is released by lysing red cells. (iii) Only a fraction of the cells are susceptible to the action of hemolysin. (iv) The hemolysin is used up in the reaction.

The following experiment was performed to demonstrate whether or not addition of calcium at the end of the period of marked hemolytic activity would stimulate additional hemolytic activity. A vessel (30 ml) which contained 1% erythrocytes, 20 mM calcium chloride, 115 mM sodium chloride, and 10 mM Tris-chloride (*p*H 7.5), and 0.6 units of hemolysin was prepared and incubated at 40 C. Samples of 2 ml each were taken at 10-min intervals, and the amount of hemoglobin released was measured. Calcium chloride was added 50 min after the start of the incubation to achieve a concentration of 30 mM. Sampling and measurement of hemoglobin release were continued at 10-min intervals for an additional 50 min. A control experiment was carried out which differed only in that no calcium was added at 50 min. The rate of release of hemoglobin followed the same pattern out to 50 min as shown for the 1% cell concentration in Fig. 8. The addition of calcium did not promote a second round of hemolysis. The rate of release of hemoglobin in both the control and the tube to which calcium was added remained at less than 0.25 nmoles/min throughout the incubation period from 50 to 100 min.

The possibility that lysed red cells or their contents inhibit alpha hemolysin was tested in the following experiment. A vessel (10 ml) was prepared as described in the preceeding experiment except that it contained approximately 0.2 unit of hemolysin. After 1 hr of incubation at 40 C, two samples of 2 ml each were taken, and the amount of hemoglobin released was measured. At the same time 0.12 unit of hemolysin was added to the remaining 6 ml of incubation mixture. At the end of the second hour, two samples of 2 ml were taken, and the amount of hemoglobin released was measured. During the first hour of incubation 24 nmoles of hemoglobin per ml of incubation mixture was released. During the second hour an additional 24 nmoles of hemoglobin per ml of incubation mixture was released. In a control tube, to which no hemolysin was added at the end of the first hour of incubation, 25 nmoles of hemoglobin per ml was released by the end of the first hour, and only 3 nmoles of hemoglobin per ml was released during the second hour of incubation. Failure of the hemolysin to release additional hemoglobin during the second hour in this control vessel indicates that it is no longer active. In addition to showing that lysed cells do not inhibit hemolysin, this experiment also demonstrates that lysis does not stop because only a fraction of the cells is susceptible to the action of alpha hemolysin. The second charge of hemolysin was essentially as effective as the first. The direct proportionality between the concentration of hemolysin and the amount of hemoglobin released under the usual conditions for assay provides further evidence that there is not a population of cells that is markedly more susceptble to hemolysis.

The experiment shown in Table 2 was performed to examine the inhibitory capacity of the red cell contents and the ghosts. Assay vessels contained 1.0 ml of the added component, 0.1 ml of hemolysin, and 1.0 ml of a 2% suspension of erythrocytes. One control vessel without hemolysin was run with each added component. The vessels were incubated at 40 C. Each vessel was removed from the water bath 60 min after erythrocytes were added. The vessels were centrifuged and the $A_{540}$ of the supernatant solutions was measured. The concentrations of the lysed-cell suspension and the cell-free lysate were one-fourth the concentration represented in the original vessel. This reduced the concentration of

TABLE 2. *Effect of erythrocyte fractions on hemolysis of erythrocytes by alpha-hemolysin*

| Added component | Time erythrocytes added (min) | Amt of hemoglobin liberated (nmoles) |
|---|---|---|
| Lysed red cell suspension | 0 | 20.2 |
| | 30 | 9.5 |
| | 60 | 4.0 |
| Cell-free lysate | 0 | 28.5 |
| | 30 | 16.3 |
| | 60 | 14.9 |
| Ghosts | 0 | 15.0 |
| | 30 | 3.4 |
| | 60 | 1.4 |
| Tris-buffered saline with 20 mM $CaCl_2$ | 0 | 25.9 |
| | 30 | 15.0 |
| | 60 | 11.6 |

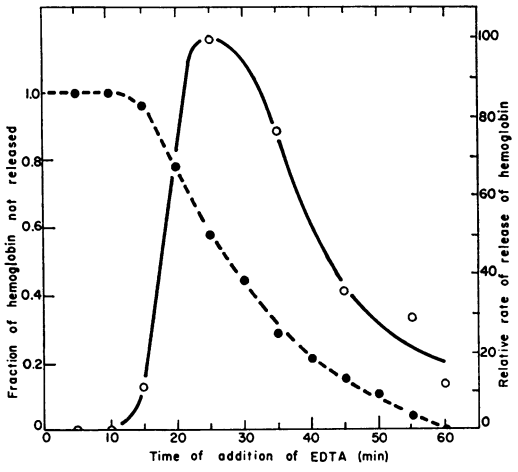


FIG. 9. *Requirement for calcium during the hemolytic period.* ●, *Fraction of hemoglobin released in 60 min that was not released in the time interval indicated.* ○, *Relative rate of release of hemoglobin.*

these components in the assay vessels to the concentration present in the usual assay vessel after sufficient hemolysis has occurred to give an $A_{540}$ of 0.4. Thus, if lysis under usual assay conditions releases sufficient inhibitor to terminate hemolysis, hemolysis should have been inhibited in these vessels. The cell-free lysate caused no inhibition. The lysed cell suspension produced a slight inhibition of hemolysis which was enhanced by preincubating the lysate with alpha hemolysin before adding the red cell suspension. The observed degree of inhibition may be accounted for on the basis that the sites on the lysed cells are able to bind hemolysin but fail to release hemoglobin. This possibility is supported by the results of the experiment in which red cell ghosts were added to assay vessels before and at the same time as the red cells were added. In these experiments the concentration of ghosts was the same as the concentration of red cells. When the ghosts and the red cells were added to the assay vessel at the same time, 15 nmoles of hemoglobin was liberated. In the control tube, without ghosts, 25.9 nmoles was liberated. It can be calculated from Fig. 7 that in the vessel containing ghosts approximately 18 nmoles of hemoglobin should have been liberated if the ghosts and the erythrocytes had the same affinity for alpha hemolysin. Preincubation of the hemolysin with ghosts for 30 min reduced the amount of hemoglobin released to 3.4 nmoles upon subsequent incubation with erythrocytes. It can be calculated from Fig. 7 and 8 that under these conditions approximately 6 nmoles should be released if ghosts reacted with hemolysin at the same rate as red cells.

**Requirement for calcium during the course of hemolysis by alpha hemolysin.** The possibility that calcium is required for the lytic action of alpha hemolysin only during some initial phase of cell damage which occurs early in the incubation period was tested as shown in Fig. 9. A series of assay vessels, volume of 2 ml, was prepared as usual except that the concentration of calcium chloride was 10 mM. At zero time 1.0 ml of 0.1 M EDTA, *p*H 7.5, was added to the first tube; and at the end of each 5-min interval EDTA was added to an additional tube. All tubes were incubated until the end of 60 min; then they were centrifuged and the amount of hemoglobin liberated in each was measured. Addition of EDTA at any time during the incubation period prevents hemolysis during the remainder of the incubation period. Thus, it appears that calcium and presumably the hemolysin-calcium-erythrocyte (HCE) complex must be maintained up to or very near to the lytic event. Since no hemolysis occurred in tubes to which EDTA was added at 0, 5, and 10 min, it is apparent that EDTA effectively inhibited the lytic action of the hemolysin. Comparison of the rate curves in Fig. 8 and 9 indicates that removal of calcium ion terminates lysis as effectively as removal of the red cells. If lysis persisted for a time after the addition of EDTA, the rate curve in Fig. 9 would be displaced to the left.

**Attempts to dissociate beta-hemolytic activity**

**from the cells.** The possibility was considered that beta- and alpha-hemolytic activity are caused by the same molcular species but differ in that one has been dissociated from the cell surface, whereas the other remains bound. If this were the case, then it might be possible to release the beta hemolysin activity in the process of disrupting the cell wall. To test this possibility, cells were grown to late log phase. Assays were performed to quantitate hemolytic activity. The cells were then disrupted by sonic treatment, freeze thawing, autolysis, and digestion by trypsin and lysozyme. Cell-free hemolysin was not obtained in the cell-free 15,000 $\times$ *g* supernatant solutions of any of these preparations. Furthermore, the cell-associated activity declined as the number of viable cells declined.

The results of the above experiments suggested the possibility that the beta-hemolytic activity is an unstable product that is liberated by the viable cell. It was also considered reasonable that such a product might be relatively small. To test the possibility that the beta-hemolytic activity was a diffusible metabolite, a growing culture ($1 \times 10^8$ cells/ml) was placed in a dialysis bag and immersed in 25 ml of a 2% suspension of erythrocytes in Tris-buffered saline containing 20 mM calcium chloride. The preparation was incubated at 40 C. Samples of 2 ml of the erythrocyte suspension were taken at 30 and 60 min, and the degree of hemolysis was determined. Under these conditions there was no apparent lysis of red cells. When the procedure was modified so that the erythrocyte suspension was inside the dialysis bag and the culture was outside, hemolysis still did not occur.

**Inhibition of beta-hemolytic activity.** Experiments were performed to assess whether agents that interfere with cellular metabolism affect beta-hemolytic activity. The agents used were potassium cyanide, 40 and 400 $\mu$M; streptomycin, 200 $\mu$g/ml; rifampin, 100 $\mu$g/ml, and nalidixic acid, 100 $\mu$g/ml. The assay vessels were prepared in the usual way except that the diluted bacteria and the interfering agent were added to the assay vessel just before the red cells.

Cells were tested in the early, middle, and late periods of exponential growth. All of the antibiotic agents completely inhibited beta-hemolytic activity. Potassium cyanide at the lower level caused 80 to 90% inhibition of beta-hemolytic activity; at the higher level it caused complete inhibition. The cell-free supernatants from the cells used in the above experiments were also assayed in the presence of the same interfering agents. The agents did not impair the alpha hemolysin. Twelve different strains of hemolytic *E. coli* that were isolated in this laboratory gave essentially the same results in this experiment.

## DISCUSSION

Cooked or uncooked meat broth and broth prepared from meat homogenate after extraction of lipid contained a nondialyzable substance that promoted the production of alpha hemolysin by certain strains of hemolytic *E. coli*. Lovell and Rees (6) and Smith (9) have previously reported that cooked meat broth was required for the production of alpha hemolysin. Snyder and Koch using a strain of *E. coli* type 06 found that defined medium was as satisfactory as meat broth for the production of alpha hemolysin (10). Snyder kindly supplied us with a culture of his strain of *E. coli*, and our results with his cells were in agreement with his findings. Thus, it appears that different strains of *E. coli* have different requirements for the production of alpha hemolysin. However, all of the strains of hemolytic *E. coli* that we have isolated from pigs have produced much higher levels of alpha hemolysin when grown in meat medium than in defined medium. The role of some component in the meat extract medium in elevating cell-free hemolytic activity is not known, but several possibilities come to mind. (i) It stabilizes a molecule that is produced and liberated by the *E. coli*. (ii) It is converted to the hemolytic substance by the action of the *E. coli*. (iii) It stimulates the liberation or production of the hemolytic substance by *E. coli*.

There are at least two molecular species of alpha hemolysin. The larger species is excluded by Sepharose 6B and the smaller by Sephadex G-100. The possibility that the smaller species is a fragment or subunit of the larger is supported by the observations that the hemolytic properties of the two are similar and the two forms are not separated by chromatography on DEAE cellulose. Cell-free hemolysin appears to contain a protein or polypeptide component which is essential for hemolytic activity as indicated by the inactivation of the hemolytic activity by trypsin. Snyder and Zwadyk (11) recently reported that the alpha hemolysin of another strain of *E. coli*, type 06, was inactivated by trypsin and chymotrypsin. Minimum solubility near *p*H 4 indicates that alpha hemolysin is an acidic substance.

The alpha hemolysin requires calcium ions for activity. The activity increases as the concentration of calcium ion increases until the concentration is approximately 10 mM. Snyder and Zwadyk (11) found that 5 mM calcium ion caused maximum activity of the alpha hemolysin produced by *E. coli*, type 06. This difference in

requirement for calcium ion and the difference in the requirement for meat extract medium for production of alpha hemolysin indicate that the hemolysins of various strains of *E. coli* are not identical.

When the concentration of alpha hemolysin is held constant and the red cell concentration is increased, the amount of hemoglobin that is released is increased (Fig. 7 and 8). This result appears to be due to the formation of an increased number of productive hemolysin erythrocyte interactions at the higher cell concentrations. The decreased number of productive interactions at lower erythrocyte concentrations is probably due to an increased frequency of multiple hits on single erythrocytes. This interpretation raises a question as to the number of hits by alpha hemolysin that are required to lyse one red cell. The data presented in Table 1 support the hypothesis that only one molecule of hemolysin is required to lyse one erythrocyte. The values calculated with the Poisson formula approximate the experimental values over a 10-fold range of erythrocyte concentrations and then diverge at the lowest concentrations. The reason for the divergence is not clear but it is possible that in the most dilute erythrocyte suspensions (i) the concentration of available reacting sites is so low that the reaction proceeds very slowly; (ii) a larger portion of the hemolysin is inactivated before it can interact with erythrocytes; and (iii) erythrocytes are less easily lysed than in the concentrated suspensions. If more than one molecule of hemolysin were required to lyse an erythrocyte, then the calculated per cent lysis would increase even more rapidly than shown in Table 1.

Preliminary evidence indicates that *E. coli* alpha hemolysin probably does not damage cells by oxidation of sulfhydryl groups. This conclusion is strengthened by the observation that dissociation of the HCE complex terminates lysis immediately. At least a portion of cells damaged by oxidation should be sufficiently damaged at any time after the prelytic period that lysis would continue for several minutes after dissociation of the HCE complex.

We conclude, from direct observation of cell lysis, that the release of hemoglobin is an all or none phenomenon for each cell. The same conclusion has been drawn for the lysis of erythrocytes by staphylococcal alpha toxin (2).

The time course of hemolysis of erythrocytes by *E. coli* alpha hemolysin is roughly similar to that of staphylococcal alpha toxin. A prelytic lag period is followed by a period of rapid release of hemoglobin which in turn is followed by a diminishing rate of release of hemoglobin. When the time course curves that are generated by the two hemolysins are inspected more closely, several important differences become apparent. The prelytic lag period has been reported to be directly proportional to the reciprocal of the concentration of staphylococcal alpha toxin (2). No direct and consistent relationship between the reciprocal of the concentration of hemolysin and lag period was detected with *E. coli* hemolysin. Cooper et al. (2) and Marucci (7) indicated that staphylococcal alpha toxin causes a period of linear release of hemoglobin which tails off asymptotically. We did not observe such a period of linear release of hemoglobin by *E. coli* alpha hemolysin. For this reason we have not been able to relate the rate of hemolysis during the period of constant rate directly to the concentration of hemolysin as has been done with staphylococcal alpha toxin.

The decrease in the rate of hemolysis after 25 min of incubation suggests that some component of the HCE complex is being used up or is being inactivated by some product of the lytic reaction. The decrease in the rate of release of hemoglobin is greater than can be accounted for by the decrease in the number of erythrocytes. For example, the rate of hemolysis decreased by 75% between 35 and 65 min in a vessel containing 2% erythrocytes, and during the same time the concentration of erythrocytes fell by only 5%. Furthermore, the addition of red cells to an assay mixture at the end of an hour followed by another hour of incubation failed to stimulate a second round of lysis. It is noteworthy that experiments of this type have given variable results with staphylococcal alpha toxin. Lominski and Arbuthnott (5) and Marucci (8) were able to demonstrate the persistence of staphylococcal alpha toxin through several cycles of cell lysis, but Cooper et al. (3) reported that they could not demonstrate persistence of alpha toxin through more than one lytic cycle. Addition of excess calcium did not stimulate lysis by alpha hemolysin. Contents of erythrocytes did not inhibit lysis. Ghosts caused no greater inhibition of lysis than could be accounted for on the basis that ghosts have about the same afinity as intact erythrocytes for hemolysin. Cell-free hemolysin was the only component that caused a second round of hemolysis when added at the end of the normal incubation period. These data suggest that the *E. coli* alpha hemolysin is used up in the lytic reaction and is not an enzyme.

We have observed that in the ordinary assay the amount of hemoglobin released is directly proportional to the amount of alpha hemolysin added to the assay vessel up to about 30 per cent

lysis. This is consistent with the hypothesis that the hemolysin is not an enzyme and only one molecule of hemolysin is required to lyse one erythrocyte. The per cent of cells hemolyzed by different amounts of hemolysin when the concentration of erythrocytes is kept constant can be calculated with the Poisson formula. When the calculated per cent hemolysis is plotted against hemolysin concentration, the theoretical curve approximates a straight line up to about 30% lysis.

When rabbit erythrocytes are exposed to staphylococcal alpha toxin during only a portion of the prelytic lag period, lysis still occurs; and the later in the prelytic lag period that the alpha toxin is inactivated, the greater the ultimate extent of lysis (3, 8). If the lysin is inactivated at the end of the prelytic lag period, lysis is not inhibited at all (8). These observations indicate that cells damaged by alpha toxin are doomed to eventual destruction even though the alpha toxin might be inactivated. In contrast, with *E. coli* alpha hemolysin lysis does not occur unless the HCE complex is maintained up to the lytic event.

The beta-hemolytic activity appears to be different from alpha hemolysin. No method of cell disruption that was tried liberated beta-hemolytic activity into the medium. Growth conditions that markedly depressed production of alpha hemolysin had little if any effect on cell-associated activity. Furthermore, Smith (9) has found that the beta-hemolytic activity was not neutralizable by alpha hemolysin antiserum. Smith (9) also reported that some *E. coli* isolated from intestines of different species of animals produce alpha but not beta hemolysin and others produce beta but not alpha hemolysin. The inhibition of beta-hemolytic activity by several compounds that inhibit nucleic acid synthesis, protein synthesis, or aerobic metabolism is interpreted to mean that beta-hemolytic activity is dependent on the metabolic status of the organism.

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