STUDIES ON THE ORIGIN OF THE α -HAEMOLYSIN PRODUCED BY *ESCHERICHIA COLI*

SALLY E. JORGENSEN, E. C. SHORT, JR, H. J. KURTZ,
HELEN K. MUSSEN AND GRACE K. WU

College of Veterinary Medicine, University of Minnesota, St Paul, Minnesota 55101 USA

THE cell-free haemolysin produced by growing certain strains of *Escherichia coli* on meat-broth medium was originally described by Lovell and Rees (1960) and has been termed α -haemolysin by Smith (1963). Since there have been a number of reports describing other filterable haemolysins (Snyder and Koch, 1966; Walton and Smith, 1969; Muranyi and Juhasz, 1971; Rennie and Arbuthnott, 1971) which might be confused with the α -haemolysin studied in these experiments, the properties that distinguish α -haemolysin from the others produced by *E. coli* are summarised here.

A review of the literature (Lovell and Rees, 1960; Smith, 1963; Snyder and Koch, 1966; Snyder and Zwadyk, 1969; Walton and Smith, 1969; Raynaud and Alouf, 1970; Muranyi and Juhasz, 1971; Short and Kurtz, 1971; Zwadyk and Snyder, 1971; Rennie and Arbuthnott, 1974) suggests that there are at least four distinct types of haemolysin produced by *E. coli*. α -Haemolysin is a cell-free, filterable, heat-labile (Lovell and Rees, 1960; Smith, 1963; Snyder and Koch, 1966) macromolecule (Short and Kurtz, 1971; Rennie and Arbuthnott, 1974), at least a portion of which is protein (Snyder and Koch, 1966; Short and Kurtz, 1971). It is produced by many strains of *E. coli* (Smith, 1963) during log-phase growth in the presence of meat extract.

A second type of filterable cell-free haemolysin, first described by Snyder and Koch (1966), differs from α -haemolysin in that it is produced on chemically defined media and is thermostable at 56°C. It is produced later in the growth phase than is α -haemolysin and its appearance is accompanied by a fall in the pH of the culture. A similar type of haemolysin, described by Rennie and Arbuthnott (1971), is produced on chemically defined medium in the presence of various carbohydrates. It is possible that the haemolytic effects described in both of these reports may be due to the lytic effect of organic acids formed from the carbohydrates in the culture medium, as reported in related studies by Muranyi and Juhasz (1971).

Walton and Smith (1969) have described a third type of filterable haemolysin, which they termed γ . This haemolysin is found in mutants resistant to nalidixic acid and either its production or its release appears to be affected by nalidixic acid.

The fourth haemolysin produced by *E. coli* is the cell-associated or β -haemolysin. β -Haemolytic activity has not been dissociated from intact cells and appears to be dependent on cellular metabolism (Smith, 1963; Short and Kurtz, 1971).

It is not possible to determine which of these four types of haemolysin are produced by various strains of *E. coli* on the basis of the appearance of colonies on blood agar plates, since both α - and β -haemolysin-producing strains give rise to a clear zone of haemolysis (Smith, 1963). Smith found that 53–76% of the faecal samples from healthy pigs, sheep, and oxen contained haemolytic *E. coli*. He found that 63–92% of the haemolytic strains

Received 7 July 1975; revised version accepted 7 Oct. 1975.

produced α -haemolysin, including some strains that produced both α - and β -haemolysin. Other strains (25–42%) produced only β -haemolysin.

The ability to produce both α - and β -haemolysin has been found to be transmissible to non-haemolytic *E. coli* and to strains of *Salmonella* and *Shigella* (Smith and Halls, 1967; Goebel and Schrempf, 1971; Goebel *et al.*, 1974).

The requirement for meat extract in the growth media for the production of α -haemolysin by *E. coli* led us to attempt to purify and characterise the meat-broth factor necessary for the synthesis. We have termed this factor haemolysin precursor (HP).

MATERIALS AND METHODS

Chemicals and other materials. Beef Extract, Bacto-Peptone and Nutrient Broth were products of Difco Laboratories (Detroit, Michigan) and Casein Hydrolysate was purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio). Trypsin was from Worthington Biochemical Corporation (Freehold, New Jersey). Sepharose 6B and Sephadex G100 were purchased from Pharmacia (Uppsala). Acetate-1-¹⁴C (56 mCi per mM) and D-glucose UL-¹⁴C (210 mCi per mM) were purchased from International Chemical and Nuclear Corporation (Cleveland, Ohio). The dialysis tubing used in these experiments was prepared as previously described (Short and Kurtz, 1971).

Organism. A strain of *E. coli*, serotype O141:85ac:NM (Minnesota Stock Culture no. 4331) was used. It was originally isolated from the intestine of a pig with oedema disease (Kurtz, Bergeland, and Barnes, 1969) and was maintained on agar slants. *E. coli* B (Biological Laboratory, Cold Spring Harbor, New York) was used as a non-haemolytic control strain. Cultures were routinely incubated at 37°C in a water bath shaking at 120 cycles per min. Bacterial growth was monitored by measuring the turbidity at 600 nm (*E*₆₀₀). The concentration of viable cells was determined by plate counts.

Media. Unless otherwise noted, meat extract broth was prepared as described by Smith (1963), except that the aqueous solution containing 0.5% of NaCl and adjusted to pH 7.4 contained 1% Casein Hydrolysate in place of Proteose Peptone. The protein-peptide concentration in the final meat broth was 15–20 mg per ml. Similar extract broths were prepared from muscle and other organs of rabbits, pigs and mice by the same procedure. Extract broths were also prepared from components of blood, in which 20 ml of whole blood, plasma, serum or blood cells were added to 100 ml of water. The solution was made 1% with respect to casein hydrolysate and 0.5% with respect to NaCl and the pH was adjusted to 7.4. The mixture was autoclaved and filtered in the same manner as described for the preparation of the meat-extract broth (Smith, 1963). Meat broth was concentrated by placing the broth inside dialysis tubing and covering the tubing with powdered polyethylene glycol 4000. A broth without HP which contained (w/v) sodium chloride 0.5%, casein hydrolysate 1% and glucose 0.2% was used as a control in some experiments. Other media used in this work were (w/v) Nutrient Broth 0.8%, Bacto-Peptone 1%, Beef Extract 0.3%, and media described by Snyder and Koch (1966) and Fraser and Jerrell (1953).

Analytical procedures. Haemolysin was measured on the basis of the amount of haemoglobin released in a solution containing haemolysin, erythrocytes, Tris-chloride buffer, calcium chloride and sodium chloride, as previously detailed (Short and Kurtz, 1971). Protein was measured by the biuret method. The protein concentrations in effluent solutions from chromatographic columns was determined by measuring the absorbance at 280 nm (*E*₂₈₀). Protein concentration (in mg per ml) was calculated by multiplying the *E*₂₈₀ reading by 0.89, a factor computed from comparison of protein concentration determinations by biuret with the *E*₂₈₀ values obtained with ammonium sulphate fractions of meat broth which were relatively free of nucleic acid. Radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer (model 3003). All samples were pipetted on to Whatman filter paper disks (no. 540, hardened ashless) 2.4 cm in diameter. The papers were dried thoroughly

and immersed in counting vials containing 10 ml of scintillation fluid. The scintillation mixture contained 8.0 g of 2-(4'-t-butyl-phenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole and 0.5 g of 2-(4'-biphenyl)-6-phenyl-benzoxole per litre of toluene.

α -Haemolysin production. Twenty ml of medium were placed in a 250-ml Erlenmeyer flask and seeded with *c.* 10^6 cells of *E. coli* strain 4331 per ml. The culture was grown for 5½ h and the cells were removed by centrifugation at 15 000 *g* for 15 min. The supernate was tested for haemolytic activity; in some cases it was filtered through a sterile membrane filter with 45-nm pores (Millipore Corp., Bedford, Mass.). Filtration did not result in loss of haemolytic activity.

Preparation of ^{14}C -labelled mouse-meat broth. Two 6-week-old male mice were given daily intraperitoneal injections of ^{14}C -glucose (0.2 ml of 100 μCi per ml) for 9 days. The mice were killed on the 10th day and the muscle (9 g in all) dissected off the limbs and vertebrae was used to prepare mouse-meat broth. This yielded 16 ml of ^{14}C -labelled mouse-meat broth which had *c.* 2500 c.p.m. per mg of protein. The broth was stored at -20°C for 2 weeks before use.

Fractionation of ^{14}C -labelled bacterial cells. The cells harvested from cultures containing either ^{14}C -labelled mouse-meat broth or ^{14}C -glucose were subjected to fractionation by the procedure of Roberts *et al.* (1955). The principal protein precipitate remaining after the extraction with hot trichloroacetic acid was dissolved in 1.5 ml of 5N HCl by heating in a boiling water bath for 20–30 min. The protein concentration and radioactivity of this solution were measured to determine the specific activity of the total bacterial protein.

Ammonium-sulphate fractionation. Increasing amounts of $(\text{NH}_4)_2\text{SO}_4$ were added to 5 ml of 5 $\times$ concentrated meat broth to separate the soluble proteins into three fractions. The proteins precipitated at 20% saturation and between 20 and 40% saturation were resuspended in 1 and 5 ml of water respectively. These two fractions and the 40%-saturated supernatant solution were dialysed for 20 h against 200 vols of a solution containing (w/v) NaCl 0.5%, glucose 0.5% and casein hydrolysate 1%. The pH of these three fractions of meat media was readjusted to 7.4 and the solutions were autoclaved for 15 min. and tested for their ability to support haemolysin production. $(\text{NH}_4)_2\text{SO}_4$ was also added to 27 ml of a cell-free supernatant solution containing α -haemolysin to precipitate proteins in fractions between 0–20% and 20–40% saturation. These precipitates were redissolved in 0.5 ml and 4.5 ml, respectively, of 0.01M Tris-chloride buffer, pH 7.5, containing 0.11M sodium chloride and 20 mM calcium chloride. The 40%-saturated supernatant solution and the redissolved precipitates were assayed for haemolytic activity.

Chromatography. Gel-filtration chromatography was done at 4°C in a column (1.8 $\text{cm}^2 \times 90$ cm). Three to 5 ml of sample (meat broth or culture supernate), with 2.5 mg of blue dextran added as a marker, were applied and 40 5-ml fractions were collected. The eluant was 0.01M Tris-chloride buffer, pH 7.5, containing 0.11M sodium chloride. The flow rate was maintained at approximately 5 ml per h.

RESULTS

Factors affecting α -haemolysin production

Filtrates from cultures of *E. coli* no. 4331 grown on meat broth contained large quantities (100–300 units per ml) of α -haemolysin, but filtrates from cultures grown on 1% Bacto Peptone, Snyder and Koch's medium, Fraser and Jerrell's medium or control broth without the postulated haemolysin precursor (HP) did not contain haemolysin (<0.5 units per ml). A small amount of haemolysin (1–2 units per ml) was produced on 0.3% Beef Extract (Difco) and on Nutrient Broth, which contains Beef Extract. Uninoculated meat broth and filtrates from cultures of non-haemolytic *E. coli* did not contain haemolytic activity.

Although the yield was somewhat lower than that obtained on meat broth prepared from beef muscle, haemolysin was produced in substantial amounts (30–100 units per ml) when the meat broth was prepared from pig muscle, heart or kidney, from rabbit muscle, heart, kidney or liver, or from mouse muscle. Haemolysin was also produced in cultures supplemented with alkaline extracts prepared from whole blood, blood cells, plasma, and serum, from either cattle or sheep. When rabbit lung or intestine was used to prepare the alkaline broth extract, very little haemolysin (5–8 unit per ml) was demonstrable in the culture filtrates. Other tissues were not tested.

The salt concentration (from 0–2.5% NaCl) used for the extraction of beef muscle did not influence the α -haemolysin yield of the meat extract broth.

Production of α -haemolysin in a typical culture of *E. coli* strain 4331 growing on meat broth is recorded in fig. 1. The initial concentration of viable cells at time 0 was 3×10^6 cells per ml. Haemolysin concentration reaches a maximum at 5 h when there are 6×10^8 cells per ml and no additional haemolysin is produced during the 20-fold increase in cell concentration that occurs over the next 4 h. Instead, haemolytic activity decreases markedly during this time of maximal cell replication. When 10% (v/v) of 10-fold concentrated meat broth was added to a similar culture at 5 h, a second peak of haemolytic activity was

FIG. 1.—Haemolysin activity and cell concentration as a function of incubation time in a culture of *Escherichia coli* strain 4331; ○—○ = haemolytic activity; ●—● = cells per ml.

produced 2 h later. No such increase occurred when a similar volume of a solution containing only sodium chloride 0.5%, casein 1% and glucose 0.2% was added at 5 h.

To examine the relationship between meat broth-concentration and haemolysin production, varying amounts of 10-fold concentrated meat broth were added to six flasks with enough solution to give a final volume of 20 ml containing sodium chloride 0.5% and casein hydrolysate 1%. The flasks were seeded with identical amounts of *E. coli* strain 4331 and incubated for 5 h at 37°. The growth rate, assessed by turbidity measurements at hourly intervals, was

FIG. 2.—Haemolysin production as a function of concentration of meat-broth precursor (HP) in the growth medium. ○—○ = haemolytic activity; ●—● = E_{600} values (turbidity as an index of growth).

approximately the same in all six flasks. Filtrates prepared from the supernates were assayed for haemolysin activity and the results are shown in fig. 2. Haemolysin production was proportional to the amount of meat broth over a five-fold change in meat-broth concentration. In the linear region of the curve, each 1-ml increment of meat-broth concentrate supported the production of an additional 44 units per ml of haemolysin activity in the supernate. The additional meat broth also supported a moderate increase in the total growth of the culture, as evidenced by the increased turbidity in the flasks with the higher concentrations of meat broth, but the relative change in optical density was about one-fiftieth the relative change in haemolysin production.

Comparison of the properties of HP and α -haemolysin

HP is obviously heat stable because the meat broth is autoclaved twice at 120°C for 20 min. in the normal course of preparation. Special preparations made without autoclaving the meat broth at all subsequently produced half the normal yield of haemolysin. Meat broth subjected to an additional autoclaving also supported the production of half as much haemolysin as was produced in normal meat broth.

The heat stability of α -haemolysin was tested by holding the haemolysin preparation at 56°C for varying lengths of time and then measuring the residual haemolytic activity by the standard assay procedure. The major portion of

FIG. 3.—Effect of heat (56°C) on haemolysin activity.

TABLE I

Precipitation of HP and of α -haemolysin by $(\text{NH}_4)_2\text{SO}_4$ fractionation of meat broth and of culture supernate respectively

Fraction*	Percentage of total yield of haemolysin	
	produced from stated fraction of meat broth	present in stated fraction of culture supernate
0–20% $(\text{NH}_4)_2\text{SO}_4$	4	1
20–40% $(\text{NH}_4)_2\text{SO}_4$	88	99
40% supernate	8	0

* The precipitates obtained at the given concentrations and the final supernate were tested.

the haemolytic activity was very unstable at this temperature, as shown in fig. 3. These data also suggest the presence of a minimal amount of another haemolytic substance that is relatively stable to heat. Similar results were obtained when the haemolysin tested was produced on broth extract prepared from blood. The heat-stable component was separated from α -haemolysin by chromatography on Sepharose 6B as described later.

Incubation of meat broth with 0.1% trypsin for 1 h at 37°C before the final autoclaving destroyed the ability of the HP to support the production of haemolysin.

Both HP and α -haemolysin were precipitated by the addition of equivalent amounts of $(\text{NH}_4)_2\text{SO}_4$ to meat broth and to a haemolysin-containing cell-free supernate. Details of the amounts of haemolysin produced from three fractions of meat broth separated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ before cell growth and the distribution of haemolysin activity in fractions of a culture supernate precipitated by the same concentrations of $(\text{NH}_4)_2\text{SO}_4$ are given in table I. Both the HP and haemolysin appear almost exclusively in the fraction precipitated between 20 and 40% saturation.

Precipitability by acid of the HP was studied by adding HCl to a series of vessels to bring the pH to values between 3.5 and 7.0. The copious precipitates that formed in each flask were removed by centrifugation at 15 000 *g* for 10 min. and the remaining supernates were readjusted to pH 7.4 and tested for their ability to support haemolysin production by the standard procedure. The results are shown in fig. 4. At pH 3.5–4.0, virtually all of the component that supports haemolysin production remained in solution. Maximal precipitation of the HP occurred between pH 4.5 and 5.0; this suggests that the precursor is an acidic protein with an isoelectric point in this region.

An estimate of the molecular weight of α -haemolysin was obtained by subjecting the haemolysin to gel-filtration chromatography on Sephadex G-100. All of the haemolytic activity was recovered in the void volume; this suggests that the molecular weight of haemolysin was in excess of 1.5×10^5 . Haemolysin

preparations were also chromatographed on Sepharose 6B and the results from a typical column are shown in fig. 5*a*. In the course of running 15 different columns, small amounts of haemolytic activity often appeared in earlier fractions of effluent, but the major peak of activity always appeared just before the final peak of material which had a high absorbance at 280 nm. The partition coefficient (K_{av}) of these fractions is 0.67 which corresponds to a protein with a molecular weight of $3-4 \times 10^4$. In an effort to resolve the discrepancy between this estimate and the higher molecular weight estimate suggested by chromatography on Sephadex G-100, the major haemolysin-containing fractions from the Sepharose 6B effluent were pooled, concentrated seven-fold and applied to a Sephadex G-100 column. The haemolytic activity again appeared in the void volume.

Similar experiments were conducted to obtain an estimate of the molecular weight of HP, but the results of chromatography on Sephadex G-100 were inconsistent and recovery was very poor. Chromatography of HP was performed on a column of Sepharose 6B identical to the one used for the haemolysin. A 5-ml sample of five-fold concentrated uninoculated meat broth was applied to the column. The meat-broth effluent was then pooled in three fractions of 60 ml each as follows: fraction I, 21–80 ml; fraction II, 81–140 ml; fraction III, 141–200 ml (see fig. 5*b*). These pooled fractions were concentrated five-fold by dialysis against polyethylene glycol. NaCl (0.5%), casein hydrolysate (1%), and glucose (0.2%) were added to the concentrated fractions.

FIG. 4.—Haemolysin production from supernates obtained after precipitation at different pH values.

The three solutions were autoclaved and tested for their ability to support haemolysin production. The results (fig. 5b) show that the only portion of the meat broth effluent capable of supporting substantial haemolysin production was the material eluted in fraction II.

In tests to determine the heat stability of the haemolysin eluted from Sepharose 6B between 105 and 150 ml (fig. 5), these fractions were pooled and held at 56°C for varying lengths of time; 98% of the initial activity was lost after 40 min. Separate assays of the individual fractions in this region revealed

FIG. 5.—Chromatography on Sepharose 6B. 5A: Meat-broth culture supernate. $\circ$ — $\circ$ = haemolytic activity; --- = E_{280} values (protein). 5B: Uninoculated meat broth, --- = E_{280} values. Fractions were pooled as I, II, and III and subsequent haemolysin production is shown as a histogram.

a small amount of a heat-stable component in the fractions eluted after the α -haemolysin peak (fig. 6).

Use of ^{14}C -labelled compounds to determine the origin of haemolysin

Haemolysin production by strain 4331 was studied in the presence of two different sources of ^{14}C -labelled compounds. The supernate from a culture in unlabelled mouse-meat broth, to which ^{14}C -glucose ($0.5\ \mu\text{Ci}$ per ml) was added at the time of inoculation, contained 8 units of haemolysin per ml. A second supernate from a culture grown in ^{14}C -labelled mouse-meat broth, with unlabelled glucose, contained 35 units of haemolysin per ml. Four ml of each of these solutions were chromatographed separately on columns of Sepharose 6B. The effluent fractions were assayed for haemolytic activity, radioactivity, and absorbance at 280 nm; the results are shown in fig. 7. In both chromatographs, the haemolytic activity and absorbance profiles are similar to those obtained by chromatography of haemolysin from beef-meat-broth cultures on Sepharose 6B (fig. 5). The major peak of haemolysin is

FIG. 6.—Heat stability of haemolysin eluted from Sepharose 6B; ○—○ = haemolysin activity; ●—● = percentage amount of the initial haemolysin activity remaining after 1 h at 56°C.

FIG. 7.—Sepharose 6B chromatography of supernates from cultures labelled with ^{14}C ; --- = E_{280} values; $\circ$ — $\circ$, haemolysin activity; $\bullet$ — $\bullet$ = c.p.m. per ml. 7a: Culture contained ^{14}C -glucose. 7b: Culture contained ^{14}C -labelled mouse-meat broth.

eluted just before the final fractions of small metabolites; the latter are marked by the large peak of radioactivity due to excess ^{14}C -glucose.

In the chromatograph of haemolysin from cells grown in the presence of ^{14}C -glucose there is very little radioactivity associated with the fraction containing the most haemolytic activity (fig. 7a). In the second chromatograph from cells grown in ^{14}C -labelled mouse-meat broth, a small peak of radioactivity was associated with the fraction containing the most haemolytic activity (fig. 7b).

In a control experiment, cells of the test strain were grown on unlabelled mouse-meat broth and removed by centrifugation. Then ^{14}C -glucose ($0.5\ \mu\text{Ci}$ per ml) was added to the supernate before chromatography on Sepharose 6B. The results showed that a small amount of ^{14}C -glucose is non-specifically absorbed to macromolecules in various effluent fractions. The fraction with maximal haemolysin activity, eluted at 125 ml, contained 60 c.p.m. per ml. These extraneous counts were subtracted from the 160 c.p.m. per ml present in the corresponding fraction from the column shown in fig. 7a before calculating the specific activity of the haemolysin fractions from the culture labelled with ^{14}C -glucose. A comparison of the specific activities of the haemolysin and of the total bacterial protein in the cultures labelled with ^{14}C -glucose and with ^{14}C -mouse-meat broth is shown in table II.

To increase the probability of detecting radioactive molecules of bacterial origin in the haemolysin-containing fractions eluted from Sepharose 6B, cells were also grown in beef-meat broth, which supports a greater amount of haemolysin production than does mouse-meat broth, and in the presence of larger amounts of ^{14}C -acetate ($20\ \mu\text{Ci}$) to increase the specific activity of the bacterial molecules. In this experiment the specific activity of the total bacterial protein fraction was 1000 times greater than that of the protein in the haemolysin-containing fraction eluted from the Sepharose 6B column.

DISCUSSION

Previous data from this laboratory (Short and Kurtz, 1971) have shown that, with 12 different isolates of haemolytic *E. coli*, no α -haemolysin is produced in liquid cultures unless non-dialysable macromolecular components from meat

TABLE II

Relative specific activities of haemolysin and total protein in cultures containing ^{14}C

Source of isotope in culture medium	C.p.m. per unit of haemolytic activity*	C.p.m. per mg of protein in haemolysin fractions	C.p.m. per mg of total bacterial protein
^{14}C -glucose	450	250	7.2×10^4
^{14}C -labelled mouse-meat broth	2500	2470	425

* Haemolysin taken from most active fractions of Sepharose 6B effluent.

extract are present in the culture medium. This finding is reaffirmed by the present experiments. In addition, blood and other tissues were shown to contain substances that support production of the haemolysin. The failure of *E. coli* to produce α -haemolysin in the absence of tissue or blood components is unusual among the various genera of bacteria that produce cytolytins and other toxins. Although toxin production is often enhanced by the presence of unidentified macromolecular components present in meat extract, dialysable media promoting moderate degrees of toxin synthesis have been described in most instances (Alouf and Raynaud, 1970). One other toxigenic system with a requirement for meat extract that appears to be analogous is the production of cereolysin—a potent haemolysin—by *Bacillus cereus*. Efforts to obtain cereolysin from *B. cereus* cultures growing on defined media have met with little success (Bernheimer and Grushoff, 1967; Bonventre and Johnson, 1970).

The present results indicate that the *E. coli* HP can be extracted from a variety of tissues from several species of animals. All of the extract fractions of blood tested supported the production of a filterable, heat-labile haemolysin. This suggests that α -haemolysin production could account for the lysis regularly observed on blood agar plates.

Measurement of haemolytic activity at various times during growth of a culture showed that the maximum activity appeared early in the growth phase when only 5% of the eventual total number of cells were present. The absence of any additional haemolysin production concomitant with the subsequent 20-fold increase in cell numbers is unlike the relationship between cell growth and toxin production observed in other bacteria. This absence of increased haemolytic activity in the later phases of growth does not appear to be due to decomposition of haemolysin by culture products because the addition of concentrated meat broth to these older cultures stimulates production of a second peak of haemolysin activity. Most toxins and enzymes of bacterial origin that are released into the medium are either produced in the late log or the stationary growth phase or are formed in approximately constant proportion to the cell mass throughout the growth cycle (Pollock, 1962; Raynaud and Alouf, 1970). The quantitative correlation between the amount of HP initially present in the growth medium and the final haemolytic activity of the culture supernate shows that the amount of haemolysin produced is limited by the quantity of HP present. This suggests that HP is not acting as an inducer or as a catalyst in promoting the synthesis of haemolysin.

The HP appears to be stable to heat, and the decrease in the amount of haemolysin produced in uncooked meat broth indicates that extraction of HP is enhanced by heating. The heat sensitivity of the α -haemolysin preparation demonstrates that most of the activity is consistent with α -haemolysin as originally defined by Smith (1963). The small amount of heat-stable activity may be attributed to the heat-stable cell-free haemolysins described by others (Snyder and Koch, 1966; Muranyi and Juhasz, 1971; Rennie and Arbuthnott, 1971).

A comparison of the physical and chemical properties of HP and α -haemolysin suggests that the two molecules are similar in several respects. The

sensitivity of both molecules to trypsin indicates that both are proteins or contain a polypeptide component. Also, the HP and α -haemolysin are both precipitated by approximately the same concentrations of ammonium sulphate. Studies of their acid precipitability indicate that the HP and α -haemolysin are acidic compounds. It is not possible to make an accurate assessment of the isoelectric points of either HP or α -haemolysin from the results presented here, but a comparison of the solubility data for α -haemolysin (Short and Kurtz, 1971) and similar data for HP presented here (fig. 4) suggests that α -haemolysin is slightly more acidic than HP.

The reasons for the discrepancy in the apparent molecular weights of α -haemolysin as indicated by gel-filtration chromatography on Sephadex G-100 ($\sim 1.5 \times 10^5$) and on Sepharose 6B ($3-4 \times 10^4$) are not clear. When the "low molecular weight" haemolysin eluted from Sepharose 6B was re-chromatographed on Sephadex G-100, it was eluted in the void volume. These data suggest that haemolysin was retarded by binding to Sepharose 6B; an alternative but less likely explanation is that small subunits were dissociated on Sepharose 6B and were reassociated on Sephadex G-100.

The physical and chemical similarities between α -haemolysin and HP may be coincidental, but they suggest that HP is a molecule that undergoes a relatively minor modification in the presence of certain strains of *E. coli* and is converted to α -haemolysin.

Radioactively labelled glucose and mouse-meat broth were used in a series of experiments to determine whether the major portion of the α -haemolysin molecule is directly derived from a precursor protein in meat broth or is a product of bacterial metabolism. In the culture labelled with ^{14}C -glucose, the specific activity of the protein eluted with the haemolytic activity was 300-fold lower than that of the total protein isolated from the bacterial cells (table II). Thus, if the haemolysin protein is of bacterial origin, it must represent less than 0.33% of the total protein present in the Sepharose 6B effluent fractions containing haemolytic activity. In the culture containing ^{14}C -labelled mouse-meat broth, the specific activity was six-fold more than that of the total protein isolated from bacterial cells and was about equal to the specific activity of the mouse protein in the mouse-broth medium. Although these findings do not rule out the possibility that the haemolytic activity is derived from a very small amount of a highly potent bacterial protein, they suggest that the major portion of the haemolysin molecule is of mammalian origin.

The cumulative evidence, including absence of increased haemolysin synthesis in the period of maximal cell replication, increased haemolysin production in the presence of increased amounts of HP, similarity of chemical and physical properties of HP and haemolysin, and the presence of mammalian but not bacterial protein in the partially purified fractions of haemolysin, suggests that the α -haemolysin in cell-free filtrates of *E. coli* may be produced by modification of an existing extraneous molecule rather than from synthesis *de novo* by growing bacterial cells. This mode of haemolysin production differs markedly from the synthesis of most cytotoxins and other extracellular toxins,

which are considered to be synthesised by the microbial cells (Kato, 1970; Raynaud and Alouf, 1970).

Inukai and Kodama (1965) suggested that the meat-broth component facilitates the release of haemolysin from the surface of the bacterial cells. Our data suggest that, if this is the case, the haemolysin that is released remains bound to the HP and that the physical and chemical properties of the HP are not significantly altered by the presence of haemolysin. In addition, the data from our experiments with ^{14}C -glucose show that the bacterial contribution to such a complex of haemolysin and meat-broth component would be very small. Our findings suggest that haemolysin production by *E. coli* results from bacterial modification of a meat-broth component rather than that the meat-broth component induces the release of a bacterial haemolysin.

Production of a lytic substance by modification of a mammalian molecule suggests a new kind of mechanism whereby bacteria may interact with the host, and this presents an additional factor to be considered in studying interactions of bacteria and host. If haemolysin is produced by modification of host factors, the amount and potency of the haemolysin synthesised will depend on the availability of the appropriate macromolecules in the host, as well as on the genetic determinants in the bacterial cell.

The lytic effect of most haemolysins is not restricted to erythrocytes (Bernheimer, 1970), and reports indicate that *E. coli* haemolysins are probably also lytic for other types of cells (Chaturvedi *et al.*, 1969) and for lysosomes (DePauw, Gill and Fried, 1971). This probable breadth in its spectrum of action and the predominance of haemolytic *E. coli* strains in various enteric diseases, such as oedema disease of swine (Kurtz *et al.*, 1969), and in non-intestinal disease processes, presents the possibility that the production of α -haemolysin may have some role in facilitating infection.

SUMMARY

Synthesis of α -haemolysin by *Escherichia coli* was proportional to the amount of meat-broth factor present in the medium and not to bacterial growth.

The meat-broth component required for the synthesis of haemolysin was found to have several physical and chemical properties in common with α -haemolysin itself. Both molecules are trypsin-sensitive, acidic substances with similar elution volumes when subjected to gel-filtration chromatography with Sepharose 6B.

Isotopic labelling experiments in which the bacteria were grown on ^{14}C -labelled meat broth showed that partially purified haemolysin preparations contained molecules of mammalian origin. Similar experiments in which ^{14}C -glucose was used to label bacterial molecules showed that little or no material of bacterial origin was present in the haemolysin preparation.

These data suggest that α -haemolysin may be produced by bacterial modification of a molecule present in meat broth rather than by synthesis *de novo* within the bacterial cell.

This work was supported by the Hatch Fund, GAR Fund and NC-62 Regional Research Funds; it is published here as paper no. 9216, Scientific Journal Series, Minnesota Agricultural Experiment Station.

We gratefully acknowledge the excellent technical assistance of Betty McKay Davis.

REFERENCES

- ALOUF, J. E. AND RAYNAUD, M. 1970. Isolation and purification of bacterial toxic proteins. In *Microbial toxins*, edited by S. J. Ajl, S. Kadis and T. C. Montie, *New York*, vol. 1, p. 119.
- BERNHEIMER, A. W. 1970. Cytolytic toxins of bacteria. In *Microbial toxins*, edited by S. J. Ajl, S. Kadis and T. C. Montie, *New York*, vol. 1, p. 183.
- BERNHEIMER, A. W. AND GRUSHOFF, P. 1967. Cereolysin: production, purification, and partial characterization. *J. gen. Microbiol.*, **46**, 143.
- BONVENTRE, P. F. AND JOHNSON, C. E. 1970. *Bacillus cereus* toxin. In *Microbial toxins*, edited by T. C. Montie, S. Kadis and S. J. Ajl, *New York*, vol. 3, p. 415.
- CHATURVEDI, U. C., MATHUR, A., KHAN, A. M. AND MEHROTRA, R. M. L. 1969. Cytotoxicity of filtrates of haemolytic *Escherichia coli*. *J. med. Microbiol.*, **2**, 211.
- DEPAUW, A. P., GILL, W. B. AND FRIED, F. A. 1971. Etiology of pyelonephritis: renal lysosome disruption by hemolytic *Escherichia coli*. *Invest. Urol.*, **9**, 230.
- FRASER, D. AND JERRELL, E. A. 1953. The amino acid composition of T3 bacteriophage. *J. biol. Chem.*, **205**, 291.
- GOEBEL, W., ROYER-POKORA, B., LINDENMAIER, W. AND BUJARD, H. 1974. Plasmids controlling synthesis of hemolysin in *Escherichia coli*: molecular properties. *J. Bact.*, **118**, 964.
- GOEBEL, W. AND SCHREMPF, H. 1971. Isolation and characterization of supercoiled circular deoxyribonucleic acid from beta-hemolytic strains of *Escherichia coli*. *J. Bact.*, **106**, 311.
- INUKAI, Y. AND KODAMA, H. 1965. Studies on hemolytic *Escherichia coli* of O-139: a certain factor essential for hemolysin production. *Jap. J. vet. Res.*, **13**, 87.
- KATO, I. 1970. Relationship of bacterial structure and metabolism to toxin production. In *Microbial toxins*, edited by S. J. Ajl, S. Kadis and T. C. Montie, *New York*, vol. 1, p. 401.
- KURTZ, H. J., BERGELAND, M. E. AND BARNES, D. M. 1969. Pathologic changes in edema disease of swine. *Am. J. vet. Res.*, **30**, 791.
- LOVELL, R. AND REES, T. A. 1960. A filterable hemolysin from *Escherichia coli*. *Nature, Lond.*, **188**, 755.
- MURANYI, F. AND JUHASZ, S. 1971. Haemolytic effect of *Escherichia coli* cultures and their filtrates. *Acta vet. hung.*, **21**, 355.
- POLLOCK, M. R. 1962. Exoenzymes. In *The bacteria*, edited by I. C. Gunsalus and R. Y. Stanier, *New York*, vol. 4, p. 121.
- RAYNAUD, M. AND ALOUF, J. E. 1970. Intracellular versus extracellular toxins. In *Microbial toxins*, edited by S. J. Ajl, S. Kadis and T. C. Montie, *New York*, vol. 1, p. 67.
- RENNIE, R. P. AND ARBUTHNOTT, J. P. 1971. Effect of carbohydrates on hemolysin production by *Escherichia coli*. *Infect. Immun.*, **3**, 849.
- RENNIE, R. P. AND ARBUTHNOTT, J. P. 1974. Partial characterisation of *Escherichia coli* haemolysin. *J. med. Microbiol.*, **7**, 179.
- ROBERTS, R. B., COWIE, D. B., ABELSON, P. H., BOLTON, E. T. AND BRITTEN, R. J. 1955. Studies of biosynthesis in *Escherichia coli*. Carnegie Institution of Washington, publication no. 607, *Washington, D.C.*, p. 13.
- SHORT, E. C. AND KURTZ, H. J. 1971. Properties of the hemolytic activities of *Escherichia coli*. *Infect. Immun.*, **3**, 678.
- SMITH, H. W. 1963. The haemolysins of *Escherichia coli*. *J. Path. Bact.*, **85**, 197.
- SMITH, H. W. AND HALLS, S. 1967. The transmissible nature of the genetic factor in *Escherichia coli* that controls haemolysin production. *J. gen. Microbiol.*, **47**, 153.

- SNYDER, I. S. AND KOCH, N. A. 1966. Production and characteristics of hemolysins of *Escherichia coli*. *J. Bact.*, **91**, 763.
- SNYDER, I. S. AND ZWADYK, P. 1969. Some factors affecting the production and assay of *Escherichia coli* haemolysins. *J. gen. Microbiol.*, **55**, 139.
- WALTON, J. R. AND SMITH, D. H. 1969. New hemolysin (γ) produced by *Escherichia coli*. *J. Bact.*, **98**, 304.
- ZWADYK, P. JR, AND SNYDER, I. S. 1971. Purification and kinetic studies of the hemolysin from *Escherichia coli*. *Can. J. Microbiol.*, **17**, 741.