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STUDIES ON HEMOLYTIC *ESCHERICHIA COLI* OF O 139
A LETHAL SUBSTANCE FOUND
IN PROLONGED CULTURE FILTRATES

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INTRODUCTION

Since 1938, when SCHANKS described edema disease of swine in northern Ireland, evidences have been presented by many workers that specific serotypes of hemolytic *Escherichia coli* are associated with the disease. For the cause of the disease, TIMONEY and other workers suggested in their reports that it is an intestinal toxemia. Problems concerning the toxin, however, have remained to be elucidated.

In June 1961, MURASE and his coworkers communicated personally that certain lethal substance(s) for mice could be found only in prolonged culture filtrates of hemolytic *Escherichia coli* of O 139 isolated from the intestine of a pig suffering from edema disease. It is generally accepted, however, that *Escherichia coli* organisms possess an endotoxin having a lethal activity for mice. In the present report, description is given of attempts made to isolate these toxic substances and to clarify their relations.

MATERIALS AND METHODS

Strain Strain used was hemolytic *E. coli* of O 139 isolated from the intestine of a pig which suffered from edema disease.

Medium Peptone broth was used.

Inoculum 16~20 hrs. peptone broth culture was used as inoculum.

Cultivation Five liters of the medium was inoculated in one experiment and incubated at 30°C for 7 days.

Preparation of lethal substance Detailed procedures for preparation of lethal substance from the culture were shown in the following scheme.

Preparation of endotoxin Endotoxin was prepared from the cells by the method of KAWAKAMI et al.

Chromatography on DEAE-cellulose column Each sample dialysed against 0.05 M phosphate buffer at pH 6.5 was adsorbed on DEAE-cellulose column equilibrated previously with the same buffer. Elution was carried out by increasing sodium chloride concentration

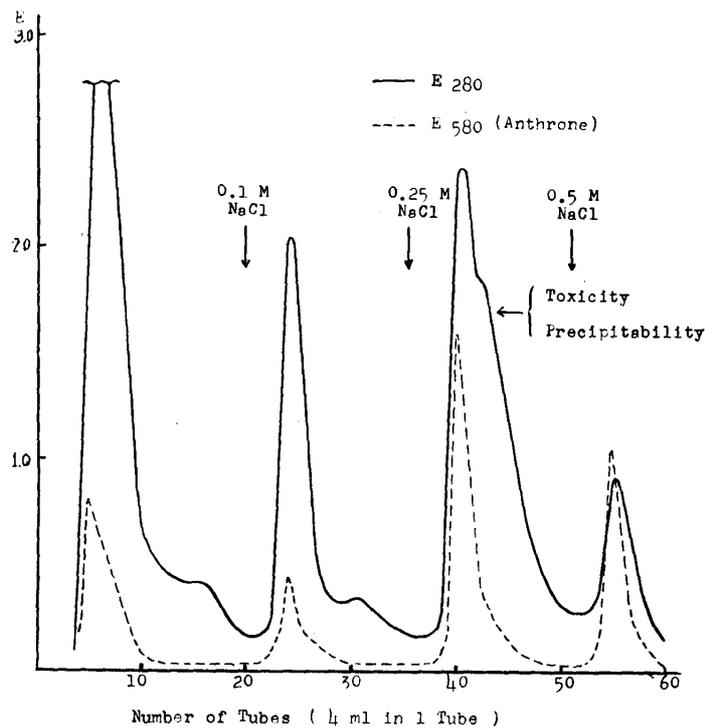
of the developing buffer stepwisely or gradiently, and 4 ml of each effluent was collected in separate tubes.

Examination of the effluent For each tube of effluents, examinations were made of protein by extinction coefficient at 280 $m\mu$, of carbohydrate by the anthrone method, of toxicity by the typical death of mice injected with 0.2 ml of sample intravenously and of precipitability against homologous anti-O serum by the ring test.

RESULTS

From a small scale experiment in which the lethal substance(s) being in the culture filtrates was precipitated quantitatively by adding suitable volume of ammonium sulfate, it seemed to be suitable to apply the salting-out technique for isolation of the lethal substance. Accordingly, fraction 1 was prepared through the procedures shown in the scheme. This fraction, dark-brownish, slightly viscous and clear solution continued to have all the lethal activity.

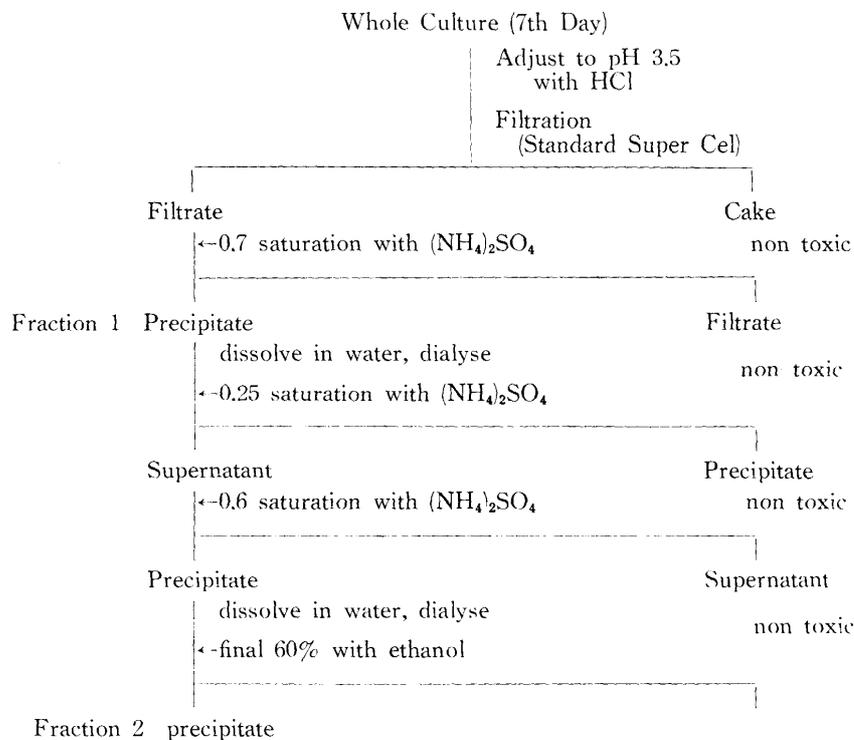
FIGURE 1. *Chromatography of Fraction 1*
(Stepwise Elution)



Chromatography of fraction 1 Chromatogram of fraction 1 is shown in figure 1, in which only one peak eluted at the concentration of 0.25 M sodium chloride presented toxicity and precipitability against homologous anti-O serum.

Chromatography of fraction 2 Fraction 1 was further purified by fractionation with ammonium sulfate followed by ethanol precipitation as shown in the scheme. Resulting

SCHEME Procedures for Isolation of Lethal Substance



fraction 2 was colorless and slightly viscous cloudy solution. Through above described procedures, some protein-like impurities were excluded considerably, moreover no decrease in toxicity was found. Chromatogram of fraction 2 is given in figure 2. As shown in the figure, both toxicity and precipitability were also found in one peak eluted at the same salt concentration as in the case of fraction 1.

Re-chromatography of the toxic peak in figure 2 The toxic peak seen in figure 2 was collected, concentrated with polyethyleneglycol and subjected to re-chromatography (linear gradient). The result is graphed in figure 3. Except a small peak eluted initially by the developing buffer, only one peak, having both toxicity and precipitability, appeared.

Chromatography of endotoxin It was considered that hemolytic *E. coli* organisms used in the present experiment also possess so-called endotoxin. Therefore, attempts were made to extract the endotoxin from the organisms and to compare its chromatographic behavior with that of the above described lethal substance. Chromatogram of the endotoxin is shown in figure 4. There appeared some fractions without toxicity, showing that the degree of purification of the endotoxin prepared was insufficient. In the figure, only one peak eluted at the concentration of 0.25 M sodium chloride was toxic and also reacted with homologous anti-O serum.

Application of zinc chloride method to isolation procedures The zinc chloride method has been used often in isolation work of some exotoxins, therefore, it was applied to isolation of the lethal substance from the toxic culture filtrates of the organisms, instead of

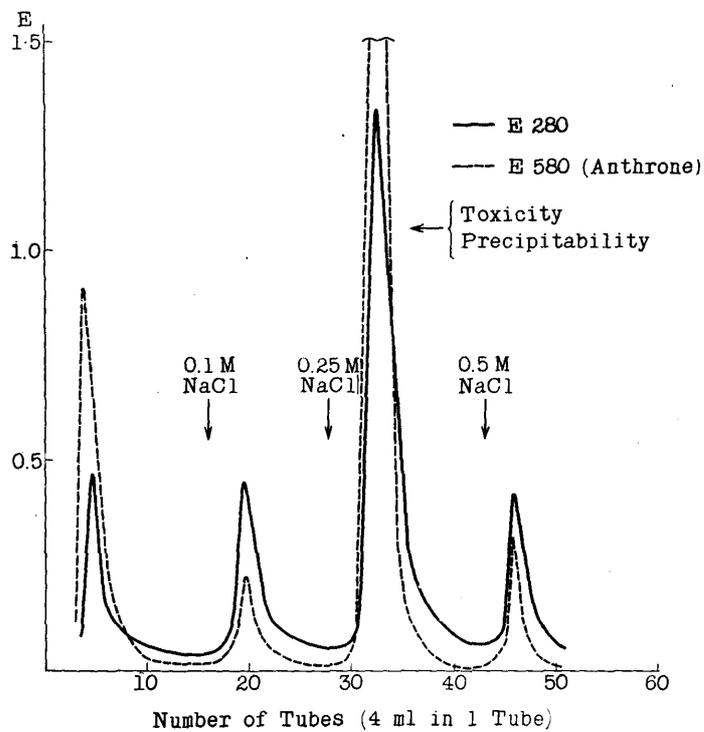
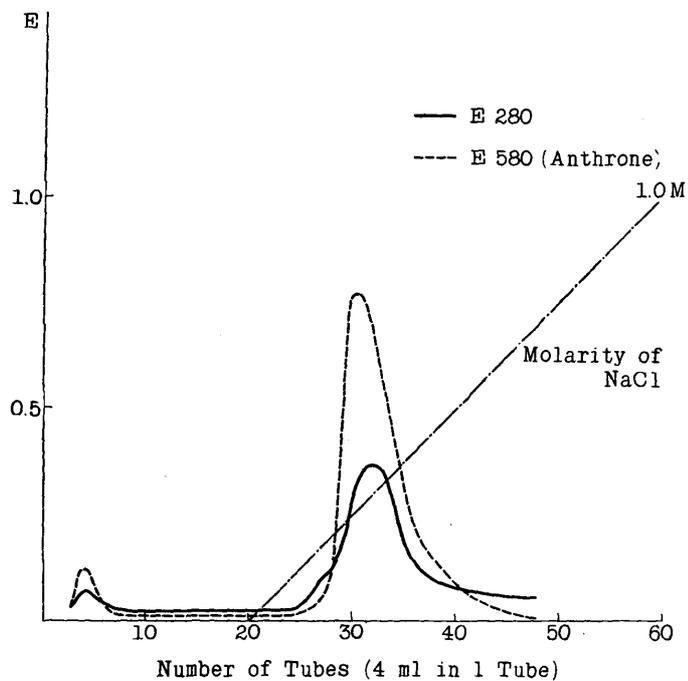


FIGURE 2.
 Chromatography of
 Fraction 2
 (Stepwise Elution)

FIGURE 3.
 Rechromatography
 of the toxic Peak
 in Figure 2
 (Linear Gradient
 Elution)



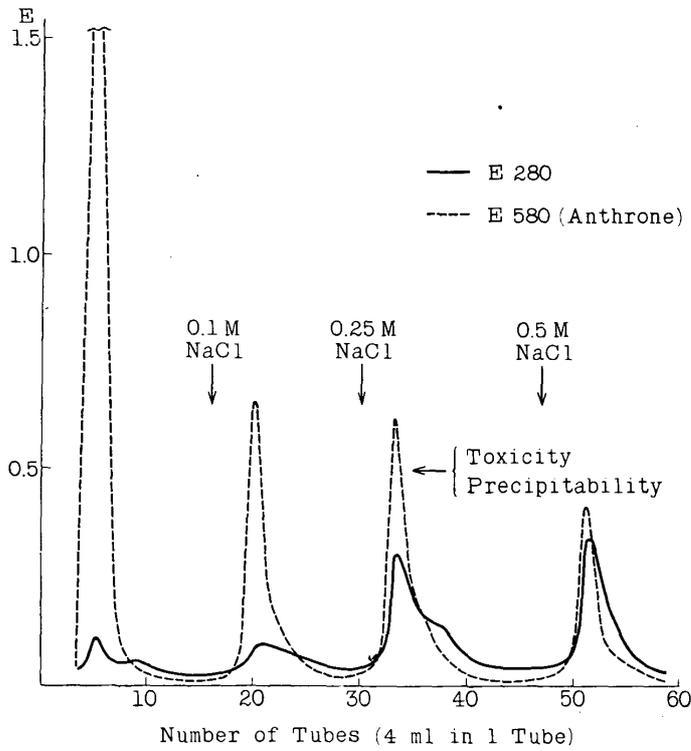


FIGURE 4.
Chromatography of Endotoxin
(Stepwise Elution)

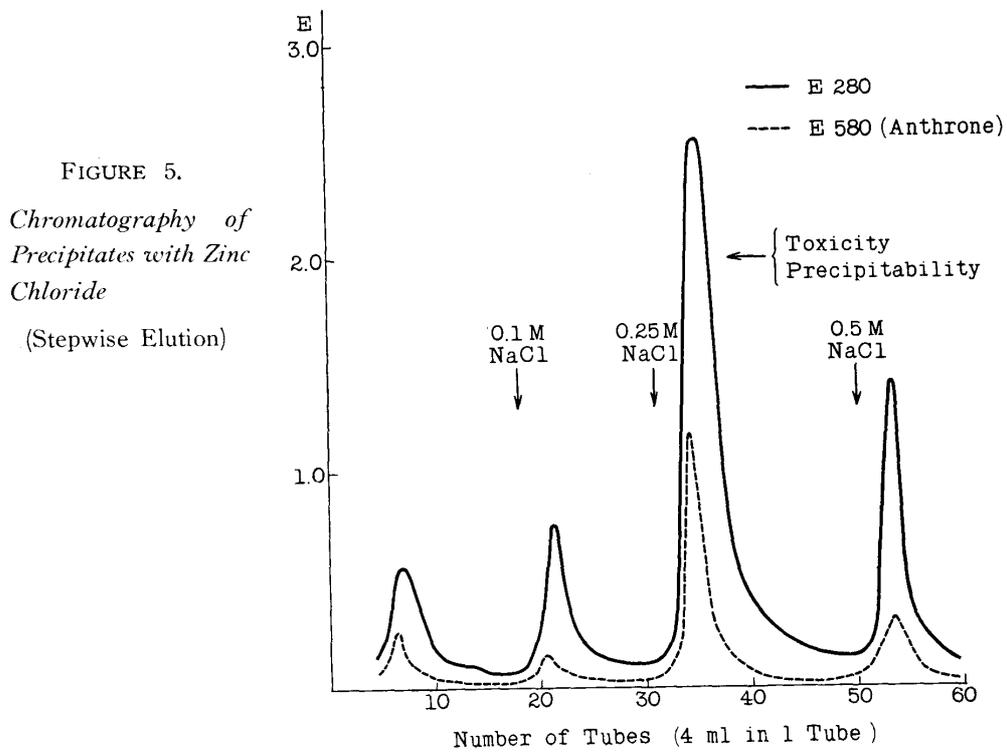


FIGURE 5.
Chromatography of Precipitates with Zinc Chloride
(Stepwise Elution)

the salting-out method already described. Ten ml of 50% zinc chloride solution was added to 1 liter of the starting toxic filtrates at pH 8.0. After centrifugation, precipitates were dissolved in a small volume of 20% sodium phosphate solution, dialysed and subjected to chromatography. Recovery of toxicity in this case was somewhat less than that in the case using ammonium sulfate. Figure 5 shows chromatogram of this fraction. There, a peak eluted by 0.25M sodium chloride had both toxicity and precipitability.

DISCUSSION

Mice injected with a certain number of living cells of hemolytic *E. coli* of O 139 are always killed by bacteriaemia within a short period. Therefore, in order to prove existence of the lethal substance, it should be required that samples to be examined are completely cell-free. Through a series of the present experiments, if necessary, samples were filtered through a glass filter (U.F.) prior to injection. Additionally, non-existence of hemolytic organisms in various organs of mice which died as a result of injection was confirmed by blood agar technique.

As the first step of isolation, procedures given in the scheme seemed to be satisfactory. That is, recovery of toxicity from starting material to fraction 2, the last product of the procedures, was almost complete, but the fraction was found to be still impure. For further purification, chromatography on DEAE-cellulose column was effective, by which the lethal substance was separated clearly from other inactive substances. As seen in figure 3, the diagram of re-chromatography presented substantially a single peak in the course of sodium chloride gradient elution, and electrophoretic pattern of the peak also suggested the absence of impurities (unpublished data).

There have been some reports supporting the presence of an edema disease producing factor. For example, TIMONEY succeeded in inducing syndrome in normal pigs experimentally using bowel extracts of pigs which had died of the disease; GREGORY also observed that in experiments with pigs, injection of bowel extracts derived from field outbreaks of the disease resulted in typical edema, and stated that similar results were obtained even in experiments with mice. Chemical nature of the factor, however, is still quite unknown. In the present experiment, mice which were killed by injection of the lethal substance, found in the culture filtrates of the organisms, indicated no obvious edema, at least externally.

Because of the fact that the behaviors of both endotoxin and the lethal substance on DEAE-cellulose chromatography are quite similar as seen in figures 1, 2 and 4, it would appear that these two toxic substances inducing the same syndrome in mice are essentially identical. This suggestion is also supported by similarities of the two toxic peaks presented in figures 2 and 4, which are found in ratio of carbohydrate to protein and in precipitabilities against homologous anti-O serum.

Further, the lethal activity in the culture filtrates occurred only when the incubation period was prolonged. Accordingly, it would be concluded that the lethal substance found extracellularly is the endotoxin, which is produced intracellularly and appears in the medium as a result of some certain lytic process of the cells.

SUMMARY

A lethal substance for mice, found in prolonged culture filtrates of a hemolytic strain of *Escherichia coli* of O 139 derived from the intestine of a pig which suffered from edema disease, was isolated by chromatography on DEAE-cellulose column. So-called endotoxin, contained in young cells of the organisms, was also isolated by the same means.

Both toxic substances behaved similarly on DEAE-cellulose column. Besides, they, when injected to mice, induced similar symptoms and each reacted against homologous anti-O serum. From these facts, identity of these two toxic substances would be emphasized.

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