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STUDIES ON HEMOLYTIC *ESCHERICHIA COLI* OF O-139
A CERTAIN FACTOR ESSENTIAL FOR
HEMOLYSIN PRODUCTION

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INTRODUCTION

Recently, LOVELL & REES and some other workers^{3,8)} have succeeded in separating a filtrable (soluble) hemolysin from young cultures of hemolytic *Escherichia coli*. In these successful attempts, particular attention was paid to the culture medium in which the organism would multiply and form the hemolysin; since most earlier workers had failed to obtain an evidence of the filtrable hemolysin using ordinary laboratory media.

In a previous work, we^{3,5)} also have demonstrated the existence of a filtrable hemolysin in the culture of hemolytic *E. coli* O-139 and further confirmed that a wide range of ordinary laboratory media does not support the appearance of filtrable hemolysin, even though it does support the bacterial growth fully. This observation may be interpreted by hypothesizing that *E. coli* O-139 requires a certain specific factor essentially for hemolysin production, in addition to some nutrient factors, and that this factor would not exist in primary laboratory media used.

The present paper gives experimental evidences of the hypothesis, and describes the factors influencing on hemolysin production by the organism. It includes observations on the appearance of hemolysin in resting cell suspension system.

MATERIALS AND METHODS

Strain Strain used was hemolytic *Escherichia coli* O-139 isolated from the intestinal contents of a pig suffering from edema disease.

Medium Peptone infusion broth (1% Proteose-Peptone, Difco) was used as the standard medium. In some case, peptone broths from different meat infusions brought inconsistent results for hemolysin production. Therefore, one lot of meat infusion, giving a good yield of hemolysin, was used through the experiment. Other media used are given on the following list.

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LIST OF MEDIA EMPLOYED

P. Y. medium:	2% Proteose peptone (Difco),	1% Yeast extract (Difco)
C. Y. medium:	2% Casamino acids (Difco),	"
M. Y. medium:	2% Mikuni peptone (Mikuni),	"
K. Y. medium:	2% Kyokuto peptone (Kyokuto),	"
T. Y. medium:	2% Polypeptone (Takeda),	"
E. Y. medium:	2% Yenjo peptone (Yenjo),	"

Titration of hemolysin Hemolytic activity was determined by the "serial dilutions" method. Each 1 ml of serial two-fold dilutions (physiological saline solution containing 0.1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 7.2) of the test fluid was placed in a series of test tubes, mixed by shaking with equal volume of 2% suspension of washed sheep erythrocytes, and the mixtures were incubated in a water bath at 37°C for 30 min. The hemolysin titre was expressed in hemolytic unit per ml (H. U./ml), which was the reciprocal of the maximum dilution of the test fluid that gave complete hemolysis.

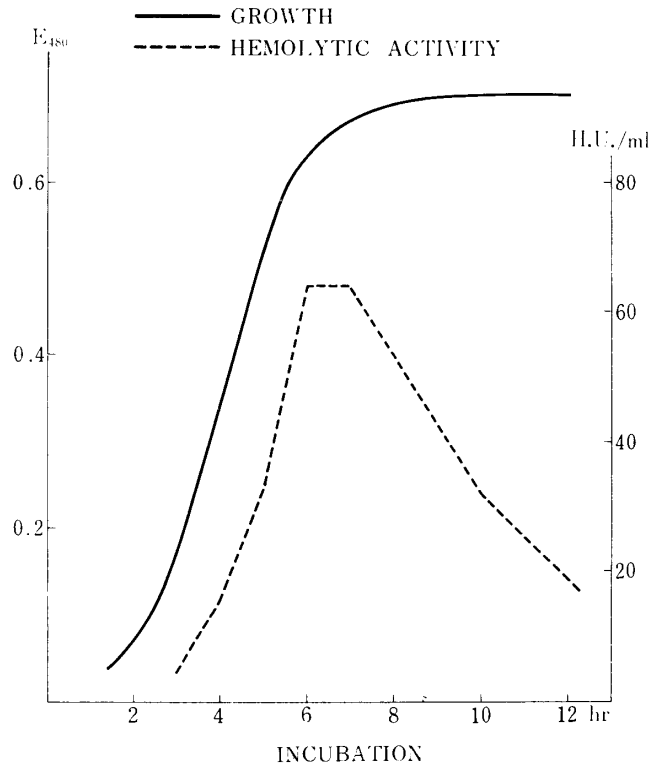
Determination of filtrable hemolysin Titration of a filtrable hemolysin produced in culture system or in cell suspension system was carried out as follows: To the test fluid containing living cells was added 1/20 volume of a 10% solution of streptomycin and the mixture was centrifuged for 10 min at 20°C (6,000 rpm). Although it was not completely free from the cell, the supernatant fluid obtained was submitted to titration of hemolysin without any further treatment, since the remaining cell had been found not to have any effect on the hemolysis.

EXPERIMENTS AND RESULTS

Hemolysin production in the culture system Growth and hemolysin production of the organism in the standard medium were estimated periodically. Hemolytic activity of the culture fluid increased for the most part in parallel with the growth of the organism and reached a maximum at the end of the logarithmic growth phase. Thereafter, the activity declined considerably. These observations are illustrated by the figure in which cell mass as measured optically, and amount of hemolysin appeared in the culture supernatant, are plotted against the time of incubation. Intracellular accumulation of the hemolysin could be almost neglected by the findings that the washed cells harvested even at the logarithmic growth phase caused little, if any, hemolysis when they were incubated with the erythrocytes, and that no hemolysin was obtained from the cells by sonic disruption or aqueous extraction with various salt solutions. It is likely, from these results, that the hemolysin is possibly synthesized in or on the surface of the cell which is actively metabolizing and successively liberated into the environment medium. In connection with this, it may be noted that certain exoenzymes of bacteria are known to follow a quantitative variation with cell mass very similar to that described by the figure, and that these are hardly detectable in the cell.

Hemolysin production in the resting cell suspension system Simplification of the hemolysin-producing system seemed to be desirable for subsequent studies on hemolysin production. Then, the cell suspension system, which modified the system employed by

FIGURE Growth and hemolysin production in the standard medium



HOSOYA et al. and BERNHEIMER for streptolysin S formation by *streptococci*, was applied to *E. coli*.

The organism grown in the standard medium was harvested at the end of exponential growth phase, washed free from the medium in physiological saline solution and suspended in 1/10 original volume of physiological saline solution. Each 0.2 ml of the cell suspension was added into tubes each containing 1 ml of the medium shown in table 1. The tubes, then, were incubated in a water bath at 37°C. Inner fluid of the test tubes was periodically titrated for hemolytic activity. The results are given in table 1. Considerable amounts of hemolysin, comparable to those found in the culture system, could be obtained only in the mixture consisting of washed cells and the standard medium. Addition of some metabolic inhibitor to the system resulted in complete loss of hemolysin appearance, which indicates that the appearance of hemolysin is closely connected with the metabolic activity of the cell. No visible difference in cell mass before and after incubation, as measured in turbidity, was observed in every tube, which seems to show that multiplication or autolysis of the cell scarcely occurs in the system during the time of incubation. These results and the fact, mentioned in the foregoing section, that the cell used did not contain appreciable amount of hemolysin, apparently indicate that the development of hemolysin in the cell suspension system depends neither upon autolysis nor upon physical extraction of preformed hemolysin, but upon de novo synthesis of hemolysin.

TABLE 1 *Hemolysin production in cell suspension system*

MEDIUM	HEMOLYTIC ACTIVITY		
	15 min	30 min	60 min
Physiological saline solution	< 2 ^{H.U./ml}	< 2 ^{H.U./ml}	< 2 ^{H.U./ml}
Standard medium	32	64	62
Standard medium+Streptomycin	< 2	< 2	< 2
Standard medium+Chloramphenicol	< 2	< 2	< 2

It was also observed that the cell was capable of forming the hemolysin repeatedly, mostly in an equal amount, provided that the fresh medium was available each time.

Physical conditions affecting the hemolysin production Effects of temperature, incubation period, and pH on hemolysin appearance in the cell suspension system were investigated. Results are summarized in tables 2~4. As seen in the tables, optimum conditions for hemolysin appearance are approximately 7 of pH, 37°C of temperature, and 30 min of incubation period, respectively. However, whether these data actually show effects of physical

TABLE 2 *Effect of temperature on hemolysin production*

TEMPERATURE	HEMOLYTIC ACTIVITY
°C	H.U./ml
4	< 2
15	4
30	32
37	64
43	8
50	< 2
60	< 2

Condition: pH 7.2, 30 min

TABLE 3 *Effect of pH (initial) on hemolysin production*

pH	HEMOLYTIC ACTIVITY
	H.U./ml
4.0	< 2
5.0	2
6.0	32
7.0	64
8.0	64
9.0	< 2

Condition: 37°C, 30 min

TABLE 4 *Effect of incubation time on hemolysin production*

INCUBATION TIME	HEMOLYTIC ACTIVITY
min	H.U./ml
5	8
15	32
30	64
60	32
120	16
180	66

Condition : pH 7.2, 37°C

condition on hemolysin production is questionable, because the hemolysin formed in the system is considerably unstable.

Substances necessary for hemolysin production It was previously known that when *E. coli* O-139 is inoculated in primary laboratory media, such as C. Y., P. Y., and M. Y. medium etc., the organism grows and multiplies very well, but nevertheless, there appears no hemolysin in environment medium. To know whether the similar figure is seen in the cell suspension system, substitution of the primary laboratory media for the standard medium in the system was carried out. The cell used was derived from the culture in standard medium. Table 5 gives the result (second column). As seen in the table, none of the media tested, even the dialyzable fraction of standard medium, could take the place of standard

TABLE 5 *Hemolysin production in cell suspension systems with various medium*

MEDIUM	HEMOLYTIC ACTIVITY	
	Original	Supplemented*
	H.U./ml	H.U./ml
Physiological saline	> 2	< 2
Standard medium	64	64
Standard medium (dialyzable fraction)	4	64
P. Y. medium	2	64
C. Y. medium	< 2	32
M. Y. medium	4	64
K. Y. medium	< 2	32
T. Y. medium	2	64
E. Y. medium	< 2	32

Condition : pH 7.2, 37°C, 30 min

* To the system (0.2 ml cell suspension, 1 ml medium), 0.1 ml of concentrated solution (1/10) of undialyzable fraction of the standard medium was added.

medium for hemolysin production. Similar result was also obtained when the cell from the culture in C. Y. medium was used. The fact, that the dialyzable fraction of standard medium does not support the hemolysin production although the standard medium does so, seems to indicate that a certain factor essential for hemolysin production may be present in the undialyzable fraction of standard medium. From this point of view, the inefficient media shown in table 5 were supplemented with the undialyzable fraction of standard medium and examined for hemolysin-production promoting activity. The result is shown in table 5 (third column). The addition of the undialyzable fraction of standard medium was significantly effective and resulted in a good yield of hemolysin to every supplemented medium, except to the physiological saline solution. Such effect, however, could not be observed when the effective fraction was added to the system after the appointed period of incubation. It, therefore, is evident that the effective fraction acts on the hemolysin production and not on the hemolytic reaction. Consequently, it would be concluded that the organism produces the hemolysin only in the presence of some nutrient factors and a certain factor which may induce the hemolysin production of *E. coli* (the I. H. P. E. factor).

Hemolysin-inducing activity of some materials Possible replacement of the undialyzable fraction of standard medium by some other materials in the cell suspension system is shown in table 6. As seen in the table, cow's milk and egg white were found to have hemolysin-inducing activity, which may suggest a wide distribution of the substance having hemolysin-inducing activity in the natural world. Hemolysin-inducing activity of the standard medium, cow's milk and egg white, was found to be associated with the globulin fraction, through the differential precipitation procedure with ammonium sulfate or ethanol, and was

TABLE 6 *Hemolysin-inducing activity of some materials*

MATERIAL	HEMOLYTIC ACTIVITY
Physiological saline	< 2
Undialyzable fraction of standard medium	32
Cow's milk original	32
" × 4 (diluted)	32
" × 11 (")	4
Egg white × 16 (diluted)	32
" × 64 (")	32
" × 256 (")	2
Egg albumin (crystal)	< 2
Casein	< 2
Egg yolk	< 2

System :	cell suspension	0.2 ml
	C. Y. medium	1.0 ml
	test material	0.1 ml

readily destroyed by trypsin. These may suggest that the I.H.P.E. factor would be a protein-like substance.

Hemolysis caused by the living cell in the absence of I.H.P.E factor In the cell suspension system without I.H.P.E. factor, as seen in table 5, filtrable hemolysin is not detected. When, however, the washed erythrocytes were added to the system and incubated, they were found to be readily lysed. If this hemolysis is due to the principle other than the filtrable hemolysin whose existence has been evidenced, it follows that the addition of I.H.P.E. factor to the system will cause increased degree of hemolysis. It follows, also, that the degree of hemolysis by the living cells in the absence of I.H.P.E. factor will be different from that of the filtrable hemolysin produced in the presence of I.H.P.E. factor under the same conditions.

Each 0.2 ml of the washed bacterial cell suspension was mixed with a) 1 ml of physiological saline solution, b) 1 ml of C.Y. medium with I.H.P.E. factor, c) 1 ml of C.Y. medium without I.H.P.E. factor, d) the same as b), and e) the same as c). To a), b), and c), 0.7 ml of packed erythrocytes was added. All the mixtures were incubated in a water bath at 37°C for 30 min and, then, centrifuged to remove bacterial cells and unlyzed erythrocytes. The supernatant fluids of d) and e) were mixed with each 0.7 ml of the erythrocytes, and the mixtures were incubated and centrifuged in the same manner as that of above described. The supernatant fluids of a), b), c), d), and e) were diluted 1:32 with the physiological saline and submitted to determination of hemoglobin. Typical results are presented in table 7. It can be seen that the degree of hemolysis under the living cells is not influenced

TABLE 7 *Comparison of the degree of hemolysis by the living cell and by the filtrable hemolysin*

SYSTEM	HEMOGLOBIN (E_{540})
a	0.015
b	0.350
c	0.342
d	0.332
e	0.010

by the added I.H.P.E. factor and is essentially in good agreement with that of the filtrable hemolysin produced under the same conditions. These results may indicate that the hemolysis observed under the presence of living cells would be due to the principle substantially identical with the filtrable hemolysin, and that, in the presence of sufficient amounts of erythrocyte, the cells could synthesize the hemolysin at the same rate as that in the presence of I.H.P.E. factor. It is very likely that the hemolysin-producing activity of the erythrocyte is closely connected with its reactivity with the hemolysin. This view is mainly based on the findings that the lysed cell, by any treatment, was not effective at all on the appearance of hemolysin and that the ghost whose reactive site with the hemolysin was specifically covered or removed (details will be described in other report), also, was ineffective. It, there-

fore, seems that the added erythrocytes to the cell suspension system participate, not directly but indirectly, in the hemolysin production of the cell.

DISCUSSION

The evidence presented here shows that under appropriate conditions washed cells of *E. coli* O-139 are capable of forming appreciable amounts of hemolysin, and that the system studied can be applied for the mass-preparation of filtrable hemolysin. The cell suspension system seems to be useful especially to study on the physiology of hemolysin production of the cell, because the use of this hemolysin producing system makes it possible to observe the hemolysin production independently of the growth and multiplication of the cell.

That the appearance of filtrable hemolysin in the cell suspension system does not depend upon autolysis or physical extraction of preformed hemolysin from the cells used but upon de novo synthesis of hemolysin during the time of incubation, is indicated by the following facts. First, there is no variation in the optical density of the system while the hemolysin is being formed. Second, the hemolysin is formed only when the cells are actively metabolizing, which is suggested by the findings; such as the necessity of nutrient factors, the inhibition of hemolysin production by some antibiotics, the effect of temperature on hemolysin production, and so on. Third, only traces of hemolysin can be found in the cells.

Appreciable quantities of filtrable (soluble) hemolysin can be obtained only when I.H.P.E. factor, named tentatively in this report, is present in hemolysin-producing system. Many workers have failed to demonstrate the existence of a filtrable hemolysin in the culture of hemolytic *E. coli*^{3,7)}, and which seems to be, at least partially, due to the absence of I.H.P.E. factor in the media used.

Hemolysin-inducing activity of the erythrocyte in the cell suspension system, from its reactivity with the hemolysin, could be interpreted by assuming as follows. The organism synthesizes the hemolysin in or upon itself, only if necessary nutrient factors are available, but can not release it in the medium, and the remaining hemolysin represses the subsequent synthesis of hemolysin. If the erythrocytes are present in the medium, they will remove the hemolysin from the cells, which will cause the successive synthesis of hemolysin.

Role of I.H.P.E. factor in hemolysin-producing systems is still obscure at the present time. However, on the basis of the information on the hemolysis by the living cell and of the fact that the I.H.P.E. factor is of high molecular weight, it will be permissible to speculate that the I.H.P.E. factor relates itself to the appearance of hemolysin in the medium from the cell rather than to the process of hemolysin synthesis in the cell.

Since all the results obtained are based upon experiments conducted with one

strain of hemolytic *E. coli*, it is uncertain whether such aspects of hemolysin production as described here could be observed commonly through other strains or not.

SUMMARY

1) Considerable amounts of filtrable (soluble) hemolysin can be obtained by a simple hemolysin-producing system, namely the cell suspension system.

2) The development of filtrable hemolysin in the cell suspension system depends neither upon autolysis nor upon physical extraction of preformed hemolysin but upon hemolysin synthesis *de novo*.

3) The appearance of filtrable hemolysin in the cell suspension system is required a special factor (the I.H.P.E. factor) essentially, in addition to some nutrient factors.

4) The I.H.P.E. factor is contained in some meat infusion broth, cow's milk and egg white, but not in ordinary peptones and yeast extracts etc., and is an undialyzable substance, presumably a protein with high molecular weight.

5) It seems that the I.H.P.E. factor may not be related to the process of hemolysin synthesis, but to the appearance of hemolysin in the environment medium.

6) The most favorable conditions for hemolysin production in the cell suspension system are approximately pH 7, temperature 37°C, incubation period 30 min, respectively.

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