TOXINS OF *PSEUDOMONAS PSEUDOMALLEI*

I. PRODUCTION *in vitro*[1]

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In studying the pathogenicity of *Pseudomonas pseudomallei* (*Malleomyces pseudomallei*), it was frequently noted that mice and hamsters which died within 1 or 2 days after inoculation of viable organisms showed no gross lesions. This observation suggested the possibility that this organism produced a lethal toxic factor. A thermolabile toxin was subsequently demonstrated in sterile culture filtrates (Nigg *et al.*, 1955). More recently, Liu (1957) confirmed the production of a lethal toxin by *P. pseudomallei* in cultures grown on tryptone-glucose-agar, using a cellophane plate technique.

Since fairly large volumes of culture filtrate were needed for the characterization of the thermolabile toxin (Heckly and Nigg, 1958), the optimal conditions for toxin production were studied. Growth media, temperature, incubation, aeration, and toxigenicity of various strains were evaluated for their effect on toxin production.

EXPERIMENTAL METHODS

Cultures. P. pseudomallei strain 111-9, derived from a single colony of a stock culture, was selected for evaluating the effect of media and physical conditions on toxin production. The colonies of this strain were typically large and cream-colored with corrugated surface (rough), although a few variants were observed from time to time. The colonial morphology was determined on 4 per cent glycerin beef extract agar (glycerin agar) plates, streaked so as to obtain well isolated colonies, and incubated 3 days at 37 C plus 4 days at room temperature. Cultures for inoculum were grown statically in 4 per cent glycerin beef extract broth, pH 6.8 (glycerin broth), at 37 C for 24 hr at which time they contained *ca.* 10^8 organisms/ml. Aliquots of this inoculum were frozen and stored in a dry ice chest to preserve the characteristics of the strain. For toxin production, 50 or 200 ml broth in 125 or 500 ml Erlenmeyer flasks, respectively, were inoculated with 0.5 to 1 ml and incubated statically. The pH of each culture was determined at the end of the incubation period.

Filtration. Broth cultures were clarified by successive filtration through Kenite[2] and a Selas[3] 02 filter. This filtrate was then sterilized by filtration through a Selas 03 filter or a hydrosol type Millipore filter. The sterility of each filtrate was checked by incubating 1 ml in 9 ml glycerin broth at 37 C for 1 week.

Titration of toxicity. The toxicity of each sterile filtrate was titrated in Namru mice (Garber and Hauth, 1950), 6 to 10 weeks old, by intraperitoneal (ip) inoculation of 1 ml of 2-fold serial dilutions in glycerin broth. A lethal unit (LU) of toxin is defined as that amount which kills within 7 days 50 per cent of the mice as calculated by the method of Reed and Muench (1938).

RESULTS

Growth medium. Toxin production *in vitro* by *P. pseudomallei* was first demonstrated in filtrates of cultures grown in glycerin broth (Nigg *et al.*, 1955). Several other media were compared subsequently for toxin production. All of the cultures were incubated for 7 days at

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[2] A diatomaceous silica produced by Kenite Corporation, Harwood Building, Scarsdale, New York.

[3] Complete removal of residual cleaning acid from the Selas filters required immersing and autoclaving the filters in dilute ammonium hydroxide solution.

TABLE 1

Effect of composition of growth medium on toxin production

Composition of Broth:			pH of 7-day Culture	LU/ml
Heart infusion broth	Glycerin	Peptone		
%	%	%		
0	4	1	6.9	2.0
1	4	0	6.7	4.0
2	4	1	6.9	8.6
2	4	0	6.8	9.2
2	0	1	7.5	2.4
2	0	0	7.3	2.6
3	4	0	6.8	9.3
4	4	0	6.7	6.2
4% Glycerin in 2% heart infusion broth containing mucin as follows:				
None			6.2	5.6
0.1% Crude			6.6	8.3
0.5% Crude			6.7	11.0
1.0% Crude			6.7	22.0
2.0% Crude			7.2	22.0
1% Crude			6.2	20.4
1% Dialyzed			6.3	19.3
1% Mucin ultrafiltrate			6.0	8.5
None			6.0	6.4
1% Crude			6.1	16.0
Dialyzed mucin ppt'd*s uccessively by:				
10% Ethanol, pH 7			6.2	22.6
20% Ethanol, pH 7			6.3	22.6
30% Ethanol, pH 7			6.2	16.0
40% Ethanol, pH 5.5			6.1	11.3
Final supernatant after ethanol precipitations			6.1	11.3

* The precipitates and final supernatant were lyophilized and tested as 1 per cent suspensions.

32 C. At this temperature, a thick, dry pellicle formed over the surface of cultures in all media which contained glycerin.

The yield of toxin in glycerin broth cultures was *ca.* 2 LU/ml. The first medium compared with glycerin broth was a heart infusion broth of the following composition: 4 per cent glycerin, 2 per cent heart infusion broth (Difco), and 1 per cent peptone (Difco), pH 7.4. This medium yielded 8.6 LU/ml (table 1). Representative results with modifications of this medium, in which the concentration of the 3 constituents was varied, are also shown in table 1. Peptone was apparently not essential for toxin production and was therefore omitted subsequently. Glycerin, on the other hand, was apparently important for maximal toxin production. Subsequent experiments showed that its role was not primarily concerned with the lowering of pH as the result of its metabolism during growth since substitution of 4 per cent glycerin by 0.5 to 4 per cent glucose resulted in approximately 50 per cent reduction in toxin production. The low yield of toxin in broth without glycerin (table 1) suggests either that the alkaline pH which developed in such cultures was unfavorable or that glycerin was a requisite metabolite for toxin production. An explanation of the direct effect of pH on toxin production was not obtained since the addition of 0.1 M sodium phosphate buffer to glycerin heart infusion broth for the purpose of controlling pH decreased toxin production, and organic buffers were metabolized by the organism. Two per cent heart infusion broth appeared to be near the optimum (table 1) and this concentration was used subsequently. Each lot was pretested since various lots gave different results.

Since growth factors had been demonstrated in mucin (Tomarelli *et al.*, 1954), varying amounts of hog gastric mucin, powdered form (Wilson Co., Chicago, Ill.), as a 5 per cent suspension prepared in a Waring Blendor, were added to glycerin heart infusion broth to determine its effect on toxin production. Increasing concentrations of mucin resulted in a progressive rise from 8.3 to 22 LU/ml (table 1). One can assume that the effect of the mucin was directly on toxin production by the organism since 1 per cent mucin did not potentiate the toxicity of sterile filtrates.

To determine the fraction of mucin responsible for increasing toxin production, crude mucin, dialyzed mucin, and an ultrafiltrate of mucin were compared. A 5 per cent suspension of mucin (600 ml) was dialyzed against 6 L of distilled water, changed daily for 8 days. The ultrafiltrate was prepared from a 5 per cent suspension by the method of Heckly and Watson (1951) and presumably contained only dialyzable sustances. Table 1 shows that the yields with dialyzed and

crude mucin were comparable. On the other hand, the yield with mucin ultrafiltrate was not significantly greater than that in broth without mucin.

Attempts were made to precipitate the specific factor from dialyzed mucin with ethanol. The various ethanol precipitates and also the final supernatant were lyophilized and reconstituted in glycerin heart infusion broth to a final concentration of 1 per cent. Cultures grown in broth containing crude mucin and 10, 20, and 30 per cent ethanol precipitates of dialyzed mucin (at pH 7) showed comparable high toxicity (table 1). The 40 per cent ethanol precipitate and the final supernatant contained little or none of the enhancing factor.

It is apparent from these results that the factor in mucin responsible for the increased toxin yields was nondialyzable and that it was precipitable from dialyzed mucin by 10 to 30 per cent ethanol.

Various other media, all containing 4 per cent glycerin, were also compared for their ability to support toxin production. These were: 2 per cent proteose peptone (Difco), ammonium gluconate[4] (Lien and Levine, 1957, *personal communication*), a blood-free medium developed for the cultivation of *Pasteurella tularensis* (Won, 1958), casein hydrolyzate (Higuchi and Carlin, 1957), and the latter medium in which 5 per cent N-Z amine type A (Sheffield) replaced the casein hydrolyzate. None of these media produced higher yields than glycerin broth. The addition of small amounts of minerals, such as $FeCl_3$, $CuSO_4$, $MnSO_4$, $ZnSO_4$, and $MgCl_2$, and 1 per cent plasma to glycerin broth also did not increase toxin production.

Physical conditions. To determine the optimal incubating conditions for toxin production, glycerin heart infusion broth cultures incubated statically for 7 days at 32 and 37 C in both a water bath and an incubator were compared. The highest average yield in 3 experiments (7.6 LU/ml) was obtained from cultures grown in an incubator at 32 C (table 2). Covering the flasks in the water bath with foil to exclude light did not improve the yield in these cultures. Neither incubator nor water bath at 37 C was favorable for toxin production. An incubator at 32 C was therefore used subsequently.

To determine the optimal incubation time for harvesting toxin, triplicate cultures in glycerin heart infusion broth with and without mucin were tested at varying intervals during incubation. The titer in each medium increased rapidly up to 7 or 10 days, and decreased at 18 to 21 days (table 2). This decrease was not referable to denaturation of the toxin at the incubation temperature since filtrate incubated 7 days at 32 C showed no loss in titer.

TABLE 2

Effect of temperature, type, and length of incubation on toxin production

Physical Condition	LU/ml	
	Glycerin heart infusion broth	Glycerin heart infusion broth containing 1% mucin
32 C, 7 days		
Incubator	7.6	
Water bath	2.9	
37 C, 7 days		
Incubator	2.7	
Water bath	2.9	
32 C Incubator		
Days		
1		<1.0
3	3.6	2.6
5	6.6	10.9
7	11.0	17.4
10	11.6	22.6
14	10.7	19.6
18	5.7	
21		11.3

The organisms of 7-day cultures, grown in glycerin heart infusion broth with mucin, centrifuged, and reinoculated into fresh broth, produced one-half as much toxin as the standard inoculum. However, when the sterile, spent broth of 5- and 7-day cultures, with and without the addition of fresh nutrients, was reinoculated with fresh inoculum, no further toxin production was demonstrable. But when the spent broth was heated, to inactivate all toxin and enzymes, more toxin was produced in these heated media following their reinoculation. These experiments show that the accumulation of heat labile metabolic products in cultures after incubation for 5 days either prevented further toxin production or inactivated toxin as it formed.

Aeration. Cultures in glycerin heart infusion broth containing 1 per cent mucin were assayed

[4] A chemically defined medium in which ammonium gluconate served as the sole carbon and nitrogen source.

TABLE 3

Comparison of colonial morphology, virulence, and toxigenicity of 4 isolates and the parent strain 111-9 of Pseudomonas pseudomallei

Strain	Colonial Morphology of Inoculum*	Organisms/ip LD_{50}†	LU/ml‡
	%		
Parent	100 RCr§	2×10^6	5.8
Isolate:			
1	100 RCr§	$<4 \times 10^2$	5.5
2	100 RCr-Y	5×10^6	2.7
3	71 RCr-Y, 25 ISCr, 4 SCr	4×10^6	5.5
4	100 RCr-Y	$>10^6$	11.9

* R-rough (corrugated), S-smooth, I-intermediate, Cr-cream-colored, Y-yellow.

† The ip LD_{50} was determined previously on cultures similar to those used for toxigenicity studies.

‡ Cultures grown in 2 per cent heart infusion broth (Difco) containing 4 per cent glycerin.

§ Colonies became brownish and produced a soluble lavender pigment.

after incubation for 1, 3, 7, and 10 days on a 32 C rotary shaker which served as a means of aeration. The highest toxin yield, obtained after incubation for 7 days, was only 1.4 LU/ml although growth was excellent and the pH was maintained at 6 to 7. Cultures grown statically in this medium at 32 C regularly produced 16 to 22 LU/ml. Aeration seemed to affect toxin production rather than its stability since similar aeration for 7 days of preformed toxin in the form of a sterile filtrate did not diminish its toxicity.

Toxigenicity of various strains. All of the preceding data were obtained with cultures of strain 111-9 because this strain had consistently produced the most toxin. A comparison of 20 additional strains, varying in colonial morphology and virulence, confirmed the initial findings of Nigg *et al.* (1955) that no apparent correlation existed between toxigenicity and colonial morphology, or virulence. Furthermore, 4 isolates of the toxigenic strain 111-9 also showed a similar lack of correlation as to these 3 properties (table 3).

DISCUSSION

The yield of toxin produced *in vitro* by *P. pseudomallei* was influenced by the culture strain, medium, conditions of incubation, and the accumulation of metabolic products.

The importance of strain selection was emphasized above. It is hoped that further search will uncover more toxigenic strains. More active culture filtrates would facilitate purification of the toxin.

Although *P. pseudomallei* grows luxuriantly in many simple media, toxin production was favored by more complex media. Glycerin, heart infusion broth, and mucin were the important constituents of these media for maximal yields. However, toxicity reached a maximum at 7 to 10 days and did not increase with further incubation. Since the organisms in 7-day cultures were still toxigenic, it would seem that higher yields would require (a) the addition during growth of other nutrient factors which would augment toxin production; or (b) the removal of heat labile substances formed during growth which either prevent continued production of toxin or inactivate it as formed. It is not yet clear whether continued toxin production was inhibited after incubation for 7 days or whether an equilibrium was established between formation and inactivation of toxin.

Histological studies on mice and hamsters infected with *P. pseudomallei* indicated that a profound toxemia occurred in melioidosis although the exact cause of death is as yet unknown (Dannenberg and Scott, 1958). Whether toxin produced *in vitro* is the same as that responsible for the toxemia *in vivo* has not yet been demonstrated.

A study by Heckly and Nigg (1958) on the characteristics of the thermolabile toxin presents evidence that the toxicity was due to more than one toxin.

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SUMMARY

Selected strains of *Pseudomonas pseudomallei* produced a thermolabile lethal toxin *in vitro*. Maximal yields were obtained by incorporating glycerin, heart infusion broth (Difco) and hog gastric mucin into a broth medium and by incubating the cultures statically at 32 C for 7 to 10 days. The active factor in mucin was nondialyzable and precipitable by ethanol.

The conditions which apparently limit continued toxin production in older cultures are discussed.

Toxigenicity was apparently not correlated with virulence or colonial morphology.

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