The Anatomical Distribution of Murine Toxin in Spheroplasts of *Pasteurella pestis*

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SUMMARY

In order to study the distribution of the murine toxin in *Pasteurella pestis*, whole organisms were converted to spheroplasts by treatment with penicillin or glycine in a sucrose medium. The spheroplasts were broken by osmotic lysis and homogenization. After centrifugation and washing, the membrane residues contained about 10% of the total activity of the spheroplast; the remainder of the toxic activity resided within the cytoplasm. Ribosomes were of low toxic activity. Magnesium ions selectively inhibited the destruction of these membranes by sonic oscillation; their treatment with trypsin resulted in the release of large amounts of non-toxic protein and peptides. Varying the temperature during spheroplast formation altered both the distribution of toxin between the anatomical components as well as the amount of toxin synthesized. Preliminary tests indicated that toxin obtained from the membrane and that found within the cytoplasm were identical.

INTRODUCTION

Ajl, Reedal, Durrum & Warren (1955) isolated, purified and described the murine toxin of *Pasteurella pestis* as a non-conjugated protein of about 74,000 molecular weight. It has been observed by a number of workers that this toxin is intracellular and that it can be released following lysis or autolysis of the organisms (Englesberg & Levy, 1954; Goodner, Pannel, Bartell & Rothstein, 1955; Warren, Walz, Reedal & Ajl, 1955). Cocking, Keppie, Witt & Smith (1960) submitted *Pasteurella pestis* grown *in vivo* to sonic oscillation. They found that toxicity for mice resided predominantly in the resulting extract while a small amount of toxic activity remained in the residue. We have observed the bulk of the toxin to be present in the extracts as well as some in the cell wall from sonically treated organisms grown *in vitro* (unpublished data). The present report presents a somewhat different approach to the problem of toxin localization within the organisms. The possibility that certain breaking procedures might lead to erroneous conclusions about toxin distribution in growing organisms prompted our use of spheroplasts, in lieu of parent organisms, in order to eliminate this possibility as well as to demonstrate whether or not toxin resided within the cell membrane, cytoplasm or ribosomes. The results described here represent an attempt to further elucidate the anatomical site of toxin biosynthesis within the bacterial cell. In these investigations we have assumed a spheroplast to be devoid of the characteristic rigid bacterial cell wall of the parent organism from which it was derived.

METHODS

Culture of cells and production of spheroplasts. Organisms of *Pasteurella pestis* strain 'Tjiwidej' used in these studies were obtained from a modified defined medium of Englesberg (1952) and Englesberg & Levy (1954) in which casein hydrolysate was replaced by sodium thiosulphate (500 mg./l.) and five L-amino acids: phenylalanine (1.0 mM), methionine (0.5 mM), isoleucine (1.0 mM), threonine (1.0 mM) and valine (1.0 mM). Vitamins were added in the following amounts ($\mu\text{g./l.}$): thiamine hydrochloride (200), biotin (10), pyridoxine hydrochloride (100), folic acid (100), and cobalamin (50) containing 3000 $\mu\text{g./g.}$ vitamin B-12. The amino acids and vitamins, sterilized by filtration, and glucose (0.2%, w/v) solution, separately sterilized by heat were added to the autoclaved salt solution.

Organisms were stored on agar slopes composed of the defined medium containing 1.6% agar. Transfers were made from slopes to liquid media and the suspension incubated at 26° for 24 hr. The resulting suspension, stored at 7°, was used for daily inoculations. An inoculum (1.5 ml.) was transferred to 150 ml. of medium to produce actively growing cultures after incubation at 26° for 16–19 hr on a rotary shaker. The organisms were centrifuged down, washed in 0.054 M-potassium phosphate buffer (pH 7.0), and standardized by resuspending in defined medium to a Klett reading of 25 with a no. 42 filter (400–465 m μ); this corresponded to a concentration of 40 $\mu\text{g.}$ Lowry protein/ml. Sucrose to 10% (w/v) and penicillin G (Parke, Davis and Co.), 200 units/ml., were then added. In certain experiments, glycine (1%, w/v) replaced the penicillin. When glycine was used, a concentration of organisms giving a Klett reading of 100 (160 $\mu\text{g.}$ protein/ml.) was achieved and was routinely used to obtain larger quantities of spheroplasts. One-litre flasks containing 250–300 ml. penicillin- or glycine-treated suspensions of organisms were incubated on a rotary shaker. After a period of 7–11 hr at 26° over 95–98% of the organisms were converted to spheroplasts as determined by haemocytometer counts.

Spheroplast fractionation. Spheroplasts were removed by centrifugation (10,000 g) from 1 l. of suspension at 4° for 10 min. All subsequent centrifugations were also performed at 4°. The spheroplasts were suspended in 30 ml. of 4×10^{-3} M- or 10^{-2} M-potassium phosphate buffer (pH 7.0) containing 2×10^{-3} M-magnesium chloride and deoxyribonuclease (beef pancreas, 1 $\times$ crystallized, Mann Research Laboratories) 10 $\mu\text{g./ml.}$ Although considerable breakage was evident, complete spheroplast destruction was obtained by further homogenization with a motor-driven Teflon pestle. The resulting homogenate was spun at 32,000 g for 10 min. The pellet obtained was resuspended in the phosphate + magnesium chloride buffer (pH 7.0) without deoxyribonuclease and homogenized until no intact spheroplasts were detected by microscopy. The membrane pellet was washed twice in 20 ml. of the phosphate buffer + magnesium chloride solution and stored at 4° until used. This membrane fraction contained an insignificant number of viable organisms as determined by agar plate counts. The combined supernatant fluids obtained after homogenization were termed the cytoplasmic fraction. Ribosomes were obtained by adjusting the supernatant fluid to 0.02 M-MgCl₂ and spinning for 90 min. at 38,000 rev./min. in a no. 40 rotor of a Model L Spinco ultracentrifuge. The ribosomal pellet was washed with 0.02 M-MgCl₂ and recentrifuged.

Total ribonucleic acid was determined by extracting the various fractions with

0.5 N-perchloric acid for 30 min. at 70° as modified after Basler & Commoner (1956). The orcinol method of Ceriotti (1955) and absorption at 260 m μ were two methods used to measure ribonucleic acid in the acid extract. Yeast ribonucleic acid (Worthington Biochemical Corporation) served as the standard.

Total protein and toxin determination. Protein was determined by a modified Lowry procedure (Oyama & Eagle, 1956) with bovine albumin (crystallized, Pentex Incorporated, Kanakee, Ill., U.S.A.) as standard. Toxin was assayed by diluting the various fractions with distilled water and injecting 0.5 ml. intraperitoneally into 16–18 g. Swiss Albino mice; deaths which occurred within 24 hr were recorded. Four mice were used for each dilution. The LD 50 dose was averaged from maximal and minimal lethal doses. Toxin concentration in a particular fraction was calculated by dividing the LD 50 dose, in μ g., into the total μ g. protein of each fraction and expressed as the total LD 50 (total toxic units).

Membrane disruption. To evaluate methods that might be used to isolate membrane-bound toxin and to gain insight into the structural relationship between the toxin and the membranes these were disrupted by various methods. In each case the treatments were concluded by centrifugation of the membrane suspension at 32,000 g for 10 min. and assaying the resulting soluble and residue fractions for total toxin. To disrupt by sonic treatment, isolated membranes were suspended in 20 ml. distilled water or dilute phosphate buffer (pH 7.0) and treated in a Raytheon 10 kc. Sonic Disintegrator for 2–6 min. at 5°. Trypsin (2 $\times$ crystallized; Worthington Biochemical Corporation) was used for digestion of isolated membranes; digestion was facilitated by slow agitation on a rotary shaker for from 2 to 5 hr. Membranes were also treated by suspending in sodium deoxycholate (0.1 %, w/v) or weak alkaline solutions (sodium hydroxide pH 9–10) and agitating the suspension for 2–5 hr. Membranes were also disrupted by rapidly freezing aqueous membrane suspensions in thin layers, grinding with a pestle for a few minutes, then slowly thawing with further grinding.

RESULTS

Toxin content of spheroplast and spheroplast fractions

The spheroplast homogenate or the recombined isolated cytoplasm + membrane fractions displayed the highest specific toxic activity (lowest LD 50 value). The LD 50 of the recombined fractions decreased 60–70 % compared with the averaged LD 50 of these two components individually. Thus, cytoplasmic and membrane toxin complemented each other in expressing a more lethal effect. Cocking *et al.* (1960) found that the combined soluble extract and residue guinea-pig toxins of *Pasteurella pestis* exhibited a similar synergistic effect.

During a typical spheroplast conversion, total protein in these fragile forms increased three- to fourfold over that of the original culture. It was noted in many experiments that spheroplasts formed with glycine tended to contain more total toxin and protein in all fractions than those obtained by the use of penicillin. Most of the toxic activity appeared to reside within the cytoplasm, but at least 10 % of the total toxin remained in the twice-washed membrane fraction (Table 1). This fraction contained 50–60 % protein on a dry-weight basis. In other experiments five subsequent washings continued to remove small amounts of protein, suggesting that the membrane was being disintegrated. The final washing contained

minute amounts of RNA. It was found that 200 μ g. RNA/mg. protein remained in the membrane fraction upon completion of the washing procedure.

A gelatinous opaque pellet consisting almost entirely of ribosomes was obtained when the cytoplasmic fraction was adjusted to 0.02M with MgCl_2 and centrifuged at 38,000 rev./min. The washed pellet contained less than 1% toxin and protein of that of the intact spheroplast.

Table 1. *Distribution of toxin and protein in Pasteurella pestis spheroplast fractions obtained with penicillin and glycine*

The final volume of spheroplast suspension was 600 ml. for each treatment. Fractionation was carried out in 10^{-2} M-potassium phosphate buffer (pH 7.0).

Treatment	Fraction	LD 50 dose (μ g. protein)	Total protein (mg.)	Total LD 50 doses
Penicillin	Cytoplasm	53	57.78	1,088
	Membrane	212	23.40	110
Glycine	Cytoplasm	50	63.00	1,260
	Membrane	162	24.60	152

Table 2. *Comparison of the relative effectiveness of various methods in removing toxic protein from spheroplast membranes of Pasteurella pestis*

The data from a series of experiments were used to estimate the relative effectiveness of a particular treatment. The methods listed below are in order of decreasing effectiveness (+ + + + = most effective).

Method	Toxic protein solubilized
Sonic treatment	+ + + +
Sodium deoxycholate (0.1%)*	+ + +
Aqueous sodium hydroxide (pH 9-10)	+ +
Trypsin	+
Freeze, grinding and thawing	—
Control	—

* Although a great deal of protein was released, the specific toxic activity (LD 50) present in the solubilized fraction was difficult to determine since deoxycholate enhanced toxin activity (also see Goodner *et al.* 1955).

Slight alkalinity favoured the solubility of toxin from lysed whole organisms. Spheroplasts were fractionated at pH 8.8 as described in the Methods by substituting sodium borate buffer for the phosphate buffer in order to avoid possible selective precipitation of cytoplasmic toxin. Membranes so obtained contained over 70% of the total toxic activity of that found in membranes fractionated at pH 7.0. Relatively larger amounts of non-toxic protein were removed from membranes at pH 8.8 as compared to pH 7.0, suggesting that non-toxic particulate protein was also being solubilized. It was concluded that toxin could not be readily removed by washing from the membrane fraction, indicating that toxin was bound to the fraction which sedimented at 32,000 g.

Release of toxin from the membranes by various agents

Attempts were made to disrupt the membrane structure to test for the presence of a membrane-bound toxin by partial isolation and to elucidate the relationship of the toxin to the membrane. Sonic treatments of membrane suspensions for 2 min. significantly increased the specific toxic activity of the protein as compared with the untreated original suspension. A comparison of the methods used to remove and solubilize the toxin is shown in Table 2. Sonic treatment for 2-6 min. was the most effective method for the release of toxin. Quick-freezing, grinding and thawing, although releasing toxin from whole organisms, did not release appreciable amounts of toxin and total protein from isolated membranes.

Table 3. *Comparison of the amounts of toxin and total protein released from 'glycine membranes' from Pasteurella pestis spheroplasts during sonic treatment, with and without 0.02 M-MgCl₂*

Membrane suspensions prepared as usual were subjected to three 2 min. sonic treatments. Control membranes were resuspended during each 2 min. treatment.

Fractions	Membranes in water			Membranes in MgCl ₂		
	LD 50 dose (μg. protein)	Total LD 50 doses (toxic units)	Protein (% of total)*	LD 50 dose (μg. protein)	Total LD 50 doses (toxic units)	Protein (% of total)*
Soluble						
Sonically treated (1)	79	80	45	83	20	15
Control	83	22	12	83	19	9
Sonically treated (2)	100	24	17	—	—	8
Control	—	—	2	—	—	2
Sonically treated (3)	—	—	8	—	—	1
Control	—	—	1	—	—	2
Final residue						
Sonically treated	> 205	—	30	250	34	81
Control	242	57	85	224	66	87

* '% of total' refers to the amount of protein present in each fraction relative to the total soluble protein + residue protein.

Release of toxin by sonic treatment

During sonic treatments it was found that an increase in MgCl₂ concentration inhibited solubilization of toxin and membrane protein. Table 3 gives the results of a typical experiment with membranes from spheroplasts produced with glycine and submitted to sequential sonic treatments in order to remove the bulk of the toxin. Membrane material was suspended in distilled water or 0.02 M-MgCl₂, sonically treated for 2 min. and centrifuged. After resuspension this procedure was repeated twice for each sample before calculation of the data presented. Over 70 % of the total protein was released from membranes suspended in water after three sonic treatments; this protein contained 104 LD 50 (total toxin units). By comparison, 20 LD 50 and 20 % protein was released in the MgCl₂ preparations. It should be emphasized that the LD 50 dose of released protein decreased about threefold below the initial membrane-bound protein. This observation and the

previously mentioned results which showed an increase in the toxic activity of the sonically treated homogenates suggest that the potential toxic activity of bound toxin cannot be adequately expressed until the toxin is solubilized.

When 0.02 M-MgSO₄ or 0.02 M-NaCl was substituted for MgCl₂ in membrane sonic-treatment experiments, only MgSO₄ afforded significant protection. It was concluded from these sonic treatments with membranes from glycine- or penicillin-derived spheroplasts that magnesium ions protect the isolated membranes of *Pasteurella pestis* against disruption by sonic treatment. This effect of Mg²⁺ is interesting in that Grossowicz (1962) reported that Mg²⁺ was needed to stabilize whole *Pasteurella pestis* organisms against lysis by lysozyme. In confirmation we have observed that whole organisms were protected against sonic treatment with higher concentrations of Mg²⁺. Sonic treatment of the cytoplasmic contents obtained from the spheroplasts and retreatment of toxic protein released from the membrane by sonic treatment did not alter the LD 50 dose of the initial preparations. It does not appear that sonic treatment affected soluble (i.e. unbound) toxin.

Table 4. *The distribution of toxic protein and/or peptides between the soluble and residue fractions of glycine spheroplast membranes of Pasteurella pestis after treatment with trypsin*

Trypsin (300 µg.) was added to a 20.75 mg. membrane protein suspension and incubated at 37° in 0.01 M-potassium phosphate buffer (pH 7.0) for 135 min. The control contained 20.25 mg. membrane protein similarly incubated but without enzyme. The 'total protein' and 'total LD 50' doses (total toxic units) are expressed as % of total soluble protein + residue protein or toxicity.

Treatment	Solubilized			Residue		
	LD 50 dose (µg. protein)	Total protein (%)	Total LD 50 doses (%) (toxic units)	LD 50 dose (µg. protein)	Total protein (%)	Total LD 50 doses (%) (toxic units)
Control	39	12	49	240	88	51
Trypsin	109	40	60	245	60	40

The effect of trypsin on membrane disruption

Ajl, Rust, Hunter, Woebke & Bent (1958) reported that incubation of partially purified murine *Pasteurella pestis* toxin with trypsin did not significantly alter the toxicity of the preparation, although ninhydrin-positive material increased. Trypsin was therefore incubated with membrane suspensions in the hope of selectively releasing membrane toxin or of removing impurities while leaving toxin bound.

The LD 50 dose of solubilized membrane protein increased 2-4 times over that of the control (membrane + buffer; Table 4). This indicated that most of the protein released by trypsin was non-toxic. These results were in contrast to those from sonic treatment experiments, where the protein released was of high toxicity. In this particular experiment (Table 4) an unusually large degree of autolysis of the control membranes occurred which resulted in the release of highly toxic protein.

It would be expected that the loss of large amounts of non-toxic protein would

be detectable as an increase in the remaining residue activity. Such an increase in residue activity was only detectable in experiments where the release of non-toxic protein was most pronounced. Incubation of toxin released by control membranes with trypsin showed no decrease in toxicity, substantiating the fact that trypsin does not destroy toxic activity. These results suggested that toxin may be bound in some manner which makes it inaccessible to trypsin action.

Toxin synthesis at 26° and 37°

It has been reported that *Pasteurella pestis* alters its metabolic characteristics and requirements as a result of temperature changes (Surgalla, 1960). Burrows (1960) stated that preliminary results showed that spheroplasts incubated at 37° contained 75% less non-diffusible antigen (apparently complexed material) than did organisms converted to spheroplasts at 28°. Experiments were made to test

Table 5. *The distribution of toxin and protein in Pasteurella pestis spheroplasts produced by penicillin or glycine at 26° or 37°*

Spheroplasts were produced at 26° and 37° with penicillin or glycine, and cytoplasm and membranes isolated. The 'total protein (%)' and 'total LD 50 (%)' refer to % of total cytoplasmic protein + membrane protein or toxicity.

Treatment	Fractions					
	Cytoplasm			Membrane		
	LD 50 dose (µg. protein)	Total protein (%)	Total LD 50 (%) (toxic units)	LD 50 dose (µg. protein)	Total protein (%)	Total LD 50 (%) (toxic units)
Penicillin						
26	53	71	90	212	30	10
37	> 140	40*	< 57	272	60	> 43
Glycine						
26	50	71	89	162	29	11
37	119	61	78	276	39	22

* Spheroplast lysis with penicillin is more pronounced at 37° than at 26°. This observation might account in part for the low % yield of cytoplasmic protein, which would be lost when harvesting the spheroplasts.

whether spheroplasts formed at 26° or at 37° differed in toxin content and distribution in the fractions studied earlier. After incubation at 37° total synthesis of toxin and protein was inhibited as compared to 26°. Total protein decreased as much as 70% at the high temperature. Membrane fractions contained a greater percentage of toxin relative to cytoplasm when incubated at 37° than at 26° (Table 5). With penicillin, membrane toxin increased to over 43% of the total toxin in both fractions. This extreme shift in the relative location of toxin activity was seen in many experiments performed with penicillin; the effect was less pronounced when glycine was used in place of penicillin (Table 5). The specific activity of the penicillin membranes decreased 30–40% at the higher temperature (Table 5), while the decrease in specific activity of the cytoplasmic fraction was more drastic, being

almost a threefold loss at 37°. From this decrease in activity of both fractions it was concluded that at 37° there was less protein synthesis and toxin production than at 26°. The increase in LD 50 dose illustrates that less toxin existed relative to total protein at 37° and suggests a selective inhibition of toxin production at this temperature. This selective effect was more pronounced in the cytoplasm than in the membrane fraction. Whether toxin was selectively degraded or its synthesis selectively inhibited remains to be determined. Synthesis inhibition seemed more likely when spheroplasts were examined microscopically (Pl. 1, figs. 1 and 2), because spheroplasts formed at 37° (fig. 2) were smaller and contained a lesser amount of dense cytoplasmic material as compared with those at 26° (fig. 1). By comparison, protein synthesis of whole organisms was inhibited over 50 % and the specific toxin activity of the sonically treated organisms decreased 40 % at 37° as compared with 26° when these suspensions were incubated under the same conditions but without penicillin or glycine. This suggests that, under the conditions used at 37°, inhibition was not an artifact of spheroplast induction but was a metabolic inhibition characteristic of the whole organisms.

DISCUSSION

The cytoplasmic toxin of *Pasteurella pestis* appears to exist as a non-conjugated non-particulate protein as deduced from the fact that a negligible amount of toxin was found in the ribosomes. In the case of the particulate toxin it may be asked whether the insoluble residue consists mainly of cell membrane. Bacterial cell membranes are composed primarily of protein and lipid (Hughes, 1962). It is, therefore, reasonable to assume that the membrane does contribute a large amount of the total protein of the residue and perhaps the bulk of the total toxin.

Clarke & Lilly (1962) recently reported that Gram-negative bacteria contain a complex consisting of cell wall bounded on both sides by the membrane structure; one of these membranes appears to be attached to the cell wall. Hughes (1962) also suggested a closely associated wall-membrane complex in Gram-negative bacteria. Such a complex in *Pasteurella pestis* would make it difficult to evaluate the results presented here, in terms of two distinct separate envelope structures found in Gram-positive bacteria. It is interesting to note that, after vigorous stirring or homogenization, the *P. pestis* membrane disintegrates into particles some of which float free. A similar fragmentation was reported by Mitchell & Moyle (1957) with *Staphylococcus aureus* membranes. The gradual loss of protein and toxin from the *P. pestis* membrane fraction during the washing procedures demonstrates the fragility of the cell wall+membrane complex in *P. pestis*. This apparent instability makes it difficult to arrive at definite conclusions concerning the precise amount of toxin and protein contained in the membrane and cytoplasm of the intact organisms.

The cytoplasmic membrane of *Pasteurella pestis* and the partially purified toxin released from autolysed whole bacteria apparently are identical. Both autolysed whole-organism toxin and cytoplasmic toxin from spheroplasts are purified by precipitation between 35 and 70 % ammonium sulphate saturation (Ajl *et al.* 1955). The toxins (cytoplasmic, membrane, and autolysed whole organism) are increased in specific toxic activity by adding sodium deoxycholate, are not decreased in

specific activity by trypsin digestion and show a similar protein band when chromatographed by acrylamide gel electrophoresis or by agar diffusion analysis (Ouchterlony, 1949). Final confirmation of the identity of the membrane and cytoplasmic toxins is being sought by further purification of each toxin so that their properties can be compared more precisely. The temperature studies suggest that the synthesis of membrane and cytoplasmic toxin, relative to total protein, decreases with an increase in temperature. Most interesting is the fact that the decrease is not proportional in both fractions. This decrease in total protein synthesis appears to be a reflexion of the increase in nutritional requirements of *P. pestis* as a function of the temperature of incubation (Higuchi & Carlin, 1958). In more recent experiments we had indications that, with phenylalanine-depleted spheroplasts, the amount of membrane toxin was decreased. Upon subsequent addition of phenylalanine for a short period of time, the membrane toxin increased 10 % to 15 % relative to total membrane protein, while an increase in cytoplasmic toxin was undetectable. These findings and the temperature studies indicate that the murine toxin of *P. pestis* may be synthesized selectively as the growth conditions are changed. If we assume that the membrane and cytoplasmic toxins are identical, a plausible hypothesis is that the toxin is synthesized within or on the membrane and is then transferred to the cytoplasm, where it accumulates as a soluble protein. That the membrane is a possible site of protein synthesis has been suggested by several authors (Butler, Crathorn & Hunter, 1958; Hunter & Godson, 1962; Mitsui, 1961; Spiegelman, 1959). Experiments are now in progress with labelled amino acids *in vitro* and *in vivo* with spheroplast fractions in an attempt to establish the exact location of toxin synthesis.

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EXPLANATION OF PLATE

Spheroplasts of *Pasteurella pestis* formed in 1 % glycine medium at 37° and 26°. Phase microscopy, × 2000.

Fig. 1. Larger spheroplasts at 26°.

Fig. 2. Spheroplasts decreased in size at 37°.

Fig. 1

Fig. 2