THE IDENTIFICATION AND ISOLATION OF TWO MOUSE-TOXIC PROTEIN COMPONENTS IN EXTRACTS FROM PASTEURELLA PESTIS*

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Gel electrophoresis has been used in studying protein components in crude cell fractions for metabolic studies (1, 2) and in determining the purity of isolated proteins (3). While determining the feasibility of this method to locate the murine toxin in crude cell fractions of *Pasteurella pestis*, we observed that toxin activity was associated with more than one protein component of the patially purified material. In this report we wish to present evidence for the existence of two proteins that are responsible for mouse toxic activity of *P. pestis* cells. In view of previous findings suggesting only a single murine toxin (4), efforts were directed toward isolating each protein as a single toxic component. Data will be presented differentiating the two proteins on the basis of electrophoretic mobility, selective reactivity to surface active agents, metabolic inhibition, and the anatomical location in the cell.

Materials and Methods

Preparation of Toxin Samples.—

Pasteurella pestis: strain "Tjiwidej" (TJW), was grown on the casein hydrolysate medium of Englesberg and Levy (5). Toxin released from the cells by autolysis after 7 days was separated from the supernatant fluid by ammonium sulfate fractionation between 35 and 70 per cent saturation as described by Kadis, Ajl, and Rust (6). Large quantities of this toxin were further purified by subjecting it to continuous-flow electrophoresis over glass microbeads as described by Winsten, Friedman, and Schwartz (7). Five hundred mg of toxin were dissolved in 100 ml of 0.003 M phosphate buffer, pH 7.4 and subjected to a current of 125 to 150 ma for 4 hours in this buffer. The collected samples containing toxin were dialyzed, lyophilized, and subsequently used for disc electrophoresis.

Growth experiments were conducted using a defined medium containing 5 amino acids, glucose, and salts as outlined elsewhere (8). Cell extracts were obtained by sonic treatment in a 10 kc Raytheon sonic oscillator for 5 minutes at 1.05 amp. Proteins in the extracts were precipitated with saturated ammonium sulfate, dialyzed and lyophilized. Spheroplasts were formed employing 1 per cent glycine in 20 per cent sucrose medium (8). Following osmotic lysis of spheroplasts and fractionation of cell components by centrifugation, cytoplasmic protein was precipitated with saturated ammonium sulfate, dialyzed and lyophilized. Membranes were lyophilized directly without prior precipitation with ammonium sulfate.

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The protein content of lyophilized samples was determined by a modified Lowry procedure of Oyama and Eagle (9). Double diffusion analysis in agar gel was modified after Ouchterlony (10). The gel contained 1 per cent agar, phosphate buffer (0.05 M, pH 7.0) in normal saline. Antisera were prepared in rabbits and LD_{50} were determined as previously described (6).

Acrylamide Gel Electrophoresis: The disc electrophoresis method of Ornstein and Davis (11) was modified and used to examine toxin preparations for heterogeneity and also as a preparative technique for obtaining small amounts of highly purified material. Runs were made on 0.5 mm x 5 cm columns for 20 to 30 minutes. The current was 4 ma/tube and a total of 160 v were supplied to the system. In some experiments, when larger quantities of toxin were required, identical apparatus was used but the upper buffer container had 6 equally spaced slots to contain larger tubes (8 mm x 5 cm gels) cut from 10 ml pipettes. The resolution on the larger gels was equivalent to that on the smaller gels. The total volts and amperes applied were the same as in the small gel runs.

A major modification in the procedure was the elimination of both the sample and the spacer gels (see reference 12). Since the sample gel did not harden adequately with higher concentrations of protein, it was substituted with 20 per cent sucrose (13). Sephadex was likewise an acceptable substitute for the sample gel (12). The toxin-sucrose solution was overlaid on the small pore gel and the tube carefully filled to the top using a capillary pipette with standard tris glycine buffer.

The gels were stained with amidoblack (1 gm/300 ml of 7 per cent acetic acid) for ½ hour. They were adequately destained with 2 or 3 changes of 7 per cent acetic acid. For elution, gels were cut into 2 or 3 mm sections and eluted with either 0.05 M maleate (pH 6.6) or 0.01 M phosphate buffer (pH 7.6). The tubes were agitated during the elution period lasting 24 or 48 hours. In some experiments gels were eluted directly in dialyzing tubing. In other experiments gels were homogenized in maleate or phosphate buffer using a motor-driven teflon pestle. The homogenates were spun and the supernatant fluids dialyzed against distilled water prior to use.

RESULTS

Resolution and Isolation of Toxin.—Toxin, partially purified by the Stubbing's glass microbead electrophoresis apparatus, was used as the starting material for acrylamide gel separation. The degree of purity of a toxin sample as demonstrated by the LD_{50} was related directly to a reduction in the total number of protein bands detected by gel electrophoresis (Fig. 1 *a*, *b*, *c*). The two heavily stained bands, more highly concentrated in the purest samples (Fig. 1 *a*), were found to be toxic when eluted and injected into mice (Table I). Increasing the run time resulted in an increase in the resolution of the two toxic bands. The upper stained band corresponding to the slower migrating toxin band (toxin A) was eluted and shown to be a single protein when resubjected to electrophoresis (Fig. 1 *d*). This indicated that the lower band (toxin B) was not a breakdown product of toxin A resulting from the separation procedure.

Determination of the Relative Activities of Toxin A and B.—Measurement of the precise amount of protein eluted from the gel slices was complicated by the dissolution of "protein-positive" materials from the acrylamide columns.

In an attempt to determine the relative toxic activities of the two protein bands 0.05 M maleate buffer, pH 6.6 was used to elute the protein from 3 mm sections of gel columns. The amount of false protein from a blank gel section was

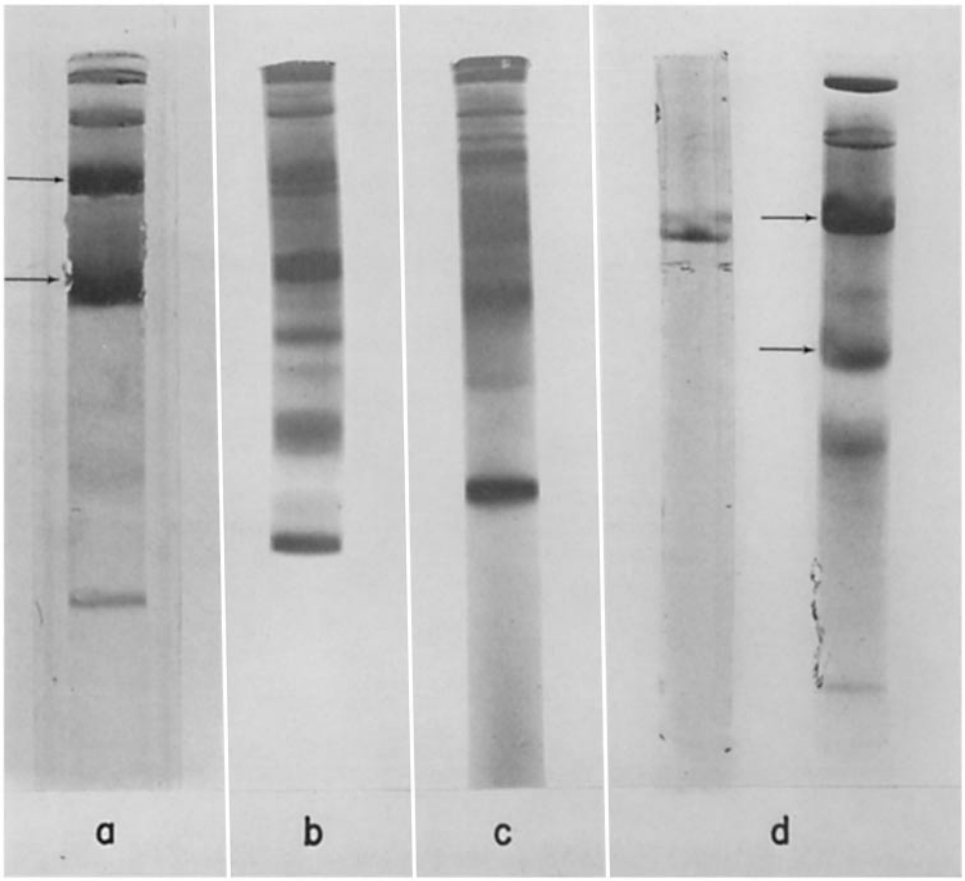

FIG. 1. Disc electrophoresis of toxin samples of varying purity. Sample LD_{50}'s a-c were as follows: (*a*) 1.9; (*b*) 4.9; (*c*) 7.4; (*d*) left, eluted toxin A, right, toxin A and B in the initial preparation. Toxin A (upper band) and toxin B (lower band) are designated with arrows.

subtracted from the unknown to obtain the actual protein content. Results obtained are shown in Table II. Intraperitoneal LD_{50}'s of 1.5 μg to slightly below 1 μg were recorded for each toxin. Thus, a 2- to 3-fold reduction in the LD_{50} was realized in each toxin from the starting preparations.

The eluted materials gave a positive Lowry reaction and absorbed at 280 mu. Tetramethylethylenediamine (TEMED) used in accelerating the polymerization of acrylamide has been previously suggested as an interfering substance by Lewis (14). We also suspected bound tris buffer and unpolymerized acrylamide to be contaminants. Substituting boric acid buffer for tris in the gel preparation did not decrease the amount of interfering substances eluted. DMAPN (E-C

TABLE I

*Separation of Toxin Proteins on Acrylamide Gel**

Gel section	Mouse assay‡
1	−
2	−
3	−
4 (Toxin A)	+
5 (Toxin A)	+
6	−
7 (Toxin B)	+
8	−
9	−
10	−
11	−
12	−

* 1.8 mg Lowry protein, LD_{50} = 4.7 was added per large gel. Each 3 mm gel section was eluted with 0.01 M phosphate buffer pH 7.6 for 24 hours in the cold room and the eluted protein dialyzed for 24 hours against distilled water.

‡ Killing, +; no killing, −.

TABLE II

*Specific Toxic Activities of Toxins A and B Eluted from the Gel Column**

Gel section‡	Protein eluted§	Total protein eluted§	LD_{50}
	μg/ml	*μg*	*μg*
1	1.0	6	−
2 Toxin A	4.4	26	1.0–1.3
3 Toxin A and B + protein impurities	5.1	28	1.5
4 Toxin B	8.9	47	0.8–1.0
5	0.5	3	−
6	0.0	0	−
7	0.9	5	−
8	0.0	1	−

* The starting sample contained 67 per cent Lowry protein by weight and had an LD_{50} of 3.8. 1.6 mg dry weight sample was added to a large gel.

‡ Each section was 3 mm.

§ After dialysis against distilled water and includes substraction of blank, 3 mm gel.

Apparatus Co., Swarthmore, Pennsylvania), another catalyst, was substituted for TEMED also with no beneficial effect. It was concluded that unpolymerized acrylamide was the interfering substance. Preliminary experiments indicated that by decreasing the pH less acrylamide was extracted from the gel.

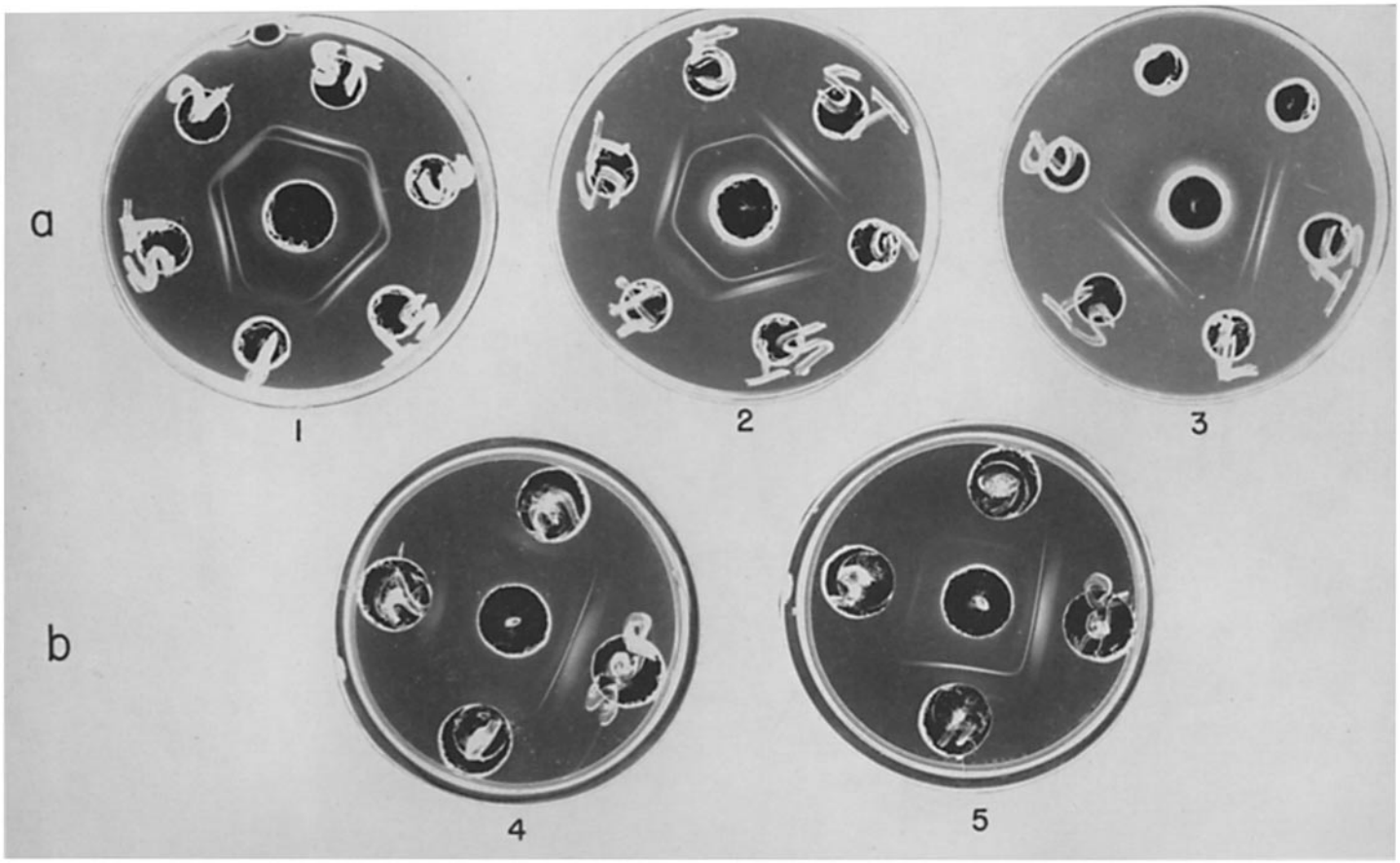

FIG. 2. Agar gel precipitin reactions of eluted toxins.

(*a*) Plates 1 to 3; 10 corresponding small gel sections were pooled and lyophilized and the concentrates alternated with the starting sample (*ST*) in the antigen wells. 0.3 ml concentrated rabbit antiserum was placed in the center well. Toxin A is the outer band (wells *2* and *3*) toxin B inner band (wells *4* and *5*).

(*b*) Plates 4 and 5; a single large gel column was sectioned and each section was placed in antigen wells. Toxin A, wells *2* and *3*, toxin B, well *4*, non toxic mpurity, wells *5* and *6*. *SEP* is the starting material.

Furthermore, the acrylamide extracted was more readily dialyzable under conditions of low pH (see reference 14).

Agar Gel Double Diffusion Precipitation of the Individual Toxins.—In our experiments we have observed almost no variation in the migration of individual protein bands in 12 gel tubes of a single run. To obtain enough protein in order to produce strong gel precipitin bands with rabbit antisera, protein was extracted from combined gel sections and concentrated by lyophilization. There was a definite correlation between the position of the major stained protein and the location of individual agar gel precipitin bands (Fig. 2 *a*). The slower migrating toxin A appeared as the outer band in the agar gel. The concave shape of the precipitin band A suggested an antigen of greater molecular weight than the antibody (15). Toxin B was identified with the innermost band and revealed a straight or slightly convex precipitin band suggesting a smaller molecular weight antigen than the corresponding antibody. If single gel columns were sectioned and an individual gel section placed in each antigen well, a distinct separation of the two toxic proteins occurs (Fig. 2 *b*). A non-toxic impurity could also be noted (Fig. 2 *b*). In addition, a similar correlation was found between gel protein bands and precipitin bands obtained by a double diffusion technique in vertical tubes. It was concluded from these experiments that each of the two major protein bands was composed of homogenous, antigenically active toxin.

Action of Steroid Derivatives.—In initial experiments to determine whether the two toxin proteins exhibited similar properties, the unresolved mixture containing both toxins was subjected to various physical and chemical treatments. Steroid derivatives were employed in an effort to distinguish bewteen the toxins (8, 16). Treatment of 2 mg of impure protein with 0.2 per cent deoxycholate appeared to destroy the integrity of toxin A but not of toxin B (Fig. 3). Similarly, toxin A was selectively attacked by 0.2 per cent digitonin. On the other hand, heating at 50°C for 45 minutes, treatment with lipid solvents, trypsin digestion or sonic oscillation produced no visible changes in either band.

Metabolic Differentiation of the Toxins.—The previous experiments were conducted entirely with toxin obtained from 7-day-old cell autolysates. Protein extracts from sonic-treated cells which had been grown on a defined medium, have been separated into single protein components directly by disc electrophoresis. Examination of the toxin content in extracts from cells treated with tryptophan analogues or incubated at 37°C, showed a selective depletion of toxin A leaving toxin B unaffected (17). We have found that the toxins of ammonium sulfate precipitated preparations can be further purified by extraction with alkaline buffer, pH 8.0–9.0. This single step resulted in preparations which were resolved with a high degree of clarity compared with unextracted samples (Fig. 4 *a*, *b*). The value of this technique appeared to be in the removal of interfering contaminants which remained in the insoluble residue after ex-

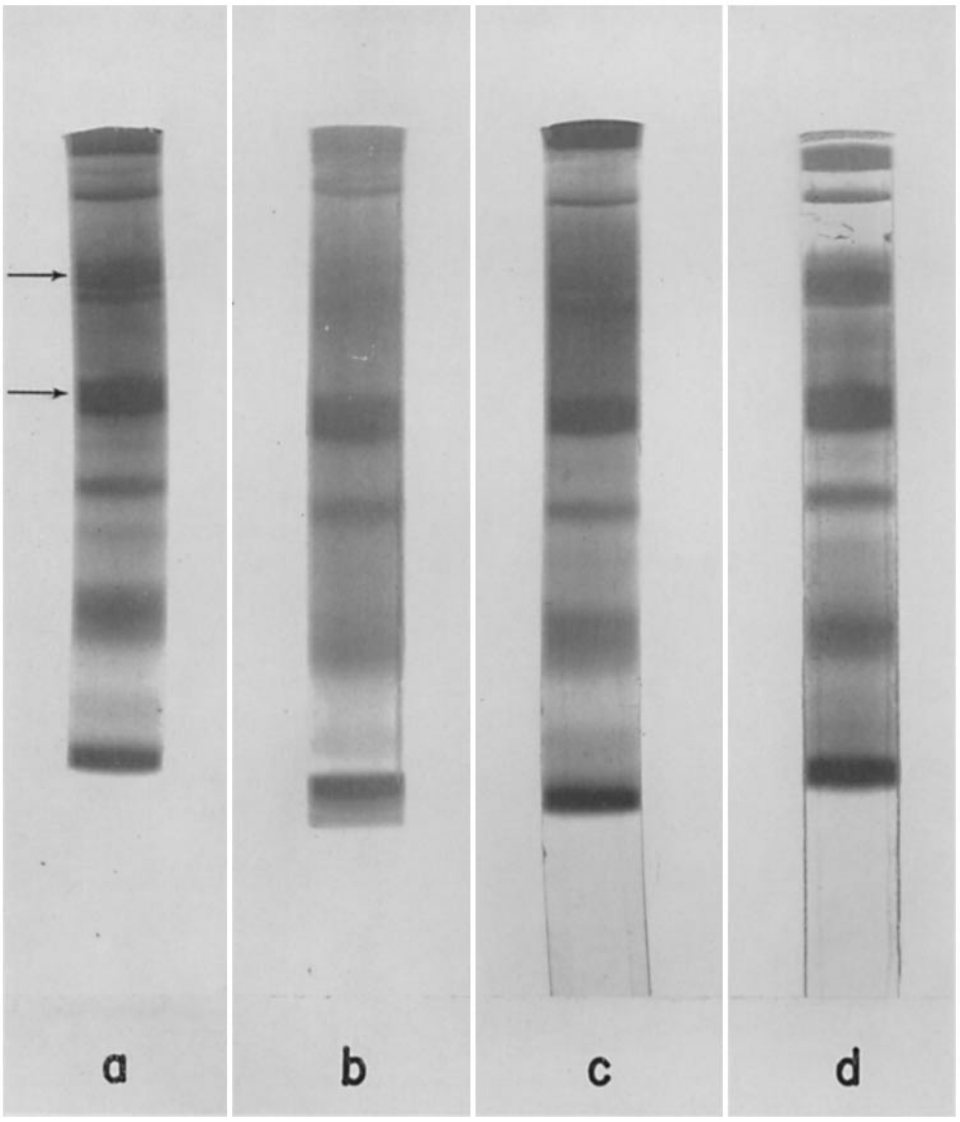

FIG. 3. Action of steroids on toxin A. The unresolved sample had an LD_{50} of 2.7 and contained 52 per cent protein, (*a*) control, 1.6 mg toxin dissolved in 0.4 ml of 0.01 M phosphate buffer (pH 7.6) plus 0.5 ml distilled water, (*b*) plus 2 mg deoxycholate (0.2 per cent), (*c*) plus 2 mg digitonin (0.2 per cent), (*d*) plus 10 μg trypsin. The treated samples were incubated for 30 minutes at room temperature followed by electrophoresis. 264 μg protein of each reaction mixture was added per gel. Toxin A (upper arrow), toxin B (lower arrow).

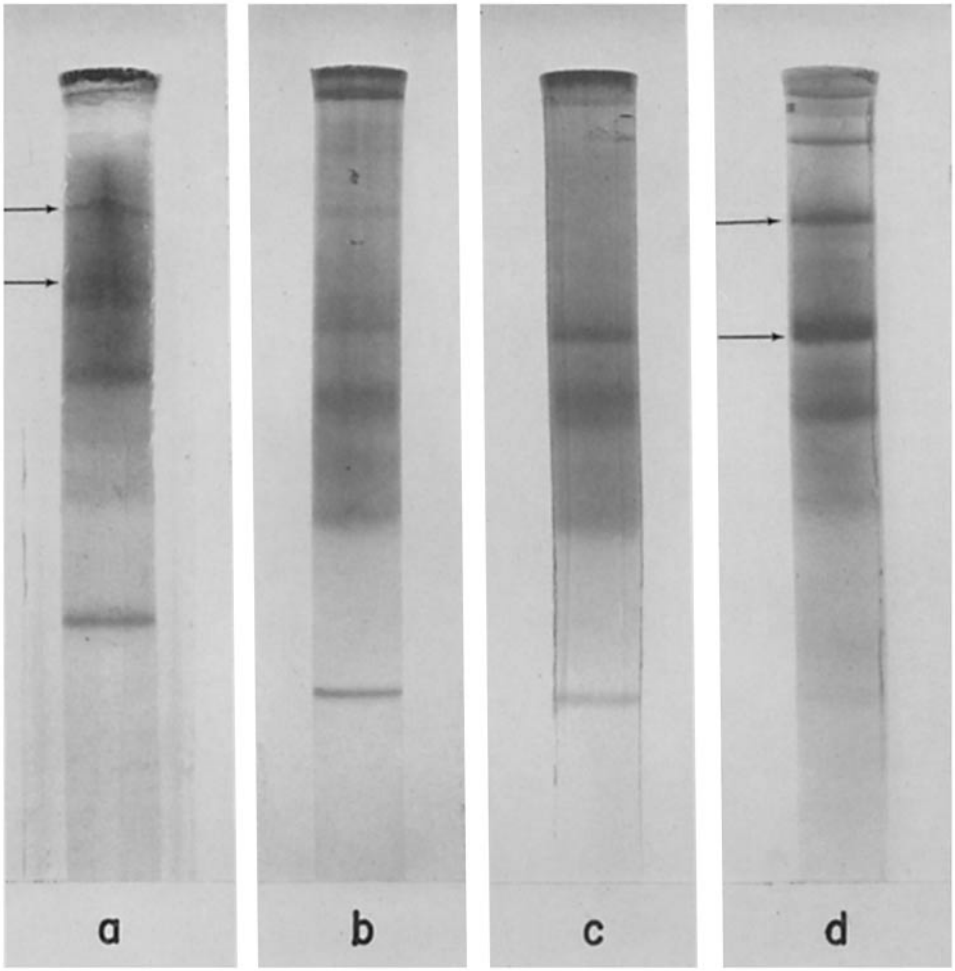

FIG. 4. Analysis of alkaline extracts from 5-fluorotryptophan-treated cells. 10 mg of lyophilized crude protein was agitated for 4 hours at room temperature in 0.5 ml pH 8.6 tris glycine buffer. 380 μg protein added per gel. (*a*) control, not extracted. (*b*) control for 5-fluorotryptophan experiment. (*c*) 5-fluorotryptophan treated. (*d*) Stubbing's sample control, LD_{50} is 4.9. Arrows designate toxin A (top band) and B (bottom band).

traction of the samples. If not removed, these impurities caused "funneling" and "blurring" of the protein bands (Fig. 4 *a*).

To further confirm earlier indications obtained by gel analyses (17), of the action of tryptophan analogues in selectively inhibiting the accumulation of toxin A, dilute suspensions of cells were exposed to 60 μg/ml of 5-fluorotrypto-

phan for 8- to 11-hour periods (approximately 10 generations). This analogue was used since it caused no growth inhibition over a number of generations. If both toxin bands were reduced in these experiments, it might be concluded that toxin A was a precursor of toxin B and that during 4- to 5-hour experiments (17) toxin A was converted to toxin B without the former being resynthesized. Mouse assay of the extracts showed a 3- to 4-fold increase in the LD_{50} of the treated cell extracts (Table III). Alkaline extracts from treated cells showed the complete absence of toxin A while toxin B was formed at rates comparable to control cells (Fig. 4 *c*). These results appeared to negate the precursor hypothesis.

TABLE III

*The Inhibiton of Toxin Formation by 5-Fluorotryptophan**

Sample	Total protein	Protein inhibition	LD_{50}	Total toxin units‡	Toxin inhibition
	mg	*per cent*	*μg*		*per cent*
Control	31.2	—	40–60	520–780	—
5-Fluorotryptophan	15.0	52	140–160	94–170	80

* 200 ml of cells initially were treated with 60 μg/ml 5-fluorotryptophan at 27°C for 11 hours. At the 6th hour 60 μg/ml 5-fluorotryptophan was added to the treated flask.

‡ Calculated from $\frac{\text{total protein}}{LD_{50}}$ = total toxin units.

Distribution of Toxin A and B in Spheroplasts.—Since toxin has been found to be located in both the cytoplasmic and membrane fractions of spheroplasts (8), this raised the question of the possible association of toxins A and B with particulate cell fractions. Mouse assay of spheroplast fractions treated with tryptophan analogues indicated that membrane toxin synthesis was preferentially inhibited while cytoplasmic toxin was inhibited only to the degree of general protein synthesis. To obtain more direct evidence for the association of toxin A with the membrane fraction and toxin B with the cytoplasmic fraction, spheroplasts were lysed by lyophilization and osmotic rupture. They were fractionated and the dried, concentrated fractions extracted with tris glycine buffer at pH 8.6. Protein patterns from these extracts revealed that toxin A was predominantly associated with the membrane and toxin B with the cytoplasmic fraction (Fig. 5). Mouse assays of sectioned homogenized gels from each fraction showed toxin activity to be associated with each band. It was noted that in the membrane fraction, although only slightly visible, toxin B, still exhibited biological activity. However equivalent traces of membrane toxin observed in cytoplasmic protein showed no biological activity. A parallel was detected in these results with the elution experiments where partially purified Stubbing's toxin yielded a smaller percentage of toxin A than toxin B

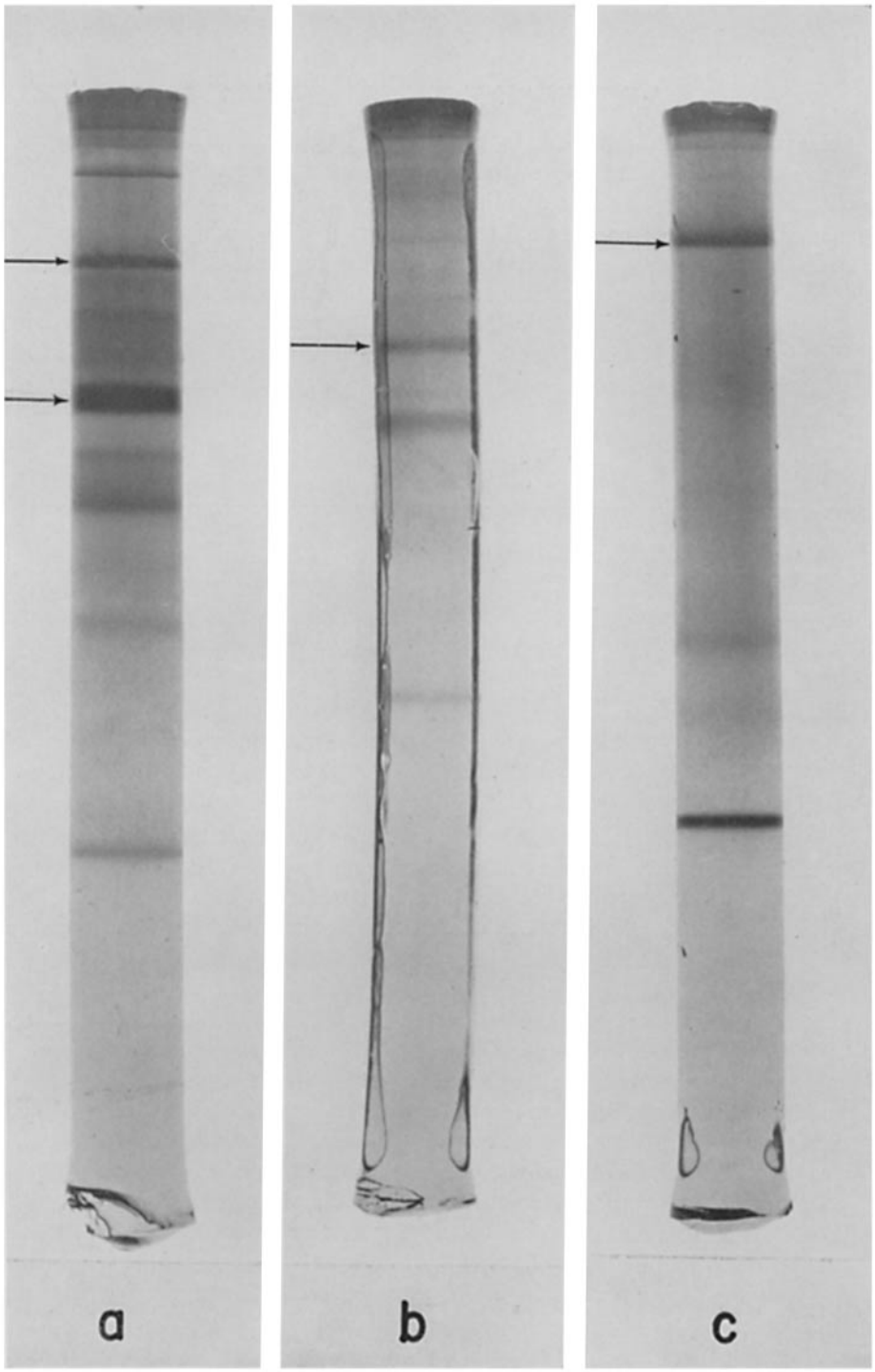

FIG. 5. Isolation of toxin A from the membrane and toxin B from the cytoplasmic fraction. 10 mg of lyophilized crude protein was agitated for 3 hours in pH 8.8 tris glycine buffer at room temperature. Cytoplasmic protein was extracted with 0.5 ml and membrane with 0.70 ml buffer. (*a*) Stubbing's sample control. (*b*) cytoplasmic extract, 226 μg/gel. (*c*) membrane extract, 290 μg/gel, LD_{50} of the unresolved sample is 20 μg.

(Table II). These results strongly suggested that toxin A was more readily absorbed to acrylamide gel than toxin B.

DISCUSSION

The evidence that only toxin A but not toxin B is inhibited by tryptophan analogues, acted upon by deoxycholate and digitonin, and bound to a particulate fraction of the cell, presumably membrane, suggests that the two toxic proteins are different molecular species. Toxin B is not a polymer of toxin A. However, it is possible that during the isolation procedure toxin A disaggregates to form an essentially "new protein" toxin B as has been observed with bovine growth hormone (14) and glutamic dehydrogenase (18). The relative similarity in specific toxin activity of the proteins would support this hypothesis. This would imply that toxins A and B have common antigenic components.

The reactivity of toxin A to deoxycholate and digitonin may be related to a lipid or phospholipid component present in combination with this protein since these compounds appear to interact with lipid-protein structures of membranous components (20, 21). Deoxycholate and other steroids known to act as surface active agents have been reported to influence hydrophobic bonding of certain proteins (19). The selective action of these compounds on toxin A may reflect a relatively higher number of non-polar amino acid end groups than toxin B. The greater availability of these end groups is a characteristic of structural proteins (22), and in toxin A this may be reflecting its derivation from the *P. pestis* membrane. The non-polar nature of toxin A may also explain its slow migrations in a charged field, and may answer the question as to why in earlier studies, using hanging curtain paper electrophoresis, the slower moving toxin A was not detected (4), since it never moved from the upper areas of the paper. The mobility of toxin B appears to correspond with the expected mobility of the earlier isolated murine toxin of 70,000 molecular weight (4), since the former runs just behind the bovine albumin band (67,000 molecular weight).

It is interesting to note that, whereas, toxin A is very susceptible to changes in the metabolic conditions of the bacterial cell, toxin B formation follows the synthesis of general cell protein more closely (see reference 17). Whether or not this reflects the degradation of protein A to supply amino acids for the synthesis of other proteins or whether this suggests a completely different mode of control for the synthesis of toxin A remains to be determined. Experiments with tryptophan analogues suggest that the intracellular level of tryptophan preferentially determines the extent of toxin A synthesis under limiting tryptophan conditions.

SUMMARY

The toxin activity of *Pasteurella pestis* cells, strain "Tjiwidej," was found to be associated with two proteins. Using a disc electrophoresis technique in con-

junction with mouse lethality, two toxic proteins were isolated exhibiting intraperitoneal LD_{50}'s of less than 1.0 to 1.5 μg protein. Each produced a single characteristic precipitin band on agar gel diffusion plates. The slower migrating toxin in gel diffusion or disc electrophoresis was designated as toxin A. It was shown to be sensitive to deoxycholate and digitonin, did not accumulate in 5-fluorotryptophan treated cells, and was associated with the membrane fraction of the cell. The faster migrating toxin B, apparently is resistant to surface-active agents, and is not affected by treatment of cells with 5-fluorotryptophan. Toxin B is associated with the soluble or cytoplasmic fraction of the cell. This evidence suggested that each toxin represented a distinctly different molecular species. The possibility is discussed that toxin B is synonymous with the murine toxin previously isolated by paper curtain electrophoresis which revealed only one antigen band in the Oudin precipitin reaction.

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