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DIFFERENTIATION OF EXOTOXIN AND OTHER BIOLOGICALLY ACTIVE SUBSTANCES IN *PSEUDOMONAS PSEUDOMALLEI* FILTRATES

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ABSTRACT

HECKLY, ROBERT J. (University of California, Berkeley). Differentiation of exotoxin and other biologically active substances in *Pseudomonas pseudomallei* filtrates. J. Bacteriol. **88**:1730–1736. 1964.—Denaturing agents such as phenol, formaldehyde, and urea reduced lethal toxicity and proteolytic activity of partially purified preparations from *Pseudomonas pseudomallei* at about the same rate. Neither toxin nor enzyme was stable at pH 11, when the solution was adjusted with sodium hydroxide, but there was a slight difference in their rates of inactivation. However, under certain conditions, ammonium hydroxide destroyed most of the enzymatic activity with only a slight effect on lethality. Conversely, toxin was less stable in acid solutions than was the enzyme. Thus, treatment with ammonium hydroxide or acetic acid yielded preparations with either a low or a high enzyme-to-toxin ratio, indicating that lethality was not dependent on enzyme activity. Although proteolysis of any one of the essential factors in the blood coagulation system can inhibit clotting of blood, the potent anticoagulant activity of culture filtrates was not associated with its proteolytic activity, but was directly correlated with lethal toxicity. It is of considerable interest that the necrotoxicity was, however, associated with enzymatic activity and not with lethality. Serological reactivity of the enzyme, as well as its proteolytic activity, was altered by ammonium hydroxide. Similarly, antigenicity and toxicity of the lethal toxin were reduced by acidification. Each acid- or alkali-treated preparation produced a single precipitin line in double diffusion in agar when reacted with antisera produced by injection of crude filtrate. Partially purified preparations, having both lethal and enzymatic activity, produced two lines, one identifiable with the enzyme preparation, and one with the toxin. Furthermore, specific precipitation with the respective antisera removed either enzyme or toxin from crude preparations. Therefore, the lethal exotoxin and proteolytic enzyme are separable entities.

Previous reports (Nigg, Heckly, and Colling, 1955; Heckly and Nigg, 1958) described some studies on the purification and characterization of toxins from culture filtrates of *Pseudomonas pseudomallei*. The heat-labile lethal toxin was closely associated with the necrotoxin, but the ratio of necrotoxicity to lethality was not constant in all preparations, especially those fractionated by use of Duolite S-30 (Chemical Process Co., Redwood City, Calif.) columns. We also described (Heckly and Nigg, 1958) the presence of a proteolytic enzyme, and no fractionation procedure used since that time has effected a separation of enzyme and toxicity. Every fraction having toxicity also exhibited proteolytic activity, and all enzymatically active fractions were toxic. Since the ratio of enzymatic activity to lethal toxicity was not constant in all preparations, it appeared that toxicity was not dependent upon the proteolytic action of the enzyme. The enzyme and lethal toxin either had very similar physical and chemical properties, or the activities were associated with different portions of the same molecule.

This report describes the results of experiments using heat and various chemicals for differential inactivation and the use of serological techniques to demonstrate that all of the biological properties are not associated with a single molecular species.

MATERIALS AND METHODS

Preparation of materials. We prepared culture filtrates by growing strain 111-14 of *P. pseudomallei* statically at 32 C for 7 days in glycerin heart infusion broth (Colling, Nigg, and Heckly, 1958). A relatively crude preparation, 420-B, was obtained by precipitation with ethanol from crude culture filtrates, by use of a countercurrent dialysis system (Heckly, 1959). A more highly purified preparation was obtained by removing

the bulk of the biologically inactive substances by column chromatography, using, first, diethylaminoethanol (DEAE), and then, carboxymethyl cellulose, as described by Heckly and Klumpp (1961).

Assays. Lethality of toxin, expressed as LD_{50} per ml, was assayed in Namru mice, as described by Colling et al. (1958).

Enzyme assays were based on the increased solubility of casein in trichloroacetic acid after digestion with a proteolytic enzyme. A 1-ml amount of an appropriate dilution of the enzyme solution was mixed with 1 ml of a 2% casein solution, both dissolved in 0.05 M phosphate buffer at pH 7.5. After 2 hr at 35 C, 3 ml of 5% trichloroacetic acid solution were added, mixed well, and allowed to stand 1 hr before centrifugation. The amount of casein digested was determined by measuring the optical density of the supernatant solution in 10-mm rectangular cells with a Beckman model DU or DK-1 spectrophotometer at 280 mμ.

One unit of enzyme was defined as that amount of enzyme which, under standard conditions, would digest casein at a rate sufficient to increase the optical density of the trichloroacetic acid-soluble portion by 1 unit per hr.

As a simple, although relatively inaccurate, measure of clotting inhibition titer (units per milliliter), we used the reciprocal of the highest dilution of a preparation which prevented coagulation of fresh rabbit blood.

Necrotoxin was assayed in normal adult albino guinea pigs by intradermal inoculation of 0.1 ml of twofold dilutions. One unit of necrotoxin was defined as the minimal amount which elicited a necrotic lesion when the animals were examined 24 hr after injection.

Ultraviolet-absorption spectra (240 to 300 mμ) were obtained with a Beckman DK-1 spectrophotometer using standard 10-mm cells. Spectra of antigen-antibody precipitates were obtained in 0.25 M acetic acid.

Antisera. Rabbits were inoculated subcutaneously with 5 to 20 mouse LD_{50} at weekly intervals for 10 to 20 weeks and bled 7 to 10 days after the last inoculation.

Precipitin test. The procedures used were based on the techniques of Ouchterlony (1949). A mold described by Miller and Heckly (1959) was used to form the wells in plates with a 1% agar containing 0.064 M phosphate buffer at pH 7.2, 0.85% sodium chloride, and 0.01% Merthiolate. After filling the wells, which had a capacity of about 0.2 ml each, the petri dishes were sealed with large rubber bands and held between 18 and 20 C. Photographs were obtained by dark-field illumination.

For specific precipitation, 9 ml of antiserum were usually mixed with 1 ml of preparation 420-B, incubated for 4 hr at room temperature, and held overnight at 2 C. The precipitate was removed by centrifugation at 2 C, washed, and resuspended in 0.85% saline solution for toxicity tests.

RESULTS

As was shown previously (Heckly and Nigg, 1958), toxin was rapidly inactivated at 100 C, but at 51 to 53 C it was possible to measure the rate of inactivation. The effect of heating on lethal toxin, enzyme, and anticoagulant, in a partially purified preparation at pH 7.5, is shown in Fig. 1. The differences in rates of inactivation shown in this figure probably are not significant. Not only at pH 7.5, but also at pH 6 and 8, lethal toxicity and enzymatic activity were reduced at essentially the same rate. Therefore, the enzyme, toxin, and clotting inhibitor have similar heat stability, and thus they cannot be separated on this basis.

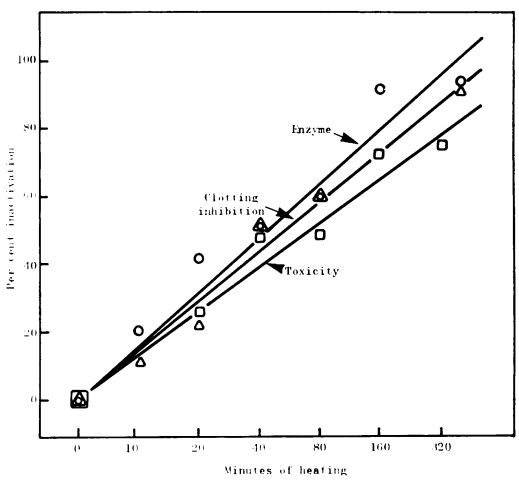

FIG. 1. *Effect of heating in a water bath at 51 to 53 C on enzymatic activity, clotting inhibition, and lethal toxicity of an ethanol-precipitated fraction (no. 415) of Pseudomonas pseudomallei filtrates. The solution was buffered at pH 7.5 by 0.03 M phosphate buffer, and each point represents the average of three separate determinations.*

TABLE 1. *Effect of denaturing agents on lethal toxicity and proteolytic activity of an ethanol-precipitated fraction (420-B) from Pseudomonas pseudomallei*

Reagent	Concn	Toxicity (LD_{50} per ml)	Enzyme (units per ml)
None (control)		45	350
Phenol*	2.5%	8 (18%)†	21 (6%)†
Urea*	6 M	22 (49%)	120 (34%)
Formaldehyde‡	0.5%	16 (35%)	120 (34%)

* After 48 hr at room temperature, the preparation was dialyzed vs. buffer before testing.

† Percentage of original activity.

‡ Tested after 4 hr at 35 C, without removing the formaldehyde.

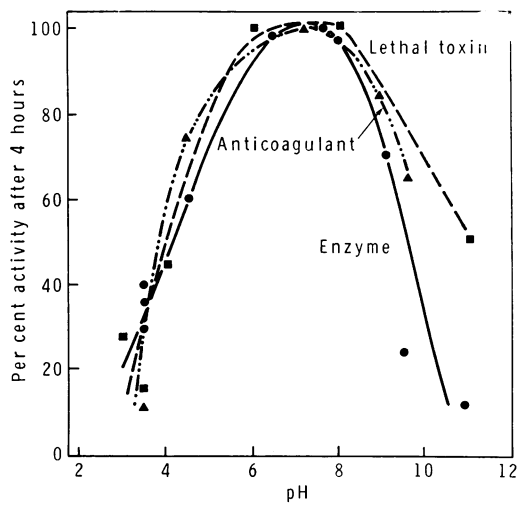

FIG. 2. *Effect of pH on lethal toxin, enzyme, and anticoagulant activity of an ethanol-precipitated preparation (420 B). After 4 hr at room temperature and at the indicated pH, the solution was neutralized and assayed.*

Similarly, denaturing agents such as phenol, formaldehyde, and urea affected both enzymatic activity and lethal toxicity to about the same extent (Table 1). Only phenol altered the ratio of lethal toxicity to enzymatic activity significantly, but, unfortunately, only a small percentage of either original activity remained.

The relative stability at room temperature of toxin and enzymatic activity between pH 3 and 11 is shown in Fig. 2. The three substances—anticoagulant, lethal toxin, and enzyme—appeared to be equally sensitive to inactivation in acid solutions. However, in alkaline solutions the toxin seemed to be appreciably more stable than the enzyme.

Figure 3 more clearly shows the difference in rate of inactivation at pH 11. Following this observation, we determined that, if conditions were carefully controlled, most of the enzyme would be inactivated with a minimal effect on lethality. As shown in Fig. 4, enzymatic activity was reduced by about 90% with only a slight

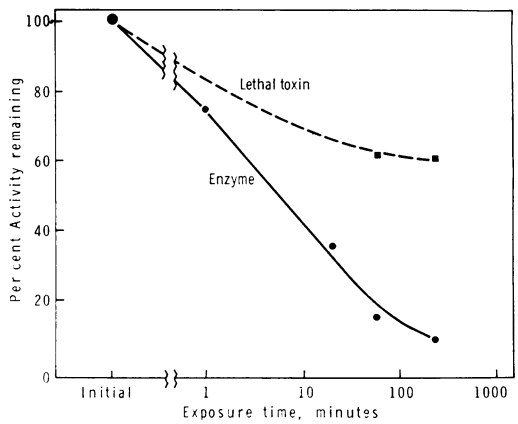

FIG. 3. *Alkaline inactivation of enzyme and lethal toxin at room temperature. An ethanol-precipitated fraction (420-B) from Pseudomonas pseudomallei filtrates was adjusted to pH 11 with 0.1 N NaOH. At intervals, samples were neutralized by mixing with an equal volume of pH 6.5, 0.1 M phosphate buffer.*

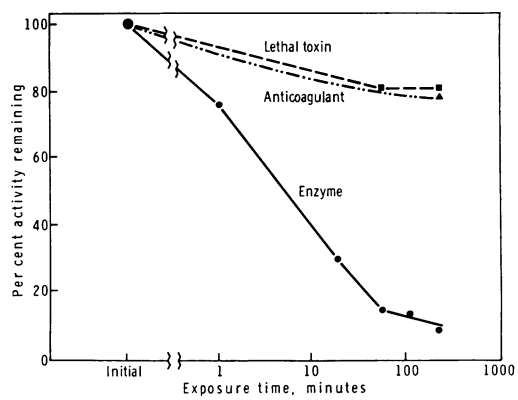

FIG. 4. *Effect of ammonium hydroxide on enzymatic, anticoagulant, and lethal toxic activities at room temperature. Five ml of 0.5 M ammonium hydroxide were added to 10 ml of an ethanol-precipitated fraction (420-B) from Pseudomonas peeudomallei. The resulting pH was 10.8, and, at intervals, samples were neutralized before testing by mixing with an equal volume of pH 6.5, 0.1 M phosphate buffer.*

reduction in lethality when the pH was adjusted with ammonium hydroxide, rather than with sodium hydroxide.

Because of the differences in stability of enzyme and lethal toxin in alkaline solutions, and because of the slight difference in stability at pH 3.5, indicated in Fig. 2, we believed that it might be possible to inactivate the toxin and retain enzyme activity. In several of the experiments at pH 2 and 3, lethal toxicity and enzymatic activity were reduced at the same rate; at pH 3.5, particularly if the acid was added slowly, the toxin and anticoagulant were inactivated more rapidly than was the enzyme (Fig. 5). Unfortunately, the differential inactivation in the acid solutions was not as great as that demonstrated with ammonium hydroxide. It did not seem to make much difference whether we used hydrochloric or acetic acid to reduce the pH. The experiments were repeated at 2 C as well as at room temperature, with various concentrations of acid, but in no instance were we able to achieve a much greater difference than that shown in Fig. 5. Various acid and ammonium hydroxide-treated preparations were retested, and, as shown in Table 2, the enzyme activity seemed to be correlated with necrotoxicity, and the clotting inhibition was correlated with lethality.

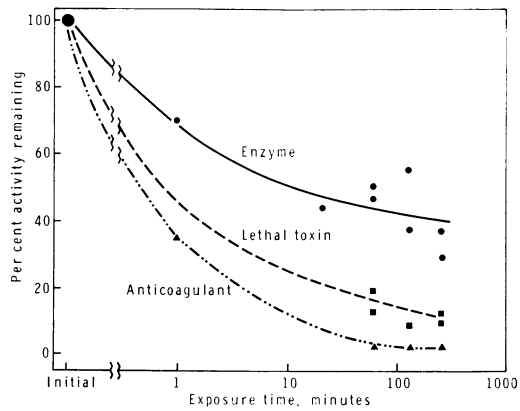

FIG. 5. *Acid inactivation of enzyme, anticoagulant, and lethal toxin at room temperature. An ethanol-precipitated fraction (420-B) from Pseudomonas pseudomallei was adjusted to pH 3.5 with 0.1 M HCl. At intervals, samples were neutralized before testing by mixing with an equal volume of pH 8.3, 0.1 M phosphate buffer.*

The effect of acid and alkali on the two major antigens in the relatively crude preparation 420-B is shown in Fig. 6. Apparently acidification altered the reactivity of one of the two antigenic components of preparation 420-B, as well as the lethality, leaving only one principal precipitin line, which we tentatively identified as that produced by the proteolytic enzyme and its specific antibody. Conversely, ammonium hydroxide destroyed serological reactivity and the activity of the enzyme, leaving only one precipitin line, which was associated with the native lethal toxin.

Figure 7 shows that the predominant antibody produced in response to injections of preparation 566 (acid treated 420-B) was that which reacted with the native enzyme. On continued immuni-

TABLE 2. *Effect of acid and alkali on exotoxin and other activities of a partially purified preparation from Pseudomonas pseudomallei*

Preparation*	Lethal toxin (LD_{50} per ml)	Clotting inhibition (units per ml)	Enzyme (units per ml)	Necrotoxin (units per ml)
No. 420-B (control—no treatment)	47	160	400	640
No. 566 (HCl added to pH 3.5 at RT†, 4 hr)	4.2 (9%)‡	—	126 (31%)	—
No. 591 (0.02 M HCl, pH 2.7, at 2 C, 24 hr)	2 (4.2%)	2 (1.2%)	125 (31%)	160 (25%)
No. 633 (0.3 M acetic acid, pH 3.5, at 2 C, 20 hr)	1.6 (3.4%)	5 (3.1%)	70 (19%)	80 (12%)
No. 565 (0.03 M NaOH, pH 11, at RT, 4 hr)	25 (53%)	—	80 (20%)	—
No. 571 (0.16 M NH_4OH, pH 10.8, at RT, 4 hr)	34 (72%)	—	46 (12%)	—
No. 619 (0.2 M NH_4OH at 2 C 24, hr)	25 (53%)	100 (63%)	140 (35%)	160 (25%)

* Solutions were neutralized prior to testing. Activity values were corrected for dilution to make results comparable with the untreated control.

† Room temperature.

‡ Percentage of original activity.

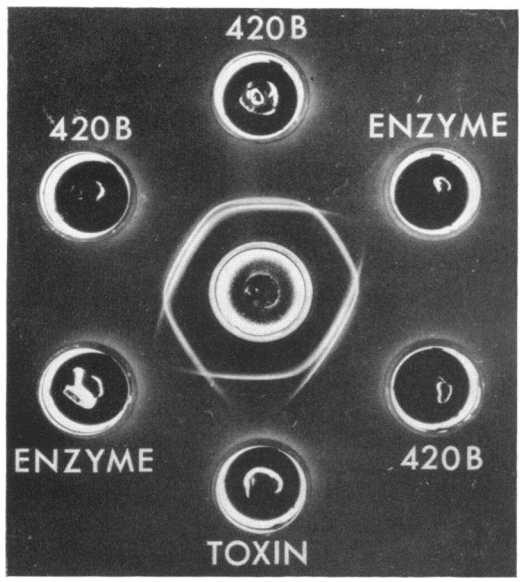

FIG. 6. *Agar diffusion plate 48 hr after introducing the reagents. The center well contained antiserum 199, which was obtained 10 days after the last of 20 weekly inoculations of 1 ml each of crude culture filtrate. Preparation 420-B, an ethanol-precipitated fraction, had both lethal and enzymatic activity. The well labeled "toxin" received preparation 565, which was obtained by alkaline treatment of preparation 420-B. The well labeled "enzyme" received preparation 566, which was obtained by acid treatment of preparation 420-B. The conditions of inactivation are given in Table 2.*

zation, some antibody was produced, which reacted to produce a faint precipitin line vs. the toxin. This is not surprising, since in no instance did we achieve 100% inactivation of toxin.

A preliminary test, in which 1 ml of 420-B (400 units of enzyme per ml) was added to 9 ml of antiserum no. 26, indicated that all of the enzyme activity was not removed in the precipitate. Therefore, enzyme and antienzyme serum were mixed in various proportions to determine the equivalence point. Even at very low enzyme-to-serum ratios, only about 70% of the enzyme was precipitated (Fig. 8). It appears as though the enzyme preparation contained a mixture of enzymes, and that antibody was effective in blocking the activity of only one of these. However, as shown in Table 3, practically all of the enzyme was removed by serum no. 41. Table 3 summarizes the results of additional studies in which lethal toxicity of the precipitates, as well as of the supernatant solutions, from the antigen-antibody mixtures were tested. From these results, it is apparent that, although sera prepared by injection of alkali-treated 420-B preparation were weak, they were indeed antitoxins and precipitated the lethal toxin. The antitoxins affected the enzymatic activity, but the antienzyme had a greater effect on the enzyme. The precipitates obtained from the antienzyme mixtures were not tested for enzyme activity, because dissociation techniques would have inactivated the enzyme and rendered results quite meaningless. The suspension of precipitate obtained with antitoxin was lethal, whereas no detectable toxicity was demonstrated with a comparable amount of precipitate obtained with antienzyme (Table 3).

DISCUSSION

Electrophoresis or analytical ultracentrifugation techniques are relatively insensitive measures of homogeneity. If two components are demonstrated by either of these methods, there is no doubt about the heterogeneity. However, obtaining a single peak, indicative of a single component, by either of these techniques, does

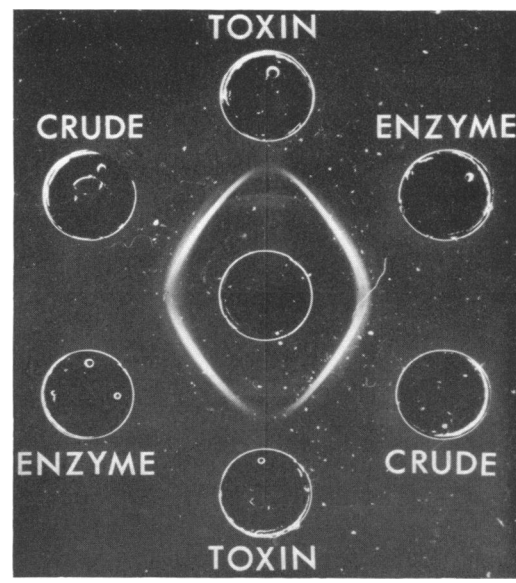

FIG. 7. *Agar diffusion plate 48 hr after introducing the reagents. The center well contained antienzyme (serum no. 26), which was obtained 10 days after the last of 10 weekly injections of 0.5 ml of an acid-treated preparation (#566). See legend of Fig. 5 for description of antigens.*

not prove that the material is pure. The fact that none of various fractionation techniques effected a separation of enzymatic activity and toxicity would indicate that the physical properties of these two must be similar in some respects. Although the effects of various inactivating procedures were comparable, it was possible to inactivate differentially the enzyme or the lethal toxin. Such differential inactivation shows that lethality was not dependent upon enzymatic activity, but it does not indicate whether there were two distinct entities or whether a single substance exhibited these two activities. On the basis of the serological tests, however, it would appear that lethal toxicity and enzymatic activity are parts of two different species of molecules.

The correlation of anticoagulant activity with lethality, rather than with proteolytic activity, is of particular interest because coagulation would be inhibited if any of the protein factors in normal blood coagulation system were altered by proteolysis. However, since it was shown that the anticoagulant activity was associated with lethal toxicity, some mode of action other than simple proteolysis will have to be considered. Some of the early studies on fractionation (Heckly and Nigg, 1958) indicated that it might be possible to separate the lethal toxin from the necrotoxin in crude culture filtrates, but because of the loss of activity and because it failed to effect an appreciable purification, this fractionation technique was abandoned. In view of the results presented in this report, it appears that the lack of necrotoxin in the first eluates from the Duolite S-30 was possibly due to inactiva-

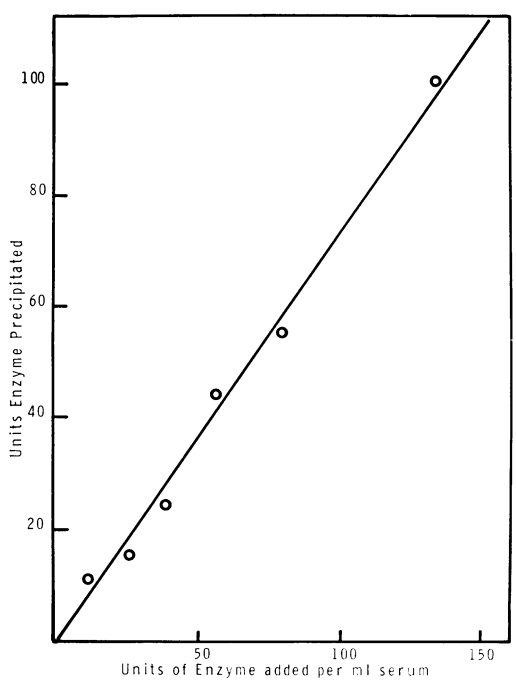

FIG. 8. *Precipitation of enzyme by specific antiserum. An ethanol-precipitated fraction (420-B) of Pseudomonas pseudomallei was mixed, in various proportions, with anti-enzyme, serum no. 26, obtained by injecting rabbits with acid-treated 420-B.*

TABLE 3. *Precipitation of Pseudomonas pseudomallei exotoxin and enzyme by specific antisera*

Mixture[a]	Enzyme (units per ml)	Lethal toxin (LD_{60} per ml)	Optical density at 280 mμ
Fraction 420-B[b] and normal serum	39	2.8	—
Fraction 420-B[b] and antitoxin no. 25[c]	15	<0.8	—
Fraction 462[b] and 0.14 M NaCl	80	5.0	—
Fraction 462[b] and antitoxin No. 40[d]	71	4.0	—
Fraction 462 and anti-enzyme No. 41[e]	3	5.0	—
Precipitate of 462 and antitoxin 40	—	1.0	1.9
Precipitate of 462 and anti-enzyme 41	—	<0.8	1.6

[a] A 1-ml amount of the fraction was added to 9 ml of serum or saline, incubated for 4 hr at room temperature, and held 16 hr at 2 C. The precipitate was removed by centrifugation at 2 C, washed, and resuspended in 10 ml of 0.14 M NaCl.

[b] Fraction 420-B is a relatively crude ethanol-precipitated preparation, and fraction 462 is the active fraction obtained from 420-B by chromatography by use of substituted celluloses.

[c] Antitoxin No. 25 was obtained from a rabbit after 10 weekly injections of preparation 565 (NaOH treated) (Table 2).

[d] Antitoxin No. 40 was obtained from a rabbit after 6 weekly injections of 0.4 ml of preparation 619 (NH_4OH treated) (Table 2).

[e] Anti-enzyme No. 41 was obtained by injecting a rabbit with 0.4 ml at weekly intervals of preparation 566 (Table 2).

tion, such as is obtained by treatment with ammonium hydroxide, and differential adsorption may have contributed little toward the fractionation that was reported.

Now that it has been determined that there are two distinct substances, efforts will be renewed to develop a procedure for isolating each of the components with minimal inactivation. The use of the precipitin test will be of considerable value in identifying the components on fractionation.

Perhaps the antisera, specific for the enzyme and the lethal toxin, may be useful in identification of pseudomonads as described by Liu (1961), particularly if either one or the other plays an essential role in the pathogenesis of the disease caused by *P. pseudomallei*.

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