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BIOLOGICAL AND IMMUNOCHEMICAL PROPERTIES OF CULTURE FILTRATES OF VIRULENT AND AVIRULENT STRAINS OF *PSEUDOMONAS AERUGINOSA*

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ON the basis of heat stable antigens strains of *Ps. aeruginosa* may be grouped into at least 13 main serotypes (Habs, 1957; Verder and Evans, 1961; Wahba, 1965; Muraschi, Bolles, Moczalski and Lindsay, 1966; Lanyi, 1967). In 1966 Jones and Lowbury, using single strains from each of the 13 serotypes, showed that not all the strains killed burned mice when they were inoculated on to the surfaces of burned mice in standardised experiments. It has also been shown that some strains of *Ps. aeruginosa* produce extracellular substances capable of causing local or systemic lesions (Liu, Abe and Bates, 1961; Mull and Callahan, 1965; Johnson, Morris and Berk, 1967) but Liu (1966) found that not all strains when grown in similar cultural conditions, produce the same range or proportions of extracellular substances. This suggests that the variation in the ability of the 13 serotypes of *Ps. aeruginosa*, used by Jones and Lowbury (1966), to kill burned mice might be attributable to differences in production of extracellular substances. To test this hypothesis we have examined culture filtrates of strains of *Ps. aeruginosa* previously shown either to cause septicaemia and death (virulent) or not to cause septicaemia and death (avirulent).

Three strains of *Ps. aeruginosa*—2 virulent and 1 avirulent for burned mice—were grown in controlled environmental conditions in a synthetic medium described by Liu (1964). Culture filtrates of these organisms were separated by gel filtration into fractions of different molecular size. The fractions were tested for toxic effects in mice and those fractions that were found to be toxic were examined for enzyme activity. The enzyme activity of unfractionated culture filtrates was also assessed. The fractionation of culture filtrates was monitored by immunodiffusion techniques.

MATERIALS AND METHODS

Strains of Ps. aeruginosa

Two strains of *Ps. aeruginosa* were obtained from the National Collection of Type Cultures: (1) Wahba's type 14 (serotype 6c), phage typing pattern 16/31/24/F8/119X/352/1214/4 referred to as P14; (2) Véron's type P2AB (serotype 5C), phage typing pattern 44/F8/109/1214 referred to as P2AB. Our third strain, B4, was isolated from a patient with burns and reacts with pooled serum from serotypes 2 and 5 but not with serum from either serotype 2 or serotype 5 (Thom—personal communication) and has the following phage typing pattern: 21/68/Col.21/+.

Strains P14 and B4 were highly virulent for burned mice and killed over 70 per cent of the mice in controlled experiments; strain P2AB killed no mice in the same series of controlled experiments (Jones and Lowbury, 1966).

Preparation of culture filtrates from Ps. aeruginosa

Strains of *Ps. aeruginosa* were grown in a synthetic liquid medium (Liu, 1964). Since all the medium constituents were dialysable, the separation of non-dialysable bacterial products from unused medium was achieved simply by dialysis.

The medium used was: Glucose 1·2 per cent; Alanine, aspartic acid, glutamic acid, sodium chloride 0·3 per cent; Calcium chloride and magnesium sulphate 0·01 per cent; Potassium dihydrogen phosphate 0·15 per cent. The constituents were dissolved in distilled water and adjusted to pH 7·5 with tris buffer. The medium was then sterilised by positive pressure filtration through a GS Millipore membrane.

A loopful of an overnight agar culture of *Ps. aeruginosa* was inoculated into the medium and cultured for 5 days at 37° in a 20 l. stainless steel container, which was aerated with air drawn through a GS Millipore membrane.

In preliminary experiments, 5 l. of 5-day cultures were centrifuged for 30 min. at 3000 rpm and the supernatant was filtered by positive pressure filtration through a RA Millipore membrane to remove bacteria. The filtrates were concentrated by ultrafiltration through cellophane tubing to 50 ml., dialysed for 24 hr. against running tap water, and lyophilised.

In later experiments 20 l. of 5-day cultures were sterilised by addition of sodium azide to give a final concentration of 0·05 per cent and the volume of the culture reduced to 1 l. in a circulatory cyclone evaporator at 25°. The concentrate was centrifuged for 30 min. at 3000 r.p.m. and filtered by positive pressure filtration through a RA Millipore membrane to remove bacteria. The filtrate was dialysed for 24 hr. against running tap water to remove unused medium constituents and sodium azide, ultrafiltered through cellophane to reduce volume to 250 ml., and lyophilised.

Fractionation of culture filtrates of Ps. aeruginosa

The lyophilised culture filtrates were separated into fractions of different molecular size by gel filtration through (1) G-200 Sephadex which excludes molecules with molecular weights ($\bar{M}w$) > 200,000; (2) Sepharose 4B which excludes molecules with $\bar{M}w$ between 1–5 million; and (3) Sepharose 2B which excludes molecules with $\bar{M}w$ between 5–50 million (Pharmacia).

UV absorption of the fractions from the chromatographic columns was measured at 280 mμ or 255 mμ in a Unicam S.P.500 spectrophotometer or automatically in a " Uvicord " (LKB) at 254 mμ.

G-200 Sephadex.—Lyophilised culture filtrates (500 mg. or 1 g.) from 3 strains of *Ps. aeruginosa*, P14, B4 and P2AB, were dissolved in 20 ml. isotonic phosphate saline, pH 7·4, containing 0·05 per cent sodium azide. After centrifuging, insoluble material was discarded and the clear supernatant was applied to a column of G-200 Sephadex, 46 cm. high with an internal diameter of 4·2 cm. Fractions (15 ml.) were eluted from the column with phosphate saline using a BTL automatic fraction collector. Pooled fractions were concentrated by ultrafiltration through cellophane, sodium azide and buffer salts were then removed by dialysis in distilled water at 4° and the remaining solution lyophilised.

Sepharose 4B.—A portion (50 or 100 mg.) of the first fraction (F1) obtained from G-200 Sephadex was dissolved in 4 ml. saline and applied to a column of Sepharose 4B, 43 cm. high and 2·5 cm. in diameter. Fractions (5 ml.) were eluted from the column with phosphate saline containing 0·05 per cent sodium azide.

Sepharose 2B.—Portions (100 mg.) of " X " fractions (molecules excluded from Sepharose 4B) from *Ps. aeruginosa*, P14, B4 and P2AB, were dissolved in 4 ml. isotonic phosphate saline and applied to a column of Sepharose 2B with internal diameter 2·2 cm. and 56·5 cm. in height. 5 ml. fractions were collected using similar techniques to those described above.

Kav values.—of the eluted fractions were calculated. Kav is the fraction of the gel phase of a chromatography column available for the molecule. It is derived from a formula by Laurent and Killander (1964).

$$\text{Kav} = \frac{\text{Ve} - \text{Vo}}{\text{Vt} - \text{Vo}}$$

Ve = elution volume of solute; Vo = volume of liquid phase (void volume of column); Vt = total volume of gel bed (Vo + Vx).

A molecule completely excluded from the gel phase, on account of its size, will be eluted immediately after the void volume, *i.e.*, Ve = Vo and will therefore have a Kav value of zero. A molecule that completely occupies the whole of the available fraction of the gel phase will have its elution volume (Ve) equal to the total bed volume Vt and have a Kav value of 1·0. From the Kav values of eluted fractions, estimations of molecule size and molecular weights may be made (Pharmacia 1966).

Toxicity tests

Before inoculation, each test solution was filtered through a syringe fitted Swinney adapter containing a GS Millipore membrane to ensure sterility. Saline (1 ml.), containing the appropriate amount of fraction, was inoculated i.p. into male albino mice (Schofield) weighing 20 g. Mice were observed and maintained in transparent plastic cages (Jones, 1964), weighed and examined daily and given food and water *ad lib.*

Burning

Burns were made on the mice by the technique described by Jones and Lawrence (1964). Brass blocks heated in boiling water were applied for 10 sec. to the dorsal surfaces of anaesthetised mice which had been depilated by plucking 4 days previously.

An autopsy was performed on all mice that died; samples of heart blood were taken after searing the surface of the heart with a hot iron; these blood samples were cultured on blood agar and on cetrimide agar (Brown and Lowbury, 1965).

Immunodiffusion techniques

Three ml. of 1 per cent Oxoid Ionagar No. 2 in veronal buffer pH 8·6 containing 0·05 per cent sodium azide was poured on to a 2 in. × 2 in. clear glass slide and placed in a petri dish having a moistened filter paper in the lid to maintain humidity. For double diffusion experiments peripheral antigen holes were cut with a No. 2 cork borer and the central serum hole with a No. 7 cork borer. The holes were arranged so that the minimum distance between the inner edge of the peripheral hole and outer edge of the serum hole was 0·5 cm.

Antigens were made up to a 1 per cent concentration in veronal buffer. After addition of antigens and antiserum, the immunodiffusion plates were kept horizontal at room temperature and examined on 3 consecutive days for appearance of precipitation bands. Photographs of precipitation lines were taken with an Exacta Varex IIb camera using Ilford FP_3 film at $\frac{1}{60}$ or $\frac{1}{30}$ sec. at f.2·8 or f.4·0 on a dark ground illuminator (Rudge, 1960).

Preparation of antiserum

Antiserum, used for immunodiffusion, was prepared in male New Zealand white rabbits weighing 4·5 kg. at the beginning of the immunisation schedule. Portions of the lyophilised fraction (0·5 mg. in 1 ml. saline) were inoculated i.v. once a week for 3 consecutive weeks. Ten days after the last inoculation, 10 ml. of blood was obtained by syringe, the serum removed and used for preliminary experiments.

When larger volumes of serum were required in later experiments the rabbits were given a booster dose (0·5 mg. in 1 ml. saline) an exsanguinated 5 days later by cannulation of the femoral artery. Serum was separated and filtered through a GS Millipore membrane and stored without preservatives at −21°.

Enzyme activity of culture filtrates of Ps. aeruginosa

Sephadex fractions.—G-200 Sephadex fractions F1 and F3 from P14, B4 and P2AB were made up to 1 mg./ml. with phosphate saline. To 0·1 ml. of doubling dilutions of the fractions, 0·1 ml. of substrate was added in the manner described below.

Broth cultures.—Amounts of 0·1 ml. of a saline suspension containing 7×10^8 organisms per ml. of an 18 hr. agar culture of 3 strains of *Ps. aeruginosa* (P14, B4 and P2AB) was inoculated into 3 rows of 7 × 4 in. × ½ in. tubes respectively containing 4 ml. of synthetic medium (Liu, 1964) which had been sterilised by positive pressure filtration through a GS Millipore membrane. Tubes were incubated at 37° in a water bath.

Daily, for 7 successive days, one tube of each of the strains was shaken at full power for 5–10 sec. on a BTL shaker, and 1 ml. of the suspension was removed and the number of organisms in 1 ml. of the culture was estimated using Brown's Opacity Tubes. The remaining 3 ml. was centrifuged for 15 min. at 3000 rpm and bacteria were removed from the supernatant by filtration through a GS Millipore membrane.

To 0·1 ml. of doubling dilutions of this filtrate 0·1 ml. of substrate was added. The following substrates were used: for estimation of lecithinase activity, a $\frac{1}{50}$ egg yolk suspension in saline; for haemolysin a $\frac{1}{100}$ saline suspension of thrice washed horse RBC; for protease, 1 mg./ml. azo-albumin (Sigma) in saline; and for elastase, 1 mg./0·1 ml. of elastin-orcein (Sigma) suspension in saline. Tubes were incubated at 37° for 2 hr. for haemolysin estimations and for 24 hr. for detection of lecithinase, protease and elastase.

The end-points of the protease and elastase assay methods were taken as the highest dilution which liberated a detectable amount of dye into solution. The end-point for haemolysin activity was the highest dilution that gave complete haemolysis, and the end-point for lecithinase assay was the highest dilution that gave a clear solution. A fat layer was not observed, probably due to the presence of lipase and esterase in the culture filtrates. Enzyme titres were expressed as reciprocals of highest dilutions giving complete reactions.

RESULTS

Gel filtration of culture filtrates of Ps. aeruginosa

G-200 Sephadex.—Fig. 1 shows elution curves, measured at a wavelength of 255 mμ, of fractions eluted from G-200 Sephadex of culture filtrates from 2 virulent strains of *Ps. aeruginosa* (P14 and B4) and 1 avirulent strain (P2AB). A similar distribution of UV absorbing materials was found in the eluted fractions from all 3 strains, although the proportions of UV absorbing materials in the absorption peaks varied from strain to strain. The first peak, included in fractions 10–17, was collected immediately after the void volume. These fractions were pooled and called F1. The fractions (36–46) forming the other large absorption peak were pooled and called F4. The fractions collected between F1 and F4 were grouped and called F2 and F3 as follows: F3 incorporated fractions (26–35) showing a small UV absorption peak, the remaining fractions (18–25) were called F2.

F1–F4 from the 3 strains were dialysed to remove buffer salts and lyophilised. Several fractionations of culture filtrates from P14, B4 and P2AB were carried out and mean Kav values for the fractions are shown in Table I. The similarity of F1, F2, F3 and F4 groupings for the 3 strains was indicated by the similarity of the

TABLE I.—*Mean Kav values for Fractions from G-200 Sephadex of 3 Strains of* Ps. aeruginosa (*P14, B4, P2AB*)

Strain	No. of runs	Fraction F1	F2	F3	F4
P14	9	.0–0·21	0·22–0·47	0·48–0·75	0·76–1·21
B4	2	.0–0·28	0·29–0·45	0·46–0·62	0·63–1·09
P2AB	17	.0–0·14	0·15–0·40	0·41–0·68	0·69–1·09

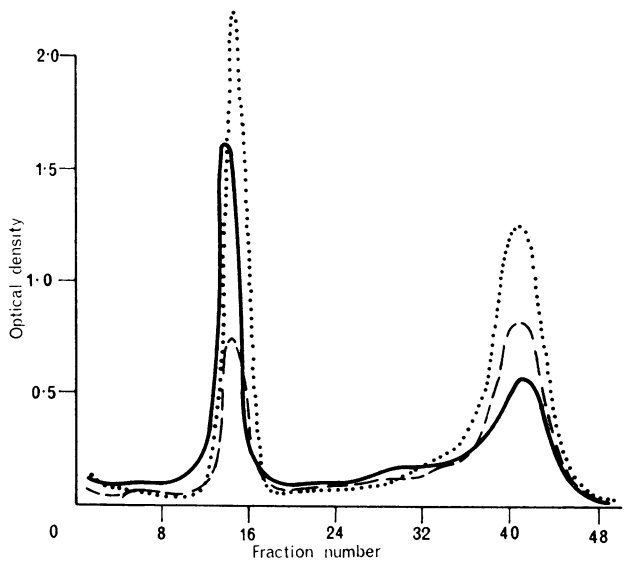


FIG. 1.—Elution curves of culture filtrates of *Ps. aeruginosa* after fractionation on G-200 Sephadex. (5 ml. fractions, $\lambda = 255$ mμ.)

——— = P. — — — = B4. ..... = P2AB

Kav values shown in Table I, and these values also show that substances of similar molecular size were included in F1–F4 fractions from the 3 strains.

TABLE II.—*Percentage Recoveries of F1 and F3 from* Ps. aeruginosa *P14, B4 and P2AB after Fractionation on G-200 Sephadex*

Strain of *Ps. aeruginosa* used for preparing fractions	Percentage of fractions recovered	
	F1	F3
P14	28·10	7·00
B4	12·75	4·90
P2AB	16·80	2·25

Table II shows the percentage recovery of F1 and F3 from P14, B4 and P2AB, expressed in terms of the amount of material added to the buffer. The amount added to the buffer was not the same as the amount applied to the column since as a result of freeze drying part of the unfractionated culture filtrates became insoluble, and only soluble material was added to the column. The proportions of F1/F3 from P14, B4 and P2AB were approximately 4/1, 2·6/1 and 7·5/1 respectively

Sepharose-4B.—Further separation of the molecules in F1 was attempted by fractionation of F1 preparations from P14, B4 and P2AB through Sepharose 4B. Fig. 2 shows elution curves measured at a wavelength of 280 mμ, of fractions eluted from a column of Sepharose-4B of F1 preparations from P14, B4 or P2AB. All 3 F1 fractions yielded a group of fractions (10–20) with similar UV absorbing properties. These fractions, collected immediately after the void volume, were called X. The remaining fractions from the 3 preparations were divided into 2

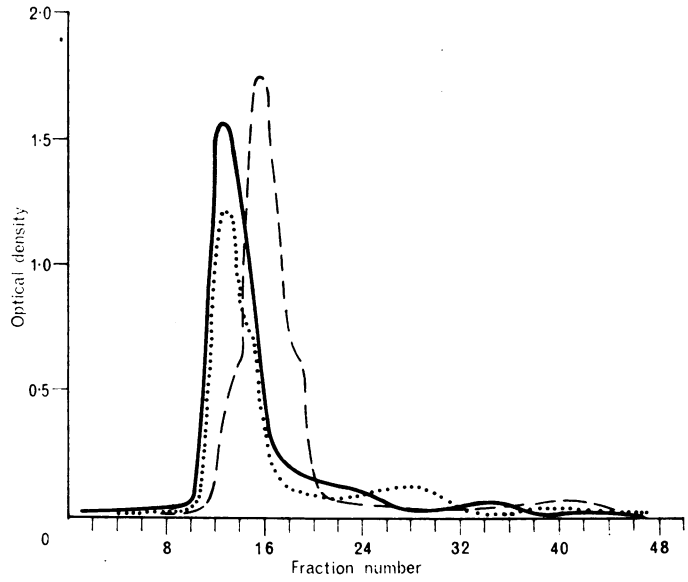


FIG. 2.—Elution curves of F1 fractions of *Ps. aeruginosa* (P14, B4 and P2AB) after fractionation on Sepharose-4B. (5 ml. fractions, $\lambda = 280$ mμ.)
— = P14. — — — = B_4. ..... = P2AB

further groups of fractions called Y and Z. " Y " incorporated fractions with slight UV absorbing properties, which followed X, and the fractions collected after Y until the column was cleared, were pooled and called Z. Fractions were freeze dried. Mean Kav values for the fractions from the 3 strains are shown in Table III.

TABLE III.—*Mean Kav Values for Fractions from Sepharose-4B of F1 Preparations of* 3 *Strains of* Ps. aeruginosa (*P14, B4, P2AB*)

Strain	No. of runs	Fraction X	Y	Z
P14 .	3	.0–0·21	0·22–0·61	0·62–1·07
B4 .	8	.0–0·27	0·28–0·67	0·68–1·11
P2AB.	8	.0–0·34	0·35–0·81	0·82–1·20

Sepharose-2B.—Fig. 3 shows elution curves for X fractions from P14, B4 and P2AB eluted from Sepharose-2B. The division of the eluted fractions into 3 pools of fractions a, b and c was decided upon, as before, by the presence of UV absorbing material in the eluted fractions. Fraction " a " incorporated fractions 13–18, fraction " b " incorporated fractions 22–37, and " c " fractions 38–46. The Kav values for a, b and c fractions are shown in Table IV and indicate that molecules of similar molecular size were present in a, b and c fractions.

Immunodiffusion of culture filtrates of Ps. aeruginosa

The degree of separation of culture filtrates of *Ps. aeruginosa* P14, B4 and P2AB into several fractions by gel filtration through G-200 Sephadex, Sepharose-4B and

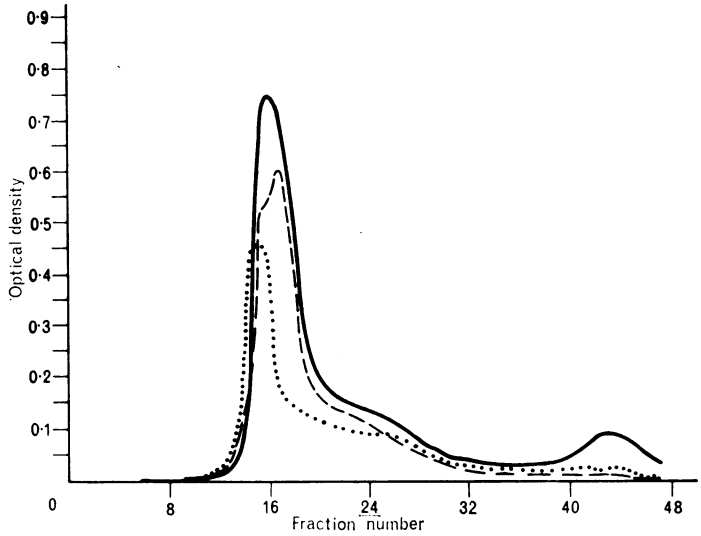


FIG. 3.—Elution curves of X fractions of *Ps. aeruginosa* (P14, B4 and P2AB) after fractionation on Sepharose-2B. (5 ml. fractions, $\lambda = 254$ mμ.
——— = P14. — — — = B4. . . . . = P2AB

TABLE IV.—*Mean Kav Values for Fractions from Sepharose-2B of X Fractions of* 3 *Strains of* Ps. aeruginosa (*P14, B4, P2AB*)

Strain	No. of runs	Fraction a	b	c
P14 .	1	.0–0·14	0·28–0·79	0·80–1·14
B4 .	1	.0–0·14	0·28–0·79	0·80–1·14
P2AB.	1	.0–0·14	0·28–0·79	0·80–1·14

Sepharose-2B, was assessed by immunodiffusion techniques. Each fraction was examined for serological homogeneity and compared with similar fractions from the other 2 strains.

F1 preparations and their corresponding subfractions (XYZ from Sepharose-4B and a, b, c from Sepharose-2B) were diffused against an antiserum prepared against P14.F1.

F3 preparations were diffused against an antiserum to P14.F3.

B4.F1 and P2AB.F1 each developed at least 2 precipitation bands against P14.F1 antiserum (Fig. 4) indicating that B4 and P2AB.F1 preparations have antigenic determinants in common with P14.F1. The dense band of precipitation formed by P14.F1 against its homologous serum demonstrates the presence of several antigenic determinants in P14.F1. The precipitation patterns of fractions from Sepharose-4B and 2B are shown in Figs. 5–8. Common antigens were found in X fractions from the 3 strains (Fig. 5) and similarly common precipitating antigens were found in the Y and Z fractions respectively. The precipitating factors from 1 fraction were not found in either of the other 2 fractions, indicating that grouping of the Sepharose-4B fractions into X, Y and Z fractions had provided a favourable separation of the antigenic determinants. The precipitation patterns

of fractions, resulting from gel filtration of fraction X from P14, B4 and P2AB, through Sepharose-2B are shown in Figs. 6, 7 and 8. The " a " fractions, from the 3 strains, each contained 2 common antigenic factors and the " c " fractions contained a single common antigenic factor. The precipitation patterns of the " b " fractions from the 3 strains (Fig. 7) illustrate a difference between the 2 virulent (P14 and B4) and avirulent (P2AB) strains of *Ps. aeruginosa* because both P14 and B4 " b " fractions contained a common antigenic factor, which was not found in P2AB " b " fraction. Fig. 6 illustrates the presence of many common antigenic determinants in F3 preparations from P14, B4 and P2AB.

Electron micrographs

Figs. 10 and 11 are electron micrographs of fraction " X " from *Ps. aeruginosa*, P2AB. The area of negative staining with phosphotungstic acid shows white, particles 100–200 Å diameter show dark.

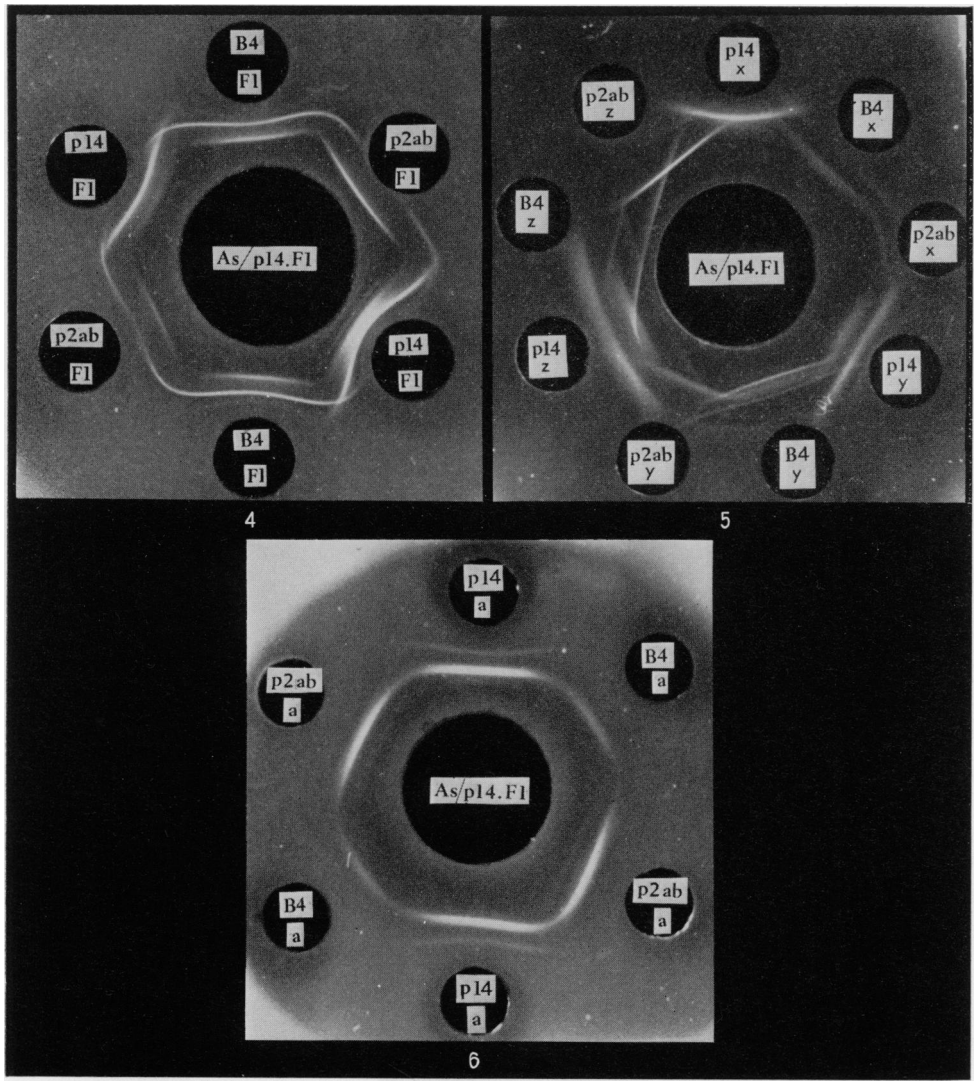
Toxicity of Sephadex separated fractions from virulent and avirulent strains of Ps. aeruginosa

Lethal effects.—The 4 preparations (F1, F2, F3 and F4) from 3 strains of *Ps. aeruginosa* (P14, B4 and P2AB) which were separated from culture filtrates by gel filtration through G-200 Sephadex, were inoculated intraperitoneally into burned and unburned mice to see if they caused any toxic effects in the mice. F2 and F4 preparations from all 3 strains failed to kill mice at the highest dosage tested—40 mg./kg. F1 and F3 preparations from the 3 strains were inoculated into groups of 10 mice at the following dosages: 40, 20, 10, 5 or 1 mg./kg. mouse. A similar range of dosages was inoculated into groups of 10 mice, 3 hr. after the mice were given a small burn.

Table V summarises the LD_{50} (calculated by Reed, Muench method (1938)) of F1 from P14, B4 and P2AB for unburned and burned mice. In unburned mice (Table V) the F1 preparations from the 2 virulent strains of *Ps. aeruginosa* (P14 and B4) were more lethal than the F1 preparation from the avirulent strain (P2AB), having respectively LD_{50} of 29 and 33·7 mg./kg. mouse for P14 and B4 compared with an LD_{50} of > 40 mg./kg. for P2AB. F1 preparations (Table V) killed approximately twice as many burned mice as they did unburned mice and the F1 preparation from the avirulent strain P2AB was found to be marginally

EXPLANATION OF PLATES

FIG. 4.—Precipitation patterns of F1 fractions from *Ps. aeruginosa*, P14, B4 and P2AB.
FIG. 5.—Precipitation patterns of X, Y and Z fractions from *Ps. aeruginosa* P14, B4 and P2AB.
FIG. 6.—Comparative precipitation pattern of " a " fractions from *Ps. aeruginosa* P14, B4 and P2AB.
FIG. 7.—Comparative precipitation pattern of " b " fractions from *Ps. aeruginosa* P14, B4 and P2AB.
FIG. 8.—Comparative precipitation pattern of " c " fractions from *Ps. aeruginosa* P14, B4 and P2AB.
FIG. 9.—Precipitation patterns of F3 fractions from *Ps. aeruginosa* P14, B4 and P2AB.
FIG. 10.—Electron micrograph of X fraction from *Ps. aeruginosa* P2AB. ×85,350. (Gradation mark = 0·25 μ).
FIG. 11.—Electron micrograph of X fraction from *Ps. aeruginosa* P2AB. ×117,350. (Gradation mark = 0·25 μ).


B4
F1
p14
F1
p2ab
F1
As/p14.F1
p2ab
F1
p14
F1
B4
F1
4
p14
x
p2ab
z
B4
x
B4
z
p2ab
x
As/p14.F1
p14
z
p14
y
p2ab
y
B4
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B4
a
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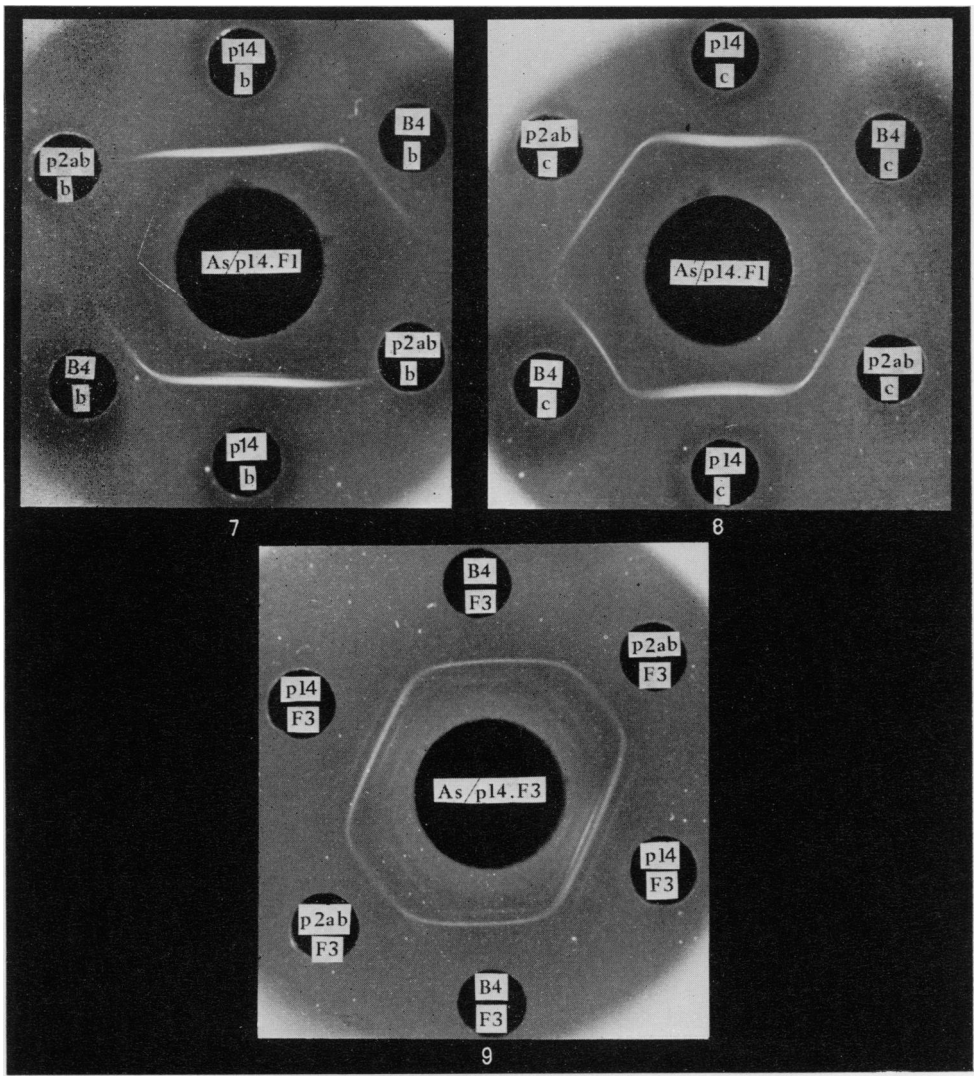
a
p14
a
6


p14 b
B4 b
p2ab b
As/p14.F1
p2ab b
B4 b
p14 b
7
p14 c
p2ab c
B4 c
As/p14.F1
B4 c
p2ab c
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p14 F3
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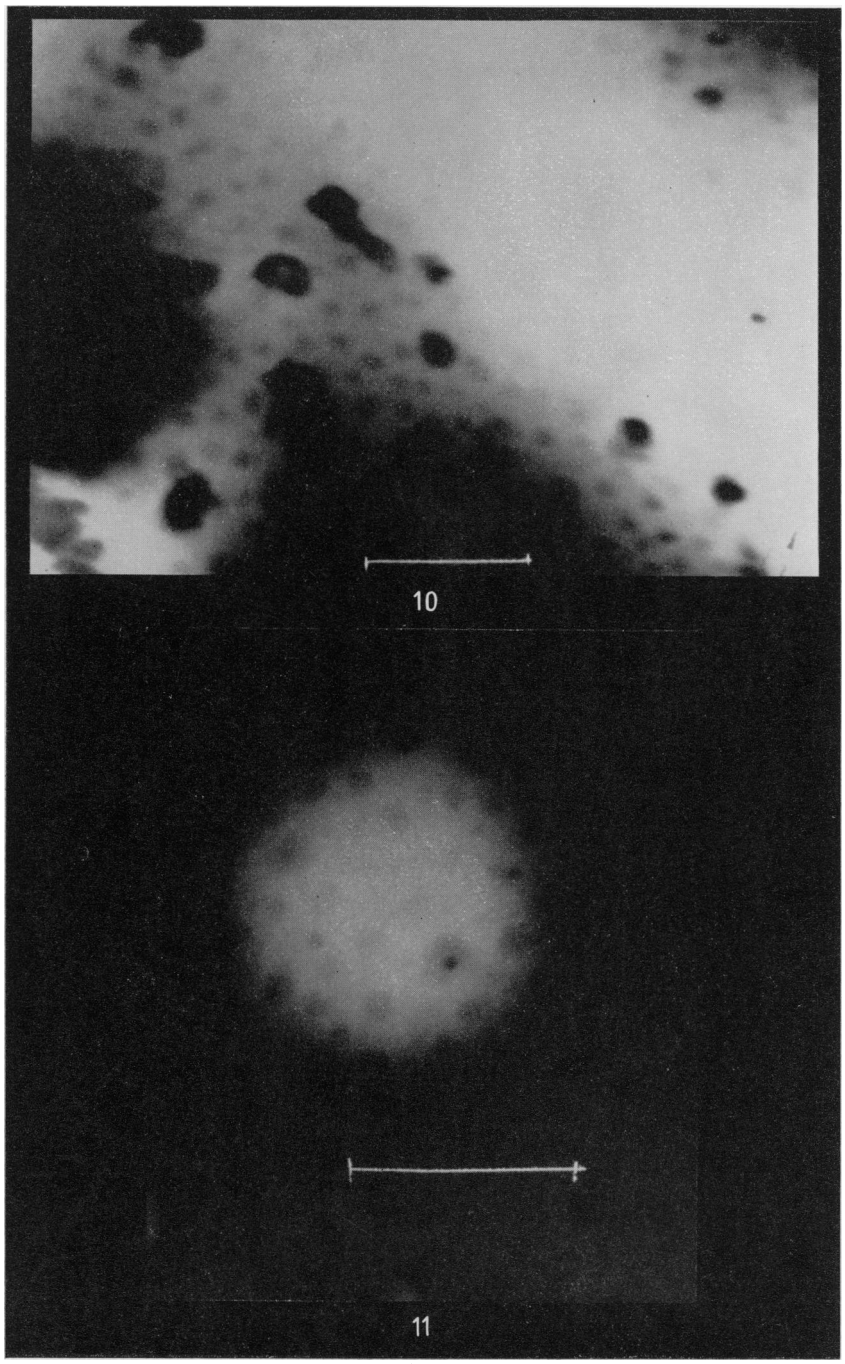

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TABLE V.—*LD_{50} for Mice of F1 Separated by G-200 Sephadex from Culture Filtrates of Virulent (P14, B4) and Avirulent (P2AB) Strains of* Ps. aeruginosa

Strain of *Ps. aeruginosa*	Calculated LD_{50} in mg./kg. mouse: unburned mice	burned mice
P14 (virulent)	33·7	17·6
B4 (virulent)	29·0	16·0
P2AB (avirulent)	>40	13·1

more toxic (LD_{50}—13·1 mg./kg.) than the F1 preparations from the 2 virulent strains (LD_{50}—17·6 and 16·0 mg./kg.). The mean survival times of mice that died after i.p. challenge of 40 mg./kg. of F1 from P14, B4 and P2AB were 3·3, 2·5 and 1·4 days respectively for burned mice and 2·0, 2·3, 1·5 days for unburned mice.

On a weight for weight basis F3 preparations from the 3 strains (P14, B4 and P2AB) were not as toxic for mice (burned or unburned) as the F1 preparations. When unburned mice were challenged with 40 mg./kg. of F3 from B4 or P2AB, no mice died, and only 2/10 mice died when challenged with same dosage of F3 from P14. F3 preparations from the 2 virulent strains of *Ps. aeruginosa* (P14 and B4) were more toxic for burned mice than they were for unburned mice. 4/10 mice were killed when 40mg./kg. of F3 from B4 was inoculated into burned mice, 2/10 mice were killed by a similar dosage of F3 from P14, but at the same dosage F3 from P2AB killed no burned mice. The mean survival times of burned mice which died after i.p. challenge with 40 mg./kg. of F3 from P14 and B4 were 1·0 day and 4·5 days respectively.

Systemic effects.—The response of the mice to challenge with F1 fractions was similar for preparations from all 3 strains. Immediately after challenge with F1 preparations at dosages > 10 mg./kg., both burned and unburned mice sat in a hunched position and became still; during the next 15 min. respiration became shallow and rapid. After 24 hr., during which time mice ate or drank very little, a heavy white exudate developed around both eyes and by 48 hr. the exudate was so profuse that the eyeball was no longer visible. About $\frac{1}{3}$ of the mice had flecks of blood in the exudate. The exudate persisted for 3–4 days in mice that survived after which time it dried and flaked off. Weight began to increase 5 days after challenge and few deaths were recorded after this time. Smears prepared from the eye exudate and stained with Leishmann's showed tetrads of cocci and a few epithelial cells. Aerobic and anaerobic bacteriological culture of the exudate in digest and cooked meat broth showed corynebacteria, micrococci and staphylococci.

At autopsy, there was no macroscopic sign of pathological change in the organs and the heart blood was sterile.

Challenge i.p. with F3 preparations from P14, B4 and P2AB were similar to those described when the mice were challenged with F1 preparations, except that the exudate around the eyes was clear and glutinous.

At autopsy, large volumes of peritoneal fluid were found which somtimes contained traces of blood. Petechiae were seen in hind leg muscles, liver and abdominal wall. The intestines were often inflamed. Heart blood was sterile.

Toxicity of Sepharose-4B separated fractions from Ps. aeruginosa *P14 and P2AB*

Table VI shows a comparison of the lethal effects for burned mice of fractions X, Y and Z separated from F1 preparations of the virulent strain of *Ps. aeruginosa*

TABLE VI.—*Mortality of Burned Mice Challenged with Fractions from Sepharose-4B from a Virulent (P14) and an Avirulent (P2AB) Strain of* Ps. aeruginosa

Strain from which fraction was made	Fraction inoculated	Mortalities of burned mice after the inoculation of the following amounts of fraction (mg./kg.)		
		40	20	10
P14 (virulent)	X	1/3	0/3	0/3
	Y	2/3	2/3	0/3
	Z	0/3	0/3	0/3
P2AB (avirulent)	X	1/3	2/3	0/3
	Y	1/3	1/3	1/3
	Z	0/3	0/3	0/3

—P14 and the avirulent strain P2AB. Even though there are only a small number of mice in this experiment, the results suggest that the X and Y fractions are more toxic than the Z fractions for both the virulent and avirulent strains of *Ps. aeruginosa*. The systemic effects of X and Y were similar to those described for F1 preparations.

Enzyme activities of culture filtrates of Ps. aeruginosa

Protease, lecithinase, haemolysin and elastase were the enzymes selected for study, as purified preparations of these enzymes are known to cause local tissue damage on s.c. injection (Liu, 1964; Johnson *et al.*, 1967) and Mull and Callahan (1965) have demonstrated the action of elastase in experimental pseudomonas infection.

A preliminary screening of F1–F4 from P14, B4 and P2AB for enzyme activity showed that most of the enzyme activity was in F3. Comparison of the enzyme titres of the freeze-dried toxic fractions (F1 and F3) is shown in Table VII.

TABLE VII.—*Enzyme Activity of F1* and *F3 G-200 Sephadex Fractions from Virulent (P14, B4) and Avirulent (P2AB) Strains of* Ps. aeruginosa

Strain of *Ps. aeruginosa* used	Enzyme titre							
	F1				F3			
	Protease	Elastase	Lecithinase	Haemolysin	Protease	Elastase	Lecithinase	Haemolysin
P14	4	0	1	0	32	4	0	0
B4	4	0	1	0	32	1	1	0
P2AB	2	0	1	0	8	1	1	0

Culture filtrates which had not been freeze-dried or fractionated were assessed in a similar way. In this series of experiments the rates of production of enzymes by virulent and avirulent strains of *Ps. aeruginosa* were compared by assaying the amount of enzyme activity present per unit volume per day for 7 successive days. The same number of organisms from each strain was inoculated into standard volumes of dialysable medium and the number of organisms per unit volume was

estimated daily. In these experiments the possibility of denaturation of enzymes by freeze-drying was avoided by using fresh filtrates which had not been freeze-dried or fractionated.

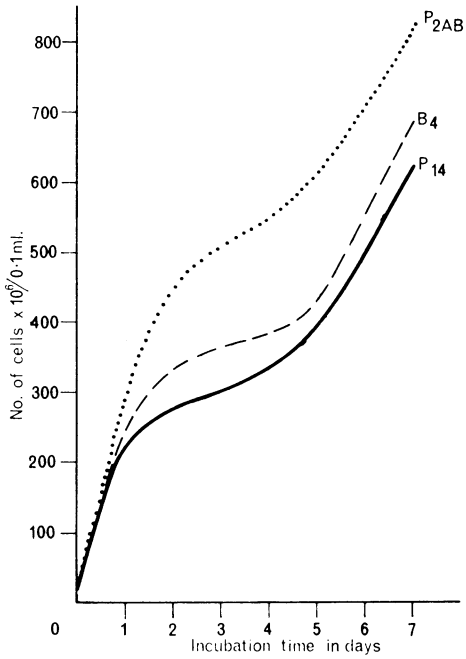

FIG. 12.—Growth rates of virulent (P14 and B4) and avirulent (P2AB) strains of *Ps. aeruginosa*.

The growth curves (Fig. 12) show that all 3 strains grew at a similar rate for approximately 4 hr. The growth rate of the 2 virulent strains (P14 and B4) then slowed down for 3 days, after which time (about the 5th day after inoculation) an increase in the growth rate was observed. The avirulent strain maintained its initial rate of growth for 2 days, the rate decreased for about 2 days and then increased again to a rate of growth similar to that of the virulent strains.

TABLE VIII.—*Production of Protease by Virulent (P14, B4) and Avirulent (P2AB) Strains of* Ps. aeruginosa

Strain of *Ps. aeruginosa* investigated	Titre of protease produced in 0·1 ml. of culture filtrate on the following days:						
	1	2	3	4	5	6	7
P14 (virulent) .	4	32	32	—	128	128	128
B4 (virulent) .	1	4	8	—	32	64	64
P2AB (avirulent) .	0	1	4	—	32	32	32

During the first 24 hr. the avirulent strain (P2AB) produced no detectable protease (Table VIII), but protease was present in culture filtrates of both virulent

strains (P14 and B4). By the 5th day of culture, the amount of protease in P14 and P2AB cultures had reached their maximum concentrations and stayed at the same level for the next 2 days of the experiment. B4 showed a 2-fold increase in amount of protease present on the 7th day having remained at a steady level since the 5th day. From the growth curve (Fig. 12) it can be seen that during part of the time when the amount of protease detected per unit volume remained steady (days 5–7), the number of organisms per unit volume increased.

TABLE IX.—*Production of Elastase by Virulent (P14, B4) and Avirulent (P2AB) Strains of* Ps. aeruginosa

Strain of *Ps. aeruginosa* investigated	Titre of elastase produced in 0·1 ml. filtrate on the following days: 1	2	3	4	5	6	7
P14 (virulent)	2	2	4	—	4	8	16
B4 (virulent)	1	1	1	—	1	2	8
P2AB (avirulent)	0	0	1	—	2	2	4

The amount of elastase (Table IX) in daily samples of culture filtrates of P14 and B4 varied from day to day and then increased steadily after the 5th day. No detectable elastase was produced by P2AB until the 3rd day of culture and the final concentration produced by this organism was lower than that of P14 and B4.

TABLE X.—*Production of Lecithinase by Virulent (P14, B4) and Avirulent (P2AB) Strains of* Ps. aeruginosa

Strain of *Ps. aeruginosa* investigated	Titre of lecithinase produced in 0·1 ml. culture filtrate on the following days: 1	2	3	4	5	6	7
P14 (virulent)	0	1	1	2	2	—	2
B4 (virulent)	0	0	0	1	1	—	1
P2AB (avirulent)	0	1	1	1	1	—	1

No lecithinase (Table X) was detected in culture filtrates from P14 and P2AB until the 2nd day, and none from B4 culture filtrates until the 4th day of culture. After 7 days of culture twice as much lecithinase was produced by P14 as by B4 and P2AB, which were similar to their initial concentrations.

Haemolysin (Table XI) was not detected in culture filtrates until the 4th day of culture and then only in cultures from the virulent strains. The avirulent strains produced no detectable haemolysin until the 7th day of culture.

DISCUSSION

The 3 strains of *Ps. aeruginosa* (P14, B4 and P2AB) used in this study showed differences in virulence for burned mice (Jones and Lowbury, 1966) and belonged to different serotypes (6C, 2/5, 5C) respectively.

TABLE XI.—*Production of Haemolysin by Virulent (P14, B4) and Avirulent (P2AB) Strains of* Ps. aeruginosa

Strain of *Ps. aeruginosa* investigated	Titre of haemolysin produced in 0·1 ml. culture filtrate on the following days: 1	2	3	4	5	6	7
P14 (virulent)	0	0	0	2	8	—	16
B4 (virulent)	0	0	0	4	16	—	16
P2AB (avirulent)	0	0	0	0	0	—	4

Our investigations have shown that freeze-dried culture filtrates of both virulent (P14 and B4) and avirulent (P2AB) strains of *Ps. aeruginosa* may be separated into 4 similar groups of fractions (F1–F4) by gel filtration through G-200 Sephadex. UV absorption curves of fractions eluted from G-200 Sephadex showed a similar distribution of UV absorbing material in the eluted fractions. Different proportions of UV absorbing material were found in corresponding fractions from the three strains, indicating different amounts of extracellular substances in culture filtrates of the 3 strains.

Immunodiffusion techniques showed that the Sephadex separated fractions F1 and F3 each contained several different antigenic factors, some of which were common to all 3 strains. Using Sepharose-4B, F1 fractions from P14, B4 and P2AB were divided into three fractions (X, Y and Z) and using Sepharose-2B the " X " fractions were subdivided into a further three fractions, a, b and c. Immunodiffusion of the Sepharose-4B fractions showed that on the basis of molecular size, the antigenic factors in F1 fractions had been separated into three groups of molecules (X, Y and Z) with different serological specificities. Two single antigenic factors were separated from the " X " fractions of the virulent strains P14 and B4 by gel filtration through Sepharose-2B. These factors were located in the b and c fractions. The avirulent strain P2AB did not possess an antigenic factor in the b fraction. The possession of the antigenic factor in the b fraction may prove a useful serological method for distinguishing virulent from avirulent strains of *Ps. aeruginosa*.

Kav values of the fractions from Sepharose-4B and Sepharose-2B enable an estimation of the molecular weights of the fractions to be made (Pharmacia 1966). F1 fractions were found to contain molecules of molecular weight ranging between 5×10^5–2×10^7. Retention of F3 fractions by G-200 Sephadex indicates that the molecular weights of these fractions is less than 200,000. Electron micrographs of fraction X from P2AB showed that spherical particles of approximately 150 Å in diameter were present. These spherical particles resemble electron micrographs of aggregates of lipopolysaccharide and muropeptides, described by Clarke, Gray and Reaveley (1967) which are thought to be derived from a murein sacculus that is associated with cell walls of *Ps. aeruginosa* (Martin, 1963; Weidel and Pelzer, 1964).

After the culture filtrates of P14, B4 and P2AB had been separated into 4 fractions by G-200 Sephadex, an examination of the toxic and enzymic properties of these fractions was attempted. The immunological properties of culture filtrates of these three strains have been reported elsewhere (Jones, 1968).

Intraperitoneal inoculation of varying concentrations of F1, F2, F3 and F4 from P14, B4 and P2AB into burned and unburned mice demonstrated that both

virulent and avirulent strains of *Ps. aeruginosa* produce extracellular substances that are potentially lethal. Fractions F2 and F4 from the 3 strains killed no mice at 40 mg./kg. The toxic substances were found in F1 and F3 fractions from each of the 3 strains.

By weight F1 fractions from P14, B4 and P2AB were more lethal than F3 fractions, and burned mice were at least twice as susceptible to the lethal action of F1 than unburned mice. Although burning increased the number of mice that died as a result of challenge with F1 fractions, it did not alter the time required for the lethal action of F1 to take effect, except in the case of mice challenged with P14.F1 which survived 1–3 days longer when burned than when unburned. F1 fractions not only had a greater lethal capacity than F3 fractions but they were present in greater amounts, the proportions of F1/F3 ranging between 2·5/1 for B4 and 7/1 for P2AB. An unexpected finding was the shorter survival times of mice that died after challenge with F1 from the avirulent strain P2AB, than those of mice challenged with F1 from P14 or B4. The reason for the apparent difference in the toxic properties of F1 from P14 and B4 and F1 from P2AB could be explained by the fact that only a small number of mice died as a result of challenge with P2AB.F1—in fact an LD_{50} for P2AB.F1 could not be calculated—and those that died happen to die immediately after challenge. The internal organs of mice which died after inoculation of F1 fractions showed no obvious signs of damage, in this respect F1 was similar to the " lethal factor " described by Liu (1966). F3, however, caused severe local damage probably due to the presence of tissue destroying enzymes in the fraction. The transient purulent conjunctivitis caused in mice after challenge with F1 from both virulent and avirulent strains, was an inexplicable feature of the toxic effects of F1 in mice.

Although it seems likely that the toxic effects of *Ps. aeruginosa* infections could be caused by fractions found in culture filtrates of pseudomonas, it is impossible to make a direct comparison of causes of death resulting from burns infected with *Ps. aeruginosa* and those resulting from experimental administration of toxins, as it is not known whether the amounts of toxic substances in the bloodstreams of infected animals would ever reach the high concentrations of toxins achieved experimentally. It is more likely that a gradual accumulation of toxins would occur in an infected animal, and the time required for a fatal level of toxin to be attained would vary from animal to animal and depend on such factors as the growth rate of the infecting strain area infected and previous immunological experience of the host: such animals could die from the results of the toxic effects of pseudomonas infection without the organism ever being isolated from the bloodstream.

Most of the enzyme activity of P14, B4 and P2AB was found in the G-200 Sephadex F3 fractions. It was observed in the preliminary screening of these freeze-dried culture filtrates, that whereas all strains possessed protease, elastase and lecithinase, the virulent strains P14 and B4 digested their substrates more rapidly than the avirulent strain, P2AB. Further investigations into the rates of production of enzyme, using unfractionated culture filtrates that had not been freeze-dried, confirmed our previous finding that all the 3 strains eventually produce haemolysin, protease, lecithinase and elastase; but virulent strains produce higher concentrations of enzymes sooner than the avirulent strain.

The rates of production of enzymes did not parallel the growth curves for the organisms, *e.g.*, the maximum protease concentration in the culture was reached

between days 4 and 5, while the number of organisms continued to increase up to the 7th day; the strain (P2AB) that produced the largest number of cells in culture also produced the smallest amounts of all 4 enzymes. The 4th to 5th days of culture was an important stage in the production of enzymes, for during this period of time the rate of growth of the organisms was accelerated, maximum concentrations of protease had been produced, elastase production increased and haemolysin was first detected in culture filtrates of the virulent strains. The 4th to 5th days is also a critical stage in the infection of burned mice with virulent strains of *Ps. aeruginosa*, for during this period the majority of burned mice die from septicaemia (Jones and Lowbury, 1966). If one can assume that the production of enzymes *in vivo* simulates enzyme production observed *in vitro* then haemolysin and elastase would not play a large part in the initial invasive process, as the maximum production of these enzymes occurs after the 5th day. As protease was being produced in ever increasing amounts up to the 5th day of culture it could be regarded as a most important invasive enzyme. Elastase and lecithinase, since they are present in constant amounts, probably act as auxiliary enzymes functioning in the wake of the main tissue destroying enzymes.

SUMMARY

Freeze-dried culture filtrates of 2 virulent (P14 and B4) and 1 avirulent (P2AB) strains of *Ps. aeruginosa* were fractionated by gel filtration through G-200 Sephadex, Sepharose-4B and Sepharose-2B. All 3 strains yielded a similar range of fractions and these were pooled on the basis of the presence or absence of UV absorbing material.

Of the 4 groups of fractions (F1–F4) from G-200 Sephadex, only F1 and F3 from all 3 strains showed toxicity for mice. F1 was more toxic than F3, but F3 was found to contain more enzyme activity than F1.

Attempts to isolate single components of F1 fractions by gel filtration through Sepharose-4B and 2B were partially successful as judged by immunodiffusion techniques. Many common antigens were found in F1 preparations from P14, B4 and P2AB, and a single antigen shared only by the 2 virulent strains was isolated.

The *in vitro* activities of protease, lecithinase, elastase and haemolysin were assayed in 7 successive daily samples of culture filtrates of P14, B4 and P2AB. The virulent strains (P14 and B4) produced more of the 4 enzymes and produced them sooner than the avirulent strain (P2AB). The avirulent strain produced more growth over the 7-day period than the virulent strains.

The relationship of toxic factors and enzyme production to the virulence of *Ps. aeruginosa* for burned mice is discussed.

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