THE CHEMICAL BASIS OF THE VIRULENCE OF *PASTEURELLA PESTIS*

III. An Immunogenic Product Obtained from *Past. pestis* which Protects both Guinea-pigs and Mice

J. KEPPIE, E. C. COCKING, K. WITT and H. SMITH

From the Microbiological Research Establishment, Porton, Salisbury, Wiltshire

Received for publication March 3, 1960

One reason for examining *Past. pestis* (and its products) from infected guinea pigs was to identify the compounds responsible for the immunizing power of living avirulent *Past. pestis* for guinea pigs, because this was greater than the protection afforded by any of the products hitherto isolated from culture *in vitro* (Smith, Keppie, Cocking and Witt, 1960). In addition, an explanation was sought for the inability of Fraction I (Baker, Sumner, Foster, Meyer and Meyer, 1952)—the currently accepted basis for immunogenicity in mice—to account for the total immunizing activity of whole (dead) organisms for that species (Smith, *et al.*, 1960).

This paper describes the properties of an immunogenic product obtained from *Past. pestis* which immunized guinea pigs as well as mice. The product was first obtained from *Past. pestis* grown *in vivo*. Later a similar material was found in *Past. pestis* grown *in vitro* and experiments are described which prepare the way for the use of preparations containing this product in an improved non-living vaccine against plague. A preliminary report of some of the work has already appeared (Keppie, Cocking and Smith, 1958).

MATERIALS AND METHODS

Past. pestis (*strain L37*) *grown* in vivo

This was obtained as previously described, (Smith, *et al.*, 1960). A suspension of organisms (1×10^{10} per ml.) was used and all *Past. pestis* were killed by treatment with alkali as described later, or by heating to 60° for 1 hr. To provide other and possibly milder methods of killing whole *Past. pestis* for tests of immunogenicity in mice and guinea pigs (see Table I) the immunizing doses were made as follows: *Dose* 1. The suspension was injected with 0·02 mg./ml. streptomycin; guinea pigs and mice received 40 mg. and 3 mg. of streptomycin respectively on each of 4 successive days. *Dose* 2. Suspension treated for 24 hr. at 18° with hexyl resorcinol (0·0002 per cent w/v); tests in mice and on plates showed complete sterility of this material. Control animals (20) receiving doses 1 and 2 without the organisms, all succumbed to subsequent challenge in the active immunity test.

Extract (US) and Residue (R) obtained from Past. pestis *grown* in vivo *by treatment with ultrasonic waves.*—These were obtained as previously described (Smith *et al.*, 1960) and used at initial concentrations (approx. 3 mg. per ml. for US and approx. 1 mg. per ml. for R) equivalent to 1×10^{10} *Past. pestis* per ml. US was filtered sterile; if R was not dissolved in alkali it was sterilized by heating to 60° for 1 hr.

Residue (left after treatment with ultrasonic waves) from Past. pestis (*strains Tjiwidej and L37*) *grown* in vitro.—The medium of Davies (1956) was inoculated with *Past. pestis* strains Tjiwidej or L37 (1×10^{8} per ml.) and shaken in Thompson bottles (250 ml. per bottle)

at 37° for 22–24 hr. Organisms were removed from the culture (7–8 l.; count 8–9 $\times$ 10^9 organisms per ml.) by centrifuging for 45 min. at 18,000 r.p.m. in the Spinco ultracentrifuge at 0–2°, and washed once with saline (1600 ml.). The bacterial deposit was suspended in distilled water (approx. 300 ml.) and aliquots (500 ml.) of the suspension (approx. 2 $\times$ 10^{11} *Past. pestis* per ml.) were treated with ultrasonic waves for 30 min. at 0–2° as previously described (Smith, *et al.*, 1960) for *Past. pestis* grown *in vivo*. The treated material (300 ml.) was diluted to 2 l. with distilled water and centrifuged at 10,000 **g**. for 45 min. at 0–4°.

The soluble material was removed and the residue washed once with distilled water (600 ml.) and finally suspended in sufficient saline (approx. 300 ml.) so that the suspension of residue was equivalent to 2 $\times$ 10^{11} *Past. pestis* per ml. before extraction with ultrasonic waves. The suspension was heated to 60° for 1 hr. to kill remaining *Past. pestis* and diluted to a concentration equivalent to 1 $\times$ 10^{10} *Past. pestis* per ml. (approx. 1 mg. dry wt. per ml.) as required for use.

Test for immunizing activity in guinea pigs and mice.—Two subcutaneous injections of the material under test were given to guinea pigs (450 g. $\pm$ 50) or to mice (20 g. $\pm$ 2) with an interval of two weeks between doses. One week after the final dose, virulent *Past. pestis* (strain L37) was given subcutaneously as a challenge ; guinea pigs received 1 $\times$ 10^4 organisms (approx. 100 LD_{50}) and mice 1 $\times$ 10_3 (approx. 100 LD_{50}). The animals were observed for 21 days and the number of survivors noted.

In a few experiments (see Table IV) the virulent strains Shasta, M.P.6, 139L and M23 were used for challenge ; we are indebted to Dr. T. W. Burrows for these strains.

Samples of Fraction I.—Three samples were tested. Sample 1 (purified) and sample 2 (crude) were kindly supplied by Dr. K. F. Meyer (*via* our colleague Dr. Burrows). Sample 3—another purified specimen was produced by our colleague Dr. D. A. L. Davies.

Serological precipitation in gels.—The techniques and antisera used have been described (Smith *et al.*, 1960).

Analytical Methods

Ultracentrifugation (in Spinco Model L machine with a synthetic boundary cell) and electrophoresis (Spinco Series 10 machine).—We are indebted to our colleagues Mr. K. Cammack and Mr. K. Grinstead for these examinations.

Nitrogen.—By the Kjeldahl and Dumas methods.

Phosphorus.—By the method of Fiske and Subbarow (1925).

Glucosamine.—By a modification (Boas, 1953) of the Elson-Morgan method.

Lipopolysaccharide.—By the heptose reaction with H_2SO_4-cysteine (Dische, 1953) using the lipopolysaccharide of Davies (1956) as standard.

Total reducing sugars.—By the method of Somogyi (1952).

Sugars (calc. as glucose).—By the benzidine method of Pridham and Jones (1954).

Lipid.—This was weighed after extracting it from the material with chloroform/methanol as described by Weibull (1957) and purifying it by the method of Folch, Lees and Sloan-Stanley, (1954).

Nucleic acid.—By the extinction of light (260 mμ.) in a Unicam Spectrophotometer.

RESULTS

Products from Past. pestis *Grown* in vivo

Preliminary experiments showed that products of *Past. pestis* found extracellularly in exudates did not immunize mice and guinea pigs effectively. Hence attention was focussed on the organisms as a source of protective antigens.

Immunizing activity for guinea pigs and mice of the ultrasonic residue (R)

Active immunity tests with a suspension of R indicated clearly immunizing activity of this preparation for guinea pigs and mice (Line 1*a* Table I). Although R showed some toxicity in mice and guinea pigs when prepared under the conditions described in the previous paper, and injected intravenously (see Table I Cocking, Keppie, Witt and Smith, 1960), this toxicity was negligible under the conditions

of the test for immunizing activity, and in relation to the amounts of R shown to be active. Thus R $\equiv 0{\cdot}5 \times 10^{10}$ and 5×10^{10} organisms, did not kill any of 50 mice or guinea pigs respectively when injected subcutaneously.

TABLE I.—*Immunizing Activity of R Compared with US and Whole* Past. pestis *grown* in vivo

Material	Survivors / Number in group							
	Guinea-pigs					Mice		
	5*	1·0	0·33	0·1	0·03	0·1*	0·03	0·01	0·003
1. Ultrasonic residue (R)									
(*a*) Heated to 60° 1 hr.; insoluble	29/30	36/49 |	4/10	16/30	..	41/59 |	9/20	23/80	5/20
(*b*) Heated to 60° and then dissolved in alkali	..	22/25	18/22	16/25 |	..	47/90 |	..	7/40	..
(*c*) Unheated then dissolved in alkali	..	9/10	26/30	28/30 |	11/30	21/30 |	6/20	2/20	2/20
2. Ultrasonic extract (US)							Toxic		
(*a*) Filtered sterile . .	4/40	2/40	5/20	3/40	..	Toxic	6/9	18/19	13/20 |
(*b*) Treated with alkali as (*c*) above†	..	Not tested			..	Toxic 15/16	18/20	12/20 |	6/20
3. Whole organisms							Toxic		
(*a*) Heated to 60° 1 hr. .	..	| 9/20	8/20	4/20	4/20	Toxic	10/11	15/19	16/20 |
(*b*) Treated with alkali as in (*c*) above‡	..	15/20	11/20 |	10/20	7/20	18/19	15/20 |	10/20	9/20
(*c*) Treated with streptomycin and hexylresorcinol§	..	19/20	17/20	14/20	13/20 |	Toxic	Toxic 16/17	15/19	12/20 |

* Figures indicate the number of organisms ($\times 10^{10}$) or equivalent amount of product in each of 2 doses given s/c 2 weeks apaert.

† US treated with an equal volume of 0·2N NaOH for 5 min. at 18° followed by addition of glycine as described in text.

‡ Whole organisms treated as described in the text for R. Under these conditions approx. 20 per cent of the bacterial substance remained undissolved. The suspension was free from live *Past. pestis* and was not centrifuged and filtered before injection.

§ See methods.

Vertical lines indicate approx. concentration producing at least 50 per cent protection

Alkali used to dissolve R without loss of immunizing activity

R ($\equiv 40 \times 10^{10}$ *Past. pestis*) was mixed with 0·1 N NaOH (10 ml.) at 18°. After 5 min. solid glycine (approx. 450 mg.) was added until the pH was 9·2 and the final volume was adjusted with water to 40 ml. The slightly turbid solution was clarified by centrifugation (10,000 *g*. for 30 min. at 4°; insoluble material 3–4 per cent of R) and was shown by culture and the injection of mice to be free of living *Past. pestis*. Table I shows at line 1*b* the results of immunity tests with

R when it was dissolved in alkali after heating to 60° for 1 hr. and at line 1*c*, the results when heating was omitted. Comparison of these results with those in line 1*a* of Table I shows that the immunizing activity of R for guinea pigs was increased by dissoving it in alkali and the activity in mice was not significantly altered by this treatment. The immunizing activity of R for guinea pigs did not drop significantly even after treatment with alkali for several hours under these conditions.

For chemical and serological analysis a more concentrated solution (approx. 0·4 — 0·5 per cent w/v) of R was prepared by treating it ($\equiv 40 \times 10^{10}$ *Past. pestis*) with 0·1 N NaOH (10 ml.) as described above but not diluting with water after the addition of glycine.

Comparison of the immunizing activity of R with that of US and of whole Past. pestis

A comparison of the results of immunity tests in guinea pigs with R, US and sterile preparations of the whole organisms (Table I) showed that the activity of R largely accounted for that of the whole organisms and that US contained negligible activity. The toxicity for guinea pigs of US and the whole organisms (Cocking *et al.*, 1960) did not interfere with the immunity tests since no deaths occurred with either preparation during the immunization course of 2 subcutaneous injections spaced 2 weeks apart.

In mice, the US and the heated preparation of whole organisms were so toxic that immunizing activity could only be compared at low dosage. However, the toxicity of these preparations for mice could be substantially reduced by treatment with alkali (Cocking *et al.*, 1960) and this enabled the immunizing activity of the various preparations to be compared also at higher concentrations. The results in Table I show that although R had appreciable immunizing activity for mice, the activity of US was greater than that of R for this species. It is also evident (Table I) that treatment of whole organisms and US with alkali destroyed to some extent their immunizing activity for mice but not so rapidly as their toxicity for this species.

Properties of the immunogenic residue (R) obtained from Past. pestis *grown* in vivo

R was insoluble in water and saline at pH7 but could be dissolved by treatment with alkali as described previously and held in solution at pH 9·2 in 0·1 N NaOH–0·6 N glycine buffer. The material tended to come out of this solution as the pH was reduced and below pH7 it was precipitated.

Analysis.—Table II shows the analysis of a sample of the immunogenic complex prepared by dialysing its solution until free of salt and freeze drying; it contained protein, lipid and carbohydrate. Almost identical results were obtained by analysing R before dissolving it by treatment with alkali. Paper chromatography after acid hydrolysis of R indicated the presence of all the usual amino acids, glucosamine, glucose and the heptose which had been found in the polysaccharide of Davies (1956).

Ultracentrifugation and electrophoresis.—In the ultracentrifuge a small amount of R appeared to sediment rapidly to the bottom of the cell but the rest sedimented as a single peak (Fig. 1*a*) with S = 4·3–4·7 (assuming a $\bar{V}$ of 0·76); the peak spread rapidly indicating high polydispersity. On electrophoresis, R showed one major and one minor component (see Fig. 1*b*) with similar mobilities (the approx.

TABLE II.—*Analysis of Immunogenic Residue (R)*

Analysis	Per cent
N	10·9 (K), 11·7 (D)
Lipid	24·1
P	0·9
Ash	0·2
Glucosamine*	1·9
Reducing sugars (as glucose)†	2·8
Sugars detected by benzidine (as glucose)‡	1·8
Lipopolysaccharide	2·8
Nucleic acid derivatives	None detectable

Material dried *in vacuo* over P_2O_5 to constant weight.
K = Kjeldahl method. D = Dumas method.
* After hydrolysis with 6 N HCl for 3 hr. 100°.
† After hydrolysis with 6 N HCl for 18 hr. at 100°; corrected for reducing action of glucosamine. Similar results from a less drastic hydrolysis.
‡ After hydrolysis with 6 N HCl for 18 hr. at 100°.

mobility of the major component was $-6{\cdot}7 \times 10^{-5}$ cm./sec./volt/cm.). The behaviour of R in the ultracentrifuge and on electrophoresis was not significantly altered by heating it to 60° for 1 hr. before dissolving it in alkali, by prolonging the alkaline treatment to 17 hr., or by washing R with alkaline buffers prior to solution in 0·1 N NaOH.

Serological precipitation in gel diffusion plates.—A solution (0·4–0·5 per cent) of R in 0·1 N NaOH/0·6 N glycine buffer at pH 9·2 was prepared as described above. This concentrated solution formed 3 lines in serological precipitation plates against antiserum prepared against *Past. pestis* organisms (serum L.R.). One line was common with that formed by Fraction I and subsequent titrations indicated 0·2–0·4 per cent of Fraction I in R (assuming " free " and " combined " Fraction I reacted similarly). The other two lines (A and B) were common with 2 lines formed by a suspension of the lipopolysaccharide of Davies (1956). Against an antiserum (L.R.A.) adsorbed with avirulent organisms the solution of R formed one line, which was not common with those formed by the V and W antigens of Burrows and Bacon (1956).

An antiserum prepared in guinea pigs by injection of a solution of R formed two lines (corresponding with A and B above) when diffused against R but did not precipitate a solution of " free " Fraction I.

Immunogenic Residues from Past. pestis *Grown* in vitro

When the virulent L37 and the avirulent immunogenic Tjiwidej strains of *Past. pestis* were grown at 37° and immediately extracted with ultrasonic waves (see " Methods "), residues were obtained which, like the product obtained from *Past. pestis* grown *in vivo*, immunized both guinea pigs and mice (Table III).

Although somewhat less effective for protecting guinea pigs than R from *in vivo* organisms (cf. Table I line 1*a* with Table III) the residue obtained from Tjiwidej strain was investigated as the potential basis for a non-living vaccine against plague. The results shown in Table IV indicate that this residue protects mice and guinea pigs against challenge with several virulent strains of *Past. pestis* which were different from the L37 used routinely in this work.

TABLE III.—*Immunizing Activity for Guinea Pigs and Mice of Residues Left After Ultrasonic Extraction of a Virulent L37 and an Avirulent Immunogenic Tjiwidej Strain of* Past. pestis *Grown* in vitro

Strain from which residue† prepared	Survivors / Number in group					
	Guinea-pigs			Mice		
	5*	1	0·1	0·1*	0·03	0·01
Virulent L37 grown at 37°	8/10	13/15	7/15	23/30	..	10/40
Avirulent Tjiwidej grown at 37°	46/65	30/70	16/65	33/60	8/20	19/60

* Figures indicate the number of organisms ($\times 10^{10}$) from which the product in each of 2 doses (given s/c 2 weeks apart) was obtained.

† Prepared as described in " Materials and Methods " and injected as a suspension after heating to 60° for 1 hr.

Vertical lines indicate approx. dose which produced at least 50 per cent protection.

TABLE IV.—*Protection of Guinea Pigs and Mice Against Challenge From Several Virulent Strains of* Past. pestis *by the Immunogenic Product from* Past. pestis *(Strain Tjiwidej) Grown* In Vitro

Strain used for challenge†	Survivors / Number in group					
	Guinea-pigs			Mice		
	Nil	5·0*	1·0	*Nil*	0·2*	0·04
MP6	1/10	6/10	8/10	0/10	7/10	3/10
139L	0/10	10/10	6/10	1/10	6/10	7/10
Shasta	0/10	8/10	6/10	0/10	8/10	8/10
M 23	0/10	10/10	10/10	0/10	5/10	4/10

* Figures indicate the number of organisms ($\times 10^{10}$) or equivalent amount of product in each of 2 doses given s.c. 2 weeks apart.

† Numbers used for challenge were for guinea-pigs 7×10^5 organisms and for mice 1×10^3 organisms ; otherwise the test for immunogenicity was the same as described in " Methods ".

Comparison of the immunogenicity of the products obtained from Past. pestis *grown* in vivo *and* in vitro *with that of Fraction I*

The results in Table V show that Fraction I injected without adjuvant had negligible immunizing activity in guinea pigs. and approx. 3–6 μg. was needed to protect 50 per cent of mice in the assay for immunizing activity. The corresponding protective dose for mice of 4 of our preparations (see Tables I and IV) are given below with their Fraction I content in brackets ; the latter was estimated by serological precipitation in diffusion plates on the assumption that " free " and " combined " Fraction I react similarly. *Past. pestis* organisms grown *in vivo* 10 μg.

TABLE V.—*The Immunogenicity of 3 Samples of Fraction I for Mice and Guinea Pigs*

Sample	Survivors / Number injected							
	Guinea-pigs				Mice			
	120*	40	12	4 μg.	6	3	0·7	0·2 μg.
1 . .	4/20	8/20	4/20	2/19	19/20	8/20	8/20	2/20
2 . .	7/20	6/20	2/20	3/20	8/20	3/20	2/20	3/20
3 . .	3/20	2/18	3/20	1/20	12/20	14/20	6/20	4/20

* Figures indicate amount (μg.) injected s/c in each of 2 immunizing doses.

(2–3 per cent); R 100 μg. (0·4 per cent); US 10 μg. (2 per cent); residue from *Past. pestis* grown *in vitro*, 100 μg. (0·9 per cent). Hence the protective activity of all preparations for mice were much greater than that warranted by their content of Fraction I.

DISCUSSION

A product has now been obtained from *Past. pestis* which effectively immunizes guinea pigs, and accounts for the immunizing activity in this species of intact *Past. pestis* organisms isolated directly from infected guinea pigs. The product was present in the residue left after treating *Past. pestis* grown *in vivo* with ultrasonic waves. This residue also immunized mice against infection with *Past. pestis*, but was less active in this respect than the material dissolved by the action of ultrasonic waves; the latter did not immunize guinea pigs.

The immunizing activity of the residue or the extract for mice could not be explained by their estimated content of Fraction I. We have confirmed that the latter injected without adjuvant produced no significant protection of guinea pigs. None of our preparations induced immunological paralysis such as that reported by Spivack, Foster, Larson, Chen, Baker and Meyer (1958), after injecting large amounts of Fraction I into guinea pigs.

The residue, immunogenic for guinea pigs and mice, contained lipid, carbohydrate (including the lipopolysaccharide of Davies. 1956) and protein (including " combined " Fraction I) but no nucleic acid. We suggest that this residue is an important part of a cell wall complex of *Past. pestis* to which Fraction I (and possibly the virulence antigens) are attached firmly near the cell wall and loosely in the body of the envelope. This cell wall complex with the attached envelope can be regarded as the best antigenic material for the protection of all species. If it is split by autolysis, acetone drying, and to a lesser extent by washing or treatment with ultrasonic waves, then the fragments lose protective power. This loss may be more apparent in some species than others. Thus, the residue from ultrasonic treatment contains a relatively large fragment which retains high immunogenicity for guinea pigs but only to a low degree for mice. The extract contains smaller fragments—ineffective for guinea pigs—but collectively more effective for immunizing mice than one of them, namely Fraction I. In fact, it appears that Fraction I is an ill-defined fragment of a more complete antigen. Thus, whole organisms

and various products therefrom have greater immunizing activity for mice than is warranted by their content of Fraction I. Furthermore this antigen was originally isolated in 2 forms, fraction IA and IB ; IA, but not IB, contained carbohydrate and if anything IA had a greater immunizing activity for mice than IB (Baker *et al.*, 1952).

It is not yet known whether the residue left after extraction of *Past. pestis* with ultrasonic waves is a single complex since it has only been examined in solution after treatment with alkali. This treatment did not destroy the immunogenicity for guinea pigs but it was relatively drastic and may have hydrolysed some linkages. However, the solution of the residues thus obtained revealed no gross heterogeneity in the ultracentrifuge, and showed only 2 components in the electrophoresis apparatus. On serological precipitation in gel diffusion plates, against unabsorbed rabbit antiserum to live *Past. pestis*, a concentrated solution of the residue formed only 3 lines in contrast to the multiplicity of lines formed by the soluble extract or whole organisms.

It appears at present, that the compounds responsible for immunogenicity in guinea pigs are not the same as those responsible for toxicity. Thus, the residue did not contain as much of the toxicity of the original organisms, as did the extract (Cocking *et al.*, 1960), which however failed to protect guinea pigs.

The results with products from organisms grown *in vivo* suggested that the development of an efficient non-toxic, sterile, vaccine was possible if corresponding immunogenic products could be obtained from *Past. pestis* grown *in vitro*. This now having been achieved, the next obvious step will be to experiment with alkali treated residue, combinations of it with alkali treated extract, and with alkali extracted whole organisms. A vaccine may thus be obtained which is effective in immunizing guinea pigs, mice and maybe man.

SUMMARY

A product has been obtained from *Past. pestis* which immunized guinea pigs and accounted for the immunizing activity in this species of killed *Past. pestis* isolated directly from infected guinea pigs.

The product was a relatively non-toxic residue—cell-wall material containing lipid, carbohydrate and protein—left after extraction of *Past. pestis* with ultrasonic waves.

The residue also immunized mice but was less active in this respect than the extract ; the latter did not immunize guinea pigs.

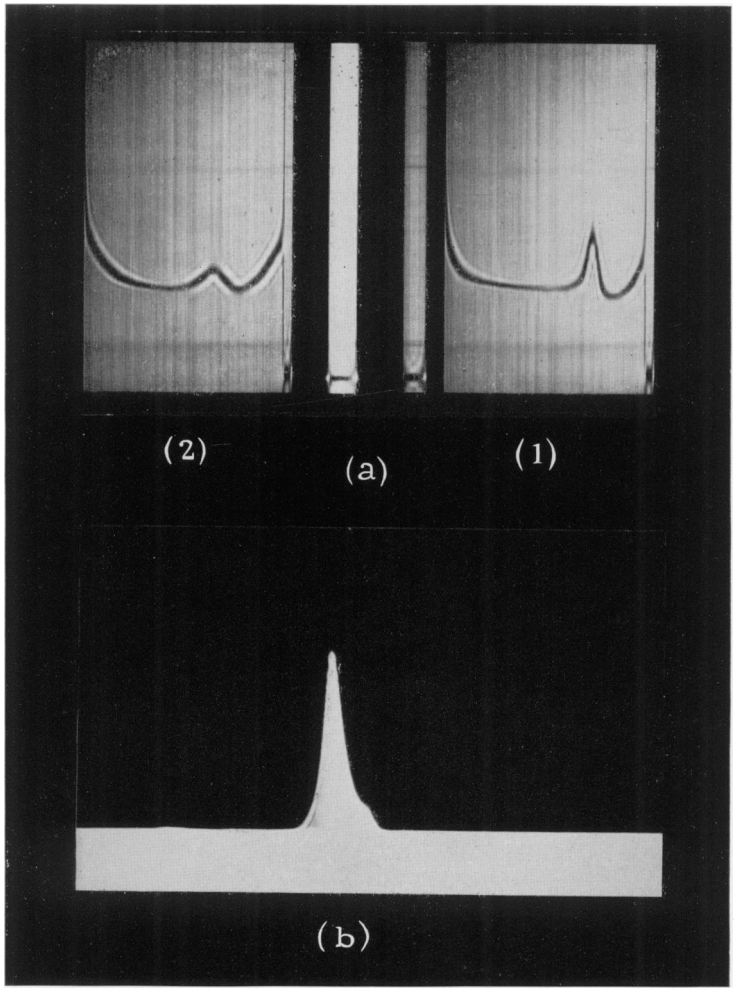
The immunizing activities for mice and guinea pigs of both residue and extract could not be satisfactorily explained on the basis of their estimated content of Fraction I.

EXPLANATION OF PLATE

FIG. 1.—Ultracentrifuge and electrophoresis diagrams on the immunogenic complex.
R (0·4–0·5 per cent w/v) was examined in 0·1 N NaOH/0·6 N glycine buffer at pH 9·2.

(*a*) Ultracentrifuge diagrams after 2 (1) and 18 (2) min. at 100,000 **g**. in a synthetic boundary cell.

(*b*) Electrophoresis diagram after 1 hr. in a field strength of 4·73 volt/cm. and a current of 12 mA ; ascending limb ; migration from left to right (anode).


(2)
(a)
(1)
(b)

It is suggested that the immunogenic residue is a relatively large fragment of a native cell wall—envelope complex which may be the best immunogen for all species.

The immunogenic product, first recognized in *Past. pestis* grown *in vivo* was later demonstrated in 2 strains of *Past. pestis* grown *in vitro*.

Various approaches now suggest themselves for the development of a sterile, non-toxic, vaccine which might be effective in immunizing man.

Our thanks are due to Mr. R. Blake and to Mr. G. Wilkins for excellent technical assistance.

REFERENCES

BAKER, E. E., SUMNER, H., FOSTER, L. E., MEYER, F. AND MEYER, K. F.—(1952) *J. Immunol.*, **68**, 131.
BOAS, N. F.—(1953) *J. biol. Chem.*, **204**, 553.
BURROWS, T. W. AND BACON, G. A.—(1956) *Brit. J. exp. Path.*, **37**, 481.
COCKING, E. C., KEPPIE, J., WITT, K. AND SMITH, H.—(1960) *Ibid.*, **41**, 460.
DAVIES, D. A. L.—(1956) *Biochem. J.*, **63**, 105.
DISCHE, Z.—(1953) *J. biol. Chem.*, **204**, 983.
FISKE, C. H. AND SUBBAROW, Y.—(1925) *Ibid.*, **66**, 375.
FOLCH, J., LEES, M. AND SLOAN-STANLEY, G. H.—(1954) *Fed. Proc.* **13**, 209.
KEPPIE, J., COCKING, E. C. AND SMITH, H.—(1958) *Lancet*, i, 246.
PRIDHAM, J. B. AND JONES, J. K. N.—(1954) *Biochem. J.*, **58**, 288.
SMITH, H., KEPPIE, J., COCKING, E. C. AND WITT, K.—(1960) *Brit. J. exp. Path.*, **41**, 452.
SOMOGYI.—(1952) *J. biol. Chem.*, **195**, 19.
SPIVACK, M. L., FOSTER, L., LARSON, A., CHEN, T. H., BAKER, E. E. AND MEYER, K.F.—(1958) *J. Immunol.*, **80**, 132.
WEIBULL, C.—(1957) *Acta chem. scand.*, **11**, 881.