NEW ANTIGENIC COMPONENT OF *PASTEURELLA PESTIS* FORMED UNDER SPECIFIED CONDITIONS OF pH AND TEMPERATURE

SHLOMO BEN-EFRAIM, MOSHE ARONSON, AND LEAH BICHOWSKY-SLOMNICKI

Israel Institute for Biological Research, Nes Ziona, Israel, and The Weizmann Institute of Science, Rehovot, Israel

Received for publication October 25, 1960

Aronson and Bichowsky-Slomnicki, (1960) reported that cells of *Pasteurella pestis* incubated at a pH below 6.7 and a temperature about 35 C undergo a change in their surface charge demonstrable by a decreased electrophoretic mobility.

The present communication presents evidence that this decrease in mobility is associated with the formation of a specific antigenic surface component. The presence of this antigen in various strains of *P. pestis*, as well as its relation to other known antigenic fractions of *P. pestis*, has been studied. Also, the possible contribution of this antigen to the pathogenesis of plague has been investigated.

MATERIALS AND METHODS

Strains. Strain TRU, avirulent, was used as the prototype strain in most of these experiments. Other strains of *P. pestis* tested were the avirulent strains A1122, EV76, TS, TSR, and the virulent strains 8776, 243, Kimberley, M23, and MP6.

The sources of these strains were as follows: strains EV76, 243, and A1122 (Jawetz) were kindly supplied by G. Girard, Institute Pasteur, Paris, France. Strain MP6 and the mutant strain M23 derived from it were obtained through the courtesy of T. W. Burrows, Porton, England. Strain TSR was kindly supplied by D. A. L. Davies, Porton, England. Strains TRU (Schultze) and TS (Otten) were supplied by the Department of Bacteriology, Hebrew University, Jerusalem, Israel, the Kimberley strain originated from the Institute Oswaldo Cruz, Rio de Janeiro, and strain 8776 was obtained from the National Type Culture Collection, England. The mouse intraperitoneal LD_{50} of the virulent strains was 10 to 200 cells.

Media. Stock cultures were maintained at 2 to 4 C on blood agar base (Difco) containing 2% defibrinated rabbit blood. Cultures for use in the experiments were grown in 2% liquid tryptose medium (Difco). Modified Englesberg medium (Englesberg and Levy), 1954; Aronson and Bichowsky-Slomnicki, 1960) was used as the standard incubation medium. Tryptose broth containing 1.5% agar (Difco) was used for plate counts.

Preparation of cultures. Test tubes containing 2 ml of liquid tryptose medium were inoculated with organisms from a stock culture and incubated under constant shaking at 28 C for approximately 20 hr. This culture was transferred in 1.5-ml amounts to Kjeldahl flasks containing 150 ml of the same medium and incubated under the same conditions. Twenty-hour-old cultures were harvested, resuspended in modified Englesberg medium to a concentration of about 5×10^8 cells/ml, and incubated in a water bath under aeration.

Measurements of electrophoretic mobility were performed as described previously (Aronson and Bichowsky-Slomnicki, 1960).

Experimental animals. Albino Swiss mice, 16 to 18 g, and rabbits, 2 to 2.5 kg, were used.

Preparation of immune sera. The immune serum used in the majority of experiments was prepared by injecting rabbits subcutaneously with living cells of the TRU strain, previously incubated at 37 C, pH 6, in modified Englesberg medium. Immune sera were also prepared by injecting rabbits with living cells of TRU, EV76, and M23 strains previously incubated at 37 C, pH 7, in modified Englesberg medium, or living cells of strains A1122 or TS from tryptose cultures grown at 28 C, pH 7.

Each rabbit received 5 to 10 injections, of about 1×10^9 cells each, at 7-day intervals. Bleeding was performed by cardiac puncture, 7 days after the last injection.

In the case of mutant M23, immunization was carried out by 5 injections of cells mixed with 5 mg of dihydrostreptomycin sulfate (RAFA, Jeru-

salem, Israel) in each injection, followed by 3 injections without the drug.

Mice were immunized by 2 subcutaneous injections of 0.5 ml each at 21-day intervals. Cells of strains TRU or EV76 at a concentration of about 1×10^7 per ml were used for each injection. The first injection was given by mixing the antigen with complete Freund's adjuvant (Difco) in proportion 1:1. The second injection contained cells only. Cells grown at 28 C, pH 7, as well as cells after incubation at 37 C, pH 6, and 37 C, pH 7, were used. Some groups of mice used for immunization received injections of living cells. All the others were immunized with cells killed by one of the following procedures: (i) ethyl alcohol 33.5%, (ii) formalin 0.5%, (iii) heating for 30 min at 56 C, and (iv) cold acetone, 9 volumes per 1 volume of cells. Treatments (i) and (ii) were carried out at 37 C for 24 hr, whereas treatment (iv) was at -20 C for the same period. All the cell suspensions were centrifuged and resuspended in physiological saline.

Preparation of adsorbed sera. Nondiluted sera were mixed with living packed cells and incubated for 3 hr at 37 C. The cells were removed by centrifugation at 4,000 rev/min for 20 min. This procedure was repeated until no reaction was detected by the gel diffusion technique between the adsorbed serum and the cells used for adsorption.

Gel diffusion tests. In gel diffusion tests, 1.5% agar (Difco), 0.5% NaCl, and 10^{-4} thiomersal (B. D. H. Laboratory Chemicals Division) were employed.

Assays were conducted by a technique of double diffusion in agar plates (Ouchterlony, 1953). In some experiments the Jennings technique was used (Jennings and Malone, 1954).

The diffusion plates were prepared by using special frames of agar cutters which permitted good reproducibility. The plates were closed by plasticine and left up to 3 weeks at 30 to 35 C. Photographs were made under standard conditions of light intensity, distance, and time exposure. Living cells in concentrations of about 1×10^{10}/ml, and undiluted sera were used. The reactants were introduced in each container in 0.1-H to 0.3-ml amounts.

Viable cell counts were made by the usual plating method.

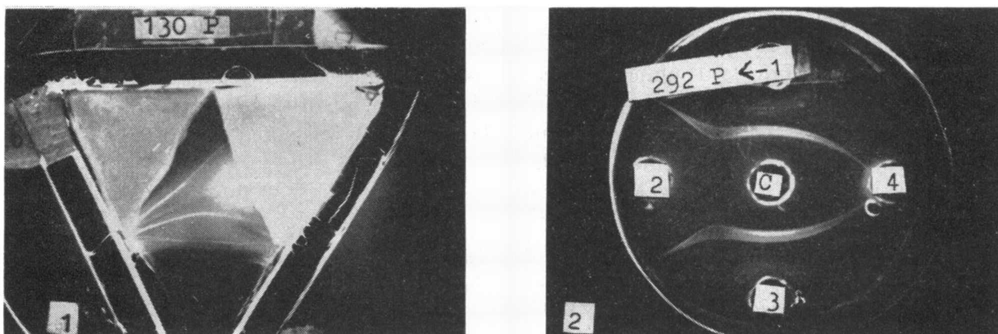
Virulence tests. The LD_{50} in mice was calculated by Karber's method (Fisher Probit analysis; Finney (1952)). Four to five groups, each containing 5 to 10 mice, were used in every experiment. The mice were inoculated intraperitoneally with 0.5-ml quantities of standard suspensions. The mortality was noted up to the 14th day.

RESULTS

Antigenic changes associated with changes in electrophoretic mobility of P. pestis. To determine whether the change in electrophoretic mobility, occurring during the incubation of *P. pestis* at 37 C, pH 6 (Aronson and Bichowsky-Slomnicki, 1960), is associated with a change in the antigenic structure of the cells, the following experiments were performed:

Cells of strain TRU were incubated at 37 C, pH 6, or 37 C, pH 7, under standard conditions. The antigenic composition of these cells, as well as of cells derived from the culture grown at 28 C, pH 7, was examined by use of the gel diffusion technique. The antiserum was prepared by immunizing rabbits with living cells of strain TRU previously incubated at 37 C, pH 6. The results of these experiments (Figs. 1, 2, 3, 4, 5, 6) show that an antigenic component was found in cells incubated at 37 C, pH 6, that was absent from cells grown or incubated under other conditions. Because the formation of this component occurred at values approaching pH 6, it was designated "pH 6 antigen." The specificity of this antigen was demonstrated by diffusion against the immune serum previously adsorbed by cells of strain TRU incubated at 37 C, pH 7. The appearance of this antigen was invariably associated with a pronounced decrease in the electrophoretic mobility of the cells.

Presence of pH 6 antigen in various strains of P. pestis. The virulent strains 8776, 243, Kimberley, MP6, M23, and the nonvirulent strains A1122, EV76, TS, TSR, were incubated at 37 C, pH 6, or 37 C, pH 7, under standard conditions. Each of the strains was examined by the gel diffusion technique against TRU 37 C, pH 6, immune serum. The pH 6 antigen was found in all the strains tested after incubation at 37 C, pH 6, but not when the strains were incubated at 37 C, pH 7, or grown at 28 C, pH 7. The antigen was formed by both smooth and rough strains. Colonies grown from cells of strains TRU and TSR that contained the pH 6 antigen were invariably rough, whereas colonies grown from



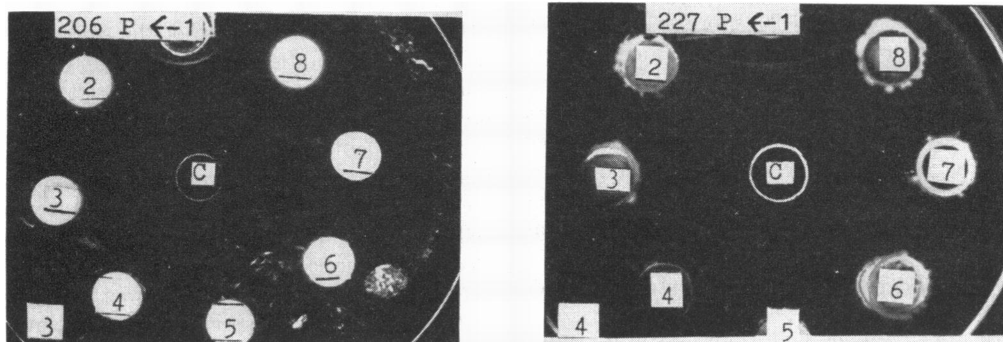
Figs. 1 and 2. Specificity of pH 6 antigen to TRU 37 C, pH 6, incubated cells. Living cells were used.

Fig. 1. Left: TRU 37 C, pH 6, cells; *right:* TRU 37 C, pH 7, cells; *bottom:* ISe.*

Fig. 2. 1: ISe; *2:* TRU 37 C, pH 6, cells; *3:* AISe;† *4:* TRU 37 C, pH 7, cells; *C* (center): TRU 37 C, pH 6, cells.

* ISe: rabbit immune serum prepared by injecting TRU living cells containing pH 6 antigen.

† AISe: ISe absorbed by *TRU* 37 C, pH 7, living cells.



Figs. 3 and 4. Presence of pH 6 antigen in various strains of *Pasteurella pestis*. Living cells were used.

Fig. 3. Lack of pH 6 antigen in 28 C grown cultures. *1:* TRU 37 C, pH 6, cells; *2–8:* 28 C cultures of strains MP6, 8776, Kimberley, A1122, M23, 243, and EV76; *C* (center): AISe (*see* Fig. 1.).

Fig. 4. Lack of pH 6 antigen in cells incubated at 37 C, pH 7. *1:* TRU 37 C, pH 6, cells; *2–4:* 37 C, pH 7, cells of strains 243, 8776, Kimberley; *5:* TRU 37 C, pH 6, cells; *6–8:* 37 C, pH 7, cells of TSR, MP6, EV76; *C* (center): AISe.

cells of the TS strain that contained the antigen were smooth in appearance (*see* Figs. 15 and 16).

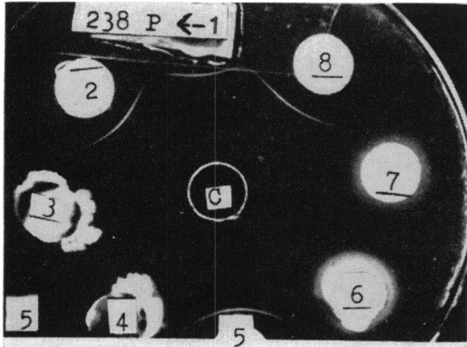
Effect of pH and temperature on the formation of pH 6 antigen. TRU cells were incubated for 3 hr at 37 C in Englesberg medium adjusted to pH levels varying from 4.7 to 7.2, and the presence or absence of the pH 6 antigen, as well as the electrophoretic mobility of the cells, was ascertained. The pH 6 antigen could only be found in cells incubated at pH values between 5.0 and 6.7.

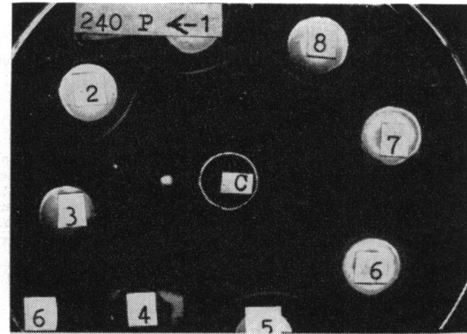
Studies on the effect of temperature showed that the pH 6 antigen was formed at 35 to 41 C within the pH limits 5.0 to 6.7. The results of these experiments are illustrated in Figs. 7 and 8.

The multiplication rate of cells incubated at 37 C, pH 6, and 37 C, pH 7, was determined by plate counts. A slight increase in the viable count of these cultures occurred within 3 hr, this being more marked in suspensions incubated at 37 C, pH 7 (Table 1).

The formation of pH 6 antigen was also studied in 20-hr-old cultures grown in modified Englesberg medium at 37 C, pH 6, or 37 C, pH 7, under constant aeration. Formation of the antigen and decrease in electric charge of the cells occurred only in cultures grown at 37 C, pH 6.

Presence of pH 6 antigen in killed cells. Cells of strains TRU and EV76 containing the pH 6 antigen were killed by one of several types of

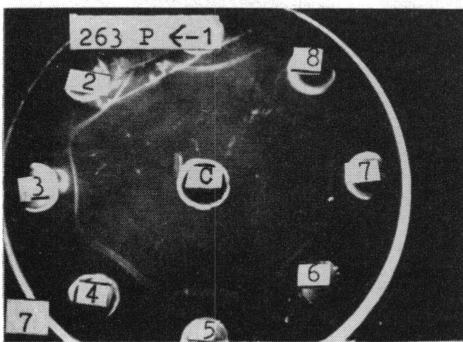


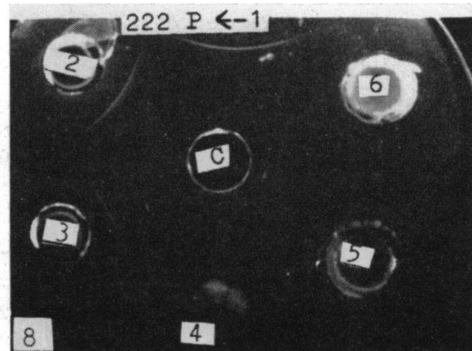


Figs. 5 and 6. Comparisons between *Pasteurella pestis* 28 C cultures, 37 C, pH 7, and 37 C, pH 6, incubated cells. Living cells were used.

Fig. 5. TRU and TSR strains. *1:* TRU 37 C, pH 6, cells; *2:* TSR 37 C, pH 6, cells; *3:* TSR 37 C, pH 7, cells; *4:* TRU 37 C, pH 7, cells; *5:* TRU 37 C, pH 6, cells; *6:* TRU 28 C culture; *7:* TSR 28 C culture; *8:* TSR 37 C, pH 6, cells; *C* (center): AISe (*see* Fig. 1).

Fig 6. TRU and MP6 strains. *1:* TRU 37 C, pH 6, cells; *2:* MP6 37 C, pH 6, cells; *3:* MP6 37 C, pH 7, cells; *4:* TRU 37 C, pH 7, cells; *5:* TRU 37 C, pH 6, cells; *6:* TRU 28 C culture; *7:* MP6 28 C culture; *8:* MP6 37 C, pH 6, cells; *C* (center): AISe.





Figs. 7 and 8. Formation of pH 6 antigen at different pH values and temperatures.

Fig. 7. Different pH levels. *1–8:* TRU living cells incubated at 37 C and pH 5.0, 5.4, 5.8, 6.2, 6.5, 6.7, 6.8, and 7.2; *C* (center): AISe (*see* Fig. 1).

Fig. 8. Different temperatures. *1–6:* TRU living cells incubated at 37 C, pH 6; 39 C, pH 6; 41 C, pH 6; 41 C, pH 7; and 39 C, pH 7; and 37 C, pH 7; *C* (center): AISe.

treatment; formalin, acetone, heat, and ethyl alcohol were used in the case of TRU cells, and formalin in the case of EV76 cells. Cells thus treated still contained the pH 6 antigen in the first days after the treatment but lost it during prolonged storage. This lability of the pH 6 antigen in killed cells was revealed by both gel diffusion assays (Figs. 9 and 10) and electrophoretic mobility measurements. Good correlation was invariably found between the presence of pH 6 antigen and the maintenance of decreased electrophoretic mobility in killed cells.

Injection into mice of killed cells still containing the pH 6 antigen elicited the formation of specific antibodies as illustrated in Fig. 11.

Formation of pH 6 antigen in animals infected with P. pestis cells. The formation of pH 6 antigen in infected animals was investigated in two sets of experiments.

In the first series, sera of immunized animals were tested for their antibody content. Antibodies against the pH 6 antigen were found in the sera of rabbits immunized with living cells of various strains of *P. pestis* not containing the antigen after growth in vitro. The cells used for immunization were from cultures either grown at 28 C,

TABLE 1

Electrophoretic mobility and rate of multiplication of TRU cells after incubation at different temperatures and pH values in modified Englesberg medium

Incubation Temp	pH	Time of Incubation	Electrophoretic Mobility	No. of Bacteria	Relative Multiplication Rate*
		hr	*μ:sec:volt:cm*	$\times 10^9/ml$	
28 C culture		0	—	1.1	1
37 C	6	1	−0.9	1.1	1
		2	−0.62	1.35	1.2
		3	−0.46	2	1.8
	7	1	−1.16	1.5	1.4
		2	−1.10	2.25	2
		3	−1.17	3.62	3.3
39 C	6	1	−0.93	1	1
		2	−0.70	1.55	1.4
		3	−0.48	2.1	1.9
	7	1	−1.15	1	1
		2	−1.19	1.95	1.8
		3	−1.17	2.85	2.6
41 C	6	1	−0.83	1.05	1
		2	−0.69	1	1
		3	−0.47	1.9	1.7
	7	1	−1.24	1.35	1.2
		2	−1.18	2.05	1.9
		3	−1.10	2.9	2.6

* As compared with the original culture.

pH 7 (as in the case of strains TS, A1122), or incubated at 37 C, pH 7 (strains TRU, M23, and EV76).

The same results were obtained when mice were immunized with living cells of strains TRU and EV76 grown at 28 C, pH 7, or incubated at 37 C, pH 7. These data are illustrated in Figs. 12 (131P) and 13 (386P).

To show that the appearance of antibody is directly related to the formation in vivo of pH 6 antigen, mice were immunized with formalinized cells. Cultures of strain EV76 grown at 28 C, pH 7, or cells of the same strains incubated at 37 C, pH 6, or 37 C, pH 7, were used. Antibodies against pH 6 antigen were found only in mice which had been immunized with formalinized cells containing the specific antigen (Fig. 11).

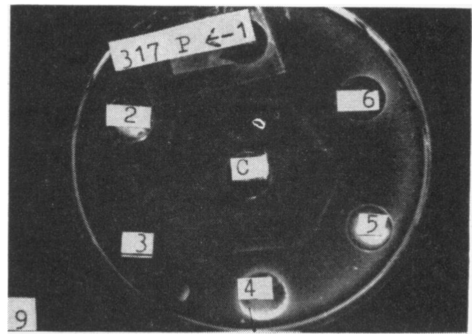


Fig. 9. Presence of pH 6 antigen in recently killed cells TRU 37 C, pH 6, incubated cells. *1:* Living cells; *2:* formalinized cells; *3:* heated cells; *4:* living cells; *5:* alcohol-killed cells; *6:* acetone-killed cells; *C* (center): AISe (*see* Fig. 1).

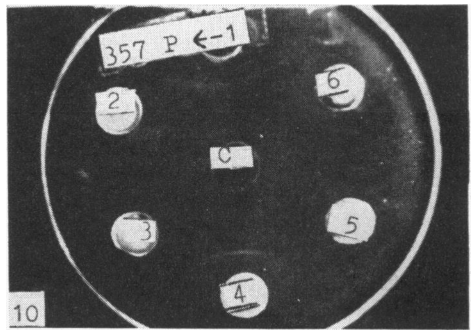


Fig. 10. Disappearance of pH 6 antigen during long storage of TRU 37 C, pH 6, killed cells. *1:* Living cells; *2:* formalinized cells; *3:* heated cells; *4:* living cells; *5:* acetone-killed cells; *6:* alcohol-killed cells; *C* (center): AISe (*see* Fig. 1).

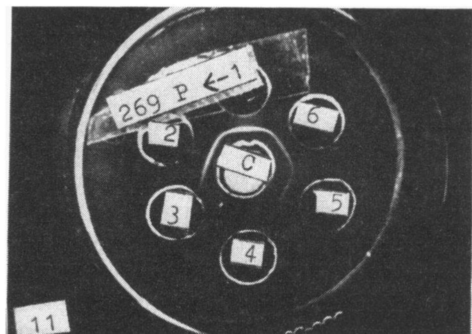


Fig. 11. Formation of antibodies against pH 6 antigen in mice injected with formalinized cells. *1:* ISe (*see* Fig. 1); *2–6:* mice immune sera prepared by injecting EV76 37 C, pH 6, cells; EV76 37 C, pH 7, cells; EV76 28 C culture; EV76 37 C, pH 6, cells; and TRU 37 C, pH 6, cells; *C* (center): TRU 37 C, pH 6 living cells.

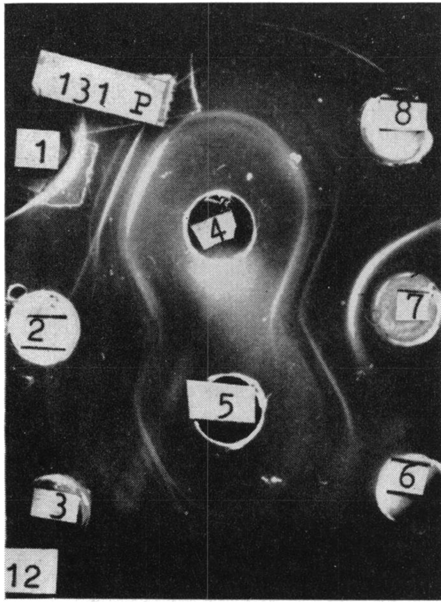


Fig. 12. Presence of antibodies against pH 6 antigen in rabbits immunized with TRU 37 C, pH 7, living cells. Diffusion against TRU living cells. *1:* 37 C, pH 6, cells; *2:* 37 C, pH 7, cells; *3:* 28 C culture; *4:* ISe (*see* Fig. 1); *5:* 37 C, pH 7, immune serum; *6:* 37 C, pH 7, cells; *7:* 37 C, pH 6, cells; *8:* 28 C culture.

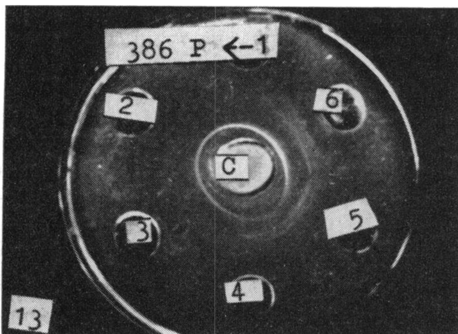


Fig. 13. Presence of antibodies against pH 6 antigen in rabbits immunized with various living cells of *Pasteurella pestis* lacking the antigen. *1:* AISe (*see* Fig. 1); *2:* TRU 37 C, pH 7, immune serum; *3:* A1122 28 C culture, immune serum; *4:* EV76 37 C, pH 7, immune serum; *5:* M23 37 C, pH 7, immune serum; *6:* TS 28 C culture, immune serum; *C* (center): TRU 37 C, pH 6, living cells.

In the second series of experiments, the presence of pH 6 antigen in *P. pestis* from the livers and spleens of infected mice was examined. Mice were inoculated intraperitoneally with about

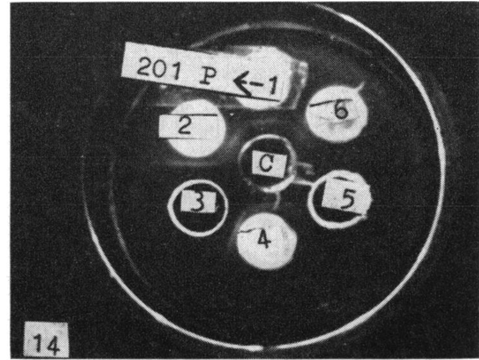


Fig. 14. Presence of pH 6 antigen in MP6 cells from homogenates of liver and spleen of infected mice. *1:* MP6 pH 6 cells, spleen and liver homogenates; *2:* MP6 pH 7 cells, spleen and liver homogenates; *3:* TRU 37 C, pH 7, serum; *4:* MP6 pH 6 cells; spleen and liver homogenates; *5:* AISe (*see* Fig. 1) *6:* MP6 pH 6 cells, spleen and liver homogenates; *C* (center): ISe. (*Note*: Presence of pH 6 antigen in MP6 cells from the homogenates; of pH 6 antibody in sera of rabbits immunized with TRU 37 C, pH 7, living cells.)

50 cells of the virulent strain MP6 which had previously been incubated at 37 C, pH 7 (i.e., cells devoid of pH 6 antigen). Four days after incubation, the animals were sacrificed and homogenates were prepared from their livers and spleens in which large numbers of *P. pestis* were found microscopically. The homogenates were examined by gel diffusion technique against TRU 37 C, pH 6, immune serum, both before and after adsorption with 37 C, pH 7, cells. A specific line of the pH 6 antigen appeared in the homogenates tested as shown in Fig. 14.

Comparative virulence tests in mice. Using cells incubated at 37 C, pH 7, or 37 C, pH 6, the LD_{50} for mice of *P. pestis* strain MP6 was determined by intraperitoneal injection. As illustrated in Tables 2 and 3, and in the statistical supplement, the rate of mortality proved to be significantly higher during the first 4 days following infection with 37 C, pH 6, incubated cells, as compared to those injected with 37 C, pH 7, cells. However, beyond the 4-day period the incidence of deaths in the two groups appeared similar.

DISCUSSION

The foregoing data provide evidence for the suggestion (Aronson and Bichowsky-Slomnicki, 1960) that the change in the surface charge occurring upon incubation of *P. pestis* at a pH

TABLE 2

Virulence for mice of Pasteurella pestis (MP6) organisms containing the pH 6 antigen

Expt No.	No. of Cells Injected	No. of Deaths in Days			Mortality					
					4 Days			14 Days		
		2	3	4	D/T*	%M†	LD_{50}	D/T	%M	LD_{50}
I	230	0	4	1	5/5	100		5/5	100	
	77	0	2	3	5/5	100		5/5	100	
	26	0	1	3	4/5	80	5	5/5	100	3
	9	0	0	3	3/5	60		4/5	80	
	3	0	0	3	3/5	60		3/5	60	
II	26	1	4	4	9/10	90		10/10	100	
	9	0	3	2	5/10	50	10	6/10	60	8
	3	0	0	0	0/10	0		0/10	0	
	1	0	0	0	0/10	0		0/10	0	
III	63	0	10		10/10	100		10/10	100	
	21	0	5	5	10/10	100		10/10	100	
	7	0	2	3	5/10	50	6	7/10	70	4
	2	0	0	1	1/10	10		3/10	30	
	<1	0	0	0	0/10	0		0/10	0	
IV	75	0	7	2	9/10	90		10/10	100	
	25	0	3	6	9/10	90		10/10	100	
	8	0	0	3	3/10	30	11	5/10	50	4
	3	0	1	0	1/10	10		6/10	60	
	1	0	0	1	1/10	10		1/10	10	
Mean LD_{50}‡							7			4

* D/T = total number dead out of total number tested.

† %M = per cent mortality.

‡ Mean LD_{50} = calculated from a total number of 165 mice.

below 6.7 and a temperature above 35 C, is due to the formation of a specific antigenic surface component. Its appearance was invariably associated with a pronounced decrease in the electrophoretic mobility of the cells in all strains of *P. pestis* tested. The synthesis of this antigen by all the strains of *P. pestis* that were tested, and the conditions required for its formation, indicate that the antigen is distinct from previously reported surface antigens of *P. pestis*, i.e., VW fraction (Burrows and Bacon, 1956), capsular antigen (fraction I, Baker et al., 1947; Chen and Meyer, 1954; Meyer, 1950), and envelope antigen (Schutze, 1932; Amies, 1951).

Burrows et al. have reported that the formation of VW antigens by *P. pestis*, closely associated with the acquisition of resistance to phagocytosis, occurs only in virulent strains (Burrows and Bacon, 1956; Burrows, 1957). In contrast the pH 6 antigen is readily produced by both virulent and avirulent strains. VW antigens are synthesized by virulent strains at 37 C, pH 7 (Burrows and Bacon, 1956), i.e., under conditions under which no formation of pH 6 antigen is observed.

Similar arguments apply against the identity of pH 6 antigen and fraction I. Strains such as TRU and M23 which cannot produce fraction I (Schutze, 1939; Meyer, 1950; Chen and Meyer, 1954; Burrows, 1957) are nevertheless capable of synthesizing the pH 6 antigen. Moreover, fraction I is formed under conditions (Fox and Higuchi, 1958) which do not allow the formation of pH 6 antigen.

The possible similarity between pH 6 antigen and the number 4 antigen of Davies et al. (Crumpton and Davies, 1956, 1957; Davies and

TABLE 3

Virulence for mice of Pasteurella pestis (MP6) organisms not containing the pH 6 antigen

Expt No.	No. of Cells Injected	No. of Deaths in Days			Mortality					
					4 Days			14 Days		
		2	3	4	D/T*	%M†	LD_{50}	D/T	%M	LD_{50}
I	380	0	2	3	5/5	100		5/5	100	
	127	0	1	3	4/5	80		5/5	100	
	42	0	0	3	3/5	60	25	4/5	80	13
	14	0	0	1	1/5	20		2/5	40	
	5	0	0	2	2/5	40		2/5	40	
II	90	0	1	3	4/10	40		10/10	100	
	30	0	2	2	4/10	40	58	6/10	60	21
	10	0	0	0	0/10	0		1/10	10	
	3	0	0	1	1/10	10		1/10	10	
III	117	0	6	2	8/10	80		10/10	100	
	39	0	0	2	2/10	20	54	6/10	60	12
	13	0	0	2	2/10	20		5/10	50	
	4	0	0	0	0/10	0		5/10	50	
IV	126	0	6	3	9/10	90		10/10	100	
	42	0	0	6	6/10	60	20	8/10	80	11
	14	0	0	5	5/10	50		7/10	70	
	5	1	1	1	2/10	20		2/10	20	
Mean LD_{50}‡							35			14

* D/T = total number dead out of total number tested.
† %M = per cent mortality.
‡ Mean LD_{50} = calculated from a total number of 145 mice.

Crumpton, 1958) has also been considered since this antigen is formed at a pH close to neutrality (Crumpton and Davies, 1957). According to these authors, "antigen 4" is associated with smooth colony forms of *P. pestis* and is never found in rough strains such as TSR (Crumpton and Davies, 1956, 1957; Davies and Crumpton, 1958). However, strains TRU and TSR, are capable of synthesizing the pH 6 antigen and the colonies isolated by plating cells of these strains which contain the antigen are invariably rough. It may be pointed out that the TSR strain tested was characterized by D. A. L. Davies as lacking antigen 4. Direct comparison by gel diffusion technique between an antigen 4 fraction kindly sent to us by D. A. L. Davies and TRU cells containing pH 6 antigen confirmed the non-identity of these antigens.

All the virulent and avirulent strains of *P. pestis* tested by us were able to synthesize the pH 6 antigen in suitable conditions. These findings lead us to suggest that the ability to synthesize this antigen is one of the inherent properties of all strains of *P. pestis*.

The observation that pH 6 antigen is formed in animals infected with *P. pestis* that did not contain the antigen when they propagated in vitro, indicates that its presence may be related to the pathogenesis of plague. This is supported by the observation that mortality was increased in those groups of mice inoculated with virulent cells which contained the pH 6 antigen as compared with the mortality following inoculation with cells lacking the antigen. The final equalization of the number of deaths in the two groups may be explained by assuming the formation of sufficient pH 6 antigen during the infection. It may also be pointed out that the intracellular pH of the lymphatic tissue and of the monocytes, i.e., the sites of proliferation of *P. pestis*, is close

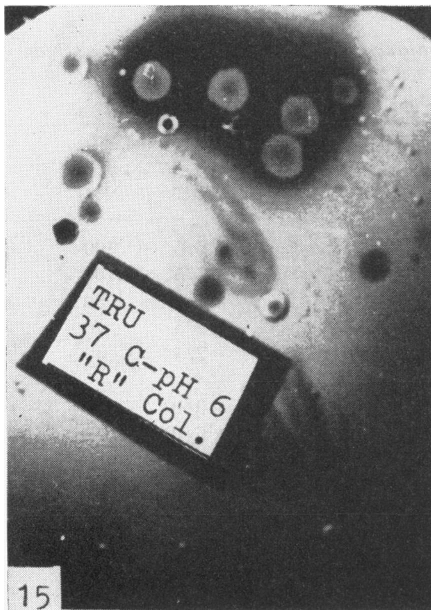


Fig. 15. Rough colonies grown from TRU cells containing the pH 6 antigen.

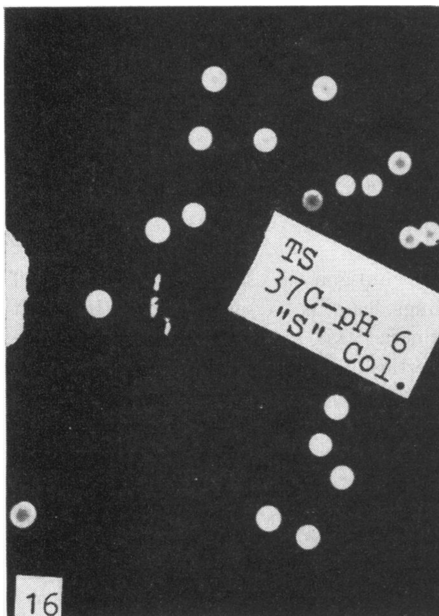


Fig. 16. Smooth colonies grown from TS cells containing the pH 6 antigen.

to that required for the formation in vitro of the pH 6 antigen (Dubos, 1954).

ACKNOWLEDGMENTS

The authors are grateful to D. A. L. Davies for the very valuable supply of "antigen 4" fraction and TSR strain, and to G. Girard and T. W. Burrows for kindly providing us with strains of *P. pestis* used in this work.

SUMMARY

An antigenic surface component called "pH 6 antigen" is formed by *Pasteurella pestis* under specified conditions of pH and temperature.

The formation of "pH 6 antigen" is closely associated with a change in the electric charge of the cells as expressed by decrease of their electrophoretic mobility.

Some aspects of the formation of this antigen have been considered, including the effect of pH and temperature, and the stability of the antigen to various treatments has been described.

Formation in vivo of pH 6 antigen was found to occur in rabbits and mice injected with *P. pestis*.

STATISTICAL ADDENDUM[1]

Analysis of difference between virulence of *Pasteurella pestis* (MP6) incubated at 37 C, pH 6, or 37 C, pH 7, is as follows.

Four paired estimates of LD_{50} were made at four different dates. Five mice per dose were used at the first estimation, whereas 10 were used later. Deaths were counted at the 4th and 14th days after injection.

Karber's method (Finney, 1952) was used for the estimations. No attempt was made to estimate the standard error (SE) in view of the plan to perform an analysis of variance.

The following results were obtained:

Days After Injection	Expt No.	Log LD_{50}		LD_{50}	
		pH 6	pH 7	pH 6	pH 7
4	1	0.69	1.39	5	25
	2	0.99	1.76	10	58
	3	0.80	1.73	6	54
	4	1.02	1.29	11	20
14	1	0.50	1.10	3	13
	2	0.89	1.33	8	21
	3	0.61	1.07	4	12
	4	0.59	1.05	4	11

The factors involved were coded as follows: A, pH; B, death times; C, experiment date; AB, interaction of pH with death times; AC, interaction of pH with experiment date; BC, interaction of death time with experiment date.

[1] D. Yassky, Israel Institute for Biological Research, Nes Ziona, Israel.

All relevant means of log LD50 were calculated:

Days	pH 6	pH 7	
4	0.8750	1.5425	1.20875
14	0.6475	1.1375	0.89250
	0.76125	1.34000	1.050625

Expt No...	1	2	3	4	
pH 6	0.595	0.940	0.705	0.805	0.76125
pH 7	1.245	1.545	1.400	1.170	1.340000
	0.9200	1.2425	1.0525	0.9875	1.050625

Expt No...	1	2	3	4	
4 days	1.040	1.375	1.265	1.155	1.20875
14 days	0.800	1.110	0.840	0.820	0.89250
	0.9200	1.2425	1.0525	0.9875	1.050625

We have also the following mean LD50 values:

Days	pH 6	pH 7	
4	7	35	16
14	4	14	8
	6	22	11

The only factors which are of interest are A, the pH values, and its interactions AB and AC. To isolate these factors a full analysis of variance (Hald, 1952) was performed with the third order interaction as an estimate of error variance:

Source	*d.f.*	*s.s.*	*m.s.*	*F*	*P*	
A	1	1.34	1.34	67.00	99.5	*P* 99.9
B	1	0.40	0.40	20.00	97.5	*P* 99.0
C	3	0.23	0.077	3.85	70	*P* 90
AB	1	0.03	0.03	1.50		
AC	3	0.06	0.02	1.00		
BC	3	0.02	0.007			
Residue	3	0.06	0.020			
Total......	15	2.15				

(*d.f.* = Degrees of freedom; *s.s.* = sums of squares; *m.s.* = mean square; *F* = variance ratio; and *P* = probability of *F*.)

A more reliable estimate of significance can be obtained by pooling:

Source	*d.f.*	*s.s.*	*m.s.*	*F*	*P*	
A	1	1.34	1.3400	4.58	90	*P* 95
B	1	0.40	0.4000	1.37		
AB	1	0.03	0.03			
Residue	12	0.38	0.2923			
Total......	15	2.15				

We can therefore conclude that the A factor (pH values) has more influence on the results than all other factors. It is also highly significant when other sources of variation are accounted for.

REFERENCES

AMIES, C. R. 1951 The envelope substance of *Pasteurella pestis*. Brit. J. Exptl. Pathol., **32**, 259–273.

ARONSON, M., AND L. BICHOWSKY-SLOMNICKI 1960 Temperature and pH dependent changes of electrophoretic mobility of *Pasteurella pestis*. J. Bacteriol., **79**, 734–740.

BAKER, E. E., H. SOMMER, L. E. FOSTER, E. MEYER, AND K. F. MEYER 1947 Antigenic structure of *Pasteurella pestis* and the isolation of a crystalline antigen. Proc. Soc. Exptl. Biol. Med., **64**, 139–141.

BURROWS, T. W. 1957 Virulence of *Pasteurella pestis*. Nature, **179**, 1246–1247.

BURROWS, T. W., AND G. A. BACON 1956 The basis of virulence in *Pasteurella pestis*; an antigen determining virulence. Brit. J. Exptl. Pathol., **37**, 481–493.

CHEN, T. H., AND K. F. MEYER 1954 Studies on immunization against plague. VII. A hemagglutination test with the protein fraction of *Pasteurella pestis*. A serologic comparison of virulent and avirulent strains with observations on the structure of the bacterial cells and its relationship to infection and immunity. J. Immunol., **72**, 282–298.

CRUMPTON, M. Y., AND D. A. L. DAVIES 1956 An antigenic analysis of *Pasteurella pestis* by diffusion of antigens and antibodies in agar. Proc. Roy. Soc. London, Ser. B, **145**, 109–134.

CRUMPTON, M. Y., AND D. A. L. DAVIES 1957 A protein antigen associated with smooth

colony forms of some species of *Pasteurella*. Nature, **180**, 863–864.

DAVIES, D. A. L., AND M. Y. CRUMPTON 1958 The protein antigens of *Pasteurella pestis*. Intern. Congr. Biochem., 4th Congr. (Vienna) Section 16–26, p. 195.

DUBOS, R. J. 1954 *Biochemical determinants of microbial diseases*. Harvard University Press, Cambridge, Mass.

ENGLESBERG, E., AND Y. B. LEVY 1954 Studies on immunization against plague. VI. Growth of *Pasteurella pestis* and the production of the envelope and other soluble antigens in a casein hydrolyzate, mineral glucose medium. J. Bacteriol., **67**, 438–449.

FINNEY, D. Y. 1952 *Probit analysis. A statistical treatment of the sigmoid response curve*. Cambridge University Press, London, England.

FOX, E. N., AND K. HIGUCHI 1958 Synthesis of the fraction I antigenic protein by *Pasteurella pestis*. J. Bacteriol., **75**, 209–216.

HALD, A. 1952 *Statistical theory with engineering applications*. John Wiley and Sons, Inc., New York.

JENNINGS, K. R., AND F. MALONE 1954 Rapid double diffusion precipitin analysis. J. Immunol., **72**, 411–418.

MEYER, K. F. 1950 Immunity in plague: a critical consideration of some recent studies. J. Immunol., **64**, 139–163.

OUCHTERLONY, O. 1953 Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. Acta Pathol. Microbiol. Scand., **32**, 231–240.

SCHUTZE, H. 1932 Studies in *B. pestis* antigens. I. The antigens and immunity reactions of *B. pestis*. Brit. J. Exptl. Pathol., **13**, 284–288.

SCHUTZE, H. 1939 Studies on *B. pestis* antigens as prophylactic agents. Brit. J. Exptl. Pathol., **20**, 235–244.