THE TYPE SPECIFICITY OF *HAEMOPHILUS PERTUSSIS*

By J. UNGAR, P. W. MUGGLETON and W. K. STEVENS
Glaxo Laboratories Ltd., Greenford, Middlesex

(With 1 Figure in the Text)

INTRODUCTION

Since Bordet & Gengou (1906) first described the morphological and cultural characteristics of *Haemophilus pertussis*, observations on the biological properties of different strains of the organism have not always been in agreement. It was early recognized by Bordet & Sleeswyck (1910) that certain variations in the characteristic appearance of the strains can occur, and they described two reversible 'states', I and II, of which only II could grow on plain nutrient agar without blood. Krumwiede, Mishulow & Oldenbusch (1923) also described two types, but Kristensen (1927) maintained that *H. pertussis* forms a single entity. Leslie & Gardner (1931), in their study of the toxigenic and agglutinogenic properties of their strains, concluded that four phases of *H. pertussis* exist, of which only phase I is virulent and toxigenic.

It is now universally recognized that under unfavourable laboratory conditions virulent strains can produce variants conforming to Leslie & Gardner's phases III and IV, but there would appear to be some lack of agreement as to the nature of the changes in the phase.

The problem of the existence of certain phases gained greater significance with attempts to put whooping-cough immunization on a solid basis. The failure in some early field trials to confer immunity was attributed partly to the use of ineffective vaccines, and this focused attention on the selection of strains for preparing a satisfactory one (Ungar, 1952).

In previous papers (Ungar & Muggleton, 1949; Ungar, James, Muggleton, Pegler & Tomich, 1950) we have described techniques (e.g. adsorption on aluminium phosphate, solubility in bile salts and sodium hydroxide solution, growth requirements in liquid semi-synthetic medium) by which freshly isolated, virulent *H. pertussis* strains can be differentiated from those avirulent forms that have been repeatedly subcultured on laboratory culture media. This work has been extended to cover the investigation of more than 200 strains from widely different sources. It was undertaken in order to study the extent of variation found in the biological characteristics of *H. pertussis* isolated from cases of whooping cough, and to see whether strains could be isolated resembling those described by Leslie & Gardner as of phase III or IV. The investigation was extended to include eleven strains of *H. parapertussis*, as first isolated by Eldering & Kendrick (1938) from children suffering from a condition resembling mild pertussis. The results of this study are recorded here.

https://doi.org/10.1017/S0022172400036949 Published online by Cambridge University Press

METHODS AND STRAINS USED

Strains

Altogether 197 strains of *H. pertussis* were used in the investigation; of these 187 were freshly isolated from cases of whooping cough in London, Manchester, Leeds, Cardiff or Oxford, together with a small number from the United States. The strains were freeze-dried at the first subculture from isolation. The remaining ten strains had been regularly subcultured in our laboratories from periods varying from 6 months to 3 years. These as well were freeze-dried at the beginning of the investigation. Eleven strains of *H. parapertussis* were included and these also were freeze-dried at the beginning of the experiments. The cultures were recovered from the freeze-dried state for each experiment.

Morphology

At the time of freeze-drying, the appearance of the cells of each culture was investigated by the microscopical examination of smears prepared from 48 hr. Bordet–Gengou medium cultures and stained by Gram's method. The cells from both 24 hr. and 48 hr. cultures on the same medium were examined for capsules by the methods of Lawson (1940), Klieneberger-Nobel (1948) and Jeffery (1948). In addition, some strains were examined by electron micrography of preparations shadowed with gold-palladium.

Growth requirements

Tests for growth of the strains in Cohen & Wheeler's (1946) liquid semi-synthetic medium with and without the addition of soluble starch were made in the way described previously (Ungar *et al.* 1950). In addition, all strains were tested for their ability to grow on plain nutrient agar without the addition of blood. All of the liquid cultures, together with those from Bordet–Gengou medium, were examined for characteristics of growth, and records were kept of colony size and appearance, characteristic pigmentation, etc.

Solubility and adsorption

The degrees of adsorption on aluminium phosphate floccules and of solubility in 1 N sodium hydroxide and 10% sodium deoxycholate solutions were measured by the methods previously described (Ungar & Muggleton, 1949).

Virulence in mice

The strains were tested by the method of Burnet & Timmins (1937) on an inbred strain of albino mice (descended from Strong A2) that we have found to give consistent results and to be uniformly susceptible to pertussis infection. Suitable dilutions of saline-washed suspensions of the growth from 48 hr. cultures on Bordet–Gengou medium were administered intranasally to groups of six mice under ether anaesthesia, the suspensions being standardized by opacity measurement on the Spekker photoelectric potentiometer. The numbers at each dose-level remaining alive were recorded for ten days.

Toxic properties of the cells

Cells from 48 hr. cultures on Bordet–Gengou medium were scraped off into saline to give suspensions that were standardized by opacity and from which serial dilutions were prepared. The live suspensions were then injected intraperitoneally into groups of six mice and intradermally into three depilated rabbits. The minimum lethal dose of cells in the mice and the minimum dose to give a standard 10 mm. diameter zone of inflammation with central necrosis in the rabbit were recorded.

Haemagglutinin estimation

Suspensions prepared from 48 hr. cultures on Bordet–Gengou medium were washed 3 times in saline in the centrifuge, standardized by opacity and diluted to $10{,}000 \times 10^6$ organisms/ml. Doubling dilutions of 0·5 ml. amounts were made in $2 \times \frac{1}{2}$ in. glass tubes and 0·1 ml. of 2% washed suspension of human erythrocytes was added to each tube, the contents of which were then mixed. The human blood was obtained aseptically by venepuncture, with 1/10,000 heparin as an anticoagulant. After 2 hr. incubation at 37° the tests were read by examining the tubes for 'sedimentation pattern', the greatest dilution of the $10{,}000 \times 10^6$ suspension to give positive agglutination being recorded.

Agglutination and agglutinin absorption tests

The strains were tested for agglutinability by the method previously described (Ungar & Muggleton, 1949). The same standard sera obtained from hyperimmunized rabbits were used throughout the tests.

In the agglutinin absorption tests, suspensions of the cells standardized to $200{,}000 \times 10^6$/ml. were mixed with equal volumes of immune sera, the sera being diluted with saline to give with a standard homologous agglutinating suspension an agglutination titre of about 1/2000. The cells were left in contact with the sera for 4 hr. at 37° C. and were then removed in the centrifuge. The remaining agglutinins in the sera were estimated by agglutination tests against the standard homologous suspensions.

Sensitivity to antibiotics

Cultures were made on plates of Bordet–Gengou medium to which penicillin, streptomycin (sulphate), chloramphenicol and aureomycin had been added in serial two-fold dilutions from 0·1 to 100 μg. or units (penicillin)/ml. The cultures were examined for inhibition of growth after 48 hr. incubation at 37° C.

RESULTS

Morphology and growth requirements

Of the 187 freshly isolated ('virulent') strains examined, all were found to produce small (0·5 mm. diameter) colonies on Bordet–Gengou medium in 48 hr. without haemolysis or discoloration of the medium. Stained films always showed the cells to be typical bacilli ($0{\cdot}3 \times 1{\cdot}5\ \mu$). None of the strains grew on plain agar without blood and none grew in liquid semi-synthetic medium from which the

starch had been omitted. When starch was included in the medium, all of the strains grew with a surface pellicle in static cultures, giving maximum growth in 6–7 days. By contrast, all of the laboratory strains ('avirulent') produced larger (2 mm. diameter) flattened colonies on Bordet–Gengou medium, although still without haemolysis or discoloration of the medium; all grew easily on plain agar and all grew in the liquid medium without starch. In the liquid medium the growth was diffuse in static cultures and reached a maximum in 4–5 days. On Bordet–Gengou medium the cells of the 'avirulent' strains were always characteristically coccoid in appearance. The *H. parapertussis* strains were obviously bacilli when

Table 1. *Growth requirements and morphology of pertussis strains*

	H. pertussis (virulent type)	*H. pertussis* (avirulent type)	*H. parapertussis*
No. of strains examined ...	187	10	11
Bordet–Gengou medium, 48 hr. cultures	Small hemispherical colonies (0·5 mm. in diameter)	Large flattened colonies (2 mm. diameter)	Large raised colonies (2–3 mm. diameter)
	No haemolysis or pigment produced	No haemolysis or pigment produced	Slight haemolysis with dark pigmentation
	Organisms show 'capsules'	Organisms show 'capsules'	Organisms show 'capsules'
	Obvious bacilli	Shortened to coccal forms	Obvious bacilli
Growth on plain agar	No growth	Good growth, no pigment	Good growth, slight pigmentation of the medium
Growth in Cohen & Wheeler's liquid medium. Static cultures	Grows with surface pellicle	Grows with diffuse turbidity	Grows with diffuse turbidity
	Maximum growth in 6–7 days	Maximum growth in 4–5 days	Maximum growth in 6–7 days
	No pigment	No pigment	Brown pigment produced
	No growth if starch omitted	Grows if starch omitted	Grows if starch omitted

grown on Bordet–Gengou medium, but in other respects they resembled the avirulent *H. pertussis* in behaviour. They could, however, be differentiated by the presence of brown pigment in the culture media.

All of the three types showed structures resembling capsules when stained by Lawson's (1940) method or by Jeffery's (1948) modification of Fleming's technique. By Klieneberger-Nobel's (1948) method, however, capsular structures could not be distinguished from slime. Examination by electron microscopy revealed in all three types structures suggesting the presence of capsules, but the significance of their appearance is uncertain, as they may have been artifacts.

The growth characteristics and nutritional requirements of the strains are summarized in Table 1.

Table 2 shows a summary of the results obtained in the tests of precipitability on aluminium phosphate floccules and solubility in 1 N sodium hydroxide and 10% sodium deoxycholate solutions. Only one of 187 freshly isolated strains failed to

be virtually completely precipitated by the insoluble phosphate. The complete precipitability and solubility of the virulent strains was a constant characteristic feature.

Virulence and toxic properties of the cells

The results of tests on 100 strains (Table 3) indicate a high virulence in the majority of those freshly isolated, seventy-five out of ninety (83 %) having an LD_{50} of 100×10^6 organisms or less. Of the others, five strains that were incompletely precipitated with $AlPO_4$ (Table 2) were of low virulence ($LD_{50} > 400 \times 10^6$). Of the repeatedly subcultured laboratory strains, however, all five tested had an

Table 2. *Adsorption on aluminium phosphate and solubility in sodium hydroxide and bile salt solutions*

Type of strain	No. of strains	Adsorption on aluminium phosphate	Solubility of cells in	
			N/ NaOH soln.	2 % sodium deoxycholate
H. pertussis, virulent type	187	100 % (182) 80 % (4) 50 % (1)	Complete (187)	Complete (187)
H. pertussis, avirulent type	10	50 % (1) 25 % (3) 0 % (6)	Not soluble (10)	Not soluble (10)
H. parapertussis	11	90 % (4) 80 % (4) 50 % (3)	Partially soluble (11)	Partially soluble (11)

Table 3. *Virulence of the strains in mice by intranasal infection*

Strains	No. examined	No. of strains having LD_{50} of dose ($\times 10^6$)					
		25	50	100	200	400	Over 400
H. pertussis, freshly isolated	90	43	12	20	3	6	6
H. pertussis, laboratory strains	5	0	0	0	0	0	5
H. parapertussis, freshly isolated	5	0	2	0	1	2	0

LD_{50} of more than 400×10^6 organisms. Mice that died and were examined *post mortem* invariably showed an extensive broncho-pneumonia, and Bordet–Gengou cultures from the lungs yielded a heavy growth of *H. pertussis*. The virulence of the *H. parapertussis* cultures was variable.

The results of tests of sixty strains for their toxic properties in relation to the virulence are shown in Table 4. Cells from all three types invariably produced some toxic reaction, as shown on intraperitoneal injection into mice and by dermonecrotic tests in rabbits. No differentiation between the three types could be made by reference to their toxic doses. The toxic effects of the cells were, however, neutralized to varying extents by the simultaneous injection of homologous, but not of heterologous, hyperimmune rabbit serum.

Haemagglutinin investigation

Table 5 shows the results of the haemagglutination tests on all strains. Cell suspensions from most of the freshly isolated strains agglutinated human erythrocytes, a finding consistent with the observations of Masry (1952). The avirulent strains did not cause any significant agglutination. The *H. parapertussis* strains gave agglutination to intermediate titres.

Table 4. *Toxic properties of cell suspensions of the strains*

Strains tested	Virulence in mice LD_{50}	Toxic dose: Mice (intraperitoneal injection)*	Toxic dose: Rabbits (intradermal injection)†
H. pertussis, virulent strains (50)	$25–100 \times 10^6$	$400–800 \times 10^6$	$100–400 \times 10^6$
H. pertussis, avirulent strains (5)	$> 400 \times 10^6$	$1000–2000 \times 10^6$	$100–400 \times 10^6$
H. parapertussis (5)	$50–400 \times 10^6$	$400–800 \times 10^6$	$100–400 \times 10^6$

* LD_{50} in mice.

† Dose required for 10 mm. diameter zone of inflammation with necrosis.

Table 5. *Haemagglutinating power of the strains against human erythrocytes*

Strains	No. of strains causing haemagglutination at: < 1/2	1/2	1/4	1/8	1/16	1/32	1/64	1/128
H. pertussis, virulent strains (187)	4	12	21	42	39	30	27	12
H. pertussis, avirulent strains (10)	6	3	1	0	0	0	0	0
H. parapertussis, (11)	2	0	3	4	2	0	0	0

Table 6. *Agglutination tests against standard rabbit antisera*

Strains	Maximum dilution of immune serum for agglutination: Virulent *H. pertussis* antiserum	Avirulent *H. pertussis* antiserum	*H. parapertussis* antiserum
H. pertussis, freshly isolated strains (41)	1/25,000 (31) 1/12,500 (4) 1/1,600 (6)	1/1600 (30) 1/800 (3) 1/100 (8)	< 1/100 (25) 1/100 (10) 1/200 (6)
H. pertussis, remaining freshly isolated strains (146)	1/25,000 (100) 1/12,800 (41) 1/6,400 (2) 1/3,200 (3)	Not tested	Not tested
H. pertussis, avirulent strains (10)	< 1/1,600 (2) 1/1,600 (8)	1/4,000 (10)	< 1/200 (6) 1/400 (2) 1/800 (2)
H. parapertussis (11)	< 1/1,600 (6) 1/1,600 (3) 1/3,200 (2)	1/100 (4) 1/200 (5) 1/400 (2)	1/3,200 (1) 1/6,400 (4) 1/12,800 (6)

Agglutination and agglutinin absorption tests

The ten avirulent *H. pertussis* strains, forty-one of the virulent *H. pertussis* and the eleven *H. parapertussis* were all tested for agglutination by *H. pertussis* virulent, *H. pertussis* avirulent and *H. parapertussis* standard antisera (rabbit).

https://doi.org/10.1017/S0022172400036949 Published online by Cambridge University Press

The remaining 146 virulent (freshly isolated) strains were tested for agglutination by the homologous serum only. The results (Table 6) show that the virulent *H. pertussis* strains are agglutinated to high titre by the homologous immune serum and to a much lower titre by the 'anti-avirulent' serum. Conversely, the avirulent strains are agglutinated to a titre of 1/4000 by the homologous antiserum, but to a lower titre by the anti-virulent serum. It is evident that the change from virulent to avirulent form is accompanied by a significant loss of agglutinogen, since the sera from rabbits immunized with avirulent vaccine never reach such high titre with the homologous agglutinating suspensions as did those corresponding to the virulent strains. The results show that the avirulent strains retain small amounts of virulent agglutinogen and moreover in the *H. parapertussis* strains, although higher titres are recorded with the homologous antiserum, there is evidence of some common agglutinogen with both the virulent and avirulent *H. pertussis* types. This relationship is further elucidated by the agglutinin absorption tests carried out on some of the strains (Table 7). For these tests the sera were diluted, as already stated, to 1/2000 in relation to the homologous agglutinating suspension. Sometimes, therefore, heterologous agglutinins in the sera were diluted to a level too low to measure.

Table 7. *Summary of results of agglutinin absorption tests*

		Agglutinins absorbed		
Immune sera against	Cells with which absorbed	*H. pertussis* virulent	*H. pertussis* avirulent	*H. parapertussis*
Virulent *H. pertussis* (9 strains)	*H. pertussis* (V)	+ +	− −	+
	H. pertussis (AV)	+	− −	±
	H. parapertussis	+	− −	+ +
Avirulent *H. pertussis* (4 strains)	*H. pertussis* (V)	− −	0	0
	H. pertussis (AV)	− −	+ +	0
	H. parapertussis	− −	0	+ +
H. parapertussis (3 strains)	*H. pertussis* (V)	− −	±	0
	H. pertussis (AV)	− −	±	0
	H. parapertussis	− −	+	+ +

(V), virulent; (AV), avirulent; + +, complete absorption of agglutinins; +, partial absorption of agglutinins; ±, slight absorption of agglutinins; 0, no absorption of agglutinins; − −, no agglutinins present in serum before absorption.

By far the strongest absorption takes place with the homologous suspension. There is, however, some cross-absorption—notably between the virulent *H. pertussis* and the *H. parapertussis* agglutinins and agglutinogens—but there is no absorption of the avirulent *H. pertussis* agglutinin by virulent *H. pertussis* cells.

For ease of interpretation of these results, Fig. 1 shows the suggested distribution of the agglutinogens in the three types of strains.

Sensitivity to antibiotics

The sensitivity of fifty strains of virulent *H. pertussis*, nine of avirulent *H. pertussis* and eleven of *H. parapertussis* was tested to penicillin, streptomycin, chloramphenicol and aureomycin, and the results are shown in Table 8. They

https://doi.org/10.1017/S0022172400036949 Published online by Cambridge University Press

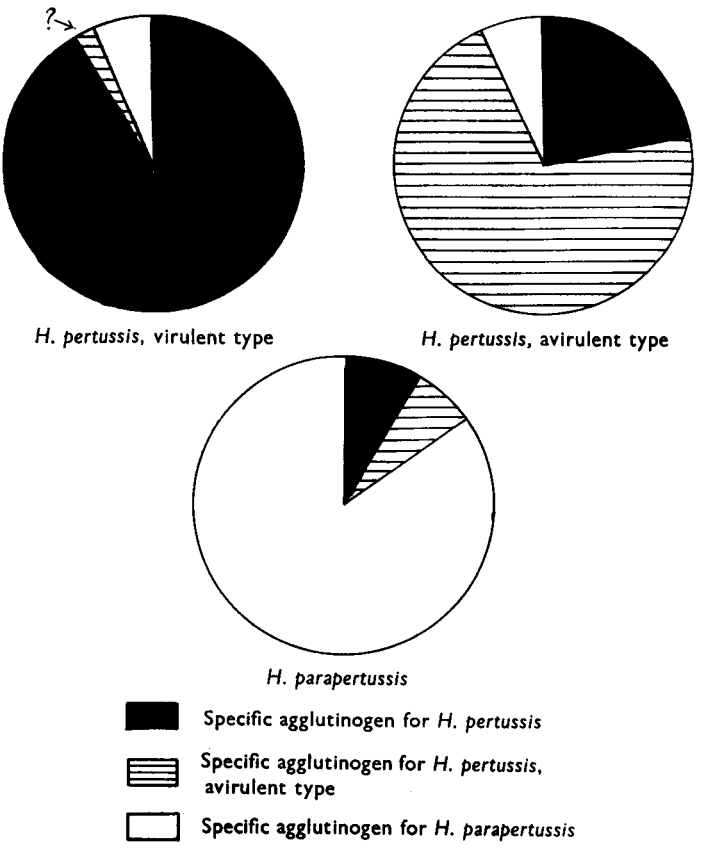

Fig. 1. Suggested agglutinogen content of the strains.

Table 8. *Sensitivity of the strains to penicillin, streptomycin (sulphate), chloramphenicol and aureomycin*

Strains tested	Antibiotic	No. of strains inhibited at (μg. or μ/ml.)*									
		0·1	0·25	0·5	1·0	2·5	5·0	10·0	25	50	100
H. pertussis virulent 50 strains	Penicillin	0	0	9	41	0	0	0	0	0	0
	Streptomycin	0	0	20	19	11	0	0	0	0	0
	Chloramphenicol	0	4	41	5	0	0	0	0	0	0
	Aureomycin	0	10	33	7	0	0	0	0	0	0
H. pertussis avirulent 9 strains	Penicillin	0	0	0	0	0	0	4	3	2	0
	Streptomycin	0	0	0	0	5	4	0	0	0	0
	Chloramphenicol	0	0	0	5	4	0	0	0	0	0
	Aureomycin	0	0	0	5	4	0	0	0	0	0
H. parapertussis 11 strains	Penicillin	0	0	0	0	0	0	0	3	4	4
	Streptomycin	0	0	0	0	0	0	0	0	0	11
	Chloramphenicol	0	0	0	7	4	0	0	0	0	0
	Aureomycin	0	0	4	7	0	0	0	0	0	0

* Sensitivity to penicillin expressed in I.U./ml. Sensitivity to streptomycin, chloramphenicol and aureomycin expressed in μg./ml.

https://doi.org/10.1017/S0022172400036949 Published online by Cambridge University Press

indicate that the change of the *H. pertussis* strains to the avirulent form is accompanied by a diminished susceptibility to penicillin, whereas the sensitivity to the other antibiotics, although rather less, is still partially retained. In marked contradistinction, the *H. parapertussis* strains were completely insensitive to penicillin and streptomycin, but were inhibited by both chloramphenicol and aureomycin at about the same concentrations as were the *H. pertussis* strains.

Attempts to reconvert the avirulent to the virulent type of Haemophilus pertussis

Attempts were made to reconvert four of the avirulent laboratory strains to the virulent type by repeated passage in mice. By giving a sufficient dose intranasally (over 1000×10^6 organisms) or intracerebrally (1×10^6 organisms) it was found possible to kill mice and recover the strain by cultivation from the lungs or brain respectively. By repeated passage strains of moderate virulence were produced (LD 50 about 200×10^6 by intranasal route or 20,000 by intracerebral route). Subsequent examimation of these strains was made by agglutination and haemagglutination test and for solubility and absorption. None of the strains was agglutinated by anti-virulent serum, none was able to cause agglutination of human red blood corpuscles and none was adsorbed by aluminium phosphate or dissolved by sodium hydroxide. All four also remained capable of growing on plain agar without blood.

DISCUSSION

From the results of the comparative tests shown in this paper, it is evident that *H. pertussis* strains isolated from the human host fall into a single well-defined type. This finding is consistent with the early observations of Bordet & Sleeswyck (1910), Krumwiede *et al.* (1923) and Kristensen (1927). It also emphasizes the important observation of Leslie & Gardner (1931) that there exist atypical forms of *H. pertussis* produced under laboratory conditions, antigenically distinct from the virulent form, the change found being irreversible.

The isolated *H. pertussis* strains show distinct morphological and cultural characteristics on Bordet–Gengou plates and in fluid culture medium are agglutinated to high titre with homologous immune serum, are virulent to mice by intranasal inoculation and produce dermonecrotic lesions in rabbit's skin. Virulent, but not avirulent, strains agglutinate red cells, grow on agar only in the presence of blood or serum and grow in liquid synthetic medium only in the presence of starch. The strains may, moreover, be differentiated by their degrees of absorption on aluminium phosphate and solubility in dilute sodium hydroxide. It has been found more recently that the virulent cells can differ chemically from avirulent in their deoxyribonucleic acid contents (Overend, Stacey, Webb & Ungar, 1951) and in their degree of susceptibility to antibiotics. The moderate degree of sensitivity to penicillin observed with some virulent strains indicates that not more than 0·1 μ/ml. should be added to media used for primary isolation.

When strains are allowed to become attenuated by repeated subculture, their properties undergo change. Their appearance on solid and in fluid media is altered and their growth requirements are simplified, for they will then grow on ordinary

agar and in fluid media in the absence of starch. Coincident with the loss of virulence to mice is loss of the virulent type-specific agglutinogen, the cells appearing to retain only a weak group agglutinogen characteristic of the attenuated strains. Avirulent strains do not display any significant haemagglutinin. They are partly precipitated with aluminium phosphate and dissolved by N sodium hydroxide; this is perhaps due to the retention of small amounts of virulent type agglutinogen.

From experiments on mice and rabbits (Kendrick, Updyke & Eldering, 1949; Ungar, 1952) it is evident that the antigenicity of the attenuated, avirulent strains is considerably inferior to that of the virulent strains, and it may be assumed that vaccines produced by these strains are poor immunizing agents in children.

In our studies we could detect no difference in the capsule formation of the virulent and avirulent cells, and indeed it is difficult to state categorically that the organism is encapsulated in the same way as, for example, the pneumococcus. It was not possible to induce in the rabbit any anti-capsular antibodies such that the sera would produce a phenomenon analogous to the Neufeld capsule reaction of the pneumococcus. As both virulent and avirulent *H. pertussis* types showed similar 'capsular' structure, the loss of antigenicity displayed by the avirulent strains cannot be associated with changes in the encapsulation. This is consistent with the findings of Evans & Adams (1952) that the capsular material of *H. pertussis* contains little antigen.

In our work we have found no evidence that *H. pertussis* exists naturally in any other than the typical virulent form constituting a single antigenic type with characteristic biological properties. We are forced to assume that the avirulent type is a collection of artificially produced variants. There is no evidence for regarding them as rough forms of smooth *H. pertussis*, as could be argued from their complete loss of virulence; moreover, the properties of these avirulent strains differ from the usual R forms encountered, e.g. among *Salmonella*.

H. parapertussis is different from either type of *H. pertussis*, particularly in its antigenic structure. Judged by the properties we have studied, however, it can rightly be regarded as closely related to *H. pertussis*.

SUMMARY

1. One hundred and eighty-seven freshly isolated strains of *Haemophilus pertussis*, together with ten laboratory strains and eleven strains of *H. parapertussis* were submitted to tests of their morphological, biochemical and antigenic properties in relation to their virulence to mice.

2. The naturally occurring *H. pertussis* strains form a single antigenic type differing from that of the attenuated laboratory strains.

3. *H. parapertussis* forms, as previously claimed, a distinctive type with characteristic properties closely related to *H. pertussis*.

Our thanks are due to Col. H. J. Bensted, Prof. J. W. McLeod and Drs A. Beck, D. G. Evans, G. Eldering, J. Marks, M. T. Parker, M. Pittman and H. Proom for supplying strains used in this investigation.

REFERENCES

BORDET, J. & GENGOU, O. (1906). Le microbe de la coqueluche. *Ann. Inst. Pasteur*, **20**, 731.

BORDET, J. & SLEESWYCK (1910). Serodiagnostic à variabilité des microbes suivant le milieu de culture. *Ann. Inst. Pasteur*, **24**, 476.

BURNET, F. M. & TIMMINS, C. (1937). Experimental infection with *Haemophilus pertussis* in the mouse by intranasal infection. *Brit. J. exp. Path.* **18**, 83.

COHEN, S. M. & WHEELER, N. W. (1946). Pertussis vaccine prepared with phase 1 cultures grown in fluid medium. *Amer. J. publ. Hlth*, **36**, 371.

ELDERING, G. & KENDRICK, P. (1938). *Bacillus parapertussis*, a species resembling both *Bacillus pertussis* and *Bacillus bronchisepticus* but identical with neither. *J. Bact.* **35**, 561.

EVANS, D. G. & ADAMS, M. O. (1952). The inability of the capsular material of *Haemophilus pertussis* to produce protective antisera. *J. gen. Microbiol.* **7**, 169.

JEFFERY, T. H. (1948). Method of demonstrating capsules. Modification of Fleming's nigrosin stain. *Bull. Inst. med. Lab. Tech.* **14**, 36.

KENDRICK, P. L., UPDYKE, E. L. & ELDERING, G. (1949). Comparison of pertussis cultures by mouse protection and virulence tests. *Amer. J. publ. Hlth*, **39**, 179.

KLIENEBERGER-NOBEL, E. (1948). Capsules and mucoid envelopes of bacteria. *J. Hyg., Camb.*, **46**, 345.

KRISTENSEN, M. (1927). Recherches serologiques sur le bacille de la coqueluche. *C.R. Soc. Biol., Paris*, **96**, 355.

KRUMWIEDE, C., MISHULOW, L. & OLDENBUSCH, C. (1923). The existence of more than one immunologic type of *B. pertussis*. *J. infect. Dis.* **32**, 22.

LAWSON, G. McL. (1940). Modified technique for staining capsules of *Haemophilus pertussis*. *J. lab. clin. Med.* **25**, 435.

LESLIE, P. H. & GARDNER, A. D. (1931). The phases of *Haemophilus pertussis*. *J. Hyg., Camb.*, **31**, 423.

MASRY, F. L. G. (1952). Production, extraction and purification of the haemagglutinin of *Haemophilus pertussis*. *J. gen. Microbiol.* **7**, 201.

OVEREND, W. G., STACEY, M., WEBB, M. & UNGAR, J. (1951). The isolation of deoxyribonucleic acid from virulent and avirulent strains of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, 268.

UNGAR, J. (1952). Recent advances in petussis immunisation. *Irish J. med. Sci.* 6th Ser., no. 316, p. 145.

UNGAR, J., JAMES, A. M., MUGGLETON, P. W., PEGLER, H. F. & TOMICH, E. G. (1950). The cultivation of *Haemophilus pertussis* in partially defined liquid media. *J. gen. Microbiol.* **4**, 345.

UNGAR, J. & MUGGLETON, P. W. (1949). The relationship of the aluminium phosphate precipitation of organims of *Haemophilus pertussis* strains to their other biological properties. *J. gen. Microbiol.* **3**, 353.

(*MS. received for publication* 27. IV. 54)