The Phase I of *Haemophilus pertussis*

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SUMMARY: An examination of freshly isolated *Haemophilus pertussis* strains for agglutinability, growth requirements, haemagglutination, virulence for mice, alum precipitability and protective properties shows that each of these characters varies quantitatively, and furthermore that they vary independently. The presence of one strongly marked character is no indication that another character will be strongly marked or even present. Not all strains necessarily have the characters to be expected in freshly isolated strains; one or possibly more may be missing. On subculture of the strains on Bordet-Gengou media the order in which these characters degenerate or are lost and the rate at which they are lost differs with the strain. Agglutinability and agglutinin content vary considerably in freshly isolated strains, and therefore the expression 'agglutinates to titre' cannot be considered as a criterion of a Phase I organism. The obvious complexity of the serology of organisms within 'Phase I' suggests that this term has served its useful purpose and should now be dropped as a description of a certain type of strain. The term '*Smooth*', which indicates the possession of the antigenic components commonly associated with the pathogenic state of the organism, is preferable.

Leslie & Gardner (1931) analysed thirty-two strains of *Haemophilus pertussis* by agglutination and agglutinin absorption and found that they fell into four well-marked groups which they called Phases I, II, III and IV. They suggested that Phases I and II probably correspond to the smooth (S) and Phases III and IV to the rough (R) phase of other bacteria. This work was confirmed by Lawson (1939) with respect to Phases I, III and IV but he did not find any strains in Phase II. He stated, however, that 'there are undoubtedly other intermediate stages in the dissociation process, their number dependent on the strain of the organism and its environmental condition', and suggested that the degradation proceeds gradually, often making it difficult to determine the border-line between the phases.

The investigation of *H. pertussis* has tended to proceed along one of two lines; either the investigation of the agglutinogenic relationship of the phases, as made by Flosdorf, Dozois & Kimball (1941), who presented evidence that variation is even more complex in minor relationships than former workers had found, or the characterization of Phase I organisms in the hope of finding a single character which would clearly distinguish organisms in this phase.

What then is Phase I, and are all organisms in Phase I identical? Historically Phase I was defined serologically, but recent work (Smolens, McLaren, McAleer & Flavell, 1947; Kendrick, Updyke & Eldering, 1949; Cohen, 1949) has shown that freshly isolated strains may vary considerably in their agglutinin content, and that a small number of typical strains are of consistently low agglutinability. Leslie & Gardner implied, though they did not

state it, that Phase I organisms only grow on Bordet-Gengou medium while Phases III and IV will grow on agar.

Lawson (1939) tabulated other characters, by which he distinguished the main phases, and later workers added more characters usually differentiating even more clearly Phase I from Phase IV; e.g. Burnet & Timmins (1937) used virulence for mice by the intranasal route, Kendrick *et al.* (1949) virulence for mice by the intracerebral route, Keogh & North (1948) haemagglutinin, Ungar & Muggleton (1949) alum precipitability and bile solubility. These investigations, while they have added to our knowledge of Phase I, have not made it any easier to define. Usually the workers were interested in the selection of strains optimal for some such purpose as vaccine manufacture, and have implied or even stated that the presence or assay of one character in a strain can be taken as a measure of another character (e.g. Smolens *et al.*, 1947, Keogh & North, 1948, Ungar & Muggleton, 1949). My investigation (Standfast, 1951) of the virulence for mice by the intranasal route and its correlation with certain other factors showed that when a large number of strains were examined there was little if any correlation between some of the characters of freshly isolated *H. pertussis*. It was felt that a re-examination of the main characters of Phase I was worth while in the hope that a more certain definition of Phase I might emerge. The characters used by most workers for differentiating strains are: (a) growth requirements, (b) agglutination and agglutinin absorption, (c) virulence, (d) protective antigenicity, (e) haemagglutinin, (f) alum precipitation and bile solubility, and to some extent (g) morphology. In the experiments described in this paper about 100 strains of *H. pertussis* freshly isolated from clinical cases of whooping cough were examined for these characters.

MATERIAL AND METHODS

Strains. Strains of *H. pertussis* were freshly isolated from patients with whooping cough, the first subculture from the original cough or swab plate being sent to the laboratory. All strains agglutinated with sera prepared against freshly isolated *H. pertussis* strains. They consisted of typical small Gram-negative cocco-bacilli. There was some variation in size from strain to strain, certain strains being smaller, $0.5-0.6 \mu. \times 0.3-0.4 \mu.$, whereas others measured $0.7-0.8 \mu. \times 0.4-0.5 \mu.$ This size variation was on the whole consistent in the strain, and though on some batches of Bordet-Gengou medium the 'smaller' strains grew rather 'larger', this increase in size was not permanent. There was no correlation between size and any other character. After prolonged subculture on laboratory media all strains tend to become larger; but the strains examined all remained cocco-bacilli and did not become 'coliform' in size even after subculture on blood agar.

Medium. Bordet-Gengou agar was used, containing 1 % Difco Proteose peptone (Baird and Tatlock Ltd., London) and 33 % horse blood. Nutrient agar was prepared from horse meat digest, to which 10 % horse blood was added to make blood agar.

A heavy inoculum on a bent glass rod was rubbed over half the Bordet-Gengou plate, then carried over to the whole of the blood plate, then the whole

of the nutrient agar plate and then the second half of the Bordet-Gengou plate. In all cases both halves of the Bordet-Gengou plate showed confluent growth after 24 hr. at 35°, indicating that there was an adequate inoculum on the blood agar and nutrient agar plates. There was never any growth on the agar plates after 5 days at 35°. Any growth on a blood agar plate was subcultured on a second blood agar plate, and only if this grew was growth considered positive on blood agar. On several occasions though growth occurred on the first blood agar plate it was not obtained on the second, indicating perhaps that the growth on the first plate was due to essential metabolites carried over with the inoculum.

Agglutination test. All agglutination tests were carried out in tubes, incubated at 55° in a water-bath for 4 hr. and then stood at room temperature overnight. The titre is the reciprocal of the highest dilution which shows obvious agglutination with a $\times 3$ hand-lens.

Sera were prepared in rabbits given three doses of 10^9 formalized bacilli subcutaneously followed by three doses of 10^9 living bacilli intravenously at weekly intervals.

Agglutinin absorptions were carried out by the method of Smolens *et al.* (1947) with certain modifications. A suspension of bacteria of any desired wet weight in a volume of 1 ml. were added to 1 ml. of a 1/250 dilution of antiserum, mixed and incubated for 2 hr. at 55° and then placed in the refrigerator overnight. Next morning the tubes were centrifuged and 0.5 ml. of dilutions of the supernatant were added to 0.5 ml. of the test suspension, incubated for 4 hr. at 55° and then stood at room temperature overnight. Serum controls were mixed with saline. The absorption titre was taken as the mg. wet weight of the strain required to absorb 90 % of the agglutinin.

Virulence tests. Intranasal virulence was titrated by the technique of Burnet & Timmins (1937). Briefly four doses of 100, 10, 1 and 0.1 million were each instilled intranasally into a group of ten mice kept under standardized conditions.

Intracerebral virulence was titrated by the method of Kendrick *et al.* (1949) using groups of ten mice on each dose. Mice of the strain Cl. used in these laboratories required doses about ten times greater than the strain of American mice, a few of which were available for comparative purposes.

The LD 50 of each strain was calculated by the method of Reed & Muench (1938).

Haemagglutinin tests. Suspensions for the haemagglutination test were prepared in saline or Difco Casamino acids (Baird and Tatlock Ltd., London) and standardized to contain 10^{10} organisms/ml., and serial twofold dilutions were prepared from these suspensions. Red blood corpuscles (R.B.C.) were prepared by centrifuging 1 ml. of fowl's blood, washing the R.B.C. three times in saline and resuspending in 50 ml. of saline. This 'strong' suspension can be kept 3 or 4 days in the refrigerator; before use it is diluted 1/4 with 0.85 % saline. It was found that 0.5 ml. of this dilute suspension was the optimum when added to 0.25 ml. of bacterial suspension.

For the test 0.25 ml. of the dilution of bacterial suspension was placed in a 12 $\times$ 55 mm. tube, and 0.5 ml. of R.B.C. suspension added. The racks were shaken

and incubated at 37° for 1 hr. The tubes were then shaken and put in the refrigerator to allow the cells to settle before reading the result.

The titre is taken as the reciprocal of the final dilution of bacterial suspension which shows obvious agglutination of the R.B.C. by eye.

Protection tests. Vaccines, prepared on horse blood Bordet-Gengou agar, were killed with 0.2 % Liq. Formaldehyd B.P. (1948) diluted to 10^{10} bacilli/ml., and tested by the method of Kendrick, Eldering, Dixon & Misner (1947). The ImD 50 calculated by the method of Reed & Muench (1938).

Aluminium phosphate precipitation was carried out by the method of Ungar & Muggleton (1949). Three-tenths ml. of 5 % Na_2HPO_4 followed by 1 ml. of 2 % potassium alum was added to 4.0 ml. of suspension of 10^{10} bacilli/ml. in saline containing 0.2 % Liq. Formaldehyd, shaken and stood for 30 min. before reading.

EXPERIMENTAL

Growth requirements

On various occasions groups of strains were plated on Bordet-Gengou agar, blood agar and nutrient agar.

One strain M7 grew abundantly on blood agar immediately after first isolation. Three cultures of this strain, all behaving in the same way, were obtained from a child in an outbreak of whooping cough in a children's ward. In all other respects this strain was typical (Table 1). This is the only strain in this series which grew, and grew profusely, on blood agar from first isolation.

Of the seventy-one strains tested, eight showed some growth on blood agar seeded with the 2nd, 3rd and 4th subculture on Bordet-Gengou. Viable counts

Table 1. *Modification of characters of four strains of Haemophilus pertussis which grew on blood agar, during subculture on Bordet-Gengou or blood agar*

Strain	Sub-culture growing on blood agar	Agglutination titre		Alum precipitability (%)		Haemagglutination titre		Protective antigen (ImD 50×10^6)		Virulence (intranasal) (LD 50×10^6)	
		N		N		N		N		N	
M 7	1	(3)	16,000	.	nt	(4)	0	.	nt	(3)	1.0
		[3]	16,000	.	nt	[4]	0	.	nt	[3]	1.0
		(17)	16,000	(17)	100	.	nt	(17)	0.20	(17)	1.0
		[17]	16,000	[17]	100	.	nt	[17]	0.21	[17]	27.0
C 5467	4	(9)	16,000	(9)	100	(2)	48	.	nt	(2)	35.0
		(4)+[5]	4,000	(4)+[5]	100	(4)+[5]	0	.	nt	(4)+[5]	40.0
C 315	6	(6)	16,000	.	nt	Always		(15)	4.0	(9)	5.0
		(41)	16,000	.	nt	negative		.	nt	.	nt
		(6)+[9]	4,000	.	nt	Always		(6)+[9]	5.0	(6)+[3]	4.0
		(6)+[19]	2,000	.	nt	negative		.	nt	.	nt
C 407	7	(15)	32,000	.	nt	(15)	12	(8)	0.85	(15)	0.63
		(32)	32,000	.	nt	(32)	12	.	nt	(32)	1.0
		(8)+[7]	16,000	.	nt	(8)+[7]	0	(8)+[7]	1.6	(8)+[7]	1.6
						(8)+[14]	0	.	nt	(8)+[14]	110.0

N, number of subculture. (), on Bordet-Gengou. [], on blood agar. (4)+[5], 5th serial subculture on blood agar following four subcultures on Bordet-Gengou. nt, not tested.

were made on Bordet-Gengou and blood agar on three of these strains. The ratio of colony counts on blood agar to Bordet-Gengou were 1:340 million, 1:940 million and 1:1270 million.

Stability of strains with respect to growth requirements on continued subculture

A group of twenty strains were plated daily on Bordet-Gengou agar and at intervals on blood agar. Five showed some growth on blood agar after 11th, 12th, 12th, 16th and 25th subcultures on Bordet-Gengou. The remaining fifteen strains did not grow on blood agar after sixty subcultures on Bordet-Gengou.

Another group of seventeen strains were plated daily on Bordet-Gengou agar for over 200 subcultures, and once a week on blood agar. Growth appeared on blood agar from seven of these strains after 38th, 114th, 190th, 197th, 200th, 203rd, and 211th subcultures on Bordet-Gengou. The other ten strains did not acquire the capacity to grow on blood agar.

The twelve strains mentioned above and the eight strains growing on blood agar soon after first isolation all produced from 10 to 100 isolated colonies on the first blood agar plate after 1-5 days at 35°. If these strains are identical with the strain isolated by Pollock (1947) it would seem that some (eight out of seventy-one) freshly isolated *H. pertussis* carry the variant. It may, however, arise spontaneously after any number of subcultures on laboratory media in some strains, and eventually all strains may produce it. One strain (C 407) of a group of five which were plated daily on blood agar suddenly produced this variant

Bordet-Gengou subculture	(5)	→	(6)	→	(7)	→	(8)
1st blood agar plate	-		+		+		+
2nd blood agar plate					-		+

the other four remaining negative. Viable counts of subcultures 7 and 8 gave a ratio of variant to parent of $1:800 \times 10^6$ on both occasions.

In other respects the strains carrying this variant appeared to be typical Phase I organisms (Table 1); the variant behaved as the parent strain for a few subcultures on blood agar, but on further culture rapidly lost some of its characters (see Table 1). In this respect it is interesting to note that the protective antigen, as measured by the ability to protect mice against an intracerebral challenge, was one of the more stable characters.

Agglutination

One strain was not agglutinable during the first four daily subcultures either by slide agglutination or by tube agglutination, but the 5th and several subsequent subcultures were agglutinated to titre. Occasionally strains were found which did not agglutinate on the slide because of a pronounced pro-zone effect which was obvious in the tube agglutination and could be avoided by diluting the serum used for the slide test, when a positive agglutination was obtained.

Table 2. *Agglutination of 105 freshly isolated strains of Haemophilus pertussis by several sera prepared against freshly isolated strains*

Titre	No. of strains
16,000-32,000	93
8,000	5
4,000	6
2,000	1

All strains examined agglutinated with Phase I serum, usually but not always to a high titre. The tests on 105 freshly isolated strains in Table 2 show that a small number of strains are of consistently low agglutinability. Other workers have found similar strains (Smolens *et al.* 1947; Kendrick *et al.* 1949; Cohen, 1949). In a further examination five of these strains (Table 3) did not differ appreciably from strains of high agglutinability in any other way.

Table 3. *General characters of five strains of Haemophilus pertussis of low agglutinability*

Strain no. and subculture	Growth on blood agar	Agglutination titre	Alum precipitability (%)	Haemagglutinin titre	Protective antigen (ImD 50 × 10 ⁶)	Virulence (intranasal) (LD 50 × 10 ⁶)
L 51-7	—	8000	nt	96	0.85	1.0
L 67-7	—	8000	100	12	0.71	nt
W 1276-3	—	4000	100	24	nt	0.1
W 1505-3	—	4000	100	nt	nt	45.0
W 1506-3	—	2000	100	12	0.45	nt

—, negative. nt, not tested. Compare also Table 7, strain P 34-7 and P 54-15.

Antisera were prepared against four freshly isolated strains, two of high agglutinability and two of low agglutinability and subjected to cross-agglutination and cross-absorption tests (Table 4). There was no difference in the major antigens of the two types of strain.

The work of Smolens *et al.* (1947) showed that strains can differ widely in their content of agglutinin. In general, they found that strains high in

Table 4. *Cross-absorption tests of two strains of Haemophilus pertussis; one (H) of high agglutinability, and the other (L) of low agglutinability*

Serum			Titres after absorption by			
Against	Absorbed by	Tested with suspension	Absorbing dose (mg. wet weight)			
			0.0	2.5	5.0	10.0
H	H	H	32,000	4,000	50	nt
		L	4,000	4,000	0	nt
	L	H	32,000	nt	2,000	100
		L	4,000	nt	1,000	100
L	H	H	16,000	2,000	0	nt
		L	4,000	1,000	0	nt
	L	H	16,000	nt	100	0
		L	4,000	nt	100	0

nt = not tested.

absorbing power were agglutinated to a high titre, and strains of low absorbing power to a low titre, but there were several exceptions. In my experiments seven strains agglutinating to 1/16,000–1/32,000 had absorbing potencies from 2·5 to 40·0 mg.; a range very similar to the 1·3–10 mg. reported by Smolens. Two strains of low agglutinability were found, one was agglutinated to a titre of 4000 and had an absorption titre of 2·5 mg., and with the other the corresponding figures were 8000 and > 20 mg. Cross-absorption tests with these strains showed that strains of high absorbing power absorb a lot of agglutinin from sera prepared with strains of high or low agglutinability and strains of low absorbing power absorb little from either type of sera. Agglutination, before and after absorption, of heterologous and homologous suspension showed that only one major antigen was involved, so that all these strains must be regarded as agglutinating to titre with Phase I serum. In this respect these strains differed from certain strains of lowered agglutinability obtained by subculture in which another antigen appeared which was demonstrated by cross-absorption tests.

From my experiments and those of Smolens *et al.* we must conclude that the agglutinability of cells and their agglutinin content vary independently.

Stability of Phase I agglutinin of strains on continued subculture

Six strains subcultured daily on Bordet-Gengou media were tested at intervals by agglutination and agglutinin absorption. By chance they were all (Table 9) strains of high agglutinability, three of them (L 46, L 49 and L 54) had a high absorbing power (titre 2·5 mg.), one (L 57) a very low absorbing power (titre *c.* 40·0 mg.). All the strains immediately lost absorbing power but not, however, in parallel with the loss of agglutinability, which occurred at different times and at different rates in four of the six strains. Furthermore, apart from the general degeneration of these strains, neither absorbing powers nor agglutinability were lost in parallel with any other character.

One of the strains, L 54, was examined by cross-absorption tests. Subculture 162 differed markedly from the freshly isolated strains of low agglutinability reported above in that another antigen was easily demonstrated which was present neither in subculture 15 nor in any freshly isolated strains of high or low agglutinability. This is presumably the Phase II or Phase III antigen.

Virulence

The virulence (LD 50) for mice by the intranasal route has been dealt with in detail elsewhere (Standfast, 1951). Briefly forty-three strains were tested at the 2nd or 3rd subculture; thirteen of these strains had a LD 50 of less than 1 million, seven between 1 million and 5 million, nine between 5 million and 25 million, thirteen between 25 million and 125 million and one more than 125 million; in fact the LD 50 ranged from 0·1 million to 152 million. These strains grew only on Bordet-Gengou medium and agglutinated with Phase I serum to a high titre.

The eight strains (p. 534) which carried (2nd–4th subculture) the variant growing on blood agar at first isolation were in no way abnormal in virulence.

The virulence of cultures grown on blood agar seems to depend on the number of times they have been subcultured, but varies somewhat from strain to strain (see Table 1), but the loss in virulence is not contemporaneous with the ability to grow on blood agar.

Kendrick *et al.* (1949) examined seventy smooth cultures for virulence by the intracerebral route and found that they fell into two groups, those having an end-point of 10^4 – 10^7 and those having an end-point of 10^3 or less. The LD 50 of the fifteen strains which fell into the latter group ranged from 100 to 1000 organisms.

I was able to test thirty-one strains for virulence by the intranasal route and the intracerebral route in parallel (Table 5). There was a wide variation in the LD 50 by the intracerebral route ranging from $<10^3$ to 10^9 .

Table 5. *Comparison of virulence by intranasal and intracerebral routes of thirty-one freshly isolated strains of Haemophilus pertussis*

(Numbers of strains in each group.)

Intracerebral LD 50	Intranasal LD 50 in millions		
	<1.0	$1.0 < 10.0$	$10.0 < 100.0$
$<10^3$	4	1	0
$10^4, <10^5$	0	2	0
$10^5, <10^6$	8	0	1
$10^6, <10^7$	8	2	2
$>10^7$	1	2	0

Virulence by the intercerebral route and virulence by the intranasal route depend on different factors. First, there is no direct correlation between results by the two routes (Table 5); secondly, though the LD 50 by the intranasal route is dependent on the weight and age of the mice (Standfast, 1951) this factor does not influence the LD 50 by the intracerebral route unless very small mice are used.

The intraperitoneal route is more a measure of the toxin content of the organisms than of their virulence. Nine strains were tested in parallel by the intranasal, intracerebral and intraperitoneal route; by the intranasal route the LD 50 varied between 0.26×10^6 and 15.5×10^6 —a 60-fold range—by the intracerebral route between 0.18×10^6 and 2.5×10^6 —a 14-fold range—and by the intraperitoneal route between 280×10^6 and 800×10^6 —a 2.85-fold range. There was no correlation between high and low value by any of these routes with any particular strain. Table 6 shows the highest and lowest values for each route for the strains tested. The results by the three routes are obviously dependent on different factors and vary independently.

Stability of virulence in strains on continued subculture

On subculture on Bordet-Gengou medium some strains retained their virulence during a large number of subcultures while others lost it quickly. Virulence once lost was not recovered by further subculture, but could sometimes be partly recovered by mouse passage. Table 9 shows some examples of

Table 6. *Comparison of virulence by three routes of five strains of Haemophilus pertussis*

Strain	LD 50 in millions		
	Intranasal	Intracerebral	Intraperitoneal
L 5,743	0.26	0.18	655
L 5,754	15.5	0.24	640
C 10,487	1.0	1.0	800
C 19,767	2.1	1.6	280
C 19,764	3.3	2.5	320

stable and unstable strains. Strains L 54 and L 58 retained their full virulence for 162 and 182 subcultures; the other four strains all lost virulence, but at different times and to a different degree. Strain L 46 has lost virulence most rapidly having an LD 50 of 39 million, at the 39th subculture while strain L 49 maintained its original virulence for at least 145 subcultures and then degenerated. Another strain, C7988, behaved like L 46, losing virulence rapidly, an LD 50 of 4.5 million at subculture 2 fell to 46.0 million by subculture 12. Other strains, such as C2188, behave like L 49, tested on five occasions, up to subculture 206, it had at each test an LD 50 of less than 0.2 million. But at the 218th subculture the LD 50 was 12.0 million and at the 286th subculture, 10.0 million. This drop in virulence followed by stability at the raised figure, as shown by strain L 46 (Table 9) and C2188 but not by L 45, suggests the sudden loss of one of the factors which determines virulence.

Haemagglutination

Seventy-one freshly isolated strains were tested for haemagglutinin on several occasions and gave consistent titres as follows: twenty strains 1/196 to 1/96, twenty-nine strains 1/48 to 1/24, eight strains 1/12 to 1/6, three strains showed traces only at 1/6 or 1/3 and eleven strains were invariably negative. As shown in a previous paper (Standfast, 1951) there is no correlation between haemagglutinin and virulence measured by the intranasal route nor does haemagglutinin tie up with any of the other characters. Table 7 shows the main characters of twelve strains arranged in order of haemagglutinin production. There is no association with any of the other characters (see also Table 9).

Stability of haemagglutinin in strains on continued subculture

Haemagglutinin appears to be one of the more labile characters of *H. pertussis*. Seventeen strains were subcultured daily on Bordet-Gengou medium and tested at various subcultures for haemagglutinin. Table 8 shows four characteristic results.

Protective antigenicity

Single strain vaccines were prepared from eighteen freshly isolated strains and tested for protective potency in mice against a reference vaccine. Most of these vaccines were tested on several occasions and an average ImD 50 obtained. Their ImD 50 varied from 0.21×10^9 to 1.32×10^9 . The errors of the test are

Table 7. *A comparison of the characters of twelve strains of Haemophilus pertussis, arranged in order of their haemagglutination titres*

Strain and subculture	Haemagglutination titre	Agglutination titre	Alum precipitability (%)	Growth on blood agar	Protective antigen ImD 50 $\times 10^9$	Virulence (intranasal) LD 50 $\times 10^6$	Quick killing factor
P 37-7	96	32,000	100	—	0.48	1.0	—
P 34-7	96	8,000	100	—	0.85	9.0	—
P 33-22	48	32,000	100	—	0.62	8.3	—
P 43-43	48	16,000	100	—	0.37	66.0	—
P 37-9	24	32,000	100	nt	1.07	1.7	+
P 54-15	24	4,000	100	—	1.32	4.6	+
P 53-8	24	32,000	100	+	0.65	3.0	—
P 32-22	12	32,000	100	—	0.58	9.4	—
P 42-43	12	32,000	100	—	1.12	34.0	—
P 31-10	Trace	16,000	100	—	0.34	0.84	+
P 26-5	0	32,000	100	—	0.57	0.64	+
P 27-5	0	32,000	100	—	1.19	0.67	+

The above characters were tested on the same suspension for each strain; after the other tests had been carried out 1/10,000 thiomersalyl was added and a sterile vaccine prepared for the mouse protection tests. Growth on blood agar: —, negative; +, positive; nt, not tested.

Table 8. *Haemagglutination titres of four typical strains of Haemophilus pertussis subcultured daily on to fresh Bordet-Gengou medium*

Strain no.											
L 59	N	4	10	15	25	.	.	.	.	.	.
		24	12	0	0	.	.	.	.	.	.
L 54	N	.	9	15	24	33	39	.	.	.	.
		.	24	24	12	0	0	.	.	.	.
L 48	N	5	.	17	20	29	40	53	69	.	.
		24	.	24	24	12	12	0	0	.	.
L 51	N	2	7	16	23	34	42	52	70	84	95
		96	96	96	48	24	12	12	6	0	0

N, numbers of subculture. Compare also strains in Table 9.

such that it is doubtful if there is any significant difference in the potency of these vaccines. But between two vaccines picked from the extremes of the range there was a suggestion of a real difference in protective properties. In four tests of vaccine C26,573, the ImD 50 was 0.16, 0.21, 0.22 and 0.26, average 0.21, and of vaccine L 57-5, the ImD 50 was 0.96, 1.27, 1.28, 0.2, average 0.93.

There is no correlation between protective properties or any other Phase I character (see Tables 1, 7 and 9), this corroborates the findings of Kendrick and her co-workers who found no marked differences in protective properties of thirty-six smooth strains or any association between protection and relative degree of virulence as estimated by the intracerebral route.

Stability of protective property of strains on continued subculture

Vaccines were prepared at different subcultures from six strains which had been subcultured daily on Bordet-Gengou medium. Table 9 shows the average ImD 50's of the vaccines prepared from the different subcultures. There is a

suggestion that the protective potency of some strains decline, though this is marked only in strain L 45. The protective property appears to be lost in different strains at different times and at different rates.

Precipitation by aluminium phosphate

Ungar & Muggleton (1949) examined thirty-four freshly isolated strains, which agglutinated to high titre (1/8000 or more) with phase I serum, for precipitation with aluminium phosphate. Because all these strains precipitated completely they concluded that precipitation with aluminium phosphate provides a quick method for distinguishing virulent Phase I strains from strains which had deteriorated. My experiments are not entirely in agreement; the majority of freshly isolated strains are positive and lose this character when they deteriorate but several freshly isolated strains which were otherwise typical were not completely precipitable, and certain strains which had lost or were losing some of their other characters were still completely precipitable with aluminium

Table 9. *Comparison of six strains tested after varying number of subcultures*

Strain	Character	Subculture no.								
		1-9	10-25	26-50	51-75	76-100	101-125	126-150	151-200	200-225
L 45	Agglutination titre	32,000	32,000	32,000	16,000	8,000	.	0	0	.
	Agglutination absorption, mg.	5.0	10.0	.	15.0	.	.	.	>40.0	.
	Alum precipitability, %	100	100	100	.	0	0	.	.	.
	Growth on blood agar	—	—	—	—	—	+	+	+	.
	Haemagglutination titre	48	48	48	12 0	0	.	.	.	.
	Protective antigen, ImD 50	0.41	0.77	1.15	.	.	.	.	3.32	.
	Virulence (intranasal), LD 50	0.64	9.4	34.0	65.0	.	.	.	80.0	.
L 46	Agglutination titre	16,000	16,000	16,000	16,000	16,000	16,000	.	.	0
	Agglutination absorption, mg.	2.5	3.75	5.0	.	.	20.0	.	.	.
	Alum precipitability, %	100	100	100	.	.	100	.	.	100
	Growth on blood agar	—	—	—	—	—	—	—	—	+
	Haemagglutination titre	48	48	48	12	6	.	.	.	.
	Protective antigen, ImD 50	0.71	0.98	0.67	.	.	1.19	.	.	.
	Virulence (intranasal), LD 50	0.46	8.3	66.0	.	.	66.0	.	.	.
L 49	Agglutination titre	16,000	16,000	.	16,000	.	16,000	16,000	16,000	0
	Agglutination absorption, mg.	.	2.5	.	.	.	10.0	.	20.0	.
	Alum precipitability, %	100	100	.	.	100	.	100	100	100
	Growth on blood agar	—	—	—	—	—	—	—	—	—
	Haemagglutination titre	48	24	24	24	6	.	0	0	.
	Protective antigen, ImD 50	0.63	0.63	.	.	.	.	0.87	1.35	.
	Virulence (intranasal), LD 50	0.26	0.64	.	1.6	.	.	3.0	26.0	.
L 54	Agglutination titre	16,000	8,000	8,000	.	.	8,000	8,000	2,000	.
	Alum precipitability, %	100	100	.	.	100	100	100	100	.
	Growth on blood agar	—	—	+	+	+	+	+	+	.
	Haemagglutination titre	24	24 12	0	0	.	.	.	.	.
	Protective antigen, ImD 50	0.71	0.77	.	.	.	.	.	0.78	.
	Virulence (intranasal), LD 50	1.2	2.6	.	.	.	.	.	1.0	.
	.	.	.	.	.	.	.	.	.	.
L 57	Agglutination titre	16,000	16,000	.	16,000	16,000	16,000	.	16,000	8,000
	Agglutination absorption, mg.	c. 40.0	.	.	.	.	>40.0	.	>40.0	.
	Alum precipitability, %	100	.	.	100	.	.	.	100	75
	Growth on blood agar	—	—	—	—	—	—	—	+	+
	Haemagglutination titre	24	24	24	24	24	.	.	.	.
	Protective antigen, ImD 50	0.93	.	.	.	.	.	.	1.27	.
	Virulence (intranasal), LD 50	0.13	.	0.53	0.66	.	.	.	3.0	.
L 58	Agglutination titre	16,000	16,000	.	16,000	.	16,000	8,000	8,000	.
	Agglutination absorption, mg.	5.0	.	.	10.0	.	20.0	c. 40.0	>40.0	.
	Alum precipitability, %	100	.	.	100	.	.	100	100	.
	Growth on blood agar	—	—	—	—	—	—	—	—	.
	Haemagglutination titre	24	24	.	24	.	0	.	.	.
	Protective antigen, ImD 50	1.13	.	.	.	.	.	1.85	0.95	.
	Virulence (intranasal), LD 50	9.3	.	.	.	.	.	10.0	10.0	.

phosphate (Table 3, Table 9, strains L 45, L 46, L 49; see also Ungar & Muggleton (1949), Table 3, strain 10,203).

In my experiments thirty-nine strains were tested at first isolation; thirty-five precipitated completely, three to about 75 % and one not at all.

Stability of aluminium phosphate precipitability in strains on continued subculture

Of forty-six strains, which had been subcultured in the laboratory on Bordet-Gengou medium up to 100 daily subcultures and were still in Phase I by the other criteria, tested with aluminium phosphate forty-one were 100 % positive, three were about 75 % and two were negative.

Six strains subcultured daily on Bordet-Gengou medium for 225 days were tested at various intervals. In one of these strains there is a complete loss of precipitability from the 48th subculture, in another some loss by the 68th subculture, the other four strains were stable up to the last subculture tested (Table 9). Four variants growing on blood agar were tested after 9, 12, 13 and 13 subcultures on blood agar. These variants were no longer agglutinated to titre with Phase I serum but two were completely precipitated and two precipitated to about 75 % (see also Table 1, strain C5467).

DISCUSSION

When freshly isolated *H. pertussis* strains are examined to determine the main characters by which so-called Phase I organisms are distinguished from the other phases, three facts come to light. (1) Any one character varies quantitatively and it is therefore impossible, except by setting up an artificial boundary, to use any character qualitatively for definition. One cannot simply say that a certain strain is 'virulent' or 'agglutinates'. (2) Not all strains have all the characters which one normally expects in freshly isolated strain of *H. pertussis*. For example, the strain M 7 in most characters was a typical *H. pertussis* strain, but from first isolation it could grow on blood agar. Portwood (1946), who investigated the catalase activity of *H. pertussis*, reported that fourteen of sixty-two smooth strains had no activity. (3) The main characters vary independently, and the presence of one character even strongly marked is no evidence that another character is also strongly marked or in fact present at all. In addition to evidence presented in the paper, Portwood (1946) reported that agglutination tests presented no evidence of differences in antigenic structure between strains with or without catalase activity.

On subculture, characters may degenerate or be lost in different strains at different times and in different orders. Diagrammatically we may view a strain of *H. pertussis*, as in Fig. 1, where the characters present in the freshly isolated strain degenerate at different rates. Fig. 2 shows a single character in a number of different strains, present in different potencies at first isolation and on culture in the laboratory degenerating at different rates and at different times.

What then is a Phase I organism? No exact definition is possible since this definition must depend to a great extent on opinion. Phase I was originally defined serologically. But Smolens *et al.* (1947), Kendrick *et al.* (1949) and Cohen

(1949) have already reported variations in the agglutinability of freshly isolated strains, and also variations in the absorbing potencies of different strains which did not go hand in hand with their agglutinability (Smolens *et al.* 1947). The serological experiments reported in this paper support the view that freshly

Fig. 1. Schematic representation of characters of a typical strain of *H. pertussis*.

Fig. 2. Schematic representation of the same character in six strains of *H. pertussis*.

isolated *H. pertussis* strains are serologically homologous. They also show that both the agglutinin content of the cells as measured by agglutinin absorption and the agglutinability of the cells as measured by the agglutination test vary, and vary independently. The significance of the consistently low agglutination titres given by some strains is not clear. By agglutination titre and agglutinin absorbing power freshly isolated strains may be divided into four groups: strains of high agglutinability and high absorbing power, high agglutinability and low absorbing power, low agglutinability and high absorbing power and low agglutinability and low absorbing power; most strains fall into the first group; strains of low agglutinability are relatively rare probably comprising not more than 10 % of strains isolated. Two of the three strains I examined were of high absorbing power and one of low.

It is obvious from cross-agglutination (Table 4) that low agglutinability is a function of the suspension and not of the antiserum, and that only one antigen is involved. Until the strain of low agglutinability and low absorption was found, I thought that strains of low agglutinability were strains with *a lot of agglutinin* which for some reason required a high concentration of serum to agglutinate them. This, however, is not always so, and furthermore it is unlikely that strains which had been kept in the laboratory and were of lowered agglutinability, would increase their agglutinin content. It is, however, difficult to understand why two strains with the same agglutinin content as measured by agglutinin absorption should behave so differently in the presence of the same dilutions of agglutinating serum unless the variation in agglutinability is determined by factors other than the amount of agglutinin on the cell surface, e.g. the distribution or availability of the receptors on the cell surface. Thus, if the active groups were clustered in discrete patches relatively distant from each other, more serum might be required to agglutinate them than if they were more evenly scattered. This hypothesis is supported, I think, by the behaviour of the old laboratory strains (Table 9). These strains all lost agglutinin as measured by absorption before they lost agglutinability, which may mean that they first lost active groups and then these groups become 'thinned out' too far. The mechanism of agglutination in this group, of course, may be complicated by the appearance of another major antigen. The behaviour of the two strains of low agglutinability and high absorption may be due to 'bad siting', resulting in lack of availability of the active groups.

These experiments, at any rate, indicate that the serology of Phase I is more complicated than originally thought and the expression 'agglutinates to titre' is not a valid criterion of a Phase I organism.

The word 'phase' with respect to Phase I *H. pertussis*, with its implication of phase as used in the salmonellas, has proved with time to be an unfortunate choice as a synonym for smooth or freshly isolated; it has served its useful purpose and should now be dropped. Leslie & Gardner's original contention that the phases of *H. pertussis* could revert has not, so far as I am aware, been confirmed.

'Freshly isolated' as a defining phrase is also open to objection, as some strains maintain their 'freshly isolated' characters for some time in the laboratory while others lose all or some rapidly.

The term 'virulent', usually meaning virulent for mice, is also open to objection, for though no avirulent, but otherwise typical strain, has yet been isolated, virulence is a quantitative term and the definition of *avirulent* must depend to some extent on dosage.

We fall back therefore on '*Smooth*' as the best description of these strains; this was first suggested by Leslie & Gardner (1931) and stressed by Shibley & Hoelscher (1934) and by Lawson (1939), who pointed out that the dissociation of *H. pertussis* was an example of the S → R variation with one or more intermediate stages, the exact characters of which will depend on the state the organism has reached.

The exact definition of the *Smooth* state with our present knowledge may be

no easier, for we are dealing with an extremely variable organism, but we are employing a word in common bacteriological usage with a recognized meaning.

Freshly isolated strains of *H. pertussis* accordingly fall into three groups, the 'ideal' or 'perfect' strains (which like other ideals may not exist in fact) containing all the characters in a fully developed state, 'normal' strains containing all the characters only some fully developed, but all adequately represented; and a third group in which one or possibly more characters are missing or are feebly displayed. All these strains will be *Smooth*.

With a highly developed parasite like *H. pertussis* the final definition of a 'freshly isolated', 'virulent', 'smooth', 'Phase I' strain will probably depend on 'virulence for human beings'. When the factors responsible for this are known a final definition may be possible.

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