THE PHASES OF *HAEMOPHILUS PERTUSSIS*

By P. H. LESLIE and A. D. GARDNER.

(*From the Sir William Dunn School of Pathology, Oxford.*)

(With one Figure.)

Introduction.

The researches of Bordet and Sleeswyck (1910) proved that agglutinating serums for the identification of freshly isolated strains of *Haemophilus pertussis* cannot be successfully made with old stock strains of the organism growing on agar, because of the serological change that occurs as the result of its adaptation to that medium. These workers considered the two serological states of the bacillus to be dependent on the medium of growth; state I being characteristic of cultures on fresh blood-medium and state II of growth on agar. The latter state was usually found to revert readily to the former when the culture was retransferred from agar to blood. In old stocks, however, a slow alteration to the second state tended to occur even on blood-medium, and toxicity to guinea-pigs was reduced or lost in the process (Bordet, 1913).

Krumwiede, Mishulow and Oldenbusch (1923) described two permanent serological varieties or types, A and B, of *H. pertussis* and were unable to confirm Bordet in his attribution of the two types to the nature of the culture medium. A close examination of their data, however, led us to doubt the validity of their conclusions. In Denmark, Kristensen (1922 and 1927) found the species to be serologically homogeneous, *i.e.* all cultures of his own isolation were of a single type. He admits the existence of Krumwiede's types but reserves his opinion as to their significance.

Finding it impossible to reconcile the conflicting views of these various workers, we embarked on a new investigation of the serological composition and variation of the species. Our analyses of thirty-two strains of *H. pertussis* by agglutination, absorption of agglutinins and tests of agglutinogenic properties have proved that they all fall into one or other of four well-marked agglutinative groups, which we call Phases I, II, III and IV.

Sixteen of the strains were freshly isolated by us, of which fifteen were in Phase I, and the sixteenth intermediate between Phases I and II. Four fresh Danish strains sent by Dr Kristensen were Phase I; one of them also showing some Phase II antigen.

All of our own strains that were tested, and all the Danish ones, were toxic to guinea-pigs.

Of seven strains from the Lister Institute, growing on egg-medium, three (nos. 760, 2471 and 2474) were in Phase III and four (nos. 366, 759, 364 and

761) in Phase IV. Six of these proved non-toxic; the seventh (no. 2474) not being tested.

Of five strains from the New York State Laboratories four were labelled Type B, and three of them proved to be Phase III and non-toxic. The fourth (no. 36), though agglutinating as a Phase III culture, had a strong toxic element which was identified by agglutination and absorption experiments with Phase II. The remaining strain, labelled Type A, was a non-toxic Phase IV. The classification of these American strains was confirmed by the testing of the American type-serums, of which "B" proved to correspond absolutely with our III and "A" with our IV.

Technique.

(1) We have used the Danish modification of the original Bordet-Gengou medium, altered in a few respects. It is made as follows: potatoes are cleaned and cut into thin slices: 1 kg. of the slices with 2 litres of distilled water and 80 c.c. glycerine are boiled until the potatoes are soft. The water lost in the boiling is made up and the whole is filtered through linen. The juice is then adjusted to *p*H 7·0. One part of the neutralised juice and 3 parts of 0·6 per cent. NaCl are mixed and distributed in flasks; 200 c.c. in each. 4 per cent. agar is added and the flasks are left in the cold store overnight. The medium is then autoclaved, and to every 200 c.c. are added 4·5 c.c. of a sterile *N*/2 solution of lactic acid (3·72 c.c. of ac. lact. of sp. gr. 1·21 to 100 c.c. of aq. dest.). When the flasks have been cooled to 45–50° C., 100 c.c. of fresh defibrinated horse's blood is added to each. The medium is then mixed well and poured into Petri dishes. It will keep good for a fortnight in the cold store.

(2) *Suspensions* were made in normal saline solution, to which 0·2 per cent. formalin had been added. In the case of cultures grown on Bordet-Gengou medium, the growth was scraped from the surface of the medium with a metal spatula and rubbed up in a small amount of normal saline solution in a mortar. On the whole the bacilli remained suspended extremely well, though it was sometimes necessary to shake thoroughly in order to obtain a good suspension. Salt sensitiveness was occasionally shown by cultures in Phase III when grown on Bordet's medium, but the phenomenon was very inconstant, for the next subculture on blood medium, or growth on egg, might be quite stable.

(3) Agglutinating serums were obtained by injecting formolised suspensions into the ear veins of rabbits. Two injections of 0·5 and 1·0 c.c. were given with a week's interval, and a blood-sample was taken on the fifth or sixth day after the last injection. If necessary, another injection was given, and the animal was bled after a further interval of 6 days. We have found that, generally speaking, two injections were sufficient to produce an agglutinating serum with a titre from 2000 to 5000.

(4) Agglutination tests were done with Dreyer's technique (Med. Res. Council 1920), and the tubes were incubated in the water-bath at 52–54° C.

Results could be read at 4½ hours, but a final reading was always taken after 18 to 24 hours. The last definite trace read with a hand-lens after incubation overnight was taken as the end-point of the reaction.

All suspensions were tested as a routine with antiserums for Phases I, III and IV, and if necessary other serums, *e.g.* Phase II, were included. The sensitivity of various suspensions varies very little, except for the occasional reduced sensitivity of Phase IV when grown on Bordet's medium. In the case of anomalous results, *e.g.* a supposed Phase IV suspension agglutinating to full titre with a Phase III serum, subsequent examination of the culture by plating and colony pickings has shown both phases to be present.

(5) Absorption tests were carried out in the usual manner. The quantities of bacilli used were in the region of 8·0 mgm. of moist culture to 1·0 c.c. of serum, diluted either 1:20 or 1:40 according to its titre. Thus, three well-grown Petri dishes of Bordet's medium emulsified in 10–12 c.c. of saline made a suitable suspension, to which the serum was added in the requisite amount. The absorption and control mixtures were incubated in corked tubes at 37° C. for 2½–3 hours, left at room temperature overnight and then centrifuged.

THE RELATIONSHIPS OF THE FOUR PHASES.

In Fig. 1 are given the averages of a large number of experiments in which the constancy of the results was remarkable; the slight variations of titre that were observed being probably due to differences in the sensitivity of the suspensions. The Phase I serum (no. 1) was made from a suspension of the colonies on a cough-plate which showed almost a pure growth of *H. pertussis*. It agglutinates Phase III very little compared with Phase I. It must, however, be mentioned that another serum (no. 2), made with the same strain after cultivation on Bordet's medium for some months, had as high a titre for Phase III as for Phase I. Another serum made a little later had a similar character. We have used Phase I, no. 1 serum throughout the whole of this work, since it distinguished the phases so sharply. The characters of the numerous other Phases II, III and IV serums, made for various purposes, agreed in all essential points with those shown in the figure.

Absorption tests were done from time to time to confirm the agglutination results. In Table I are given the results of some experiments to determine the cross-absorptive powers of Phases I, II, III and IV suspensions. For the sake of brevity only one representative set of experiments is given, though the results have been confirmed on other occasions with different strains.

The chief points demonstrated are: that Phase I agglutinins are untouched by absorption with Phases II, III and IV. Phase I reduces the homologous titre of Phase II serum and removes Phase III agglutinins from all the phase serums. It is likely that freshly isolated Phase I bacilli would have less, if any, affinity for the Phase III antibody. Phases I and III have a definite though partial absorptive action on Phase IV serum, and finally Phase IV has little or no action on heterologous serums.

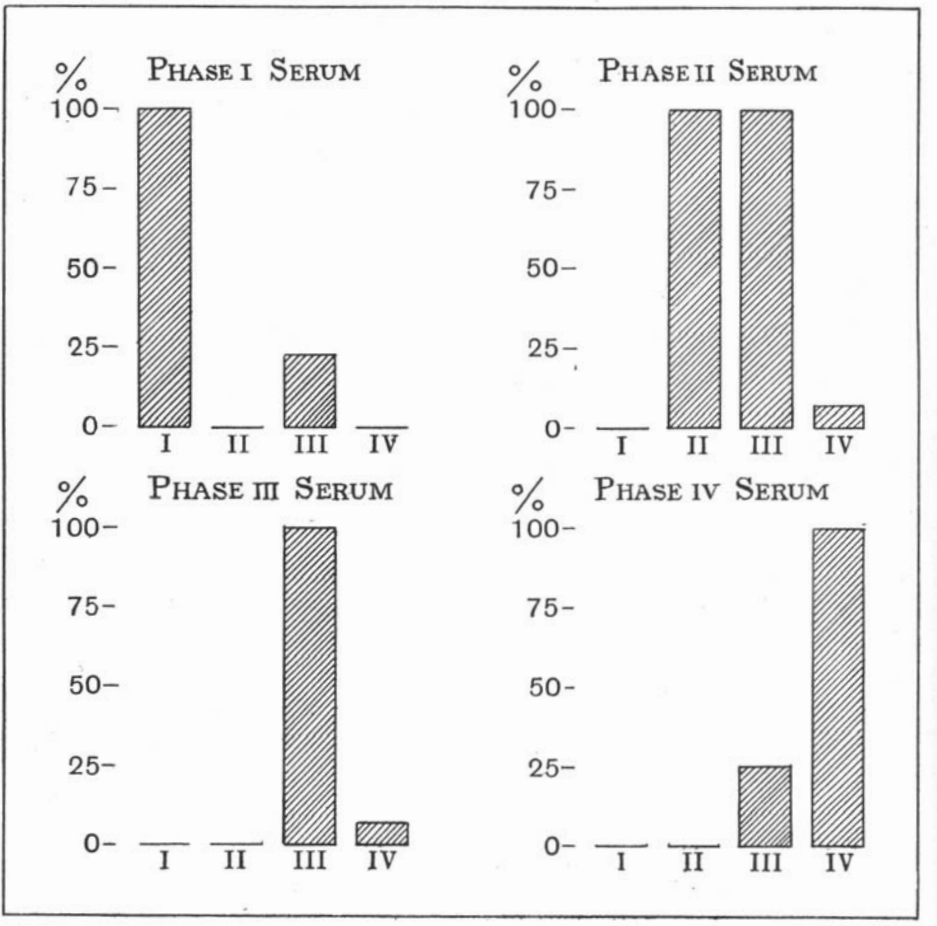

Fig. 1. *Cross-agglutination Tests with Phase Serums.* Minor agglutinins expressed as percentages of the titre to the homologous serum-strain.

Table I. *Cross-absorption tests with Phases I, II, III and IV.*

Serum	Organism agglutinated Phase and Strain	Titre before absorption	Titre after absorption with Phase I (H.W.)	Phase II (Am. 36)	Phase III (2471)	Phase IV (364)
Phase I, No. 3	I (H.W.)	4000	100	—	4000	4000
	I (Ruth)	2000	—	1000	—	—
	III (2471)	2000	<100	—	<100	1000
Phase II	II (H.W.)	2–4000	500	50	4000	—
	II (Am. 36)	5000	—	<50	—	—
	III (2471)	4000	<100	—	<100	—
Phase III	III (2471)	1000	50	—	<50	1000
Phase IV	III (2471)	500	<100	—	<100	<100
	IV (364)	1000	400	—	300	<100

All cultures were grown on Bordet's medium and the absorption doses were in the region of 7–8 mgm. of moist culture per c.c. of diluted serum.

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Comparing these results with Bordet's and Krumwiede's records, we find that they are all in accord if Bordet's first serological state corresponds with our Phase I, and if his second state, together with Krumwiede's type B, is our Phase III. Krumwiede's type A corresponds completely with our Phase IV.

TRANSFORMATION OF PHASES.

By transplantation on to appropriate media, addition of immune serum to a fluid substrate, selection of colonies and other simple procedures, we have shown that a culture derived from a single colony can produce in turn all the antigens characteristic of the four phases. Moreover, the change is to a considerable degree reversible, though there is evidence that Phase IV may become firmly and irrevocably established in some cultures. The details of the experiments are sufficiently shown in Table II.

It was in the course of these experiments that we first recognised the antigen characteristic of Phase II, and therefore an agglutinating serum for it was available only in the later stages. This phase does not seem to occur regularly as an intermediate stage between I and III, and many points about it remain obscure; but we have had no difficulty in proving its reality by the usual cross-agglutination and absorption tests (Table I and Fig. 1). It is toxic and tends to undergo transformation to Phase III.

THE SIGNIFICANCE OF THE TRANSFORMATION OF PHASES.

We believe that Phases I and II correspond to the smooth or pathogenic form of other bacteria, Phases III and IV to the rough, relatively harmless, saprophytic form. Under cultivation there is a general tendency to pass from the smooth to the rough state, but the addition of sufficient fresh blood to the medium makes it possible for the parasitic phase to persist unaltered for long periods. This accounts for Kristensen's belief in the serological uniformity of the species, for his strains were grown and stored exclusively on Bordet's medium. It appears from the work of Debré, Marie and Pretet (1928) that small quantities of blood in the medium are not so efficacious, for their cultures isolated a year previously were agglutinated by the American type B serum, *i.e.* had changed to Phase III.

Of our "rough" phases, no. IV is the more complete, being apparently irreversible in some cultures. Phase III may be considered as a transitional or partial rough, with the power either to revert to I or pass on to IV.

The direct, visual qualities of roughness and smoothness are not very obvious in *H. pertussis*, nor is there any constant and measurable difference in salt-sensitiveness. But there is no doubt that Phases III and IV are on the average rougher and have a greater tendency to instability in NaCl solutions than Phase I.

Table II. *Transformation of phases.*

Exp.	Strain	Course of cultivation on media named	% agglutination with phase serums I	II	III	IV	Remarks
1	H.W.	Bordet (single colony)	100	0	0	0	Isolated by us 1929. Toxic to guinea-pigs (Table III)
		subcultured Bordet 18 *months*	100	0	0	0	
		S.B. (3 months at R.T.)					
		S.B. (4 months at R.T.)					
		Bordet (3 colonies agglutinated)	100	—	0	0	
		S.B. (1 generation)					
		S.A.	25–50	—	0	0	No Phase II serum available
		S.A. several generations	50–100	—	0	0	
		Egg (3rd subculture)	15–20	—	100	20	Agglutinogenically Phase III and non-toxic to guinea-pigs (Table IV)
		Egg (1 colony on old slope at R.T.) 2nd subculture agglutinated	0	0	0	100	Agglutinogenically Phase IV and non-toxic to guinea-pigs (Table V)
2	H.W.	Bordet (single colony)	100	0	0	0	Toxic to guinea-pigs (Table III)
		5 % Phase I immune S.B. (2 subcultures)					Agglutinogenically Phase III. Very early experiment, when no Phase III serum was available
		S.B. (6 days' growth)	20	—	—	25	
		5 % Phase I immune S.B. (3 subcultures)	0	50	0	0	Agglutinogenically Phase II
		1 colony on S.A.					
		Egg (8 subcultures)	20	—	100	25	
		Broth and agar (several single colonies agglutinated)	20	—	100	25	
3	Holland	Bordet (single colony)	100	0	0	0	Isolated by us 1929. Toxic to guinea-pigs (Table III)
		5 % Phase I immune S.B. (3 subcultures)					
		S.A. (single colony)	0	100	0	0	
		1 colony					
		Egg (2nd subculture)	20	—	100	25	
4	Lister 761	Bordet (single colony)	0	—	0	100	Received on egg from Lister Institute; obtained from America 1920
		Bordet (10 daily subcultures)	0		0	100	Confirmed by absorption test
		S.B. (24 hours)					
		S.A. (2 generations)	20	—	100	50	Confirmed by absorption test
			20	—	100	25	
5	Lister 761	Bordet (single colony)	0	—	0	100	
		S.B. (24 hours)					
		Bordet (plating)					
		3 colonies agglutinated	0	—	0	100	
		1 colony	? 25	—	?100	? 25	Very salt-agglutinable suspension-readings only approximate
		Egg	50	—	100	25	Non-toxic to guinea-pigs (Table IV)
6	Lister 2471	Egg	25	—	100	25	Received on egg from Lister Institute, isolated 1927
		Bordet (plating)					
		majority of colonies	25	—	100	25	Non-toxic to guinea-pigs (Table IV)
		1 colony	100	—	0	0	Toxic to guinea-pigs (Table III)
		Egg (one generation)	20	—	100	25	Second generation on egg proved non-toxic to guinea-pigs (Table IV)

Abbreviations: S.B. = 5 % sheep-serum broth. S.A. = 5 % sheep-serum agar. R.T. = room temperature.

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A NOTE ON THE RELATIONSHIP BETWEEN *H. PERTUSSIS* AND *B. BRONCHISEPTICUS*.

Ferry and Noble (1918) have described a one-sided serological relationship between *B. bronchisepticus* and *H. pertussis*, in that an antiserum of the former agglutinated suspensions of the latter organism to a considerable extent, whereas their *pertussis* antiserum had no effect upon suspensions of *bronchisepticus*. Further, normal serums of a rabbit stock which had an epidemic due to the latter organism agglutinated *pertussis* sometimes up to a titre of 1:800.

We have found that an agglutinating serum made from a strain of *bronchisepticus* (N.T.C. Man) reacted on our *pertussis* Phase IV to 20–100 per cent. of the homologous titre, and on Phase III to 5–25 per cent.; the suspensions varying greatly in their agglutinability by this serum. *Bronchisepticus* suspensions were agglutinated partially by Phase IV serums and were completely unaffected by all our other phase-serums. Evidently there is a close serological relationship between this strain of *bronchisepticus* and our *pertussis* Phase IV. Since *bronchisepticus* infection is unknown in our animal houses we are satisfied that no error due to this infection has affected our serological results.

TOXICITY EXPERIMENTS.

With the two exceptions mentioned in the tables all strains tested for toxicity were grown for 48 hours on Bordet's medium in Petri dishes after sufficient subcultures had been made to induce free growth. The bacilli were removed from the surface of the medium, weighed wet, suspended in either saline or Ringer's solution, and injected intraperitoneally into guinea-pigs as soon as possible.

The doses were given in milligrams irrespective of the weight of the animal, and were subsequently calculated in relation to the blood volume of each animal, according to Dreyer's (1911) formula, thus:

$$\frac{\text{Dose in mgms.} \times 1000}{(\text{Weight in grams})^{0{\cdot}72}}.$$

The results are expressed as whole numbers.

In Tables III and IV the results of injection of Phases I, III and IV are given. In the case of the type strain (H.W.) in Phase I it will be seen that, when the doses are arranged in decreasing amounts, a point is reached where some of the animals live and others die. The same effect is shown by our own recently isolated strains. This dose, which may be termed the approximate M.L.D., lies in the region of 25–30. Bordet's view that the death is purely toxic and that there is no multiplication of the culture within the guinea-pig is fully corroborated by our observations. The percentage loss of weight, calculated for all animals that lived for four days or upwards, is roughly propor-

tional to the size of the dose. The post-mortem changes in the animals that died from toxaemia varied greatly according to whether the death was acute or delayed. In those dying within three days the changes were similar to those described by Bordet, viz. blood-stained peritoneal effusion with petechial haemorrhages in the peritoneal wall, commencing necrosis in the liver and intense injection of the testicles and sometimes of other organs, *e.g.* the spleen. In our experience the pleural effusion described by Bordet has not occurred frequently. When death is delayed for 30 days these acute reactions have

Table III. *Toxicity experiments with Phase I cultures.*

Type strain H.W.					Other Phase I strains					
Sex	Guinea-pig weight	Dose	Death in days	% loss in weight	Sex	Strain	Guinea-pig weight	Dose	Death in days	% loss in weight
♀	206	87	2	—	Own recently isolated strains:					
♂	312	64	Lived	0*						
♂	320	63	2	—	♂	Hart	245	76	1	—
♀	336	61	5	35	♂	Holland	245	76	2	—
♂	350	59	6	31	♂	McMahon	247	76	2	—
♂	353	59	2	—	♂	McMahon	229	60	1	—
♀	415	58	2	—	♂	Hart	271	36	9	26
o?	360	58	6	27	♂	Holland	280	35	7	28
♂	361	58	6	40	♂	Mace	325	31	8	33
♀	363	58	2	—	♀	Christine	445	25	7	38
♀	368	58	2	—	♀	Low	561	21	Lived	37
♂	372	56	2	—	♀	Low	576	21	Lived	20
♂	375	56	2	—	♀	Low	584	10	Lived	9
♀	376	56	2	—						
♂	380	55	5	26	Danish strains:					
♂	382	55	2	—	♂	E. Chr.	229	80	2	—
♂	400	54	3	—	♂	Else†	230	80	4	—
♂	443	50	6	33	♂	Ruth	252	75	1	—
♂	464	48	13	41	♂	1778	256	74	2	—
♂	473	47	2	—	♂	E. Chr.	222	61	Lived	0
♂	401	27	Lived	29	♂	Else	226	61	7	27
♀	412	26	26	38	♂	Ruth	228	60	2	—
♀	432	25	10	37	♂	1778	243	57	Lived	38
♀	264	18	Lived	22						
♂	403	13	Lived	18	Reverted strains:					
♂	238	5	Lived	0	♀	Lister 2471	263	72	3	—
♂	267	1	Lived	0	0?	Lister 2471	326	62	11	27
					♂	Lister 2471	260	37	Lived	23

$$\text{Dose} = \frac{\text{mgm.} \times 1000}{\text{Wt. (gm.)}^{0 \cdot 73}} \text{ expressed as nearest whole number.}$$

* Subsequently injected with a dose of 59—death in 3 days.
† "Else" was a mixture of Phase I and a small amount of Phase II.

cleared up, but there is evidence of extensive damage to the liver in the shape of numerous focal necroses in all stages of degeneration and repair.

Table IV shows that guinea-pigs tolerate a twenty or thirty times larger dose of Phases III and IV than of Phase I. We have therefore felt justified in using the term toxic for the latter, and non-toxic for the former, although it is not impossible that Phase III may possess a residue of toxicity, demonstrable perhaps in other animals or by some more delicate method.

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Table IV. *Toxicity experiments with Phase III and IV cultures.*

	Guinea-pig sex	Strain	Guinea-pig weight	Dose	Result	% loss in weight
PHASE III.						
Lister strains:	♀	2471	243	610	Lived	11
	♂	760	240	290	Lived	10
	♂	2471	272	140	Lived	0
American Type B strains:	♂	35	199	440	Lived	9
	♂	34	317	320	Lived	10
	♂	33	344	300	Lived	7
	♂	35	193	230	Lived	0
	♂	34	315	160	Lived	0
	♂	33	353	150	Lived	5
Transformed strains:	♂	Lister 761	190	460	Lived	2
	♂	H.W.	340	300	Lived	19
	♂	Lister 761	214	210	Lived	0
	♂	H.W.	362	140	Lived	3
	♀	Lister 2471	274	70	Lived	7
	♀	Ph. 1–111 (Egg)	332	31	Lived	2
PHASE IV.						
Lister strains:	♀	364	242	770	Lived	0
	♂	366	259	370	Lived	0
	♂	759	259	370	Lived	12
	♀	364	300	260	Lived	0
Transformed strains:	♂	H.W.	320	310	Lived	10
	♂	H.W.	338	150	Lived	3

$$\text{Dose} = \frac{\text{mgm.} \times 1000}{\text{Wt. (gm.)}^{0 \cdot 72}}.$$

IMMUNITY EXPERIMENTS.

The following experiments were performed in order to discover whether it is possible to protect guinea-pigs by means of vaccines against a lethal dose of living Phase I organisms, and also whether there is any difference in the immunising powers of the various phases. All vaccines and test cultures were grown on Bordet's medium and their serological state was determined by agglutination. The normal animals used as controls are all included in Table III (strain H.W.).

Exp. no. 1 was the comparison between killed Phase I and living Phase III vaccines. A Phase I vaccine (strain H.W. 4·0 mgm. per c.c.) was divided into two portions; one killed by heating to 55° C. for ½ hour, the other killed in the cold with 0·4 per cent. formalin. Both were tested for sterility before use. Phase III vaccine consisted of a living suspension of strain 2471, 4·0 mgm. per c.c. Animals received subcutaneously two injections of the same dose, with a 7 days' interval; and 14 days after the second a test dose of living Phase I organisms (strain H.W.) was given intraperitoneally. The results are shown in Table V. The twelve guinea-pigs that had received Phase I vaccine survived, while those injected with Phase III living vaccine showed no immunity at all. None of the controls survived.

In order to compare our phase vaccines with those on sale for human use, some prophylactic *B. pertussis* vaccine was obtained from Parke Davis and Co. It was found to be serologically in Phase III.

Exp. no. 2. *Vaccines of Phases I, III and IV were made by suspending*

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the bacilli in physiological saline to which 0·2 per cent. of formalin had been added. They were then diluted to the same opacity as the Parke Davis vaccine and were left in the cold store, being shaken daily until sterile.

Table V. *Immunity experiment no.* 1.

Phase I vaccine:						Phase III vaccine (living):				
Nature	Two doses of mgm.	Guinea-pig weight	Test dose	Result	% loss in weight	Two doses of mgm.	Guinea-pig weight	Test dose	Death in days	% loss in weight
Heated 55° C.	2·0	320	63	Lived	31	1·0	292	67	4	—
,,	1·0	359	58	,,	33	2·0	311	64	2	—
,,	4·0	369	57	,,	29	4·0	354	59	5	—
0·4 % formalin	4·0	366	57	Lived	38					
,,	1·0	377	56	,,	19					
,,	2·0	397	54	,,	26					
,,	4·0	309	32	,,	8	4·0	304	33	14	32
,,	1·0	328	31	,,	22	2·0	352	29	14	34*
,,	2·0	362	29	,,	17	1·0	385	28	46	31
Heated 55° C.	4·0	357	29	Lived	13					
,,	1·0	378	28	,,	11					
,,	2·0	403	27	,,	0					

All the guinea-pigs were females.

$\text{Dose} = \frac{\text{mgm.} \times 1000}{\text{Wt. (gm.)}^{0\cdot7\cdot2}}$ expressed as nearest whole number.

* Killed in a moribund condition.

The animals received an initial dose of 0·25 c.c. subcutaneously followed at intervals of 6 and 5 days by doses of 0·5 and 1·0 c.c. respectively. Twenty-one days after the last injection they received a test dose of a living Phase I (strain H.W.) suspension intraperitoneally.

The results are given in Table VI. None of the animals vaccinated with Phases III and IV or with the Parke Davis vaccine survived, but of the four animals which received Phase I two lived. The remaining two and all the controls died.

Exp. no. 3 was similar, except that other strains were used for making the vaccines, which, as before, were made up in physiological saline solution containing 0·2 per cent. of formalin to the same density as the Parke Davis vaccine. The animals received an initial dose of 0·25 c.c. followed at intervals of 5 days by 0·5 and 1·0 c.c. Twenty-two days after the last injection they received a test dose of a living Phase I (strain H.W.) suspension intraperitoneally.

The results are given in Table VI. Out of eight animals vaccinated with the Phase I suspensions two survived. All those receiving the Parke Davis and the Phase III vaccine, as well as the controls, died.

Summarising the last two experiments: out of twelve guinea-pigs vaccinated with Phase I four survived, and of nineteen animals treated either with Parke Davis vaccine or with Phases III and IV none survived. Eight control animals also died.

A further experiment was done by immunising ten animals with a vaccine made with a recently isolated Phase I strain. These, with an equal number

of controls, were then injected intraperitoneally with a dose of 55 of the same toxic strain (H.W.) used in the previous experiments. The experiment was a failure, since out of the ten controls, four survived with no loss of weight. This was completely opposed to our previous experience in which out of a total of twenty-seven animals injected with our own toxic strains in doses greater than 30, only one had survived. Whether this increase in percentage of animals unaffected by a toxic dose of this strain is an indication of a commencing loss of Phase I antigen, we are unable to say. Although the strain had been under cultivation for some 18 months, it was still serologically in Phase I and we had no reason to expect a falling off in toxicity. Of the immunised group of animals just as many died as of the controls, so that no evidence of immunity was obtained.

Table VI.

Immunity experiment no. 2.

Vaccine. Doses: 0·25, 0·5, 1·0 c.c.

Guinea-pig sex	Strain	Phase	Guinea-pig weight	Test dose	Death in days	% loss in weight
♂	Low	I	314	64	Lived	0*
♀	,,	,,	362	58	6	30
♀	,,	,,	369	57	2	—
♂	,,	,,	381	56	Lived	0†
♂	2471	III	395	54	2	—
♂	,,	,,	400	54	3	—
♂	,,	,,	447	49	3	—
♂	,,	,,	452	49	3	—
♀	Parke Davis	III	420	52	3	—
♀	,,	,,	426	51	31	53
♀	,,	,,	449	49	23	48
♀	,,	,,	‡			
♀	364	IV	380	56	2	—
♀	,,	,,	400	54	2	—
♀	,,	,,	411	52	2	—
♀	,,	,,	431	51	2	—

Immunity experiment no. 3.

Vaccine. Doses: 0·25, 0·5, 1·0 c.c.

Guinea-pig sex	Strain	Phase	Guinea-pig weight	Test dose	Death in days	% loss in weight
♀	Low	I	318	63	2	—
♂	,,	,,	360	58	9	41
♀	,,	,,	371	57	10	37
♂	,,	,,	467	47	6	39
♂	H.W.	I	375	56	Lived	0
♂	,,	,,	396	54	12	40
♂	,,	,,	416	52	Lived	37
♂	,,	,,	434	50	6	38
♂	Parke Davis	III	346	60	5	34
♂	,,	,,	368	57	5	36
♂	,,	,,	398	54	5	30
♂	,,	,,	394	54	6	37
♂	H.W.	III	349	59	3	—
♀	,,	,,	369	57	10	43
♂	,,	,,	406	53	4	—
♀	,,	,,	490	46	11	49

$$\text{Dose} = \frac{\text{mgm.} \times 1000}{\text{Wt.}\ (\text{gm.})^{0\cdot72}}$$ expressed as nearest whole number.

* 3½ months afterwards reinjected with Dose 59—death in 3 days.
† 3½ months afterwards reinjected with Dose 56—lived with 4 % loss weight.
‡ Died during vaccination. P.M. cause of death unknown—cultures negative.

The general results of our immunity experiments therefore are irregular, and further work is clearly needed. But the evidence so far obtained, coupled with the serological findings, indicates that for prophylactic vaccination in the human subject Phase I vaccines made from recently isolated strains are more promising than those made from old cultures.

The work of Teissier, Reilly, Rivalier and Cambassédès (1929) on the immunising properties of *pertussis* endotoxin, which came to our notice after the completion of this work, shows that a more solid protection can be obtained with formolised endotoxin than with formolised bacilli. It also suggests that older cultures, presumably some of them in Phase III, are by no means devoid of endotoxin. Some experiments we have done on the effects of intradermal injections of cultures in rabbits have also led us to suspect this. These authors are pessimistic about the possibilities of immunising human beings,

owing to the intensity of the reactions and the apparent failure to produce antibodies; but there is no reason to believe that the last word on this subject has yet been said.

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ADDENDUM. Since this paper was written we have isolated 11 more strains of *H. pertussis*, all of which were in Phase I.

SUMMARY.

Haemophilus pertussis is a uniform species, without fixed varieties or "types."

After isolation from the human subject it tends to pass through a series of antigenically distinct phases, of which the first two are toxic to guinea-pigs, whereas the last two are relatively harmless. The former probably correspond to the smooth, the latter to the rough phase of other bacteria.

The phase-changes are, up to a point, reversible.

Experiments on guinea-pigs have produced some evidence that the toxic Phase I is the best and perhaps the only antigen for the production of active immunity in guinea-pigs.

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