SOME SURFACE COMPONENTS OF *HÆMOPHILUS PERTUSSIS*: IMMUNISING ANTIGEN, HISTAMINE - SENSITISING FACTOR AND AGGLUTINOGEN

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IT has been shown by Frappier and Guérault (1954, 1955-56) and Frappier, Guérault and de Repentigny (1955) that a fraction of *Hæmophilus pertussis* obtained by washing the bacilli from liquid cultures in saline or distilled water will immunise mice against intracerebral challenge. The washings were practically devoid of toxicity. Not all the immunising antigen was removed; washed bacteria were almost as effective in protecting mice as the washings. An extract prepared by mechanical disintegration of the bacteria also protected mice. This extract was strongly toxic, but its toxicity or that of a bacterial suspension could be removed by heating at 56° C. for 1 hr with 0·16 per cent. formaldehyde. Detoxification did not decrease the immunising potency. The washings and the washed bacteria as well as the extract produced agglutinin in rabbits.

The investigations reported here have confirmed in general the data relating to washings and washed bacteria and have extended the earlier work in several directions. Cultures have been grown on cellophane-covered Bordet-Gengou (BG) medium. Bacterial suspensions, washings, washed bacteria, a chemical extract and the extracted residue have been compared with regard to mouse-protective antigen, histamine-sensitising factor (HSF) and agglutinogen. Antibodies in human hyperimmune serum have been investigated. A preliminary chemical examination of washings and extract has been made.

MATERIALS AND METHODS

Strains used. Strain 360E was received from the Glaxo Laboratories. It had relatively high values in sensitising mice to histamine and in protecting them against intracerebral challenge. Strains H35 and H36 were obtained from Dr B. W. Lacey. Strain GL353 was obtained from Mr A. F. B. Standfast as being suitable for intranasal challenge. Strain 18323 was the standard Kendrick strain used for intracerebral challenge.

Strains were preserved by freeze-drying from cultures on BG medium. During the period of experiments they were maintained in subcultures on BG medium.

Cultivation. Unless otherwise stated, cultures for experiments were grown aerobically at 35° C. for 3-4 days on cellophane-covered BG medium. For such cultures, a cellophane disc, sterilised by autoclaving, was aseptically applied to the surface of the medium in a Petri dish before inoculation.

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Strains used for preparing challenge suspensions were recovered from the freeze-dried state and cultured on bare BG medium.

Preparation of bacterial suspension. Unless otherwise stated, strain 360E was used for making suspensions as well as for washings and extract, which are described in the next sections. Growth was carefully scraped off into 0·85 per cent. NaCl to make a suspension of 25×10^9 organisms per ml.; these were left in contact with 0·1 per cent. formaldehyde solution at 4° C. for at least 10 days.

Preparation of washings and washed bacteria. Growth was emulsified in saline to a concentration of 18×10^{10} organisms per ml. This suspension was shaken vigorously by hand for 2-3 min. at room temperature and immediately centrifuged to deposit all the bacteria. The supernatant liquid was dialysed against distilled water at 4° C. until free from chlorine ions. It was a clear solution free from precipitate and is referred to as " washings ". The washings were freeze-dried before physico-chemical investigations were made.

The deposited bacteria were resuspended in saline at the original concentration and stored at 4° C. This preparation is referred to as " washed bacteria ".

Preparation of extract and residue. We are indebted to Dr B. W. Lacey for hitherto unpublished details of the method he used for removing the surface antigen of *H. pertussis* (Lacey, 1958). The growth was scraped off and transferred to physiological saline acidified with *N*/20 HCl to *p*H 5·5-6·5. This was shaken vigorously by hand for 2-3 min. at room temperature to suspend the bacteria, at a concentration of about 18×10^{10} per ml. To one volume of suspension one volume of " TUF " mixture (thiourea 30 g., urea 260 g., formamide 127 ml., water to 550 ml., *p*H adjusted if necessary to $<7 \cdot 0$) was added slowly with rapid mixing. After adding TUF the suspension was again shaken vigorously for 1 min. and centrifuged to deposit the extracted bacteria. They were washed once in saline, resuspended in saline and stored at 4° C. They are referred to as " residue ". The supernatant liquid obtained after extraction was dialysed at 4° C. overnight against physiological saline buffered at *p*H 6·5 with *M*/200 acetate (Walpole's). During dialysis a fine precipitate formed so that a faintly cloudy suspension was produced which settled out slowly on standing. This entire product is referred to as " extract ". It was dialysed further against distilled water, freeze-dried and stored in that form.

Preparation of antisera. Rabbits were immunised with bacterial suspension, washings, extract, and residue. The last three preparations contained the amount of material which would be derived from a similar volume of bacterial suspension. Rabbits were inoculated intravenously on three successive days in each of three weeks. The dose of inoculum was 0·5 ml. for the first week and 1·0 ml. thereafter. Serum was tested for agglutinin before inoculation and at the beginning of the second and third week. In the fourth week a preliminary bleeding to harvest serum was made; three injections were given in the fifth week, and one week later a further amount of serum was collected. For use in the experiments approximately equal amounts of serum taken at 4 and 6 weeks were mixed. Serum from two to four rabbits was pooled.

Technique of agglutination. Living suspensions of strain 360E were made in saline by emulsifying growth from BG medium, without cellophane, incubated at 35° C. for 2 days.

Killed suspensions were made from 4-day cultures by adding 0·1 per cent. formaldehyde and storing at 4° C. until the bacteria were dead, the minimum period of storage before use being about 10 days. Instead of storage some formalinised suspensions were heated at 56° C. for 1 hr. In all suspensions the number of organisms was about 3×10^{10} per ml.

Agglutination tests in Dreyer tubes were placed in a water-bath at 37° C. for 2 hr and read after standing overnight at room temperature.

Methods of testing for histamine-sensitisation and anti-HSF. Female white

mice of the Schofield strain, weighing 23-25 g., were used. To detect histamine-sensitisation 1 ml. of the test material was injected intraperitoneally. Five days later the mice were given intraperitoneally 6 mg. histamine dihydrochloride (British Drug Houses, London) in 0·5 ml. *M*/15 phosphate buffer, *p*H 7·4 (150 mg. histamine dihydrochloride per kg. body-weight). The number dead within 24 hr was recorded. Normal mice of the same weight, used as controls in each experiment, were shown not to be killed by 6 mg. or 30 mg. histamine ; 60 mg. always killed every mouse. These controls are not shown in the tables.

In testing for antibody to the histamine-sensitising factor (anti-HSF) the same procedure was followed except that the mice were pretreated by injecting 0·5 ml. of serum, or dilutions of it, intraperitoneally 1-2 hr before they were sensitised with a dose of *H. pertussis* vaccine which, if given to normal mice, would result in about 70 per cent. mortality when the mice were challenged with histamine. One group of normal mice was put up as control in each experiment to verify the effect of the sensitising dose.

Intracerebral challenge of actively and passively immunised mice

Active immunisation. Mice weighing 14-16 g. were given one intraperitoneal injection of 0·5 ml. of test material and challenged 14 days later.

Passive immunisation. Mice weighing 16-18 g. were given one intraperitoneal injection of 0·5 ml. of serum or diluted serum 1-2 hr before challenge.

The *challenge dose* was 0·03 ml. containing 100-150 LD50 of *H. pertussis* strain 18323, approximately 10^5 bacteria by opacity measurement. Deaths were recorded up to 14 days.

Intratracheal challenge of passively immunised mice. Mice weighing 14-16 g. were passively immunised with 0·5 ml. of serum or diluted serum given intraperitoneally 1-2 hr before challenge with strain G1353. Deaths were recorded up to 3 weeks after infection.

The intratracheal infection of mice was performed by Mr W. A. Te Punga who developed the technique (Te Punga and Preston, 1958). It is a more precise alternative to intranasal infection.

RESULTS

Examination of fractions of H. pertussis *for agglutinogen, histamine-sensitising factor, and protective antigen*

Agglutinogen. Four rabbits were inoculated with bacterial suspension and two each with washings, extract and residue. For each material the development of antibody in individual rabbits was uniform. Similar end-points were obtained when the sera were titrated with living suspensions and suspensions killed with formalin at 4° C. The pooled sera from the 4th and 6th week bleedings (see Methods) had the following titres : anti-bacterial suspension 1 in 12,800 ; anti-washings 1 in 640 ; anti-extract 1 in 3200 ; anti-residue (6th week bleeding only) 1 in 1600. These sera were used in subsequent experiments.

Histamine-sensitising factor (HSF). The histamine-sensitising properties of bacterial suspensions, washings and washed bacteria, extract and residue are compared in table I. Each experiment represents material from a separate batch of cultures.

TABLE I

Histamine-sensitisation with strain 360E

No. of organisms ($\times 10^9$) corresponding to dose (in 1 ml.)	Test material	No. mice dead/no. mice challenged with histamine, after injection of test material in experiment no.											Total	Per cent.
		1	2	3	4	5	6	7	8	9	10	11		
5	Bacterial suspension	10/10	10/10	7/7	9/10	7/9	6/9	8/8	3/10	9/9	8/10	9/10	86/102	84
20	Washings . .	9/10	6/6	6/10	9/10	6/9	4/10	9/10	7/10	9/10	10/10	...	75/95	79
20	Washed bacteria .	...	...	6/10	...	5/10	6/10	6/8	2/10	9/9	10/10	...	44/67	66
20	Extract . .	...	...	...	...	...	...	...	...	8/10	9/9	8/10	25/29	86
50		...	...	...	...	...	...	...	...	10/10	...	...	10/10	100
20	Residue . .	...	...	...	...	...	...	...	...	0/10	0/9	0/10	0/29	0
50		...	...	...	...	...	...	...	...	0/10	...	...	0/10	0

Bacterial suspension, washings and washed bacteria contained HSF. Extract also contained HSF but extraction appeared to be more efficient than washing in removing HSF as residue had no detectable HSF even when the dose was increased to an equivalent of 50×10^9 bacteria. A quantitative assessment of HSF was not attempted. The dose of suspension was chosen to give a high percentage of deaths in challenged mice. The doses of the other preparations were chosen arbitrarily as likely to indicate possible differences between them.

Strains H35 and H36 were each used in one experiment ; suspension, washings and washed bacteria were tested and results were similar to those noted for strain 360E.

In experiment 4 the effect of filtration on washings was tested. The unfiltered washings sensitised mice so that 9/10 died after challenge (table I). Filtrates gave the following results :—of 10 mice injected with the filtrate from 35 ml. washings passed through a 5 cm. Seitz EK filter, none died when challenged with histamine ; 30 ml. passed through a sintered glass filter of 0·9 μ A.P.D., 9/9 died ; 30 ml. passed through a gradocol membrane of 0·8 μ A.P.D., 6/10 died. Thus a Seitz filter pad was very active in adsorbing HSF, and in this experiment HSF was removed to below the level of detection in the filtrate.

In one experiment the sensitising potency of washings or washed bacilli was not affected by preparing them at 56° C. instead of room temperature.

The sensitising potency of washings was not affected, in two experiments, by adding 1 mg. per ml. of aluminium phosphate as adjuvant, or by dialysis against distilled water, or by freeze-drying.

Anti-HSF. The antisera made against bacterial suspension, washings, extract and residue were tested for anti-HSF. The results are summarised in table II. The sera against bacterial suspension, washings and extract had anti-HSF. Antiserum against residue had no detectable anti-HSF thus confirming the apparent absence of HSF in the residue by direct test (table I).

Of the three sera containing anti-HSF the order of decreasing potency was antiserum against bacterial suspension, against extract and against washings, which corresponded with the order of their agglutinin titres. The anti-residue serum although devoid of anti-HSF did have considerable agglutinin.

Active immunisation of mice against intracerebral challenge. The results of ten experiments are shown in table III. The experiments and their numbering are the same as those on HSF shown in table I. It will be seen that bacterial suspension, washings, washed bacteria, extract and residue all gave good protection against intracerebral challenge. The high immunising potency of extract is evident although exact quantitative comparisons cannot be made. Residue, which did not sensitise mice to histamine at a concentration up to

50×10^9 (table I), produced substantial immunity to intracerebral challenge.

Bacterial suspension, washings and washed bacteria from strains H35 and H36 were each tested once similarly, and gave some protection, but less than that given by strain 360E.

The effect of filtration, the addition of adjuvant, dialysis, freeze-drying and preparation at 56° C. on the immunising capacity of washings was tested concurrently with the similar experiments on HSF detailed in the previous section. The same materials were used for both sets of observations. In experiment 4 unfiltered washings protected 7 of 14 mice against intracerebral challenge (table III) whereas Seitz-filtered washings did not protect any mouse out of 14; washings filtered through sintered glass and a gradocol membrane

TABLE II

Neutralisation of HSF

Dilution of serum	No. mice dead/no. mice challenged with histamine, after injection with sensitising agent mixed with rabbit antiserum against			
	bacterial suspension	washings	extract	residue
1 in 2	...	0/10	1/10	8/10
1 in 5	0/10	...	...	...
1 in 10	...	3/10	0/10	6/10
1 in 25	0/10	...	...	...
1 in 50	...	4/10	3/10	6/10
1 in 125	4/10	...	...	...
1 in 250	...	6/10	7/10	7/10
1 in 625	6/10	...	...	...

Controls: injected with sensitising agent only: 8/10 died on challenge; of those injected with sensitising agent mixed with normal rabbit serum, 9/10 died on challenge.

protected 5/14 and 3/12 respectively. Thus the Seitz filter was markedly adsorbent for protective antigen and the other filters appeared to be less active, a result in conformity with the findings for HSF. Dialysis, freeze-drying, preparation at 56° C. and the addition of adjuvant, did not alter the immunising capacity appreciably, results which also conform to those for HSF.

Passive immunisation of mice against intracerebral and intratracheal challenge. The sera prepared in rabbits against bacterial suspension, washings, extract, and residue, and tested for agglutinin and anti-HSF as detailed earlier, were also tested for protective antibody by passive immunisation of mice. The results are shown in table IV. The residue antiserum was tested for intracerebral challenge only, and appeared to afford little if any protection to the mouse. The other sera gave protection against both intracerebral and intratracheal routes of challenge. The order of potency of the sera for passive protection was the same as for anti-HSF (table II).

TABLE III

Active immunisation of mice against intracerebral challenge

No. of organisms ($\times 10^9$) corresponding to immunising dose (in 1 ml.)	Test material	No. mice surviving/no. challenged intracerebrally, after immunisation with test material in experiment										Total	Per cent.
		1	2	4	5	6	7	8	9	10	11		
2·5	Bacterial suspension	11/13	10/14	13/14	14/15	9/14	12/15	5/14	9/11	14/15	14/14	111/139	80
10	Washings . .	8/14	3/7	7/14	7/14	13/15	8/14	2/14	12/15	9/15	...	69/122	57
10	Washed bacteria .	...	...	...	13/15	13/15	10/15	12/14	11/14	12/15	...	71/88	81
10 25	Extract . .	... ...	... ...	... ...	... ...	... ...	... ...	... ...	15/15 15/15	14/15 ...	15/15 ...	44/45 15/15	98 100
10 25	Residue . .	... ...	... ...	... ...	... ...	... ...	... ...	... ...	12/15 6/11	10/15 ...	8/14 ...	30/44 6/11	68 55
0	Controls . .	1/13	0/13	1/14	1/14	0/15	1/14	1/15	1/15	2/15	1/15	9/143	6

Antibodies to H. pertussis *in hyperimmune serum*

In view of the uncertainty regarding the identity of the antigen, or antigens, in *H. pertussis* concerned in establishing immunity in man the opportunity was taken to examine samples of human hyperimmune serum for agglutinin, anti-HSF, passive protection of mice, and anti-infective action on the bacteria. It was of particular interest to examine the sera for anti-HSF as no report on this topic was available.

Anti-pertussis serum was supplied by the Cutter Laboratories, Berkeley, California, U.S.A. This was a refined globulin fraction from the blood of adults having a history of pertussis in childhood, who had been hyperimmunised with Cutter pertussis vaccine. The globulin solution represented a ten-fold concentration of serum.

TABLE IV

Passive immunisation of mice against intracerebral and intratracheal challenge

Dilution of serum	No. mice surviving/no. mice challenged, after passive intraperitoneal immunisation with rabbit serum against						
	bacterial suspension		washings		extract		residue
	intra-cerebrally	intra-tracheally	intra-cerebrally	intra-tracheally	intra-cerebrally	intra-tracheally	intra-cerebrally
Undil.	...	...	9/12	8/10	26/27	10/10	3/15
1 in 2	11/12	...	...	...	...	...	...
1 in 5	...	10/10	9/12	2/10	17/26	7/10	1/14
1 in 10	12/12	...	...	...	...	...	...
1 in 25	...	5/10	1/12	2/10	7/27	0/10	0/15
1 in 50	8/12	...	...	...	...	...	...
1 in 125	...	4/10	2/12	0/10	1/27	1/10	0/15
1 in 250	2/12	...	...	...	...	...	...
1 in 625	...	0/10	...	...	...	...	...

Controls receiving no serum : { intracerebral challenge 1/32 survived ; intratracheal challenge 0/10 survived.

Serum was also received from the Institut Mérieux, Lyon, France. This was in two forms :—(*a*) pooled serum not processed, from donors who had been hyperimmunised with injections of pertussis vaccine, and (*b*) gamma globulin prepared from pooled hyperimmune serum and representing a ten-fold concentration.

These sera were compared with a control immune rabbit serum prepared against Pillemer's stromata protective antigen (SPA) which contained anti-HSF (Maitland, Kohn and Macdonald, 1955) ; it also protected mice against intracerebral challenge. A negative control of normal human serum was also included.

It was shown, as a preliminary, that the Cutter and Mérieux vaccines, as supplied by the respective laboratories, sensitised mice to histamine and actively immunised mice against intracerebral challenge.

The hyperimmune human sera against these vaccines contained anti-HSF (table V). End-points were not attempted, but the results indicated clearly that in man anti-HSF is produced in response to

repeated doses of pertussis vaccine. The human antisera appeared to be somewhat weaker than the known positive rabbit serum.

TABLE V

Anti-HSF property of human hyperimmune serum

Dilution of serum	No. mice dead/no. mice challenged with histamine 5 days after intraperitoneal injection of a mixture of HSF and the stated dilution of						
	Cutter globulin	Mérieux serum		Mérieux globulin		anti-SPA rabbit serum	normal human serum
		Lot 369	Lot 1610	Lot 169	Lot 269		
Undil.	0/19	1/9	0/10	...	...	...	6/10
1 in 1·5	...	...	...	...	0/9	...	...
1 in 2	0/9	...	...	...	...	...	...
1 in 3	...	...	...	0/10	...	...	...
1 in 5	0/10	...	...	...	...	0/30	...
1 in 10	0/10	3/10	1/10	...	...	...	...
1 in 20	...	...	...	...	...	0/10	...
1 in 40	0/10	...	...	...	...	...	...
1 in 50	2/10	5/10	7/10	...	...	...	...
1 in 80	...	...	...	...	...	4/10	...
1 in 100	5/10	6/10	7/10	...	...	...	...
1 in 160	4/10	...	...	...	...	...	...
∞	21/29	15/19		6/9		21/29	6/9

Cutter globulin and Mérieux unprocessed sera protected mice passively against intracerebral challenge (table VI). In addition the Cutter globulin reduced the infectivity of *H. pertussis*. Mixtures of

TABLE VI

Effect of hyperimmune human serum on intracerebral infection of mice

Dilution of serum	No. mice surviving/no. mice challenged with *H. pertussis*					
	after passive protection with the stated dilution of				previously mixed with the stated dilution of	
	Mérieux serum		Cutter globulin	anti-SPA rabbit serum	Cutter globulin	anti-SPA rabbit serum
	Lot 369	Lot 1610				
1 in 5	...	...	...	25/30	...	13/15
1 in 10	4/15	7/15	25/30	...	17/30	...
1 in 20	...	...	...	3/15	...	8/15
1 in 40	...	...	8/15	...	8/15	...
1 in 50	...	...	6/15	...	...	...
1 in 80	...	...	...	3/15	...	5/15
1 in 100	...	...	4/15	...	...	...
1 in 160	...	...	4/15	...	6/15	...
∞	1/15		1/30		1/15	

bacteria (strain 18323) and serum were kept at room temperature for 1 hr; 0·03 ml. of the mixture, so arranged that this volume contained the required challenge dose for mice and the desired dilution of serum, was injected intracerebrally into 16-18 g. mice. The globulin

had considerable activity at a dilution of 1 in 160 (which represented a 1 in 16 dilution of unprocessed serum) and appeared by this test also to be somewhat weaker than the anti-SPA serum (table VI).

The agglutinin titre of four of the five sera used for these investigations was obtained with living and killed suspensions. The results were the same with each suspension :—Cutter globulin, 1 in 4000 ; Mérieux serum (lots 369 and 1610), 1 in 800 ; rabbit antiserum to Pillemer's SPA, 1 in 1600.

TABLE VII

Histamine-sensitising factor in cultures of H. pertussis *grown on Bordet-Gengou medium with and without cellophane*

Test material	No. organisms in, or corresponding to, immunising dose (in 1 ml.)	Per cent. wet weight (w/v) Dose 1 ml.	No. of experiments	No. mice dying/no. challenged with histamine after immunisation with test material from culture	
				with cellophane	without cellophane
Bacterial suspension .	25×10^9	...	4	34/40	10/39
	10×10^9	...	3	27/30	12/20*
	5×10^9	...	3	11/28	1/29
	1×10^9	...	1	0/10	0/10
Medium after removal of culture	...	25	3	8/30	26/30
	...	15	3	3/30	15/30
	...	6	1	0/10	5/10
	...	1·5	1	0/10	1/10
Washings . . .	20×10^9	...	3	20/30	4/29
Washed cells . .	20×10^9	...	3	12/30	14/30

* Two experiments only.

The properties of Hæmophilus pertussis *grown on cellophane in contact with Bordet-Gengou medium*

During the course of these investigations some results were obtained which indicated that *H. pertussis* grown on cellophane differed in some respects from organisms grown on the bare medium. Further studies were made to determine the extent of these differences.

Growth was slower and less profuse on cellophane and strains differed markedly in the readiness with which they grew. With strain 360E the ratio of viable counts in cultures 4 days old was 5 : 1 in favour of bare medium. Cellophane may have affected growth adversely because of interference with diffusion into the medium and absorption of substances produced by the bacteria which may be inimical to their multiplication (Pollock, 1947).

Histamine sensitisation. The results shown in table VII summarise the findings of three experiments under this heading. Organisms

grown on cellophane contained more HSF than those grown directly on the medium. One reason for this appeared to be that some HSF diffused into the medium during incubation of the culture and could be demonstrated there when cellophane was not present, whereas cellophane largely prevented this diffusion, so that only relatively small amounts were found in the medium when organisms were grown on cellophane.

TABLE VIII

Active immunisation of mice against intracerebral challenge with H. pertussis *grown on Bordet-Gengou medium with and without cellophane*

Test material	No. organisms in, or corresponding to, immunising dose (in 0·5 ml.)	Per cent. wet weight (w/v) Dose, 0·5 ml.	No. of experiments	No. mice surviving/no. challenged after immunisation with test material from culture	
				with cellophane	without cellophane
Bacterial suspension .	$2{\cdot}5\times10^9$	...	1	14/14	14/15
	$1{\cdot}0\times10^9$	...	2	9/30	5/30
	$0{\cdot}5\times10^9$	...	3	17/42	15/43
	$0{\cdot}1\times10^9$	...	1	1/13	2/15
Medium after removal of culture	...	25	3	3/40	11/42
	...	15	2	8/30	16/28
	...	6	1	1/15	3/15
	...	1·5	1	1/15	0/15
Washings . . .	10×10^9	...	3	10/44	10/42
Washed cells . .	10×10^9	...	3	31/43	34/44
Nil (controls) . .	0	...	5	3/75	

The medium was tested for HSF by removing the growth and cutting out a block from the centre of the Petri dish. This block was washed in three changes of saline, and ground in a mortar with saline to make a 25 per cent. w/v suspension. To this 0·1 per cent. formaldehyde was added and it was kept at 4° C. for a week or more before testing. Uninoculated medium was inactive as regards HSF and protective antigen.

The presence of more HSF in washings obtained from bacteria grown on cellophane compared with washings from bacteria grown directly on the medium is in conformity with the foregoing results. The washed bacteria from the two types of culture were, however, similar in HSF content; the HSF which remained was not readily detached by washing in saline and probably it would not diffuse readily into the medium.

Active immunisation against intracerebral challenge. The results, shown in table VIII, indicate that no significant difference was detectable in immunising capacity between suspensions, washings and

washed bacilli prepared respectively from cultures with or without cellophane. Some immunising antigen had, however, diffused into the medium in cultures without cellophane and although the amount was greater than when cellophane was present it was not so large as to reduce detectably the efficiency of the bacteria as immunising agents.

Antibody production. Bacterial suspensions from cultures with and without cellophane were compared by using them to prepare antisera in rabbits and mice and testing the properties of these sera. No differences were found in the rate of development and final titre of agglutinin, the titre of anti-HSF, the capacity to protect mice passively against intracerebral or intratracheal challenge and the qualitative character of the antibodies judged by cross-agglutination and agglutinin-absorption.

Similar results were obtained with sera prepared in mice, but with these sera no passive immunisation and agglutinin-absorption tests were done.

Agglutinability. No difference was found in agglutination titre between suspensions, either living or dead, prepared from BG cultures with and without cellophane, tested with five rabbit antisera made against a variety of preparations of *H. pertussis* and with 3 samples of hyperimmune human serum or globulin.

Precipitin reactions

Precipitin reactions in agar gel by the methods of Oakley and Fulthorpe (1953) and Ouchterlony (1953) have amplified or confirmed some of the other findings.

A suspension of uninoculated BG medium reacting in tubes with antiserum to bacteria grown on bare BG medium gave four lines of precipitate, whereas only one line was formed with antiserum to bacteria grown on cellophane over BG medium. Thus less antigenic material derived from the medium was present in suspensions of bacteria grown on cellophane, which was an advantage in preparing growth for antigenic or chemical analysis.

Bacterial suspension, washings, washed bacteria and extract from bacteria grown on cellophane, reacted with 5-6 lines. Residue was less complex and gave a maximum of 3-4 lines thus agreeing with its lack of HSF and its weakness in producing a protective antiserum in rabbits. The missing lines of precipitate could not however be associated with absence of particular properties. Antiserum to residue gave fewer lines than antisera to other fractions when reacting with any of the antigenic fractions and with bacterial suspension.

Tests in Petri dishes indicated at least two common antigens in washings, washed bacteria and extract from cellophane cultures; washings and extract each had one component which was not found in the other.

Physico-chemical studies of washings and extract

Standard techniques of ultraviolet spectroscopy and paper chromatography were employed in examining the ultraviolet absorption spectrum of the washings, and the amino acid, amino sugar and sugar

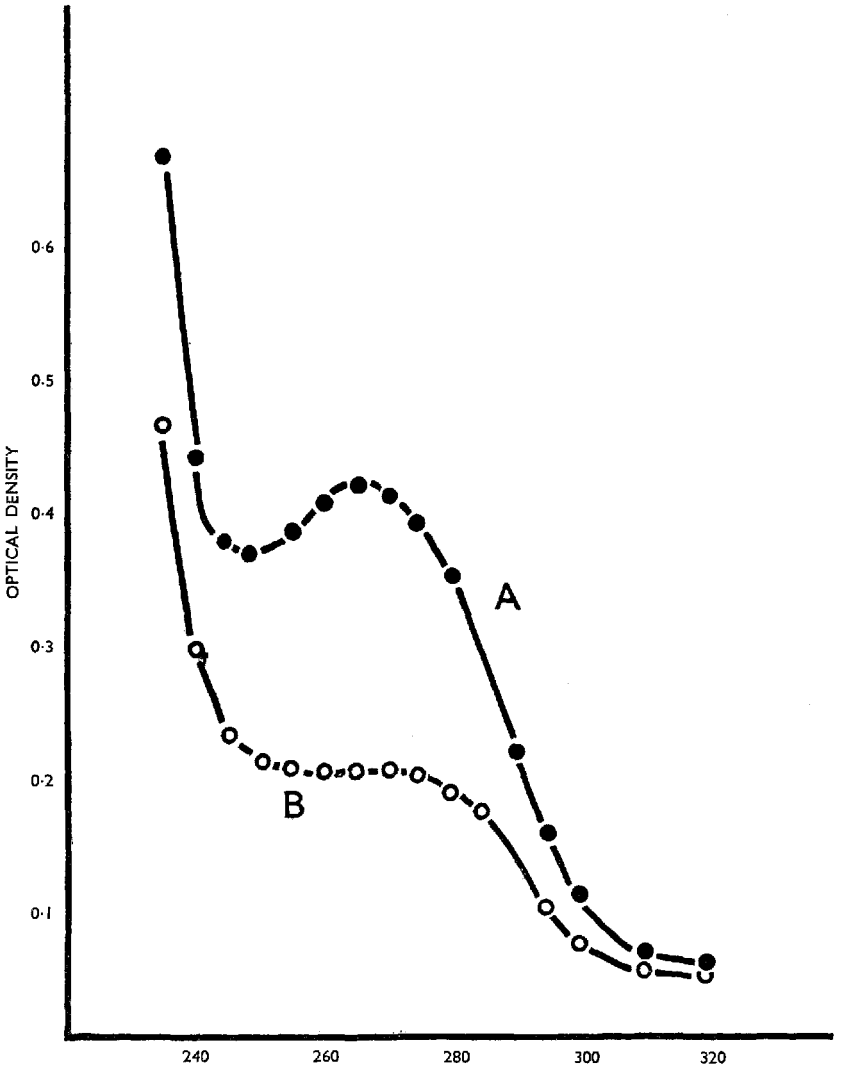

FIGURE.—Ultraviolet absorption spectrum of *Hæmophilus pertussis* saline washings. A : before dialysis ; B : after dialysis.

constituents of the hydrolysates of washings and extract. Washings contained 0·65 mg./ml. of solids as compared with 0·97 mg./ml. for extract.

The washings showed an ultraviolet absorption maximum at 265 mμ corresponding to nucleic acids or purine-pyrimidine constituents. Dialysis of the washings removed these substances, the dialysed material retaining the biological properties (HSF, protective antigen) of the

original and possessing an absorption spectrum similar to that of proteins (figure).

Paper chromatography of both dialysed washings and extract after hydrolysis with 6*N* HCl for identification of amino acids showed a typical protein amino acid composition, i.e., most of the amino acids occurring in the majority of proteins were detectable. Glucosamine, often present in protein-lipid-carbohydrate complexes of bacterial origin, was also found. Nitrogen was determined colorimetrically with Nessler's reagent as described by Johnson (1941) after digestion of samples by the Kjeldahl method under the conditions recommended by Chibnall *et al.* (1943). Freeze-dried preparations of washings and extract contained 9·5-14 per cent. total nitrogen. Estimation of the amounts of reducing substances after hydrolysis for 2 hr with 2*N* HCl at 100° C., by the Hagedorn and Jensen method modified by Hanes (1929) showed that they accounted for 5-10 per cent. of the weight of the dialysed extract; small amounts of glucose and galactose were detected by paper chromatography. These results would be in conformity with the presence of a protein-lipid-carbohydrate complex in the dialysed material.

Discussion

Washing *Hæmophilus pertussis* with saline affords a simple method of obtaining, free from the bulk of the bacterial cell, the antigen that immunises mice against intracerebral challenge, and presumably therefore the component of pertussis vaccine that immunises children against whooping cough; the washings contain as well the histamine-sensitising factor and agglutinogen. These antigens would therefore appear to be surface components of *H. pertussis* although they were not all removed by washing for the short period adopted in the method employed. De Repentigny and Frappier (1956) have shown by using fluorescent antibody that rabbit antiserum against washings reacts with the surface of *H. pertussis* from liquid or solid media, and that the surface reaction persisted after the bacteria had been washed with saline, thus supporting by direct evidence the view that washings contain surface components. In view of the possibility of some bacteriolysis or escape of cell contents, it cannot at present be stated with certainty that all of the antigenic material found in washings comes from the superficial part of the cell, but it would seem likely that the bulk of it is what is ordinarily called surface antigen.

The antigens found in washings were also obtained by extraction with TUF, which was more efficient in removing material from the bacteria than was washing with saline. Whether this efficiency was confined to the antigens under study or included extraneous material is not known. The high mouse-protective value of extract suggests that it might be a successful immunising agent for children, and that it could be used as starting material for purification of the protective antigen.

Rabbit sera against suspension, washings and extract all contained antibody which passively protected mice against intratracheal as well as intracerebral challenge. Te Punga and Preston (1958, and personal communication) found that these suspensions and washings which we have shown to protect mice actively against intracerebral challenge also protected against intratracheal challenge. Immunity to infection by these two routes may therefore have some common basis. The experiments reported here do not settle this point. The antisera which passively protected contained anti-HSF and agglutinin, but no evidence is presented for or against the participation of these antibodies in immunity.

The antiserum against residue was different from the others in having no anti-HSF and being weak in passively protecting mice, thus reflecting the greater loss of antigens by bacteria after extraction with TUF than after washing with saline.

The absence of HSF in extracted bacilli (residue), which contained protective antigen and agglutinogen, might suggest that HSF was separate from the other two antigens. This may eventually prove to be the case, but the result just noted could be due to a difference in the threshold of detection of different biological effects rather than to the operation of different entities. Protective antigen appeared to have been reduced in these bacilli, as rabbit antiserum against them was weak in passive protection of mice. Further investigations are required to elucidate the relationship of the antigens under consideration.

In this connexion it is of interest that man produces anti-HSF in response to pertussis vaccine. The human hyperimmune sera also passively protected mice and contained agglutinin.

In preparing growth for antigenic analysis or chemical examination it is desirable to have it as free from extraneous material as possible, and to avoid loss of antigenic material through washing. In employing BG medium it is therefore an advantage to grow cultures on cellophane as this reduces contamination with the material from the medium to a minimum. The washings described by Frappier and his colleagues (1954, 1955, 1955-56) may have even less extraneous material as they were derived from bacteria grown in a liquid medium and were prepared in a somewhat different manner; they showed only two lines of precipitate when tested in agar gel against an anti-bacterial serum. In the experiments recorded here the washings from BG (cellophane) cultures have not been compared with those from liquid cultures.

The indications obtained from preliminary experiments that a Seitz filter could adsorb a considerable amount of HSF and mouse-protective antigen applies to the technique of preparing materials containing them. Sintered glass and gradocol filters were less adsorptive.

The presence in washings of substances having an ultraviolet

absorption maximum corresponding with that of nucleic acids or their derivatives suggests the possibility that some material from inside the bacterial cell may occur in the washings, and by analogy possibly in extract, owing perhaps to some autolysis or altered permeability of cells in the 3-4-day-old cultures from which washings were prepared. There was no evidence of any marked bacteriolysis during washing. On the other hand such substances are known to be surface components of some species (Catlin, 1956) and it has been suggested that DNA may possibly be a surface component of *H. pertussis* (Overend *et al.*, 1951). However, nucleic acids or their derivatives, whatever their origin, appeared not to account for the immunising or histamine-sensitising properties of the washings. Further examination of washings or extract should assist in characterising chemically and biologically the antigenic entities they contain. The antigens under examination in this paper appear to be what are ordinarily termed surface antigens and it is probable that they are largely surface components although the experiments do not exclude the possibility that some part of them may be present inside the cell.

Summary

Saline washings of *Hœmophilus pertussis* protected mice against intracerebral challenge and contained histamine-sensitising factor and agglutinogen. Washed bacteria still protected mice and retained HSF, so that washing by the method employed did not remove all of the substances responsible for these effects.

By treating the bacteria with a mixture of thiourea, urea and formamide (TUF) an extract was obtained which, like saline washings, contained mouse-protective antigen, HSF and agglutinogen. Extraction with TUF removed more material than washing with saline. The extracted bacteria had no detectable HSF but they protected mice against intracerebral challenge and were agglutinogenic. The mouse-protective antigen was probably reduced in amount, as rabbit antiserum made against extracted bacteria was poor in mouse-protective antibody. The extract on the other hand had a high mouse-protective value.

Rabbit antisera to bacterial suspension, washings and extract passively protected mice against both intracerebral and intratracheal challenge and contained anti-HSF and agglutinin. The rabbit antiserum to extracted bacteria (" residue ") had no anti-HSF, a low value in passively protecting mice and a moderate agglutinin titre.

When *H. pertussis* was grown on bare BG medium, HSF, and to a lesser extent mouse-protective antigen, diffused into the medium; conversely, antigens derived from the medium were found in bacterial suspensions made from the cultures. By growing *H. pertussis* on cellophane applied to the surface of BG medium it was found, in comparison with cultures on the bare medium, that (i) more HSF

was present in the bacteria, (ii) there was no appreciable increase in the mouse-protective potency of the bacteria, (iii) bacterial suspensions and washings were much less contaminated with antigens from the medium. Growth on cellophane was less profuse than on bare medium.

Preliminary experiments indicated that a Seitz filter adsorbed considerable amounts of HSF and mouse-protective antigen from saline washings. Sintered glass and gradocol filters were less active in this respect.

Dialysis of washings, freeze-drying of washings and washed bacilli, and preparation of these materials at 56° C. did not appreciably affect their content of HSF or their capacity to protect mice against intracerebral challenge.

Human serum from persons hyperimmunised by repeated injections of commercial pertussis vaccines contained anti-HSF and agglutinin and passively protected mice against intracerebral challenge.

Substances giving an ultraviolet absorption spectrum which corresponded with nucleic acids or purine-pyrimidine constituents were present in washings. These substances could be removed by dialysis. The dialysed washings had an absorption spectrum similar to that of proteins and retained their HSF and mouse-protective properties.

Hydrolysis of dialysed washings and extract yielded most of the amino acids found in the majority of proteins, and estimates of the total nitrogen indicated the presence of a substantial amount of protein. Glucosamine and small amounts of glucose and galactose were also present. The chemical findings were consistent with the presence in washings and extract of a protein-lipid-carbohydrate complex.

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