A SEROLOGICAL STUDY OF *HAEMOPHILUS INFLUENZAE*

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Until recently the observations of most workers were in agreement regarding the extreme antigenic heterogeneity of *Haemophilus influenzae*. In 1919 Valentine & Cooper investigated a large number of strains isolated from various sources by agglutination and absorption of agglutinins, and reported a marked heterogeneity, but considered that there was some evidence of the existence of small groups. Small & Dickson (1920), using a similar technique, were able to classify a small number of strains into four groups, but Provitzky & Denny (1921) were not quite so successful in an examination of about 100 old strains which had been isolated during the pandemic of 1918 and ninety newly isolated strains. Using thirty antisera they found that four meningeal strains fell into one group while three others were of quite different types. Five respiratory strains were of one type, but all the rest fell into a number of groups none of which contained more than two members.

A considerable advance was made in 1931 when Pittman described two types of colony formation in this species. When freshly isolated strains were grown on a suitable transparent medium like Levinthal's medium, some of them produced large smooth mucoid colonies which were slightly opaque and markedly iridescent. When these strains were subcultured there frequently appeared among these large colonies smaller ones which were more translucent and were never iridescent. These were the usual "typical" Pfeiffer's bacillus colony. The morphology of the organisms constituting the two types of colony differed also. A film taken from a large iridescent colony showed the organisms to be short rods with very little variation in size or shape, and when suitably stained appeared to be capsulated. The organisms from the "typical" colonies showed considerable pleomorphism, filamentous forms were quite common, and no capsule was visible. Pittman considered that the organisms constituting the large iridescent colony were the normal "smooth" or S forms, and that those which constituted the "typical" colony were the "rough" or R variants. Continuing the examination of these strains Pittman prepared antisera against both S and R strains, and by agglutination reactions carried out at 37° C. she was able to determine the presence of two sharply defined groups. When the

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reaction was carried out at 47° C., however, the same specificity was not observed. A point of considerable interest was that washings of an S strain gave a precipitate when added to the homologous antiserum; and by means of precipitin reactions the same specificity could be demonstrated as when the agglutination reactions were carried out at 37° C.

The soluble substance of one of these S strains was studied by Goebel (1931) and found to be carbohydrate in nature, and thus to resemble the soluble specific substance of the pneumococci. From analogy Pittman concluded that this soluble specific substance was related to the capsule which she demonstrated in connexion with the S forms.

In 1934, in a letter to the author, Miss Pittman stated that she had discovered other groups to the number of six which she designated *a*, *b*, *c*, *d*, *e* and *f*, and she kindly supplied me with cultures of type species of groups *a*, *b*, *e* and *f*, for which I now express my thanks.

The experiments described in this paper were carried out in an endeavour to see whether strains of *Haem. influenzae* isolated from the naso-pharynx of apparently healthy persons could be similarly classified, and also to determine their relationship to the strains recovered from pathological sources.

EXPERIMENTAL WORK

During the course of these investigations eighty-seven strains were examined. Eighty-six of them were supplied to me by Dr Edith Straker of the London School of Hygiene and Tropical Medicine, to whom I am greatly indebted. These strains were isolated from the naso-pharynx of healthy boys either directly from the primary culture or after passage through a mouse. Of these eighty-six strains fifty-two were recovered from that number of individuals, while the remaining thirty-four were obtained at subsequent swabbings from nine of the above mentioned fifty-two individuals.

One strain, recovered from a fatal case of influenzal meningitis, was also included for study. This strain, designated MW 1, was procured for me by Dr T. McClurkin from Group Captain Whittingham, to both of whom I desire to express my thanks.

All strains were dependent upon the presence of both X and V accessory food factors for growth (see Fildes, 1923).

When plated out on the peptic digest of blood medium described by Fildes (1920), the meningeal strain MW 1 and twenty of the naso-pharyngeal strains gave rise to large iridescent colonies, films from which showed little evidence of pleomorphism among the constituent organisms. According to the criteria laid down by Pittman these strains appeared to be "smooth", but attempts to stain a capsule by Muir's method were not successful. Sometimes a few of the organisms in a film appeared to be surrounded by a faintly stained irregular halo, but one could never be certain that a true capsule was present.

Of the twenty naso-pharyngeal strains, sixteen were isolated from that

number of individuals, while the remaining four were recovered from four of those sixteen individuals at a subsequent swabbing.

Antisera were prepared against all the "smooth" naso-pharyngeal strains, the meningeal strain MW 1 and the four type cultures supplied by Miss Pittman. Rabbits were injected intravenously, first with heat-killed organisms followed by living organisms, the injections being given regularly on the first three days of every week. At the end of a month or 5 weeks a trial bleeding was made. Rarely a second course of injections was needed. When a rabbit failed to produce a satisfactory titre in 5 weeks a fresh animal was immunized. All sera were preserved with 0·3 per cent phenol.

In the experiments described below the antisera prepared against the four "smooth" naso-pharyngeal strains recovered at subsequent swabbings were not used, as those strains were shown to be identical with the original "smooth" strain recovered from these carriers.

Agglutination reaction

As a preliminary examination all strains were tested by the agglutination technique with the antisera prepared against the seventeen "smooth" strains. Agglutination suspensions were prepared by washing off 24 hour Fildes' agar plate cultures, centrifuging to recover the organisms, and resuspending them in normal saline to make a suspension of an opacity equivalent to that of a 1000 million *Bact. coli* standard.

In the earlier experiments both living and heat-killed suspensions were used in duplicate tests, but as no difference could be detected between the two results, it was decided to use as a routine suspensions of organisms killed by heating to 55° C. for 30 min.

The agglutination reactions were carried out in the usual way, 0·3 c.c. volumes of suspension and diluted antiserum being incubated in Dreyer tubes in a water bath. The reactions were carried out at 37° and 55° C.

During the course of the examination the seventeen "smooth" antisera were tested against the corresponding seventeen "smooth" strains and sixty-six "rough" strains of the normal naso-pharyngeal type. It is not practicable to tabulate all the results obtained. It was found that each antiserum agglutinated most of the strains tested, but the strains not agglutinated by one antiserum were always found to be agglutinable by one or more of the remaining antisera.

All the "smooth" strains were agglutinated by all the smooth antisera, but not all to the same titre. The end titres after 24 hours' incubation varied from 1 : 20 to 1 : 5120. With the "rough" strains there was a similar variation in end titre of the reaction when such occurred, the range being from 1 : 20 to 1 : 1280. Thus by direct agglutination, using "smooth" antisera, it was not possible to differentiate between the "smooth" and "rough" naso-pharyngeal strains, nor was it possible by this means to divide these strains into serological groups.

If readings were made at frequent intervals during the process of incubation, evidence of some degree of specificity was obtained. In Tables I and II are recorded the results of reactions carried out at 37° and 55° C. with the first six "smooth" strains received and the corresponding antisera. With short incubation at 37° C. there was an indication of some degree of specificity, but with long incubation at this temperature, or, when the test was carried out at 55° C., this evidence of specificity was lost and the end result after 24 hours' incubation at 37° C. was practically the same as that obtained at 55° C.

As a control on this finding, tests were carried out with Miss Pittman's type strains and the corresponding antisera. The results of such tests are given in Table III. The marked specificity of these four strains is apparent even when the reaction is carried out at 55° C. When, however, suspensions of these type strains were set up against the antisera prepared from the "smooth" naso-pharyngeal and meningeal strains and vice versa, the results obtained were more confusing. All four type strains were agglutinated by the meningeal and fifteen of the naso-pharyngeal antisera with titres ranging from 1 : 20 to 1 : 1280 when the reaction was carried out at 55° C. Antiserum L 95 agglutinated types *b*, *e* and *f* only. When the test was made at 37° C. and the results recorded after 1 hour, it was noticed that some of the antisera had not agglutinated any of the type strains while the others had agglutinated one or two suspensions. With longer incubation further suspensions were agglutinated, so that, with one exception, after 24 hours the results were almost identical with those obtained at 55° C. The exception was that antiserum L 95 agglutinated type *b* strain only.

When the type antisera were set up against the meningeal and naso-pharyngeal strains at 37° and 55° C. they agglutinated both "smooth" and "rough" strains. With the exception of strain L 95 all "smooth" strains were agglutinated by each type antiserum. The results obtained with the "rough" strains were similar to those obtained when these strains were put up against the "smooth" naso-pharyngeal antisera, each antiserum agglutinating the majority of the suspensions. Every "rough" strain was found to be agglutinable by one or more of the type antisera.

When the technique of absorption of agglutinins was used, the results were just as obscure. When one of the "smooth" naso-pharyngeal strain antisera was absorbed with the homologous strain, agglutinins for all strains, both "smooth" and "rough", were removed, but when absorbed with any of the heterologous strains, in many cases, agglutinins for that strain alone were removed. When a "smooth" strain was used as the absorbing one agglutinins for several other "smooth" strains were removed also, but most "rough" strains could only remove agglutinins for the particular one used in the absorption and occasionally for one or two others, also, especially when these strains were those obtained from subsequent swabbings of a carrier.

It, therefore, seemed that the agglutination technique could not be used to differentiate between "smooth" and "rough" strains, nor did it seem likely

Table I. *Results of agglutination reactions carried out at 37° C.*

Antisera	Readings at end of 1 hour Suspensions						Readings at end of 24 hours Suspensions					
	Ly 98	MW 1	Ly 94	P 35 S	H 25	H 37	Ly 98	MW 1	Ly 94	P 35 S	H 25	H 37
Ly 98	1280	320	0	160	0	0	5120	1280	20	1280	80	80
MW 1	0	640	0	40	0	0	320	1280	20	160	160	20
Ly 94	320	0	1280	1280	0	0	2560	80	1280	1280	320	320
P 35 S	640	40	40	5120	2560	0	2560	40	80	5120	2560	320
H 25	0	0	0	1280	1280	0	2560	640	20	2560	5120	2560
H 37	0	20	160	320	0	640	1280	320	320	640	80	2560

The figures indicate the end titre of the reactions.

Table II. *Results of agglutination reactions carried out at 55° C.*

Antisera	Readings at end of 4 hours Suspensions						Readings at end of 24 hours Suspensions					
	Ly 98	MW 1	Ly 94	P 35 S	H 25	H 37	Ly 98	MW 1	Ly 94	P 35 S	H 25	H 37
Ly 98	2560	1280	40	1280	640	160	5120	1280	40	1280	640	160
MW 1	320	2560	20	160	320	40	320	2560	40	160	320	80
Ly 94	1280	160	1280	640	320	640	2560	640	1280	640	320	640
P 35 S	1280	20	20	5120	2560	160	2560	20	20	5120	2560	640
H 25	0	0	0	1280	1280	320	1280	320	80	2560	2560	640
H 37	20	20	40	1280	640	1280	2560	320	320	1280	1280	2560

The figures indicate the end titre of the reactions.

that these strains could be divided into serologically distinct groups by this method.

Table III. *Results of agglutinations with Pittman type strains and homologous antisera*

	4 hours at 37° C. Suspensions				4 hours at 55° C. Suspensions			
Antisera	*a*	*b*	*e*	*f*	*a*	*b*	*e*	*f*
a	1280	10	0	10	1280	80	20	320
b	0	320	0	0	0	1280	0	160
e	0	0	160	0	0	320	1280	640
f	0	0	0	1280	20	20	0	5120

The figures indicate the end titre of the reactions.

COMPLEMENT FIXATION REACTIONS

For the performance of complement fixation tests bacterial suspensions were prepared as for agglutination reactions. A "five-volume" technique was used. To a range of dilutions of antiserum were added equal volumes of bacterial suspension and fresh guinea-pig serum diluted to contain 3 M.H.D. of haemolytic complement per volume, and 2 hours at bench temperature were allowed for fixation. The sensitized red cells were then added, and after incubation at 37° C. for 1 hour readings were made.

Six antisera were tested in this way against all the "smooth" and forty "rough" strains. Where positive reactions were obtained the highest dilutions of the sera giving complete fixation ranged from 1 : 20 to 1 : 320. The results were similar to those obtained with the agglutination technique.

PREPARATION OF SOLUBLE SUBSTANCE

In her paper Pittman showed that the specificity of the groups depended upon the presence of a soluble substance which she concluded was related to a capsule.

It was decided therefore to isolate this soluble substance with the object of using a precipitin reaction to determine the presence of subgroups.

Several methods were used, but only the two which gave the best results are described here. In this part of the work the writer was given a great deal of help and advice by Mr Pirie of the Department of Biochemistry of the University of Cambridge, to whom he is greatly indebted.

Method I. The organism was grown at 37° C. in Rivers & Kohn's (1921) haemo-peptone water distributed in 2 litre quantities in a number of 5 litre flasks. After 48 hours' incubation the culture was concentrated *in vacuo* to one-tenth its original volume. The insoluble material and the organisms were then removed by centrifuging at 3500 r.p.m. for 2 hours. The transparent brownish supernatant was then decanted and 2 g. of sodium acetate crystals added, followed by two volumes of absolute alcohol which threw out a flocculent precipitate. This was allowed to settle out overnight and recovered next day

by centrifugation. The precipitate was taken up in the smallest volume of water necessary and any insoluble material thrown down in the centrifuge. The soluble substance was precipitated twice more as described above, and finally taken up in distilled water and frozen out *in vacuo* from a concentrated solution.

Method II. The growth was washed off a number of Roux bottle Fildes' agar 24 hour cultures and the resultant thick suspension placed in a water bath at 100° C. for 30 min. When cold, sufficient $N/10$ sodium hydroxide was added to make a final concentration of $N/500$. The suspension was then placed in the ice chest overnight. A little sterile sand was added and the suspension agitated in a mechanical shaker at 1150 complete excursions per min. for half an hour. It was then placed in the ice chest overnight and the next morning all insoluble matter was removed by centrifugation. 5 per cent acetic acid was then cautiously added to the clear supernatant until no further precipitate formed. This precipitate was thrown down in the centrifuge and the soluble substance obtained from the supernatant by precipitation with absolute alcohol as described in Method I.

The product obtained by both these methods was readily soluble in water and was perfectly white in colour, and no difference could be detected in the immunological reactions of the two products from any particular strain.

Nitrogen determinations indicated an average nitrogen figure of 6·7 per cent.

Precipitin reactions

In the preliminary investigations, washings from plate cultures were used as antigens (Pittman, 1931). 24 hour Fildes' plate cultures of the "smooth" strains were washed off in normal saline and the organisms thrown down in the centrifuge. The supernatant was then used as antigen in precipitin tests.

A range of dilutions of antigen was set up against equal volumes of antiserum diluted 1 : 5. In the earliest experiments the test was always carried out in duplicate, one set of tubes being incubated at 37° C. and the other at 55° C. in water baths, readings being made at 1 hour, 4 hours and again after standing overnight in the ice chest. The final readings in both tests were always the same and agreed with the results of the agglutination tests. The large number of cross reactions made it impossible to subdivide the strains.

As a control on this portion of the work, tests were set up with washings from Miss Pittman's type strains against the corresponding antisera and nine other sera selected at random from among those prepared against the "smooth" naso-pharyngeal strains. The results of these reactions are given in Table IV. Cross reactions were common, and it will be noticed that the washings from strain *f* gave a precipitate with all antisera used. By examining the tubes at short intervals of time evidence of specificity could be noted. Precipitates formed most rapidly in tubes containing washings from type strains and the homologous antiserum and perhaps one or two other sera.

Table IV. *Results of precipitin reactions using washings of Pittman type strains as antigens*

	Washings from types			
Antisera	*a*	*b*	*e*	*f*
P 35S	+	−	−	+
H 25	−	+	+	+
MW 1	−	+	−	+
L 23	−	+	−	+
H 53	+	+	−	+
Ly 98	−	−	−	+
Ly 94	−	−	−	+
L 95	−	+	−	+
H 37	−	+	+	+
a	+	+	−	+
b	+	−	−	+
e	−	+	+	+
f	+	+	−	+

When, however, solutions of purified soluble substances were used in the precipitin tests, clear-cut results were obtained. The solutions were made up in saline to a strength of 2 mg. per c.c. The tests were set up in the same way as when the crude washings were used and readings were made after 4 hours' incubation in a water bath at 55° C., and again after standing overnight in the ice chest. The results of these reactions are given in Table V. Of nine soluble

Table V. *Results of precipitin reactions using solutions of soluble substances as antigens*

	Soluble substances from strains								
Antisera	P 35S	H 25	MW 1	L 95	H 37	*a*	*b*	*e*	*f*
P 35S	+	−	−	−	−	+	−	−	−
H 25	−	+	+	−	−	−	−	+	−
MW 1	−	+	+	−	−	−	−	+	−
L 95	−	−	−	+	−	−	−	−	−
H 37	−	−	−	−	+	−	−	+	−
a	+	−	−	−	−	+	−	−	−
b	−	−	−	−	−	−	+	−	−
e	−	+	+	−	+	−	−	+	−
f	−	−	−	−	−	−	−	−	+
H 53	−	+	+	−	−	−	−	+	−
L 23	−	−	−	−	−	−	−	−	−
Ly 94	−	−	−	−	−	−	−	−	−
Ly 98	−	−	−	−	−	−	−	−	−

substances examined eight gave positive reactions when tested against Pittman type sera. Four fell in group *e*, and two in group *a*. The type strains *b* and *f* were the only members of those two groups. Although the soluble substance was not obtained from strains H 53, the results suggest that this strain most probably belongs in group *e*. The classification of the naso-pharyngeal and meningeal strains from which soluble substances were extracted would be as follows:

Types

a	*e*
P 35S	H 25
	H 37
	MW 1
	H 53

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Strains Ly 98, Ly 94 amd L 23 failed to react with any of the type specific substances and probably belong to other groups.

An attempt was made to isolate a fraction which was common to all strains. An alkali-soluble–acid-precipitable substance was obtained from a "smooth" strain which, when frozen out *in vacuo*, was soluble in water but was quite insoluble when dried out *in vacuo* with the gentle application of heat. This substance gave a precipitate when mixed with immune serum, but reacted with only a small number of such antisera.

Discussion

In her paper Pittman (1931) stated that her "smooth" strains were readily divisible into serologically distinct subgroups by agglutination and precipitin tests, provided that the former reaction was carried out at 37° C. The results of agglutination experiments with the American type strains recorded above not only confirmed these findings but also showed that these groups could be detected even when the reaction was carried out at 55° C.

Using the same technique it was impossible similarly to subdivide the "smooth" naso-pharyngeal strains, although with short incubation at 37° C. evidence of the possible existence of serological groups was obtained. When, however, partially purified soluble substances from "smooth" strains were tested against antisera prepared against "smooth" naso-pharyngeal and the Pittman type strains, their differentiation into distinct serological groups was evident.

The results of the agglutination reactions with "rough" strains against "smooth" antisera call for comment. The fact that the "rough" naso-pharyngeal strains showed evidence of a division into groups contrasted sharply with Miss Pittman's observation that "rough" strains formed a homogeneous group. A satisfactory explanation of this difference is not apparent. If it is assumed that the cross reactions between the groups of the "smooth" strains were probably due to the presence in the antisera of antibodies to the protein fraction of the organism, it might be necessary to assume that this fraction was common to all strains. Miss Pittman's experiences suggest that this is the case, but the observations recorded above do not accord with this view.

It seems likely that the species *Haem. influenzae* is complex antigenically, but that a common antigen or number of antigens near the surface of the organisms renders most strains agglutinable by any single antiserum especially at high temperatures or after long incubation at low temperatures. A surface antigen of a carbohydrate nature is responsible for differentiating the "smooth" strains into a number of subgroups.

Summary

1. Of eighty-six strains of *Haem. influenzae* isolated from the naso-pharynx of normal persons, sixteen had the general characters of Pittman's "smooth" type.

2. These sixteen strains and one "smooth" strain isolated from a case of meningitis were submitted to various serological tests, including the separation and partial purification of a carbohydrate fraction from the meningeal strain and from four of the sixteen strains.

3. By precipitin tests carried out with these purified fractions, it was possible to identify three of the five "smooth" naso-pharyngeal strains and the meningeal strain, as belonging to Pittman's type *e*, and one of the "smooth" naso-pharyngeal strains to Pittman's type *a*.

4. Some indication of grouping, within this small sample of smooth strains, was obtained by various other methods, such as precipitin tests carried out with simple saline washings or agglutination reactions; but, apart from the four type strains *a*, *b*, *e* and *f*, received from Miss Pittman, no strain could be satisfactorily typed by any of these methods. Only by using partially purified polysaccharide fractions was it possible to assign any of the "smooth" naso-pharyngeal strains to their correct type.

5. The testing of these naso-pharyngeal strains against antisera prepared against types *a*, *b*, *e* and *f* by direct agglutination, showed that either many of these strains contained some proportion of the smooth antigens, or that the antisera contained antibodies acting on other antigenic components, although, in relation to the type strains themselves, they appeared to be specific.

6. The examination of the eighty-six strains, as a whole, revealed the extreme antigenic heterogeneity that has been noted by many previous workers.

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REFERENCES

FILDES, P. (1920). *Brit. J. Exp. Path.* **1**, 129.
—— (1923). *Ibid.* **4**, 265.
GOEBEL, W. F. (Quoted by Pittman (1931).) *V. infra.*
PITTMAN (1931). *J. Exp. Med.* **53**, 471.
PROVITZKY, O. R. & DENNY, H. F. (1921). *J. Immunol.* **6**, 65.
RIVERS, T. M. & KOHN, L. S. (1921). *J. Exp. Med.* **34**, 477.
SMALL, J. C. & DICKSON, G. K. (1920). *J. Inf. Dis.* **26**, 230.
VALENTINE, E. & COOPER, G. M. (1919). *J. Immunol.* **4**, 359.

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