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# The stability of the serotypes of *Bordetella pertussis* with particular reference to serotype 1,2,3,4

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SUMMARY

Strains of *Bordetella pertussis* in which all the organisms contain agglutinogens 1 and 3 or 1, 2 and 4 are easy to identify as serotypes 1,0,3,0 and 1,2,0,4 respectively; and similarly, stable strains of serotype 1,0,3,4 are occasionally found. During repeated subcultures, passage *in vivo*, and lyophilization and preservation for many years, these serotypes do not change. Mixing 1,0,3,0 and 1,2,0,4 serotypes and culturing them together *in vivo* and *in vitro* produces cultures from which organisms of the same two serotypes can be isolated.

In contrast, strains which type as 1,2,3,4 are often a heterogeneous group. We have attempted to classify these as 'stable', 'variable' and 'mixed' cultures. Some strains comprise organisms all of which contain the four agglutinogens and are as easy to type as the strains described above. These we have called 'stable' 1,2,3,4 strains. Other 1,2,3,4 strains are made up of colonies possessing all four agglutinogens, as shown by agglutinin production, but in amounts varying from day to day so that direct typing is inconsistent. These we have called 'variable' 1,2,3,4 strains. The last category, 'mixed', is made up of organisms most of which give rise to stable 1,2,3,4 cultures; a few of the component organisms, however, have one or two of the four agglutinogens missing.

The importance of the 'variable' cultures is emphasized for work on apparent change of serotype, e.g. during infection.

## INTRODUCTION

The serotyping system for *Bordetella pertussis* based on the possession of agglutinogens has been in use since their identification by Andersen (1953) and Eldering, Hornbeck & Baker (1957). Most isolates type easily, but 209 of the 1293 isolates typed in the Public Health Laboratory Service (PHLS) survey (1973) did not type consistently. The isolates over which typing difficulty is experienced usually fall into the agglutinogen group 1,2,3,4. Some workers have called these cultures 'mixed'. This concept has now been extended by Stanbridge & Preston (1974*b*) to suggest that all serotypes are capable of becoming mixtures on subculture.

This paper deals with the typing experiences in our laboratory of isolates received during the PHLS investigation from 1966 to 1968, sent by courtesy of the reference laboratories, and also of isolates collected several years earlier in

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Britain during Medical Research Council investigations and kept in the lyophilized state (Bronne-Shanbury, Miller & Standfast, 1976). In our laboratory the stability of types 1,0,3,0 and 1,2,0,4 contrasts strongly with that of 1,2,3,4. Our results indicate that these last are far more complex than mere mixtures of serotypes. They are based on a more sensitive method of detection of agglutinogens than direct serotyping can provide, namely that of agglutinin production in mice and subsequent identification of the agglutinins.

Correct determination of the serotypes of strains is important in extended epidemiological investigations, e.g. when considering possible changes in serotype during convalescence and between source and contact patients.

## METHODS

### *Strains and isolates*

Strains used as control suspensions in the typing of isolates by direct agglutination and agglutinin production were as described previously (Bronne-Shanbury, 1976; Bronne-Shanbury *et al.* 1976). In addition mouse-virulent strains 18-323 and B106 were used. Strain 18-323, originally from Washington and passaged at the Lister Institute for many years, is a 1,2,0,4 strain, unlike the cultures described by Cameron (1967). Strain B106 (1,0,3,0), passaged to become mouse-virulent, was derived from B5533 by Dr Haire, Belfast (Dolby & Bronne-Shanbury, 1975). Cultures were grown on modified Bordet-Gengou (BG) medium at 36° C.

### *Typing of the isolates*

Each isolate was investigated by three methods. These were (*a*) direct agglutination of the whole culture by monospecific typing sera 1, 2, 3 and 4 to identify the antigens (Bronne-Shanbury *et al.* 1976), (*b*) agglutinin production and identification of the agglutinins (Bronne-Shanbury, 1976) and (*c*) isolation of single colonies and subsequent testing.

### *Isolation of single colonies*

Several methods of producing cultures from single colonies were used. Initially, a little of an 18–24 hr. culture of a given isolate was emulsified in 1 % casamino acids (CA) and diluted to an equivalent of 300 organisms (of which 10 % were viable) per 0·02 ml. ($1{\cdot}5 \times 10^4$/ml.). Five 0·02 ml. drops were pipetted on each of two Bordet-Gengou (BG) plates, which were incubated for 2–3 days at 36° C. Six to 24 colonies were selected and each was emulsified on the side of a 25 ml. screw-capped glass bottle containing 9 ml. CA and a glass bead, the bottle was thoroughly shaken and a loopful of the liquid was spread on one-fifth of a BG plate and incubated at 36° C. to give further single colonies. One colony from each culture so obtained was emulsified as before and spread on one-fifth of a further BG plate. This culture was used for serotyping or alternatively one colony from each culture was treated in one of the three ways shown in Fig. 1. Cultures from colonies thus obtained were tested by agglutinin production in mice and analysis of the sera produced ((*b*) above).

*Mixing of strains of serotype* 1,0,3,0 *and* 1,2,0,4

*On solid medium.* The strains were grown separately on BG plates at 36° C. An 18–24 hr. growth from each plate was suspended in CA and diluted to an equivalent of $1{\cdot}5 \times 10^4$ organisms/ml. by opacity. Equal volumes of the two serotypes were mixed and 1 ml. of the mixture spread on BG plates and incubated at 36° C. for 24 hr. Six subcultures were made from the resultant culture. From the last subculture, single colonies were isolated as described above.

*In liquid medium.* The strains were grown separately on BG plates at 36° C. The 18–24 hr. growth from one plate of each strain was harvested in Cohen & Wheeler (1946) medium (CW), modified by the addition of 0·5 % glutamic acid. An equal volume of each harvest was mixed, 20 ml. was inoculated into 250 ml. of the CW medium in a depth of 1–2 cm. in flasks incubated at 36° C. for 48 hr. on a shaking machine.

*In the mouse brain.* The mouse-virulent strains 18-323 (1,2,0,4) and B106 (1,0,3,0) were grown separately on BG plates at 36° C. An 18–24 hr. growth of each was harvested separately in CA and diluted to an equivalent of 50,000 organisms per 0·03 ml. Equal volumes of each were mixed and the mixture was injected intracerebrally into five mice. Two groups of five mice were injected with each strain to act as controls. The experiments were performed in normal mice and in mice which had been inoculated 14 days before infection with 1 i.u. of pertussis vaccine capable of protecting 100 % of the mice.

## RESULTS

*Isolates typing consistently as* 1,0,3,0 *or* 1,2,0,4

Isolates of serotype 1,2,0,4 and 1,0,3,0 have been found to type consistently after repeated subculture and after lyophilization. Twenty cultures grown from single colonies from each of twelve 1,2,0,4 isolates all typed as 1,2,0,4 after subculture. Vaccines made from ten of these cultures were used for agglutinin production in mice and all gave rise to agglutinins 1, 2 and 4.

Twenty-three 1,0,3,0 isolates were similarly investigated. Twenty cultures from single colonies each serotyped as 1,0,3,0 and vaccines of ten of these elicited agglutinins 1 and 3 only.

*Isolates typing as* 1,2,3,4

Thirty-one isolates typing as 1,2,3,4 were investigated in detail. They were found to be a heterogeneous group and were subdivided into 'stable', 'variable' and 'mixed' according to the results.

'*Stable*' *isolates.* Five isolates serotyped consistently with all four monospecific agglutinating sera. Cultures grown from single colonies of these typed consistently and strongly with all four antisera.

'*Variable*' *isolates.* These were isolates which typed differently from subculture to subculture but agglutinated with all four antisera at least once in three or four tests, though not necessarily with all antisera on the same day. Thirteen were

Table 1. '*Variable*' 1,2,3,4 *isolates showing colonies with variable agglutinogens*

| Isolate no. | No. of colonies tested | No. of colonies variable | No. of colonies with variable agglutinogens | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 2 | 3 | 4 | 2,3 | 2,4 | 3,4 | 2,3,4 |
| L13 | 10 | 7 | 1 | — | 4 | — | 2 | — | — |
| L70 | 20 | 15 | 1 | — | 5 | — | 8 | — | 1 |
| D1217 | 20 | 20 | 1 | — | — | 1 | 15 | — | 3 |
| D4613 | 20 | 11 | 1 | 3 | — | 4 | 2 | — | 1 |
| D4810 | 6 | 6 | — | 6 | — | — | — | — | — |
| D4919 | 6 | 6 | 1 | 1 | — | 2 | 1 | — | 1 |
| D9335 | 13 | 12 | 11 | — | — | — | — | — | 1 |
| D11085 | 39 | 17 | 1 | 10 | — | — | 5 | — | 1 |
| D11464 | 20 | 10 | 7 | 2 | — | — | 1 | — | — |
| D13318 | 20 | 14 | 7 | 3 | — | — | — | — | 4 |
| D17602 | 6 | 6 | — | 6 | — | — | — | — | — |
| D25120 | 18 | 17 | 6 | — | — | — | 8 | — | 3 |
| D56079 | 17 | 10 | 8 | 2 | — | — | — | — | — |

Table 2. '*Mixed*' 1,2,3,4 *isolates showing the serotype of component colonies*

| Isolate no. | No. of colonies tested | No. of colonies of stable serotype | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1,2,3,4 | 1,2,3,0 | 1,2,0,4 | 1,0,3,4 | 1,0,3,0 | 1,0,0,0 |
| C4486 | 18 | 15 | — | 1 | — | — | 2 |
| C5468 | 14 | 11 | — | 3 | — | — | — |
| G352 | 20 | 17 | 2 | — | — | 1 | — |
| G724 | 38 | 28 | — | 10 | — | — | — |
| L16 | 18 | 15 | 3 | — | — | — | — |
| L19 | 12 | 10 | — | 2 | — | — | — |
| M12 | 18 | 14 | — | 2 | — | — | 2 |
| D2928 | 17 | 16 | — | — | 1 | — | — |
| D2960 | 6 | 3 | — | — | — | 3 | — |
| D8127 | 18 | 15 | — | — | 3 | — | — |
| D8214 | 25 | 16 | — | — | 8 | 1 | — |
| D9774 | 44 | 27 | — | 10 | 6 | — | 1 |
| D25525 | 20 | 19 | — | — | 1 | — | — |

investigated. Colonies from these isolates gave rise to cultures in which the agglutination titre varied from day to day but all four agglutinogens were always present as shown by agglutinin production, even if not by direct typing. Results are shown in Table 1.

'*Mixed*' *isolates*. In this group 10–20 % of the colonies which were separated gave rise to stable cultures which were of a type other than 1,2,3,4, e.g. 1,0,3,4 or 1,2,0,4; examples of such mixtures are shown for 13 isolates in Table 2. Usually, less than half of the isolated colonies were of a serotype other than 1,2,3,4. In the examples given in Table 2, isolates D8214 and D9774 had the largest mixed populations (and perhaps D2960 on the few colonies tested). Part of the detailed analysis of D9774 is illustrated in Fig. 2. In such an analysis a culture was grown from one colony; from this two were selected, and from each of these another two (see Fig. 1C). This was done on six colonies from the whole culture of the isolate, resulting in a total of 24 cultures from single colonies. These cultures were homogeneous and remained stable as described below. The usefulness of agglutinin

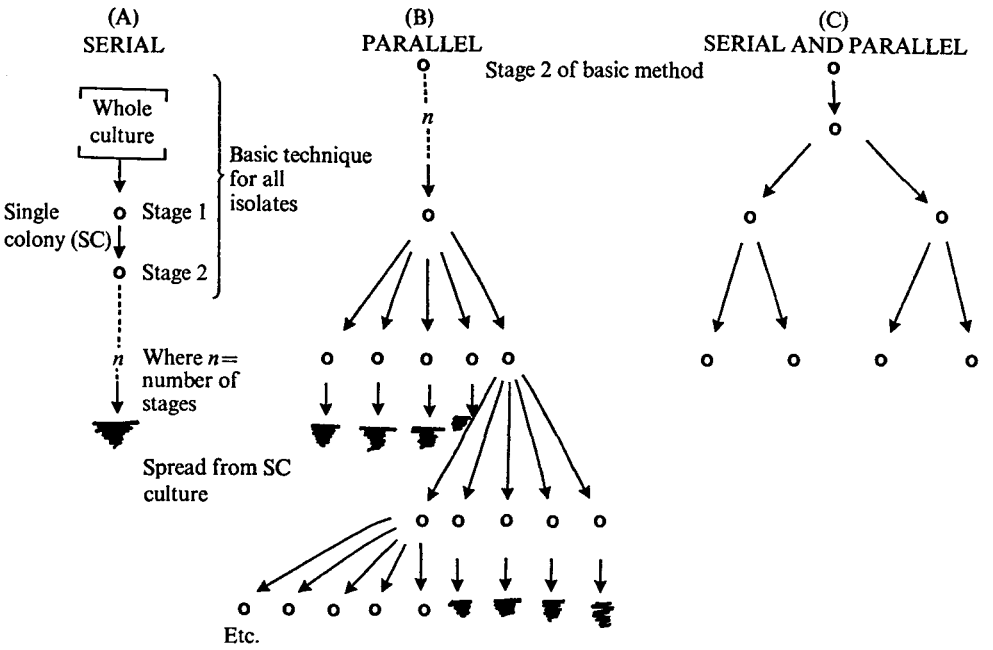


Fig. 1. Analysis of isolates by single colony separation.

production in determining serotype is shown by results for single colonies (sc) 7 and 10, detecting agglutinogens 3 and 2 respectively; agglutinogen 4 was a weak antigen in mice and was often detected better by direct typing (colony 6). The appearance of the colonies and their rate of growth was not related to their serotype.

*Stability of the component colonies*

Several isolates were tested over several single colony separations using various methods (see Fig. 1). Cultures grown from single colonies were serotyped by direct agglutination and by agglutinin production in as many cases as possible. Isolates of 1,0,3,0 and 1,2,0,4 were used for comparison. Twelve isolates were tested over several serial single colony separations (Fig. 1A). Twenty colonies from each were subcultured and tested after 6, 10 and 26 separations. From Table 3 it can be seen that after six or more serial single colony isolations the serotype remained constant.

A 1,2,3,4 isolate was taken and eight serial subcultures were performed, on each occasion the subcultures being made from a single colony. One colony was emulsified in CA and spread on a BG plate (Fig. 1B). Thereafter four of the resulting colonies were cultured and serotyped. A fifth colony was emulsified in CA medium and spread on to BG medium. Four colonies were cultured and serotyped and a fifth again subcultured to give single colonies. This process was repeated four times. A 1,0,3,0 colony and a 1,2,3,4 colony, both from different strains, were used. The serotypes of both remained constant throughout testing.

Table 3. *Stability of the component colonies of isolates of different serotype*

| | | No. serial single colony cultures | | | | | |
|---|---|---|---|---|---|---|---|
| | | 6 | | 10 | | 26 | |
| Serotype | No. isolates tested | No. of colonies | Serotype | No. of colonies | Serotype | No. of colonies | Serotype |
| 1,2,3,4 | | | | | | | |
| (*a*) Stable | 2 | 40 | 1,2,3,4 | 40 | 1,2,3,4 | 40 | 1,2,3,4 |
| (*b*) Variable | 2 | 40 | 1,2,3,4 | 40 | 1,2,3,4 | 40 | 1,2,3,4 |
| (*c*) Mixed | 2 | 33 | 1,2,3,4 | 33 | 1,2,3,4 | 33 | 1,2,3,4 |
| | | 5 | 1,0,3,0 | 5 | 1,0,3,0 | 5 | 1,0,3,0 |
| | | 2 | 1,2,0,4 | 2 | 1,2,0,4 | 2 | 1,2,0,4 |
| 1,2,0,4 | 3 | 60 | 1,2,0,4 | 60 | 1,2,0,4 | 60 | 1,2,0,4 |
| 1,0,3,0 | 3 | 60 | 1,0,3,0 | 60 | 1,0,3,0 | 60 | 1,0,3,0 |

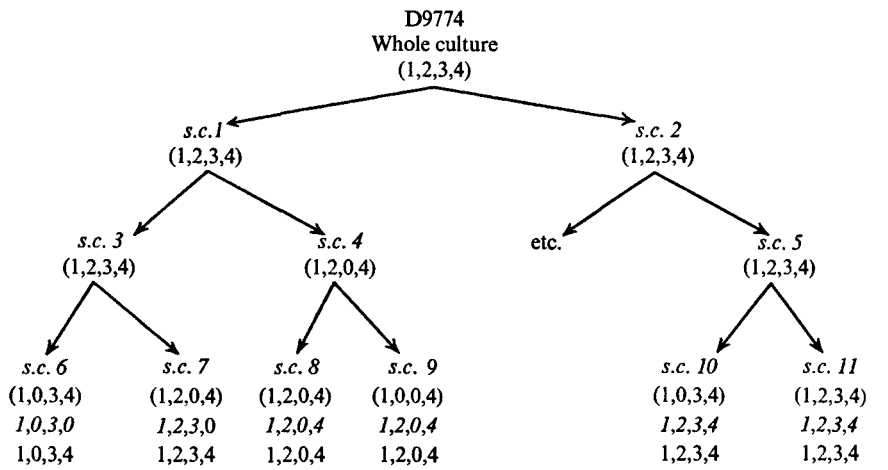


Fig. 2. The agglutinogens of cultures derived from a 'mixed' 1,2,3,4 isolate D9774 by serial and parallel single colony culture. Expression of serotype: direct agglutination (in parentheses); agglutinin production (italic type); combined information (ordinary type).

*Isolates typing as* 1,0,3,4

Of the many isolates appearing to type as 1,0,3,4 on direct typing, only one, D3096, was shown by agglutinin production in mice to be type 1,0,3,4. The others were either 'variable' or 'mixed' 1,2,3,4. This isolate was as stable on single colony culture as were isolates typing as 1,0,3,0 and 1,2,0,4. Although we found only one such isolate from a child, organisms typing as 1,0,3,4 do occur in 'mixed' 1,2,3,4 isolates, as can be seen in Table 2 and Fig. 2.

*Attempts to alter the serotype by mixing two serotypes*

Strains shown to be homogeneous by serotyping cultures from single colonies were used. The 1,2,0,4 strains were D14105, 3865 and the mouse-virulent 18-323. The 1,0,3,0 strains were D11044, B16 and the mouse-virulent B106. One strain of

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each serotype was mixed in equal quantities and grown together as described in Methods.

*On solid and liquid medium.* Although single colonies were more difficult to separate than those arising in cultures isolated from children, requiring at least three or four single colony isolations before cultures would type consistently, the final result showed that there were almost equal numbers of 1,2,0,4 and 1,0,3,0 colonies present, with no evidence of any colonies of other serotypes.

*In the mouse brain.* Analysis of the mixed infection in mouse brain with 18-323 and B106 gave an easy separation into colonies of serotype 1,2,0,4 and 1,0,3,0. Serotype 1,2,3,4 was not found at all in the mixed infection.

The experiments were first carried out in normal mice. Analysis of the growth from the homogenized brains of mice after three days infection showed that the mixed infection of 18-323 and B106 separated into colonies which could easily be distinguished by their size and colour. Those of 18-323 were grey and were larger, whilst those of B106 were whiter and smaller; 68 % of the colonies were 1,2,0,4 and 32 % were 1,0,3,0.

To determine if a state of immunity (as in vaccinated or convalescent children) would alter the result, vaccinated mice were next used. The brains were removed from the mice at five days after challenge, i.e. 2 days after the blood–brain barrier had broken (Holt, Spasojevic, Dolby & Standfast, 1961). The brains were homogenized and diluted and the suspension was seeded on BG medium; the resultant colonies were tested for their serotype. Preliminary experiments showed that the colonies typed easily as one or other of the serotypes of the infecting strains. Thus serotype 1,2,3,4 was not produced from mixtures of 1,2,0,4 and 1,0,3,0 strains *in vitro* or *in vivo*.

*Stability of the serotypes in source-contact isolations*

In the PHLS survey of 1966–8 some isolates from source and contact patients were available. In eight instances routine typing of the isolates by the reference and area laboratories suggested a change of type on passage from the source to the contact patient. Six pairs were available for retesting, the results are shown in Table 4.

In the three cases in which it was reported that the isolate from the source patient was type 1,2,3,4 and the contact was type 1,0,3,0, agglutinin production showed that only in one of the three pairs was there a difference in serotype. In the three cases in which the isolate from the source patient was reported to be 1,0,3,0 and from the contact was 1,2,3,4, agglutinin production showed that only one source patient was infected with 1,0,3,0, the other two source isolates being 1,2,3,4. Thus in only two of the six reported instances were there actually differences in serotype of source and contact patient. Before attributing this to a change of serotype much more information about the environment of the patients would need to be known. This could be a real change of serotype but other explanations are discussed below.

Table 4. *Suspected changes in serotype of isolates from six contact patients*

(Tested by direct agglutination and agglutinin production.)

| Patient nos. | | Isolate from source patient | | Isolate from contact patient | | |
|---|---|---|---|---|---|---|
| Source | Contact* | Reported type† | Agglutinins produced | Reported type† | Agglutinins produced | Type difference |
| 1 | 2 | 1,2,3 | — | 1,0,3 | 1,0,3,0,0,0 | Yes |
| 3 | 4 | 1,2,3 | — | 1,0,3 | 1,2,3,4,0,0 | No |
| 5 | 6 | 1,2,3 | — | 1,0,3 | 1,2,3,4,0,6 | No |
| 7 | 8 | 1,0,3 | 1,2,3,4,0,0 | 1,2,3 | — | No |
| 9 | 10 | 1,0,3 | 1,0,3,0,0,6 | 1,2,3 | — | Yes |
| 11 | 12 | 1,0,3 | 1,2,3,4,0,0 | 1,2,3 | — | No |

* Contact patients 2, 4 and 6 were vaccinated but developed whooping cough; contact patients 8, 10 and 12 had not been vaccinated.

† PHLS survey result obtained by Area or Reference laboratories by direct agglutination (routine).

*Stability of serotypes of vaccines*

Some 60 vaccines, made from single strains of *B. pertussis*, killed by formalin and stored in merthiolate saline at 4° C. have been tested. Single strain 1,0,3,0 vaccines retained their 1 and 3 agglutinability for 5 years, the maximum time such vaccines were stored. Vaccines made from 1,2,3,4 strains were, however, stored without loss of any agglutinogens for 12 years, and 1,2,0,4 for 15 years. Vaccines containing agglutinogens 5 and 6 were stored for 4 years without loss of agglutinogens.

## DISCUSSION

Strains possessing agglutinogens 1,0,3,0 and 1,2,0,4 behave consistently in agglutination tests on cultures grown from single colonies. Only some strains of serotype 1,2,3,4 do so; the others produce cultures in which the component colonies possess variable but positive amounts of all four agglutinogens or the cultures consist of colonies with a mixture of serotypes. Difficulties experienced in serotyping are nearly always caused by isolates reported on at least one occasion to be 1,2,3,4 (Public Health Laboratory Service, 1973). We have shown that agglutinogens of a strain, present in variable amounts from subculture to subculture, can be better detected by agglutinin production than by direct agglutination. Single colony culture and agglutinin production reveal the 'mixed' and 'variable' categories into which these isolates fall, e.g. Fig. 2.

Reports of change in serotype of organisms during illness and convalescence of one patient (Zakharova, Gorbunova, Kuznetsov & Denisova, 1970) and on passage from source to contact patient (Public Health Laboratory Service, 1973) should be critically examined. Often a 1,2,3,4 strain is implicated. We have found that investigation by agglutinin production revealed that of the six reported changes in serotype on source-contact passage, four had not changed after all. Our results showed that two strains might have changed serotype on human passage, one

from 1,2,3 to 1,0,3; the other from 1,0,3 to 1,2,3. Emphasis must be laid on the possibility of multiple or alternative source infections of the contact patient however. For a conclusion to be drawn, there must be no doubt that the contact was indeed infected by the source and a thorough investigation of the isolates from the source patient should be made. The experiments of Stanbridge & Preston in marmosets (1974*a*) have been interpreted as a change in serotype during nasopharyngeal infection. The strain which they used, 41633, in our hands was a 'variable' 1,2,3,4 strain when tested by single colony cultivation and agglutinin production. Their derivative cultures would therefore be difficult to serotype by direct agglutination; it would have been interesting to see if agglutinins to all four agglutinogens could be found on agglutinin production.

Attempts to alter the serotype of a strain by growing two cultures together in normal and vaccinated mice have failed, but we used only stable cultures for infection and the brain as the only site of investigation. It is much more likely that new strains are derived from the 1,2,3,4 serotype. The changes in serotype by the suppression of one agglutinogen, as a result of the nasopharyngeal infection of marmosets, reported by Stanbridge & Preston (1974*a*) with less stable strains mentioned above, may be real but the results with the strains they used are difficult to interpret as we have explained. A reason for a change could be that antiserum containing bactericidal antibody together with complement can affect the cell walls of *B. pertussis* growing *in vitro* so that some survivors from the lethal action are permanently changed with respect to their agglutinogens (Dolby & Bronne-Shanbury, 1975). Children recovering from whooping cough might have high enough bactericidal antibody titres (Dolby & Stephens, 1973) to achieve this *in vivo* during convalescence; so might marmosets.

In our hands, continual subculture *in vitro* of 'variable' and 'stable' strains and the component colonies of 'mixed' strains have not caused the change of serotype reported by Stanbridge & Preston (1974*b*) and which they ascribed to mutations. Since three of their four strains were serotype 1,2,3 from children and the other was the strain continually passaged through mice of varying degrees of immunity conferred by vaccination, it may be that they were dealing with 'variable' and 'mixed' cultures initially, whose serotypes were difficult to determine by direct typing.

The reason for the complete change of infecting serotype as happens over a period of years (Preston, 1963; Bronne-Shanbury *et al.* 1976) is unknown. Virulence and rate of growth are not related to the serotype of an isolate. Is it possible that strains of serotype 1,2,3,4 growing *in vivo* may be the source of strains of other serotypes? Fig. 2 demonstrates that stable cultures of different serotypes can be achieved by *in vitro* separation from a 'mixed' strain (Cameron, 1967; E. K. Andersen, personal communication). Such a mixture may result from a host–parasite relationship as suggested above; very little is known about the carrier state in *B. pertussis* infections.

Other strains can be derived from 1,2,3,4 strains by loss of agglutinogens but what is the source of 1,2,3,4 cultures? There always appear to be at least some 1,2,3,4 strains in any community; in Britain the numbers of these isolated have

been constant for 30 years, whilst there were wide fluctuations of incidence of isolates of other serotypes (Bronne-Shanbury *et al.* 1976).

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