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FREDERICK GRIFFITH, 1879–1941

The main part of this issue is published as a memorial to Frederick Griffith. To commemorate the twenty-fifth anniversary of his death we are reprinting one of his most important papers on 'The Significance of Pneumococcal Types' (*J. Hyg. Camb.* (1928), **27**, 113–59). This is followed by contributions by some of his friends.

(*Facing p.* 129)

THE SIGNIFICANCE OF PNEUMOCOCCAL TYPES.

By FRED. GRIFFITH, M.B.

(*A Medical Officer of the Ministry of Health.*)

(*From the Ministry's Pathological Laboratory.*)

CONTENTS.

I. Observations on Clinical Material.

Since communicating my report[1] on the distribution of pneumococcal types in a series of 150 cases of lobar pneumonia occurring in the period from April, 1920 to January, 1922, I have not made any special investigation of this subject. In the course, however, of other inquiries and of the routine examination of sputum during the period from the end of January, 1922, to March, 1927, some further data have been accumulated[2].

Table I gives the results in two series and, for comparison, those previously published.

[1] *Reports on Public Health and Medical Subjects* (1922), No. 13.

[2] I owe many thanks to Dr J. Bell Ferguson, formerly Medical Officer of Health for Smethwick, for sending me many specimens from cases of lobar pneumonia.

Table I. *Types of Pneumococci in Lobar Pneumonia.*

Period of inquiry	Total cases examined	Percentage incidence of types			
		Type I	Type II	Type III	Group IV
Apr. 1920–Jan. 1922	150	30·6	32·6	6·6	30·0
Feb. 1922–Oct. 1924	61	42·6	21·3	3·2	32·7
Nov. 1924–Mar. 1927	67	34·3	7·4	4·4	53·7

The main point of interest, since the beginning of the inquiry, is the progressive diminution in the number of cases of pneumonia attributable to Type II pneumococcus. The great majority of the cases occurred in the Smethwick district, and the figures may reveal a real local decrease of Type II, and a corresponding increase of Group IV cases. It must, however, be remembered that the isolation on a single occasion of a Group IV strain from a sputum, especially in the later stages of the pneumonia, does not prove that strain to be the cause of the disease. This is clearly shown by the examination of several samples of sputum taken at different times from the same case; in these a Group IV strain was often found in addition to one or other of the chief types. There may be a slight element of uncertainty regarding causal connection of the Group IV strains with the pneumonia, since the cultures of pneumococci in this series were derived from sputum (except in four cases where the material was pneumonic lung) and some of the samples of sputum were obtained when the disease had been in progress for some time—from 4 to 11 days after the onset.

Occurrence of a Variety of Serological Types in the Sputum from an individual case of pneumonia.

In my report (1922 *loc. cit.*) I described a number of instances where several serological varieties of pneumococci were found in the sputum of a pneumonia patient. One instance was particularly striking, where the sputum, No. 112, taken on the sixth day of the disease (a crisis had not occurred), yielded a Type I culture and three strains of Group IV, *i.e.* four distinct serological races. On other occasions different specimens of sputum from the same case, taken at varying periods after the onset of pneumonia, were found to contain two or more serological types.

Three alternative explanations, at least, are possible.

1. The patient having previously been a carrier of several Group IV strains became infected with a Type I strain which produced pneumonia. There is no evidence to show which of the types was present in the pneumonic lung, but I think that the Type I may be assumed to have caused the disease.

2. The patient when normal was a naso-pharyngeal carrier of a Group IV strain. Owing to a condition being produced favourable to mutation, a type of pneumococcus, in this instance Type I, was evolved in his air-passages which was able to set up pneumonia. On this hypothesis, the different serological types would be evidence of the progressive evolution.

3. On the other hand, the Group IV strains might be derived from the Type I in the course of successful resistance against the latter strain. With the increase of immune

substances or tissue resistance the Type I would be finally eliminated, and there would remain only the Group IV strains which are almost certainly of lower infectivity and perhaps of less complex antigenic structure.

In the hope of gaining further information on these points I continued the analysis of the types yielded by the same patient, employing the following method:

The sputum was preserved in the ice-chest until the preliminary diagnosis of the infecting pneumococcus had been made in the usual way, viz. by the intraperitoneal inoculation of a mouse and by testing the peritoneal washings versus the type sera. The following day some of the sputum was inoculated together with the type serum corresponding to the strain identified. Frequently the second mouse died from an infection with a pneumococcus of a different type from that first obtained. If a serum corresponding to the fresh type was available a third mouse was inoculated together with the sera appropriate to the two types already identified. The following examples will make the procedure clear.

(1) A specimen of sputum, No. 239, from a case of pneumonia of four days' duration was sown on plates, and five colonies of pneumococci were examined, all of which proved to be Type I; the mouse test also gave Type I. The next day the sputum was inoculated into a mouse together with Type I serum. The mouse died and the peritoneal washing reacted only with Pn. 41 (Group IV) serum; the blood of the mouse was plated and of five colonies examined two were Pn. 41 and three were strains of Group IV which could not be identified. The sputum was inoculated a third time plus a mixture of Type I and Pn. 41 sera. The third mouse died within 24 hours and its blood yielded a virulent culture of a Group IV strain which did not react with any of the available agglutinating sera.

(2) A more complete examination was made of the sputum from a second case, No. 273, of lobar pneumonia, specimens being taken at different periods after the onset of the disease. The details are given in Table II.

Table II.

		Types of pneumococci obtained		
			Through mouse	
Specimen of sputum	Day of disease	On direct plate	Sputum alone	+ Type I serum
1	4th	Type I (3 colonies)	Type I	Pn. 41
2	6th	Type I (7 colonies)	Type I	Pn. 160 and Group IV?
3	8th	Not done	Type I and Pn. 160	—
4	12th	Not done	Type I	—
5	15th	Group IV (1 colony)	Group IV	—
6	17th	Pn. 160 (2 colonies)	Group IV	—
7	19th	Not done	Type I and Pn. 160	—
8	21st	No Pn. colonies	Type I	—

The first specimen of sputum collected on the 4th day of the disease yielded pneumococci of Type I, both on the direct plate and through the mouse. The same specimen of sputum, which had been kept on ice in the meantime, was inoculated into a mouse together with a protective dose of Type I serum; the mouse died within 24 hours and a pure culture of Group IV, viz. Pn. 41, was obtained from the blood. The specimen collected on the 6th day of the disease was examined in the same way and gave a similar result, but on

this occasion the protected mouse yielded two different strains of Group IV. One of the latter was identified with Pn. 160; I had no serum corresponding to the other.

An interesting result was obtained with the 8th day specimen. The mouse inoculated with the sputum died of a mixed infection, and on a plate from the blood it was possible to pick out only three pneumococcus colonies. Two of these colonies reacted with Pn. 160 serum alone, while the third gave equally good specific clumping (firm masses) with both Type I and Pn. 160 sera. This third colony culture was plated and the plate showed two varieties of smooth pneumococcus colonies differing slightly in appearance but easily distinguishable. Several of each variety were grown in broth and the agglutination reactions were tested; rounded dome-shaped pearly colonies were found to be Type I, and larger, flatter and more translucent colonies were Pn. 160. In addition to the above there were on the plate a few typical rough pneumococcus colonies, four of which were subcultivated and tested on mice. Three were avirulent but the fourth caused septicaemia in mice and produced a peritoneal washing which agglutinated specifically with Pn. 160 serum. The culture obtained from the blood remained rough in character and thus possessed a combination of rough cultural characteristics and virulence which had not previously been noted. Further experiments were made with this culture (see p. 117).

The above instance may be simply an example of a mixed colony and nothing more. On the other hand there is the possibility that this mixed colony was derived from a single coccus possessing double antigenic properties. The culture being perhaps in an unstable condition may have separated in the course of growth into two elements, Type I and Pn. 160, in each of which the second antigen was suppressed.

On the 12th day the sputum yielded Type I through the mouse. In a specimen taken three days later, Type I was not found, the direct plate cultures as well as the cultures through two mice belonging to Group IV but not reacting with any of the available sera.

On the 17th day of the disease there was again no evidence of Type I, each of two mice being infected with an unknown Group IV strain, while on the direct plate Pn. 160 reappeared. Type I was again found on the 19th day and still persisted in the sputum on the 21st day after the onset of the pneumonia.

It is curious that the Pn. 41 culture was never again found after the first test. The strain produced large capsules in the blood of the mouse and a peritoneal washing reacted vigorously with the type serum. The Pn. 160 culture on the other hand appeared frequently in the course of the investigation.

(3) Sputum 218 came from a man who had been ill with pneumonia and had not yet had a crisis. This specimen yielded Type I colonies both on plates made direct from the sputum and through the mouse. On the 10th day of the disease, three days after the crisis, a second specimen of sputum was taken. The sputum alone inoculated into a mouse caused fatal septicaemia, and plates from the blood gave a pure growth of pneumococci; three colonies belonged to Type I and seven to an unidentified strain of Group IV. Inoculated plus Type I serum this 10th day sputum killed a mouse and ten plate colonies from the blood belonged to an unidentified Group IV strain.

On the 14th day of the pneumonia the sputum alone killed a mouse, and four colonies on the plate from the blood were identified as Type I. Sputum together with Type I serum yielded through the mouse an apparently pure culture of Type II B (12 plate colonies were identified). The sputum which had been preserved in the ice-chest was then inoculated into a mouse together with a mixture of Types I and II B sera; this mouse died of a pure Type III infection.

A final specimen of sputum taken on the 16th day of disease was inoculated into three mice; (1) with sputum alone and (2) sputum plus Type I serum both yielded Type II B only, (3) sputum plus a mixture of Types I and II B sera yielded an unidentified strain of Group IV.

(4) Six other cases of pneumonia were investigated in a similar manner to the above.

Type I pneumococci were grown from each case and, in addition, Group IV strains were obtained from five and a Type III strain from one.

(5) A few cases only of lobar pneumonia due to Type II pneumococci have been studied in the above manner. Sputum No. 267, which killed a mouse with a Type II infection, was re-inoculated plus Type II serum and the mouse died of a Type III infection.

(6) The lung from a fatal case of pneumonia, No. 230, was plated directly and 34 colonies were examined, all of which proved to be Type II; 12 colonies from a mouse inoculated with the lung were also Type II.

I have not had an opportunity of ascertaining whether more than one type of pneumococcus can be obtained from the lung in a fatal case of pneumonia due to Type I; only in sputum has a mixture of several types been demonstrated. This latter fact might suggest that the secondary strains, viz. Group IV and Type III, were present in the upper air passages prior to the infection with the more invasive strains of Types I and II. On a balance of probabilities interchangeability of type seems a no more unlikely hypothesis than multiple infection with four or five different and unalterable serological varieties of pneumococci. Moreover, failure to find more than one type in the lung of a fatal case of pneumonia would not be conclusive evidence against the modification hypothesis, since the fatal termination would in itself indicate an absence of those protective antibodies which may be necessary to initiate an alteration in the type of the infecting pneumococcus. Lung puncture in a case of resolving pneumonia might furnish more precise indications.

The above findings, taken alone, are not decisive in favour of either of the two hypotheses, but they assume greater significance when considered together with the laboratory experiments on alteration of type described later.

Further remarks on a Pneumococcus Strain from Sputum producing rough colonies yet virulent for mice.

A distinguishing feature of an avirulent pneumococcus is the rough appearance of the colonies after 24 hours' growth on a blood agar plate. Until the appearance of the strain already referred to on p. 116 the above morphological character of a pneumococcus colony has been found invariably associated with absence of virulence. The strain in question produced very typical rough colonies, but nevertheless was able to multiply in the mouse and cause fatal septicaemia. The blood of the mouse showed pneumococci with well marked capsules, and on plate cultures rough colonies grew, identical in appearance, except in one instance, with those of the original strain.

There are some points of interest both in regard to the origin of the strain and in the experiments which proved it to combine roughness of colony with virulence for mice.

The strain was derived from the sputum, No. 273, of a case of lobar pneumonia. Several specimens of sputum from this case were examined at different stages of the disease and the results are given on p. 116. The sputum which yielded this strain was the third specimen and was taken on the 8th day after the onset of pneumonia; it produced pneumococcal

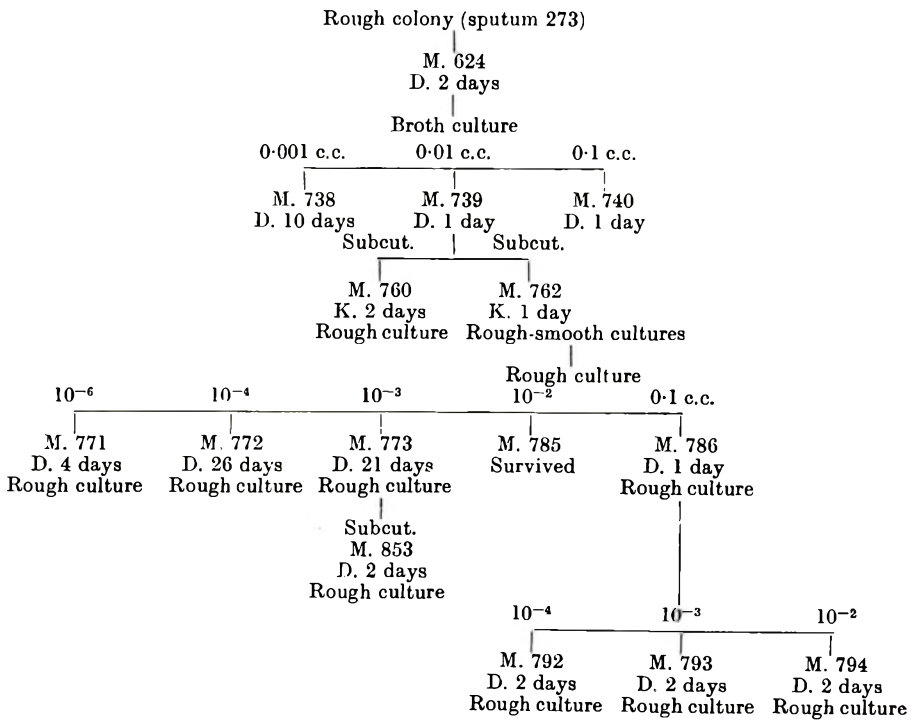
septicaemia in a mouse and a smooth colony was subcultivated in broth from the blood. The broth culture on being tested against all the available pneumococcus agglutinating sera gave a positive reaction with two sera, viz. Type I and Group IV (Pn. 160); in both cases the coarse masses characteristic of a reaction with soluble substance were formed. This unusual occurrence was investigated in the following ways.

The original colony culture in broth was plated and on the plate three different varieties of colonies were identified. Two varieties were smooth, one of which was found to agglutinate with Type I serum and the other with Group IV (Pn. 160) serum; the third variety was rough. Four of the rough colonies were subcultivated and each was inoculated subcutaneously into a mouse in a dose of 0·25 c.c. of broth culture. Three of the mice were well when killed three days later and cultures were grown from the seat of inoculation; each culture thus obtained was inoculated intraperitoneally into a second mouse without causing any ill effects. The mouse inoculated with the fourth colony died of pneumococcal septicaemia. The blood yielded colonies which were wholly of the rough variety and which, nevertheless, when grown in broth agglutinated typically with Pn. 160 serum.

The broth culture of the original colony was also inoculated intraperitoneally in a dose of 0·01 c.c. into three mice which had received preliminary treatment with protective sera. The mouse immunised with Type I serum died 6 days after inoculation and a pure culture of Pn. 160 was obtained from the blood; the mouse protected with Pn. 160 serum died within 24 hours of a Type I infection; the third mouse treated with the two sera, Type I and Pn. 160, survived the culture inoculation. The colonies of the two strains obtained as above were smooth; the rough Pn. 160 element was eliminated.

A series of passage experiments, the details of which are given in Table III, was made with the rough virulent culture of Pn. 160 to ascertain whether a change into the smooth form might be induced.

Table III.



Throughout a long series of passage experiments, of which those given in Table III are about half the total, the majority of the mice died of pneumococcal septicaemia and showed capsulated diplococci in the blood; the cultures obtained from the blood retained the original rough appearance of colonies with a single exception. The survival of some of the mice for prolonged periods, up to 26 days, is noteworthy; in the end such mice succumbed to pneumococcal septicaemia and yielded rough colonies from the blood. All the rough cultures in broth agglutinated with Pn. 160 serum.

At one stage of the passage experiment, as mentioned above, a change from rough to smooth occurred and this was the only instance observed, although plate cultures had been made from every mouse in the series. The change from the rather large coherent colony into a much smaller shiny colony of almost watery consistency was very striking.

The circumstances of the conversion have some interest. The mouse, No. 762, from which the smooth variety was obtained, had been inoculated subcutaneously with 0·25 c.c. of broth culture of a rough passage strain and was killed the next day. The blood of the mouse yielded numerous colonies of the usual rough character, among which was detected one with a small smooth segment. The latter was touched with a fine needle and from it a second plate culture was made on which grew a mixture of rough and smooth colonies. From one of the latter a pure smooth colony culture was obtained and this agglutinated like the original rough colony with Pn. 160 serum.

The virulence of the smooth colony resembled that of the rough colony and after passage through four mice in series the same chronic infection occurred ending in death from septicaemia; for example, one mouse which received 10^{-5} dose of the smooth broth culture died 25 days after inoculation. At the end of the passage the smooth colony still retained its small size, being definitely smaller than the normal Group IV colony.

Both the above cultures, the rough and the smooth, were grown in the antiserum of their type. From the serum cultures rough and smooth strains were obtained and it was found that each variety had become attenuated. Neither would kill mice in intraperitoneal doses ranging from 0·1 c.c. to 0·2 c.c., the mice being kept under observation for two months.

I record the foregoing observations without attempting at present to interpret them.

A Strain agglutinating specifically with Sera of two Different Types.

The strains of Group IV comprise many different types which are remarkably well defined and exhibit no cross-agglutination amongst themselves, provided one takes as the criterion of type agglutination the formation of firm clumps, either with cultures or with peritoneal exudates. This specificity is no doubt due to the secretion of soluble substances peculiar to each type. The strain which I am about to describe gives with two different type sera the firm clumps characteristic of the reaction between soluble substance and agglutinin.

The source of the culture was the lung of a woman who died 9 days after the onset of broncho-pneumonia. A plate made directly from the lung showed large smooth pneumococcus colonies. Seven colonies were subcultured separately and were inoculated intraperitoneally into mice, which died within 24 hours. The peritoneal washings from these mice were found to react with two different Group IV sera, viz. II B and Pn. 87; the reactions were equal with six of the washings, while the seventh gave a heavier precipitate with II B serum than with Pn. 87 serum.

Comparative tests were made with the new strain and with the stock II B and Pn. 87 strains.

Strain		II B serum	Pn. 87 serum
II B:	peritoneal washing	+	−
Pn. 87:	,,	−	+
New strain:	,,	+	+

The culture of II B agglutinated up to 1 in 320 with II B serum and not at all with Pn. 87 serum. Pn. 87 culture agglutinated to 1 in 160 with Pn. 87 serum and gave a trace of 1 in 20 with II B serum. The new strain in culture agglutinated with both sera but, unlike the two homologous strains, did not form firm masses characteristic of virulent pneumococci. Instead there was produced a turbidity made up of fine granules, showing that there was probably a deficiency of soluble substance. In the peritoneal cavity of the mouse, on the other hand, soluble substance is more readily produced and in consequence the washings gave with both sera the typical reaction of a virulent strain.

It may be remarked that such behaviour has been observed on a few other occasions; a pneumococcus strain in culture, obtained direct from sputum, has not reacted with the type serum while the same strain in the peritoneal exudate of a mouse has reacted typically. Such a result is no doubt an indication of reduced virulence with associated deficiency in the production of soluble substance.

A series of experiments were made to prove that the new strain contained two antigens and was not merely a mixture of II B and Pn. 87. A preliminary test showed that II B and Pn. 87 sera each protected mice against 0·0001 c.c. of the new strain but not against 0·001 c.c. Plate cultures were made from the blood of (1) a mouse injected with II B serum, and (2) a mouse injected with Pn. 87 serum along with the new strain, and four colonies from each were studied. Seven of the colony cultures reacted equally well with both sera; one gave a stronger reaction with II B serum than with Pn. 87 serum. Cultures were made in II B serum and were inoculated into mice treated with II B serum; from the mice which died strains with the double antigens were recovered, whereas if the new strain had been a simple mixture one would have expected the II B constituent to be eliminated. In point of fact the II B antigen was probably the major antigen, since some single colony strains were obtained which reacted only slightly with Pn. 87 serum.

I have recorded my observations on this strain rather fully since it is the only exception I have found to the rule that a pneumococcus has only one well-developed antigen. The observation may however be significant as indicating that this rule is not absolute and that the purity of the specific antigen in virulent pneumococci may only be apparent.

II. Experimental Modification.

Production of Attenuated Strains of Pneumococci.

(1) *By growth in immune serum.*

Culture in homologous immune serum is perhaps the most convenient method of producing attenuated strains of pneumococci, recognisable by the morphological appearances of the colonies and termed the R form of the pneumococcus. Complete attenuation of a virulent pneumococcus culture is secured only after several passages in series, the first and second serum cultures generally being composed of a mixture of S and R forms while either the third or fourth cultures may contain purely R forms. During my first

observations[1] on this matter I found that the more stable R forms were obtained from the later cultures in series and I concluded that this was the effect of the several passages in serum.

As, however, very stable R forms may be isolated from a first serum culture, and, on the other hand, unstable R strains, those which readily regain virulence in the mouse, may retain this property after repeated passages in immune serum, my first experience may have been a matter of chance. It appears, as the tests on p. 123 show, that R colonies on a plate from an immune serum culture are not equally attenuated, the capacity to revert to the smooth type on inoculation into the mouse being more pronounced in some colonies than in others. Thus it is true that several passages in series are required to eliminate the smooth form but it cannot be predicted what the effect of repeated exposure to the action of immune serum may be once the R state has been reached.

(2) *By growth on solid media.*

Attenuated R strains can also be obtained from virulent cultures by growth on chocolate blood agar. A virulent pneumococcus culture in blood broth, plated on this medium and examined after 24 hours' incubation at 37° C., yields as a rule only smooth colonies. If the plate is left in the incubator for two days, some of the smooth discs, which after the first night's incubation were perfectly regular in outline, develop small rough patches in their margins. The rough patches may develop into a wedge with the base at the periphery and the apex at the centre of the colony. They may be either raised above or depressed below the level of the original smooth growth, and generally they project beyond its margin. Sometimes the rough area forms a rounded projection extending well beyond the margin of the S colony and sending a process like a single root towards the centre. Usually a colony shows only a single rough focus but cultures are variable in this respect, and some produce colonies which become studded with rough areas. But many cultures, especially highly virulent strains of Type II, fail entirely to produce colonies with rough patches.

Rough foci have never, in my experience, become visible in smooth colonies after a single night's incubation; it is essential that the culture medium should be sufficiently favourable to allow growth to continue for at least 48 hours. The following is an instance. The stock virulent strains of Types I and II were plated and produced completely smooth colonies after 48 hours' incubation. After three days incubation Type I colonies showed occasional small rough areas but none was seen in the Type II colonies even on the 4th day. One of the Type II colonies was subcultured in blood broth and then plated; the majority of the colonies produced were smooth but there were also a few R colonies.

Apparently a few R pneumococci are formed in a culture which is allowed to age on blood agar and these may multiply and produce a rough area or colony in and perhaps at the expense of the smooth growth.

When the patch is large it may be touched with the point of a spatula and a pure

[1] *Reports on Public Health and Medical Subjects* (1923), No. 18.

rough strain may be obtained. Generally, however, when a rough patch is subcultured in blood broth and plated a mixture of R and S colonies grows.

R cultures from rough areas are, so far as I have ascertained, identical with those obtained by growth in immune serum.

The tendency to produce colonies with rough patches seems to be inherent in some strains and may perhaps indicate deterioration in virulence. It is not removed by a single animal passage; a Type I strain of medium virulence which produced colonies with many rough patches was inoculated into a mouse and caused fatal septicaemia. A culture from the blood was plated and produced a pure growth of smooth colonies after 24 hours' incubation; many of the latter developed rough patches after a second night's incubation.

Rough patches in colonies have been produced only on the chocolate blood medium which contains fresh horse serum and every batch of this medium has not been equally favourable. It is possible that the rough change may be due to the presence of immune bodies in the horse serum. On agar plates without blood pneumococcus colonies quickly lyse and become almost invisible. If such plates are left in the incubator daughter colonies may grow out from the lysed colonies, but when these are subcultivated and plated they almost invariably produce smooth colonies only.

The formation of individual R pneumococci in a smooth culture does not apparently take place when the culture has ceased to grow. This was shown in an experiment made to test the viability of pneumococci on ordinary agar.

Fourteen cultures of pneumococci, each of a different serological type, on nutrient agar slopes were incubated at 37° C. in tubes sealed with paraffined plugs. In 24 hours the growth had become almost invisible from lysis. The tubes were left undisturbed in the incubator for two months when they were scraped and subcultivated in blood broth. All the cultures were viable and the colonies grown on blood agar plates were smooth. After 5½ months' incubation twelve of the cultures were still alive, and on plates, while most of the colonies produced were smooth; occasional rough ones were detected. After 15 months in the incubator four cultures still survived; two were completely rough and had lost their virulence for mice, while the other two produced a mixture of R and S colonies. It will be observed that the surviving pneumococci remained in their original smooth condition in the tubes which had not been disturbed for two months. At the end of that period I suggest that the scraping of the surface and the transference of pneumococci to fresh parts of the medium caused further growth, with the result that R forms appeared. For the same reason the change to the R state was still more advanced at the conclusion of the experiment.

Similarly, as the pneumococci do not grow, no attenuation occurs when the spleens of mice which have died of pneumococcal septicaemia are dried and preserved for prolonged periods. I have recovered strains from dried spleens after 3½ years and have found the virulence unaltered. The surviving pneumococci may be very few in number and may be recovered in the following way. The whole of the spleen is ground to a fine powder in a mortar and emulsified in a small quantity of blood broth. This may be plated directly or after a few hours' incubation. The colonies produced have always been of the smooth form; the pneumococci have remained dormant and there has, therefore, been no opportunity for the production of the R forms.

(3) *Differences between individual R and S colonies.*

Virulent pneumococci which have been grown in homologous immune serum and have undergone the change from the S to the R form are not all equally affected. Pure R colony cultures show differences amongst themselves in (1) capacity to revert, (2) type of agglutination and (3) immunising properties.

A virulent Type I strain was grown in Type I serum for two generations, the second of which was plated. Six R colonies were taken and grown in small quantities of blood broth; these colonies were identical in appearance and gave non-specific agglutination in pneumococcal type sera. Each colony culture was subcultivated in 10 c.c. of broth; this was centrifuged and the deposits were inoculated into mice subcutaneously.

1st inoculation experiments.

No. of colony culture	Mouse	Result
1	923	Died 2 days. S colonies from blood
2	924	,, ,, ,,
3	925	,, ,, ,,
4	926	Survived 12 days. Nil in blood
5	927	Died 4 days. S colonies from blood
6	928	Survived

Each of the six colony cultures was then plated and an isolated colony was grown which was again inoculated subcutaneously in a dose of 10 c.c. of broth culture deposit.

2nd inoculation experiments.

No. of colony culture	Mouse	Result
1	967	Died 2 days. S colonies from blood
2	968	,, ,, ,,
3	969	,, ,, ,,
4	970	Survived
5	971	Died 3 days. S colonies from blood
6	972	Survived

In order to make certain that the colony cultures were free from any S forms of pneumococci, each culture was again plated and an isolated R colony was grown in blood broth. This procedure (plating and selection of colonies) was carried out six times in succession. Broth cultures were made from colonies on the final plates and these were inoculated subcutaneously into mice in the same doses as before.

3rd inoculation experiments.

No. of colony culture	Mouse	Result
1	20	Died 2 days. Pneumococcal septicaemia, culture overgrown
2	21	Died 2 days. S colonies from blood
3	22	Died 2¼ days. ,, ,,
4	23	Survived
5	24	Died 2 days. S colonies from blood
6	25	Survived

The results were practically identical in each of the three series of inoculation experiments; four of the R strains reverted in the mouse to the smooth type, while two, Nos. 4 and 6, were more completely attenuated. The different degrees of virulence were retained after seven successive platings, thus showing that the characters were stable and were the property of the whole strain in each case.

The following experiment reveals similar differences between individual R colonies.

Four cultures made from different colonies of a rough Type I strain were inoculated into mice in doses of 0·15 c.c. intraperitoneally. Cultures 1 and 2 killed the mice in two days and S colonies were grown from the blood. The mice inoculated with cultures 3 and 4 survived. The latter two mice were reinoculated on the 6th day with 0·25 c.c. and on the 13th day with 0·5 c.c., each with the same living broth culture as before. They were tested on the 26th day after the first inoculation with 10^{-6} c.c. of a virulent Type I culture. Both mice died, thus showing absence of immunity.

The immunising capacity of the other two rough cultures, 1 and 2, was tested in the following way.

No. 1 was inoculated intravenously into three mice, each with 0·2 c.c. of living broth culture. They resisted this inoculation and a test dose of virulent culture was given 18 days later. All of these survived. A similar intravenous experiment with No. 2 failed to produce immunity against the same test dose. No. 2 was then used to vaccinate 3 mice, inoculated intraperitoneally twice at 7 days' interval. Tested 21 days after the first vaccinating dose, two out of these mice survived.

Thus two of the rough cultures produced immunity and two failed. The properties of the four strains are given in the following table:

No. of rough culture	Reversible to S type	Immunising capacity	Agglutinability
1	Yes	Positive	Type specific
2	,,	,,	,,
3	No	Negative	Non-specific
4	,,	,,	,,

In this instance by type specific in regard to agglutinability is meant that though agglutination was of the rough character, *i.e.* the deposit was readily shaken up, it occurred up to the full titre 1 in 160 of a smooth Type I serum. The non-specific cultures agglutinated to 1 in 10 only with both Types I and II sera.

An experiment on similar lines was made with a rough Type II culture.

Six rough strains from individual colonies were inoculated into mice in doses of 0·75 c.c. and 0·1 c.c. of broth culture intraperitoneally. All the mice which received the larger dose died within 24 hours, but in no case was there any reversion to the smooth type. The peritoneal washings were tested against Type II serum and several gave a slight precipitate showing probably the formation of a small amount of soluble substance; two gave no precipitate. The mice injected with 0·1 c.c. all remained well; they were reinoculated after 6 days with 0·2 c.c. and after a further 7 days with 0·3 c.c. of the same cultures as before. The immunity of five mice (one died accidentally) was tested 26 days after the first injection with 10^{-6} c.c. of a virulent Type II culture. Three mice resisted and two succumbed.

It is interesting that the two mice which died were immunised with the two cultures which failed to produce a trace of soluble substance in the peritoneal washings. The change to the R form is apparently less complete in some pneumococci than in others, and it is possible that the retention of a small amount of the original S antigen in their composition may explain the capability of certain R strains to immunise against a virulent pneumococcus as well as their tendency to revert to the S form.

On the other hand, while diminution of virulence in a pneumococcus culture may be due to a proportion of the individual organisms composing it having undergone the change into the R form, this is not invariably the case. Cultures which produce only smooth colonies may possess intermediate grades of virulence, killing mice in doses not less than 0·1 c.c. or 0·01 c.c. of broth culture. This has been observed in cultures immediately after reversion from the rough to the smooth form. In such instances the change from the

one form of antigen to the other may not have been complete, and thus larger numbers of organisms are required to produce a sufficient concentration of those substances which neutralise the protective fluids of the animal and enable the bacteria to multiply.

Reversion from Rough to Smooth.

A. *Origin of the rough strains used; effect of different sera.*

The rough Type II strain was obtained in the following way. A virulent culture of Type II, which killed mice when inoculated intraperitoneally in a dose of 10^{-8} c.c., was sown into undiluted Type II serum and was passed from serum to serum for six generations, each of which was incubated at 37° C. overnight. The final culture was plated, and five rough colonies were selected and subcultured.

A test was made to show that these five subcultures were free from smooth virulent pneumococci; the procedure being the same for each, a single description is applicable to all. Each rough culture was plated and an isolated colony was grown in blood broth. A mouse was inoculated intraperitoneally from the blood broth culture and a plate was made. This procedure, plating followed by selection of rough colony and mouse test, was repeated six times in succession. In no case did any of the plates show a smooth colony, and all the mice (a total of 30) survived the intraperitoneal inoculation of culture, the doses of which ranged from 0·1 c.c. to 1·0 c.c. The final cultures were tested against agglutinating sera of Types I and II with both of which only minute clumps were formed, thus showing that the type characteristics had been lost.

A virulent culture of Type I was treated as above, except that the transferences were limited to five. The final rough cultures were tested on mice in the same way as those of the Type II. Although the appearance of the colonies was typically rough, they were found to revert readily in the mouse to the smooth virulent variety. One culture was then passed through five more generations of the same batch of Type I serum. The fifth serum culture was plated and several rough colonies were subcultured and these also were found to revert readily to the smooth form on being inoculated into mice.

Another protective Type I serum was then taken and a culture was started with a trace of blood from a mouse which had died from a Type I pneumococcal septicaemia. Four generations of serum cultures were made in succession, a night's incubation intervening between each. In the first two generations the culture grew in the form of a firm mass at the bottom of the serum; the third culture was partly granular and the fourth was quite diffuse. Plate cultures were made from the first, third and fourth generations and five colony cultures from each plate. These cultures were tested on mice, 27 mice with the first generation, six with the third and six with the fourth, the doses ranging from 1 c.c. of broth culture up to the deposit of 50 c.c.

All the strains, those from the first as well as the fourth serum cultures, were avirulent and none reverted to the smooth form.

It was clear that the second batch of Type I serum used was more efficient in producing attenuation than the first. In spite of eleven passages, the latter did not succeed in removing from the rough strain its ability to revert to the smooth on inoculation into mice.

A test was made to discover whether growth in the second more potent serum would further attenuate the readily reverting rough strain. After a night's incubation in the serum a plate was made and three rough colonies were subcultivated. These were tested on mice in subcutaneous doses of 1 c.c. of blood broth; two of the mice died and smooth colonies were recovered from the blood; the third mouse survived.

Thus the more active serum did not attenuate the rough strain so completely as it did the virulent capsulated pneumococcus sown from the blood. It is possible that pneumococci may to some extent become habituated to the action of the serum. For example, treatment with too low a concentration of protective antibodies seems to have induced the formation of a rough but reversible strain upon which the serum could no longer act.

The R culture of Type I most frequently used in the subsequent experiments was made from a rough colony (No. 3) grown from the first generation in the more powerful Type I serum referred to above.

B. *Passage experiments through mice.*

The following is an example of many similar experiments which I have made to discover whether an avirulent R pneumococcus can be transformed into the virulent S form by growth in the body of the mouse. As a rule, the experiment has been started with the inoculation of one mouse and several lines of passage have subsequently developed, in only an occasional one of which has the transformation into the virulent form been effected. This irregularity of reversion has been a feature of the experiments where the culture has been passed through a succession of mice in small doses and by the intraperitoneal method of inoculation. Instances will be given later to show that a greater regularity may be attained when very large doses, viz. the centrifuged deposit of 50 to 100 c.c. of broth culture, are inoculated under the skin, though even then only a small proportion of the mice succumb to pneumococcal septicaemia where a thoroughly attenuated strain has been used.

This particular passage experiment was begun with rough Type II strains obtained as described on p. 125 and was continued along five separate lines. Pure line strains from single organisms were not used, but the preliminary tests on mice showed that the highly virulent pneumococcus had been eliminated by growth in the immune serum. As a further precaution, each of the five strains was plated and an isolated rough colony was grown in blood broth; this procedure was repeated six times and colonies from the final plates were made the starting point of the passage.

One of the five lines of passage is shown in Table IV.

Table IV.

Rough colony culture No. 1.
Deposit of 4 c.c. of broth culture inoculated subcutaneously

M. 690	M. 691
Killed 2 days	Survived
Local lesion culture	
Subcut.	
M. 713	
Killed 2 days	
Local lesion culture	
Rough colony	
Subcut.	
Deposit of 8 c.c.	

M. 723	M. 724
Killed 2 days	Survived
Local lesion culture	
Rough colony	
Subcut.	
Deposit of 100 c.c.	

M. 747	M. 746
Died 3 days	Died 6 days
Smooth culture of T II virulent in a dose of 10^{-8}	Rough colonies from local lesion
	Intrap.
	M. 801
	Killed 9 days
	P.M. culture
	Nil

The first two mice each received the centrifuged deposit of 4 c.c. of broth culture under the skin of the inguinal region. One mouse survived; the other was killed after two days, and from the tissue around the inguinal gland a blood plate and a blood broth culture were sown. Both cultures were pure, the plate showing rough colonies only, and the blood broth was inoculated into M. 713 in a dose of 0·5 c.c. Mouse 713 appeared well when killed two days later and a plate culture was made from the local lesion. A few small rough colonies grew and one of these was grown in broth. From this culture two mice were inoculated, each with the centrifugalised deposit of 8 c.c., under the skin of the inguinal region. One mouse, No. 723, was killed when well two days later and a plate culture from the local lesion yielded a few rough colonies with one of which the passage was continued. The fellow mouse was allowed to survive.

M. 723 culture was grown in 100 c.c. of broth and the centrifuged deposit was divided equally between two mice, both being inoculated subcutaneously. One mouse, No. 747, died in 3 days of pneumococcal septicaemia, the blood showing numerous capsulated diplococci. A smooth culture was obtained from the blood and this killed mice, inoculated intraperitoneally, in a dose of 10^{-8} c.c. of broth culture.

The fellow mouse, No. 746, died six days after inoculation; the blood was sterile and a few rough colonies were grown on a plate from the seat of inoculation. One of these colonies was grown in blood broth and a mouse inoculated intraperitoneally with 0·5 c.c. remained

well. The passage experiments with the other four rough colony cultures ended, as in the example described above, with the subcutaneous inoculation of two mice, each with the centrifuged deposit of 50 c.c. of broth culture. The results are as follows: in No. 2 passage both mice yielded smooth virulent cultures; Nos. 3 and 4 were like that first described, one mouse yielding rough colonies and the other smooth; in No. 5 the final cultures obtained from both mice were still rough and avirulent.

An intermediate rough culture in the fifth line of passage was taken and was again inoculated into two mice. One received subcutaneously the deposit of 66 c.c. of broth and the other the deposit of 33 c.c. The former died of pneumococcal septicaemia in three days, the blood yielding a smooth culture; the latter was killed after two days and rough colonies only were obtained from the local lesion.

In the above experiments it will be noted that the reversion from rough to smooth occurred in those mice which were inoculated subcutaneously with large amounts of culture. It seemed possible that this latter circumstance in affording a favourable nidus may have had more influence on the development of smooth characteristics than the transference from mouse to mouse. In order to test this view, four of the original rough cultures were inoculated subcutaneously into mice, each of which received the deposit of 50 c.c. Five survived and were healthy when killed ten days after inoculation; one died in three days and yielded a few rough colonies; only one died of septicaemia with numerous capsulated diplococci in the blood, from which a smooth culture of Type II was obtained. It may be mentioned that none of these cultures reverted when inoculated in a small dose, so it appears that a large dose favours the reversion, but does not produce it infallibly.

In the following experiment the passage was begun with two R strains of Type II which had been subcultivated for over a year and had been repeatedly both plated and tested on mice without producing any S colonies or showing any virulence for mice in moderate doses. Each culture was inoculated intraperitoneally into a mouse in a dose of 1 c.c. of broth culture. The mice were ill when killed the next day and plate cultures from the blood yielded sparse rough colonies. An R colony strain was made from each mouse (for convenience of description these have been designated A and B respectively); each was inoculated subcutaneously into three mice in doses of 1 c.c. of blood broth. All the mice survived for three days when they were killed apparently well and plate cultures were made from the seat of inoculation.

Result of inoculation of rough culture A.

Mouse 1 yielded a mixture of R and S colonies from the seat of inoculation. A subculture was made from each variety and tested on mice. The R strain failed to kill in a dose of 0·5 c.c. intraperitoneally; fatal septicaemia was produced by the S strain in a dose of 0·1 c.c. intraperitoneally and 0·5 c.c. subcutaneously, but not in smaller doses.

Mice 2 and 3 yielded R colonies only which were not virulent when inoculated into other mice.

Result of inoculation of rough culture B.

Mouse 1 gave only R colonies on the plate from the local lesion. A culture from one was inoculated subcutaneously in a dose of 1 c.c. into a mouse which died in two days from pneumococcal septicaemia; typical S colonies of Type II were grown from the blood.

Mouse 2 yielded a majority of R colonies and one S colony which agglutinated with Type II serum, an R colony was avirulent; the S colony killed mice in a subcutaneous dose of 0·1 c.c. but not in smaller doses.

Mouse 3 yielded R colonies which after two further passages in series through mice remained rough and avirulent.

This experiment shows that the ability of an attenuated R strain to revert to the virulent S form persists during prolonged periods of subculture in the R form.

heated Type I culture, and (3) with heated Type III culture; the results with the last only appear in the table.

The R strains were each tested subcutaneously on two mice in a dose of about 1 c.c. of blood broth culture; all the mice were well when killed in 9–16 days and no pneumococci were recovered from the seat of inoculation.

The virulent cultures were killed by heating for two hours at 60° C.; in the case of Types II and III, cultures heated for 3 hours were also used. As a test of sterility the Types II and III cultures (heated 2 hours) were injected into two mice. Each mouse received subcutaneously the deposit of 130 c.c. of glucose broth; both remained well and were killed 16 days later. Cultures from the seat of inoculation remained sterile; in one of the two mice the culture was made from an encapsulated abscess which had formed under the skin and still contained fairly well staining diplococci.

From the majority of the mice inoculated with the mixture of the above heated culture and the living R strains of Type I, virulent S colonies of Type II were obtained. In the case of the R colony 1 both mice were negative while only one of the pair inoculated with R colony 6 yielded S colonies of Type II.

It is interesting that of all the mice inoculated with the twice heated Type I culture together with the R strains only the one which received R colony 1 failed to develop fatal Type I septicaemia. This result certainly suggests that R strains may differ in their suitability for mutation in the same way as they differ for reversion experiments.

There is a similar indication in the experiments with Type III heated culture. Only one R strain, R colony 4, yielded the watery colonies of Type III and the positive results occurred with the suspension heated for two hours and three hours respectively.

It will be noticed that the heating for an additional hour has lowered the proportion of positive results with the killed virulent Type II culture. The experiments with heated Type II culture and attenuated R strains derived from Type I have not always been so successful as the above in producing an apparent change of type, as will be seen from the following example.

A suspension of virulent Type II was heated for one hour at 60° C. and again for a second hour and was injected into mice (doses = deposit of 90 c.c.) together with (1) R colony 3 culture of Type I, (2) R colony 2 culture of Type I.

Eight mice were used for each R strain and one mouse out of each series died of Type II septicaemia in 4 and 10 days respectively. Excepting one which died prematurely and one which died in 8 days (only R colonies at the seat of inoculation) the rest were killed 16 days after inoculation and no pneumococci of any form were obtained from the subcutaneous tissues.

In another experiment the Type II S culture was heated for three hours at 60° C. and was injected in doses equivalent to 60 c.c. of broth culture. Four mice were injected with the heated cultures alone and were killed in

10 to 14 days; plate cultures from the seat of inoculation yielded no pneumococci.

The attenuated R cultures of Type I employed in this experiment were different from those used previously. They were six different colony strains from a plate from the fourth successive culture of Type I in homologous immune serum. Three mice were inoculated with 0·25 c.c. of each (total of 18 mice) together with the above mentioned heated culture. Two inoculated with different R colonies were killed, ill in 7 and 10 days respectively, and S colonies of Type II were grown from the blood. The remaining 16 were killed when well after 10 to 14 days and cultures from the subcutaneous seat of inoculation were negative except in three instances where R colonies alone were grown.

These experiments may be summarised as follows:

The injection of virulent S culture of Type II killed by heat at 60° C. together with living R strains of Type I has resulted in the formation of a virulent S culture of Type II.

The transformation has taken place when the virulent culture has been heated at 60° C. for 2 and 3 hours respectively, but the positive results were less frequent in the case of the culture heated for the longer period. Different R strains appear to vary in their ability to develop into a new S form under the influence of the heated virulent culture.

Inoculation experiments with heated virulent culture of Types I and II together with living attenuated strains of Group IV.

The living R strain of Group IV in the experiments in Table XIV was derived from Type II A by growth in homologous immune serum. The latter

Table XIV.

Killed S pneumococci	Living R pneumococci	No. of mice	Result	Type of culture obtained from mice
Type I heated at 60° C. for 2 hours	R 1, Type II A. Dose =0·25 c.c. of blood broth culture	5	All died in 2–5 days	S culture of Type I from each
Type II heated as above	R 1, Type II A, as above	5	All died in 2 days	S culture of Type II from each
Type II A heated as above	R 1, Type II A, as above	4	All died in 3–5 days	S culture of Type II A from each
None	R 1, Type II A. Doses =0·5–1·0 c.c. of blood broth culture	3	All survived	—
Type I heated at 60° C. for 2 hours	R 1, Pn. 41. Dose =0·2 c.c. of blood broth culture	5	1 killed in 9 days 4 died in 4–8 days	S cultures of Pn. 41 from 4, nil from 1
Type II heated at 60° C. for 2 hours	R 1, Pn. 41, as above	5	2 killed in 9 days 3 died in 3–6 days	S cultures of Pn. 41 from 4; nil from 1
Pn. 41 heated as above	R 1, Pn. 41 as above	4	3 died 2–4 days 1 died prematurely	S cultures of Pn. 41
None	R 1, Pn. 41. Dose =0·5 c.c. to 1 c.c. of culture	3	1 killed in 9 days 2 died in 6–7 days	S culture of Pn. 41 from 2; nil from 1

was effective in producing attenuation, since none of five colonies selected reverted when inoculated subcutaneously in mice in doses of 10 c.c.; R colony 1 was chosen for this experiment and three control mice were inoculated.

The results show that the R strain of II A was readily transformed either into the S form of Type I or into the S form of Type II, and that reversion to its original S form occurred when it was inoculated with heated culture of that S form. The R strain of II A inoculated alone has not reverted, though larger doses than 10 c.c. have not been tried.

The second rough Group IV strain, Pn. 41, gives an interesting result and, in addition, provides a useful control for the heated cultures since, as will be seen, the heated Types I and II suspensions which were the same as those used with the rough Type II A never caused the appearance of an S strain either of Type I or Type II, thus showing that the heating had been effective in killing the S cultures.

There was no transformation of the R strain of Pn. 41 and this fact may perhaps be connected in some way with the insufficient attenuation of the strain which, as will be observed, reverts readily unaided. The R colony culture used was from one of four colonies which were picked off the plate sown from the culture in homologous serum. Evidently the serum was weak in protective substances, since two out of the four reverted on the preliminary testing. R colony 1 which failed to revert when first tested, also reverted too readily unaided when tested later.

Inoculation of living and dead R cultures.

The experiments in Table XV are negative with one exception where an R strain derived from Type II reverted to the S form of II when inoculated into a mouse together with heated rough Type I culture.

The experiments were repeated except that six mice were used in each series = total of 24 mice. All of the mice survived.

Table XV.

Killed R pneumococci	Living R pneumococci	No. of mice	Result	Type of culture obtained from the mouse
Rough Type I heated at 60° C. for 2 hours. Dose = deposit of 100 c.c. of broth culture	R 4, Type II. Dose = 0·25 c.c. of blood broth culture	3	Killed 16 days (2) Died 4 days (1)	None S colonies of Type II
As above	R 3, Type I. Dose = 0·25 c.c.	4	Killed 16 days	None
Rough Type II, as above	R 4, Type II. Dose = 0·25 c.c.	4	,, 16 ,,	,,
As above	R 3, Type I. Dose = 0·25 c.c.	3	,, 16 ,,	,,

The heated R culture, although the doses were very large, viz. the deposit of 170 c.c. of broth, exerted no effect on the living R strains either in the direction of reversion or transformation of type. This is consistent with

the view that in both cases the result depends on the presence of S antigen of which there are only traces in an R strain. The heating to which the R culture had been subjected would diminish the activity of the small amount still further or destroy it entirely.

III. Discussion.

The serological analysis of a bacterial species has an obvious practical application in bacteriological diagnosis as well as in the preparation of antibacterial therapeutic sera. There are, however, other issues, probably of greater importance, which have to do with the occurrence and remission of epidemics, the appearance of epidemic types in certain diseases and the attenuation of the infecting agent in others. It must have occurred to every serologist to ask himself the meaning of the types he has defined. Do serological types represent stages in the normal life history of a bacterium or are they the response on the part of the bacterium to changes in the immunological state of the animal host? If it is a question of altered environment, are the influences which initiated the divergence of type still at work, *i.e.* are the type characters still in a state of flux, or have the different varieties become stabilised?

On considering the above questions one cannot fail to realise that their solution would be a valuable contribution to the epidemiology of disease and would explain some of the phenomena in the rise and fall of epidemics. In certain bacterial infections, of which lobar pneumonia serves as an example, it is possible that even the most potent antisera will not avail to cut short the disease once the organisms are established in the tissues. Attention must, therefore, be directed to prevention of infection, and to this end a close study of bacterial virulence and its relation to variations in serological type is essential.

Virulence and type characters are closely related in the pneumococcus. When pneumococci are grown in homologous immune serum, some descendants become attenuated in virulence and these can be recognised by their formation on solid media of a distinctive variety of colony known as the R form of the pneumococcus. The virulent or S form of pneumococcus produces in fluid media, and still more abundantly in the peritoneal cavity of the mouse, a soluble substance which, though not itself antigenic, gives a copious precipitate with the appropriate antiserum. Each type of pneumococcus forms a special soluble substance which has no affinity for an antiserum prepared with any other pneumococcal type and it is to this property that the remarkably clear definition of the serological races of pneumococci is due. These substances have been shown[1] to consist chiefly of carbohydrate and, though highly reactive, to be non-antigenic. As a result of its change to the R form, the pneumococcus generally loses this power of producing soluble

[1] Heidelberger, M. and Avery, O. T. *J. Exp. Med.* **38**, 73 and **40**, 301.

substance, though individual strains differ in the degree of this loss. In addition to the exceptional R variety of a Group IV strain described pp. 116, 117, which produced a considerable amount and was virulent for mice, I have shown that quite attenuated R strains may form traces in the peritoneal cavity of the mouse. Cultures of R strains, however, rarely contain sufficient soluble substance to give a demonstrable precipitation with the appropriate antiserum, and, in consequence, the agglutination test no longer serves to identify a strain with the virulent type from which it was derived. Since virulence and the capacity to form soluble substances are attributes of the S strain, their possession may for convenience be ascribed to a special antigen which may be termed the S antigen. Thus an attenuated R strain which has no demonstrable S antigen has lost the serological characters of its type, but, if virulence is restored by passage through mice, the strain reverts to the S form of the type from which it was derived.

Some attenuated R strains revert readily to the virulent form and this feature is correlated with demonstrable traces of S antigen in their composition. Other strains have been found in which the R state is much more stable. In a series of peritoneal passage experiments beginning with one strain and carried on with its descendants reversion has occurred in one branch of the descent and not in another.

The acquirement of the typical characters of a virulent pneumococcus by an R strain from which the S antigen has been almost eliminated by growth in immune serum recalls some experiments by Bail on the anthrax bacillus[1]. By exposing a culture of anthrax bacilli to a temperature of 42° C. he obtained strains which were almost deprived of their power of producing capsules. Such a strain might produce a mixture of colonies some of which on subculture invariably failed to form capsules while others showed a small minority of capsule-forming bacilli. This result he ascribed to a deficient inheritance of the capsule-forming substance, so that an individual bacillus was able to endow only one of its descendants with a sufficient amount to produce a typical capsule-forming strain.

That there might be some principle underlying these infrequent and apparently haphazard positive results was suggested by the following observation. An attenuated R strain which regularly became virulent when inoculated intraperitoneally into a mouse failed to revert when the same dose was introduced into a vein. Apparently attenuated pneumococci require a protected situation in which to multiply and acquire virulence, and this they find occasionally when inoculated into the peritoneal cavity. If they are put directly into the blood stream in a dose which does not overwhelm the animal, it would appear that they do not find such suitable conditions and are readily disposed of.

This view has been confirmed by subsequent experiments and it has been found that a more certain method of ascertaining whether an R strain is capable of reverting is by the inoculation of a large dose of culture under the skin of a mouse. The mass of culture, I suppose, forms a nidus in which the

[1] *Centralbl. f. Bakt.* Orig. **79**, p. 425, 1917.

attenuated pneumococci are protected from the bactericidal action of the tissues. Since, however, attenuated pneumococci may remain alive and unaltered in the subcutaneous tissues for two or three weeks, local protection is clearly not the only factor in this method of restoring virulence. It seemed possible that the mass of R pneumococci, disintegrating under the action of the animal tissues, might furnish some substance which was utilisable by the survivors to build up their virulent structure. Acting on the assumption that this material might be the S antigenic substance, which in varying amounts persists in the R form, I inoculated into the mouse's subcutaneous tissues a very much smaller dose of living R pneumococci together with a mass of killed virulent culture. The result of this greater concentration of S antigen, or perhaps of some substance derived from it, was to make the conditions still more favourable and reversion of the R strain to the virulent form was secured with great regularity.

The observation that a sublethal dose of a bacterium may cause a fatal effect when inoculated together with the sterile products of that bacterium has long been known and forms the basis of the theory of aggressins. It was at first maintained that aggressins could only be obtained from the bacterium through contact with living animal tissues, *e.g.* from the peritoneal exudate of an animal inoculated with virulent culture, though it was finally conceded that they might be present to a slight degree in disintegrating cultures.

The action of the killed pneumococcus culture in enhancing the virulence of the R strain *in vivo* though not *in vitro* is certainly analogous to that of the hypothetical aggressin, and these results may throw fresh light on an obscure subject.

The principle of the action of aggressins was held to be their toxic influence on the leucocytes which were thus rendered incapable of attack. This is no doubt partly the function of the mass of killed virulent culture injected together with the attenuated R variety of pneumococcus, but there are other considerations which support the view already put forward that the attenuated organisms actually make use of the products of the dead culture for the synthesis of their S antigen.

An R strain is most readily transformed into the S variety when the killed culture used is of the same serological type as that from which the R strain was derived. For example, Type II S culture killed by steaming at 100° C. readily causes the R strain of Type II to revert in the mouse, whereas Type I S culture, similarly treated, does not, though it may when heated to 60° C. (*vide infra*).

An exception to the statement above is that certain Group IV strains are practically as effective as Type II in causing the R form of the latter type to revert to the S variety. The same Group IV strains, however, have no effect when injected together with an R strain of Type I. Apparently the antigens of these Group IV strains are more closely related to Type II than to Type I

and the results are further evidence of the specific selective action of different pneumococcal antigens in causing reversion.

The specific effect of the killed culture is at first sight less evident where the culture is heated to a lower temperature than 100° C. For instance, cultures of Type I heated at 60°, 70° or 75° C. frequently cause the R form derived from Type II to revert to the S form of Type II. How does this result affect the hypothesis suggested above that an attenuated R strain with deficient S substance requires the products of an S culture of the same type with which to rebuild its former type characters and virulence? One must consider first the effect of heat on the two types, I and II. Type II virulent culture, heated for so short a period as 15 minutes at 60° C., has so far never caused the R form derived from Type I to revert to the S form of Type I, although steamed cultures of Type II are effective in inducing reversion of its own R form to the corresponding S form. On the other hand, Type I while effective after heating at temperatures of 60°–75° C. in producing the R to S change with its own type loses this property when heated at 80° C. or higher.

These observations suggest that the specific S substance of Type I suffers more by exposure to heat, that is to say, a greater proportion of it is destroyed, than that of Type II.

By S substance I mean that specific protein structure of the virulent pneumococcus which enables it to manufacture a specific soluble carbohydrate. This protein seems to be necessary as material which enables the R form to build up the specific protein structure of the S form. But it appears that this material may be modified by heat in such a way that the R form cannot utilise it for the reconstruction of its own internal structure. (The specific carbohydrate which is the product of the S form is unaffected by heat.)

In order to reconcile the experimental data referred to above with the hypothesis that the R pneumococcus which reverts in the mouse to the S form has synthesised its S antigen from similar material in the heated virulent culture injected at the same time, it is necessary to assume that a virulent Type I pneumococcus contains some S antigen of Type II. An alternative hypothesis would be that the R form of Type II is able to reconstruct its virulent S form from either the S substance of Type I or that of Type II. One is then faced with the difficulty of accounting for the failure of an R form of Type I to build up its S form from Type II S substance.

The amount of S antigen of Type II in Type I must obviously be small in proportion to the Type I, since the serological tests give no indication of its presence, and it is legitimate to suppose that heating to a temperature which would not greatly diminish the total amount of Type II S antigen in a Type II pneumococcus might conceivably destroy it entirely in a Type I. This is supported by the experiments which show that after heating to 80° C. the capacity to induce reversion of the R form of Type II is lost by the Type I culture and retained by the Type II culture.

The relationship between Types I and II pneumococci just suggested, in which the major antigen of one type is represented as a subsidiary antigen in the other is not without parallel in other bacterial groups, *e.g.* meningococci. There are special circumstances, related no doubt to the formation of soluble substance, which in the ordinary serological test keep this relationship in the background.

If there is a reciprocal relationship between Types I and II, as one would expect, what is the explanation of the failure of the reversion experiments to show evidence of the presence of Type I antigen in a Type II pneumococcus? It is, I think, a question of the difference in heat resistance between the two antigens. As Type I antigen is the more heat sensitive, the small amount assumed to be present as a subsidiary antigen in a Type II pneumococcus might be destroyed by a temperature which would not affect the Type II antigen in a Type I pneumococcus.

These considerations, I think, afford a reasonable explanation of the experimental data in connection with the restoration of virulence to an attenuated pneumococcus. The chief points are:—(1) in the change from the S to the R form some of the S antigen may persist; the amount, rarely demonstrable by *in vitro* tests, varies in different R strains; (2) the S antigen remaining in an R strain may be regenerated and reach its original abundance under suitable conditions, *e.g.* inoculation subcutaneously into a mouse in large doses or in small doses plus a mass of heated culture containing the particular S antigen; (3) an S strain of one type (I or II) may contain in addition to its major antigen a remnant of the other type antigen.

Application of the principles underlying these observations to the question of transformation of one type into another has given results of considerable interest.

When pneumococci of Types I and II are reduced to their respective R forms by growth in homologous immune sera, they lose nearly all their major S antigen though they may retain their minor S antigens which are presumably not affected by the heterologous immune substances. But the major S antigen apparently still preponderates, since an R strain on reversion to the S form regains its original type characters. Some R strains, however, do not revert even when inoculated in large amounts under the skin of a mouse, and it is not unlikely that in such strains the major antigen has been reduced to the same insignificant amount as the minor antigen.

In such circumstances an R strain derived from Type I would be identical with an R strain from Type II, and under suitable conditions the development from it of a virulent form of either type might be anticipated.

This has been shown actually to occur; a virulent Type I pneumococcus can be derived through the intermediary R form from a virulent Type II pneumococcus, and *vice versa*.

Up to the present I have maintained a distinction between the R forms derived from Types I and II respectively, though, as I have stated earlier,

they can be identified only if they revert unaided to the S type from which they originated.

When the R form of either type is furnished under suitable experimental conditions with a mass of the S form of the other type, it appears to utilise that antigen as a pabulum from which to build up a similar antigen and thus to develop into an S strain of that type. Therefore the R form of Type II, when inoculated together with a heated suspension of Type I, uses the antigen of the latter strain and an S pneumococcus of Type I makes its appearance. There is a further complication since, as it appears, the heated Type I suspension contains a subsidiary S antigen of Type II, and some of the R pneumococci may use this to develop an S strain of Type II. As a result one mouse may yield the S form of Type I and another the S form of Type II, while quite frequently the same mouse may yield both types.

Similarly, if the R form of Type I is inoculated together with the heated S culture of Type II, a virulent S form of Type II is developed. (The S culture of Type II never causes the R form of Type I to change into the corresponding S form, because, as I have already explained, the subsidiary Type I antigen is destroyed by the heating.)

These observations suggest that there is no essential distinction between the two R varieties. In fact, there are certain indications that the R pneumococcus in its ultimate form is the same, no matter from what type it is derived; it possesses both Types I and II antigens in a rudimentary form or, as it may be differently expressed, it is able to develop either S form according to the material available.

If Type III substance is offered as a pabulum, either form is able to build up a typical S strain of Type III, though it appears that the R form derived from Type II is more readily converted into Type III than is the R form of Type I. Why there should be this difference is not clear, though it may be assumed to have some association with the fact that this particular R strain from Type II has not reached the same stage of attenuation as the R strain of Type I. (The latter in the relatively few tests made has not reverted, as does the R Type II, to the S form when inoculated unaided, *i.e.* alone in large doses.) There is also a further point of distinction in that injection of heated Group IV cultures has caused the R form of Type II to revert but not the R form of Type I. The S substance of the Group IV strains is evidently closely related to that of Type II since it provides a suitable pabulum for the regeneration of the virulent S strain. In this connection I may recall that Group IV strains appear in the sputum during convalescence from Type I pneumonia, and it is suggested that they are formed from the Type I after suppression of the major antigen through the action of the immune substances and by the development of the subsidiary Type II antigen, though not to its full complexity.

It may be that the minor antigen in a Type I is not actually a fully developed Type II S antigen in small amount but a less differentiated substance

which serves indifferently as a foundation for the building up of either Type II or a Group IV strain.

The R form derived from a Group IV strain, viz. Type II A, can be transformed into the S forms of Type I or Type II or changed back to its original S form according to the particular S substance which is injected along with it. On the other hand, another Group IV strain which was incompletely attenuated invariably reverted to its original S form no matter what type of S culture was injected with it. It seems that, if a pneumococcus has a moderately well-developed S structure, there is no tendency to develop into an S variety of any other type.

The method by which transformation of type has been secured consists in heating to 60° C. for 15 minutes up to 3 hours a virulent culture of one type and inoculating a large amount of the heated culture under the skin of a mouse together with a small dose of the R strain derived from another type.

Experiments with culture heated at temperatures higher than 60° C. have rarely been successful in causing transformation of type. In one instance the S form of Type I was obtained from a mouse which had been inoculated with the R form derived from Type II together with a suspension of Type I heated to 70° C. for 15 minutes, in a second the S form of Type II was obtained from a mouse inoculated with the R form of Type I together with a virulent Type II culture heated to 65° C. for 15 minutes.

The question arises whether heating at the above temperatures had in fact killed all the individual pneumococci in the mass of virulent culture or whether the apparent change of type was due to the occurrence of a survivor. I have given this question careful consideration and I have never been able by the ordinary methods of culture and animal inoculation to demonstrate the presence of viable organisms in the heated cultures. Since there is no reason to suppose, and I have had no evidence to show, that the R strains used were mixed, there seems to be no alternative to the hypothesis of transformation of type.

A few years ago the statement that a Type I strain could be changed into a Type II or a Type III would have been received with greater scepticism than at the present day. Since, however, it has been shown that a pneumococcus can readily be deprived of its type characters and virulence, and that under favourable conditions these can be restored, the possibility appears less unlikely.

The apparent transformation is not an abrupt change of one type into another, but a process of evolution through an intermediate stage, the R form, from which the type characters have been obliterated. Mutation of type among disease-producing bacteria is a subject of obvious importance in the study of epidemiological problems. If it can be proved to occur in the pneumococcus group with its sharply defined immunological races, the possibility can hardly be denied to other bacterial groups where the serological

types cannot be differentiated without the help of agglutinin-absorption experiments.

The position in regard to the causal relation of different types of pneumococci to lobar pneumonia presents certain difficulties. Types I and II pneumococci, which cause 60 to 70 per cent. of the total cases of lobar pneumonia, are rarely found in the normal nasopharynx except in close contacts of the disease. Whilst this latter observation indicates some power of epidemic spread, it is not often demonstrable that a case of pneumonia acts as a focus for fresh cases. On the other hand, Group IV pneumococci are of common occurrence in the nasopharynx and about 25 to 30 per cent. of pneumonia cases are attributable to various types in this group; these cases are generally considered to be of autogenous origin.

It is a very remarkable fact that the incidence of the chief types of pneumococci in lobar pneumonia is almost identical in countries where the climatic and social conditions are similar. While this occurrence is not easily explained on the supposition that the disease is partly infectious and partly autogenous, it is not inconsistent with the evolution in the individual of special types most suited to set up pneumonia, *i.e.* the similar distribution is due to similar composition of the population as regards susceptibility to the pneumococcus and not to similarity in the diffusion of pneumococcal types.

In convalescence from pneumonia Types I and II tend to disappear from the respiratory tract and are replaced by the common Group IV strains. According to the more generally accepted view, the chief types die out and the Group IV pneumococci, the normal inhabitants of the nasopharynx, again come into prominence. An alternative hypothesis which was purely speculative in the absence of evidence of the instability of pneumococcal types is that the chief types revert to the Group IV varieties from which they were derived during the development of the disease in the individual.

On the lines of my previous argument as to the process at work in the development of a virulent S strain from an attenuated R pneumococcus, it may be surmised that the immune substances formed during recovery suppress the S antigens of the chief type and under suitable conditions the subsidiary antigens are developed to form a new virulent type—in this case one of the varieties of Group IV. As mentioned earlier, I have shown that the sputum of a case of pneumonia due to Type I almost invariably contains, in addition, one or more virulent strains of Group IV, and as many as four distinct types have been isolated from a single case.

This latter instance certainly suggests that the Group IV strains are variants of the Type I, and it is of some significance in this connection that the antigens of the Group IV strains have been shown to be related to that of Type II which is represented as a subsidiary antigen in a Type I pneumococcus. It would appear that the Type I antigen no longer serves its purpose in the presence of the immune substances formed during convalescence, and

the pneumococcus consequently develops its Type II side. I have not so far been able to find a Group IV strain in a case of pneumonia due to Type II (in one instance a Type III strain was found in association with the Type II), but my observations have not been sufficiently numerous to justify a conclusion on this point.

While these suggestions of a regular sequence of changes in the type of pneumococcus before the development of pneumonia and during recovery are necessarily tentative in character, they are in harmony with the experimental data and amplify this line of thought.

The formation of a Group IV strain from a Type I might be considered as an adaptation on the part of the Type I pneumococcus to the altered conditions consequent on the development of immune bodies. These make it difficult for the Type I to survive as such (*in vitro* the R form is the response), and in assuming Group IV characters it makes some sacrifice of its antigenic complexity and, with that, of infectivity in exchange for a greater degree of resisting power to the animal tissues. Type III exhibits a still greater instance of change in that direction, since it is very slightly invasive but fatal in its effects once it is established in the body. It is more difficult to produce protective sera in rabbits with Group IV and Type III strains than with Type I, while Type II occupies an intermediate position.

In the interaction between the animal tissues and the bacterium one is apt to consider the bacterium as playing a purely passive part and to overlook the possibility that the various forms and types may be assumed by it to meet alterations in its environment.

What, for instance, is the meaning of the change to the R form? Most writers have regarded it as a degenerative change due to unfavourable conditions for growth. While this is true in a sense, since in the R form the bacterium lacks certain important attributes characteristic of the S form, there is some evidence of its being rather a vital adaptation, as P. Hadley[1] has suggested.

In the case of the pneumococcus the change to the R form is brought about most rapidly in immune serum which, nevertheless, provides an excellent medium for the growth of both the R and the S form. The effect of the serum is due to the specific immune substances, and, as a result, the pneumococcus becomes susceptible to phagocytic action. If, as is probable, the immune bodies exercise a similar influence *in vivo* during successful resistance, the animal has achieved its end in rendering the invader harmless. By assuming the R form the pneumococcus has admitted defeat, but has made such efforts as are possible to retain the potentiality to develop afresh into a virulent organism. The immune substances do not apparently continue to act on the pneumococcus after it has reached the R stage, and it is thus able to preserve remnants of its important S antigens and with them the capacity to revert to the virulent form.

[1] *The Journal of Infectious Diseases*, 1927, **40**, pp. 1–312.

While the R form may be the final stage in the struggle of the bacterium to preserve its individuality, I look upon the occurrence of the various serological races as evidence of similar efforts to contend against adverse circumstances. These are more successful in that the S form is retained and, in addition, increased powers of resistance are acquired but at the sacrifice of invasive properties.

The experiments on enhancement of virulence and transformation of type suggest an explanation of the manner in which a pneumococcus residing as an apparently harmless saprophyte in the nasopharynx acquires disease-producing powers. So long as it retains certain potentialities, indicated by the possession of traces of S antigen, the most attenuated pneumococcus may develop the full equipment of virulence. The first essential is a situation in which it can multiply, unchecked by the inhibitory action of a healthy mucous membrane. In the nidus thus formed the pneumococcus gradually builds up from material furnished by its disintegrating companions an antigenic structure with invasive properties sufficient to cope with the resistance of its host.

When recovery from pneumonia takes place, the formation of immune substances initiates the retrogressive changes in antigen structure resulting in the production of the Group IV and Type III pneumococci which probably have increased resisting powers but diminished capacity for invasion.

These considerations which relate to an individual case of pneumonia are capable of application to an outbreak of epidemic disease in a community. Thus the consequences which ensue on the decline of an epidemic are not only an increase in the number of insusceptible individuals but also an alteration in the character of the infective organism.

IV. SUMMARY.

1. In the course of the examination of sputum from cases of lobar pneumonia, observations have been made on the incidence of the chief types of pneumococci. In the district from which the material was obtained, there was an apparent local diminution in the number of cases of lobar pneumonia due to Type II; the figures were 32·6 per cent. of Type II cases in the period 1920–22, and only 7·4 per cent. in the period 1924–27. The incidence of Type I was approximately the same in the two periods, the percentages being 30·6 and 34·3.

2. Several different serological varieties of pneumococci have been obtained from the sputum of each of several cases of pneumonia examined at various stages of the disease. This has occurred most frequently in cases of pneumonia due to Type I, and in two instances four different types of Group IV were found in addition to the chief types. The recovery of different types is facilitated by the inoculation of the sputum (preserved in the refrigerator), together with protective sera corresponding to the various types in the order of their appearance.

3. Two interesting strains of Group IV pneumococci have been obtained from pneumonic sputum.

One was an R strain which produced typical rough colonies, yet preserved its virulence for mice and its capacity to form soluble substance. This R pneumococcus developed a large capsule in the mice, which died of a chronic type of septicaemia. A strain producing smooth colonies was obtained from it in the course of a prolonged series of passage experiments.

The second strain, which was proved not to be a mixture, agglutinated specifically with the sera of two different types. In the peritoneal cavity of the mouse the specific soluble substance of each type was produced.

4. A method of producing the S to R change through ageing of colonies on chocolate blood medium containing horse serum is described. After two to three days' incubation small rough patches appear in the margins of the smooth colonies, and from these pure R strains can be isolated.

5. It has been shown that the R change is not equally advanced in the descendants of virulent pneumococci which have been exposed to the action of homologous immune serum. Some R strains form traces of soluble substance in the peritoneal cavity of the mouse; these revert readily to the virulent S form and, in addition, are able to produce active immunity. Others show no evidence of S antigen; spontaneous reversion takes place with difficulty, if at all, and they are incapable of producing active immunity. The stronger the immune serum used, the more permanent and complete is the change to the R form.

6. Restoration of virulence to an attenuated R strain, with recovery of the S form of colony and of the original serological type characters may be obtained by passage through mice. The change from the R to the S form is favoured by the inoculation of the R culture in large doses into the subcutaneous tissues; but the most certain method of procuring reversion is by the inoculation of the R culture, subcutaneously into a mouse, together with a large dose of virulent culture of the same type killed by heat.

Incubation of such a mixture *in vitro* does not induce reversion.

7. Reversion of an R strain to its S form may occasionally be brought about by the simultaneous inoculation of virulent culture of another type, especially when this has been heated for only a short period to 60° C., *e.g.* R Type II to its S form when inoculated with heated Type I culture.

8. Type I antigen appears to be more sensitive to exposure to heat than Type II antigen, since the former loses the power to cause reversion when heated to 80° C., whereas Type II culture remains effective even after steaming at 100° C.

9. The antigens of certain Group IV strains appear to be closely related to that of Type II, and are equally resistant to heat. Steamed cultures of these Group IV strains cause the R form derived from Type II to revert to its S form, while they fail to produce reversion of the R form derived from Type I.

10. The inoculation into the subcutaneous tissues of mice of an attenuated R strain derived from one type, together with a large dose of virulent culture of another type killed by heating to 60° C., has resulted in the formation of a virulent S pneumococcus of the same type as that of the heated culture.

The newly formed S strain may remain localised at the seat of inoculation, or it may disseminate and cause fatal septicaemia.

The S form of Type I has been produced from the R form of Type II, and the R form of Type I has been transformed into the S form of Type II.

The clear mucinous colonies of Type III have been derived both from the R form of Type I and from the R form of Type II, though they appear to be produced more readily from the latter. The newly formed strains of Type III have been of relatively low virulence, and have frequently remained localised at the subcutaneous seat of inoculation.

Virulent strains of Types I and II have been obtained from an R strain of Group IV.

11. Heated R cultures injected in large doses, together with small doses of living R culture have never caused transformation of type, and only rarely produced a reversion of the R form of Type II to its virulent S form.

12. The results of the experiments on enhancement of virulence and on transformation of type are discussed and their significance in regard to questions of epidemiology is indicated.

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The discovery of pneumococcal type transformation: an appreciation

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The remarkable paper by Fred. Griffith on the significance of pneumococcal types, reproduced in the preceding pages of this *Journal*, in which it was first published 38 years ago (Griffith, 1928), describes a series of careful and painstaking experiments which show beyond doubt that the ability to produce a polysaccharide capsule can regularly be restored to 'rough' (R) strains of pneumococci which have lost it, by the subcutaneous inoculation of mice with a mixture of a small number of the living R bacteria and an excess of heat-killed, capsulated (S) virulent bacteria. The inoculated mice frequently died from septicaemia and virulent, capsulated S organisms could be isolated from the blood. On the other hand, in control experiments, the injection of suspensions of heat-killed S bacteria alone never yielded living organisms, while recovery of virulent S organisms by (mutational) reversion, following inoculation of living R bacteria alone, was a rare event. Griffith called this phenomenon 'transformation' and, at least in the field of bacterial genetics, this name is still specifically used to describe it.

Griffith found that transformation occurred most frequently when the R strain to be transformed originated from the same capsular type as the transforming bacteria, the R bacteria being merely restored to their original capsular type. However, the main interest of the phenomenon, both then and subsequently, centred on the discovery that R pneumococci originating from one type (say, type II) could be permanently transmuted into another type (say, type I or type III) corresponding to that of the heat-killed bacteria with which it was inoculated.

Griffith's abiding interest, and his life's work, was the epidemiology of infectious disease and he believed that the proper understanding of epidemiological problems lay in more detailed and discriminating knowledge of infectious bacterial species and of the nature of bacterial variation. For him, therefore, as for nearly all medical bacteriologists of his time and for many years thereafter, the importance of transformation of pneumococcal types rested on the light it might throw on such problems as the evolution of bacterial virulence, the rise and fall of epidemics, and variations in the incidence of type infections. By any yardstick, the demonstration that such dramatic and specifically directed transmutations of both type and virulence might occur with considerable frequency, in epidemiologically well-defined types of bacteria, was a startling enough revelation.

Griffith concentrated on providing convincing evidence that the phenomenon of transformation was a fact although, as he says: 'A few years ago the statement

that a type I strain could be changed into a type II or a type III would have been received with greater scepticism than at the present day' (Griffith, 1928), largely because of his own prior studies of the mutational loss and recovery of virulence and type character. Nevertheless, he appears to have hesitated for some time before publishing his results (see Obituary, 1941). He did attempt, and failed, to demonstrate *in vitro* transformation as well as the transforming activity of cell-free extracts, later established by Dawson & Sia (1931) and Alloway (1933) respectively, but it must be admitted that he does not seem to have tried very hard; nor, so far as I can find, did he publish any further papers on the subject.

Despite his preoccupation with the epidemiological aspects of his discovery, it is strange that Griffith does not draw attention to, much less stress, what now seems to us the most striking feature of transformation, namely, that it results in an *hereditary* alteration of character wherein, of course, lies its real biological significance. The nearest he got to analysis of the phenomenon in depth was the postulation, based on the relative thermolability of the capacity of certain (type I) heated suspensions to transform, that it might be mediated by a 'specific protein structure of the virulent pneumococcus which enables it to manufacture a specific soluble carbohydrate' (Griffith, 1928), rather than by the polysaccharide itself. It is most probable that this hypothesis is simply a descriptive one, and was not intended to carry any enzymic or genetical overtones. Griffith can hardly be criticized for failing to carry his analysis further for, apart from his over-riding interest in the epidemiological aspects of transformation, the climate of knowledge of his day offered few clues which could have served him as a basis for further work or even speculation.

I began by describing Griffith's paper as remarkable, and have already given some reasons why it should have been so regarded by his contemporaries. What Griffith did not know and, sadly, did not live to see, was that his work was the fuse of a time bomb whose explosion 16 years later ushered in the greatest revolution in biological knowledge of the twentieth century. Following the demonstration by Alloway (1933) that the transforming principle was present in cell-free extracts of donor strains of pneumococci, O. T. Avery and his colleagues began a systematic analysis of its chemical nature, culminating in a convincing mass of evidence that it is composed of pure, highly polymerized deoxyribonucleic acid (DNA) (Avery, MacLeod & McCarty, 1944). Apart from the chemical evidence, it was shown that the activity of transforming preparations remains unaffected by treatment with proteolytic enzymes and ribonuclease (RN-ase), but is rapidly and completely destroyed by the enzyme deoxyribonuclease (DN-ase) (McCarty & Avery, 1946). Subsequent purification studies virtually excluded the possibility that the active agent in transforming preparations could be any substance other than DNA (Hotchkiss, 1952). Finally, in more recent years, it has been shown not only that radioactive phosphorus incorporated into transforming DNA is transferred irreversibly to the DNA of the transformed bacteria, but also that the amount of transfer is proportional to the number of transformants (Goodgal & Herriot, 1957; Lerman & Tolmach, 1957).

In 1944, when transforming principle and DNA were first equated, the chemical

nature of genetic material was still a mystery. It was known, of course, that both DNA and protein were intimately associated with the chromosomes, but only protein had been shown to display specificity and was thought to possess enough complexity of structure to carry the vast number of specifications which are the inheritance of even the simplest of creatures. Accordingly there was widespread reluctance to draw general conclusions from the facts about transformation. Progress developed along two lines. First, it soon became apparent that transformation is far from being a very special phenomenon restricted to capsule formation in pneumococci, but occurs in many bacterial genera and species, and involves as many characters as can be recognized and selected for. It should also be remembered that the occurrence of transformation may be difficult to observe. It depends on the ability of the recipient bacteria to take up the transforming DNA molecules; the state of competence, during which they can do this, may exist for only a small fraction of the growth cycle, or be dependent on subtle environmental factors, while the conditions for its expression may differ widely between different species. Nevertheless, apart from *Diplococcus pneumoniae*, the occurrence of transformation has now been reliably reported in various species of *Agrobacterium*, *Bacillus*, *Escherichia* (one strain only), *Haemophilus*, *Neisseria*, *Rhizobium*, *Staphylococcus*, *Streptococcus* and *Xanthomonas*, while a wide variety of characters, including morphological features such as filament, capsule and spore formation, the production of specific antigens, resistance to a considerable range of antibacterial agents and synthesis of a large number of specific enzymes mediating steps in amino acid synthesis and in the fermentation of sugars, have been shown to be transformable (see Ravin, 1961).

The second line of development was the increasing evidence that transformation behaves as if it were due to the transfer of fragments of genetic material (chromosome) from donor to recipient bacterium, where it pairs with the homologous region of the recipient chromosome; part of the immigrant fragment then replaces the corresponding region of resident chromosome by a more or less random process of genetic exchange or 'crossing-over' (Ephrussi-Taylor, 1951). The evidence for this is briefly as follows:

(1) Transformation can occur in both directions; thus, for example, not only can R strains of pneumococci be transformed to S, but S pneumococci yield R transformants if treated with DNA from an R donor strain.

(2) The transformed character is not simply added to the phenotype of the recipient bacteria, but replaces its corresponding, or allelic, character.

(3) Wild-type recombinant bacteria can be obtained by transformation between two parental strains which are defective in the same character. A probable instance of this was first described by Griffith (1928: table XV) who obtained smooth type II pneumococci by inoculating mice with a mixture of living R bacteria derived from type II and heat-killed R bacteria derived from type I (see below).

(4) With regard to certain pairs of characters, joint transformation is possible. In pneumococci, for example, the characters of streptomycin-resistance (or sensitivity) and mannitol fermentation (or non-fermentation) are inherited together in transformation with a very much higher probability than can be accounted for by

chance, and irrespective of which combination of alleles (*str-s.mtl*$^+$ and *str-r.mtl*$^-$, or *str-s.mtl*$^-$ and *str-r.mtl*$^+$) characterizes the donor and recipient strains (Hotchkiss & Marmur, 1954; for other examples see Ravin (1961). This means that the determinants of such pairs of transformable characters must be arranged in a fixed relationship to one another, and sufficiently closely as to be often transferred together on the same molecule of DNA. This type of genetic 'linkage' is also a feature of transformation in *Haemophilus* and *B. subtilis* although quite different kinds of characters are involved, and is irrefutable evidence that the DNA involved in transformation constitutes fragments of the genetic material itself.

It is interesting to look, in retrospect, at Griffith's original transformation results and to interpret them in the light of genetical analysis and of what we now know of the biochemistry of pneumococcal polysaccharide synthesis (Jackson, 1962; Mills & Smith, 1962). In general it turns out that capsular synthesis involves a pathway mediated by a number of different enzymes and, therefore, of different genes, and that some at least of these genes are not only transferred on the same DNA fragment but are also very closely linked. Moreover, part of the pathway is common to different pneumococcal types so that genetic blocks there, leading to failure of polysaccharide synthesis, can be repaired by transforming DNA from the other types. In contrast, genes determining enzymes responsible for the specificity of type polysaccharides are usually mutually exclusive alternatives, or alleles, and so cannot coexist or participate in mutual repair, but can only substitute for one another.

In Fig. 1, the various categories of transformation discovered by Griffith are expressed in terms of genetic exchanges. Two genetic regions are shown, of which *A* determines that part of the synthetic pathway common to the synthesis of types I and II polysaccharide, while *B* is a region (or gene) conferring type specificity. In transformation, as indeed in all types of bacterial sexuality, because the donor genetic contribution is fragmentary, at least two (and in any case an even number) of genetic exchanges are required to yield a complete recombinant chromosome. As Fig. 1 shows, the production of a particular transformant type depends on whether the mutation in the recipient is in the *A* or *B* region as well as on the positions of the genetic exchanges in relation to these regions.

In the case of one of Griffith's strains (R 4, type II; tables VII–XII) we can be sure that the mutation leading to loss of capacity to produce type II polysaccharide involved region *A*, mediating that part of the pathway common to types I, II and III polysaccharide. The transformation of this strain to type II capsulation by heterologous types indicates that its *B* region, determining type II specificity, must have remained intact, while the concomitant production of types I and III transformants by killed, capsulated type I (table XI) and type III (table XII) donors respectively, reveals the interchangeability and, therefore, the similarity of the *A* regions of the three types. Continuing this line of reasoning, let us examine the last result shown in Fig. 1 where a single mouse, receiving a mixture of heat-killed, non-capsulated type I bacteria (R I) and living, non-capsulated type II (R II) recipients, yielded capsulated type II organisms. Let us also assume, as is likely, that this single result was due to transformation. In this case the original

loss of type II capsulation must have been due to mutation in the *A* region since type II capsule was restored, but, as Fig. 1 shows, we cannot say whether the *A* or *B* region of the R I donor strain was defective. Nor do we get much help from Griffith's paper since the particular R I mutant used is not specified and might have been any one of a number of independent R I isolates (see table XIII). If we assume that both of the non-capsulated strains under discussion were defective in the *A* region, then the production of capsulated progeny probably resulted from recombination between mutational sites in the same or two very closely linked

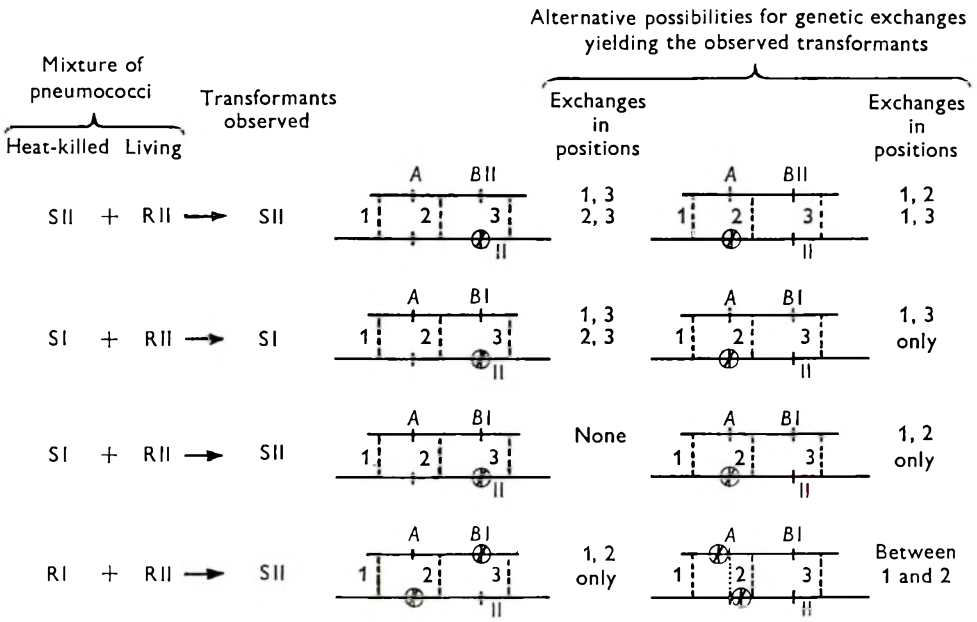

Fig. 1. An interpretation of some of Griffith's (1928) transformations of pneumococci in genetical terms. The pneumococcal strains used in each experiment, and the types of the resulting transformants, are shown on the left; S I and S II indicate capsulated strains of types I and II respectively, while R I and R II are non-capsulated, rough variants (mutants) derived respectively from types I and II pneumococci. The diagrams on the right half of the figure show, for each experiment, the positions of genetic exchanges which could yield the observed transformants. The lower and longer of each pair of lines represents the chromosome region of recipient bacteria which determines capsular polysaccharide synthesis; the upper and shorter line represents the corresponding fragment of donor chromosome (DNA). The chromosomal regions marked *A* are concerned with that part of the biosynthetic pathway common to types I and II polysaccharide; the regions marked *B* determine the type specificity of the polysaccharide, indicated by the suffix I or II. The alternatives shown for each experiment depend on whether the mutation resulting in loss of capsulation involved a gene in the *A* or the *B* region. The site of mutation is indicated by the symbol —⊗—. The interrupted, vertical lines represent the positions of genetic exchanges, of which two are necessary to produce a viable transformant. The type of transformant resulting from these exchanges depends on the particular part of the donor fragment which they incorporate into the recipient chromosome. This can be found for any pair of exchanges by tracing along the recipient (lower) chromosome from the left, then up to the donor fragment at the first exchange point and, finally, down to the recipient chromosome at the second exchange point. Further explanation in text.

genes. Thus, in addition to being the first to perform and record the results of genetic crosses in bacteria, Griffith may also, however unwittingly, have made the first analysis in any living creature of what is now called the genetic fine structure. The order of scale involved is set by the fact that all recombination events leading to transformation of any kind occur within the dimensions of transforming fragments of DNA, which usually have a mean molecular weight of about 10^7 and are long enough to carry some twenty genes; this is about one-hundredth the length of the whole bacterial chromosome and corresponds to about one-hundred-thousandth the total chromosomal DNA of a mouse cell.

The new certainty arising from chemical and genetical research into the nature of transformation, that the genetic material, at least of bacteria, consists of DNA, stimulated increasing study of the chemical and physical structure of this nucleic acid. Pre-eminent among these studies were the X-ray diffraction analyses of M. F. H. Wilkins and his colleagues (Wilkins, Stokes & Wilson, 1953; Franklin & Gosling, 1953). Finally, precisely a quarter century after the publication of Griffith's paper, the culminating step was taken by Watson & Crick (1953*a*), who, by a brilliant synthesis, fitted together the chemical and diffraction data into a symmetrical, double-helical structure which not only conformed to all the known facts but also possessed all the inherent properties one would expect of genetic material. Once the model of DNA was constructed, it immediately became obvious how the genetic material replicates, how it carries genetic information, and why it suffers heritable mutation (Watson & Crick, 1953*b*). This model, of an elegant and beautiful simplicity, has stood the test of time and there is now no doubt that the genetic material of all cells, whether animal, plant or bacterial, consists fundamentally of DNA. Only in the genetic material of some viruses is DNA substituted by its close but single-stranded analogue, ribonucleic acid (RNA).

The effect which this discovery has had on our basic biological conceptions has been so rapid and profound as to constitute a revolution. However this is another story, which although perhaps derivative from Griffith and his work is no longer of direct relevance to it. On the other hand, it would be relevant, and might be of interest to some, to conclude this retrospective appreciation with a brief summary of a few of the ways in which transformation itself has proved of value to medical and biological research.

Speaking broadly, transformation may play two distinct types of role in biological research. First, it can be used more or less directly in the analysis of bacterial behaviour and, indeed, there are some genera and species in which it is the only method of genetic analysis. I will give two examples of the kind of information it can provide. First, when DNA from strains of penicillin-resistant pneumococci is used to transform sensitive strains to resistance, the transformants show only a fraction of the degree of resistance of the donor strain. However, the low-level resistance of the transformants can be further increased in a stepwise manner by subsequent, successive exposures to the same DNA preparation until the donor level of resistance is reached (Hotchkiss, 1951). This pattern of acquisition of resistance has been shown to be characteristic of the majority of antibiotics. It confirms what had previously been inferred about penicillin from mutation studies

(Demerec, 1945), that high resistance results from the summation of a series of independent mutations, usually in unlinked genes; in transformation these genes are carried on separate DNA molecules so that normally only one is taken up at a time by any particular recipient bacterium. In contrast, transformation to high-level resistance to streptomycin, due to a single mutation, is achieved in one step.

Transformation has recently been put to rather different use in demonstrating that replication of the chromosomal DNA of *B. subtilis* begins at a particular point on the chromosome and is polarized, that is, proceeds in the same direction in all the bacteria of a culture (Sueoka & Yoshikawa, 1963; Oishi, Yoshikawa & Sueoka, 1964). Stationary phase bacteria or spores, both strands of whose DNA have been made denser than normal by growth in a medium containing heavy nitrogen (^{15}N) and heavy water (D_2O), are seeded into light medium containing ^{14}N and H_2O. At intervals during the first division cycle thereafter, samples of the bacteria are removed and their DNA extracted. The newly synthesized DNA, having one old, heavy and one new, light strand and, therefore, of intermediate density, is then separated from the initial heavy DNA molecules in a density gradient, and tested for its ability to transform recipient bacteria with respect to a range of genes known to be distributed along the chromosome. The genes are found to appear in the newly synthesized, transforming DNA in a strict and reproducible sequence as replication of the chromosome proceeds; only at the end of the cycle can the preparation of newly synthesized DNA transform with respect to all the genes.

The second role of transformation in biological research rests on the fact that it is the only way in which the effects of defined physical or chemical alterations, or of radiations, on the biological activity of DNA can be measured. For example, the phenomenon of photoreactivation has been found to be due to an enzyme which, *in vitro* and in the presence of visible light, can restore transforming activity to DNA which has previously been inactivated by ultraviolet light (Rupert, 1961). There is no doubt that when biologically active DNA is finally synthesized in the test tube, the criterion of its activity will be its transforming ability.

In conclusion we may note the discovery of another kind of transformation, provoked by the prior knowledge that large molecules of nucleic acid can penetrate semi-permeable cell membranes. This is the ability of purified, viral nucleic acid to infect cells and promote the synthesis of both new viral nucleic acid and protein, and the liberation of complete, infective virus particles (Gierer & Schramm, 1956; Fraenkel-Conrat, Singer & Williams, 1957). Just as in the case of bacterial transformation, viral infection turns out to be a genetic phenomenon. Very recently an extraordinary collaboration between these two processes was achieved by the growth of vaccinia virus in *B. subtilis* bacteria which had been exposed, in the competent state, to the viral DNA (Abel & Trautner, 1964). Similarly, using a special technique, it has proved possible to infect *E. coli* with RNA from encephalomyocarditis virus, with the resulting formation of complete virus particles (Ben-Gurion & Ginsburg-Tietz, 1965). Although it is too early to speculate on the future implications of these astounding experiments, enough has been said to show that, in this twenty-fifth anniversary year of Fred. Griffith's death, the phenomenon which he discovered is far from being forgotten.

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With 2 plates
Printed in Great Britain

The nature of the opaque colony variation in group A streptococci*

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INTRODUCTION

Griffith (1934) described the occurrence of opaque colony variants of streptococci from human sources and made extensive use of them in his serological studies. He found the selection of these variations to be useful in obtaining type-specific organisms for slide-agglutination tests. It has subsequently been suggested that the opaque colony character is a reflexion of a high content of type-specific M protein on the group A streptococcal cell-wall (Gooder & Maxted, 1961). However, selection of high M-producing variants by this procedure has given inconstant results (Wilson, 1959), and unpublished studies carried out in this laboratory some years ago indicated that there is no consistent relationship between the presence of M protein and the opaque colony form. In fact, several different opaque colony strains were isolated in which M protein was not detectable. The present investigation was undertaken to determine what property of the organism is responsible for this striking colonial variation. Although the initial working hypothesis was based on the assumption that some constituent of the bacterial surface was involved, no evidence was obtained for the presence of a new component or for a quantitative increase in a known component. Rather, it was concluded that the opaque colony results from an altered growth pattern of the organisms that depends on an unusually tenacious union between individual cocci following cell division.

MATERIALS AND METHODS

Streptococcal strains

Opaque colony lines were established with sixteen different group A streptococcal strains of the stock laboratory collection. These were of twelve different M types. In each case, the translucent (or blue) variant was isolated for comparison. Most of the detailed chemical and serological studies were carried out with opaque and blue variants of strain S 43 (type 6) and strain S 23 (type 14).

Selection of variants

The organisms were grown on a clear medium composed of nutrient agar to which 5% horse serum and 0·1 mg./ml. bovine testicular hyaluronidase† were added at 45–50° C. just prior to pouring the plates. The presence of hyaluronidase prevented occurrence of mucoid colonies which tend to obscure the other colonial

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† Worthington Biochemical Corporation, Freehold, New Jersey.

characteristics. The further addition of 0·05 mg./ml. lyophilized beef liver catalase* is useful in enhancing growth and increasing colony size. Plates were streaked so as to give good distribution of colonies and grown at 37° C. overnight.

A colony microscope with obliquely transmitted light was used for examining colonies. The several stock strains varied in the degree to which opaque colonies or sectors were apparent on initial plating, but opaque variants were isolated from every strain in which the attempt was made. Repeated transfers of selected opaque and blue colonies were carried out until pure lines were established. The opaque colonies under these conditions appear white or yellowish and coarsely granular. The non-opaque colonies show some fine granulation in most cases, but are characteristically blue in appearance and translucent (Pl. 1, fig. 1).

Antisera

Agar-grown organisms were used for the preparation of immunizing vaccines. The suspensions of washed cells in physiological saline were heated at 56° C. for 30 min. Rabbits were injected intravenously on four successive days a week for 8–10 weeks. After a rest period of 1–2 months, a second course of injections was given.

Extraction and fractionation

Test antigenic extracts were prepared by a wide variety of techniques, for example, simple buffer extraction; heating at pH 2; treatment with trypsin or chymotrypsin; dissolution with *Streptomyces albus* enzymes and group C streptococcal phage lysin. Capillary precipitin tests, gel diffusion and immunoelectrophoresis were employed in tests of the extracts and antisera.

Cell-wall, cell membrane and mucopeptide fractions were prepared for serological and chemical analysis by the procedures previously described (Freimer, Krause & McCarty, 1959; Krause & McCarty, 1961).

Chemical analyses

Rhamnose, total hexosamine, glucosamine, muramic acid, nitrogen and phosphorus were determined quantitatively as in previous cell-wall studies. Paper chromatography was used in the analysis of the amino acid content of mucopeptide (Krause & McCarty, 1961).

RESULTS

Occurrence of known surface antigens

In the initial serological studies, the content of M protein of paired opaque and blue strains was estimated semiquantitatively by the capillary precipitin test. Serial dilutions of acid extracts were reacted with type-specific rabbit antisera. In certain instances the opaque colony variant showed a higher titre of M protein than either the blue variant or the original stock strain, although there was frequently no significant difference between them. Of more significance is the fact that four strains yielded opaque variants which showed no detectable M protein,

* Worthington Biochemical Corporation, Freehold, New Jersey

in contrast to the parent strains from which the variants were isolated. These results led to the conclusion that this surface antigen could not be responsible for the opaque colonial characteristic.

The content of T antigen was assessed in the opaque and blue variants of a type 1 strain (T 1). No difference was demonstrable in precipitin tests with trypsin extracts. Similarly, the opaque variant of T 28 showed no increase in 28 R antigen. These explorations, therefore, yielded no evidence for a relationship between colony form and the known surface protein antigens.

Search for new serological components

The possibility was entertained that the opaque character is dependent on an unidentified antigenic component of the cell. In order to test this possibility, a systematic analysis was carried out employing sera obtained from rabbits at intervals during immunization with opaque and blue strains. The soluble antigens used in the precipitin analysis were extracted by widely varying techniques to increase the likelihood that the hypothetical antigen would be present in at least one preparation. Repeated double diffusion and immunoelectrophoretic tests produced no evidence for the occurrence of an antigen in opaque strains that was absent (or present in reduced amounts) in the blue variants. Exploratory agglutination and complement-fixation tests were equally unpromising.

Chemical analysis of cell fractions

The foregoing results would not have uncovered a non-antigenic component nor an antigen that was not readily detected by the methods employed. Since the chemical composition of the surface structures of group A streptococci is reasonably well established, it was feasible to use chemical analysis in an effort to detect the presence of an unidentified component. Thus, the presence of an appreciable quantity of an unknown substance in the cell-wall of opaque variants would depress the analytical values of the known components. However, there was no significant difference in the content of protein, rhamnose, glucosamine, nitrogen or phosphorus between the walls of paired opaque and blue strains. The mucopeptide fractions were alike in glucosamine and muramic acid content and had the same amino acid composition both qualitatively and quantitatively. In short, the chemical findings, like the serological studies, failed to suggest a basis for the colonial variations.

Morphological observations

The first clue to a possible explanation for the colonial differences came from the routine examination of Gram-stained bacterial smears. Suspensions of agar-grown opaque colony variants, even after repeated washing in saline, were found microscopically to consist of tangled masses of extremely long streptococcal chains (Pl. 1, fig. 3). In contrast, suspensions from blue colonies showed only clusters and short chains, the more common pattern in smears of streptococci from surface colonies (Pl. 1, fig. 4). These differences were consistent for all of the opaque and

blue pairs examined. It must be emphasized that these findings relate specifically to organisms grown on agar surface, and the difference is much less apparent in broth-grown organisms because of the enhanced chaining of blue variants under these conditions.

The exaggerated chaining of opaque variants was sufficiently striking to warrant further study in an attempt to determine its basis. The chains are extraordinarily resistant to disruption. They are not affected by mechanical agitation. They survive extraction with lipid solvents and drying from acetone and ether. A variety of reagents, including 6 M urea, 50% pyridine, 10% trichloracetic acid, 90% phenol, and glacial acetic acid leave them unchanged. Proteolytic enzymes (trypsin, chymotrypsin, pepsin and streptococcal proteinase) cause no appreciable shortening of the chains. Even cell disintegration by shaking with glass beads does not always result in complete disruption, so that preparations were obtained in which isolated cell-walls occurred in chains.

The most illuminating information came from formamide extraction, which has been shown to leave as an insoluble residue the mucopeptide fraction of the cell wall (Krause & McCarty, 1961). Acetone-dried cells from agar-grown opaque variants were subjected to two successive extractions with formamide at 180° C. for 20 min. The washed residue had the composition of typical mucopeptide, except for the retention of somewhat more cell-wall carbohydrate (3–5% rhamnose) than in preparations previously described. Microscopy revealed that the mucopeptide, devoid of nearly all of the other cellular constituents, still retained the chain configuration of the original cells. This is illustrated in the electron-micrograph in Pl. 1, fig. 2. It appears, therefore, that the property of tenacious chaining of streptococci in opaque colonies resides in this rigid structural layer of the cell wall.

Relationship of chaining to genesis of the opaque colony character

It is proposed that the property of rugged chain formation, brought about by the elaboration of an exceptionally massive intercellular bridge of the structural mucopeptide, leads to spatial orientation of cells growing on an agar surface which results in the appearance of granularity and opacity. It is significant that the colonial differences are readily apparent only with obliquely transmitted light, suggesting that special optical properties in the dispersion of light are responsible for the effect. That chain formation exerts a direct effect on the pattern of surface growth and colony configuration can be readily demonstrated by the examination of microcolonies.

For the production of microcolonies, sterile microscope slides covered with a thin layer of nutrient agar were inoculated by streaking with cultures of opaque and blue variants and incubated at 37° C. for 3–4 hr. Colonies composed of a few dozen to a few thousand cells were readily visualized microscopically at a magnification of ×500. At this stage the opaque colonies (Pl. 2, figs. 5–7) give the appearance of being formed from a single convoluted chain of cocci, and the edges are scalloped by the peripheral occurrence of frequent open loops of the chain. The blue colonies, on the other hand, are more regular in contour and appear to be

composed of closely packed spheres (Pl. 2, figs. 8, 9). These growth characteristics seem to be quite adequate to account for the differences in dispersion of transmitted light by the macrocolonies.

DISCUSSION

The observations reported in this paper suggest that the opaque variants of group A streptococci owe their colonial appearance to persistent chaining during growth on an agar surface. The rigid mucopeptide layer of the cell-wall is clearly involved in the formation of the exaggerated intercellular bridges, but there is no information on the nature of the process that leads to this result. One must assume that the architecture of cell-wall synthesis, at least at the site of the cross-wall, is modified so that greater continuity between cells is retained after division. The possibility that the overall thickness of the basic cell-wall is greater in the opaque colony variants is not eliminated in these studies, since the data do not permit a quantitative estimate of the weight of cell-wall or mucopeptide per coccus.

This proposed mechanism for the genesis of opaque colonies leaves unexplained the success that was encountered in the selection of type-specific strains on the basis of this character. Even though the results of this kind of selection were inconstant, as noted in the introduction, it is clear that M-containing strains are obtained more often by this procedure than could be due to chance alone. Thus, there may be some relationship, although not a consistent one, between the altered cell-wall synthesis resulting in exaggerated chaining and the synthesis of M protein. This is reminiscent of the association between the production of hyaluronate capsules (mucoid colonies) and M protein. As in the case of opaque colonies, the selection of mucoid colonies is frequently useful in the isolation of strains with enhanced M protein production, although mucoid M-negative strains are well known.

It is likely that the opaque variants employed in the present study are comparable to those which Griffith (1934) referred to as *very opaque* and which gave granular suspensions unsuitable for slide agglutination. However, the same properties are found in the somewhat less opaque and granular colonies which we studied, and they differed primarily in being composed of rather shorter and more fragile chains. The degree of opacity of a colony appears to depend on the durability of the intercellular bridges between the cocci.

SUMMARY

Opaque colonies of group A streptococci, detected on clear agar plates with obliquely transmitted light, are composed of extremely long chains of cocci. The mucopeptide layer of the cell-wall is involved in the formation of enhanced intercellular bridges. It is suggested that this exaggerated chaining results in an altered growth pattern which gives the appearance of granular opacity to surface colonies.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Appearance of the opaque and blue colonies in mixed culture from strain S43/137 (type 6). The granularity of the opaque colonies is apparent. Microphotograph taken by oblique transmitted light (approx. 15° from perpendicular). Magnification, × 10.

Fig. 2. Electronmicrograph of streptococcal chain extracted twice with formamide at 180° C. Magnification, × 6000. Micrograph taken by Dr Earl H. Freimer.

Fig. 3. Gram-stained smear of suspension of organisms from opaque colony of S43. Magnification, × 1600.

Fig. 4. Gram-stained smear of organisms from blue (translucent) colony of S43. Magnification, × 1600.

PLATE 2

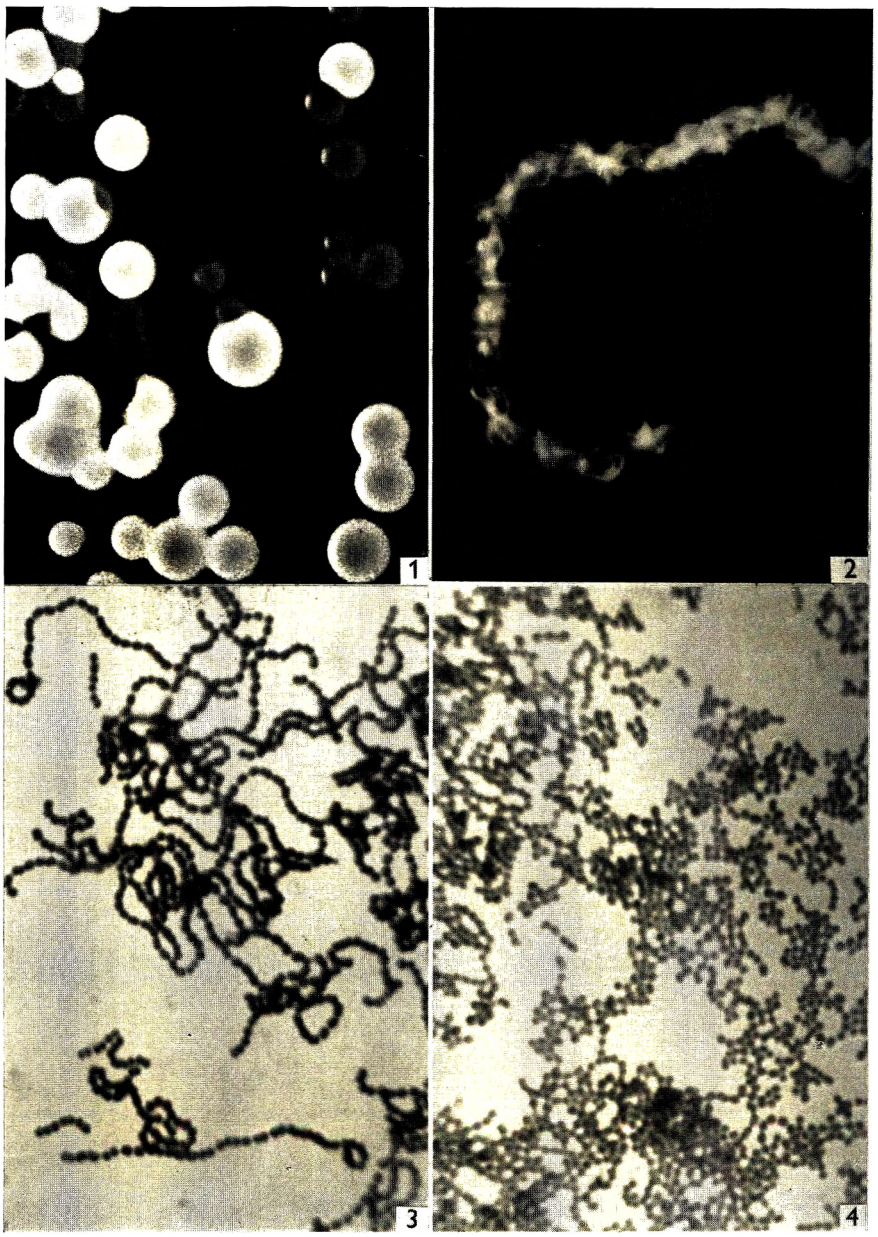
Figs. 5–7. Microcolonies of opaque variant (S43) after 3–4 hr. growth. Magnification, × 1100.

Fig. 5. Small colony showing continuous chain.

Fig. 6. Larger, more compact colony still retaining loops of chains at periphery of colony.

Fig. 7. Moderately large colony illustrating the marked effect of chaining on colony form.

Figs. 8, 9. Microcolonies of blue variant (S43) after 3–4 hr. growth. Magnification, × 1100. The greater uniformity of these colonies and close packing of cocci is in contrast to the appearance of the opaque colonies.



MACLYN McCARTY

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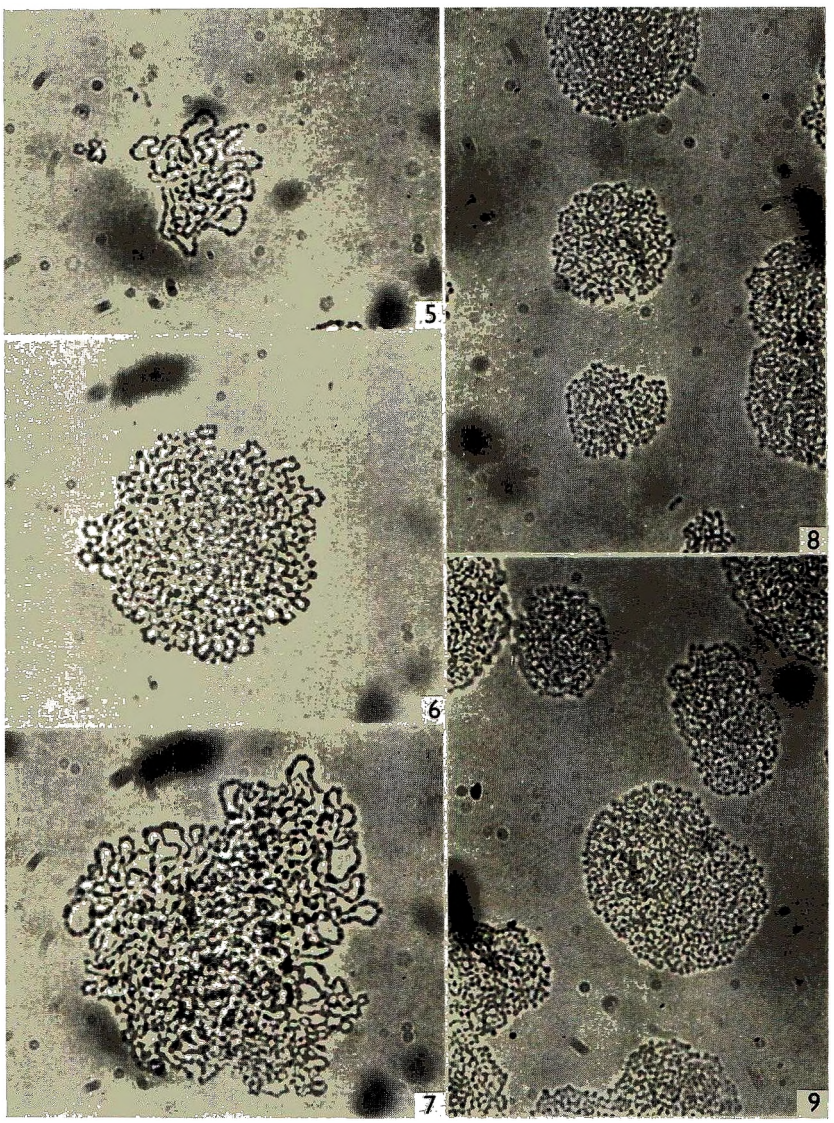
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With 1 *plate*
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Type-specific polysaccharide antigens of group B streptococci*

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(*Received* 7 *February* 1966)

INTRODUCTION

Since the earliest studies on the serological reactions of group B streptococci, it has been recognized that individual types occur among these micro-organisms and that the antigens differentiating them are probably polysaccharides. These polysaccharides appear to be capsular antigens chemically and serologically distinct from the group-specific carbohydrate contained in the cell-walls. By means of the cell-wall polysaccharide, these streptococci are classified into a single serological group. Antibodies directed against the type-specific polysaccharides of group B streptococci not only give type-specific precipitin reactions, but are also capable of passively protecting mice against infection with mouse-virulent strains of the homologous type.

Investigations of the type-specific antigens of group B streptococci have been almost entirely limited to immunological reactions. Stableforth (1959) was one of the first to study the serological reactions of these organisms in bovine infections. Whether the same serological types occur among strains of human and bovine origin has been the concern of many investigators (Daniels-Bosman, 1959; de Moor, 1959; Jelínková, 1963; Pattison, Matthews & Howell, 1955; Stableforth, 1932, 1959; Stewart, 1937).

Lancefield (1934) prepared and partially purified group- and type-specific substances of group B strains, using alcohol precipitation as a means of separating the group and type-specific polysaccharides. Griffith (1935) also published an account of his experiments with strain Aronson N (090 in our series), one of the type Ia strains still in use in the present experiments. His work was concerned with characteristics of this strain, such as its virulence, pathogenicity, and ability to induce protective antibodies. Pattison and associates reported studies in cattle and goats of active immunity which was related to serological types; and they studied a serologically active protein antigen in addition to the type-specific polysaccharides (Pattison & Smith, 1953; Pattison *et al.* 1955). Recently, interest in possible human pathogenicity of group B strains has been revived and the evidence reviewed by several groups of investigators (Eickhoff *et al.* 1964; Hood, Janney & Dameron, 1961).

Further studies on group B streptococci were undertaken in order to define the group- and type-specific antigens more accurately than was previously possible. It became unnecessary to investigate the group-specific carbohydrate in more

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detail because a thorough study of this antigen was carried out recently by Curtis & Krause (1964). They isolated the cell-wall from a strain which had lost its capsule by serial subculture in type-specific serum, and showed that the cell-wall still contained the group-specific polysaccharide. It was similar to other streptococcal cell-wall antigens in that it contained rhamnose. This was the principal determinant sugar, but it was in a different linkage from those rhamnose determinants found in group A-variant and group G streptococci. Wittner & Hayashi (1965) have reported chemical analyses of fractions isolated from type II strains in preparations extracted with either hot HCl or *Streptomyces albus* enzymes. They also found that the group polysaccharide contained a high proportion of rhamnose, and suggested that type-specific activity was found primarily in a galactose-rich fraction.

Our studies of the immunological reactions and chemical components of the type-specific antigen of group B, type II streptococci are presented in the following report.

MATERIALS AND METHODS

Strains of group B streptococci

Representatives of the four specific types, Ia, Ib, II, and III, previously studied in this laboratory, were used (Lancefield, 1934). Mouse-virulent strains were employed in order to include the biologically important mouse-protection test in the study. One type II strain, 18RS 21/19, originally isolated from a human throat culture, had become mouse-virulent after nineteen serial passages through mice. A second type II strain, V8, isolated from milk by Dr Theobald Smith in 1915, was not mouse-virulent. Strain H36 B/7 (type Ib), isolated from the blood of a new-born infant, was highly mouse-virulent.

Preparation of antisera

Sera containing antibodies to the group-specific and type-specific polysaccharides were prepared as previously described (Lancefield, 1938*b*). In general, group-specific antisera were obtained from strains devoid of the outer capsular layer of type-specific antigen. Strains rich in type-specific polysaccharide were used to obtain sera with high titres of type-specific antibodies.

Preparation of antigens

Streptococci grown for 16 hr. in 15 l. lots of Arnold-sterilized Todd-Hewitt broth were collected and lyophilized. A typical yield from 45 l. was 22·0 g. of dry bacteria.

Extraction methods

(1) Hot HCl: 10 g. of lyophilized bacteria were suspended in 100 ml. of HCl (pH adjusted to 2·0), immersed in a boiling-water bath, and maintained for 10 min. at 96° C. with continuous stirring. Two additional extractions of the bacterial residue removed most of the serologically active material.

(2) Cold TCA: 12 g. of the same bacteria were suspended in 125 ml. of 2·5 %

trichloracetic acid, bringing the pH to 2·0. Extraction was carried out just above the freezing point in a Braun cell disintegrator for 5 min. (Bleiweis, Karakawa & Krause, 1964). Four or five successive extractions removed most of the TCA antigen. After washing the TCA extracted bacterial residue with saline, further extraction at 96° C. with HCl at pH 2 yielded HCl antigen but no further TCA antigen.

Method of purification

Each HCl extract was neutralized (pH 7), and the active substances were precipitated at 4° C. by adding 4 vol. of absolute ethanol. Each TCA extract was precipitated while still acid, and then neutralized. All precipitates were collected by centrifugation, and were dissolved in saline, since electrolytes are essential for alcohol precipitation. At each step in the purification process, insoluble residues were discarded. By testing dilutions of the extracts in capillary precipitin tubes, assays of group- and type-specific activity were obtained. Active HCl extracts were pooled, as were active TCA extracts; but the pools were kept separate. Each polysaccharide solution was dialysed while being treated first with DNase and RNase, and then with trypsin. Residual proteins, including the enzymes added above, were removed by shaking the solution with chloroform, according to the Sevag technique (Sevag, 1934; Sevag, Lackman & Smolens, 1938). Residual nucleic acids were removed by adding the anionic exchange resin, Dowex 1–X8 in the Cl^- form. The final solutions, which still contained both the group and the type antigens, no longer contained substances giving significant readings at 260 mμ or 280 mμ as measured by the Beckman spectrophotometer.

Method of separation of the type and group antigens

The purified HCl extracts contained large amounts of group-specific antigen, while TCA extracts contained much less. This group-specific polysaccharide could be eliminated from either extract by alcohol fractionation because it did not precipitate until more than 2 vol. of alcohol were added. In general, the type-specific HCl antigen was precipitated with 1 vol. of ethanol, while the type-specific TCA antigen required 1½ vol. Each antigen was refractionated until capillary precipitin reactions showed that the removal of group antigen was essentially complete. Finally, each polysaccharide was precipitated with ethanol, and dried with alcohol and ether *in vacuo*. In one experiment (lot no. 13), 10 g. of bacteria yielded 150 mg. of purified HCl antigen. Twelve g. of bacteria yielded 40 mg. of purified TCA antigen, and an additional 55 mg. of HCl antigen on re-extraction of this bacterial residue.

Precipitin analysis

Quantitative precipitin analyses were done by a spectrophotometric procedure (McCarty & Lancefield, 1955).

Immunodiffusion

Double diffusion was performed by a modification of the Ouchterlony method (Freimer, 1963). Slides for immunoelectrophoresis were prepared by the Sheidegger technique (Sheidegger, 1955).

Analytical methods

Analyses for rhamnose, glucose, glucosamine, nitrogen and phosphorus were performed as previously described (Freimer, 1963). Galactose was determined by a modified method employing galactose oxidase (galactostat) available from the Worthington Biochemical Corporation, Freehold, New Jersey.

Other techniques are described with the respective experiments.

RESULTS

Relationship of the mouse protective antibody to the serologically type-specific polysaccharide

Chemical analysis of the type-specific material, obtained by HCl extraction, showed that three sugars, galactose, glucose, and *N*-acetyl glucosamine, accounted for more than 75 % of its weight. The chemistry of this preparation (referred to as the HCl antigen), will be considered in detail in a later paper. However, since high concentrations of this antigen did not react with group-specific antisera, and did not contain rhamnose, the principal determinant of the group B antigen, the preparation was essentially free of the group-specific polysaccharide. In contrast, small amounts of this HCl antigen gave type-specific precipitin reactions with antisera prepared by immunizing rabbits with type II streptococci. High dilutions of the same antisera were shown to protect mice against infection with homologous group B strains, and it became essential to establish the relationship of HCl antigen to type-specific protection.

Table 1 contains the results of a typical experiment in which the protective effect of an unabsorbed type II antiserum was compared with that of the same serum after it was absorbed with streptococci of homologous or heterologous type. In these passive protection tests, mice were inoculated intraperitoneally with 0·5 ml. of a 1/200 dilution of the virulent type II strain, 18RS21/19, containing approximately one million chains of living streptococci. The same syringe contained 0·5 ml. of a dilution of serum in saline or of saline alone. Unabsorbed serum and serum absorbed with a suspension of either homologous type II or heterologous type Ib heat-killed streptococci were examined with normal rabbit serum as a control. The results show that the titre of protective antibodies in unabsorbed serum EE was as high as 9000, and that this protective property, as well as the precipitins for HCl antigen, were both eliminated by absorption with streptococci of homologous type. The type-specificity of this phenomenon was demonstrated by the failure of a heterologous strain to remove either the protective or the precipitating antibody. Experiments using other antisera led to similar results, and confirmed the earlier studies with group B streptococci (Lancefield, 1934).

In contrast to these results, when antisera were absorbed with a solution of the

HCl polysaccharide, only part of the protective antibody was removed. In these experiments, each serum was absorbed with HCl antigen at its equivalence point determined by quantitative precipitin analysis with the HCl polysaccharide. The unabsorbed and absorbed sera were compared for the level of protection they afforded mice inoculated with a virulent type II strain. Although antibody that precipitated with HCl polysaccharide had been completely absorbed from the serum, protective antibody was only partially removed. This finding suggested that a second antigen-antibody system might also be involved in type-specific protection.

Table 1. *Passive protection tests in mice showing the effect of absorbing type II antiserum with homologous and heterologous group B streptococci.*

(Challenged with 0·5 ml. of a 1/200 dilution of type II streptococci.)

	Serum given to mice						
	Dilution*						
	1	10	100	1000	6000	9000	10,000
Serum EE (type II)†							
Unabsorbed	S	S	S	S	S	S	D (1)
Absorbed with bacteria of							
Type II (18RS21/19)	D (1)	D (1)	D (5)	D (1)	—	—	D (1)
Type II (V8)	D (1)	D (3)	D (2)	D (1)	D (1)	D (1)	D (1)
Type Ib (H36B/7)	S	S	S	S	—	—	S
Normal rabbit serum	D (1)	D (1)	D (1)	D (1)	—	—	D (1)

* Expressed as reciprocal of serum dilution.

† Serum EE was from a rabbit immunized with strain 18RS21/19.

D represents death of one mouse with day of death in ( ). S indicates survival for 7 days.

Virulence controls: strain 18RS21/19 killed control mice in doses which contained from 10^{-1} to 10^{-7} ml. of the original culture.

Table 2. *Precipitin reactions*

	HCl antibody	TCA antibody
HCl antigen	+ + + +	0
TCA antigen	+ + + +	+ + + +

As it was possible that the capsule of the streptococcus contained two distinct antigens, and that HCl extraction had degraded one of the antigens, other methods of extraction were tried. Extracts made with cold TCA were found to react in precipitin tests with antisera which had been previously absorbed with HCl antigen. Thus, a second antigen was present in the TCA extract. Furthermore, this 'TCA antigen' was able to absorb both antibodies, the HCl as well as the TCA, from previously unabsorbed serum, in contrast to the fact that the HCl antigen only absorbed the HCl antibody. These relationships, shown in Table 2, indicate that rabbits immunized with type II streptococci produce two distinct type-specific antibodies: the HCl antibody which reacts with a determinant present in both the HCl and the TCA antigens, and 'the TCA antibody' reactive with a determinant present solely in TCA antigen.

Although all of the rabbits immunized with type II strains produced both the HCl and the TCA antibodies, quantitative precipitin analyses showed that the relative amounts of each antibody varied from serum to serum. The results of an analysis of serum E4 are shown in Fig. 1. In this experiment, increasing amounts of each antigen were added to 0·1 ml. samples of the serum, and the volume of each was adjusted to 1·0 ml. Equivalence was reached with 60 μg. of HCl antigen. With TCA antigen, the equivalence point is not shown in Fig. 1, but more than twice as much antibody globulin precipitated with 60 μg. of the TCA antigen.

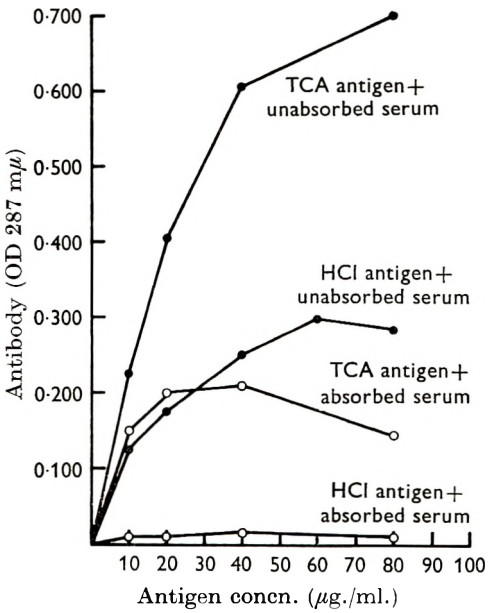

Fig. 1. Quantitative precipitin analysis showing the amount of antibody globulin precipitated with TCA and with HCl antigens from unabsorbed serum E4, and from serum E4 absorbed at equivalence with HCl antigen. The antigens were added to 0·1 ml. of serum, and the volume adjusted to 1·0 ml.

In order to determine how much of this globulin was TCA antibody, a sample of serum E4 was absorbed near equivalence with HCl antigen. This absorbed serum was tested with each of the antigens in the manner just described. These results are also plotted in Fig. 1. It is clear that while this absorbed serum no longer precipitated with HCl antigen, it still reacted with TCA antigen. The amount of antibody precipitated from the absorbed serum with TCA antigen was less than that expected from the results with unabsorbed serum. Recent evidence to be reported later suggests that this was due to inhibition.

The type II specificity of these reactions was tested by preparing HCl antigens from strains of three heterologous types, and in one instance a TCA antigen (type III from strain D136C). None of these gave type-specific reactions with type II antisera, although all reacted specifically with their homologous type antisera.

To investigate the relationships suggested by the observations with type II

polysaccharides, a solution of TCA antigen was brought to pH 2 with HCl, and maintained at 96° C. for 10 min., the procedure used for extracting HCl antigen from intact streptococci. This treatment altered the TCA antigen, and resulted in a polysaccharide identical in chemical composition and in immunological reactivity with that of the HCl antigen. In a parallel experiment, TCA antigen was brought to pH 2 with TCA and heated, but no change was detected. Thus the HCl antigen appears to be a degraded form of the more complete TCA antigen. TCA antigen contains an additional and more labile component which is lost by heating in the presence of the mineral acid.

Immunodiffusion and immunoelectrophoresis afforded other ways of examining the relationships between these two antigens. When the HCl and TCA antigens were compared by double diffusion in agar, each formed a precipitin line with unabsorbed type-specific serum, and these precipitin lines joined to form a band of identity. The TCA precipitin line extended beyond the merger, forming a spur which ran toward the well containing the HCl antigen. This pattern, shown in Pl. 1*a*, supported the concept of two TCA determinants, and demonstrated that they were both part of the same molecule. In addition, the pattern showed that one of these determinants was identical with the HCl antigen. Two preparations of TCA antigen produced a single band of identity (Pl. 1*b*). Furthermore, TCA antigen which had been treated with hot HCl now formed a continuous band of identity with HCl antigen and a spur with the untreated TCA antigen from which it had been derived (see Pl. 1*c*, *d*). Another pattern which is not illustrated showed that only a single band of precipitate formed between TCA antigen and serum previously absorbed with HCl antigen. No precipitin line developed between this absorbed serum and HCl antigen. These patterns confirmed the presence of two antibodies in unabsorbed serum, one reactive with both antigens, and one reactive only with TCA antigen.

The HCl and the TCA antigens also were compared by immunoelectrophoresis, and a typical pattern is shown in Pl. 1*e*. In this system, using Veronal buffer (pH 9·3), the TCA antigen has migrated toward the anode while the HCl antigen appears to have moved toward the cathode. It is also apparent that after HCl treatment of TCA antigen, the treated antigen behaves like HCl antigen. Other electrophoresis experiments in the same buffer, using Pekivon as a supporting medium, resulted in a more rapid movement of TCA antigen toward the anode, than that of the HCl antigen which remained essentially at the origin. Thus the TCA antigen clearly has a net negative charge when compared with HCl antigen.

The isolation of the TCA antigen and its reactivity with two antibodies raised the possibility that both antibodies might play a role in protecting mice against infection with a virulent organism. The observed failure of the HCl antigen to remove the protective properties of an antiserum completely could thus be explained by the presence of a second protective antibody, the TCA antibody.

In order to examine this hypothesis, passive mouse protection tests were set up, using unabsorbed serum and serum absorbed with each polysaccharide antigen at equivalence, as described in Fig. 1. The results of a typical experiment are recorded in Table 3. When the 50% end-point of survival was calculated, serum H was

found to have a protective titre of 8333. Serum H absorbed with HCl antigen still protected to a 50% end-point of 2685, and TCA absorption reduced the titre to 1333. Although it is evident that neither antigen absorbed all of the protective antibody, the TCA antigen did reduce the protective titre by 84% and the HCl antigen by 68%. The failure of TCA polysaccharide to absorb all of the protective antibody, although it did remove all of the type-specific precipitins, requires additional consideration. Factors which may play a role are the unusually high titres of protective antibodies in type II sera, coupled with the difficulty of precise achievement of the equivalence point with a soluble antigen. At either side of this point, some antibody remains in solution. When particulate antigens such as bacteria or cell walls are used for absorption, it is obvious that these difficulties do not occur. It is also possible that an additional determinant, not yet discovered, may be responsible for the residual protective antibody.

Table 3. *Passive protection tests in mice showing the effect of absorbing type II antiserum with homologous polysaccharides extracted with* HCl *or with* TCA

(Challenged with 0·5 ml. of a 1/200 dilution of type II streptococci.)

	Serum given to mice: Dilution*							
	30	100	300	1000	3000	10,000	30,000	50% end-point of survival expressed as serum titre
	Survivors/deaths‡							
Serum H (Type II)†								
Unabsorbed	5/0	5/0	5/0	5/0	5/0	2/3	0/5	8333
Absorbed with								
HCl antigen	5/0	5/0	5/0	4/1	2/3	1/4	0/5	2685
TCA antigen	5/0	5/0	5/0	3/2	0/5	0/5	0/5	1333
Normal rabbit serum	1/4	—	—	1/4	—	—	—	—

* Expressed as reciprocal of serum dilution.
† Serum H was from a rabbit immunized with strain 18RS21/19.
‡ No. of mice surviving/no. dying.
Techniques: see Table 1.

In other passive protection tests, unabsorbed type II antisera had 50% end-points at serum titres that ranged from 2000 to 10,000. In one such experiment, serum EE protected at a serum titre of 2000; after absorption with HCl antigen the level of protection was still 700, while absorption with TCA antigen reduced the level to 100.

Since the proportion of these two antibodies varied from serum to serum, it seemed desirable to compare their relative protective properties on the basis of equal weights of γ-globulin. This was accomplished by the formation of specific antigen-antibody precipitates at equivalence, and the subsequent dissociation of a specific γ-globulin from each complex. As HCl antigen combined with only one antibody, a specific precipitate containing HCl antigen and its specific antibody could be prepared from an antiserum containing both antibodies. The preparation of specific globulins was carried out at 4° C. HCl antigen, dissolved in saline, was

mixed with serum H, and after 16 hr. the specific precipitate was collected by centrifugation. The serum supernatant then contained only the TCA antibody which was precipitated by adding TCA antigen in small increments. When no further precipitate developed, this TCA precipitate was collected. Each antigen-antibody complex was washed with cold 0·05 M phosphate-buffered saline (pH 7·3) until free of soluble protein, and then dissolved in 0·1 M glycine buffer (pH 3). After removing insoluble material by centrifugation, the dissociated γ-globulin was precipitated by 0·33 saturation of the solution with ammonium sulphate. Each precipitated globulin was dissolved in saline, and concentrated by vacuum dialysis against phosphate-buffered saline. In addition to the HCl (3E) and TCA (3K) specific globulins, a globulin solution containing both antibodies (4E) was prepared from a specific precipitate of TCA antigen and serum H. In order to begin with equal weights of globulin, solutions of the three specific antibody globulins were diluted with phosphate-buffered saline so that each contained the same protein concentration, O.D. of 0·100 (at 287 mμ).

Table 4. *Passive protection tests in mice showing the protective effect of equal weights of* HCl *and* TCA *antibody globulins*

(Challenged with 0·5 ml. of a 1/200 dilution of type II streptococci.)

	γ-Globulin solutions given to mice							
		Dilution†						
		1	2	4	10	20	40	100	50 % end-point of survival expressed as O.D.
Fraction*	O.D. undiluted	Survivors/deaths‡							
Specific HCl antibody globulin (3E)	0·100	5/0	5/0	5/0	5/0	1/4	0/5	1/4	0·006
Specific TCA antibody	0·100	5/0	5/0	5/0	2/3	0/5	0/5	0/5	0·012
Specific HCl and TCA antibody globulins (4E)	0·100	5/0	5/0	5/0	5/0	3/2	3/2	0/5	0·003
Total immune globulin (2B)	0·400	—	10/0	10/0	10/0	9/1	3/7	3/7	0·011
Total normal globulin (1B)	§	0/5	—	—		—	—	—	—

* The antibody globulins were from serum H (see Table 3).
† Expressed as reciprocal of globulin dilution.
‡ No. of mice surviving/no. dying.
§ The volume of normal globulin was adjusted to that of the original normal rabbit serum.
Techniques: See Table 1.

Finally, two additional solutions were prepared with ammonium sulphate: a positive control which contained the total gamma globulin fraction (2B) of serum H; and a negative control containing the same fraction (1B) of a normal rabbit serum. Each of these globulin solutions was reconstituted to the original serum volume with phosphate buffer. The protein concentration of the immune globulin solution (2B) had an O.D. reading of 0·400 (at 287 mμ).

Serial dilutions of the immune globulins were prepared at 1/3 log intervals, and each dilution was given to five mice in combination with a virulent type II culture. The undiluted normal globulin was given to five mice. The results of this experiment are shown in Table 4, in which the 50 % end point of survival is expressed as the O.D. The variability was within the experimental limits of the biological nature of the test system. Relatively small amounts of each specific globulin protected mice from infection with homologous strains, and the results indicated that the HCl and the TCA globulins are equally protective. These findings suggest that when either HCl or TCA antibody binds to its specific antigenic site it so alters the bacterial surface that phagocytosis can occur and mice challenged with these streptococci are protected from infection.

These experiments led to renewed studies of the chemical basis for serological specificity of these two type-specific polysaccharides. Although a detailed report of these findings will appear in a subsequent publication, it may be briefly stated that the type II HCl antigen contains 26 % of glucose, 14 % of glucosamine, and 35 % of galactose. The galactose, in a β linkage, is the principal determinant of the serological specificity of the HCl antigen. In addition to the same proportion of these three sugars, the TCA antigen also contains other components which represent 30 % of its weight. These contribute the negative charge to this molecule as well as the additional serological specificity.

DISCUSSION

The unusual finding of two distinct protective type-specific antibodies directed against different determinants in the capsule of the same group B streptococcal strain led to an investigation of the immunological and chemical relationships of the antigens involved. Both antibodies are specific for type II, and both protect mice against infection with a mouse-virulent strain of the same serological type. Moreover, equal weights of the two specific globulins are equally protective. Three sugars, galactose, glucose and glucosamine, comprise more than 75 % of the HCl extracted antigen. The same three sugars are present in the TCA extracted antigen as well as an additional serologically active component not yet identified chemically. This component is more labile than the HCl antigen, and is split off by heating with dilute HCl. By this hydrolysis the TCA antigen is converted to an antigen which appears to be immunologically and chemically identical with the HCl extracted antigen.

These results are reminiscent of the studies of Avery & Goebel in which they found that the specific polysaccharide of the type I Pneumococcus was either acetylated or deacetylated depending upon the methods of extraction and purification (Avery & Goebel, 1933). The acetyl radical of this polysaccharide was exceedingly labile when exposed to strong alkali. They reported two serologically distinct reactions: one antibody was directed against the deacetylated polysaccharide; the specificity of the other antibody was determined by the presence of an acetyl radical. As the acetyl-polysaccharide absorbed all of the protective antibody, and the deacetylated polysaccharide only absorbed a small part of the

protective antibody, these authors reasoned that the antibody directed against the acetyl-polysaccharide was the major one involved in type I protection. Methods were not available at the time of these studies to isolate specific antibody globulins in order to compare their protective value. Consequently a close parallel between their results and those which we have obtained with the two group B, type II protective systems can only be suggested as being possible. The tentative nature of this comparison is also dependent upon the identification of the chemical nature of the TCA determinant in the group B, type II antigen.

Several other antigen-antibody systems with a similar dual specificity but without protective properties, are known. They include: (1) the polyglycerophosphate antigen described by McCarty which exists either with or without an ester-linked D-alanine, each form being serologically distinctive (McCarty, 1964); (2) the group A and A-variant cell-wall polysaccharides in which the terminal serologically active radical in group A strains is *N*-acetyl-glucosamine, with the underlying serologically distinct rhamnose-rhamnose linkages characteristic of the A-variant streptococcus (McCarty, 1956); and (3) the intermediate cell-wall polysaccharides found in groups C and G by Krause (1963).

What may represent another of these relationships has long been known in group B. Strains placed in types now designated as Ia and Ib were originally all classified as type I on the basis of precipitin reactions and reciprocal protection (Lancefield, 1938*a*). Discrepancies began to appear as numerous antisera were tested, and reciprocal absorption experiments using whole streptococci as absorbing agents distinguished cross-reactive antibodies from those specific for either Ia or Ib strains. As has been shown in preliminary experiments not yet reported, these differences are reflected in the serological reactions of the purified types Ia and Ib polysaccharides. The complex relationships of types Ia and Ib, have not yet been clarified.

SUMMARY

Rabbits immunized with whole streptococci of group B, type II, produce two immunologically distinct type-specific antibodies which are essentially equal by weight in protecting mice against infection with homologous type strains.

The capsular antigen with which these antibodies react is a polysaccharide containing galactose, glucose, glucosamine, and a labile component which has not been chemically identified. Extraction of the bacteria with TCA yields this 'complete' antigen, whereas extraction with HCl yields a partial antigen without the labile component. This degraded antigen can also be derived from the TCA antigen by treating the latter with hot HCl, and is indistinguishable from that extracted directly from the bacteria with HCl.

One of the antibodies produced, the TCA antibody, is directed against the labile component of the polysaccharide. The other, the HCl antibody, is directed against a β-D-galactoside determinant; and the precipitin reaction with this antibody is not masked in the 'complete' TCA antigen by the presence of the labile component.

The group-specific polysaccharide, which is located in the cell-wall, is also extracted with either TCA or HCl but can be eliminated from the preparations by

fractional precipitation with ethanol. Although it is known that the type-specific polysaccharide is located in the streptococcal capsule, it is not at present clear in what form this substance occurs in the living streptococcal cell. It may be present partially as the degraded HCl form, or possibly wholly as the intact TCA form. Further immunological and chemical studies of these type-specific polysaccharides are in progress, and will be presented in another communication.

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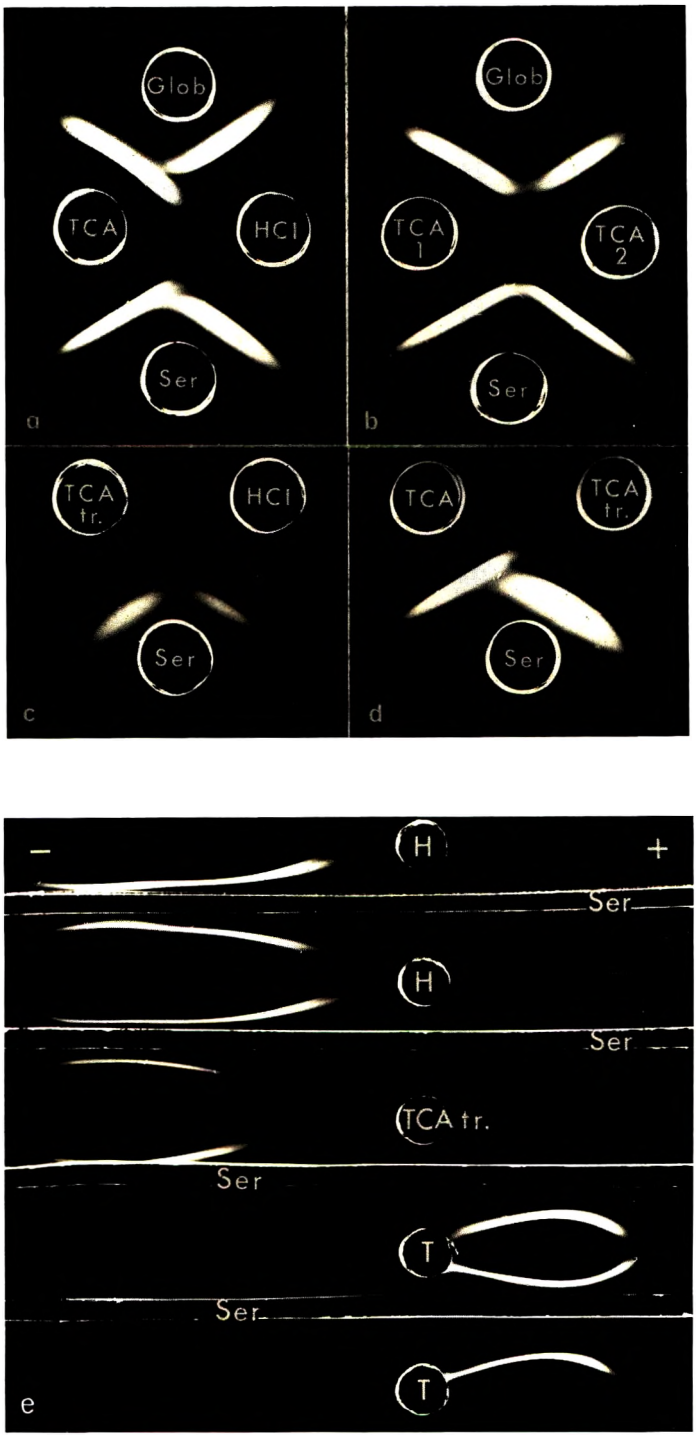
Glob
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EXPLANATION OF PLATE

(*a*)–(*d*) Microimmunodiffusion slides demonstrating the relationships between TCA and HCl antigens. TCA antigens are in wells marked TCA 1 and TCA 2, while HCl antigen is in wells marked HCl.

(*a*) The precipitin lines of the TCA and the HCl antigens have joined to form a band of identity. The TCA line shows a spur which indicates the presence of a second determinant as part of this molecule. No essential difference is noted between the unabsorbed type II serum EE (Ser.) and the concentrated globulin fraction (Glob) obtained from another unabsorbed type II antiserum H.

(*b*) The identity of two preparations of TCA antigens.

(*c*) The identity of TCA antigen treated with hot HCl (TCA tr.) and HCl antigen.

(*d*) The formation of a spur between (TCA tr.) and the original TCA antigen.

(*e*) Immunoelectrophoretic comparison of HCl antigen (H), TCA antigen (T), and TCA antigen treated with hot HCl (TCA tr.). The patterns have been developed with unabsorbed type II antiserum EE (Ser)

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Streptococcal infection in young pigs

I. An immunochemical study of the causative agent (PM streptococcus)

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Outbreaks of streptococcal infection in pigs 2–6 weeks after birth are fairly common both in England (Field, Buntain & Done, 1954) and in the Netherlands (de Moor, 1963). The infection takes the form of a bacteriaemia frequently involving the brain and joints. In the early stages it responds to treatment with antibiotics, but some animals recover spontaneously, while others, untreated, die in the early stages of the disease.

The general characteristics of the causative streptococcus have already been described by Field *et al.* (1954). It is a haemolytic diplococcus, non-pathogenic for mice, guinea-pigs and rabbits, but causing arthritis and meningitis on intravenous or subdural inoculation into 24-day-old pigs. Field and his co-workers were unable to assign the streptococcus to any of the established serological groups (A to O) and this was also the experience of de Moor, who examined strains isolated from the disease in Holland. Some of the strains isolated by Field and by de Moor appeared to be identical (de Moor, 1963).

It is the object of this report to describe the serological relationships of streptococci isolated from nineteen outbreaks of the disease in England, mostly in East Anglia. The streptococci, here designated PM streptococci, were isolated at autopsy from the heart blood, joints and brains of pigs dying from the infection. The PM streptococci belong to Lancefield's group D and to a single serological type within that group. Cocci serologically identical with these were found in ante-mortem throat and blood cultures from some of the affected animals as well as from throat cultures of apparently normal litter-mates. Serological matches for these streptococci were also found in throat and nose cultures from a small number of sows without manifest infection (Elliott, Alexander & Thomas, 1966).

Through the co-operation of Mr Field and Dr de Moor I have been able to compare their strains with the PM streptococci to be described in this report. I have found that Field's strain 428 and de Moor's strains designated group S are serologically identical with the PM streptococcus.

METHODS

Serological methods

Streptococcal cultures and extracts. The PM streptococci were grown either in Todd Hewitt broth containing 10% of horse serum or in dialysate broth (Dole, 1946) made with Pfanstiehl peptone and containing 1% glucose.

Saline extracts were made by resuspending in a small volume of 0·85 % NaCl the centrifuged cocci from broth cultures. The suspensions were incubated at 37° C. for 2 hr., after which the supernatant fluids were separated from the cocci by centrifugation.

Acid extracts were made either by heating the cocci to 100° C. for 10 min. in hydrochloric acid at pH 2 or by stirring the cocci in 5 % trichloracetic acid for 24 hr. at 4° C.

Streptococcal antisera were prepared in rabbits by the method previously described (Elliott, 1960).

Precipitin tests were carried out using the capillary technique (Swift, Wilson & Lancefield, 1943).

Analytical methods

Electrophoresis was carried out on Pevikon in 0·1 M veronal buffer pH 8·6 at 400 V. for 12–14 hr. at 4° C. The Pevikon block (45 cm. × 7 cm.) was then cut transversely into 1 cm. strips and each suspended in 1 ml. H_2O. The 'antigens' thus eluted were identified by precipitin tests with appropriate antisera.

Paper chromatography was used for the identification of monosaccharides after hydrolysis of carbohydrates in 4 N-HCl at 100° C. for 6 hr. in sealed ampoules. The following solvents were used: butanol, ethanol, water (4:1:1); butanol, pyridine, water (3:2:1·5); isopropyl ether, formic acid for phosphorus compounds (9:6). Spraying agents were aniline hydrogen phthalate and, for phosphorus compounds, amidol reagent followed by ultraviolet irradiation. Glucosamine and galactosamine were differentiated chromatographically after conversion to pentoses (Stoffyn & Jeanloz, 1954). Glucose was estimated by the glucose oxidase reaction (Glucostat), xylose and methylpentose by the cysteine–sulphuric acid methods (Dische, 1949; Dische & Shettles, 1948), and hexosamines by a modification of Elson & Morgan's procedure (1933).

GROUP IDENTIFICATION OF PM STREPTOCOCCI

Serological reactivity of extracts from PM streptococci

Saline and hydrochloric acid extracts made from PM streptococci as described under Methods were examined by precipitin tests with streptococcal group antisera kindly provided by Dr R. C. Lancefield. From the results shown in Table 1, it can be seen that extracts prepared from all strains precipitated with group D antisera; these reactions occurred regardless of the type specificity of the antisera and were interpreted as being group specific. Positive reactions also occurred with some, but not all, group E and group N antisera; these reactions were probably due to type- rather than group-specific antibodies and their significance is unknown. Some of the PM streptococcal extracts reacted with antisera to the C polysaccharide of pneumococcus. Preliminary study indicates that this reaction is probably due to a serological relationship between cell-wall components of the PM streptococci and pneumococci.

Gel diffusion tests

In gel diffusion tests using the Ouchterlony plate method, extracts from PM streptococci, from *Streptococcus faecalis* (cell-wall types 1, 4) and from *Strep. durans* (cell-wall type 26) when tested against group D antiserum (cell-wall type 1) formed precipitin lines that showed continuity and indicated probable identity of the antigenic determinants involved in the reactions. The same appearances were obtained when a group D reactive antiserum made against a porcine strain, A 227, was substituted for the *Strep. faecalis* antiserum. Strain A 227 was isolated from the throat of a 5-week-old piglet convalescent from an experimental infection produced 3 weeks previously with a PM streptococcus, strain PM 23. Culturally and serologically the two strains resembled one another except that PM 23 was encapsulated and pathogenic for piglets, whereas A 227 had no capsule and was not pathogenic (Elliott *et al.* 1966). It is possible that A 227 was an attenuated variant of PM 23 produced through residence in the throat of the convalescent, immune piglet.

Table 1. *Precipitin reactions of crude acid extracts from PM streptococci with streptococcal group antisera*

	Rabbit antisera to streptococci of stated group and type							
	Group D			Group E	Group N			Groups A, B, C, G, L, M, O
Antigen	Type 1	Type 3	Type 40	Strain K 129	Strain 6681	Strain R7	Strain R9	
Acid extracts from PM strep. (19 strains)	+	+	+	±	+	−	−	−

± Indicates positive reactions with three out of seven rabbit antisera.

Chemical analysis of the group D reactive component of PM streptococci

It has previously been shown that streptococci that compose Lancefield's group D are characterized by a serologically reactive polymer consisting of glucose and α-glycerophosphate, the so-called 'intracellular teichoic acid' (Elliott, 1962; Wicken, Elliott & Baddiley, 1963). In order to obtain additional evidence supporting the relationship of PM streptococci to group D, partially purified group extracts from four strains were analysed for the presence of glucosyl glycerophosphate. The four strains included two, PM 1 and PM 23, isolated from diseased piglets, a serologically identical strain, C 22 N, isolated from the nose of a normal sow and strain A 227 described in the preceding paragraph.

Extraction and purification of group substances

The streptococci were grown in 20 l. amounts of dialysate broth incubated for 18 hr. at 37° C. Because the yield of purified group substance from PM streptococci was small (from 1 to 5 mg./l. of culture), group reactive material extracted from

the cocci by a variety of methods was pooled and then partially purified before chemical analysis. Preliminary extraction in saline was carried out as described under Methods. The cocci were then disrupted by shaking with glass beads (Ballotini no. 13) in a Braun disintegrator; the resulting supernatant fluid was rich in group substance and could be separated from the disrupted cocci by high-speed centrifugation. Finally, the residual bacterial debris was extracted several times with trichloracetic acid as described under Methods.

To the pooled extracts was added ethanol (2 vol.) and the resulting precipitate, which contained the group reactive component, was re-dissolved in a small volume of water. Concentrated extracts thus prepared contained, in addition to the group D reactive substance, a large admixture of type-specific material probably derived from the bacterial capsule (see below) and from the bacterial cell walls. As shown in Table 2, separation of the type and group components was readily achieved by electrophoresis on Pevikon at pH 8·6. Under these conditions the group substance was negatively charged and moved to the positive end of the Pevikon block, whence it could be eluted free from contamination with type-specific material. The group reactive material eluted in water from the Pevikon was dialysed against water and finally frozen and dried. The lyophilized material was submitted to chemical analysis without further purification.

Table 2. *Precipitin reactions of electrophoretically separated components of extracts from PM streptococci*

	Rabbit antisera to group D streptococci				
	Group reactive			Type specific	
Antigen (extracts from PM. streptococci)	Type 1*	Type 3*	Type 10*	PM 1†	PM 23†
Fraction A (group specific)‡	+	+	+	−	−
Fraction B (type specific)§	−	−	−	+	+

* Group D cell-wall types.
† PM streptococci.
‡ Fast-moving component negatively charged at pH 8·6.
§ Slow-moving component negatively charged at pH 8·6.

Chemical analysis

The methods employed in analysing the group reactive material from strains PM 1, PM 23, C 22 N and A 227 are described under Methods. Examination of acid hydrolysates by paper chromatography revealed the presence of glycerophosphate, glucose and xylose in all four strains. Table 3 shows the results obtained from quantitative chemical analysis of these partially purified group D extracts. In addition, analytical data derived from a previous study of group antigen from three known group D strains (D 76, C 1 and C 3) are given for comparison (Elliott, 1962).

It can be seen that the chemical data support the serological findings and confirm the presence of group D antigen in strains PM 1, PM 23, C 22 N and A 227. The presence of xylose is distinctive but does not appear to affect the serological

specificity of the antigen for, as already shown, gel diffusion precipitin tests indicated identity between group substances from all the strains listed in Table 3.

Table 3. *Carbohydrate content of group D substances isolated from streptococci of porcine and non-porcine origin*

Strain designation	Source	Glucose (%)	Xylose (%)	Glycerophosphate
PM 1	Bacteriaemia in piglet	17·5	11·0	+
PM 23	Bacteriaemia in piglet	26·9	3·2	+
C 22N	Nose of normal sow	12·3	2·4	+
A 227	Throat of piglet convalescent from streptococcal bacteriaemia	26·2	2·2	+
D 76 (type 1)	Cheese	25·6	.	+
C 1 (type 4)	Cheese	30	.	+
C 3 (type 26)	Cheese	30	.	+

Strains D 76, C 1 and C 3 were obtained from Dr R. C. Lancefield.

Glycerophosphate was identified by paper chromatography but not determined quantitatively.

THE TYPE-SPECIFIC CAPSULAR COMPONENT OF PM STREPTOCOCCI

Formalized PM streptococcal vaccines elicit in rabbits the production of antibody specifically reactive with some component of these micro-organisms. Such antisera reacted strongly with saline or acid extracts prepared from PM streptococci isolated in all nineteen outbreaks investigated. They reacted minimally or not at all with extracts of other group D streptococci. By precipitation reactions with these antisera the type-specific component of the PM streptococci could be identified in unconcentrated filtrates of 18 hr. broth cultures. More concentrated preparations of the type-specific substance were obtained by saline extraction of the living cocci as described under Methods. The ease with which the type-specific substance could be extracted without apparent injury to the cocci suggested a superficial location, possibly in the form of a capsule. Further evidence for this was found in the 'swelling' reaction shown by the cocci in the presence of type-specific immune serum and by the characteristic precipitin haloes that surrounded colonies of PM cocci growing on nutrient agar incorporating such serum.

Characterization of the type-specific (capsular) component of PM streptococci

Extraction methods

The PM streptococci to be used as a source of type-specific substance were grown in 20 l. amounts of dialysate broth from which they were harvested after 18 hr. incubation at 37° C. Three methods were used to separate the type-specific substance. First, the soluble type-specific material was recovered from culture filtrates by precipitation with ammonium sulphate 0·8 saturation (fraction 1). Secondly, the cocci harvested from the cultures were allowed to autolyse in the presence of sodium deoxycholate (0·1 %, w/v) and the soluble type and group substances thus liberated separated from the residual bacterial debris by centrifugation (fraction 2). Finally, after autolysis, the residual bacterial debris was

extracted with 5 % trichloracetic acid at 4° C. Several extractions, each of 24 hr. duration, were usually required; the extracts were pooled (fraction 3).

Purification procedures

From the three fractions much of the contaminating nucleic acid was removed by treatment first with ethanol (0·2 vol.) in the presence of 1 % $CaCl_2$ (Anderson & McCarty, 1951) and then by digestion with deoxyribonuclease. Protein was removed by digestion with streptococcal proteinase followed by prolonged dialysis against distilled water. After these preliminary measures the fractions invariably contained a mixture of type- and group-specific substances. These were separable by electrophoresis on Pevikon at pH 8·6. In the case of the capsulated streptococci (strains PM 1 and PM 23) both group- and type-specific components migrated towards the anode, but the former moved more rapidly than did the latter and separation was usually achieved after electrophoresis for 12–14 hr. Immobile cell-wall material was excluded from the electrophoretically separated components of the capsulated strains, PM 1 and PM 23. In the case of the non-capsulated strain, A 227, the type-specific component was immobile on electrophoresis at pH 8·6. It seems probable that this component was derived from the streptococcal cell wall. The electrophoretically separated type-specific components were eluted from the Pevikon in water, dialysed free from buffer and finally frozen and dried before chemical analysis. The yield of type-specific was greater than that of group-specific substances and usually amounted to between 10 and 20 mg./l. of culture.

Table 4. *Chemical analysis of type-specific substance (capsular 'antigen') from two serologically identical strains of PM streptococci and type-specific cell-wall antigen from a non-capsulated strain*

Strain designation	Source of type-specific fraction	Carbohydrates in type specific fractions (acid hydrolysate) Glucose (%)	Hexosamine (%)*	Rhamnose (%)
PM 1 (capsulated)	Culture filtrate (fraction 1)	7·6	18·0	.
PM 1 (capsulated)	Autolysate + TCA† extract of residual debris (fractions 2 and 3)	10·8	23·0	.
PM 23 (capsulated)	TCA extract (fraction 3)	12·0	20·0	.
A 227 (non-capsulated)	TCA extract from cell walls	27·2	23·0	20·0

* Hexosamine = glucosamine + galactosamine.
† TCA denotes trichloracetic acid (5%).

Chemical analysis of type-specific substances

The carbohydrate components of the purified type-specific substances were identified by paper chromatography of acid hydrolysates and their concentrations determined as described under Methods. No attempt was made to identify protein constituents in the purified preparations. The results are shown in Table 4.

DISCUSSION

The streptococci here described as the cause of neonatal infection in pigs belong to a single serological type within Lancefield's group D. The type-specific component appears to be a capsular polysaccharide composed of glucose, glucosamine and galactosamine. The group reactive component is an 'intracellular' teichoic acid immunologically related to and probably identical with those characteristic of *Strep. faecalis* and *Strep. durans*. Chemically, it resembles them in containing glucose and glycerophosphate but differs from them in containing a small amount of xylose. This pentose does not appear to affect the serological specificity of the group substance, although examination of a larger number of antisera to the porcine streptococci might reveal a xylose specificity. In this connexion it will be recalled that some but not all rabbit antisera to group A streptococcal glycerophosphate contain antibody to the alanine component (McCarty, 1964). It has previously been shown that the group substances (glucosyl glycerophosphates) produced by different strains of group D streptococci differ in their glucose content (Wicken *et al.* 1963). The demonstration of xylose as a component of the porcine streptococcal group substance provides further evidence of the variable composition of the group D 'antigen'. Such variability is in keeping with the heterogeneous nature of group D, which includes streptococci diverse in habitat and physiological characteristics. As shown in Table 5 the porcine strains here

Table 5. *Comparison of PM streptococcus with other group D streptococci*

Characterization by	PM streptococci	*Strep. faecalis*	*Strep. bovis*
Group D 'antigen'	+ Glucose-xylose-glycerophosphate	+ Glucose-glycero-phosphate	+ Not analysed
Capsule (serologically type-specific)	+	−	+
Slime production (dextran)*	−	−	+
Growth in 40% bile*	± Slow	+	+
Growth in penicillin (5 units per ml.)	−	+	Not tested
Heat resistance (60° C.)	−	+	Variable

* E. Barnes (personal communication).

described differ in many respects from *Strep. faecalis* and *Strep. bovis*. For this reason there would appear to be some justification for establishing within group D an additional subgroup with status equivalent to that of *Strep. faecalis* and *Strep. bovis* but to include streptococci with the characteristics of the PM strain. The PM streptococci appear to be identical with those isolated from piglet infections by Field and by de Moor, who designated his strains 'group S'. We share the experience of de Moor (1963) in that these micro-organisms have been isolated only from pigs so that the new subgroup within group D might appropriately be designated *Streptococcus suis* and the PM strains, Capsular Type 1.

SUMMARY

1. Streptococci causing neonatal infection in piglets have been identified serologically as belonging to group D.

2. Glycerophosphate, glucose and xylose have been identified as components of the group substance isolated from these micro-organisms.

3. The streptococci isolated from piglets with this disease in England appear to belong to a single serological type characterized by a capsular polysaccharide.

4. Glucose, glucosamine and galactosamine have been identified as components of the type-specific substance.

5. It is suggested that these streptococci should be designated Capsular Type 1 in *Streptococcus suis*, a new subgroup in group D.

I thank the Veterinary Investigation Officers, particularly those in Cambridge and Reading, who have supplied me with streptococci from pigs with neonatal infections and I acknowledge with gratitude the skilled technical assistance of Mrs Christa Levine of the Rockefeller University, N.Y.

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Streptococcal infection in young pigs

II. Epidemiology and experimental production of the disease

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INTRODUCTION

In their studies on piglet mortality, Field, Buntain & Done (1954) noted that outbreaks of streptococcal meningitis and arthritis were usually confined to animals in a single litter. From this they concluded that the sow was the probable source of infection. In the work reported here we found evidence supporting this theory. Furthermore, our study of the disease in the field suggests that the causative organism (PM streptococcus) is probably transmitted from the nose or throat of the sow to the upper respiratory tract of the piglet. This is supported by the results of experiments in which a condition indistinguishable from the naturally occurring disease was produced in young pigs by spraying cultures of the PM streptococcus into the nose and throat. Complete protection against such infection was afforded by the prior administration of serum from piglets convalescent from experimental infection.

METHODS

Bacteriological examination of sows and piglets

Throat and nose cultures

Throat and nose swabs were plated on 5% horse blood agar with and without the addition of crystal violet and thallous acetate (Edwards, 1933). The plates were incubated aerobically for 18 hr. at 37° C. and haemolytic streptococcal colonies subcultured in Todd Hewitt broth containing 10% horse serum.

Saline or acid extracts from these streptococci were tested by precipitin reactions with type-specific rabbit antisera prepared with PM streptococci (Elliott, 1966).

Blood cultures

Five ml. samples of blood from the anterior vena cava were mixed with 500 units of heparin. Two 1 ml. volumes of the heparinized sample were plated with 15 ml. molten nutrient agar for colony counts; the remainder was added to 15 ml. Todd Hewitt broth. Plate and broth cultures were incubated aerobically at 37° C. for 7 days.

Experimental animals

Piglets to be used in the experimental production of the disease were weaned at 7 days. Each group of animals was reared in semi-isolation in a loose-box and fed

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on a diet consisting of antibiotic-free milk substitute (Amvilac) and pellets. Convalescent serum used in protection experiments was obtained by bleeding the animals from the axillary vessels while under barbital anaesthesia.

EXPERIMENTS AND RESULTS

Incidence of PM streptococci in infected litters

Throat cultures were examined from both sick and healthy piglets of litters in which outbreaks of streptococcal meningitis or arthritis had occurred within the preceding 3 weeks. All the animals were less than 8 weeks old.

In two consecutive outbreaks of infection cultures positive for PM streptococci were obtained from the throats of five out of five animals with the overt disease, either arthritis or meningitis; blood cultures from these animals were also positive. Throat cultures were also positive in fourteen of twenty-three apparently normal piglets from five litters in which cases of infection had occurred during the preceding 3 weeks (Table 1). This probably gives a low estimate of the number actually infected for colonies of the PM streptococci are not easily identified on blood agar plates.

The finding of PM streptococci in the throats of infected piglets and their littermates suggested two possibilities: first, that the reservoir of infection might be the porcine upper respiratory tract, and secondly, that in susceptible piglets the upper respiratory tract might provide the portal of entry for the PM streptococci. Further investigations were therefore undertaken to investigate these possibilities.

The incidence of PM streptococci in the upper respiratory tract of normal sows

We were unsuccessful in preliminary attempts to isolate PM streptococci from sows in whose young streptococcal infection had recently occurred; cultures from the rectum, vagina and from the skin overlying the teats were negative. Bacteriological examination of the upper respiratory tract of forty-four normal non-pregnant sows (gilts), however, yielded positive cultures from three. From the noses of three sows streptococci serologically indistinguishable from the PM strain were recovered; in one of these animals the PM streptococcus was also recovered from the throat. Experiments described later in this report showed that at least one of these strains (C 22 N) was pathogenic for piglets up to the age of 12 days.

Experimental production of the disease in piglets by infection of the upper respiratory tract with PM streptococci

Infection with PM streptococci from diseased piglets

The same general procedure was adopted in all the following experiments. PM streptococci were grown for 12–14 hr. at 37° C. in modified Todd Hewitt broth containing 10% normal horse serum. In most experiments approx. 5 ml. of the culture were sprayed from an ordinary throat spray (atomiser) into the nose and throat of the piglets. In two cases the centrifuged deposit from 100 ml. of culture

was resuspended in milk which was rapidly gulped down by the piglets. Littermates serving as controls received the same dose contained in a gelatin capsule from which the cocci were subsequently liberated during digestion in the stomach and intestine. After inoculation of the animals, serial 5 ml. samples of blood were withdrawn from the anterior vena cava and cultured for PM streptococci. In some instances throat cultures were also taken and in animals dying from the infection post-mortem cultures were taken from the heart blood, joints and brain.

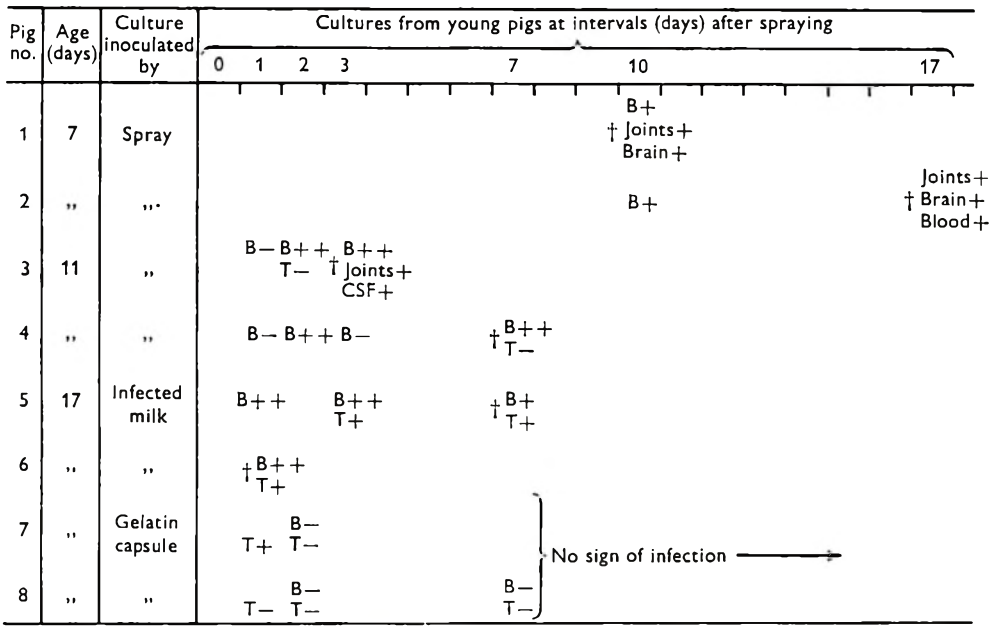

Fig. 1. Result of inoculating PM streptococci* into nose and throat of young pigs.
* Strain PM 23 B isolated from blood of naturally infected piglet.
† indicates death of piglet.
T, throat culture; B, blood culture; +, positive for PM streptococci; + +, positive for PM streptococci > 300 colonies per ml. blood.

The results of these experiments are summarized in Fig. 1. In all six piglets, aged 7–17 days, inoculation of the upper respiratory tract with strain PM 23 resulted in a streptococcal bacteriaemia which terminated fatally in four animals; of the two remaining, one (piglet 5) died as a result of cardiac puncture and the other (piglet 2) was killed after 17 days. In the two animals which received the cocci in a gelatin capsule no bacteriaemia resulted. In two additional piglets, not included in Fig. 1, an attempt to produce the disease by spraying at the age of 21 days proved unsuccessful although one of these animals yielded a positive throat culture 21 days later.

Infection with PM streptococci from a normal sow

In the next experiment the invasiveness of two different streptococcal strains of porcine origin was compared. Strain C 22 N had been isolated from the nose of a 2-year-old sow with no history of disease. This strain was serologically identical

with the PM streptococcus used in the preceding experiments. The second strain, A 227, was isolated from the throat of an 8-week-old pig which had recovered from an experimental bacteriaemia produced with PM streptococcus strain PM 23. Strain A 227 belonged to group D and resembled strain PM 23 in all respects other than the possession of a capsule (Elliott, 1966).

The upper respiratory tracts of two 12-day-old pigs (S 1 and S 2) were sprayed with strain C 22 N and two litter-mates (C 1 and C 2) with strain A 227. The results are summarized in Fig. 2. Piglets S 1 and S 2 developed a streptococcal bacteriaemia the duration of which was at least 14 days in S 1 and 21 days in S 2. Both animals appeared fully recovered when they were killed after 38 days. No positive blood cultures were obtained from the two piglets C 1 and C 2 sprayed with the non-capsulated streptococcus strain A 227.

From the results of this experiment it was concluded that PM streptococci in

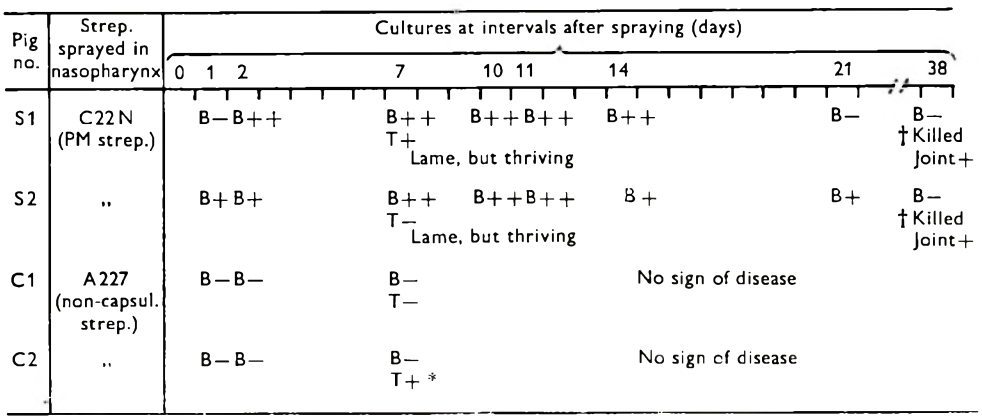

Fig. 2. Result of spraying PM strep. (C22N)** and non-capsulated GpD strep. (A 227)* in 12-day-old piglets' nasopharynx.

** Strain C 22N was isolated from nose of a normal sow.

* Strain A 227 was isolated from throat of convalescent piglet 9 weeks after infection.

T, throat culture; B, blood culture; +, positive for PM streptococci; + +, positive for PM streptococci > 300 colonies per ml. blood.

Table 1. *Streptococcal infection in five litters of piglets. Throat cultures from healthy litter-mates* 3 *weeks after onset*

Piglets in five litters		
Piglets in litter	Survivors	Survivors with positive throat cultures*
5	3	3
8	3	2
10	4	1
10	7	2
7	6	6

* Positive throat cultures were those from which PM streptococci were isolated.

the upper respiratory tract of normal sows are capable of infecting young piglets. It seems possible that the invasiveness of these streptococci is associated, in part at least, with their possession of a capsule.

Expt. 1. Nine 12-day-old litter-mates			Expt. 2. Ten 12-day-old litter-mates		
Pig no.	Serum*	Cultures at intervals after spraying (days) 0 7 14	Pig no.	Serum*	Cultures at intervals after spraying (days) 0 7 10
1	From convalescent piglets (S1+S2)	B− B− B− B− T+	11	From convalescent piglets (S1+S2)	B− B− B− T−
2	,,	B− B− B− B− T+	12	,,	B− B− B− T−
3	,,	B− B− B− T+	13	,,	B− B− B− T−
4	From refractory piglets	B+ B++ B+ T− lame	14	,,	B− B− B− T−
5	,,	B− B− B− T−	15	,,	B− B− B− T−
6	,,	B+ B++ B+ T+	16	No serum	B− B+ B++ T− lame
7	No serum	B− B− B− T+	17	,,	B++ B++ † lame CSF+
8	,,	B++ B++ B++ B+ T+ lame	18	,,	B++ B++ lame
9	,,	B++ B? B++ B+ T+	19	,,	B++ B++ lame
			20	,,	B+ † lame

Fig. 3. Effect of convalescent serum on piglets sprayed in nasopharynx with PM streptococci (strain C22N isolated from nose of normal sow).

* 20 ml. convalescent serum was given subcutaneously 24 hr. before spraying nasopharynx.

† Indicates death of piglet;

T, throat culture; B, blood culture; +, positive for PM streptococci; ++, positive for PM streptococci > 300 colonies per ml. blood; ?, contaminated blood sample.

Passive immunization with serum from convalescent piglets

Two experiments were carried out to determine whether, by the prior administration of serum from convalescent piglets, susceptible animals could be protected from infection with PM streptococci (strain C 22 N). The convalescent serum was a pool of samples taken from piglets S 1 and S 2 38 days after experimental infection with strain C 22 N (Fig. 2).

In the first experiment nine 12-day-old litter-mates were divided into three groups: piglets 1 to 3 each received subcutaneously 20 ml. of convalescent serum; piglets 4 to 6 received 20 ml. of pooled serum from three 38-day-old piglets, litter

mates which, for some unknown reason, had proved refractory to infection with strain C 22 N; piglets 7 to 9 received no serum.

In the second experiment, ten 12-day-old litter-mates were divided into two groups each of five piglets. Those of one group each received subcutaneously 20 ml. of the convalescent serum; those of the other received no serum.

On the day following the administration of serum all the piglets were sprayed with PM streptococci, strain C 22 N. The results are summarized in Fig. 3 from which it can be seen that none of the animals that had received convalescent serum developed a bacteriaemia. Of the animals that did not receive convalescent serum, in the first experiment four out of six and in the second, five out of five developed a streptococcal bacteriaemia. In most cases this occurred within 24 hr. of spraying.

DISCUSSION

Streptococcal infections are a common hazard of the neonatal period. In addition to causing the disease of pigs here described, streptococci are responsible for neonatal infections in babies, lambs and foals. In babies, group B streptococci cause meningitis and have also been isolated from the blood (Hood, Janney & Dameron, 1961). At Boston City Hospital they were the commonest single cause of neonatal sepsis during the period December 1961 to June 1963 (Eikhoff *et al.* 1964). In lambs, group C streptococci cause 'joint-ill' a neonatal septicaemia with involvement of the joints and, less commonly, the heart valves (Blakemore, Elliott & Hart Mercer, 1941).

In none of these conditions is there definite information concerning the source or primary focus of the infection, although in foals it is thought that the invading micro-organisms gain entry by way of the umbilicus (Gunning, 1947). An investigation of streptococcal 'joint-ill' in lambs produced no evidence of umbilical infection (Elliott, unpublished observation). In the present report on neonatal infection in pigs we give reasons for considering the throat of the piglet a likely portal of entry and the upper respiratory tract of the sow a possible source of the streptococci.

Bacteriaemia is a finding common to the human, ovine and porcine varieties of streptococcal infection in the newborn. In experimental infections of the upper respiratory tract of piglets we were impressed by the speed and regularity with which the PM streptococci invaded the blood stream: positive blood cultures were usually obtained within 24 hr. of spraying the naso-pharynx. Of equal interest was the observation that the presence of bacteria in the blood stream did not, of itself, appear to incapacitate the piglets. In poorly developed animals, diarrhoea was sometimes an early sequel to infection but usually, unless the joints or brain were involved, the animals continued to thrive regardless of the large numbers of streptococci circulating in the blood. After 2–3 weeks, the circulating bacteria gradually diminished in number and eventually disappeared, presumably in response to a developing active immunity. As shown here, serum taken from convalescent animals 3–4 weeks after infection and administered to susceptible piglets afforded complete protection against subsequent infection with the PM streptococcus. The results of one experiment (Fig. 3, Expt. 2) suggested that infection of

the naso-pharynx by spray was more difficult to achieve in passively immunized than in normal piglets: attempts to recover PM streptococci from the throats of passively immunized piglets 3 days after spraying were unsuccessful, although by then all of their non-immune litter-mates infected at the same time had developed a bacteriaemia. In the previous experiment no difficulty had been experienced in recovering the PM streptococci from the throats of passively immunized piglets 48 hr. after spraying. These results are inconclusive owing to the small number of animals involved but they suggest that passive immunity prevents not only invasion of the blood stream but also infection of the naso-pharynx by streptococci sprayed into the upper respiratory tract. Of the nature of this immunity we have at present no information but we assume it to be directed against the type-specific, capsular polysaccharide of the PM streptococcus (Elliott, 1966).

The implication of relatively non-pathogenic micro-organisms to the exclusion of more invasive bacteria in the examples of neonatal infection cited above merits consideration. Although sometimes associated with subacute endocarditis (Fry, 1938), the group B streptococci concerned in human neonatal infections are non-pathogenic for normal human adults, the strain of group C streptococci that causes 'joint-ill' in lambs is not pathogenic for adult sheep or for laboratory animals and the PM streptococci isolated from piglets resembles other members of group D in its lack of invasiveness for adult pigs, mice, guinea-pigs and rabbits (Field *et al.* 1954). Clearly, lack of immunity in the newborn animal is a factor in the causation of disease by these relatively harmless bacteria. Is it possible that such passive immunity as the young animal receives from its mother is directed, not against these relatively non-pathogenic micro-organisms, but against agents responsible for infectious diseases of adult life? If such were indeed the case, the newborn animal might be vulnerable to micro-organisms of borderline invasiveness but immune to common pathogens. This reasoning affords no explanation for our experience that all group D streptococcal infections of the newborn pig have been caused by cocci of a single serological type. A similar observation was made in 'joint-ill' of lambs caused by group C streptococci; one serological type, only, was implicated (Blakemore *et al.* 1941). In both porcine and ovine diseases our experience has been drawn almost exclusively from outbreaks in East Anglia, but in so far as the piglet infection is concerned, streptococci of identical serological type have been isolated in Holland (de Moor, 1963). The small number of types—one only, in our experience—incriminated in the disease together with the protective effect of convalescent serum against experimental infection suggests that active immunization of the sow with PM streptococci might be effective in preventing this form of neonatal infection in piglets.

SUMMARY

1. In streptococcal infection of piglets the causative agent (PM streptococcus) was isolated from the throats of a high proportion of infected animals and from their apparently healthy litter-mates.

2. The PM streptococcus was isolated from the noses of three out of forty-three normal sows. In one sow the streptococcus was also isolated from the throat.

3. Bacteriaemia was induced in piglets up to the age of 17 days by inoculating into the nose and throat broth cultures of the PM streptococcus. Blood cultures were usually positive within 24 hr., and secondary involvement of the joints and meninges frequently occurred during the ensuing few days.

4. Serum taken from piglets 5 weeks after experimental infection and administered subcutaneously protected susceptible piglets against subsequent infection with PM streptococci.

5. The possibility of preventing streptococcal infection in piglets by active immunization of the sow is discussed.

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Streptococcal school outbreaks: a method of investigation and control

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Effective control of contagious epidemic disease lies in the recognition and neutralization of the source of infection or of the principal means of transmission, or in protection of the population at risk, or in some combination of these three. Where infections of the upper respiratory tract are concerned, that is, where the principal means of transmission is by contaminated air, purification is not generally possible apart from special environments. Control of spread of infection must therefore reside in dealing effectively either with the source or with the population at risk. This is the problem confronting the investigator into outbreaks of streptococcal disease in schools and training establishments—communities, often residential, notoriously prone to such infection and to its serious complication, rheumatic fever.

With the exception of immunization against the Dick toxin streptococcal infections have not proved amenable to artificial forms of protection other than by mass treatment with sulphonamides or penicillin. It has been claimed that penicillin in therapeutic doses for '10 or more days' for all at risk, regardless of their carrier state, is a certain method of terminating an outbreak (Seal, 1955). In considering this procedure, in so far as it halts an epidemic, it seems reasonable to ask why it should succeed when treatment of infected persons only does not necessarily do so. There can only be one answer, that the methods of investigation do not disclose all the important sources of infection, and failure to include them all in the treatment group spells failure in control. But is every patient or carrier of equal importance as a source of infection? If not—and we think not—can the important, in this sense, be distinguished from the unimportant? It is here that the aim of the investigation must be defined with precision and a distinction drawn between the detection of an infected person for his own sake and his detection as a disperser for the sake of the community. The distinction is fundamental for it needs to be understood that the kind of inquiry we have in mind is aimed at recognizing only those carriers who are also dispersers.

In the spread of streptococcal infections from the upper respiratory tract it is axiomatic that the infecting organisms must be expelled in some way from the original host in order to reach and infect a new host. Apart from streptococcal infections of the skin and ear, which are obvious sources of environmental contamination, the two common sites of infection in the host are the tonsil and the nose. Nasal infections are often ignored, or at least not looked for, though it is

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obvious that infected nasal discharges can easily, and will inevitably leave the host and be dispersed in his surroundings via the hands or the handkerchief or by post-nasal discharge contaminating the saliva.

We first became interested in this problem while investigating epidemics of diphtheria associated with evacuees also infected with a variety of streptococcal types (Boissard & Fry, 1941). Nasal infections, ear infections and infected skin lesions of all kinds quickly impressed us with their importance as sources of infection, and we concluded that wherever infected secretions or discharges could be shed into the environment they were potent disseminators of disease. To these we soon added a fourth. Observations on cross-infections in the isolation hospitals arising from children with coughs led to an investigation of the saliva from acute and convalescent patients with either scarlet fever or diphtheria or both. The patients were mostly very young, very sick children, and the saliva specimen was in fact a mouth swab—taken all round the mouth and under the tongue but avoiding the tonsils and back of the throat. We found that in almost half the patients tested in a period of 4 months we were able to isolate group A streptococci from the mouth swab in the first week of illness. Thereafter, although streptococci could be grown from a tonsillar swab—often for very many weeks—none could be recovered from cough plates or mouth swabs in patients whose infection was confined to the tonsil. Hamburger (1944), working with Army recruits and collecting samples of saliva, was able to isolate streptococci from a much larger proportion of his samples, 80%, for a much longer period, up to 3 weeks. It is probable that our mouth swab discloses only very heavily contaminated salivas, and that, in the absence of a nasal infection, contamination of such a degree is a feature of only the early stages of a tonsillar infection and may be taken as indicative of it. Thus, in assessing the capacity of an infected person to act as a disperser, it seemed to us that a salivary swab, in combination with a nasal swab, might well be substituted for the customary throat swab in the clearance from infectivity of convalescents and contacts. Ear and skin lesions apart, only persons with streptococci in the nose or saliva need consideration as sources of infection.

These are the general considerations which gradually led us to the use of nose and mouth swabs, at first, in 1946, in persistent tonsillar carriers after an acute infection for clearance from infectivity, and later, in 1959, for mass investigations to disclose significant sources of infection. The advantages of mouth over throat swabs are that they are quick and easy to take, no spatulas are required and the number likely to be positive in the mouth alone—as compared with the throat alone—is small, usually less than 5% of the population swabbed. Moreover, by disclosing only those carriers who are also dispersers, all the positive results are significant.

In 1937 sulphanilamide powder, applied locally, was used to treat a streptococcal infection in multiple sinuses which had been discharging for 3 years. A permanent cure was effected in 3 weeks (Purdie & Fry, 1937). We found local applications of sulphanilamide powder equally effective in streptococcal impetigo. These results encouraged us to try the effect of intranasal sulphanilamide powder, by insufflation on two longstanding nasal carriers of 11 and 5 months duration. This treat-

ment was completely successful and was successfully repeated on twenty-six patients, all in hospital, who had been carrying group A streptococci in the nose for periods varying from 4 to 21 weeks (Boissard & Fry, 1942). These results are detailed with the follow-up histories of these patients in table II and fig. 4 of the paper referred to. The details of treatment can be modified to suit particular circumstances, but the basic course consists of insufflation of both nostrils twice a day, morning and evening for a week. In schools which disperse for the weekend it is better to carry insufflations on for two 5-day weeks. There is no need to exclude children from school while being treated. At the end of the period of insufflation there should be an interval of one clear week before carriers are swabbed for clearance. The treatment can be repeated if necessary, although in the majority one course of insufflation, properly given, is adequate.

RESULTS

Since 1941 we have dealt with several dozen school outbreaks of streptococcal infection. The seven described below and summarized in Table 1 have been chosen to illustrate points we think are of interest and importance.

Table 1. *A summary of the main points of the illustrative outbreaks*

Out-break	School	Year	Term	Approx. no. in school	Type of investi-gation	Swabs taken*	Epidemic type
1	L.C.	1943	Summer	300	Limited	N & T	8/25
2	L.S.	1964	Autumn	300	Limited	N & T	4/28
3	L.S.	1954	Spring	300	Mass†	N & T	3
4	L.S.	1955	Spring	300	Mass†	N & T	18
5	K.C.S.	1962	Spring	150	Mass	N & M	28
6	L.H.S.	1965	Spring	66	Mass	N & M	3
7	F.S.SW.	1965	Spring	340	Mass	N & M	6

* N = nose, T = throat, M = mouth.
† School swabbed in two parts.

The first two, widely separated in time, have in common the importance of a chronic disperser as a source of infection. The history in such outbreaks is often so suggestive that it is possible to undertake a limited investigation with a good prospect of success.

Outbreak 1

This occurred in a boarding school of about 300 boys, and covered one school year. It began with cases of scarlet fever and sore throat in the autumn term of 1942. In the spring term of 1943 there were no cases of scarlet fever and very few sore throats, but in the summer term, up to the time of our first visit to the school, there had been three cases of scarlet fever and seven of acute tonsillitis. Enquiry for a boy with a chronic cough or catarrh at once produced the name of one boy, whose personal history had a remarkable bearing on the incidence of infection in the school. Eighteen months earlier he had had scarlet fever, which left him with a persistent rhinorrhoea, still present when he first entered the school in September

1942. During the spring term of 1943 he was kept at home for treatment, and he rejoined the school for the summer term with his rhinorrhoea as bad as ever. Cultures from this boy, from the most recent case of scarlet fever and from five of the seven cases of acute sore throat all showed infection with group A streptococci of type 8/25. The disperser's infection was confined to his nose. He was treated with sulphanilamide insufflation and cured. Enquiry a year later confirmed that no further cases had occurred, and the boy had remained free from symptoms.

An account of this outbreak has been published (Boissard & Fry, 1944), but the details are summarized here because the original publication is somewhat inaccessible.

Outbreak 2

This outbreak, summarized in Table 2, occurred in a boarding school of over 300 boys in the autumn term of 1964. The school re-assembled on 17 September, and all the new boys had routine nose and throat swabs examined. Five were found positive for group A streptococci in the throat alone. No further action was taken.

Table 2. *The results of bacteriological examination in outbreak* 2

Date	Name	Disease*	Culture from N	Culture from T	Type	Form	House	Dormitory	Remarks
17. ix.	A.M.P.	.	−	+	4/28	V_2B	W	1	New boy
6. x.	J.K.G.B.	Tons	−	+ + +	4/28	V_2A	S	.	.
15. x.	D.J.R.	Tons.	−	+ + +	4/28	VI	W	1	.
16. x.	Q.B.P.	Sinus.	−	+ + +	4/28	V_1B	W	2	.
20. x.	N.C.	Tons.	+	+ + +	4/28	IVA	W	1	.
20. x.	E.G.	Tons.	−	+ + +	4/28	V_2C	W	1	.
21. x.	Mrs S.	Tons.	−	+ + + +	4/28	Cleaner	W	.	Worked in dormitories 1 and 2
23. x.	A.M.P.	.	+	−	4/28	V_2B	W	1	The only positive found on swabbing dormitories 1 and 2

* Tons, = tonsillitis; Sinus. = sinusitis.

The first case of acute tonsillitis occurred on 6 October, the second on 15 October and the third on 16 October. Cases 4 and 5 occurred on 20 October by which date it was known that the earlier cases were all type 4/28. Moreover, cases 2 to 5 were all in one house (W), three in dormitory 1 and one in dormitory 2 on the same top floor across a passage. This drew attention to a new boy, A.M.P., swabbed on admission to the school on 17 September and at that time found to have type 4/28 in his throat. He was in dormitory 1 of W house where three of the four infections had occurred, and he could have had class-room contact with case 1.

An initial limited investigation was advised, confined to the thirty-two in dormitories 1 and 2 in W house, and boys with colds or catarrh anywhere in the school. A total of forty-one boys was swabbed on 23 October and a single positive was found, the new boy A.M.P., now positive in the nose alone with the same type 4/28. On the same day swabs were received from Mrs S., a cleaner whose

duties were confined to the top floor of W house, who had developed an acute tonsillitis on 21 October, and was also found to be infected with type 4/28. A.M.P. gave a history of severe acute tonsillitis at his preparatory school the previous spring for which he was kept in bed for a week, a story which added to the possibility that he was a chronic nasal carrier from that time and that the admission swabs

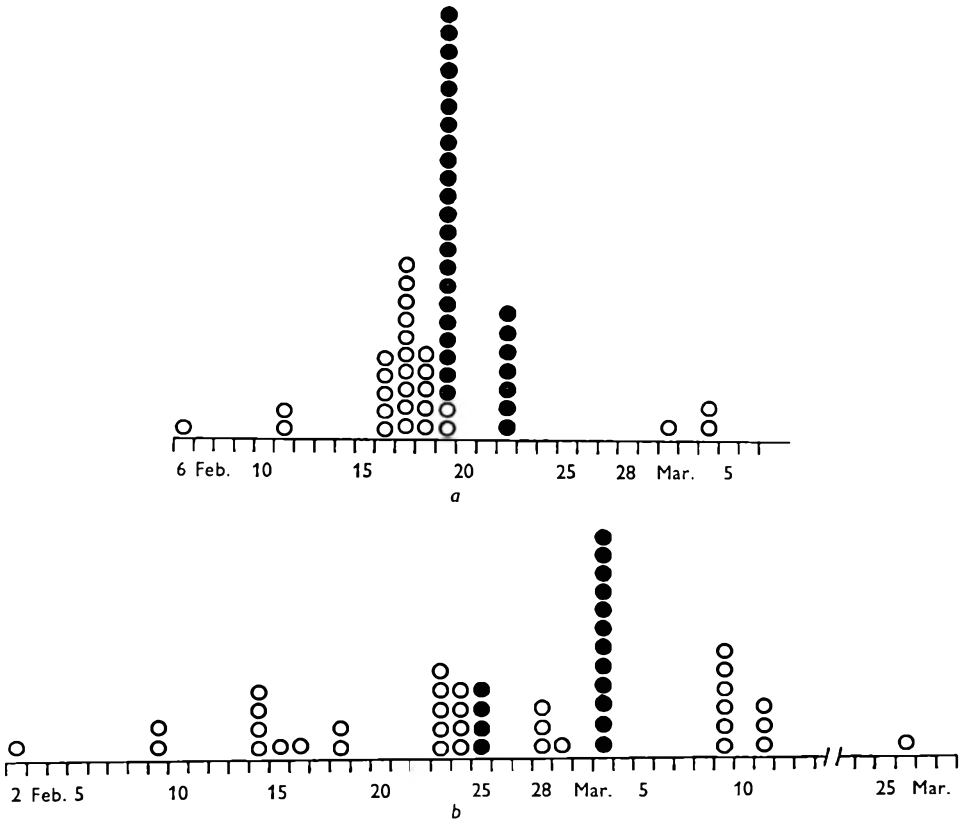

Fig. 1. (*a*) Outbreak 3, (*b*) outbreak 4. ○ = cases of acute streptococcal infection; ● = dispersers found at mass swabbing.

were perhaps mislabelled. He and the other boy with a positive nasal swab were treated with sulphanilamide insufflation and cleared of nasal infection. No more infections with this type occurred in the school for the rest of the term.

Outbreaks 1 and 2 are strikingly similar; both appear to have been started by a single individual with a chronic infection following an acute attack. In both recognition and treatment of the disperser brought the outbreak to an abrupt end.

Outbreaks 3 *and* 4

These two outbreaks occurred in the same boarding school in the spring terms of 1954 and 1955. In each year the first case of tonsillitis occurred in the first week of February, followed by a build-up of cases in the next 2–3 weeks as shown by the open circles in Fig. 1*a*, *b*.

By the time investigations could be undertaken it was clear that mass swabbing

alone offered hope of control. For administrative reasons the swabbing was done in two parts, the junior school first in each year followed by the senior school 3 days later in 1954, and 6 days later in 1955. Nose and throat swabs were taken each time, and the numbers of nasal carriers are shown by the black circles in Fig. 1. All the nasal carriers were treated with sulphanilamide insufflation for a week without exclusion from school.

In 1954 where the interval between the two swabbings was short, and where the first investigation gave the larger number of dispersers, the outcome was reasonably satisfactory. Only three more cases of infection occurred, all within 10 days of the second swabbing, and there were no more infections in that or the next term.

In 1955, when the interval between the swabbings was longer, and when the number of dispersers eliminated by the first swabbing was small, the effect of the intervention was less decisive. Four infections occurred between the two swabbings and nine more within 10 days of the second swabbing. These are probably linked, more or less directly, with the dispersers found at the second swabbing, but there is another possibility. On each occasion the number of carriers found positive in the throat alone exceeded the nasal positives found at the same time. Some of these would almost certainly have shown positive mouth swabs, had these been taken, and such temporary dispersers could have given rise to some of the post-swabbing infections. That no chronic dispersers were responsible is strongly suggested by the fact that, apart from a single case near the end of term, there were no further cases after 10 days, and there were no further cases in the next term.

If mass swabbing is considered necessary, the whole community should be swabbed at the same time, and this outbreak illustrates the drawback of dividing the swabbing. Any division is likely to be an artificial one from the epidemiological point of view, as here, where division into lower and upper school bears no relation to the division into houses and dormitories.

Outbreaks 5, 6 *and* 7

These three outbreaks, one in 1962 and two in 1965, have several points in common. They all occurred in the spring term, and were all investigated by mass swabbing of the whole school on one day, using nose and mouth swabs instead of nose and throat swabs. The schools however differered greatly in size, and the epidemic type of streptococcus was different in each. All dispersers, whether nose or mouth positives, were treated with sulphanilamide insufflation as before, and penicillin lozenges to suck for two days were advised for those whose mouth swabs were positive.

Outbreak 5 was in a preparatory school of 149 boys, seventy-five of whom were boarders. Fig. 2*a* shows that there were nine infections up to the day of the mass swabbing, the last two of which appeared on that day. Five dispersers were found and treated without exclusion from school. All the acute cases and all but one of the dispersers were boarders. No further cases due to type 28 occurred that term or in the next.

Outbreak 6 was in a boarding school of sixty-six boys. The school re-assembled

after Christmas on 12 January; the first case of tonsillitis occurred on 21 January and the second on 25 January. On the same day a swab was taken from a boy with a 10-day history of a discharging ear, shown in Fig. 2*b* in black, for 15 January, the day the discharge started. By 27 January it was known that all the strains belonged to type 3, and as the likely source of infection had been operating since 15 January, mass swabbing was advised. For administrative reasons this was

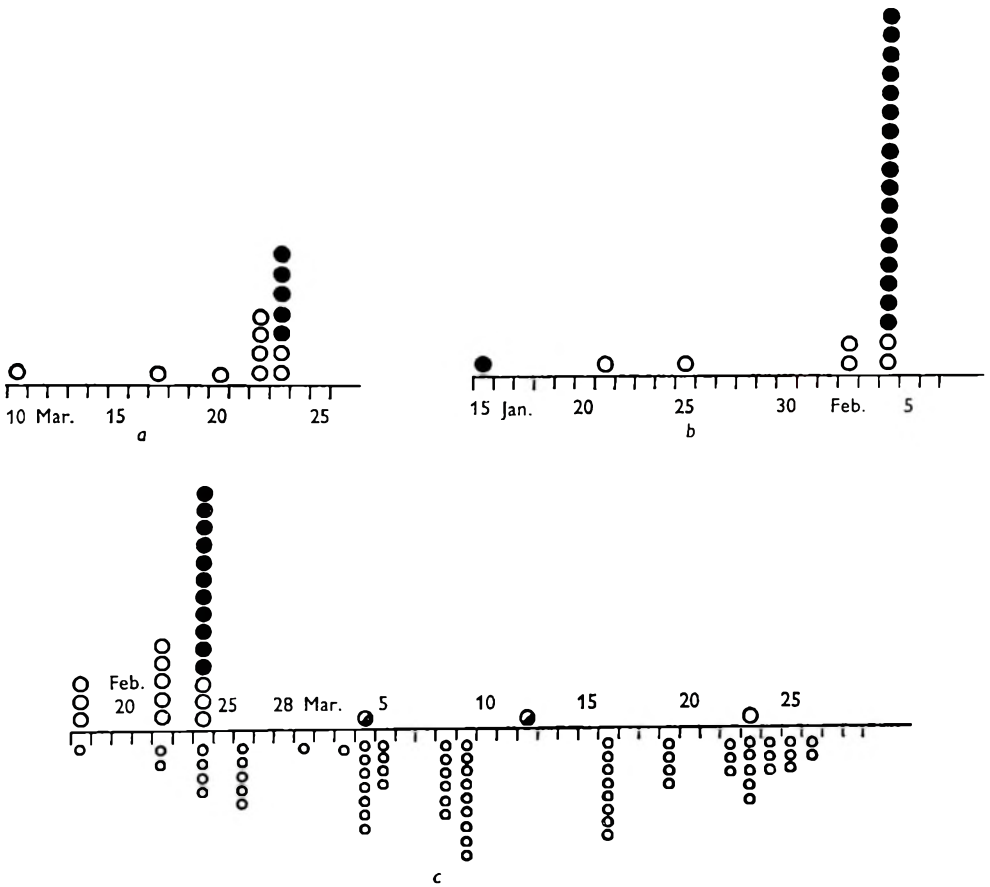

Fig. 2. (*a*) Outbreak 5; (*b*) outbreak 6; (*c*) outbreak 7. Acute infections and dispersers shown as in Fig. 1. ○ (below the line) = cases of non-streptococcal respiratory illness. ◕ see text, page 228.

postponed until 4 February, by which time four more cases had occurred, two on 2 February, and two on 4 February. Seventeen dispersers were found on 4 February. They were treated, and no new cases occurred. About the middle of March, however, there were fourteen cases of upper respiratory disease from whom no bacterial pathogens were isolated, except for one boy with a throat culture positive for type 3 streptococci. It appears highly probable that his symptoms were due to whatever was causing the illness in his fellows at the time and that he was a symptomless throat carrier who would not have been recognized at the mass swabbing, when throat swabs were not taken.

Outbreak 7 was in a boarding school of 340 children. This, like the previous outbreak, was complicated by a non-streptococcal upper respiratory illness, this time concurrent with the streptococcal infections, and the number of such cases was so large that we have shown them in Fig. 2*c* by the small open circles below the zero line. In the figure it is seen that there were eleven cases of acute streptococcal tonsillitis between 18 and 24 February, the day of the mass swabbing. On that day nose and mouth swabs were taken from 324 children and eleven dispersers were found. These were treated. Meanwhile, many cases of sore throat with negative swabs were occurring, but two children, one on 4 March and one on 12 March showed a scanty growth of type 6 streptococci in cultures from their throats. These two are shown in Fig. 2*c* above the line, as half-black circles, to indicate that they were probably cases of non-streptococcal sore throat who happened to be undetected throat carriers. The single case shown on 23 March, however, was a heavy nasal positive and must be considered a new infection. The possible source of this new infection is of some interest. A child who developed an acute infection on 22 February, was treated with penicillin and sent home for a period of convalescence. She returned to school without being re-swabbed, and on 15 March was found to be a heavy nasal carrier. She was then given sulphanilamide insufflation *for the first time*, combined with a second course of penicillin for a suspected infection of the antrum, and was cured. This child may represent the early stage of a chronic disperser of the type found in outbreaks 1 and 2.

No further cases occurred during that term or the next.

DISCUSSION

We have in these seven examples illustrations of two fundamentally different types of outbreak. In one, which we call the limited epidemic, virtually all persons infected from the primary source develop symptoms of acute infection and are as a result removed or treated. The symptomless disperser does not seem to arise. An example of this is seen in outbreak 2, where complete investigation of the disperser's surroundings showed no symptomless infections. The same is probably true of outbreak 1. In both these the elimination of the initiator brought the infections to an end.

This is in contrast to the second type, the unlimited outbreak, in which, whether the initiator is recognizable or not, factors which we believe to be associated with the organisms rapidly give rise to many symptomless dispersers so that the epidemic becomes self-propagating. Removal of the initiator alone is unlikely to end the outbreak. This type is shown in all its details in outbreak 6. In such an outbreak we think that only mass swabbing offers a good prospect of control, but the control still depends on the neutralization of the human dispersers. The environmental contamination appears to be self-limiting, and although a few cases may arise after the neutralization of the dispersers (see Fig. 1*a*, *b*) we would not expect to see any beyond about 10 days. It is infections arising beyond this period that suggest that an undetected disperser is at large. This has been discussed under outbreak 7, and is shown in Fig. 2*c* where, because of the large concurrent

outbreak of non-streptococcal upper respiratory infection, the bacteriological control could hardly have been more extensive.

We have here been considering conditions in which early information about the history of the outbreak and the epidemic type of the infecting organism is available. But it must be made clear that the method of control does not depend on this information, though its availability may on occasions make it possible to reduce the scale of the investigation with safety. Control depends on mass swabbing to recognize dispersers, and where epidemic conditions exist almost all the dispersers found will be of the epidemic type. Though a few persons dispersing strains of non-epidemic type may be included in the treatment group the number is likely to be very small, and treatment can do them no harm.

It is of importance to determine the type of the epidemic strain at some stage in order to establish that control has been effected. Twice we have seen one outbreak succeed another almost without an interval, and only the knowledge of the streptococcal types enabled us to show with certainty that the second epidemic was not a recrudescence due to failure to control the first.

In treating nasal infections, with or without an associated positive culture from the mouth, we have used a well-tried method because we have found that it succeeds. We do not believe that this is the only method, and would welcome an equally effective alternative to sulphanilamide powder, if only for use when we meet a sulphonamide-resistant strain of streptococcus. But we have not yet failed to clear up a nasal infection, and this may be because of the very high concentration achieved by local application of a sulphonamide with a relatively high solubility (1/170 in water for sulphanilamide). It is reasonable to suppose that organisms that are resistant to the concentrations reached in the body fluids during oral treatment (1/20,000–1/50,000 approx. in the blood) may yet respond to the much higher concentrations achieved by local application of sulphanilamide.

The numbers of dispersers positive in the mouth alone is relatively small, and though they are undoubtedly of importance as sources of infection while the saliva is heavily infected this degree of infection diminishes very rapidly with the passage of time alone. In most of our work we have included them in the nasal insufflation group without additional therapy because it was the simplest thing to do administratively, because treatment could do them no harm and might do some good by preventing a nasal or a middle ear infection from arising and because at the end of their week of treatment they would in any case be nearing the end of the period when they would be recognized, by our methods, as dispersers. We have in certain circumstances, such as children sitting a public examination, advocated penicillin lozenges for 2 days, but this is entirely in the interests of the contacts. We doubt its value as a therapeutic measure in effecting the cure of a tonsillar infection. An efficient alternative capable of making the saliva non-infective would be welcomed.

We have, throughout this account, advocated chemotherapeutic measures of control because we know of no other, except strict exclusion from school of all dispersers. Since nasal carriers may remain positive for weeks, months or even years this is clearly an unsuitable method. In general terms, however, we state

emphatically that, in our opinion, control can be achieved by finding all dispersers as we have defined them and by dealing with them by any means available that will prevent them from acting as dispersers.

SUMMARY

A method of controlling outbreaks of streptococcal infection of the upper respiratory tract in communities such as boarding schools is described.

It is suggested that, in a search for carriers, nose and mouth swabs should be taken in place of the customary nose and throat swabs. These swabs will detect those who are dispersing streptococci into the environment, as distinct from the carriers who are not dispersers. It is essential that infections of the skin and ears should also be looked for.

It is recommended that all dispersers, whether nose or mouth positive, should receive sulphanilamide insufflation in both nostrils twice daily for a week, or longer if necessary, without exclusion from school. In addition, penicillin lozenges for 2 days may be recommended for the mouth positives only.

The history is considered to be an important factor in deciding the extent of the initial investigation, and examples are given to illustrate this. When mass swabbing is considered necessary, it is highly desirable that the whole community should be swabbed at the same time, as early as possible in the outbreak. It is also desirable that bacteriological follow-up should be available to check that control has been effected.

Our gratitude is due to all School Medical Officers and Matrons who have helped us in this work by providing facilities for our investigations. In particular we thank Dr M. E. Hocken, Dr C. H. Budd, Dr A. A. Craigen, Dr Margaret Reed and Dr K. Lumsden for their help and encouragement, and Mrs Rita Spaxman for typing the many thousands of strains of streptococci on which our observations are based.

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Quantal and graded dose-responses of bluetongue virus: a comparison of their sensitivity as assay methods for neutralizing antibody*

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INTRODUCTION

For the quantification of virus-neutralizing antibodies, various procedures for obtaining quantal, graded or enumerative responses have been employed (Bryan, 1957). When an enumerative response, based on quantitative pock or plaque-counting methods, is not available, a 50% quantal response, recording an all-or-none reaction, is commonly used. In such a response, based on extinction dilution titrations, each animal or test tube provides only a positive or negative reading. In contrast, in a graded response, which uses a continuous variate such as survival times, each host unit allows a numerical estimation of the virus, or indirectly antibody quantity (Smith & Westgarth, 1957). Therefore, almost twice as many host units are required for bioassays of a certain accuracy in the quantal as in the graded response from which the quantal response is derived.

The purpose of the present study was first to compare the sensitivity of quantal and graded responses to bluetongue virus for the detection of homologous antibody. Secondly, we wanted to analyse these responses to three bluetongue virus variants and in particular to determine the adherence of the quantal responses to the one-or-more particle curve of the Poisson distribution. When comparing the sensitivity of the two responses for antibody detection, the data were analysed by the probit (Bliss, 1952; Finney, 1952) and rankit (Ipsen & Jerne, 1944) methods.

MATERIALS AND METHODS

Viruses

The test virus, California isolate BT_8, was obtained from Dr C. J. York, Indianapolis, Indiana, at the 80th passage level as a suspension of infected chicken embryos. White Leghorn eggs were supplied from a single hatchery.

Embryonated eggs, incubated at 37° C. for 7 days, were inoculated by the yolk-sac or stab method (Gorham, 1957) with a virus suspension containing $10^{2\cdot7}$ egg lethal doses for 50% (ELD 50). After incubation at 34° C. for 72 hr., whole embryos

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were harvested, and a 1/5 tissue suspension (v/v) was prepared in Difco nutrient broth pH 6·8 containing 250 i.u. penicillin and 100 μg. streptomycin per ml. The suspension was clarified by low-speed centrifugation and the supernatant fluid stored at $-60°$ C. in sealed glass ampoules. This stock virus preparation represented the 180th embryonated egg passage.

The egg-propagated bluetongue virus (EBTV) was adapted to the intracerebral route in unweaned mice (Svehag, 1962). For preparation of a mouse-adapted bluetongue virus (MBTV) stock, Webster Swiss albino mice of 3–4 days of age were inoculated intracerebrally with an estimated $10^{2·7}$ mouse lethal doses for 50% (MLD 50) of virus and killed when moribund. A 1/5 dilution of brain material was prepared in nutrient broth, the suspension was clarified and the supernatant stored at $-60°$ C. The stock virus used in this study represented the 53rd serial mouse passage.

Infectivity assays

Two inoculation techniques were used for the chicken embryos: the stab method (Gorham, 1957), in which several embryonic structures received virus, or the yolk-sac route. The eggs were inoculated with 0·2 ml. virus and incubated at 37° C. for 7 days, at which time all eggs were opened and inspected so that non-specific deaths could be differentiated from deaths caused by the virus. Embryos succumbing to EBTV infection usually have a cherry-red colour. For plotting the graded response infected eggs were candled at 6 hr. intervals from 30 to 180 hr. post-inoculation (p.i.). Infectivity titres were calculated by the method of Reed & Muench (1938) or by probit analysis (Bliss, 1952; Finney, 1952) and are expressed in $\log_{10}$ ELD 50 per ml. undiluted chicken embryo homogenate.

Mouse-adapted virus was propagated by inoculation (0·02 ml.) into the left cerebral hemisphere of 3- to 4-day-old mice. In determining the graded response to MBTV, the mortalities were recorded every 4th hour from 36 to 124 hr. p.i. The quantal response as recorded by death or survival of inoculated mice was obtained 9 days p.i. The methods of Reed and Muench or probit analysis were used for calculating 50% end-points and titres are expressed in $\log_{10}$ MLD 50 per ml. undiluted mouse brain homogenate. The virus stock had a geometric mean titre in $\log_{10}$ MLD 50 per ml. of 8·5 and the standard error of the mean was 0·056.

Sera

Antiserum was prepared in a sheep by three subcutaneous inoculations of about 50,000 MLD 50/dose of MBTV. The animal was bled before immunization and 2 weeks after the final virus injection. The sera were stored at $-25°$ C. without preservatives.

EXPERIMENTS AND RESULTS

I. *Analysis of quantal and graded dose-responses to bluetongue virus*

(1) *Quantal dose-responses for bluetongue virus variants.*

Quantal dose-responses obtained with BTV in three different *in vivo* systems were compared with theoretical dose-response curves, constructed according to the Poisson distribution for the random variation of particles in virus inocula

(Fig. 1). The latter curves represent the frequencies of samples containing at least 1, 2, 3, 4 and 5 virus particles for various average numbers of particles per inoculum. The responses in the three *in vivo* systems are plotted so that the fitted curves coincide at 50 %.

The response data fit best to the 'one-or-more particle' Poissonian curve. The results obtained with mouse-adapted virus show a high degree of goodness of fit to this curve when the differences between observed and expected frequencies are

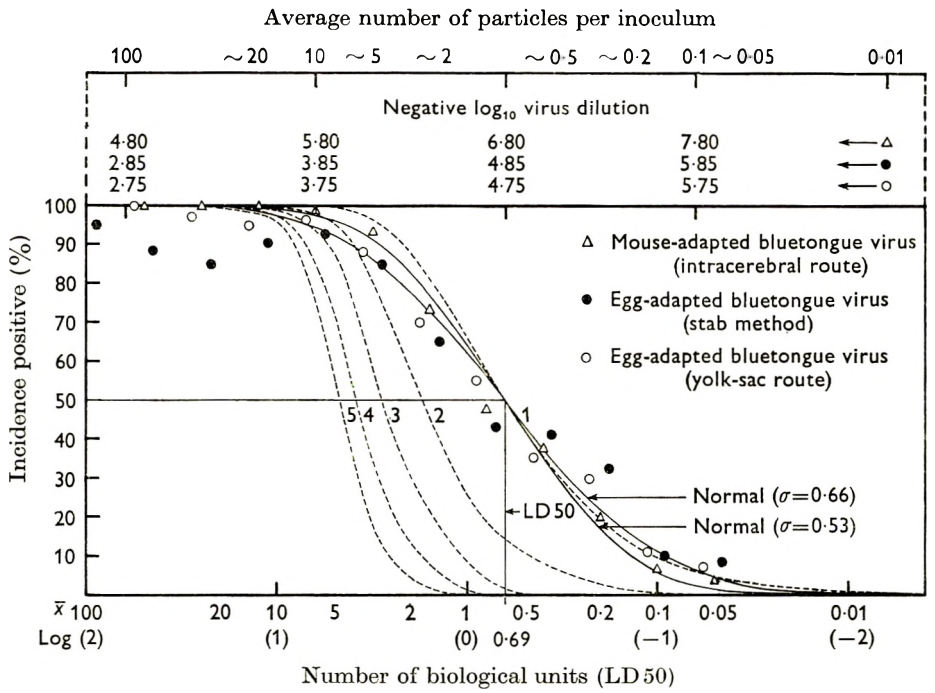

Fig. 1. Quantal dose-response curves of three bluetongue virus variants compared with the theoretical Poisson curves for samples containing at least 1, at least 2, etc., virus particles and with integrated normal distributions with standard deviations (σ) of 0·53 and 0·66. Number of animals: (△) 165, (●) 180, (○) 176.

tested with the χ^2-test. However, a satisfactory approximation to the same data is also given by a cumulative normal distribution curve with a standard deviation of about 0·5 in $\log_{10}$ dose units. The curves fitted to the responses of the other two virus variants are flatter and the responses are more irregular. Moran (1954*a*, *b*) has described a test specifically designed for the detection of heterogeneity. This test is particularly useful when twofold dilution series, which cover a response range from zero to nearly 100 %, are employed. When this test is applied to the data for the yolk-sac route, the fit to the theoretical 'one particle' curve is not acceptable (T = 334, $E(T)$ = 240, S.E. (T) = 35·14 and $\{T-E(T)\}$/S.E. (T) = 2·7; $P < 0{\cdot}01$). However, the results obtained with the two egg-adapted virus variants show a good fit to normal distribution curves with slightly greater standard deviations (0·66 for the yolk-sac route and 0·76 for the stab method).

The response to virus propagated by the stab method fits a normal curve only within the response range 0–80 %.

A good fit to the first term of the Poisson distribution, as illustrated by data for MBTV, has usually been interpreted to indicate that a single virus particle is capable of initiating infection (Bryan, 1959). The assumption is made that the response curve only reflects the distribution of virus particles in the inoculum and is unaffected by variations in virus strains or host factors. An experiment was designed to test the hypothesis that the chance variation of one-or-more virus particles in inocula is the only random variable that can account for a curve of this type.

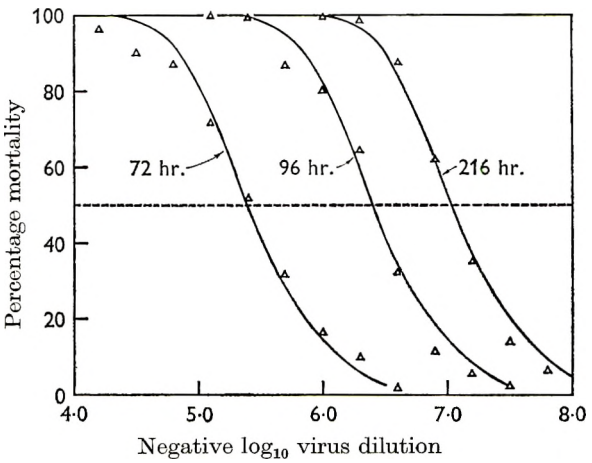

Fig. 2. Quantal responses (△) to mouse-adapted bluetongue virus recorded at 72, 96 and 216 hr. post-inoculation. Solid lines represent Poisson 'one-or-more' particle curves superposed at the observed 50 % mortality levels. Number of mice: 143.

The quantal response (percentage mortality) to serial twofold dilutions of MBTV, inoculated intracerebrally in 143 mice, was recorded at 72, 96 and 216 hr. after inoculation (Fig. 2). The character of the response curve was essentially the same at these successive readings although the 50 % infectivity end-point increased with time. The concept that the lower tail of the response is due to the rare distribution of single virus particles in the inoculum does not seem to be compatible with this gradual change in the position of the response curve. For instance, the 72 hr. reading shows that the probability of having at least 1 virus particle in an inoculum from the 10^{-6} dilution is quite low, while according to the 216 hr. reading this occurs with a probability of 1 (causing a 100 % mortality). Therefore, the curves cannot be explained by the random variation of one-or-more virus particles in virus inocula.

The main data obtained with all three virus variants are fitted by integrated normal distribution curves. Such curves can be converted to linear form by the use of probits as response metameter (Fig. 3). The curves for the egg-adapted virus variants are more shallow and the responses more irregular than for the mouse-adapted variant. Rather than fit a curvilinear regression to the data of the egg-adapted variant (yolk-sac), the curve was divided into two linear segments to

obtain a so-called truncated normal distribution (Bliss, 1937; Ipsen, 1949; Bryan, 1956; Svehag, 1962). Possible explanations for the truncation are the presence of a few highly refractory animals in an otherwise uniformly susceptible group of

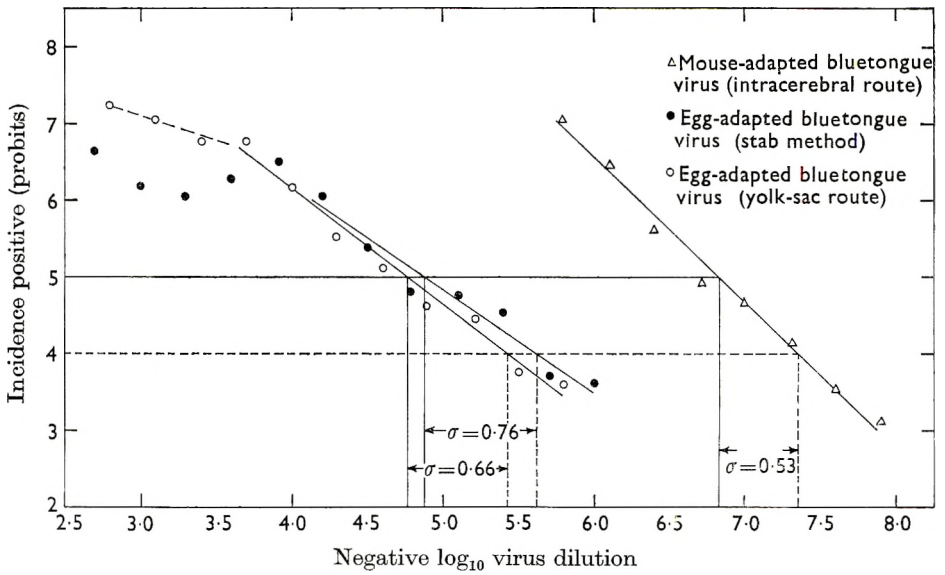

Fig. 3. Quantal dose-response curves of three bluetongue virus variants converted to linear form by the use of probit transformation. σ = standard deviation. Number of animals: (△) 120, (●) 180, (○) 176.

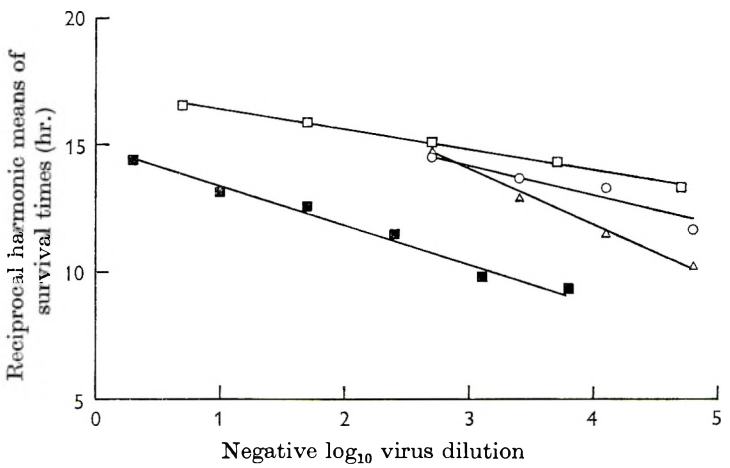

Fig. 4. Graded dose responses for two bluetongue virus variants plotting reciprocal harmonic means of survival times against virus dilutions. ■, Egg-adapted virus (yolk-sac route); □, mouse-adapted virus; ○, mouse-adapted virus and normal serum ($10^{-5\cdot5}$); △, mouse-adapted virus and antiserum ($10^{-5\cdot5}$). Number of animals: (■) 75, (□) 95, (○) 60, (△) 60.

animals or of reversible aggregates of virus in the lower virus dilutions. The second possibility is less likely since the mouse-adapted virus variant, when assayed in eggs (yolk sac), also gave a truncated distribution as did a partly purified EBTV

preparation. Thus, the truncation appeared to be associated with the assay system and was best explained by the existence of a few highly resistant chicken embryos.

(2) *Graded dose-responses for bluetongue virus variants.*

Before we could proceed with the experiments described in section II, it was necessary to analyse the graded responses with respect to survival times to the virus variants. Fig. 4 illustrates such graded responses to varying amounts of virus. In an attempt to detect a function of the response which would yield a straight line, the plotting of reciprocal harmonic mean times against log virus dilution (Gard, 1940; Mandel & Racker, 1953) was found to give the best fit to such a line. A rectilinear relationship simplifies statistical analysis, and the slope of the response lines was a necessary parameter for the subsequent studies.

Table 1. *Summary of results graphically illustrated in Figs.* 5 *and* 6

Test	Serum dilution	No. animals	No. positives*
Virus† + IOS	$10^{-4\cdot3}$	48	9
	$10^{-4\cdot6}$	47	18
	$10^{-5\cdot5}$ $10^{-5\cdot8}$ $10^{-6\cdot1}$	48	30
	$10^{-6\cdot4}$	48	31
Virus + NOS	$10^{-4\cdot3}$	48	29
Virus‡ + IOS	$10^{-5\cdot5}$	95	25
Virus + NOS	$10^{-5\cdot5}$	60	16

* At five different dilutions of the virus-serum mixtures.
† Test dose: 100 MLD 50.
‡ Test dose: 2 MLD 50.
IOS, immune ovine serum; NOS, normal ovine serum.

II. *A comparison of the sensitivity of quantal and graded responses for the detection of antibody*

(1) *The quantal response.*

The approach used was simply to find the lowest dilution of a reference antiserum that showed no antibody activity in an assay based on a quantal response and then retest this particular dilution by the graded response method. It was therefore important that the quantal response data be obtained under conditions which were optimal for the detection of antibody.

An ovine antiserum was used as reference serum. Eight serial twofold dilutions of this serum ($10^{-4\cdot3}$ to $10^{-6\cdot4}$) were each incubated with 100 MLD 50 of virus at 37° C. for 24 hr. The virus control consisted of a pre-immunization serum sample from the sheep (diluted $10^{-4\cdot3}$) and 100 MLD 50 of virus (Table 1). After incubation all virus-serum mixtures were placed in an ice bath, diluted in six twofold increments and immediately titrated in unweaned mice using eight animals per dilution.

The resultant quantal responses to the different virus-serum mixtures were compared when the percentage death was transformed into probits and plotted against the logarithm of the dilution of the virus-serum mixtures (Fig. 5). The reasonably good fit to straight lines in the probit analysis indicated that the

response to each virus-serum mixture reflected a host susceptibility that followed a normal distribution. This was in agreement with the results for the MBTV preparation in Fig. 3. The regression coefficients (b) of the lines in Fig. 5, and the corresponding standard deviations of the tolerance distributions (σ) were determined graphically and found not to be significantly different. Therefore, the response lines could be considered parallel and differences in virus titres directly given by

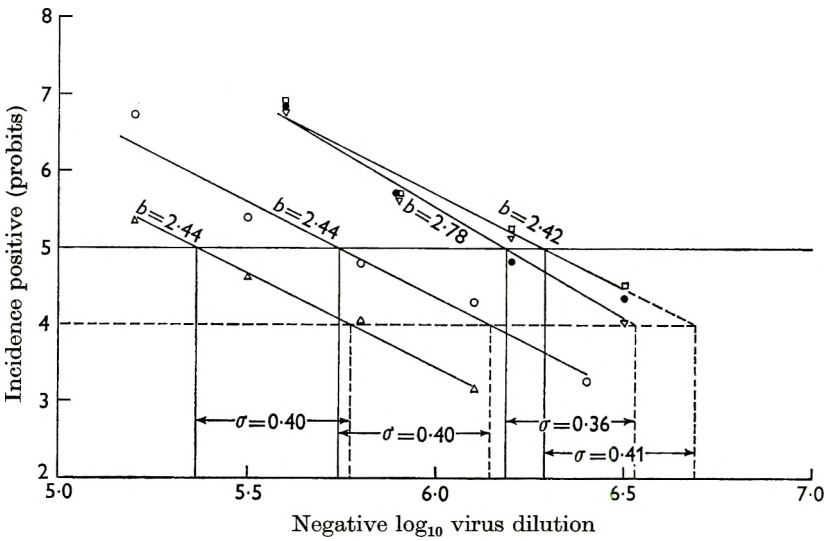

Fig. 5. Probit analysis of quantal response data to seven mouse-adapted bluetongue virus-serum mixtures. △, Virus and antiserum ($10^{-4\cdot3}$) ○, virus and anti serum ($10^{-4\cdot6}$), ▽ virus and antiserum ($10^{-5\cdot5}$, $10^{-5\cdot8}$, $10^{-6\cdot1}$); □, virus and antiserum ($10^{-6\cdot4}$); ●, virus and normal serum ($10^{-4\cdot3}$); σ = standard deviation. The virus test dose was 100 MLD 50. Number of mice: (△) 48, (○) 47, (▽) 48, (□) 48, (●) 48.

the displacements of the lines at the probit value 5. The probit slope values of all four responses were higher than those expected according to the independent active theory (Meynell, 1957) as under this theory the slopes should be equal to or less than 2.

In the presence of the lowest dilution of antiserum ($10^{-4\cdot3}$) virus did not produce a 100% positive response and the 50% infectivity endpoint was as low as $10^{-5\cdot3}$. With higher dilutions of antiserum the response lines moved closer to the virus control (virus plus normal serum). For the sake of clarity, the lines representing virus plus the serum dilutions $10^{-4\cdot9}$ and $10^{-5\cdot1}$ were not included in the figure but fell between the $10^{-4\cdot6}$ and $10^{-5\cdot5}$ lines. The straight line representing the best fit to the responses of virus plus antiserum of the final dilutions $10^{-5\cdot5}$, $10^{-5\cdot8}$ and $10^{-6\cdot1}$ was almost identical with the line for the virus control. Thus, these virus-serum mixtures evoked the same percentage death and the graphically estimated 50% end-points were the same, indicating that in the quantal response antibody was not detectable when the antiserum was diluted equal to or more than $10^{-5\cdot5}$.

The test dose of virus used in quantal responses influences the sensitivity of the test for detection of antibody; a smaller virus dose increasing the sensitivity. The $10^{-5\cdot5}$ dilution of antiserum was therefore retested by the quantal response method

against an estimated 2 MLD 50 of virus (Table 1). This experiment was run as a concurrent control with the graded response test described in the next section of this paper. A probit analysis of the results (Fig. 6) revealed no differences between the responses to this virus dose and normal serum and antiserum of the $10^{-5\cdot5}$ dilution respectively. Thus, with this small test dose of virus no neutralizing antibody was demonstrable in this serum sample by the quantal response method.

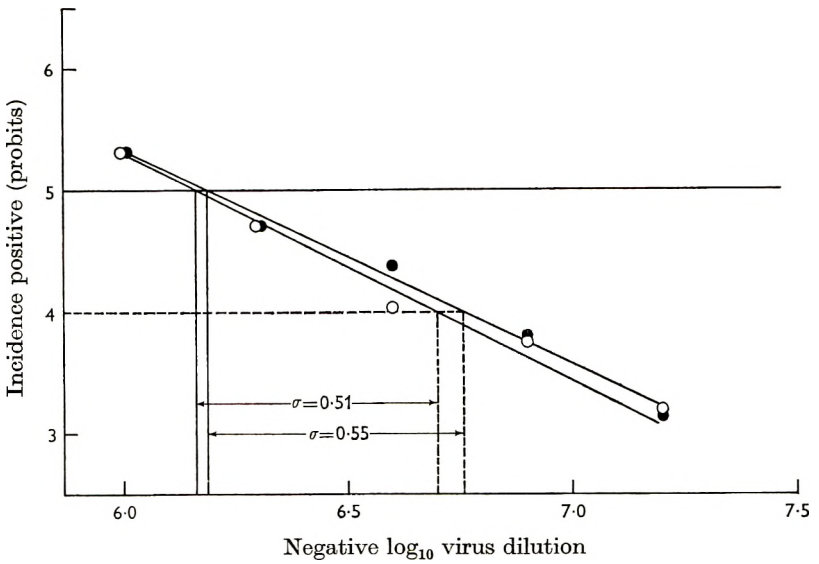

Fig. 6. Probit analysis of quantal response data to two mouse-adapted bluetongue virus-serum mixtures. ○, Virus and antiserum ($10^{-5\cdot5}$); ● virus and normal serum ($10^{-5\cdot5}$). The virus test dose was 2 MLD 50. σ = standard deviation. Number of mice: (○) 95, (●) 60.

(2) *The graded response.*

The $10^{-5\cdot5}$ dilution of the antiserum, saved in a frozen state from the previous experiments, was also tested for the presence of viral neutralizing antibody by the graded response method. Four fivefold virus dilutions were each divided into two portions and incubated for 24 hr. at 37° C. with antiserum and normal serum (both diluted $10^{-5\cdot5}$) respectively. After incubation all serum-virus mixtures were placed in an ice-bath and immediately assayed in unweaned mice using sixteen animals per reaction mixture. The time of mouse death was recorded at 4 hr. intervals until all mice had succumbed. To allow a graphical and statistical evaluation, the data had to be tested to ascertain whether all the graded responses to time after inoculation with serum-virus mixture showed normal and similar distributions. Therefore, the arithmetic means and standard deviations of the survival times for the responses to virus plus normal serum or antiserum were calculated and multiples of the standard deviations were used as abscissae for two histograms (Fig. 7). Two other histograms representing normal distributions with the same standard deviations and number of observations as in the two test groups were superimposed upon the experimental data. When the observed frequencies were compared in the

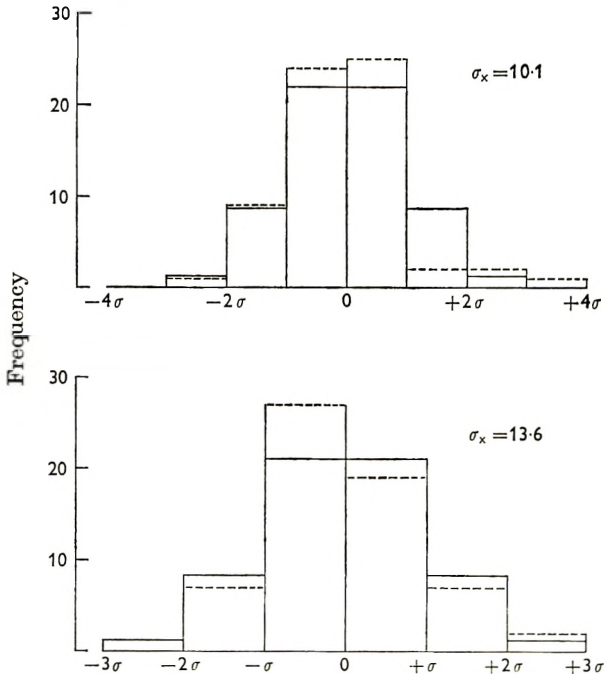

Fig. 7. Variation of susceptibility between mice. The histograms show the distributions of survival times to mouse-adapted virus and normal serum (top part) and virus and antiserum (bottom part) respectively. The solid lines represent normal distributions with the same standard deviations (σ) and number of observations as in the two test groups. Number of mice: top histogram 64, bottom histogram 62.

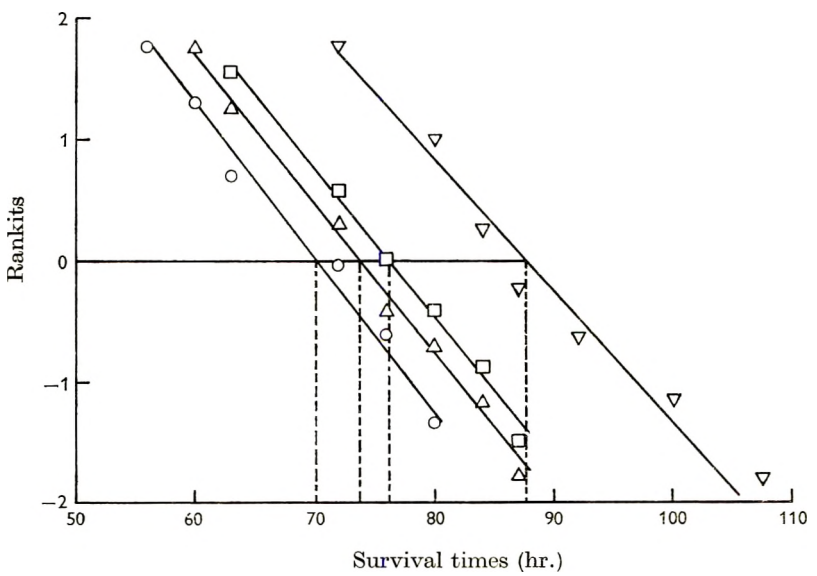

Fig. 8. Rankit analysis of survival times to different dilutions of mouse-adapted virus and normal serum ($10^{-5\cdot5}$). ○, Virus dilution $10^{-2\cdot7}$; △, dilution $10^{-3\cdot4}$; □, dilution $10^{-4\cdot1}$; ▽, dilution $10^{-4\cdot8}$. Total number of mice 63.

χ^2-test with those expected according to the corresponding normal distributions, there was no significant difference ($\chi^2_{(3)} = 4{\cdot}3$; $0{\cdot}3 > P > 0{\cdot}2$ and $\chi^2_{(3)} = 2{\cdot}7$; $0{\cdot}5 > P > 0{\cdot}4$) indicating that the survival times followed distributions indistinguishable from normal distributions. The mean survival time for responses to virus plus antiserum was 5 hr. longer than for virus plus normal serum, a difference which was significant at the 5 % level ($t = 2{\cdot}34$; $0{\cdot}05 > P > 0{\cdot}01$). The response to virus and antiserum showed the highest standard deviation.

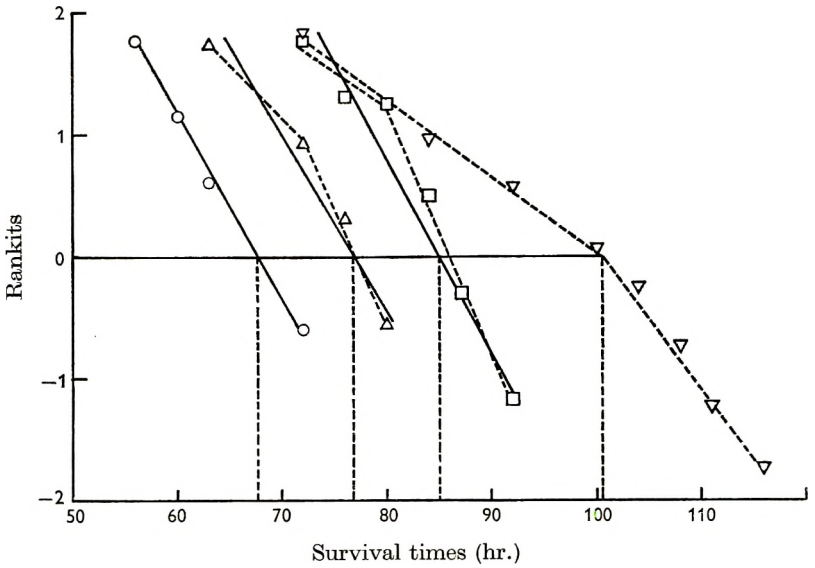

Fig. 9. Rankit analysis of survival times to different dilutions of mouse-adapted virus and antiserum ($10^{-5{\cdot}5}$). ○, Virus dilution $10^{-2{\cdot}7}$; △, dilution $10^{-3{\cdot}4}$; □, dilution $10^{-4{\cdot}1}$; ▽. dilution $10^{-4{\cdot}8}$. Total number of mice 63.

It was then tested whether the graded response data within varying dilutions of virus also reflected normal distributions of susceptibility and if a certain virus antiserum mixture was mainly responsible for the change in the overall mean survival time. Therefore, a rankit-analysis (Ipsen & Jerne, 1944) of the time-frequency responses to virus plus normal serum or antiserum was performed (Figs. 8, 9). For virus plus normal serum (Fig. 8), the relationships between time and rankit appeared to be linear, showing that the times were approximately normally distributed. Further, since the standard deviations of the different responses were similar (parallel lines), the means of the survival times could be directly compared. In the analysis of the responses to virus and antiserum (Fig. 9), dilute virus inocula appeared to give slightly truncated response curves. This suggested change from a linear to a truncated response indicated a change in the action of the inoculum. At the $10^{-4{\cdot}8}$ dilution, the range and the variance of the response was much greater than at the lower virus dilutions. With this virus dose, the first death occurred as early as in the corresponding group in the control with normal serum (Fig. 8), but most animals exhibited distinctly prolonged survival times. Table 2 gives the number of observations, the mean survival times, their

standard errors and the standard deviations of the survival times. When the mean survival times of the responses to virus plus normal and immune serum respectively were compared by the *t*-test, significantly prolonged survival times were observed in response to the two highest virus dilutions plus antiserum.

In the graded response to time with mouse-adapted bluetongue virus, a linear relationship exists, over an intermediate dose range, between the reciprocal harmonic means of survival times and virus dilutions (Svehag, 1962). Also, in this kind of plot (Fig. 4), the effect of the antiserum was observed only in the higher virus dilutions.

Table 2. *Analysis of the survival times to various test doses of bluetongue virus and dilute normal and immune sheep serum respectively*

($\bar{Y}$, Mean survival time; σ, standard deviation; $\sigma_{\bar{Y}}$, standard error of the mean; N.S., not significant; S., significant.)

	Virus plus normal sheep serum				Virus plus immune sheep serum				
Log_{10} virus dilution	No. observations	$\bar{Y}_1$	σ	$\sigma_{\bar{Y}_1}$	No. observations	$\bar{Y}_2$	σ	$\sigma_{\bar{Y}_2}$	*t*-test for $\bar{Y}_1 = \bar{Y}_2$
−2·7	16	70	8	2	16	68	7	1·8	N.S.*
−3·4	16	73·5	7·5	1·9	16	77·5	7	1·8	N.S.**
−4·1	16	76	8	2	16	85	6·5	1·6	S.**
−4·8	16	87·5	9	2·3	15	101	8·5	0·94	S.***

DISCUSSION

When samples are taken at random from a homogeneous virus suspension and every sample containing at least one infectious virus particle produces a visible sign of infection, the quantal dose response curve is expected to be Poissonian. The fact that only a fraction of the virus particles may be infectious does not affect the Poissonian character of the curve. The assumption is made that the host system is uniformly susceptible.

Deviation of observed quantal dose-response curves from the theoretical one-or-more particle Poisson curve have, however, been observed in some virus-host systems (Bryan & Beard, 1940; Meynell, 1957; Fazekas de St Groth & Cairns, 1952; Armitage & Spicer, 1956). In particular, the slopes have been significantly less than that of the Poisson curve. Parker's response curves with vaccinia virus (1938) also showed shallower slopes than those expected from the Poisson distribution. In contrast, his data gave a very good approximation to normal distribution curves. Similar results were obtained with the two egg-adapted variants of bluetongue virus in the present study.

Factors considered responsible for the deviations from the theoretical exponential curve were reversible aggregation of virus particles and variations in susceptibility among host units. It has been suggested that a shallow slope could be due to the presence of reversible aggregates of virus particles that dissociate into smaller aggregates on stepwise dilution (Price, 1946). This explanation was unlikely in the

present study since one and the same MBTV preparation yielded data which in one system (intracerebral route) agreed well with the theoretical 'one particle curve', while in another (yolk-sac route) the response curve was significantly shallower. Further, both ultracentrifugally purified and unpurified EBTV gave curves with slopes less than the Poisson curve when administered by the yolk-sac route.

The type of truncation illustrated in Fig. 3 is probably due to the existence of a few more resistant chicken embryos. The presence of individuals with increased resistance will show up particularly in the upper tail of the curve as 100% mortality is expected here. This heterogeneity in the test population will mask the effect of the statistical distribution of infectious virus particles. Had an interferon effect been involved, this would have been expected to affect the response curve in the high dilution range.

There are only a few reports in the literature of dose-response curves which are steeper than the 'one particle curve'. They have been associated with complicating factors in the assay system, e.g. toxic effects of influenza (Henle & Henle, 1946) and adenovirus preparations (Pereira & Kelly, 1957) in mice and HeLa cell cultures respectively. Thus, the theoretical one-or-more particle curve came to be generally recognized as the limiting curve of error for quantal responses to viruses. Yet, too much stress should not be laid on the fact that good agreement exists between an experimental dose-response curve and a theoretical curve based on the assumption that at least one infectious virus particle is necessary to produce visible signs of infection. Factors other than the statistical distribution of virus particles may be responsible for or affect the observed distribution. The finding in the present study that the character of the quantal responses to MBTV was the same at three successive readings even though the infectivity end-point gradually increased did not support the assumption that the response curves only reflected the distribution of virus particles in inocula. The data are, however, consistent with the more general version of the independent active theory (Meynell, 1957) which assumes that each particle has a probability P of initiating an infection. The different curves in Fig. 2 would correspond to different values of P. Pereira & Kelly (1957) similarly reported that the character of quantal dose-response curves to adenovirus in HeLa cell cultures remained the same at three successive readings but varied in position with time.

The quantal dose-response curves to the EBTV variants had the appearance of cumulative normal distribution curves and could consequently be converted to linear forms by the use of probits (probability units) as the response metameter. Probit transformation (Bliss, 1952) is merely a statistical device for transforming the bell-shaped or sigmoidal normal distribution curve into a straight line by the proper choice of coordinates. The probit method permits good utilization of the data, including the 5–95% response range and easy graphic estimation of the 50% dose effect and the standard deviation of the distribution. Further, the slope of the response line represents a valuable new parameter.

Normality must be assumed when probits are used. Although the MBTV data showed no exact fit to a normal distribution, the approximation was sufficiently

close to justify the application of probit transformation also to results obtained with this virus variant. The relatively steep probit line obtained with MBTV was advantageous as the accuracy of a dose-response assay largely depends on the steepness and reproducibility of the probit-regression line—a steep slope permitting more precise quantification. Therefore, in the comparison of the sensitivity of quantal and graded responses to BTV for the detection of specific antibody, the response to the MBTV variant was employed.

It is advantageous for statistical analysis to work with response data which are linearly related to dosage or dosage transformed to other units. When the percentage response to MBTV was expressed as probits and virus doses as their logarithms, the relationship was linear. In the graded response a linear relationship was obtained when the rank order of observed data was transformed into rank units (Ipsen & Jerne, 1944). The plotting of reciprocal harmonic mean times against the logarithms of the virus doses was also found to result in a linear relationship. For comparing the sensitivity of quantal and graded responses, the rankit transformation was preferred.

The distributions of individual graded responses about their mean value are frequently unsymmetrical and the frequency distribution may tail out toward the higher measurement values as in Fig. 7. The survival times to MBTV, however, did not deviate significantly from a normal distribution. Further, as the standard deviations of the responses to different doses of virus were similar, the amount of antibody present in a certain virus-serum mixture could be estimated by reference to a control preparation of virus and normal serum. The average test dose of virus used in the quantal response (100 MLD 50) was less than that in the graded response (equal to or more than 1000 MLD 50) which might be expected to render the quantal response more sensitive for the detection of antibody. In spite of this, the graded response to time was found to be superior for the detection of minor amounts of antibody to BTV.

SUMMARY

The sensitivity of quantal and graded responses to mouse-adapted bluetongue virus for the detection of neutralizing antibody was compared using probit and rankit analysis. The graded response, based on survival times, allowed the demonstration of antibody in highly dilute serum, in which antibody was not detected by the quantal response recording percentage death.

Quantal responses to bluetongue virus variants were compared with theoretical dose-response curves constructed according to the Poisson distribution for the random variation of virus particles in inocula. Of these theoretical curves the first term in the Poisson distribution gave the best approximation to the experimental data but the fit to normal distribution curves was better. The quantal responses to bluetongue virus did not appear to reflect the random variation of one-or-more infectious virus particles in inocula.

In graded responses to bluetongue virus, a rectilinear relationship was observed between reciprocal harmonic means of survival times and log virus dilutions.

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Salmonellae and shigellae in a group of urban South African Bantu school children

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INTRODUCTION

In South Africa, as elsewhere, salmonellosis and shigellosis are recognized public health problems. These infections are usually claimed to predominate in the underprivileged section of the population. In view of this, Bokkenheuser & Richardson (1960) examined at regular intervals a group of rural Bantu school children living under primitive conditions approximately 13 miles from Rustenburg, a town 80 miles west of Johannesburg. Water was obtained from shallow, heavily infected surface wells. Medical clinics were not available within 8–10 miles. Many of the children were infected with salmonellae and shigellae and it was concluded that every child experienced at least one annual infection with these pathogens. Many had multiple infections.

A similar study was undertaken in a group of periurban Bantu school children from poor though economically somewhat better homes in the Witkoppen area about 15 miles north of Johannesburg (Richardson & Bokkenheuser, 1963). The water was unsatisfactory bacteriologically, but a clinic was available one morning per week. Among the children examined the prevalence of salmonellae and shigellae was only slightly lower than among the children in the Rustenburg district and, once again, it appeared that all children experienced at least one annual infection, and many of them several infections.

This paper records a third study among Bantu school children living under urbanized conditions in a Johannesburg township with excellent water and ample clinic facilities.

MATERIAL AND METHODS

Two schools, situated in the Dube township, were chosen for this investigation. The township belongs to the Soweto complex; a housing scheme built by the Government, Municipality and other bodies, to accommodate a population of 480,200 employed in various walks of life in Johannesburg. Soweto is about 15–20 miles south-west of the centre of the city and essential services are administered by the Johannesburg Municipality. Piped water from the Rand Water Board is available in each house. A water-borne sewerage system serves both schools and the homes of the children. Clinics and Baragwanath Hospital (2300 beds) are within easy access.

According to Dr A. R. P. Walker of this Institute, although the diet of the

children varies from family to family, the following may be regarded as fairly representative. *Breakfast* (taken about 7 a.m.): soft porridge (maize or 'Kaffir corn'), with sugar but seldom with milk; occasionally sour porridge (*magou*); half a slice of bread with margarine, butter or peanut butter, or sometimes jam; tea with sugar and condensed milk (occasionally fresh milk). *Mid-morning* (at school, 11 a.m.): fat cakes, or doughnuts (bought locally), one slice of polony occasionally. *Lunch* (after school, 3 p.m.): 'stiff' porridge, soup (beans, or other vegetables). *Evening meal* (8 p.m.): 'stiff' porridge, meat or occasionally fish, vegetables, and tea, usually with condensed milk. According to season, fruit is consumed fairly frequently, usually at weekends. Speaking generally, the diet consumed is adequate, both with regard to calories and gross protein; it is low in animal protein and fat. According to orthodox recommended allowances, it is low in certain mineral elements (e.g. calcium), and certain vitamins (e.g. B complex and D).

For comparison with the two previous studies, 130 children were randomly selected, fifty-six, aged between 7 and 10 years, from a primary school called Sizaneni, and seventy-four, aged 11–18 years, from a junior school, Sholom Manne. The schools are within half a mile of each other.

Faeces from the children were examined 8 times during the year at fairly regular intervals. On each occasion oral temperatures were taken and the consistency of the stools noted. The specimens were then planted on S.S. agar and in selenite F medium, and a portion was placed in a sterile bottle for parasitological examination. Plates and enrichment media were taken to the laboratory in Johannesburg and incubated overnight. The following morning, a loopful from the selenite F medium was planted on S.S. agar and incubated. From each plate three non-lactose-fermenting colonies, if present, were tested for biochemical reactions and those conforming to salmonellae were typed serologically. Shigellae were classified according to their group antigen. A maximum of 6 colonies per specimen could thus be examined.

Using the disk method, the organisms were tested for sensitivity to the following antibiotics: ampicillin (25 μg.), cloxacillin (5 μg.), streptomycin (100 μg.), tetracycline hydrochloride (50 μg.), chloramphenicol (25 μg.), erythromycin (50 μg.), colistin (10 μg.), novobiocin (50 μg.), kanamycin (50 μg.), nitrofurantoin (100 μg.). A zone of inhibition of less than 2 mm. from the edge of the disk was taken to indicate a resistant organism. An Oxford strain of *Staphylococcus aureus* was used routinely as a control.

For parasitological investigation, the stools were examined by the merthiolate–iodine–formaldehyde (MIF) method (Sapero & Lawless, 1953).

On each occasion the schools were visited, water samples were taken from taps in the school grounds, placed in an insulated ice box and taken to the laboratory for bacterial counts and investigated for faecal *Escherichia coli*, using the methods recommended by the South African Bureau of Standards (1951). It was assumed that, since each house drew its water from the municipal water supply, this analysis would be representative of the general condition of the water.

Table 1. *Salmonella and shigella infections by age and sex*

Age (years)	No. of individuals	No. of specimens	Specimens infected with: Salmonellae No.	Salmonellae %	Shigellae No.	Shigellae %	Individuals infected with: Salmonellae No.	Salmonellae %	Shigellae No.	Shigellae %	All infected individuals No.	All infected individuals %
7–10	56	372	22	5·9	2	0·5	19	33·9	2	3·6	20	35·7
11–18	74	493	19	3·9	6	0·4	15	20·2	6	8·1	21	28·4
Total	130	865	41	4·7	8	0·5	34	26·2	8	6·2	41	31·5
Females	64	439	24	5·5	2	0·5	21	32·8	2	3·1	22	34·4
Males	66	426	17	4·0	6	1·4	13	19·7	6	9·1	19	28·8

Table 2. *Seasonal distribution of salmonella and shigella infections in 55 children*

Month of examination	*S. labadi*	*S. typhimurium*	*S. london*	*S. abortus bovis*	*S. derby*	*S. johannesburg*	*S. cerro*	*S. pomona*	*S. adelaide*	Percentage salmonella-infected individuals*	*Sh. flexneri*	*Sh. flexneri newcastle*	Percentage shigella-infected individuals	Total infected individuals No.	Total infected individuals %
Feb.	2	1	2	1	2	—	—	—	—	14·5	—	—	—	8	14·5
Mar.	1	2	1	—	1	—	1	—	1	12·7	—	—	—	7	12·7
May	1	1	—	—	—	—	—	1	—	5·5	1	—	1·8	4	7·3
June	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Aug.	—	—	—	—	—	1	—	—	—	1·8	—	—	—	1	1·8
Sept.	—	2	—	—	1	—	—	—	—	5·5	—	—	—	3	5·5
Oct.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Jan.	—	2	1	—	—	1	—	—	—	7·3	—	4	7·3	8	14·5
Total	4	8	4	1	4	2	1	1	1	—	1	4	—	—	—

* Because of salmonellae being isolated from the same children on different occasions, twenty individuals over the year had twenty-six infections (see text).

RESULTS

With an even distribution of males and females, the 130 children in the group selected passed 865 specimens of which 41 (4·7 %) yielded a growth of salmonellae and 8 (0·5 %) of shigellae (Table 1). Over the year of the investigation 41 (31·5 %) children had either salmonellosis or shigellosis. Of these, 34 (26·2 %) had a salmonella and 8 (6·2 %) a shigella infection. One child produced a salmonella in one specimen and a shigella in another. There were slightly more infections among females and the younger age group.

Because some were absent on the day of collection and others had left the school only 55 children were present for all eight investigations. The data of these are presented in Table 2. A total of 26 strains of salmonellae was isolated from 20 (36·4%) of these children and 5 shigellae from 5 (9·1 %) of them. From one child *S. typhimurium* was isolated three times, in March on the first occasion and January on the last. This was the only child from whom the same organism was isolated over a long period. Two others had the same salmonella in successive specimens, and another two children had different salmonellae in different specimens. The highest yield of salmonellae occurred in February and March (late summer), being 14·5 and 12·7 % respectively; there were no pathogens isolated in June (winter) or October (spring). From Table 3 it can be seen that only 5 (0·6 %) stools showed evidence of diarrhoea and these came from the group in which no pathogens were recovered. The consistency of the stools was thus unrelated to the presence or absence of pathogens. On the other hand, a temperature of 100° F. or higher was found in over 12 % of those who were infected with salmonellae or shigellae, but in less than 6 % of those from whom no pathogens were recovered. However, temperatures below 99·9° F. were no guarantee of absence of infection. Thus, from the consistency of the faeces and the temperature observations, it appears that most of the children from whom salmonella and shigella organisms were isolated were in apparent good health.

During the year *S. labadi* and *S. typhimurium* were most frequently isolated, with the group B–E constituting 75 % of the total isolations. On no occasion was *S. typhi*, *S. paratyphi A*, *B* or *C* found.

Of the 120 strains of salmonellae and shigellae tested against a range of antibiotics (Table 4) all were sensitive to streptomycin (100 μg./disk), tetracycline (50 μg./disk) and nitrofurantoin (100 μg./disk). A few strains were resistant to chloramphenicol, colistin and kanamycin. About a quarter of the strains were resistant to ampicillin and almost all to cloxacillin and novobiocin.

The results of bacteriological analysis of water samples taken at the schools (Table 5) show that the water was of good quality, and generally fulfilled the standards laid down by the South African Bureau of Standards (1951) for large towns. The three occasions (March, August, October) when counts of over 100 organisms/ml. were noted were probably due to constructional work which is prevalent in the township. On no occasion were any presumptive coliforms or faecal *E. coli* isolated.

During the period of the investigation, 64 of the 130 children (49·3 %) showed

Table 3. *Clinical observations related to infection*

	No. of specimens	Consistency of faeces: Hard No.	Hard %	Soft No.	Soft %	Liquid No.	Liquid %
Pathogens not recovered	816	487	59·7	324	39·7	5	0·6
Salmonella infected	41	23	56·1	18	43·9	—	—
Shigella infected	8	6	75·0	2	25·0	—	—
Total infected	49	29	59·2	20	40·8	—	—

	No. of specimens	Temperature (° F): < 98·4 No.	< 98·4 %	98·4–99·8 No.	98·4–99·8 %	100+ No.	100+ %
Pathogens not recovered	816	406	49·8	363	44·5	47	5·7
Salmonella infected	41	19	46·3	17	41·5	5	12·2
Shigella infected	8	—	—	7	87·5	1	12·5
Total infected	49	19	38·8	24	49·0	6	12·2

Table 4. In vitro *resistance to antibiotics of isolated strains*

Genus	No. of strains	Percentage of strains resistant to: Ampicillin	Cloxacillin	Streptomycin	Tetracycline HCl	Chloramphenicol	Erythromycin	Coliston	Novobiocin	Kanamycin	Nitrofurantoin
Salmonella	91	24·1	98·9	0	0	6·6	47·3	4·4	96·7	1·1	0
Shigella	29	24·1	100·0	0	0	0	6·9	6·9	93·1	0	0

the presence of parasites (Table 6). Flagellates were observed but were not reported, while *Entamoeba coli* was found in 73·0 % of the children. In addition, 13 children showed multiple parasitic infections. Stool consistency appeared to have no relationship with parasitic infections.

Table 5. *Water testing*

Months ...	Feb.	Mar.	May	June	Aug.	Sept.	Oct.	Jan.
Sholom Manne, no. of colonies/ml.	60	149	3	38	47	0	155	62
Sizaneni, no. of colonies/ml.	53	23	5	40	225	1	27	7

On no occasion were there any presumptive or faecal *E. coli*.

Table 6. *Incidence of faecal parasites in* 130 *Dube children*

Individuals infected			Children with double infestation		
Parasite	No.	%	Parasite	No.	%
Hymenolepis nana	14	10·8	*Hymanolepis nana* + *Giardia*	3	2·3
Ascaris lumbricoides	31	23·8	*H. nana* + *Ascaris lumbricoides*	3	2·3
Enterobius vermicularis	2*	1·5	*A. lumbricoides* + *Giardia*	2	1·5
Giardia	15	11·5	*A. lumbricoides* + *Necator americanus*	1	0·8
Trichuris trichiura	4	3·1	*A. lumbricoides* + *Enterobius vermicularis*	1	0·8
Necator americanus	8	6·2	*Necator americanus* + *Trichuris trichiura*	3	2·3
Taenia	5	3·8			

* See text for explanation of low figure.

DISCUSSION

The main cause of human salmonellosis as a public health problem is attributable to foods of animal origin, egg products, human carriers, food handlers and contaminated water (Sickenga, 1964; Bowmer, 1964; American Public Health Association, 1963). Shigellosis, on the other hand, usually occurs under conditions where there is inadequate sanitation with the resulting abundance of flies and poor personal hygiene. Occasionally water may be incriminated (Wilson & Miles, 1964). Thus, persons living under low socio-economic conditions would be more prone to infections by both these groups of organisms.

The three surveys, Rustenburg, Witkoppen and the present one, Dube, were chosen to represent, in that order, increasing earning capacities of the families, with the resulting improvements in housing, clinic and hospital facilities, food and water supply. It was expected that the improved conditions of urbanization of the Bantu would result in noticeable decrease in the number of salmonellae and shigellae isolated. However, this was not the case (Table 7) since over the period of the survey 26·2 % of the children had salmonella infections and 6·2 % shigella infec-

tions. It is apparent that there is no clear-cut differentiation between the three socio-economic groups as far as the prevalence of these organisms is concerned. It is possible that the Dube children, although in the higher category, still lack some undefined essential nutritional factors, as well as basic education relating to personal hygienic habits.

Table 7. *Salmonellosis and shigellosis among Bantu children of different socio-economic standards in three comparable surveys*

	Rustenburg	Witkoppen	Dube
Socio-economic standard	Very poor	Poor	Better but inadequate
Water supply	Highly unsatisfactory	Unsatisfactory	Satisfactory
% salmonella-infected individuals	35·5	28·9	26·2
% shigella-infected individuals	25·0	4·7	6·2

In the group of fifty-five children who were present at each collection, just under half had an infection of either of the two pathogens at some time during the year. While in agreement with the Witkoppen finding, this is 25 % less than the Rustenburg figure. As found in other studies (Bokkenheuser & Greenberg, 1959; Richardson & Koornhof, 1965) there were more isolations in the summer months than in winter but on no occasion were there signs of outbreaks.

Clinical observations on the children indicated the mildness of the infections. None of the children from whom salmonellae and shigellae were isolated had watery diarrhoeal stools. The small group with oral temperatures over 100° F. yielded twice as many pathogens as the control group.

Taking into consideration the amounts of the antibiotics in the disks, which may vary in different laboratories, the antibiotic sensitivity findings were as expected. The relatively large amounts of streptomycin (100 μg.), tetracycline (50 μg.) and erythromycin (50 μg.) per disk may account for the higher percentage of strains sensitive to these drugs as compared with the findings of some authors (Wilson & Miles, 1964; Cruickshank, 1965).

The investigations of Ramsey & Edwards (1961) show that over the years from 1948 to 1960 there was a gradual increase from 0 to 29·0 % in the resistance of *S. typhimurium* to tetracycline. Manten, Kampelmacher & Guinée (1964) found a similar increase of from 2·1 % in 1959 to 24·4 % in 1962. This increase is attributed to the extensive use of tetracycline in these areas. It is interesting that our findings show that all the *S. typhimurium* strains were sensitive to tetracycline, probably because this drug is not used as frequently as in the above population groups.

Rustenberg and Witkoppen surveys showed that the water used in the homes was of inferior quality. It was suggested that this water was a factor in the transmission of salmonellosis and shigellosis. Furthermore, in a survey conducted in winter and summer at a school at Komatipoort in the Eastern Transvaal Lowveld (Richardson & Koornhof, 1965) it was shown that, of eight children from whom pathogens were isolated, six came from an area where water was drawn from a river from which numerous salmonellae were isolated. It was therefore interesting to find the same prevalence of salmonellae and shigellae in the present study, carried

out in an area with excellent municipal water supply. Although it is reasonable to assume that water contamination plays some part in the transmission of these organisms it does not appear to be the most important factor in these surveys.

All the parasites implicated in this investigation (see Table 6), except taenia, are transmitted from person to person or by self-infection. Supplementing the bacteriological findings, this suggests a lack of personal hygiene. The low incidence of *Enterobius vermicularis* can be explained by the method used for examining the stools, as the ova are seldom found in them (Belding, 1958). One would expect a higher incidence, since the Bantu on urbanization are exposed more and more to the type of indoor micro-environment necessary for the maintenance of the enterobic life cycle.

An investigation carried out in 1962 (H. J. Heinz, personal communication) showed that a considerable amount of meat was available in the township from areas outside the control of the Municipal abattoirs. This was sold at lower prices than in the controlled shops and has a ready market because of the low salaries earned by the majority of the families. From a parasitological point of view this meat was inferior and explains the taenia infestations in children who have probably never left the area. Meat being a possible source of salmonella infections in these children, an investigation into the extent of salmonella infections in abattoirs, butchers' shops and uncontrolled sources, similar to that conducted by a Working Party of the Public Health Laboratory Service (1964), would provide valuable information.

From this study and its comparison with the previous surveys, it is apparent that there is a need for more efficient food control and for the education of the Bantu as to the importance of personal hygienic habits. Furthermore, as the socio-economic position of the Bantu improves, more judicious spending of money on food of higher nutritional value may have a bearing on their susceptibility to diarrhoeal diseases (Report, 1965).

SUMMARY

1. Faeces from apparently healthy Bantu children from schools in Soweto township near Johannesburg were examined eight times at fairly regular intervals over a period of 1 year.

2. Only fifty-five children were present for all eight investigations and, of these, 36·4% yielded salmonellae and 9·1% shigellae. Taking the group as a whole, 26·2% of the children had salmonella infections and 6·2% shigella infections.

3. Parasites were shown to be present in sixty-four of the 130 children during the period of the investigation.

4. As in other surveys, there were more isolations in summer than in winter.

5. *S. labadi* and *S. typhimurium* were most frequently isolated, with the group B–E constituting 75% of the total isolations. *S. typhi*, *S. paratyphi A*, *B* and *C* were not found.

6. Water supplied to each house by the Johannesburg municipality was of good quality, yet it did not affect the incidence of salmonellosis and shigellosis.

7. In spite of better socio-economic conditions the incidence of salmonellosis is comparable to the two previously mentioned surveys. These findings indicate a lack of instruction on personal hygiene and the importance of public health measures.

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