THE SIGNIFICANCE OF PNEUMOCOCCAL TYPES.

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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.

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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.

Specimen of sputum	Day of disease	Types of pneumococci obtained: On direct plate	Through mouse: Sputum alone	Through mouse: +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

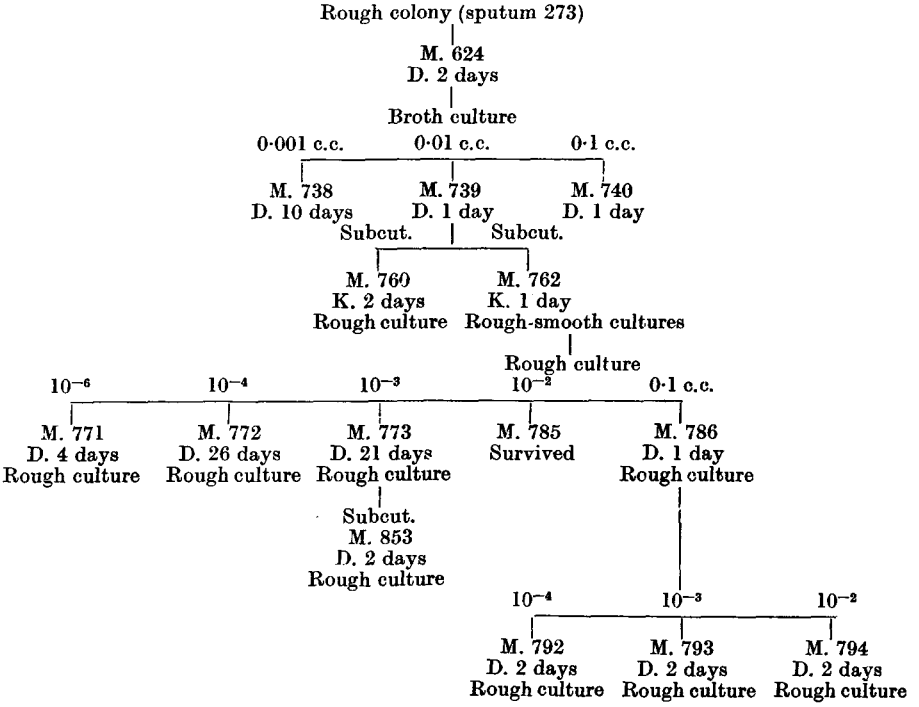
septicaemia in a mouse and a smooth colony was subcultivated in broth from the blood. The broth culture on being tested against all the available pneumococcus agglutinating sera gave a positive reaction with two sera, viz. Type I and Group IV (Pn. 160); in both cases the coarse masses characteristic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



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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 $5\frac{1}{2}$ 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 $3\frac{1}{2}$ 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.

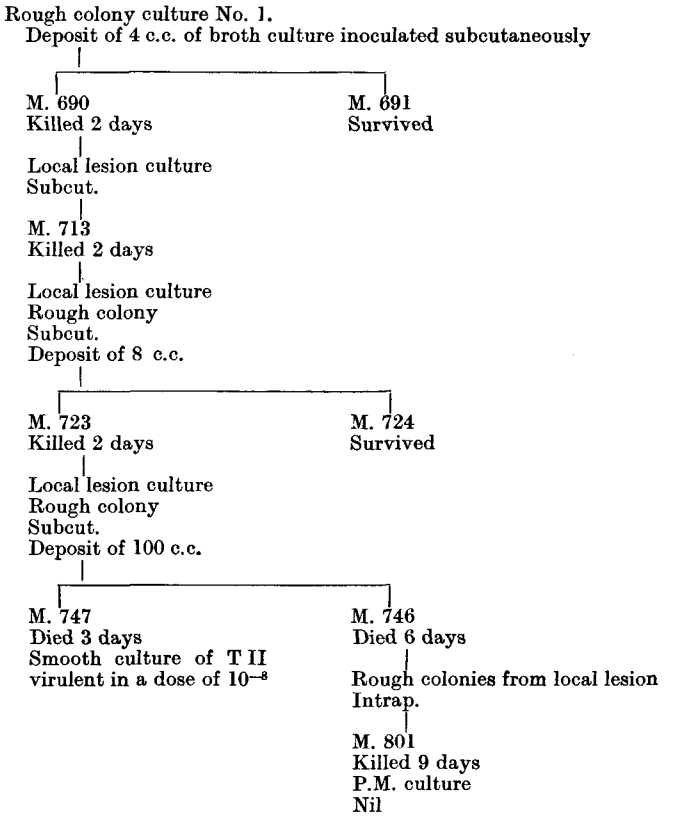
B. *Passage experiments through mice.*

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.



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.

C. *Inoculation of large doses of R pneumococci subcutaneously into mice.*

The following table gives the results of inoculating the deposits of broth culture of a single strain of attenuated R pneumococcus under the skin of mice.

Mouse	Culture	Dose	Result
744	Rough Type II	Deposit of 50 c.c.	Died 3 days. R colonies only
745	,,	,, ,, ,,	,, 3 ,, S colonies, Type II
746	,,	,, ,, ,,	,, 6 ,, R colonies only
747	,,	,, ,, ,,	,, 3 ,, S colonies, Type II
748	,,	,, ,, ,,	,, 5 ,, S colonies, Type II
749	,,	,, ,, ,,	Killed 10 ,, R colonies only
750	,,	,, ,, ,,	Died 4 ,, S colonies, Type II
751	,,	,, ,, ,,	Killed 13 ,, Culture—nil
752	,,	,, ,, ,,	Died 3 ,, S colonies, Type II
753	,,	,, ,, ,,	Killed 13 ,, R colonies only
759	,,	,, ,, ,,	Died 2 ,, S colonies, Type II
760	,,	,, 30 ,,	Died 2 ,, S colonies, Type II
761	,,	,, 70 ,,	Survived
762	,,	,, 70 ,,	Died 1 day. S colonies, Type II
763	,,	,, 10 ,,	,, 2 days. S colonies, Type II
764	,,	,, 20 ,,	Killed 6 ,, Culture—nil

These experiments support the view expressed earlier that the subcutaneous inoculation of a mass of culture under the skin furnishes a nidus in which the R pneumococcus is able to develop into the virulent capsulated form and thence invade the blood stream. As mentioned earlier, passage experiments with smaller doses up to 1 c.c. of broth culture have also resulted in reversion to the virulent S form, but the occurrence is infrequent and irregular. The effect of the larger doses inoculated subcutaneously is more certain and in only 7 out of the 16 examples given has the R strain remained unaltered.

Inoculation of Attenuated R pneumococci together with virulent S culture killed by heat.

Preliminary Experiments.

The development of the virulent S form of pneumococcus from the R form inoculated in large doses under the skin of the mouse is no doubt favoured by the mass of culture forming a nidus. This protection from the normal defensive mechanism of the animal tissues cannot, however, be the sole factor in the production of the change, since the attenuated R pneumococcus may survive unaltered in the subcutaneous tissues for two or three weeks without any such protection.

Some R strains revert to the virulent type much more readily than others, and it is possible, as I have suggested above, that such strains may have retained in their structure a remnant of the original S antigen insufficient in ordinary circumstances to enable them to exert a pathogenic effect in the animal body. When a strain of this character is inoculated in a considerable mass under the skin, the majority of the cocci break up and the liberated

S antigen may furnish a pabulum which the viable R pneumococci can utilise to build up their rudimentary S structure. The amount of S antigen in an R strain, even one only partially attenuated, might not be very large, and it might happen that such an R strain did not liberate in sufficient concentration the stimulating or nutrient substances necessary to produce reversion. It appeared possible that suitable conditions could be arranged if the mass of culture was derived from killed virulent pneumococci, while the living R culture was reduced to an amount which, unaided, was invariably ineffective. There would be thus provided a nidus and a high concentration of S antigen to serve as a stimulus or a food, as the case may be.

A number of experiments on the above lines have been done and the results have shown quite conclusively that reversion of an attenuated R pneumococcus to its virulent prototype can be induced with much greater regularity than after inoculation of large amounts of the living R strain alone. The details of the procedure are given in the following example. A virulent culture of Type II was grown for a few hours in glucose broth, a fairly dense growth being produced, and was then killed by steaming at 100° C. The culture was concentrated by centrifuging and four mice were inoculated subcutaneously each with the deposit of 50 c.c. together with 0·5 c.c. of a living serum broth culture of R Type II. All four mice died in 3 to 5 days with numerous capsulated diplococci in the blood, cultures from which gave the typical agglutination reaction of a virulent Type II strain. A control experiment was made at the same time. Ten mice were inoculated subcutaneously each with the same amount of the living R strain as above together with the steamed deposit of 40 c.c. of broth culture of Type I. One of the mice died 2 days later from an infection with Gram-negative bacilli; the rest were killed healthy in 7–10 days. Cultures made from the tissues at the seat of inoculation remained sterile except in two mice, killed after 7 days, which yielded a few R colonies of pneumococci. This control experiment shows

(1) that the R pneumococcus of Type II remained attenuated in the absence of steamed virulent Type II, and

(2) that it was not assisted to regain virulence by the presence of the steamed Type I culture.

The reverse of the above experiment was then tried, the attenuated culture being the R form of Type I, but in this case there was found a difference in the results according to the temperature to which the heated virulent culture was raised. In an experiment with four mice steamed Type I (deposit of 70 c.c. of broth culture) together with 0·25 c.c. of the R cultures had no effect, all the mice being perfectly healthy when killed 17 days later. The same amount of Type I culture heated to 80° C. for one hour caused one mouse to die 10 days later and a virulent Type I culture was recovered from the blood; the other three mice were killed after 17 days and cultures from the seat of inoculation showed that the R pneumococci had perished.

The effect of Type I culture heated at 60° C. for two hours was then tried.

Six mice received subcutaneously the deposit of 60 c.c. of heated culture together with 0·25 c.c. of the rough Type I culture; five out of the six died of Type I pneumococcal septicaemia. In a repetition of the last experiment the same R culture of Type I in a dose of 0·25 c.c. was administered together with the deposit of 110 c.c. of virulent Type I culture which had been heated for two hours at 60° C. Ten mice were inoculated subcutaneously; one died within a few hours; the rest succumbed to pneumococcal septicaemia in 2–6 days and smooth Type I cultures were grown from the blood in every case.

A control experiment with the above rough Type I, inoculated plus the deposit of 100 c.c. of virulent Type II culture which had been steamed for 15 minutes, was negative. Ten mice were used and all were healthy when killed after 10–13 days. The R strain had disappeared except in one mouse where a few R colonies grew from the seat of inoculation.

In the preceding experiments the steamed Type II antigen exerted a specific influence since it caused only the R form of Type II to increase in virulence and had no effect on that of Type I. On the other hand, virulent Type I antigen appears to be injured by steaming at 100° C. and produces no effect either on the corresponding R form or upon the R form derived from Type II.

If the virulent Type I culture is heated only to 60° C. it very readily changes the R form of Type I into the virulent S form. But the lowering of the temperature at which the virulent culture is killed has other important results, as will be shown later. It may be mentioned now that the shorter the period during which the culture is heated to 60° C. (lower temperatures have not been tried), the more powerful and the less confined to its own type is the effect of the virulent antigen on the attenuated R form when the two are injected simultaneously into mice. For example, virulent cultures of Type I heated to 60° C. may cause the attenuated R strain of Type II to assume the capsulated S form in the animal body.

Short exposures at 60° C. introduce a risk which can be excluded when the cultures are killed by steaming at 100° C., the risk namely that some of the pneumococci in the culture may have survived the heating and be still viable. To ensure that the culture deposits have been uniformly heated, they have been enclosed in a sealed glass capsule and immersed in a large water-bath, the whole capsule being kept below the level of the water which has been carefully maintained at the required temperature. The heated deposits have been tested by incubation followed by plating and by the injection of large amounts subcutaneously into mice. These tests of viability have invariably been negative and exposure for so short a period as 15 minutes to a temperature of 60° C. appears to be sufficient to kill pneumococci. But the results of inoculating attenuated R strains into mice together with heated suspensions of virulent cultures of different types have been so remarkable as to raise the question whether the ordinary tests of viability are sufficiently comprehensive. It becomes necessary to consider whether heating to 60° C.

may produce resistant forms of pneumococci which do not multiply except when injected into a mouse together with a living attenuated pneumococcus. On account of the importance of the correct interpretation of the experiments which I am about to describe, I have given in detail a number of examples varying slightly in the experimental conditions and in the method of control.

As the heated S culture appears to be the determining factor, each series of experiments is headed by the particular type of virulent pneumococcus furnishing it. All inoculations designed to increase virulence or alter type have been done subcutaneously in mice.

Inoculation experiments with heated virulent Group IV and attenuated R Type I and R Type II pneumococci.

Table V shows that three different strains of Group IV killed by steam at 100° C., when injected into mice together with living attenuated R pneumococci derived from Type II by growth in homologous immune serum, caused the R form to revert to the virulent capsulated S form. R 4, Type II, *i.e.*

Table V.

Killed S pneumococci	Living R pneumococci	No. of mouse	Result	Type of culture obtained from mouse
Pn. 85, Group IV, steamed 20 mins. Dose = deposit of 60 c.c. of broth culture	R 4, Type II. Dose = 0·25 c.c. of blood broth culture	405	Died 4 days	S colonies, Type II
		406	Killed 7 ,,	None
		407	,, 7 ,,	R colonies
		408	Died 4 ,,	S colonies, Type II
Pn. 160, Group IV, as above	R 4, Type II as above	409	Killed 7 days	S colonies, Type II
		410	Died 4 ,,	,, ,,
		411	,, 4 ,,	,, ,,
		412	,, 3 ,,	,, ,,
II B, Group IV, as above	R 4, Type II as above	413	Died 3 days	S colonies, Type II
		414	,, 2 ,,	,, ,,
		415	,, 3 ,,	,, ,,
		416	Killed 7 ,,	R colonies
None	R 4, Type II. Doses = 0·75, 1·0, 1·0 c.c. of blood broth culture	462	Killed 19 days	None
		463	,, 19 ,,	,,
		464	,, 19 ,,	,,

rough colony 4, Type II, which has been used throughout these experiments is so much attenuated as never to kill mice in doses of 1·0 c.c. of blood broth culture. The three control mice remained well and were killed 19 days after inoculation. As mentioned earlier, all the inoculation experiments to increase virulence have been done subcutaneously and it has been the custom in the case of mice killed when apparently well to make plate cultures from the tissues at the seat of inoculation, viz., the right groin. Generally the plates are either sterile or purely pneumococcal, but care must be taken should the inoculated material cause the skin to ulcerate. Extraneous organisms are usually staphylococci which cause no difficulty. There were additional controls to the above experiments. These have not been given in the table, but they show that the stimulus to reversion of the R strain was not possessed by

steamed Type III culture (deposit of 60 c.c. for each of 12 mice) or by scarlatinal streptococci (4 mice).

Table VI.

Killed S pneumococci	Living R pneumococci	No. of mouse	Result	Type of culture obtained from mouse
Pn. 85, Group IV, heated 2 hours at 60° C. Dose = deposit of 50 c.c. of broth culture	R 4, Type II. Dose = 0·25 c.c. of blood broth culture	791	Killed 7 days	None
		792	Died 3 ,,	S colonies, Type II
		793	,, 3 ,,	,, ,,
Pn. 85, Group IV, as above	R 6, Type I, as above	794	Killed 16 days	None
		795	,, 16 ,,	,,
		796	,, 16 ,,	,,
Pn. 160, Group IV, as above	R 4, Type II, as above	773	Died 2 days	S colonies, Type II
		774	,, 3 ,,	,, ,,
		775	,, 2 ,,	,, ,,
Pn. 160, Group IV, as above	R 6, Type I, as above	776	Killed 15 days	None
		777	,, 15 ,,	,,
		778	,, 15 ,,	,,
II B, Group IV, as above	R 4, Type II, as above	785	Died 3 days	S colonies, Type II
		786	,, 3 ,,	,, ,,
		787	,, 3 ,,	,, ,,
II B, Group IV, as above	R 6, Type I, as above	788	Killed 15 days	None
		789	,, 15 ,,	,,
		790	,, 15 ,,	,,

Table VI confirms the conclusions drawn from the experiments in Table V, that Group IV S antigen enables attenuated Type II pneumococci to become virulent in the mouse.

In addition it shows that Group IV antigens, contained in three virulent strains, after heating to 60° C. for an hour on two occasions, did not produce reversion of the R form of Type I; the mice inoculated remained well and no culture could be obtained from the seat of inoculation 15–16 days later.

The latter part of the experiment, which shows that an attenuated R culture of Type I is not increased in virulence through the influence of heated S antigens of Group IV, was repeated with identical results. The same three Group IV strains, heated at 60° C., were injected in doses of 100 c.c. of broth culture deposit together with a different rough culture, R 3, Type I (this signifies rough colony 3, derived from Type I and this strain has been most frequently employed in these experiments). All the mice, a total of 21, were healthy when killed 22–28 days later.

These experiments may be summarised as follows.

Group IV virulent pneumococci, killed either by heating to 60° C., or by steam at 100° C., when injected in large quantities of culture deposit into mice together with an attenuated R strain of Type II caused the latter to revert to the capsulated virulent form and set up fatal septicaemia in the mice.

Under similar conditions three Group IV cultures failed to increase the virulence of attenuated R strains derived from Type I.

Inoculation experiments with heated virulent Type I culture and attenuated R strains of Types I and II.

Conversion of R Type II into S Type I. In the experiment in Table VII two out of eight mice injected with heated virulent Type I culture together with an attenuated R culture derived from Type II died of pneumococcal septicaemia and yielded pure S colonies of Type I from the blood; plates from the lesions at the seat of inoculation showed a mixture of R and S colonies.

Table VII.

Killed S pneumococci	Living R pneumococci	No. of mouse	Result	Type of culture obtained from mouse
Type I heated 2 hours at 60° C. Dose = deposit of 50 c.c. of broth culture	None	641	Killed 5 days	None
	,,	642	,, 6 ,,	,,
	,,	643	,, 6 ,,	,,
	,,	644	,, 6 ,,	,,
As above	R 4, Type II. Dose = 0·25 c.c. of blood broth culture	645	Died 3 days	S colonies, Type I
		646	Killed 5 ,,	R cols. from local lesion
		647	,, 6 ,,	,, ,,
		648	,, 6 ,,	,, ,,
As above	R 4, Type II, grown in the heated Type I deposit. Dose = 0·36 c.c.	649	Killed 5 days	R cols. from local lesion
		650	Died 4 ,,	S colonies, Type I
		651	Killed 6 ,,	None
		652	,, 6 ,,	One R colony

The remaining six mice were killed, apparently healthy, after 5–6 days and plate cultures made from the subcutaneous seat of inoculation either produced only R colonies or remained sterile.

The four mice which received the heated Type I culture alone were well when killed 5–6 days later, and plates made from the subcutaneous tissues at the seat of injection remained sterile.

The rough attenuated Type II culture has apparently in two instances been changed into a virulent Type I. The most obvious alternative to this presumption is that a few Type I pneumococci in the heated culture remained viable. It is therefore desirable to give in detail the cultural tests and the treatment to which the virulent culture had been submitted. After heating for one hour at 60° C. a large loopful of suspension was sown on blood agar plates. The tube containing the thick suspension (the deposit of 600 c.c. concentrated by centrifuging to 12 c.c.) was resealed and again heated to 60° C. for one hour. The suspension was then incubated at 37° C. overnight and plated the next day. All the above plates remained sterile. In order to show that the thick suspension would not inhibit the growth of viable pneumococci 4 c.c. of it were sown with the R strain of Type II, incubated overnight and plated; a profuse growth of R colonies only was obtained. This culture in the suspension was used for the last four mice in the table.

In view of these precautions, I think it may be assumed that the heated suspension contained no viable Type I pneumococci. The R strain of Type II, although not tested alone on this occasion, has been used throughout these

experiments and numerous tests have shown that it is a pure attenuated rough culture.

A second experiment (see Table VIII) on the same lines was made and the cultural tests of viability of the heated culture were as rigid as possible. The thick suspension of the concentrated broth culture after heating was distributed in ten small tubes so that each tube contained the deposit of 100 c.c. and to each of them was added 1 c.c. of sterile bovine serum. The mixtures were incubated overnight and subcultures were made the next day both upon fresh blood agar plates and in blood broth; the latter were also plated after incubation at 37° C. The cultures from every tube remained sterile.

Table VIII.

Killed S pneumococci	Living R pneumococci	No. of mice	Result	Type of culture obtained from mouse
Type I heated 3 hours at 60° C. Dose = deposit of 50 c.c. in salt solution	None	4	Killed 7 days	None
Type I as above. Dose = deposit of 100 c.c.	None	2	Killed 7 days	None
Type I as above. Dose = deposit of 50 c.c.	R 4, Type II. Dose = 0·2 c.c. of blood broth culture	8	Killed 7 days	R colonies from 4 and none from the rest
None	R 4, Type II. Dose = 0·5 c.c.	2	Killed 13 days	None
Type I as above. Dose = deposit of 100 c.c.	R 4, Type II. Grown in heated culture	2	Killed 13 days	None
Type I deposit of 100 c.c. (bovine serum not removed)	R 4, Type II. Dose = 0·25 c.c.	1	Died 3 days	S colonies, Type I

Seven of the tubes of suspension were centrifuged; the supernatant serum was removed and replaced by 0·5 c.c. of salt solution. Eight mice were inoculated subcutaneously each with the deposit of 50 c.c. of the heated culture together with 0·2 c.c. of blood broth culture of the living R strain of Type II. Six mice were similarly injected with the heated culture alone, four receiving the deposit of 50 c.c. and two the deposit of 100 c.c. of the broth culture.

All of the above mice were killed seven days later; cultures made from the seat of inoculation were completely negative in the case of the control mice and all of those receiving the mixtures except four which yielded a few R colonies only.

Failure of attempt to induce reversion in vitro. Two tubes containing heated suspension (the concentrated deposit of 100 c.c.) in serum were used as culture media. They were sown with the R strain of Type II to ascertain if it could be changed into the S form *in vitro*. After incubation overnight plates were made and the tubes were then centrifuged. The supernatant serum was pipetted off, leaving the deposit to which was added a fresh quantity of sterile bovine serum. This procedure was repeated eight times and the final as well as the intermediate plate cultures yielded only R colonies.

The whole of the two deposits, each containing the heated Type I pneumococci from 100 c.c. plus the growth of the R strain, were inoculated into two mice. These were killed 13 days later and found to be healthy.

Importance of the presence of all the products in the smooth culture. There was one tube of Type I suspension remaining from which the bovine serum added a week earlier had not been removed. This was inoculated into a mouse together with 0·25 c.c. of an 18-hour old blood broth culture of the R II strain. The mouse was killed when ill 3 days later and smooth virulent Type I pneumococci were grown from the blood and from the subcutaneous seat of inoculation.

The negative result in the first part of the experiment may have been due to the removal of some important substance from the heated culture deposit. As mentioned above, 1 c.c. of sterile bovine serum was added to each tube of deposit in order to make it a favourable medium for the growth of any viable pneumococci possibly remaining. The total bulk, being now over 1·5 c.c. and being mainly serum, was too great for subcutaneous inoculation into a mouse; so each tube was centrifuged, the supernatant serum was removed and salt solution was substituted.

The experiment shown in Table IX was done to test the hypothesis suggested above.

Table IX.

Killed S pneumococci	Living R pneumococci	No. of mouse	Result	Type of culture obtained from mouse
Type I heated 1½ hours at 60° C. Dose = deposit of 70 c.c. in salt solution	R 4, Type II. Dose = 0·25 c.c. of blood broth culture	817	Killed 13 days	None
		818	,, 13 ,,	,,
		819	Died 3 ,,	? S colonies, Type I
		820	Killed 13 ,,	None
		825	,, 13 ,,	,,
Supernatant bovine serum removed from the above deposits	As above	826	Killed 16 days	None
		827	,, 16 ,,	,,
		828	,, 16 ,,	,,
		829	,, 16 ,,	,,
		830	,, 16 ,,	,,
Type I heated as above. Dose = deposit of 70 c.c. with bovine serum	As above	821	Died 4 days	S colonies, Type I
		822	,, 3 ,,	,, ,,
		823	Killed 13 ,,	None
		824	,, 13 ,,	,,

A glucose broth culture of Type I, after concentration by centrifuging, was heated to 60° C. in a sealed tube for half an hour and again for one hour. The whole of the culture (700 c.c. reduced to 5 c.c.) was distributed in small tubes, 0·5 c.c. in a tube, and to each was added 0·5 c.c. of sterile bovine serum. The tubes were incubated overnight at 37° C. and each was plated; they were plated again after a second incubation. All were sterile. The tubes were then divided into two sets. Five were centrifuged and the deposit was separated from the supernatant serum broth. Four of the remaining were retained whole.

To each of the 14 tubes thus obtained 0·25 c.c. of the attenuated R strain was added and the mixtures were injected into mice.

Two of the four mice which received the whole culture died of pneumococcal septicaemia and yielded S colonies of Type I.

One of the five mice inoculated with the deposit resuspended in salt solution died and numerous small colonies, not definitely smooth, grew from the blood. These colonies, however, formed firm masses with Type I serum and one colony subculture killed a mouse from which typical S colonies of Type I were obtained.

There is a strong suggestion that the whole deposit is more effective in converting the R strain into a virulent form than the deposit from which the serum was removed after incubation, *i.e.* the deposit which had been washed with serum. The supernatant fluid, however, did not contain any substance in sufficient concentration to increase the virulence of the R culture. Although the experiment is not decisive, it rather indicates that the essential material for the building up of a virulent form from the R form may be associated with the capsule of the pneumococcus and may be to some extent washed off.

Conditions affecting the efficacy of the S antigen in inducing reversion. It is certain that the efficacy of the S antigen in providing the conditions necessary for recovery of virulence is variable and, for example, differences in temperature and period of heating may exert considerable influence, as will be seen from the following experiments.

In Table X are given the results of heating the S culture at 60° C. for different periods, ranging from 15 minutes to 50 minutes.

Each heated deposit alone was injected into 3 mice, while 2 mice received

Table X.

Killed S pneumococci	Living R pneumococci	No. of mouse	Result	Type of culture obtained from mouse
Type I. Dose = deposit of 100 c.c. glucose broth culture heated 15 mins. at 60° C.	None	978	Killed 13 days	None
	,,	979	,, 13 ,,	,,
	,,	980	,, 13 ,,	,,
As above	R 4, Type II. Dose = 0·25 c.c.	994	Died 2 days	S colonies, Types II and I
		995	,, 3 ,,	S colonies, Type I
Type I heated 25 minutes at 60° C.	None	981	Killed 13 days	None
	,,	982	,, 13 ,,	,,
	,,	983	,, 13 ,,	,,
As above	R 4, Type II	996	Died 3 days	S colonies, Type I
		997	,, 2 ,,	,, Type II
Type I heated 40 minutes at 60° C.	None	984	Killed 13 days	None
	,,	985	,, 13 ,,	,,
	,,	986	,, 13 ,,	,,
As above	R 4, Type II	998	Died 2 days	S colonies, Type II
		999	,, 2 ,,	,, Type I
Type I heated 50 minutes at 60° C.	None	987	Killed 13 days	None
	,,	988	,, 13 ,,	,,
	,,	989	,, 13 ,,	,,
As above	R 4, Type II	1000	Killed 12 days	None
		1	Died 3 ,, (Pn. in blood)	Culture overgrown

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the combined inoculation. All the control mice were killed 13 days later and plate cultures from the subcutaneous seat of inoculation remained sterile. Culture tests also showed the heated culture to be sterile.

On the other hand, all the mice, except one, inoculated with the attenuated strain in addition died of pneumococcal septicaemia within 3 days. The mouse which survived had received the deposit heated for 50 minutes. The fellow mouse died and the culture from the blood became overgrown. The infection was most probably due to Type II pneumococci, since microscopical examination of the blood showed diplococci with well-marked capsules. (As a rule a Type I pneumococcus appears in the mouse's blood in short chains and the capsules are rarely as large and well stained as those of Type II pneumococci.)

The S culture heated for 15, 25 and 40 minutes had almost identical effect on each pair of mice, one yielding a virulent Type I culture and the other a virulent Type II. From one mouse injected with the 15 minutes heated deposit Type II colonies were grown from the blood, while on the plate from the local lesion among the majority of translucent Type II colonies a single slightly opalescent colony was identified as Type I.

The above results are more striking than the examples already given with cultures heated at 60° C. for an hour or more and there is an indication of a falling off as the period of heating approaches the hour.

An experiment was therefore made to compare at the same time cultures heated for over and under an hour.

A virulent Type I culture was grown for a few hours in 2 litres of glucose broth. It was then centrifuged and the deposit was collected into two sealed tubes each containing 10 c.c. Both were heated in a water-bath at 60° C. for half an hour. The next day one was heated to the same temperature as before for a further period of $2\frac{1}{2}$ hours. The tubes were then opened and the thick suspension was distributed into tubes in 1 c.c. quantities to each of which was added 0·25 c.c. of blood broth. The tubes were incubated overnight and plated the next day. One had become contaminated with a bacillus; the rest were sterile. The heated cultures alone were not tested on mice. Ten mice were inoculated with the culture heated for 3 hours and nine with the portion heated for half an hour. Each mouse received the deposit of 100 c.c. together with 0·25 c.c. of the attenuated R strain of Type II.

The results showed very definitely that there was a considerable difference in character between the two differently heated suspensions.

Of the ten mice which received the 3-hour heated culture three died of septicaemia and S colonies of Type II were grown from the blood. One died prematurely (3 days) and a few R colonies were grown both from the blood and from the local lesion. The rest were killed in 6–7 days and R colonies only were obtained from the seat of inoculation.

Of the nine mice which received the $\frac{1}{2}$-hour heated culture five died of septicaemia in 3–6 days and S colonies of Type I were grown from the blood.

One mouse died in 4 days; the blood yielded S colonies of Type II, but among these was observed a colony a little whiter than the rest. A subculture of the latter inoculated into a mouse intraperitoneally produced septicaemia; a culture and the peritoneal washing gave positive agglutino-precipitation with Type I serum. The seventh mouse died in 4 days of Type II septicaemia. The remaining two were killed in 6 days; they showed nothing microscopically in the blood and a few R colonies only were grown from the seat of inoculation.

The question of intermediate stages in the transformation of type. An attempt has been made, from time to time, to ascertain whether, if it is the case that the virulent S form is built up from the R form, the change is gradual, *i.e.* whether the R form before developing the virulent S form of another type passes through a stage when, though still R, it resembles the R of the new type. The only way to differentiate with certainty between the R strain derived from Type I and the R strain derived from Type II is by producing reversion to the S form of the original type without the assistance of any heated S antigen. The experiment just described offered a suitable occasion for testing this point. An R colony from the local lesion from one of the above mice which died of Type I septicaemia was grown in 100 c.c. of glucose broth. The centrifuged deposit was divided equally between two mice by subcutaneous inoculation. One died of Type II septicaemia, the other remained unaffected. The R colony chosen was therefore the same as that inoculated, viz. the R colony of Type II. Colonies were also taken from five of the mice in which no virulent S form had made its appearance and they were treated as above. Two of the colony cultures, inoculated in 50 c.c. doses, caused Type II septicaemia in one of each pair of mice; all the other mice survived.

Positive evidence of an intermediate R form between the attenuated Type II and the possibly newly formed virulent Type I thus fails.

Further experiments on the destructive effect of heat on Type I S antigen. The effect of heating the S culture to temperatures above 60° C. has been given some attention. It has already been noted that Type I virulent culture after exposure to 100° C. loses the property possessed by cultures heated to 60° C. of restoring the virulence of the R strain of Type I inoculated at the same time and does not cause the appearance of S cultures of Type I when injected along with an attenuated R strain of Type II.

The results of injecting Type I culture heated for 15 minutes at temperatures ranging from 60° C. to 80° C. are given in Table XI. Only the deposit heated at 60° C. was tested on mice for the presence of viable pneumococci. Four mice received each the deposit of 65 c.c. of glucose broth and were killed 10 days later. Plates made from the seat of inoculation were sterile; the subcutaneous tissue from the groin of each mouse was removed, emulsified and injected into a second mouse intraperitoneally; all these mice survived. It seems clear that no viable pneumococci remained in the culture heated to 60° C. for 15 minutes.

Table XI.

Killed S pneumococci	Living R pneumococci	No. of mice	Result	Type of culture obtained from the mouse
Type I heated at 60° C. for 15 minutes. Dose = deposit of 65 c.c. of culture	None	4	Killed 10 days	None
Type I as above. Dose = deposit of 30 c.c.	R 4, Type II. Dose = 0·25 c.c. of blood broth culture	2	Died 3 and 5 days	S colonies of Type I from blood
Type I heated at 65° C. for 15 minutes. Dose = deposit of 65 c.c.	R 4, Type II, as above	4	Killed 10 days	None
Type I heated at 70° C. as above	R 4, Type II, as above	4	Killed 10 days (2) Died 4·5 days (2)	None (2) S colonies of Type II (i) S colonies of Types I and II (1)
Type I heated at 75° C. as above	R 4, Type II, as above	4	Killed 10 days	None
Type I heated at 80° C. as above	R 4, Type II, as above	4	Killed 10 days (3) Died 3 ,, (1)	None (3) R colonies only (1)

Both mice inoculated with the R strain of Type II together with the deposit of 30 c.c. of this broth culture of Type I heated at 60° C. developed Type I septicaemia.

The results with the R Type II plus Type I culture heated at 70° C. were interesting. Two mice died of pneumococcal septicaemia. S colonies of Type II were grown from the blood of one and S colonies of Type I from the other. The local lesion of the latter mouse was plated and among a majority of R colonies a few S colonies grew, two of which were tested and found to be Type II.

From the remaining mice, most of them being killed healthy, no S cultures were obtained.

The Type I cultures heated at different temperatures were also tested on mice together with an R strain derived from Type I; the dose of heated culture was only half the amount used in combination with the R strain of Type II. All the mice which received the culture heated at 65° and 70° C. respectively died of Type I septicaemia. At 75° C. the culture was effective in causing reversion to the S form of Type I in two out of three mice, whilst the culture heated at 80° C. produced no effect, the mice remaining well and the R pneumococci having disappeared from the seat of inoculation. The destructive effect of the increased temperature on the Type I antigen is thus exhibited as in the preceding experiments.

These experiments may be summarised as follows.

The injection of heated virulent S culture of Type I into the subcutaneous tissues of mice together with an attenuated R strain derived from Type II apparently results in the conversion of the latter into a virulent S culture of Type I or of Type II. Cultures heated for 15 minutes at 60° C. are more

effective in producing the transformation than cultures heated for longer periods at 60° C. or at higher temperatures.

The chances of an R strain of Type II reverting to its original S form or being converted into the S form of Type I after inoculation together with virulent Type I culture heated at 60° C. for 15 minutes appear to be about equal.

At 80° C. the active substance in the Type I pneumococcus is so much altered that the heated culture causes neither reversion of the R form of Type I to its S form nor transformation of the R form of Type II into the S form of Type I.

Cultures of Type I heated at 70° C. and 75° C. respectively are still effective in producing reversion of the R form of Type I, but transformation of the R form of Type II is rarely brought about by cultures heated higher than 60° C. One positive result with culture heated at 70° C. for 15 minutes was obtained.

Neither reversion to the S form nor transformation of type has been obtained *in vitro*.

Inoculation experiments with heated virulent Type III culture and (1) *an attenuated R strain derived from Type I, and* (2) *an attenuated R strain of Type II.*

The Type III culture used in the experiments shown in Table XII was grown for a few hours in a flask of glucose broth which on removal from the incubator was immersed in a water-bath at 60° C. for one hour to arrest

Table XII.

Killed S pneumococci	Living R pneumococci	No. of mouse	Result	Type of culture obtained from the mouse
Type III heated 2 hours at 60° C. Dose = deposit of 100 c.c. of glucose broth culture	R 4, Type II. Dose = 0·25 c.c. of blood broth culture	869	Killed 14 days	None
		870	,, 14 ,,	,,
		871	Died 7 ,,	S colonies, Type III
		872	,, 10 ,,	,, ,,
		873	Killed 14 ,,	,, ,,
		874	,, 14 ,,	None
		875	Died 2 ,,	S colony, Type III
		876	,, 8 ,,	S colonies, Type III
Type III, as above	R 3, Type I. Dose = 0·25 c.c. of blood broth culture	877	Killed 14 days	None
		878	,, 14 ,,	,,
		879	,, 14 ,,	S colonies, Type III
		880	,, 14 ,,	None
		881	,, 14 ,,	,,
		882	,, 14 ,,	,,
		883	,, 14 ,,	,,
		884	,, 14 ,,	,,
Type III steamed at 100° C. for 12 minutes. Dose = deposit of 100 c.c.	R 4, Type II. Dose = 0·25 c.c.	885	Died 3 days	S colonies, Type II
		886	Killed 14 ,,	None
		887	,, 14 ,,	,,
		888	Died 3 ,,	S colonies, Type II
Type III, as above	R 3, Type I. Dose = 0·25 c.c.	889	Killed 14 days	R colonies only
		890	Died 12 ,,	None
		891	Killed 14 ,,	,,
		892	,, 14 ,,	R colonies and one S colony

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autolysis. On the following day the 2400 c.c. of broth were concentrated by centrifuging to 24 c.c.; 16 c.c. were placed in a sealed tube and heated for one hour at 60° C., and the remaining 8 c.c. were steamed at 100° C. for a full 12 minutes.

In this instance mice were not injected with the heated culture alone.

(*a*) *Heated to* 60° *C.* Eight mice were inoculated with the R strain of Type I and eight with the R strain of Type II, both accompanied by a dose equivalent to 100 c.c. of Type III S culture heated to 60° C. It will be seen from Table XII that the watery colonies of Type III appeared more frequently in the mice inoculated with the attenuated R strain of Type II than in those which received the R strain of Type I, viz. five times in the former and once in the latter. This fact lends some support to the view that the particular type of R strain is the important factor in the production of the S colonies of Type III. Incidentally it is further evidence against the hypothesis that viable Type III pneumococci persisted in the culture after heating.

The low virulence of these newly formed Type III pneumococci, as one might term them, is noteworthy. The mouse inoculated with the R strain of Type I and the heated Type III culture was apparently well when killed 14 days later, and the S colonies came to light on the plate made from the subcutaneous seat of inoculation. These S pneumococci, which were in pure culture, were certainly multiplying in the local lesion and, though evidently of low virulence, might ultimately have caused the death of the mouse. Some of the Type III pneumococci from the mice inoculated with the R strain of Type II showed more virulence, since three out of the five positive mice died with pneumococcal septicaemia in 7–10 days. Mouse 875, which probably died prematurely from shock, yielded a single S colony among numerous R colonies from the seat of inoculation; the blood culture was contaminated. The fifth mouse, 873, was, like that in the first mentioned series, perfectly well when killed, and S colonies were grown from the seat of inoculation.

The large watery colonies of Type III on blood agar plates were very typical in appearance, and, in addition, the blood smears from the mice which died showed the characteristic picture of round cocci with large well-stained capsules. I have had to rely for the identification of Type III pneumococci on the above appearances in conjunction with negative agglutination reactions to both Types I and II sera, since, in spite of several trials, I have not recently been able to prepare a serum which gives the characteristic agglutination reaction with a virulent Type III culture.

(*b*) *Heated to* 100° *C.* The steamed Type III culture injected with the attenuated R strain of Type II caused the latter to revert to the virulent S form in two out of four mice.

An interesting result was obtained in one of the four mice which received the R strain of Type I together with the steamed Type III culture. The plate culture from an abscess at the seat of inoculation produced numerous small apparently rough colonies and one typical smooth disc-shaped colony.

Subcultures from the latter were virulent for mice; pneumococci multiplied in the capsulated form in the blood, and cultures from the blood yielded typical smooth discs. I was unable to identify the strain as Type III, since the typical watery colony was not produced; also no agglutination was obtained with Types I and II sera or with any of the available group IV sera.

In either case, whether it is a Group IV strain or a Type III, the result is important enough to encourage further experiments with steamed culture.

One must however bear in mind the possibility of accidental contamination, especially in view of a somewhat similar circumstance which is recorded in the description of the next experiment.

A virulent Type III culture in glucose broth was heated for 3 hours at 60° C. in a sealed tube and was injected subcutaneously into seven mice together with an R strain of Type II. The dose of the former was the deposit of 70 c.c. and of the latter 0·2 c.c. of blood broth. Four mice died in 4–7 days of pneumococcal septicaemia and watery colonies of Type III were grown from the blood; the remaining three mice were killed in 7 days and in each case similar colonies were grown from the seat of inoculation. The above was an exceptional result and fortunately an equal number of mice were injected with the same dose of the heated Type III culture alone. All the latter seven mice were killed on the 7th day after injection and cultures were made from the seat of inoculation. All the plates were sterile with one exception in which a pneumococcus colony appeared.

(In view of this disconcerting result in one of the control mice, I will describe in detail the procedure in making cultures from the seat of injection.

The mouse is pinned out and the abdominal skin is seared with a hot iron, but not exactly over the seat of injection. Through the seared area an incision is made with a sterile knife and the edge of the skin is grasped with a pair of forceps. The skin, held firmly, is reflected back until the seat of injection is exposed and is then pinned down. A fresh pair of sterile instruments is used to scrape the subcutaneous tissues and usually the gland in the groin is removed as well. The material is placed in a small tube with a few drops of broth in which it is rubbed up. A loopful of the fluid is spread on a plate and to the remainder a little blood broth is added. If the next day after incubation the fluid culture shows on microscopical examination of smears any diplococci it also is plated.)

In the case of the mouse referred to above there were no diplococci in the tissue fluid after incubation and the plate made subsequently from it produced no growth. The plate direct from the tissue emulsion was sterile except for a single smooth disc shaped colony which was bile soluble and virulent for mice. At no time did this strain produce watery colonies like Type III and there was no agglutination with Types I and II sera.

This result occurred some three months before the writing of this article and in spite of numerous tests designed to reveal the presence of any viable pneumococci in heated cultures I have never again found any evidence of viability either by culture or through the mouse, even in cultures heated at 60° C. for so short a period as 15 minutes.

On the whole I am inclined to think that the pneumococcus responsible for the colony fell on the plate while it was being spread.

Still it is often useful to record unexpected occurrences when there is no absolutely certain explanation. A result which appears to the worker concentrated on a particular issue to be a regrettable flaw in his working may be significant to another considering the subject from a different point of view.

These experiments may be summarised as follows:

The injection of S culture of Type III, heated at 60° C. for two periods

of one hour each, along with living R strains derived from Type I or Type II results in the appearance of an S pneumococcus of Type III.

This transformation of type occurs more frequently with the R form of Type II than with the R form of Type I.

The newly formed strains of Type III sometimes kill the mice from septicaemia in 7–10 days and at other times are only discovered at the seat of inoculation when the mouse is killed, apparently well, 14 days after inoculation.

Inoculation experiments with heated virulent Type II culture and different attenuated R colony strains of Type I.

The results of inoculating attenuated R strains of Type I together with heated Type II culture are shown in Table XIII. A comparison is made between different R colonies (developed on a plate from a virulent Type I culture grown in homologous immune serum).

The R strains were also inoculated into mice (1) alone, (2) together with

Table XIII.

Killed S pneumococci	Living R Type I pneumococci	No. of mouse	Result	Type of culture obtained from the mouse
Type II heated 2 hours at 60° C. Dose = deposit of 90 c.c.	R colony 1	724	Killed 12 days	None
	,, 1	725	,, 12 ,,	,,
	,, 2	726	Died 11 ,,	S colonies, Type II
	,, 2	727	,, 10 ,,	,, ,,
	,, 3	728	,, 4 ,,	,, ,,
	,, 3	729	,, 4 ,,	,, ,,
	,, 4	730	Killed 11 ,,	,, ,,
	,, 4	731	Died 4 ,,	,, ,,
	,, 5	732	Killed 13 ,,	,, ,,
	,, 5	733	,, 6 ,,	,, ,,
	,, 6	734	Died 9 ,,	,, ,,
	,, 6	735	Killed 13 ,,	None
Type III heated 2 hours at 60° C. Dose = deposit of 80 c.c.	R colony 1	736	Killed 11 days	None
	,, 1	737	,, 11 ,,	,,
	,, 2	738	,, 9 ,,	,,
	,, 2	739	,, 11 ,,	,,
	,, 3	740	,, 9 ,,	,,
	,, 3	741	,, 11 ,,	,,
	,, 4	742	,, 10 ,,	S colonies, Type III
	,, 4	743	,, 11 ,,	None
	,, 5	744	,, 10 ,,	,,
	,, 5	745	,, 11 ,,	,,
	,, 6	746	,, 11 ,,	,,
	,, 6	747	,, 9 ,,	,,
Type II heated 3 hours at 60° C. Dose = deposit of 90 c.c.	R colony 1	748	Killed 12 days	None
	,, 1	749	,, 12 ,,	,,
	,, 2	750	,, 13 ,,	,,
	,, 2	751	,, 6 ,,	S colonies, Type II
	,, 3	752	Died 3 ,,	,, ,,
	,, 3	753	Killed 13 ,,	None
Type III heated 3 hours at 60° C. Dose = deposit of 80 c.c.	R colony 4	754	Killed 10 days	None
	,, 4	755	,, 9 ,,	S colonies, Type III
	,, 5	756	,, 9 ,,	None
	,, 5	757	,, 9 ,,	,,
	,, 6	758	,, 11 ,,	,,
	,, 6	759	,, 10 ,,	,,

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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 IIA 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

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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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