SOME STUDIES ON THE PNEUMOCOCCUS

WITH SPECIAL REFERENCE TO MUTATION OF TYPE.

by

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P R E F A C E.

This work was carried out during my appointment as Resident Physician in Knightswood Hospital.

It has been to some extent the realisation of an ambition since reading the article on transmutation of Pneumococcus types by F. Griffith.

The work was commenced in November 1935 and, after prolonged preliminary preparations, rapid progress was possible during the Influenza epidemic of late 1936 and early 1937 which was followed by the constant arrival of pneumonia patients into this hospital.

I am largely indebted to Dr. William Dow, Medical Superintendent of Knightswood Hospital, for his great interest throughout and his encouragement during the slow preliminaries and to Miss A.A. McIntosh for her most capable and ever willing assistance in the more monotonous laboratory preparations for this work.

CONTENTS.

INTRODUCTION.

The epidemiology and therapeutics of pneumococcal infections entered upon a new phase when it was demonstrated in 1909 by Neufeld and Handel that the pneumococcus could be separated into distinct serological types.

Confirmation of these results was given by numerous workers in different countries and it was discovered that the common types prevalent in one locality were also found in other countries though their incidence was not necessarily the same.

In more recent years Cooper and her co-workers have found it possible to classify further into separate types most of the pneumococci hitherto classified as Group IV.

In 1923 Griffith found it possible to change the virulent Smooth form into the avirulent Rough form with resulting loss of the pneumococcus capsule and later, in 1927 during a considerable number of experiments, he found that it was frequently possible to convert one type of pneumococcus into another serological type in the mouse.

Griffith's work opened up a new field of bacteriological research and Dawson and Sia found it possible in 1930 to effect this mutation of type more readily by conducting in vitro experiments under special conditions.

Although the experiments of Griffith and Dawson and Sia gave ample scope for a new conception of the role of pneumococci in health and in pathological processes, it is surprising that little further work has been done to ascertain the possibility of the occurrence of this mutation under suitable conditions in man.

Certain arguments and experimental evidence in favour of this possibility are presented in this work.

No reference has been found in the literature to the specificity of the different types of pneumococci and investigations have been made into the agglutination and cross agglutination reactions in Type I and Type II.

The Eosin Relief Method of demonstrating bacterial capsules has recently given a rapid and simple method of capsule examination and afforded the opportunity of making comparisons in the capsules of different types.

Virulence Tests have been performed on a number of pneumococci isolated and comparisons have been made with the different types of pneumococci and the results compared and contrasted with the size of the capsule observed in using the Eosin Relief Method.

In the selection of suitable material for isolation of pneumococci much necessary delay was avoided by the use of the rapid method of pneumococcus typing as recommended by Sabin.

Experiments demonstrating the value of Sabin's method are also submitted.

PART I.

THE TRANSMUTATION OF TYPES.

1. Media used for cultivation of pneumococci.

A preliminary investigation was performed to determine the possibility of using the medium recommended by H.D. Wright in 1936 in preference to Hartley's broth for the routine culture of pneumococci and the suitability of alternative media in the event of an unexpected shortage of the most favourable preparation.

For this purpose 6 - 10 cc of the following preparations were stored in sterile test tubes:-

1. Hartley's broth.
2. Hartley's broth containing 1/2,000,000 acriflavine.
3. Peptone broth.
4. 5% Serum broth.
5. Wright's Medium.
6. Blood Agar.
7. Blood broth.

Hartley's broth and Peptone broth and Blood Agar were prepared as described in the text book of Muir and Ritchie.

Acriflavine solution 1/1000 was diluted by adding 1 part to 19 parts distilled water and the addition of 1 part of this solution to each 99 parts of Hartley's broth resulted in the final required dilution of 1/2,000,000 acriflavine in Hartley's broth.

The serum broth was prepared by the addition of 5 cc. horse serum obtained from Burroughs Wellcome & Co. to each 95 cc. of Hartley's broth.

Wright's medium was prepared as follows:-
To 1 litre distilled water were added 10 grammes peptone, 5 grammes sodium chloride and 500 grammes veal finely minced after removal of excess fat. This was then well mixed and heated at 68° C. for 20 minutes, stirring at intervals. The flask was then well shaken and steam sterilised for 30 minutes, filtered through paper and p H. adjusted to 7.8 - 8.0

After a further 30 minutes steaming it was again filtered through paper. The p H. was finally checked (7.6 - 7.8) and 1.5 gramme glucose per litre added. It was then distributed in amounts of 6 - 10 cc. into test tubes and autoclaved for 10 minutes at 10 lb. pressure.

Blood broth was prepared by the addition of 5 cc. defibrinated horse blood (B.W. & Co.) to 95 cc. Hartley's broth.

Hartley's broth gave a rapid and good growth of pneumococci and on subculturing every 36 hours it was possible to maintain growth almost indefinitely.

Hartley's broth with added Acriflavine showed a delayed and poorer growth which could be maintained in subculture every 36 hours for many days.

Peptone broth showed delayed and poor growth, rapidly dying out on subculture.

Serum broth afforded rapid and good growth which could be maintained almost indefinitely but there was frequent contamination of the growth.

Wright's medium gave a very rapid growth with much greater opacity than that obtained in Hartley's broth but, even on subculturing every 24 hours, growth could not be maintained for many days.

Blood Agar gave very good growth which could be maintained indefinitely on subculture every 3-4 days.

Blood broth gave rapid and very good growth maintained indefinitely with little trouble from contamination on subculture every 2 days.

As a result of these preliminary experiments it was decided to adopt Hartley's broth, Blood Agar and Blood broth as routine media.

2. Recovery of pneumococci from sputum.

Sputum in amounts of 0.5 - 1.0 cc. was injected intraperitoneally into mice and on the death of the mouse, usually within 24 hours, the peritoneal cavity was washed out with normal saline and the resulting washings typed with agglutinating sera for types I, II, and III. The mouse heart blood was plated on blood agar and colonies subcultured in Hartley's broth. Confirmation of the type of pneumococcus was then made by Sabin's rapid typing method and subcultures made from the growth in broth to blood agar slopes.

3. Preparation of Rough Strains.

The antipneumococcus serum was obtained from Burroughs Wellcome & Co. 10 cc. of Type I anti-pneumococcus serum were added to 90 cc Hartley's broth and to another 90 cc. of Hartley's broth 10 cc. of Type II antipneumococcus serum were added. The resulting 100 cc. of 10% serum broth were then distributed in amounts of 10 cc. into test tubes.

Subcultures of pneumococcus Type I and Type II were made into the broth containing the homologous antiserum. A uniformly turbid growth resulted and, at intervals of 2 days, subcultures were repeated. In the case of the Type II subculture a flocculent deposit appeared in the serum broth after the third subculture but only on the fourth subculture in the case of Type I. After six subcultures of Type II the growth was entirely of flocculent type with flocculent deposit and flocculi adhering to the sides of the test tube. Examination of the pneumococci by the Eosin Relief Method of capsule demonstration which is described on page 48 showed the absence of capsules

and the intraperitoneal injection of 0.25 cc. of an 18 hour subculture in Hartley's broth failed to kill mice in less than 4-5 days. The pneumococci, in addition, failed to agglutinate with antiserum for their original Smooth type. Subculture from the serum broth to Blood agar resulted in a growth of typical rough colonies.

Identical results were obtained with the Type I pneumococcus after 8 subcultures.

4. Preparation of Heat-killed Pneumococci.

The pneumococci of different types were inoculated into flasks containing 100 cc. Hartley's broth and incubated at 37° C. for 18 hours. A loopful of the growth was spread on a slide and stained to verify the absence of contamination. The flasks were then immersed in a water bath at a temperature of 65°C with the water level at least one inch above the level of the broth and allowed to remain in the bath for 30 mins. then removed and allowed to cool.

Under all sterile precautions the contents of the flask were poured into a series of 10 cc. centrifuge tubes and plugged with cotton wool transfixed with a sterile pin to prevent the wool being forced down the tube and centrifuged at 3000 revs. per minute for 30 minutes. The almost clear supernatant fluid in each tube was then poured off and the deposit shaken with the remaining few drops of fluid in the tube and transferred to a sterile test tube. In this way about 1.2 cc. of creamy fluid was obtained, consisting of a highly concentrated suspension of pneumococci.

Examination of the suspension by means of the Eosin Relief Method showed that the capsules were unaffected by the heating process.

5. Transmutation Experiments.

A. Griffith's Method.

On the evening preceding the day on which the experiments were due to be performed a subculture of S pneumococci was made into 100 cc. Hartley's broth. After an interval of 18 hours the heat-killed suspension of S pneumococci was prepared from the resulting growth.

Several control tests were performed to ensure that all Type II S pneumococci had been killed by heat. Strict control tests such as these are essential in all experiments of this kind:-

(i) A large loopful of the concentrated suspension was spread on a blood agar plate,

(ii) 0.1 cc. of the suspension was inoculated into 6 - 10 cc. of Hartley's broth,

(iii) The remainder of the suspension, usually 0.4 - 0.5 cc., was injected intraperitoneally into a white mouse.

If no growth resulted on Blood Agar or in Hartley's broth and the control mouse lived as long as the two mice injected with 0.5 cc. of the mixed S and R pneumococci, it was considered justifiable to conclude that all controls were satisfactory.

Also, on the evening preceding the day on which the experiments were due to be performed, a subculture of R pneumococci was made into 10 cc. Hartley's broth.

It will suffice to describe one typical experiment, an attempt to convert Type I pneumococci into Type II pneumococci. 0.75 cc. of the concentrated suspension of heat-killed Type II S pneumococci was transferred

to a sterile 5" x ½" test tube and to it was added 0.25 cc. of an 18 hour subculture of Type I R pneumococci. The contents of the tube were then thoroughly mixed by shaking and 0.5 cc. of this mixture injected, by means of a fine intradermal needle, subcutaneously into the abdominal wall of each of two white mice.

Nine experiments were performed. In three experiments it was attempted to cause mutation of Type I to Type II, in three it was attempted to convert Type II to Type I and in three it was attempted to convert Type II to Type III.

Results.

In one experiment (No. 7) in which it was attempted to convert Type II to Type III, all three controls proved that the supposedly heat-killed concentrated suspension of Type III contained living Type III pneumococci.

In the eight other experiments the controls were all negative.

In these eight experiments it was found that, after four or five days, a small superficial ulcer appeared on the abdominal wall of all the mice injected with the mixed S & R suspension and this ulcer tended to spread for some days.

Only in one experiment was it possible to cause mutation of type (Type I to Type II) and the result of this experiment will be described in detail later.

In the seven other experiments the mice remained active for periods varying from 10-18 days. On the death of the mice they were at once pinned out on a board and the abdominal wall shaved with a razor and

the condition of the ulcer noted. Cultures were then made on blood agar plates from the centre of the ulcer, from underneath the edge of the ulcer, from the peritoneal cavity and from the heart blood.

The findings are given in Table I.

TABLE I.

Experiment.	Attempt to cause conversion of Type.	Mice died on.	Condition of Ulcer.	Culture results.
1.	I to II.	1.12th day) 2.15th day)	Small, healing.	No pneumococci found.
2.	I to II.	1.10th day.	large, not healing.	few colonies of R I pneumococci from edge of ulcer only.
		2.18th day.	healed	No pneumococci found.
4.	II to I.	1.14th day.	medium, healing)	No pneumococci found.
		2.17th day	small, healing)	
5.	II to I.	1.12th day	medium, healing)	No pneumococci found.
		2.17th day	small, healing)	
6.	II to I.	1.14th day.	small, healing)	No pneumococci found.
		2.16th day.	healed)	
8.	II to III.	1.10th day.	large, not healing.	few colonies of R II pneumococci from edge of ulcer only.
		2.17th day.	small, healing.	no pneumococci found.
9.	II to III.	1.13th day) 2.14th day)	small, healing)	No pneumococci found.

Experiment 3.

All control experiments showed the absence of living pneumococci, the control mouse living until killed on the 18th day and cultures from the peritoneal cavity and heart blood proved to be sterile.

The two mice used for mutation of Type I and Type II died on the 6th and 8th day respectively.

The first mouse to die showed a medium sized, spreading ulcer and cultures from the four sites resulted in an apparently pure growth of Type II S pneumococci. The second mouse showed a medium sized ulcer, cultures from the heart blood and peritoneum showing a few colonies, all of Type II S pneumococci, and cultures from the ulcer and under the ulcer edge showing the presence mainly of Type II S pneumococci and a few Type I R pneumococci.

The Type II S pneumococci obtained from the mice in this experiment were further examined to discover whether or not they differed in any way from the original Type II S pneumococci used in the preparation of the heat-killed suspension. It was found that the size of their capsules was identical with that of the original, they agreed similarly in having maximum virulence for mice, both killing mice in two days after the injection of 0.5 cc. of a dilution of a Hartley's broth subculture containing less than twelve colonies in each 0.5 cc. They also agreed in their macroscopic agglutination reactions, both just giving a slight flocculated deposit in a dilution of Type II antiserum 1 part to 63 parts saline and of 1 part Type I antiserum to 7 parts saline.

The subcutaneous injection of a 1-10,000,000 dilution of Hartley's broth culture of the original Type II S pneumococci used for preparation of the heat-killed suspension produced death of a mouse from septicaemia in three days. The short period of survival of the mouse in this case serves as an added control of the non-existence of live Type II S pneumococci in the mixed II S & I R suspension.

From these results it was concluded that a Type I S pneumococcus had been converted to a Type II S pneumococcus having all the characteristics of an organism of that Type.

These experiments were all performed at intervals of 2 - 3 weeks and it is possible that owing to prolonged subculture of the R strains of pneumococci on artificial media their characteristics were finally so altered, perhaps by losing the remnant of their S antigen, that they failed to cause mutation in the later experiments.

B. Method of Dawson & Sia.

The original investigators found this method to be more successful in causing mutation of type.

The experiments to be described were conducted during the last month of my appointment. The type I R strain had recently become contaminated and only the Type II R strain remained.

In these experiments the indication that mutation has occurred is obtained by examination of colonies on blood agar and, to simplify the identification of colonies it was attempted to cause mutation of Type II to Type III with their typical large, mucoid colonies.

Two experiments were conducted.

The preliminary procedures consisted in the collection of the following materials:-

1. Heat-killed suspension of Type III S pneumococci.
2. Hartley's broth culture of Type II R pneumococci.
3. Rabbit serum.
4. Blood broth.

The heat-killed suspension of Type III S pneumococci and the Hartley's broth culture of Type II R pneumococci were prepared as described in Griffith's method and the blood broth was prepared as described in the preparation of media.

The same strict controls of death of the Type III S pneumococci as described in the previous method were used in these experiments.

Rabbit serum was prepared by defibrinating fresh rabbit blood obtained from the Marginal Vein of a rabbit's ear. Venesection was performed a few hours before the commencement of the experiments and the blood allowed to stand in the ice chest until required. The serum was then pipetted off when required.

Twelve sterile 3" x $\frac{1}{2}$" tubes were placed in a rack. To each tube was added 0.5 cc. Blood broth, 0.1 cc. Rabbit serum and 0.05 cc. heat killed suspension of Type III S pneumococci. These tubes were then stored until ready for use.

A suspension of 1 part Hartley's broth subculture of Type II R pneumococci in 1,000,000 parts Hartley's broth was then prepared and one drop of this dilution was added to one of the twelve tubes which was then incubated at 37° C. Definite growth resulted next day. A loopful of the contents of this tube was then spread on a blood agar plate and the minutest possible amount was also transferred to another of the original twelve tubes and incubated. This procedure of plating and subculturing was performed daily and the growth on the plates examined for possible typical Type III S pneumococci.

In the first experiment the subcultures were continued for 9 days without the appearance of Type III S colonies on blood agar plates. Contamination of the tubes occurred on the 10th day.

In the second experiment subcultures were continued for 16 days without the appearance of Type III S colonies and the intraperitoneal injection into a mouse of 0.5 cc. of the 18 hour growth in tube 16 failed to cause death of the mouse in 14 days. The failure to occasion mutation of type in these experiments may thus have been due, as in the later experiments following Griffith's method, to the loss of II S antigen in the Rough strain owing to repeated subculture.

PART II.

THE SPECIFICITY OF PNEUMOCOCCAL TYPES.

As a result of his experiments on transmutation of pneumococcal types, Griffith considered that in the case of Type I and Type II pneumococci the major antigen of one type is represented as a subsidiary antigen in the other. Georgia Cooper and co-workers, dealing with the further differentiation of pneumococcal types in the previously unclassified Gp. IV pneumococci, has discovered that marked cross-agglutination occurs with antisera for Type II and Type V, Type III and Type VIII, Type VII and Type XVIII and Type XV and Type XXX and that the serum of horses immunised with one or other of these related types has the property of agglutinating the closely allied type in a high dilution.

The work of Georgia Cooper emphasises the presence in pneumococci of an antigenic complex but no reference in the literature has been found relating to the agglutination and cross-agglutination reactions of pneumococci of Type I and Type II. Interest in these unknown points was increased after the mutation experiments had been performed with success in these types.

Technique.

On the growth on blood agar plates of colonies of Type I and II pneumococci from the heart blood of mice injected with sputum, a colony was subcultured into 6 cc. of Hartley's broth for 18 hours. In early experiments the pneumococci were killed by heating at 60° C. for 30 minutes but later experiments conducted without the previous heating showed that the results in both cases were identical. The agglutinating sera used throughout were obtained from Burroughs Wellcome

& Co. and were of the same batch in each type. Agglutination and cross-agglutination experiments were carried out on each culture at the same time. The method of dilution and agglutination was that described by Muir and Ritchie, using 3" x ½" test tubes and finally transferring their contents to agglutination tubes. After a few initial experiments it was found that a dilution of 1 part homologous antiserum to 3 parts normal saline gave finer end points than a dilution of 1 part antiserum to 4 parts saline. In the cross-agglutination experiments the heterologous antiserum was used undiluted. In preliminary experiments the agglutination tubes were incubated at 37° C. but later a more clear cut end result was obtained by heating in a water bath for 2 hours at 50-56° C. Saline controls were incorporated in each experiment.

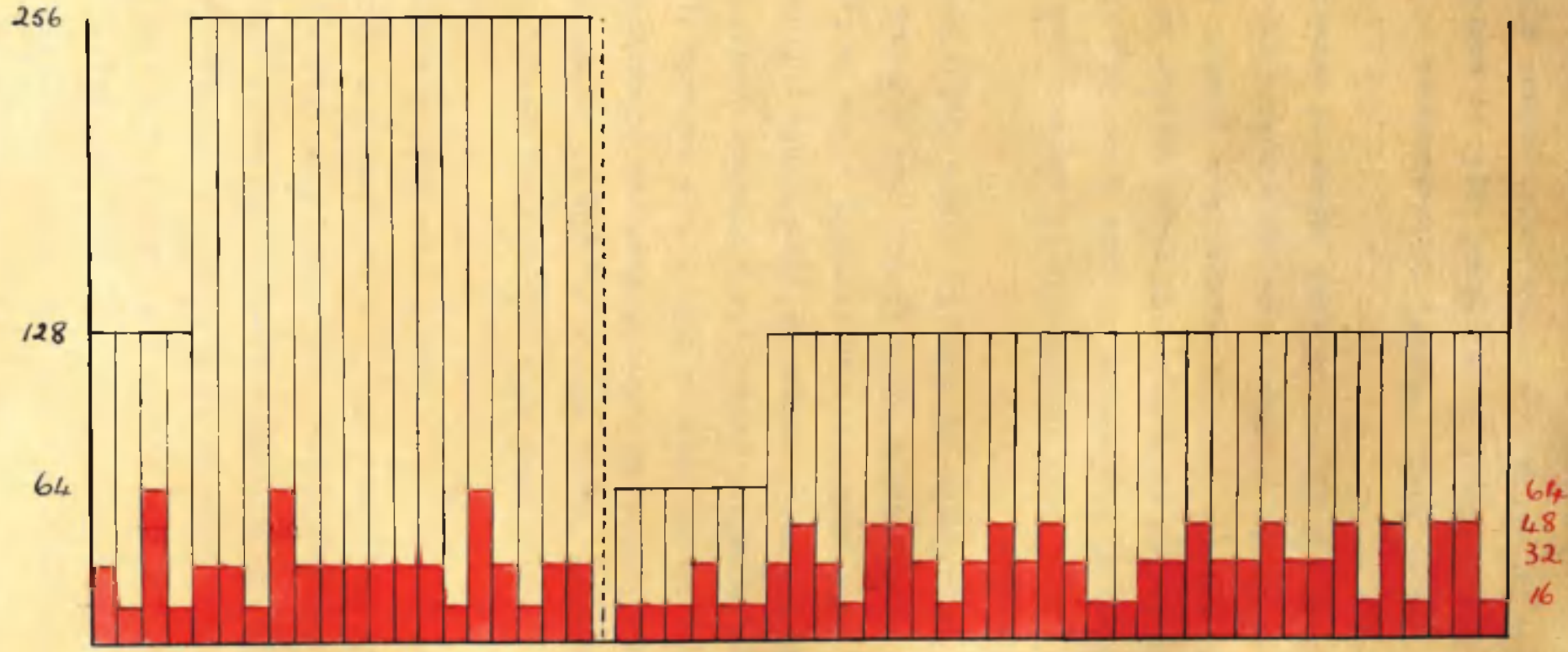
Results.

Considerable uniformity of results was obtained and in the few experiments in which deviation from the average occurred it is probable that such differences are solely due to slight experimental error. A total of 56 experiments was performed on 20 Type I and 36 Type II cultures. A remarkably low percentage of pneumonia patients admitted to hospital gave Type I pneumococci thus accounting for the relatively few experiments possible with this Type.

The results are given in the accompanying histogram in which the dilution of heterologous antiserum is multiplied by four and thus affords easier contrast. For each type the observations are arranged in rising order of titre of homologous antiserum. From this histogram it is seen that Type

DILUTION OF HOMOLOGOUS ANTISERUM

■ = 4x DILUTION OF HETEROLOGOUS ANTISERUM



I antiserum agglutinated Type I pneumococci in a dilution of 1 in 256 and also agglutinated Type II pneumococci in a dilution of 1 in 12. One Type I strain obtained from the Lister Institute, though relatively avirulent and possessing a very small capsule, probably as a result of repeated subculture, agglutinated nevertheless in the maximum dilutions of the antisera. Type II antiserum agglutinated Type II pneumcccccci in a dilution of 1 in 128 and also agglutinated Type I organisms in a dilution of 1 in 16.

Serum taken from patients recovering from pneumonia due to Type I or Type II pneumococci agglutinated only the pneumococci of corresponding type, in the case of Type I to a maximum dilution of 1 in 16 and of Type II to a maximum dilution of 1 in 4 in the small number of experiments performed.

The agglutinating property shown to develop in the blood during convalescence was probably too small to demonstrate any possible cross-agglutination.

Conclusion.

From these experiments it appears that all the strains of pneumococci of Type I and Type II which were tested agree with the other strains of the same type and the antigenic composition of each type shows no variation on examination by a method which necessarily permits of some degree of experimental error.

It is seen, however, on examination of the cross-agglutination reactions that, in addition to the major antigen, a considerable amount of minor antigen is present in the two types. Type I appears to be purer in its antigenic composition and antisera

for this type contain but little antibody for Type II pneumococci, whereas Type II appears to contain relatively large amounts of the minor antigen and antisera for this type contain an appreciable amount of antibodies for Type I.

No antisera were available for investigating the cross-agglutination reactions of Type I and Type II with other known types but considerable antigenic complexity might have been shown to exist if it had been possible to pursue further experiments on these lines.

In a series of experiments Griffith found that the interaction of heat-killed S I and R II pneumococci in mice occasionally resulted not in mutation but in the death of the mouse from S II septicaemia and suggested the possibility of the presence of some S II antigen in S I pneumococci. He failed, however, to bring about the development of S.I. from the interaction of R I with heat killed S II pneumococci.

The agglutination experiments described give support to Griffith's theory in demonstrating the small amount of S II in S I pneumococci and of relatively more of S I antigen in S II pneumococci.

PART III.

THE POSSIBILITY OF MUTATION OCCURRING IN MAN.

Although it must be admitted that mutation has been produced under circumstances not met with under natural conditions in man, the first experiments conducted by Griffith using massive amounts of pneumococci and resulting in mutation in mice were followed by more successful and less complicated experiments with smaller amounts of pneumococci in vitro. Mutation experiments have been neglected for some years but a resumption of research in this subject may result in the discovery of an easier method of producing mutation and suggest a way in which this may occur in the human being.

Cooper has found that over 80% of pneumococcal strains may be grouped in 32 different types and other types will probably be identified later. From the results of mutation experiments a possible deduction is that these pneumococcal types represent stages in the normal life history of the pneumococcus or possibly represent the response of the bacterium to changes in the immunological state of the person harbouring it. In an investigation into the types of pneumococci present in the respiratory passages in normal and pathological conditions it is frequently found that the type of pneumococcus isolated may differ on examinations conducted at short intervals. In this case it is possible that the influences causing the alteration in type are still at work. In a large proportion of cases it is found that the type of pneumococcus remains constant over prolonged periods, particularly in persons with some persistent abnormality in the respiratory tract, the persons harbouring these pneumococci being chronic carriers of this particular type. This apparent stabilisation

of type may be due to the persistence of certain specific local or general immunological stimuli.

Group IV pneumococci are commonly found in the sputum during convalescence from pneumonia due to Types I, II and III. During the present investigation and routine typing of the pneumococci present in the sputum of pneumonia patients admitted to hospital many examples of this occurrence were found and three examples may be given.

Case I. Female, 19 years, left apical pneumonia.

Admitted on 3rd. day of illness. Two hours after admission the rapid typing method revealed the presence of Type I pneumococci in the sputum. A mouse was injected and, on its death, the heart blood was plated. A colony was then subcultured on blood agar and virulence tests performed. The low degree of virulence aroused suspicions and a Hartley's broth subculture of this organism was injected into a mouse which proved the organism to belong to Group IV on investigating the peritoneal exudate with antisera for Types I, II and III. Another colony was then taken from the original heart blood streaked plate and proved to be a Type I pneumococcus, two organisms killing mice in three days. On the sixth day of illness no Group IV organisms could be recovered from the sputum.

During convalescence prior to dismissal, only Group IV organisms were found in the sputum. No case of Type I pneumonia was present in the ward at this time.

Case 2. Male, 42 years, right apical pneumonia.

Admitted on the 5th day of illness. One hour

after admission investigation revealed the presence of Type II pneumococci by rapid typing, and investigation identical with that in case 1 proved the presence of both Type II and Group IV pneumococci in the sputum. During convalescence only Group IV organisms were recovered from the sputum on two occasions.

Case 3. Male, 69 years, right apical pneumonia.

Admitted on 5th day of illness. Three hours after admission the sputum was injected into a mouse and a plating from the heart blood revealed the presence of typical Type III pneumococci. During convalescence only Group IV organisms could be found. No other case of Type III pneumonia was present in the ward at this time.

In several other instances it was found that, although no Group IV organisms could be found in the heart blood of mice injected with sputum of pneumonia patients during the progress of the disease, the sputum during convalescence contained only Group IV pneumococci. It is possible that the pneumococci of Type I, II or III die out and become replaced by the normal Group IV inhabitants of the respiratory tract but Griffith suggests that these types may revert to pneumococci of Group IV from which they were formed or that the Group IV organisms are formed from the other types after suppression of the major antigen by immune bodies developed during the course of the illness. Griffith was unable to stress the possibility of the latter occurrence owing to failure to find Group IV organisms in a sputum containing Type II pneumococci but evidence in support of this possibility is given in Case 2 example above.

Six instances of the simultaneous occurrence of Type I and Group IV pneumococci were met with during the present investigation. Similar results were frequently noted by Griffith. Is it possible that a reason can be found for this frequent occurrence in the suggestion that it is easiest for Group IV organisms to mutate to Type I organisms in the course of infections and that the slightly varying virulence of Type I organisms, as evidenced by Table IV. is indicative of a varying maturity in the strains investigated?

In some cases mouse heart blood platings revealed colonies of Group IV pneumococci of markedly dissimilar appearance but the lack of specific agglutinating sera for the several types prevented the further investigation of these colonies.

It is an observed fact that, unlike many of the infectious diseases, one attack of pneumonia does not lessen the possibility of further attacks but when infection again occurs this is almost invariably due to another type of pneumococcus. Two examples of second attacks occurred recently and a few details are given.

Case 4. Male, 29 years, right apical pneumonia.

Admitted on the 5th day of illness. Examination on two occasions revealed the presence of Group IV pneumococci. Two weeks after dismissal he returned on the third day of pneumonia, the same lobe being involved and a pure growth of Type II pneumococci was obtained from the heart blood of the injected mouse.

It is possible that the Group IV organisms

originally present had mutated to Type II but, as the patient was exposed to infection by Type II pneumococci liberated by other patients during his first stay in hospital, too much reliance cannot be placed upon this possibility.

Case 5. Male, 50 years, left basal pneumonia.

The sputum contained Type III pneumococci. Three years previously he had a right basal pneumonia and Group IV pneumococci were recovered from the sputum. Eight days after the crisis in the first attack he developed acute arthritis of his left ankle due to Type I pneumococci.

In these cases it is possible that the immunity resulting from a previous attack due to one type prevented the development of the same type but not that of another type in the second attack.

In a large percentage of healthy human beings, the percentage varying from 30-90 per cent, Group IV pneumococci can be isolated from the respiratory passages. The sputum of patients suffering from pneumococcal lobar pneumonia contains pneumococci which in 93 per cent of cases agrees with the type of pneumococcus present in the blood or lung puncture cultures or in complications developing during the course of the illness. Where discrepancy occurs it is generally considered that the pneumococcus found in the blood or lung juice or complications has been the cause of the illness but these discrepancies may be explained in another way. Griffiths considers that the apparently harmless saprophyte with traces of S antigen, localised in a situation in which it can multiply in a suitable nidus, gradually builds

up antigenic structures with invasive properties from the material furnished by its disintegrating companions sufficient to overcome the host's resistance and resulting in mutation of pneumococcal type. Thus it appears possible that, in the cases where other types of pneumococci have been isolated later in the course of the disease or from other sites during the disease, these other types have been derived by mutation from the type originally found.

The development of Type I arthritis after the crisis of a lobar pneumonia in Case 5 may have been due to mutation of Group IV pneumococci to Type I. Similarly, in Cases 1 and 2, the Group IV pneumococci may have mutated to the other types and set up severe infections. Cruikshank found that four patients, in a total of forty-three investigated, gave Group IV pneumococci repeatedly in their sputum and Type I or II in their blood on typing by mouse injection. Further examples may be given.

Case 6. (Christie, 1933).

Sputum on admission contained Type II pneumococci. A subsequent empyema revealed the presence of Type I pneumococci. Retyping of sputum and empyema fluid confirmed these findings.

Case 7. Female, 42 years, Influenzal Bronchopneumonia.

The heart blood of a mouse injected with sputum three hours after admission revealed the presence of Type III and Group IV pneumococci. During convalescence only Type III pneumococci could be isolated from sputum injected into mice. No case of Type III pneumonia was present in the ward during her stay.

This patient had long been subject to Chronic Bronchitis and it appears possible that Group IV

organisms were present in her sputum until the attack of Influenza initiated a process resulting in mutation of the type of pneumococcus which later persisted in the diseased respiratory passages.

Case 8. Female. 40 years, right basal pneumonia.

This patient also had been subject to Bronchitis and typing of her sputum on admission showed the presence of Group IV pneumococci. Intercurrent empyema developed, the fluid showing Type I pneumococci and a repeat examination of the sputum showed Type I pneumococci at this time. During convalescence only Group IV organisms were found in the sputum.

Case 9. Male. 20 years, left basal pneumonia.

Sputum revealed the presence of Group IV pneumococci and on the day appointed for dismissal the patient developed a sore throat. Three days later signs of empyema developed, the fluid showing the presence of Type III pneumococci.

The factors causing this possible mutation of Type are unknown. It is possible that the general condition and age of the patient play considerable part and that local or general changes produced in the defence mechanism of the host are also of importance.

Table II shows the incidence of the different types in a series of 577 cases of lobar pneumonia admitted to Knightswood Hospital during the past four years, the average age of patients harbouring each type and the death rate in each type. It is seen that the pneumococci causing the more severe infections occur generally in older persons.

A large number of patients under 15 years

produced no sputum for typing and, since the percentage of Type I infections is high at these ages, the average age for Type I patients would have been considerably less. The low death rate in children suffering from Type I lobar pneumonia would have further helped to show the contrast between the average age of fatal cases and the average age for this type.

TABLE II.

	Type I.	Type II.	Type III.	Group IV.
No. of cases.	153	243	34	147
Frequency %.	26.5	42.1	5.9	25.5
No. of Deaths.	14	70	19	14
Death Rate %.	9.2	28.8	55.9	9.5
Average age (in years).	28.9	32.4	54.1	31.7
Average age of fatal cases.	34.1	40.5	53.6	45.0

A similar commentary applies in the case of Group IV infections and the greater age at death implies a lessened severity of Group IV pneumonias possibly due to a lower virulence of Group IV pneumococci. Further reference to this table will be made in Part IV.

Further evidence of the influence of general conditions may be given in the fact that the percentage incidence of the different types of pneumococci varies from year to year in the same place and varies in the same year in different districts and in different countries.

The possible importance of local or general changes in the host's defence mechanism may be exemplified by a study of 77 cases of pneumococcal

lobar pneumonia admitted to Knightswood Hospital during and for three months after the Influenza epidemic of 1937. From Table III it is seen that there was an increased incidence of Type II and Type III infections with a marked decrease in the incidence of pneumonia due to Type I pneumococci and a slightly decreased incidence of Group IV infections. These results agree with those published by Christie after the 1933 epidemic.

TABLE III.

	Type I.	Type II.	Type III	Group IV.
No. of Cases.	14	40	6	17
% Incidence.	18.6	51.9	8.1	22.1
Christie's Figures.	23.3	53.3	11.7	11.7
Average % incidence in past four years.	26.5	42.1	5.9	25.5

It is interesting to note that, in addition to the change in the percentage incidence of types in the lobar pneumonias of the Influenza epidemic, other differences were noted which appeared to indicate an alteration in the local and general body defence mechanism. Empyema was a less common complication, possibly due, however, to the small proportion of infections due to the invasive Type I organisms and serous effusion was more common and occurred solely after lobar pneumonia due to Type II pneumococci. In the early part of the epidemic Type II infections were very severe (12 cases with 8 deaths) and in the latter part the death rate approached the 30% average for Type II (28 cases with 9 deaths). There was an increased incidence of double lobar infections,

especially involving both bases and an increase of cases dying on the third and fourth days of the illness. Hoarseness was much more common during and after the illness and Laryngeal Diphtheria, usually a very rare complication, occurred in four cases, an incidence of 5.2%.

Griffith's theory on the mutation of types certainly deserves further investigation and consideration. It is impossible to dogmatise but in all the examples described, and in other instances in which difficulty occurred in assigning to the different types of pneumococci isolated their exact importance in the disease process, the application of his views permitted of a simple and logical reasoning. It is noteworthy to observe that further research in this subject appears now to be progressing and within the past few months Barnes and Wright have described the spontaneous transformation of Type V pneumococcus to Type II.

PART IV.

VIRULENCE TESTS.

It is apparent from a study of Table II that lobar pneumonias due to infections by Type I and Group IV pneumococci show a low mortality rate and that infections due to Type II and Type III are of much graver outlook. The differences in the average severity of the illnesses caused by the different types are apparent to all who have had the opportunity of studying pneumococcal infections. The differences in the mortality rates cannot be explained solely by the differences in the average age of incidence of these types for at all age groups the mortality rates are higher in Type II and Type III infections than in those due to Type I and Group IV pneumococci.

Virulence tests were performed to see if it was possible to explain this difference by differences in the virulence of the pneumococci in susceptible animals.

Technique.

After the death of a sputum-injected mouse the pneumococci in the peritoneal exudate were typed and the heart blood was plated on blood agar. After 24 - 36 hours a colony was subcultured in 6 cc. of Hartley's broth and allowed to grow for 15-18 hours. The growth was then examined and confirmatory typing performed in the cultures of Types I, II and VII by Sabin's method following the earlier discovery of Group IV pneumococci on plates considered to contain only Type I or Type II pneumococci. Successive decimal dilutions of the culture were made in Hartley's broth, using 0.3 cc. of the culture to 2.7 cc. of broth. Usually, in the case of Type I, II and III pneumococci, dilutions number 7, 8 and 9 containing 10^{-7}, 10^{-8} and 10^{-9} cc. of the original culture were used and, in the case of Group

IV pneumococci, dilutions number 5, 6 and 7 were used.

Two poured blood agar plates were then prepared, using 12 cc. agar to each of which was added 0.6 - 0.7 cc. defibrinated rabbit's blood prepared from the laboratory rabbits or obtained from Burroughs Wellcome & Co. Finally was added 0.5 cc. of the dilution to be tested. The three dilutions thus required six poured plates.

Two mice were each then injected intraperitoneally with 0.5 cc. of a dilution, six mice thus being used to each virulence test.

After 36 - 48 hours the plates were then counted and an average of the counts in the two plates of each dilution gave an estimate of the number of pneumococci injected into each mouse for each dilution used. On the death of a mouse the peritoneal exudate was examined for the presence of pneumococci and only on this positive evidence was death considered to be due to pneumococcal invasion.

This method, though not ideal, is considered to be sufficiently accurate for the purpose and little disappointment occurred in the series of experiments to discourage its continuance.

The results were generally read according to the method used by Cooper and given in the table below but in a number of cases death of the mice resulted from pneumococcal invasion up to a period of eight days and a modification of this table was then used in these circumstances.

Degree of Virulence.	Number of pneumococci required to kill within three days.
Fully.	1 - 5
Very highly.	5 - 50
Highly	50 - 500
Moderately	500 - 500,000.

Results.

The results of several experiments are given as examples and appended on accompanying pages. These results show a very satisfactory degree of accuracy in the dilutions and the close correspondence of the counts in the two plates used for each dilution.

In the experiments in which the virulence of Group IV strains was being tested it was occasionally found that only one mouse of the pair injected died of pneumococcal invasion and occasionally others injected with a greater number of pneumococci survived. In these cases accurate estimation of the virulence was not possible owing, apparently, to the differing susceptibility of the mice used. The examples given show instances of these difficulties encountered during the experiments.

TYPE I.

Expt.I. Source - empyema pus.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	17 and 12	15	Survived	over 18th	-
" 8	87 and 106	96	both died	6th	positive
" 7	763 and 824	793	" "	5th	"

Classified - Highly virulent.

Expt.II. Source - empyema pus.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	10 and 6	8	both died	3rd. & 4th.	positive
" 8	48 and 62	55	" "	6th. & 7th.	"
" 7	446 and 481	463	" "	4th	"

Classified - Very highly virulent.

Expt.III. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	36 and 36	36	both died	6th & 8th	positive.
" 8	150 and 182	166	" "	5th & 6th.	"
" 7	over 1,000	-	" "	6th	"

Classified - Highly virulent.

Expt. IV. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	0 and 0	0	survived	over 14th.	-
" 8	0 and 0	0	"	" "	-
" 7	1 and 1	1	both died	3rd. & 7th.	positive.

Classified - Fully virulent.

Expt. V. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	0 and 7	3	both died	4th & 8th.	positive.
" 8	22 and 44	33	" "	6th & 9th.	"
" 7	307 and 323	315	" "	5th & 6th.	"

Classified - Highly virulent.

Expt. VI.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	3 and 7	5	both died	5th & 6th	positive.
" 8	37 and 51	44	" "	6th	"
" 7	392 and 421	406	" "	5th	"

Classified - Very highly virulent.

TYPE II.

Expt. VII. Source - sputum, lobar pneumonia.

	Colony counts.	Average	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 6	1 and 0	1	both died	3rd & 7th	positive.
" 5	7 and 9	8	" "	2nd.	"
" 4	82 and 94	88	" "	2nd.	"

Classified - Fully virulent.

Expt. VIII. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9.	2 and 3	2	both died	2nd.	positive.
" 8	24 and 31	27	" "	2nd.	"
" 7	over 200	-	" "	2nd.	"

Classified - Fully virulent.

Expt. IX. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	0 and 0	0	both survived	over 14th.	-
" 8	0 and 0	0	"	"	-
" 7	0 and 1	1	"	2nd. & 3rd.	positive.

Classified, Fully virulent.

Expt. X. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 7	1 and 5	3	both died	2nd.	positive.
" 6	15 and 29	22	" "	2nd.	"
" 5	over 200	-	" "	2nd.	"

Classified - Fully virulent.

Expt. XI. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 6	13 and 8	10	both died	2nd.	positive.
" 5	53 and 61	57	" "	2nd.	"
" 4	over 500	-	" "	2nd.	"

Classified - Fully virulent.

Expt. XII. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 8	9 and 4	6	both died	2nd.	positive.
" 7	42 and 51	46	" "	2nd.	"
" 6	approx. 400	-	" "	2nd.	"

Classified - Fully virulent.

TYPE III.

Expt. XIII. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice	Day.	Examination of exudate for pneumococci.
Dilution 7	1 and 2	1	both died	2nd.	positive.
" 6	6 and 9	7	" "	2nd.	"
" 5	42 and 47	44	" "	2nd.	"

Classified - Fully virulent.

Expt. XIV. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 7	1 and 3	2	both died	2nd.	positive.
" 6	9 and 24	16	" "	2nd.	"
" 5	88 and 96	92	" "	2nd.	"

Classified - Fully virulent.

Expt. XV. Source - sputum, broncho pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 6	8 and 3	5	both died	2nd.	positive.
" 5	27 and 31	29	" "	2nd.	"
" 4	over 200	-	" "	2nd.	"

Classified - Fully virulent.

Expt. XVI. Source - empyema pus.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 7	0 and 0	0	both survived	over 14th	-
" 6	0 and 0	0	" "	"	-
" 5	5 and 2	3	both died	2nd.	positive.

Classified - Fully virulent.

Expt. XVII. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	3 and 5	4	both died	2nd.	Positive.
" 8	37 and 41	39	" "	2nd.	"
" 7	over 300	-	" "	2nd.	"

Classified - Fully virulent.

Expt. XVIII. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 9	0 and 0	0	both survived	over 14th.	-
" 8	0 and 0	0	" "	" "	
" 7	0 and 2	1	both died	2nd.	positive.

Classified - Fully virulent.

GROUP IV.

Expt. XIX. Source - sputum, bronchiectasis.

	Colony counts.	Average.	Mice	Day.	Examination of exudate for pneumococci.
Dilution 6	2 and 1	1	both survived	over 14th	-
" 5	8 and 6	7	" "	" "	-
" 4	61 and 63	62	both died	2nd.	positive.

Classified - Highly virulent.

Expt. XX. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice	Day.	Examination of exudate for pneumococci.
Dilution 7	11 and 13	12	both died	9th & 11th.	Negative
" 6	67 and 72	69	" "(1)	3rd.	positive.
			(2)	6th	negative.
" 5	over 500	-	" "	3rd & 6th	positive.

Classified - Highly virulent.

Expt. XXI. Source - sputum, mild influenza.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 7	9 and 7	8	both died	2nd.	positive.
" 6	63 and 53	58	" "	2nd.	"
" 5	over 500	-	" "	2nd.	"

Classified - Very highly virulent.

Expt. XXII. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 6	100 & 104	102	both survived	over 18th	-
" 5	approx.1000.	1000	both died.	6th	positive.
" 4	-	-	" "	6th & 7th.	"

Classified - Moderately virulent.

Expt. XXIII. Source - sputum, bronchitis.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 6	237 & 276	255	both survived	over 14th.	-
" 5	approx. 2000.	2000	both died	(1) 7th.	positive.
				(2) 9th.	negative.
" 4	-	-	" "	6th	negative.

Classified - Moderately virulent.

Expt. XXIV. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice.	Day.	Examination of exudate for pneumococci.
Dilution 6.	326 & 372.	349	both survived	over 14th.	-
" 5.	approx. 3000.	3000	" "	" "	
" 4.	-	-	both survived	" "	

Classified - Slightly virulent.

TYPE VII.

Expt. XXV. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice	Day	Examination of exudate for pneumococci.
Dilution 8.	23 & 18	20	both survived	over 14th.	-
" 7.	108 & 137	122	one died	7th.	positive.
" 6.	over 1000	over 1000	both survived		-

Classified - Moderately or slightly virulent.

Expt. XXVI. Source . sputum, lobar pneumonia.

	Colony counts.	Average.	Mice	Day	Examination of exudate for pneumococci.
Dilution 6	30 & 26	28	both died	10th & 16th.	negative.
" 5	over 200.	200	" "	13th & 14th.	"
" 4	over 2000.	2000	" "	15th and 16th.	"

Classified - Moderately or slightly virulent.

Expt. XXVII. Source - sputum, bronchitis.

	Colony counts.	Average.	Mice	Day	Examination of exudate for pneumococci.
Dilution 7.	164 & 128.	146.	both survived	over 14th.	
" 6.	over 1000		" "	" "	
" 5.	approx. 10,000		one died	2nd.	positive.

Classified - Moderately virulent.

Expt. XXVIII. Source - sputum, lobar pneumonia.

	Colony counts.	Average.	Mice	Day	Examination of exudate for pneumococci.
Dilution 6	87 and 76.	81	both survived	over 14th.	-
" 5	over 500		" "	" "	-
" 4	over 5,000		" "	" "	

Classified - Moderately or slightly virulent.

Six examples are given of Types I, II, III and Group IV and the details of the virulence tests performed on the Type VII strains identified by Sabin's method are also included.

A total of 77 virulence tests was performed and the results are given in Table IV.

TABLE IV.

Degree of Virulence.	Type I.	Type II.	Type III.	Type VII.	Group IV.
Fully virulent.	3.	13.	15.		2.
Very highly virulent.	8.				2.
Highly virulent.	3.				7.
Moderately or Slightly virulent.				4.	20.
Total:-	14.	13.	15.	4.	31.

In many of the examples given, the colony counts in the decimal dilutions are more often approximately one-eighth of the count of the next lower dilution. This is perhaps accounted for by the fact that the vigorous shaking to which dilution tubes were subjected resulted in the breaking up of some chains and, in the case of the higher counts, it is possible that the greater separation of colonies encouraged the growth of less viable organisms.

It is generally considered that Type I pneumococci are fully virulent. Christie found that the four strains investigated by him were fully virulent but Table IV shows that only approximately 20% were fully virulent and over half were very highly virulent, the remaining 20% being highly virulent. It is seen from

the examples given, that, even in fairly low dilutions containing a hundred or more colonies in each 0.5 cc. injected, the mice survived for many days in some experiments. This fact was also noted by Cruikshank.

From a study of the incidence of bacteraemia in lobar pneumonia due to the different types of pneumococci Cruikshank considers that Type I, as indicated by its frequent presence in the blood stream, has greater invasive properties than the other types and possesses less toxigenicity. The results of the thirteen virulence tests also tend to support this view.

Type II pneumococci were fully virulent in all strains investigated, the mice dying in 36-60 hours even after the injection of dilutions containing only one or two pneumococci.

All Type III strains were fully virulent, more so, if possible, than Type II pneumococci since mice injected with the highest possible dilution died within 30-48 hours. Christie found that seven strains of the eleven Type III pneumococci investigated by him were not quite fully virulent and Blake stated that Type III pneumococci may be less virulent than is usually considered, the high death rate in Type III infections being due to the greater age at which these infections occurred but Cruikshank found that the death rate for Type III infections was greater at all age groups than that of other types and the results of the fifteen virulence tests are in accordance with this opinion.

Type VII strains appear to be of low virulence.

Group IV pneumococci are of many types and show differing degrees of virulence. Some possess maximum

virulence and others considerably less, many being of low virulence. In many virulence tests performed on Group IV pneumococci it was frequently found that although the mice died in six or more days, examination of the peritoneal exudate failed to reveal the presence of pneumococci. Possibly a chronic septicaemia was caused in these cases, perhaps in the nature of an infective endocarditis.

The 77 strains of pneumococci investigated were mainly recovered from patients suffering from lobar pneumonia or its direct complications but in some cases, particularly in Type III and Group IV, were recovered from much milder infections. The results tend to show that, no matter what the source, the virulence remains unaltered.

In many organisms it is impossible to increase the virulence by passage through susceptible animals (Webster). This appears to be the case with reference to pneumococci newly isolated from man. The pneumococcus investigated in Expt. XXIV was passaged through mice on eight occasions without any alteration occurring in its virulence for mice.

Loss of virulence, however, apparently occurs on repeated subculture. A Type I strain obtained from the Lister Institute, probably maintained in subculture for a long time, was found to kill mice only by the intraperitoneal injection of broth containing over 1,000 pneumococci. On the other hand, a Type II strain subcultured on blood agar at intervals of four days for four months was still fully virulent for mice at the end of this time. By the Eosin Relief method of capsule demonstration the Type I strain had only a very poor capsule whereas the Type II

pneumococcus had still a large capsule. The relatively low virulence of Type I organisms in the virulence tests detailed above could not be attributed to too long maintenance in artificial culture since the tests were performed within 24 - 48 hours of isolation from the heart blood of mice injected with sputum.

A comparison of Tables II and III and the results of the virulence tests justifies certain conclusions:-

The greater invasiveness of Type I pneumococci probably accounts for the greater incidence in young persons and the low death rate in these infections is due to the good condition of the patients and the relatively low virulence and toxigenicity of the pneumococcus.

Type II and Type III pneumococci are of maximum virulence and the greater age of the patients is an added factor in the high death rates in these infections.

Group IV strains are generally of low virulence with little toxigenicity and the death rate in pneumonia caused by these strains is low except in older people with lessened resistance.

PART V.

THE EOSIN RELIEF METHOD OF CAPSULE DEMONSTRATION.

In preceding pages the Eosin Relief method of capsule demonstration has frequently been mentioned. The results of the use of this method are incorporated in Part VI of this investigation and the comparatively recent discovery of this method of capsule demonstration also justifies a description of the technique and results obtained in its use.

The general methods used for the demonstration of capsules involve the staining of the capsule and necessitated the use of special stains and usually required a considerable degree of skill. The Eosin Relief method is easy and rapid to perform and the stains used are simple in preparation.

Technique.

The special stains required are (1) Dilute (1/10) Carbol Fuchsin and (2) 10% solution of Eosin. By means of a small platinum loop, one loopful of the material to be examined e.g. culture of pneumococci or the peritoneal washings of a mouse injected with sputum, is placed on a slide and to it is added a loopful of the Dilute Carbol Fuchsin. This is allowed to act for ½ - 1 minute and then one loopful of Eosin solution is added. By means of a piece of cigarette paper, or any thin, straight-edged paper, this small amount of fluid is drawn evenly over the slide and allowed to dry. It is then examined microscopically using the oil immersion lens.

Results.

The dilute Carbol Fuchsin stains the bodies of the pneumococci and the Eosin forms an even, red background around the organisms leaving the capsule unstained and demonstrated as a clear area round the deeply stained body. The contrast is very marked and striking.

It soon became possible to differentiate with great accuracy the different types of pneumococci. It was whilst using this method as routine in the examination of the peritoneal washings of sputum injected mice that the simultaneous presence of two different types of pneumococci was detected and later proved by the investigation of colonies growing on blood agar after spreading with mouse heart blood.

Type I pneumococci, examined by this method, reveal a moderately large, fairly well-defined capsule of very slightly pink colour and often appear in chains. The chain appearance, when present is highly characteristic, having an appearance suggestive of a concertina - the bodies are close together, the margins of the capsule scalloped and dark transverse bands traverse the capsule between the indentations. In diplococcal form the capsule and body appear rather pointed and the transverse marking is usually well marked. Though usually moderately large, definite differences were noted in the size of the capsule.

Type II pneumococci show very large, white capsules, often with bulbous ends, this latter appearance being highly characteristic. Transverse markings are not present.

Type III pneumococci do not show the pale or white capsule but a smooth red halo surrounds a rather large, rounded body and merges rather gradually into the slightly granular Eosin background.

Group IV pneumococci, being of many types, show considerable difference in appearance. It may be stated generally that the capsule is rather small, of light red appearance and occasionally is surrounded by a thin clear area perhaps resulting from retraction

of the Eosin background from the capsule but not noticed in other types. The bodies are usually much smaller than those of the other types.

Type VII pneumococci possess the general characters of Group IV.

Tinted drawings of examples of the different types of pneumococci as seen under the microscope are given to illustrate their different characteristics.

This method was also highly satisfactory in the detection of Friedländer's bacillus occasionally found in the peritoneal exudate of mice injected with sputum of Influenza patients. On one occasion the peritoneal exudate of a mouse injected with sputum of an Influenza patient showed an almost pure collection large-chained streptococci possessing a definite, fairly large white capsule. A pure growth of markedly haemolytic colonies with typical streptococcal appearance resulted on plating the heart blood on blood agar. These organisms did not agglutinate in antipneumococcal serum and Hartley's broth subcultures were not bile-soluble.

The peritoneal washings of sputum-injected mice were investigated in 139 successive instances, the sputum being obtained from successive patients admitted to the pneumonia wards of Knightswood Hospital. The capsule was classified according to its size as very large, large, moderately large, fairly small and small. Table V gives a summary of the findings further classified according to type.

TABLE V.

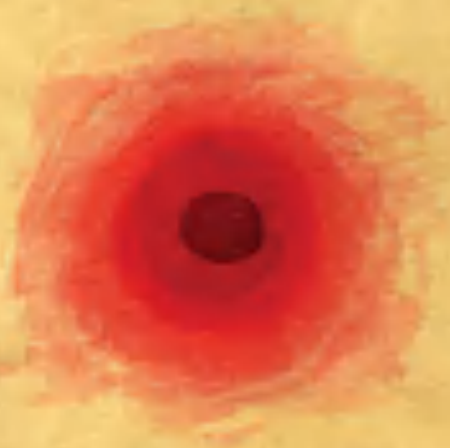
Capsule.	Type I.	Type II.	Type III.	Type VII.	Group IV.
Very large.	3	40	13		
Large.	4				4
Moderate.	13				13
Fairly small.				4	35
Small.					10

From this table it is seen that the capsule is uniformly very large in Type II and Type III pneumococci, generally moderate in Type I but showing definite differences in size and usually fairly small in Group IV pneumococci.

TYPE I

TYPE II

TYPE III



GROUP IV

PART VI.

THE CORRELATION OF VIRULENCE AND SIZE OF CAPSULE.

The pneumococcus loses its virulence when the capsule disappears, the rough, non-capsulated strains being avirulent. The Type I strain obtained from the Lister Institute was of low virulence and the capsule was very small.

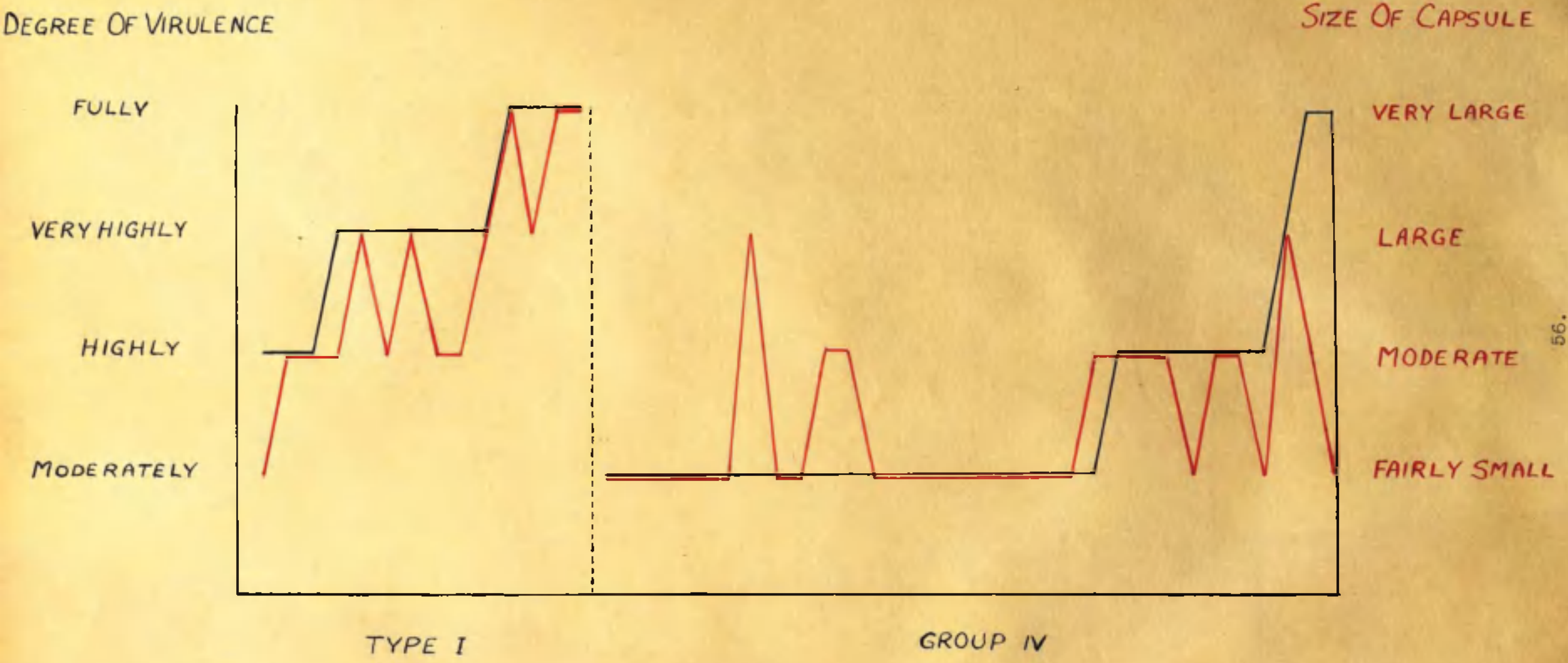
A study of Tables IV and V reveals close similarities in their general appearance and suggests that pneumococci possessing the larger capsules are more virulent for mice than those possessing smaller capsules. This possibility can not be satisfactorily investigated by the comparison of the virulence and size of capsule in groups of pneumococci but only by observation of these points in individual strains.

Prior to the performance of virulence tests, the size of capsule was classified as in Table V and the results are given in Table VI. It is superfluous to include in this table Type II and Type III pneumococci for all strains belonging to these types possess very large capsules and were fully virulent. Similarly, the Type VII strains investigated were of moderate or slight virulence and possessed fairly small capsules. The results of Type I and Group IV investigations are given in detail.

TABLE VI.

Pneumococcus.		Degree of Virulence.	Size of Capsule.
Type 1.	1.	Highly.	Moderate.
	2.	Fully.	Very large.
	3.	Highly.	Moderate.
	4.	Very highly.	Moderate.
	5.	Very highly.	Large.
	6.	Very highly.	Moderate.
	7.	Highly.	Moderate.
	8.	Very highly.	Large.
	9.	Fully.	Large.
	10.	Fully.	Very large.
	11.	Very highly.	Moderate.
	12.	Very highly.	Moderate.
	13.	Very highly.	Large.
	14.	Fully.	Very large.
Group IV.	15.	Fully.	Moderate.
	16.	Moderate.	Fairly small.
	17.	Moderate.	Fairly small.
	18.	Moderate.	Fairly small.
	19.	Moderate.	Fairly small.
	20.	Moderate.	Fairly small.
	21.	Highly.	Moderate.
	22.	Highly.	Moderate.
	23.	Moderate.	Fairly small.
	24.	Moderate.	Large.
	25.	Moderate.	Fairly small.
	26.	Moderate.	Fairly small.
	27.	Highly.	Moderate.
	28.	Moderate.	Moderate.
	29.	Very highly.	Large.
	30.	Moderate.	Moderate.
	31.	Highly.	Moderate.
	32.	Moderate.	Fairly small.
	33.	Highly.	Fairly small.
	34.	Moderate.	Fairly small.
	35.	Moderate.	Fairly small.
	36.	Fully.	Fairly small.
	37.	Moderate.	Fairly small.
	38.	Highly.	Moderate.
	39.	Moderate.	Fairly small.
	40.	Moderate.	Fairly small.
	41.	Moderate.	Fairly small.
	42.	Highly.	Fairly small.
	43.	Moderate.	Fairly small.
	44.	Moderate.	Fairly small.
	45.	Moderate.	Moderate.

These details are given in graphical form which illustrates the general trend more clearly. The observations are grouped in rising order of virulence with the size of capsule superimposed for comparison.


DEGREE OF VIRULENCE
SIZE OF CAPSULE
FULLY
VERY HIGHLY
HIGHLY
MODERATELY
VERY LARGE
LARGE
MODERATE
FAIRLY SMALL
TYPE I
GROUP IV

Since it was difficult to ascertain in almost all cases whether a Group IV strain was moderately or only slightly virulent owing to the small series of broth dilutions used in each experiment and as it was difficult to ascertain with a great degree of accuracy the size of capsule of these strains with little capsule visible, for the purpose of the graph, the virulence in these cases has been entered as 'moderate' and the size of capsule as 'fairly small'.

It is not always possible to decide accurately the correct classification of the size of capsule but, taken in general, the graph gives a definite appearance of a high correlation between the virulence and size of capsule. This is most marked in Type II, III and VII. In Type I and Group IV pneumococci there is less high correlation and in two instances in Group IV two notable contrasts are seen.

In one instance a fully virulent organism possessed a fairly small capsule and in the other instance a moderately or slightly virulent organism possessed a large capsule. There appeared to be no explanation for these two departures from the general rule and there is only the possibility that two different types of Group IV pneumococci were present in the sputum and that the colony subcultured from the plated heart blood belonged to the type present in great minority in the peritoneal washings and thus overlooked in the Eosin method of examination.

PART VII.

RAPID TYPING OF PNEUMOCOCCI.

The Neufeld reaction was described in 1902 and although Etinger-Tulczynska had used rapid typing for some years and a new method was proposed simultaneously by Armstrong and by Logan and Smeal in 1932, it was the result of the work of Goodner and of Sabin in 1933 which showed its value and the unsuitability of horse serum.

During the present investigation considerable use has been made of Sabin's method as an aid to the selection of sputa for further bacteriological investigations and in the verification of the type of pneumococcus recovered from blood agar plated with sputum-injected mouse blood.

The sputum of all pneumonia patients in Knightswood Hospital is typed for statistical purposes and the information obtained is of considerable prognostic value in the individual cases. Serum treatment is not performed in the hospital but it is possible that such treatment will soon be available now that it has been found to be of value and potent antisera have been prepared at a reasonable and rapidly falling cost. In this event it is important that the type of the infecting pneumococcus should be discovered with the minimum delay in order to obtain maximum benefit from treatment. Using the mouse technique, the type is usually only discovered in twenty-four hours and in several cases it may be some hours before sputum is available for injection into a mouse. It frequently happens that the mouseries cannot supply sudden demands and sputum cannot be typed for some days.

It has frequently been stated that the rapid typing of pneumococci by Sabin's method required special skill, and detailed instructions for optimum

results in its performance often mislead a possible convert to this method. Contrary to original fears, this method has proved exceedingly simple and accurate and became the routine method of typing in use in the hospital in the last few months. It can be performed by all who possess a microscope and obviates the necessity of obtaining a Home Office licence for animal experiments.

Technique.

Antipneumococcic serum prepared from rabbits was obtained from the Lederle Laboratories. It was supplied in small, stoppered bottles containing 0.5 cc. antiserum already tinted with Methylene Blue.

A fleck of sputum is transferred to a glass slide by means of a platinum loop and one or two loopsful of tinted antiserum added to the sputum and thoroughly mixed. The tinted serum is most convenient for use but many laboratories prepare the antiserum for distribution untinted and in capillary tubes. In this case a small loopful of Methylene Blue is also added to the sputum and the antiserum on the slide and thoroughly mixed. A coverslip is then applied and the slide examined under the oil-immersion lens. It is almost essential to possess a daylight illuminating lamp or, failing this, to place a blue glass between the lamp and the microscope mirror. The illumination of the field must be reduced by means of the diaphragm.

Erythrocytes are plainly recognised and, by means of the Methylene Blue, it is possible to recognise and distinguish polymorphonuclear and epithelial cells. Bacteria are stained a light blue

and a search is made for diplococcal or chain forms of pneumococci. A few seconds search is usually rewarded by success but in cases of difficulty it was advisable to stain a smear of sputum with Dilute Carbol Fuchsin to show the bacterial flora and relative abundance or scarcity of pneumococci. It was usually more difficult to find pneumococci in the sputum obtained in the first two days of the infection than at any later period.

A negative reaction was recorded if the pneumococci showed only a light halo due to refraction from the capsule. Alterations to the illumination by means of the diaphragm resulted in an alteration in size of the halo and cleared the mind of all doubt in cases showing large haloes.

A positive reaction was recorded on the appearance of a sharply demarcated, greyish swelling of the capsule ("quellung" phenomenon) at the first examination of the preparation and rarely delayed more than a minute, though American writers generally state that the reaction may only appear after many minutes have lapsed.

A search was made for not less than fifteen minutes in every case in which a negative result was recorded.

Results.

Only antisera for Types I, II and VII were available. Type I pneumococci possess a rather large body and the capsular swelling in a positive reaction is of fairly clear appearance and moderate size, the breadth of the swelling being approximately the breadth of the body. For this reason a careful

search must be conducted.

Type II pneumococci possess a large body and a positive reaction is indicated by a bulky, thick, grey swelling almost twice the breadth of the body. When present, the result is striking and noticeable on even cursory examination.

The marked difference in the degree of the swelling of the capsules in Type I and Type II pneumococci and the greater size of the capsule in Type II as demonstrated by the Eosin Relief method is in contrast to the statement by Cruikshank that he found it difficult to distinguish any difference in the amount of capsular materials in Type I and II.

No recently prepared antiserum for Type III pneumococci was available but a four-year-old supply of untinted diagnostic antiserum for Type III was found in the laboratory. This serum was put up for use in capillary tubes and the majority of them gave negative reactions with sputum containing Type III pneumococci. A few tubes, however, contained antiserum which had retained its potency to some degree and certain observations have been obtained in its use. In the presence of a potent serum the capsular swelling was very marked, with a breadth three times that of the body, rather pale and giving an appearance of flatness. Even without the Type III antiserum the appearance of a sputum containing Type III pneumococci was highly suggestive when examined under the microscope. The pneumococci were present in abundance and in chains of an average of 6-10 cocci, the bodies being large and the halo of much greater size than that of pneumococci of any other type.

Type VII pneumococci possessed a body of moderate

size and the capsular swelling was distinct and greyer but slightly less broad than that of Type I pneumococci.

In the absence of antisera for other types in Group IV, conclusions could only be drawn from negative results in the employment of the other antisera. It was possible, after a time, to predict with considerable accuracy, after the examination of a sputum with any one antiserum, that the pneumococci observed belonged to Group IV. Though sometimes large, the bodies were usually smaller than those of Type I, II and III and their haloes smaller. The presence of chains was sometimes noticed but these lacked the large haloes of Type III pneumococci. On the exhaustion of the small stock of Type III antiserum, definite determination of Type was not possible in sputa failing to give positive reactions with antisera for Type I, II and VII and mouse injection was necessary to determine whether the infection was due to Type III or Group IV. In these cases the opinion previously formed as to Type or Group was found to be remarkably accurate.

The method was found to be just as satisfactory in typing pneumococci in pleural fluids.

In young broth cultures the Neufeld reaction was well marked but in older cultures many of the pneumococci failed to show swelling of the capsule or showed it in small degree only.

In cases in which no sputum was available, particularly in children, it was usually possible to give within eight hours a definite statement as to the type of pneumococcus present, often much earlier. The posterior pharyngeal wall was rubbed with a swab

stick and mucus removed by the swab or coughed on to the swab through the reflex irritation. The swab was put into 6 cc. Hartley's broth and incubated in the broth until growth was seen, usually in four to six hours. A film was prepared and stained and, if pneumococci were seen, the culture was typed by Sabin's method.

In the earlier period of the use of Sabin's method, notes were taken to compare the accuracy of the mouse and rapid typing methods. The sputum of 136 consecutive patients admitted to the pneumonia wards was first typed by the rapid method in so far as the available antisera allowed and then mice were injected intraperitoneally with the sputum. The results obtained with the available Type III antiserum are not included in the results of typing since the antiserum available was only sufficient to perform a few tests in suspicious cases and only at the beginning of the series.

From Table VIII it is seen that of the 136 sputa examined 63 gave a definite type on using Sabin's method and 73 failed to type.

Table IX gives the results obtained by typing by the mouse technique and a comparison of Table VIII and IX reveals that accurate comparisons may be made only in the case of Types I and II.

TABLE VIII (SABIN'S METHOD).

	Lobar.	Non-Lobar.
Type I.	19	0
Type II.	38	2
Type III.	3 } 33	1 } 44
Failed to type.	30 }	43 }
Total:-	90	46

TABLE IX. (MOUSE TECHNIQUE).		
	Lobar.	Non-Lobar.
Type I.	17	0
Type II.	40	2
Type III.	8)	5)
Group IV.	22) 33	39) 44
Non-pneumococcal	3)	0)
Total:-	90	46

In both Sabin's and the mouse typing method a total of 59 sputa gave definite types but a detailed comparison reveals the fact that discrepancies in the results appear on four occasions. On two occasions Sabin's method detected the presence of Type I pneumococci in the sputum when the mouse technique revealed the presence of only Group IV organisms but the rapid typing of the mouse peritoneal exudate revealed the presence of a few Type I pneumococci in addition. The mouse technique proved the presence of Type II pneumococci in a varied bacterial flora from the peritoneal washings in two cases in which, after fifteen minutes search by Sabin's method for the specific reaction in each type, the result was recorded as negative. Repeat examinations of fresh sputum from these two cases were performed by Sabin's method on the finding of Type II pneumococci by the mouse technique and in one case a positive result was obtained one day later after a search of ten minutes and in the other case the positive result was obtained only after three days. The mouse technique detected the presence of only Group IV organisms in the four instances in which Type VII pneumococci were identified by Sabin's method.

These results indicate that Sabin's method of typing pneumococci is as accurate as the mouse typing method and in the present investigation the results show that for the purpose of serum treatment Sabin's method would have detected two early cases of Type I lobar infections which were most suitable for treatment and would have failed to detect two cases of Type II infection in which serum treatment is of less value.