# A SEROLOGICAL INVESTIGATION OF NEISSERIA GONORRHOEAE USING A RED CELL SENSITISATION TECHNIQUE.

A thesis presented to the University of Cape Town in part fulfilment of the requirements for the degree of Doctor of Medicine.

by

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CONTENTS.

CONTENTS.



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# SECTION I

## INTRODUCTION.

# INTRODUCTION.

At a time when such great strides have been made in the elucidation of antigenic relationships in so many groups of bacteria, it is perhaps startling to reflect that in the field of the gonococcus there has been no major advance for about fifty years.

Undoubtedly we have a far greater assortment of culture media at our disposal, but even in 1885 Bumm[1] had no difficulty in growing the gonococcus, and in maintaining it on subculture.

Its biochemical behaviour was accurately recorded at the turn of the century by Gordon[2] (1905) and others.

Müller and Oppenheim[3] in 1906, followed in the same year by Bruck[4], obtained positive gonococcal complement-fixation tests in a number of their cases, and not even the most optimistic among us can claim that we have obtained significantly better results.

## The Nature of the Problem.

Two major problems in relation to the gonococcus confront us today.

The Problem of Identification. The presence of the typical Gram-negative diplococci in the leucocytes from a urethral, vaginal, or ophthalmic discharge is, in practice, sufficient to establish a gonococcal aetiology with a reasonable degree of certainty.

On the other hand identification of a culture on the basis of its colonial morphology, biochemical and serological behaviour as N. gonorrhoeae, is a far more formidable problem.

Some of the less pigmented, non-pathogenic, Gram-negative cocci are difficult to distinguish colonially; all show a striking preference for enriched culture media, although the gonococcus is undoubtedly the most fastidious in this respect; and all share a positive oxidase reaction. One is therefore reduced to acceptance of the results of fermentation

reactions, and, on present day criteria, a Gram-negative coccus which ferments glucose only, is a gonococcus.

On this basis a non-glucose fermenting gonococcus does not exist. Such an organism is N.catarrhalis. Yet strains of N. gonorrhoeae which ferment glucose weakly and as late as four to five days are not uncommon, and we have encountered organisms morphologically and serologically indistinguishable from N. gonorrhoeae which have not fermented glucose after ten days.

If maltose is fermented as well, the organism becomes a meningococcus. Carpenter and Charles[5] (1942) reported the isolation of seven meningococci from the genital tract. In one of these patients a previous culture had been identified as a gonococcus. In a second patient a gonococcus was isolated subsequently. To complicate matters still further Johnston[6] (1950) labelled her twelve glucose and maltose fermenting strains isolated from the genito-urinary tract as N. subflava.

The Problem of Antigenic Variants. The second important problem in relation to the gonococcus is that of antigenic variants within the group. Are all gonococci serologically identical, or can antigenically distinct types be defined within the group ?

Although a considerable amount of effort has been devoted to this problem, no really acceptable results have emerged. There is a large body of opinion that supports the view that all strains are serologically identical. Others claim to be able to differentiate definite antigenic groups, and these range from two, three, four, to as high as fifty two.

Some workers have interpreted small differences in agglutinin titres as indicative of an antigenic difference. Others consider strains to be identical if they are able to remove the greater part of the agglutinins from an antiserum in absorption tests. The position, therefore, remains as uncertain today as it was in 1907 when Torrey[7] hazarded the first attempt at a serological grouping of these organisms.

## The Red Cell Sensitisation Technique.

In this thesis an attempt has been made to apply a red cell sensitisation technique to the elucidation of some of these problems.

Middlebrook and Dubos[8] in 1948 published an account of the sensitisation of sheep red cells with a fraction obtained from M. tuberculosis. The technique was simply to incubate a dilution of Koch's old tuberculin with a suspension of sheep cells, and, under these circumstances, a polysaccharide fraction from the tubercle bacillus became adsorbed on to the red cells. Cells sensitised in this manner became specifically agglutinable by tuberculous antisera.

The test itself, which became known as the Middlebrook-Dubos test, was not of great value since it lacked specificity producing a large number of apparently false positive results. The technique, however, represented a major advance in serological methods, and in the last few years has found wide application in such diverse fields as brucellosis[9], plague[10], investigation of escherichia strains[11], the detection of antibodies against trichomonas infections[12], and in the field of salmonella serology[13,14,15,16].

The application of these techniques to the field of gonococcal infections was first mentioned by Thomas and Mennie[17] (1950). Using various methods of extraction, they were able to sensitise cells with a gonococcal extract, and they obtained positive reactions with the sera of a number of cases of chronic gonococcal infection.

The work described in this thesis is an extension of some of the original observations of these workers. The technique of red cell sensitisation was adapted both to the performance of an absorption test, as well as to the direct estimation of antibody titres. Using the absorption method a serological study of a series of freshly isolated gonococcal strains was carried out. This thesis concerns itself with a description of these methods, and with the results of their application to the problems discussed.

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# SECTION II

# METHODS.

# CHAPTER II.

## GENERAL.

Saline. Saline containing 0.85 % sodium chloride in distilled water was used.

Sheep cells. Sheep were bled via the external jugular vein into an M.R.C. blood transfusion bottle containing 50 ml. of a 5.0 % sodium citrate solution in saline. Approximately 250 ml. to 350 ml. of blood was taken at a time. On a few occasions blood was collected in citrate saline from sheep killed at the abbatoir by severing the neck vessels. Before use the cells were washed three times with five volumes of saline, and finally packed for 25 minutes at 2500 r.p.m.

Complement. For complement-fixation tests not less than twelve guinea pigs were heart punctured and ten ml. blood taken from each animal. The sera were pooled. In summer it was necessary to boost this complement with high titre complement which had been lyophilised during the colder months of the year. When not in use the complement was stored at -20° C.

For the gonococcal haemolysis test an excess of complement was required, and this was provided by using a 1 in 15 dilution of the complement remaining from the previous day.

Tubes and racks. Round bottom 75 x 13 mm. tubes were used for all haemolysis and complement-fixation tests. Dreyer tubes were used for agglutination tests.

Forty eight hole racks of the usual Wassermann pattern made of perspex were used. The transparent base permitted the direct reading of haemolysis and complement-fixation tests and proved of the utmost value.

Titres. All titres will be expressed in terms of the reciprocal of the initial serum dilution.

Haemolysin. An antiserum against sheep erythrocytes was prepared in rabbits and preserved with an equal volume of glycerol. The method of preparation and titration employed was that described by Kolmer[18].

Nomenclature. The various gonococcal strains and the antigens prepared from them will be referred to by ordinary capital letters; the corresponding antisera by small letters.

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## CULTURE MEDIA.

After investigating various media, a chocolate agar prepared in the following manner was adopted for routine use.

| | | |
|---|---|---|
| Base. | Bacto beef extract | 3.0 grams |
| | Bacto tryptose | 10.0 grams |
| | Sodium chloride | 5.0 grams |
| | Agar | 25.0 grams |
| | Water | 1000 mls. |
| | pH adjusted to 7.4. | |

This was autoclaved at fifteen pounds pressure for thirty minutes and stored in 500 ml. amounts.

Enrichment. Outdated human blood bank blood was laked by freezing (-20° C.), and then thawing at room temperature.

For use the base was melted, and then cooled to approximately 50° C. Approximately 15 % laked blood was then added and the mixture placed in a boiling water bath. The final colour of medium aimed at was a dark reddish brown. The average time in the water bath was about three minutes. Plates were then poured using not less than 25 ml. of medium to a 100 mm. diameter petri dish.

The use of human blood was determined by its availability in almost unlimited amounts. Sterile horse blood in the quantities used (average 150 ml. per day), was not readily available. Human blood, however, proved extremely satisfactory. Laked blood was preferred since it appeared to give a medium of superior quality.

The type of growth obtained was largely dependant on the quality of the medium. Overheating after the addition of the blood, using too little medium per plate, or using too little blood in proportion to base, all having striking adverse effects on the growth of the gonococcus.

With the best medium a luxuriant growth was obtained in eighteen hours. The medium proved eminently suitable for overnight primary isolation, as well as for the maintenance of stock cultures. For the latter purpose the medium was dispensed in screw capped bottles and stored at 37° C. They were subcultured weekly.

For the investigation of fermentation reactions the following medium was used.

| | | |
|---|---|---|
| Base. | Bacto beef extract | 1.0 gram |
| | Proteose peptone No. 3 (Difco) | 10.0 grams |
| | Sodium chloride | 5.0 grams |
| | Bacto agar | 12.5 grams |
| | Phenol red (0.025 %) | 5.0 mls. |
| | Water | 1000 mls. |
| | pH adjusted to 7.4. | |

This was autoclaved at fifteen pounds pressure for fifteen minutes, and stored in 100 ml. amounts.

Sugars. 10.0 % solutions of dextrose, maltose, sucrose, and lactose were prepared, and sterilised by Seitz filtration.

Enrichment. Ascitic fluid was used.

For use 100 ml. of the base was melted, cooled to 50° C., 12.0 ml. of carbohydrate and 30.0 ml. of ascitic fluid added, and the medium distributed and sloped in 2.0 ml. amounts in screw capped bottles (bijou bottles).

This medium, having a final agar concentration of about 1.0 %, provided a soft but sufficiently solid medium readily supporting the growth of the gonococcus.

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## THE GONOCOCCAL STRAINS INVESTIGATED.

The strains of N.gonorrhoeae used in this investigation were obtained from untreated cases of acute gonococcal urethritis in males attending the Venereal Diseases Clinic at the King Edward VIII Hospital, Durban.

Smears were made and stained by Gram's method. At the same time a previously warmed chocolate agar plate was inoculated, and the plates then incubated overnight in an atmosphere of $CO_2$ which was simply provided by leaving them in a closed gallon tin with a lighted candle. Single colonies were then subcultured on to further chocolate agar plates.

Each strain of N.gonorrhoeae investigated originated from a single colony from the original culture plate, or from a single colony from the primary subculture.All the strains fulfilled the following criteria.

1.In direct smears the organisms presented as Gram-negative intracellular diplococci. A few smears, however, had numerous pus cells but no organisms on direct examination, the gonococcus being recovered by culture.

2.Growth occurred only on enriched media, and, generally, only in the presence of an atmosphere of $CO_2$. After a few weeks on subculture some of the strains grew on blood agar under ordinary aerobic conditions, the growth under these circumstances, however, being poor.

3.Three types of colonial appearance were observed on chocolate agar after approximately eighteen hours.

i. The commoner was a colony one to two mms. in diameter, with a convex surface, and an entire edge. The surface was smooth, shiny, and translucent.

ii. The less common colony was umbonate and one to three mms. in diameter with an undulate edge. Both these appearances have been observed on the same plate with the same gonococcal strain. Recently isolated strains were typically mucoid when manipulated with a loop.

iii.The third and least common type of colony observed

was a tiny colony approximately 0.3 mms. in diameter after eighteen hours incubation, showing a convex surface and an entire edge. It tended to lack the mucoid properties of the other types, and occasionally required more vigorous decolorisation in the Gram stain.

4. When tested with a fresh 1.0 % aqueous solution of dimethylparaphenylenediamine hydrochloride, the colonies gave a positive oxidase reaction. The colonies became purple in about thirty seconds, and black in five to ten minutes.

5. All the strains fermented glucose, but not lactose, maltose, or saccharose. The fermentation of glucose was usually obvious overnight, but four strains required two, three, four, and five days respectively before the fermentation was apparent.

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## PREPARATION OF ANTIGENS.

Suspensions for the preparation of antigens were prepared by harvesting the organisms from chocolate agar plates into 20.0 ml. saline in a McCartney bottle. Between three to six plates of each strain were harvested daily, and the antigens extracted within ten to fourteen days of the isolation of the organism. Thus all the strains investigated could reasonably be described as freshly isolated. The suspensions were stored at -20° C. prior to extraction.

### Extraction of the Type Specific Sensitising Antigen.

The suspension harvested from fifty petri dishes was washed once in saline, resuspended in 10.0 ml. saline, and 0.5 ml. N/1 sodium hydroxide solution added. The suspension was then placed in a 37° C. water bath until solution was complete. If the organisms did not readily dissolve a further 0.5 ml. N/1 sodium hydroxide solution was added.

Excess alkali was then cautiously neutralised by the addition of N/1 hydrochloric acid, 0.1 ml. at a time, since an excess produced precipitation. This precipitate could be redissolved by adding alkali. Five volumes of absolute alcohol was then added, and the flocculant precipitate which formed recovered by centrifugation. The supernatant was discarded and the precipitate allowed to drain.

The precipite was resuspended with vigorous shaking in 20 ml. of a phosphate buffer (pH 6.9). Insoluble material was removed by centrifuging. The clear supernatant contained two antigenic fractions:

i. A fraction which was adsorbable on to red cells (type specific antigen).
ii. A fraction which was not adsorbable on to red cells but which was active in a complement-fixation test (group antigen).

Since this solution is used in the sensitisation of red cells in the gonococcal haemolysis test, the term "sensitising antigen" will be used when referring to the solution containing the type specific, and, generally, the group antigen as well.

The final deposit was also retained and used either in absorption tests, or in the preparation of a complement-fixing antigen.

Comment. In general the gonococcal antigens are soluble in alkali and precipitated by acid. In order to dissolve the sensitising antigen in a slightly alkaline solvent (pH 7.2) a pH of 6.9 was selected as the precipitate to be resuspended was strongly alkaline.

After a variable period of storage the sensitising antigen deteriorated as indicated by a loss of its capacity to sensitise cells. This loss was associated with a fall in pH. Although its activity was not restored on readjusting the pH, it was hoped by the use of a buffer to circumvent this fall in pH and hence the deterioration of the antigen. Thus a 6.9 buffer was selected as a solvent for the sensitising antigen.

## Preparation of the Complement-Fixing Antigen.

This was prepared from either a suspension harvested from approximately twenty petri dishes, or from the deposit obtained following the extraction of the sensitising antigen. The method followed was that described by Price[19].

The suspension was standardised to an opacity of 500 million organisms per ml. (between tubes 1 and 2 Burroughs Welcome Opacity Standards). To every 100 ml. suspension 1.0 ml. N/1 sodium hydroxide solution was added and the suspension placed in a 37° C. water bath till solution was complete (approximately 30 minutes). One and a half ml. N/1 hydrochloric acid for every 1.0 ml. sodium hydroxide solution used, was added. The resulting precipitate was harvested by centrifugation, suspended in 4.0 ml. saline, and the pH adjusted to 7.6 with N/10 sodium hydroxide solution using phenol red as indicator. One ml. formol-saline was added and the antigen heated at 60° C. for one hour.

## Preparation of Agglutinable Suspensions.

A saline suspension of gonococci of a density of $1 \times 10^9$ organisms per ml. was prepared by dilution from a concentrated suspension stored at -20° C. As this temperature was invariably lethal to the organisms, no other attempt was made to "kill" the organisms. Methiolate (1:10,000) was added as a preservative.

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## PREPARATION OF ANTISERA.

Very satisfactory gonococcal antisera were prepared in rabbits using the following schedule:

1st day --- 0.3 ml. of a heat killed suspension.
4th day --- 1.0 ml. of a live suspension.
25th day --- 1.0 ml. of a live suspension.
30th day --- Bleed for stock.

The suspension, containing 1 x $10^9$ organisms per ml. was given intravenously via the marginal vein of the ear. The initial inoculum was heated at 56° C. for thirty minutes.

Sera prepared in this way gave very satisfactory titres in both the agglutination and gonococcal haemolysis tests, and proved superior to sera prepared by the more usual course of immunisation of increasing doses at three to four day intervals.

An initial injection of live organisms, however small, proved rapidly and invariably fatal. If, however, a heat killed suspension was given initially, subsequent live injections in the doses used were very well tolerated.

All antisera were freeze dried in two ml. amounts and reconstituted with distilled water as required.

Antisera were also prepared with the sensitising antigen, the group antigen, the type specific antigen, and with various rough gonococcal strains. These will be discussed later.

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## SENSITISATION OF SHEEP ERYTHROCYTES WITH A GONOCOCCAL EXTRACT.

Sensitisation of red cells with the type specific gonococcal antigen was effected by incubating a saline solution of the antigen with red cells at 37° C.

An appropriate dilution of the sensitising antigen in saline was made, and this depended on the nature of the test to be performed. In general, a dilution of 1 in 25 was satisfactory.

Packed sheep cells were added to the diluted sensitising antigen to give a 2.0 % suspension, and the cells evenly suspended by inverting the tube a few times. The suspension was then incubated in a 37° C. water bath for one hour with occasional shaking.

The suspension was next centrifuged, the supernatant discarded, and the deposited cells washed three times with saline using ten mls. for each wash. Finally, the cells were resuspended in saline to give a 1.0 % suspension. This suspension served as the "antigen" in the gonococcal haemolysis test.

Comment. Sensitised cells were used on the day of sensitisation. With storage it was occasionally found that the cells became more easily haemolysed so that the titre with a given gonococcal antiserum tended to rise by one to two tubes, usually with a trace of haemolysis in the sensitised cell control.

Washing of the red cells after sensitisation was an essential step in the procedure. The supernatant generally contained "non-adsorbable" gonococcal fractions and these were able to "block" the action of a gonococcal antiserum giving lower titres than expected. This blocking action was readily demonstrated by preliminary incubation of the serum with the supernatant obtained after sensitisation, and using this serum in the gonococcal haemolysis test. This will be discussed later.

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## THE GONOCOCCAL HAEMOLYSIS TEST.

Sera. Sera were inactivated at 56° C. for thirty minutes, and, in order to remove naturally occurring heterophile antibody, absorbed for two hours at 37° C. with half the volume of packed sheep cells.

Normal cell suspension. A 1.0 % saline suspension of sheep cells of the same batch which had been sensitised for use in the gonococcal haemolysis test, was made.

The test. Serial doubling dilutions of the serum were made using 0.1 ml. quantities, the initial dilution with rabbit antisera being generally 1 in 32, and the dilutions were carried through for twelve tubes. To each tube was then added 0.1 ml. of the sensitised red cell suspension, and 0.1 ml. of an excess of complement. The rack was shaken and incubated for one hour in a 37° C. water bath.

The titre was taken as the highest dilution showing clear, sparkling haemolysis. Negatives showed the usual button of deposited cells.

Table 1: Controls in the gonococcal haemolysis test.

| | Serum mls. | Saline mls. | Sensitised cells mls. | Normal cells mls. | Complement excess mls. |
|---|---|---|---|---|---|
| Serum control | 0.1 * | - | - | 0.1 | 0.1 |
| Sensitised cell control | - | 0.1 | 0.1 | - | 0.1 |
| Normal cell control | - | 0.2 | - | 0.1 | - |
| Complement control | - | 0.1 | - | 0.1 | 0.1 |

*0.1 ml. of the initial serum dilution was used

Controls. The controls included are set out in table 1. All these controls should show no haemolysis.

Haemolysis in the serum control indicated that the heterophile antibodies had not been completely absorbed out.

Even a trace of haemolysis in the sensitised cell control alone, indicated that the sensitising antigen was probably haemolytic, and hence unsuitable for use.

Haemolysis in the normal cell control indicated an unsuitable batch of sheep cells (either infected, or in the case of abbatoir cells, possibly mixed with gastric contents). Sheep cells showing some haemolysis after one to two saline washes were obviously unsuitable, and gave that type of picture.

Haemolysis in the complement control alone has not been encountered.

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# THE ABSORPTION TEST.

## General Description.

In carrying out the absorption test a gonococcal antiserum is absorbed with sensitised red cells.

Prior to the absorption the titre of the serum to be absorbed (a) is determined by carrying out the gonococcal haemolysis test using as antigen red cells sensitised with the homologous sensitising antigen (A). These cells will be referred to as the test cells, and the titre as the initial titre.

If the absorption is to be carried out with an antigen from gonococcus B, B sensitised red cells (hereafter referred to as the absorbing cells) are prepared.

The antiserum to be absorbed (a) is now added to the absorbing cells (B), and the suspension allowed to interact in the water bath for thirty minutes (figure 1). The suspension is then centrifuged, and both serum and cells retained.

Gonococcal antiserum (a) + B sensitised red cells (absorbing cells)

figure 1.

To follow the progress of the absorption two steps are now carried out.

1. The serum (a) is titrated in the gonococcal haemolysis test to follow the decline in titre, if any, using the homologous test cells (A) as the antigen.
2. The absorbing cells (B) are tested to see whether they have abstracted antibodies from serum (a). This is simply done by the addition of complement. Where absorption has taken place, and the absorbing cells have combined with antibody, haemolysis will occur on the addition of complement (figure 2).

[B sensitised red cells (absorbing cells) + Antibody from serum (a)] + Complement ⟶ Haemolysis.

Figure 2.

If no absorption has occurred, it will be indicated by the absence of haemolysis on the addition of complement (figure 3).

[B sensitised red cells (absorbing cells) and NO antibody taken up] + Complement ⟶ No haemolysis

Figure 3.

The absorption is then repeated by the addition of the partially absorbed antiserum (a) to a further volume of absorbing cells (B), and the process repeated.

Absorption is considered complete when:

1. The antiserum (a) shows no further fall in titre after repeated absorption when tested with the test cells (A), or when the titre of the serum becomes zero.
2. The absorbing cells (B) show no haemolysis on the addition of complement indicating that they are no longer taking up antibodies from serum (a).

## Method.

Sensitisation of the absorbing cells. To six centrifuge tubes were added 10.0 ml. saline, 0.2 ml. of the sensitising antigen to be used in the absorption, and 0.2 ml. packed sheep cells. These cells were allowed to sensitise in the usual way. After the final saline wash the suspension was centrifuged, and the supernatant saline not removed until the cells were required.

Sensitisation of the test cells. This was carried out as described using a 1 in 25 dilution of sensitising antigen. The sensitising antigen should preferably be derived from the same organism as was used to prepare the antiserum to be absorbed, or failing that to belong to the same gonococcal type.

The antiserum. The serum was inactivated, and absorbed with sheep cells as described for the gonococcal haemolysis test. It was diluted to give a titre of 64 to 256, i.e. seven to nine tubes; a dilution of 1 in 250 was generally adequate. The diluted serum was then titrated in the gonococcal haemolysis test to determine the initial titre.

The absorption. The supernatant saline from the packed absorbing cells was removed, and 2.5 mls. of the diluted serum added. The cells were evenly suspended by shaking, and placed in a 37° C. water bath for thirty minutes. The tube was then centrifuged, and both serum and absorbing cells tested as follows:

Serum. The serum was titrated in the gonococcal haemolysis test using the test cells as an antigen.

Absorbing cells. The cells were resuspended in 10.0 ml. saline. Two tubes were then set up as indicated in table 2.

Table 2: The test carried out on the absorbing cells.

| Tube | 1 | 2 |
|---|---|---|
| Saline | 0.3 ml | 0.7 ml |
| Absorbing cells | 0.1 ml | 0.1 ml |
| Complement (excess) | 0.4 ml | - |

After shaking these were incubated at 37° C. for one hour.

The serum was then reabsorbed with a second lot of absorbing cells, and the process repeated.

Absorption was considered complete when the absorbing cells showed no further evidence of having taken up antibody, i.e. showed no haemolysis in tube 1, tube 2 serving as a control (table 2). In practice, however, once the titre of the serum became zero (usually after two to three absorptions under the conditions cited), the absorbing cells inevitably became negative after a further one to two absorptions, and in such a case the absorption was considered complete after the serum became negative when tested with the test cells.

When the serum was not absorbed out, however, the reaction of the absorbing cells was the only indication that the absorption was complete, and the test was carried on until that stage was reached. This usually required between four to six absorptions.

Examples of two absorption tests are set out in table 3. A type I gonococcal antiserum was absorbed with cells sensitised with the type I gonococcal antigen, and with cells sensitised with the type II gonococcal antigen.

Table 3: An absorption of a type I antiserum (n) by
i. a type I antigen (J),
ii. a type II antigen (E).

| Antiserum / Absorbing cells | Type I (n) | | | |
|---|---|---|---|---|
| | Type I (J) | | Type II (E) | |
| Stage | Serum titre | Absorbing cells | Serum titre | Absorbing cells |
| Initial | 128 | | 128 | |
| Absorption 1 | 8 | + | 64 | + |
| Absorption 2 | 0 | + | 32 | + |
| Absorption 3 | 0 | - | 32 | ± |
| Absorption 4 | | | 32 | - |

+=haemolysis; ±= partial haemolysis; -= no haemolysis.

Thus a type I gonococcus has completely exhauted a type I gonococcal antiserum.

A type II gonococcus, however, is unable to absorb out a type I antiserum.

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## THE GONOCOCCAL AGGLUTINATION TEST.

Serial doubling dilutions of the serum to be tested were made in Dreyer tubes using 0.25 ml. quantities. Again the initial serum dilution depended on the anticipated titre and varied from neat serum to one in sixteen, the dilutions being carried through for twelve tubes.

An equal volume of gonococcal bacterial suspension was added to each tube, the rack shaken, and incubated at 56° C. for eighteen hours.

A control with saline replacing serum was included.

The titre was taken as the highest dilution showing macroscopic agglutination.

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## THE GONOCOCCAL COMPLEMENT-FIXATION TEST.

In general the Kolmer[18] technique of overnight fixation in the cold was followed.

### Antigenic and Anticomplementary Titration of the Antigen.

Two rows of twelve tubes were set up. To each row was added:

1. Antigen. Serial doubling dilutions using 0.25 ml. quantities starting with undiluted antigen.
2. Serum. To each tube of the antigenic titration was added 0.25 ml. of an inactivated gonococcal rabbit antiserum diluted one in fifty. In the anticomplementary titration saline was added instead of serum.

Table 4: Controls in the antigenic and anticomplementary titration of a gonococcal antigen, and in the gonococcal complement-fixation test.

| | Antigen (ml.) | Saline (ml.) | Serum (ml.) | Complement (ml.) | Overnight fixation in the cold | Haemolysin (ml.) | Sheep cells (ml.) |
|---|---|---|---|---|---|---|---|
| Haemolytic system control | - | 0.5 | - | 0.5 | | 0.25 | 0.25 |
| Corpuscle control | - | 1.0 | - | - | | 0.25 | 0.25 |
| Serum control | - | 0.25 | 0.25 | 0.5 | | 0.25 | 0.25 |
| Antigen * control | 0.25 | 0.25 | - | 0.5 | | 0.25 | 0.25 |

* This is not required in the antigenic and anticomplementary titration of the antigen.

3. Complement. Two full units of complement contained in 0.5 ml. saline was added to each tube. The complement titre for the purpose of standardisation of the antigen was that obtained when titrated against the Kolmer Wassermann antigen.

The rack was then gently shaken and left at approximately 6° C. overnight. It was then incubated at 37° C. for thirty

minutes.

4. Haemolysin. Two units of haemolysin in 0.25 ml. saline was added to each tube.
5. Sheep cell suspension (2.0 %). Finally 0.25 ml. of a 2.0 % sheep cell suspension was added to each tube. The rack was shaken and incubated in a 37° C. water bath for one hour and read in the usual way.
6. Controls. The controls included are set out in table 4.

The antigen was used in an amount equivalent to not less than one third or one fourth of its anticomplementary unit. Table 5 shows the results obtained with various antigens.

Table 5: Antigenic and anticomplementary titrations of four gonococcal antigens.

| Antigen | Antigenic in a dilution of: | A.C. in a dilution of: | Dilution for use: |
|---|---|---|---|
| A | 64 | 2 | 16 |
| C | 32 | 4 | 16 |
| M | 128 | 4 | 30 |
| J | 1024+ | 16 | 250 |

There was no detectable difference in antigens prepared from a suspension of whole organisms, or from the deposit following the extraction of the sensitising antigen.

It is noteworthy that all the antigens were anticomplementary to a greater or lesser extent.

## The Complement Titration.

Details of the complement titration are set out in table 6. The last tube (11) serves as a control.

The smallest amount of complement giving complete sparkling haemolysis was the exact unit (Kolmer). The next tube contained the full unit. Two full units were employed in the test.

Table 6: The complement titration for the gonococcal complement-fixation test.

| Tube | Complement 1 in 30 (ml.) | Antigen (ml.) | Saline (ml.) | | Haemolysin 2 units (ml.) | Sheep cells 2.0 %(ml.) | |
|---|---|---|---|---|---|---|---|
| 1 | 0.2 | 0.5 | 1.3 | 37° C. for one hour. | 0.5 | 0.5 | 37° C. for one hour. |
| 2 | 0.25 | 0.5 | 1.3 | | 0.5 | 0.5 | |
| 3 | 0.3 | 0.5 | 1.2 | | 0.5 | 0.5 | |
| 4 | 0.35 | 0.5 | 1.2 | | 0.5 | 0.5 | |
| 5 | 0.4 | 0.5 | 1.1 | | 0.5 | 0.5 | |
| 6 | 0.45 | 0.5 | 1.1 | | 0.5 | 0.5 | |
| 7 | 0.5 | 0.5 | 1.0 | | 0.5 | 0.5 | |
| 8 | 0.55 | 0.5 | 1.0 | | 0.5 | 0.5 | |
| 9 | 0.6 | 0.5 | 0.9 | | 0.5 | 0.5 | |
| 10 | 0.65 | 0.5 | 0.9 | | 0.5 | 0.5 | |
| 11 | - | - | 2.0 | | 0.5 | 0.5 | |

The Gonococcal Complement-Fixation Test.

Sera were inactivated at 56° C. for thirty minutes. Serial doubling dilutions of the serum were made, using 0.25 ml. quantities for ten tubes.

Antigen (0.25 ml.) and two units of complement in 0.5 ml. saline were added, the rack shaken, and left at approximately 6° C. overnight. After incubation at 37° C. for fifteen minutes, two units of haemolysin in 0.25 ml. saline and 0.25 ml. 2.0 % sheep cell suspension were added, the rack shaken, and incubated at 37° C. for thirty minutes.

The controls are those set out in Table 4.

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# SECTION III

# EXPERIMENTAL INVESTIGATIONS.

## FACTORS CONCERNED IN THE ADSORPTION OF A GONOCOCCAL ANTIGEN ON TO RED BLOOD CELLS.

There are a number of variables involved in the process of adsorption of an antigen on to red cells. These are:
1. Variation in the concentration of the sensitising antigen.
2. Variation in the time of interaction.
3. The effect of temperature.
4. The capacity of the red cells to adsorb antigen.

The following experiments were devised to investigate the importance of each of these factors in the gonococcal haemolysis test. The sensitising antigen employed was one extracted from fifteen pooled newly isolated gonococcal strains. The same rabbit gonococcal antiserum was employed throughout.

The only variation from the technique described was that the sensitised cells were used in an 0.5 % suspension instead of the 1.0 % suspension finally adopted for routine use.

### Variation in the Concentration of the Sensitising Antigen.

In this experiment the amount of antigen adsorbed on to the red cells was varied by sensitising cells with dilutions of antigen from 1 in a 100 to 1 in 25.

Table 7: The effect of variation in the dilution of the antigen used in sensitising the red cells on the titre in the gonococcal haemolysis test.

| Dilution of Sensitising Antigen | Titres in gonococcal haemolysis test. | | | | | |
|---|---|---|---|---|---|---|
| | 512 | 1024 | 2048 | 4096 | 8192 | 16384 |
| 1 in 100 | + | + | ± | - | - | - |
| 1 in 90 | + | + | + | ± | - | - |
| 1 in 70 | + | + | + | + | - | - |
| 1 in 50 | + | + | + | + | ± | - |
| 1 in 25 | + | + | + | + | ± | - |

+ = haemolysis; ± = partial haemolysis; - = no haemolysis.

As will be shown subsequently the red cells are capable of adsorbing all the antigen to which they are exposed, and, thus, increasing the concentration of the sensitising antigen automatically increased the degree of sensitisation. The effect of this progressive increase in sensitisation was determined by testing these sensitised cells in the gonococcal haemolysis test against aliquot portions of the gonococcal antiserum.

It will be seen from table 7 that as the degree of sensitisation was increased from 1 in a 100 to 1 in 70, the titre rose correspondingly from 1024 to 4096. Further increase in sensitisation to 1 in 25, however, resulted in no corresponding titre rise.

Thus as the degree of sensitisation was increased, there was a corresponding rise in titre until a maximum was reached which may be interpreted as the titre of that particular antiserum.

## Variation in the Time of Interaction.

The effect of variation in the period of sensitisation was next investigated.

Two dilutions of the sensitising antigen were made, 1 in 50, and 1 in a 100. Cells were added to make the usual 2.0 % suspension. Each suspension was then divided into five portions, and placed in the 37° C. water bath.

Table 8: The effect of variation in the period of sensitisation on the titre in the gonococcal haemolysis test.

| | 1 in 100 sensitised cells | | | 1 in 50 sensitised cells | | |
|---|---|---|---|---|---|---|
| Time in water bath | Titres in the gonococcal haemolysis test | | | Titres in the gonococcal haemolysis test | | |
| | 1024 | 2048 | 4096 | 4096 | 8192 | 16384 |
| ½ hour | + | ± | - | + | ± | - |
| 1 hour | + | ± | - | + | ± | - |
| 2 hour | + | ± | - | + | ± | - |
| 3 hour | + | ± | - | + | ± | - |
| 5 hour | + | ± | - | + | ± | - |

+ = haemolysis; ± = partial haemolysis; - = no haemolysis.

One tube from each batch was removed after ½, 1, 2, 3, and 5 hours, and the cells washed and resuspended in the usual way. They were then tested in the gonococcal haemolysis test against the rabbit gonococcal antiserum.

The results are set out in table 8. After thirty minutes sensitisation the titre in the gonococcal haemolysis test was 1024 in the case of the 1 in 100 sensitised cells, and 4096 in the case of the 1 in 50 sensitised cells. Prolonging the period of incubation to as long as five hours did not result in a further rise in titre.

Thus sensitisation of the red cells occurred rapidly, and prolonging the period of sensitisation did not influence the titre attained with a particular antiserum.

## The Effect of Temperature on Sensitisation.

A batch of red cells was sensitised with a 1 in 50 dilution of sensitising antigen. One portion was left at 4° C. for one hour, and the second at 37° C. for one hour. These two batches of cells were then washed, resuspended in the usual way, and tested in the gonococcal haemolysis test. The results are shown in table 9.

Table 9: The effect of temperature on the sensitisation of red cells.

| Temperature of sensitisation. | Titre in the gonococcal haemolysis test |
|---|---|
| 4° C. | 32 |
| 37° C. | 4096 |

Thus an adequate temperature was necessary for the rapid adsorption of a gonococcal antigen on to the red cells. A temperature of 37° C. was adequate for this purpose.

## The Capacity of the Red Cells to Adsorb Antigen.

In this experiment a single batch of red cells was exposed to six successive portions of sensitising antigen. The effect of this repeated sensitisation was investigated by testing a portion of these cells after each sensitisation in the gonococcal haemolysis test using the same antiserum as in the previous experiments.

In addition the supernatant fluid after each sensitisation was investigated to see whether all the antigen had been removed by the red cells, or whether there was residual antigen in the supernatant. This was simply done by adding fresh red cells to the supernatant, and allowing sensitisation to proceed in the usual way. These cells were then tested against the gonococcal antiserum.

The detailed experiment was as follows:

The sensitising antigen was diluted 1 in 50.

Step 1. 0.1 ml. packed sheep cells was added to 5.0 ml. sensitising antigen, and placed in the 37° C. water bath for thirty minutes. The suspension was then centrifuged, and both the cells and supernatant retained.

a. Cells. These were resuspended in 20 ml. saline. Two ml. were removed, washed three times, and tested with the gonococcal antiserum in the gonococcal haemolysis test.

b. Supernatant. To the 5.0 ml. supernatant 0.1 ml. sheep cells was added, and the suspension incubated at 37° C. for thirty minutes. The cells were then washed three times, resuspended in 20 ml. saline, and tested with the gonococcal antiserum in the gonococcal haemolysis test.

Step 2. The 18 ml. cell suspension remaining from stage 1a was centrifuged, the supernatant saline discarded, and 4.5 ml. of fresh sensitising antigen added to these already once sensitised cells. They were then incubated at 37° C. for thirty minutes, and then centrifuged.

a. Cells. The cells were suspended in 18 ml. saline and 2.0 ml. removed for testing in the gonococcal haemolysis test.

b. Supernatant. To the 4.5 ml. supernatant was added 0.09 ml. of packed sheep cells. After further incubation these were washed, suspended in 18 ml. saline and tested as described.

This process was repeated in steps 3 to 6 using the following quantities.

Step 3. The 16 ml. residue from step 2a was again centrifuged and 4.0 ml. fresh sensitising antigen added.

a. Cells. The cells were suspended in 16 ml. saline and 2.0 ml. abstracted.

b. Supernatant. To the 4.0 ml. supernatant was added 0.08 packed sheep cells. These were finally suspended in 16 ml. saline.

Step 4. 3.5 ml. sensitising antigen was employed.
a. Cells. These were resuspended in 14 ml. saline.
b. Supernatant. To the 3.5 ml. supernatant was added 0.07 ml. packed sheep cells. These were finally resuspended in 14 ml. saline.

Step 5. 3.0 ml. sensitising antigen was employed.
a. Cells. These were resuspended in 12 ml. saline.
b. Supernatant. To the 3.0 ml. supernatant was added 0.06 ml. packed sheep cells. These were finally resuspended in 12 ml. saline.

Step 6. 2.5 ml. sensitising antigen was employed.
a. Cells. These were resuspended in 10 ml. saline.
b. Supernatant. To the 2.5 ml. supernatant was added 0.05 ml. packed sheep cells. These were finally resuspended in 10 ml. saline.

Thus throughout the experiment the cell suspension during sensitisation was maintained at 2.0 %, and the suspension for testing in the gonococcal haemolysis test was maintained at 0.5 %.

Table 10: The effect of six sensitisations on a single batch of red cells in the gonococcal haemolysis test, and the estimation of residual antigen in the supernatant following each of these sensitisations.

| Sensitisations | Titres in the gonococcal haemolysis test with cells sensitised with the supernatants. | | | following successive resensitisation of a batch of red cells. | | | | |
|---|---|---|---|---|---|---|---|---|
| | Neat | 2 | 4 | 1024 | 2048 | 4096 | 8192 | 16384 |
| 1 | - | - | - | + | + | - | - | - |
| 2 | - | - | - | + | + | + | ± | - |
| 3 | - | - | - | + | + | + | ± | - |
| 4 | - | - | - | + | + | + | ± | - |
| 5 | - | - | - | + | + | + | ± | - |
| 6 | - | - | - | + | + | + | ± | - |

+ = haemolysis; ± = partial haemolysis; - = no haemolysis.

The results are set out in table 10. The supernatant removed after sensitising a batch of red cells contained no residual antigen capable of being adsorbed on to red cells and detectable in the gonococcal haemolysis test, and this

was still the case after the red cells had been subjected to five previous sensitisations.

Thus the red cells were able to take up all the adsorbable antigen to which they were exposed; once the titre of the serum was reached, however, this enhanced antigen content was not reflected in the titration.

## Summary

To sum up, adsorption of a gonococcal antigen on to red cells takes place rapidly at 37° C. The red cell will take up all the adsorbable antigen available in the solution.

Thus the only variable will be the degree of sensitisation and this is determined solely by the amount of antigen present during the process of sensitisation.

This latter factor has an important influence as far as the specificity of the reaction is concerned, especially when dealing with human antisera. In applying the gonococcal haemolysis test to the detection of gonococcal antibodies in patients, it was found that oversensitisation resulted in low titre reactions with a proportion of apparently normal sera.

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## THE S-R CHANGE IN THE GONOCOCCUS.

Fifteen gonococcal strains which were isolated in October, 1951, were maintained on chocolate agar slopes, and subcultured at seven to ten day intervals. A sensitising antigen was extracted as described, and, when used to sensitise cells at a dilution of 1 in 50, reacted to titre with various rabbit antisera. These titres varied from 1024 to 16,384.

After four months on subculture further suspensions of these organisms were made, and extracted in the identical manner. This extract, however, even when used 1 in 5, failed to sensitise cells in the gonococcal haemolysis test when tested with the same sera used originally. These observations have been confirmed with further gonococcal strains.

Thus, there is an antigen present in recently isolated gonococcal strains which can be adsorbed on to red cells, and which sensitises these cells in the gonococcal haemolysis test. This antigen is gradually lost on subculture, and, under the conditions described, this loss is complete after four to five months.

The loss of this antigen is associated with a change in the cultural character of the organism. The recently isolated gonococcus is typically sticky and mucoid. It tends to stick to the agar so that when attempting to transfer the culture to a platinum loop it is tenuous and tends to snap back, either to the agar or on to the loop. With the loss of the sensitising antigen, however, this stickiness of the culture disappears, it becomes far drier, and it is harvested with much less difficulty.

There is a third associated change, and that is a loss of agglutinability of the organism with gonococcal antisera prepared with recent strains. This will be discussed at greater length subsequently.

It is suggested that this change in the gonococcus is exactly analogous to the Smooth-Rough change first demonstrated by Arkwright[20] in relation to the enterobacteriaceae, and since then in many other groups of bacteria.

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## THE DEMONSTRATION OF TWO ANTIGENICALLY DISTINCT GONOCOCCAL TYPES.

Eighteen gonococcal strains were obtained from cases of acute urethritis and antisera prepared with each of these strains.

It was soon apparant, when these strains were investigated by the gonococcal haemolysis, agglutination, or complement-fixation tests, that there was marked cross reaction, and no obvious antigenic differences could be distinguished. These results are discussed more fully subsequently. Absorption tests using sensitised cells were then carried out.

Table 11: The results of absorption tests with red cells sensitised with a gonococcal antigen using eighteen gonococcal strains and their antisera.

| Gonococcal antigens | Gonococcal antisera b | c | d | e | f | h | i | j | k | m | n | p | r | s | t | u | v | w |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| D | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| E | + | + | + | - | + | + | - | + | + | + | + | - | + | + | + | + | + | + |
| F | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| H | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| I | + | + | + | - | + | + | - | + | + | + | + | - | + | + | + | + | + | + |
| J | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| K | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| M | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| N | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| P | + | + | + | - | + | + | - | + | + | + | + | - | + | + | + | + | + | + |
| R | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| U | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| V | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| W | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

- = Antigen will absorb out the corresponding antiserum.
+ = Antigen will not absorb out the corresponding antiserum.

The method was to take two strains and their corresponding antisera. If these two strains were able to exhaust each others' antisera they were accepted as being identical. One of these two strains and its antiserum was then cross absorbed with a third strain and the antiserum corresponding to the third strain. If these latter two mutually absorbed out each others' sera, all three strains were accepted as identical.

The results of cross absorbing eighteen gonococcal strains and eighteen corresponding antisera are set out in table 11. Less than half the cross absorptions listed in the table were actually carried out. Where two strains were proved identical within the limits of the technique, the anticipated result for the second strain has been entered in the table. The strains fell into two groups.

Type I. Fifteen strains which were able to exhaust all eighteen antisera.

Type II. Three strains, E, I, and P, which were able to exhaust each others' sera but not the sera corresponding to the fifteen strains listed as type I.

Some examples of these absorptions are set out in tables 12 and 13.

Table 12: The absorption of a type I gonococcal antiserum (c) by i.a type I antigen (N), ii.a type II antigen (I).

| Antiserum | Type I (c) | | | |
|---|---|---|---|---|
| Absorbing cells | Type I (N) | | Type II (I) | |
| Stage | Serum titre | Absorbing cells | Serum titre | Absorbing cells |
| Initial | 128 | | 128 | |
| Absorption 1 | 8 | + | 64 | + |
| Absorption 2 | 0 | + | 32 | + |
| Absorption 3 | 0 | - | 32 | ± |
| Absorption 4 | | | 32 | - |

+ = haemolysis; ± = partial haemolysis; - = no haemolysis.

These absorptions were performed with sensitised cells. That the identical results were obtained when the absorptions were performed with whole organisms was shown in the following experiment.

Table 13: The absorption of a type II gonococcal antiserum (e) by
i. a type I antigen (N),
ii. a type II antigen (P).

| Antiserum Absorbing cells | Type II (e) Type I (N) | | Type II (P) | |
|---|---|---|---|---|
| Stage | Serum titre | Absorbing cells | Serum titre | Absorbing cells |
| Initial | 256 | | 256 | |
| Absorption 1 | 4 | + | 8 | + |
| Absorption 2 | 0 | + | 0 | + |
| Absorption 3 | 0 | + | 0 | + |
| Absorption 4 | 0 | - | 0 | - |

+ = haemolysis; ± = partial haemolysis; - = no haemolysis.

Fifteen plates of a smooth type II strain (I) were harvested into saline and the suspension centrifuged. The supernatant was discarded. The organisms were then resuspended in 5.0 ml. of an undiluted type I antiserum (n), and absorption allowed to proceed at 37° C. for one hour. The absorbed serum was then recovered by centrifugation.

This n gonococcal antiserum was then tested in the gonococcal haemolysis before and after absorption using red cells sensitised with a 1 in 25 dilution of a type I, and a type II sensitising antigen. The results are set out in Table 14.

Table 14: The effect of absorption of a type I gonococcal antiserum (n) with a suspension of N. gonorrhoeae (typeII) on the titre in the gonococcal haemolysis test using type I and type II sensitised test cells.

| Type I antiserum (n) | Titres in the gonococcal haemolysis test Type I sensitised cells | Type II sensitised cells |
|---|---|---|
| Unabsorbed | 8192 | 4096 |
| Absorbed | 512 | 0 |

Thus a type I serum after absorption with type II gonococci had no further activity against type II sensitised cells in the gonococcal haemolysis test, but was still able to react to high titre with type I sensitised cells. These results then are the same as those obtained when a type I antiserum was absorbed out with type II sensitised cells.

It is clear from these results that these two gonococcal types possess an antigen in common. The type I gonococcus, however, must possess an additional antigen not present in the type II gonococcus. The situation can be simply represented as follows (table 15):

Table 15: Representation of the antigenic structure of type I and type II gonococci as shown by absorption tests with sensitised red cells.

| N. gonorrhoeae | Antigen | Antiserum |
|---|---|---|
| Type I | A + B | a + b |
| Type II | A | a |

Thus a type I gonococcus can completely absorb out both a type I and a type II antiserum. The type II gonococcus, however, lacking antigen B, is unable to completely absorb out a type I antiserum.

It must be emphasised that all these strains were extracted and tested within ten to fourteen days of primary isolation so that type II is not a transition stage in the S-R change. The progression to the rough stage is associated with the extraction of an antigen of decreasing potency as determined by its capacity to sensitise cells in the gonococcal haemolysis test. Eventually a sensitising antigen can no longer be obtained. A transition from one type to another has never been observed.

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## THE GROUP ANTIGEN.

By the group antigen is meant an antigen which is not adsorbable on to red cells, and is common to all gonococcal strains. The group antigen can be investigated in various ways.

1. A gonococcal antiserum contains antibodies against the group antigen, and the type specific antigen. By absorbing the antiserum with sensitised cells antibodies against the type specific antigen can be completely removed. Such an absorbed antiserum will be active only against the group antigen.
2. The type specific antigen can be removed from a preparation which contains both the group antigen and the adsorbable type specific antigen.
   i. The sensitising antigen often contains both type specific and group antigens. The type specific antigen is completely removed by adsorption on to red cells.
   ii. The bacterial residue following alkali extraction and alcoholic precipitation of the gonococcal suspension can be washed free from the water soluble type specific antigen.

   Thus antigens containing only the group factor can be prepared.

The methods employed were agglutination and complement-fixation using both the absorbed and unabsorbed antisera and antigens.

### The Use of Absorbed Antisera.

Antisera representing both gonococcal types were absorbed out with red cells sensitised with the homologous sensitising antigen. These antisera were:

Type I antiserum (j) diluted 1 in 25.
Type II antiserum (i) diluted 1 in 20.

These dilutions were selected so as to permit a reasonably easy absorption, and still give a positive complement-fixation titre of at least two to three tubes. Even so, twelve to nineteen absorptions using 0.5 ml. sensitising antigen for each absorption, were required before the sera gave negative gonococcal haemolysis tests, and before the absorbing cells no longer haemolysed in the presence of complement.

The original unabsorbed serum and the absorbed serum were then tested in the gonococcal haemolysis test, agglutination test, and complement-fixation test. All the titres have been expressed in terms of an undiluted serum, i.e. the titres in the case of serum j have been multiplied by 25, and in the case of serum i by 20.

Table 16: The results of the gonococcal haemolysis, agglutination, and complement-fixation tests with gonococcal antisera before and after absorption with sensitised red cells.

| | Sera | Titres with antigen J using | | Titres with antigen I using | |
|---|---|---|---|---|---|
| | | Unabsorbed serum | Absorbed serum | Unabsorbed serum | Absorbed serum |
| Gonococcal haemolysis test | j | 16384 | 0 | 8192 | 0 |
| | i | 16384 | 0 | 4096 | 0 |
| Agglutination test | j | 400 | 400 | 200 | 200 |
| | i | 160 | 320 | 1280 | 1280 |
| Complement-fixation test | j | 100 | 200 | 25 | 25 |
| | i | 40 | 40 | 40 | 20 |

The results (table 16) show that when all antibodies reacting in the gonococcal haemolysis test were absorbed out of a gonococcal antiserum, the absorbed serum showed no loss of titre whatsoever in agglutination and complement-fixation tests. Comparison of titres with absorbed and unabsorbed sera in agglutination and complement-fixation tests show a variation no greater than one tube; a variation well within the margin of error of a serological test. Some exceptions to these observations will be discussed later.

Thus a gonococcal antiserum prepared with a smooth gonococcal strain contains two distinct antibodies:

1. One which is active in the gonococcal haemolysis test.
2. One which is active in an agglutination, and complement-fixation test.

The assumption is that these two antibodies are active against two distinct antigens present in the gonococcus.

## The Use of Absorbed Antigens.

The problem can be approached from another angle, viz. to observe the effect of removal of the type specific fraction from an antigen containing fractions active in both the gonococcal haemolysis test and the complement-fixation test.

Method. One ml. of sensitising antigen was added to nine. mls. saline.

i. Five mls. were retained, and will be referred to as the unabsorbed antigen.
ii. To the second five mls. were added 1.0 ml. of packed sheep cells. The suspension was incubated in a 37° C. water bath for one hour. The sheep cells were then removed by centrifugation. In this way the type specific antigen was completely removed. This fraction will be referred to as the absorbed antigen.

These two fractions were then tested as follows:

i. For their capacity to sensitise cells in the gonococcal haemolysis test by testing cells so sensitised with the homologous antisera.
ii. For their antigenicity in a complement-fixation test by performing an antigenic titration as described.
iii. For anticomplementary activity as described.

Table 17: The effect of removal of the type specific fraction from a gonococcal antigen on the gonococcal haemolysis test, on its complement-fixing activity, and on its anticomplementary activity.

| | Titres in the gonococcal haemolysis test | | Complement-fixing activity* | | Anti-complementary activity | |
|---|---|---|---|---|---|---|
| Antigen | Unabsorbed antigen | Absorbed antigen | Unabsorbed antigen | Absorbed antigen | Unabsorbed antigen | Absorbed antigen |
| I | 4096 | 0 | 0 | 0 | 0 | 0 |
| J | 16384 | 0 | 1280 | 1280 | 0 | 0 |
| K | 32768 | 0 | 640 | 320 | 0 | 0 |
| M | 16384 | 0 | 1280 | 1280 | 0 | 0 |
| N | 16384 | 0 | 1280 | 1280 | 0 | 0 |
| R | 4096 | 0 | 0 | 0 | 0 | 0 |
| S | 16384 | 0 | 0 | 0 | 0 | 0 |
| T | 8192 | 0 | 40 | 40 | 0 | 0 |
| U | 16384 | 0 | 0 | 0 | 0 | 0 |

*The complement-fixing activity has been expressed as the highest initial dilution of the sensitising antigen which deviated complement.

The results are set out in table 17 and they bring out a number of points.

1. Various sensitising antigens differ in their content of the complement-fixing antigen, and this varies from nil to 1 in 1280.
2. Complement-fixing activity of the antigen is uninfluenced by removing the type specific antigen by adsorbing it on to red cells (antigens J, K, M, N, and T).
3. The type specific antigen itself has no complement-fixing activity (the unabsorbed I, R, S, and U antigens).

The anti-complementary titration serves as a control. It is, however, interesting to observe the complete absence of anticomplementary activity in these antigens as compared with the antigens prepared by Price's method.

These experiments with absorbed sera and absorbed antigens therefore establish that there are at least two antigens in the gonococcus, and that a gonococcal antiserum prepared with a smooth strain contains antibodies which correspond to these antigens. One of these antigens is detectable by its capacity to sensitise cells in the gonococcal haemolysis test, and has been termed the type specific antigen. The second antigen is active in agglutination and complement-fixation.

## The Serological Identity of the Agglutinin-binding Antigen in the Type I and Type II Gonococcus.

It has been demonstrated by absorption tests that the type specific antigen is of at least two antigenic types. It remains to be shown that these two gonococcal types share a common antigen active in agglutination and complement-fixation.

Method. The absorbed type I antiserum (j), and the absorbed type II antiserum (i) previously described were used.

The antigen was prepared from the residues following extraction of the sensitising antigen. The residues obtained from the extraction of about 250 petri dishes of type I and type II strains were pooled separately. These residues were then washed in saline until the saline washings were no longer able to sensitise cells in the gonococcal haemolysis test.

In this way antigens and antisera for the type I and type II gonococcus respectively were prepared which were completely free from type specific activity.

These absorbed antisera were then reabsorbed with the residues containing only the group antigen. Thus:

The type I serum was reabsorbed with the type II residue.
The type II serum was reabsorbed with the type I residue.

These reabsorbed sera were then tested in agglutination tests with the homologous suspensions. Two such absorptions were required in each case. The results are set out in table 18.

Table 18: Absorption of type I and type II gonococcal antisera with the heterologous agglutinin-binding (group) antigens.

| Antiserum | Agglutinin titre with serum after absorption of type specific antibodies with homologous sensitised cells. | Agglutinin titre after re-absorption of these sera with the heterologous agglutinin-binding (group) antigens. |
|---|---|---|
| Type I (j) | 16 | 0 |
| Type II (i) | 64 | 0 |

Thus the type I and type II group antigens are capable of exhausting each others sera in absorption tests when tested by agglutination, and are therefore acceptable as being serologically identical. It is proposed to refer to this antigen as the group antigen since it is common to the type I and type II gonococcus.

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## CROSS REACTIONS BETWEEN THE TYPE SPECIFIC AND GROUP ANTIGENS AND THEIR ANTISERA.

A cross reaction between the type specific antibody in a gonococcal antiserum and the group antigen can be demonstrated under at least two sets of circumstances.

### In an Agglutination and Complement-fixation Test.

This reaction was observed with three antisera which had been absorbed out with sensitised cells. The unabsorbed and absorbed sera were tested in agglutination and complement-fixation tests as described. The results are shown in table 19.

Table 19: To demonstrate the augmented agglutinin and complement-fixing titre after absorption of certain gonococcal antisera with sensitised red cells.

| Antiserum | Titres in the gonococcal agglutination test with | | Titres in the gonococcal complement-fixation test with | |
|---|---|---|---|---|
| | Unabsorbed serum | Absorbed serum | Unabsorbed serum | Absorbed serum |
| b | 512 | 4096 | 64 | 1024 |
| c | 256 | 2048 | 128 | 1024 |
| k | 256 | 1024 | 32 | 64 |

The absorbed sera show a higher titre in agglutination and complement-fixation tests than the unabsorbed sera.

The explanation suggested is that the type specific antibody present in the unabsorbed serum has combined with some of the group antigen, and so blocked the action of the agglutinating group antibody. The type specific antibody itself, however, is presumably unable to produce agglutination, just as it is unable to fix complement in the presence of an immune serum.

## In a Gonococcal Haemolysis Test.

The same type of cross reaction can be demonstrated more directly in the following way.

Red cells were sensitised in the usual way, the suspension centrifuged, and the supernatant was removed. Serial doubling dilutions of the supernatant were made using 0.2 ml. volumes for ten tubes (table 20). Tubes 11 and 12 serve as controls. To each tube was then added 0.2 ml. of a 1 in 50 dilution of an absorbed and inactivated gonococcal antiserum. In tube 12 saline replaced the serum. The rack was then placed in a 37° C. water bath for thirty minutes.

Table 20: To demonstrate the blocking effect of the group antigen which is present in the supernatant after the sensitisation of red cells, on the type specific antibody in a gonococcal antiserum.

| Tube | Supernatant 0.2 ml. | Antiserum 1 in 50 ml. | 37° C. for thirty minutes. | Sensitised cells - ml. | Complement excess - ml. | 37° C. for one hour. | Result |
|---|---|---|---|---|---|---|---|
| 1 | Neat | 0.2 | | 0.2 | 0.2 | | - |
| 2 | 1 in 2 | 0.2 | | 0.2 | 0.2 | | + |
| 3 | 1 in 4 | 0.2 | | 0.2 | 0.2 | | + |
| 4 | 1 in 8 | 0.2 | | 0.2 | 0.2 | | + |
| 5 | 1 in 16 | 0.2 | | 0.2 | 0.2 | | + |
| 6 | 1 in 32 | 0.2 | | 0.2 | 0.2 | | + |
| 7 | 1 in 64 | 0.2 | | 0.2 | 0.2 | | + |
| 8 | 1 in 128 | 0.2 | | 0.2 | 0.2 | | ± |
| 9 | 1 in 256 | 0.2 | | 0.2 | 0.2 | | - |
| 10 | 1 in 512 | 0.2 | | 0.2 | 0.2 | | - |
| 11 | sal.* | 0.2 | | 0.2 | 0.2 | | - |
| 12 | neat | sal.* | | 0.2 | 0.2 | | - |

+ = haemolysis; ± = partial haemolysis;
- = no haemolysis.
* 0.2 ml. saline.

In this way the group antigen present in the supernatant was allowed to react directly with the type specific antibody in the serum. In order to detect a reaction between the group antigen and the type specific antibody sensitised cells and complement were now added as in the gonococcal haemolysis test,

and the rack read for haemolysis after an hour incubation at 37° C. In addition to the usual controls, the controls indicated in table 20 were included.

The results (table 20) show an inhibition of the gonococcal haemolysis test in the lower dilutions of the supernatant. There the group antigen has combined with the type specific antibody and prevented its action on the sensitised red cells.

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## THE ANTIGENICITY OF THE GONOCOCCAL FRACTIONS.

Animals were immunised with the various gonococcal fractions.

1. The sensitising antigen. Six rabbits were immunised, 0.5 ml. inoculations being given intravenously at 3 to 4 day intervals. The total course varied from 4 to 10 injections.
2. The type specific antigen. Human (group O) red cells were sensitised with the type specific antigen, and the sensitised cells, after washing, injected intravenously into a rabbit. In this way it was hoped to investigate the effect of the adsorbed antigen only. As sheep cells, and not human cells, are used in the gonococcal haemolysis test the effect of the human cells was probably not important. Six injections at four day intervals were given, the amount of antigen used in sensitisation increasing from 0.1 ml. to 0.6 ml.
3. The group antigen. A 1 in 10 dilution of the sensitising antigen was absorbed with sheep cells to remove all adsorbable type specific antigen. This "absorbed" antigen was used for immunisation. This antigen proved extraordinarily toxic. Thus 0.1 ml. of the diluted and absorbed sensitising antigen was fatal to a 2500 gram rabbit. The most toxic preparations were J and H, and these both showed high complement-fixing titres indicating a high content of the group antigen. A 1 in a 1000 dilution of the absorbed sensitising antigen was made (giving a final dilution of 1 in 10,000), and a course of 6 injections given starting with 0.1 ml. The dose was increased every three to four days.

The results of testing the antisera prepared in this manner are set out in table 21.

Table 21: Antibody titres following immunisation of rabbits with various gonococcal fractions.

| Antigenic fraction used in immunisation | Titre in the gonococcal haemolysis test | Titre in the agglutination test |
|---|---|---|
| Sensitising antigen | 65536 | 2048 |
| Type specific antigen | 512 | 16 |
| Group antigen | 32 | 512 |

The results given are representative of the sera prepared with each antigen.

Immunisation with the sensitising antigen, and with the type specific antigen adsorbed on to human red cells, produced the same results as obtained when whole organisms were used in immunisation. The sensitising antigen proved a very potent antigen and is probably the antigen of choice in the production of high titre antisera.

Immunisation with the group antigen active in an agglutination test, on the other hand, produced a serum with a relatively high agglutinin titre and a very low gonococcal haemolysis test titre, i.e. the opposite to that obtained when using the whole organism or the sensitising antigen in immunisation.

The result of immunisation with the type specific antigen proved disappointing in so far as antibodies against both the group and type specific factors were produced. In view of the very small doses of the group antigen required for immunisation, it is probable that the group factor was incompletely removed in washing the sensitised cells prior to inoculation.

The results obtained in immunisation with the group antigen, on the other hand, supports the concept of two distinct antigens in the gonococcus, the high agglutinin titre contrasting strikingly with the low haemolysis test titre. The extreme toxicity of the group antigen has been mentioned. It was also a very potent antigen, the total course of antigen administered being 1.5 ml. of a 1 in 10,000 dilution divided into six doses.

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# THE INVESTIGATION OF GONOCOCCAL STRAINS BY THE GONOCOCCAL HAEMOLYSIS TEST.

In comparing titres in the gonococcal haemolysis test, one is faced with the problem of standardisation of the antigen system, in this case the sensitised cell suspension. It has been demonstrated that the titre with a particular gonococcal antiserum increased as the degree of sensitisation was increased, until a limit was reached for that serum. The red cells were then fully sensitised as far as that serum was concerned, since a further increase in sensitisation was not reflected in an increased titre. Thus the problem can be approached in two ways:

1. Titres may be compared at increasing degrees of sensitisation.
2. Fully sensitised cells may be used. In general a 1 in 25 dilution of sensitising antigen was adequate to produce maximum titres.

## The Use of "Partially" Sensitised Cells

Table 22 shows the results of titrating a gonococcal antiserum (c) against cells sensitised with seventeen different antigens, the degree of sensitisation varying from 1 in 50 to 1 in 600.

The results in general reflect the potency of the antigen used in sensitisation, and do not help the elucidation of possible antigenic variations in the organisms. Three antigens were of relatively low potency, viz. those extracted from the homologous strain C, and from E and H. C and H were type I gonococci, and E was a type II strain.

The failure of this method to distinguish gonococcal types is further illustrated in table 23 where the results of the gonococcal haemolysis test using cells of increasing degree of sensitisation with two type I antigens and two type II antigens and type I and type II antisera, are set out.

Table 22: A comparison of titres in the gonococcal haemolysis test using the sensitising antigens from seventeen gonococcal strains and a single type I antiserum (c). The cells were sensitised with dilutions of sensitising antigen varying from 1 in 600 to 1 in 50.

| Degree of sensitisation | Titres in the gonococcal haemolysis test with a type I antiserum (c). Antigens | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q |
| 1 in 50 | 8192 | 8192 | 4096 | 4096 | 1024 | 32768 | 2048 | 1024 | 4096 | 16384 | 32768 | 32768 | 32768 | 32768 | 4096 | 4096 | 8192 |
| 1 in 100 | 8192 | 8192 | 2048 | 4096 | 256 | 32768 | 2048 | 64 | 2048 | 16384 | 16384 | 32768 | 32768 | 32768 | 4096 | 4096 | 8192 |
| 1 in 150 | 4096 | 8192 | 256 | 4096 | 128 | 32768 | 1024 | 0 | 1024 | 8192 | 16384 | 32768 | 32768 | 32768 | 4096 | 2048 | 8192 |
| 1 in 200 | 4096 | 8192 | 0 | 4096 | 0 | 32768 | 512 | 0 | 1024 | 8192 | 16384 | 32768 | 32768 | 32768 | 4096 | 2048 | 8192 |
| 1 in 250 | 4096 | 4096 | 0 | 2048 | 0 | 32768 | 512 | 0 | 256 | 8192 | 16384 | 32768 | 32768 | 32768 | 2048 | 2048 | 8192 |
| 1 in 300 | 4096 | 4096 | 0 | 2048 | 0 | 32768 | 512 | 0 | 128 | 8192 | 8192 | 8192 | 32768 | 32768 | 2048 | 2048 | 8192 |
| 1 in 350 | 4096 | 2048 | 0 | 2048 | 0 | 32768 | 512 | 0 | 64 | 4096 | 8192 | 8192 | 32768 | 32768 | 2048 | 512 | 8192 |
| 1 in 400 | 0 | 2048 | 0 | 2048 | 0 | 16384 | 512 | 0 | 64 | 4096 | 8192 | 8192 | 32768 | 32768 | 2048 | 512 | 8192 |
| 1 in 450 | 0 | 2048 | 0 | 2048 | 0 | 16384 | 512 | 0 | 32 | 2048 | 8192 | 8192 | 32768 | 16384 | 2048 | 512 | 8192 |
| 1 in 500 | 0 | 0 | 0 | 2048 | 0 | 8192 | 512 | 0 | 16 | 2048 | 8192 | 8192 | 32768 | 16384 | 2048 | 512 | 8192 |
| 1 in 550 | 0 | 0 | 0 | 0 | 0 | 8192 | 512 | 0 | 0 | 2048 | 8192 | 8192 | 32768 | 8192 | 2048 | 0 | 8192 |
| 1 in 600 | 0 | 0 | 0 | 0 | 0 | 8192 | 256 | 0 | 0 | 2048 | 8192 | 8192 | 8192 | 8192 | 2048 | 0 | 8192 |

Table 23: Titres in the gonococcal haemolysis test using cells sensitised with varying dilutions of two type I antigens and two type II antigens and their corresponding antisera.

| Degree of sensitisation | Titres in the gonococcal haemolysis test with a type I antiserum (c) | | | | Titres in the gonococcal haemolysis test with a type II antiserum (i) | | | |
|---|---|---|---|---|---|---|---|---|
| | Antigen* | | | | Antigen* | | | |
| | O | Q | P | I | O | Q | P | I |
| 1 in 50 | 4096 | 8192 | 4096 | 4096 | 16384 | 16384 | 8192 | 4096 |
| 1 in 100 | 4096 | 8192 | 4096 | 2048 | 16384 | 16384 | 4096 | 4096 |
| 1 in 150 | 4096 | 8192 | 2048 | 1024 | 16384 | 16384 | 4096 | 4096 |
| 1 in 200 | 4096 | 8192 | 2048 | 1024 | 16384 | 8192 | 4096 | 4096 |
| 1 in 250 | 2048 | 8192 | 2048 | 256 | 16384 | 8192 | 2048 | 4096 |
| 1 in 300 | 2048 | 8192 | 2048 | 128 | 16384 | 8192 | 1024 | 2048 |
| 1 in 350 | 2048 | 8192 | 512 | 64 | 8192 | 8192 | 1024 | 1024 |
| 1 in 400 | 2048 | 8192 | 512 | 64 | 8192 | 8192 | 1024 | 1024 |
| 1 in 450 | 2048 | 8192 | 512 | 32 | 8192 | 8192 | 1024 | 1024 |
| 1 in 500 | 2048 | 8192 | 512 | 16 | 8192 | 8192 | 1024 | 512 |
| 1 in 550 | 2048 | 8192 | 0 | 0 | 4096 | 8192 | 0 | 256 |
| 1 in 600 | 2048 | 8192 | 0 | 0 | 4096 | 8192 | 0 | 0 |

*O and Q are type I strains; P and I are type II strains.

In general the titres with serum i tend to be one to two tubes higher than with serum c. Otherwise there are no significant differences in the titrations.

Table 24: A prozone phenomenon in the gonococcal haemolysis test.

| Degree of sensitisation | Tube * | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1 in 50 | + | + | + | + | + | + | + | + | + | + | + | ± |
| 1 in 100 | + | + | + | + | + | + | + | + | + | + | + | ± |
| 1 in 150 | + | + | + | + | + | + | + | + | + | + | + | ± |
| 1 in 200 | + | + | + | + | + | + | + | + | + | + | + | ± |
| 1 in 250 | + | + | + | + | + | + | + | + | + | + | + | ± |
| 1 in 300 | - | + | + | + | + | + | + | + | + | + | + | ± |
| 1 in 350 | - | - | ± | + | + | + | + | + | + | + | + | ± |
| 1 in 400 | - | - | - | ± | + | + | + | + | + | + | ± | - |
| 1 in 450 | - | - | - | ± | + | + | + | + | + | + | ± | - |
| 1 in 500 | - | - | - | - | ± | ± | + | + | + | ± | - | - |
| 1 in 550 | - | - | - | - | - | ± | + | + | + | ± | - | - |
| 1 in 600 | - | - | - | - | - | ± | ± | + | + | ± | - | - |

*Serial doubling dilutions of serum (f) starting at 1 in 32.
+=haemolysis; ±=partial haemolysis; -=no haemolysis.

Reading of the results when using "partially" sensitised cells was rendered more difficult by a type of prozone which was encountered with some of the sera. An example of this is given in table 24. A similar prozone is not uncommonly seen in sera from patients with gonococcal infections.

## The Use of "Fully" Sensitised Cells.

The second approach, viz. the use of fully sensitised cells, was found to be of equally little value as a means of grouping gonococci. Table 25 shows the titres with fully sensitised cells using various type I and type II antisera. There was complete cross reaction between type I (B,C,D,H), and type II (P,I,E) antigens and antisera.

Table 25: Titres in the gonococcal haemolysis test using "fully" sensitised cells.

| Antiserum | Antigens | | | | | |
|---|---|---|---|---|---|---|
| | B | C | D | H | P | I |
| b | 16384 | 1024 | 16384 | 4096 | 16384 | 8192 |
| c | 16384 | 4096 | 8192 | 4096 | 4096 | 4096 |
| d | 16384 | 2048 | 8192 | 2048 | 8192 | 8192 |
| h | 4096 | 512 | 4096 | 1024 | 4096 | 4096 |
| p | 16384 | 16384 | 16384 | 16384 | 16384 | 8192 |
| i | 8192 | 8192 | 4096 | 8192 | 8192 | 4096 |
| e | 16384 | 8192 | 8192 | 8192 | 16384 | 16384 |

On the other hand the gonococcal haemolysis test, using a type I serum which had been absorbed out with type II sensitised cells, provided clear cut evidence of these two gonococcal types.

Table 26: The differentiation of type I and type II gonococcal types by the gonococcal haemolysis test using a type I antiserum which had been absorbed out with type II sensitised cells.

| Type I serum | Absorbed with *Type II cells (E or I) | Titres with type I sensitised cells | | | Titres with type II sensitised cells | | |
|---|---|---|---|---|---|---|---|
| | | Antigen | | | Antigen | | |
| | | K | L | M | E | I | P |
| b | I | 16 | 16 | 16 | 0 | 0 | 0 |
| b | E | 8 | 16 | 16 | 0 | 0 | 0 |
| h | I | 16 | 16 | 16 | 0 | 0 | 0 |
| h | E | 64 | 64 | 64 | 0 | 0 | 0 |
| n | I | 8 | 16 | 16 | 0 | 0 | 0 |
| n | E | 16 | 16 | 16 | 0 | 0 | 0 |

*Diluted 1 in 250.

Table 26 shows the results of the gonococcal haemolysis test with type I antisera (b,h, and n), which had each been absorbed out with type II sensitised cells (I and E). The absorbed antisera reacted with the type I strains only, and not with the type II.

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# THE INVESTIGATION OF GONOCOCCAL STRAINS BY THE AGGLUTINATION TEST.

The agglutination test has been used fairly extensively by many workers in the investigation of antigenic variations in the gonococcus.

Table 27 shows the results of agglutination tests with nineteen gonococcal suspensions, and eighteen antisera. It is obvious that there are undoubted differences in the agglutinability of various gonococcal strains. Thus the H agglutinable suspension gave uniformly high titres with all the antisera. Suspensions T and N, on the other hand, tended to give lower titres. There are strong reasons, however, for not regarding this difference in agglutinability between various strains as being indicative of basic antigenic differences between the strains. Thus those suspensions that tended to give low titres behaved in that manner with all the eighteen antisera used. Suspensions giving higher titres such as H, similarly showed a uniform behaviour with all the sera.

A discussion of the reasons for the variation in the agglutinability of different gonococcal strains will be deferred to a later section of this thesis. One aspect, however, has been subject to experimental investigation and will be discussed here.

## The Influence of the S-R Change on the Agglutinin Titre.

Various gonococcal strains were maintained on stock culture and agglutinable suspensions prepared from these strains at the following times:

1. Within three weeks of primary isolation.
2. After three to four months on stock culture.
3. After six months on stock culture.

During this period the organisms had become rough in so far as a type specific antigen could no longer be extracted. These suspensions were then tested in an agglutination test against various antisera which had been prepared with recently isolated strains. The titrations were not performed simultaneously, but with each suspension as it became available. The first suspension with strain C was unfortunately not prepared.

Table 27: The results of the gonococcal agglutination test with nineteen gonococcal agglutinable suspensions, and eighteen gonococcal antisera.

| Antiserum | Gonococcal agglutinable suspensions | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E | H | I | J | K | M | N | P | R | S | T | U | V | W | A | G | L | O | Q |
| e | 512 | 4096 | 2048 | 1024 | 256 | 512 | 128 | 256 | 512 | 128 | 128 | 64 | 256 | 256 | 512 | 512 | 256 | 512 | 256 |
| h | 512 | 32768 | 1024 | 256 | 256 | 512 | 64 | 256 | 4096 | 2048 | 64 | 512 | 256 | 256 | 512 | 1024 | 2048 | 1024 | 512 |
| i | 256 | 4096 | 2048 | 256 | 64 | 256 | 32 | 64 | 2048 | 512 | 32 | 512 | 64 | 64 | 512 | 512 | 512 | 512 | 256 |
| j | 256 | 1024 | 256 | 512 | 256 | 1024 | 32 | 128 | 1024 | 512 | 32 | 512 | 512 | 64 | 512 | 128 | 256 | 512 | 256 |
| k | 256 | 512 | 256 | 32 | 256 | 512 | 32 | 64 | 256 | 256 | 32 | 64 | 32 | 16 | 128 | 256 | 64 | 64 | 256 |
| m | 128 | 2048 | 256 | 128 | 64 | 1024 | 32 | 128 | 1024 | 256 | 128 | 128 | 128 | 32 | 256 | 512 | 512 | 64 | 256 |
| n | 256 | 1024 | 128 | 128 | 128 | 512 | 32 | 256 | 256 | 128 | 64 | 32 | 64 | 16 | 512 | 128 | 128 | 128 | 256 |
| p | 256 | 2048 | 2048 | 512 | 256 | 1024 | 64 | 1024 | 512 | 512 | 32 | 128 | 128 | 512 | 512 | 128 | 1024 | 1024 | 256 |
| r | 256 | 2048 | 4096 | 1024 | 256 | 512 | 64 | 512 | 512 | 256 | 32 | 64 | 128 | 512 | 512 | 256 | 512 | 512 | 256 |
| s | 512 | 2048 | 1024 | 256 | 128 | 512 | 128 | 64 | 512 | 512 | 32 | 64 | 64 | 128 | 512 | 512 | 512 | 512 | 512 |
| t | 256 | 1024 | 2048 | 512 | 256 | 512 | 128 | 128 | 256 | 256 | 128 | 16 | 32 | 128 | 512 | 256 | 256 | 512 | 128 |
| u | 256 | 2048 | 2048 | 512 | 256 | 512 | 32 | 128 | 1024 | 128 | 64 | 512 | 64 | 512 | 512 | 512 | 256 | 512 | 128 |
| v | 8 | 64 | - | 32 | 16 | 16 | 8 | 8 | 8 | 16 | 4 | 32 | 64 | 32 | 16 | 16 | 16 | 16 | 16 |
| w | 256 | 2048 | - | 256 | 128 | 256 | 128 | 256 | 512 | 256 | 32 | 32 | 128 | 512 | 512 | 256 | 512 | 256 | 256 |
| b | 256 | 8192 | 1024 | 256 | 256 | 1024 | 64 | 64 | 2048 | 512 | 128 | 512 | 256 | 64 | 512 | 1024 | 1024 | 512 | 256 |
| o | 1024 | 2048 | 1024 | 256 | 512 | 2048 | 128 | 1024 | 1024 | 512 | 64 | 256 | 128 | 128 | 512 | 1024 | 256 | 512 | 512 |
| d | 512 | 4096 | 256 | 128 | 256 | 1024 | 128 | 256 | 512 | 256 | 64 | 128 | 128 | 64 | 1024 | 256 | 256 | 128 | 512 |
| f | 2048 | 65536 | 4096 | 1024 | 512 | 2048 | 1024 | 256 | 8192 | 4096 | 256 | 1024 | 1024 | 512 | 4096 | 4096 | 4096 | 1024 | 1024 |

The results of the agglutination tests with these suspensions are set out in table 28.

Table 28: Titres in a gonococcal agglutination test using suspensions prepared from smooth and rough gonococcal strains, and antisera prepared with smooth strains.

| Antiserum | Agglutinable suspensions* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | H1 | H2 | H3 | J1 | J2 | J3 | G1 | G2 | G3 |
| b | - | 128 | 16 | 8192 | 8192 | 1024 | 256 | 128 | 32 | 1024 | 32 | 4 |
| c | - | 32 | 32 | 2048 | 4096 | 1024 | 256 | 32 | 32 | 1024 | 32 | 32 |
| d | - | 128 | 32 | 4096 | 1024 | 512 | 128 | 128 | 64 | 256 | 128 | 64 |
| e | - | 256 | 16 | 4096 | 1024 | 1024 | 1024 | 64 | 64 | 512 | 16 | 16 |
| f | - | 512 | 8 | 65536 | 16384 | 256 | 1024 | 256 | 64 | 4096 | 512 | 32 |
| h | - | 64 | 8 | 32768 | 4096 | 4096 | 256 | 128 | 16 | 1024 | 128 | 8 |
| i | - | 64 | 8 | 4096 | 2048 | 1024 | 256 | 64 | 16 | 512 | 256 | 8 |
| j | - | 256 | 16 | 1024 | 1024 | 128 | 512 | 256 | 128 | 128 | 256 | 16 |
| k | - | 64 | 8 | 512 | 128 | 64 | 32 | 64 | 16 | 256 | 64 | 4 |
| m | - | 32 | 16 | 2048 | 2048 | 1024 | 128 | 64 | 32 | 512 | 128 | 16 |
| n | - | 32 | 16 | 1024 | 256 | 256 | 128 | 64 | 16 | 128 | 16 | 8 |

*Suspensions H1,J1,G1 prepared with recent strains.
Suspensions C2,H2,J2,G2 with strains 3-4 months old.
Suspensions C3,H3,J3,G3 with strains 6 months old.

It will be seen that as the strains become rough there is a striking loss of agglutinability when tested with an antiserum prepared with an organism in the smooth phase.

Table 29:Titres in a gonococcal agglutination test with antisera prepared from smooth strains (cS,hS,and jS),and from rough strains (cR, gR,hR,and jR), and with agglutinable suspensions prepared from smooth and rough gonococcal strains.

| Antiserum | Agglutinable suspensions* | | | | | |
|---|---|---|---|---|---|---|
| | G1 | G2 | G3 | H1 | H2 | H3 |
| cS | 1024 | 32 | 32 | 2048 | 4096 | 1024 |
| cR | 32 | 128 | 128 | 32 | 128 | 256 |
| gR | 8 | 256 | 512 | 128 | 512 | 512 |
| hS | 1024 | 128 | 8 | 32768 | 4096 | 4096 |
| hR | 128 | 512 | 512 | 4096 | 1024 | 2048 |
| jS | 128 | 256 | 16 | 1024 | 1024 | 128 |
| jR | 64 | 64 | 64 | 256 | 256 | 256 |

*Suspensions G1,H1 prepared with recent strains.
Suspensions G2,H2 with strains 3-4 months old.
Suspensions C3,H3 with strains 6 months old.

On the other hand if rough organisms are used in the preparation of antisera, the tendency is for the maximum titres to occur with the "rough" suspensions, and the lower titres with the "smooth" suspensions.

This is illustrated in Table 29 which shows the results of agglutination tests with antisera prepared soon after the organisms had been isolated, and with antisera prepared with the same organisms after they had been on subculture for six months. These antisera referred to as S and R in the table were tested against agglutinable suspensions prepared with smooth and rough strains. It will be seen that the tendency is for the titres with the S antisera to be highest against the recently isolated strains, and lower against older strains. On the other hand, the antisera prepared with the rough strains (R) show maximum titres with older gonococcal strains, and lower titres with the recently isolated strains. This is particularly well shown by gonococcus G.

Thus the behaviour of an antiserum, and the agglutinability of a gonococcal suspension, both are dependant upon the same factor, viz. whether the gonococcus used in their preparation was a recently isolated smooth strain, or an older rough strain.

## The Agglutination Test, the Gonococcal Haemolysis Test, and the S-R change.

As has been indicated the S-R change is associated with two detectable antigenic changes.

1. The loss of the type specific antigen detectable in the gonococcal haemolysis test.
2. A loss of agglutinability.

It is tempting to correlate these two changes, and to suggest that the loss of agglutinability is due to the loss of the type specific antigen.

However, evidence has been presented to show that the agglutination test is directed against an antigen other that the type specific antigen which is detected by the gonococcal haemolysis test. These two antigens could be quite clearly separated by their behaviour.

It therefore must be concluded that the S-R change in the gonococcus results in the loss of at least two antigenic factors.

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## THE GONOCOCCAL COMPLEMENT-FIXATION TEST.

Of the various serological techniques available for the investigation of gonococcal strains, the complement-fixation test proved the least satisfactory, and for that reason it was not pursued to the same extent as the other methods. The reasons for this are as follows:

1. Lack of sensitivity. The relative insensitivity of the gonococcal complement-fixation test is illustrated in table 30. Complement-fixation and agglutination tests were carried out with antigens prepared from four gonococcal strains using a single antiserum (b), and the titres compared.

Table 30: A comparison of titres in agglutination and complement-fixation tests with four antigens, and a single serum.

| | Antigens | | | |
|---|---|---|---|---|
| | H | I | J | K |
| Titres in a complement-fixation test | 256 | 64 | 32 | 16 |
| Titres in an agglutination test | 8192 | 1024 | 256 | 256 |

It will be seen that the titres in the agglutination test are considerably higher than in the complement-fixation.

2. Anticomplementary activity. The majority of the complement-fixing antigens prepared by Price's method are anticomplementary to a greater or lesser degree, and at the same time not very highly antigenic.
3. Loss of antigenicity. There is a steady loss of antigenicity of the complement-fixing antigen on storage, the anticomplementary unit, however, remaining unchanged. This is illustrated in Table 31.

Probably as a result of a combination of these factors, quantitative results are not readily reproducable, and in practice the technique proved highly unsatisfactory.

Table 31: The antigenic and anticomplementary titres of three gonococcal complement-fixation antigens before and after storage for four months.

| Antigen | Initial titres | | Titres after storage for four months | |
|---|---|---|---|---|
| | Antigenic unit | A.C. unit | Antigenic unit | A.C. unit |
| B | 32 | 2 | 8 | 2 |
| E | 64 | 2 | 16 | 2 |
| N | 128 | 2 | 16 | 2 |

In general, however, as indicated in table 30, the results of complement-fixation tests tended to parallel those obtained by the agglutination test.

This is not the case with the gonococcal haemolysis test. Thus strain H which showed very high agglutinin titres, proved a poor antigen in the gonococcal haemolysis test (table 27). On the other hand strain N which gave relatively low titres in an agglutination test, provided a very potent sensitising antigen.

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## THE CHEMICAL NATURE OF THE TYPE SPECIFIC ANTIGEN

The sensitising antigen which was extracted from a gonococcal suspension, was a watery clear solution. Occasional antigens showed a slight turbidity, but this could always be removed by centrifugation. The chemical nature of the type specific antigen was investigated and the results are set out in table 32.

Table 32: The results of the Molisch, Biuret, and xanthoproteic tests performed on the sensitising antigen.

| Antigen | Molisch's test | Biuret test | Xanthoproteic test |
|---|---|---|---|
| A | + | - | - |
| C | + | - | - |
| E | + | - | - |
| G | + | - | - |
| H | + | - | - |
| I | + | - | - |
| L | + | - | - |
| O | + | - | - |
| P | + | - | - |
| Q | + | - | - |
| R | + | - | - |
| S | + | - | - |

+ = positive; - = negative.

All the antigens gave strongly positive Molisch's tests and negative biuret tests and xanthoproteic reactions, indicating a probable carbohydrate nature.

The heat stability of the antigen was next investigated. This was carried out by making 25 ml. of a 1 in 25 dilution of a pooled sensitising antigen. The antigen was then heated in a water bath at 95° C. for three hours.

A portion of the antigen was removed before the commencement of the heating, and at varying intervals thereafter, cooled to room temperature, and then used to sensitise sheep cells which were then tested in the gonococcal haemolysis test. These cells were tested with the same antiserum which had been prepared with fifteen pooled strains.

The results of testing these cells in the gonococcal haemolysis test are set out in table 33.

Table 33: The effect of heat on the capacity of the type specific antigen to sensitise cells in the gonococcal haemolysis test.

| Sensitising antigen heated at 95° C. for: | Titre in the gonococcal haemolysis test |
|---|---|
| nil | 4096 |
| ¼ hour | 4096 |
| ½ hour | 4096 |
| 1 hour | 4096 |
| 1½ hours | 4096 |
| 2 hours | 4096 |
| 2½ hours | 4096 |
| 3 hours | 4096 |

The type specific antigen proved to be heat stable showing no loss of its capacity to sensitise red cells in the gonococcal haemolysis test even after three hours at 95° C.

Thus, to summarise, the type specific antigen extracted from a gonococcal suspension was a heat stable polysaccharide which was detected by its capacity to sensitise cells in the gonococcal haemolysis test.

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## THE GONOCOCCAL TYPES.

After the investigations described had been completed a second series of gonococcal strains were investigated.

The technique was simplified in so far as extraction of the type specific polysaccharide was carried out on the suspension obtained from eight to ten petri dishes (instead of fifty), using appropriately smaller volumes of reagents, and dissolving the final product in five ml. buffer. Cells sensitised with the antigen were then tested in absorption tests against antisera prepared with type I and type II gonococcal strains.

In all a series of sixty seven recently isolated strains were investigated. Their distribution among the two gonococcal types was as follows:

Table 34: The distribution of gonococcal types among sixty seven recently isolated strains.

| Type | Frequency |
|---|---|
| Gonococcus type I | 59 |
| Gonococcus type II | 8 |

### A Third Gonococcal Type

During the course of this investigation a strain was encountered which did not fall into either of these two types.

The strain was probably partially rough when first isolated and after a few weeks on subculture an active polysaccharide could no longer be extracted. The antisera prepared with this strain were of relatively low titre.For these reasons the strain was not included in the series listed in table 34, and the suggestion that it represents a third type is advanced somewhat tentatively.

Morphologically and biochemically the strain showed the usual behaviour of a gonococcus (Chapter IV).

It was agglutinated by type I and type II antisera, and an antiserum prepared with this strain agglutinated type I and type II gonococcal suspensions.

Its behaviour in absorption tests was as follows:

1. Red cells sensitised with the polysaccharide extracted from this strain partially absorbed out a type I and a type II gonococcal antiserum, but were unable to remove completely the antibodies from either.

Table 35: The absorption of a type I and a type II gonococcal antiserum by a new gonococcal type.

| Antiserum / Absorbing cells | Type I / Type III | | Type II / Type III | |
|---|---|---|---|---|
| Stage | Serum titre | Absorbing cells | Serum titre | Absorbing cells |
| Initial | 128 | | 64 | |
| Absorption 1 | 64 | + | 32 | ± |
| Absorption 2 | 32 | ± | 32 | - |
| Absorption 3 | 32 | - | 32 | - |
| Absorption 4 | 32 | - | | |

+ = haemolysis; ± = partial haemolysis; - = no haemolysis.

2. If a type I antiserum was absorbed out as far as possible with type II sensitised cells, the remaining antibodies could be removed by absorption with red cells sensitised with the polysaccharide obtained from this strain. Similarly, if the type I antiserum was first absorbed out with cells sensitised with this strain, the absorption could be completed by the type II gonococcus. Thus if the type I gonococcus is represented by A + B, and the type II gonococcus by A, this strain must possess the antigen B.

3. An antiserum prepared with this strain was only partially absorbed out by type I, or type II sensitised cells so that this strain possessed a further antigen other than B.

This strain has been called gonococcus type III.

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## CROSS REACTIONS WITH OTHER NEISSERIAE.

Although this investigation was primarily concerned with the problem of antigenic relationships within the gonococcus group, it was obviously pertinent to enquire whether these antigens were also present in other neisseriae.

### Non-pathogenic Neisseriae.

Twenty three strains were investigated by the methods already described. These strains included four strains, N.catarrhalis, N.sicca, N.haemolysans, and N.flavus, obtained from the National Collection of Type Cultures, Colindale, London. The remainder were local strains isolated from the nasopharynx. Their biochemical behaviour was variable and it was not possible to assign the majority of these strains into the biochemical groups listed by Bergey.

Cells sensitised with extracts from these organisms were tested with gonococcal antisera as described in the gonococcal haemolysis test. Twelve of these extracts proved to be haemolytic. The remainder did not cross react with a gonococcal antiserum.

### Neisseria meningitidis.

Five strains, all of which were harvested and extracted within a few days of primary isolation, were investigated. The results showed an exciting relationship to the gonococcus group.

In an Agglutination Test. The gonococcal antisera which were used in the agglutination tests set out in table 27, were tested in agglutination tests against agglutinable suspensions prepared with five recently isolated meningococcal strains.

The results showed no agglutination whatsoever. Thus the meningococcus possesses a distinctive antigen not detectable by agglutination with gonococcal antisera.

In the Absorption Test. Extracts were prepared with the five meningococcal strains, and red cells sensitised with these strains were used to absorb out type I and type II gonococcal antisera. The results were as follows:

a. Three meningococcal strains completely removed all antibodies from both type I and type II gonococcal antisera, i.e. they showed the behaviour of a type I gonococcal polysaccharide.

b. One meningococcal strain removed all the antibodies from a type II gonococcal antiserum, but only partially exhausted a type I antiserum, i.e. it showed the behaviour of a type II gonococcal polysaccharide.

c. One strain partially removed the antibodies from both type I and type II gonococcal antisera but was unable to exhaust either, i.e. it showed a behaviour similar to that which has been termed a type III gonococcus.

Thus the three polysaccharide types encountered in this investigation of N.gonorrhoeae, are also present in N.meningitidis. The two organisms differ in the possession of distinctive antigens detectable in an agglutination test, i.e. they have distinctive group antigens. Further division into types can be made on the basis of the type specific polysaccharide.

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# SECTION IV

## A REVIEW OF THE LITERATURE.

# CHAPTER XXIV.

## SEROLOGICAL INVESTIGATIONS ON THE GONOCOCCUS.

The gonococcus was first observed by Neisser[21] in 1879 in the pus cells of thirty five cases of gonorrhoea, and in nine cases of ophthalmia, both neonatorum and adult. Shortly afterwards the organisms were successfully cultured by Leistikow[22] (1882), and by Bumm[1] (1885). Bumm demonstrated the pathogenicity of his cultures by inoculation into human subjects, and so completed the requirements of Koch's Postulates.

The next advance came with the accurate demonstration of the biochemical behaviour of the gonococcus by Gordon[2] (1905), Rothe[23] (1908), Elser and Huntoon[24] (1909), Watabiki[30] (1910), and Martin[25] (1910), and the differentiation of the gonococcus, both by its cultural and biochemical characters, from the meningococcus and catarrhalis group.

The application of serological methods to the investigation of the gonococcus may be said to have commenced with the demonstration by MÜLLER and OPPENHEIM[3] (1906) of a positive complement-fixation test on the serum of a patient with a gonococcal arthritis.

This observation was confirmed by BRUCK[4] (1906) who obtained three positive complement-fixation tests in eight cases. Using both complement-fixation and agglutination methods Bruck also showed that rabbits could be immunised by the inoculation both of the live gonococcus as well as by aqueous extracts of the organism.

BRUCKNER and CRISTÉANU[26] (1906) at the same time were attempting to distinguish the gonococcus from the meningococcus by serological methods. They prepared gonococcal antisera in horses, but found that their sera agglutinated both a gonococcal and a meningococcal suspension to equal titre (about 1 in 2000). A similar result was obtained with a precipitin technique.

WOLLSTEIN[27] (1907) similarly failed to differentiate the gonococcus from the meningococcus by agglutination.

VANNOD[28] (1906), however, using gonococcal rabbit antisera showed that the titre with the meningococcal suspension was considerably lower than with the gonococcus, and this observation was soon confirmed by TORREY[7] (1907), ELSER and HUNTOON[24] (1909), and MARTIN[25] (1910).

The first attempt at differentiation within the gonococcal group was made by TORREY[7] (1907). He investigated ten strains by agglutination and agglutinin absorption methods. Eight gonococcal antisera were employed.

Torrey showed that there were differences in the agglutinability of various gonococci, and he concluded that the gonococcus group embraced a heterogeneous collection of types. Of the ten strains studied, three types were recognised to which, however, only six of these strains could be referred.

Similar conclusions were arrived at using a complement-fixation technique (Teague and Torrey[29],1907) although there was less difference between their type 1 and type 2 as there was when using an agglutination technique.

Torrey's major contribution in this field was made in a second and larger investigation published in 1922. The chief interest of his earlier work, however, lies in the fact that six of his original strains plus a further six added to them at that time, were maintained on subculture in various laboratories and re-examined by Torrey 14 years later, as well as by other workers with somewhat different results. This will be discussed later with Torrey's second paper.

WATABIKI[30] (1910) investigated eight recently isolated strains by simple agglutination. He used eight rabbit antisera prepared with these strains, as well as an antiserum obtained from Torrey.

He found that all the strains were agglutinated by all the antisera. Two of the strains, however, showed a fourfold (two tube) difference in titre as compared with the other six, and on this basis Watabiki divided the strains into two groups. He points out that his experiments showed no distinct difference among the various strains of gonococcus, only a comparative difference. Torrey's antiserum similarly agglutinated all eight strains, the larger group of six strains having a slightly higher titre than the remaining two strains.

Essentially similar results were recorded by VANNOD[28] (1907), and by ELSER AND HUNTOON[24] (1909) using simple agglutination. These latter workers, however, commented on some of the difficulties inherent in this approach. Thus the proviso is laid down that the "strains chosen must not be highly inagglutinable". Not only is agglutination affected by the nature of the culture medium, but old cultures were twice as easily agglutinable as recent ones.

PEARCE[31] (1915) investigated eighteen strains, nine of which were isolated from adults, six from cases of vulvo-vaginitis in children, and three from cases of ophthalmia. All the strains were described as "recently isolated" with the exception of one of the Torrey strains which had been under cultivation for seven years. At least six rabbit antisera prepared with strains obtained from adults and infants were used, her methods being agglutination and complement-fixation.

Pearce suggested that there was a significant difference in titre in agglutination tests between the strains obtained from adult sources, and those from cases of vulvovaginitis. The figures given in her paper, however, show a fair degree of overlapping, and the titres of eight of the fifteen strains in these two groups were either identical, or within one tube of each other. The three ophthalmic strains were taken to occupy an intermediate position serologically between the adult and infant strains. Similar results were obtained by complement-fixation.

The conclusions are: "From the agglutination and complement-fixation tests just described it is evident that within the large group of organisms known as the gonococcus two more or less distinct types may be differentiated. These types correspond to the clinical source of the strains".

Two additional points in her work are of interest. The one is the presence of a prozone in her agglutination tests, this prozone being greatest with those strains which agglutinated to lower titre. The second is a comment on the complement-fixing antigens, some of which became too anticomplementary for use if stored overnight.

WARREN[32] (1921) investigated twenty three gonococcal strains, most of which had been kept on culture for some time before being investigated. Seventeen of these strains

had been isolated by Warren, and six were obtained from other laboratories. Four gonococcal rabbit antisera were used.

Using an agglutination technique he found that all his sera readily agglutinated all his gonococcal strains, and he concluded that the "agglutination test does not serve to differentiate strains of gonococci into serological groups".

COOK and STAFFORD[33] (1921) investigated sixteen strains ten of which they had isolated, and six of which they had obtained from another laboratory. The strains had all been subcultured for some time prior to investigation. Eight rabbit antisera were used, and their techniques were complement-fixation, agglutination, and agglutinin-absorption.

Their results showed that " all serums gave positive reactions with all the gonococcus antigens, and whatever differences there were between the individual strains were not sufficiently marked or consistent to justify a classification on this basis. Any differences in titer of the individual antigens seemed to be due to the efficiency or lack of efficiency of the individual antigens rather than to any specific relationship between serum and antigens. Certain antigens gave a uniformly high titer with all serums, while certain others were uniformly low ".

Their agglutinin absorption tests followed the technique described by Hermanies[37]. They found that there was no uniformity in the manner in which agglutinins were absorbed by various strains from their antisera and that there was no evidence of grouping among the sixteen strains of gonococci.

JÖTTEN[34] (1921) investigated twenty seven gonococcal strains of which ten were obtained from severe cases with complications, and the remainder from uncomplicated cases.

Using direct agglutination, he classified twenty of these strains into four groups containing five, five, seven, and three strains respectively. The remaining seven strains did not fit into any group. His first two groups comprised the strains isolated from the more severe cases, and these strains, it is claimed, were more toxic for mice. Similar results were obtained with a complement-fixation technique.

GORDON[35] (1921), quoted by Tulloch, examined thirty strains by agglutinin absorption and found that twenty five of these could be regarded as constituting a fairly well defined subgroup. He states that the results,however, were not clear cut.

THOMSEN and VOLLMOND[36] (1921) investigated twenty six recently isolated gonococcal strains. Simple agglutination, using presumably rabbit antisera, failed to differentiate the various strains. Complement-fixation, however, when combined with an absorption technique proved more satisfactory.

On this basis they divided their strains into four groups. The fourteen strains comprising their first group showed well marked serological variation within the group. The other three groups consisted of five, five, and two strains respectively.

HERMANIES[37] (1921) investigated eighty five gonococcal strains all of which had been maintained on culture for some time. These strains included Torrey's strains which had been originally isolated in 1907. His stocks were maintained at room temperature and subcultured every eight to ten days.

Rabbit antisera were prepared with strains which the author terms were "representative of three different types of infection".

The technique employed was that of agglutinin absorption. Gonococci for absorption were grown on a slant in a 15 x 1.7 cmm. tube, and washed off with 1.0 ml. of a dilution (generally 1 in 250) of the serum to be absorbed. The suspension was left at 56° C. for four to six hours and then overnight in the cold. Only a single absorption was performed on each serum. After absorption the absorbed serum was tested by agglutination with a suspension of the homologous organism.

Thus forty one strains were able to more or less remove the agglutinins from 1 ml. of a 1 in 250 dilution of an antiserum prepared with his gonococcus strain 1.Absorption of this serum with the remaining forty four strains failed to remove all the agglutinins for the homologous gonococcus (strain 1) after a single absorption. On this basis the forty one strains which were effective in the absorption were grouped together as group 1.

Many of the strains not included in this group, however, showed a partial removal of agglutinins. Moreover, results were less clear cut if a different serum dilution was employed. Thus only what Hermanies refers to as the " more decisive dilutions " are presented in the paper. The technique was further complicated by the observation that the "agglutinability of the same strain varied somewhat from day to day and at times this variability was quite marked, and more pronounced in some strains than in others".

Having thus separated forty one strains into a single group, the same experiment was repeated using an antiserum prepared from one of the remaining forty four strains. In this way a further five types were established, giving six gonococcal types in all.

These experiments, to quote Hermanies, " prove that that the gonococci are a collection of organisms that fall into distinct clear cut immunological types, having very little relation with one another".

The Torrey strains, six of which Torrey (1907) classified into three groups all fell into Hermanies' type 1.

In a second paper Hermanies[38] (1921) further subdivided the thirty six strains present in his type 2 into four different races which he referred to as a,b,c, and d strains. This subdivision he found necessary due to the variation in the agglutinogenic and absorptive capacity of the strains grouped as type 2.

Thus of the eighty five strains, forty one fell into type 1, thirty six into type 2 (with its four subdivisions), and the remaining eight into a further four groups.

TORREY[39] working with BUCKELL made a second investigation into the problem of the serology of the gonococcus in 1922.

They used seventy seven strains representing gonococcal infection occurring in various countries, and all the strains had been on subculture for some time. Twelve of the strains including the ten Torrey strains had been under cultivation for fourteen years.

Simple agglutination tests were carried out with forty seven strains and eight antisera. Their figures clearly illustrate the futility of attempting classification by direct agglutination, titres with heterologous strains often greatly exceeding the homologous titres. They conclude that "straight agglutination tests are not of any definite value in indicating possible type relations between gonococcus strains".

They then proceeded to carry out agglutinin absorption tests. The method of absorption was as follows:

> The absorbing strain was harvested from ascitic agar slopes with saline, centrifuged, and 0.2 ml. of the loosely packed organisms used for the absorption. The serum to be absorbed was appropriately diluted (generally 1 in a 100 to 1 in 250), and 5.0 ml. added to the 0.2 ml. of the absorbing organisms. Absorption was allowed to proceed at 45° to 50° C. for two hours. The agglutinin titre of both the absorbed and unabsorbed serum against the homologous organism was then determined.

Some of the results obtained by Torrey and Buckell in carrying out these absorptions have been abstracted from their paper and are set out in table 36.

Table 36: The results obtained by Torrey and Buckell in absorption of a gonococcal antiserum with various gonococcal strains.

| Strain | Titre unabsorbed serum | Titre absorbing strain with absorbed serum | Titre homologous strain with unabsorbed serum | Titre homologous strain with absorbed serum | Percentage absorption of specific agglutinins |
|---|---|---|---|---|---|
| 2 | 1000 | <500 | 8000 | 8000 | 0 |
| 74 | 8000 | <500 | 8000 | 6000 | 25 |
| 21 | 500 | <500 | 6000 | 4000 | 33 |
| 4 | 1000 | <500 | 8000 | 4000 | 50 |
| 28 | 2000 | <500 | 6000 | 2000 | 66 |
| 3 | 4000 | <500 | 8000 | 2000 | 75 |
| 31 | 8000 | <500 | 6000 | 1000 | 84 |
| 12 | 500 | <500 | 8000 | 1000 | 87 |
| 19 | 1000 | <500 | 8000 | 500 | 93 |
| 11 | 4000 | <500 | 8000 | <500 | 100 |

The results have been expressed as the percentage of agglutinins removed from the serum (the last column in table 36).

Nine antisera were used in the larger investigation and a single absorption was carried out on each of these sera with the available gonococcal strains. In table 37 the results of absorption tests with fourteen strains on the nine antisera employed have been tabulated. The figures have been abstracted from Torrey and Buckell's paper, and the strains selected include the nine strains used in preparing the antisera. The grouping of the strain has been included in the last column.

Table 37: The results of agglutinin absorptions carried out by Torrey and Buckell (1922) showing the percentage absorption of the nine antisera used in their investigation by the nine homologous gonococcal strains and five other strains.

| Gonococcal strains | The percentage of agglutinins removed from gonococcal antisera | | | | | | | | | Group |
|---|---|---|---|---|---|---|---|---|---|---|
| | 29* | 33* | 15 | 8 | 42 | 7 | 5 | 18 | 41 | |
| 29 | 75+ | 75+ | 75+ | 75+ | 75+ | 75+ | ±50 | 75+ | -33 | Regular |
| 33 | 75+ | 75+ | 75+ | 75+ | 75+ | 75+ | ±50 | -33 | -33 | Regular |
| 15 | ±50 | 75+ | 75+ | 75+ | 75+ | 75+ | ±50 | ±50 | -33 | Regular |
| 8 | 75+ | 75+ | 75+ | 75+ | ±50 | 75+ | 75+ | ±50 | 75+ | Regular |
| 42 | 75+ | 75+ | 75+ | 75+ | 75+ | 75+ | -33 | -33 | -33 | Regular |
| 7 | ±50 | 75+ | ±50 | -33 | 75+ | 75+ | 75+ | ±50 | ±50 | Intermediate |
| 5 | -33 | -33 | ±50 | ±50 | ±50 | -33 | 75+ | ±50 | -33 | Intermediate |
| 18 | -33 | -33 | 75+ | 75+ | 75+ | ±50 | -33 | 75+ | 75+ | Irregular |
| 41 | -33 | -33 | -33 | ±50 | 75+ | -33 | -33 | ±50 | ? | Irregular |
| 1 | -33 | ±50 | 75+ | ±50 | 75+ | -33 | ±50 | 75+ | 75+ | Irregular |
| 2 | -33 | -33 | -33 | ±50 | -33 | -33 | 75+ | -33 | -33 | Irregular |
| 12 | -33 | 75+ | 75+ | -33 | -33 | ±50 | ±50 | ±50 | -33 | Intermediate |
| 4 | 75+ | 75+ | ±50 | ±50 | 75+ | -33 | 75+ | ±50 | -33 | Regular |
| 13 | ±50 | 75+ | -33 | ±50 | -33 | -33 | ±50 | ±50 | -33 | Intermediate |

* These antisera were prepared with the "Torrey strains" which had been maintained on artificial culture for fourteen years.

Torrey and Buckell's conclusions were that the "strains could not be distributed among a number of distinct serological types which were sharply separated from one another".

Rather than leave the matter at that, however, they then proceeded to divide their strains into three groups.

1. Regular group (thirty nine strains) which on the whole tended to absorb out more than 75 % of the agglutinins from five of the nine antisera. There were, however, many exceptions.
2. Intermediate group (sixteen strains) which though showing a close relationship to the regular group tended to show more variation in the behaviour of the strains.
3. Irregular group (nineteen strains) which exhibited individual antigenic variations to a marked degree.

A number of significant points emerge from Torrey and Buckell's paper.

a. They encountered occasional strains which were agglutinated to titre by various antisera, yet absorbed little or no agglutinins from that serum in absorption tests. Other strains showed poor agglutinin titres in agglutination tests, but proved highly effective in absorption.

b. Re-examination of five of the six strains originally examined in 1907 and which then appeared to comprise three distinct serological types, now appeared to be serologically indistinguishable and were placed in the regular group.

c. Many recently isolated strains were relatively inagglutinable, but became agglutinable after a period on artificial media. It may be noted that their sera were prepared with strains which had been on subculture for some time. Two antisera were prepared with strains fourteen years old.

d. Day to day variations in agglutinability of strains were observed.

e. Two antisera prepared with the same strain showed striking variation in their behaviour in absorption tests. Thus the same gonococcus when used to absorb out these two antisera prepared with the identical strain, in one case almost removed all the agglutinins from the serum, and in the other failed to effect any absorption whatsoever (table 38).

Table 38: Two antisera were prepared with each of two gonococcal strains ( 5 and 29). The figures, taken from Torrey and Buckell's paper, illustrate the varation in the percentage absorption when twelve gonococcal strains (1 to 49) were used to absorb out these two antisera.

| Antiserum against gonococcus | Percentage absorption with gonococcal strains | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 7 | 11 | 15 | 18 | 19 | 25 | 30 | 34 | 38 | 41 | 49 |
| 5 (251) | 50 | 90 | 50 | 50 | 0 | 50 | 0 | 80 | 80 | 80 | 0 | 0 |
| 5 (121) | 25 | 50 | 25 | 50 | 50 | 75 | 0 | 100 | 75 | 90 | 0 | 0 |
| 29 (217) | 0 | 50 | 50 | 50 | 0 | 75 | 75 | 0 | 100 | 90 | 50 | 0 |
| 29 (236) | 94 | 88 | 88 | 88 | 0 | 88 | 88 | 88 | 100 | 100 | 63 | 0 |

f. No definite serological distinction could be drawn between infant and adult strains as suggested by Pearce[31] (1915).

g. In an attempt to find out whether the use of a larger number of sera would provide a more clear cut division of their strains, they absorbed twenty seven antisera with twelve selected strains. Again, the results showed that some sera were more regularly absorbed out under these conditions than others, but it was obvious that no grouping of strains was present. Indeed, it seems probable that the more sera employed, the more obvious does the degree of overlap become.

Torrey and Buckell ascribed the differences between the gonococcal strains as due to a phenomenon of antigenic lability, a term which Hermanies also employed in relation to his type 2.

TULLOCH[40] (1923) investigated one hundred gonococcal strains isolated from cases of urethritis in males. Unlike almost all other workers in this field his strains were all freshly isolated. Five gonococcal rabbit antisera were used.

The technique Tulloch employed was agglutination and agglutinin absorption. Absorption was carried out by adding a small volume of serum to 2.0 ml. of a suspension of the absorbing organism, so as to provide a known dilution of the serum. Absorption was then allowed to proceed at 37° C. for 24 hours.

Preliminary agglutination of ten strains with three antisera produced "unexpectedly homogeneous" results, and no antigenic grouping appeared possible. Absorption tests were then carried out.

Of the one hundred strains, seventy two substantially reduced the titre of a single antiserum (termed a type 1 antiserum), and these strains were grouped together as type 1. The original serum was accidentally lost and was replaced with another showing similar behaviour during the investigation.

Four further groups were demarcated by the capacity of the strains to absorb four other antisera, and these groups comprised seven, three, five and five strains respectively. Eight strains were finally listed as unclassifiable.

Some other points require comment.

a. Like Torrey and Buckell, Tulloch found that a number of relatively inagglutinable strains proved highly effective in absorption of the same antiserum.

b. A rise in agglutinin titre as the organism was subcultured was noted.

c. Absorption of the strains constituting his minor groups showed rather remarkable behaviour, viz. the absorbed serum showed a considerably higher agglutinin titre against the homologous organism than the unabsorbed serum. Thus absorption did not result in a fall in titre of the serum, but in a rise!
The explanation of this lies in the blocking effect of the polysaccharide (Tulloch was using only recently isolated strains), as described in Chapter XVI. Table 39 has been abstracted from Tulloch's paper and illustrates the results of absorption tests with the strains constituting his four minor groups.

Table 39: The results of absorption tests on serum "1887" with some of the strains constituting Tulloch's minor groups.

| Reference no. of strain | Absorption of "1887" serum | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Unabsorbed serum | | | | Absorbed serum plus homologue | | | | Absorbed serum plus test coccus | | | |
| | 8T* | 4T | 3T | 2T | 8T | 4T | 3T | 2T | 8T | 4T | 3T | 2T |
| 1889 | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | - | - | - | - |
| 1900 | ++ | ++ | ++ | - | ++ | ++ | ++ | + | - | - | - | - |
| 2026 | ++ | + | - | - | ++ | ++ | ++ | ++ | - | - | - | - |
| 2053 | ++ | - | - | - | ++ | ++ | ++ | ++ | - | - | - | - |

* T = titre of antiserum.

ATKIN[41] (1925) noted a variation in the colonial appearance of gonococci after they had been allowed to grow for five to seven days or longer. The one type which Atkin found in recently isolated strains, grew as a thin sheet with papillae (type 1).

The second type, present in strains which had been on subculture for two years was a dense thick growth without papillae (type 11). Atkin then prepared two antisera, one with each strain, and noted that recently isolated strains were agglutinated by the antiserum prepared from the recently isolated strain. Stock strains, however, were agglutinated by the type 11 antiserum.

Just as these two colony types were the extremes of a wide range of intermediate types, so the agglutinin titres of the corresponding antisera overlapped, and a number of strains were well agglutinated by both antisera. In all seventy four strains were investigated.

The suggestion was that the type I colony by selection of the longer lived papillae eventually changed into the type II. This transition, however, was not directly observed by Atkin in any strain maintained on stock culture. What Atkin did observe was a loss of agglutinability of a recently isolated strain with his type I serum.

On the basis of a small number of strains isolated from the cervix and which had features in common with his type 11, Atkin then suggested that the type 1 to type 11 transformation also occured in such sites as the cervix, and that the type 11 strains were responsible for the more chronic types of gonococcal infection.

The conclusions were that strains isolated from cases of urethritis were type 1 strains; those from the cervix, joints, etc. were predominantly type 11 strains.

MIRAVENT, QUIROGA, and NEGRONI[42] (1926) investigated forty seven gonococcal strains with four selected antisera. All the strains had been subcultured for not less than three months. The technique employed was agglutination and agglutinin absorption the method being largely that used by Torrey and Buckell[39].

Forty five of their strains were divided into four groups containing sixteen, eight, nine, and twelve strains respectively. Two strains could not be placed.

They concluded that their division into groups was definite, and they had no doubts as to the identity of the various strains. Unfortunately their paper is limited to a general description of technique and details are not given so that a critical evaluation of their work is not possible.

SEGAWA[43] (1932) examined sixty four freshly isolated strains with thirty five rabbit gonococcal antisera.

Simple agglutination tests with thirty five gonococcal strains and the corresponding thirty five antisera revealed no grouping of strains, and the presence of a complete cross reaction.

Some strains which agglutinated to a lower titre were then selected and agglutinin absorption tests performed. Neither the technique, nor the results are given in any detail. However, the results of absorptions of twenty two antisera with thirty gonococcal strains were reported to be as good as the absorptions with the homologous gonococcal strains. A variation in absorption was noted and was ascribed to a lability ("Labilität") in the gonococcus.

Segawa's conclusions were that the investigation disclosed "no variants which could be looked upon with certainty as a distinct immunological type".

It is unfortunate that this important paper was so inadequately documented as it appears to be the only investigation where an adequate number of apparently recently isolated strains were investigated with a large range of antisera.

ABDOOSH[44] (1934) investigated one hundred gonococcal strains which he had isolated from cases of acute urethritis in Cairo. Three rabbit antisera were used.

The technique was agglutination as well as agglutinin absorption. The absorption was performed by incubating a 1 in 50 dilution of gonococcal antiserum with an equal volume of a thick suspension of the absorbing gonococcal strain. Absorption was allowed to proceed at 37° C. for two hours, and with a further volume of emulsion overnight in the cold.

Simple agglutination tests revealed that eighty four of the strains agglutinated to high titre with the three antisera. Thirty of these strains were then selected for absorption tests, and it was found that twenty six of these completely removed the agglutinins from the three antisera in absorption tests. An antiserum prepared with one of the four remaining strains was exhausted in an absorption test by the homologous gonococcus but not by the three remaining strains.

Of the sixteen remaining strains, six were described as auto-agglutinable. Colonially these strains presented as a small colony (0.5 mms. in 24 hours) using blood or serum agar, with little tendency to grow larger on further incubation. Abdoosh describes this group as antigenically heterogeneous.

The final ten strains failed to agglutinate with the three antisera. An antiserum prepared with one of these strains agglutinated the homologous strain only to any appreciable titre.

Thus the final grouping of his one hundred strains was as follows:

Group A - 73 % ) 84 %
Group B - 11 % )

Group A formed a homogeneous group; group B was closely related to group A.

Group C - 6 %. This group was auto-agglutinable.
Group D - 10 %. A heterogeneous group.

Like Atkin, Abdoosh suggested that in the body the gonococcus underwent a change which was manifested by a variation in the antigens of the organism, drifting gradually to a heterogeneity with the auto-agglutinable rough variants as the final stage. The process was supposed to be a gradual one and therefore all sorts of gradations in antigenic structure could be encountered.

No information is available as to the period of time the strains were maintained on artificial media, nor whether the antisera were prepared with fresh or old gonococcal strains.

CASPER[45] (1937) investigated one hundred and nine gonococcal strains. He emphasised the importance of using recently isolated strains. However, it is obvious from the figures given in the paper that many strains were more than a month old when he commenced preparing the antisera. One strain was two years old.

Two selected rabbit gonococcal antisera were used, and these were selected as representing two gonococcal types. The technique employed was simple agglutination of each gonococcal strain against the two antisera. A two tube difference in agglutinin titre between these two antisera was considered adequate to assign an organism as belonging to one or other type. Agglutinin absorption was also used, but was seldom required.

Thirty five of these strains were placed as type 1, and twelve as type 11. Five were described as "heterologous", but after absorption four of these were placed in these two groups. The fifth absorbed both sera equally well and was called a type 1 + 2, or a "bivalent" strain.

Twenty one strains were described as "irregular" since they agglutinated with neither serum to a titre above 1 in 200.

Thirty five strains were described as "degenerated" since they showed traces of agglutination in normal serum and saline controls. Subsequently, however, two of these strains were spoken of as having "recovered from their degeneration" since Casper was able to use them satisfactorily in absorption tests.

Casper then investigated the gonococcal strain which Tulloch[40] (1923) had described as his predominant strain comprising seventy two of his hundred strains. This strain had been maintained on artificial media for about fifteen years. On analysis it corresponded with the least common of Casper's strains, the bivalent strain!

STOKINGER, CARPENTER, and PLACK[46] (1944) investigated nine strains which had been under cultivation for a period varying from one month to thirty four years.

The technique was to perform agglutination tests, and the quantity of antibody nitrogen absorbed was estimated by the method described by Heidelberger and Kabat[47] (1934).

They found that the amount of antibody nitrogen taken up by nine strains from a single antigonococcal serum varied with each strain investigated, and as the degrees of cross-reactivity equalled the number of strains employed, no evidence of gonococcal types was established.

REYN[48] (1944,1949) working in Ørskov's laboratory in Copenhagen, attempted to apply the methods so successfully used in the salmonella group by Kauffmann[49] and others, to the gonococcus.

Thus by absorbing a gonococcal antiserum with a heterologous gonococcal strain some residual activity was left against the homologous strain. This absorbed serum was then referred to as a "factor serum" in the sense of the single factor salmonella "O" antisera which are produced by appropriate absorption. In this way a large number of gonococcal "factor" sera were produced.

Reyn used the relatively insensitive complement-fixation technique in preference to agglutination. Because the absorbed antisera were often anticomplementary to a greater or lesser degree (Reyn gives a figure of 60 % as the percentage of anticomplementary "factor" sera), and as the antigen was equally anticomplementary, many strains (even as high as 50 %) could not be typed. Attempts to overcome these difficulties by adding inactivated guinea pig serum to the antigen, and by preparing gonococcal antisera in guinea pigs instead of rabbits, were unsuccessful.

The more "factor" sera employed, the more gonococcal types appeared. Thus with one hundred gonococcal strains, and five absorbed antisera, twenty seven types were obtained including eighteen with more than one representative. If nine "factor" sera were used the number of types increased to fifty two, eighteen of which had more than one representative and thirty four of which had only one strain representing each type.

That these "types" were far from clear cut is obvious from her statement that "the types could be established only with some difficulty, small deviations being left out of consideration". Further in the same paper she writes that "the types obtained were not very clearly defined, as the fixing capacity of the individual strains varied considerably within these types".

Investigation of strains repeatedly isolated from the same patient, or from couples, where it would be anticipated that the strains be serologically identical, were, in fact, found to be serologically different in 34 % of cases using five "factor" sera.

Reyn suggests a mutation as the most probable explanation of this discrepancy, but it seems far more probable that they were due to unsatisfactory technical methods.

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# CHAPTER XXV.

## GONOCOCCAL POLYSACCHARIDES.

BOOR and MILLER[50] (1931) extracted a gonococcal suspension with sodium hydroxide, and then acidified the clear product with acetic acid. The precipitate which formed contained what they termed the nucleoprotein.

The clear supernatant was concentrated, boiled to coagulate any protein which was removed, and then precipitated with ten volumes of alcohol. The precipitate was redissolved in water.

This solution, on testing, gave a positive Molisch test, but tests for protein were negative. This product also gave positive precipitin tests with gonococcal antisera prepared with whole organisms, with antisera prepared with the nucleoprotein fraction, and with a pneumococcus type III antiserum.

In a second series of papers BOOR and MILLER[51] (1934) carried this work further, and characterised the carbohydrate as being a light yellow powder, water soluble and precipitated by alcohol. It was resistent to the action of acid and alkali. Micro-Kjeldahl gave a nitrogen content of 4.2 %, and the product was optically inactive. The product was non-toxic for rabbits and mice, and was non-antigenic. However, a delayed type of skin reaction was obtained in immunised rabbits on intracutaneous injection.

Positive precipitin tests were obtained with gonococcal, meningococcal, and pneumococcal type III antisera, but not with N.catarrhalis antisera.

There was no evidence of strain specificity in so far as carbohydrate fractions prepared from six strains of gonococcus all cross reacted with five gonococcal antisera.

They concluded that since it was impossible to demonstrate capsules on gonococci, the carbohydrate fraction was of somatic origin.

MUTERMILCH and GRIMBERG[52] (1935) obtained a product similar to that of Boor and Miller. It gave a positive Fehling reaction after hydrolysis, about 35 to 40 % being converted to reducing sugars.

Their method of preparation differed somewhat, in that they dissolved the organisms in 10 % sodium taurocholate solution. The acid precipitable fraction was then discarded, protein removed by boiling, and the carbohydrate separated by alcoholic precipitation.

Their product was not antigenic in rabbits, but was able to fix complement in the presence of an immune serum.

CASPER[53] (1937) extracted the gonococcus by solution in sodium taurocholate to which excess alkali had been added. The clear fluid was precipitated with alcohol, the precipitate re-dissolved in water, and again precipitated with acetic acid. Following further repeated alcoholic precipitations from aqueous solution, a product was finally obtained which was protein free and gave the usual tests for a carbohydrate.

The fraction gave positive precipitin tests with the antisera of the corresponding gonococcal type (either type 1 or type 11). There was no cross reaction with meningococcal and pneumococcal antisera.

The carbohydrate prepared from his type 11 gonococcus was heat labile; that from type 1 apparently heat stable. He claims that his products gave a specific skin reaction in patients with gonococcal infection, patients infected with the type 1 gonococcus only reacting with a type 1 carbohydrate. Any cross reactions he obtained i.e. a patient infected with a type 1 gonococcus reacting to a type 11 carbohydrate, he attributed to the presence of a "species specific" carbohydrate.

PINETTI[54] (1937) attempted to isolate a type specific polysaccharide from the gonococcus. He followed various techniques of extraction, the final product being a yellow solution, non-toxic, and non-antigenic. Used intradermally positive reactions were obtained in 65 % of gonococcal cases as compared with 20 % of normal controls.

ROSSETT[55] (1939) obtained a carbohydrate from the broth of a six day gonococcal culture, again using alcoholic precipitation as the method of extraction. It gave the usual chemical tests for a carbohydrate, and a wheal on intradermal injection of patients.

MILLER and BOOR[56] (1944) in a further series of investigations, suggested that the carbohydrate in the gonococcus was bound as glucoprotein and glucolipoid. Using trichloracetic acid precipitation they obtained a glucolipoid complex which was toxic to animals, and also antigenic in rabbits.

Chemically their fraction was a polysaccharide containing phosphorus and nitrogen, combined loosely with a phospholipid.

STOKINGER, ACKERMAN, and CARPENTER[57] (1944) found that carbohydrate constituted from 5 to 9 % of the weight of the dried cell. They obtained a carbohydrate showing group specific reactions as reported by Miller and Boor but their analytical values failed to establish the presence or absence of an immunologically specific carbohydrate.

They then proceeded to investigate the fraction obtained by Rossett in broth cultures and claimed that it was a protein-like material containing a variable amount of carbohydrate. It was toxic, antigenic, and fixed complement in the presence of an immune serum. This latter property was lost after denaturation. It was destroyed by proteolytic enzymes. The carbohydrate portion alone was non-antigenic. They concluded that it was a degraded protein derived from the nucleoprotein of the gonococcal cell.

THOMAS and MENNIE[17] (1950) prepared fractions active in the gonococcal haemolysis test by rapid freezing (dry ice alcohol) and thawing (56° C.) twenty times; by papain digestion; and by alkaline hydrolysis followed by alcoholic precipitation.

It seems probable that some of the techniques described would prove adequate in preparing a polysaccharide similar to that employed in this investigation. This is particularly true of the earlier work of Boor and Miller had they employed smooth gonococcal strains.

Other fractions, however, were active in complement-fixation, and cross reacted with other neisseriae, and probably do not correspond to what has been termed the type specific antigen investigated in this work. They may, however, correspond to what has been termed the group antigen.

The technique of extraction described by Casper was repeated but failed to yield a fraction active in the gonococcal haemolysis test.

The methods described by Thomas and Mennie were investigated but only their third method, when used with freshly isolated gonococcal strains, produced a fraction active in the gonococcal haemolysis test. An active fraction was not obtained by freeze-thawing alone, nor by papain digestion.

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# SECTION V.

# DISCUSSION.

# THE SIGNIFICANCE OF THE S-R CHANGE AND OTHER FACTORS.

## The S-R Change.

Arkwright[20,58] (1920,1921,1924), working with the enterobacteriaceae first described the smooth-rough (S-R) variation in these organisms as characterised by the formation of rough granular colonies on solid media, by the presence of granular growths in fluid media, and by spontaneous agglutination in saline. The change was associated with a loss of the polysaccharide antigen that characterised the surface of the normal smooth form, and with a loss of virulence; a feature well illustrated by the lack of virulence of the rough pneumococcus for mice.

Subsequently Arkwright[59] (1927) demonstrated that a vaccine prepared with the rough form failed to provide protection to guinea pigs when challenged with a dose of the smooth form.

The S-R change was a gradual one and intermediate forms appeared between the typical S and the fully degraded R when a culture was plated out.

Although at first the emphasis was placed on the change in the colonial appearance of the organism, it was soon realised that the essential change was not the changed colonial morphology, but the loss of an antigenic component characterising the surface of the bacterial cell in the normal smooth form. Indeed, an obvious change in colony form need not be present.

Although the possibility of some degenerative change in relation to the gonococcus had been suggested by many workers, the recognition of an S-R change could not be made with any degree of certainty because of the absence of an obvious morphological change, and because of the lack of a method for detecting the loss of a surface antigen.

The gonococcal haemolysis test is directed against a heat stable polysaccharide antigen present on the surface of smooth gonococcal strains. After the gonococcus has been maintained for a period on artificial media, this antigen is

lost, and after a few months an antigen active in the gonococcal haemolysis test can no longer be extracted.

It is suggested that this change is in every way analogous to the S-R change described in other organisms, and is offered as unequivocal proof of the S-R change in the gonococcus.

As with other organisms, the rapidity with which the gonococcus becomes rough varies from strain to strain. In one strain (Z12) the type specific polysaccharide was lost after ten weeks; strain E still possessed a detectable antigen after six months. Even when the antigen can no longer be detected in the gonococcal haemolysis test, its presence may be demonstrated by animal immunisation after at least nine months on subculture.

## The Morphological Change.

It has been stated that the S-R change is not associated with an obvious morphological change. A morphological change does, however, occur, and it consists of a loss of the typical stickiness of the recently isolated gonococcus.

There is very little reference in the literature to this quality in the smooth gonococcus. Tulloch[40], who was one of the few workers to restrict himself to recently isolated strains, described this feature very well. He states:"The most constant feature of the growth is its peculiar mucus-like quality, which is readily appreciated when a colony is picked off for isolation, for the growth tends to hang to the edge of the loop and to the agar at the same time leaving quite an appreciable string of growth between loop and medium". Similarly in saline the gonococcal suspension appears as long strings of sticky mucoid material.

As the strain is subcultured these characteristics become less well marked, the culture appears to become drier, and more easily manipulated with a loop. This change has not the obviousness of the S-R change in organisms such as the salmonellae, but is none the less a valid observation that has been made by a number of workers in this laboratory who have been engaged on daily subculture and harvesting of gonococcal strains over a long period.

## The Influence on Antiserum Production.

The change of the gonococcus to the rough state is associated with two important changes.

1. The first is a change in the agglutinability of the organism.
2. The second is related to antiserum production by smooth and rough strains.

Using an antiserum prepared with a smooth strain, a relatively high agglutinin titre was obtained when tested against other smooth gonococcal strains. After a few months on subculture, and a gradual change to the R form, there was a fall in agglutinin titre, sometimes a very marked fall, when these same strains were tested with the original antiserum.

On the other hand, when the antiserum is produced with an older and presumably relatively rough gonococcal strain, agglutinin titres tended to be high against older strains and lower against suspensions prepared from the freshly isolated gonococcus.

## The Loss of a Second Antigen.

It has been indicated that with the S-R change there is a loss of the type specific polysaccharide. There is also a loss of an agglutinin binding antigen.

It has been demonstrated that an antiserum against a smooth strain possessed at least two components.

1. One active in the gonococcal haemolysis test against the type specific polysaccharide.
2. The second active in agglutination against an antigen shared by all gonococcal strains, and which has been termed the group antigen.

The evidence for the separation of these two components was that removal of antibodies against the type specific antigen (by absorption of the serum with sensitised cells) did not result in any loss of titre in an agglutination test. Removal of the type specific polysaccharide from an antigen similarly did not in any way interfere with the capacity of that antigen to fix complement in the presence of the immune serum.

There is thus convincing evidence that the agglutination and complement-fixation tests are directed against an antigen present in the gonococcus other than the polysaccharide fraction reacting in the gonococcal haemolysis test. The loss of agglutinability of gonococcal strains (when tested with an antiserum prepared with a smooth strain), and the loss of activity in the gonococcal haemolysis test, must therefore be ascribed to the loss of two distinct antigens.

## The "Blocking" Effect.

The importance of the S-R change and its influence on antiserum production has been mentioned. There is a third important factor, however, which can markedly influence agglutinin titres in relation to the gonococcus, and that is the presence of type specific antibodies in the serum.

The type specific antibody can combine with the group antigen, and so block the action of the agglutinating antibody. Removal of these type specific antibodies by absorption of the serum with sensitised cells, may result in markedly augmented agglutinin titres. This blocking effect was only apparent with some gonococcal strains, and at the moment no explanation can be offered as to why this should be the case.

## Confirmatory Views.

All of these variations in agglutinability have been recorded and discussed by other workers in this field.

Thus as far back as 1909 Elser and Huntoon[24] noted the presence of "highly inagglutinable strains", and that old cultures were twice as easily agglutinable as recent ones.

Torrey and Buckell[39] (1922) using antisera prepared with old strains noted that many recently isolated strains were relatively inagglutinable but became agglutinable after a period on artificial media. Tulloch[40] (1923) recorded the same observation.

The opposite observation, namely the loss of agglutinability of a recently isolated strain with a serum prepared with a recent strain was made by Atkin[41] (1925). What Atkin referred to as his type 1 and type 11 strains were smooth and rough forms, and his serological classification is

in fact, merely a division into smooth and rough variants. Thus his type 1 antiserum (prepared with a smooth strain) agglutinated other recently isolated strains, and hence were grouped together as type 1. His type 11 serum prepared with a rough gonococcal strain, agglutinated other old stock strains but not recently isolated ones to equivalent titre.

The transition from type 1 into type 11 is the S-R change. Atkin,however, considered that this transition took place in the body as well as on artificial media, and that the type 11 strains which arose in this manner were responsible for the chronic types of gonococcal infection. There is no evidence in favour of this point of view. Neither is there any evidence for his correlation of his two gonococcal types with distinctive colonial appearance.

The rise in agglutinin titre following absorption was recorded by Tulloch[40] (1923), without any comment as to its significance. Again it occurred only in a proportion of his strains, and,notably, in that proportion that failed to fit into his major group. That it was not encountered by other workers in this field is almost certainly due to the use of rough gonococcal strains in the preparation of their antisera.

Variation in the gonococcus has been postulated by other workers as an explanation of their inability to obtain rigid serological grouping of their strains. Thus both Hermanies[37] (1921) and Torrey and Buckell[39] (1922), as well as Segawa[43] (1932),used the term "lability", although none of these workers explain what precisely is meant by it. The term tends to convey the idea of a reversible antigenic change, rather than of an irreversible degenerative change of the S-R type. Reyn[48] (1949) postulated a mutation as the reason for her failure to obtain reproducable results in her typing of gonococcal strains.

It is rather surprising that more attention has not been paid to the problem of S-R variation in relation to the gonococcus, especially when one bears in mind its strictly human pathogenicity, and its most fastidious cultural requirements.

The problem of the antigenic relationships of the gonococcus bears a striking resemblance to the situation that prevailed in relation to Haemophilus influenzae.

The earlier work on the serological analysis of this organism revealed a striking heterogeneity. Thus Park, Williams, and Cooper[60] (1918), using an agglutination and agglutinin absorption technique with one hundred and sixty strains and twenty antisera, found only four identical pairs. Even strains repeatedly isolated from the same individual were not always identical. This pattern of serological behaviour was confirmed by many other workers.

Pittman[61] (1931) then observed that if smooth strains only were used, six clear cut types were established one of which (type b) was the most common. These smooth strains all possessed a type specific carbohydrate. These smooth forms, however, rapidly gave rise to the rough variant on artificial media, with the loss of the specific carbohydrate.

The problem of the S-R change has been deliberately discussed before embarking on a critical assessment of the results of the various papers that have been reviewed in the earlier section, since this change throws important light on some of the difficulties encountered in this field.

There are some other factors, however, which should be considered in interpreting differences in agglutinin titres.

## The Question of Optimum Proportions.

Certain antigen-antibody systems are notoriously liable to prozone phenomena, the best known example perhaps being the brucella agglutination where a prozone of 1 in a 1000 is not uncommon.

An inhibition of agglutination in the zone of antibody excess is also seen in a very high proportion of gonococcal agglutination tests. The majority of reactions show a prozone of two to three tubes, and occasionally it is as high as seven.

An inhibition of agglutination in the zone of antigen excess has received far less consideration, and the possibility of its occurence is very conveniently ignored in reading agglutination tests. That it is a major problem has been recently demonstrated by Morgan and Schütze[62] (1946), and Stewart and McKeever[63] (1950) in relation to the enteric group of organisms, and by Wilson and Merrifield[64] (1951) in relation to brucellosis.

By the addition of an anti-human globulin serum to the thrice washed suspension in the "negative" tubes of an agglutination test they were able to demonstrate considerably augmented titres. The technique in the case of a bacterial suspension, however, is extremely laborious.

Apart from Pearce[31] (1915) there is no mention of a prozone in gonococcal agglutination tests. The explanation undoubtedly lies in the fact that agglutination reactions were almost invariably commenced at an initial serum dilution of 1 in 50 or higher; a dilution generally beyond the range of the prozone.

Significantly enough in the tables included in Pearce's paper the prozone is confined to those agglutination tests giving the lower agglutinin titres so that it is highly probable that an antigen-antibody reaction occurred beyond the macroscopically visible titre.

The Degree of Development of Various Antigens.

Differences in agglutinability may exist which depend on the degree of development of the various surface antigens. This is well known in the salmonella and shigella groups.

Thus strains of S.typhi may be O, H, and Vi deficient, such as the well known 901-O, 901-H, and Vi 1 strains.

Somatic antigens such as the salmonella antigen XII may be developed to various degrees (expressed as $XII_1$, $XII_2$, and $XII_3$), and, according to Kauffmann[49], these forms even undergo a phasic variation analogous to that encountered with the H antigens.

Similarly an inhibition of agglutination in the Shigella group was finally traced to the presence of the so-called heat labile K antigens.

Thus variations in agglutinability do exist which can be explained by reasons other than basic antigenic differences between strains.

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## SEROLOGICAL GROUPING OF GONOCOCCI BY AGGLUTINATION AND AGGLUTININ ABSORPTION TECHNIQUES.

Having considered these variables, is there then any evidence of serological grouping among gonococci by direct agglutination?

The concensus of opinion is that no grouping of gonococci into serologically distinct types is possible by simple agglutination.

Certainly a number of papers do suggest that such grouping is possible.

Thus Watabiki[30] (1910), on a two tube difference in titre, suggested that two of his eight strains showed a comparative but not a distinct difference. Vannod[28] (1906), and Elser and Huntoon[24] (1909) recorded similar results. Pearce[31] (1915) although eight of her fifteen strains showed no significant difference in agglutinin titre, still suggested that two distinct types of gonococci may be differentiated. Jötten[34] (1921) placed twenty of twenty seven strains into four groups by simple agglutination. Casper[45] (1937) in a rather confused paper, placed forty seven of one hundred and nine strains into two types by simple agglutination, the remaining being described as either irregular or degenerated.

These workers have not appreciated the wide variability of agglutinin titres in relation to the gonococcus.

Warren[32] (1921), Cook and Stafford[33] (1921), Thomsen and Vollmond[36] (1921), Torrey and Buckell[39] (1922), Tulloch[40] (1923), Segawa[43] (1932), and Stokinger, Carpenter, and Plack[46] (1944) carried out direct agglutination tests and agree that no distinction between strains can be made by this method. Most of these papers too, comment on the variability of results obtained by this technique.

Similar conclusions were arrived at as a result of the agglutination tests described in Chapter XVIII.

Thus agglutination tests will only be of differential value where the results are clear cut and there is no cross reaction. Once cross reaction occurs a common antigenic fraction is present, and the results of serological differentiation by simple agglutination are no longer valid.

Under these circumstances the only proof of serological identity is that the strains in question are capable of completely exhauting each others'sera in absorption tests. The emphasis should be on the words "completely exhausting".

Where, however, after adequate absorption the two strains have exhausted their own sera, but not each others', the two strains must have different antigens. This difference in antigen content may indicate a true serological difference in the sense that they are distinct types, or it may indicate that the one strain has undergone a greater change of the S-R type relative to the other.

This latter point will be of little importance in relation to such organisms as the salmonellas; it has provided an explanation for some of the confusion prevalent in the field of shigella serology; it presents a major problem in relation to the gonococcus.

Of the various papers published on the differentiation of gonococcal strains by agglutinin absorption methods, unfortunately only two are sufficiently well documented for an intelligent assessment of their results. These are the papers by Torrey and Buckell[39] (1922), and Tulloch[40] (1923).

In none of these papers were the criteria set out above fulfilled. In all cases a single absorption of a standard volume of diluted serum by a standard amount of gonococcal suspension was carried out.

Torrey and Buckell regarded strains as identical if they removed 75 % of the agglutinins from a number of gonococcal antisera. If one bears in mind that all gonococcal strains cross react to a greater or lesser extent in agglutination tests, and hence share a common antigen, such a criterion is obviously unsatisfactory. Nevertheless these workers found a considerable degree of cross absorption with all their strains, although some behaved in a more uniform manner in this respect than others, and they were unable to determine any distinct serological types amongst them.

All the strains used by Torrey and Buckell were probably rough to a greater or lesser degree. Their antisera too were prepared with strains of various ages, two indeed with the "Torrey strains" which at that stage had been maintained on subculture for fourteen years.

It is suggested that these factors are more than enough to account for the irregularities in the results obtained, and a more adequate interpretation of their results is that no serological grouping of strains whatsoever was evident from their figures.

The most disturbing feature of this work is that two antisera prepared with the same organism, showed a totally different behaviour in respect to certain gonococcal strains in absorption tests. Indeed it casts doubt on whether any of the results recorded by methods other than those where the absorptions were carried to their logical conclusions, can be considered to be of any value.

Tulloch[40] (1923) on the other hand investigated one hundred recently isolated strains and the objections raised in respect to Torrey and Buckell's work, namely that they employed rough strains, do not apply here.

Of these strains seventy two absorbed out a single antiserum to a greater or lesser extent in a single absorption, and were grouped together.

The majority of the remaining strains (twenty seven out of twenty eight) showed not a fall in titre after absorption, but a rise. The explanation, of course, is that the absorption removed antibodies against the type specific polysaccharide and so removed the factor which had blocked the agglutination reaction.

Unfortunately Tulloch did not perform more than one absorption with these strains. An agglutination test with the absorbed serum against the absorbing strain was performed and was always negative. But, as both Tulloch,and Torrey and Buckell point out, strains were encountered which though not agglutinating to any appreciable titre were yet effective in absorption. The sole criterion that absorption is complete is that there is no further fall in titre of the serum when tested against the homologous organism in spite of repeated absorption. This was not carried out.

Two other approaches are possible today. The first would be to test the absorbing suspension with an anti-rabbit globulin serum (if rabbit antigonococcal sera were being used). If the test were negative it would indicate that the absorbing suspension was no longer taking up antibodies from the serum and absorption could be considered complete.

A second approach well worth trying would be a preliminary absorption of the serum with sensitised cells to remove the blocking type specific antibody. Neither of these approaches, however, were available to Tulloch.

Another major criticism of Tulloch's work is the use of an insufficient number of antisera. The ideal would be to have a serum corresponding to each strain and perform complete cross absorptions. Tulloch went to the other extreme and used a single antiserum. Four more antisera were subsequently selected from the strains not fitting into his major group, and used to group the remaining strains into four groups. Eight organisms finally remained unplaced.

To summarise, Tulloch found seventy two strains to be similar. He was not able to place the remainder convincingly and the suggestion advanced is that it was due to inadequate absorption. The major defect of his work is the use of insufficient antisera.

Hermanies[37,38] (1921) investigated eighty five strains, all rough, with an inadequate number of antisera, and performed single absorptions. Unlike Torrey and Buckell, and Tulloch, he claimed to have demonstrated six distinct groups.

His grouping is entirely the result of the method employed. Thus his first serum was more or less absorbed out by forty one strains. These were then segregated as his first group. A second serum was prepared from the remaining strains and the process repeated. The strains absorbing out this serum were group two. A third serum was prepared from one of the still unclassified strains, and strains absorbing out the third serum were group three. This was continued until no strains were left.

At first sight it is difficult to reconcile the results obtained by Hermanies with those obtained by other workers, especially Torrey and Buckell, and Tulloch. Hermanies gives the results of absorptions only with what he has termed the "more decisive dilutions". It is probable therefore that if

a fuller range of serum dilutions were quoted a greater amount of cross reaction would become apparent.

Even at the "more decisive dilutions" nineteen of his type one strains were still unable to absorb out a 1 in a 1000 dilution of his type one antiserum. There was only a one tube difference (a titre of 400 and 800) between some of his type two strains and some type one and type four strains; and only a fourfold (two tube) difference separated a further seven type two strains from other groups.

Two of his type two strains (77 and 78) were less effective in removing agglutinins from the type two antiserum than at least fourteen type one strains. Thus there is even from the limited data given in Hermanies' tables a considerable degree of overlap between his type one and type two.

If a relationship between these two types is accepted then seventy eight of his eighty five strains fall into one related group leaving four groups containing three, two, one, and one strains respectively. Two of these also partially absorbed out a type two antiserum.

Hermanies' results on analysis are not clear cut but show a gradation from a fairly uniform type one (including the Torrey strains) into a more heterogeneous type two (Hermanies described four subgroups) and type four. Interpreted in this way it may be stated that he has failed to produce any evidence of serological grouping of his eighty five gonococcal strains, and this viewpoint is confirmed by his statement that if his type one antiserum were diluted 1 in a 100 instead of 1 in a 1000 it was then not absorbed out by about half of his type one strains.

It serves to indicate that strains cannot be accepted as identical unless they completely exhaust each others sera and anything less than that is a bad second best. Thus the picture recorded by Hermanies is in essentials similar to that recorded by Torrey and Buckell, namely a variable degree of cross reaction in absorption tests with most strains and no evidence of distinct types. The explanation of the variability in behaviour is that rough gonococcal strains were used.

One of the more impressive papers employing the agglutinin absorption technique was that of Segawa[43] (1932). Using thirty recently isolated strains and twenty two antisera he found a variability in absorption but no

evidence of distinct serological types. Unfortunately no details of technique, or of results are given in this paper.

Most of the papers in this field have been described in the previous section and they do not contribute to any degree to the solution of the problem, other than by the failure of their results to fit into a particular pattern.

To summarise, the agglutinin absorption tests that have been performed on gonococcal strains have failed to reveal the presence of clear cut antigenic groupings.

On the contrary, the diversity of results recorded, the variability and overlapping of the results of absorption tests, all tend to emphasise the essential similarity of all gonococcal strains when investigated by these methods, and that the reason for the variability in their behaviour should be looked for elsewhere than in the hypothesis that these differences represent distinct antigenic types.

Indeed, the suggestion can reasonably be advanced that all strains of N.gonorrhoeae share a common group antigen active in agglutination and complement-fixation, and for the evidence for this statement one need look no further than their complete cross reaction in direct agglutination tests, a cross reaction most striking when only recently isolated strains are used as was done in this work, and also by Tulloch. It is to this common antigen that the term group antigen has been employed in this thesis.

In stating that all gonococci share a common group antigen, it is not implied that this antigen is identical in all strains. This, though highly probable, is as yet, unproven, and would require extremely laborious absorption tests before a final answer was found.

If any antigenic differences are found, however, their relationship will be rather that of Salmonella paratyphi A (I,II,XII) to Salmonella paratyphi A var.durazzo (II,XII), or of Salmonella derby (I,IV,XII) to Salmonella bredeny (I,IV,V,XII), rather than distinct antigenic variants. As yet, however, no evidence of such variants exist.

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# CHAPTER XXVIII.

## SEROLOGICAL GROUPING OF GONOCOCCI BY A RED CELL SENSITISATION TECHNIQUE.

Whereas the techniques of agglutination and agglutinin absorption are directed against a group antigen common to all gonococcal strains, the antigen reacting in the gonococcal haemolysis test is a polysaccharide which is not detectable by agglutination, and which does not fix complement.

The situation, therefore, is in many respects analogous to that present in the pneumococcus. All pneumococci share a common somatic antigen and a differentiation into types is made on the basis of a surface polysaccharide of which over forty types have been recorded. However, whereas the polysaccharide in the pneumococcus is in the form of a wide zone so that identification can be conveniently performed by the Quellung reaction, such an approach was not possible with regard to the gonococcus, and the absorption test with polysaccharide sensitised cells was devised to serve a similar purpose.

Absorptions with sensitised cells differ from absorptions with whole organisms, in that a particular antigen is absorbing out only its specific antibody. Provided, however, that the principle of such an absorption is accepted, there is no reason why the same significance cannot be attached to the results as to the results of absorption with whole organisms. That the principle is valid can be shown by the consistency with which the homologous antiserum is exhausted in such absorption tests.

The technique of absorption with sensitised cells in practice has proved extremely satisfactory. Not only is the absorption rapid, but seven or more successive absorptions on the same serum can be carried out in a single day.

The progress of the absorption can be easily followed, both by the falling titre of antibodies in the serum, as well as by the extremely elegant method of testing the absorbing cells for combined antibody. It is thus a technique that enables an absorption to be readily carried out to its logical conclusion, namely to the state where the absorbing antigen has completely exhausted the serum being absorbed

of the corresponding antibody.

With cells sensitised with the gonococcal polysaccharide the testing of the absorbing cells merely required the addition of complement. With other systems, however, such as erythrocytes sensitised with the salmonella Vi antigen, haemolysis did not occur with regularity on the addition of complement. An alternative technique was therefore adopted in Vi haemagglutination, namely the addition of an anti-globulin serum.

The appropriate antiglobulin serum had to be used depending on whether the serum being tested was human, rabbit, or horse, and, since sheep cells were sensitised in the test, natural antibodies against these cells had to be removed by preliminary absorption of the Coombs serum with sheep cells.

In carrying out the test the Vi sensitised cells which had taken part in the antigen-antibody reaction were washed three times in saline to remove all traces of uncombined globulin and the diluted antiglobulin serum added. A positive reaction was indicated, after incubation, by a strong agglutination pattern; a negative by the usual button of deposited cells.

The use of an antiglobulin serum introduced a further complication, namely the presence of an "incomplete" heterophile antibody. To remove this antibody absorption of antisera, particularly human, with normal sheep cells had to be prolonged and often repeated, the control of the serum with a normal sheep cell suspension being also tested with a Coombs serum before being considered negative. The presence of this "incomplete" heterophile antibody could be demonstrated even more convincingly by performing a titration for hetero-phile antibodies with papain treated sheep cells.

Of these two methods, the addition of complement, and the addition of an antiglobulin serum, the former is far simpler, and to be preferred where it can be used. It was routinely employed in gonococcal work.

In attempting to apply these principles to the inves-tigation of N.gonorrhoeae, the preliminary work was restricted to eighteen strains. With each strain the preparation of the antigens was completed within two weeks of primary isolation, and animal inoculation was commenced as soon as the strain was obtained in pure culture, i.e. within 48 to 72 hours of primary isolation. A large range of cross

absorptions were performed, and the absorptions were continued until it was demonstrated that they were complete.

Of these eighteen strains, fifteen were serologically identical and were grouped as type I. Three, although exhausting each others' sera in absorption tests, were unable to absorb out the type I antisera, and were grouped together as type II.

A larger series of strains were then investigated by performing absorption tests against type I and type II antisera. In this way a total of sixty seven gonococcal strains were tested. Of these, fifty nine proved to be type I strains, and eight type II strains.

One strain not included in this series partially removed the antibodies from type I and type II antisera, but was unable to exhaust either, and was provisionally termed a type III gonococcus.

## The Relationship of N.meningitidis.

The present day status of meningococcal typing by agglutination methods is much the same as that prevailing in relation to the gonococcus. There has been a tendency to distinguish between epidemic strains which appear homogeneous, and those responsible for sporadic cases which are said to be heterogeneous. Wilson and Miles[65] (1946) have summarised the position by stating that "no sharp line of demarcation can be drawn between different types of meningococci".

It is probable that much of what has been already said in relation to the gonococcus is equally applicable to the meningococcus.

Using smooth strains, and antisera prepared with smooth gonococcal strains, no cross reaction in an agglutination test was demonstrated. It was concluded therefore, that the two organisms have different group antigens. Further differentiation into types may be made on the basis of a surface polysaccharide of which three have been identified in relationship to sixty eight gonococcal strains, and three similar if not identical polysaccharides in relationship to five meningococcal strains.

## The Serological Identification of the Gonococcus.

This problem has only been partially answered. The polysaccharide antigens are also shared by the meningococcus so that the gonococcal haemolysis test will be of limited value.

The variability of the agglutination test renders it unsatisfactory for routine work although this variability may be of less importance when only smooth strains and antisera prepared with smooth strains, are used.

In practice, however, differentiation of the meningococcus offers little problem. Apart from fermentation reactions, the colonial appearances on chocolate agar are characteristic. The meningococcus appears as a large, shiny, mucoid, dome-shaped colony rather like a large coliform colony, as contrasted with the smaller more delicate gonococcus.

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A Serological Investigation of Neisseria Gonorrhoeae Using a Red Cell Sensitisation Technique.


## SUMMARY

Investigation of a series of recently isolated, smooth, strains of N. gonorrhoeae revealed the presence of two antigens.

1. A surface polysaccharide which was type specific.
2. A somatic group antigen.

The polysaccharide fraction was obtained by alkaline solution and alcoholic precipitation and had the following features:

1. It was heat stable, gave a positive molisch, and a negative biuret test.
2. It could be extracted from recently isolated gonococcal strains, but was lost by these same strains after they had been on artificial media for a few months.
3. The polysaccharide was unable to fix complement in the presence of a gonococcal antiserum. Its presence, however, could be detected by adsorption on to red blood cells. Cells sensitised in this manner reacted specifically with gonococcal antisera (gonococcal haemolysis test).
4. A gonococcal antiserum could be absorbed out with red cells sensitised with the gonococcal polysaccharide. A serum which was absorbed out in this manner was now negative in the gonococcal haemolysis test. The absorbed serum, however, showed no loss of activity whatsoever in an agglutination or complement-fixation test.

It was concluded, therefore, that gonococcal antisera prepared with smooth strains have at least two demonstrable antibodies; the one active against the polysaccharide fraction in the gonococcal haemolysis test, and the other active in an agglutination and complement-fixation test against the somatic antigen.

After a variable period on artificial media the gonococcus undergoes an S-R change with the loss of the polysaccharide antigen.

Using an absorption technique with red cells sensitised with the gonococcal polysaccharide sixty eight smooth gonococcal strains were investigated, and three gonococcal types demonstrated. These types shared a common group antigen detectable by agglutination and complement-fixation.

Five freshly isolated strains of N. meningitidis were investigated and found to possess similar, if not identical, type specific polysaccharides to the gonococcus. The two organisms differed in the possession of distinctive group antigens since there was no cross reaction in agglutination tests.

The literature on the serology of the gonococcus is reviewed and the failure of other workers to obtain reproducable results was largely ascribed to the use of rough gonococcal strains.


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