A CONTRIBUTION TO THE STUDY OF MENINGOCOCCI.

By E. W. AINLEY WALKER, D.M., Capt. R.A.M.C. (T.),
Lecturer in Pathology in the University of Oxford.

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(With 5 Charts.)

It has been shown by numerous observers that well-marked serological differences are found between different strains of meningococci. And since the work of Dopter on the relation of "parameningococci" to meningococci and to cerebro-spinal fever, there has arisen a fairly general agreement that among meningococci capable of causing cerebro-spinal meningitis two broadly separable groups can be defined by immunological methods. But beyond this point considerable divergences of opinion appear. On the one hand there are a number of recent workers who have failed to convince themselves that the two groups of meningococci are in reality clearly delimited, permanent and independent entities. On the other hand certain other workers claim to have still further subdivided these micro-organisms into four definite and independent types, each of which possesses in itself the value of a bacterial species [cp. Andrewes (1917)].

The extended investigations carried out by Dr A. Eastwood, Dr Fred Griffith, and Dr W. M. Scott, for the Medical Officer of the Local Government Board (1917), have led them to the conclusion that any attempt to establish further subdivisions among meningococci, beyond the broad division into the two groups already mentioned, would necessitate the recognition of a large and uncertain number of ill-defined sub-groups. These sub-groups would lack individuality. They would fail to be clearly and decidedly marked off from one another, but would merge gradually one into another by means of intermediate varieties. Moreover even the two main groups themselves seem to be linked by less determinate strains whose serological reactions exhibit intermediate characters.

On the other hand Lieut.-Colonel M. H. Gordon (1917) and his co-workers at the Central Cerebro-Spinal Fever Laboratory have obtained results in their investigations which they interpret as evidence for the existence of four specifically different types of meningococcus. And though their Types I and II and their Types II and IV are described as being somewhat closely allied, each of these serologically distinguishable types is provisionally held to constitute a definite bacterial species. As the result of a very laborious and extended inquiry carried out in the study of these four "epidemic types," it is claimed that substantially all meningococci capable of producing epidemic cerebro-spinal meningitis among troops belong to one or other of these four specifically different types of organism.

Now the question whether the causal agent of cerebro-spinal fever is bacteriologically one or two, or four different organisms is clearly one of fundamental importance as a scientific problem. Its practical bearing acquires significance in relation to treatment, particularly as regards sero-therapeutic measures.

In the course of certain experiments upon the agglutination of meningococci, my attention was more particularly directed to this aspect of the subject by results obtained by Major A. G. Gibson and Mrs Ludlow Hewitt. During their investigation of meningococci recovered from the cerebro-spinal fluid of cases of cerebro-spinal fever they found:

(1) That when meningococci, which on first isolation had fallen more or less readily under one or other type, were employed in the immunisation of rabbits, they gave rise to the development of agglutinating serums whose specificity of type was often very much less clearly defined.

(2) That on inoculation in the human subject two particular types of meningococci, which had been similarly defined, gave rise to the development in the serums of the individuals thus immunised of agglutinins *for different types and in one instance for the different type only*, within the limits of their observations.

Mrs Hewitt and Major Gibson give an account of their investigations in an accompanying Report. Results such as they record bring very sharply into consideration the question how far serological differences of a particular character defined in relation to the serum of immunised rabbits, between micro-organisms which are morphologically and culturally indistinguishable, have any necessary or assured application to the human subject. They suggest that, so far at any rate as concerns disease in man, criteria of differentiation which may possibly hold in

relation to the rabbit, but have not at present been proved to hold for man, may perhaps not possess any primary or fundamental importance in relation to human infection. This aspect of the question appears to acquire additional weight not only from the absence of any notable differences in pathogenicity among the four types, but also from the fact, referred to several times by Lieut.-Colonel Gordon himself, that when a different animal—the horse—is made use of in the preparation of immune serums, the agglutinins produced show *much less specificity* of type than seems to be the case with those of the rabbit.

That this statement of Lieut.-Colonel Gordon represents a moderate presentation of the facts is shown by the results obtained on testing Lister Institute Therapeutic type serums of the horse against Central C.S.F. Laboratory emulsions of type cocci (Table I). For it becomes clear that in some cases, at any rate, the phrase “much less specificity” would be better replaced by the words “complete change of type.”

TABLE I.

Agglutination tests of Lister Institute Therapeutic Anti-Meningococcus Serums against Central C.S.F. Laboratory Emulsions.

Coccus		25	50	100	200	400	800	1600	3200	Control
Serum Type I Lister Insti- tute 58 D.	Type I, Batch E	0	0	0	0	0	0	0	0	0
	Type II, Batch E	0	0	0	0	0	0	0	0	0
	Type III, Batch D	0	0	0	0	0	0	0	0	0
	Type IV, Batch C	<i>t</i>	<i>t</i> -	<i>p</i> +	<i>p</i> -	0	0	0	0	0
Serum Type III Lister Insti- tute 57 D.	Type I, Batch F	<i>t</i>	<i>t</i> -	<i>p</i> -	<i>tr</i> -	? <i>tr</i>	0	0	0	0
	Type II, Batch D	0	0	0	0	0	0	0	0	0
	Type III, Batch D	? <i>tr</i>	0	0	0	0	0	0	0	0
	Type IV, Batch C	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i> -	<i>tr</i>	0	0	0	0

t = total agglutination, *p* = partial, *tr* = trace. The exact notation used is described on p. 395.

But even as regards the rabbit, it may fairly be urged on the information at present available, that the evidence put forward in favour of the specific character of the serological differences between the four types of meningococci remains inconclusive. And for the reasons which will be discussed immediately, the observations and experiments on which reliance has been placed by Lieut.-Colonel Gordon for the specific differentiation of his four types may still be regarded as at any rate indecisive.

Before any so far-reaching a conclusion should receive assent, or be accepted as a final basis for therapeutic measures, it ought to be established on the most secure foundations. Until this has been achieved

the view that there are four clinically identical, but bacteriologically distinct epidemic cerebro-spinal fevers must be received with a good deal of caution. Undoubtedly it is the case that were this proved, a sufficiently striking parallel exists in the three bacteriologically distinct enteric fevers. But in that connection it is necessary to note that while recovery from infection with any one of the three typhoid-paratyphoid organisms leaves the individual susceptible to infection (even immediate infection) with either of the other two, one does not see or hear of second attacks of cerebro-spinal fever. A single proved instance of a second attack of meningitis due to a type of meningococcus different from that which caused the first attack would afford very weighty evidence in support of the theory of specificity of type. But until such evidence may be forthcoming it would appear that an individual who recovers from cerebro-spinal fever has probably become immune to all four types.

In suggesting the propriety of an attitude of doubt and hesitation in regard to the alleged bacteriological specificity of the four types of meningococci, it is important to state clearly that it is not thereby intended for a moment to imply any doubt regarding the very great value of the practical measures which have been introduced and so successfully carried out in relation to cerebro-spinal fever among troops. For it cannot fail to be universally recognised that these measures have achieved remarkable success in the detection and isolation of infected persons, in the limitation of epidemic outbreaks, and in the recognition of the source and origin of particular meningococcal infections, as well as in the actual treatment of the disease.

The only question which is raised at the moment is the question whether the evidence is at present adequate to justify the claim put forward. Namely that it "would appear to indicate in no uncertain manner that the four types of meningococcus...are stable entities, that they are specifically distinct from one another," and that the cases of cerebro-spinal fever investigated were due "not to transient and unstable variants of a single micro-organism, but to a group of individual species of meningococcus." The definition of types serologically different in their reaction with the immune serums of rabbits, though otherwise similar, may afford information of great practical utility, in particular circumstances. But it does not necessarily afford an evidence that the differences in question are specific unless those serological reactions are constant. It is moreover of relatively little importance how many strains, or how great a percentage of all strains investigated fall into the classes proposed, if instances are found in which some strains so

placed subsequently exhibit variation of type, and refuse to remain in their original class. A few such instances would suffice to prove that the classification lacks specific value.

THE QUESTION OF FIXITY OF TYPE.

It is therefore clearly fundamental to the theory of specificity of type in the four groups of meningococcus, that these types should be demonstrably "fixed" and in no circumstances interchangeable.

Upon this point Lieut.-Colonel Gordon records the fact that neither under prolonged sub-cultivation, nor otherwise, has he met with change of type or gross variation in the very numerous meningococcal cultures which have come under his observation. In agreement with this statement most other workers appear to have found a considerable degree of constancy in their strains; though differences in agglutinability in different subcultures has quite frequently been marked, and differences of degree in sugar fermentation have often been noted. But while, as might be expected, this is undoubtedly the general trend of recorded observations, a study of the literature at once brings out the fact that the majority of recent workers have drawn attention either to a single isolated instance, or sometimes to several cases, in which particular strains of meningococcus failed to maintain fixity of type during the period of observation, or even at one and the same moment belonged equally to two different types.

A few examples may be quoted in illustration:

1. Gordon and Murray (1917) have referred to the occurrence of an "amphoteric" strain—Types I and III, which absorbed the specific agglutinin of both these types.
2. Arkwright (1915) found a strain which agglutinated in both his groups, and another which changed serologically from one group to the other during the period of observation.
3. W. M. Scott (1917) has derived two different strains from a particular culture, the one possessing the serological characters of his Group 1, the other the serological characters of his Group 2.
4. Walker, Hall and Peters (1916) found in two cases that the serological reactions of the meningococci isolated from the cerebro-spinal fluid of a patient on successive days differed in type.
5. On two occasions I have had the opportunity of examining the serum of two human subjects who had been repeatedly inoculated by Major A. G. Gibson with Type I and Type III meningococcus respectively. On each occasion agglutinins were present in small amount for

Type III and Type IV meningococcus respectively, but not for any other type (the tests being made against emulsions issued from the Central C.S.F. Laboratory). That is to say a Type I antigen led to the production of Type III agglutinins, and a Type III antigen to the production of Type IV agglutinins.

Fixity of type—that is to say the existence of different species among meningococci—could therefore only be maintained on the assumption that all the observers here referred to had fallen into error, and were mistaken in their observations. And the more extensive the record of such observations becomes, the greater is the probability that the differences which exist between the four types are insufficient to amount to differences of species. It is, therefore, of importance that among 356 strains of meningococci W. J. Tulloch found no less than 23 (6·5 per cent.) which could not be placed either as specifically type cocci, or even as cocci showing the common group relationship of Types I–III, or Types II–IV by agglutination tests; and three out of the 107 to which the absorption test was also applied (2·8 per cent.) which still failed to qualify in respect of specificity in relation to rabbit serum.

THE METHOD OF “SATURATION.”

The most weighty experimental evidence adduced by Lieut.-Colonel Gordon in favour of the specificity of the four types has been derived from two series of investigations, the first carried out by Major Hine and himself, the second by Major Hine and Captain Tulloch.

In the first of these, immunity tests were made on animals immunised with one or other of the four types of meningococcus by determining in each case the fatal dose of a particular type when the animal was treated by the “saturation” method of Gordon and Horder (1907–8). The results so obtained are regarded as lending support, so far as they go, to the view that the protection obtained by immunisation with type meningococci is of a *univalent* character. The inference drawn is that they help to justify the claim for the specificity of the types. But a consideration of the data presented seems to lend weight to a contrary opinion concerning the validity of this inference.

The two experiments under discussion are quoted in Table II below where the two series of results have been placed side by side for ease of comparison.

In each experiment four rabbits were immunised against each of the four types of meningococcus. The animals thus immunised are represented in four horizontal rows in the table, one row for each type

of coccus. In each experiment one rabbit died during immunisation. Ten days after the last immunising dose the animals, along with suitable control rabbits (represented in the lowest row), were "saturated" with living meningococci of appropriate type in correspondence with the vertical columns of the table. The procedure was carried out by injecting into a vein of the animal the living cocci from one slope culture of the type concerned every hour until death occurred, or until all the animals but one in any given column had succumbed.

The numerals indicate the number of hourly intravenous inoculations given, the sign minus (−) signifies that the animal dies, the plus (+) sign that it survived the last dose administered. The bracketed figure indicates that though the animal concerned did not die it became ill and remained so for some time.

TABLE II.
Gordon and Hine's "Saturation" Tests.

Protecting Coccus	Saturating Coccus							
	Type 1		Type 2		Type 3		Type 4	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Type I	−6	−2	−12	−8	−5	(+8)	...	−9
Type II	−8	−8	+12	+8	−5	−8	−9	−9
Type III	−8	−6	−12	...	+8	+8	−9	−9
Type IV	−3	−8	−8	−4	−8	−7	+9	+9
Control	−8	−6	−6	−6	−8	−7	−8	−8

The following considerations appear to be relevant in regard to these results:

1. The critical margin of dosage between animals that lived and those that died is a very small one upon which to base a far-reaching conclusion. It amounts to no more than one slope culture in a total of 8, 9 or 12; that is to say from 8 per cent. to 12 per cent. of the total dosage. A difference of this magnitude would fail to carry conviction even had the content of the doses been more accurately measured, and the weights of the animals recorded in relation to the dosage. And it is evident that doses reckoned only in "slope cultures" are open to variations which might easily outweigh this slight margin.

In the animals saturated with Type III coccus the two controls show a difference of one hour and one slope culture in the fatal result. Yet a conclusion is based on a difference of only this extent between homologously and heterologously immunised animals. And under Type I there is a range of fatal dose for the controls of two slope cultures.

It is therefore much to be regretted that experiments of so laborious and protracted a character as these undoubtedly are, were not rendered more convincing by continuing the inoculations in the animals which survived to a point which might have placed the results entirely beyond question, had it been reached successfully. For as the matter stands it is legitimate to suggest that some or all of the surviving rabbits might have succumbed to the next inoculation.

2. If the four types of cocci are in reality specifically different, and if, as is claimed, the immunity conferred is therefore "mainly of a univalent character," the *heterologously* immunised rabbits should fail to show evidence of any significant degree of protection. This is held to be the case.

It will, however, be observed that many of these animals easily outlived their controls, as shown in the accompanying table (Table III).

TABLE III.

Compiled from Gordon and Hine's Saturation Tests.

Saturating coccus	Heterologously immunised rabbits which outlived their control animals		Number of slope cultures by which they outlived the controls
	Number	Percentage	
Type I	2 out of 6	33	2
Type II	2 out of 5	40	6
"	4 out of 5	80	2 or more
Type III	2 out of 6	33	1
Type IV	5 out of 5	100	1

The experiments in fact afford much more convincing evidence of some degree of general protection of the heterologous animals, than any which they offer in support of a specific univalent immunity in the homologously immunised individuals. For the latter in no case amounts to more than one slope culture, while in one experiment two individuals out of three survive until they have received a dose which is *double* that which killed the corresponding control.

3. In other directions also the experiments appear to prove too much or too little for the security of the view which its authors are at present inclined to maintain. For if immunisation with heterologous meningococci were in the main indifferent as regards protection, the animals thus treated *should behave like control animals* when submitted to the test by "saturation." But this is not the case as can be seen by comparing the range of the fatal dose in the two series (Table IV).

The heterologously immunised rabbits show a wide range of fatal dose with three of the types, namely 300 per cent., 200 per cent. and

60 per cent. respectively; while the greatest variation in fatal dose for control rabbits is 33 per cent.

TABLE IV.
Compiled from Gordon and Hine's Saturation Tests.

Rabbits	Fatal dose expressed in slope cultures, for control rabbits and for the heterologously immunised rabbits			
	Saturating Coccus			
	Type I	Type II	Type III	Type IV
Control	6 to 8	6	7 to 8	8
Immunised	2 to 8	4 to 12	5 to 8	9

Only two possible explanations suggest themselves: either (1) that the true range of fatal dosage for unprotected animals possessed the wide limits shown by the heterologously immunised rabbits (on the supposition that they were really unprotected as is claimed). This is improbable, but if true it would at once deprive the whole of the observations of the significance intended. Or (2) that some of the immunised animals under discussion were in fact moderately protected by their heterologous immunisation, as already suggested (i.e. the types are not *specifically* different); while others, like the two homologously immunised *Type I* rabbits (see Table II) had actually become more susceptible than normal.

As shown in Table V *no less than 4 rabbits out of 15 (26.7 per cent.) in each experiment succumbed to doses less (often much less) than those required to kill their control animals.* An explanation of this fact should be forthcoming. It may be sought along the following lines.

TABLE V.
Compiled from Gordon and Hine's Saturation Test.

Saturating coccus	Heterologously immunised rabbits which died before their control rabbits		Number of slope cultures by which their controls out- lived them
	Number	Percentage	
Type I	2 out of 6	33	4 to 5
Type II	1 out of 5	20	2
Type III	2 out of 6	33	3
Type IV	0 out of 5	3	—

4. At the time when the "saturation" method of testing virulence in meningococcus was introduced by Gordon and Horder, these authors showed that while after a single massive intravenous inoculation cocci could be cultivated from the peripheral circulation for 12 hours or more, they could never be grown from the blood when the "saturation" method was used. They also found that in the latter case a marked leucopenia

appeared, instead of the usual leucocytosis. But neither then nor in the present connection have data been recorded regarding the manner of death of these animals and the post-mortem findings.

The possibility at once presents itself to the mind that these animals (or some of them) when inoculated hourly with one whole slope culture intravenously, die from mechanical causes, rather than from bacterial intoxication. It was shown many years ago by Jörgensen and Madsen that the introduction of massive doses of *B. typhosus* intravenously in animals possessing high titre agglutinating serums might lead to death by the production of multiple embolisms. And it is of importance to observe that the two homologously immunised animals which died early in the experiments now under consideration were the Type I rabbits. The interest of this observation lies in the fact, noted by Gordon, that Type I meningococcus "is perhaps the best type of all for exciting the production of agglutinin by the rabbit."

In view of these facts I have devoted some attention to the "Saturation" method, and have found with three different micro-organisms, one of them *non-pathogenetic*, that the immediate cause of death in the cases hitherto observed has appeared to be a *widespread thrombosis of capillaries and veins in the pulmonary circulation*. In the course of these observations three rabbits have been killed by the method in question; the first with *Meningococcus*, the second with *B. typhosus*, and the third with *Sarcina lutea*. In each case a careful post-mortem examination was made, and numerous microscopical sections prepared from the various organs. The whole material was subsequently handed over to my friend Major A. G. Gibson who has kindly consented to append a Report on the histological appearances.

In the experiments of Gordon and Hine the number of meningococci introduced in the more prolonged tests was probably of the same order of magnitude as the total number of red corpuscles in the blood, and almost certainly many times greater than the total number of leucocytes in the circulation. In the third of my own experiments the total number of cocci (*Sarcina*) introduced was doubtless still greater.

PROTOCOLS OF SATURATION EXPERIMENTS.

In each case a large number of slope cultures was grown for twenty-four hours. They were then carefully emulsified in normal saline solution, mixed together to secure uniformity of dosage, and made up to such a volume that 1 c.c. represented one slope culture (or in the case of *Sarcina* 1.1 slope culture and 1.5 slope culture on two different occasions).

Experiment 1. Meningococcus.

A healthy male rabbit of 2500 grammes weight, which had been under immunisation with meningococci for seventeen days, but received its last inoculation nine days before the day of saturation, and had been gaining weight during the whole period of observation, was saturated by means of hourly intravenous injections of 1 c.c. (one slope) of emulsified living meningococci.

The animal was obviously in distress after eight inoculations. After the tenth it was helpless and in a dying condition. No further inoculation was given. The animal died between the eleventh and twelfth hour.

At the post-mortem examination there was marked patchy congestion of the lungs with sub-pleural haemorrhages. There was one sub-peritoneal haemorrhage in the small intestine; marked congestion and a number of small haemorrhages in the vermiform appendix.

Microscopically the immediate cause of death was extensive pulmonary thrombosis.

Experiment 2. B. typhosus.

A healthy female rabbit of 2250 grammes weight was saturated by means of hourly intravenous injections of 1 c.c. (one slope) of emulsified living *B. typhosus*. .

A few minutes after the third inoculation convulsive struggling occurred followed by extensor spasm, and the animal died within a minute or two.

An immediate post-mortem examination was made with due precautions. There was a serous effusion in the pericardial sac, a marked patchy congestion of the lungs, a small sub-epicardial haemorrhage, and a small area of sub-capsular haemorrhage in the liver. The cause of death was widespread thrombosis of vessels in the lungs.

Experiment 3. Sarcina lutea.

A healthy male rabbit of 2850 grammes weight was saturated by means of hourly intravenous injections of 1 c.c. (1·1 slope) of emulsified living *Sarcina*. Eight doses were given without producing any recognisable effect whatever, except that almost immediate clotting occurred in the vein at the site of each successive inoculation. The animal's weight fell 50 grammes for one day only, and it appeared to remain in perfect health.

Ten days later, its serum then showing evidence of some agglutinating action on a formalised bouillon culture of *Sarcina*, it was again saturated,

receiving intravenous inoculations of 1 c.c. (1·5 slope) of emulsified living *Sarcina* hourly for three doses, and then at intervals of three quarters of an hour until the tenth dose. On this occasion it was observed that no clotting occurred in the veins at the sites of inoculation, in striking contrast to the result seen on the earlier occasion.

From the seventh inoculation onwards the condition of the animal began to fail; and thirty-five minutes after the tenth inoculation it fell into convulsions, which passed on into a rigid extensor spasm, death occurring in about five minutes.

An immediate post-mortem was made. There was serous effusion in the pericardium, and extreme patchy congestion with oedema of the lungs.

The cause of death was extensive thrombosis of the pulmonary capillaries and veins.

In none of the foregoing experiments were blood cultures made from the peripheral circulation. But in each case a number of films of blood from a peripheral vein were examined. No organisms could be found in any of these films, except for a single small group of half a dozen *Sarcina* seen in one of the films from the third experiment. But examination of the histological sections at once suggests an explanation both of the leucopenia and of the absence of cocci in the peripheral blood noted by Gordon and Horder. For there is an enormous accumulation of leucocytes and micro-organisms in the capillaries and veins of internal organs; particularly the lungs, which constitute a first filter for the micro-organisms injected into the peripheral veins, and eventually become obstructed by the extending thrombosis. Accordingly it seems not unreasonable to suggest that the comparatively early occurrence of pulmonary obstruction and thrombosis affords a probable explanation of the fact that so many of the immunised rabbits in Gordon's and Hine's experiments died before the control animals. Possibly it is a common cause of death in animals treated by the "saturation" method; and it may be the usual cause. But if this is so the results obtained in such experiments require to be interpreted with extreme caution. In any case it is a fair supposition that we may thus account for the death of those rabbits which died *earlier* than any one of the eight control rabbits. If therefore we omit these individuals from consideration for the moment, and calculate the average death time for all other heterologously immunised animals, and the average death time for the eight controls an interesting result emerges. For this purpose I take the lethal dose for the first animal in column 6 as 9 slope cultures, since this animal did

not die with a dose of 8 although it became ill. The lethal dose at any rate could not have been *less* than 9 slope cultures, though it might have been more.

On making the calculation just referred to it appears that while the average lethal dosage for control rabbits is 7.1 slope cultures, that for the remaining heterologously immunised rabbits is 8.6 slope cultures. That is to say the fatal dose for the latter is 21 per cent. greater than that for the controls. The margin is not large, but so far as it goes it is in opposition to the view that the immunity conferred by type meningococci is monovalent. It is at any rate a greater margin of dosage than that upon which Gordon's conclusion was founded (about double), since the latter never exceeded 8 to 12 per cent. of the total dosage.

SUPERIMPOSITION TESTS.

Under this term Lieut.-Colonel Gordon records experiments in *successive immunisation* with different types of meningococci. The experiments were carried out by Major Hine and Captain Tulloch. Only the first of them is given in any detail in the Report. In this experiment five rabbits were immunised with Type I coccus as primary antigen. When their blood had acquired agglutinating power for Type I they were inoculated with a second antigen as shown in the table below (Table VI).

The records demonstrate the fact that each secondary antigen produced agglutinins for itself, and that its effect did not (in two cases out of three) prevent the steady fall of the agglutinins for Type I which were being formed as the result of the earlier inoculations with Type I. In the third case a marked secondary rise of the Type I agglutinins to at least double their previous maximum titre was induced.

The conclusion drawn is that the agglutinins for the different types are *entirely* independent; and the inference is that the four types are four distinct though to some extent allied species.

The first point of importance in regard to these results is the fact that the titre of agglutinins for the secondary antigens of Types II, III and IV never exceeded a dilution of 1 in 500, yet the lower limit taken for agglutinin readings has been arbitrarily fixed at 1 in 100. Normal rabbit serum, within the limits of my present experience, does not in ordinary circumstances give more than traces of agglutination, if so much, with any of the four types at 1 in 20 dilution, and only infrequently gives complete or nearly complete agglutination at 1 in 10

dilution. Occasionally, however, there may be nearly complete agglutination at 1 in 20 and traces up to 1 in 40. But seeing that one always begins an experiment by measuring accurately the normal agglutinins of the animal before inoculation it follows that there is usually in these observations a range of dilutions of valid significance¹ from 1 in 20 up to 1 in 100; a range as extensive as the range from 1 in 100 to 1 in 500, on which reliance is placed, but one apparently unexplored in Hine and Tulloch's observations.

Nevertheless in *two* different cases where the maximum titre shown for the primary antigen was 1 in 600 and that for the secondary antigen 1 in 500, agglutination was present for another type in a dilution of 1 in 200. In the third case the agglutination titre for another type was such as to give a "slight" reaction at 1 in 100. These reactions are dismissed as being due to "group-agglutinins," a point to which reference will be made immediately. Table VI gives a digest of the experiment of Major Hine and Captain Tulloch.

TABLE VI.

Compiled from Hine and Tulloch's Superimposition Experiment.

Rabbit	Primary Antigen Type	Secondary Antigen Type	Titre for primary Antigen 1 in	Titre for secondary Antigen 1 in	Type II agglutinated 1 in		Type III agglutinated 1 in		Type IV agglutinated 1 in	
					100	200	100	200	100	200
A	I	—	600	—	0	0	0	0	0	0
B	I	I	600	600	0	0	0	0	0	0
C	I	II	600	400	—	—	0	0	slight twice	0
D	I	III	600	500	slight twice	slight once	—	—	0	0
E	I	IV	600	500	some 5 times	some 4 times	0	0	—	—

It thus appears that in some cases "group agglutinins" were present to a titre of from *one-third to one-half* the titre of the "specific" agglutinins.

Now whatever theory be held as to the real meaning of "group agglutinins," it will be admitted at any rate that they probably represent some sort of common factor of the agglutinable properties of the different bacterial cultures concerned with the particular sample of serum employed under the conditions provided. And wherever group agglutinins are found to be present opinion will remain divided as to

¹ For the purposes of investigation of known meningococci, as distinguished from the diagnosis of unknown cocci.

the justifiability or wisdom of distinguishing species among the micro-organisms thus grouped, if they are shown to be otherwise closely related. Where bacteria are clearly distinguishable by other biological characters the phenomenon of their "group-agglutination" by serum specific for only one of them tends to become less frequently observed as technique advances. Thus, for example, in the case of *B. typhosus*, *B. paratyphosus* A, and *B. paratyphosus* B, one has never seen any trace of "group agglutination" in thousands of agglutination tests made with standardised agglutinable cultures of these bacteria. The titre of a serum may be many thousand for T. A or B, as the case may be, without the least trace of agglutination for the other two at 1 in 25 dilution and lower.

The facts regarding group agglutination may perhaps have a different basis in the case of meningococcus, and some other organisms. But until much more conclusive evidence is available, it is clearly permissible to hold the view that in low titre serums (such as those under discussion) with a maximum agglutination titre for the homologous coccus of 1 in 400 or so, agglutination in dilutions of 1 in 20, and 1 in 40 (in marked excess over the normal agglutinating power of the serum of the animal before immunisation), and rising to 1 in 80, 1 in 100 or more is evidence to cause hesitation in concluding that the types are specifically distinct.

By the courtesy of Lieut.-Colonel Gordon and Major Hine I have been supplied with samples of the four type emulsions and serums, as well as with living cultures of the four type cocci. I am indebted to their kindness for the opportunity of bringing my experiments and materials obtained from other workers into their proper relation to their standards. I am also indebted to Major Gibson who was good enough to place all his meningococci of cerebro-spinal origin at my free disposal; and to Dr A. Eastwood by whose kindness I have been enabled to make use of a number of attested meningococcal cultures, and their corresponding serums, sent me by himself and by Dr Fred Griffith and Dr W. M. Scott.

A brief account may now be given of experiments bearing on the meaning of the "superimposition tests" (or successive immunisations) carried out for Lieut.-Colonel Gordon by Major Hine and Capt. Tulloch. And it may here be stated that while all the tests recorded in the present communication have been made by himself, all the more important agglutination results were kindly read for me independently by Dr A. D. Gardner, who was kept in ignorance of the dilutions and particular types before him until the readings had been made and recorded.

After determining the limits of agglutination of the four types with normal rabbit serum the first step was to make cross agglutinations with the four type cocci and the four type serums as issued from the Central C.S.F. Laboratory. The results are given below in Table VII, as read after 24 hours in a water-bath at 55° C. In some experiments the tubes were read again after standing in the cold for a further 24 hours. This carries the titre somewhat higher, but it necessitates bringing into use a very large number of stands and tubes. The emulsions gave very excellent results indeed, in so far that the controls did not begin to show any sign of sedimenting spontaneously until they had stood for 36 to 48 hours following the 24 hours in the water-bath.

The drop method was used with small agglutination tubes, as employed for enteric groups and dysentery agglutinations. Ten drops of emulsion of cocci were always added to 10 drops of appropriately diluted serum in normal saline solution.

The notation used in the readings is as follows:

- t = total or complete agglutination with complete sedimentation.
- $t -$ = total agglutination with fluid almost clear, but sedimentation incomplete.
- p = partial agglutination; marked sedimentation, but considerable opalescence of the fluid remaining.
- $p+$ = more than p , and less than $t -$.
- $p-$ = less than p .
- tr = traces of agglutination; some deposit, and slight clearing of opalescence.
- $tr+$ = larger traces, but less than $p-$.
- $tr-$ = a very slight deposit, and opalescence distinctly less than in the control titre.
- $?tr$ = doubtful.

The cross agglutinations shown in Table VI were carried out with the materials named below obtained from the Central C.S.F. Laboratory.

Serums: Type I, Batch B.	Emulsions: Type I, Batch D.
Type II, Batch B.	Type II, Batch C.
Type III, Batch B.	Type III, Batch B.
Type IV, Batch B.	Type IV, Batch C.

The readings recorded in Table VII show the extent to which agglutination occurred with each type serum against the four type emulsions. With serum Type I and serum Type II coccus Type IV shows no higher

agglutination than might have been found with normal rabbit serum. The same is possibly true of coccus Type II with serums Type III and Type IV. But in a number of cases cocci of Types I, II or III agglutinate with heterologous serums up to a titre from one-twentieth to as much as one-fifth or more of the titre given with the homologous coccus.

TABLE VII.
Cross Agglutinations.

Serum	Emulsion of cocci	Dilution 1 in								Control
		20	40	100	200	400	800	2000	4000	
Type I	Type I	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i>	<i>tr</i> +	0	0	0
	„ II	<i>t</i>	<i>t</i>	<i>p</i>	<i>tr</i> -	—	—	—	—	0
	„ III	<i>p</i> +	<i>p</i>	0	0	—	—	—	—	0
	„ IV	<i>tr</i> -	0	0	0	—	—	—	—	0
Type II	„ I	<i>t</i>	<i>t</i>	<i>tr</i> -	0	—	—	—	—	0
	„ II	* <i>p</i>	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i>	<i>p</i>	? <i>tr</i>	0	0
	„ III	<i>tr</i> +	<i>tr</i>	? <i>tr</i>	0	—	—	—	—	0
	„ IV	<i>t</i> -	<i>p</i> -	<i>tr</i> -	0	—	—	—	—	0
Type III	„ I	* <i>t</i> -	<i>t</i>	<i>t</i>	<i>p</i>	—	—	—	—	0
	„ II	<i>tr</i>	<i>tr</i> -	0	0	—	—	—	—	0
	„ III	* <i>t</i> -	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i> -	<i>tr</i>	0	0	0
	„ IV	? <i>tr</i>	0	0	0	—	—	—	—	0
Type IV	„ I	<i>t</i> -	<i>p</i> +	0	0	—	—	—	—	0
	„ II	<i>t</i> -	<i>tr</i> +	0	0	—	—	—	—	0
	„ III	* <i>t</i> -	<i>t</i> -	<i>tr</i> -	0	—	—	—	—	0
	„ IV	* <i>t</i> -	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i>	0	0	0

A — signifies that the test concerned was not made.

* = clear evidence of the existence of a zone of inhibition.

Now it is stated that of the four types, Types I and III and Types II and IV are the most nearly allied. But in the present test it is seen that:

(a) Type I serum carried up Type II coccus much more than Type III coccus.

(b) Type II serum carried up Type I coccus at least as much as Type IV coccus.

(c) Type III serum carried up Type I coccus most, and acted little on Type II and very little if at all on Type IV coccus.

(d) Type IV serum carried up Type III coccus most, and acted more on Type I than on Type II coccus.

The Type III serum was, therefore, the only one of this batch which gave results at all suggestive of the relation claimed to exist.

In order to present further data bearing on this question the results of two immunisation experiments may be quoted. In the first of these a male rabbit of 1900 grammes weight was inoculated intravenously with 0.5 c.c. (approximately 1000 million cocci) of the Type I emulsion named above on the *first, third* and *fifth* day of experimentation. Agglutination determinations of its serum against Central C.S.F. Laboratory emulsions are given in Table VIII for day 1 (before inoculation) and days 5, 6 and 8. They show a fairly rapid production of agglutinins for Type I, and a synchronous gradual development of increasing, though much less, amounts of agglutinin for all four types.

By day 8—seven days from the first inoculation—the agglutination of the homologous coccus is complete up to 1 in 800 dilution, and partial at 1 in 2000. The titre for Types II and III is pretty nearly equal, reaching 1 in 100. But (for what it is worth) Type II is somewhat more agglutinated than Type III, though earlier on the agglutinins for Type III had developed more rapidly than those for Type II. Type IV agglutinins are laggard and very feeble, as was also the case in two instances in Table VI.

TABLE VIII.

Immunisation with Type I Coccus. Agglutination Determinations.

Coccus Dilution 1 in	Type I										Type II					
	10	20	40	80	100	200	400	800	2000	Control	10	20	40	80	100	Control
Day 1	tr +	? tr	0	0	0	—	—	—	—	0	? tr	0	0	0	0	0
Day 5	t	t -	p +	tr	0	—	—	—	—	0	? tr	0	0	0	0	0
Day 6	t	t	t -	p	tr +	tr	0	—	—	0	t -	tr	0	0	0	0
Day 8	*tr -	*t -	*t -	t	t	t	t	t -	p	0	t	t	t	t -	p -	0

Coccus Dilution 1 in	Type III							Type IV			
	10	20	40	80	100	Control		10	20	40	Control
Day 1	tr	? tr	0	0	0	0		0	0	0	0
Day 5	p	p -	tr	? tr	0	0		p	tr	0	0
Day 6	t	t -	tr	? tr	0	0		p +	tr	0	0
Day 8	*p	*t -	t	p	tr +	0		t -	p -	0	0

— signifies test not made.

* indicates clear evidence of a zone of inhibition.

In the second experiment a male rabbit of 2450 grammes weight was inoculated on the *first, third*, and *fifth* day of experimentation with 0.5 c.c. (approximately 1000 million) of the Type II emulsion, and on the *ninth* day with 1 c.c. (approximately 2000 million) of the Type III emulsion. Agglutination determinations against Central C.S.F. Laboratory emulsions are given in Chart I for day 1 (before inoculation) and for days 7, 9, 12, 14 and 16.

The Chart illustrates the course of the development of agglutinins for all four types during successive immunisation with two of the types.

Many points of interest appear and among them may be noted the comparative ease with which the titre of agglutination for the homologous coccus can be made to rise.

Keeping the foregoing evidence in view, the "superimposition tests" may now be considered in somewhat greater detail. And it now seems probable that the appearance given in Lieut.-Colonel Gordon's charts

Chart I. Agglutination Titre of Serum of Rabbit for the four Types of Meningococcus during successive immunisation with two of the Types.

of clear and sharply defined *qualitative* differences in the agglutinin response to inoculations with different types of the meningococcus is in reality due to the omission of all measurements of titre below the 1 in 100 dilution. Though even so, agglutination for types other than those used for inoculation was frequently detected at 1 in 100, and sometimes also at 1 in 200 dilution, that is to say, up to one half or nearly half the titre found for the second antigenic type.

Nevertheless, it might perhaps remain a matter of doubt whether these results, along with the results of the Absorption tests, to which

decisive value is attached by Lieut.-Colonel Gordon, did not entitle the four types of meningococcus to be ranked as "independent entities" were no collateral evidence available.

But if it is shown that a series of different strains of *B. typhosus*, for example, may sometimes yield precisely similar results, the interpretation to be placed on evidence of this character will become much clearer. For presumably no bacteriologist will suggest that different strains of *B. typhosus* constitute different bacterial species.

The "superimposition tests" are in principle closely similar to experiments in successive immunisation carried out by myself (1901) with different strains of *B. typhosus* in the Swiss Serum Institute at Berne (1899-1900). In the experiments material to the present discussions four strains of *B. typhosus* were employed, denoted F, K, Z, and T and rabbits were immunised successively with three of the strains (F, K, Z) in different orders of succession. At the end of each stage, or period of immunisation the serum of each rabbit was tested to determine its titre of agglutination against each of the four strains.

The results obtained are shown in Charts II, III and IV, which have been re-drawn from my original paper. They appear to me to show in greater or less degree all the same points as are emphasised by Lieut.-Colonel Gordon in the charts obtained by Major Hine and Capt. Tulloch. And if one chose to draw a line across the charts and to ignore agglutination at 1 in 4000 (*about one-third of the maximum titre obtained*) and all below, very misleading conclusions might be drawn from the remaining readings. For example, the four strains might be regarded as specifically independent organisms, of which Types F and Z showed some affinity to each other, while Type K, and to an even greater extent Type T, were more widely differentiated by the serological reaction of agglutination. And it may be noted particularly that there is frequently shown a fall in titre for an earlier antigen, while the titre for the most recently inoculated strain is rising.

But since the experiments referred to are now rather old, and were carried out before the introduction of the present accurately standardized methods, I have recently repeated them with the four strains of *B. typhosus* denoted below as T.E., T.L., T.O., and T.T. These four strains were plated out and carried on from single colonies. They were put through the appropriate tests, and were shown by them and by agglutination tests to be genuine *B. typhosus*. Agglutinable cultures of standardized and equal opacity were prepared from the strains for use in subsequent agglutination tests. A suitable rabbit was then inoculated

intravenously with a small dose of the strain T.L. (day 1), and on the eighth day was inoculated with an equal dose of the strain T.T. The results of agglutination tests of its serum are presented in Chart V.

It will at once be seen that as the result of these tests the four strains of *B. typhosus* chosen at random divide themselves at once into two groups, T.E. and T.L. forming one group, and T.O. and T.T. the other. The groups are widely differentiated by the agglutination tests made during the period of primary immunisation. They remain equally

Charts II, III, IV. Agglutination Titre of Serum in three Rabbits under successive immunisation with three strains of *B. typhosus* in different orders of succession.

differentiated after the secondary antigen has been introduced. The agglutination titre rises to about 1 in 8000. And it will be seen that if in view of this fact one decided to ignore agglutination below 1 in 1000 (*about one-eighth of the maximum titre*) or to put it aside as "group-agglutination," one might claim that T.E. and T.L. were specifically different types from T.O. and T.T.

But if, as is to be presumed, that claim would absolutely fail to gain

support among bacteriologists, it seems not unreasonable to refuse to accept similar evidence as adequate in the case of meningococcus. In the latter case great weight appeared to be attached to the fact that the titre for Type I, the primary antigen, continued to fall when the secondary antigen was introduced, and the curve of its agglutination showed a progressive rise. Precisely the same observation holds for

Chart V. Agglutination Titre of Serum of Rabbit tested against four strains of *B. typhosus* during successive immunisation with two of the strains.

the titre of T.L. (and its ally T.E.) when the titre for T.T. (and its ally T.O.) is rising.

It is not any part of my present intention to discuss the meaning of the very striking differences in agglutinability exhibited by these four strains of *B. typhosus* under the experimental conditions just described. They are associated with other interesting and well-marked characteristics, which do not bear immediately upon the question at

issue. But it would seem obvious that the results recorded above go a long way to deprive similar observations on the meningococcal types of conclusive value in support of the theory that the four types constitute four independent bacterial species.

ABSORPTION TESTS.

The Absorption test is applied by Lieut.-Colonel Gordon as the final and decisive means of establishing the specificity of the four types of meningococcus, and as the method to be employed for properly placing strains which are found to agglutinate with two or more type serums. For this purpose the test has been extensively made use of by Captain Tulloch who carried out an important series of laborious tests on 356 strains of meningococcus, almost all of which he succeeded in placing by this method.

How great is the value attributed to this test as a criterion of differentiation is shown by the fact that Lieut.-Colonel Gordon dismisses the opinion of M. Nicolle of the Pasteur Institute that Types I and III belong to the meningococcus group, and Types II and IV to the group of parameningococcus solely on the ground that no absorption tests were performed. On the other hand the results of absorption tests recorded by the expert workers for the Local Government Board are somewhat hastily put aside chiefly on the grounds, as it would seem, that the test is a difficult one to perform accurately, and that certain pitfalls may have been overlooked by these observers. It is, however, impossible to ignore so great an accumulation of experimental data, especially since the results of the several observers show a substantial agreement. These results are the more important because they include the investigation of certain phenomena which Lieut.-Colonel Gordon either disregards or dismisses briefly as "irregularities."

Much, of course, depends upon what is meant by an "irregularity." If it implies an error of technique it will disappear on improving the technique and repeating the observation several times. But if it means that the result obtained came out contrary to expectation, it may be that the expectation was fallacious. Such irregularities, if numerous enough, may necessitate a modification of our working hypothesis, or may invalidate a whole theory.

If the four types of meningococcus are to be regarded as "independent entities" one of the most striking irregularities is that recorded by Lieut.-Colonel Gordon himself, in which a particular strain of meningococcus absorbed the agglutinins of two separate types. It was apparently two independent entities at once.

My own experiments have revealed a number of irregularities of interest. Whether they represented reality, or were due to technical error, must remain for the time being a matter of opinion.

But for what it is worth one of the most highly paradoxical (on the view that the types are fixed and independent) is recorded in Table IX.

Two portions of the serum of a rabbit immunised against Type II and Type III were diluted to 1 in 20, the one with normal saline solution, the other with a formalised emulsion of Type II (Franklin) meningococcus of three times the opacity of the Central C.S.F. Laboratory emulsions used in the subsequent agglutination tests. The tubes were properly protected, and placed in a water-bath at 55° C. for 24 hours, and then in the cold chamber for another 24 hours. Both tubes were then centrifugalised to complete the deposition of the cocci in the second sample. Parallel agglutination tests were then made.

TABLE IX.

*Immune Serum for Type II and Type III absorbed with Type II.
Agglutination Test.*

Coccus		Serum 1 in 40	80	100	200	400	800	2000	Control
Type I	before absorption	<i>p</i> -	<i>tr</i> +	<i>tr</i>	0	0	0	0	0
Batch F	after „	<i>p</i> -	? <i>tr</i>	0	0	0	0	0	...
Type II	before „	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i> -	<i>p</i> -	0	0
Batch E	after „	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i> -	<i>tr</i>	0	...
Type III	before „	<i>t</i>	<i>t</i>	<i>t</i>	<i>t</i>	<i>p</i>	<i>tr</i>	0	0
Batch D	after „	<i>p</i> -	? <i>tr</i>	0	0	0	0	0	...
Type IV	before „	<i>t</i>	<i>p</i> -	<i>tr</i> +	? <i>tr</i>	0	0	0	0
Batch C	after „	<i>tr</i>	0	0	0	0	0	0	...

The titre of the serum for all four types of meningococcus was somewhat reduced by absorption with Type II. But the reduction was only slight¹ in the case of Type II itself, and in that of Type I. It was considerably greater in the case of Type IV, and in that of Type III, it was great enough to remove all traces of agglutination above 1 in 80. In fact were significance attached only to "complete" agglutination, one could be entitled to say that absorption with Type II coccus had left the agglutinins for Type II unaltered, while entirely removing those for Type III and Type IV. It may be added that the readings found for the non-absorbed sample of serum were substantially the same as those originally given by the same serum a fortnight earlier.

In regard to the foregoing experiments the only possible alternatives

¹ Where agglutination tests are not carried to an end-point such degrees of absorption will often be missed.

in explanation are either that the whole result was due to error of technique, or that the absorbing coccus Type II had ceased to be specifically Type II, and reacted in the manner expected of Type III. The former explanation might be accepted if the experiment stood alone. But in view of all the evidence presented above against the theory of specificity of the types, it seems quite possible that the results were reliable.

CONCLUSIONS.

1. Fixity of type among meningococci of Types I, II, III and IV (Gordon) is non-proven. Exceptions are on record and evidence is accumulating in a contrary sense.

2. Results obtained by the method of "saturation" are rendered dubious by the fact that death may be due to mechanical complications such as widespread thrombosis in the circulation. But so far as it goes it tends to show that the protection afforded by immunisation with meningococci of any type is multivalent rather than univalent.

3. The method of "superimposition" tests gives results with the four types of meningococcus, which can be paralleled with four strains of *B. typhosus*. It, therefore, affords no support to the theory of specificity of type in meningococci.

4. The serological results obtained with type meningococci in different animals (horse, man) may entirely fail to accord with types employed as antigens. Accordingly the types differentiated in relation to the rabbit do not appear to represent independent entities.

REFERENCES.

- ANDREWES, F. W. (1917). *The Lancet*, II. 847.
 ARKWRIGHT, J. A. (1915). *Brit. Med. Journ.* II. 881-885.
 EASTWOOD, A., GRIFFITH, F., and SCOTT, W. M. (1917). *Report to the Local Government Board on Public Health and Medical Subjects*, New Series, No. 114.
 GORDON, M. H. (1917). *Medical Research Committee*, Special Report Series, No. 3.
 GORDON, M. H. and HORDER, T. J. (1907-8). Report of the Medical Officer for 1907-8: *37th Annual Report of the Local Government Board*, No. 37 Supplement (1909), Appendix B, No. 3, 341.
 GORDON, M. H. and MURRAY, E. G. (1915). *Journ. Roy. Army Med. Corps*, xxv. 411.
 HALL, I. WALKER, and PETERS, B. A. I. (1916). *Journ. Roy. Army Med. Corps*, xxvii. 399.
 SCOTT, W. M. (1917). *Report to the Local Government Board on Public Health and Medical Subjects*, New Series, No. 114, 121.
 WALKER, E. W. AINLEY (1901). *Journ. of Pathol. and Bacteriol.* VII. 250.

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