THE SEROLOGICAL CLASSIFICATION OF *BACILLUS DIPHTHERIAE*

By A. J. EAGLETON, M.D., M.R.C.P. and EDITH M. BAXTER, M.Sc.

HISTORICAL INTRODUCTION.

The fact that diphtheria bacilli can be agglutinated by an anti-diphtherial serum has been known for many years.

Nicholas in 1896 demonstrated the agglutination of one strain of diphtheria bacillus by homologous antitoxic serum; and in 1898 (Nicholas, 1898 *a*, *b* and *c*) he found that by means of this serum, he could divide his strains of *B. diphtheriae* into two groups—an agglutinable and an inagglutinable. He showed, moreover, that agglutinability and virulence did not correspond, and that the same antitoxin protected animals against both groups of virulent *B. diphtheriae*.

Meanwhile, Nicolle (1898) attempted to make agglutinating sera in rabbits and white rats, using for injection the same strain as had been employed by Nicholas (Park 8, the American bacillus). From his negative results, he concluded that this particular strain did not produce agglutinins. In 1898, Bruno tested human sera for the presence of agglutinins. His sera were derived from cases of diphtheria and from healthy subjects, and he used an antigen made from a single strain of *B. diphtheriae*. His results persuaded him that a diagnostic test on these lines was of no value. In 1900, Lubowski published a paper on the diagnosis of diphtheria. He found that all his virulent strains fell into one serological group, whereas the avirulent strains and *B. Hoffmann* were not agglutinated by his sera. He laid stress on the difficulty caused by non-specific sedimentation of diphtherial emulsions and, to prevent this, he treated his antigens with glycerin.

In 1902, Wassermann reported his work on the use of precipitating sera for diagnosis of *B. diphtheriae*. He found that by this means he could differentiate this organism from *B. Hoffmann*. In the same year, Schwoner (1902) using a polyvalent horse serum, was able to differentiate *B. diphtheriae* from *B. Hoffmann*. He noted, in opposition to previous workers, that antitoxic serum did not agglutinate his diphtherial strains. It must, however, be remembered that the earlier antitoxin was made by injecting unfiltered broth cultures. Meanwhile, Mervyn Gordon (1901) attempted to differentiate *B. diphtheriae* and *B. Hoffmann* by means of specific guinea-pig plasma. He demonstrated definite serological differences between these two organisms, but finding to his surprise that all *B. diphtheriae* were not of the same type, concluded that as a means of identifying *B. diphtheriae* "the agglutination test promises to be of more use in its positive than in its negative aspects."

https://doi.org/10.1017/S002217240000810X Published online by Cambridge University Press

In 1903, Lipstein, continuing some earlier work, attempted to separate a few strains of *B. diphtheriae* into serological groups. He used rabbit sera, the animals being injected intraperitoneally with mixtures of virulent bacilli and antitoxin. He summed up his results by suggesting that all diphtheria bacilli had a common basal receptor mechanism, but differed in having in addition partial receptors peculiar to each strain. This far-reaching conclusion, though no doubt essentially true or very near the truth, was founded on the study of five strains, three of which appear to have been identical.

In the same year, Schick and Ersetting (1903) using serum prepared by Schwoner and others, confirmed its value in the differential diagnosis of *B. diphtheriae* and *B. Hoffmann.* The most interesting part of their paper deals with their description of the formation of "rough" and "smooth" colonies on agar by certain strains of *B. diphtheriae.* They extended the earlier work of Zupnik and showed that of the cases examined, 22 per cent. showed "rough" colonies only, 72 per cent. "smooth" only, while in 6 per cent. both types were found. They demonstrated, moreover, that the same antitoxin would protect against toxin, prepared from "rough" or "smooth" strains.

In 1912, V. Przewoski collected 50 strains of *B. diphtheriae* and five of *B. Hoffmann.* He made sera from two of the diphtherial strains and one Hoffmann. He found that the two diphtherial sera were identical and agglutinated all the strains of *B. diphtheriae* to 1/9600, but agglutinated the Hoffmann only to 1/150. The reverse held with the anti-Hoffmann serum. He used rabbits, injecting intravenously at first dead and then living cultures.

In 1914, Van Riemsdijk made univalent rabbit sera by injecting intravenously cultures killed at 60° C. He recognised different types of diphtheria bacilli, but, as he was only seeking a method of diagnosis from *B. Hoffmann,* he then made his sera polyvalent. He also noted the difficulty of spontaneous agglutination and advises prolonged heating of the suspension at 60° C.

In 1916, Langer made a univalent agglutinating serum by injecting rabbits with living diphtheria bacilli, suspended in antitoxin. He sums up his results by saying that there are two groups of *B. diphtheriae,* an agglutinable and an inagglutinable. Strains of the first group are all agglutinated by one type serum; the second group when injected into rabbits give rise to sera which do not agglutinate the strain used for injection but will agglutinate all the members of the first group. We can only suppose that he was dealing with one type to which both his groups belonged; and that the inagglutinability of certain strains was due to the unsuitable nature of the antigens employed (*vide infra*).

In 1919, Mason, working in an army laboratory, attempted to differentiate *B. diphtheriae* from organisms which resembled it morphologically. He made a monovalent rabbit serum by injecting heat-killed cultures of *B. diphtheriae.* He obtained a serum with a titre of 1/320, and found that 64 of 65 strains of *B. diphtheriae* were agglutinated at a serum dilution of 1/40 or higher. The diphtheroids tested did not agglutinate.

In 1920, Havens published the result of his study of 206 strains of virulent

https://doi.org/10.1017/S002217240000810X Published online by Cambridge University Press

diphtheria cultures, derived from cases, persistent carriers ("release cultures") and healthy carriers. His strains fell into two groups. His larger group (comprising 169) corresponds with that of earlier workers (Park 8).

In the same year, Durand (1918 and 1920) published papers on the serological types of *B. diphtheriae.* In an earlier paper in 1918, he had made horse and rabbit agglutinating sera and had shown that of 96 strains which he could emulsify, 70 fell into four groups. Later, he brought his total to 255 strains, 18 of which were nôn-emulsifiable. His horses were injected intravenously with living bacilli. He also tried the same process with goats, but the results were disappointing. He states that "les injections intraveineuses de bacilles diphthériques tués par la chaleur, sensibilisés ou vivants à doses progressives amènent le plus souvent la mort plus ou moins rapidement avec ou sans paralysie." He therefore used a mixture of antitoxin and bacilli for injection into these animals. He finally determined five types, which he found absolutely specific by the method of absorption. In a paper written in conjunction with Jean Guérin (1921) he investigated the relation of serological type to family incidence and epidemiology, and concludes that "the unity of type in an epidemic seems established." As these writers say, this may explain results such as those obtained by Langer. In a final paper, in 1921, Durand studied the fermentative reactions of 224 strains. He finds glucose and laevulose are always fermented; mannite, dulcite, sorbite, xylose, mannose, lactose, starch inulin and glycogen never; glycerin, galactose, maltose, saccharose and dextrin variable.

His types I and II differ from the remainder in sugar reaction, and his type II ferments saccharose.

In a recent paper, Park (1922) and his colleagues who examined Durand's strain "corroborated fully" his findings as far as they relate to the serological type.

Early in 1922 a paper appeared by Bell on this subject. He examined 133 strains, three of which he was unable to emulsify. Of the remainder, 80 per cent. belonged to three types; 13 per cent. were included in type I, which was Havens' larger group and included Park 8, the strain which has been used for so much experimental work. His second type corresponds to Havens' smaller group and claims 6 per cent., while the remaining 61 per cent. were included in type III. 20 per cent. were left untyped. He used rabbit sera, having tried guinea-pigs without success; he gave intravenous injections at five-day intervals, using at first heat killed and later living bacilli together with antitoxin. The mortality among his animals was high. His agglutinations were carried out with suspensions of bacilli grown on pea flour agar; the mixtures of sera and bacillary suspensions were heated at 55° C. for four hours before reading.

This brief summary of the literature shows that most of the earlier work was carried out with a view to finding a serological means of distinguishing *B. diphtheriae* and *B. Hoffmann*, or between virulent and avirulent strains of

https://doi.org/10.1017/S002217240000810X Published online by Cambridge University Press

B. diphtheriae. In recent years, the distinction between *B. diphtheriae* and *B. Hoffmann* has ceased to be a matter of great difficulty. Moreover, as far as clinical medicine is concerned, a virulence test gives more valuable information than serological study. Thus we find that Durand and Guérin stress the epidemiological value of their work which, though commenced earlier than that of Havens and Bell, may be said to present the most modern aspect of the subject. The object with which a research is undertaken, though not influencing the facts elucidated in the course of that research, must alter the direction of the work, and the value attached to its different phases. And thus the earlier tendency to unify all types of *B. diphtheriae* in an effort to provide a basis for differential diagnosis is, later, replaced by a schismatic phase—a stressing of differences.

SCOPE OF THE WORK.

In the course of his research Major A. S. W. Bell very kindly grouped, by means of his type sera, several strains of virulent and avirulent *B. diphtheriae*, which we had in our collection. Certain of these strains, already classified by him, we chose for making sera. In this way our first three groups correspond with his (*vide infra*). From the strains which failed to react with the three-type sera originally used, haphazard selection was made for the preparation of univalent sera. In many cases several of the chosen strains proved to be identical, so that we were able to test the homogeneity of each group by means of cross agglutination and absorption experiments. In this communication we are dealing with 348 cultures, all virulent. Some of the strains were used in an earlier investigation on the value of the intracutaneous test (1921 and 1922) and their virulence checked by the subcutaneous method. The remainder have been tested by the intracutaneous method only. Sera have been made from 33 of these strains, 36 rabbits and four horses having been employed in their preparation. The number of individual experiments carried out prevents the presentation of complete protocols. We propose, therefore, to give full technical details of the methods employed but content ourselves with a summary of the results obtained.

TECHNIQUE.

(1) *Antigens*. We deliberately selected a medium in common use, and as Loeffler's serum was found to be suitable for every stage of our work, we used this throughout. The organisms were isolated on Loeffler's serum inspissated in Petri dishes. Antigens were made from 24 hour growths on the same medium. Small quantities, suitable for routine typing, were obtained from two slopes. For preparing large amounts we proceed as follows: the serum mixture is poured into a 100 c.c. flat-sided bottle. The bottle is then placed on its side in the oven, the serum inspissated by heating at 85° C. for one hour; the fluid which exudes is then poured off, and a further amount of serum mixture added. The bottle is then placed on the opposite side in the oven, and heated again

https://doi.org/10.1017/S002217240000810X Published online by Cambridge University Press

for one hour. The fluid is again poured off and the bottle incubated at 37° C. for one day to test its sterility. We have thus two opposing surfaces covered with Loeffler's medium. An overnight slope culture is emulsified in broth. This is poured into the bottle and carefully washed all over both prepared surfaces. The bottle is then placed upright in the incubator. The next day the fluid contents are carefully poured out down one of the bare sides of the bottle. Saline (0·9 per cent.) is then added and the growth emulsified. The emulsion is heated at 60° C. for 30 minutes and diluted to contain 10 thousand million or 2 thousand million organisms per c.c. (judged by an opacity standard). Phenol is added to a concentration of 0·5 per cent. The former "thick" antigen is used for absorption experiments, and for injection into horses. The "thin" antigen is used for agglutination and injection into rabbits. The "thick" antigen can be diluted with Phenol saline to make thin antigen as required. From each bottle nearly 20 c.c. of "thick" antigen can be obtained. Large amounts of "thin" antigen were generally made from two bottles, and can be kept in stock for serial experiments. Owing to the variability in agglutinating properties of antigens prepared from the same strain at different times, this is fortunate.

(2) *Sera.* In view of previous work on this subject, and for convenience, rabbits were selected as the animals to be used. At first we employed living avirulent cultures, being deterred from using virulent strains by the high mortality reported by previous workers (Durand, Bell, etc.) and thinking it unwise to complicate matters by injecting our animals with antitoxin. We soon found, however, that we could safely employ killed virulent strains and in this communication we shall only mention sera so made. Rabbits were injected intravenously with 1, 2 and 3 c.c. of "thin" antigen on consecutive days. After an interval of eight or ten days an experimental bleeding was taken; 3 c.c. were then injected, and after an interval of ten or twelve days another experimental bleeding; if the titre of the serum was 1/400 or higher the animal was bled twice with a day's interval between the bleedings; the animal was then rested for four or five days and its serum re-tested, subsequent procedure depending on the titre found. Of the 36 rabbits used only one died in the course of immunisation, and two others never produced serum of a higher titre than 1/100. The titres produced by the remainder may be tabulated as follows:

Titre	No.	Titre	No.
1/500	1	1/1000	4
1/600	4	1/1200	2
1/800	15	1/1600	7

The time needed for immunisation was generally very short. Serum was obtained from 14 rabbits in 16–21 days after the first injection; from 11 rabbits in 22–28 days after the first injection; from four rabbits in 29–35 days after the first injection; while four rabbits did not produce usable serum until the 54th–60th day. It will be noticed that on the whole the sera produced were of low titre. They were, however, extremely specific as can be seen from Tables

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III and IV. For routine agglutination we employed them in a final dilution of 1/100, 1/200 and 1/400.

Our colleague Mr Buxton injected four horses with four separate strains. "Thick" antigens were employed. Three of the horses produced sera of a titre of 1/1500 or higher. One never reacted at all, though a rabbit injected with the same strain produced a serum of 1/800 titre. These sera are considered later.

(3) *Agglutination.* After trial had been made of different apparatus, temperature, etc. the following method was finally adopted. Small tubes, 7·5 × 0·5 centimetres are employed. 0·9 per cent. saline is employed for all dilutions. Each tube contains 0·5 c.c. diluted serum and an equal quantity of "thin" antigen. For routine work at least four sera are tested at one time against each antigen. We have found this forms a safer control than the inclusion of normal serum dilutions and saline in the test. The serum antigen mixtures are placed in a water bath at about 55° C. for one hour, the tubes being partially immersed after Topley's method. Readings are then made by naked eye, the agglutination being very definite.

(4) *Absorption.* To 2 c.c. of each antigen is added 0·1 c.c. of serum diluted according to its agglutinating titre. Thus, if the titre of the serum to be tested is 1/800, 0·4 c.c. of serum is diluted to 1 c.c. with saline. 0·1 c.c. of this (containing 0·04 c.c. of serum) dilution is added to each 2 c.c. of "thick" antigen, so that the final concentration of serum is 0·04 in 2·1 c.c. = 1/50.

The mixture is incubated at 37° C. for two hours, centrifugalised (or allowed to sediment) and the supernatant fluid removed and tested for agglutination against the appropriate antigen in final dilutions of 1/100, 1/200, 1/400, 1/800. We have found recently that it is unnecessary to employ centrifugalisation. After two hours' incubation the racks containing the tubes are placed in cold water over-night. The next morning the supernatant fluid is sufficiently free of organisms to admit of being pipetted off and tested. This saves a great deal of time when 30 or more absorption tests are being made. We always include in the test at least four positive and negative controls. That is, sera mixed with their corresponding antigen and with saline. We make a preliminary test on one positive and one negative control. If the former shows that absorption is not quite complete, we re-incubate for one hour; if the absorption is very slight we add more antigen and re-incubate; if the negative control does not show agglutination in a sufficiently high dilution (three or four tubes completely positive in a cent. per cent. series), *i.e.* in a dilution eight or sixteen times the lowest of the series, the experiment is discarded and much time saved.

(5) *Difficulties.* As far as sera are concerned we have had little trouble. We are, however, convinced that they deteriorate rapidly even when kept in the cold room. They certainly compare very unfavourably, in this respect, with other agglutinating sera. Fortunately being so specific, they can be used in low dilutions. It is the antigens that have caused us most anxiety. In the

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vast majority of cases the growth on a 24-hour Loeffler culture can be emulsified easily in 0·9 per cent. saline. Antigens made as described above remain suspended for many hours, and, when the organisms settle only need a thorough shaking before use. Certain cultures, however, yield antigens that are not completely homogeneous; sufficient material, however, remains in suspension for use in the test. In a very few instances (as is noted by Durand, Bell, etc.) the culture cannot be emulsified at all. Thus we find every stage—a perfect, a usable and a useless antigen. Some antigens again though apparently homogeneous, sediment when diluted with serum or saline and heated. This "non-specific" sedimentation can generally be distinguished from specific agglutination in three ways. In the first place, the aggregation of the organisms is much less compact than in the latter, in the second the sedimentation takes place in the higher rather than lower dilutions (the serum acting as a protective colloid), and lastly the reaction affects several, perhaps four or five sera. With some sera, however, this non-specific reaction is more marked than with others, so that unless several sera (three at least) are employed in each test erroneous conclusions may be drawn. It must be emphasised that controls of normal serum and saline are not of much practical value. They may show no agglutination, and yet non-specific reactions may occur with the same antigen and other sera. This tendency to non-specific reaction with serum and difficulty in emulsification seem to be due to physical causes, being different degrees of instability. We tried to overcome these difficulties by heating, reducing the salt content, the action of acid and alkali, etc. but with no success.

In the course of quite a different set of experiments, however, we found that glycerine in a final concentration of 1/16 completely inhibited the specific agglutination of a Mutton strain of *B. aertrycke*, and after trial of different strengths of glycerine we finally added one part of glycerine to 19 parts of diptherial antigen prepared in the usual way. The mixture was well shaken and heated at 55° C. in a water bath for eight to ten hours, being shaken repeatedly. By this means suitable antigens have been obtained from all except three of the unstable strains. Specific agglutination is not interfered with; the positive results obtained have been checked by absorption experiments. Antigens that sediment non-specifically do not absorb agglutinin, except from their homologous sera. The three strains, above-mentioned, failed to absorb the agglutinin from any of our sera and are included amongst the "unclassified" in Table I.

These phenomena may be partially explained by the presence of "rough" and "smooth" variants in the cultures. We have had more than one strain which, after providing suitable antigens for a long time, has gradually developed the tendency to clump naturally and we have watched one particular strain pass through every phase until it became impossible to emulsify it in the ordinary way, though glycerination and heating proved successful. At this stage it was plated and several colonies found to be all equally refractory.

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These changes may also take place in old stock antigens; which can be restored to usefulness by glycerination and heat. It is interesting to note that we were able to produce an agglutinating serum in response to intravenous injection of one most refractory strain. The serum so made was tested later against the same emulsion after treatment with glycerine. It is the strain 668 in Tables III and IV, where it is seen to act quite specifically in direct agglutination and absorption.

Our other difficulty was of an entirely opposite character. Certain antigens do not agglutinate with the corresponding sera, and for this reason we always make it a rule to prepare a second antigen if the first one made from an unknown strain reacts with none of our type sera. In spite of this precaution we have twice prepared sera from cultures classified as "unknown" only to find that the sera so obtained were identical with one or other that we already possessed. Such antigens will absorb the agglutinins from their own type sera and can thus be classified (*vide infra*).

Nicholas (1900) records the fact that one strain originally inagglutinable became agglutinable after being subcultured in the laboratory over a period of a year. This inagglutinable tendency is more frequently met with in antigens from old laboratory cultures than recently isolated strains. We have every reason to believe that the relative agglutinability of these antigens depends upon the proportion of agglutinable individuals present, though we have no direct evidence on this point with regard to *B. diphtheriae*. We have, however, demonstrated it clearly for certain strains of *V. cholerae*.

SOURCE OF MATERIAL AND GENERAL RESULTS OBTAINED.

The 348 strains were derived from cases, contacts, convalescent carriers, and healthy carriers together with a few cultures of unknown origin transferred to us from other laboratories. The general results are shown in Tables I and II.

It will be seen that by direct agglutination we were able to classify in ten groups 323 strains—including 13 which showed agglutination with the sera of groups 3 and 4. Of the 25 unclassified by this means nine absorbed the agglutinins from one or other of the type sera. Unfortunately, only three of the 13 in groups 3 and 4 were preserved and tested by absorption; two were type III, one type IV. These are included in Table II, which gives details of the origin of the cultures. It will be seen that group 3 contains the largest number of strains; group 5 about half the number; group 2 is the third most important. Group 1 is probably over-represented as 14 of its members were of unknown origin and there are almost certainly several strains of Park 8, under different designations. In different series the relative preponderance of each group varies with the source of the material. Thus, even in large series of strains the influence of local epidemic conditions may obscure the vision of the worker who does not search far and wide in his attempt to achieve catholicity. Langer and Mason found one group while Havens' strains are represented by our groups 1 and 2. He missed entirely our largest collection. It follows that

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it is of no value to work out the percentages of each type found in a miscellaneous collection such as ours, most of which were derived from the London area, some from Scotland and the provinces, and some from America.

What is important is to realise that organisms of every group can be cultivated from "cases" or carriers, and there is every reason to believe in the existence of still further groups which we have not discovered. Nor would there seem to be any evidence for the view that one type is responsible mainly for cases or exists chiefly in carriers. At first sight it might appear that types V and IX were specially associated with cases, III and II being relatively more frequent in the carrier. But as the carriers were mainly taken from an area in which a type III epidemic was raging, and were many of them more or less direct contacts, any such conclusion would be unjustified. In the same way it appeared at first sight as if types V and IX were specially associated with nasal infection. Of 14 nasal cases, six were associated with type V and four with type IX. Here again three of the latter occurred in one institution and four of the former in another, the cases being so mild in character that they could be regarded as "Bacteriological Diphtheria," *i.e.* carriers. We have examined only five cultures from the ear. Two belonged to type III, two to type V, while one, though showing affinities to other groups, was classified by absorption as type I (*vide* No. 502, Tables III and IV).

SEROLOGICAL RELATIONSHIPS.

If we examine Tables III and IV we find that, as far as direct agglutination is concerned we have ten absolutely specific type sera. When we turn to the absorption results we find a variable relationship between types III and IV. These were tested several times and sometimes partial cross-absorption was demonstrated, sometimes not. Otherwise the ten type sera are specific by this test also. The five "Intermediate" sera are of great interest:

502	by agglutination = type	I (VII, VIII)	by absorption = type	I
301	,, ,,	III (IV, V, I, VII)	,, ,,	III (I)
525	,, ,,	III, V, IV	,, ,,	IV
506	,, ,,	III, IV, VII	,, ,,	III
519, 46	,, ,,	II, VII	,, ,,	II, VII.

We have also three sera which react only with their homologous strain.

The "mongrel" sera will be seen to be mainly associated with groups 3 and 4, the strains chosen for producing the sera having been difficult to assign to one of these types (301, 525, 506). As will be seen from Table II this is the most important association with which we have met. Moreover, there is a slight association between these two type sera (*vide* Table IV). Now most of our strains have come from an area where type III organisms are epidemic. It is therefore suggestive that the "mongrel" strains should show a relationship with type III compatible with the theory that they are either ancestors or descendants of this strain. We must also expect to find in other areas strains more nearly related to types IX and X, etc. and to the unknown groups which may be found elsewhere. Table VI shows the relationship between the sero-

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logical types of *B. diphtheriae* found by previous workers and ourselves. Bell's sera and our own show interesting differences. In each case type I was made by injection of the same strain. The difference in type specificity is curious and probably due to different methods of immunisation. Dr W. M. Scott kindly sent me some serum which agglutinated all our ten types though the strain with which it was prepared, was, when tested against our own sera, specific type III. The second point of special interest is the fact that Bell regarded his three sera, Andrews, Mulaney and Blackburn, as identical; our results are shown in the table.

AGGLUTINOGENESIS.

We have seen that, with our method of immunisation in the rabbit, we obtain from most strains highly specific sera, while a few strains give more catholic results. Presupposing that horse sera would be less specific, we decided to study the production of agglutinins in the horse in response to injections of types I, II and III. We hoped that the horse sera would gradually become non-specific, and that the relationship of the strain used to the strains of the other groups could be demonstrated by this lack of specificity.

The results obtained with the sera of three horses are shown in Table VI. *H* 1154, injected with type I, shows at first relationship with III, IV, VIII and IX; then with II, V and X.

H 1156 injected with type II was never found to be specific but showed relationship between II and I, III, V and IX, and later VIII, VII and VI but never with X.

H 1173, injected with type III, remained specific for a long time and then showed relationship between III and II and VIII, then I, IV, V, IX.

Now if we consider the strains 55 and 301 we find that the former produces an almost specific rabbit serum, and if injected into the horse, only after a long while shows a tendency to catholicism. 301, on the other hand, even in the rabbit, produces a serum with broad sympathies. 55 is more specialised, but both strains contain in common antigenic components = III, I, IV, V, VII (*vide* Tables III and IV). It is in the relative predominance of antigenic component III that they differ.

We may then assume as a working hypothesis that all diphtheria bacilli have the same qualitative structure, and that every strain contains antigen elements I, II, III, IV, V, etc. In group 1, element I predominates and sera can be produced which show group 1 agglutinins only. But the other elements are there and when sufficient of them have been injected the animal will respond. This latter suggestion is strongly supported by Table VI, which shows the agglutinogenesis in the three horses. It will be seen that the non-specific group reaction is a miniature of the specific and comes on later. This would accord with the hypothesis of quantitative variations but qualitative identity in composition as judged by agglutinogenetic power.

It may perhaps be objected that, if this qualitative identity exists, it could

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be established by means of absorption tests. Thus, any diphtherial agglutinating serum could be absorbed by any one antigen provided enough of the antigen be employed. We have tried such an experiment, treating all the ten type sera with large amounts of antigen of one strain (15 times the amount usually employed). But no cross-absorption could be demonstrated. It may be that we could not use sufficient antigen; this, however, is unlikely as we should have found faint indications of absorption at any rate.

That the agglutinogenetic capacity of two antigens might be qualitatively similar even though they show no connection by agglutinating or absorption tests is easy to conceive; for in the former case the actual agglutinogen may be formed from the antigen in the body of the animal injected.

VALUE OF SEROLOGICAL CLASSIFICATION OF *B. DIPHTHERIAE.*

We have seen that the previous work on this subject has been actuated by different motives. Our own object in undertaking this study was primarily concerned with an enquiry into the relationship between virulent and avirulent strains of *B. diphtheriae*. We hoped that, by studying their agglutinating properties we might find a clue to their relationship. We soon realised, however, that it was wiser to limit ourselves at first to the classification of virulent strains, and this for two reasons:

(1) We had a sure test for the genuineness of our strains, *i.e.* a virulence test.

(2) We found that, though we could make sera from avirulent strains and thereby classify virulent strains and *vice versa,* still the serological reactions of the avirulent organisms were much less clean cut than the virulent. We, therefore, postponed the consideration of the former until we had established, to our satisfaction, the relationships of the latter.

From a practical point of view the main value of this method of investigation should be found in epidemiology. In agreement with Durand and Guérin, our work suggests that, broadly speaking, in one epidemic one type prevails. This fact is also eloquently shown by the results of Langer, Mason and others.

SIMULTANEOUS AND REPEATED SWABS.

Positive cultures from the nose and throat of the same patient at the same time have in our hands always shown the same type of organism. Organisms cultured from the same patient at different times have almost always shown the same type of organism. We have followed several individuals in this way for many months, and this serological constancy is the rule in the vast majority of cases. We have, however, met with a few interesting variations. Thus one patient with a persistent otorrhea gave us one strain allied to types III and IV and a month later another mongrel 502 (*vide* Tables III and IV). Likewise, of six swabs from one patient, three were pure type III and three mongrel III and IV. This variability has been specially marked with type III carriers. Thus we find III and IV, III and V.

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We have also found type II followed by VII and V; in one case we found IV followed one month later by V.

As we pointed out before, we are dealing mainly with an area in which type III is dominant; we should therefore expect the mongrel strains to show their relationship to this type.

FAMILIES.

Organisms cultivated from members of the same family, and contacts of one case show in general the same type organism. We have examples of this from types II, III and V. No variation between types has been found in one family at one time; the variation may occur later in the organisms cultivated from individual members.

EPIDEMICS.

We have examined series of organisms from two sources. The first is a school near London in which an epidemic was followed by sporadic cases. The second is a London borough, the organisms being isolated mainly from direct contacts, but including a few cases. The results are best arranged chronologically as under:

The Institution gives 23 virulent cultures of which

15 are type III; 1 is type IV; 4 are type V; 3 are type IX:

all the avirulent cultures examined were nasal and type V.

The London Borough series gives 33 organisms all virulent, all from the throat:

29 are type III; 1 a mongrel III and IV; 3 are type V.

Our material so far is scanty, but it suggests that, though an epidemic may start with one type, other types will appear. This also would be expected if we believe that the agglutinative types of *B. diphtheriae* are not fixed, but to borrow Prof. Andrewes' expression, "kaleidoscopic."

The typing of the few avirulent cultures in the School series is also of interest as suggesting their nearness of kin to the prevailing virulent type. Most of these nasal cases were regarded by us as carriers.

SUMMARY.

1. Evidence is given that there exists a multiplicity of serological groups of *B. diphtheriae*.

2. Ten of these groups have been studied in this communication.

3. Into these ten groups could be placed all except 16 of 348 strains of virulent *B. diphtheriae* investigated.

4. A description of the technical difficulties encountered is included in the hope that this may prove of service to other workers.

Some evidence on the antigenic structures of different strains is advanced.

5. The application of this method of investigation to the problems of epidemiology is briefly discussed, and evidence of its value brought forward.

https://doi.org/10.1017/S002217240000810X Published online by Cambridge University Press

Table I. *Direct agglutination.*

	Types												
	1	2	3	4	5	6	7	8	9	10	3 and 4	Un-classified	Total
Cases	4	5	32	4	20	2	2	2	6	1	3	3	84
Persistents	1	0	4	1	5	0	0	0	0	0	1	0	12
Contacts	0	0	26	1	3	0	0	0	0	0	1	0	31
Carriers	6	20	80	6	31	8	5	4	1	8	7	13	189
Unknown	11	4	3	2	2	0	0	0	0	0	1	9	32
Total	22	29	145	14	61	10	7	6	7	9	13	25	348
	310										13	25	
	323											25	

Table II. *Final results.*

	1	2	3	4	5	6	7	8	9	10	3 and 4	Un-classified	Total
Cases	4	5	33	4	21	2	2	2	6	1	2	2	84
Persistents	1	0	4	1	5	0	0	0	0	0	1	0	12
Contacts	0	0	26	1	3	0	0	0	0	0	1	0	31
Carriers	6	20	83	7	31	8	5	5	1	8	5	10	189
Unknown	14	5	3	2	2	0	0	1	0	0	1	4	32
Total	25	30	149	15	62	10	7	8	7	9	10	16	348
	332												

Table III. *Direct agglutination.*

	Sera	Antigens																	
		1	2	3	4	5	6	7	8	9	10	301	502	525	506	519 46	666	668	77
1	34, Δ79, Δ85	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
2	26	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
3	55, 325, 477	–	–	+	–	–	–	–	–	–	–	1/2	–	1/5	+	–	–	–	–
4	Δ 90, 663	–	–	–	+	–	–	–	–	–	–	1/40	–	1/40	–	–	–	–	–
5	36, 186, 317, Δ 91, 296	–	–	–	–	+	–	–	–	–	–	–	–	1/16	–	–	–	–	–
6	459, 393	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
7	516, 227, 784	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
8	61, 98, 613	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
9	851	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
10	35	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
"Mongrel"	301	1/16	–	+	1/16	1/16	–	1/16	–	–	–	+	–	1/8	+	–	–	–	–
"Mongrel"	502	+	–	–	–	–	–	1/4	1/16	–	–	–	+	–	–	–	–	–	–
"Mongrel"	525	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
"Mongrel"	506	–	–	+	1/2	–	–	1/8	–	–	–	+	–	1/32	+	–	–	–	–
"Mongrel"	519, 46	–	+	–	–	–	–	1/8	–	–	–	–	–	–	–	+	–	–	–
Unknown	666	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Unknown	668	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Unknown	77	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+

– = No agglutination at 1/20 final dilution.
+ = Full.
1/2, 1/4, etc. = Fraction of full titre.

https://doi.org/10.1017/S002217240000810X Published online by Cambridge University Press

Table IV. *Absorptions*

	1	2	3	4	5	6	7	8	9	10	301	502	525	506	519 46	666	668	77
1	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
2	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
3	–	–	+	– or 1/4	–	–	–	–	–	–	1/2	–	–	+	–	–	–	–
4	–	–	– or 1/2	+	–	–	–	–	–	–	–	–	+	1/2	–	–	–	–
5	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
301	– or 1/2	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
502	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
525	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–
506	–	–	+	–	–	–	–	–	–	–	(+)	–	–	+	–	–	–	–
519, 46	–	+	–	–	–	–	(+)	–	–	–	–	–	–	–	+	–	–	–
666	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
668	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
77	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+

+ = full absorption.
(+) = almost complete absorption.
1/2 and 1/4 denote relative completeness, compared with homologous strain.

Table V. *Table of comparative results.*

1	2	3	4	5	6	7	8	9	10		
+	–	–	–	–	–	–	–	–	–	Nicholas and C.	A
+	–	–	–	–	–	–	–	–	–	Havens: type I	B
–	+	–	–	–	–	–	–	–	–	,, ,, II	B
+	tr.	tr.	tr.	1/16	1/16	1/8	1/8	?	?	Bell ,, I	C
1/8	+	–	–	–	–	–	1/8	?	?	,, ,, II	D
–	–	tr.	–	+	1/16	1/4	–	?	?	,, ,, III "Blackburn"	D
–	–	+	–	tr.	1/4	+	–	?	?	,, ,, III "Andrews"	D
–	–	+	–	–	–	–	–	?	?	,, ,, III "Mulaney"	D
+	–	–	–	–	–	–	–	–	–	Durand (from Park): type I	C
–	–	–	–	–	–	–	–	–	–	,, ,, ,, II A.V.	B
–	–	–	+	–	–	–	–	–	–	,, ,, ,, III	B
–	–	–	+	–	–	–	–	–	–	,, ,, ,, IV	C
–	–	–	–	–	–	–	–	–	–	,, ,, ,, V	B

A = deduced from literature.
B = culture tested.
C = serum and culture tested.
D = serum tested.
A.V. = avirulent.

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Table VI. *Horse sera (agglutinations).*

	Type cultures 1	2	3	4	5	6	7	8	9	10	
H 1154. Type I	–	–	–	–	–	–	–	–	–	–	23. vii. 22 before inj.
	1000	tr.	–	–	–	–	–	–	–	–	28. viii. 22
	800	–	–	–	–	–	–	–	–	–	6. ix. 22
	2000	–	40	10	–	–	–	10	50	–	11. ix. 22
	2000	150	80	tr.	80	–	tr.	–	80	20	22. ix. 22
	640	30	10	–	–	–	–	–	20	–	3. x. 22
H 1156. Type II	–	–	–	–	–	–	–	–	–	–	28. vii. 22 before inj.
	64	500	128	–	25	–	–	–	48	–	28. viii. 22
	–	1000	60	–	tr.	–	–	30	60	–	6. ix. 22
	tr.	40	40	20	10	tr.	15	15	20	–	11. ix. 22
	30	2000	100	30	40	20	20	40	40	–	22. ix. 22
	–	2000	100	–	30	–	–	–	40	–	3. x. 22
H 1173. Type III	–	–	–	–	–	–	–	–	–	–	28. vii. 22 before inj.
	–	–	1500	–	–	–	–	–	–	–	28. viii. 22
	–	–	1500	–	–	–	–	–	–	–	6. ix. 22
	–	50	800	tr.	tr.	–	tr.	20	–	–	11. ix. 22
	20	60	1000	10	40	tr.	–	30	30	tr.	22. ix. 22
	–	–	1000	–	–	–	–	–	–	–	3. x. 22

Table VII. *A school.*

Types of organisms occurring in a prolonged series of cases

Date	Virulent	A.V.	3	4	5	9
14. ii. 22	2	0	2	0	0	0
15. ii. 22	2	0	2	0	0	0
19. ii. 22	1	0	0	0	1	0
21. ii. 22	1	0	1	0	0	0
24. ii. 22	2	0	2	0	0	0
27. ii. 22	1	0	0	0	0	1
6. iii. 22	1	0	0	0	0	1
16. iii. 22	1	0	0	1	0	0
27. iii. 22	1	0	0	0	1	0
10. iv. 22	1	0	1	0	0	0
10. iv. 22	0	2	0	0	2	0
11. iv. 22	0	1	0	0	1	0
13. iv. 22	0	1	0	0	1	0
18. iv. 22	2	0	1	0	1	0
25. iv. 22	1	0	1	0	0	0
27. iv. 22	1	0	1	0	0	0
1. v. 22—2. vi. 22	4	0	4	0	0	0
23. vi. 22	1	0	0	0	1	0
24. viii. 22	1	0	0	0	0	1
Total	23	4	15	1	8	3

Table VIII. *A London Borough.*

Date	Cases	Contacts	Type 3	3 and 4	5
September and October, 1921	4	2	6	0	0
November, 1921	0	16	12	1	3
December, 1921	0	2	2	0	0
January, 1922	0	3	3	0	0
February and May, 1922	0	2	2	0	0
July, 1922	0	4	4	0	0
Total	4	29	29	1	3

https://doi.org/10.1017/S002217240000810X Published online by Cambridge University Press

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https://doi.org/10.1017/S002217240000810X Published online by Cambridge University Press