THE ABSORPTION OR SATURATION TEST OF CASTELLANI: ITS APPLICATIONS IN SERO-DIAGNOSIS, AND IN THE RECOGNITION OF BACTERIAL SPECIES.

A CRITICAL REVIEW.

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The Absorption or Saturation Test published by Castellani in 1902 is now becoming more extensively used by serological and bacteriological workers in European and American laboratories. Its use is also essential for the accurate study of certain tropical infections, so that it behoves all tropical workers to be familiar with this test. It is therefore believed that an account of the method together with a general review of the literature of the subject may not be out of place in this *Journal*.

The phenomenon of absorption of agglutinins from serum on saturation with homologous organisms was first observed by Bordet (1899) in the case of normal serum. Bordet's observation was soon confirmed by Eisenberg and Volk (1902).

Castellani (1902) next discovered that the serum of rabbits immunised with *B. typhosus* agglutinating not only the typhoid bacillus, but also to a less extent certain strains of *B. coli*, on saturation with an excess of typhoid bacilli not only lost its specific (primary homologous) typhoid agglutinin, but also the non-specific (secondary heterologous or co-agglutinin) coli agglutinin. Saturation of the same serum with *B. coli* was found to remove the non-specific coli agglutinin, but the specific typhoid agglutinin was not removed, or only to a very small extent. Further, in a serum derived from rabbits immunised both for *B. typhosus* and *B. coli*, saturation either with typhoid bacilli alone or with *B. coli* alone failed to remove the whole of the specific agglutinins, whilst this was achieved by saturation with both organisms either simultaneously or successively.

The following experiments selected from those recorded by Castellani will illustrate these findings—a rabbit immunised to *B. typhosus* agglutinated *B. typhosus* 1:5000 and *B. coli* (31) 1:600. After saturation

with typhoid bacilli, all agglutinins were removed for both micro-organisms. A rabbit immunised to both *B. typhosus* and *B. coli* (31) agglutinated typhoid bacilli 1: 4000 and *B. coli* (31) 1: 1000. After saturation with typhoid bacilli the serum no longer agglutinated typhoid, but did agglutinate *B. coli* (31) 1: 900. After saturation with the colon bacillus (31) it failed to agglutinate *B. coli* (31), but still agglutinated *B. typhosus* 1: 4000.

From these and other experiments Castellani drew the following important conclusions:—(1) The serum of an animal immunised against a certain micro-organism, when saturated with that micro-organism, loses not only its agglutinating power for that organism, but also for all the other varieties that it acted upon; when saturated with the others, its agglutinating power upon the first is reduced little or not at all. (2) The serum of an animal immunised against two micro-organisms, *A* and *B*, loses its agglutination when saturated with *A* only for *A*. Saturated with *A* and *B* it loses its agglutinating power for both. (3) These facts may be applied to the diagnosis of mixed infections, and to the differentiation of closely allied germs. Suppose for instance the serum from a typhoid case agglutinates with the laboratory culture of *B. typhosus* and those of a variety of *B. coli*, saturate the serum with typhoid bacilli. If the serum loses its agglutinating power for the typhoid bacillus only, it is a case of mixed infection with both the typhoid and colon bacilli. If the serum loses its agglutination for both the typhoid and coli organisms, it is a pure typhoid infection, the *B. coli* having been agglutinated by the group agglutinins produced by the typhoid infection.

These findings of Castellani were not immediately accepted without criticism. Thus, Posselt and Sagasser (1903) showed that in immunising there is not only an increase in the amount of primary agglutinins for the organism used but also of secondary agglutinins which act on other organisms. As regards absorption these secondary agglutinins behave like the special agglutinins in cases of mixed infection and as the agglutinins in normal serum, being often increased to a high degree. Thus the serum of a guinea-pig immunised against *B. typhosus* with a titre of 1: 12,000 for this bacillus had also secondary agglutinins for *B. cholerae* 1: 4500 and for *B. dysenteriae* 1: 4000.

Ballner and Sagasser (1904) also showed that a homologous bacterial species can withdraw from an immune serum only its own primary agglutinins but not the secondary agglutinins which act on other bacteria, and that a heterologous bacterial species binds only its own partial

agglutinins and no other portion of the total agglutinins; hence they conclude that the absorption of agglutinins through homologous and heterologous micro-organisms must be regarded as a strong specific reaction. They give examples showing that the co-agglutinins are at times markedly increased, and that inoculation with *B. tetani* and *B. pneumoniae* of Friedländer lead to the formation of few primary agglutinins, but numerous co-agglutinins.

Again, Hetsch and Lentz (1903) by employing the method of absorption to genuine cholera bacilli and cholera-like vibrios, demonstrated the specificity of the agglutinins in normal horse serum and in that of an animal immunised against the *B. cholerae*. Saturation with *B. cholerae* diminished the agglutinins for this organism whilst the co-agglutinins remained either the same or were only slightly diminished.

Levy and Fornet (1908) also insisted on the value of Castellani's saturation method of distinguishing primary and secondary agglutinins, both in patients' and in animal sera, provided always that high serum dilutions were repeatedly treated with large amounts of bacteria.

D'Amato (1910) also, as the result of a very extensive investigation, came to the conclusion that, by means of the saturation test it is possible to determine with considerable certainty which is the real infecting organism in doubtful cases of typhoid infection.

The importance of co-agglutinins and of their investigation in cases of mixed infection was also recognised by Dreyer (1916) who made use of standard agglutinable cultures in testing the agglutinating power of serum against *B. dysenteriae* (Shiga, Flexner and Y), *B. typhosus*, *B. paratyphosus* A, *B. paratyphosus* B, *B. enteritidis* (Gaertner), *B. coli* and *Vib. cholerae.*

When in the course of a differential diagnosis it is found that the patient shows a high agglutination titre for two or more of the dysenteric bacilli, the question arises whether we are dealing with co-agglutination, or with agglutination persisting from a part infection with another form of bacillary dysentery or with a mixed infection. In the former case the rise and fall in agglutination to the different microbes will be synchronous. Dreyer considers that it is more common to see a co-agglutination of *B. Flexner* in a *B. Shiga* infection than of *B. Shiga* in a *B. Flexner* infection. When the case is one of mixed infection the agglutination curves for the different infecting organisms are usually not synchronous, and pursue their ordinary course independently of each other.

TYPHOID-PARATYPHOID GROUP.

Conradi (1904) confirmed Castellani's findings in the case of typhoid and paratyphoid infections.

Park and Collins (1904) in a Study of "Specific and Non-Specific Agglutinins" successively applied this method to a serum mixture obtained by adding equal parts of serum from an animal injected with a maltose fermenting paradysentery culture (Manila) and from an animal injected with a paratyphoid bacillus. Their results are set forth in Table I.

This was followed by Boycott (1906) in an elaborate series of "Observations on the Bacteriology of Paratyphoid Fever and on the Reactions of Typhoid and Paratyphoid Sera."

The accuracy and value of the Castellani test he stated to be generally acknowledged and he considers that absorption tests are necessary for the positive or negative diagnosis of mixed infections. Some of his results are thus set forth (Table II).

These figures, and others given, he considers require little comment.

Bainbridge (1909) published a paper "On the Paratyphoid and Food-poisoning Bacilli, and the Nature and Efficiency of Certain Rat Viruses," in which he employed this method extensively "in the hope of obtaining more complete differentiation of these bacilli than is afforded by their agglutination reactions."

Some of his results are exhibited in Table III.

Harvey (1909) investigated the enteric fever convalescents at the depôt at Naina Tal and found that a certain number of these were *B. paratyphosus* A infections. In one case where *B. paratyphosus* A was isolated from the blood and faeces he performed absorption experiments (Table IV) in order to differentiate the specific and group agglutinins.

It will be noted that the high agglutinins for *B. typhosus* were almost removed by absorption with *B. paratyphosus* A isolated from the patient's own blood. If these agglutinins had been due to the *B. typhosus* then absorption with *B. paratyphosus* A should have only slightly reduced the agglutination titre. Absorption with the *B. typhosus* reduced but did not remove the low agglutinins for the *B. paratyphosus* A.

In another case which was proved by culture to be a double infection with the *B. typhosus* and the *B. paratyphosus* A, absorption with the latter bacillus had no effect on the agglutinins for the former.

Notwithstanding these results Harvey concludes that the only certain method by which cases of infection by paratyphoid bacilli can be differentiated from cases due to infection by the *B. typhosus* is by careful

TABLE I.

Absorption by the paratyphoid bacillus.

	Agglutination	
	Before Absorption	After Absorption
Paratyphoid bacillus	1 : 500	<1 : 10
Dysentery bacillus	1 : 1000	1 : 800
Colon bacillus X	1 : 500	1 : 10

TABLE II.

Nature of serum	Dilution	Absorbed with	Agglutination (after absorption) with: *Typhoid Guy's*	*Brion and Kayser*	*Schott B.*	*Aertryke*	*Gaertner < I.P.M.*	*Schott A.*
I. Typhoid: human	1 : 10	Original titre	2000	1000	50	50	500	50
		Typhoid Guy's	+++	O	O	O	+++	–
			O	O	O	O	O	–
		Brion and Kayser	+++	++	O	O	+++	–
			+++	O	O	O	+++	–
		Schott B.	+++	++	O	O	++	–
			+++	O	O	O	++	–
		B. coli communis	+++	++	+++	++	+++	–
II. Paratyphoid B: human case "Barklay"	1 : 10	Original titre	200	500	75,000	1000	200	50
		Typhoid Guy's	O	+++	+++	+++	+	–
		Brion and Kayser	++	+++	+++	+++	+++	–
			++	O	+++	+++	+++	
		Schott B.	O	O	+++	+++	O	–
			O	O	+++	+++	O	–
			O	O	O	O	O	-
		Aertryke	O	O	+++	O	O	–
		Gaertner < I.P.M.	O	+++	+++	+++	O	–
		B. coli communis	+++	+++	+++	+++	+++	–
		Guy's and Brion and Kayser + Aertryke + Gaertner	O	O	+++	O	O	–
III. Gaertner: original rabbit	1 : 25	Original titre	1:1000	200	20	<20	5000	50
		Typhoid Guy's	O	O	O	–	+++	–
		Brion and Kayser	++	O	O	–	+++	–
		Schott B.	+++	O	O	–	+++	–
		Gaertner < I.P.M.	O	O	O	–	O	–
		Aertryke	+++	O	O	–	+++	–
		B. coli	+++	+	O	–	+++	–
IV. Aertryke: rabbit	1 : 50	Original titre	200	500	2000	2000	20	–
		Typhoid Guy's	O	+++	+++	+++	–	–
		Brion and Kayser	O	O	+++	+++	–	–
		Schott B.	O	+	O	+++	–	–
		Aertryke	O	O	O	O	–	–

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Table III.

Serum			Agglutination at 1—200 after absorption		
			B. Aertryke	*B. Paratyphoid* B	*B. Suipestifer*
B. Aertryke	Original titre		5000	5000	2000
	Absorbed with:				
	B Aertryke		O	O	O
	B. Suipestifer		O	O	O
	B. Paratyphoid B	1 : 2000	+	O	+
		1 : 1000	+	O	+
B. Paratyphoid B	Original titre		5000	1000	1000
	Absorbed with:				
	B. Paratyphoid B		O	O	O
	B. Aertryke		O	+	O
	B. Suipestifer		O	+	O
B. Suipestifer	Original titre		5000	4000	5000
	Absorbed with:				
	B. Suipestifer		O	O	O
	B. Aertryke		O	O	O
	B. Paratyphoid B	1 : 200	+	O	+
		1 : 2000	+	O	+
B. Aertryke	Original titre		4000	4000	4000
	Absorbed with:				
	B. Aertryke		O	O	O
	B. Suipestifer		O	O	O
	B. Paratyphoid B	1 : 200	+	O	+
		1 : 1000	+	O	+
B. Paratyphoid B	Original titre		—	10,000	10,000
	Absorbed with:				
	B. Paratyphoid B		—	O	O
	B. Suipestifer		—	+	O

Table IV.

Pte *P*'s serum untreated.

Widal reaction	20	40	100	200	400
Stock typhoid	+	+	+	±	∓
Para A own strain	±	∓	Trace	–	–

Pte *P*'s serum after absorption for one hour with own bacillus:

Widal reaction	20	40	100	200	400
Stock typhoid	∓	Trace	–	...	...
Para A own strain	Trace	Trace	–	...	...

Pte *P*'s serum after absorption for one hour with the *B. typhosus*:

Widal reaction	20	40	100	200	400
Stock typhoid	Trace	–	...	...	...
Para A own strain	±	Trace	–	...	...

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cultural examinations of the blood and excreta, and that this differentiation has a practical significance in view of the statistics for anti-enteric inoculation as this prophylaxis cannot be expected to protect against paratyphoid infection, which would appear to be becoming more common in India.

In a later communication Harvey (1915) considered that blood culture should be attempted in every case at the earliest opportunity, but if this fails (as often happens) it is quite possible to diagnose paratyphoid fever on clinical grounds in combination with a close study of the Widal (including absorption) reactions. He also found that in cases due to infection with the *B. paratyphosus* A whose serum agglutinated both the paratyphoid A and typhoid, absorption with A removed all the agglutinins, or if the man had been inoculated reduced those for typhoid to the level that obtained before the fever. Absorption with *B. typhosus* removed only the group agglutinins for this bacillus but not those for *B. paratyphosus* A, even although these had only been present in a 1 in 20 dilution. This rule, however, is not invariable, as in some cases the titre for paratyphoid A was considerably reduced by absorption with the *B. typhosus* although the cases had been proved by blood culture to be pure paratyphoids; but this was exceptional.

The method was then assiduously employed by Officers of the Royal Army Medical Corps working in India.

Gratton and Wood (1911) investigating Paratyphoid Fever in India differentiated the typhoid from the paratyphoid bacilli by means of the absorption method. They found that if the organism tested were the *B. paratyphosus* A the specific agglutinins were completely removed. Before accepting a suspected organism as *B. paratyphosus* A they required that it should completely remove the agglutinins specific for *B. paratyphosus* A; and they frequently tested heterologous organisms as controls, such as *B. typhosus*, *B. paratyphosus* B and *B. coli* against their paratyphosus A serum, and by this means have never removed the specific agglutinins for *B. paratyphosus* A.

Gratton and Harvey (1911) investigated a small epidemic of Typhoid Fever in India caused by an "acute carrier," and identified *B. paratyphosus* A in the stools of the carrier by means of the absorption test, and state that "to accept any bacillus *isolated* from faeces as a paratyphoid bacillus without putting it through some such series of tests (as agglutination and absorption) is to run great risks of making mistakes."

Harvey (1911) in discussing "The Causation and Prevention of Enteric Fever in Military Service, with a Special Reference to the

Importance of the Carrier" made use of the absorption method in the identification of the typhoid and paratyphoid bacilli in the faeces of convalescents and carriers.

Firth (1911) in discussing "The Para-typhoid Problem in India" considers that "it is only by the adoption of critical methods such as Castellani's Absorption Reaction that differentiation (of the paratyphoid bacilli) is possible.

Sacquépée (1916) calls attention to the great help which may be obtained by the use of the saturation test in the diagnosis of certain cases of typhoid in which the serum contains high amounts of agglutinin.

Chantemesse and Grimberg (1916) also call attention to the great practical importance of the saturation test in certain cases of typhoid and paratyphoid fever.

Gautier and Weissenbach (1916), as the result of an elaborate study came to the conclusion that the saturation test is extremely useful in determining which is the specific agglutinin, when two or more agglutinins are present in the serum. The results obtained by the saturation test in cases of typhoid and paratyphoid infections have always been identical with the results obtained by haemoculture.

Three of the less frequent and less known members of the typhoid group discovered by Castellani, namely *B. Asiaticus* I, *B. Asiaticus* II, and *Bact. Columbensis* were experimentally differentiated from each other and from the other members of the typhoid-coli group by the use of the absorption method by Fulle (1914).

DYSENTERY.

The absorption test has also been applied to the dysentery group of bacilli and has yielded somewhat discordant results in the hands of various workers, and very different opinions have been expressed as to its value in the investigation of this group of organisms.

Thus Morgan (1911) investigated a large number of strains of the mannite-fermenting group of *B. dysenteriae* isolated in this country from sources not obviously connected with clinical dysentery. He concluded that these indigenous strains, like certain foreign strains isolated from definitely dysenteric sources, could not be completely identified with any of the well-known types of the group by the application of extensive fermentation and absorption tests. He believes that when a sufficiently extended series of carbohydrate media is tested, the fermentation properties of the mannite-fermenting group afford an indication of

differences between the members of this group which are not brought to light by agglutination and absorption tests.

Again, Park (1904) and Händel (1908) found that by the absorption method they could distinguish the Flexner and Y types from each other, and by the extensive use of homologous and heterologous immune sera showed that they each possessed, in great part, homologous agglutinins for the other types.

Somewhat similar uncertain results were also obtained by Posselt and von Sagasser (1903), by Knox and Shorer (1906) by Lüdke (1911) and by Winter (1911). The uncertain results obtained by these various investigators may be explained partly by the variable agglutinating powers usually shown by the bacillus and sometimes also by Flexner's bacillus, and partly to the fact that they consider that the method of Castellani is subject to a whole series of experimental errors due to its complicated character.

Kruse (1907), who considers the Shiga-Kruse bacillus to be the only true dysentery bacillus, and includes all the other varieties—Flexner, Strong and Hiss—under the term pseudo-dysentery bacilli, the former being spoken of as toxic and the latter as atoxic dysentery bacilli, successfully employed the absorption method in the differentiation of the atoxic or pseudo-dysentery group of bacilli. He found the Y bacillus to comprise seven types, whilst the Flexner and Strong bacilli consist only of one of each type. Nine types were thus produced, and these were designated by the capital letters A to H. In Kruse's absorption experiments, however, doubtful or anomalous results were not infrequently obtained, being ascribed by him to experimental errors.

Winter (1911) also examined thirty-one strains of the Flexner type of dysentery bacilli. Castellani's method was found unsatisfactory and cross agglutination experiments were relied upon, with the following results: Eleven strains appeared to correspond to Kruse's *B. pseudo-dysenteriae* D; seven to Kruse's group A; three were so feebly agglutinated by any of his sera, that they could not be classed with any certainty, but appeared to belong to group A; two were intermediate between groups A and D; whilst the remaining twelve could not be grouped by any of the sera employed.

Wassermann (1912), too, in employing the absorption test for the differentiation of the dysentery bacilli found similar anomalous results to occur so frequently, that he considers the method unsuitable for their differential diagnosis.

Lösener (1909) also encountered great difficulties in applying the

results of the absorption test to the differentiation of the atoxic group of dysentery bacilli. Thus, a dysentery bacillus which he isolated in Königsberg, and which gave all the cultural characters of the Y bacillus, and was agglutinated in a dilution of 1 in 5000 by a Y serum with a 1 in 10,000 titre, could not be placed by the method of absorption into any of Kruse's groups.

Lentz (1913) also isolated an atoxic Y bacillus which could not be placed by the absorption test in any of Kruse's groups and must therefore be considered as a new type, if the results of the absorption test can be taken as the criterion.

Ruffer and Willmore (1909) isolated a bacillus causing dysentery at El Tor and designated it *B. dysentericus* El Tor, No. 1. By agglutination they demonstrated the close relationship of this organism to the *B. pseudo-dysentericus* D of Kruse, and could differentiate them from each other only by saturation.

Mayer (1910) also obtained similar results.

Hutt (1913) confirmed Kruse's opinion of the futility of grouping dysentery bacilli according to their action on carbohydrates. By the use of the absorption method he also confirmed Kruse's groupings and even increased their number.

The above experimental findings would indicate that if we are to take the absorption test as our guide, the number of types of dysentery bacilli must be considerably increased—a view for which Lenz (1913) however considers that at present there is not sufficient justification. Obviously this point can only be settled by a much more extensive and systematic investigation of the subject than has yet been attempted.

CHOLERA AND ALLIED VIBRIOS.

The absorption method was applied to cholera and El Tor Vibrios by Ruffer (1907), in an attempt to decide whether the El Tor Vibrios should be considered to be true cholera vibrios or not. A thorough and complete serological examination was undertaken including the agglutination, saturation, and complement fixation tests and Pfeiffer's reaction. Undoubted cholera vibrios were positive to all these tests and were not haemolytic. The El Tor vibrios gave positive agglutination and saturation tests and Pfeiffer's reaction with cholera serum, but did not fix the cholera immune body and were strongly haemolytic. A third group of vibrios fixed the cholera immune body but were not agglutinated by immune serum and did not give the saturation test or Pfeiffer's reaction and were feebly haemolytic; whilst a fourth group of strongly

haemolytic vibrios were negative to all the serological tests. From a consideration of all the serological data Ruffer came to the conclusion that the El Tor vibrios were to be distinguished from true cholera vibrios, and considers that the agglutination, saturation, and Pfeiffer's tests are not in themselves of absolute diagnostic value for cholera vibrios.

Neufeld and Händel (1907) however after a re-examination of some of the El Tor vibrios consider that they are true cholera vibrios.

Crendiropoulo (1912) examined the stools of large numbers of passengers arriving in Alexandria from infected parts for vibrios and subjected the vibrios so obtained to thorough cultural and serological investigations, including agglutination, saturation, Pfeiffer's, and the complement-fixation tests, haemolytic power and pathogenicity to pigeons. The saturation tests gave similar results to the agglutination tests.

PNEUMOCOCCUS.

Many years ago Eyre and Washbourn (1899) showed that a given antipneumococcic serum would protect rabbits only against certain strains of pneumococci. Bezançon and Griffon (1900) confirmed this observation by experiments in both agglutination and protection.

Using the methods of Cole (1914) a similar serological study was made by Mathers (1915) of the cases of lobar pneumonia entering the Cork County Hospital during the season of 1914–15 and his results may be seen at a glance in Table V.

TABLE V.

Classification of Different Strains of Pneumococci.

Group	No. of Cases	Percentage of total	Mortality
I	50	45·0	26 %
II	25	22·5	28 %
III	5	4·5	80 %
IV	31	28·0	25·8 %

Thus Group I was the dominant and typical type, but its members are less virulent than those of Groups II and III, whilst Group IV, consisting of the heterologous types, was the least virulent of the four types.

Dochez and Avery (1915), working in New York, also found four serological types of pneumococci obtained from cases of lobar pneumonia.

Their results for 1912–13 and 1913–14 may be tabulated thus (Table VI):

TABLE VI.

	1912–13	1913–14
Group I	35 = 47 %	21 = 30 %
Group II	13 = 18 %	28 = 39 %
Group III (*mucosus*)	10 = 13 %	6 = 8 %
Group IV (heterogeneous)	16 = 22 %	16 = 23 %
Total typical	58 = 78 %	55 = 77 %
Total heterogeneous	16 = 22 %	16 = 23 %
Total	74	71

Neufeld and Händel (1910) demonstrated the existence of certain varieties of pneumococcus which were fundamentally different in their immunological reactions. Dochez and Gillespie (1913) applied the methods of protection and agglutination to the investigation of the pneumococci obtained from a series of human cases of pneumonia. In this way they were enabled to classify the pneumococci associated with lobar pneumonia in man into four groups. Groups I and II consisted of members closely related immunologically to other members of their respective groups; Group III was formed of the *Pneumococcus* or *Streptococcus mucosus*; and in Group IV the remainder were combined to form a heterogeneous group. In 35 instances they found that in 4 per cent. the organisms belonged to Group I; in 13 instances 18 per cent. belonged to Group II; in ten instances 13 per cent. to Group III and in 16 instances 22 per cent. to Group IV.

TABLE VII.

	Pneumococcus										
	Subgroup II A				Subgroup II B			Subgroup II X			Group II
Anti-pneumococcus Serum II absorbed by	Jn.	As.	L.	M.	W.	Ar.	J.	S. 13	F.C.B.	H.	Type II
Subgroup A. Jn.	−	−	−	−	++	+±	++	++	+	++	++
„ B. W.	++	++	++	+±	−	−	−	++	+	++	++
„ X. S 13	++	++	++	+±	++	++	++	−	+	++	++
Group II	−	−	−	−	−	−	−	−	−	−	−

Cole (1914) was also able to differentiate pneumococci into four groups according to their agglutination reactions and their protective power, and the methods of absorption were then applied to the pneumococci so grouped. Table VII gives the results of some of his absorption experiments with these three sub-groups of Type II which he designated sub-groups II A, II B, and II X respectively.

The organisms in these three sub-groups gave: (*a*) Agglutination with anti-pneumococcus serum II: (*b*) Protection with anti-pneumococcus serum II, except sub-group II X, and (*c*) Absorption of anti-pneumococcus serum II with a member of sub-groups II A or II B removes only the antibodies for the homologous sub-group and absorption of anti-pneumococcus serum II with any given number of sub-group II X removes the antibodies for that particular strain only.

That these three sub-groups possess specific differential characters is shown by the following facts: (*a*) the organisms of any sub-group are not agglutinated by the anti-sera of the other two-groups: (*b*) they are not protected against by the sera of other sub-groups: (*c*) they do not absorb from anti-pneumococcus serum II the specific immune bodies of the other sub-groups.

Chickering (1915) went further and demonstrated the specific absorption of immune substances from polyvalent anti-pneumococcus serum, not only by living bacteria, but also by bacterial extracts. He showed that a polyvalent anti-pneumococcus serum may be specifically exhausted of its immune bodies for one of the types of the pneumococcus by the addition of a bacterial extract of the corresponding type, and that the immune substances of the other type remain intact and can be removed subsequently by the addition of the appropriate antigen.

MENINGOCOCCUS AND ALLIED ORGANISMS.

Albrecht and Gohn (1901) were the first to apply the methods of agglutination to the meningococcal group of micro-organisms and showed that it was possible to obtain a specific agglutinating serum for the meningococcus by the intraperitoneal injection of the meningococcal cultures. This work was confirmed and extended by others especially by Lieberknecht (1909) who showed conclusively that, by the employment of serological methods the true meningococcus could be identified and differentiated from the other gram-negative cocci that are frequently found inhabiting the nasopharynx of normal individuals.

The close relationship that exists amongst the Gram-negative group of diplococci is well shown by a study of their morphological cultural and agglutinating properties, which makes them an admirable group for investigation by the absorption method.

Dopter and Koch (1908) showed that anti-meningococcus serum will agglutinate both meningococci and gonococci, and similarly anti-gonococcal serum will agglutinate both gonococci and meningococci. By the absorption method they showed the anti-meningococcus serum

contains a specific agglutinin for meningococci and a non-specific agglutinin for gonococci and that the inverse proposition is true for anti-gonococcal serum. Hence this method demonstrates the fact that these organisms are two specifically distinct germs.

Elser and Huntoon (1909) repeatedly confirmed and amplified Lieberknecht's work and came to the conclusion that gram-negative cocci, having the cultural and fermentative reactions of the meningococcus, are not necessarily that organism. They also imply, although they do not definitely state, that the identification of the meningococcus by means of the agglutination and absorption tests is not always reliable. Elser and Huntoon (1909) further suggested that the term meningococcus was applied to a group of organisms though they did not go so far as to divide that group into definite sub-groups or "types." Tulloch (1917) suggests that their failure to indicate definitely the sub-divisions of the groups may have depended to some extent upon the fact that the sera which they used in their agglutination and absorption tests were of very variable titre, thus introducing possible errors of technique which are difficult to control.

By adopting the absorption test, Darre and Dumas (1914) found a new species of parameningococcus, and came to the conclusion that several varieties certainly exist, just in the same way as there exist several varieties of the paratyphoid bacilli. They state that it is only by the application of the saturation of agglutinins that the differentiation of these diverse species becomes possible.

Dopter and Pauron (1914) applied the saturation test both of agglutinins and precipitins to the differentiation of the meningococcus and parameningococcus.

Dopter and Pauron (1914) also differentiated the parameningococci into three types by means of the saturation of agglutinins, and named these types parameningococcus α, parameningococcus β and parameningococcus γ, respectively.

Gordon (1915) applied the absorption test in order to determine whether the capacity of the meningococcus for absorbing its own ag glutinin could be used practically for the purpose of identifying the micro-organisms of the outbreak of that year. The preliminary observations dealt with in this communication appeared to suggest that the capacity of the meningococcus for absorbing its own agglutinin can usefully be employed for identifying that organism. He states that it must be borne in mind that possibly and even probably there are several different strains of the meningococcus capable of producing meningitis,

and that some of these meningococci may not absorb the specific agglutinin of others. How many different strains there are at work at present is a matter of prime importance and further observations are needed for the practical application and limits of the absorption test for the purpose of identifying the meningococcus can be regarded as defined.

These further observations have now been supplied by Gordon and Murray (1915) and by Tulloch (1917). The former observers isolated meningococci from the cerebro-spinal fluid of cases of cerebro-spinal fever, and found that the organisms so obtained could be resolved by the absorption of agglutinin test into four groups, viz. I, II, III, IV. Of the 32 meningococci in question 19 belonged to Group I, 8 to Group II, 4 to Group III and 1 to Group IV. Further, of 9 specimens of gram-negative cocci isolated from the nasopharynx of contacts and suspected cases, and closely resembling the meningococcus in morphological, cultural, staining, and fermentative characters, by the application of the absorption test five were found to be identical with Group II and one with Group I of the cerebro-spinal fluid meningococci.

TABLE VIII.

Type II Serum.

	Agglut. 1/50 for 24 hrs. at 37° C.	Unsaturated — Test coccus added 1/100	1/200	1/300	1/400	Saturated — Homologous coccus added 1/100	1/200	1/300	1/400	Saturated — Test coccus added 1/100	1/200	1/300	1/400
2 Gar.	−	−	−	−	−	+++	+++	+++	+++	−	−	−	−
11 „	−	−	−	−	−	+++	+++	+++	+++	−	−	−	−
(*g*) Br. F.	(+)	−	−	−	−	+++	+++	+++	+++	−	−	−	−
(*f*) Bow.	−	−	−	−	−	+++	+++	+++	+++	−	−	−	−
(*h*) Cr.	++	−	−	−	−	+++	+++	+++	+++	−	−	−	−
(*i*) Y.	−	−	−	−	−	+++	+++	+++	+++	−	−	−	−
(*j*) Pon.	+	−	−	−	−	+++	+++	+++	+++	−	−	−	−
Control type I	−	−	−	−	−	+++	+++	+++	+++	−	−	−	−
„ „ II	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−
„ „ III	−	−	−	−	−	+++	+++	+++	+++	−	−	−	−
„ „ IV	++	+	−	−	−	+++	+++	+++	+++	−	−	−	−

By the use of similar methods in the investigation of the meningococci obtained from the cerebro-spinal fluid in 107 cases during the current outbreak of cerebro-spinal fever, Tulloch (1917) found that the organisms described by Gordon as Types I, II, III and IV meningococcus comprise all the organisms responsible for all the cases of cerebro-spinal fever with the exception of three, in which the organism could not definitely be typed, and three others, in which the investigation could

not be completed owing to the organisms having died before the complete series of tests was applied to them. The subdivision of the group meningococci into the types described finds full justification. He calls special attention to the fact that the technique employed was, as far as possible, standardized, and the following is one of the many tables given showing the kind of results obtained.

TETANUS.

Tulloch (1917) in an investigation on the bacteriology of wound infections in cases of tetanus applied serological methods to the differentiation of *B. tetani*. Simple agglutination showed that at least three serological types of this organism exist capable of producing a tetanizing poison. Absorption of agglutinin tests were therefore carried out.

TABLE IX.

Saturation of "U.S.A." Agglutinating Serum.

	Unsaturated serum			Saturated serum, homologous bacilli added			Saturated serum, test bacillus added		
	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400
U.S.A. II	++	++	++	−	−	−	−	−	−
U.S.A. (whole)	++	+	+	+	−	−	−	−	−
T 67 R viii	−	−	−	++	++	++	−	−	−
T 80 B i	−	−	−	++	++	++	−	−	−
R 220 Sp	−	−	−	++	++	++	−	−	−
T 72 B.R.A.	−	−	−	++	++	++	−	−	−

Saturation of "T 67" Agglutinating Serum.

	Untreated serum			Saturated serum, homologous bacillus added			Saturated serum, test bacillus added		
	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400
U.S.A. II	−	−	−	++	++	++	−	−	−
U.S.A. (whole)	−	−	−	++	++	++	−	−	−
T 67 R viii	++	++	++	−	−	−	−	−	−
T 80 B i	++	++	+	−	−	−	−	−	−
R 220 Sp	−	−	−	++	++	++	−	−	−
T 72 B.R.A.	−	−	−	++	++	++	−	−	−

Saturation of "R 220" Agglutinating Serum.

	Untreated serum			Saturated serum, homologous bacillus added			Saturated serum, test bacillus added		
	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400
U.S.A. II	−	−	−	++	++	++	−	−	−
U.S.A. (whole)	−	−	−	++	++	++	−	−	−
T 67 R viii	−	−	−	++	++	++	−	−	
T 80 B i	−	−	−	++	++	++	−	−	−
R 220 Sp	++	++	++	−	−	−	−	−	−
T 72 B.R.A.	++	++	++	−	−	−	−	−	−

Each serum was absorbed by adding to it emulsions of six organisms —two representatives of each serological type—2·5 cc. of 2000 million emulsion being allowed for each 0·5 cc. of serum. The tubes were incubated for twenty-four hours at 37° C. were then spun and the clear supernatant fluid was used to agglutinate the homologous organism and also the test organism in dilutions of $\frac{1}{100}$, $\frac{1}{200}$, and $\frac{1}{400}$. The results were controlled by reactions with unabsorbed serum in each case.

Table IX giving the results obtained, clearly proves the existence of three types of *B. tetani* serologically distinct from each other.

PLAGUE.

Agglutination and absorption tests have also been employed in the investigation of plague bacilli in Malta by Zammit and Broughton-Alcock (1917), making use of the time measurement slide method of the latter investigator (*vide infra*). Five human cases of plague were studied, one ending fatally, and of 1000 rats examined 15 were found to be infected with *B. pestis*. The human serum agglutinated the *B. pestis* isolated from man more readily than either of two strains isolated from rats and the serum of the fatal case gave a higher specific agglutination action than the others.

Castellani's absorption test was carried out by first determining the degree of agglutinating action present in the sera. The dilution giving complete agglutination in one minute was employed. Three drops of the serum diluted to half this dilution factor was mixed in a sedimentation tube with three drops of the growth formalinized in broth or agar emulsion. At the end of 24 hours at the laboratory temperature it was found that the *B. pestis* isolated from man and rats removed the specific agglutinin previously present for all in human or prepared animal anti-plague serum.

DIAGNOSIS OF MIXED INFECTIONS OF CLOSELY ALLIED BACTERIAL SPECIES PHENOMENON.

Having discovered the absorption of agglutinins as a method of distinguishing between specific and non-specific agglutinins Castellani (1902) then applied it to:

(1) The sero-diagnosis of mixed infections, and

(2) The differentiation of closely allied species and types of bacteria.

It now remains to point out the precise manner in which the test may be applied to these two conditions and finally to give a brief description of the technique which is employed in the performance of the test.

THE SERO-DIAGNOSIS OF MIXED INFECTIONS.

The blood of a patient, suspected to be suffering from enteric, contains a fairly large amount of agglutinins for *B. typhosus* and *B. paratyphosus* B. Is it a case of mixed infection typhoid + paratyphoid B? Or is it a case of typhoid with secondary, non-specific agglutinins for *B. paratyphosus* B? Or a case of paratyphoid B, with secondary non-specific agglutinins for *B. typhosus*? Castellani's absorption method will generally enable us to answer these questions, as it will show which are the *specific* agglutinins present.

(1) Let us assume we have had the following results:

After saturation with *B. typhosus*, the typhoid agglutinin and the paratyphoid agglutinin have disappeared completely or nearly so; after saturation with *B. paratyphosus* B, the paratyphoid agglutinin has disappeared completely or nearly so, while the typhoid agglutinin remains in practically the same amount. These results show that the typhoid agglutinin is the specific one, and therefore the case is one of typhoid with non-specific agglutinins (co-agglutinins), for *B. paratyphosus* B. It is not a case of mixed infection of typhoid + paratyphoid B.

(2) Let us assume we have had the following results:

After saturation with *B. typhosus*, the typhoid agglutinin has disappeared or nearly so, while the paratyphoid B agglutinin remains in practically the same amount. After saturation with *B. paratyphosus* B, both the paratyphoid B agglutinin and the typhoid agglutinin have disappeared completely or nearly so. These results mean that the paratyphoid B agglutinin is the specific one and that the case is one of paratyphoid B with secondary non-specific agglutinins for *B. typhosus*.

(3) Let us assume that we have had the following results:

After saturation with *B. typhosus* the typhoid agglutinin has disappeared completely or nearly so—while the paratyphoid B agglutinin remains in the same amount or nearly so; after saturation with *B. paratyphosus* B, the typhoid agglutinin remains practically unchanged. The two agglutinins disappear on saturation with *B. typhosus* and *B. paratyphosus* B. These results show that both the typhoid and the paratyphoid agglutinins present are specific, and that the case is according to all probabilities one of mixed infection typhoid + paratyphoid B.

THE DIFFERENTIAL DIAGNOSIS OF CLOSELY ALLIED BACTERIAL SPECIES AND TYPES.

Suppose we have isolated a bacillus with the cultural and biochemical characters of *B. paratyphoid* B, and that the bacillus is well agglutinated by a paratyphoid B serum: is it *B. paratyphosus* B? Or is it *B. aertryke*, which as is well known has all the cultural and biochemical characters of *B. paratyphosus* B, and is well agglutinated by paratyphoid B serum? Castellani's absorption method will enable us to give a definite answer. If the bacillus we have isolated is really *paratyphosus* B, we shall have the following results: paratyphoid B serum saturated with organism we have isolated will lose, completely or nearly so its agglutinin for *paratyphosus* B, and also its agglutinin for *B. aertryke*. If the germ we have isolated is *B. aertryke*, paratyphoid B serum saturated with the germ we have isolated will lose its agglutinating power completely or very nearly so on *B. aertryke*, but will not lose or only to a slight extent its agglutinating power on *B. paratyphosus* B.

TECHNIQUE.

I. THE SATURATION METHOD.

The test should be carried out by employing the technique described by Taylor (1918) as follows: Take (say) a paratyphoid B serum agglutinating powerfully both the *B. paratyphosus* B and *B. aertryke,* the agglutination titre for both germs being > 1 in 10,000, and proceed as follows:

(1) Dilute the serum with normal saline to have 1 in 50 dilution; put 2·5 cc. of the diluted serum in a sterile centrifuge tube, which label "tube No. 1." Put the same amount of diluted serum (2·5 cc.) in another centrifuge tube which label "tube No. 2."

(2) Scrape off with a platinum wire the growth of 4 or 5 agar slope cultures of *B. paratyphosus* B and add it to the serum in No. 1 tube. Do not wash off the growth with the diluted serum.

(3) Scrape off, with a platinum wire, the growth of 4 or 5 agar slope cultures of *B. aertryke* and add it to the serum in tube 2. Do not wash off the growth with the diluted serum.

(4) After incubating both tubes at 37° C. for two hours centrifuge them until the whole of the bacilli are precipitated and the supernatant fluids are clear.

(5) Pipette off the supernatant fluids into two separate sterile tubes and test the agglutinating action of both fluids up to a dilution of 1 in

10,000 against both *B. paratyphosus* B and *B. aertryke* remembering of course that the serum is already diluted 1 in 50.

The serum before absorption agglutinated both *B. paratyphosus* B and the *B. aertryke* in very high dilution (> 10,000). After absorption with *B. paratyphosus* B the titre for both organisms will be practically reduced to nil or nearly so (> 400). After absorption with *B. aertryke* the titre for this bacillus will be practically reduced to nil or nearly so (> 400), while the titre for the paratyphoid B bacillus will be unchanged or only slightly lessened.

Finally, the dosage of bacilli necessary to produce saturation or complete absorption of the homologous agglutinin will vary with the agglutination titre of the serum employed. This is well exemplified in the following table from Bainbridge and O'Brien (1911):

TABLE X. *The effect of absorbing a serum with varying amounts of heterologous bacilli.*

The volume of serum used was constant, namely 2 cc. of 1 in 10 dilution; the amount of bacilli added varied from 2 to 8 agar slopes of approximately uniform size and density of growth.

A *B. suipestifer* serum was absorbed with *B. paratyphosus* B.

Serum	Agglutination limits after absorption	
	B. suipestifer	*B. paratyphosus* B.
B. suipestifer serum. Original titre	20,000	5000
„ absorbed with 2 slopes	20,000	<100
„ „ „ 4 „	20,000	<100
„ „ „ 8 „	20,000	—

From this and other similar experiments it is seen that the amount of bacilli to be added must vary according to the agglutinating titre of the serum; the higher the titre the greater the quantity of bacilli to be added.

II. THE SUPERSATURATION METHOD.

By supersaturation the principle of Castellani's absorption method is carried one step further.

Harvey and Wood (1911) showed that an excess of micro-organisms other than *B. paratyphosus* A removed or reduced the amount of detectable specific agglutinin for *B. paratyphosus* A present in the serum.

Broughton-Alcock (1917) confirmed and extended this observation and showed that in a similar manner supersaturation of a serum with emulsions of other than the corresponding micro-organisms removes or

reduces the detectable specific agglutinins for *B. dysenteriae*, Shiga, Flexner-Hiss and M. Melitensis. Based on these facts he has evolved a quantitative method which includes in its technique the utilisation of a practically defined amount of micro-organisms and a practically defined agglutination content of the serum.

By the employment of this method he was able to distinguish the specific agglutinin for *B. aertryke* from the agglutinin for a *B. paratyphosus* B strain due to previous T.A.B. inoculation, whilst an emulsion of *B. Gaertner* did not remove either agglutinin.

This method also showed that the specific agglutinin for *B. typhosus* developed in serum in response to anti-typhoid vaccine inoculation might be temporarily increased by inoculation with anti-paratyphoid vaccine. It was also found that specific agglutinins for *B. typhosus* or *B. paratyphosus* B were not reduced by supersaturation with any other than their respective emulsions. This fact permits the use of the supersaturation absorption test to determine whether specific agglutinins for *B. typhosus* or *B. paratyphosus* B are present in a given serum.

In conclusion, having shown what a large amount of valuable work has already been done and how extensive is the field of investigation opened up by the method of absorption of agglutinins, there can be little doubt that this method will be more and more extensively employed by serological and bacteriological workers in the future.

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