THE IMMUNITY REACTIONS OF AN INAGGLUTINABLE STRAIN OF *B. TYPHOSUS.*

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(With 2 Charts.)

INAGGLUTINABLE strains of *B. typhosus* are of considerable interest to bacteriologists both from a practical and theoretical aspect. These strains present the usual characteristics of the typhoid bacillus, with the exception that they are not agglutinated by anti-typhoid sera, and if such a contingency be overlooked one may fail to recognise that one is dealing with a typhoid infection. This feature persists in the sub-cultures for many months, thus differentiating the inagglutinable strains from the many strains of *B. typhosus* which agglutinate with difficulty when freshly isolated but which regain their full agglutinability after one or two subcultures. Since their discovery these inagglutinable strains have been investigated on several occasions without any definite agreement as to the condition being arrived at.

The isolation of inagglutinable or feebly agglutinating strains of *B. typhosus* has been recorded by Achard and Bensaude (1896), Kolle (1897), Johnston and Taggart (1897), Van de Velde (1897), Sacquépée (1901), Remy (1901), Rodet (1902), Nicolle and Trenel (1902), Lipschütz (1904), Klinger (1902), Lesieur (1903) and others. Although a certain number of the above were obtained from chronic typhoid lesions, there is no evidence to show that there is any relation between inagglutin-ability and chronicity of infection. At present there is no record of the isolation of an inagglutinable strain from a "typhoid carrier." The strain on which the following observations were made was isolated from an acute case of typhoid fever.

Nicolle considers that there is a close relation between agglutination and motility, and the production of agglutinins. He worked with a non-motile strain which on injection into rabbits produced agglutinins neither for itself nor for the laboratory strain. On the other hand Remy states that inagglutinable strains readily produce agglutinins while Rodet doubts whether there is any relation between the agglutinogenic function and agglutinability. The latter found that an inagglutinable strain of *B. prodigiosus* was capable of producing on injection agglutinins for normal *B. prodigiosus*.

It would therefore appear that inagglutinability arises as the result of an increased resistance on the part of the bacilli to anti-bodies secreted against them. The artificial production of inagglutinable strains by Sacquépée, Walker (1902), and Müller (1903), support this view.

The inagglutinable strain, which forms the subject of this paper, was isolated from a patient whose clinical history was briefly as follows:

The patient was a boy (J. S.) aged 12 who had been out of sorts for a week or so previously to being seen on Sept. 30th, 1912. He was one of the earlier patients to be attacked in the recent Aberdeen epidemic of typhoid fever (1912–1913).

The clinical features of the case may be briefly summed up as being those of a continued fever with rapid pulse, showing symptoms and physical signs of capillary bronchitis with enlarged spleen. During the second week of high fever, diarrhoea set in with pea-soup stools. Deafness intervened during the third week but gradually disappeared during the ensuing weeks. The Widal reaction was first found to be positive on Nov. 4th, 1912. Previous tests had been negative while the blood also had failed to react to Paratyphoid B. We present a temperature chart of the case. On Oct. 16th a sample of blood was taken aseptically (10 c.c.) from the right median basilic vein and sown into sterile broth. The sample was incubated overnight at 37° C. A transplantation was made on to MacConkey bile salt agar plates and ultimately a bacillus was isolated from the blood with the cultural reactions of *B. typhosus*.

The patient's serum agglutinated the laboratory strain of *B. typhosus* in a dilution of 1 in 50 within five minutes, but failed altogether to agglutinate the bacillus recovered from the veno-puncture in a large series of dilutions. The infection was considered then a case of typhoid fever arising from an inagglutinable strain of *B. typhosus*.

Characters of the strain isolated from the case.

The organism isolated presented all the characteristics of *B. typhosus* with the single exception that it was not agglutinated by an anti-typhoid serum, as the following experiments show.
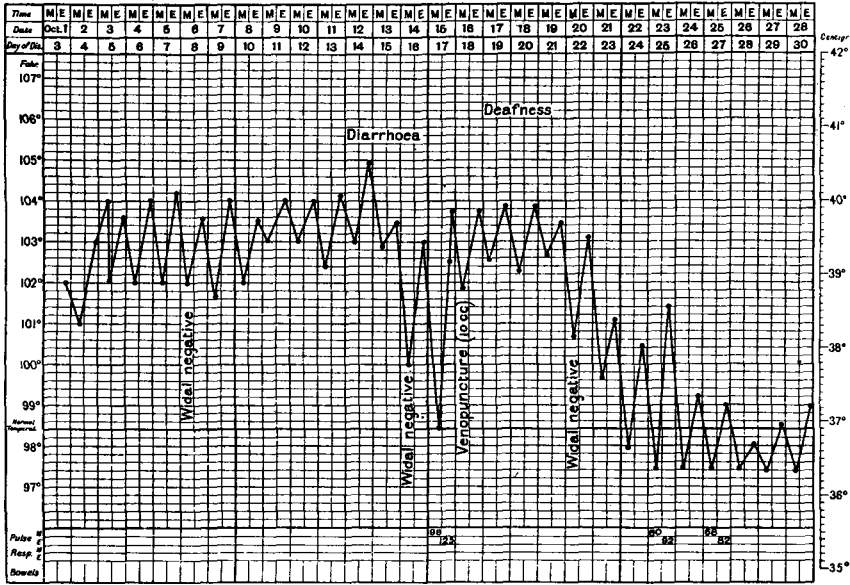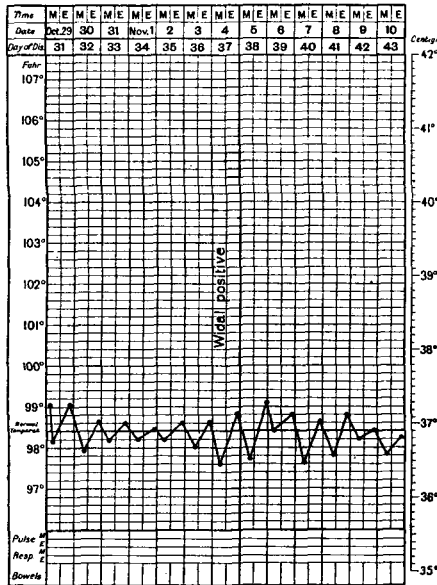
Chart 1.

Chart 2.

The following table gives the fermentation reactions of the in-agglutinable typhoid bacillus. (Tested on five occasions.)

	Nov. 1 (24 hrs.)	Nov. 4	Nov. 5	Nov. 7	Nov. 14
Glucose	A	A	A	A	A
Lactose	—	—	—	—	—
Dulcite	—	—	—	—	—
Saccharose	—	—	—	—	—
Mannite	A	A	A	A	A
Maltose	A	A	A	A	A
Dextrine	A ?	A	A	A	A
Galactose	A ?	A	A	A	A
Sorbite	A	A	A	A	A
Inulin	—	—	—	—	—
Raffinose	—	—	—	—	—
Arabinose	—	—	—	—	—
Laevulose	A	A	A	A	A
Salicin	—	—	—	—	—
Erythrite	—	—	—	—	—
Milk	A ?				
Milk (first sub-culture)	A	A (pinkish colour, no clot)			

Subcultures made four months later showed no change in the fermentation reactions.

Agglutinability.

An anti-typhoid serum with a titre of about 1 in 5000 failed to agglutinate the bacillus in dilutions beyond 1 in 40, even after 24 hours.

Experimental Part.

Agglutinogenic function.

The problem that suggested itself to us was :—Could an inagglutin-able strain of *B. typhosus* injected into an animal produce an anti-typhoid serum which would fail to agglutinate its own antigen yet agglutinate easily a normal strain of *B. typhosus*?

Injections were made into rabbits. The first injection consisted of an emulsion in sterile normal saline of half an agar slope culture (24 hours growth) of the inagglutinable *B. typhosus* killed by heating to 60° C. for 45 minutes. The inoculation was made intraperitoneally. The second injection made eight days later was similar but consisted of two agar slopes; the third injection made a week later consisted of

three agar slopes. Other two rabbits which were treated intravenously with an emulsion of the bacillus unfortunately died before their sera had acquired any great agglutinating power. Samples of blood were taken from the animals from time to time and tested. It was noted that the serum always failed to agglutinate its own antigen in a dilution of 1 in 50 and upwards, while an increasing capacity to agglutinate the normal *B. typhosus* was observed. Ultimately when the serum of a rabbit had acquired a titre of 1 in 1600 towards the normal typhoid bacillus, the animal was killed, the blood gathered aseptically, and the serum separated and stored.

The following tables give the reactions of the serum to its own antigen and to a normal agglutinating strain of *B. typhosus*. The emulsions of bacilli used in the test were prepared by emulsifying a 24 hours agar slope growth in normal saline or in carbolic saline (0·5 %) and the test carried out in small tubes.

The signs + and − denote positive and negative reactions respectively.

Macroscopic method.

B. typhosus (inagglutinable).

Serum dilutions	1–30	1–40	1–50	1–100	1–200	1–400	1–800	1–1600	1–3200
Agglut.	+	−	−	−	−	−	−	−	−

B. typhosus (agglutinating strain).

Serum dilutions	1–30	1–40	1–50	1–100	1–200	1–400	1–800	1–1600	1–3200
Agglut.	+	+	+	+	+	+	+	+	+

Microscopic method.

Tested by the hanging drop method where the criterion of clumping was taken to be the picture presented of obvious clumping under the low power of the microscope.

Reading after 30 mins.

B. typhosus (inagglutinable).

Serum dilutions	1–20	1–30	1–40	1–100	1–200	1–400	1–800	1–1600
Agglut.	+	−	−	−	−	−	−	−

B. typhosus (agglutinating strain).

Serum dilutions	1–20	1–30	1–50	1–100	1–200	1–400	1–800
Agglut.	+	+	+	+	+	+	+

Reading after 2 hours.

B. typhosus (inagglutinable).

Serum dilutions	1–20	1–30	1–40	1–100	1–200	1–400	1–800	1–1600
Agglut.	+	+	−	−	−	−	−	−

B. typhosus (agglutinating).

Serum dilutions	1–20	1–30	1–50	1–100	1–200	1–400	1–800
Agglut.	+	+	+	+	+	+	+

This serum therefore, prepared by the injection of the inagglutinable strain of *B. typhosus*, only agglutinates its own antigen in dilutions up to 1 in 30 in 24 hours by the macroscopic method, while the same serum can agglutinate the normal agglutinable strains of *B. typhosus* up to 1 in 1600. Consequently we may conclude that by the injection of the inagglutinable strain of typhoid bacilli an anti-serum can be produced which will not agglutinate its homologous strain but will agglutinate a heterologous agglutinable laboratory strain.

Accordingly an inagglutinable strain of *B. typhosus* can on injection elicit the production of agglutinins for agglutinable strains of the bacillus and not for itself; in other words the inagglutinable strain has agglutinogenic functions.

We then proceeded to determine the presence or absence of group agglutinins in the anti-serum.

B. coli.

Serum dilutions	1–50	1–100	1–200	1–400	1–800	1–1600	1–3200
Agglut.	–	–	–	–	–	–	–

B. paratyphosus (B).

Serum dilutions	1–50	1–100	1–200	1–400	1–800	1–1600	1–3200
Agglut.	–	–	–	–	–	–	–

B. gaertner.

Serum dilutions	1–40	1–80	1–160	1–320	1–640	1–1280	1–2560
Agglut.	+	+	+	+	–	–	–

B. paratyphosus (A).

Serum dilutions	1–25	1–50	1–100	1–200	1–400	1–800	1–1600	1–3200
Agglut.	–	–	–	–	–	–	–	–

B. dysenteriae (Flexner).

Serum dilutions	1–25	1–50	1–100	1–200	1–400	1–800	1–1600	1–3200
Agglut.	+	+	+	–	–	–	–	–

The injection therefore of an inagglutinable strain of typhoid into an animal produces an anti-serum which contains the group agglutinins usually present in an anti-typhoid serum. The peculiarity here is that the inagglutinable typhoid bacillus functionating as an antigen can produce a group agglutinin of higher specificity for a heterologous bacillus than for the homologous bacillus.

Inagglutinability.

It is now usually recognised that the mechanism of agglutination, as first pointed out by Bordet, consists of two stages (1) fixation, (2) aggregation. The question then presents itself: Does the inagglutinable strain of *B. typhosus* fix (absorb) typhoid agglutinin in a manner similar to that of the normal agglutinating typhoid bacillus?

(1) *Absorption.* The first investigation on this problem was made with the idea of ascertaining whether the agglutinin in the inagglutinable serum is capable of being absorbed by the inagglutinable strain.

Experiment 1. Equal quantities of the serum were measured out in sterile tubes and labelled "A" and "B."

To "A" four loops of culture of *B. typhosus* (inaggl.) were added and emulsified. To "B" no addition was made. Both were put in the incubator at 37° C. for two hours, then centrifugalised and equal quantities of the clear supernatant fluid pipetted off and dilutions made with sterile normal saline.

"A." Saturated with *B. typhosus* (inagglutinable).

Serum dilutions	1–80	1–160	1–320	1–640	1–1280	1–2560	1–5120
Agglut. of normal bac.	−	−	−	−	−	−	−

"B." Same serum not saturated.

Serum dilutions	1–80	1–160	1–320	1–640	1–1280	1–2560	1–5120
Agglut. of normal bac.	+	+	+	+	+	−	−

As the inagglutinable strain, therefore, absorbed the agglutinin from the homologous anti-serum, it was next resolved to test whether the absorptive power of the inagglutinable strain was greater or less than that of the normal strain.

Experiment 2. In this experiment the absorptive power both for homologous and heterologous agglutinins was tested: 1 c.c. of the immune serum was mixed with an equal bulk of the bacillary emulsion. The emulsion was prepared by adding 4 c.c. of normal saline to a 24 hours agar slope growth, and shaking till thoroughly broken up. The emulsions were standardised either by counting or by the opalescence method so that the number of bacteria present corresponded in each test as far as possible. The mixtures were kept for $2\frac{1}{2}$ hours in the incubator, then centrifugalised, the clear fluid pipetted off and amount of agglutinin present estimated.

The anti-typhoid serum used had a titre of 1 in 5000 and the homologous serum a titre of 1 in 1250 for a normal strain of *B. typhosus*.

	Agglut. before absorption	After absorption with normal typhoid	After absorption with inagglut. typhoid
Anti-typhoid serum (dil. 1 in 4)	1–5000	1–320	1–160
Anti-inagglut. serum ...	1–1250	1–160	1–80
Anti-typhoid serum (dil. 1 in 10)	—	1–600	1–320
Anti-inagglut. serum ...	—	1–320	1–160
Anti-typhoid serum (dil. 1 in 30)	—	1–880	1–220
Anti-inagglut. serum ...	—	1–440	1–220

The above experiments show that there is no loss of the absorptive power for agglutinin. There is indeed some evidence of increased absorptive power.

It was also found that the inagglutinable bacillus could absorb the group agglutinins for the *B. gaertner*, as the following experiment shows.

Anti-inagglutinable bacillus serum (after absorption by the inagglut. bacillus):

Serum dilutions	1–80	1–160	1–320	1–640	1–1280	1–2560
Agglut. of *B. gaertner*	–	–	–	–	–	–

Ditto (before absorption by the inagglut. bacillus):

Serum dilutions	1–80	1–160	1–320	1–640	1–1280	1–2560
Agglut. of *B. gaertner*	+	+	+	–	–	–

An attempt was then made to discover whether the converse was true, namely whether the inagglutinable bacillus after complete saturation with agglutinin behaved as the normal bacillus on digestion with saline.

Exp. Equal quantities of an anti-typhoid serum (1·0 c.c. of a 1 in 4 dilution) were placed in two centrifuge tubes; to one tube "A," 0·25 c.c. of an emulsion of the inagglutinable bacillus were added, and to the other, "B," a corresponding amount of the agglutinable bacillus. The tubes were then placed in the incubator at 37° C. for 2½ hours, at the end of which time they were centrifugalised and the fluid pipetted off. About 1·0 c.c. of saline was added to each, the tubes shaken and immediately centrifugalised and the saline pipetted off. In this way the greater part of the remaining anti-serum was removed. To each deposit of bacilli 1·0 c.c. of saline was added, the tubes well shaken and then allowed to digest at 37° C. for three hours. They were then centrifugalised and the agglutinating power of the fluid tested. Finally the deposit was digested with 1·0 c.c. of saline overnight and the agglutinating power of the supernatant liquid tested.

First digestion of saturated inagglut. bacillus. (A):

Dilutions	1–8	1–16	1–32	1–64	1–128	1–256
Agglut.	++++	++++	+++	++	0	0

First digestion of saturated agglut. bacillus. (B) :

Dilutions	1–8	1–16	1–32	1–64	1–128	1–256
Agglut.	++++	++++	++++	+++	+	0

Digestion over night.

(A)

Dilutions	1–8	1–16	1–32	1–64	1–128	1–256
Agglut.	++++	++++	++	0	0	0

(B)

Agglut.	+++	++	+	0	0	0

In the above experiment both the inagglutinable and agglutinable bacillus behave with regard to the liberation of absorbed agglutinin in a similar manner, perhaps with the inagglutinable bacillus the fixation is not quite so firm, as in the digestion overnight slightly more agglutinin was liberated.

(2) *Aggregation.* From Bordet's and Gengou's researches it would appear that the determining factor in the aggregation of bacteria in agglutination is a change in the surface tension of the fluid in which they are suspended. The addition of certain substances which bring about agglutination (chemicals or specific sera) do so by modifying the molecular relations between the particles and the fluid.

More recently it has been suggested that all particles suspended in a liquid remain separate on account of a repellant electrical charge carried by the particles. Many bacteria and electro-negative colloids are precipitated by acids, that is, by the positively laden H-ions.

On the above hypothesis Michaelis (1911) and his pupil Beniasch (1912) showed that certain bacteria, especially those of the *typhoid-colon* group, were precipitated by definite H-ion concentrations, each having an optimum concentration of precipitation. This reaction they say is of a highly specific nature, and depends on the same phenomena as serum agglutination.

The following experiments show the behaviour of the inagglutinable strain of typhoid towards these chemical agglutinants.

Saffranin. Both the normal and the inagglutinable strains were agglutinated by saffranin in dilutions up to 1 in 3000.

Formalin. With formalin no difference was observed between the two strains. With a 50 % dilution no apparent agglutination beyond a slight formation of clumps was seen.

Acid agglutination. In applying the acid agglutination test concentrations of acetic and lactic acids were employed according to the formulae of Michaelis, Beniasch, and Heinmann (1913).

Exp. Acetic acid. A series of six concentrations of H-ions was made up according to Michaelis and their effects on the inagglutinable and normal bacilli tested. About 1 c.c. of each concentration was placed in a series of small test tubes and then 0·1 of the constant bacillary emulsion added to each.

Series :—	1	2	3	4	5	6
n. NaOH	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.
n. CH_3COOH	7·5 c.c.	10 c.c.	15 c.c.	25 c.c.	45 c.c.	85 c.c.
Water dis.	87·5 c.c.	85 c.c.	80 c.c.	70 c.c.	50 c.c.	10 c.c.
H-ion concn.	$1·0 \times 10^{-5}$	2×10^{-5}	4×10^{-5}	8×10^{-5}	$1·6 \times 10^{-4}$	$3·2 \times 10^{-4}$
B. typhosus inaggl.	0	0	++++	+++	+	0
B. typhosus lab.	0	+	++++	+++	++	0
B. typhosus Smith	0	+	++++	+++	++	0

++++ = complete sedimentation. The results were read after 2 hours at 37° C.

Exp. Lactic acid. The concentrations in this series were made up according to the formula of Heinmann, the technique being otherwise similar to that of the previous experiment.

Series :—	1	2	3	4	5	6	7	8
n/10 Sod. lactate	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.
n/10 Lactic acid	0·12 c.c.	0·25 c.c.	0·5 c.c.	1·0 c.c.	—	—	—	—
n/1 Lactic acid	—	—	—	—	0·2 c.c.	0·4 c.c.	0·8 c.c.	1·6 c.c.
Water dist.	1·48 c.c.	1·35 c.c.	1·1 c.c.	0·6 c.c.	1·4 c.c.	1·2 c.c.	0·8 c.c.	0·0 c.c.
(H)	$3·5 \times 10^{-5}$	7×10^{-5}	$1·4 \times 10^{-4}$	$2·8 \times 10^{-4}$	$5·5 \times 10^{-4}$	$1·1 \times 10^{-3}$	$2·2 \times 10^{-3}$	$4·4 \times 10^{-3}$
B. typhosus inagglut.	++	++++	++++	+++	0	0	0	0
B. typhosus lab.	++++	++++	+++	+++	?	0	0	0
B. typhosus Smith	++	+++	+++	++	0	0	0	0

In the above and preceding experiment no evidence of any sort was observed which indicated that the inagglutinable bacillus was more or less susceptible to precipitation by acids than the normal bacillus, the bacillus in question behaving like the control strains. The optimum concentration of H-ions which precipitate the inagglutinable bacillus is the same as that for the normal *B. typhosus*, namely $3·5 \times 10^{-5}$—8×10^{-5}.

Complement fixation. Recent work has shown that by the use of suitable technique it is possible to distinguish between one member of the typhoid-colon group and another even though very closely related. This experiment was made in order that the antigenic power

of the inagglutinable bacillus in the fixation of the complement reaction might be compared with that of the normal bacillus.

Technique. The technique employed was based on that used in the Bacteriological Laboratory of the London Hospital, in performing the Wassermann reaction. The antigen was prepared by emulsifying a 24-hour agar slope growth in 4 c.c. of carbolic saline (0.5%). The necessary amount for the test was taken to be one-quarter of that amount which just gave complete inhibition of haemolysis after incubation for one hour at 37° C.; usually 0.02 c.c. In the haemolytic system a 5% suspension of sheep corpuscles was used and 3 units of amboceptor; of complement $2\frac{1}{2}$ units were used. In the test the total volume was 1.5 c.c. of which 0.5 c.c. was haemolytic system.

Exp. With anti-typhoid serum:

Serum quantity	Inagglutinable strain	Agglutinable strain
0·05	++++	++++
0·025	++++	++++
0·02	++	++++
0·01	++	++++
0·006	+	+++
0·003	+	+++
0·001	0	+++
0·0005	0	++

With anti-inagglutinable typhoid serum:

Serum quantity	Inagglutinable strain	Agglutinable lab. strain
0·05	++++	++++
0·025	++++	++++
0·02	++++	++++
0·01	++++	++++
0·006	++++	++++
0·003	++++	+++
0·001	++++	+++
0·0005	+++	.++

Argument. Inagglutinable strains or as they are sometimes called serum-fast strains of *B. typhosus* are undoubtedly of the same nature as the immune or fast races of protozoa. The foregoing experiments were made with the idea of finding some clue as to the mechanism by which such strains are able to resist the specific agglutin. There is reason to believe that the resistant strains arise from mere variants of the normal bacillus.

It has been suggested that the inagglutinable bacilli owe their peculiarity to a loss of their agglutinin receptors. Our absorption experiments however failed to demonstrate any loss or weakening of

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the absorptive power for homologous or heterologous typhoid agglutinin. Müller, and Eisenberg and Volk found however with their strains some loss of absorptive power. There was some evidence in our experiments that the fixation of the agglutinin was not so firm, as it was more easily removed by washing than was the case with the normal bacillus.

Again complement fixation experiments failed to show any marked difference with regard to the antigenic power of the inagglutinable bacillus and the normal bacillus, though it seemed as if the inagglutinable bacillus was slightly more resistant to the heterologous than to the homologous amboceptor—a result frequently observed in complement fixation experiments.

It would therefore appear from the inoculation and other experiments that there is not a sufficient loss of agglutinin receptor or other receptors to account for practically a complete inagglutinability towards the specific agglutinin.

As regards the second part of agglutination—aggregation a purely physico-chemical phenomenon—no striking difference was found between the inagglutinable bacilli and the normal agglutinating strains of *B. typhosus*. They both behaved towards the chemical agglutinants in much the same way with the exception that occasionally the inagglutinable bacillus was slower in showing complete agglutination. The time limit in the acid agglutination experiments was $2\frac{1}{2}$ hours but the normal bacilli were often completely agglutinated within $1\frac{1}{2}$ hours while the inagglutinable bacillus only showed commencing aggregation. The physical agglutination is therefore apparently slightly delayed though there was no complete resistance as was the case with the specific anti-serum. Our experiments in this connexion do not support the view of Michaelis and his pupils, that acid agglutination and serum agglutination depend on the same factors.

Though our results are necessarily somewhat indefinite there is reason to believe that the phenomenon of inagglutinability is of a physical nature, the difference between the strains being of quality and not of kind.

CONCLUSIONS.

(1) By injections into rabbits, an inagglutinable strain of *B. typhosus* can produce specific agglutinin to the species *B. typhosus* though not to itself, except in the slightest degree.

(2) The injections also produce group-agglutinins for other members of the typhoid-colon group.

(3)   The inagglutinable bacillus absorbs the agglutinin from both the homologous and heterologous antisera.

(4)   The group agglutinins (for *B. gaertner*) are also removed by absorption with the inagglutinable strain.

(5)   There is little or no difference quantitatively between the absorptive power of the inagglutinable bacillus and the normal bacillus.

(6)   The antigenic properties of the inagglutinable bacillus in the fixation of the complement reaction do not appear to be impaired to any extent.

(7)   Chemical agglutinants, and acids in particular, act very similarly on both strains, though the reaction is slightly delayed in the case of the inagglutinable strain.

(8)   The inagglutinable typhoid bacillus seems therefore to owe its peculiarity to some alteration of a physical character rather than to a loss of receptors which makes it more resistant to certain physico-chemical states which affect the normal bacillus in a certain definite manner.

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