OBSERVATIONS ON THE OCCURRENCE, CHARACTERISTICS AND SPECIFICITY OF NATURAL AGGLUTININS.

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Introduction.

The ability of normal serum to agglutinate bacteria in suspension was observed by many of those early workers who studied the properties of the immune agglutinins (Bordet (1899), Kraus and Low (1899), Gengou (1899), Rodet (1899), Jatta (1900)). No systematic investigation of this natural phenomenon was made at that time. Points of difference between natural and immune agglutinins were described by Hahn and Tromsdorf (1900), Landsteiner and Calvo (1902), Landsteiner and Reich (1905) and others. In the serological study of particular organisms their agglutination by normal sera was mentioned and its extent noted as, for example, in the case of the streptococci by Moser and v. Pirquet (1902) and Rossiwall and Schick (1905). Lambotte and Maréchal (1899) made similar observations with *B. anthracis*.

That the sera of young animals showed little or no agglutinating activity was noted by Kraus and Low (1899), Lüdke (1905) and Braun (1909). Bail (1902) demonstrated the greater thermostability of the natural agglutinins as compared with the lytic functions of normal sera. Further study of the inactivation temperature of the normal agglutinating principle was made by Romberg (1902), Eisenberg and Volk (1902) and Joos (1903). This subject has been recently reopened by Felix and Olitzki (1929) who have shown the heat resistance of the natural agglutinins to approximate to that of the O-immune agglutinins when tested under strictly comparable conditions.

Without demonstrating conclusively the specificity or otherwise of the natural agglutinins, agglutinin absorption experiments were carried out by Hetsch and Lentz (1903), Scheller (1904), Lüdke (1905) and Braun (1909). Burgi (1907) and Mamlock (1909) reviewed the occurrence of agglutinins for a wide variety of organisms in the sera of many animal species. Little account was taken of variations among individuals within a given species. Burgi concluded that visible agglutination was the result of a non-specific physico-chemical process exactly paralleled by flocculation of a colloidal sol. Bruce White (1927), in his work on spontaneous agglutination of "rough" strains of organisms of the *Salmonella* group and others, suggested that agglutination by normal sera may be analogous to salt agglutination. He described a type of agglutination which might be caused by the serum of any animal species, the effect being specially pronounced in the case of "rough" strains of the intestinal organisms.

In the studies recorded in this paper a large number of specimens of normal sera from various species have been tested with a variety of bacteria, with a view to surveying as completely as possible the occurrence of natural bacterial agglutinins, in this way to ascertain for which types of bacteria such agglutinins are most frequent and whether they are related to various animal species. For this purpose careful quantitative tests were carried out in each case. In order to correlate these principles with agglutinins resulting from immunisation and with other "immune" and "natural" antibodies, their thermostability was

investigated and also their specificity as judged by the absorption method. The distribution of the natural agglutinating principle in the serum protein fractions was studied by the method of carbonic acid precipitation. As a result of this work the view is put forward that two factors are concerned in the agglutination of a bacterial suspension by normal animal serum. It seems probable that the chief factor is a "natural antibody" which is strictly specific for the particular organism. Superimposed on this, yet separable from it, is a non-specific factor acting on all organisms in common. The natural specific agglutinins show many points of resemblance to other natural antibodies and it seems reasonable to suppose that they are the precursors in normal serum of the "immune" agglutinins.

Methods.

Bacterial strains used. These were chosen for the most part from the stock laboratory cultures, choice being limited to those which normally form a stable emulsion in 0·85 per cent. salt solution or are capable of variation to such a form. Those which have been most fully investigated belong to the group of Gram-negative intestinal aerobes. The full list of organisms will be seen in the tables. With regard to the strains of *B. coli* designated X, F 1, F 2, F 3 and F 4, strains X and F 4 are motile varieties, the remainder are non-motile. *B. coli* X and F 2 are typical, while F 1, F 3 and F 4 are atypical in not producing indol.

Bacterial suspension. Fresh suspensions were prepared for each experiment, the living organisms for a 24 hours' agar slope culture being suspended in 0·8 per cent. salt solution. These suspensions were all standardised by opacity and corresponded to Brown's opacity standard number 5.

Technique. The usual technique for macroscopic agglutination reactions was employed. The series of serum dilutions tested ranged from 1 : 4 to 1 : 256 (or higher). The mixtures of bacterial emulsion and serum were incubated for 1½ hours at 37° C. in an air oven, and then allowed to stand at room temperature. As the formation of visible floccules was slow, the end-titre shown by the 18-hour reading is that recorded. In every case the stability of the emulsion was carefully controlled, and all results were rejected if the emulsion showed more than a slight tendency to sedimentation after 18 hours.

Agglutinin absorption. The absorbing emulsion was made by suspending the surface growth of a varying number of 24 hours' agar slope cultures of the organism to be used in 1–3 c.c. of 0·85 per cent. salt solution. Equal parts of the resulting dense emulsion and undiluted serum were well mixed and incubated for 3 hours at 37° C. with repeated shaking. The organisms were removed by centrifuging and the supernatant serum, now diluted 1 : 2, was pipetted off. This serum was then diluted further and agglutination reactions were carried out as in the case of untreated serum. Difficulty was sometimes experienced in estimating an adequate absorbing dose and many experiments had to be repeated after failure to absorb completely the particular agglutinins. Dilution of the serum prior to absorption was not practicable in view of the low dilutions required for the subsequent reactions. In some cases as many as twelve agar slope cultures were required for absorption of agglutinins from 1·2 c.c. of serum. In no case, however, was it found impossible to remove all trace of agglutinin for the absorbing organism when the cultures used were of the "smooth" form. In every case the untreated serum was tested simultaneously with the absorbed, the same emulsions being used. In the later work large (6 in.) agar plate cultures were used. The 24 hours' growth on one of these plates was emulsified in 2–3 c.c. of normal salt solution, and the emulsion produced was usually found adequate for the absorption of an equal quantity of normal serum.

In cases where a sample of serum was absorbed consecutively by two different organisms the first absorption was carried out by emulsifying the growth from large plates in undiluted serum. The subsequent treatment of the serum consisted in the addition to it of an equal volume of a dense emulsion in saline. In this way the first dilution of serum in the series tested was always 1 : 4 even after double absorption.

In experiments where non-specific absorption was attempted, Kieselguhr or charcoal were used. These materials in a very fine state of division were made up into a thick suspension in normal saline. Using this suspension in place of the dense bacterial emulsion absorption was carried out as above.

Heating of sera was carried out in the water bath, the temperature indicated being maintained for 30 minutes. For temperatures above 57° C. the serum was first diluted 1 : 2 with saline. Higher dilutions would have restricted the series of dilutions tested in the agglutination reaction. No tendency to coagulation was observed in this dilution at temperatures up to 67·5° C.

GENERAL SURVEY OF THE OCCURRENCE OF NATURAL BACTERIAL AGGLUTININS AMONG VARIOUS SPECIES.

Table I summarises the results of direct agglutination tests with a series of bacteria and the sera of various animals. Under the name of the organism and opposite the name of each animal species four data are tabulated.

(1) The number of specimens of serum which have been examined from the species in question.

(2) Agglutination end-titres most frequently noted.

(3) The range of end-titres observed with different samples of serum.

(4) The temperature which completely annulled the action of the serum in 30 minutes.

From this tabular statement of results it will be seen that the natural bacterial agglutinins are widely yet unequally distributed in the sera of animals. Some sera reacted strongly with a majority of the organisms used. Ox, pig and horse sera gave the most consistently strong results. Other animal sera were generally less active, both as regards the number of organisms which they agglutinated and in their agglutination end-titres. Pig serum yielded the highest end-titres encountered, viz. 1 : 1024 with *Pneumobacillus* and with *B. dysenteriae* Y. Ox serum gave the most uniform results, but even these were subject to considerable variation.

An attempt was made to place the animal species in a definite order according to the agglutinating power of their sera. The most frequently recorded end-titre of the serum of each species for a given organism could be used to group the various animal sera in descending order of activity.

It was found that, for all organisms, the order of activity of different animal sera was almost constant. Ox serum was strongest, pig and horse sera were less active and almost equal, sheep serum was next in order, while human, cat, rabbit and guinea-pig sera, in succession, showed markedly weaker effects. Rat serum gave the lowest end-titres of all.

One point which study of the results with individual specimens of serum emphasised was that the strength or weakness of a specimen was general for

Table I. *General results.*

Number of samples of serum for each species.
The most frequent end-titre. The range of titres. Temperature of inactivation.

Serum	No. of samples tested	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (° C.)	No. of samples tested	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (° C.)
	B. proteus X 19.				*B. coli* F 2.			
Ox ...	20	1 : 32	1 : 16–1 : 128	65	8	1 : 32	1 : 4–1 : 64	55
Rabbit ...	6	0	0–1 : 16	.	2	0	0–0	.
Guinea-pig	5	0	0–0	.	2	0	0–0	.
Horse ...	5	1 : 32	1 : 16–1 : 64	62	2	0	0–1 : 16	.
Sheep ...	9	1 : 32	1 : 8–1 : 32	65	5	0	0–1 : 8	.
Pig ...	10	1 : 32	1 : 16–1 : 128	65	4	0	0–1 : 8	.
Rat ...	5	0	0–1 : 8	.	2	0	0–0	.
Cat ...	2	0	0–0	.	2	0	0–0	.
Human ...	7	0	0–1 : 8	.	6	0	0–1 : 8	.
	B. pyocyaneus				*B. coli* F 3.			
Ox ...	15	1 : 256	1 : 64–1 : 256	65	18	1 : 32	1 : 16–1 : 64	60
Rabbit ...	6	1 : 16	0–1 : 64	.	3	0	0–0	.
Guinea-pig	4	1 : 8–1 : 16	1 : 8–1 : 16	.	2	0	0–0	.
Horse ...	4	1 : 64	1 : 16–1 : 256	62	4	0	0–1 : 32	.
Sheep ...	6	1 : 64	1 : 64–1 : 256	67·5	5	0	0–1 : 32	.
Pig ...	8	1 : 256	1 : 128–1 : 1024	65	7	1 : 16	0–1 : 16	65
Rat ...	3	0	0–1 : 8	.	3	0	0–0	.
Cat ...	2	1 : 16–1 : 64	1 : 16–1 : 64	65	2	0	0–0	.
Human ...	15	1 : 16	0–1 : 32	.	5	0	0–1 : 8	.
	B. coli X.				*B. coli* F 4.			
Ox ...	23	1 : 32	1 : 16–1 : 128	50	11	1 : 32	1 : 16–1 : 256	53
Rabbit ...	7	0	0 : 1 : 16	.	2	0	0–0	.
Guinea-pig	5	1 : 8	0–1 : 8	.	2	1 : 8–1 : 16	1 : 8–1 : 16	.
Horse ...	5	1 : 32	0–1 : 32	.	3	0	0–1 : 16	.
Sheep ...	11	1 : 16	1 : 4–1 : 64	67·5	3	1 : 16	0–1 : 16	.
Pig ...	11	1 : 8	0–1 : 256	50	7	1 : 8	1 : 8–1 : 32	60
Rat ...	5	0	0–1 : 4	.	2	0	0–0	.
Cat ...	2	0	0–1 : 32	62·5	2	0	0–0	.
Human ...	12	1 : 8	0–1 : 32	.	6	0	0–0	.
	B. coli F 1.				*Pneumobacillus* (Friedländer).			
Ox ...	8	1 : 32	1 : 4–1 : 64	53	11	1 : 256	1 : 64–1 : 256	65
Rabbit ...	3	0	0	.	5	1 : 128	1 : 64–1 : 128	.
Guinea-pig	2	0	0–0	.	4	1 : 32	1 : 32	.
Horse ...	3	1 : 4	0–1 : 8	.	4	1 : 64	1 : 64–1 : 128	62·5
Sheep ...	5	0	0–1 : 16	65	7	1 : 128	1 : 16–1 : 128	65
Pig ...	6	1 : 16	0–1 : 16	.	9	1 : 256	1 : 64–1 : 512	67·5
Rat ...	2	0	0–0	.	4	1 : 32	1 : 16–1 : 64	.
Cat ...	2	0	0–0	.	2	1 : 64	1 : 64–1 : 128	67·5
Human ...	8	0	0–1 : 16	.	14	1 : 128	0–1 : 256	.
	B. typhosus R.C.P.				*B. dysenteriae* Y.			
Ox ...	20	1 : 64	1 : 32–1 : 256	65	13	1 : 256	1 : 64–1 : 256	65
Rabbit ...	6	1 : 32	1 : 4–1 : 32	.	5	1 : 128	0–1 : 128	.
Guinea-pig	4	1 : 8–1 : 16	1 : 8–1 : 16	.	4	1 : 16	1 : 8–1 : 32	.
Horse ...	4	1 : 32	1 : 64–1 : 256	62	4	1 : 256	1 : 64–1 : 256	.
Sheep ...	8	1 : 64	0–1 : 128	67·5	6	1 : 256	1 : 64–1 : 256	65
Pig ...	10	1 : 128	1 : 32–1 : 256	65	7	1 : 256	1 : 32–1 : 1024	65
Rat ...	4	0	0–1 : 8	.	3	1 : 8	1 : 8–1 : 32	.
Cat ...	2	1 : 8	1 : 8	.	2	1 : 64	1 : 64–1 : 128	67·5
Human ...	10	1 : 32	0–1 : 32	.	10	1 : 256	1 : 128–1 : 256	.

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Table I—*continued.*

Serum	No. of samples tested	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (° C.)	No. of samples tested	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (° C.)
		B. paratyphosus A.				*B. dysenteriae* Shiga.		
Ox ...	17	1 : 32	1 : 16–1 : 128	62·5	13	1 : 32	1 : 32–1 : 128	65
Rabbit ...	3	0	0–0	.	6	1 : 8	0–1 : 8	.
Guinea-pig	4	0	0–1 : 4	.	3	1 : 8	1 : 8–1 : 16	.
Horse ...	3	1 : 32	1 : 32–1 : 128	62·5	5	1 : 16	1 : 16–1 : 64	60
Sheep ...	5	1 : 16	0–1 : 16	.	8	1 : 16	1 : 16–1 : 64	65
Pig ...	8	1 : 32	1 : 16–1 : 64	65	9	1 : 32	0–1 : 64	62·5
Rat ...	3	0	0–0	.	3	0	0–0	.
Cat ...	2	0	0–0	.	2	0	0–0	.
Human ...	2	0	0–0	.	8	0	0–1 : 32	.
		B. paratyphosus B.				*B. morgan* I.		
Ox ...	22	1 : 128	1 : 32–1 : 256	62	8	1 : 64	1 : 32–1 : 256	62
Rabbit ...	6	0	0–0	.	5	1 : 16	0–1 : 16	.
Guinea-pig	5	1 : 8–1 : 16	1 : 8–1 : 16	.	4	1 : 8	0–1 : 8	.
Horse ...	5	1 : 64	1 : 32–1 : 256	62	3	1 : 64	1 : 32–1 : 64	62
Sheep ...	9	1 : 64	1 : 16–1 : 128	65	6	1 : 32	1 : 8–1 : 64	65
Pig ...	10	1 : 128	1 : 32–1 : 128	65	7	1 : 32	1 : 16–1 : 128	65
Rat ...	5	0	0–1 : 8	.	2	0	0–0	.
Cat ...	2	0	0–0	.	2	0	0–1 : 4	.
Human ...	7	1 : 8–1 : 16	0–1 : 16	.	5	0	0–1 : 8	.
		B. enteritidis Gaertner.				*V. cholerae.*		
Ox ...	12	1 : 128	1 : 32–1 : 128	62	19	1 : 64	1 : 32–1 : 256	65
Rabbit ...	4	0	0–1 : 8	.	5	0	0–1 : 4	.
Guinea-pig	4	1 : 8	1 : 8	.	5	1 : 16	1 : 8–1 : 16	.
Horse ...	3	1 : 64	1 : 16–1 : 64	65	6	1 : 64	1 : 32–1 : 256	65
Sheep ...	6	1 : 32	1 : 8–1 : 16	65	9	1 : 64	1 : 16–1 : 128	67·5
Pig ...	8	1 : 32	1 : 16–1 : 128	65	10	1 : 32–1 : 64	1 : 16–1 : 128	65
Rat ...	2	0	0–0	.	4	1 : 16	0–1 : 32	.
Cat ...	2	0	0–0	.	2	1 : 16	1 : 16–1 : 32	65
Human ...	9	0	0–1 : 16	.	4	0	0–1 : 16	.

Note. In this and in all subsequent tables the figure 0 denoting an end-titre signifies that no agglutination occurred in dilution 1 : 4 or higher dilution. A single dot (.) indicates that no observation was made.

all the organisms tested. This applied to each animal species. No serum has been encountered which gave abnormally strong or weak reactions with one organism only.

Table I shows clearly that certain organisms were agglutinated in a consistently higher dilution of all sera than were others. *B. dysenteriae* Y, *Pneumobacillus* and *B. pyocyaneus* were outstanding in this respect. They would appear to be highly susceptible to the agglutinating action of normal serum. The end-titres of agglutination of these organisms varied markedly according to the species of animal and the sample of serum, but they were always higher than the corresponding end-titre for a less susceptible organism. When weakly active samples of serum were used the agglutination titre of these organisms dropped in common with others, but always remained relatively higher.

As in the case of the activity of sera it was possible to group the strains employed according to their susceptibility to agglutination by normal sera. Four well-defined groups could be differentiated.

(1) *B. dysenteriae* Y, *Pneumobacillus* and *B. pyocyaneus*—most susceptible.

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(2) *B. typhosus*, *B. paratyphosus* B, *V. cholerae*, *B. enteritidis* Gaertner, *B. morgan* I and *B. dysenteriae* Shiga—less susceptible.

(3) *B. paratyphosus* A, *B. proteus* X 19, *B. coli* X—still less susceptible.

(4) *B. coli* F 1, F 2, F 3, F 4, showing reactions only in very low dilutions of all sera.

It may here be mentioned that *B. lactis aerogenes* was used in reactions with all the sera employed, and gave absolutely negative results throughout. This strain would therefore form a fifth group, being uniformly and completely resistant to natural agglutinins.

In view of the great variations among individual specimens of serum any degree of mathematical accuracy in assessing results may be attained only after a very large number of samples from each species have been examined. The foregoing conclusions are, therefore, drawn with all reserve, and the volume of data upon which they are founded is supplied in the various tables so that their limitations may be readily appreciated.

Influence of the age of the animal.

Brief reference is made by many of the earlier workers to the influence of the age of the animal on the agglutinating activity of its serum.

The present work included observations of three samples of calf serum. Negative results were obtained with a majority of organisms. The exceptions in all cases were *B. pyocyaneus*, *Pneumobacillus* and *V. cholerae*, and with these an end-titre of only 1 : 16 was recorded. It will be noted that these are the organisms which give most marked effects with adult ox serum.

A litter of rabbits was studied, the mother's serum acting as control. The serum of the mother reacted to an appreciable extent with only three organisms, viz. *B. pyocyaneus*, *Pneumobacillus* and *B. dysenteriae* Y. The results obtained are summarised in Table II.

Table II. *Influence of age upon agglutinating activity of rabbit serum.*

End-titres noted in agglutination reactions with serum from two young rabbits.

	Young rabbit A		
Age (days)	*B. pyocyaneus*	*Pneumobacillus*	*B. dysenteriae* Y
24	0	1 : 4	0
44	0	1 : 32	1 : 4
85	1 : 16	1 : 64	1 : 32

	Young rabbit B	
Age (days)	*Pneumobacillus*	*B. dysenteriae* Y
24	1 : 4	0
44	1 : 16	1 : 8
85	Animal dead	

	Mother of litter		
Days after birth of young	*B. pyocyaneus*	*Pneumobacillus*	*B. dysenteriae*
24	1 : 16	1 : 128	1 : 128
44	1 : 16	1 : 128	1 : 128
85	1 : 16	1 : 128	1 : 64

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It will be seen that the agglutinating activity of the serum increased with age, the increase being steadily progressive.

Observations of this kind are hampered by the fact that the sera of the small laboratory animals react weakly, and with few of the organisms of the series used.

THERMOLABILITY OF NATURAL AGGLUTININS.

Only those sera giving consistent agglutination effects have been systematically studied with a view to determining the thermolability of the agglutinating principle. The results recorded (Table I) for the sera of ox, horse, pig and sheep are as a rule the findings with several samples from each species. In the case of other, weakly acting, animal sera the results refer to single samples.

The temperature of inactivation recorded was arrived at by a series of experiments involving the heating of serum to graded temperatures differing by 2° C. or 2·5° C. In each case the serum was heated for 30 minutes.

The temperature zone 60–65° C. was that at which complete inactivation was most frequently obtained. It will be seen (Table I) that the sera of all animal species show abolition of their agglutinating effect for one or more organisms in this zone. Thermostability at 65° C. was rare but, exceptionally, it was found that a temperature above this was required to complete the inactivation, *e.g.* 67·5° C. for sheep serum with *B. pyocyaneus*, *B. dysenteriae* Y, and *B. typhosus*.

In general terms, therefore, it may be stated that the natural agglutinins of animal sera are inactivated at 60–65° C. The exceptions to this and the variations within this range of 5° are significant in indicating the complexity of normal serum from the point of view of bacterial agglutination. It will also be noted (Table I) that in the case of a given serum the degree of thermostability of agglutinins for all organisms tested was not uniform. Ox serum exemplifies this.

Further, agglutinins for the same organism in sera of different animal species were not inactivated at a constant temperature. Thus the normal serum principle responsible for agglutinating *B. coli* X is thermolabile at 55° C., 65° C. and 62·5° C. in the sera of ox, sheep and cat respectively.

No correlation could be discerned between the quantity of the natural antibody present (as estimated by end-titre) and its thermolability. The following examples are illustrative. A sample of pig serum which was fully investigated gave end-titres of 1 : 32 for *B. proteus* X 19 and 1 : 256 for *B. pyocyaneus*. Inactivation of the serum for both organisms occurred at 65° C. A specimen of horse serum in the unheated state yielded an end-titre of 1 : 64 for *B. enteritidis* Gaertner. All trace of agglutination was abolished at 65° C. The fresh serum agglutinated *B. pyocyaneus* in dilutions up to 1 : 256, but this effect was annulled at 62° C. It, therefore, seems probable that the relative thermolability of the natural agglutinins as compared with immune agglutinins is not dependent entirely on the small quantities in which they are present.

THERMOLABILITY CURVES.

A study of the behaviour of serum which had been exposed to temperature lower than that causing final and complete inactivation revealed interesting features. With few exceptions the final inactivation was rapid, a sudden drop in titre being noted at temperatures over 60° C. The specimens of serum showing extreme lability of the *B. coli* agglutinins (see Table I) were the exceptions to this rule.

In the temperature zone, 50–60° C., the thermolability curves were of three types. The first as exemplified by the reaction of *B. enteritidis* Gaertner with the sera of horse, sheep and ox, exhibited a fall in end-titre at 55° C. followed by a rise up to or above that obtained with fresh unheated serum. The temperature at which this apparent reactivation occurred lay between 55° C. and 60° C., the peak in most cases being apparently midway between these two points.

The *second type* seen with *B. morgan* I and the sera of ox, pig, horse and sheep showed a comparative stability at temperatures below 55° C. Higher temperatures produced a slowly progressive fall which reached zero at 62° C.–65° C.

A *third type* of curve was noted, though rarely, in which increasing activity occurred at temperatures up to 55° C. Higher temperatures produced a rather slowly progressive fall as in the second type.

The first form was most frequent and all strongly acting sera showed a curve of this type in reactions with one or more organisms. It is of interest by comparison with the findings of Mackie and Finkelstein (1928) in their work on non-specific complement-fixation.

DISTRIBUTION OF NATURAL AGGLUTININS IN THE SERUM PROTEIN FRACTIONS.

The method of fractioning by carbon dioxide (Liefmann, 1909) was adopted. This method has been utilised by Mackie and Watson (1926) and Mackie and Finkelstein (1928, 1930) in analysing complement-fixation phenomena produced by normal sera and certain "pseudoantigens." The carbonic acid insoluble and soluble fractions are designated respectively *A* and *B*. The *A* fraction contains the euglobulin and part of the pseudoglobulin of the serum, the *B* represents the remainder of the pseudoglobulin and the albumen.

The difficulty of the high initial dilution of the *B* fraction (the serum was diluted 1 : 10 preparatory to carbon dioxide treatment) was surmounted by taking appropriate larger quantities—a method which was found in practice to give results quite comparable with those obtained by low dilutions.

The results are summarised in Table III.

For the purposes of comparison specimens of various high-titre agglutinating sera were subjected to the same treatment with carbon dioxide in order to determine the distribution of the specific immune agglutinins. Table IV

Table III. *Carbon dioxide fractioning of normal sera.*

Titres recorded in agglutination reactions using whole serum, *A* (CO_2 insoluble) and *B* (CO_2 soluble) fractions.

Organisms used in agglutination reactions	Ox serum			Sheep serum		
	S	*A*	*B*	*S*	*A*	*B*
B. proteus X 19 ...	1 : 128	1 : 128	0	.	.	.
B. pyocyaneus ...	1 : 64	1 : 256	1 : 10	1 : 128	1 : 64	1 : 5
B. typhosus R.C.P. ...	1 : 256	1 : 128	1 : 80	.	.	.
B. paratyphosus A ...	.	.	.	1 : 32	0	1 : 10
B. paratyphosus B ...	.	.	.	1 : 64	1 : 32	0
B. enteritidis Gaertner	1 : 64	1 : 64	1 : 5	1 : 32	1 : 16	0
B. coli X	1 : 32	1 : 16	1 : 20	1 : 16	0	0
B. dysenteriae Y ...	1 : 256	1 : 128	1 : 40	1 : 256	1 : 64	1 : 20
B. dysenteriae Shiga ...	1 : 64	1 : 64	0	.	.	.
B. morgan I	1 : 128	1 : 128	0	1 : 64	1 : 32	0
V. cholerae	1 : 64	1 : 128	1 : 20	1 : 64	1 : 16	1 : 10
Pneumobacillus ...	1 : 128	1 : 128	1 : 40	1 : 64	1 : 64	1 : 10

Organisms used in agglutination reactions	Pig serum			Horse serum			Guinea-pig serum		
	S	*A*	*B*	*S*	*A*	*B*	*S*	*A*	*B*
B. proteus X 19 ...	1 : 128	1 : 32	1 : 80	1 : 16	1 : 4	0	.	.	.
B. pyocyaneus ...	1 : 256	1 : 64	1 : 80	1 : 128	1 : 64	1 : 20	1 : 8	1 : 4	0
B. typhosus R.C.P. ...	1 : 64	1 : 64	1 : 20	1 : 32	1 : 16	1 : 5	1 : 16	1 : 8	0
B. paratyphosus A ...	1 : 32	1 : 8	1 : 5	.	.	.	1 : 8	0	0
B. paratyphosus B ...	1 : 128	1 : 64	1 : 40	1 : 16	1 : 16	0	1 : 16	0	0
B. enteritidis Gaertner	1 : 128	1 : 64	1 : 10	1 : 16	1 : 8	0	1 : 8	0	0
B. coli X	1 : 64	1 : 32	1 : 80	1 : 32	0	0	1 : 8	0	0
B. dysenteriae Y ...	1 : 512	1 : 256	1 : 160	1 : 256	1 : 128	1 : 40	1 : 16	1 : 4	0
B. dysenteriae Shiga ...	.	.	.	.	.	.	.	.	.
B. morgan I	1 : 128	1 : 128	1 : 20	1 : 32	1 : 16	0	.	.	.
V. cholerae	1 : 64	1 : 32	1 : 10	1 : 32	1 : 8	1 : 5	1 : 16	1 : 8	0
Pneumobacillus ...	1 : 512	1 : 64	1 : 160	1 : 64	1 : 16	1 : 40	.	.	.

S = Whole serum. *A* = *A* fraction. *B* = *B* fraction.

Table IV. *Carbonic acid fractioning of immune sera.*

End-titres of sera and fractions for the homologous organisms. In all cases serum was from rabbit.

Homologous organism	Whole serum	*A* fraction (CO_2 insoluble)	*B* fraction (CO_2 soluble)
V. cholerae (Bombay)	1 : 50,000	1 : 800	1 : 25,000
B. paratyphosus A (Schottmüller)	1 : 51,200	1 : 1,600	1 : 51,200
B. dysenteriae Y (Hiss and Russell)	1 : 6,400	1 : 200	1 : 6,400
B. typhosus (R.C.P.)	1 : 6,400	1 : 200	1 : 6,400

illustrates the results of four such experiments. The end-titres are recorded for the whole serum, and for each of the fractions.

In the case of normal serum the agglutinating property is located mainly in the *A* (carbonic acid insoluble) fraction. Pig serum gave certain results which showed divergence from this, but they were in a minority and no general tendency was noted for the activity of the *B* fraction to be markedly greater than the *A*.

This distribution differs from that of the immune agglutinins, which appear to reside almost entirely in the carbonic acid soluble moiety (see Table IV). It is of particular interest that, in normal sera, the *A* fraction was in some cases more active than the whole serum, *e.g.* ox—*B. pyocyaneus* and ox—*V. cholerae* (Table III). This appears to be analogous to the unmasking effect

of the *B* fraction described by Mackie and Watson (1926) in relation to complement-fixation by normal sera and the Wassermann antigen.

Agglutinin-absorption experiments.

By the technique described above, specimens of serum were subjected to treatment with bacterial cultures with a view to absorbing the homologous agglutinins[1] and determining whether the agglutinating principles of normal sera were specific.

It was at once found that an organism could absorb its agglutinins from normal serum, provided the absorbing dosage were adequate. Experiments were carried out to estimate the necessary dose and to exclude the possibility of a zone of diminished agglutinin binding power in the presence of excess of antigen. An example of such an experiment is as follows:

Amounts of 1·2 c.c. of normal horse serum were treated with varying quantities of *B. pyocyaneus* culture, computed in terms of the number of agar slope cultures from which it was derived—viz. one, two, four, six and eight cultures. The untreated serum agglutinated this organism in dilutions up to 1 : 64. Each successive increase of absorbing dosage halved the activity of the serum as estimated by the end-titre, the agglutinins being completely removed by six cultures. Still larger absorbing doses also removed the agglutinin completely. Absorption did not, therefore, depend on optimal proportions. The amount of culture required to remove such natural agglutinin seemed vastly greater than that necessary to produce a corresponding lowering of titre in the case of an immune serum.

The results of agglutination reactions between such absorbed serum and organisms other than that used for absorption were extremely variable. They had this in common, however, that treatment with an organism never removed the agglutinins for all heterologous bacteria. Complete loss of agglutinating power by a treated serum for a heterologous organism was exceptional and never occurred when the initial end-titre of the untreated serum for such organism was relatively high.

While varying results were thus obtained in the reactions of absorbed serum with heterologous organisms, by far the most frequent was some reduction in titre though, as a rule, this was not pronounced; more rarely the absorbed serum showed no loss of strength, while in some cases the paradoxical result of a rise in titre was noted.

In a manner quite analogous to the results of direct agglutination reactions the absorption experiments showed considerable variations among specimens of serum from different individuals of the same species, and among different species. The results of agglutinin absorption experiments are summarised in Tables V–IX.

[1] The terminology applicable to immune agglutinins is used throughout in the description of the results of this work. This is done with all reserve and without the implication that natural and immune agglutinins are identical.

These tables all show the essential fact that the homologous organism could absorb its own agglutinins in every case. In addition, the alteration of the serum end-titre for heterologous organisms is seen. Such a result might be explained on the assumption that two factors are present in the process of natural agglutination. Absorption of its own agglutinins by any reacting organism shows a "specific" element, while the removal of part of the effect for other organisms would suggest a "non-specific" factor. It was with a view to the further study of the latter that physical absorbents, such as charcoal and Kieselguhr, were employed in similar experiments. The results of such experiments are recorded in Tables V–VII, and it will be seen that such treatment had the effect of lowering or raising the serum end-titre for organisms in an irregular way. If the "specific" absorption by organisms of their homologous agglutinins be excluded for the moment, the results of treatment with such physical agents are similar to those following bacterial absorption.

Table V. *Agglutinin absorption experiments. Normal ox serum—3 samples.*

Agglutination reactions with	Sample *A*		Sample *B*		Sample *C*	
	Untreated serum	Serum absorbed *V. cholerae*	Untreated serum	Serum absorbed *V. cholerae*	Untreated serum	Serum absorbed Kieselguhr
cholerae	1 : 128	0	1 : 64	0	1 : 32	1 : 16
proteus X 19 ...	1 : 32	1 : 32	1 : 32	1 : 32	1 : 16	1 : 16
pyocyaneus	1 : 128	1 : 64	1 : 256	1 : 256	1 : 128	1 : 64
coli X	1 : 32	0	1 : 32	1 : 64	1 : 32	1 : 32
coli F 1	1 : 4	0	.	.	1 : 32	1 : 16
coli F 2	1 : 32	1 : 16	.	.	1 : 16	1 : 4
coli F 3	1 : 32	1 : 16	.	.	1 : 16	1 : 16
coli F 4	1 : 16	0	.	.	1 : 32	1 : 16
cumobacillus ...	.	.	1 : 32	1 : 32	1 : 256	1 : 128
typhosus R.C.P. ...	1 : 64	1 : 8	1 : 128	1 : 64	1 : 128	1 : 128
paratyphosus A ...	1 : 16	0	1 : 32	1 : 32	1 : 16	1 : 8
paratyphosus B ...	1 : 64	1 : 4	1 : 128	1 : 64	1 : 64	1 : 16
enteritidis Gaertner	.	.	1 : 16	1 : 16	1 : 32	1 : 16
dysenteriae Y ...	1 : 128	1 : 128 (zone)	1 : 256	1 : 256 (zone)	1 : 64	1 : 128 (zone)
dysenteriae Shiga ...	1 : 64	1 : 32	1 : 32	1 : 16	1 : 32	1 : 16
morgan I ...	.	.	1 : 64	1 : 32	.	.

Table VI. *Agglutinin absorption experiments. Normal sheep serum—3 samples.*

Agglutination reactions with	Sample *A*		Sample *B*		Sample *C*	
	Untreated Serum	Serum absorbed by charcoal	Untreated Serum	Serum absorbed by *B. proteus* X 19	Untreated Serum	Serum absorbed by *B. morgan* I
proteus X 19 ...	1 : 32	1 : 32	1 : 32	0	1 : 32	1 : 16
pyocyaneus ...	1 : 256	1 : 128	1 : 256	1 : 64	1 : 256	1 : 256
coli X	1 : 64	1 : 64	1 : 32	1 : 64	1 : 64	1 : 64
cumobacillus ...	1 : 64	1 : 32	1 : 64	1 : 128	1 : 64	1 : 64
typhosus R.C.P. ...	1 : 64	1 : 32	1 : 64	1 : 64	1 : 64	1 : 64
paratyphosus A ...	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
paratyphosus B ...	1 : 32	1 : 16	1 : 128	1 : 64	1 : 32	1 : 128
enteritidis Gaertner	1 : 32	1 : 16	1 : 32	1 : 64	1 : 64	1 : 64
dysenteriae Y ...	1 : 256	1 : 256 (zone)	1 : 256	1 : 256 (zone)	1 : 256	1 : 256 (zone)
dysenteriae Shiga...	1 : 32	1 : 16	1 : 32	1 : 32	1 : 16	1 : 8
morgan I	1 : 64	1 : 64	1 : 64	1 : 32	1 : 32	0
cholerae	1 : 32	1 : 32	1 : 64	1 : 32	1 : 128	1 : 128

Table VII. *Agglutinin absorption experiments. Normal pig serum—2 samples.*

Agglutination reactions with	Sample *A*		Sample *B*	
	Untreated serum	Absorbed by *B. pyocyaneus*	Untreated serum	Absorbed by charcoal
B. proteus X 19 ...	1 : 256	1 : 256	1 : 128	1 : 512
B. pyocyaneus ...	1 : 128	0	1 : 512	1 : 256
B. coli X	1 : 128	1 : 128	1 : 256	1 : 64
Pneumobacillus ...	1 : 256	1 : 256	1 : 512	1 : 256
B. typhosus R.C.P. ...	1 : 64	1 : 64	1 : 128	1 : 128
B. paratyphosus A ...	1 : 32	1 : 64	1 : 128	1 : 64
B. paratyphosus B ...	1 : 512	1 : 64	1 : 512	1 : 128
B. enteritidis Gaertner	1 : 64	1 : 64	1 : 256	1 : 256
B. dysenteriae Y ...	1 : 512	1 : 256	1 : 512	1 : 512
B. dysenteriae Shiga...	1 : 128	1 : 16	1 : 16	1 : 16
B. morgan I	1 : 256	1 : 128	1 : 256	1 : 32
V. cholerae	1 : 32	1 : 32	1 : 64	1 : 32

Table VIII. *Agglutinin absorption experiments.*

Normal *Horse* serum absorbed *B. proteus* X 19.
Normal *Human* serum absorbed *B. dysenteriae* Y.

Agglutination reactions with	Normal horse serum		Normal human serum	
	Untreated serum	Serum absorbed *B. proteus* X 19	Untreated serum	Serum absorbed *B. dysenteriae* Y
B. proteus X 19 ...	1 : 16	0	.	.
B. pyocyaneus ...	1 : 128	1 : 256	1 : 16	1 : 16
B. coli X	1 : 64	1 : 64	1 : 16	1 : 8
B. coli F 4	1 : 64	1 : 64	.	.
Pneumobacillus ...	1 : 128	1 : 128	1 : 64	1 : 64
B. typhosus R.C.P. ...	1 : 64	1 : 32	.	.
B. paratyphosus A ...	1 : 16	1 : 16	.	.
B. paratyphosus B ...	1 : 64	1 : 64	.	.
B. enteritidis Gaertner	1 : 16	1 : 16	.	.
B. dysenteriae Y ...	1 : 128	1 : 256	1 : 256	0
B. dysenteriae Shiga...	1 : 256	1 : 64	1 : 32	0
B. morgan I	1 : 16	1 : 16	.	.
V. cholerae	1 : 16	1 : 16	.	.

Table IX. *Absorption of one sample of ox serum by organisms and by charcoal.*

Agglutination reactions	End-titres of reactions with serum absorbed by			Unabsorbed serum
	B. typhosus R.C.P.	*B. paratyphosus* A	Charcoal	
V. cholerae	1 : 64	1 : 64	1 : 64	1 : 128
B. proteus X 19 ...	1 : 16	1 : 16	1 : 16	1 : 64
B. pyocyaneus ...	1 : 256	1 : 256	1 : 256	1 : 256
B. coli X	1 : 32	1 : 32	1 : 32	1 : 64
Pneumobacillus ...	1 : 64	1 : 64	1 : 64	1 : 128
B. typhosus R.C.P. ...	0	1 : 32	1 : 32	1 : 64
B. paratyphosus A ...	1 : 16	0	1 : 16	1 : 32
B. paratyphosus B ...	1 : 32	1 : 32	1 : 32	1 : 64
B. enteritidis Gaertner	1 : 32	1 : 32	1 : 32	1 : 64
B. dysenteriae Y ...	1 : 256 (zone)	1 : 256 (zone)	1 : 256 (zone)	1 : 256
B. dysenteriae Shiga...	1 : 8	1 : 8	1 : 8	1 : 64
B. morgan I	1 : 128	1 : 128	1 : 128	1 : 128

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The variation between one specimen of serum and another from the same species, as shown by the results of agglutinin absorption, is exemplified by the samples of ox serum *A* and *B* (Table V) following absorption by *V. cholerae*. The content of "specific" and "non-specific" agglutinins both vary among individuals of the same species. In this way it is possible to explain the lack of exact parallelism between the results of absorption by one organism and another, and between absorption by organisms and that by physical agents.

Much light was thrown on this question by a series of experiments, in each case of which a single specimen of serum was subjected to bacterial and non-specific physical absorption. Table IX records the result of such an experiment. The end-titres of the serum for a series of organisms are recorded before and after absorption by two organisms and by charcoal. It will be noted that, in its action on natural agglutinins for heterologous organisms, a bacterial emulsion produces results identical with those following the use of a physical suspension. The experiment illustrates the use of the terms "specific" and "non-specific" as applied to natural agglutinins. For example, in the case of agglutinins for *B. typhosus* R.C.P., the unabsorbed serum produced agglutination in a dilution of 1 : 64. The end-titre fell to 1 : 32 following absorption by *B. paratyphosus* A or charcoal in virtue of the loss of "non-specific" effect. The "specific" titre of 1 : 32 was exhausted by homologous absorption. It thus appears probable that absorption by an organism removes both "specific" and "non-specific" agglutinins for itself, the heterologous agglutinins absorbed being quite "non-specific" and capable of being removed by non-bacterial suspensions. Similar experiments with single samples of pig and sheep sera showed the same features.

FURTHER ENQUIRY INTO THE QUESTION OF SPECIFICITY BY A TECHNIQUE OF DOUBLE ABSORPTION.

The results of the simple agglutinin absorption experiments left the question of specificity somewhat in doubt. To obtain further light on the question double absorption was resorted to. A sample of serum was tested against a series of organisms and absorbed by one of them, when its agglutinating power for all was again recorded. The absorbed serum was then subjected to further absorption by another of the series. The absorbing organisms were chosen quite at random from those reacting with the animal serum in question.

The object of such experiments was to show that treatment of a serum with a dose of any organism sufficient to absorb completely all homologous agglutinins left unabsorbed in the serum strictly specific agglutinins for other organisms. Table X gives an example of such an experiment.

It will be seen from Table X that simple absorption by *B. proteus* X 19 demonstrated a high degree of specificity of the agglutinins in normal horse serum. Of the fourteen organisms tested, nine were agglutinated up to their original end-titre by the absorbed serum. The reactions with *B. dysenteriae*

Table X. *Double absorption.*

Horse serum absorbed: (1) *B. proteus* X 19; (2) *B. typhosus* R.C.P.
Agglutination end-titres of serum before and after absorptions.

Organism	Horse serum untreated	Horse serum absorbed by *B. proteus* X 19	Horse serum absorbed: (1) *B. proteus* X 19; (2) *B. typhosus* R.C.P.
B. proteus X 19 ...	1 : 16	0	0
B. typhosus R.C.P. ...	1 : 64	1 : 32	0
B. typhosus R.L.L. ...	1 : 64	1 : 64	0
B. paratyphosus A ...	1 : 16	1 : 8	1 : 16
B. paratyphosus B ...	1 : 64	1 : 64	1 : 64
B. enteritidis Gaertner	1 : 16	1 : 16	1 : 16
Pneumobacillus ...	1 : 128	1 : 64	1 : 128
B. dysenteriae Y ...	1 : 128	1 : 256	1 : 256
B. dysenteriae Shiga...	1 : 256	1 : 64	1 : 64
B. pyocyaneus ...	1 : 128	1 : 256	1 : 256
B. morgan I	1 : 16	1 : 16	1 : 16
B. coli X	1 : 64	1 : 64	1 : 64
B. coli F 4	1 : 64	1 : 64	1 : 64
V. cholerae	1 : 16	1 : 16	1 : 16

Shiga illustrate the fall in titre which a serum may sustain for a heterologous organism. This has been discussed in a previous section. On further treatment of the serum with *B. typhosus* R.C.P., however, no further loss occurred, the titre remaining 1 : 64.

A further fact illustrated by these results was that where a rise in titre follows the initial absorption it is maintained after the second treatment (seen in the cases of *B. dysenteriae* Y and *B. pyocyaneus*).

The two *B. typhosus* strains used showed that the once-treated serum behaved in a way precisely analogous to an immune serum. The absorbing strain removed the agglutinins for another homologous strain while agglutinins for all other bacterial species were unimpaired. Admittedly there are other factors in these reactions, as for example the absorption of an inhibitory factor causing a rise in titre after treatment, but the presence of a large specific factor is strongly suggested by these results.

Samples of serum of other animal species gave similar results.

Thus a strongly reacting sample of pig's serum was absorbed successively by *B. pyocyaneus* and *V. cholerae*. The preliminary treatment with *B. pyocyaneus* removed its own agglutinating principle and reduced the end-titre for certain other organisms. The superimposed absorption by *V. cholerae* removed all agglutinins for this organism but produced no further diminution in the end-titres for others.

In the case of pig's serum a further manipulation of some interest was carried out with *B. lactis aerogenes*, an organism which is not agglutinated by this or any other of the animal sera investigated[1]. Pig serum was treated with *B. lactis aerogenes* and equal parts of the resulting serum were absorbed by *B. paratyphosus* B and *B. coli* X. The initial treatment by an organism not itself agglutinated by the serum was found to have removed part of the agglu-

[1] The viscid character of an emulsion of this organism made from an agar slope culture may be a factor in preventing agglutination. The removal of this organism by centrifugalisation is a matter of some difficulty.

tinating effect for other organisms. Further treatment by either *B. paratyphosus* B or *B. coli* X failed to produce any diminution in titre for organisms other than themselves.

Double absorption experiment using a heated emulsion.

It was found that heating the organisms used for primary absorption did not alter the results. The full significance of this experiment will be considered in a later study of antigenic structure in relation to the agglutination reactions of normal sera with bacteria.

Method. The growth from five 6 in. agar plate cultures of *V. cholerae* was emulsified in 12 c.c. of normal saline and the resultant dense emulsion heated for 2 hours at 100° C. in boiling water. The emulsion was then cooled and 12 c.c. of normal ox serum were added, the whole being incubated for 3 hours at 37° C. with repeated shaking.

The vibrios were separated by rapid centrifugalisation and of the supernatant absorbed serum (now diluted 1 : 2) part was used in agglutination reactions with a series of organisms. The remainder was used to emulsify the growth of *B. proteus* X 19 from three 6 in. agar plates, absorption being carried out under the same conditions as before.

The results of this experiment are shown in Table XI.

Table XI. *Double absorption.*

Ox serum absorbed: (1) *V. cholerae* heated 100° C.; (2) *B. proteus* X 19.
End-titres of serum before and after absorptions in reactions with twelve strains.

Organism	Ox serum untreated	Ox serum absorbed *V. cholerae* 100° C.	Ox serum absorbed: (1) *V. cholerae* 100° C.; (2) *B. proteus* X 19
V. cholerae	1 : 128	1 : 16	1 : 16
B. proteus X 19 ...	1 : 64	1 : 32	0
B. typhosus R.C.P. ...	1 : 64	1 : 16	1 : 32
B. paratyphosus A ...	1 : 64	1 : 32	1 : 32
B. paratyphosus B ...	1 : 128	1 : 32	1 : 32
B. pyocyaneus ...	1 : 128	1 : 128	1 : 128
Pneumobacillus ...	1 : 64	1 : 32	1 : 32
B. dysenteriae Y ...	1 : 256	1 : 256	1 : 256
B. dysenteriae Shiga	1 : 128	1 : 4	1 : 4
B. morgan I	1 : 64	1 : 32	1 : 32
B. coli X	1 : 32	1 : 64	1 : 64
B. enteritidis Gaertner	1 : 128	1 : 32	1 : 32

It will be noted that the heated emulsion of *V. cholerae* did not completely absorb agglutinins for the homologous organism in the unheated form as used in the subsequent reaction. Otherwise the experiment presents the same features as before.

Double absorption experiment using a physical agent.

A sample of pig serum was used in a double absorption experiment involving the application of charcoal as the preliminary absorbent. This agent had been found by previous work to give results comparable to Kieselguhr and organisms in its power of reducing in an irregular way the titres of a serum for certain organisms of the series used.

Method. 5 grm. of animal charcoal were suspended in 10 c.c. of normal saline. To this was added 10 c.c. of pig's serum. Absorption was allowed to proceed at room temperature

for 2 hours with repeated stirring. The serum was separated by centrifuging and part used in agglutination reactions. The remainder was now absorbed by the growth from two 6 in. agar plate cultures of *V. cholerae* for 3 hours at 37° C.

The doubly absorbed serum was now used in agglutination reactions with the organism as noted in Table XII.

As in all the experiments quoted, the untreated serum was tested simultaneously with the absorbed, the same emulsions being used throughout in order that the results should not be vitiated by variation in serum or organisms through keeping.

Table XII. *Double absorption using a physical agent.*

Pig serum absorbed: (1) charcoal; (2) *V. cholerae.*
End-titres of agglutination reactions with twelve strains using untreated and absorbed serum.

Organism	Pig serum untreated	Pig serum absorbed charcoal	Pig serum absorbed: (1) charcoal; (2) *V. cholerae*
V. cholerae	1 : 64	1 : 32	0
B. proteus X 19 ...	1 : 128	1 : 512	1 : 512
B. typhosus R.C.P. ...	1 : 128	1 : 128	1 : 128
B. paratyphosus A ...	1 : 128	1 : 64	1 : 64
B. paratyphosus B ...	1 : 512	1 : 128	1 : 256
B. enteritidis Gaertner	1 : 256	1 : 256	1 : 256
Pneumobacillus ...	1 : 512	1 : 256	1 : 256
B. pyocyaneus ...	1 : 512	1 : 256	1 : 256
B. dysenteriae Y ...	1 : 512	1 : 512	1 : 512
B. dysenteriae Shiga ...	1 : 32	1 : 8	1 : 16
B. morgan I	1 : 256	1 : 64	1 : 128
B. coli X	1 : 256	1 : 64	1 : 64

The results recorded in Table XII show clearly that treatment with charcoal has produced a serum which contains agglutinins capable of demonstration as specific by the test of further absorption by any organism selected quite at random.

Discussion.

The power of agglutinating organisms in suspension is present in the serum of normal animals of many species. In some (*e.g.* ox, pig, horse, sheep) the effect is relatively marked, while in others (*e.g.* human, rabbit, guinea-pig and rat) little or no agglutinin can be demonstrated for any organism. Though individual specimens vary greatly in activity, it has been possible, by examining a large number of specimens, to determine an order of activity of the sera of different animal species. This result is in accordance with the findings of Burgi (1907).

The general results show considerable parallelism with those of Mackie and Finkelstein (1930) in their work on the natural complement-fixation effects of normal animal sera with bacterial antigens. They found similar variations among individuals and among species. Complete correlation is not possible as some sera which yielded consistently strong complement-fixation effects are deficient in natural agglutinins and *vice-versa*. Thus pig serum, a weak reactor *quâ* complement-fixation, shows marked agglutinating activity while the reverse is noted in the case of human serum. Weil and Felix (1920) have adduced

considerable evidence that complement-fixation runs parallel with the content of stabilotropic O agglutinins in immune serum. A similar correlation in normal serum may explain discrepancies between results of experiments involving the use of unheated organisms of the normal form. The O and H antigenic mechanism of organisms in its relation to agglutination reactions with normal serum has been the subject of a separate study which will be published at a later date.

The influence of the age of the animal on the agglutinin content of its serum is of some interest. Absence of the effect from the serum of young animals has been previously noted in the literature and experiments carried out in this work confirm those findings. The same increase with age was noted in the complement-fixation reactions of normal animal sera with Wassermann antigen (Mackie and Watson, 1926) and with "pseudo-antigens" (Mackie and Finkelstein, 1928), the development of this "natural antibody" in rabbit serum running parallel with that of the natural anti-sheep haemolysin. Friedberger, Bock and Fürstenheim (1929) in a recent study of natural antibodies in human serum at different ages, have shown that the natural anti-sheep haemolysin and the natural agglutinin for rabbits' red corpuscles increase progressively with age and that the curve produced resembles in many respects that of the proportion of Schick negative reactors in the population at age periods. In their work on complement-fixation by normal sera with bacterial antigens Mackie and Finkelstein (1930) were unable to demonstrate this feature. The serum of young rabbits, guinea-pigs and bovines possessed the property to a marked degree.

Study of the thermolability of the serum principle shows in general that the natural agglutinins are more labile than are the specific immune agglutinins. This corresponds to the difference between natural and immune haemolysin. The results suggest that the difference is not simply quantitative. Again, the temperature of inactivation most frequently noted, viz. 60° C.–65° C., would exclude complement-action as a factor in the process of natural agglutination. Felix and Olitzki (1929) considered that normal and immune agglutinins possess the same resistance to heat when investigated under strictly comparable conditions. Thus they used an O-immune serum the agglutinins of which were compared with those of normal serum for the same O strain. They emphasised the importance of the changes in the globulin which result from heating. Normal serum was used as diluent for immune serum to secure a protein content equal to that in the normal serum dilutions. Heuer (1922) showed that normal serum as diluent behaved as a protective colloid, tending to inhibit agglutination, and this factor, in association with the known thermolability of O agglutinins as compared with the H type, would explain the low inactivation temperatures for immune agglutinins arrived at by these workers.

The lability curves showing a zone of partial inactivation in the region of 55° C. are of great interest as they indicate a marked resemblance to those obtained in the complement-fixation work with "pseudo-antigens" and bacterial antigens to which reference has already been made.

The wide variety of temperatures which produce inactivation of natural agglutinins, varying greatly with the type or strain of organism used, is strong evidence that the active component of the serum is not a single non-specific property. The results suggest the presence of a series of specialised "antibodies."

Investigation of the distribution of natural agglutinin in the serum protein fractions gives results which are in accordance with previous studies in the natural antibodies (see Mackie and Finkelstein, 1928). The general conclusion seems permissible that these normal serum properties are located mainly in the carbonic acid insoluble fraction while one of the modifications consequent on immunisation is an altered distribution of the antibody in the serum protein.

The results of agglutinin absorption experiments recorded bring out clearly the presence of specific and non-specific elements in the reaction of natural agglutination. It is seen that where an organism is capable of removing in whole or part agglutinin for another, such a result may be produced in an exactly similar way by a non-specific agent such as charcoal. But underlying this non-specific process there is a distinctly specific factor. The experiments involving double absorption of a specimen of serum demonstrate this in a striking way. On removal of the non-specific moiety the serum is shown to contain a series of bacterial agglutinins which are strictly specific. Absorption by any one organism leaves the end-titre of the serum for all others undiminished. Mackie and Finkelstein (1930) also noted that the natural complement-fixing antibody, reacting with bacterial antigens, showed a marked though relative specificity.

Bruce White (1927) considers that natural agglutination probably covers a heterogeneous collection of phenomena, and cites as the dominant factor the interaction between serum colloids and a lipoid hydrophobe component of the organism. The specific soluble substance of smooth races was considered by him to be inhibitory. The agglutination produced is apparently quite non-specific. The phenomenon he describes thus appears to differ in many respects from that which has been investigated in this work.

The present studies appear to offer evidence that agglutination of bacterial suspensions by normal animal sera is largely due to the presence of a series of specific natural antibodies presenting considerable resemblances to other normal antibodies which have been investigated by other workers. Thus, further support has been given to the hypothesis that within normal serum there are present the precursors of all those antibodies which may arise in response to a specific immunising stimulus.

Summary and conclusions.

1. A study has been made of natural agglutination as exemplified by the reactions of the serum of nine animal species with a variety of bacteria.

2. End-titres are recorded, and the fact is noted that sera of different animal species show an order of agglutinating activity which is almost constant

for all organisms used. Ox, pig and horse sera give consistently strong reactions, while specimens from rabbit, guinea-pig and rat react weakly or not at all. Sheep, human and cat sera occupy an intermediate position. Variations are noted, however, with different individual specimens of serum from the same species.

3. Organisms of the series tested can also be grouped in order according to their apparent susceptibility to agglutination by normal sera.

4. The serum of young animals is found to be deficient in the agglutinating principle.

5. The agglutinating effect shows a thermolability intermediate between that of complement and the immune agglutinins. Complete inactivation occurs as a rule after exposure to 60° C.–65° C. for half-an-hour. For certain strains the serum principle is inactivated at much lower temperatures.

6. Lability curves show marked irregularity. In certain cases a zone of relative inactivation is produced at a temperature of 55° C.

7. The natural agglutinating substance is found to be present in greater degree in the carbonic acid insoluble fraction of serum than in the carbonic acid soluble fraction. In this respect it differs from the immune agglutinins, which are chiefly located in the carbonic acid soluble moiety.

8. The agglutinating principle for each organism can be absorbed completely by the homologous strain, when a variable lowering of the end-titre for other unrelated organisms results. A similar lowering of activity for these organisms may be produced by treating the serum with non-specific physical absorbents. Charcoal and Kieselguhr were used to demonstrate this.

9. By the technique of double absorption it can be shown that agglutination depends on non-specific and specific factors and it is concluded that normal serum agglutinates bacteria in virtue of a twofold mechanism:

(*a*) A non-specific effect reacting in varying degree with all organisms and removable by treatment with a finely divided absorbent.

(*b*) A series of specific effects reacting as true "natural antibodies." These specific antibody-like principles exist for a wide variety of organisms. Absorption of any one organism removes the homologous effect leaving the remainder quantitatively unimpaired.

10. The question of bacterial variation and receptor analysis in relation to the natural agglutinins is being studied and will be reported on at a later date.

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