THE NATURAL BACTERIAL AGGLUTININS

T H E S I S

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by

H.J. GIBSON,

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INTRODUCTION

The object of the work described in this thesis was to carry out a survey of natural bacterial agglut-:inins as found in the serum of normal animals. This inquiry was instituted in the first place with a view to elucidating the nature of these normal serum prin-:ciples, their relation to other natural antibodies and to "immune" agglutinins.

Historical.

The antibodies of normal human and animal sera attracted considerable attention among European work-:ers during the opening years of the present century. The power of normal sera to agglutinate micro-organisms in suspension was that chiefly studied. Dieudonné (1898) described agglutination of B.typhosus, V. chol-:erae and other bacteria. Bordet (1899) in his classical paper on the mechanism of agglutination made a brief reference to agglutination of B. typhosus and V. cholerae by normal ox serum. He carried out ag-:glutinin-absorption tests with these organisms in turn and suggested the specificity of the respective natural agglutinins. The possibility of such natural antibodies being the precursors of the "immune" anti-:bodies was also alluded to. Kraus and Low (1899) observed/

observed agglutination of a series of organisms which included staphylococci, B. anthracis, V. cholerae and B. coli by normal ox, rabbit, rat, human and horse sera. Gengou (1899) described the agglutination of B. anthracis by the normal serum of various animals. Lambotte and Maréchal (1899) studied the agglutinins of normal human and animal sera for the same organism and extended their observations to include serum of patients suffering from a variety of diseases. They recorded higher end-titres with human than with animal sera. Emulsions of anthrax "premier vaccin" were ag-:glutinated by normal human serum in dilutions up to 1:500. The end-titre showed increase in the case of patients suffering from active tuberculosis or typhoid fever. Rodet (1899) found that sheep serum failed to agglutinate B. coli. Further study of agglutination by sheep serum showed it to be relatively inactive. Thus Jatta (1900) reported negative results with two strains of B. typhosus and with 41 out of 57 strains of B. coli, the end-titre for the remainder being 1:10 - 1:30.

The delay in the formation of visible floccules by normal as compared with immune sera attracted the attention of Hahn and Tromsdorf (1900). Further dif-:ferences/

differences between natural and immune agglutinins were described by Landsteiner and Reich (1905). By a study of the physical properties of the floccules form-:ed by normal sera and the facility with which they could be broken up by mechanical means with re-appear-:ance of the agglutinating effect in the suspending fluid, these workers postulated a greater avidity of the immune agglutinin for its antigen. The distribut-:ion of the agglutinating principle over the serum protein fractions was the subject of investigation by Landsteiner and Calvo (1902) who separated normal ag-:glutinin in the euglobulin.

Streptococci were found to be agglutinable by horse serum and to a less extent by sheep serum (Moser and v. Pirquet (1902); Rossiwall and Schick (1905); and others).

The important observation was made by Müller (1901) that the serum of young horses showed little or no ag-:glutinating power. Kraus and Low (1899) who included streptococci in their series of organisms also noted this deficiency in the serum of young animals. Lam-:botte and Maréchal (1899) had previously recorded in the case of B. anthracis that the serum of newly born children possessed some agglutinating activity, titres up/

up to 1:60 being recorded in the first three days of life. The end-titres of sera from adults ranged from 1:300 to 1:500 for this organism. Lüdke (1904) found that serum of calves aged 1-4½ weeks lacked the agglut-:inating effect found in ox serum. Similar observat-:ions by other workers have been quoted by Rissling (1907). According to Braun (1909) sera from young animals are generally poor in natural antibodies though not deficient in complement.

The intimately associated questions of thermo-:lability, specificity, and relation to "immune" agglutinins were considered by many of the early work-:ers.

Bail (1902) found the natural agglutinins to be more thermostable than the lytic functions of normal sera while Romberg (1902) observed an unusual thermo-:lability of the natural agglutinins for B. tuberculos-:is, their effect being abolished at 56°C. Eisenberg and Volk (1902) found that horse serum lost its agglut-:inating power for B. typhosus at a temperature of 60°C. to 65°C. Joos (1903) suggested the existence of thermolabile and thermostable components in the natural agglutinins.

The question of specificity was studied by Hetsch and Lentz (1903). From agglutinin absorption experi-:ments they concluded that horse serum contains specific/

specific agglutinins for V. cholerae and for certain other vibrios. Scheller (1904) repeated this work and concluded that the binding group (haptophore) of a normal agglutinin is identical with that of an immune agglutinin. Lüdke (1905) undertook a considerable in-:vestigation into the absorption of agglutinins from normal ox serum by organisms. No decision was reached on the question of specificity. He noted, however, the difficulty of securing complete absorption of ag-:glutinins for the homologous organism and also a low-:ering of the end-titre for heterologous organisms which resulted. Streng (1909) in a systematic examin-:ation of the antibacterial power of normal ox serum noted that organisms fell into two classes according to the thermolability of the serum principles responsible for their agglutination. Those for B. tuberculosis, B. diphtheriae, B. mallei and B. pertussis were marked-:ly labile (56° C.) while those for B. typhosus, B. coli and others resisted heating up to 62° C. The reactions of the former group with bovine sera in presence of complement gave rise to the hypothesis of conglutinat-:ion.

Braun (1909) investigating agglutinins for V. cholerae in normal ox serum proposed a division in-:to thermostable and thermolabile factors, visible ag-:glutination/

agglutination being due to the thermolabile moiety, union with the receptor mechanism of the organism per-:taining to the thermostable group. He was able to demonstrate in inactivated serum agglutinoids with greater combining properties than the unheated agglut-:inins. Pronounced agglutination was found to result from the combined action of inactivated and absorbed serum each of which separately produced no visible re-:actions. Bürgi (1907) had attempted to explain nor-:mal agglutination in a similar way. Agglutination experiments with organisms were carried out in parallel with flocculation tests with a gum-mastic sol. The results with specimens of serum from the same species showed considerable similarity. It was suggested that the specific principle in both normal and immune agglut-:ination is the haptophore group of the agglutinin, visible agglutination being the result of a non-specific physical process. He arranged various animal species in order of potency as regards their natural bacterial agglutinins and found that this order held also in the colloid flocculation experiments.

Mamlock (1909) concluded that normal agglutinins are specific but full details of his results are not recorded. Bruce White (1927) in his work on spontan-:eous agglutination of "rough" salmonella-group organ-:isms and others suggested that agglutination by normal sera/

sera may be analogous to salt agglutination. He found that treatment of the organisms with lipoid solvents abolished the effect. He concluded that a hydrophobe lipoid is present in the cell-body of organisms and that its interaction with the colloids of normal sera results in agglutination. The effects were specially marked in the case of "rough" strains of the intestinal organisms. This worker inferred that a great deal of "normal agglutination" is probably a physical process of this non-specific nature.

It is now well recognised that motile organisms possess a double antigenic structure which comprises flagellar "H" and somatic "O" components, the former being thermolabile, the latter heat-stable (Smith and Reagh, 1903). The corresponding agglutinins of an immune serum react conversely to heat, the agglutinin for the flagellar component being more stable than that for the somatic antigen (Beyer and Reagh, 1904).

A further object of this study was to investigate the relationship of natural agglutinins to the flagellar and somatic antigens. Schiff (1922) has stated that natural agglutinins for members of the Salmonella group and also for B. proteus X and B. pyocyaneus strains are exclusively of the "O" type, and Felix and Olitzki (1929) consider that this accounts for the greater thermolability/

thermolability of natural agglutinins as compared with those of immune serum. Breinl (1920) on the other hand found evidence of agglutinins in normal ox serum reacting with both antigenic components of B. proteus strains. Timmerman (1930) has reported the occurrence of agglutinins for "O" and "H" forms of B. typhosus in the serum of different animals, and has observed that, in those sera which give marked reactions with organisms in the normal form, agglutinins for both types can be demonstrated.

Since the publication of part of the material embodied in this thesis, Lovell (1932, 1934) has studied the natural agglutinins in considerable detail with special reference to the "H" and "O" antigenic structure of the Salmonella group. By the use of "H" suspensions of S. aertrycke representing both type and group phases he was able to demonstrate specific agglutinins in the normal sera of healthy pigs, cattle, sheep and horses. This specificity was not confined to whole bacteria, but was also apparent for the antigenic components of the strains used. Thus specific "H" type agglutins could be clearly differentiated from those reacting with "H" group antigens, a remarkable demonstration of the specificity which natural antibodies may display. Lovell's work bearing on the origin of such antibodies will be more fully considered in/

in the discussion.

In the studies recorded in this thesis 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 occur-:rence of natural bacterial agglutinins, in this way to ascertain for which types of bacteria such agglutin-:ins are most frequent and whether they are specially associated with particular 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 thermostabil-:ity was investigated and also their specificity as judg-:ed 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 ag-:glutination 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 separ-:able from it, is a non-specific factor acting on all organisms in common. The natural specific agglutinins show/

show many points of resemblance to other natural anti-:bodies and it seems reasonable to suppose that they are the precursors in normal serum of the "immune" agglutinins.

EXPERIMENTAL WORK/

EXPERIMENTAL WORK

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 from 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/

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 emul-:sion showed more than a slight tendency to sedimentat-:ion 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 undil-:uted serum were well mixed and incubated for 3 hours at 37°C. with repeated shaking. The organisms were re-:moved 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 absorb-:ing dose and many experiments had to be repeated after failure to absorb completely the agglutinins for the absorbing strain. Dilution of the serum prior to ab-:sorption was not practicable in view of the low dilut-:ions required for the subsequent reactions. In some

cases/

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/

absorption was carried out as above. Certain absorp-:tion tests involved the use of red cell stromata pre-:pared by heating a 3 per cent. suspension of washed corpuscles overnight at 55°C. The fluid was removed and the sediment washed with saline before use.

Heating of sera was carried out in the water bath, the temperature indicated being maintained for 30 min-:utes. 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.

Methods of treatment of motile organisms to annul the "H" antigen were as follows:

(1) Heating:- A suspension of greater opacity than that required in the subsequent tests was heated in boiling water for two hours. Standardization to an opacity corresponding to Brown's tube 5 was then carried out by addition of saline.

(2) Treatment with Alcohol (see Bruce White (1928)):- Growth from plate cultures was emulsified in absolute alcohol and transferred to stoppered test tubes. The alcohol suspension was heated at 55°C. for 3 hours and repeatedly shaken. The organisms were then precipitat-:ed/

precipitated by centrifuging and washed once in fresh absolute alcohol after which they were suspended in 50 per cent. alcohol to form a dense suspension. Sus-:pensions of standard opacity for agglutination react-:ion were prepared by adding a small quantity of this to normal saline.

(3) Growth on 1:1000 Phenol Agar (see Braun and Schaeffer (1919)):- After repeated subculture the majority of the organisms used grew well on this medium and provided enough growth for making serviceable emulsions.

Flagellar suspensions were prepared (as described by Balteanu (1926)) from agar plates by emulsifying the growth in a small volume of 0.85 per cent. salt solut-:ion. The suspension was then vigorously mixed by drawing it up into a 10 c.c. pipette and rapidly ex-:pelling it. This pipetting was carried out for at least 5 minutes when the suspension was agitated in a shaking machine for half an hour. It was then centri-:fuged at high speed till the supernatant fluid was completely free from any obvious turbidity. The clear fluid constituted the flagellar suspension. The poss-:ible presence of bacterial bodies in such a fluid did not affect the results as readings were made within a short time after removal of the reactions from the incubator when only large loose floccules were noted.

Results/

Results

General Survey of the Occurrence of Natural Bacterial Agglutinins among various Animal Species.

Table I summarises the results of direct agglutin-:ation 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 act-:ion 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 react strongly with a majority of the organ-:isms used. Ox, pig and horse sera give the most con-:sistently strong results. Other animal sera are generally less active both as regards the number of organisms which they agglutinate and in their agglutin-:ation 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/

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.)
		B. proteus X 19.		
Ox	20	1:32	1:16 - 1:128	65
Rabbit .	6	0	0 - 1:16	.
Guinea-pig	5	0	0 - 0	.
Horse ...	5	1:32	1:16 - 1:64	62
Sheep ...	9	1:32	1:8 - 1:32	65
Pig	10	1:32	1:16 - 1:128	65
Rat	5	0	0 - 1:8	.
Cat	2	0	0 - 0	.
Human ...	7	0	0 - 1:8	.
		B. pyocyaneus.		
Ox	15	1:256	1:64 - 1:256	65
Rabbit ..	6	1:16	0 - 1:64	.
Guinea-pig	4	1:8-1:16	1:8 - 1:16	.
Horse ...	4	1:64	1:16 - 1:256	62
Sheep ...	6	1:64	1:64 - 1:256	67.5
Pig	8	1:256	1:128-1:1024	65
Rat	3	0	0 - 1:8	.
Cat	2	1:16-1:64	1:16 - 1:64	65
Human ...	15	1:16	0 - 1:32	.

B. coli X/

TABLE I - continued.

Serum	No. of samples tested.	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (°C.)
		B. coli X.		
Ox	23	1:32	1:16 - 1:128	50
Rabbit ..	7	0	0 - 1:16	.
Guinea-pig	5	1:8	0 - 1:8	.
Horse ...	5	1:32	0 - 1:32	.
Sheep ...	11	1:16	1:4 - 1:64	67.5
Pig	11	1:8	0 - 1:256	50
Rat	5	0	0 - 1:4	.
Cat	2	0	0 - 1:32	62.5
Human ...	12	1:8	0 - 1:32	.
		B. coli F 1.		
Ox	8	1:32	1:4 - 1:64	53
Rabbit ...	3	0	0	.
Guinea-pig	2	0	0 - 0	.
Horse ...	3	1:4	0 - 1:8	.
Sheep ...	5	0	0 - 1:16	65
Pig	6	1:16	0 - 1:16	.
Rat	2	0	0 - 0	.
Cat	2	0	0 - 0	.
Human ...	8	0	0 - 1:16	.

B. coli F 2/

TABLE I - continued.

Serum	No. of samples tested.	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (°C.)
		B. coli F 2.		
Ox	8	1:32	1:4 - 1:64	55
Rabbit .	2	0	0 - 0	.
Guinea-pig	2	0	0 - 0	.
Horse ..	2	0	0 - 1:16	.
Sheep ..	5	0	0 - 1:8	.
Pig	4	0	0 - 1:8	.
Rat	2	0	0 - 0	.
Cat	2	0	0 - 0	.
Human ..	6	0	0 - 1:8	.
		B. coli F 3.		
Ox	18	1:32	1:16 - 1:64	60
Rabbit .	3	0	0 - 0	.
Guinea-pig	2	0	0 - 0	.
Horse ...	4	0	0 - 1:32	.
Sheep ...	5	0	0 - 1:32	.
Pig	7	1:16	0 - 1:16	65
Rat	3	0	0 - 0	.
Cat	2	0	0 - 0	.
Human ...	5	0	0 - 1:8	.

B. coli F 4/

TABLE I - continued.

Serum	No. of samples tested	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (°C.)
		B. coli F 4.		
Ox	11	1:32	1:16 - 1:256	53
Rabbit .	2	0	0 - 0	.
Guinea-pig	2	1:8-1:16	1:8 - 1:16	.
Horse ..	3	0	0 - 1:16	.
Sheep ..	3	1:16	0 - 1:16	.
Pig	7	1:8	1:8 - 1:32	60
Rat	2	0	0 - 0	.
Cat	2	0	0 - 0	.
Human ..	6	0	0 - 0	.
		Pneumobacillus (Friedländer).		
Ox	11	1:256	1:64 - 1:256	65
Rabbit .	5	1:128	1:64 - 1:128	.
Guinea-pig	4	1:32	1:32	.
Horse ...	4	1:64	1:64 - 1:128	62.5
Sheep ...	7	1:128	1:16 - 1:128	65
Pig	9	1:256	1:64 - 1:512	67.5
Rat	4	1:32	1:16 - 1:64	.
Cat	2	1:64	1:64 - 1:128	67.5
Human ...	14	1:128	0 - 1:256	.

B. typhosus R.C.P./

TABLE I - continued.

Serum	No. of samples tested.	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (°C.)
		B. typhosus R.C.P.		
Ox	20	1:64	1:32 - 1:256	65
Rabbit ..	6	1:32	1:4 - 1:32	.
Guinea-pig	4	1:8-1:16	1:8 - 1:16	.
Horse ...	4	1:32	1:64 - 1:256	62
Sheep ...	8	1:64	0 - 1:128	67.5
Pig	10	1:128	1:32 - 1:256	65
Rat	4	0	0 - 1:8	.
Cat	2	1:8	1:8	.
Human ...	10	1:32	0 - 1:32	.
		B. paratyphosus A.		
Ox	17	1:32	1:16 - 1:128	62.5
Rabbit .	3	0	0 - 0	.
Guinea-pig	4	0	0 - 1:4	.
Horse ..	3	1:32	1:32 - 1:128	62.5
Sheep ..	5	1:16	0 - 1:16	.
Pig	8	1:32	1:16 - 1:64	65
Rat	3	0	0 - 0	.
Cat	2	0	0 - 0	.
Human ..	2	0	0 - 0	.

B. paratyphosus B./

TABLE I - continued.

Serum	No. of samples tested.	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (°C.)
		B. paratyphosus B.		
Ox	22	1:128	1:32 - 1:256	62
Rabbit .	6	0	0 - 0	.
Guinea-pig	5	1:8-1:16	1:8 - 1:16	.
Horse ...	5	1:64	1:32 - 1:256	62
Sheep ...	9	1:64	1:16 - 1:128	65
Pig	10	1:128	1:32 - 1:128	65
Rat	5	0	0 - 1:8	.
Cat	2	0	0 - 0	.
Human ...	7	1:8-1:16	0 - 1:16	.
		B. enteritidis Gaertner.		
Ox	12	1:128	1:32 - 1:128	62
Rabbit ..	4	0	0 - 1:8	.
Guinea-pig	4	1:8	1:8	.
Horse ...	3	1:64	1:16 - 1:64	65
Sheep ...	6	1:32	1:8 - 1:32	65
Pig	8	1:32	1:16 - 1:128	65
Rat	2	0	0 - 0	.
Cat	2	0	0 - 0	.
Human ...	9	0	0 - 1:16	.

B. dysenteriae Y./

TABLE I - continued.

Serum	No. of samples tested.	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (°C.)
		B. dysenteriae Y.		
Ox	13	1:256	1:64 - 1:256	65
Rabbit .	5	1:128	0 - 1:128	.
Guinea-pig	4	1:16	1:8 - 1:32	.
Horse ...	4	1:256	1:64 - 1:256	.
Sheep ...	6	1:256	1:64 - 1:256	65
Pig	7	1:256	1:32 - 1:1024	65
Rat	3	1:8	1:8 - 1:32	.
Cat	2	1:64	1:64 - 1:128	67.5
Human ...	10	1:256	1:128-1:256	.
		B. dysenteriae Shiga.		
Ox	13	1:32	1:32 - 1:128	65
Rabbit .	6	1:8	0 - 1:8	.
Guinea-pig	3	1:8	1:8 - 1:16	.
Horse ...	5	1:16	1:16 - 1:64	60
Sheep ...	8	1:16	1:16 - 1:64	65
Pig	9	1:32	0 - 1:64	62.5
Rat	3	0	0 - 0	.
Cat	2	0	0 - 0	.
Human ...	8	0	0 - 1:32	.

B. morgan I/

TABLE I - continued.

Serum.	No. of samples tested.	Most frequent end-titre	Range of titres noted	Temp. producing inactivation (°C.)
		B. morgan I.		
Ox	8	1:64	1:32 - 1:256	62
Rabbit .	5	1:16	0 - 1:16	.
Guinea-pig	4	1:8	0 - 1:8	.
Horse ...	3	1:64	1:32 - 1:64	62
Sheep ...	6	1:32	1:8 - 1:64	65
Pig	7	1:32	1:16 - 1:128	65
Rat	2	0	0 - 0	.
Cat	2	0	0 - 1:4	.
Human ...	5	0	0 - 1:8	.
		V. cholerae.		
Ox	19	1:64	1:32 - 1:256	65
Rabbit .	5	0	0 - 1:4	.
Guinea-pig	5	1:16	1:8 - 1:16	.
Horse ...	6	1:64	1:32 - 1:256	65
Sheep ...	9	1:64	1:16 - 1:128	67.5
Pig	10	1:32-1:64	1:16 - 1:128	65
Rat	4	1:16	0 - 1:32	.
Cat	2	1:16	1:16 - 1:32	65
Human ...	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.

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. The position of each animal species in such a series could be expressed numerically. In the same way for each other organism a similar series could be constructed. From a consideration of the numerical expressions of the positions of any animal species in all of the sixteen such series corresponding to the sixteen organisms used, a crude estimate might be made of its position in a "mean order of activity". It was at once apparent that with minor variations this mean order held for all organisms. For each organism the order of activity of different animal sera was al-:most 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.

In Table II the animal sera are arranged in des-:cending order of activity for four organisms. The end-titres on which the order is based are quoted. They are in every case those most frequently noted.

For/

TABLE II

Animal sera arranged in order of activity for four organisms. The end-titre recorded is that most frequently noted among specimens examined.

Serum	End-titre	Serum	End-titre
B. proteus X 19.		**B. pyocyaneus**	
Ox	1:64	Ox, Pig	1:256
Pig, Horse, Sheep	1:32	Horse, Sheep, Cat	1:64
Human, Cat, Rabbit, Guinea-pig, Rat	0	Human, Rabbit, Guinea-pig	1:16
		Rat	0
B. paratyphosus A.		**B. dysenteriae Y.**	
Ox, Pig, Horse	1:32	Ox, Pig, Horse, Sheep, Human	1:256
Sheep	1:16	Cat, Rabbit	1:128
Human, Cat, Rabbit, Guinea-pig, Rat	0	Guinea-pig	1:16
		Rat	1:8

Average order of potency

Ox
Pig
Horse
Sheep
Human
Cat
Rabbit
Guinea-pig
Rat

For comparison, the average order of activity, based on the results of agglutination reactions with all sixteen organisms, is recorded.

The four strains selected for Table II illustrate two examples of organisms which are relatively resistant to agglutination by normal sera, viz. B. proteus X19 and B. paratyphosus A. Contrasted with these in their greater susceptibility to the action of natural agglut-:inins are B. pyocyaneus and B. dysenteriae Y. Yet, despite this, variations in the order of activity among sera of different species will be seen to be slight.

One point which study of the results with individ-:ual specimens of serum emphasised was that the strength or weakness of a specimen was general for all the organ-:isms tested. This applied to each animal species. No serum has been encountered which gave abnormally strong or weak reactions with one organism only. This is illustrated in Table III in which two samples of pig serum are compared, one being markedly more active than the other towards all organisms.

Table I shows clearly that certain organisms were agglutinated in a consistently higher dilution of all sera than were others. B. dysenteriae Y, Pneumo-:bacillus and B. pyocyaneus were outstanding in this respect. They would appear to be highly susceptible to/

TABLE III

End-titres recorded in agglutination reactions with two specimens of *Pig* serum.

Organism	Serum	
	Pig I	Pig II
B. proteus X 19	1:64	1:16
B. pyocyaneus	1:256	1:128
B. coli X	1:32	1:8
B. coli F 4.	1:32	1:8
Pneumobacillus	1:256	1:64
B. typhosus	1:32	1:16
B. paratyphosus A	1:32	1:8
B. paratyphosus B	1:128	1:8
B. enteritidis Gaertner	1:32	1:16
B. dysenteriae Y	1:1024	1:32
B. dysenteriae Shiga	1:32	1:32
B. morgan I	1:64	1:16

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 individual 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.

With the object of ascertaining the mean order of susceptibility to agglutination of the organisms used a similar method was applied as in evaluating the order of activity of the sera. The organisms were arranged in nine series according to the most frequent end-titre recorded with the serum of each of the nine animal species under consideration. The relative susceptibility of an organism could then be crudely estimated by its position in an average series derived from these. A comparison could then readily be made between the order of agglutinability by any one serum and the mean series. It was found that with minor variations this average order of susceptibility was a fairly accurate indication of the results with sera of each animal species.

Table IV shows the most frequent titres noted in agglutination/

TABLE IV

Organisms arranged in order of the most frequent end-titres noted in their reactions with numerous samples of serum from four animal species.
Their average position obtained after consideration of results with serum of all species of animals investigated is recorded for comparison.

Organism	End-titre
Sheep Serum	
B. dysenteriae Y	1:256
Pneumobacillus *B. pyocyaneus*	1:128
B. typhosus R.C.P *B. paratyphosus* B *V. cholerae* *B. Gaertner*	1:64
B. morgan I *B. proteus* X 19	1:32
B. dysenteriae Shiga *B. paratyphosus* A *B. coli* X *B. coli* F 4	1:16
B. coli, F1, F3, F2	0
Pig Serum	
B. dysenteriae Y *Pneumobacillus*	1:256
B. pyocyaneus *B. typhosus* R.C.P *B. paratyphosus* B	1:128
V. cholerae	1:64
B. Gaertner *B. morgan* I *B. proteus* X 19 *B. dysenteriae* Shiga *B. paratyphosus* A	1:32
B. coli F 1 *B. coli* F 3	1:16
B. coli X and F4	1:8
B. coli F2	0
Guinea-pig Serum	
Pneumobacillus	1:32
B. dysenteriae Y *B. pyocyaneus* *B. typhosus* R.CP *B. paratyphosus*B *V. cholerae*	1:16
B. Gaertner *B. morgan* I *B. dysenteriae* Shiga *B. coli* X & F4	1:8
B. proteus X19 *B. paratyphosus* A *B. coli* F1, F3, F2	0
Ox Serum	
B. dysenteriae Y *Pneumobacillus* *B. pyocyaneus*	1:256
B. paratyphosus B *B. Gaertner*	1:128
B. typhosus R.C.P. *V. cholerae* *B. morgan* I	1:64
B. dysenteriae Shiga *B. proteus* X19 *B. paratyphosus* A *B. coli* X, F4, F1 *B. coli* F3, F2	1:32

Average/

TABLE IV - continued

Average order for sera of all animal species

B. dysenteriae Y

Pneumobacillus

B. pyocyaneus

B. typhosus R.C.P.

B. paratyphosus B

V. cholerae

B. Gaertner

B. morgan I

B. dysenteriae Shiga

B. proteus X 19

B. paratyphosus A

B. coli X

B. coli F 4

B. coli F 1

B. coli F 3

B. coli F 2

agglutination reactions between the sixteen organisms of the present series and the sera of sheep, pig, guinea-pig and ox. Sheep and guinea-pig exemplify the weaker sera, pig and ox those giving stronger reactions.

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.

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

(3) B. paratyphosus A, B. proteus X19, 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, probably because of its dense mucoid capsular material.

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 significance may be readily estimated.

Influence of the Age of the Animal on the Agglutinin Content of its Serum.

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 ser-:um 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 V.

It will be seen that the agglutinating activity of the serum increased with age, the increase being stead-:ily progressive.

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

Thermolability/

TABLE V

Influence of age upon agglutinating activity of rabbit serum.

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

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

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

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

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 ar-:rived 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 in the constant-temperature water-bath.

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. Thermostabil-:ity 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.

Furthermore, agglutinins for the same organism in sera of different animal species were not inactivated at the same temperature. Thus the normal serum prin-:ciple 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 X19 and 1:256 for B. pyocyaneus. In-:activation 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 being abolished at 65°C.; the fresh serum agglutinated B. pyocyaneus in dilutions up to 1:256 but this effect was annulled at 62°/

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 temperatures 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 No. I and the sera of ox, pig, horse and sheep showed a comparative stability at temperatures below 55°C. Higher temperatures/

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 provided 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.

Braun (1909) suggested that thermal inactivation of natural agglutinins suppressed merely the group responsible for visible clumping of the organism - the agglutinophore (Ehrlich) - leaving the binding group with heightened affinity for the organisms (pro-agglutinoid).

Experiments were carried out in which organisms were treated with serum inactivated by heat and subsequently used in agglutination reactions with fresh unheated serum of the same species. If "pro-agglutinoids" of greater binding power than that of the agglutinins were present in heated serum they would attach themselves strongly to the organisms, block their receptor mechanism and so render them inagglutinable by fresh serum.

The/

The following is an example of such an experiment:-

Two well-grown 24 hours' agar slope cultures of B. pyocyaneus and B. dysenteriae Y were emulsified in 0.85 per cent. salt solution. The growths were separated by centrifuging and emulsified each in 5 c.c. of a 1:4 dilution of pig's serum which had been previously inactivated by heating for 30 minutes at 66°C. Organisms and serum were incubated for 3 hours at 37°C. when the organisms were again separated and washed in 0.85 per cent. saline.

The treated and washed organisms were then resuspended in salt solution and used in agglutination reactions with fresh pig's serum. Controls were introduced in parallel with these reactions, untreated organisms being tested with fresh and inactivated serum, and treated organisms with inactivated serum.

The results were as shown in Table VI.

This experiment shows that previous treatment with inactivated serum did not render the organism inagglutinable by fresh serum. It may therefore be concluded that pro-agglutinoids in the sense understood by the earlier workers are not formed in normal sera exposed to heat. In the case of B. pyocyaneus it will be noted that a higher titre was recorded after treating the organisms than before.

The/

TABLE VI

Agglutination reactions involving the use of organisms treated with serum which had been inactivated by heat. Treated and untreated organisms used in agglutination reactions with fresh and inactivated serum.

Organism	Pig's serum			
	Fresh		66°C. - 30 minutes	
	Organisms		Organisms	
	Treated	Untreated	Treated	Untreated
B. pyocyaneus	1:1024	1:512	0	0
B. dysenteriae Y	1:512	1:512	0	0

The reason for this was provided by an experiment on similar lines carried out with pig's serum and Pneumobacilli. The serum was heated at 66°C., a temperature insufficient in this case to produce complete inactivation. The results were as illustrated in Table VII.

It will be seen that the temperature applied left a residue of agglutinins giving an end-titre of 1:32, as compared with 1:128 in the fresh serum. This appears to have been sufficient to sensitize the treated bacilli so that the unheated serum agglutinated to a higher end-titre than with the untreated organisms. A further noteworthy result is that the heated serum acting on the treated organisms yielded as high an end-titre as that noted in the test with fresh serum.

It seems probable therefore that the rise in titre noted in the experiment with B. pyocyaneus (Table VI) was due to the fact that the degree of heat applied was insufficient to abolish completely the agglutinins for this organism. The small residue, insufficient to produce obvious agglutination in a 1:4 dilution of serum, was still able to bring about an apparent "sensitising" effect.

Distribution/

TABLE VII

Agglutination reactions involving the use of organisms treated with serum which had been heated to a temper-:ature insufficient to secure complete inactivation.

Organism	Pig's serum			
	Fresh		66°C.	
	Organisms		Organisms	
	Treated	Untreated	Treated	Untreated
Pneumobacillus	1:512	1:128	1:512	1:32

Distribution of Natural Agglutinins in the Serum Protein Fractions.

The method of fractioning by carbon-dioxide (Liefmann, 1909) was adopted. This technique has been utilised by Mackie and Watson (1926) and Mackie and Finkelstein (1928, 1930) in analysing complement fix-:ation phenomena produced by normal sera and certain "pseudo-antigens".

The method was as follows. One volume of serum was diluted with nine volumes of ice-cold distilled water and carbon dioxide gas was bubbled through the mixture for a period of ten minutes. The whole was then allowed to stand for an hour, the tube containing the fluid being kept cool by immersion in chopped ice. The precipitate which formed was removed by centri-:fugalising. The clear supernatant fluid, the CO_2 soluble portion, constitutes the B fraction. It was rendered isotonic by the addition of concentrated NaCl solution. The CO_2 insoluble precipitate (A fraction) was dissolved in an amount of 0.85 per cent. salt sol-:ution equal to twice the volume of the original serum (Browning and Mackie, 1925). The A fraction contains the euglobulin and part of the pseudoglobulin of the serum, the B represents the remainder of the pseudo-:globulin and the albumen.

The/

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 ap-:propriately 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 VIII.

For purposes of comparison specimens of various high-titre agglutinating sera were subjected to the same treatment with carbon-dioxide in order to deter-:mine the distribution of the specific immune agglutin-:ins. Table IX 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 pro-:perty is located mainly in the A (carbonic acid-in-:soluble) 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 act-:ivity 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 IX). It is of particular interest that in normal sera the A fraction/

TABLE VIII

Carbon dioxide fractioning of normal sera.
Titres recorded in agglutination reactions using whole serum, A (CO_2 insoluble) and B(CO_2 soluble) fractions.

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

Organisms used in agglutination reactions	Ox serum Sample I			Ox serum Sample II		
	S	A	B	S	A	B
B. proteus X 19 ..	1:128	1:128	0	1:32	1:32	0
B. pyocyaneus	1:64	1:256	1:10	1:64	1:32	0
B. typhosus R.C.P.	1:256	1:128	1:80	1:64	1:32	0
B. paratyphosus A	.	.	.	1:16	1:8	0
B. paratyphosus B	.	.	.	1:32	1:16	0
B. enteritidis Gaertner...	1:64	1:64	1:5	.	.	.
B. coli X	1:32	1:16	1:20	1:8	0	0
B. dysenteriae Y .	1:256	1:128	1:40	1:128	1:128	1:80
B. dysenteriae Shiga	1:64	1:64	0	1:64	1:16	0
B. morgan I	1:128	1:128	0	.	.	.
V. cholerae	1:64	1:128	1:20	1:64	1:16	0
Pneumobacillus ...	1:128	1:128	1:40	.	.	.

	Sheep serum			Pig serum		
	S	A	B	S	A	B
B. proteus X 19 ..	.	.	.	1:128	1:32	1:80
B. pyocyaneus	1:128	1:64	1:5	1:256	1:64	1:80
B. typhosus R.C.P.	.	.	.	1:64	1:64	1:20
B. paratyphosus A.	1:32	0	1:10	1:32	1:8	1:5
B. paratyphosus B	1:64	1:32	0	1:128	1:64	1:40
B. enteritidis Gaertner..	1:32	1:16	0	1:128	1:64	1:10
B. coli X	1:16	0	0	1:64	1:32	1:80
B. dysenteriae Y	1:256	1:64	1:20	1:512	1:256	1:160
B. dysenteriae Shiga ...	.	.	.	.	.	.
B. morgan I	1:64	1:32	0	1:128	1:128	1:20
V. cholerae	1:64	1:16	1:10	1:64	1:32	1:10
Pneumobacillus ..	1:64	1:64	1:10	1:512	1:64	1:160

Horse/

TABLE VIII - continued

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

TABLE IX

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 insol-:uble)	B fraction (CO_2 sol-:uble)
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

fraction was in some cases more active than the whole serum, e.g. ox - B. pyocyaneus and ox - V. cholerae (Table VIII). 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/

Agglutinin-Absorption Experiments

By the technique described above, specimens of serum were subjected to treatment with bacterial cult-:ures with a view to absorbing the homologous agglutin-:ins* and determining whether the agglutinating prin-:ciples of normal sera were specific.

It was at once found that an organism could absorb its agglutinins from normal serum, provided the absorb-:ing 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 ex-:ample 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 cult-:ure, computed in terms of the number of agar slopes 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/

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*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.

being completely removed by six cultures. Still larg-:er absorbing doses also removed the agglutinin com-:pletely. 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.

Similar experiments were carried out with other sera. Thus, 1.2 c.c. of normal human serum required eleven agar slope cultures of B. dysenteriae Y, the original titre in this case being 1:128. This experi-:ment was of interest in showing some tendency to a "zone" effect. After treatment by three agar slope cultures the end-titre was 1:16, with six cultures the end-titre was 1:64.

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 exception-:al and never occurred when the initial end-titre of the untreated serum for such organism was relatively high.

While/

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 dir-:ect 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 X - XIII.

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 ag-:glutination. 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 ab-:sorbents, such as charcoal and Kieselguhr, were employed/

TABLE X

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
V. cholerae....	1:128	0	1:64	0	1:32	1:16
B. proteus X19	1:32	1:32	1:32	1:32	1:16	1:16
B. pyocyaneus	1:128	1:64	1:256	1:256	1:128	1:64
B. coli X	1:32	0	1:32	1:64	1:32	1:32
B. coli F 1 ..	1:4	0	.	.	1:32	1:16
B. coli F 2 ..	1:32	1:16	.	.	1:16	1:4
B. coli F 3 ..	1:32	1:16	.	.	1:16	1:16
B. coli F 4 ..	1:16	0	.	.	1:32	1:16
Pneumobacillus	.	.	1:32	1:32	1:256	1:128
B. typhosus R.C.P.	1:64	1:8	1:128	1:64	1:128	1:128
B. paratyphosus A ..	1:16	0	1:32	1:32	1:16	1:8
B. paratyphosus B	1:64	1:4	1:128	1:64	1:64	1:16
B. enteritidis Gaertner.	.	.	1:16	1:16	1:32	1:16
B. dysenteriae Y	1:128	1:128 (zone)	1:256	1:256 (zone)	1:64	1:128 (zone)
B. dysenteriae Shiga .	1:64	1:32	1:32	1:16	1:32	1:16
B. morgan I ..	.	.	1:64	1:32	.	.

TABLE XI

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 *B. proteus* X 19	Untreated serum	Serum absorbed *B. morgan* I
B. proteus X19	1:32	1:32	1:32	0	1:32	1:16
B. pyocyaneus	1:256	1:128	1:256	1:64	1:256	1:256
B. coli X	1:64	1:64	1:32	1:64	1:64	1:64
Pneumobacillus	1:64	1:32	1:64	1:128	1:64	1:64
B. typhosus R.C.P. .	1:64	1:32	1:64	1:64	1:64	1:64
B. paratyphos-:us A ..	1:16	1:16	1:16	1:16	1:16	1:16
B. paratyphos-:us B ..	1:32	1:16	1:128	1:64	1:32	1:128
B. enteritidis Gaertner .	1:32	1:16	1:32	1:64	1:64	1:64
B. dysenteriae Y	1:256	1:256 (zone)	1:256	1:256 (zone)	1:256	1:256 (zone)
B. dysenteriae Shiga .	1:32	1:16	1:32	1:32	1:16	1:8
B. morgan I ...	1:64	1:64	1:64	1:32	1:32	0
V. cholerae ...	1:32	1:32	1:64	1:32	1:128	1:128

TABLE XII

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 X19 ..	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. paratyphos-:us A ...	1:32	1:64	1:128	1:64
B. paratyphos-:us 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 XIII

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 X19	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. paratyphos-:us A ..	1:16	1:16	.	.
B. paratyphos-:us 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	.	.

employed in similar experiments. The results of such experiments are recorded in Tables X - XII, 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.

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 X) 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 by physical agents.

Much light was thrown on this question by a series of experiments in each of which a single specimen of serum was subjected to bacterial and non-specific physical absorption. Table XIV records the result of such an experiment. The end-titres of the serum for a series of organisms are recorded before and after absorption/

TABLE XIV

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. paratyphos-:us A	1:16	0	1:16	1:32
B. paratyphos-:us 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

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 the homologous organism removes both "specific" and "non-specific" agglutinins, the heterologous agglutinins absorbed being quite "non-specific" and capable of being removed by non-bacterial suspensions.

Tables XV and XVI show the same features in the sera of sheep and pig. The specimen of sheep serum used illustrates the possibility of the whole of the agglutinins for a particular organism being "non-specific" as shown in the case of the B. dysenteriae Shiga reactions. Treatment by each of four unrelated organisms and by charcoal removed all agglutinating effect/

TABLE XV

Absorption of a sample of normal sheep serum by organisms and by a physical agent (charcoal).

Agglutination reactions with	Absorption carried out with organisms and charcoal as under. End-titres as noted.					Titre before absorption
	B. paratyphosus B.	Pneumobacillus	B. Gaertner	V. cholerae	Charcoal	
B. proteus X 19 ..	1:8	1:16	1:8	1:8	1:16	1:16
B. pyocyaneus	1:64	1:64	1:64	1:64	1:64	1:64
B. coli X ...	1:8	1:8	1:8	1:8	1:8	1:8
Pneumobacillus	1:64	0	1:64	1:64	1:64	1:64
B. typhosus .	1:16	1:16	1:16	1:16	1:16	1:16
B. paratyphosus A	1:4	1:8	1:4	1:4	1:4	1:8
B. paratyphosus B	0	1:16	1:16	1:16	1:16	1:16
B. enteritidis Gaertner	1:16	1:32	0	1:32	1:32	1:64
B. dysenteriae Y ...	1:64	1:64	1:128	1:128	1:64	1:64
B. dysenteriae Shiga	0	0	0	0	0	1:32
B. morgan I ..	0	0	0	1:4 (tr)	0	1:8
V. cholerae ..	1:16	1:16	1:16	0	1:16	1:32

TABLE XVI

Absorption of a sample of pig's serum by organisms and by charcoal.

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

effect for this strain.

There was considerable evidence that the "specific" agglutinin was more constant among individuals of a species than the "non-specific". Thus the reactions of ox serum absorbed by various organisms and physical agents showed a constant end-titre of 1:16 or 1:32, for V. cholerae, even though the initial end-titre of the untreated serum varied from 1:64 to 1;256. This also applied to sera of other species. Sheep serum absorb-:ed by B. paratyphosus B, B. proteus X19, or by char-:coal agglutinated V. cholerae in a titre of 1:32. The titres of samples of untreated sheep serum for this organism are as a rule lower than those of ox but the loss sustained on non-specific absorption is less. Pig and horse serum showed similar features. See Table XVII.

In the same way it was found that serum treated by heterologous organisms and by physical absorbents re-:tained the power of agglutinating B. dysenteriae Y in a titre varying from 1:128 to 1:256. It was also noted with great regularity that such treated serum failed to agglutinate this organism in low dilutions. The reactions showed a zone of maximal agglutination in dilutions 1:32 and 1:64, the effect becoming progress-:ively weaker in higher and lower concentrations of serum/

TABLE XVII

End-titres of agglutination of *V. cholerae* by unabsorb-:ed and absorbed samples of serum from four animal species, showing the relatively constant end-titre of "specific" agglutinin which remains after absorption by an unrelated strain.

Serum	Titres of agglutination of V. cholerae	
	Before absorp-:tion	After absorp-:tion
Ox serum treated		
B. coli X	1:128	1:32
B. paratyphosus B	1:128	1:32
B. proteus X 19	1:256	1:32
R.B.C. stromata	1:128	1:32
Kieselguhr	1:64	1:16
Sheep serum treated		
B. paratyphosus B	1:64	1:32
B. proteus X 19	1:64	1:32
Charcoal	1:32	1:32
Pig serum treated		
B. pyocyaneus	1:32	1:32
R.B.C. stromata	1:64	1:32
Charcoal	1:64	1:32
Horse serum treated		
B. proteus X 19	1:16	1:16

serum. In certain cases where the titre of the original specimen was low, treatment of the serum resulted in an unexpected rise in titre, reaching 1:128 - 1:256, the reaction again showing a "zone" effect. This held for all sera examined, viz. ox, horse, pig, and sheep (see Table XVIII).

It would seem probable that, on removal of the "non-specific" principle, specimens tend to show more equality in their activity towards certain strains.

Absorption at Low Temperatures.

A series of experiments was carried out in which moieties of the same specimen of serum were absorbed at 0°C. or less and at 37°C. Subsequent reactions showed similar results in both cases.

Further/

TABLE XVIII

To show in the case of *B. dysenteriae* Y the relative uniformity of end-titre of sera following heterologous absorption.

Serum	Titres of agglutination of B. dysenteriae Y	
	Before absorption	After absorption
Ox serum treated		
B. proteus X19, sample I	1:64	1:128 (zone)
sample II	1:256	1:256 (")
V. cholerae, sample I	1:128	1:128 (zone)
sample II	1:256	1:256 (")
Kieselguhr, sample I	1:256	1:128 (zone)
sample II	1:64	1:128 (")
R.B.C. stromata	1:256	1:128 (zone)
Sheep serum treated		
B. paratyphosus B	1:256	1:128 (zone)
B. proteus X 19	1:256	1:256 (zone)
B. morgan I	1:256	1:256 (zone)
Charcoal	1:256	1:256 (zone)
Pig serum treated		
B. pyocyaneus, sample I	1:512	1:256
sample II	1:256	1:128
R.B.C. stromata	1:1024	1:256
Charcoal	1:512	1:512 (zone)
Horse serum treated		
B. proteus X 19	1:128	1:256

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 XIX gives an example of such an experiment.

It will be seen from Table XIX 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 Shiga illustrate the fall in titre which a serum may sustain/

TABLE XIX

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

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 similar 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/

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 aerog-:enes, an organism which is not agglutinated by this or any other of the animal sera investigated. 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 agglutinating effect for other org-:anisms. 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 section on 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/

added, the whole being incubated for three hours at 37° C. with repeated shaking.

The vibrios were separated by rapid centrifugalis-:ation 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 XX.

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 pre-:sents the same features as before.

Double Absorption Experiment Using a Physical Agent.

A sample of pig serum was used in a double ab-:sorption experiment involving the application of char-:coal 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 org-:anisms of the series used.

Method/

TABLE XX

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

Method. 5 gm. 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 XX.

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.

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

Occurrence/

TABLE XXI

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

Occurrence of Natural "H" and "O" Agglutinins in the Serum of Various Animals.

For the demonstration of natural O-agglutinins normal serum from the following animal species was ex-:amined: man, ox, pig, horse, sheep, guinea-pig, rabbit and cat. The following organisms were employ-:ed:- B. pyocyaneus, V. cholerae ("Bombay" and "3134" strains), B. proteus X19, B. morgan No. 1, B. typhosus (various strains), B. paratyphosus A, B. paratyphosus B, B. enteritidis Gaertner, Derby type of Salmonella group, B. abortus equi, B. coli (various strains).

By examination of a sufficient number of specimens of serum from the various animal species it was found possible to demonstrate the composite nature of the natural agglutinins for all the above organisms. As in the case of untreated live organisms, when the "O" agglutinogen alone was tested marked differences were noted between one sample of serum and another from the same animal species. It was concluded that without examining a very large number of samples of serum from each animal species, it would be unjustifiable to state any order of agglutinating strength quâ O-agglutinin among the various species.

In some cases the end-titre for a heated suspens-:ion was greater than that for the normal unheated organisms/

organisms, in others it was less, while in still other cases both were equal. The relative preponderance of "H" and "O" agglutinins varied in different samples of serum from the same species, and in different animal species.

Table XXII illustrates the results obtained. The suspensions from growths on phenol-agar, heated and alcoholised suspensions gave only small-flake agglutinat-:ion. The granules formed slowly and settled as a fine powdery deposit at the foot and on the side of the agglutination tube exactly as in O-agglutination with immune sera. In all cases the end-titre was lower with formolised than with fresh suspensions. Assoc-:iated with this there was a relative preponderance of large flakes when formolised suspensions were used. It was apparent that the presence of formalin to some extent inhibited the action of the O-agglutinins or interfered with "O" antigen. A comparison of the end-titres for heated suspensions with those for phenol-agar and alcoholised suspensions showed that heating does not produce exactly the same result as growth in presence of phenol or treatment with alcohol. By absorption methods Timmerman (1930) found that heating destroys part of the "O" antigen as well as the "H", while alcoholisation is a less drastic procedure and leaves/

TABLE XXII

Ox serum

Agglutination Reactions with "O" and "H" antigens of certain Gram-negative bacilli.

Bacterial suspension used	B. typhosus	B. enteritidis Gaertner	B. paratyphosus A	B. abortus equi
Unheated	1:64 L + S	1:64 L + S	1:64 L+S	1:16 L + S
Formolised	1:32 L + S	1:32 L	1:64 L	1:32 L + S
0.1% Phenol agar suspension ...	1:64 S	1:64 S	1:64 S	1:64 S
Heated (100° C.) suspension	1:32 S	1:32 S	1:32 S	1:32 S
Alcoholised	1:64 S	1:64 S	1:64 S	1:64 S

In this and subsequent tables,
L = Large-flake or flocculent agglutination;
S = Small-flake or granular agglutination.

leaves more of the somatic antigen intact.

While the end-titres of "H" and "O" agglutination as shown in Table XXII were often similar this was not constantly found. In certain cases the end-titre for the "O" antigen of an organism was markedly greater than that noted before destruction of the "H" antigen. Table XXIII illustrates this in the case of a strain of B. coli. This strain had been recently isolated and showed no tendency to "rough" variation which might have produced a similar effect after heating, as has been observed by Bruce White (1928).

In other cases the titre for a heated antigen was lower than that for unheated organisms (see Table XXIV). Thus, the serum of different animal species gave lower end-titres with B. pyocyaneus after removal of its "H" antigen.

The subsequent tables also illustrate the end-titre of samples of serum from various species in their reactions with "H" and "O" forms of organisms.

Agglutination/

TABLE XXIII

Serum from animals of various species in agglutination reactions with unheated and heated suspensions of a *B. coli* strain (F 3).

B. coli	Horse (1)	Horse (2)	Ox	Pig	Rabbit	Sheep	Cat
Unheated	0	1:32 L+S	1:16 L+S	1:32 L+S	0	1:8 L	0
Heated (100°C.)	1:256 S	1:128 S	1:256 S	1:128 S	1:8	1:256 S	1:8 S

TABLE XXIV

Serum from animals of various species in agglutination reactions with unheated and heated suspensions of *B. pyocyaneus*.

B. pyocyaneus	Horse (1)	Horse (2)	Ox (1)	Ox (2)
Unheated	1:64 L+S	1:64 L+S	1:128 L + S	1:128 L + S
Heated (100°C.)	1:16 S	1:16 S	1:64 S	1:16 S
	Pig	Guinea-pig	Cat	Sheep
Unheated	1:1024 L + S	1:16 L + S	1:16 L+S	1:256 L + S
Heated (100°C.)	1:32 S	1:8 S	0	1:32 S

Agglutination Reactions with Flagellar Suspensions

Balteanu (1926) and others have shown how the clear supernatant fluid of vigorously shaken suspensions of V. cholerae may be employed as an "H" antigen in agglutination reactions. By the technique described above various motile organisms were treated to obtain such flagellar suspensions. The results were quite definite, large flocculent masses appearing rapidly in such tests. It was found that such appearances could be produced in all cases where a sample of serum re-:acted in even moderately high dilution with an un-:treated suspension of a motile organism. Heating the suspension before separation of flagella, or heating the flagellar suspension, abolished the effect. It was observed, however, that in reactions with heated "flagella" a very fine granular type of agglutination appeared late (18 hours). This was probably due to a failure to remove all bacterial bodies by the method of separation employed. Such granular agglutination con-:trasted sharply with the flocculent flagellar type in its appearance and in the time at which it was observed (Table XXV). In some cases an early reading half an hour after removal from the incubator showed the first tube (dilution 1:4) to be completely opalescent, the large floccules merging into each other. Horse and pig/

TABLE XXV

Ox serum.

Agglutination reactions with flagella from two strains of *B. typhosus*.

	Reading at 4 hours	
	End-titre	Type of agglutination
B. typhosus ("CB") unheated "flagella"	1:32	Floccules only
heated (100° C.) "flagella"	0	
B. typhosus ("Cole") unheated "flagella"	1:32	Floccules only
heated (100° C.) "flagella"	0	
	Reading at 18 hours	
	End-titre	Type of agglutination
B. typhosus ("CB") unheated "flagella"	1:32	Floccules + granules
heated (100° C.) "flagella"	1:16	Granules only
B. typhosus ("Cole") unheated "flagella"	1:32	Floccules + granules
heated (100° C.) "flagella"	1:16	Granules only

pig sera gave similar results with B. typhosus and other organisms including V. cholerae (2 strains), B. paratyphosus A, B. paratyphosus B, B. proteus X19, B. morgan No. 1, and B. pyocyaneus.

Films were made from flocculi and their structure was shown by appropriate staining methods to consist of a loose network of bacterial flagella.

Agglutinin/

Agglutinin Absorption Experiments Involving "H" and "O" Antigens.

Schiff (1922) observed what appeared to be large-flake agglutination of B. proteus and other strains with normal animal sera but as a result of agglutinin absorption tests suggested that such appearances were simply the result of the coalescing of small flakes in low dilutions of serum. In order to determine whether the results described above could be explained in this way a series of experiments was undertaken in which ser-:um was absorbed by unheated and by heated suspensions of the same organism, the result being analysed by the use of unheated living organisms, alcoholised suspens-:ions and flagella in the subsequent agglutination re-:actions (Table XXVI). The absorbing dose of organisms in each case was the 24 hours' growth from one 6 ins. agar plate for 2 c.c. of serum. The results suggested that agglutinins are present in normal serum which re-:act specifically with the somatic and flagellar antigens of the organism.

An attempt was made to find if any relation exists between the specific and non-specific effects on the one hand and the somatic and flagellar agglutinins on the other. Table XXVII records the results of an ex-:periment/

TABLE XXVI

Horse serum

Agglutination absorption by B. typhosus ("Cole") unheated and heated at 100°C. for two hours.

Suspension B. typhosus	Serum untreated	Serum absorbed	
		B. typhosus ("Cole")	B. typhosus ("Cole") 100°C.
Unheated	1:64 L+S	0	1:16 L only
Heated 100°C.	1:32 S	0	0
Alcoholised	1:32 S	0	0
Flagella	1:32 L only	0	1:32 L only

TABLE XXVII

Ox serum

Double absorption of serum by:
(1) V. cholerae ("Bombay") heated at 100°C.;
(2) B. proteus X 19 (unheated).

Suspensions used in agglutination reactions	Serum unabsorbed	Serum absorbed	
		V. cholerae 100°C.	1.V.cholerae 100°C. 2.B.proteus X19 (unheated)
Unheated			
V. cholerae	1:128 L+S	1:16 L	16 L
B. proteus X 19	1:64 L+S	1:32 L + S	0
B. paratyphosus A	1:64 L+S	1:32 L + S	32 L + S
B. paratyphosus B	1:128 L+S	1:32 L + S	32 L + S
B. enteritidis Gaertner	1:128 L+S	1:32 L + S	32 L
B. morgan I	1:64 L+S	1:32 L + S	32 L (+ S)
Heated 100°C.			
V. cholerae ...	1:64 S	0	0
B. proteus X 19	1:128 S	1:32 S	0
B. paratyphosus A	1:32 S	1:16 S	1:4 S
B. paratyphosus B	1:128 S	1:16 S	1:8 S
B. enteritidis Gaertner	1:128 S	1:16 S	0
B. morgan I	1:32 S	1:32 S	1:8 S

experiment carried out to throw light on this question. Previous work had shown that absorption of normal ser-:um by organisms heated or unheated removed a non-specific agglutinating effect from the serum. In this case V. cholerae (heated at 100°C.) was used for the removal of O-agglutinin for V. cholerae and the "non-specific" agglutinins for the other organisms tested. It is then seen that the once-treated serum contained only "specific" agglutinins since a further absorption by B. proteus X19 removed only its own agglutinins, leaving those for unheated suspensions of unrelated strains quantitatively undiminished. The type of ag-:glutination noted after the second absorption was, however, changed in that a preponderance of "H" or flagellar type appeared.

The second part of Table XXVII in which heated suspensions were used after each absorption shows that the somatic agglutinins for all organisms were reduced by each absorption. B. proteus X19, the second ab-:sorbing organism, not only removed its own somatic agglutinins but removed wholely or partly those for the other organisms used. The organisms used for absorp-:tion in this experiment were selected at random from those which reacted with the specimen of serum used.

This experiment and others of the same type which were/

were carried out seemed to show that the non-specific agglutinins reacted with both "H" and "O" agglutinins but to a greater extent with the latter.

The suggestion is made that the large-flaking agglutinins of normal serum are of two types.

1. Non-specific, absorbable by any organism (including heated organisms) or physical absorbent such as charcoal.

2. Specific - each for an individual organism.

The small-flaking agglutinins appear to react differently, having affinities for the "O" antigens of a large number of unrelated organisms. It was found impossible by any treatment of the serum to demonstrate a residium of O-agglutinins, each specific for an individual organism and unaffected by absorption by an unrelated strain. Repeated absorption had a tendency to remove small-flaking agglutinins for all organisms from the serum.

That the H-flocculating agglutinins were not entirely specific was also shown by absorption of samples of serum of various species by unheated organ-:isms or by flagellar suspensions, titres for suspens-:ions of flagella from the absorbing and from unrelated organisms being estimated before and after absorption, see Table XXVIII. The absorbing suspension was the supernatant/

TABLE XXVIII

Ox serum

Absorption by flagella of *V. cholerae* (Bombay).

Serum before and after absorption used in agglut-:ination reactions with flagella washings of other motile organisms.

Flagella of	Serum unabsorbed	Serum absorbed by flag-:ella of *V. cholerae* (Bombay)
V. cholerae (Bombay)	1:32 L	1:4 (tr) L
V. cholerae (3134)	1:32 L	1:16 L
B. paratyphosus B	1:64 L	1:16 L
B. proteus X 19 ..	1:64 L	1:32 L
B. paratyphosus A	1:32 L	1:16 L

supernatant fluid after agitating the growth from two large (6") agar plates in 5 c.c. of 0.85 per cent. saline and centrifuging it for two hours. Equal parts of serum and the "flagellar" suspensions were mixed and incubated for four hours. Large floccules appeared which were removed by centrifuging. It will be seen that V. cholerae absorbed its own "H" agglutinins and reduced to some extent those for other organisms.

Normal/

Normal Serum Reactions with "H" and "O" Strains of Bacteria.

Evidence of the antigenic differences between "O" and "H" strains of B. proteus X19 could be elicited by means of normal serum reactions. Table XXIX illustrates the result of agglutinin absorption tests with the B. proteus X19 strains.

In the case of certain strains of the Salmonella group it was found that their serological relationships could not be definitely demonstrated by normal serum reactions. Monophasic types were selected to avoid complication. Table XXX exemplifies an agglutinin absorption experiment using B. typhosus (Cole), B. enteritidis Gaertner (McNee), B. derby 1728 and B. abortus equi. The results are similar to those which might have been obtained with unrelated strains. Unheated B. typhosus absorbed its own agglutinins completely and reduced the serum titre for flagellar and somatic antigens of the other organisms. Alcoholised B. typhosus absorbed B. typhosus "O" agglutinins but did not remove completely the agglutinins reacting with the somatic antigen of B. enteritidis (Gaertner), as might have been expected from a knowledge of the antigenic structure of these organisms as shown by immune sera.

Thermolability/

TABLE XXIX

Ox serum

Absorption by B. proteus HX 19 and OX 19.

Suspensions unheated and heated 100° C. for two hours.

B. proteus	Serum unabsorbed	Serum absorbed			
		HX 19	OX 19	heated HX 19	heated OX 19
Unheated HX 19	1:64 L+S	0	1:16L	1:8 L	1:16 L
Unheated OX 19	1:64 S	0	0	0	0
Heated HX 19	1:64 S	0	0	0	0
Heated OX 19	1:64 S	0	0	0	0

TABLE XXX

Horse serum

Absorption by an unheated and by an alcoholised suspension of *B. typhosus* ("Cole").
Agglutination reactions
with four monophasic *Salmonella* strains.

Organism	Serum unabsorbed	Serum absorbed	
		B. typhosus	*B. typhosus* (alcoholised)
Normal			
B. typhosus ("Cole")	1:32 L+S	0	1:16 L
B. enteritidis Gaertner ..	1:64 L+S	1:16 L	1:8 L + S
Salmonella Derby ..	1:8 L+S	1:4 L	1:4 L + S
Salmonella abortus equi	1:64 L+S	1:64 L+S	1:32 L + S
Alcoholised			
B. typhosus ("Cole")	1:32 S	0	0
B. enteritidis Gaertner ..	1:64 S	1:8 S	1:8 S
Salmonella Derby ..	1:8 S	1:4 S	1:4 S
Salmonella abortus equi	1:64 S	1:32 S	1:16 S
Flagella of :-			
B. typhosus ("Cole")	1:64 L	0	1:64 L
B. enteritidis Gaertner ..	1:64 L	1:8 L	1:8 L
Salmonella Derby ..	1:16 L	1:8 L	1:16 L
Salmonella abortus equi	1:128 L	1:32 L	1:64 L

Thermolability of O-Agglutinins in Normal Serum.

In the previous section of this work it was found that the thermolability of agglutinins for normal unheated suspensions of organisms varied considerably. In some cases inactivation of the serum occurred at 55°C. but for the majority of organisms inactivation occurred at 60°C. to 65°C.

Experiments have been carried out to determine the lability of normal agglutinins in the "O" form, alcoholised suspensions being used in parallel with normal suspensions. In all cases it has been found that inactivation of the O-agglutinins occurred at a lower temperature than that required to remove the effect for suspensions containing "H" antigen (Tables XXXI and XXXII).

Previous work has shown that in some cases there was an apparent reactivation of the agglutinating principle at 55°C. Results obtained suggest strongly that this is largely due to the O-agglutinin effect. Table XXXII shows examples in the case of B. typhosus (Cole) and B. proteus X19. In some cases this was extreme. A sample of pig serum in the unheated state did not agglutinate an alcoholised B. paratyphosus B suspension. On heating the serum to 55°C. relatively marked agglutination occurred which was abolished at 62°/

TABLE XXXI

Ox serum

Thermolability of "O" agglutinins.
Normal "H" and alcoholised "O" suspensions of motile organisms in agglutination reactions with unheated and heated serum.

Suspension	Unheated serum	Temperatures at which serum was heated for 30 minutes		
		55°C.	60°C.	65°C.
B. typhosus ("Cole") "H"	1:32 L + S	32 L + S	16 L	0
B. typhosus ("Cole") "O"	1:32 S	16 S	0	0
B. enteritidis Gaertner "H"	1:64 L + S	64 L + S	32 L	0
B. enteritidis Gaertner "O"	1:16 S	8 S	0	0
V. cholerae (Bombay) "H"	1:32 L + S	64 L	16 L	0
V. cholerae (Bombay) "O"	1:8 S	0	0	0

TABLE XXXII

Pig serum

Thermolability of "O" agglutinins.
Normal "H" and alcoholised "O" suspensions in agglutination reactions with unheated and heated serum.

Suspension	Unheated serum	Temperatures at which serum was heated for 30 minutes			
		55°C.	60°C.	62.5°C.	65°C.
B. pyocyaneus					
"H" ...	1:128 L + S	1:128 L + S	1:64 L + S	1:16 L	0
"O" ...	1:64 S	1:128 S	1:8 S	0	0
B. typhosus ("Cole")					
"H" ...	1:32 L + S	1:32 L + S	1:32 L + S	1:8 L	0
"O" ...	1:16 S	1:32 S	1:4 S	0	0
B. proteus X19					
"H" ...	1:256 L + S	1:128 L + S	1:128 L + S	1:128 L	0
"O" ...	1:4 S	1:16 S	1:8 S	0	0

62° to 65° C., at which temperature reactions still occurred with a normal "H" suspension. This result closely parallels the findings of Mackie and Finkelstein (1930) in their observations on the complement-fixing antibodies of normal sera.

Normal/

Normal Agglutinins in Relation to Rough Variation of Bacteria.

By plating out old broth cultures of a strain of B. typhosus (CB) a rough colony variant was obtained which agglutinated spontaneously in 0.85 per cent. salt solution. Both "S" and "R" organisms were motile. In carrying out agglutination reactions involving rough and smooth variants of the same strain all emulsions were made up in 0.1 per cent. salt. The initial 1:2 dilution of serum was made up in 0.1 per cent. salt and for subsequent dilutions in the series a solution was used of 0.4 per cent. strength. In this way, after mixture of equal parts of serum dilution and suspension the salt concentration was approximately 0.25 per cent. in all tubes. In this strength the unheated "R" form was relatively stable. Suspensions of such variants, however, were invariably subject to spontaneous ag-:glutination after heating to 100°C.

The results of agglutinin absorption tests using the rough and smooth races of the same strain with vari-:ous animal sera were somewhat irregular. In the case of heated "R" suspensions salt agglutination was com-:plete in the control tube at the end of 18 hours. A reading taken 4 hours after the addition of suspension showed, however, that agglutination took place in the tubes containing serum, the degree being proportional to/

to the serum dilution. Absorption experiments indic-:ated that this was a phenomenon not previously ob-:served in work with "S" variants. Previous absorption of the serum by "S" or "R" organisms of the same strain unheated or heated did not affect the end-titre of the reaction. This phenomenon has been pre-:viously described by Bruce White (1928).

An example of the results obtained is seen in Table XXXIII. It will be seen that serum in dilution 1:32 agglutinated the heated "R" suspension and that this effect was not removed by absorption. Another feature which was constantly noted was that the un-:heated organism in the "R" form did not absorb all agglutinins for the "S", while serum treated with smooth organisms lost all agglutinins for the unheated "R" form. In the same way it was noted that heated "R" organisms did not remove agglutinins for heated "S" forms.

Similar experiments were carried out with samples of serum from other animal species and the results showed considerable resemblances. The suggestion is made that in the case of B. typhosus normal animal sera contain principles which react with the flagellar "H" antigens, the "O" antigen of smooth organisms and the "R" antigens of rough races. The "O" antigen can absorb the "R" agglutinins but the reverse does not hold/

TABLE XXXIII

Horse serum

Serum absorbed by: (1) unheated "R" variant of B. typhosus CB;
(2) heated "R" variant of B. typhosus CB;
(3) unheated normal "S" form of B. typhosus CB;
(4) heated normal "S" form of B. typhosus CB.

Serum untreated and absorbed used in agglutination reactions with suspensions of unheated and heated "R" and "S" B. typhosus.

Suspension	Horse serum unabsorbed	Horse serum absorbed by B. typhosus			
		CB "R"	CB "R" (100° C.)	CB "S"	CB "S" (100° C.)
B. typhosus					
CB "R"	32 L	0	4 L	0	0
CB "R" (100° C).	32 S	32 S	32 S	32 S	32 S
CB "S"	32 L+S	8 L+S	16 L+S	0	4 L
CB "S" (100° C).	16 S	0	8 S	0	0

hold. The analogy with the findings of investigations involving the use of high titre immune sera is not complete and the results are difficult to interpret in view of the many non-specific factors which appear in agglutination reactions with rough organisms.

Influence/

Influence of the Pre-Existing Natural Agglutinins on the Response to Immunisation.

Glenny and Südmersen (1921) pointed out the important fact that horses whose serum contained "natural" diphtheria antitoxin responded more rapidly to immunising injections of toxin than did those without pre-existing antibody. Animals which lacked any trace of antibody, when immunised by an injection of toxin reacted sluggishly yielding a serum of very low titre after a prolonged interval of about three weeks. This response they classified as that to the "primary stimulus". A second dose of toxin in such an animal produced a massive and rapid response which they described as characteristic of the "secondary stimulus". Animals whose serum contained an appreciable amount of "natural" antitoxin reacted to the first dose of antigen in a way which suggested that it was, for them, a "secondary stimulus".

Experiments were undertaken to determine whether a parallel observation could be made in the case of the agglutinin response. Rabbits were tested for serum agglutinins before and after immunisation. In all some thirty rabbits were used, additional information regarding the bactericidal power of the serum being studied at the same time. In all cases the response to immunisation was immediate. Tests performed three days/

days after immunisation revealed an appreciable in-:crease in antibody. This is the response character-:istic of the "secondary stimulus" of Glenny and Südmersen (1921). The results are illustrated in Table XXXIV.

The curve of antibody production was always of the same type whether the animal's serum contained pre-existing agglutinin or not. The curve of O-agglutinin titre did not rise so steeply as that for H-antibodies but the initial rise was never delayed.

In a number of further experiments rabbits were immunised with a course of injections of "H" and "O" vaccines, the agglutinin titre being recorded before and after immunisation. No parallelism could be dis-:cerned between the activity of serum before, and the antibody response after, immunisation. In individual animals the response to the same course of immunising injections varied widely but it could not be correlated with the previous natural agglutinin titre.

TABLE XXXIV

The response to immunisation with a dose of 5000 million *V. cholerae* (living). Agglutination tests before and after immunisation carried out with formolised "H" and boiled "O" antigens.

Time of test	Result of agglutination reactions	
	"H" titre (formolised suspension)	"O" titre (boiled suspension)
Before immunisation	1:16	0 < 1:4
4 days after immunisation	1:64	1:8
10 " " "	1:1024	1:32
19 " " "	1:1024	1:64
26 " " "	1:256	1:256

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 agglutin-:in 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 differ-:ent 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 activ-:ity while the reverse is noted in the case of human serum. Weil and Felix (1920) have adduced consider-:able/

considerable evidence that complement-fixation runs parallel with the content of stabilotropic O-agglutinins in immune serum. The studies in which O-agglutinins were investigated provided no evidence that this cor-:relation held for normal serum reactions. In general the O-titres of specimens of serum from the same species were extremely variable but the order of activity of the various animal species for O-agglutination showed no parallelism with that for complement-fixing activity reported by Mackie and Finkelstein (1930).

The influence of the age of the animal on the ag-:glutinin 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 experi-:ments 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 Wasser-:mann antigen (Mackie and Watson, 1926) and with "pseudo-antigens" (Mackie and Finkelstein, 1928), the develop-:ment of this "natural antibody" in rabbit serum running parallel with that of the natural anti-sheep haemolysin. Friedberger, Boch 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/

increase progressively with age and that the curve pro-:duced 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 Finkel-:stein (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 im-:mune haemolysin. The results suggest that the differ-:ence is not simply quantitative. Again, the temperature of inactivation most frequently noted, viz. 60°C. to 65°C., would exclude complement-action as a factor in the process of natural agglutination. Felix and Olitzki (1929) considered that normal and im-:mune 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/

serum dilutions. Heuer (1922) showed that normal ser-:um as diluent behaved as a protective colloid, tending to inhibit agglutination and this factor, in associat-:ion with the known thermolability of O-agglutinins as compared with the "H" type would explain the low in-:activation temperatures for immune agglutinins arrived at by these workers.

The lability curves exhibiting a zone of partial inactivation in the region of 55°C. are of great inter-:est as they resemble closely 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 in-:activation 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 ag-:glutinin 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/

carbonic acid insoluble fraction while one of the modifications consequent on immunisation is an altered distribution of the antibody in the serum protein. The carbonic acid insoluble fraction consists of the whole of the euglobulin and part of the pseudoglobulin while the carbonic acid soluble fraction is made up of the remainder of the pseudoglobulin and the albumin. Lewis and Wells (1922) and others have reported a globulin deficiency in the serum of newly born animals. This involves particularly the euglobulin. With age the serum protein-complex undergoes a change with an increase of euglobulin. This biochemical development may be intimately associated with the natural antibody increase. That it is not, however, applicable to all antibodies is suggested by the fact that the natural antibacterial complement-fixing and bactericidal principles are already well developed in early life. It is also impossible to state with certainty that the euglobulin increase is the cause and not the effect of the apparent development of antibodies in the cases where this occurs.

The results of agglutinin absorption experiments recorded bring out clearly the presence of specific and non-specific elements in the reaction of natural ag-:glutination. It is seen that where an organism is capable/

capable of removing in whole or in 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-spec-:ific 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 comple-:ment-fixing antibody, reacting with bacterial anti-:gens, showed a marked though relative specificity.

Bruce White (1927) considers that natural agglut-:ination probably covers a heterogeneous collection of phenomena, and cites as the dominant factor the inter-:action between serum colloids and a lipoid hydrophobe component of the organism. The specific soluble sub-:stance 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 section of this study devoted to "H", "O" and "R"/

"R" antigens in their reactions to natural agglutinins illustrates further analogies between the mode of action of these normal serum principles and that of the corresponding immune agglutinins. It shows that an analysis of natural agglutination is possible by the use of bacterial suspensions modified to contain the various antigenic components of the organisms in as pure a form as possible. Schiff (1922) concluded that normal agglutinins are all of the stabilotropic "O" type. He has stated that absorption by a heated sus-:pension specifically absorbed all agglutinins for the organism in the unheated form. He used serum from a number of animal species along with B. proteus X19 and X2 in the "O" and "H" form, and also B. pyocyaneus. In this inquiry, by the use of flagellar suspensions as pure "H" antigen and heated or alcoholised suspensions as pure "O" antigen, strong evidence has been obtained that normal sera contains both types of agglutinins. Direct agglutination reactions have enabled a distinct-:ion to be drawn between large-flaking agglutination with unheated motile organisms and the fine granular type shown by heated and alcoholised suspensions. Ag-:glutinin absorption seemed to give results confirming the results of direct agglutination.

The/

The rate of formation of the floccules, their appearance when fully formed and the absorption findings suggest a very close analogy with agglutination by an immune serum. This must not be stressed too much since it was not found possible in every case to demonstrate known antigenic relationships, as for example that between B. typhosus and B. enteritidis Gaertner.

Experiments carried out to demonstrate the relation of "O" and "H" agglutinins to the specific and non-specific effects previously observed revealed a further difficulty. It had been shown that treatment of serum by any organism, heated or unheated, removed the so-called "non-specific" agglutinins for organisms other than that used for absorption. The later experiments have shown that only the "H" agglutinin is concerned in this effect. The results of absorption tests suggest that O-agglutinins of normal serum have marked affinities for unrelated organisms. It was found impossible to demonstrate a series of strictly specific "O" agglutinins reacting with the O-antigens of organisms. The non-specific element of natural agglutination is largely associated with the small-flaking agglutinins. The method used was to absorb the serum by one organism in the "O" form and to test the/

the specificity of the agglutinins not so removed by further absorption with another organism. It appeared probable that absorption by a limited number of organ-:isms would remove from the serum O-agglutinins for all organisms.

This finding would suggest that quâ natural agglut-:ination bacteria are widely related through their "O" antigens and this may be the normal physiological basis of those relationships which may be demonstrated in immune sera between antibodies to the heat-stable com-:ponents of organisms of various groups. Thus in the Salmonella group the "O" antigens have a great deal in common while the heat-stable "R" antigens of apparently unrelated strains possess some community in antigenic structure. Specificity in the motile races is largely a function of the "H" (specific phase) antigens.

Lovell (1934) has confirmed the existence of "O" and "H" agglutinins in a study of the natural agglutin-:ins with special reference to those for members of the Salmonella group in animal sera. He was able to de-:monstrate the selective absorption of agglutinins reacting with specific antigens of both "O" and "H" type. He disagrees with my findings on the question of the presence of a non-specific element in the pro-:cess. He did not observe any significant reduction in/

in the serum titre following absorption with Kieselguhr. It should be noted that in my experiments such non-specific absorption was somewhat inconstant and small in degree. It was in most cases quite overshadowed by the more significant specific affinity of antigens for their own particular antibodies. Lovell states that the non-specific element was only sought for in the case of agglutinins for members of the Salmonella group. His failure to observe any element of non-specificity in the process is contrary to the results of all the early workers on this subject. [Lüdke (1905), Mamlock (1909) and others.] Indeed among the complement-fixing natural antibodies the element of non-specificity reported has lead many immunologists, including Browning (1927) and Dunlop (1928), to regard the process as entirely non-specific. This view is still maintained in the case of the natural bactericid-:al antibodies by Gordon. (See Gordon and Carter, 1932.)

A study of the thermolability of the agglutinins of normal sera which react with "H" and "O" antigens of bacteria adds further support to the possibility of the existence of separate "H" and "O" agglutinins. Felix and Olitzki (1929) have suggested that the re-:latively low temperature of inactivation of normal serum/

serum is due to the preponderance of "O" agglutinins. It can, however, be shown that the normal serum prin-:ciple reacting with heated or alcoholised suspensions of organisms is more labile than that which reacts with the flagellar antigen. It may be noted that both are more labile than the corresponding agglutinins of specific immune sera.

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.

Origin of Natural Antibodies.

The origin of natural antibodies in the serum of animals of many, if not all, species is a problem for which no completely satisfactory solution has yet been proposed. In view of the fact that little is known as to the mechanism of production of the much more precisely defined immune antibodies, this is not unexpected.

Broadly speaking two opposing views are held

(Topley/

(Topley, 1933). The first regards all natural anti-:bodies as arising from the previous presence of a specific immunising stimulus. This may take a number of forms which include the actual organism or toxin with which the antibody is shown to react, an organism which contains a related antigen, or finally, antigenically active material which gains access to the tissues by absorption from the bowel or other mucous membrane and which is immunologically allied to the bacterial anti-:gen for which the natural antibody has affinity. The holders of the second view, of which the chief protagonist is Hirszfeld (1926), maintain that pre-:vious exposure to the antigen is not essential for the development of the natural antibodies. They suggest that the antibody-producing mechanism appears at a cer-:tain stage in the development of the animal in a way analogous to the appearance of certain of the internal secretions, for example those of the secondary sex characters. The demonstrable manifestation of the presence of such a "biochemical reflex" is the natural antibody which possibly indicates a capacity to develop the true immune antibodies in response to overt or latent infection or artificial immunisation. In this connection it is of interest to recall that in 1905 Lüdke described the high agglutinin titres of normal ox serum/

serum as being "due to cell-free receptors arising in the course of metabolism". The conception is further developed by the observations of Hirszfeld, Hirszfeld and Brokman (1924) who reported a correlation between the inheritance of blood-groups and of immunity to diphtheria. Hirszfeld (1926) maintained that the genetic factor responsible for ability to produce diphtheria antitoxin was linked to that determining the blood-group. The accuracy of this particular observ-:ation has, however, been questioned by Rosling (1928).

When the problem is approached it is at once seen that the term "natural antibody" probably describes a variety of unrelated phenomena which show superficial resemblances one to another. Any argument by analogy is liable to lead to fallacious conclusions.

On the one hand it cannot be disputed that, apart from frank infection, exposure to subinfective doses of pathogenic micro-organisms may lead to the develop-:ment of serum antibodies. In the absence of history of specific disease, such serum may be labelled normal. The studies of Sheldon Dudley (1923, 1926) leave no doubt, for example, that exposure to B. diphtheriae accelerates the production of specific antitoxin in a concentration sufficient to establish the Schick-negative state. It would be manifestly wrong to re-:gard/

regard such an antitoxin as a "natural antibody" in the true sense. In other cases the presence of "natural" antitoxin is not so readily explained, as for example the antitoxins of diphtheria, tetanus or B. welchii in horse serum, although there is at least a possibility that the homologous antigen may have been present to stimulate their production. The same possibility exists to explain the presence of diphtheria antitoxin in the serum of adults in countries where the disease in humans is rare. /Heinbecker and Irvine-Jones (1928), Kleine and Kroo (1930)./ On the other hand, such findings as a high concentration of agglutinins for V. cholerae in the serum of a normal pig obviously requires a somewhat different explanation. Topley (1933), while admitting that genetic and evolutionary factors play a part, inclines to the view that such anomalies may be explained on the grounds that organisms pathogenic and commensal for the animal species in question may share antigens for those foreign bacteria for which antibodies may be demonstrated. He points out that comparative studies have rarely been made of the antigenic structure of unrelated bacterial species. The data of the heterogenic antibody phenomena, such as the Forssman reaction, the Weil-Felix and Wassermann reactions, point to the possibility of the existence/

existence of such cross-relationships. The identity of the S.S.S. of Pneumococcus Type II and of Pneumo-:bacillus (Friedlander) Type B is an even more striking example (Avery, Heidelberger and Goebel, 1925).

Mackie and Finkelstein (1928) pointed out that the development of natural complement-fixing antibodies re-:acting with various pseudo-antigens (such as lipoid suspensions, alcohol and peptone) ran parallel with that of anti-sheep haemolysin in the serum of young rabbits. The two properties were, however, shown to be independent by agglutinin-absorption tests. Friedeman (1917) has suggested that the natural anti-sheep haemolysin of rabbit and human sera is of the heterophile type. Thus the antigen described by Forssman (1911) and now known to be of the nature of a lipoid hapten is a factor which is relevent to the pre-:sent discussion, especially as it is known to occur in certain bacteria. There is at least a prima facie case for the suggestion than an inciting agent of this type may stimulate the production of those "normal" serum antibodies which react with bacteria, red cells, antigens and pseudo-antigens of all kinds. This ex-:planation is an attractive one but it appears to be invalid for the following reasons.

1./

1. The presence and amount of natural antibody in the serum of the various animal species cannot be correlated with their known capacity to develop the Forssman antibody. The horse does not respond to the injection of heterophile antigen and yet it is one of the stronger agglutinin reactors. On the other hand, the rat, whose serum is relatively deficient in natural agglutinins, is capable of producing the Forssman antibody.
2. With regard to bacteria, the Forssman antigen is found in a very few species, e.g. B. dysenteriae (Shiga), B. anthracis, Pneumococcus and certain races of the Salmonella and Pasteurella groups. Again no correlation can be discerned between susceptibility to the action of normal agglutinin and content of heterophile antigen.

It might be argued that the Forssman phenomenon may not be an isolated case and that another antigen of heterophile type might be operative in stimulating natural antibody production. Among animal species a varying capacity to react to such a stimulus, or a varying opportunity of coming into contact with it, might account for the order of activity noted in this work. All explanations along these lines fail to take into account the marked specificity of the natural agglutinins. The striking feature of heterophile antigen is its immunological homogeneity under whatever circumstances/

circumstances it may be found. Antibody arising in response to such a stimulus would always be the same serological entity even though it might give reactions with widely different materials which had in common the specific antigen. Absorption experiments would at once expose the unity of the essential antibody. It is possible that a heterophile stimulus might account for the relatively unimportant element of non-specific-:ity which absorption tests reveal. To account for the specific antibodies the suggestion of multiple heterophile antigens of wide distribution does not simplify the problem but rather leads to very great complication.

The proposition that natural antibodies are the response to an immunising stimulus, however occult, appears to neglect the essential difference between them and the immune antibodies. If the mechanism of production of both were the same there would appear to be no reason to anticipate such differences. But, in addition to such physical properties as thermolability and distribution over the serum proteins, there is the cardinal difference that the natural antibodies proper-:ly so-called, do not confer immunity. Numerous examples could be cited but one which is specially striking is the high agglutinin titre for B. mallei in normal/

normal horse serum (Lovell, 1935). The order in which the animal species may be arranged, having regard to the natural agglutinin content of their sera, is not paral-:leled by their known resistance to disease. The rat occupies a lowly place in the series but it certainly displays a general resistance to infection equal to, or greater than, that of the horse, pig or ox.

Among the natural antibodies which might be regard-:ed as indicative of ability to resist infection are the complement-fixing principles which react with bact-:erial antigens (Mackie and Finkelstein, 1930), and the natural bactericidal antibodies (Mackie and Finkelstein, 1931). These serum properties are exceptional in be-:ing present from an early age. Thus the antibodies which are most likely to have a protective function are just those which lack an important attribute of the naturally acquired immune antibodies, viz. progressive increase with age during early life.

Lovell (1934), who expresses the opinion that "it seems impossible to accept the view that these anti-:bodies are purely evolutionary and arise irrespective of any specific stimulus", has sought such specific stimuli among the commensal bacteria of animals. He reported that coliform bacilli, isolated from the mesenteric glands of pigs whose serum contained Salmon-:ella/

Salmonella agglutinins, on inoculation into rabbits, induced the formation of agglutinins acting on certain bacilli of the Salmonella group. Such a finding is of interest as throwing light on the antigenic relation-:ship of the strains in question as shown by immune agglutinins but it does not necessarily illuminate the origin of natural agglutinins. It remains to be prov-:ed that the agglutinins so formed possess the charact-:ers typical of the natural antibodies.

Widespread serological relationships among unrel-:ated bacterial species is easier to suggest than to prove. In diagnostic work high-titre specific sera have now been constantly and widely employed for many years, and any important antigenic relationship between, for example, B. coli strains and the intestinal patho-:gens would readily have been revealed by such potent reagents. The suggestion that a minimal absorption of antigenic material from commensal intestinal bacteria can account for the appearance of agglutinins to re-:latively high titre for bacteria quite foreign to the animal species in question is a proposition which re-:quires the most rigid proof for its acceptance.

In this connection a highly significant fact noted in the early stages of this study may be recalled. In describing the marked variation in activity among in-:dividuals/

individuals of the same species the observation was made that a weak sample was invariably deficient in agglutinins for all organisms. No sample of serum was found which gave abnormally strong or weak reactions with one organism only. To reconcile this finding with the conception of natural antibodies as a response to specific stimuli, one would require to postulate that all animals within a species had been influenced by the same stimuli, some to a greater degree by all, some to a less; that each individual had had the same antigenic experience, differences being merely quantitative, not qualitative; and that no one stimulus, whatever its nature, had dominated the development of the natural agglutinin complex of any animal.

Throughout the experimental work described the results have repeatedly suggested that the existence of this series of specific antibodies is conditioned by one underlying influence. In some animal species and in some individuals within species the influence has been more potent or the response more effective and their serum reacts more strongly than that of others.

Now this apparent unity of mechanism is a strong argument against the conception of natural antibodies as a response to specific antigenic stimuli. No one antigen will stimulate the production of a series of immunologically//

immunologically unrelated antibodies and if separate antigens were responsible their presence would almost certainly have been betrayed by an unusual preponder-:ance or absence of the antibody corresponding to one of them in the serum of individuals. To regard the natural specific antibodies as a manifestation of an inborn capacity for their development, varying quantit-:atively in individuals perhaps as a result of genetic influences, would appear to be a view of their origin more in keeping with this observation.

To sum up, it is necessary to classify the anti-:bodies encountered in normal serum into at least two groups, each characterised by a different mechanism of production. Thus in any normal serum specific immune antibodies may be present as a result of unrecognised infection or the threat of infection involved by con-:tact with the specific antigens. The presence of such antibodies may be correlated with resistance to the specific disease. Sharply contrasted with these are the natural antibodies of which the agglutinins may be taken as an example. While largely specific for their respective antigens, they differ in their properties and functions from the immune agglutinins. The haem-:agglutinins which determine blood-groups in man and

many/

many animal species are certainly the products of bio-
:chemical development influenced by genetic factors.
With them the natural agglutinins appear to have much
in common and the conclusions arrived at as to their
origin are in accordance with this similarity.

SUMMARY AND CONCLUSIONS/

SUMMARY AND CONCLUSIONS

1. A study has been made of natural agglutination as exemplified by the reactions of the serum of nine ani-:mal 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 consist-:ently 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 individ-:ual 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 defic-:ient 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/

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

7. The natural agglutinating substance is found to be present in greater degree in the carbonic acid in-:soluble 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/

principles exist for a wide variety of organisms. Ab-:sorption of any one organism removes the homologous effect leaving the remainder quantitatively unimpaired.

10. Normal serum from various mammalian animals con-:tains agglutinins which react with the "H" and "O" antigenic constituents of motile bacteria. Flagellar suspensions have been used to demonstrate H-agglutinins.

11. Agglutinin-absorption experiments show that the specificity of natural agglutinins depends chiefly on the "H" type. The "O" type agglutinins appear to possess affinities for antigenic constituents which are more widely shared by different organisms.

12. It was not found possible to demonstrate the anti-:genic relationship among members of the Salmonella and B. proteus X groups so precisely with normal sera as with immune sera.

13. The thermolability of the O-agglutinins was found to be greater than that of the "H" type in the normal serum of a number of animal species. Both showed greater lability than the corresponding immune agglut-:inins.

14. "Rough" and "smooth" variants of the same bacter-:ial strain showed antigenic differences in their re-:actions with normal sera.

15. The origin of natural agglutinins is discussed in its/

its relation to other natural immunity reactions.

16. The suggestion is made that true natural anti-:bodies must be contrasted with specific immune anti-:bodies in the serum of apparently normal animals. After reviewing the available evidence, the conclusion is reached that the former cannot be regarded as a response to a specific stimulus. The most satis-:factory explanation of their presence is one which suggests that they arise spontaneously in the course of the serological development of the animal.

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