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COMPLEMENT-FIXATION BY THE INTERACTION OF NORMAL SERUM AND BACTERIAL SUSPENSIONS A CONTRIBUTION TO THE STUDY OF NATURAL IMMUNITY PHENOMENA.

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(With 1 Chart.)

INTRODUCTION.

IN a previous communication the authors drew attention to the occurrence of complement-fixation by normal serum along with various non-antigenic substances, and recorded the results of a study of this phenomenon with particular reference to the serum principle or principles concerned in the reaction (Mackie and Finkelstein, 1928). Though no conclusive interpretation of the results could be offered, a close analogy was established between the reacting agent of the serum and a natural antibody, *e.g.* a natural haemolysin, and the observations appeared to be of significance in relation to natural immunity phenomena.

Similar reactions had been studied by Takenomata (1924), and substances capable of yielding such effects with normal serum were designated by him "pseudo-antigens." Among these he included bacterial suspensions, and drew attention to the occurrence of non-specific complement-fixation through the interaction of normal serum and bacteria.

A preliminary investigation of this reaction was made in the course of our previous inquiry into non-specific complement-fixation but the question of its relationship to the reaction with non-antigenic agents was not fully studied.

The occurrence of non-specific complement-fixation effects by the interaction of various bacterial antigens along with the normal serum of the rabbit and dog was noted by Kolmer and his co-workers (1916). They used suspensions of *Staphylococcus aureus*, *B. typhosus* and *B. coli communis* which had been heated at 60° C. and stated that the effect occurred with serum heated at 55° C. as well as with fresh serum.

A closely related phenomenon has also been recorded and studied by Dunlop (1928), namely, complement-fixation by guinea-pig serum interacting in certain concentrations with suspensions of *B. typhosus* and other organisms, and thus characterised by a "zone phenomenon" when varying amounts of the serum (containing both the fixing agent and complement) are tested. This "zonal fixation" was observed with the majority of strains of *B. typhosus*,

though individual strains varied in their fixing power. Dunlop suggested that the reaction was explicable by the presence in guinea-pig's serum of a natural antibody. This substance resembled antibodies generally in its ability to "sensitise" the organisms but differed from antibodies resulting from immunisation in its thermolability and lack of specificity. It proved sometimes more labile even than complement. Though sensitisation occurred at 0° C., complement was not fixed at this temperature. Dunlop was able to absorb this antibody from serum not only by bacteria capable of reacting with it but also by other organisms and by coal dust, charcoal and powdered glass. Coliform bacilli, staphylococci and *B. anthracoides* were also found to produce similar zonal fixation.

This reaction, though zonal in character, may nevertheless be regarded as a simple complement-fixation effect such as that elicited by fixed amounts of antigen and an antibody-containing serum along with varying doses of complement. Thus, the zonal nature of the reaction may be due to the fact that both the antibody and complement are being varied simultaneously and proportionately, whereas the amount of complement fixed is not directly proportional to the quantity of antibody present, and a minimum quantity of antibody is also necessary before complement-fixation becomes manifest.

The system adopted by us in carrying out complement-fixation reactions with non-antigenic substances was to test fixed amounts of the substance and the serum along with varying doses of guinea-pig's serum as complement. Guinea-pig's serum sometimes, though infrequently, gave zonal fixation with certain of these substances independently of the serum tested for fixing properties, showing that the power of reacting with such agents was not entirely absent from the fresh serum of this species though more frequently present in that of certain other animals (*e.g.* ox, sheep, horse). In our preliminary study of complement-fixation by bacterial suspensions and animal serum, a similar system of testing was employed. A strain of a typical *B. coli* (designated "x") was used; zonal fixation was found to be a frequent occurrence with the guinea-pig serum *per se* used as complement and this introduced a complicating factor. Under these conditions it was difficult to estimate the fixation produced by the serum tested as apart from that of the complement-serum itself. The fixation by guinea-pig's serum could, however, be reduced to a minimum or abolished by incubating it for several hours at 37° C. (and that without serious loss in the haemolytic action of the complement). In this way it was possible to carry out, with various sera, fixation tests in which guinea-pig's serum was used as complement. It was found that the serum of different species might yield pronounced fixation reactions with certain selected bacterial strains, but the property was markedly thermolabile, being usually annulled within half-an-hour at 55° C.

Since this preliminary work was recorded, a more extended study of these reactions has been made with the particular object in view of correlating them with other normal serum reactions and of ascertaining the nature of the

reacting agent in the serum. The results are detailed in this paper and are of considerable interest in their bearing on the general question of natural immunity phenomena.

METHODS.

The general system adopted for estimating complement-fixation quantitatively was that previously described (Mackie and Finkelstein, 1928).

The *bacterial suspensions* were generally prepared by emulsifying in normal saline solution, 24 hours' growths on plain nutrient agar, and were standardised by opacity. The usual densities employed were equivalent to Brown's opacity standards Nos. 5 or 6. Only bacteria capable of growing on plain agar were used. In the earlier experiments the suspensions were prepared without any previous washing of the growths; in later work the organisms were washed once or twice with saline before final suspension. It was found that in this way anticomplementary effects by the suspensions *per se* were considerably reduced. The test amount was usually 0·5 c.c. The concentration and quantity used in the general tests were determined as a result of preliminary tests with organisms of the coli-typhoid group, the object being to employ the maximum amount of bacterial suspension without excessive anticomplementary effects.

The test amount of *serum* was usually 0·05 c.c. and the antigen and serum were mixed and allowed to stand at room temperature for 1 hour before addition of complement.

In experiments in which varying concentrations of serum and bacterial antigen were tested with a view to determining whether the reaction depended on optimal proportions, the results showed that the maximum effects occurred with the maximum amounts of serum and antigen that could be used conveniently for a complement-fixation test, *i.e.* without exhibiting excessive anticomplementary effects in the control tests.

Guinea-pig serum, 18–24 hours after withdrawal of the animal's blood, was employed as *complement*, and varying quantities (computed in M.H.D.) were added to the selected amounts of antigen and serum.

The necessary controls were included, *and in all "antigen controls" the same series of doses of complement-serum were tested as in the actual fixation reaction to exclude the possibility of the results noted in the fixation test with other sera being due to fixation by an antibody-like principle in the guinea-pig's serum* (*v. supra*). As pointed out above, zonal fixation was noted with untreated guinea-pig serum *per se* and certain of the bacteria tested; it was found possible, however, to annul this effect by incubating the serum at 37° C. for 4 to 5 hours before using it as complement, thus enabling other sera to be tested without this complicating factor. The earlier experiments were carried out in this way, but for certain organisms it was found that this treatment did not entirely inactivate the fixing properties of the complement-serum. In later tests, the fixing agent of the guinea-pig serum was removed by treatment with Bragg's charcoal according to the method described by Dunlop (1928). This was found to be uniformly effective though sometimes lessening the haemolytic value of the serum.

The *haemolytic system* was a 3 per cent. suspension of ox red corpuscles sensitised with a rabbit *v.* ox haemolytic antiserum: 0·5 c.c. was the quantity used.

Other methods used in the course of the work are described later.

SERA OF DIFFERENT ANIMAL SPECIES.

A large number of specimens of ox, sheep, horse, pig, rat, rabbit and human serum were examined for their complement-fixing properties with various bacteria. The organisms selected for most of the tests were: typical *B. coli* (strains designated "x" and "F 3"), *B. typhosus* (strains designated "CB"

and "Cole"), *B. paratyphosus* B ("Tidy"), *B. dysenteriae Shiga* (strain designated "109"), *B. proteus* X 19, *Vibrio cholerae* (strain designated "Bombay"), *Staphylococcus aureus* (Laboratory strain), and *B. anthracoides* (Laboratory strain). In certain experiments other strains (of the same or different species) were included, *e.g. B. typhosus* (strain "RLL")[1], *B. enteritidis* Gaertner (Laboratory strain), *B. aertrycke* ("Mutton")[2].

Tables I to VIII illustrate the general results with fresh unheated sera of the different species. The chart shows typical results in detail. The results with a specimen of cat's serum are included (Table VIII). Any complement-fixing properties pertaining to the guinea-pig serum used as haemolytic complement had been annulled by appropriate treatment (*vide* technique). The results of the "antigen-control" tests showed that the fixation results

Table I. *Ox sera.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum: specimen (*a*) Unheated	Antigen control	Serum: specimen (*b*) Unheated	Heated 55° C.	Antigen control
B. coli x	>18	0	>18	0	0
B. coli F 3	8	0	.	.	.
B. typhosus CB	12	0	>18	0	0
B. typhosus Cole	.	.	12	0	0
B. typhosus RLL	8	0	.	.	.
B. paratyphosus B, Tidy	>18	0	>18	0	0
B. proteus X 19	8	0	8	0	0
B. anthracoides Lab. strain	8	2	4	0	0
B. dysenteriae Shiga 109	12	0	.	.	.
Staph. aureus Lab. strain	8	2	12	0	0
V. cholerae Bombay	12	0	8	0	0
B. aertrycke Mutton	.	.	8	0	0
B. enteritidis Lab. strain	.	.	12	0	0
Serum control	2	.	2	0	.

Table II. *Sheep sera.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum: specimen (*a*) Unheated	Antigen control	Serum: specimen (*b*) Unheated	Heated 55° C.	Antigen control
B. coli x	12	0	>18	2	0
B. coli F 3	>18	0	4	0	0
B. typhosus CB	.	.	>18	2	0
B. typhosus Cole	>18	0	.	.	.
B. typhosus RLL	.	.	8	2	0
B. paratyphosus B, Tidy	>18	0	8	2	0
B. proteus X 19	.	.	4	2	0
B. anthracoides Lab. strain	>18	2	4	0	2
B. dysenteriae Shiga 109	>18	0	12	0	0
Staph. aureus Lab. strain	>18	2	>18	0	0
V. cholerae Bombay	>18	4	2	0	0
Serum control	0	.	2	0	.

[1] The strain used by Dunlop (1928) in his experiments on zonal fixation.

[2] The organisms used were devoid of haemolytic effects *per se* under the conditions pertaining to the complement-fixation test.

Table III. *Horse sera.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum: specimen (*a*) Unheated	Antigen control	Serum: specimen (*b*) Unheated	Heated 55° C.	Antigen control
B. coli x	>18	2	>18	0	0
B. coli F 3	8	2	.	.	.
B. typhosus CB	>12	4	12	0	0
B. typhosus Cole	.	.	>18	0	0
B. typhosus RLL	>18	4	.	.	.
B. paratyphosus B, Tidy	12	2	4	0	0
B. proteus X 19	12	2	4	0	0
B. anthracoides Lab. strain	12	2	4	0	0
B. dysenteriae Shiga 109	12	2	.	.	.
Staph. aureus Lab. strain	8	2	4	0	0
V. cholerae Bombay	12	4	12	0	0
B. aertrycke Mutton	.	.	4	0	0
B. enteritidis Gaertner, Lab. strain	.	.	8	0	0
Serum control	2	.	2	0	.

Table IV. *Normal human sera.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum: specimen (*a*) Unheated	Antigen control	Serum: specimen (*b*) Unheated	Heated 55° C.	Antigen control
B. coli x	>18	2	>18	0	2
B. coli F 3	4	2	.	.	.
B. typhosus CB	12	4	>18	0	4
B. paratyphosus B, Tidy	>18	4	>18	2	2
B. dysenteriae Shiga 109	>18	2	12	2	0
Staph. aureus Lab. strain	>18	4	12	0	0
B. anthracoides Lab. strain	.	.	>18	0	0
Serum control	2	.	2	0	.

Table V. *Pig sera.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum: specimen (*a*) Unheated	Antigen control	Serum: specimen (*b*) Unheated	Heated 55° C.	Antigen control
B. coli x	12	2	>18	0	0
B. coli F 3	0	2	0	0	0
B. typhosus CB	12	4	12	0	0
B. typhosus RLL	8	2	4	0	0
B. paratyphosus B, Tidy	0	4	4	0	0
B. proteus X 19	0	2	2	0	0
B. anthracoides Lab. strain	2	2	4	0	2
B. dysenteriae Shiga 109	4	2	0	0	0
Staph. aureus Lab. strain	4	2	8	0	2
V. cholerae Bombay	0	2	4	0	0
Serum control	0	.	0	0	.

Table VI. *Rabbit sera.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum: specimen (*a*) Unheated	Antigen control	Serum: specimen (*b*) Unheated	Heated 55° C.	Antigen control
B. coli x	2	0	>18	2	2
B. coli F 3	12	0	4	4	4
B. typhosus CB	12	2	8	8	8
B. typhosus Cole	12	0	.	.	.
B. typhosus RLL	.	.	12	4	4
B. paratyphosus B, Tidy	2	0	8	2	4
B. proteus X 19	4	0	4	2	4
B. anthracoides Lab. strain	0	2	4	2	0
B. dysenteriae Shiga 109	0	0	8	4	2
Staph. aureus Lab. strain	4	2	4	2	2
V. cholerae Bombay	4	4	.	.	.
Serum control	0	.	0	0	.

Table VII. *Rat sera.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum: specimen (*a*) Unheated	Antigen control	Serum: specimen (*b*) Unheated	Heated 55° C.	Antigen control
B. coli x	4	2	>16	0	0
B. coli F 3	4	2	4	0	0
B. typhosus CB	>18	4	>12	2	2
B. typhosus Cole	.	.	12	0	0
B. paratyphosus B, Tidy	4	2	12	0	0
B. dysenteriae Shiga 109	4	2	4	0	0
B. proteus X 19	4	2	4	0	0
V. cholerae Bombay	4	4	4	0	4
B. anthracoides Lab. strain	8	2	4	0	2
Staph. aureus Lab. strain	4	2	>16	0	2
B. typhosus RLL	12	4	.	.	.
Serum control	2	.	2	0	.

Table VIII. *Cat serum.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Unheated serum	Serum heated 55° C.	Antigen control
B. coli x	>18	0	0
B. coli F 3	4	0	0
B. typhosus CB	12	0	2
B. typhosus RLL	8	0	0
B. paratyphosus B, Tidy	4	0	2
B. proteus X 19	4	0	0
B. anthracoides Lab. strain	4	0	2
B. dysenteriae Shiga 109	4	0	0
Staph. aureus Lab. strain	8	0	0
V. cholerae Bombay	4	0	0
Serum control	0	0	

recorded were independent of any fixing property of the complement-serum. As will be noted later average specimens of guinea-pig serum possessed complement-fixing properties for only a minority of the bacteria used in these tests. It will be observed from the tables that certain sera produced fixation

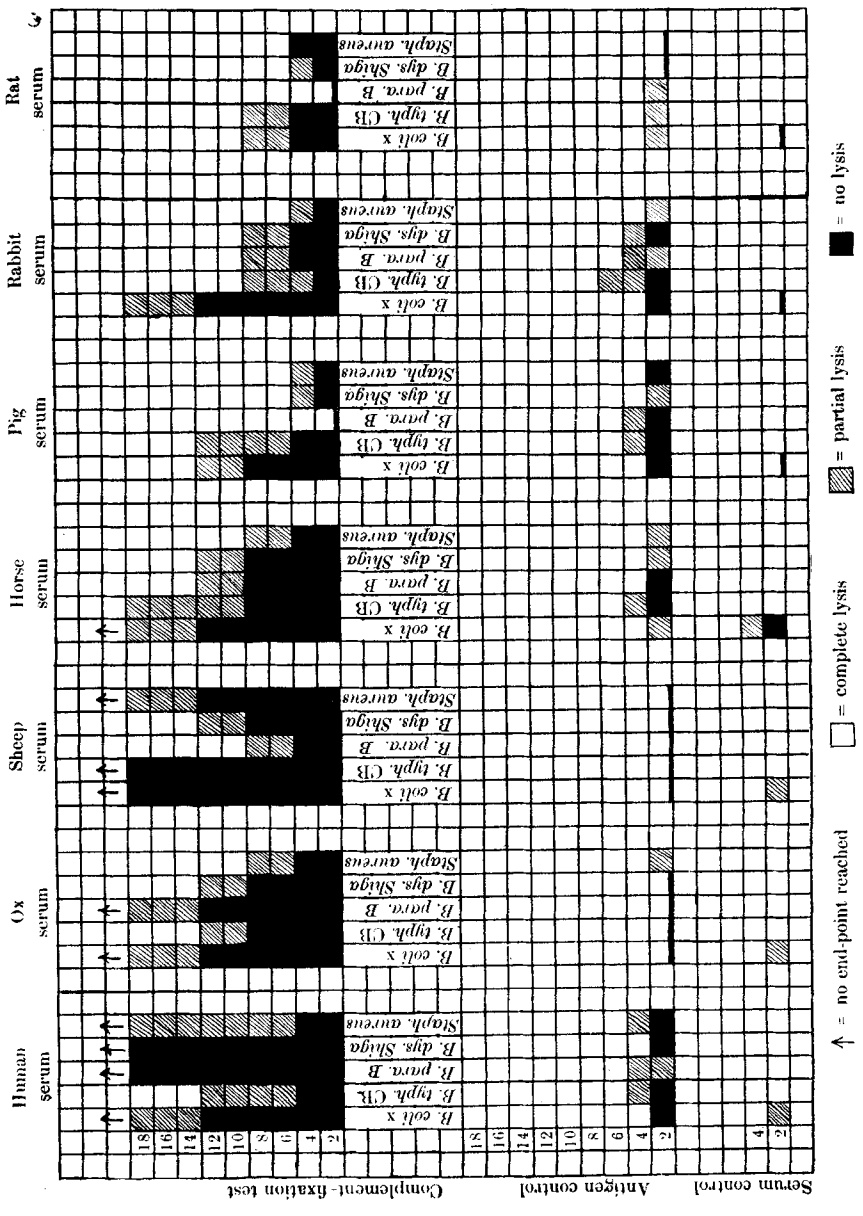
Complement-fixation test
Antigen control
Serum control
Human serum
Ox serum
Sheep serum
Horse serum
Pig serum
Rabbit serum
Rat serum
B. coli x
B. typh. CB
B. para. B
B. dys. Shiga
Staph. aureus
↑ = no end-point reached
□ = complete lysis
▨ = partial lysis
■ = no lysis

Chart 1.

effects with all the organisms tested and that all the sera reacted with certain organisms. The results were, however, variable in degree, varying both with different specimens of serum from the same animal species and with the different organisms. In some cases the effects were pronounced, over 18 M.H.D. of complement being fixed. Certain strains seemed to be more active than others, *e.g. B. coli* x, *B. typhosus* Cole and CB, while some were generally weakly active, *e.g. B. proteus* X 19, *B. coli* F 3, *V. cholerae* and *B. anthracoides*. The relative strength or weakness of the different strains was specially noticeable with weakly reacting sera.

The sera of certain species seemed to possess the reacting property more strongly than others: thus ox, sheep, horse and human sera generally gave strong reactions, while pig, rabbit and rat sera yielded lesser effects. In all cases serum heated for half-an-hour at 55° C. was practically inactive.

Zonal fixation with untreated guinea-pig serum similar to that described by Dunlop was obtained on occasions with various strains; it occurred more regularly with *B. coli* x, *B. typhosus* CB and Cole, *i.e.* the strains which reacted most strongly with the serum of other animals (see Table IX).

Specimens of pigeon serum were tested in the same way as that of ox, sheep, etc., guinea-pig serum treated with charcoal being used as complement. Though the treated complement-serum was devoid of any fixing effect (as evidenced in the antigen control tests), zonal fixation with certain organisms

Table IX. *Guinea-pig sera.*

Zonal fixation by untreated serum.

Serum	*B. coli* x	*B. coli* F 3	*B. typhosus* CB	*B. typhosus* RLL	*B. typhosus* Cole	*B. paratyphosus* B, Tidy	*B. proteus* X 19	*B. dysenteriae Shiga* 109	*Staph. aureus* Lab. strain	*B. anthracoides* Lab. strain
1	Z 4–18	–	–	Z 4–18	–	–	–	–	–	–
2	Z 4–14	–	–	–	–	–	–	–	–	–
3	–	–	Z 4–8	–	Z 4–14	–	–	–	–	–

Z indicates that zonal fixation occurred and the figures indicate the range of this zone in terms of the number of M.H.D. of the complement.

Table X. *Pigeon serum.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum: unheated	Serum: heated 55° C.	Antigen controls (guinea-pig serum treated with charcoal)	Zonal fixation by untreated guinea-pig's serum
B. coli x	Z 4–18	Z 4–18	No Z	Z 8–18
B. typhosus CB	0	0	No Z	Nil
B. typhosus RLL	Z 12–18	Z 12–18	No Z	Z 4–8
B. paratyphosus B, Tidy *B. dysenteriae Shiga* 109 *B. proteus* X 19 *Staph. aureus* Lab. strain *B. anthracoides* Lab. strain *V. cholerae* Bombay	0	0	No Z	Nil

Z signifies zonal fixation; see Table IX.

Table XI. *Ox serum.*

Antigen	Complement-fixation stated in terms of number of M.H.D. of complement fixed
B. coli x	>16
B. coli F 3	6
B. typhosus CB	10
B. paratyphosus B, Tidy	16
B. dysenteriae Shiga 109	10
Staph. aureus Lab. strain	10
B. anthracoides Lab. strain	6
B. proteus X 19	10
V. cholerae Bombay	10
B. pyocyaneus Lab. strain	6
B. Morgan No. 1 Lab. strain	>16
B. enteritidis Lab. strain	10
B. dysenteriae Y, Lab. strain	16
B. paratyphosus A, Schottmüller	16
Pneumobacillus Lab. strain	16
B. suipestifer Hog Cholera	10
B. xerosis Lab. strain	10
Micrococcus tetragenus Lab. strain	10
Sarcina lutea Lab. strain	10
B. aertrycke Mutton	10

In this and subsequent tables the number of M.H.D. of complement fixed by antigen and by serum *separately* (*i.e.* in the "antigen control" and "serum control") have been deducted from the number of M.H.D. of complement fixed by antigen *plus* serum.

Table XII. *Various strains of* B. typhosus.

Designation of strain	Sheep serum: No. of M.H.D. of complement fixed	Zonal fixation effect with guinea-pig serum	Predominant type of colony
Howard	>20	Z 4–>24	S
Summers	>20	Z 4–>24	S
Cole	20	Nil	S
Typhique 8	20	Nil	S
Harmer	>20	Z 4–>24	S
Horan	20	Z 4–>24	S+R
Price	>20	Z 4–>24	S
Western	4	Nil	R almost entirely
Lister	10	Nil	S
John Hopkins	20	Z 4–>24	S
Richards	10	Z 4–>24	S
1361	>20	Z 4–>24	S
CB	20	Z 4–8	S+R
RLL	>20	Z 4–>24	S

Z indicates that zonal fixation occurred and the figures indicate the range of this zone in terms of the number of M.H.D. of the complement.

S signifies "smooth" type. R signifies "rough" type.

occurred in the presence of pigeon serum, both fresh and heated. This serum seemed to reactivate the property of the guinea-pig serum annulled by treatment with charcoal, but exerted no effect which could be regarded as independent of the complement-serum (Table X).

A specimen of ox serum was tested with a wider range of organisms representative of various bacterial groups (Table XI) and definite reactions were obtained with each.

The various tables show how strains of the same species vary in their reactivity and the variation noted among the organisms used depends

probably as much on the strains selected as the species to which they belong. Fourteen strains of *B. typhosus* (Table XII) were tested with a specimen of sheep serum: all reacted (the majority strongly) but to a varying degree.

Thus the property of fixing complement with normal mammalian serum is widely distributed among the bacteria; variation is probably dependent more on the strain than the species; among the various mammals tested the reacting power of the serum is a constant one, varying with different individuals but more strongly marked in man, ox, sheep and horse than in pig, rabbit and rat. Taking zonal fixation of guinea-pig serum as an index of the reacting effect of this animal, the property is weakly developed, being only elicited with strongly reacting organisms.

Pigeon serum does not apparently possess the property elicited with mammalian sera but is able, both in the unheated and heated state, to reactivate the fixing property of guinea-pig serum annulled by treatment with charcoal. It seems possible that charcoal absorption may leave intact a "fraction" of the reacting substance. The result with pigeon's serum might then be explained by assuming that this serum contains a thermostable principle homologous with the absorbable moiety of guinea-pig serum but lacks some other essential component.

Quantities of antigen and serum required for the reaction.

While the general tests have been carried out with fixed amounts of bacterial suspension and of serum, a number of tests have also been made in which a fixed quantity of suspension was tested with varying quantities of serum, and a fixed quantity of serum with varying quantities of antigen. In these experiments no evidence was elicited that the reaction depended on optimal proportions of the reagents. It was found, however, that even minute amounts of serum (*e.g.* 0·05 c.c. of a 1 in 4 dilution of sheep serum) were sufficient to yield marked effects (*e.g.* with *B. coli* x) and even lesser concentrations (0·05 c.c. of a 1 in 64 dilution) still gave definite reactions. Effects were also produced by relatively small quantities of antigen (0·5 c.c. of the standard suspension of *B. coli* x diluted 1 in 8). It should therefore be emphasised that these reactions are frequently possessed in marked degree by normal serum and are not to be regarded generally as feeble properties.

Thermolability of the serum principle.

In the earlier experiments it was noted that serum was completely or almost completely inactivated at 55° C. (within half-an-hour) and the lability of the effect explains why the reaction has not previously attracted special attention in the course of systematic complement-fixation tests with immune sera which are usually tested only after inactivation or preservation. The thermolability of different sera were studied in more detail, viz. by testing in parallel specimens of a serum in the unheated state and after heating for half-an-hour at temperatures ranging from 40° to 100° C. (see Tables XIII–XVI).

Table XIII. *Thermolability of the reacting properties of ox serum (two specimens—not tested simultaneously).*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum unheated	Serum heated 40° C.	50° C.	52° C.	55° C.	60° C.	70° C.	75° C.	100° C.
B. coli x	>14	.	>14	8	0	0	.	0	0
B. typhosus CB	>14	.	>14	8	2	0	.	0	0
B. paratyphosus B, Tidy	>14	.	>14	8	2	0	.	0	0
B. dysenteriae Shiga 109	>14	.	>14	8	2	0	.	0	0
Staph. aureus Lab. strain	>14	.	8	8	0	0	.	0	0
B. coli x	>10	>10	>10	4	0	0	0	.	.
B. typhosus CB	>10	>10	>10	4	0	0	0	.	.
B. paratyphosus B, Tidy	>10	>10	>10	4	0	0	0	.	.
B. anthracoides Lab. strain	>10	>10	>10	4	0	0	0	.	.
B. dysenteriae Shiga 109	>10	>10	4	4	0	0	0	.	.

Table XIV. *Thermolability of the reacting properties of sheep serum (two specimens—not tested simultaneously).*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum unheated	Serum heated 50° C.	52° C.	55° C.	60° C.	75° C.	100° C.
B. coli x	10	2	2	0	0	0	0
B. typhosus CB	16	6	2	0	0	0	0
B. paratyphosus B, Tidy	10	6	2	0	0	0	0
B. coli x	>14	4	0	0	0	0	0
B. typhosus CB	14	4	0	0	0	0	0
B. paratyphosus B, Tidy	14	0	0	0	0	0	0
B. dysenteriae Shiga 109	>14	0	0	0	0	0	0

Table XV. *Thermolability of the reacting properties of horse serum.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum unheated	Serum heated 50° C.	52° C.	55° C.	60° C.	75° C.	100° C.
B. coli x	>12	>12	>12	Zonal fixation	0	0	0
B. typhosus CB	>12	>12	>12	0	0	0	0
B. paratyphosus B, Tidy	>12	>12	>12	0	0	0	0
B. dysenteriae Shiga 109	>12	>12	>12	Zonal fixation	0	0	0
Staph. aureus Lab. strain	>12	>12	>12	0	0	0	0

(Guinea-pig serum used as complement treated with charcoal to remove its fixing properties: *B. coli* x and *B. dysenteriae Shiga* 109 showed zonal fixation with the untreated complement.)

Table XVI. *Thermolability of reacting properties of ox serum.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Serum unheated	Serum heated 40° C.	45° C.	50° C.	52° C.	55° C.	60° C.
B. coli x	>14	>14	>14	14	4	0	>14
B. typhosus CB	>14	>14	>14	14	4	0	>14
B. paratyphosus B, Tidy	>14	>14	>14	4	4	0	0
B. anthracoides Lab. strain	>14	>14	>14	4	4	0	0
B. dysenteriae Shiga 109	>14	>14	>14	14	8	8	>14
Staph. aureus Lab. strain	8	4	4	4	4	0	0

(In this experiment the guinea-pig's serum used as complement had been incubated at 37° C. for 5 hours to annul its fixing properties.)

The samples were diluted 1 in 4 with saline to prevent coagulation at the higher temperatures. Inactivation generally occurred between 52° and 55° C. but in some cases between 50° and 52° C. and even at 50° C. Partial inactivation was noted sometimes at 40° C.

Reference has already been made to the inactivation of the fixing property of guinea-pig's serum by incubation at 37° C. for several hours, which also indicates the pronounced thermolability of this complement-fixing principle.

On certain occasions when guinea-pig serum previously incubated for several hours at 37° C. (to annul its own fixing effect) was used as complement in these tests, though the serum under investigation was inactivated at 55° C., fixation occurred with specimens heated at 60° C. and 70° C. (Table XVI). Still higher temperatures (*e.g.* 100° C.) annulled the reaction.

Similar results were not obtained when the complement-serum was previously treated with charcoal. Table XV illustrates, however, the reactivation of the fixing property of the complement-serum (as evidenced by zonal fixation) by horse serum heated at 55° C. The guinea-pig serum in this case had been treated with charcoal to remove its fixing properties. The unheated serum gave zonal fixation with *B. coli* x and *B. dysenteriae Shiga*. A similar effect by pigeon serum has been referred to and has been observed also with heated rabbit serum.

It is apparent that the fixing agent in serum differs in its thermolability from antibodies formed in response to immunisation. It is more labile even than complement. This has already been alluded to by Dunlop in the case of guinea-pig serum. Mackie and Finkelstein (1928) have also demonstrated a corresponding thermolability of the constituents of certain sera (pig, rat, etc.) responsible for complement-fixation along with non-antigenic substances.

Serum fractions.

It has been shown that the principle in serum reacting with "pseudo-antigens" is resident in the carbonic-acid insoluble globulins (Mackie and Watson, 1926: Mackie and Finkelstein, 1928). The same also applies to a natural haemolytic antibody (*e.g.* rabbit *versus* sheep haemolysin). Specimens of ox, sheep, rabbit and rat sera were fractioned by Liefman's method and tested with *B. coli* x and various organisms. In the specimens of rabbit and sheep serum examined, the complement-fixing property was contained for the most part in the carbonic-acid insoluble moiety. Specimens of ox and rat serum, however, yielded carbonic-acid soluble and insoluble fractions respectively which were equally active. Thus the active principle of these reactions was not restricted to the carbonic-acid insoluble fraction of the serum.

Sensitisation of bacteria by normal serum.

The fixing agent in serum, like specific antibodies, could be absorbed at 0° C. from the serum by bacteria which then became "sensitised" so that even after removal of the serum and thorough washing with saline solution

the bacterial substance fixed complement to a degree practically equal with the organisms when in contact with serum.

Technique.

Bacterial suspension in the required bulk was mixed with serum in the proportion of 0·5 c.c. suspension to 0·05 c.c. serum and placed at 0° C. for 2 hours; part of the mixture was centrifugalised so as to separate the organisms from the serum; the bacterial sediment was washed twice with saline and then resuspended in saline to the original volume of the mixture from which it was derived; this constituted the "sensitised suspension." 0·55 c.c. of the original mixture of bacteria *plus* serum and the same quantity of "sensitised" bacterial suspension were then tested for complement-fixation with varying doses of complement. The usual antigen and serum controls were included.

Table XVII illustrates the results with *B. coli* x and *B. typhosus* CB.

Table XVII. *"Sensitisation" experiment.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

		Antigen control	Serum control
B. coli x *plus* sheep serum	>18	2	2
B. coli x "sensitised" by sheep serum	>18	.	.
B. typhosus CB *plus* sheep serum	12	0	.
B. typhosus CB "sensitised" by sheep serum	12	.	.

ABSORPTION TESTS.

It has been shown how bacteria remove the fixing agent from serum and become "sensitised" in this way. A considerable series of absorption tests were carried out in which serum was saturated with various bacteria and then tested for its complement-fixing action with the same organisms and also other types.

Technique.

In the case of the typhoid-paratyphoid-dysentery group, 24 hours' growths on agar slopes were emulsified in undiluted serum, in the proportion of one culture to 1 c.c. of the serum; with more abundantly growing organisms, *e.g. B. coli*, *B. anthracoides*, *Staphylococcus aureus*, etc., such amount of culture was emulsified in the serum as to yield a density comparable in opacity with that of the emulsion prepared with *B. typhosus* and allied organisms. The mixtures were placed at 0° C. for 4 hours and then centrifugalised; the separated serum was tested for complement-fixation in the usual way.

Dunlop (1928) has observed in absorption tests with guinea-pig serum that the active principle is removed non-specifically and reference has already been made to the fact of its removal by such absorptive agents as charcoal. We have found similarly that charcoal, stromata of red corpuscles and bacteria generally may exert a non-specific absorptive action removing in whole or part the principle or principles that react with various bacteria. But apart from this non-specific absorption which is often partial, even when large amounts of the absorbing agent is used, a more complete specific absorption can be demonstrated. Thus a particular organism removes from the serum the property of fixation with itself but leaves unaltered or only partially reduced the power of reacting with heterologous organisms. Tables XVIII–

XXI illustrate these effects. Only the strongly reacting sera were satisfactory for eliciting such specific absorption; in the case of weakly active sera, the non-specific action was often sufficient to annul completely the complement-fixation reaction.

Table XVIII. *Sheep serum (two specimens—not tested simultaneously). Absorption experiments.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Serum	*B. paratyphosus* B, Tidy	*B. coli* x	*B. typhosus* CB	*V. cholerae* Bombay	*B. anthracoides* Lab. strain.	*B. enteritidis* Gaertner, Lab. strain	*B. typhosus* Cole	*B. coli* F 3	*B. dysenteriae Shiga* 109	*Staph. aureus* Lab. strain
Untreated	>14	>14	>14	8	8	4	14	.	.	.
Absorbed with										
B. coli x	>14	0	14	14	4	4	14	.	.	.
B. anthracoides Lab. strain	>14	>14	>14	14	0	>14	>14	.	.	.
Untreated	>14	.	14	.	8	.	14	14	>14	14
Absorbed with										
B. coli F 3	14	.	14	.	8	.	14	0	>14	14
B. typhosus CB	10	.	0	.	8	.	0	10	10	10
B. typhosus Cole	8	.	0	.	0	.	0	8	8	8
B. paratyphosus B, Tidy	0	.	8	.	8	.	8	8	8	8
B. dysenteriae Shiga 109	8	.	8	.	8	.	8	8	0	8
Charcoal	8	.	8	.	0	.	8	8	0	8

Table XIX. *Ox serum. Absorption experiment.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Serum	*B. paratyphosus* B, Tidy	*B. coli* x	*B. typhosus* CB	*V. cholerae* Bombay	*B. proteus* X 19	*B. enteritidis* Gaertner	*B. aertryche* "Mutton"
Untreated	>10	>10	>10	4	>10	10	10
Absorbed with							
B. paratyphosus B, Tidy	0	10	>10	10	0	0	0

Table XX. *Ox serum (two specimens—not tested simultaneously). Absorption experiments.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Serum	*B. typhosus* CB	*Staph. aureus* Lab. strain	*B. coli* F 3	*B. typhosus* Cole	*B. paratyphosus* B, Tidy
Untreated	>12	>12	.	.	.
Absorbed with					
Charcoal	8	8	.	.	.
B. typhosus CB	0	8	.	.	.
Staph. aureus Lab. strain	8	0	.	.	.
Untreated	4	.	4	8	8
Absorbed with					
Charcoal	0	.	0	4	0
B. coli F 3	4	.	0	8	8
B. typhosus CB	0	.	4	0	8
B. typhosus Cole	0	.	4	0	8
B. paratyphosus B, Tidy	4	.	4	8	0

Table XXI. *Horse serum (four specimens—not tested simultaneously). Absorption experiments.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Serum	*B. typhosus* CB	*B. paratyphosus* B, Tidy	*B. dysenteriae Shiga* 109	*Staph. aureus* Lab. strain	*B. coli* F 3
Untreated	14	15	8	8	.
Absorbed with					
B. typhosus CB	0	>15	8	8	.
B. paratyphosus B, Tidy	8	0	14	8	.
B. dysenteriae Shiga 109	8	>15	0	8	.
Staph. aureus Lab. strain	8	>15	8	0	.
Untreated	>15	>15	.	.	.
Absorbed with					
Charcoal	>15	>15	.	.	.
B. typhosus CB	4	>15	.	.	.
B. paratyphosus B, Tidy	>15	4	.	.	.
Untreated	14	>14	.	.	.
Absorbed with					
Charcoal	8	>14	.	.	.
B. typhosus CB	0	>14	.	.	.
B. paratyphosus B, Tidy	8	0	.	.	.
Untreated	.	.	.	10	>10
Absorbed with					
Charcoal	.	.	.	10	>10
B. coli F 3	.	.	.	10	0
Staph. aureus Lab. strain	.	.	.	0	>10

Table XXII shows the results of absorption tests with guinea-pig serum. The complement-fixing principle in this serum proved highly susceptible to any absorptive agent and specific effects could not be demonstrated.

Table XXII. *Guinea-pig serum. Absorption experiment.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Serum	*B. coli* x	*B. typhosus* RLL
Absorbed with		
Charcoal	0	0
B. coli x	0	0
B. coli F 3	4	0
B. typhosus CB	0	0
B. typhosus RLL	12	0
B. paratyphosus B, Tidy	0	0
Untreated	12	12

In this experiment 0·05 c.c. of guinea-pig serum (untreated and after absorption with the various organisms shown) along with the usual amount of antigen was tested with varying quantities of guinea-pig serum from which fixing properties had been removed by treatment with charcoal. In the former case the serum was tested for its fixing properties: in the latter it was used as complement.

THERMOLABILITY OF BACTERIAL ANTIGENS.

The most pronounced fixation effects were obtained with unheated bacterial suspensions. Heating almost invariably reduced the reacting power of the organisms. Thus one hour's exposure at 55° C. was sufficient in most instances to lower the value of the suspension and often produced complete inactivation. In many of the tests there was partial weakening even at 50° C. The exact degree of thermolability, however, was extremely variable. Tables

XXIII–XXV illustrate the results. In the experiment with ox serum shown in Table XXIII, all the antigens tested were completely inactivated at temperatures over 60° C. Certain antigens were inactivated at 55° C. and most were weakened at 50° C. Table XXIV illustrates two experiments with horse serum: in the first, *B. coli* x and *B. typhosus* CB were inactivated at 55° C., *B. paratyphosus* B at 50° C.; in the second experiment *B. coli* x was only

Table XXIII. *Thermolability of antigens. Ox serum.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Unheated	Antigen heated					
		50° C.	55° C.	60° C.	70° C.	80° C.	100° C.
B. coli x	>14	>14	14	4	0	0	0
B. typhosus CB	>14	14	14	0	0	0	0
B. paratyphosus B, Tidy	>14	14	0	0	0	0	0
B. anthracoides Lab. strain	14	4	0	0	0	0	0
B. dysenteriae Shiga 109	14	4	4	4	0	0	0

Table XXIV. *Thermolability of antigens. Horse serum (two specimens—not tested simultaneously).*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Unheated	Antigen heated					
		50° C.	55° C.	60° C.	70° C.	100° C.	120° C.
B. coli x	10	10	0	0	0	0	0
B. typhosus CB	10	4	0	0	0	0	0
B. paratyphosus B, Tidy	10	0	0	0	0	0	0
B. coli x	>10	>10	>10	>10	>10	4	0
B. typhosus RLL	>10	>10	>10	>10	>10	4	0

Table XXV. *Thermolability of antigens. Sheep serum (two specimens—not tested simultaneously).*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Unheated	Antigen heated							
		50° C.	55° C.	60° C.	65° C.	75° C.	80° C.	100° C.	120° C.
B. coli x	>10	4	4	4	0	.	0	0	0
B. typhosus CB	10	0	0	0	0	.	0	0	0
B. typhosus Cole	10	0	0	0	0	.	0	0	0
B. paratyphosus B, Tidy	>10	6	6	6	6	.	6	4	0
B. dysenteriae Shiga 109	>10	6	6	6	6	.	6	6	0
Staph. aureus Lab. strain	>10	0	0	0	0	.	0	0	0
B. coli x	>16	16	10	10	.	10	.	0	.
B. typhosus CB	10	10	6	6	.	6	.	0	.
B. typhosus RLL	>16	16	10	10	.	6	.	0	.
B. paratyphosus B, Tidy	10	6	0	0	.	0	.	0	.
B. dysenteriae Shiga 109	>16	10	0	0	.	0	.	0	.

completely inactivated when heated to a temperature over 100° C. In the first experiment shown in Table XXV with sheep's serum there was inactivation of certain organisms at 50° C. and partial weakening of others; two of the strains tested, however, were only inactivated completely above 100° C. The second experiment with sheep's serum (Table XXV) showed the same lack of uniformity. The thermolability temperature of *B. coli* x suspensions prepared on different occasions and tested with the sera of different species and different

specimens from the same species ranged from 50° C. to 100° C. and over. The same applied to *B. typhosus* CB and most of the other organisms examined.

The organism (*B. typhosus* RLL) used by Dunlop in his study of zonal fixation (*v. supra*) also varied in the same way in our tests. Dunlop, however, found it to be regularly thermostable at 120° C. when tested for zonal fixation with guinea-pig serum.

It was apparent that thermostability depended on some variable factor irregularly affecting the organism.

It has been recognised as regards the agglutinogens of bacteria that different antigenic constituents vary in thermostability: thus the H antigen of Weil and Felix is labile at 80°–100° C. while the O antigen is stable at this temperature (see Arkwright, 1927). The lability of the antigens in our complement-fixation experiments with normal serum considerably exceeded even that of the H antigen.

Most of the organisms used were from cultures that had been cultivated artificially over long periods and certain of them showed colony variants. It seemed not unlikely, therefore, that individual strains represented different types of antigen associated with these colony variants. If these differed in thermostability and if individual cultures varied in their relative content of their constituent antigens, the variations noted in the experiments might be susceptible of explanation. This requires, however, further investigation.

It is possible also that the type of medium and duration of the culture may be a factor influencing antigen stability. Dunlop used a casein-digest medium and 48 hours' cultures. In our experiments 24 hours' growths on a plain nutrient agar were employed. We have used cultures on a meat-digest medium but without different results.

The thermostability of antigens when tested for zonal fixation with guinea-pig's serum was also examined and similar results were obtained: in some experiments inactivation occurred at 55° C. and inactivation was always complete at 100° C. It was noted that when the bacteria were washed with saline before the preparation of the suspension the stability was frequently increased but inactivation at 100° C. still occurred (Tables XXVI and XXVII).

Preliminary washing of the cultures did not materially influence stability in tests with other sera.

Table XXVI. *Guinea-pig serum (two specimens). Zonal fixation.*

			B. typhosus RLL	
Antigen	*B. typhosus* CB	*B. coli* x	Bacteria washed with saline	Unwashed
Unheated	Z 8–24 D	Z 8–24 D	.	.
Heated 55° C.	Nil	Z 8–12 D	.	.
„ 70° C.	„	Nil	.	.
„ 100° C.	„	„	.	.
Unheated	.	.	Z 4–18 D	Z 4–18 D
Heated 55° C.	.	.	Z 4–8 D	Z 4 D
„ 100° C.	.	.	Nil	Nil

Table XXVII. *Guinea-pig serum. Zonal fixation.*

Antigen	*B. typhosus* CB		*B. typhosus* RLL		*B. coli* x	
	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed
Unheated	Z 4–18 D	Nil	Z 4–30 D	Z 4–24 D	Z 4–24 D	Z 4–30 D
Heated 53° C.	Z 4–18 D	,,	Z 4–24 D	Nil	Nil	Nil

Comparative thermolability tests were carried out with bacterial suspensions from growths on plain agar and casein-digest medium. Sheep's serum was used with charcoal-treated guinea-pig serum as complement, and zonal fixation was also tested for with untreated guinea-pig serum. Tables XXVIII and XXIX show the results. With *B. coli* x and sheep serum no difference was

Table XXVIII. *Sheep serum.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

Antigen	Grown on ordinary agar	Grown on Casein-digest agar
B. coli x:		
Unheated	>16	>16
Heated 55° C.	6	6
,, 70° C.	6	6
,, 100° C.	0	0
,, 120° C.	0	0
B. paratyphosus B:		
Unheated	10	6
Heated 55° C.	6	6
,, 70° C.	2	6
,, 100° C.	0	0
,, 120° C.	0	0
B. typhosus RLL:		
Unheated	0	10
Heated 55° C.	0	10
,, 70° C.	0	6
,, 100° C.	0	0
,, 120° C.	0	0

Table XXIX. *Zonal fixation by guinea-pig serum.*

Antigen	Grown on ordinary agar	Grown on Casein-digest agar
B. coli x:		
Unheated	Z 4–>14 D	Z 4–>14 D
Heated 55° C.	Nil	Z 4–8 D
,, 70° C.	,,	Nil
,, 100° C.	,,	,,
,, 120° C.	,,	,,
B. typhosus RLL:		
Unheated	Z 4–>14 D	Z 2–>14 D
Heated 55° C.	Nil	Z 2–>14 D
,, 70° C.	,,	Z 4–>14 D
,, 100° C.	,,	Z 4–>14 D
,, 120° C.	,,	Z 4–>14 D

elicited. The same also applied to *B. paratyphosus* B. *B. typhosus* RLL was inactive when grown on ordinary agar; when cultivated on the digest medium it showed a distinct reaction but was inactivated between 70° and 100° C. In the zonal fixation tests *B. coli* x grown on ordinary agar was inactivated at

55° C.; grown on the digest medium it was slightly more stable but inactivation still occurred at 70° C. *B. typhosus* RLL, however, showed a profound difference; grown on ordinary medium it proved labile at 55° C. but on the digest medium was stable even at 120° C. This accords with Dunlop's results and shows how this particular strain quâ fixation with guinea-pig's serum possesses a high degree of stability when cultivated on a casein-digest medium though distinctly labile when grown on a plain medium or a meat-digest agar.

Such results seem to indicate that the nature of the antigenic substance of an organism may vary greatly according to the substrate on which it is growing —a finding of great significance in relation to antigenic composition.

The reacting power is apparently not related to variations in the colony appearances as regards "roughness" or "smoothness." Pure "rough" and "smooth" variants of *V. cholerae* were tested in parallel without appreciable quantitative difference being noted. This is also illustrated among the various typhoid strains examined (see Table XII).

REACTING POWER OF THE SERUM IN RELATION TO THE AGE OF THE ANIMAL.

It has been shown by Mackie and Watson (1926) and Mackie and Finkelstein (1928) how the natural property of reacting with various "pseudo-antigens" (lipoids, peptone, cholesterol) is absent from the serum of young animals. The same also applies to the natural haemolysin of the rabbit. This is not true, however, for the natural complement-fixation reactions with bacterial antigens. Young rabbits even 18 days from the time of birth, guinea-pigs 10 days old and calves 6 to 8 weeks old were found to possess the reacting property as strongly developed as adult animals (see Tables XXX and XXXI). In the case of the guinea-pig litter referred to in Table XXXI, the mother's serum was devoid of the fixation property while marked zonal fixation occurred with the serum of the young animals.

Table XXX. *Rabbits—litter* 18 *days.*

Complement-fixation stated in terms of number of M.H.D. of complement fixed.

	Antigens						
		B. typhosus					
Serum	*B. coli* x	CB	Cole	RLL	*B. para-typhosus* B	*B. pyo-cyaneus*	*Staph. aureus*
Rabbit 1	>16	>16	6	10	6	4	0
„ 2	>16	>16	>16	10	4	4	6
Mother	10	>16	6	6	4	0	4

Table XXXI. *Guinea-pigs—litter* 10 *days. Zonal fixation.*

	Antigens				
Serum	*B. coli* x	*B. typhosus* RLL	*B. typhosus* CB	*B. typhosus* Cole	*V. cholerae* Bombay
Guinea-pig 1	Z 10–28 D	Z 10–28 D	Z 10–28 D	Z 10–28 D	Z 10–16 D
„ 2	Z 10–22 D	Z 10–28 D	Z 10–28 D	Z 10–28 D	Z 10–16 D
Mother	Nil	Nil	Nil	Nil	Nil

THE QUESTION OF CORRELATION OF NATURAL COMPLEMENT-FIXATION AND AGGLUTINATION EFFECTS.

A number of parallel observations of complement-fixing and agglutination reactions have been made with specimens of serum from different species and various bacteria. These tests were carried out with a view to ascertaining whether the complement-fixation results correspond to the occurrence in the serum of natural agglutinins for particular organisms.

Tables XXXII and XXXIII illustrate results with horse and rabbit serum. Though there was some correspondence in the occurrence of the two reactions, the correlation was incomplete. Further, while the complement-fixation reaction was annulled when the serum was heated at 55° C. (*v. supra*), this did not apply to the natural agglutinin[1].

Table XXXII. *Horse serum.*

Bacterium	Complement-fixation stated in terms of number of M.H.D. of complement fixed		Agglutination end-titre	
	Unheated serum	Serum heated at 55° C.	Unheated serum	Serum heated at 55° C.
B. coli x	>16	0	1 in 16	1 in 16
B. coli F 3	16	0	–	–
B. typhosus CB	>16	0	1 in 256	1 in 64
B. typhosus RLL	16	0	1 in 64	1 in 32
B. paratyphosus B	>16	0	1 in 32	1 in 32
B. proteus X 19	16	0	1 in 16	1 in 64
B. dysenteriae Shiga	>16	0	1 in 16	1 in 16
V. cholerae	16	0	1 in 64	1 in 32

The – sign under agglutination signifies absence of any demonstrable reaction by a dilution of 1 in 2 of the serum.

Table XXXIII. *Rabbit serum.*

Bacterium	Complement-fixation stated in terms of number of M.H.D. of complement fixed		Agglutination end-titre	
	Unheated serum	Serum heated at 55° C.	Unheated serum	Serum heated at 55° C.
B. coli x	4	0	1 in 4	–
B. coli F 3	4	0	–	1 in 4
B. typhosus CB	8	0	1 in 64	1 in 16
B. typhosus RLL	4	0	1 in 32	1 in 8
B. paratyphosus B	0	0	–	–
B. proteus X 19	0	0	–	–
B. dysenteriae Shiga	4	0	1 in 16	1 in 32
V. cholerae	0	0	–	–

DISCUSSION.

The observations recorded form a contribution to the study of natural immunity phenomena. It has been long recognised that specific antibodies for various types of antigens occur naturally in the serum of particular animal species. The complement-fixation reactions described in this paper reveal a

[1] We are indebted to Dr H. J. Gibson for these data taken from his unpublished studies on natural agglutinins.

remarkable multiplicity of natural antibacterial principles which are widely distributed among mammalian animals. Analogous reactions have been studied by others (see Takenomata, 1924; Kolmer and co-workers, 1916; Browning, 1927; Dunlop, 1928) and have been regarded as "non-specific," but our observations indicate a distinct though relative specificity. Thus multiple specific antibody-like principles for a wide variety of bacteria can be demonstrated in animal sera. Among the mammals tested the occurrence of such principles is almost a constant one, varying with different species, different individuals of the same species and for different organisms. These substances (as judged by quantitative complement-fixation tests) are strongly developed in man, ox, sheep and horse and are less pronounced in pig, rabbit, white rat and guinea-pig; they are either more reactive or more markedly developed for certain bacteria, but this variation according to organisms may depend on the bacterial strain rather than the species. While these principles correspond, in virtue of their specific absorption by homologous antigens, to antibodies formed as a result of immunisation, they certainly differ from "immune" antibodies in thermolability. The lability of these substances seems at first to correspond to that of complement but is even greater: for example, inactivation occurs sometimes at 50° C. within half-an-hour, and partial inactivation may result even at 40° C. In the case of the guinea-pig a few hours' incubation at 37° C. may be sufficient to produce inactivation while haemolytic complement shows little deterioration.

The content of these substances in serum, while relatively low as compared with an "immune" antibody in the serum of an immunised animal, is still considerable and in some cases quantitatively marked.

The complement-fixation reaction of guinea-pig serum with *B. typhosus* and certain other organisms, described by Dunlop, and the reactions observed by Takenomata are probably analogous to the phenomena we have studied and admit of a similar interpretation. Dunlop found no evidence of specificity in the reaction of guinea-pig's serum. He pointed out the extreme susceptibility of the reacting substance to absorptive agents—bacteria, charcoal, etc., and this makes the demonstration of specific absorption difficult. Our claim that these antibody-like agents are specific, rests on observations with sera and organisms which yield the strongest fixation reactions. The labile antityphoid antibody described by Dunlop in guinea-pig sera is probably one example of these multiple antibody-like substances we have demonstrated in a variety of animals.

Our findings parallel to some extent observations of the occurrence of natural agglutination of bacteria by normal serum. This phenomenon has been studied by various workers and has been generally attributed to natural agglutinating antibodies analogous to immune agglutinins. Bordet (1899) as a result of absorption tests indicated the specificity of such natural agglutinins and also suggested the possibility of their being the precursors of immune antibodies. Kraus and Low (1899) observed the occurrence of natural

agglutinins for a variety of bacteria in different animal species, and the study of natural agglutinins has been extended by other workers (see Gengou, 1899; Goldberg, 1901; Rissling, 1907; Braun, 1909).

While there is some degree of correspondence between the complement-fixation phenomena recorded and agglutination of bacteria by normal serum, this is not complete. Thus, natural agglutinins are distinctly more thermostable than the complement-fixing principles.

The absence of natural antibodies, *e.g.* haemolysin, agglutinin, from the serum of young animals (see Lüdke, 1904; Braun, 1909; Mackie and Watson, 1926; Mackie and Finkelstein, 1928) is not paralleled in the case of the complement-fixing agents we have studied.

Browning (1927) has put forward the hypothesis based on Dunlop's observations that a common "prototype" of immune antibodies may exist normally in blood serum in an undifferentiated state. The specificity we have noted indicates that such precursors of immune antibodies are already highly differentiated, and correspond to the pre-existing differentiated receptors of the protoplasm molecule originally postulated by Erhlich. He supposed that such receptors, naturally sessile, became "free" as a result of overproduction following a specific immunising stimulus. Whether these natural antibody-like principles are to be interpreted in this way, or identified with immune antibodies, their difference from the latter lies mainly in their lability. In this connection it is noteworthy how a natural haemolysin and haemagglutinin differs similarly in thermolability from the corresponding immune lysin (see Landsteiner and Reich, 1908; Thiele and Embleton, 1914; and Mackie and Finkelstein, 1928).

Certain aspects of the phenomena we have described present considerable difficulties in interpretation, viz. inactivation at 55° C. associated with activation at higher temperatures, and the reactivation (following inactivation by charcoal) of the fixing agent in guinea-pig serum by pigeons' sera, heated horse and rabbit serum. Activation at high temperatures (with inactivation at a lower temperature) has been noted by the authors in a previous study of complement-fixation along with non-antigenic substances; and the influence of heat on serum principles generally presents problems which in the present state of knowledge of the nature and constitution of these agents and of the physical factors involved are difficult to elucidate. It seems possible, on the analogy of the constitution of complement, that these substances comprise different "fractions" including a thermostable component.

The variability in the heat resistance of the bacterial antigen also requires further study, but it has been specially noteworthy how stability may depend on the type of substrate on which the organism is growing. This observation seems of fundamental importance in regard to the study of the antigenic constitution of bacteria.

The special interest of our observations lies in their demonstration of the existence naturally in animal serum of multiple specific antibodies for a wide

variety of bacterial antigens and seems to open up many further questions that require study in natural immunity.

CONCLUSIONS.

1. The normal sera of various mammalian species (*e.g.* man, ox, sheep, horse, pig, white rat, rabbit, guinea-pig) possess the property of fixing complement with a wide variety of bacteria.

2. This property is most strongly developed in man, ox, sheep and horse of the animal species examined but varies in degree with different individual specimens of serum from the same species.

3. The reactions vary in degree with the different bacteria tested and with different strains of the same bacterial species. Such variation probably depends more on the strains than the species.

4. The reactions with certain sera and bacteria are quantitatively marked.

5. The reacting principle of the serum is highly thermolabile, being uniformly inactivated at 55° C. within half-an-hour. Complete inactivation frequently occurs at 50°–52° C. and partial inactivation is sometimes noted at 40° C.

6. The complement-fixing agent in the serum is "absorbed" at 0° C. by bacteria which became "sensitised" in this way.

7. The complement-fixing agent is susceptible to absorption by various substances including bacterial suspensions, but apart from this non-specific absorption a more complete specific absorption by bacteria can be demonstrated.

8. As a rule, the reacting power of bacterial suspensions (prepared by the method described) is partially or completely labile at 55° C. but the thermolability temperature may vary considerably under different conditions. The type of medium on which the organism is grown may influence the thermostability.

9. The reacting power of the serum is fully developed in young animals (contrasting in this respect with certain natural antibody-like principles).

10. There is some relationship between these complement-fixation reactions and bacterial agglutination by normal serum but the correspondence is incomplete.

11. It is concluded from the observations recorded that among mammalian animals multiple specific antibody-like principles for a variety of bacteria occur in the serum. While these principles correspond in some respect to antibodies formed as a result of immunisation, they differ from "immune-antibodies" in thermolability.

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