NATURAL BACTERICIDAL ANTIBODIES: OBSERVATIONS ON THE BACTERICIDAL MECHANISM OF NORMAL SERUM.

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Introduction.

The natural occurrence of antibody-like principles in the serum of various animals has been the subject of recent systematic studies by Mackie and Watson (1926), Mackie and Finkelstein (1928, 1930) and Gibson (1930). An important result of this work has been the demonstration in normal sera of specific complement-fixing and agglutinating agents for a wide variety of bacterial antigens. Different animal species vary, however, as regards the occurrence and degree of activity of these principles. Bacteria vary also in their reactivity. Serological analyses have established the antibody-like character of the serum principles responsible for these reactions, though they undoubtedly differ in certain respects from the antibodies produced by immunisation.

The data collected in this way regarding the occurrence and characteristics of "natural" antibodies are of interest from a general biological standpoint, and have an important bearing on immunological theory. These questions have been discussed in the publications cited above.

As a corollary to such studies and in the light of their results, a systematic inquiry has been instituted into the bactericidal action of normal serum. The immediate object in view has been to analyse the mechanism of this phenomenon, with particular reference to the part played by antibody-like principles, and the characters and behaviour of these substances.

Since the classical observations of Nuttall (1888), Buchner (1889) and others, first contributed to our knowledge of the subject, it has been recognised that this property of serum is a variable one, differing according to the animal species and the type of bacterium, but, except in certain particular instances, the variations have not been fully defined and interpreted from the biological and immunological standpoints. There has also been considerable uncertainty regarding the mechanisms involved in the bactericidal property of normal serum, though the factors concerned in the same effect produced by a specific immune serum have been more clearly defined.

The thermolability of the normal bactericidin of blood serum towards certain bacteria was demonstrated by the early workers on the subject, and later work on immune cytolytic sera has identified the thermolabile bactericidal principle in normal serum with "complement" as defined by Ehrlich. On this basis, the mechanism of normal bactericidal action towards certain

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bacteria was carefully studied by Muir and Browning (1908). They concluded that this reaction may, in certain cases, differ from the same effect produced by an immune serum, but they left the question open whether the effect is mediated by a natural sensitising immune body or is due to the direct action of a "bacteriophilic" complement. It has been generally assumed, however, that the reaction is analogous to that of an immune serum and that a sensitising antibody plays a part in the phenomenon, though the possibility of bactericidal action by complement alone has not been definitely excluded. While the existence of certain natural bactericidal antibodies has been proved, there has been no clear evidence to show that the corresponding action of normal serum generally is dependent on such principles.

There has also been uncertainty regarding the specificity or non-specificity of natural bactericidal effects. Muir and Browning (1908) reviewed the literature on this question prior to their own work, and studied the specificity of these reactions by absorption methods. They found that treatment of a normal serum with increasing amounts of bacterial emulsion produced first a diminution of the bactericidal action towards the homologous bacterium, and then a decrease in the effect of the serum on other bacteria. The studies of natural complement-fixing and agglutinating antibodies (referred to above) have suggested the likelihood that the bactericidal effects of normal serum may be due to multiple specific antibodies sensitising bacteria to the lytic action of complement. Recently Gordon and Wormall (1928) have shown how bacteriolysis of *B. dysenteriae* (Flexner) by normal guinea-pig serum depends on the combined action of complement and a thermostable factor removed from the serum by absorption with the particular organism.

The question is further complicated by the fact that different mechanisms may be concerned in the bactericidal action of normal sera, and that the factors may vary with different organisms. The killing of anthrax bacilli by normal serum was attributed by Gruber and Futaki (1907) to a product of blood platelets ("plakanthrakocidine") which is independent of complement. Leucocytic extracts ("leukins") have been shown to possess marked bactericidal properties for certain bacteria (see Ledingham, 1922). Pettersson (1926, 1927–8) has classified the bactericidal agents of serum into α-lysins and β-lysins. The former apparently represents the complement acting along with a sensitising agent analogous to an immune body. The latter, according to Pettersson, consists of a stable "activating" agent (resisting a temperature of 63° C. for half an hour) and an "activable" principle which unites with the bacteria in the presence of the activating agent. He differentiates the organisms that are susceptible to the α- and β-lysins respectively. The occurrence of heat-stable bactericidal properties in serum has also been studied by Selter (1918), who inclined to the view that different organisms are affected by a common agent and not by multiple specific substances. It is noteworthy that Seiffert (1912, 1917) regarded the bactericidal properties of a serum as due to specific agents.

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The whole subject of the bactericidal action of normal serum has been reviewed recently by Knorr (1929).

In the studies recorded in this paper attention has been directed mainly to those bactericidal properties which have proved labile at 55° C. and dependent, therefore, on the action of a labile complement-like agent. The essential questions at issue have been whether these effects are mediated by antibodies and the properties and reactions of such substances.

METHODS.

The following methods of estimating bactericidal action have been devised for the special purpose of carrying out large numbers of quantitative and comparative tests with the maximum facility compatible with accuracy and reliability, and were used in this investigation.

METHOD I.

In testing organisms which did not rapidly lose viability when suspended in salt solution, a given quantity of serum was mixed with a given volume of varying dilutions of bacteria made up in 0·85 per cent. sodium chloride solution; after incubation at 37° C. the relative sterilising action of the serum was determined by cultivating a standard loopful of each mixture, a stroke inoculation being made on a plate of the appropriate medium. A parallel control test was carried out by cultivating similarly loopfuls of the varying bacterial dilutions, to the given volume of which saline solution had been added instead of serum, these suspensions having been incubated previously along with the test series. The degree of bactericidal effect could in this way be determined by comparison of the end-points of growth in the two series, growth being absent from concentrations in the test series which yielded growth in the control (see Table I). Lack of bactericidal action was indicated by coincidence of the two end-points (see Table II). The bacterial dilutions were prepared from 18–24 hours' cultures.

This method gave satisfactory results with certain organisms, *e.g.* Staphylococci, the *coli*-typhoid and allied groups, *V. cholerae*, *B. pyocyaneus*, *B. proteus*, etc., which maintained their viability in saline solution during short periods of incubation (*e.g.* 3 hours) so that no appreciable alteration in the number of viable organisms in the bacterial dilutions occurred during the course of the test (see Tables III and IV). It had the advantage that the comparative bactericidal power of a large number of specimens of serum towards a particular organism could be estimated by one set of loop-transfers made at the end of the period of incubation (cp. Method II *infra*).

The mixtures were all carefully shaken before making transfers to ensure uniformity of the bacterial suspensions. The possibility of agglutination of the suspended organisms influencing results and introducing a fallacy was considered but did not appear to affect the validity of these tests; for example, certain organisms were strongly agglutinated by various sera (evidenced by visible clumping when serum was added to the standard suspension) without the least sign of bactericidal effect as judged by the results of the test.

The comparison of end-points was also checked by estimates of the approximate amount of growth resulting from each transfer. The relative sterility of the serum was controlled by a loop-transfer from the given quantity of serum mixed with an amount of saline equal to that of the bacterial dilutions and incubated along with the other mixtures.

This system differs from methods in vogue in which bactericidal action is determined by comparative counts of colonies developing from mixtures of a bacterial suspension and varying quantities of serum. When a serum also agglutinates the organism tested, methods dependent on colony counts must yield often fallacious results, and the complete sterilisation of a suspension containing a known number of bacteria by a known quantity of serum

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Table I. B. typhosus *and human serum.*

Bacterial dilutions	1 $S/1$	2 $S/10^2$	3 $S/10^4$	4 $S/10^6$	5 $S/10^8$	6 $S/10^{10}$
Control	+ + + +	+ + +	+ +	+	f.c.	–
Bacteria + serum	+ + +	–	–	–	–	–

Bactericidal effect stated as +8.

Table II. B. pyocyaneus *and human serum.*

Bacterial dilutions	1	2	3	4	5	6
Control	+ + + +	+ + +	+ +	f.c.	–	–
Bacteria + serum	+ + + +	+ + +	+ +	f.c.	–	–

No bactericidal effect.

Table III. B. typhosus *and rabbit serum.*

	Bacterial dilutions	1	2	3	4	5	6
Before incubation	Control	+ + + +	+ + +	+ +	+	f.c.	–
	Bacteria + serum	+ + + +	+ + +	+ +	+	f.c.	–
After incubation	Control	+ + + +	+ + +	+ +	+	f.c.	–
(3 hours)	Bacteria + serum	+ + +	+ +	+	–	–	–

Bactericidal effect stated as +4; no appreciable alteration in the number of viable organisms in saline.

Table IV. Staphylococcus aureus *and rabbit serum.*

	Bacterial dilutions	1	2	3	4	5	6
Before incubation	Control	+ + + +	+ + +	+ +	+	f.c.	–
	Bacteria + serum	+ + + +	+ + +	+ +	+	f.c.	–
After incubation	Control	+ + + +	+ + +	+ +	+	f.c.	–
(3 hours)	Bacteria + serum	+ + + +	+ + +	+ +	f.c.	–	–

Bactericidal effect stated as +2; no appreciable alteration in number of viable organisms in saline.

Table V. B. diphtheriae *and guinea-pig serum.*

	Bacterial dilutions	1	2	3	4	5	6
Before incubation	Control	+ + + +	+ +	–	–	–	–
	Bacteria + serum	+ + + +	+ + +	+ +	+	f.c.	–
After incubation	Control	+ + + +	–	–	–	–	–
(3 hours)	Bacteria + serum	+ + + +	+ + +	+ +	+ +	+	f.c.

Increased viability of organisms on transfer from serum as compared with saline; after incubation, end-point higher in test than control series; diminution in number of viable organisms in control series; increase in number in test series; no bactericidal effect.

Table VI. Streptococcus pyogenes *and pig serum.*

	Bacterial dilution	1	2	3	4	5	6
Before incubation	Bacteria + serum	+ + + +	+ +	+	–	–	–
After incubation (3 hours)	Bacteria + serum	+ + + +	+ +	+	–	–	–

End-points in test series same before and after incubation; no bactericidal effect.

affords a more reliable index of bactericidal action. In the system we have adopted, instead of testing complete sterilisation by cultivating the whole volume of each mixture, the relative degree of sterilisation was ascertained by loop-transfers. Our experience of this technique has justified the validity of the results obtained by it.

At first the series of bacterial concentrations used for the test consisted of successive decimal dilutions prepared from a standard concentration determined by comparison with Brown's opacity standards. In order to elicit definite end-points in both the control and test series when a serum was strongly bactericidal, an extensive range of such decimal

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dilutions was found necessary—$S/1$ to $S/10^{10}$ (S = standard concentration). This series did not yield a distinctly visible gradation in the relative amounts of growth obtained on loop-transfer from two successive dilutions unless near the end-point. Centimal dilutions from the standard, $S/1$, $S/10^2$, $S/10^4$, $S/10^6$, $S/10^8$, $S/10^{10}$, gave, as a rule, a distinct gradation in the successive growths and proved adequate for comparative tests. This series was therefore generally used; in certain cases, to determine lesser differences, a decimal series was adopted.

The initial standard was varied (according to trial tests) for different organisms, the object being to arrange the series so that an end-point was obtained in the control at the fourth or fifth dilution. For organisms of the *coli*-typhoid group Brown's opacity standard No. 2 represented a suitable initial suspension.

The mixtures of bacteria and serum were made in small sterile stoppered test-tubes. The volume of bacterial suspensions was usually 0·5 c.c. and of serum 0·15 c.c. Even minute amounts (*e.g.* 0·025 c.c.) of an active bactericidal serum yielded distinct results by the method described.

The serum was either from defibrinated or coagulated blood, all precautions being taken to obtain a sterile product, and was used within a few hours of the withdrawal of the blood.

The mixtures were incubated usually for 3 hours. In tests with rabbit serum and *B. typhosus* and certain other organisms it was found that the full effect was developed in this time and further incubation did not elicit appreciably greater killing.

The transfers were made with a loop of 4 mm. diameter and each loopful was stroked out on a marked division of the plate.

The degree of bactericidal action was recorded as follows: for example, if, in the control series, growth resulted from all concentrations up to the fifth ($S/10^8$), while in the test series growth occurred only from the first ($S/1$), the result (a bactericidal effect) was stated as + 8; if in the control the end-point was at the fifth dilution ($S/10^8$) and in the test at the third ($S/10^4$), the result was recorded as + 4, *i.e.* the difference between the indices of the two dilutions.

In the detailed records the relative amount of growth from each transfer was recorded by + signs and other symbols as follows: + + + + (a confluent growth on stroke inoculation), + + +, + +, +, f.c. (a few colonies).

METHOD II.

In testing certain organisms by the above method (*e.g.* Streptococci, Pneumococcus, diphtheroid bacilli, *Pasteurella* group, etc.) which rapidly lost viability in saline, it was noted that growth often occurred from higher dilutions in the test series than the control. In the case of *B. diphtheriae*, a comparison of the results of loop-transfers from both series before and after incubation, showed that the effect was due to the following factors: (1) loss of viability of the organisms in saline suspension; (2) maintenance of viability in the presence of serum and even some degree of multiplication; (3) greater viability on transfer to culture medium from serum as compared with transfer from saline solution; thus a proportion of the individual bacteria in saline were incapable of multiplying on culture medium while the presence of serum rendered them viable (see Table V). In this case a bactericidal effect could be excluded and the action of the serum was actually growth-promoting, but in testing organisms which rapidly lost viability in saline solution, and, in fact, all organisms which gave negative results by Method I, transfers were made from the mixtures of serum and bacteria both before and after incubation and the respective end-points of growth compared (see Table VI). This involved a double set of transfers for each specimen of serum tested.

For estimating bactericidal effects in Method II, the transfers before incubation provided the control, and the same system of notation was used as in Method I.

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Analyses of the mechanism of natural bactericidal action by serum.

As a preliminary to the analytical study which was the main object in view, an extensive survey was made of the natural bactericidal properties of the serum of various animal species. Specimens of ox, sheep, horse, pig, rabbit, rat, guinea-pig, pigeon and human serum were tested with organisms representative of various bacterial groups.

It is not intended in this communication to detail or discuss the observations made in the course of such survey. Some of the data elicited were confirmatory of observations recorded by previous workers on the subject; many of the results have opened up questions which are still being studied and require further investigation.

The serum of all these animal species exhibited in a specially marked degree and most uniform manner bactericidal properties towards certain organisms, exemplified by the typhoid-paratyphoid and dysentery groups and the cholera and paracholera vibrios. The bacterial types selected, therefore, for the enquiry recorded in this paper were drawn mainly from these groups.

For the analyses of the bactericidal mechanism, ox, sheep, pig, rabbit, horse, guinea-pig and human sera were used, and the following organisms were selected for the experiments: *B. typhosus* (strain "CB"), *B. dysenteriae* Shiga, *V. cholerae* (strains "Bombay" and "3134") and *B. proteus* X 19.

In all cases inactivation of the serum occurred at 55° C. within half an hour, and the bactericidal reactions with these organisms were apparently dependent on a principle analogous in thermolability to serum-complement. To determine whether a sensitising antibody-like agent plays an essential part in bactericidal action along with this labile principle (assumed to be complement), a series of tests were carried out as follows:

Living organisms from agar cultures (18–24 hours) were treated for 2 hours at 0° C. with a large volume of serum (usually one slope-culture to 10 c.c. serum), the object being to sensitise them, if an antibody were present in the serum, without fixation of complement or bacteriolysis resulting (see Mackie and Finkelstein, 1930); thereafter the organisms were separated by centrifuging and washed in saline solution; a suspension of standard density was then prepared. In the text and tables such organisms are described as "sensitised." Serum was also treated with heat-killed (65° C.) cultures for 2 hours at 0° C. with the object of absorbing and removing the antibody, if present, without affecting the complement; the serum was separated by centrifuging. For absorption the minimum amount of growth found sufficient in preliminary tests to inactivate the serum was used (usually 12 agar slope cultures to 5 c.c. serum). Serum treated in this way is described as "absorbed." A bactericidal effect on the "sensitised" organisms by "absorbed" serum (if inactive *per se* towards unsensitised organisms) was taken as evidence of the existence of a sensitising antibody in the serum. The actual tests were carried out by Method I described above and the necessary controls were included.

In many cases, the existence of a sensitising antibody was demonstrated in this way without difficulty or dubiety. This held for all sera tested with *B. typhosus* (Table VII). The thermostability of this antibody was estimated

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Table VII.

Serum	*B. typhosus* ("CB")		*B. proteus* X 19		*B. dysenteriae* Shiga	
Pig	Sensitised by unheated and heated (55° C.) serum	Bactericidal effect by heated serum *plus* absorbed serum	Sensitised by unheated and heated (55° C.) serum	Bactericidal effect by heated serum *plus* absorbed serum	Sensitised by unheated and heated (55° C.) serum	.
Ox	Do.	.	Do.	Do.	Do.	.
Horse	Do.	.	Not sensitised by unheated or heated serum	Bactericidal effect by heated serum *plus* absorbed serum	Do.	.
Sheep	Do.	.	Do.	Do.	Usually not sensitised by unheated or heated serum; sometimes partially	Usually no bactericidal effect by heated serum *plus* absorbed serum; sometimes a partial effect
Rabbit	Do.	Bactericidal effect by heated serum *plus* absorbed serum	Do.	.	Not sensitised by unheated or heated serum	No bactericidal effect by heated serum *plus* absorbed serum
Human	Do.	.	Not sensitised by unheated serum	.	Sensitised by unheated and heated serum	.
Guinea-pig	Do.	.	.	.	Sensitised by unheated and heated serum but effects variable	.

Serum	*V. cholerae* ("Bombay")		*V. cholerae* ("3134")	
Pig	Sensitised by unheated and heated (55° C.) serum	Bactericidal effect by heated serum *plus* absorbed serum	.	.
Ox	Sensitised by unheated serum, not by heated serum (variable)	Bactericidal effect by heated serum *plus* absorbed serum (variable)	Sensitised by unheated serum not by heated serum	Bactericidal effect by heated serum *plus* absorbed serum
Horse	Do.	Do.	Do.	Do.
Sheep	Usually not sensitised by unheated or heated serum	Usually no bactericidal effect by heated serum *plus* absorbed serum	Do.	Do.
Rabbit	Do.	Do.	.	.
Human	Do.	Do.	.	.
Guinea-pig	Do.	.	.	.

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by "sensitising" organisms with serum which had been heated at varying temperatures; it was found to be stable usually at 55° C., completely or partially stable at 60° C. and entirely inactivated at 70° C. (Table VIII).

In those cases in which sensitisation occurred, serum heated at 55° C. along with "absorbed" serum also yielded the full bactericidal effect of the native serum, though neither was active *per se* (Tables VII and IX). The lytic effect was clearly due to an antibody-like agent stable at 55° C., absorbed from the serum or inactivated by the organisms at 0° C., and capable of sensitising the organisms to the thermolabile complement which was not bound by the organisms at 0° C.

Table VIII. B. typhosus *"CB" and pig serum.*

			Bactericidal effect
Bacteria	(untreated)	+ serum (untreated)	+6
„	(untreated)	+ „ (absorbed)	0
„	(sensitised with unheated serum)	+ „ (absorbed)	+6
„	(sensitised with serum, 55° C.)	+ „ (absorbed)	+6
„	(sensitised with serum, 60° C.)	+ „ (absorbed)	+6
„	(sensitised with serum, 70° C.)	+ „ (absorbed)	0

(To obviate coagulation, serum was diluted 1 in 4 with saline before heating.)

Table IX. B. typhosus *"CB" and ox serum.*

				Bactericidal effect
Bacteria	(untreated)	+ serum	(untreated)	+4
„	(untreated)	+ „	(absorbed)	0
„	(untreated)	+ „	(heated 55° C.)	0
„	(untreated)	+ „	(heated 55° C.) + serum (absorbed)	+4
„	(untreated)	+ „	(heated 60° C.)	0
„	(untreated)	+ „	(heated 60° C.) + serum (absorbed)	+2
„	(untreated)	+ „	(heated 65° C.)	0
„	(untreated)	+ „	(heated 65° C.) + serum (absorbed)	0

Further tests of the thermostability of the antibody were made in which serum heated at varying temperatures above 55° C. was used as antibody and "absorbed" serum as complement. It was noted that inactivation occurred between 60° C. and 65° C. (Table IX). The antibody corresponded, therefore, in thermolability to the natural haemolytic antibodies and natural agglutinins, though more labile than "immune" antibodies (see Mackie and Finkelstein, 1930).

With *B. typhosus* and various animal sera, no difficulty was experienced in demonstrating a bactericidal antibody, but the results with other organisms varied (see Table VII). *B. proteus* gave results similar to those of *B. typhosus* in the case of ox and pig serum, but with sheep, rabbit, horse and human serum an antibody could not be demonstrated by "sensitising" the organism as in the other experiments, though serum heated at 55° C. along with absorbed serum produced a bactericidal effect equal to that of the native serum, each being individually inactive (Table X).

In certain cases an antibody could not be demonstrated either by "sensitisation" or by testing heated serum along with "absorbed" serum, and such

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Table X. B. proteus *X* 19 *and sheep serum.*

Bacteria		Serum	Bactericidal effect
Bacteria (untreated)	+ serum	(untreated)	+6
„ (untreated)	+ „	(absorbed)	0
„ (untreated)	+ „	(heated 55° C.)	0
„ (untreated)	+ „	(heated 55° C.) + serum (absorbed)	+6
„ (sensitised with unheated serum)	+ „	(untreated)	+6
„ (sensitised with unheated serum)	+ „	(absorbed)	0

Table XI. V. cholerae "*Bombay*" *and sheep serum.*

Bacteria		Serum	Bactericidal effect
Bacteria (untreated)	+ serum	(untreated)	+10
„ (untreated)	+ „	(absorbed)	0
„ (untreated)	+ „	(heated 55° C.)	0
„ (untreated)	+ „	(heated 55° C.) + serum (absorbed)	0
„ (sensitised with unheated serum)	+ „	(absorbed)	·0
„ (sensitised with serum 55° C.)	+ „	(absorbed)	0

results might have been taken to indicate that the bactericidal effect was due directly to complement and that "absorption" inactivated it (Table XI). In other cases partial sensitisation effects were noted and also bactericidal effects by heated serum *plus* absorbed serum falling short of the full action of the native serum. In some cases also sensitisation was produced by unheated serum but failed with serum heated at 55° C., while the full effect of the original serum resulted from the combined action of heated *plus* absorbed serum—an apparently contradictory result (Table XII).

With certain organisms and serum from particular animal species the results varied with individual samples of serum.

The general results (Table VII) seemed to indicate, however, that these bactericidal effects were due to antibody-like agents (stable at 55° C.) acting along with serum-complement. It was apparent that, in many cases, the failure to demonstrate an antibody was due to its inability to sensitise the organisms under the conditions of the experiment. Various modifications in the technique of sensitisation were tried, *e.g.* varying the time of treatment at 0° C. from 2 to 18 hours, varying the temperature of treatment from 0° C. to room temperature, varying the amount of serum used. None of these modifications overcame the failure of certain sera to sensitise particular organisms, *e.g.* sheep serum and *V. cholerae* ("Bombay").

In tests with pig serum and *B. typhosus* it was noted that the antibody, like other natural antibodies (see Mackie and Finkelstein, 1928, 1930), was resident mainly in the carbonic acid-insoluble fraction of the serum (Table XIII). Sensitisation was attempted with this serum-fraction where difficulty occurred in sensitising with whole serum, but without further success.

An attempt was also made to demonstrate in certain cases an antibody by filtering the serum through a Berkefeld filter with a view to separating it from complement in this way. It was shown originally by Muir and Browning (1909) that an immune body passes such filter while complement is non-

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Table XII. V. cholerae "*Bombay*" *and ox serum.*

Bacteria	Serum	Bactericidal effect
Bacteria (untreated)	+ serum (untreated)	+10
„ (untreated)	+ „ (absorbed)	0
„ (untreated)	+ „ (heated 55° C.)	0
„ (untreated)	+ „ (heated 55° C.) + serum (absorbed)	+10
„ (sensitised with unheated serum)	+ „ (absorbed)	+10
„ (sensitised with serum 55° C.)	+ „ (absorbed)	0

Table XIII. B. typhosus "*CB*" *and pig serum.*

	Bactericidal effect
Bacteria + serum (untreated)	+6
„ + „ (heated 55° C.)	0
„ + „ (absorbed)	0
„ + „ (heated 55° C.) + serum (absorbed)	+6
„ + CO_2-insol. fraction (heated 55° C.)	0
„ + „ (heated 55° C.) + serum (absorbed)	+8
„ + CO_2-sol. fraction (heated 55° C.)	0
„ + „ (heated 55° C.) + serum (absorbed)	+2

filterable. This method has been used by Yoshinare (1921, 1922) for demonstrating the natural haemolytic antibody of ox serum for guinea-pig erythrocytes. Filtered sheep serum was tested on *V. cholerae* along with "absorbed" serum, but proved inactive.

It will be noted from Table VII that this interference with sensitisation varied according to the organism and the animal species from which the serum was obtained. Pig serum gave definite sensitisation effects with all the organisms tested. While a sensitising effect towards a particular organism (*e.g. V. cholerae* "Bombay") could not be elicited with the serum of one species (*e.g.* sheep), another serum proved effective (*e.g.* ox).

Experiments were carried out in which organisms "sensitised" by ox serum were tested with absorbed sheep serum. The results showed that the negative effects with sheep serum were not due to absorption of complement in addition to the antibody (*v. supra*). Thus absorbed sheep serum produced a bactericidal effect on organisms sensitised with ox serum though inactive towards the same organism "sensitised" with sheep serum (Table XIV). The failure to sensitise depended on some factor affecting the antibody which varied with the organism and the serum tested.

In a later section of this paper it is shown how cultures of various organisms (especially after heating at high temperatures) contain an extracellular agent which interferes in a non-specific manner with bactericidal antibodies. The non-specific neutralising action of killed organisms on the bactericidal power of human serum as described by Georgevitch (1926) is probably of the same nature. This inhibitory substance can be removed from cultures by repeated washing with saline solution, and in this way it has been possible to show that the failure to demonstrate a lytic antibody in the experiments described above was due to such agent. This was well illustrated in further experiments with sheep serum and *B. dysenteriae* Shiga (Table XV) and *B. proteus.* When

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Table XIV. V. cholerae "*Bombay*" *with ox and sheep serum.*

Bacteria		Serum		Bactericidal effect
Bacteria	(untreated)	+ ox serum	(untreated)	+6
,,	(untreated)	+ sheep serum	(untreated)	+4
,,	(untreated)	+ ox serum	(absorbed)	0
,,	(untreated)	+ sheep serum	(absorbed)	0
,,	(sensitised with ox serum)	+ ox serum	(absorbed)	+8
,,	(sensitised with ox serum)	+ sheep serum	(absorbed)	+8
,,	(sensitised with sheep serum)	+ ox serum	(absorbed)	0
,,	(sensitised with sheep serum)	+ sheep serum	(absorbed)	0

Table XV. B. dysenteriae *Shiga and sheep serum.*

Bacteria		Serum		Bactericidal effect
Bacteria	(untreated)	+ serum	(untreated)	+8
,,	(untreated)	+ ,,	(absorbed)	0
,,	(sensitised in usual way)	+ ,,	(untreated)	+8
,,	(sensitised in usual way)	+ ,,	(absorbed)	0
,,	(washed with saline and then sensitised)	+ ,,	(untreated)	+8
,,	(washed with saline and then sensitised)	+ ,,	(absorbed)	+6

the culture used for sensitisation was washed with saline beforehand, sensitisation could be effected whereas the unwashed organisms were not sensitised.

In the case of *V. cholerae* ("Bombay") and sheep serum, a somewhat different result was obtained: washing of the culture used for sensitisation did not alter the previous result, but when the culture used for absorption was thoroughly washed, the "absorbed" serum produced a bactericidal effect on the "sensitised" organisms whereas serum absorbed with unwashed culture was inactive (Table XVI). Analogous results were obtained when serum heated at 55° C. was used as antibody and tested along with "absorbed" serum.

Table XVI. V. cholerae "*Bombay*" *and sheep serum.*

Bacteria		Serum		Bactericidal effect
Bacteria	(untreated)	+ serum	(untreated)	+10
,,	(untreated)	+ ,,	(absorbed in usual way)	0
,,	(untreated)	+ ,,	(absorbed with growth previously washed in saline)	0
,,	(sensitised in usual way)	+ ,,	(untreated)	+8
,,	(sensitised in usual way)	+ ,,	(absorbed in usual way)	0
,,	(sensitised in usual way)	+ ,,	(absorbed with washed growth)	+6
,,	(washed with saline and then sensitised)	+ ,,	(untreated)	+10
,,	(washed with saline and then sensitised)	+ ,,	(absorbed in usual way)	0
,,	(washed with saline and then sensitised)	+ ,,	(absorbed with washed growth)	+8

In the case of *B. dysenteriae* Shiga and *B. proteus* the inhibitory agent was derived from the living culture used for sensitisation; in the experiment with *V. cholerae* it was derived from the organisms used for absorption, probably diffusing into the serum and later in the actual test interfering with the action of the antibody. It has been shown that it has no action on the complement of the absorbed serum. The negative results with heated serum *plus* absorbed serum (*e.g.* sheep serum and *V. cholerae*) could also be explained by the presence of the inhibitory agent in the absorbed serum.

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It is of further interest that the inhibitory agent referred to may inactivate a particular bactericidin in the serum of one animal species (sheep) though not in another (pig). This is also illustrated in the results given in Table XIV. While unable in some cases to inactivate the bactericidin in unheated serum, it may affect the heated serum (*e.g.* ox serum and *V. cholerae* "Bombay"—Table XII).

It is difficult to explain these various differences. It will be shown later how the bactericidins for certain organisms (*e.g. B. proteus*) are more susceptible to this neutralising agent than others (*e.g. V. cholerae*). In the former case there may be a greater combining affinity between the antibody and this substance so that the latter is completely fixed in the "absorbed" serum and none is left uncombined to interfere with bactericidal action in the ultimate test. In the case of the *V. cholerae* bactericidin, there may be a lesser combining affinity so that no neutralisation occurs in the "sensitisation" process (the amount of the inhibitory substance being small in relation to the quantity of serum) whereas a large amount is left free in the absorbed serum to interfere with bactericidal action in the actual test. The different susceptibility of a particular bactericidin according to the animal species from which it is derived and according to the physical state of the serum would suggest that some other factor in serum, possibly thermolabile and varying according to species, is concerned in the neutralisation phenomenon.

It may be noted also that two strains of the same species are not identical in regard to their production of this substance: *e.g. V. cholerae* "Bombay" and "3134." The latter was readily sensitised with unheated sheep serum, and heated serum *plus* absorbed serum yielded the bactericidal action of the native serum (Table VII).

By removing from cultures used for sensitisation and absorption this extracellular inhibitory agent, it has been possible to demonstrate sensitising bactericidal antibodies where otherwise their presence could not be proved.

Interchangeability of the complements of different animals.

It has been shown above (Table XIV) how the absorbed serum of the sheep acts as complement along with the natural bactericidal antibody of the ox for *V. cholerae.* A number of experiments were carried out to ascertain whether the complements of different species were interchangeable in these bactericidal reactions. In such tests the "absorbed" serum from one species was found capable of acting as complement with the natural antibody derived from another, irrespective of the species and the type of organism (Table XVII). This applied even when the untreated serum, which was used as a complement, proved inactive by itself towards the particular organism (Table XVIII). Such results suggest that lack of bactericidal action is due to deficiency in antibody rather than in complement.

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Table XVII. V. cholerae "*Bombay*" *with ox and rabbit serum.*

		Bactericidal effect
Bacteria (untreated)	+ ox serum	+10
,, (untreated)	+ rabbit serum	+10
,, (untreated)	+ ox serum (absorbed)	0
,, (untreated)	+ rabbit serum (absorbed)	0
,, (sensitised with ox serum)	+ ox serum	+10
,, (sensitised with ox serum)	+ rabbit serum	+10
,, (sensitised with ox serum)	+ ox serum (absorbed)	+ 8
,, (sensitised with ox serum)	+ rabbit serum (absorbed)	+ 8

Table XVIII. B. typhosus "*CB*" *with pig and guinea-pig serum.*

	Bactericidal effect
Bacteria + pig serum	+4
,, + guinea-pig serum	0
,, + pig serum (heated 55° C.)	0
,, + guinea-pig serum (absorbed)	0
,, + pig serum (55° C.) + guinea-pig serum (absorbed)	+4

Absorption of serum by charcoal.

It has been shown (Dunlop, 1928; Mackie and Finkelstein, 1930) how treatment of serum by charcoal under certain conditions removes natural complement-fixing antibodies without affecting the haemolytic complement. A series of experiments was carried out, in which the bactericidal properties of serum after such treatment with charcoal were tested with unsensitised and sensitised bacteria. In this way it was found that absorption with charcoal, according to the method described by Dunlop, generally inactivated the serum (tested with unsensitised bacteria), but the absorbed serum was also inactive with sensitised organisms. "Bactericidal" complement, unlike "haemolytic" complement was, therefore, absorbed by charcoal. This effect was not studied further, as charcoal absorption did not provide a means of removing selectively the bactericidal antibody as in the case of the complement-fixing substance, but the result quoted would indicate that different complements or complement-moieties are concerned in haemolysis and bacteriolysis respectively (see Muir and Browning, 1908).

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

It has been shown how the serum of young animals (*e.g.* rabbits 3 or 4 weeks from the time of birth) fail to exhibit certain natural immunity reactions which are characteristic of adult animals. A litter of five young rabbits, 14 days old, was tested for the bactericidal power of their serum towards *B. typhosus* and *V. cholerae.* The serum of each proved practically equal in action to that of the mother (+ 6). The bactericidal property was, therefore, well developed at this stage of life, unlike natural agglutination, though similar, in this respect, to the natural property of fixing complement along with bacterial antigens (see Mackie and Finkelstein, 1928, 1930; and Gibson, 1930).

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Observations on the specificity of natural bactericidal antibodies.

With the object of determining whether these natural bacteriolytic antibodies are specific substances, a series of absorption tests was carried out. Normal serum was treated with heat-killed cultures for 2 hours at 0° C. (as in the experiments described in a previous section), separated by centrifuging and then tested for its bactericidal effect on the organism used for absorption and on representative strains of other species. Preliminary tests were made to ascertain the amount of culture required to annul completely the bactericidal effect towards the homologous organism.

In the first tests carried out, results seemed to indicate that absorption was non-specific: treatment of serum with a particular bacterium annulled its bactericidal power for some entirely unrelated species, although its effect on other unrelated organisms remained unaltered. After a series of such tests, it became apparent that these non-specific effects pertained more to the bactericidins for some organisms than others. For example, irrespective of the organism used for absorption and the serum absorbed, the bactericidin for *B. proteus* X 19 was invariably affected non-specifically whereas the bactericidal effect on *B. typhosus* "CB" and *B. dysenteriae* Y was only annulled by treatment of serum with the homologous organism. As regards the bactericidins for other organisms, results varied. It was also noted that such non-specific effects were often more marked quantitatively (when varying doses of the absorbing organism were used) than the absorption of the homologous bactericidin. When the organisms used for absorption had been heated to 120° C., treatment of the serum resulted in the complete abolition of its bactericidal action for all species tested. Organisms which had been heated and then washed several times with a considerable volume of salt-solution did not exert this effect. Thus, the substance responsible for such non-specific action was found to be present in the fluid of the heated bacterial suspension, and apparently heating at high temperatures liberated it in large amount from the organisms. It seemed likely that this agent corresponded to that which interfered with sensitisation (*v. supra*), and subsequent absorption tests were carried out with heat-killed cultures which had been washed three times with salt solution. The amount of growth required to produce complete absorption of the particular lysin was considerable. The method used was as follows:

A 24 hours' growth on a 6 in. plate of nutrient agar was emulsified in a small volume of saline solution, and heated at 65° C. for 1 hour; it was then washed three times with a large volume of saline; after the final washing the growth was emulsified in the serum to be treated; in general the growth from one plate was required to absorb completely the bactericidin in 2 to 3 c.c. of serum and, in some cases, even this did not effect complete absorption (see Tables XXII, XXV, XXVI).

Absorption tests carried out in this way revealed the highly specific nature of these bactericidal agents. Tables XIX–XXIX give a number of illustrative

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examples of the results of such tests. It will be noted that, occasionally, the bactericidal effect for certain organisms, *e.g. B. proteus* X 19, was completely or partially affected in a non-specific manner. It has been shown how the bactericidins for this and certain other organisms are susceptible to a non-specific effect by unwashed cultures, and it is not surprising that in using a large amount of culture for absorption, washing failed at times to eliminate this influence. In Tables XIX–XXII are shown results of tests in which a

Table XIX. *Horse serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. paratyphosus* B	*B. aertrycke*	*B. dysenteriae* Shiga	*B. dysenteriae* Y	*V. cholerae* "Bombay"
Untreated	+4	+6	+6	+8	+8	+6
Absorbed by *B. dysenteriae* Shiga	+4	+6	+6	0	+8	+6

Table XX. *Ox serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. dysenteriae* Shiga	*B. faecalis alkaligenes*	*Pneumobacillus*	*B. proteus* X 19	*V. cholerae* "Bombay"
Untreated	+8	+4	+8	+4	+8	+10
Absorbed by *B. proteus* X 19	+8	+4	+8	+4	0	+10

Table XXI. *Ox serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. paratyphosus* A	*B. dysenteriae* Y	*V. cholerae* "Bombay"	*V. cholerae* "3134"
Untreated	+2	+6	+10	+10	+6
Absorbed by *V. cholerae* "3134"	+2	+4	+10	+10	0

Table XXII. *Sheep serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. paratyphosus* A	*B. paratyphosus* B	*B. aertrycke*	*B. dysenteriae* Shiga	*B. dysenteriae* Y	*B. proteus* X 19	*V. cholerae* "Bombay"
ıtreated	+ 8	+10	+4	+6	+8	+8	+4	+10
›sorbed by *ʾ. dysenteriae* Shiga	+10	+10	+6	+6	+4	+8	+4	+10

serum was absorbed with one type of organism and then tested with a number of others. The organisms chosen for testing were those which were actively killed by the particular serum. In Table XIX it is noted how absorption of horse serum with *B. dysenteriae* Shiga removed the bactericidin for this organism, but had no influence on the bactericidal action of the serum towards *B. typhosus*, *B. paratyphosus* B, *B. aertrycke*, *B. dysenteriae* Y and *V. cholerae*. In the experiment shown in Table XX absorption of ox serum with *B. proteus* X 19 completely annulled the bactericidal effect towards this organism but did not reduce the value of the serum for *B. typhosus*, *B. dysenteriae* Shiga, etc. Table XXI shows analogous results after absorption of ox serum with *V. cholerae* (strain "3134"), and it is of special interest how absorption of the serum while annulling its effect on this strain, had no influence on the bactericidal action towards another strain ("Bombay") of the same species. These two strains of *V. cholerae* were similar in general characters and were both

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Table XXIII. *Ox serum.*

Bactericidal effects.

Serum	*B. dysenteriae* Shiga	*V. cholerae* "Bombay"
Untreated	+8	+10
Absorbed by *B. dysenteriae* Shiga	+0	+10
Absorbed by *V. cholerae* "Bombay"	+8	0

Table XXIV. *Pig serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. dysenteriae* Shiga
Untreated	+8	+4
Absorbed by *B. typhosus* "CB"	0	+4
Absorbed by *B. dysenteriae* Shiga	+8	0

Table XXV. *Pig serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. paratyphosus* A	*B. paratyphosus* B	*B. dysenteriae* Shiga	*B. dysenteriae* Y	*B. proteus* X19	*V. cholerae* "Bombay"
Untreated	+6	+10	+4	+10	+10	+4	+12
Absorbed by *B. paratyphosus* B	+8	+ 8	0	+10	+10	+6	+10
Absorbed by *B. dysenteriae* Shiga	+8	+ 8	+4	+ 2	+10	+6	+10

Table XXVI. *Sheep serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. paratyphosus* A	*B. paratyphosus* B	*B. aertrycke*	*B. dysenteriae* Shiga	*B. dysenteriae* Y	*B. proteus* X19	*V. cholerae* "Bombay"
Untreated	+8	+6	+4	+6	+10	+ 8	+2	+10
Absorbed by *B. typhosus* "CB"	+2	+6	+4	+6	+10	+10	0	+10
Absorbed by *V. cholerae* "Bombay"	+8	+6	+6	+6	+10	+10	+2	0

Table XXVII. *Sheep serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. paratyphosus* A	*B. paratyphosus* B	*B. aertrycke*	*B. dysenteriae* Shiga	*B. dysenteriae* Y	*V. cholerae* "Bombay"
Untreated	+4	+8	+2	+8	+10	+10	+6
Absorbed by *B. paratyphosus* A	0	0	+2	+8	+10	+10	+8
Absorbed by *B. dysenteriae* Y	+2	+8	+2	+8	+10	0	+8

Table XXVIII. *Sheep serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. paratyphosus* A	*B. paratyphosus* B	*B. aertrycke*	*B. proteus* X19	*V. cholerae* "Bombay"	*V. cholerae* "3134"
Untreated	+6	+8	+6	+10	+4	+8	+12
Absorbed by *B. paratyphosus* A	0	0	+6	+ 8	+4	+8	+12
Absorbed by *V. cholerae* "Bombay"	+6	+8	+6	+ 8	+2	0	+10

Table XXIX. *Ox serum.*

Bactericidal effects.

Serum	*B. typhosus* "CB"	*B. typhosus* "Cole"	*B. paratyphosus* A	*B. paratyphosus* B	*B. dysenteriae* Shiga	*B. dysenteriae* Y	*B. aertrycke*	*V. cholerae* "3134"
Untreated	+4	+6	+4	+4	+6	+10	+8	+10
Absorbed by *B. typhosus* "CB"	0	+6	+4	+4	+6	+10	+8	+ 8
Absorbed by *B. typhosus* "Cole"	0	0	0	+4	+6	+10	+8	+ 8

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agglutinated by an anti-cholera serum. In Table XXVIII it is shown how absorption of a serum with *V. cholerae* ("Bombay") had no effect on the bactericidal action towards *V. cholerae* ("3134")—the converse result to that of Table XXI. When these results were obtained, agglutinin-absorption tests were carried out with an immune serum for a known strain of *V. cholerae.* Such tests elicited a difference in antigenic structure between the strains, though they apparently contained constituents in common. The bactericidin-absorption tests recorded above demonstrated even more sharply the difference between these strains than the agglutinin-absorption reaction with an immune serum.

Table XXII illustrates results similar to those in the previous tables, but in this case absorption of the homologous bactericidin was only partial; no quantitative reduction of the bactericidal values from other organisms was noticeable.

Tables XXIII and XXIV illustrate the results of absorption of serum with two organisms, the absorbed serum being tested with each. The effects were completely specific.

Tables XXV–XXIX afford further illustrations of the results of absorption tests. Occasional non-specific effects were noted, possibly due to incomplete removal (by washing) of the inhibitory agent referred to above, but apart from such results absorption was highly specific. The bactericidal antibodies for even closely related organisms (with the exception of *B. typhosus* and *B. paratyphosus* A—Tables XXVII, XXVIII and XXIX), seemed to be sharply differentiated. Absorption of a serum with *B. typhosus* ("CB") did not abolish its bactericidal effect on another typhoid strain ("Cole") though absorption with the latter annulled its action on both strains and also *B. paratyphosus* A (Table XXIX). Agglutinin-absorption tests with an anti-typhoid serum elicited no difference in the reactions of these two typhoid strains.

Such results along with those obtained with the two strains of *V. cholerae* indicate further the high grade of specificity of such natural bactericidal antibodies—their specificity being even more restricted than that of immune agglutinins.

Discussion.

This study of the mechanism involved in the bactericidal action of normal serum contributes further information regarding the occurrence and characteristics of natural antibodies.

The data elicited are of particular interest in correlation with those obtained in previous studies of natural complement-fixing and agglutinating antibodies for bacteria (see Mackie and Finkelstein, 1930; Gibson, 1930). These studies showed how the normal serum of various mammalian animals contain specific antibodies for a wide variety of bacterial antigens, different animal species varying as regards the occurrence and reactivity of these agents, and a remarkable multiplicity of such natural antibodies was demonstrated.

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The present enquiry was instituted primarily to ascertain whether bactericidal action by normal serum is dependent on antibody-like principles, and whether these are specific. Bacteria vary profoundly in their apparent susceptibility to this effect, and our analytical observations have been limited, in the first place, to the examination of the most highly reactive types, *e.g.* typhoid-paratyphoid bacilli, *V. cholerae*, etc. As noted by earlier workers, the bactericidal action of serum for such organisms is annulled by heating at 55° C., and, as long recognised, serum-complement plays an essential part in the process. Our observations leave no doubt as to the inactivity of complement *per se*, and an antibody-like principle invariably acts as an intermediary agent. In short, the natural bactericidal reactions studied are definitely analogous in their mechanism to that of an immune serum. As proof of the antibody nature of these intermediary agents, we have taken their capacity to "sensitise" bacteria to the bactericidal action of serum-complement and their "absorption" by bacteria from serum of 0° C. Their degree of thermostability is interesting in comparison with that of other natural antibodies: they are stable at 55° C. and are only inactivated at 60°–65° C.; in this respect they correspond closely to natural haemolysins and agglutinins (see Mackie and Finkelstein, 1928, 1930; Gibson, 1930). They contrast, however, with immune antibodies which are stable at higher temperatures. They differ also from natural complement-fixing antibodies, which are labile at 55° C. or even lower temperatures (40°–50° C.). It is of particular interest that natural bactericidal and complement-fixing antibodies should differ; the bactericidal reaction involves combination of complement with the antigen *plus* antibody, and it might seem reasonable to assume that complement-fixation is due to the same antibody. The difference in thermostability would tend to contra-indicate this.

Our results in attempted sensitisation of certain bacteria (*e.g. V. cholerae*) with this type of antibody in the serum of particular animals (*e.g.* sheep) are specially noteworthy. The failure to sensitise might have led to the conclusion that, in such cases, complement acted without any intermediary agent. The general results obtained by testing a variety of organisms with sera from different animals indicated by analogy, if not by direct proof, that in all cases an antibody-like substance was part of the bactericidal mechanism. The demonstration of a neutralising or inhibitory substance in bacterial cultures affecting the antibody explained such failures to sensitise. This neutralisation varied according to the particular organism, and the animal species from which the serum was obtained. It constitutes a phenomenon of some interest and importance, and requires further investigation. The neutralising agent is liberated in large amount from cultures heated at high temperatures, it is markedly thermostable (at 120° C.) and is not apparently related to the specific antigen of the organism in view of its non-specific action. It was shown by Georgevitch (1926) that killed organisms incubated with serum neutralised non-specifically the bactericidal action of the serum, and it seems

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possible that the inhibitory effect we have noted corresponds to that described by this author. It is noteworthy that the neutralising substance acts at 0° C., but we have found it is more active at 37° C. This agent, irrespective of the organism from which it is produced or the serum used for the bactericidal test, affects strongly the bactericidal antibodies for certain organisms, and is less active towards others. It can be removed by repeated washing of cultures with large volumes of saline solution, and this has rendered it possible to demonstrate sensitising antibodies when otherwise these would have been effectively obscured in analytical tests. The peculiar differences in the results of sensitisation experiments with washed and unwashed cultures of different organisms suggest that the neutralising substance has a varying combining affinity for different bactericidal antibodies. It may also inactivate heated serum, though it exerts no effect on the fresh serum; it may inactivate the serum of one species but not another. These facts suggest the possibility of some property in serum which is protective against this agent. Two strains of the same bacterial species may differ in their production of the substance. Such results all serve to illustrate the great complexity of the serum-reaction studied and the many factors involved.

The sensitising antibody of the bactericidal reaction was found to be resident mainly in the carbonic-acid-insoluble fraction of serum, and in this respect resembles other natural antibodies.

It was found to be present in the serum of young animals at a stage when other natural antibodies (*e.g.* haemolysins, agglutinins) are absent, though it corresponds in this respect to the natural antibody responsible for complement-fixation along with bacterial antigens (see Mackie and Finkelstein, 1928; 1930; Gibson, 1930).

The complements of different animal species were found to be interchangeable in these bactericidal reactions and certain observations recorded have led us to assume that differences in the bactericidal properties of sera towards particular organisms depend on variation in the antibody rather than the complement.

It is also of interest that, unlike haemolytic complement, the complement concerned in bactericidal action is absorbed by charcoal (see Dunlop, 1928; Mackie and Finkelstein, 1930). It was originally suggested by Muir and Browning (1908) that different moieties of complement are concerned in haemolysis and bacteriolysis respectively.

The absorption tests clearly prove the specificity of these natural antibodies and the results of such tests support the analogous observations in the case of natural complement-fixing and agglutinating antibodies (referred to above). It is noteworthy that in our initial absorption experiments the bactericidal antibody appeared to be a non-specific principle and the existence of a non-specific natural antibody-like principle has in fact been suggested by Browning (1927). This apparent non-specific absorption was found to be due to the neutralising agent previously encountered in sensitisation experi-

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ments. When carefully washed growths were used for absorption, highly specific effects were obtained. It would appear that the specificity of these antibodies may be even more restricted than that of an immune antibody (agglutinin), as evidenced by tests with different strains of *B. typhosus* and *V. cholerae*. It has been suggested by Felix and Olitzki (1929) that the bactericidal action of an immune serum is closely related to the O-agglutinin. The differences noted between the specificity of natural bactericidal antibodies as compared with immune agglutinins may be dependent on the different types of antigenic constituents (and their corresponding antibodies) concerned in the two reactions respectively. This requires further investigation, but the results cited provide a specially marked illustration of the high degree of specificity of natural bactericidal antibodies.

Correlating the observations recorded in this paper with previous observations on natural antibacterial antibodies, we would suggest that the various types of immune antibacterial antibodies have their precursors specifically differentiated in the serum of normal animals, certain types appearing at a very early stage of life, others later, and that specific immunisation enhances the output of the particular antisubstance and at the same time leads to its increased stability. In general, immune antibodies cannot be regarded, therefore, as substances formed *de novo* in response to a special stimulus. It would be of great interest to ascertain how far the response to immunisation with a particular antigen depends on the pre-existing power of the tissues to form antibodies for this antigenic substance. The work we have recorded opens up, therefore, many questions of great theoretical and also practical importance in immunity, and the multiplicity of natural antibodies which can be inferred from our work is of the greatest interest from a biological as well as the immunological standpoint.

Summary and Conclusions.

1. An analytical study has been made of the mechanism of natural bactericidal action by the serum of various animals (ox, sheep, horse, rabbit, guinea-pig, rat, man) towards certain organisms (*B. typhosus*, *B. dysenteriae* Shiga, *B. proteus*, *V. cholerae*) exhibiting the maximum reactivity to this effect.

2. Serum-complement has no bactericidal action *per se*, and an antibody-like agent invariably acts as an intermediary agent, "sensitising" the particular organism to the action of the complement and capable of being "absorbed" by it from serum at 0° C.

3. This sensitising agent is stable at 55° C. but labile at 60°–65° C. In this respect it resembles natural haemolysins and agglutinins, but contrasts with the more stable immune antibodies and the more labile natural complement-fixing antibodies (for bacterial antigens). It is resident mainly in the carbonic-acid-insoluble fraction of the serum. It is present in the serum of young animals before certain other natural antibodies have developed.

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4. Absorption tests demonstrate the high degree of specificity of these natural bactericidal antibodies for particular bacteria.

5. A non-specific extracellular substance occurs in bacterial cultures which may neutralise or inhibit these antibodies, and interfere with their sensitising action even at 0° C.

6. This substance is liberated in large amount in cultures heated at high temperatures (120° C). It can be removed by repeated washing of growths in saline solution. It may inactivate a bactericidal antibody in heated serum, though not in fresh unheated serum, and may inactivate a particular antibody in the serum of one animal species but not in another. Strains of bacteria vary in their production of this substance.

7. The observations submitted in this paper, correlated with previous studies of natural antibodies by the authors and others, indicate that immune antibodies have their precursors specifically differentiated in the serum of normal animals and that, in general, immune antibodies are not substances formed *de novo*.

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