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THE BACTERICIDINS OF NORMAL SERUM: THEIR CHARACTERS, OCCURRENCE IN VARIOUS ANIMALS AND THE SUSCEPTIBILITY OF DIFFERENT BACTERIA TO THEIR ACTION.

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CONTENTS.

INTRODUCTION.

SINCE the early work of Nuttall (1888), Buchner (1889), and others first disclosed the bactericidal property of normal serum, a considerable amount of attention has been devoted to this important biological phenomenon. The literature (which has been reviewed recently by Knorr, 1929) lacks, however, a comprehensive and co-ordinated study of the subject, and in fact presents a good many confusing data in regard to the nature of the active principles concerned and their immunological significance.

It has long been recognised that the bactericidal action of normal serum towards certain bacteria (*e.g. B. typhosus*, *V. cholerae*, etc.) depends on a

mechanism in which a thermolabile complement-like principle (originally designated "alexin") plays an essential part. Recently we have made an analytical study of this mechanism and have cited the earlier work (Mackie and Finkelstein, 1931). In certain cases, however, serum-bacteriolysis may be independent of complement, and due to a more stable principle.

In the earlier studies of the subject, special attention was paid to the killing of *B. anthracis* by normal serum. Gruber and Futaki (1907) associated this property with a product of blood platelets ("plakanthracocidin"). Bail (1903) clearly established the thermostability of the substance responsible for the killing of the anthrax bacillus and stated that its action was only annulled on heating the serum at 63° C. for half an hour, as contrasted with the thermolability of complement. Pettersson (1902) drew attention to the essential difference between the lysis of *B. anthracis* and of such organisms as *B. typhosus* and *V. cholerae* which are susceptible to the labile "alexin." Pirenne (1904) showed how the thermostable bactericidin of rat's serum acts on *B. anthracis* and certain other biologically allied organisms (*e.g. B. subtilis*) while inactive towards *B. coli*, *V. cholerae* and similar types. According to Gonzenbach and Uemura (1916) the absorption of a serum by *B. typhosus* does not affect its killing power towards the anthrax bacillus. Such observations demonstrated the existence of two different bactericidal mechanisms in serum, one inactivated at 55° C., the other stable at this temperature, and for convenience the respective bactericidins may be designated differentially as "thermolabile" and "thermostable."

The labile bactericidin consists of two separate principles, and a certain amount of evidence has been adduced that the stable bactericidin is also dual in its composition. Bail (1900) claimed that, as regards the lysis of *B. anthracis*, inactive rabbit's serum could be reactivated by adding such minute amount of active serum as would be ineffective *per se*. According to Gruber and Futaki (1907) inactive rabbit's plasma could be activated by a thrombocyte extract; Kling (1910) attributed similar activating effects to a leucocytic product. Pettersson (1926) stated that a small amount of serum (inactive *per se*) could reactivate rabbit's serum which had been heated at 70° C.; he observed the same reactivating effect with thrombocyte extracts. Pettersson thus formulated the view that the thermostable bactericidin consists of two constituents—"activating" and "activable" principles respectively. In 1927 he classified the types of organisms acted on by the thermolabile and thermostable bactericidins (named by him "α" and "β" lysins respectively).

Dresel and Keller (1922–3) observed no correlation between the activity of "anthracocidin" and platelet counts in the human serum, and concluded the reaction was not due to a platelet product.

The subject is further complicated by the reputed bactericidal action of leucocyte extracts (Pettersson, 1905; Zinsser, 1910; Manwaring, 1912; and others) and the question is still an open one, whether a leucocyte product is responsible for the bacteriolytic action of serum towards certain bacteria.

Knorr (1929), in discussing the relative activity of leucocyte extracts and serum bactericidins, shows how these extracts kill such organisms as streptococci, staphylococci, Pneumococcus, *B. anthracis* and *B. diphtheriae*, whereas the "alexin" of serum acts on *B. typhosus*, *B. coli*, *V. cholerae* and similar organisms. Schattenfroh (1897) stated that leucocyte extracts are only destroyed at 75–80° C., and this high degree of stability has been confirmed by Zinsser (1910) and by Manwaring (1912).

A bacteriolytic agent "lysozyme" has been demonstrated by Fleming (1922) in various secretions and tissues and is reported to be present in serum (see also Fleming and Allison, 1922). The relationship of this agent to the bactericidal principles of serum requires to be determined.

In the course of certain studies of natural antibodies, an inquiry was made by the writers into the part played by such principles in the bactericidal action of normal serum. A survey was made of the natural bactericidal action of the serum of various animal species towards different bacteria and it was apparent that this serum reaction is most pronounced with certain bacteria, particularly the typhoid-paratyphoid, dysentery, cholera and paracholera groups. Careful analyses of the bactericidal mechanism showed that the reaction is due to a natural "sensitising" antibody acting along with serum-complement which is inactive *per se*. A striking result of this work was the high degree of specificity exhibited by these antibodies (see Mackie and Finkelstein, 1931).

These observations led to an extended study of the bactericidal reactions of normal serum, and the object of our present communication is to record the more important results of this work and draw attention particularly to certain comparative characteristics of the "thermolabile" and "thermostable" bactericidins of animal serum.

METHODS.

The methods used were adopted in order to facilitate the carrying out of large numbers of quantitative bactericidal tests (see Mackie and Finkelstein, 1931). They have the advantage of reducing to a minimum the expenditure of culture medium required for such tests and of obviating tedious colony counts as in certain of the methods that have been in vogue. The possible fallacy of a technique in which reliance is placed on comparative colony counts is well recognised.

Method I. A certain quantity of serum was mixed with a given volume of each of a series of graded bacterial dilutions (prepared in 0·85 per cent. saline solution from a 24 hours' culture) and after incubation at 37° C. for 3 hours, the relative sterilising effect was determined by loop-transfers from each mixture to a plate of culture medium; a control test was carried out in parallel by means of loop-transfers from the bacterial dilutions, the given volume of each being made up with saline to that of the mixtures and similarly incubated. The bactericidal effect was estimated by comparison of the end-points of growth in the test and control series. In this way, with the same control, the bactericidal action of a number of specimens of serum towards a particular organism could be tested simultaneously and quantitatively compared.

This method, however, was limited to organisms which maintained their viability in

saline suspension during the period of incubation and whose viability on transfer from a saline suspension to culture medium was not influenced by the presence of serum, *e.g. B. coli, B. typhosus, V. cholerae* (see Mackie and Finkelstein, 1931).

The series of bacterial concentrations consisted of decimal or centimal dilutions commencing with a standard concentration comparable with one of Brown's opacity standards. A series of six centimal dilutions proved adequate in many cases and allowed of a wide range in the quantitative test particularly where the bactericidal effect was pronounced. The initial or standard concentration varied, the object being to arrange the series so that an end-point was obtained in the control test at the fifth dilution. The volume of bacterial dilution was 0·5 c.c. and of serum 0·15 c.c. The serum was always tested within a few hours after the withdrawal of the blood.

Table I. *Illustrative results of tests carried out by Method II.*

			Bacterial dilutions					
			$S/1$	$S/10^2$	$S/10^4$	$S/10^6$	$S/10^8$	$S/10^{10}$
B. abortus "Bang" + rabbit serum	Before incubation		C	+ + +	+ + +	+ +	f.c.	1 c.
	After	„	+ + +	2 c.	−	−	−	−
			Bactericidal effect − 8.					
Strept. haemolyticus "SS 1" + rat serum	Before	„	+ + +	+ +	+	f.c.	−	−
	After	„	+ + +	+	−	−	−	−
			Bactericidal effect − 4.					
B. anthracis + rabbit serum	Before	„	C	+ + +	+	f.c.	1 c.	−
	After	„	C	+ +	f.c.	2 c.	−	−
			Bactericidal effect − 2.					

			Bacterial dilutions					
			$S/10^2$	$S/10^3$	$S/10^4$	$S/10^5$	$S/10^6$	$S/10^7$
Pneumococcus I + human serum	Before incubation		+ + +	+ +	+	f.c.	−	−
	After	„	+ + +	+ +	f.c.	−	−	−
			Bactericidal effect − 1.					
B. diphtheriae + rabbit serum	Before	„	C	+ + +	+ + +	+ +	+	f.c.
	After	„	C	+ + +	+ + +	+ +	+	+
			Bactericidal effect − 0.					
Staph. albus + rabbit serum	Before	„	C	+ + +	+ + +	+ +	+	3 c.
	After	„	C	+ + +	+ +	+	−	−
			Bactericidal effect − 2.					

Symbols. S = initial or standard suspension; C = a confluent growth; + + + = abundant growth but showing discrete colonies; + = colonies along whole line of inoculation but well separated; + + = intermediate between + + + and +; f.c. = a few scattered colonies; 1 c., 2 c., etc. = one colony, two colonies, etc.

The system of notation of results consisted in stating the degree of bactericidal effect by a number representing the difference between the indices of the dilutions which constituted the end-points of growth in the two series respectively. The approximate amount of growth from each transfer was recorded in the protocols of experiments by the symbols illustrated in Table II. Absence of bactericidal action was indicated by coincidence of the two end-points and by approximately equal amounts of growth from the corresponding loop-transfers in the two series respectively. Illustrative results of tests carried out by this method have been given in the paper cited above.

Method II. In testing organisms which rapidly lost viability in saline suspension or whose viability on transfer from saline to culture medium was enhanced by the presence of serum (*e.g.* streptococci, Pneumococcus, *B. diphtheriae*, Pasteurella group, etc.), Method I did not elicit bactericidal effects unless they were pronounced. In such cases the following modification was adopted, and was also used in testing all Gram-positive organisms and

those Gram-negative types which gave uniformly weak or negative results by the first method.

The mixtures of serum and bacterial dilutions were prepared as before, but loop-transfers were made both *before* and *after* incubation, and the respective end-points of growth compared. This of course necessitated a double set of transfers for each specimen of serum as compared with the single set in the first method. The transfers before incubation provided the control. The amount of serum used was 0·5 c.c. and of bacterial dilution 0·1 c.c. At first a series of six centimal dilutions were employed starting with a concentration equivalent to Brown's opacity standard No. 5; later, six decimal dilutions were used, the initial concentration being equivalent to 1/100th of this concentration. This latter series proved most suitable for estimating relatively weak bactericidal effects. The period of incubation was usually 4 hours, but in many cases loop-transfers were also made after 20–24 hours.

THE OCCURRENCE OF NATURAL BACTERICIDAL PROPERTIES IN ANIMAL SERUM.

With a view to ascertaining the comparative distribution of bactericidal properties among different animals and the reactions of various types of bacteria to these properties, specimens of ox, sheep, horse, pig, rabbit, white rat, guinea-pig, pigeon and human serum were tested with a series of organisms representative of various bacterial groups. The organisms used are indicated in Tables II–IV. Most of the Gram-negative types (Table II) were tested by the first method described above and positive reactions were readily elicited by this technique. Some, however, did not give satisfactory results by this method and were tested by Method II (Table III). Method I was inapplicable to most of the Gram-positive organisms and the alternative technique was employed in all cases (Table IV). The applicability of these methods has been discussed above. The number of specimens of serum examined from each species varied: when the results with one or two samples from each of the different species fell into line and when the results with biologically allied organisms corresponded closely, the number of samples tested was limited. When differences emerged in these respects multiple samples were examined so as to ascertain the range of variation and whether the average for each animal species and each organism was a bactericidal effect ("B" in the Tables). At the outset it was noted that individual specimens varied qualitatively and quantitatively, particularly for certain organisms. The variation was in some cases considerable; in others there was more uniformity. The results stated are of course relative, *i.e.* a negative result does not necessarily indicate complete absence of the bactericidal property, but only absence of any effect by the quantity of serum tested. It can hardly be claimed that the averages stated are statistically correct: to ensure such accuracy would possibly have entailed a greater number of tests than have actually been carried out. In several instances as many as twenty separate specimens were examined; in most cases, however, the number was limited (*e.g.* three to six). It was hoped, without carrying out such exceedingly large numbers of tests as to ensure statistical accuracy, to obtain a fairly representative survey of the occurrence of natural bactericidal properties for different bacteria.

With each organism, specimens of serum from one or more of the animal species were tested after heating for half an hour at 55° C. (the temperature being carefully controlled by a standard thermometer). This was to determine whether the respective bactericidins were labile or stable at this temperature. The results of this observation at once differentiated the Gram-negative from the Gram-positive organisms: when the former were acted on, the bactericidal property of the serum was completely annulled at 55° C.; when the latter were killed by serum, the bactericidal effect proved completely stable at this temperature. In the tables the various organisms are separated according to the lability of their respective bactericidins. Tables II and III include those acted on by the labile bactericidin: Table IV includes those affected by the stable bactericidin, and it will be seen that the differentiation corresponds exactly with the Gram reaction. A certain number of organisms gave uniformly negative results, but they are shown in the tables along with the other members of the biological groups to which they belong. It was noted also that bactericidal effects were, on the whole, more frequent and more pronounced towards Gram-negative than Gram-positive bacteria (see Tables II–IV).

Table II. *Bactericidal action of serum of various animal species—determined by Method I.*

	Sheep	Ox	Human	Rat	Pig	Horse	Rabbit	Guinea-pig	Pigeon
Meningococcus	2–10	4–6	*2*	4	*8*	6–10	*2*	*4*	10
M. catarrhalis	*8*	*0*	2–6	0–2	*2*	*2*	0	*2*	0–6
Gonococcus	*2*	—	—	2	—	*2*	—	—	2
B. proteus X19	0–8B	0–8B	0–2	4–6	2–8	0–6B	0–4B	0	0
B. coli "X"	0–4b	0–4b	0–2	0–2b	0	0–2B	0	0	0
B. coli "F"	0–4B	0	0–2	0–2b	0–2	0–2	0	0	0
Pneumobacillus	0–4B	0–8B	*0*	4	*0*	4	*0*	0	0–2
B. faecalis alk.	2	0–10B	*0*	2	0–2	4	0	0	4–6
B. typhosus "CB"	4–6	2–10	2–8	0	2–6	0–6B	0–8B	0–6B	0
B. typhosus "Cole"	*6*	8	*8*	6	6–8	0–10	6	4	2
B. paratyphosus A	4	2–8	*10*	2	4–8	0–6B	4	6	4
B. paratyphosus B	2–6	0–6b	*4*	0	0–4B	0–2	2–4	0–4B	2
B. enteritidis	0–8b	2–6	*8*	2	4–6	0–4	4–6	2–6	0
B. aertrycke	*6*	*8*	*8*	8	6	2–8	6	*8*	4
B. dysenteriae Shiga	0–8B	0–10B	*4*	6	0–10B	0–8B	2–8	0–8B	2
B. dysenteriae Y	2–8	4–6	*4*	4	*2*	0–8B	4	*4*	6
B. Morgan No. 1	*4*	0–4B	2–4	6	0–2B	2–4	0–8B	0	0
B. influenzae	0–2b	2–8	*2*	0–6B	*2*	*0*	2–4	0	0
V. cholerae "Bombay"	0–10B	0–10B	4–10	6	0–10B	4–10	2–10	0–10B	0–10B
V. paracholerae	8–10	8	*6*	6–8	*4*	8	*8*	0–2B	6

Those organisms in the table which have no strain designation were standard laboratory strains of the species named.

In Tables II, III and IV where only one specimen of serum was tested from a particular animal species the result is italicised.

Where results with multiple specimens varied, the range of variation is indicated by two figures representing the extremes of this variation, *e.g.* 0–6 indicates that results varied from nil to 6 according to the notation described on p. 4.

"B" indicates that the average result was a bactericidal effect; "b" indicates an approximately equal number of positive and negative effects.

In the case of the rat and pigeon, blood from two or three animals was pooled and the mixed serum tested. This also applied to a number of specimens of guinea-pig serum.

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Table III. *Bactericidal action of serum of various animal species—determined by Method II.*

	Sheep	Ox	Human	Rat	Pig	Horse	Rabbit	Guinea-pig	Pigeon
B. melitensis "Arkwright"	*6*	*6*	*8*	0	*4*	*6*	*8*	*4*	2
B. melitensis Lab. strain	0	0	0	0	0	0	0	0	0
B. abortus (Bang)	*8*	*8*	*6*	6	*4*	*6*	*10*	*8*	4
B. abortus (Barker)	0	0	0	0	0	0	0	0	0
B. abortus (Hog)	*6*	*6*	*0*	4	*6*	*4*	*2*	*4*	4
B. pyocyaneus	4	0	2	4	0–6 B	0	0–2	0	0–2
B. suisepticus	0–4 B	2–8	6	4	0–6 B	2–6	0–6 B	2	2
B. avisepticus	0–4 b	0–10 B	4	0	0	2	0–4	0	0
B. pseudotuberculosis rodentium	0–4 b	0–4 b	4	0	0–6	2	0–4 b	0	0–2
B. pestis	0	0	0	0	0	0	0	0	0

In most of the tests recorded in this table and Table IV the bactericidal action was estimated both after 4 hours' and 20–24 hours' incubation: in some cases the maximum effect was elicited after 4 hours, in others after 20–24 hours; the results stated are the maxima obtained.

Table IV. *Bactericidal action of serum of various animal species—determined by Method II.*

	Rabbit	Rat	Horse	Human	Pig	Sheep	Ox	Guinea-pig	Pigeon
Staph. aureus "Lab."	0–6 B	4–6	0	0	0	0	0	0	0
Staph. aureus "II"	0–2 B	0	0	0	0	0	0	0	0
Staph. aureus "III"	4	4	0	0–2	0	0	0	0	0–1
Staph. albus	2–6	2–4	0	0	0	0	0	0	0
M. lysodeikticus	3–6	2	*3*	0–1 b	*2*	*1*	*3*	1–3	*0*
Sarcina lutea	2–6	4–6	0	0	2	0	0	0	0
Strept. haemolyticus "SS 1"	4–6	2–4	6	0–2 b	0	0–4	0–1 b	0	0
Strept. haemolyticus "E 4"	0–1 B	1–2	0	0	0–4 b	0–4 b	0	0	0
Strept. viridans "Lab."	0	0	0	0	0	0	0	0	0
Strept. viridans "I"	0–1 b	0	2	0	2–4	0	0	2–4	0
Strept. viridans "II"	0	0	0–4 b	0	0–4 B	0–4 B	0–2 B	0	0
Strept. viridans "III"	2–8	0–2 b	0–2	0–4 b	0	0–4 b	0	0	0
Enterococcus	0	0	0	0	0	0	0	0	0
Pneumococcus I	0–1	0–1	0	0–4 B	0–1	0	0	0	0
Pneumococcus II	0	0	0	0–1 B	0	0	0	0	0
Pneumococcus III	0–4 B	2	4	1	0	0	0	0	0
Pneumococcus IV	0	0	0	0	0	0	0	0	0
Pneumococcus I Rough variant	0–2 B	2	0–2	4	0–2 b	2	0–4 B	0–2 b	—
M. crassus	0–1	0	0	0	0	0	0	0	0
B. diphtheriae	0–2	0	0	0	0	0	0	0	0
B. of Hofmann and *B. xerosis*	0	0	0	0	0	0	0	0	0
B. smegmatis and *B. phlei*	0	0	0	0	0	0	0	0	0
B. anthracis (virulent)	2–3	2–3	3–4	0–2	0	0	0–2	0	0–2
B. anthracis (attenuated)	2–3	4	4	4	0–4 b	0–6 B	4	3	0
B. anthracoides	2	2–4 B	4	0	0	0	0–2	0	0
B. subtilis	2	4	2	0	0	0	0	0	0
B. welchii	0–4 B	0–4 B	0–3 b	1–4	2	0	0	0–2 b	0–4 b
B. sporogenes	0	0	0	0	0	0	0	0	0

Previous workers (*e.g.* Pettersson, 1902, 1926, 1927; Pirenne, 1904; Gonzenbach and Uemura, 1916; and others) have indicated the difference in bactericidal mechanisms for certain Gram-positive and Gram-negative organisms respectively, and Pettersson (1927) has classified the various types of bacteria acted on by the "α" (labile) and "β" (stable) lysins. His results do not show the sharp distinction which we have elicited between the Gram-

positive and negative types, though as indicated in his paper the majority of organisms susceptible to the "α" lysin are Gram-negative, and the majority acted on by the "β" lysin are Gram-positive.

Bactericidal effects towards Gram-negative bacteria.

The maximum and most frequent effects were obtained with the following organisms: *V. cholerae* and *V. paracholerae*, *B. typhosus*, *B. paratyphosus* A and B, *B. enteritidis*, *B. aertrycke*, *B. dysenteriae* Shiga and Y types, *B. Morgan* No. 1, *B. proteus*, Meningococcus, *B. influenzae*, *B. suisepticus*, *B. abortus* "Bang" and "Hog" strains and *B. melitensis* "Arkwright." With *B. abortus* "Hog" and *B. melitensis* "Arkwright," bactericidal reactions were only noted after 20–24 hours' incubation, not after 4 hours. With certain organisms the average for all animal species was a bactericidal effect, though the results with individual samples of serum varied quantitatively, and were occasionally negative. This applied to Meningococcus, *B. paratyphosus* A, *B. aertrycke*, *B. dysenteriae* Shiga and Y types, *V. cholerae*, *V. paracholerae* and *B. suisepticus*. *B. abortus* "Bang" was tested only with single specimens of serum from most of the animals but the results were uniformly positive.

The organisms which gave quantitatively the most pronounced effects were *V. cholerae*, *V. paracholerae* and *B. dysenteriae* Shiga.

Among the Gram-negative bacteria a certain number of types showed distinctly less frequent and less marked reactions: *B. coli* (two typical strains), *B. pyocyaneus*, *B. faecalis alkaligenes*, Pneumobacillus, *M. catarrhalis*, *B. avisepticus* and *B. pseudotuberculosis rodentium*. These organisms were either less susceptible to lysis or their particular bactericidins were less developed in the various animals.

Certain Gram-negative organisms gave uniformly negative results: *B. melitensis* ("Lab. strain"), *B. abortus* "Barker" and *B. pestis*.

The Gonococcus was only tested with a few specimens of serum: it gave uniformly positive but relatively weak reactions.

Reactions of various types of Gram-negative bacteria and of different strains of the same type.

In the course of this survey it became apparent that qualitative and quantitative differences might occur with different strains of the same bacterial species. Thus *B. melitensis* "Arkwright" and *B. melitensis* "Lab." gave quite divergent results, and the same applied to different strains of *B. abortus*. The two strains of *B. typhosus*, "CB" and "Cole," did not behave in an identical manner, and the two strains of *B. coli* also showed different reactions (Tables II and III). Thirteen strains of *B. typhosus* were tested simultaneously with the same specimen of ox serum: the bactericidal effects showed a considerable quantitative range of variation (Table V). An interesting observation was also made regarding the comparative reactions of the R and S types

of the same strain (*B. typhosus* "CB"). In plate culture this organism showed an approximately equal number of rough and smooth colonies. Pure R and S types were separated and tested comparatively. The R variant proved more susceptible, as it were, to serum bacteriolysis than the S form (Table VI).

Table V. *Various strains of* B. typhosus *and ox serum.*

Strain	Bactericidal effect	Relative number of "rough" colonies
"CB"	4	+ +
"Cole"	6	+ + +
3	6	+ +
4	8	+ + + +
5	8	+ + +
6	8	+ + +
7	8	–
8	10	–
9	6	+ + +
10	8	+ + +
11	6	+
12	10	+
13	8	+

Table VI.

	Bactericidal effect		
B. typhosus "CB"	Sheep serum	Pig serum	Ox serum
R type	10	8	8
S type	6	4	4

The proportion of R colonies in the various strains of *B. typhosus* tested was then estimated (Table V), but no correlation could be established between the degree of bactericidal effect and the "roughness" of the strain. Strains which were mainly "rough" were not more susceptible than the "smooth" strains. Similar R and S types of a *B. dysenteriae* Shiga were also separated and tested comparatively but showed no difference in their reactions. No rule, therefore, could be drawn as regards the relative susceptibility of R and S types.

Seven strains representing different fermentative varieties of typical coliform bacilli were tested simultaneously with specimens of sheep, rabbit and guinea-pig serum. These showed a similarity in their behaviour with sheep and guinea-pig serum but irregularity with rabbit serum (Table VII). *B. coli anaerogenes* and *B. lactis aerogenes* were also tested along with these organisms. The former proved highly susceptible even to guinea-pig serum which was quite inactive towards the typical coliform bacilli. *B. lactis aerogenes* was also more reactive (to sheep and rabbit serum) than the other coliform types.

Two strains of *B. paratyphosus* B were tested simultaneously with samples of active serum and showed quantitatively different results. The same difference was noted with two strains of *V. cholerae.*

The results all tend to show that an essential factor in bactericidal effects is the reactivity or susceptibility of the particular strain to such lytic action, apart altogether from its biological group or species.

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Table VII. *Comparison of various strains of* B. coli.

Typical strains of *B. coli*	Bactericidal effects		
	Sheep serum	Rabbit serum	Guinea-pig serum
X	2	0	0
F	2	0	0
N	0	4	0
G	2	0	0
S	2	0	0
C	2	4	0
V	2	2	0
P	2	0	0
B. lactis aerogenes	6	6	0
B. coli anaerogenes	8	8	8

Taking the results as a whole biologically allied organisms showed a general similarity in their reactions to the natural bactericidal properties of serum, *e.g.* the Gram-negative diplococci, the vibrios, the typhoid-paratyphoid-dysentery bacilli. On the other hand, marked differences occurred among closely related types. *B. pestis* was exceptional among the Pasteurella group in its negative reactions, and one strain of *B. melitensis* and a strain of *B. abortus* differed from the other organisms of the melitensis-abortus group.

It seems possible that many of the differences depend more on strain peculiarities than on the biological group or species. Torrey (1908) in a study of serum-bactericidal reactions with the Gonococcus elicited differences among strains which he concluded were dependent on varying susceptibility to lysis.

The bactericidal mechanism.

In our previous study (Mackie and Finkelstein, 1931) of the mechanism of bactericidal action, certain organisms representing three different biological groups of Gram-negative bacteria were selected for analytical tests: *B. typhosus*, *B. dysenteriae* Shiga, *B. proteus* and *V. cholerae*. These tests were carried out with ox, sheep, horse, pig, rabbit, guinea-pig and human serum, and it was shown that for each organism the bactericidin consisted of serum-complement acting along with a sensitising antibody-like agent which could be absorbed from serum at 0° C. by the particular organism. Neither complement nor antibody was active *per se*.

Absorption tests demonstrated a multiplicity of such bactericidal antibodies highly specific for particular organisms. The lability at 55° C. of the bactericidal property was shown to be due to the thermolability of the complement, the antibody being stable at 60° C.

Comparison of different animals.

With certain organisms, *e.g. V. cholerae*, *B. dysenteriae*, the sera of the various animals were practically similar in their average reactions, both as regards the frequency of positive results (when multiple specimens were tested) and the quantitative degree of these reactions. With other organisms considerable differences were elicited among the various animals particularly as

regards the frequency of bactericidal effects (see Tables II and III). Sheep and ox were the most active in this respect, guinea-pig and pigeon least active, while man, horse, pig, white rat and rabbit occupied an intermediate position.

The difference between sheep and guinea-pig serum was well marked and is illustrated further in Table VII, showing the effects of these sera towards different types of *B. coli*. It is to be noted that in these tests, as in the general results, rabbit serum occupied an intermediate position.

The question arose whether the serum of lower vertebrates possesses similar properties, taking into account the fact that the species of bird examined (pigeon) exhibited relatively infrequent positive reactions. Frog serum was tested with a series of organisms, each specimen being obtained from the pooled blood of four or five individuals. The results were fairly similar to those obtained with guinea-pig and pigeon serum, *i.e. V. cholerae*, *B. typhosus* were strongly lysed, while *B. coli* and *B. pyocyaneus* gave negative results.

BACTERICIDAL EFFECTS TOWARDS GRAM-POSITIVE BACTERIA.

When bactericidal effects were elicited with Gram-positive organisms, the active principle in the serum proved stable at 55° C., and apparently the thermolabile complement, essential for the lysis of Gram-negative bacteria, is not concerned in the killing of Gram-positive types. On the whole the bactericidal reactions with the latter were less frequent and less pronounced than in the case of the former (Table IV). As with the Gram-negative organisms, great variation was noted when multiple specimens of serum were examined from each animal species. The most frequent and most pronounced effects were obtained with an attenuated strain of *B. anthracis*, an avirulent Pneumococcus (originally of Type I), and *M. lysodeikticus* (the Gram-positive Staphylococcus described by Fleming (1922) as highly sensitive to "lysozyme"). A considerable number of the organisms examined gave consistently negative results, *e.g. Strept. viridans* "Lab.", Enterococcus, Pneumococcus Group IV, *M. crassus*, *B. diphtheriae*, Hofmann's bacillus, *B. xerosis*, *B. smegmatis*, *B. phlei* and *B. sporogenes*. Most of the organisms which yielded positive results reacted with the serum of only a limited number of the animal species examined: *Staph. aureus* (three strains), *Staph. albus*, *Sarcina lutea*, *Strept. haemolyticus* (two strains), *Strept. viridans* (three strains), Pneumococcus Types I, II and III, *B. anthracis* (virulent strain), *B. anthracoides*, *B. subtilis* and *B. welchii*.

Comparison of different animal species.

The occurrence of the thermostable type of bactericidin among the various animals showed a considerable difference from the complement-antibody lysin. As judged by the frequency of bactericidal effects, the rabbit and white rat were most active; reactions with pigeon serum were very infrequent; the guinea-pig also (as in the case of the labile bactericidin) showed a low fre-

quency of activity and the same applied to ox and sheep sera. Between the two extremes horse, human and pig sera occupied an intermediate position.

Reactions of various types of Gram-positive bacteria and different strains of the same type.

Two strains of *Staph. aureus* and a strain of *Staph. albus* all behaved similarly, being acted on uniformly by rabbit and rat serum though unaffected usually by the serum of other animals. *Staph. aureus* "strain II" was apparently of lesser susceptibility: while usually killed by rabbit serum it was insusceptible to rat serum. As mentioned above *M. lysodeikticus* proved the most reactive of all the Gram-positive cocci. The Sarcina tested resembled the staphylococci except for its reactions with pig serum. All the strains of streptococci behaved differently as regards their reactions with the various sera, and the same was true for the pneumococci. The avirulent Pneumococcus was a derivative of the strain of Pneumococcus Type I shown in the table; the difference in its susceptibility to serum-lysis as compared with the original strain is marked. It is noteworthy also how the attenuated strain of *B. anthracis* was acted on in a fairly uniform manner by the serum of all the mammalian species, whereas the virulent strain of this organism resisted pig, sheep and guinea-pig sera, *i.e.* the sera of species which are highly susceptible to anthrax infection. *B. anthracoides* and *B. subtilis* reacted like one another, being killed by rabbit, rat and horse serum.

The results all tended to show the variations among strains in their susceptibility to this type of bactericidin.

While rabbit and rat serum seemed to be the most frequently active in killing certain organisms such as the staphylococci and the haemolytic streptococci, in the case of the other susceptible organisms the serum of man, pig, horse and sheep were as frequently active as rabbit and rat serum and in some instances more so. Thus, horse serum which invariably killed the sporing aerobes did not as a rule act on the staphylococci. Horse, pig and sheep sera were as frequently bactericidal towards the *viridans* streptococci as rabbit serum and more active than rat serum. As regards the pneumococci human serum was the most active among all the species.

These findings seemed to indicate that the thermostable bactericidal mechanism is to some extent differentiated as regards various animals and bacterial groups, and is not an entirely homogeneous agent.

Thermostability of the bactericidal property.

Some of the earlier workers (*e.g.* Bail, 1903) stated that the bactericidal action of serum towards the anthrax bacillus was stable up to 63° C. A considerable number of detailed tests have been carried out with various Gram-positive organisms in which unheated serum and serum heated at varying temperatures were tested. When these tests were first made, the serum was diluted 1 in 4 with saline to obviate coagulation at the higher temperatures

and it was found that, though the bactericidal power for *Staph. aureus* was unaltered when the undiluted serum was heated at 55° C. for half an hour, the property was lost at this temperature in diluted serum; this was the case even when the dilution was only 1 in 2. In the detailed tests of stability, therefore, the serum was heated at varying temperatures in the undiluted state; it was not, of course, possible to exceed 65° C. owing to inspissation or coagulation at this or higher temperatures. The results (illustrated in Table VIII) showed a markedly uniform stability, inactivation taking place between 57·5° C. and 60° C. In some cases a weakening of the effect was noted at 57·5° C. The active principle was therefore more labile than the natural bactericidal antibodies for the Gram-negative organisms though more stable than complement. The lability at 55° C. in diluted serum seems a characteristic feature of this principle and in thermolability it falls into a category by itself among the various antibacterial principles in serum.

Table VIII. *Thermostability test.*

Staph. aureus and rabbit serum.

	Bactericidal effect
Serum unheated	4
„ heated 55° C.	4
„ „ 57° C.	3
„ „ 60° C.	0
„ „ 65° C.	0
Serum diluted 1 in 2:	
Unheated	4
Heated 55° C.	0
„ 60° C.	0

B. anthracis and horse serum.

	Bactericidal effect
Serum unheated	2
„ heated 55° C.	2
„ „ 57·5° C.	1
„ „ 60° C.	0

Streptococcus haemolyticus "SS 1" and rabbit serum.

Serum unheated	2
„ heated 55° C.	2
„ „ 57·5° C.	1
„ „ 60° C.	0

The greater thermolability of the bactericidin on dilution of the serum bears some resemblance to the results obtained by Mellanby and Woolley (1913–14) with trypsin. They showed that pancreatic juice diluted with an equal volume of water loses all activity on heating for 5 minutes at 60° C. It was also noted that trypsin in pancreatic juice is still active after heating at 100° C. for 5 minutes, provided the juice is slightly acid. On the other hand, trypsin in neutral or alkaline solution loses its activity when so heated. In alkaline solution trypsin is rapidly destroyed at 50° C.

Rabbit serum was rendered acid (*p*H 6·7) by the addition of *N*/10 HCl and the thermostability of its bactericidin (for *Staph. aureus*) was then compared with that of the normal serum (*p*H 7·7). No increased stability was noted: in both cases inactivation occurred between 55° and 60° C. (30 minutes). A similar comparison was made between normal serum and serum rendered alkaline (*p*H 8·6) by the addition of *N*/10 sodium hydroxide, the sera being tested with *Staph. aureus* and *B. anthracoides*. The alkalinisation greatly increased the lability, the serum becoming inactivated at 50° C. in 5 minutes, whereas the neutral serum was unaltered in its activity.

Dilution of the serum with an equal volume of saline did not render the

bactericidin (for *Staph. aureus* and *B. anthracoides*) labile on heating for 5 minutes at 60° C., though as stated above lability of diluted serum at 55° C. was noted after 30 minutes.

In its greater thermolability on dilution and in an alkaline medium, the bactericidin for the Gram-positive organisms shows some analogy with trypsin, but the similarity in its reactions to heat is incomplete.

Absorption tests.

It has been shown that the natural antibody concerned in the lysis of the Gram-negative bacteria is absorbed from serum by the particular organism at 0° C. The stable bactericidin showed a similar reaction. A culture of *B. anthracoides* was mixed with a given volume of serum (*e.g.* one agar slope culture to 10 c.c. serum), kept at 0° C. for 2 hours, then separated from the serum by centrifuging, and washed twice with saline solution; the organisms were then re-suspended in saline to the standard density for the bactericidal test and decimal dilutions were prepared in the usual way, loop-transfers being made from them immediately and after 4 hours at 37° C. For comparison the usual test was carried out with the untreated organisms and the same specimen of serum. The bacteria apparently absorbed the bactericidin as judged by the occurrence of lysis of the treated bacteria on incubation, though no serum was present. The same result was obtained with *Staph. aureus*. Table IX illustrates these results.

Table IX. *Absorption of bactericidin by organisms at 0° C.*

		$S/10^2$	$S/10^3$	$S/10^4$	$S/10^5$	$S/10^6$	$S/10^7$
B. anthracoides + rabbit serum	Before incubation	C	+ +	+	2 c.	1 c.	–
	After „	2 c.	–	–	–	–	–
B. anthracoides treated with serum at 0° C., separated from serum and suspended in saline	Before „	C	+ + +	+ +	f.c.	3 c.	1 c.
	After „	+ +	–	–	–	–	–

A large number of absorption tests were carried out in which serum was treated with various organisms at 0° C., separated by centrifuging and then tested with other Gram-positive species. Rabbit serum was used in virtue of its more uniform activity as compared with the serum of other animals. In some cases Gram-negative bacteria, *e.g. B. typhosus*, *V. cholerae* and *B. suisepticus* were included and a few experiments were carried out in which serum was absorbed with *B. typhosus* and then tested with Gram-positive organisms. The results elicited the essential difference between the bactericidins for Gram-positive and negative organisms respectively (Table X). A serum absorbed with a Gram-positive organism was unaltered in its bactericidal action towards the Gram-negative types and *vice versa*. No definite specificity was noted, however, as regards the lysis of different Gram-positive bacteria: a serum absorbed with one type generally lost its killing power not only for this organism but also for others. Small quantities of culture sufficed as a rule to produce complete absorption as contrasted with the large quantities re-

quired for the absorption of the bactericidal antibody for Gram-negative bacteria. The growths were washed three times with saline before being used for absorption in view of our previous finding of a non-specific extracellular agent in cultures capable of inhibiting the lysis of Gram-negative bacteria.

Table X. *Absorption tests.*

Strept. haemolyticus "SS 1," growth from three phosphate-broth (10 c.c.) cultures, heated 67° C. 1 hour, washed thrice with saline, suspended in 11 c.c. rabbit serum, kept at 0° C. for 2 hours; serum separated by centrifuging.

	Bactericidal effects						
	S. haemolyticus "SS 1"	Pneumococcus III	*Staph. albus*	*Staph. aureus* "Lab."	*Staph. aureus* III	*B. typhosus*	*B. anthracoides*
Untreated serum	2	2	2	4	2	4	2
Absorbed ,,	0	0	0	0	0	4	0

Rabbit serum 11 c.c. absorbed with *Staph. aureus* (growth from three agar slope cultures, heated 67° C.).

	Bactericidal effects				
	Staph. aureus "Lab."	*Staph. albus*	*Strept. haemolyticus* "SS 1"	*B. suisepticus*	*B. anthracoides*
Untreated serum	4	2	2	2	2
Absorbed ,,	0	2	0	2	0

Rabbit serum 6·5 c.c. absorbed with *B. typhosus* (growth from three 6 in. plates of agar, the organisms having been heated at 67° C.).

	Bactericidal effects	
	B. typhosus	*Staph. aureus*
Untreated serum	4	4
Absorbed ,,	0	4

Human serum treated as above.

	Bactericidal effects	
	B. typhosus	*Strept. haemolyticus* "SS 1"
Untreated serum	6	2
Absorbed ,,	0	2

From such results it might appear that the bactericidin for Gram-positive bacteria is a single homogeneous agent as contrasted with the highly differentiated bactericidal antibodies for the Gram-negative organisms, though certain observations already referred to seemed to indicate lack of complete homogeneity. In certain of the absorption tests this apparent homogeneity was also incomplete. Thus in some experiments absorption with *Staph. aureus*, while annulling the bactericidal effect for the homologous organism, *Strept. haemolyticus* and *B. anthracoides* did not reduce the lytic action of the serum towards *Staph. albus*. In other experiments absorption with *Staph. aureus*, while annulling the effect on *Staph. albus*, did not alter the bactericidal property of the serum for Pneumococcus Type III. Such results were, however, in a minority among all those obtained; on the whole the results were non-specific, though uniform and complete non-specificity could not be demonstrated. It was concluded that the bactericidal effect of serum for Gram-

positive organisms is due to a single agent only slightly differentiated as regards its action on the various types and identifiable with the principle which has been described as "anthracocidin" in virtue of its action on the anthrax bacillus and allied organisms.

Occurrence of the thermostable bactericidin in young animals.

It is characteristic of certain natural antibodies, *e.g.* haemolysins, agglutinins, etc., that they are absent from the serum of very young animals, *e.g.* rabbits during the first 3 weeks of life, and begin to appear at a certain stage of development (see Mackie and Finkelstein, 1930; 1931). Some types of natural antibodies, however, are demonstrable in young animals at an earlier stage than those instanced above, and this applies to the bactericidal antibodies for the Gram-negative organisms. The thermostable bactericidin is apparently almost coincident with the bactericidal antibodies in this respect. A litter of five young rabbits 20 days from birth were examined for their bactericidal properties towards *Staph. aureus*, *B. anthracoides* and *B. typhosus*. The results are shown in Table XI.

The reactions with *B. typhosus* varied quantitatively among the litter: with one exception the bactericidal power for *Staph. aureus* was already developed, though with *B. anthracoides*, three of the animals gave negative results. Gózony (1913) found the property of killing *B. anthracis* present in foetal serum. On the other hand, Behring and Nissen (1890) originally noted its absence from young animals, *e.g.* rabbits.

Table XI. *Bactericidal effects of serum of young animals.*

	Bactericidal effects		
	Staph. aureus	*B. anthracoides*	*B. typhosus*
Litter of rabbits 20 days:			
1	2	2	6
2	4	2	5
3	2	0	3
4	1	0	4
5	0	0	2
Mother	2	2	6

The question of the dual constitution of the thermostable bactericidin.

Reference has been made to the observation of Bail, Gruber and Futaki, and Pettersson, which seemed to indicate that the bactericidal agent for certain Gram-positive organisms is dual in constitution. Thus, Pettersson claimed that a small amount of serum which was inactive *per se* could reactivate serum which had been heated at 70° C., and he observed the same reactivation with thrombocyte extracts. On this basis he assumed there are two principles involved, one "activating" and the other "activable."

We have carried out a number of experiments to test this and have been unable to confirm his findings. Rabbits' serum has been used, with *Staphylococcus aureus* and *B. anthracoides* as the test organisms. Serum inactivated

by heating at 65°–68° C. was not reactivated to the slightest degree by small amounts of serum which were inactive *per se* nor by a platelet extract. The platelets were separated from 50 c.c. rabbits' blood according to the method described by Bacot and Segal (1922). A large yield of platelets practically unmixed with other cells was obtained and extracted in 5 c.c. saline overnight after freezing and thawing alternately on several occasions. The extract possessed no bactericidal activity *per se*. Pettersson determined bactericidal action by colony counts; we have used the technique employed throughout this investigation.

Leucocyte extracts also failed to reactivate heated serum. These extracts were prepared as follows: guinea-pigs were injected intraperitoneally with peptone solution; the exudate was removed and centrifuged to separate the leucocytes, and the cells were then extracted in saline solution after repeated freezing and thawing.

The question whether the thermostable bactericidin is limited in its action to Gram-positive organisms.

The exact limitation of the effect of the thermostable bactericidin to the Gram-positive bacteria and the similar restriction of the antibody-complement bacteriolysin to the Gram-negative types is of special interest. In our work, without exception, the bactericidal action of serum for Gram-negative organisms proved labile (due apparently to the lability of the complement which is essential for this effect) whereas the effect on Gram-positive bacteria was invariably stable at 55° C.

Selter (1918) has indicated that certain Gram-negative bacteria may be killed by heated serum, the effect apparently depending on a thermostable agent. Pettersson (1927) includes Gram-positive organisms (*e.g. Streptococcus pyogenes*) among those acted on by the thermolabile or "α" lysin and Gram-negative types (*e.g. B. proteus vulgaris*) among those affected by the thermostable or "β" lysin. We have carried out a large number of most careful quantitative tests by Method II with one or more representatives of each of the various groups of Gram-negative organisms and heated sera from various animals, particularly the rabbit, in view of its high grade of activity towards the Gram-positive organisms. In no instance was any trace of bactericidal action detected with such sera after heating at 55° C. for half an hour. (The temperature was carefully controlled by means of a certified standard thermometer.) Apparently the thermostable bactericidin *per se* is quite devoid of effect on Gram-negative organisms. Further, without exception, serum complement which constitutes part of the mechanism for the killing of Gram-negative bacteria is not concerned in the lysis of the Gram-positive organisms.

In this connection it is interesting to note that Smith (1922) elicited a remarkable difference between Gram-positive and negative bacteria, the latter being susceptible to lysis by trypsin and by alkali, the former resistant. (In his experiments the Gonococcus was an exception to this rule.) In serum-lysis,

on the contrary, the Gram-negative organisms are peculiarly resistant to an agent to which other organisms are susceptible.

The question of the relationship of the thermostable bactericidin of serum to "lysozyme" and "leukins."

According to Fleming and Allison (1922) "lysozyme" occurs in blood serum (*vide supra*). It is a relatively thermostable principle and the question arose as to its possible relationship to the thermostable bactericidin.

Lysozyme has usually been demonstrated by its rapid and visibly lytic action in suspensions of a Gram-positive staphylococcus ("*M. lysodeikticus*") which exhibits a high susceptibility to its action. It has been shown (see Table IV) how this organism is also highly sensitive to lysis by serum when tested according to the technique we have used. This property of serum is stable at 55° C. It seemed likely that the lysis of this organism by serum was due to the same bactericidin as that affecting other Gram-positive organisms, though this effect by serum has been attributed by Fleming and Allison to the presence of "lysozyme."

The thermostability of "lysozyme" in a 1 per cent. saline dilution of human tears (tested with *M. lysodeikticus*) was compared with that of the bactericidal property of serum towards the same organism. Rabbit serum was selected in view of its pronounced and uniform lytic action on *M. lysodeikticus*. The "lysozyme" withstood heating at 70° C. for half an hour. The lytic action of the serum was annulled between 57·5° and 60° C. and it would appear that the lysis of *M. lysodeikticus* by serum is due to the bactericidin that acts on other Gram-positive bacteria. The results of the thermostability tests recorded above indicate that this bactericidin and lysozyme are different principles.

Schattenfroh (1897), Zinsser (1910) and Manwaring (1912) all agree as regards the high thermostability of the leukins which are apparently only destroyed at 75°–80° C. The serum bactericidin for Gram-positive organisms differs considerably in this respect from the leukins and cannot be identified with them. It is noteworthy, however, that the organisms susceptible to the bactericidal action of leucocyte extracts are mainly Gram-positive organisms, *i.e.* those types sensitive to the thermostable bactericidin (see Knorr, 1929).

It might be objected to these comparisons on a basis of thermostability that the physical conditions of the fluids in which thermostability has been tested differ and that this characteristic is influenced by such factors. The "lysozyme" was tested in a dilute form in saline solution and still showed marked thermostability, whereas the effect of dilution on the serum bactericidin was to increase its lability.

Discussion.

The studies recorded in this paper were based on a systematic survey of the bactericidal properties of the serum of different animal species (including man) towards representative types of bacteria drawn from various biological

groups. It has been recognised that these properties vary according to the animal species and the type of bacterium acted on, but the variations have not been fully defined with a view to their ultimate interpretation. This survey elicited also considerable differences dependent on the individual sample of serum tested and the bacterial strain examined. An attempt was made to ascertain the extent of these variations so that the results of the survey could be presented in as comprehensive a form as possible, though we cannot claim complete statistical accuracy of the averages we have drawn. The tests were sufficiently distributed over the calendar year to obviate any influence that seasonal fluctuation might have in determining the averages. Season variations in the bactericidal power of the serum have been demonstrated by Kraus and Clairmont (1900) and Tromsdorff (1902), and we have noted some indication of this in the case of the thermostable bactericidin of rabbit serum tested with *Staphylococcus aureus*. This factor, however, requires more detailed study.

One of the most definite results of the work has been the sharp differentiation between the Gram-positive and negative bacteria as regards the natural bactericidal principles in serum which act on them. Thus the property for the Gram-negative organisms is invariably labile at 55° C. (30 minutes) and, as shown in a previous paper (Mackie and Finkelstein, 1931), is dependent on a specific sensitising antibody acting along with serum-complement. The thermolability of this mechanism is due to the lability of the complement. The antibody is stable up to 60°–65° C. The bactericidal activity of normal serum towards the Gram-positive organisms is invariably stable at 55° C. but is annulled between 57·5° C. and 60° C. (30 minutes). Complement does not apparently play any part in the killing of these organisms. Thus, two entirely different mechanisms are concerned in the killing of Gram-positive and negative bacteria respectively. It is also noteworthy that the bactericidal power for Gram-negative types is more developed among all the animals examined than the bactericidal effect on Gram-positive organisms. Previous workers (see Pettersson, 1926, 1927) have drawn attention to the existence in serum of two different bactericidal mechanisms. Pettersson designated the thermolabile mechanism the "α" lysin, the stable bactericidin the "β" lysin. He also classified the organisms susceptible to these lysins, but his classification does not show the differentiation which we have elicited.

Among the Gram-negative bacteria considerable variation was noted in their reactions to the bactericidal properties of normal serum. The most markedly reactive groups were the pathogenic vibrios and Gram-negative bacilli (typhoid-paratyphoid-dysentery groups). Few Gram-negative types gave uniformly negative results, the only examples being particular strains of the *B. melitensis-abortus* group and *B. pestis*.

We have shown that these effects are due to highly differentiated and specific sensitising antibodies, and the variations noted may depend on the content of the particular antibody in the serum, the antigenic composition of the organism or its susceptibility to such lytic effects. Different strains of

the same species may give divergent results and it seems likely that the inherent sensitiveness of a strain to the bactericidal effect of serum is a determining factor. While biologically allied organisms showed some general similarity in their reactions, marked differences were noted among closely related types and probably many of the differences elicited depend more on peculiarities of the strains used than their biological species. Interesting differences were found among the various animals as regards the frequency of positive reactions when samples of serum from several individuals were examined. Differences in the degree of positive reactions were less obvious. Sheep and ox sera were the most frequently active: guinea-pig and pigeon sera least so, while the sera of man, horse, white rat, pig, and rabbit were intermediate.

It is of interest to correlate such differences with those elicited from the study of natural agglutinins and complement-fixing antibodies for the Gram-negative bacteria. Gibson (1930) has described the following order among these species in regard to the activity of their natural agglutinins: ox (most active), pig, horse, sheep, man, rabbit, guinea-pig, rat (least active). Mackie and Finkelstein (1930) have also shown how species vary as regards the activity of their serum in fixing complement along with various bacterial antigens: ox, sheep, horse, and human serum generally react strongly in this respect while rabbit, pig, rat and guinea-pig are less active. In natural agglutinating, complement-fixing and bactericidal properties guinea-pig serum contrasts with that of other species, particularly sheep and ox, in its low grade of activity. It is interesting that the serum of an amphibian should possess bactericidal properties for certain organisms, *e.g. B. typhosus*, *V. cholerae*, etc.

The distribution among the various animals of bactericidal properties for Gram-positive organisms is quite different from those for other bacteria. Thus, the serum of the rabbit and white rat were the most frequently bactericidal, whereas ox and sheep sera were much less active. Guinea-pig serum, as in the case of the labile bactericidin, was also infrequently bactericidal and pigeon serum was very rarely active.

In the early work on the serum-lysis of the anthrax bacillus, the existence of a type of bactericidin active towards this organism and closely allied species was postulated and spoken of as "anthracocidin." Knorr (1929), in reviewing the occurrence of "anthracocidin" in various animals, has quoted certain authors to show that it is practically limited to rabbit, rat and horse serum, other animals, *e.g.* ox, dog, guinea-pig, mouse, fowl and pigeon lacking this principle in their sera. Dresel and Keller (1922–3) studied "anthracocidin" in human blood and found it to be absent from healthy persons: they noted, however, its appearance in acute and chronic infections, blood diseases, etc. Absorption tests, while differentiating the bactericidins for Gram-positive and negative organisms respectively, did not elicit any definite specificity of the thermostable bactericidin for particular organisms. Apparently all the Gram-positive organisms are acted on by a common principle which is practically undifferentiated. The "anthracocidin" of previous workers may, therefore,

be identified with the thermostable bactericidin as defined in this paper and Pettersson's "β" lysin. Its distribution among animals is apparently much wider than that indicated by Knorr.

As among the Gram-negative bacteria great variations were noted in the reactions of different groups, species and strains of Gram-positive organisms and apparently the inherent susceptibility or resistance of the particular strain to the bactericidal effect is a determining factor in these variations. There was some indication that among strains of the same species such varying resistance may be dependent on virulence. Thus a virulent Type I Pneumococcus was found to be insusceptible to most of the sera examined, whereas an avirulent "rough" derivative of this strain was killed by rabbit, rat, pig, sheep, ox, guinea-pig and human sera. An analogous difference was noted between a virulent and an attenuated anthrax bacillus. This requires further study.

The thermostability of the bactericidin for Gram-positive organisms was carefully determined: in undiluted serum it was stable at 57·5° C. but inactivated at 60° C. (30 minutes). This was noted with great regularity when serum from different animals was tested with various organisms. The thermostability, however, was diminished in diluted serum and slight alkalinisation of the serum had a similar effect. In these respects it shows some analogy with trypsin (Mellanby and Woolley, 1913–14). Slight acidity, however, did not increase the thermostability, whereas in a slightly acid medium the stability of trypsin is increased to a high degree. The similarity to trypsin is incomplete. The question arose whether the bactericidin in question is a natural proteolytic enzyme of serum capable of acting in a bacteriolytic capacity. It has been shown, however, by Smith (1922) that the Gram-positive organisms are insusceptible to trypsin-lysis as compared with the Gram-negative types which are sensitive to this effect. The thermostable bactericidin is limited apparently in its action to the Gram-positive organisms, the converse of the reactions to trypsin described by Smith.

We have found no evidence of the dual constitution of this principle nor any relationship to extractable products of blood platelets or leucocytes as recorded by Pettersson and others.

The question was also considered whether the stable bactericidin might be a principle of the same nature as the bactericidal antibodies for the Gram-negative bacteria, but capable of acting on the Gram-positive organisms without serum-complement (like an agglutinating antibody). In this connection it is noteworthy that the bactericidin, like antibodies generally, is absorbed at 0° C. by the bacteria acted on. The lability points, however, of the two principles are different: 60–65° C. in the case of the bactericidal antibodies and 57·5–60° C. in the case of the stable bactericidin. Absorption of serum by a Gram-positive organism while removing the stable lysin (which we have found undifferentiated as regards various Gram-positive types) did not affect the bactericidal antibodies for Gram-negative species. On the other

hand, these have been found themselves to be highly differentiated multiple principles. The thermostable lysin definitely contrasts with the natural bactericidal antibodies in its ability to act on Gram-positive organisms without complement, its greater thermostability, and its lack of specific differentiation as regards the bacteria susceptible to its action.

The bactericidin for Gram-positive organisms cannot be identified with either the leukins or lysozyme. Both these principles possess a degree of thermostability which is greatly in excess of that pertaining to this substance.

While the bactericidal mechanism for the Gram-negative bacteria consists of well-recognised types of antibacterial substances, the thermostable bactericidin seems to be in a category by itself among serum principles. If it were of antibody nature one would expect its content to increase as a result of immunisation. It has been generally observed, however, that immunisation by Gram-positive organisms does not lead to the formation of a lytic immune substance.

The question is therefore unsettled as to the immunological nature of the thermostable bactericidin, though the facts might suggest it is a lytic enzyme with a special relationship to the category of Gram-positive organisms.

The peculiar distribution of these bactericidins among various animals is difficult to interpret, and there is no definite correlation between the possession of bactericidal properties for particular organisms and natural resistance to the corresponding infection. The relatively infrequent reactions with guinea-pig serum are noteworthy, however, in view of the marked susceptibility of this animal to various infections under experimental conditions. The variation among individual animals and the varying susceptibility of individual bacterial strains illustrate, however, the extreme difficulty of establishing any such correlation or dissociating bactericidal properties from natural immunity. Furthermore, the virulence of an organism is a deciding factor irrespective of the degree of natural resistance, and the question whether virulence and insusceptibility to bactericidal action are associated requires further study.

This work opens up a number of questions requiring extended study, but the observations we have recorded are of interest in affording a further definition of the bactericidal properties of normal serum and in analysing the mechanisms concerned. The data presented also illustrate not only the potentialities of these mechanisms but also their limitations as defences against microbic infection.

Conclusions.

1. The bactericidal property of normal serum towards the Gram-negative bacteria is labile at 55° C. (30 minutes); the corresponding effect on Gram-positive bacteria is stable at this temperature. The Gram-negative and positive organisms are acted on by separate mechanisms, the "thermolabile" and "thermostable" bactericidins respectively. Bactericidal effects are more frequent and pronounced towards Gram-negative than Gram-positive bacteria.

2. The "thermolabile" bactericidin consists of complement and a sensi-

tising antibody. The lability of the bactericidin is due to the lability of the complement. The antibody is stable at 60° C. and specific for the particular organism acted on.

3. The "thermostable" bactericidin in undiluted serum withstands a temperature of 57·5° C. though labile at 60° C.; its lability is considerably increased in diluted serum and in slightly alkalinised serum though unaltered by slight acidity.

4. This principle shows little or no differentiation as regards the various Gram-positive organisms acted on and corresponds to the "anthracocidin" of the earlier literature.

5. No evidence has been obtained of its relationship to "plakins," "leukins" or "lysozyme," nor of its dual constitution as described by other workers. It differs from other recognised antibacterial principles.

6. Various animal species can be arranged in order as regards the activity of their bactericidins. "*Thermolabile*" *bactericidins*: sheep (most active); ox; man, white rat, pig and horse; rabbit; guinea-pig; pigeon (least active). "*Thermostable*" *bactericidin*: rabbit (most active); rat; horse; human; pig; sheep; ox and guinea-pig; pigeon (rarely active). Individual animals of the same species vary considerably in the bactericidal activity of their serum.

7. Bacteria vary greatly in their reactivity or susceptibility to these bactericidal properties. Individual strains of the same species also vary in this respect. The most reactive Gram-negative organisms are the vibrios and the typhoid-paratyphoid-dysentery group. Few Gram-negative organisms are uniformly non-reactive. Among Gram-positive types studied the most susceptible are an attenuated anthrax bacillus, an avirulent Pneumococcus and "*M. lysodeikticus*."

8. The "thermostable" bactericidin is practically coincident with the natural bactericidal antibodies for Gram-negative bacteria in its appearance in the serum of young animals.

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