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The Antibacterial Action of Spleen and Liver Extracts on Pathogenic Staphylococci *

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SUMMARY

An antistaphylococcal substance was demonstrated in extracts of guinea pig spleen and liver but not of kidney. The distribution of this substance was in good accord with the fate of pathogenic staphylococci in these organs, as described by Smith and Dubos (1956).

The active substance was shown to be in cells of RES, because it was found in the Kupffer's cells of the liver. The activity was assayed by measuring the decrease in the number of colonies, but it was shown that this decrease in the number of colonies was the result of a bactericidal effect and not of agglutination by staphylococcal agglutinins.

The active substance was precipitated at between 25 and 50 per cent saturation of ammonium sulfate. It was resistant to heat treatment at 56°C for 30 minutes, but it was inactivated by heat treatment at 70°C for 30 minutes or at 100°C for 10 minutes. It had an isoelectric point at pH 4.5-5.0 and the resulting precipitate recovered activity on solution at pH 7.0.

Escherichia coli B, *Klebsiella pneumoniae* and *Shigella flexneri* type 2a were sensitive to it, while salmonellae were not.

INTRODUCTION

Rogers and Tompsett (1952) reported that pathogenic staphylococci could multiply actively in human leucocytes but that nonpathogenic strains were killed and rendered Gram negative in the leucocytes. The same phenomena were also observed in guinea pig leucocytes and the active substance was identified in this laboratory as "leucozyme A", one of the antibacterial substances in guinea pig leucocytes (Amano *et al.*, 1956). No antibacterial substance could be found in guinea pig leucocytes even when the extraction method was changed.

Smith and Dubos (1956), reported that when the pathogenic staphylococci were injected intravenously into mice there was a decrease in the viable count in the spleen and liver and marked increase in the kidney. Therefore further attempts to demonstrate an antistaphylococcal substance in the spleen and liver were made *in vitro*, using a modification of the most sensitive method of Hirsch (1958). The present study is on this antistaphylococcal substance which was found in the spleen and liver.

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MATERIALS AND METHODS

1. *Bacteria used*

The following bacterial strains were used in this study.

1) *Staphylococci*

a) *Staphylococcus aureus* Newman strain (designated as the N-1 strain in this laboratory). It is a typical pathogenic strain, kindly supplied by Dr. E.S. Duthie. It possesses free and bound coagulases.

b) *N-3 strain.* A mutant obtained from the N-1 strain. It is free coagulase negative and bound coagulase positive and is streptomycin resistant (independent) (Kashiba *et al.*, 1959).

c) *N-4 strain.* A mutant isolated from the N-3 strain which is devoid of both coagulases (Kashiba *et al.*, 1959).

d) *Air-1 strain.* This strain was isolated in our laboratory. It lacks both coagulases and is non-pathogenic.

2) *Escherichia coli* B, *Klebsiella pneumoniae*, *Shigella flexneri* type 2a, *Salmonella typhosa* H 901W, *Salmonella paratyphi* 1015 and *Salmonella schottmuelleri* 8006, all well known strains kept in this Institute.

2. *Preparation of organ extracts*

Normal guinea pigs weighing 400-500 g were killed by exsanguination. The peritoneal cavity was opened, and the lungs, liver, spleen and kidneys were removed. The organs were well washed with saline, weighed, cut into small pieces and suspended in an equal weight of M/15 phosphate buffer at pH 7.0. The suspensions were homogenized in a Waring blender, and the homogenates were frozen and thawed twice and then centrifuged at 4000 rpm for 20 minutes at 5°C. The supernatant was again centrifuged at 15000 rpm for 30 minutes by a Servall high speed centrifuge at 5°C. The clear supernatant was sterilized by passage through a Millipore filter membrane. The filtrate was named the crude extract. Serial dilutions were made with M/15 phosphate buffer at pH 7.0.

3. *Assay method for antistaphylococcal activity*

An over night broth culture of staphylococci was diluted 1:50 and the new culture was incubated at 37°C, with shaking. After 3 hours the culture (1×10^9 viable units per ml) was diluted $1:10^6$ with 1:2 diluted broth containing 0.5 per cent gelatin. 0.2 ml aliquots of the bacterial suspension were distributed into small sterile metal capped tubes. An appropriate amount (usually 0.2 ml) of serial dilutions of the organ extracts was added to each tube. The total volume was adjust to 1.0 ml with M/15 phosphate buffer at pH 7.0. Two tubes containing 0.2 ml and 0.1 ml of the bacterial suspension were also included as controls to compare the number of colonies. The total volume was adjusted to 1.0 ml. All the tubes were incubated at 37°C for 2 hours. After incubation, 2 ml of melted nutrient agar (1 per cent agar) were added to each tube, and the contents mixed well and cooled. The surface of the solidified agar was covered with 0.5 ml of melted 1 per cent plain agar and the tubes cooled. They were then incubated at 37°C overnight. The number of colonies in the test tubes was compared with that in the two control tubes. When no decrease was observed and approximately 200 colonies were detected, the tube was given the value of 4. When the colony number was intermediate between the two control tubes (200 and 100), the value was expressed as 3. When approximately 100 colonies were found, the value was expressed as 2. When less than 100 colonies were found, it was expressed as 1.

EXPERIMENTAL

1. *Fate of intravenously injected pathogenic staphylococci in guinea pig*

Smith and Dubos (1956) stated that, after four days of intravenous injections into mice, the number of the pathogenic staphylococci decreased markedly in the

spleen and liver and that, on the contrary, they increased greatly in the kidney resulting in renal abscesses. We repeated their experiments in guinea pigs, because antibacterial substances in guinea pig leucocytes have been extensively studied in this laboratory and thus it is possible for us to differentiate antistaphylococcal substances from leucocytic antibacterial substances.

Ten healthy guinea pigs were injected intravenously with 0.1 ml of an actively growing broth culture (1×10^8 per ml) of the pathogenic N-1 strain. After 5 days the guinea pigs were sacrificed and various organs were removed aseptically. The organs were weighed and homogenized with equal weights of sterile M/15 phosphate buffer at pH 7.0 in a Waring blender. The viable units (clusters of cells) were estimated by plating. The results are shown in Table 1.

Table 1. Pathogenic staphylococci recovered from guinea pig organs 5 days after intravenous injection.

	Spleen	Liver	Kidney
Bacteria (units) found in 1.0 ml of organ homogenate	1.8×10^3	1.5×10^5	1.4×10^7

As can be seen, the ratio of viable units in the spleen to those in the liver and kidney was approximately 1:100: 10000. In the spleen the decrease in viable units was most marked. To exclude an error due to air-born contamination by micrococci, the experiment was repeated using the streptomycin-resistant strain N-3 and adding streptomycin to the agar medium used for measuring the viable count. The same results were obtained.

2. *Antibacterial action on pathogenic staphylococci of crude organ extracts*

Table 2. Antibacterial action of a crude spleen extract on pathogenic staphylococci (N-1)

	1	2	3	4	5	6	7	8	Control 1	Control 2
	ml	ml	ml	ml	ml	ml	ml	ml		
Crude spleen extract	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	—	—
Dilutions of extract	1:1	1:1	1:2	1:4	1:8	1:16	1:32	1:64		
M/15 phosphate buffer, pH 7.0	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.8	0.9
Bacterial suspension* (N-1)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1
After incubation at 37°C for 2 hrs., 2 ml of melted nutrient agar (1% agar) were added to each tube and the contents mixed well, and then incubated at 37°C overnight										
Number of colonies**	1	1	1	1	1	2	3	4	4	2

* The bacterial suspension contained ca. 1000 viable units per ml.

** The number of colonies was expressed as follows
4, ca. 200 colonies; 3, ca. 150 colonies; 2, ca. 100 colonies;
1, ca. 50 colonies or fewer

Table 3. Antibacterial action of crude liver, lung and kidney extracts on pathogenic staphylococci (N-1)

	1	2	3	4	5	6	7	8	9	Control 1	Control 2
	ml	ml	ml	ml	ml	ml	ml	ml	ml		
Crude liver extract	0.4	0.2	0.1	—	—	—	—	—	—	—	—
Crude lung extract	—	—	—	0.4	0.2	0.1	—	—	—	—	—
Crude kidney extract	—	—	—	—	—	—	0.4	0.2	0.1	—	—
M/15 phosphate buffer, pH 7.0	0.4	0.6	0.7	0.4	0.6	0.7	0.4	0.6	0.7	0.8	0.9
Bacterial suspension (N-1)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1
Results	2	4	4	4	4	4	4	4	4	4	2

Studies were made on the antibacterial action of crude spleen, liver, lung and kidney extracts on the pathogenic staphylococci strain N-1.

The crude extracts were prepared as described in the Methods section. The assay method used in this study was a modification of the plastic tray technique of Hirsch (1958), the details of which are described in Methods. The tube contents and results are shown in Tables 2 and 3.

The results indicate that the spleen extract was the most active of the organ extracts tested. The liver extract showed very weak but definite activity against pathogenic strain N-1. On the other hand kidney and lung extracts were completely inactive. As these findings are in good accord with the observations made by Smith and Dubos (1956), it can be assumed that the active substance shown in these experiments, plays a major role in the phenomena which they described.

3. *The physico-chemical nature of the antistaphylococcal substance*

1) *Salting out by ammonium sulfate*

To purify the substance the effect of salting out by ammonium sulfate was studied.

A crude extract of guinea pig spleen was divided by ammonium sulfate into four fractions, i.e. those precipitating at 0-25, 25-50, 50-75, 75-100 per cent saturation. Each precipitate was dissolved in a small amount of M/15 phosphate buffer at pH 7.0 and then dialyzed against saline containing M/75 phosphate buffer, pH 7.0, in the cold overnight, with constant shaking. The volume of the dialyzed solutions was adjusted to the original volume of the crude extract with M/15 phosphate buffer at pH 7.0. Each fraction was sterilized by passage through a Millipore filter membrane. The activity of each fraction was examined using pathogenic strains N-1, N-3 and N-4 and a nonpathogenic strain Air-1. The results are presented in Table 4.

The activity was found in the fraction precipitating between 25 and 50 per cent saturation of ammonium sulfate. The antistaphylococcal substance was effective on strains N-3 (free coagulase negative, bound coagulase positive and pathogenic), N-4

(free and bound coagulase negative and pathogenic) and the nonpathogenic Air-1 strain.

Table 4. Salting out of the antistaphylococcal substance by ammonium sulfate in a crude spleen extract

	1	2	3	4	5	6	7	8	Control 1	Control 2
	ml	ml	ml	ml	ml	ml	ml	ml		
0-25 per cent saturation	0.4	0.2	—	—	—	—	—	—	—	—
25-50 per cent saturation	—	—	0.4	0.2	—	—	—	—	—	—
50-75 per cent saturation	—	—	—	—	0.4	0.2	—	—	—	—
75-100 per cent saturation	—	—	—	—	—	—	0.4	0.2	—	—
M/15 phosphate buffer, pH 7.0	0.4	0.6	0.4	0.6	0.4	0.6	0.4	0.6	0.8	0.9
Bacterial suspension	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1
(Strain used)										
N - 1	4	4	1	1	4	4	4	4	4	2
N - 3	4	4	1	1	4	4	4	4	4	2
N - 4	4	4	1	1	4	4	4	4	4	2
Air - 1	4	4	1	1	4	4	4	4	4	2

The active substance in the liver extract was found in the same fraction as that of the spleen after ammonium sulfate fractionation. On the other hand, no active substance could be found even in 10 fold concentrations of fractions of kidney extract prepared from precipitates formed between 25 and 50 per cent saturation of ammonium sulfate. From these results it can be concluded that the active substance is a protein and is present in the spleen and liver but not in the kidney.

2) *Heat stability*

Though staphylococci are quite resistant to the bactericidal action of antibody and complement, studies were made on the requirement of the complement system for this antistaphylococcal activity. The experimental conditions and results are

Table 5. Heat stability of the antistaphylococcal substance in a crude spleen extract

	1	2	3	4	5	6	Control 1	Control 2
Crude spleen extract (0.2 ml)	un-treated	un-treated	heated at 56°C for 30 min.	heated at 56°C for 30 min.	heated at 70°C for 30 min.	heated at 100°C for 10 min.	—	—
Dilution of the extract	1:16	1:32	1:16	1:32	1:1	1:1		
M/15 phosphate buffer, pH 7.0	0.6	0.6	0.6	0.6	0.6	0.6	0.8	0.9
Bacterial suspension (N-1)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1
Results	2	3	2	4	4	4	4	2

shown in Table 5.

As can be seen, the antistaphylococcal activity was not affected by heating at 56°C for 30 minutes at neutrality but was inactivated by heating at 70°C for 30 minutes or at 100°C for 10 minutes at neutrality. Therefore the complement system is not required for antistaphylococcal activity.

3) *Isoelectric precipitation at pH 4.0*

When the pH of the crude spleen extract was adjusted to 4.0, a large precipitate formed. The supernatant was neutralized and centrifuged and its activity estimated. The precipitate formed at pH 4.0 were suspended in M/15 phosphate buffer at pH 7.0 and the pH of the solution was adjusted to 7.0. After mixing and centrifugation, the resulting supernatant was also tested.

Table 6. Isoelectric precipitation of the antistaphylococcal substance in a crude spleen extract at pH 4.0

	1	2	3	4	5	Control 1	Control 2
	ml	ml	ml	ml	ml	ml	ml
Preparation*	0.4 (A)	0.2 (A)	0.4 (B)	0.2 (B)	0.1 (B)	—	—
M/15 phosphate buffer, pH 7.0	0.4	0.6	0.4	0.6	0.7	0.8	0.9
Bacterial suspension (N-1)	0.2	0.2	0.2	0.2	0.2	0.2	0.1
Results	4	4	1	1	1	4	2

* Preparation A was obtained as follows: A crude spleen extract was adjusted to pH 4.0 and centrifuged. The supernatant was neutralized and the supernatant after centrifugation was used as Preparation A.

* Preparation B was obtained as follows: The precipitate obtained at pH 4.0 was suspended in M/15 phosphate buffer at pH 7.0 and the pH was adjusted to 7.0. After solution and centrifugation, the supernatant was used as Preparation B.

As shown in Table 6, the active substance was precipitated at pH 4.0 and could be brought into solution again at pH 7.0. This property is very useful for purification of the active substance.

4. *Cellular distribution of the active substance in the liver*

As liver consists mainly of liver cells and Kupffer's cells, these two kinds of cells were fractionated by the method of Kamahora *et al.*, (1954) and the antistaphylococcal activity of each examined.

10 guinea pigs were killed by exsanguination and their peritoneal cavities were opened. After cutting the hepatic vein, the liver was perfused with Lock's solution containing no calcium, at pH 7.4. Then the liver was weighed, cut into small pieces and suspended in 5 volumes of Robinson's solution. Homogenates were prepared in a Waring blender run at lowest speed for 4 minutes, and were filtered through 6 layers of gauze. The cell suspension was fractionated by differential centrifugation as shown in Table 7. Thus three cell fractions A, B and C were obtained. The cells of fraction A were mainly liver cells while fraction C was rich in Kupffer's cells. The yield of cells was

greatest in fraction A and least in fraction C. These cells were resuspended in 5.0 ml of chilled Robinson's solution. Extracts of these fractions were prepared as described above, and their antistaphylococcal activity assayed. The results are presented in Table 8.

Table 7. Differential centrifugation of liver cells

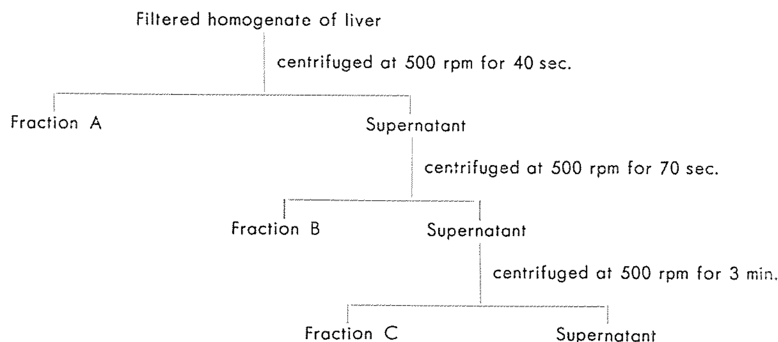

Table 8. Cellular distribution of the active substance in liver

	1	2	3	Control 1	Control 2
Extract of fraction (0.4 ml)	A	B	C	—	—
M/15 phosphate buffer, pH 7.0	0.4	0.4	0.4	0.8	0.9
Bacterial suspension (N-1)	0.2	0.2	0.2	0.2	0.1
Results	4	4	1	4	2

As can be seen, fraction A contained no antistaphylococcal activity, although the concentration of cells before extraction was highest in this fraction. To the contrary, fraction C rich in Kupffer's cells had activity although the cell concentration before extraction was lowest in this fraction. Therefore the Kupffer's cells contain the antistaphylococcal substance. Thus the cells which contain the active substance in the spleen may also be cells of RES.

5. *Antibacterial spectrum of the active substance*

The susceptibility of various gram negative bacteria to the antibacterial substance was examined.

Escherichia coli B, *Klebsiella pneumoniae*, *Shigella flexneri* type 2a, *Salmonella typhosa*, *Salmonella paratyphi* and *Salmonella schottmuelleri* were tested. The assay method used was the same as described above. The growth of flagellated bacteria was restricted to isolated colonies by the soft agar used in this experiment. Fractions of spleen extracts obtained by ammonium sulfate fractionation were used for the assay, as shown in Table 9.

E. coli B, *Klebsiella pneumoniae* and *Shigella flexneri* type 2a were found be susceptible to the active substance in the same fraction which was active with pathogenic staphylococci. It is very interesting, that salmonellae were insensitive to this substance, though the chemical composition of the somatic 0 antigen of these sensitive and insensitive enteric bacteria is quite similar.

Table 9. Antibacterial spectrum and salting out of the active substance in the crude spleen extract

	1	2	3	4	5	6	7	8	Control 1	Control 2
	ml	ml	ml	ml	ml	ml	ml	ml	ml	ml
0–25 per cent saturation $(NH_4)_2SO_4$	0.4	0.2	—	—	—	—	—	—	—	—
25–50 per cent saturation $(NH_4)_2SO_4$	—	—	0.4	0.2	—	—	—	—	—	—
50–75 per cent saturation $(NH_4)_2SO_4$	—	—	—	—	0.4	0.2	—	—	—	—
75–100 per cent saturation $(NH_4)_2SO_4$	—	—	—	—	—	—	0.4	0.2	—	—
M/15 phosphate buffer, pH 7.0	0.4	0.6	0.4	0.6	0.4	0.6	0.4	0.6	0.8	0.9
Bacterial suspension	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1
Strain used										
E. coil B	4	4	1	1	4	4	4	4	4	2
Kl. pneumoniae	4	4	1	1	4	4	4	4	4	2
Sh. flexneri	4	4	1	1	4	4	4	4	4	2
Sal. typhosa	4	4	4	4	4	4	4	4	4	2
Sal. paratyphi	4	4	4	4	4	4	4	4	4	2
Sal. schottmuelleri	4	4	4	4	4	4	4	4	4	2

6. *Effect of an extract of ascitic mononuclear cells of guinea pigs*

It was important to examine the activity of extracts of large mononuclear ascitic cells (histiocytes or monocytes), because these cells are macrophages, like the cells of RES which had been shown to contain the active substance.

Ascitic large mononuclear cells were collected from the peritoneal cavities of 25 guinea pigs, which had been 5 days previously administered intraperitoneally with 5 ml of 5 per cent broth. The peritoneal cavities were washed with saline containing 0.5 per cent sodium citrate and the cells were washed twice with saline. The cells were suspended at a concentration of 1×10^8 cells per ml in M/15 phosphate buffer at pH 7.0 and an extract was prepared by freezing and thawing the suspension 4 times. After centrifugation at 8000 rpm for 20 minutes, the clear supernatant was named extract A. Large mononuclear ascitic cells were also lyophilized and extracted with M/15 phosphate buffer at pH 7.0 after suspension at a concentration of 1×10^8 cells per ml. This supernatant after centrifugation was designated as extract B. The assay method was the same as described above.

Extracts A and B from the large mononuclear ascitic cells were completely devoid of antistaphylococcal activity. Therefore it can be assumed that the antistaphylococcal substance was present only in cells of RES and not in macrophages in general.

7. *Distinction of antistaphylococcal substance from normal staphylococcal agglutinins*

It was very important to exclude the possibility of an apparent decrease in the number of colonies due to agglutination by the staphylococcal agglutinins in normal sera and tissue fluids. There are three reasons for excluding this possibility.

The first reason is that mutual collisions of bacterial clusters causing agglutina-

tion are unlikely, because a cell concentration of 200 per ml was used in this assay method. Several authors, and especially Maaløe (1946) stated that at such a low concentration bacteria could not be agglutinated by addition of specific antibodies.

The second reason for excluding the possibility of agglutination was given by studies on the effect of normal guinea pig sera. A pool of fresh sera from 50 normal guinea pigs showed an agglutination titer of up to 1:1280 and this dropped to 1:64 after heat treatment at 56°C for 30 minutes. However, neither fresh nor heated sera exhibited antistaphylococcal activity. The agglutination titer of crude spleen extracts was also tested at dilutions of up to $1:10^4$. No agglutination could be detected. Therefore the apparent decrease in the number of colonies cannot be explained as being due to normal agglutinins in the spleen extract.

The third reason was obtained from an experiment on the clumping of bound coagulase positive and free coagulase negative staphylococci in rabbit plasma.

0.2 ml of varying dilutions (1:10, 1:20, 1:40 and 1:80) of fresh citrated rabbit plasma were added to tubes containing 0.6 ml of M/15 phosphate buffer at pH 7.0 and 0.2 ml of the staphylococcal suspension containing about 200 viable units. The medium of the bacterial suspension was prepared in the same way as for the assay of the antistaphylococcal activity. As a control, 0.2 ml of crude guinea pig spleen extract was used in place of the diluted rabbit plasma. Two further control tubes containing 0.2 (200 viable units) and 0.1 ml (100 viable units) of bacterial suspension and 0.8 and 0.9 ml of the phosphate buffer were also included. The tubes were incubated for 2 hours at 37°C and then treated in the same way as for assay of antistaphylococcal activity. The tube contents and results are presented in Table 10.

Table 10. Effect of rabbit plasma on the bound coagulase positive and free coagulase negative strain N-3

	1	2	3	4	5	6	Control 1	Control 2
	ml	ml	ml	ml	ml	ml	ml	ml
Diluted rabbit plasma (Dilution)	0.2 (1:10)	0.2 (1:20)	0.2 (1:40)	0.2 (1:80)	—	—	—	—
Crude spleen extract (Dilution)	—	—	—	—	0.2 (1:1)	0.2 (1:16)		
M/15 phosphate buffer, pH 7.0	0.6	0.6	0.6	0.6	0.6	0.6	0.8	0.9
Bacterial suspension (N-3)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1
Results	4	4	4	4	1	2	4	2

The results indicate that clusters of bound coagulase positive bacteria could not be clumped by the fibrinogen in fresh rabbit plasma, because no decrease in the number of colonies was observed. The clumping activity of rabbit plasma was demonstrated by the fact that clusters in a turbid suspension (1×10^{10} per ml) of bound coagulase positive staphylococci were clumped together by addition of 0.2 ml of a 1:10 dilution of the same rabbit plasma. Therefore a bacterial cluster had no chance to collide with other clusters to form aggregates even after all the cells in the suspension had been sensitized by the fibrinogen in the rabbit plasma. Nevertheless, the number of colonies definitely decreased when the spleen extract was mixed with 200 viable units.

These three reasons proved that the decrease in the number of colonies was not caused by normal agglutinins in the active extracts.

8. *Effect of aseptic peritonitis on the antistaphylococcal substance in guinea pig spleen*

After the leucocytes had been removed from guinea pig peritoneal cavities having aseptic inflammations the spleens, which had increased in weight, were tested. However, only a very weak activity could be demonstrated. It is very interesting that such a rapid change in concentration of the antistaphylococcal substance should occur when inflammation took place in some tissue of the body.

9. *Precipitation of the antistaphylococcal substance with ethyl alchol*

As far as the authors are aware there has only been one report on an antistaphylococcal substance extracted from human and bovine RES tissues (Nutini *et al.*, 1942, 1945). This active substance was soluble in 80 per cent (vol./vol.) ethanol and active against *Staphylococcus aureus* (pathogenic?) exclusively. It was not active against *Staphylococcus albus* (nonpathogenic?). Thus it was necessary to compare our substance with Nutini's substance.

40 ml of absolute ethanol were added to 10 ml of the spleen extract at −5°C and then the mixture was centrifuged at −5°C. The precipitate was dissolved in 5.0 ml of chilled M/15 phosphate buffer at pH 7.0 and dialyzed against the same buffer in the cold. The supernatant was concentrated to dryness *in vacuo* on a water bath in the way used by Nutini. The dried material was dissolved in 5.0 ml of chilled buffer and dialyzed against the same buffer in the cold. Two dialyzed solutions were adjusted to 10 ml with buffer. After sterilization by passage through a Millipore membrane, the antistaphylococcal activity was assayed. The results are shown in Table 11.

Table 11. Precipitation of the antistaphylococcal substance by ethanol

	1	2	3	4	5	6	7	8	Control 1	Control 2
	ml	ml	ml	ml	ml	ml	ml	ml		
Fraction*	A	A	B	B	B	B	B	B		
Volume	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	—	—
Dilution	1:1	1:1	1:1	1:2	1:4	1:8	1:16	1:32		
M/15 phosphate buffer, pH 7.0	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.8	0.9
Bacterial suspension (N-1)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1
Results	4	4	1	1	1	2	2	4	4	2

* Fraction A of the crude spleen extract was soluble in 80% ethanol and Fraction B was insoluble in 80% ethanol. These preparations were obtained as described in the text.

No antistaphylococcal activity could be found in the 80 per cent ethanol soluble fraction, in which Nutini detected an active substance from human and bovine spleen and brain. Moreover, our substance was active against pathogenic as well as nonpathogenic staphylococci. Therefore no substance similar to the antistaphylococcal substance of Nutini was present in guinea pig spleen.

DISCUSSION

It was important to see whether animal tissues contain some bacteriostatic or bactericidal substance which was active against pathogenic staphylococci. Since staphylococci can infect and invade some animals, it may be assumed that the tissues of susceptible animals do not possess any antistaphylococcal substance. However such a concept cannot explain the natural healing of staphylococcal infections in man. Therefore the present studies on an antistaphylococcal substance active against pathogenic staphylococci were made in animal tissues. First attempts were made to find such a substance in polymorphonuclear leucocytes of the guinea pig, an animal in which antibacterial substances have been extensively studied in this laboratory. However, no active substance was demonstrated in the leucocytes.

Our attention was turned to various organs, because Smith and Dubos (1956) described the very interesting phenomenon that staphylococci, injected intravenously into mice and trapped in the spleen and liver, showed a decrease in viable units while those in the kidney increased in viable units during the course of infection. As the antistaphylococcal activity of the spleen and liver was supposedly very weak, the most sensitive assay method had to be employed. For this purpose, the plastic tray technique of Hirsch (1958) was modified using small glass tubes in place of holes in a plastic tray. Using this assay method, a new antistaphylococcal substance was demonstrated in spleen and liver extracts of normal guinea pigs, and it was proved to be a protein. Though its activity was weak or undetectable when 1×10^8 viable units were tested with the active extract, it was definitely demonstrated when 200 viable units were tested. As some viable units of bacterial suspensions were composed of several cocci and moreover a single coccus had been divided by transverse septa into two or four cells, 200 viable units were equivalent to approximately 2000 viable cells. Nevertheless, the activity of the substance was far weaker than that of leucozyme A which is only active against nonpathogenic staphylococci. If the activity of the substance had been as strong as that of leucozyme A, all the staphylococci would have been nonpathogenic to guinea pigs.

It was also very important to see what kind of cells in the spleen and liver contained the antistaphylococcal substance. As the spleen contains many kinds of cells, liver cells were separated into three fractions by low speed differential centrifugation. One fraction, very rich in Kupffer's cells, was active. The fraction containing liver cells, which had the highest cell count of the three fractions, had no activity. Results suggest that in spleen also the active substance was present in cells of RES. On the antistaphylococcal substance in RES tissue only one report was published (Nutini *et al.*, 1942, 1945). Nutini found it in human and bovine brain and spleen. The nature of the active substance in guinea pig spleen differs from that of Nutini and no substance similar to Nutini's could be demonstrated in guinea pig spleen.

It might be that the decrease in the number of colonies was due only to agglutination by normal staphylococcal agglutinins in the active extracts, because, 1) the

activity of the antistaphylococcal substance was assayed by measuring the decrease in number of colonies, 2) the heat stability of the active substance was similar as that of the antibodies and 3) no other direct evidence could be gained on the death of the bacteria because of the few cells in the reaction mixture. However as described in the Experimental section, three indirect lines of evidence exclude this possibility. First, several authors, especially Maaløe (1946), have shown that, at as low a concentration as was used in this work, bacteria could not be agglutinated by homologous antibodies, because the sensitized bacteria had a negligible chance of collision to form agglutinates. Second, guinea pig sera did not contain the active substance although they contained staphylococcal antibodies capable of agglutinating turbid suspensions of the bacteria. Third, fresh rabbit plasma, capable of clumping a turbid suspension of bound coagulase positive and free coagulase negative staphylococci, did not decrease the number of bacterial colonies. Nevertheless the number of viable units in the bacterial suspension decreased when tested with an active spleen extract. From these reasons the spleen and liver extracts must contain a bactericidal substance active against pathogenic as well as nonpathogenic staphylococci.

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