PRODUCTION OF BACTERIOCINES BY GROUP A STREPTOCOCCI WITH SPECIAL REFERENCE TO THE NEPHRITOGENIC TYPES*

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It has been known for many years that some strains of *Escherichia coli*, *Bacillus megaterium*, and Group D streptococci secrete substances called colicines, or bacteriocines, which inhibit the growth of other strains of closely related microorganisms (1–6).

In 1949 Sherwood and his coworkers (7) reported observations on the inhibitory action of certain strains of beta hemolytic streptococci on other strains of streptococci. Seventeen of 61 strains of beta hemolytic streptococci inhibited the growth of one or more strains of Group A and non–Group A streptococci. Five of 29 strains of Group A streptococci had this inhibitory effect, but the serologic types of these strains were not stated.

In this paper the inhibitory action of Group A streptococci on other strains of Group A and non–Group A streptococci was investigated. It was found that these inhibitory substances are produced more frequently by the nephritogenic types than by other serologic types of Group A streptococci.

Materials and Methods

Strains of Streptococci.—Strain Type 18 (T18W) isolated during the Westinghouse Valley rheumatic fever outbreak was kindly supplied by Dr. Max C. Moody and strain Type 25 (T25-41) by Dr. John B. Zabriskie.

Strains of the nephritogenic types and strains isolated from patients with rheumatic fever were obtained from The Rockefeller University collection and from the Communicable Disease Center, Atlanta. Other strains were isolated at the Streptococcal Disease Laboratory, Department of Pediatrics, Sinai Hospital of Baltimore. Strains of various non–Group A streptococci were supplied by Dr. Rebecca C. Lancefield.

Grouping and typing sera were obtained from Dr. Lancefield and Dr. Moody.

Broth.—Difco bacto brain heart infusion supplemented with 0.5% yeast extract and 10% horse serum.

Agar.—Difco bacto brain heart infusion supplemented with 1.1% agar, 0.5% yeast extract and 10% horse serum.

Ultraviolet Light.—Agar plates were exposed for 20 to 40 sec at a distance of 30 cm to a 15 watt General Electric Germicidal lamp.

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Optical Density.—Optical densities of bacterial suspensions were read at 650 μ in a Coleman junior spectrophotometer. Standard 10 × 75 mm tubes were used for all determinations.

EXPERIMENTAL

Group A streptococcal bacteriocines were first detected in the course of bacteriophage studies. A Group A streptococcus Type 18, T18W, isolated from

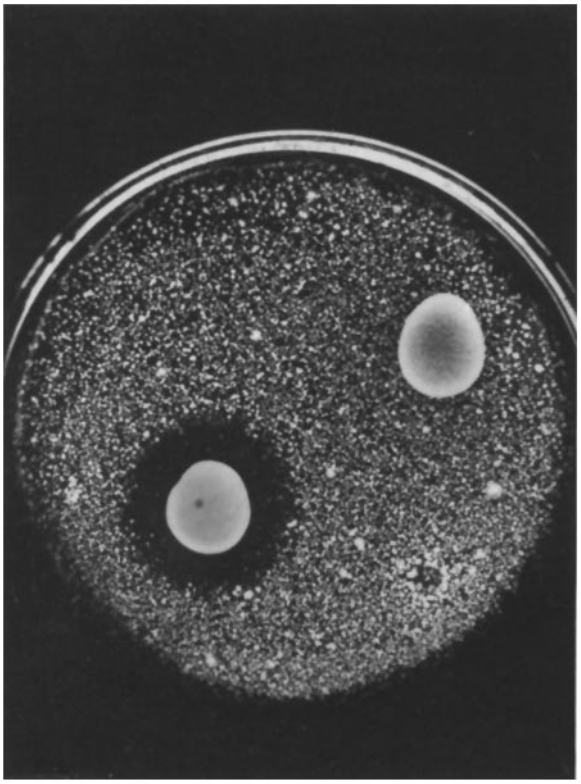

FIG. 1. T18W lawn UV 20 sec. Growth of lawn inhibited by drop of T18W. Drop of bacteriophage indicator strain negative.

a patient with acute rheumatic fever, was investigated for bacteriophage with techniques similar to those employed by Zabriskie (8).

The T18W strain was grown for 4 hr in serum broth. 1 ml of this culture was placed on each of 2 serum agar plates. The plates were rotated to obtain a uniform bacterial lawn. The excess fluid was removed with a pipette and the plates dried in the incubator for 20 min. One plate was exposed to ultraviolet light for 20 sec, the other plate was not irradiated.

Drops of known bacteriophage-sensitive strains as well as drops of the

homologous T18W strain were placed on different segments of both plates. The drops were allowed to dry and the plates were then incubated overnight in a candle jar.

On the following day none of the drops of the known bacteriophage-sensitive strains on either plate showed plaques indicative of bacteriophage. Likewise no plaques were visible in the drop of the T18W strain. However on the irradiated plate, the drop of the T18W strain was surrounded by a well defined, clear area in which the growth of the T18W lawn was completely inhibited (Fig. 1).

The initial experiment showed that the T18W strain produced inhibitory substances which diffused into the agar and prevented the growth of the homologous T18W lawn. Attempts were made to transfer these inhibitory substances by emulsifying a block of agar cut out from the clear zone and placing drops of this emulsion on an irradiated T18W lawn. The plate was incubated overnight. On the following day no zones of inhibition were observed. These findings suggested that the inhibitory substances produced by the T18W strain resembled the well known colicines and bacteriocines produced by many different kinds of microorganisms. These inhibitory substances vary depending on the nature of the bacteria from which they are derived, but it is generally agreed that their chief characteristics are similar.

Effect of T18W Strain on Lawns of Heterologous Group A Streptococci.—Bacterial lawns were prepared with a number of strains of Group A streptococci to investigate whether the T18W bacteriocines were active against heterologous strains. Only one strain, serologic Type 25, T25-41, proved susceptible. After overnight incubation drops of T18W on an irradiated T25-41 lawn were surrounded by well demarcated area in which the growth of the T25-41 lawn was completely inhibited.

In the initial studies, it was thought that exposure of the indicator lawns to ultraviolet light was essential for the demonstration of bacteriocine activity. Subsequently, however, it was found that the chief effect of irradiation was to reduce the density of the bacterial lawn. Equally good zones of inhibition were obtained without exposure to ultraviolet light, if young broth cultures of the indicator strains with an OD of 0.10 to 0.12 were diluted 10^{-4} and 1.5 ml of this dilution inoculated on the surface of the agar plate. Thus, 2 strains, T18W and T25-41, were available which could serve as indicator lawns to test the production of bacteriocines by other strains of Group A streptococci.

The Production of Bacteriocines by Group A Streptococci.—Strains of different serologic types of Group A streptococci were tested. Drops of young broth cultures of the strains were placed on lawns of the 2 indicator strains, T18W and T25-41. The drops were allowed to dry and the plates incubated overnight in a candle jar. On the following day bacteriocine production was indicated by clear zones surrounding the drops in which the growth of the indicator lawns was inhibited. The results on the two indicator lawns were identical.

In a preliminary survey, 39 strains of Group A streptococci isolated from a variety of sources, were examined. Seventeen different serologic types were represented. Twenty-one of these 39 strains produced bacteriocines and 18 did not. In most of the 17 types both bacteriocine-positive and bacteriocine-nega-

TABLE I

Bacteriocine Production by Group A Streptococci

Clinical status	Production of bacteriocines			
	Serologic type	No. of strains		Positive/ total
		Positive	Negative	
Acute glomerulonephritis	12	18	0	32/32
	4	6	0	
	49	8	0	
Uncomplicated streptococcal pharyngitis	12	11	1	16/18
	4	5	1	
	1	1	4	
	2	1	3	
	3	1	4	7/28
	5	2	5	
	6	2	3	
	11	0	1	
	13	0	1	
Rheumatic fever	3	1	1	
	5	0	5	
	6	2	0	
	13	0	1	4/16
	14	0	1	
	18	1	0	
	19*	0	1	
	24*	0	1	
	32*	0	1	
	36*	0	1	

* Types 19, 24, 32, and 36 were epidemic strains isolated from outbreaks of pharyngitis followed by multiple cases of rheumatic fever.

tive strains occurred. It was noted, however, that the nephritogenic types 12, 4, and 49 produced bacteriocine more consistently than other types. Therefore, 32 strains of these types, isolated from patients with acute glomerulonephritis, were investigated. Of these 32 strains 18 were identified as Type 12, 6 as Type 4, and 8 as Type 49. Without exception these 32 strains produced bacteriocines

(Table I). The inhibitory zones produced by strains of the nephritogenic Types 12, 4, and 49 on a lawn of indicator strain T25-41 are illustrated in Fig. 2.

Forty-six strains of Group A streptococci isolated from children with uncomplicated streptococcal pharyngitis were also studied. Of these 46 strains, 12 strains were identified as Type 12, and 6 as Type 4. The remaining 28 strains

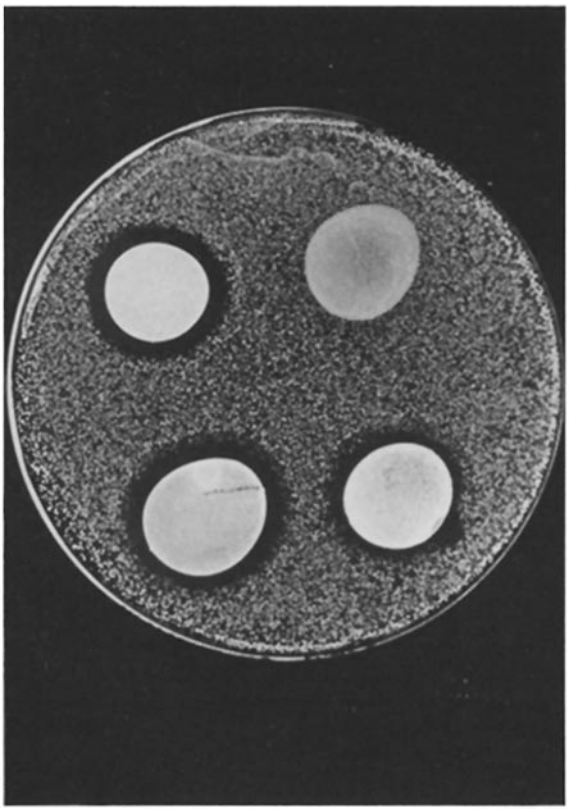

FIG. 2. Drops of nephritogenic types 12, 4, and 49 on a lawn of indicator strain T25-41 produced typical zones of inhibition. Strain T5 Tritt was negative.

included 7 different types other than Types 12 and 4. The 18 strains of Types 12 and 4 with only two exceptions produced bacteriocines. Of the 28 strains of the other types, 7 produced bacteriocines and 21 did not (Table I).

Bacteriocine production by 16 strains of Group A streptococci isolated from patients with acute rheumatic fever, representing 10 different serologic types, was also investigated. Four of these 16 strains produced bacteriocine and 12 did not (Table I).

These findings indicated that bacteriocine production by the nephritogenic

Types 12, 4, and 49 was a consistent characteristic of strains isolated from patients with acute glomerulonephritis. Strains of Types 12 and 4 isolated from children with uncomplicated streptococcal pharyngitis also produce bacteriocine frequently with only occasional exceptions.

On the other hand, among strains of Group A streptococci isolated from patients with acute rheumatic fever, bacteriocine production occurred infrequently. The frequency was the same as that observed in children with uncomplicated streptococcal pharyngitis due to types other than Type 12 and 4.

TABLE II

Bacteriocine Production by Non-Group A Streptococci

Serologic group	Production of bacteriocine		
	No. of strains		Positive/total
	Positive	Negative	
B	3	5	3/8
C	5	2	5/7
D	2	2	2/4
E	0	3	0/3
F	0	3	0/3
G	5	0	5/5
H	3	0	3/3
L	1	2	1/3
M	1	2	1/3
N	1	2	1/3
O	1	2	1/3
Total.....	22	23	22/45

Bacteriocine Production by Non-Group A Streptococci.—Bacteriocine production by streptococci belonging to groups other than Group A was also investigated. It was found that some strains of the groups listed in Table II produced bacteriocine which inhibited the growth of the group A indicator strains. T18W and T25-41. Three strains of Group E and three strains of Group F were negative.

Thus, it appears that bacteriocine production is a common characteristic not only of Group A streptococci but also of non-Group A streptococci. It, therefore, is unlikely that bacteriocines play a role in determining the pathogenicity of Group A streptococci for human beings.

Indicator Strains.—Indicator strains susceptible to streptococcal bacteriocine are uncommon. The susceptibility of 2 indicator strains, T18W and T25-41 employed throughout these studies, remained essentially the same during a 10 month period. During the course of these studies only one other strain,

Johnson, kindly identified by Dr. Max D. Moody as a Type 9 by the slide agglutination technique, was susceptible to streptococcal bacteriocine. However, with this strain the zones of inhibited growth were poorly defined as compared with the standard indicator strains, T18W and T25-41. The Johnson strain, therefore, was not used as a routine.

Bacteriocine-producing strains killed only a limited number of the microorganisms in the indicator lawn. If the lawn was too dense, no inhibitory effect was observed. Well defined zones of inhibition were obtained if the amount of growth was reduced either by exposure to ultraviolet light, or by preparing the lawn with a diluted culture to obtain approximately equivalent density. These results are summarized in Table III and are illustrated in Figs. 3, 4, and 5.

TABLE III

Effect of Bacteriocine on Indicator Lawns as Related to Density of Growth

Bacteriocines	Bacterial lawn: seed culture*		
	Undiluted confluent growth	Exposed to UV discrete colonies	Diluted 10^{-4} discrete colonies
	Zones of inhibition		
	mm	*mm*	*mm*
Bacteriocine T18W‡	0	6	8
T4	0	7	8

* 1.5 ml/plate of 5 hr broth culture OD 0.12.

‡ Same strain used for the indicator lawn.

Bacteriocine Production.—Although strains susceptible to bacteriocine and suitable to serve as indicators were uncommon, many strains of Group A streptococci produced bacteriocines, and in most instances it was a stable characteristic. Occasionally a strain maintained for several weeks in a stock culture of blood broth, no longer produced bacteriocine. Variations in bacteriocine production were also noted in strains isolated at different times from the same child following uncomplicated streptococcal pharyngitis treated with penicillin. In some cases the initial culture obtained during the acute stage did not produce bacteriocine. However, if the streptococci were not eliminated and a positive culture of the same serologic type was obtained on the 10th day, the second culture sometimes showed bacteriocine production. The reverse also occurred: the initial culture was bacteriocine positive and the second negative.

Properties of Group A Streptococcal Bacteriocines.—Strains to be tested for bacteriocine production were grown in broth containing 10% horse serum for 4 to 5 hr. Rapid vigorous growth was essential. Once a good growth was obtained, longer incubation usually did not increase bacteriocine production. The

most clearly defined and largest inhibitory zones were obtained if both the bacteriocine-producing strain and the indicator lawn were actively growing in the logarithmic phase.

Bacteriocine present in whole young broth cultures diffused readily into agar.

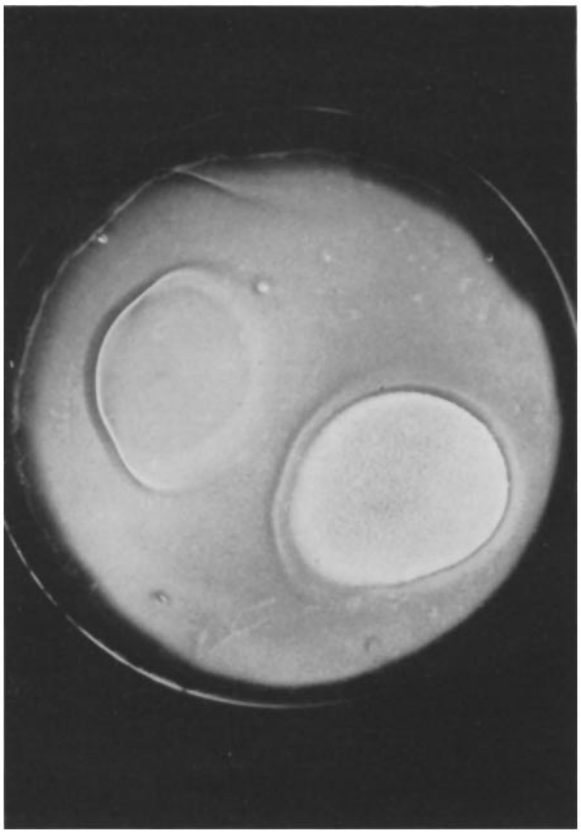

FIG. 3. T18W lawn, seed culture undiluted, drops of T18W and T4 did not cause inhibition.

If the broth cultures were centrifuged, the supernates had no inhibitory effect. Drops of the resuspended sediment, however, placed on the indicator lawn and incubated overnight caused inhibition only as the result of active growth. Active material could not be separated from the microorganisms by repeated washings. Bacteriocine was not liberated by the disintegration of the bacteria by freezing and thawing, by sonification of the bacterial suspension or by the lytic action of Group C phage associated enzyme.[1]

[1] This enzyme was kindly supplied by Dr. John B. Zabriskie.

Heating young active broth culture to temperatures of 50 to 56°C, which were sufficient to kill the microorganisms or delay their growth, rendered them inactive. Cultures killed with chloroform likewise showed no activity. Tryptic digestion of suspensions of bacteriocine-producing strains did not inhibit

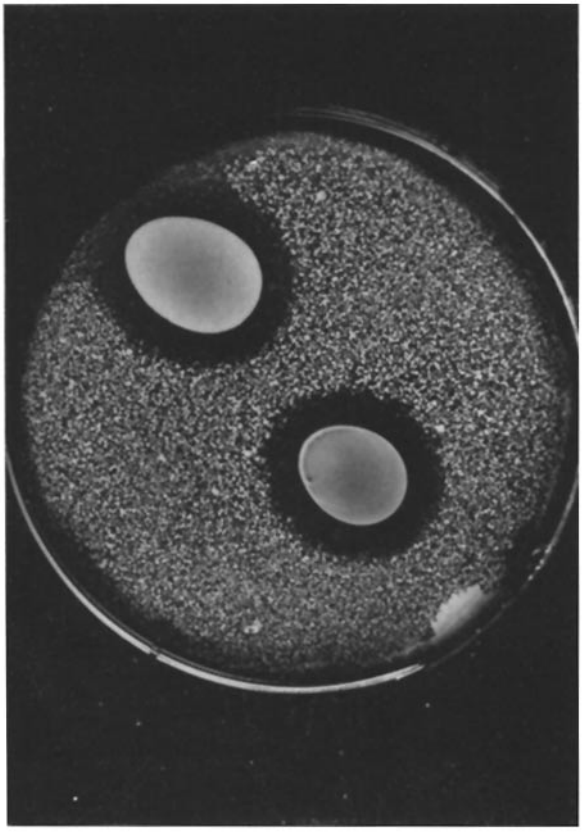

FIG. 4. T18W lawn; exposed to UV for 20 sec. T18W and T4 produced zones of inhibition.

bacteriocine production because the bacteria were not killed, and grew vigorously when placed on the indicator lawn.

DISCUSSION

In 1953, a significant advance was made in our understanding of the epidemiology of acute glomerulonephritis by Rammelkamp and Weaver (9). These investigators showed that only a small proportion of the 50 known serologic types of Group A streptococci have nephritogenic properties. These findings not only clarified the epidemiology of nephritis, but also stimulated investigations

of these special types. Numerous studies were undertaken to determine whether the nephritogenic types, chiefly Types 12 and 4, had special characteristics which might explain their association with nephritis and differentiate them from other types of Group A streptococci.

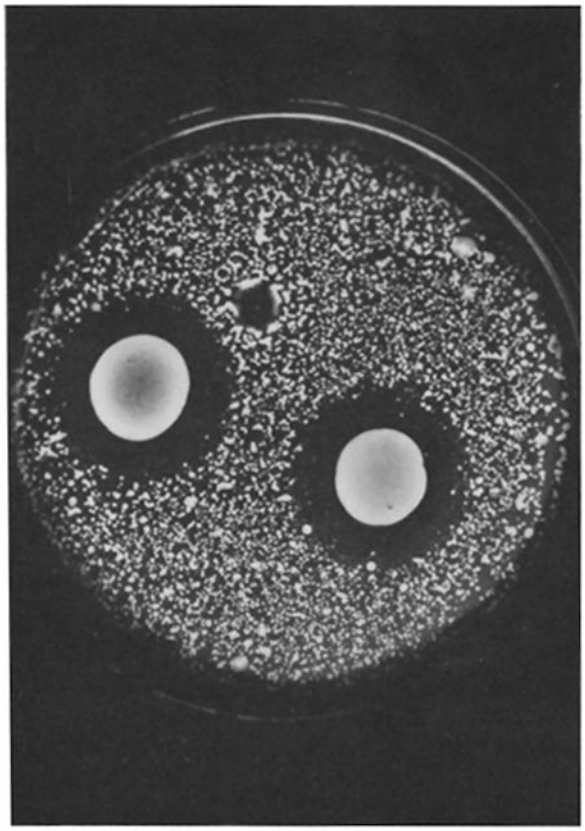

FIG. 5. T18W lawn seed culture diluted 10^{-4}: T18W and T4 produced zones of inhibition.

Although the importance of the nephritogenic types is generally recognized, it is apparent that different strains of these special types vary greatly. In the general population pharyngitis due to Type 12 is extremely common. Yet only a small proportion of these infections are followed by nephritis. Infections due to Type 4 also are not always followed by nephritis (10). In an outbreak of 32 cases of pharyngitis due to streptococcus Type 4, observed in a convalescent home for rheumatic children, none of the patients developed nephritis or rheumatic recurrences, suggesting that this particular strain of Type 4 lacked both nephritogenic and rheumatogenic properties.

In 1956 Hardin et al. (11) studied the production of streptolysins O and S, streptokinase, hyaluronic acid, hyaluronidase, proteinase, and erythrogenic toxin by 14 strains of the nephritogenic Types 12 and 4 isolated from patients with acute glomerulonephritis. These investigators were unable to detect any significant differences between these strains and other strains isolated from patients with rheumatic fever, uncomplicated streptococcal pharyngitis, or from healthy carriers.

In 1957 Wilson (12) reported a new and interesting property of hemolytic streptococci. He found that following phagocytosis of certain strains of Group A streptococci, human leukocytes disintegrated rapidly while with other strains the leukocytes remained intact. Some strains of Group C and Group G had a similar effect on human leukocytes. Streptococci, therefore, were classified as leukotoxic or nonleukotoxic.

Wilson studied the leukotoxicity of strains of the nephritogenic Types 12 and 4. Eleven strains of Type 12, isolated from nephritic patients were leukotoxic as were 12 strains of Type 12 isolated from other sources. With strains of Type 4 leukotoxicity was variable. Of 5 strains of Type 4 isolated from nephritic patients, 2 strains were leukotoxic and 3 were not. Leukotoxic strains of types not associated with nephritis also occurred. Five strains of Type 6 were all leukotoxic. Of 9 strains of Type 3, 7 were leukotoxic and 2 were not. The nephritogenic types, therefore, could not be differentiated from other serologic types of Group A streptococci on the basis of this property.

Lazarides and Bernheimer in 1957 (13) investigated the production of diphosphopyridine nucleotidase, DPNase, by different serologic types of Group A streptococci. They reported that strains of Types 3, 4, 6, and 12 frequently produced DPNase, whereas with strains of Types 1, 5, 14, 19, and 24 DPNase production was rarely observed.

Further studies by Bernheimer, Lazarides, and Wilson (14) showed that there was a striking correlation between leukotoxicity and DPNase production. Of 22 strains of Types 12 and 4, which produced DPNase only 2 strains were not leukotoxic.

In this paper the production of colicinelike substances, bacteriocines, by Group A streptococci is described. Strains of different serologic types were investigated. It was found that bacteriocine production occurred more commonly and consistently with strains of the nephritogenic Types 12, 4, and 49, than with other types. The possible correlation of this property with leukotoxicity and DPNase production was not studied.

Thirty-two strains of Types 12, 4, and 49 isolated from patients with acute glomerulonephritis uniformly produced these inhibitory substances (Table I). Strains of Types 12 and 4 isolated from children with uncomplicated streptococcal pharyngitis also produced bacteriocines frequently: among 18 strains of Types 12 and 4, there were only two exceptions.

Among strains of serologic types isolated from patients with acute rheu-

matic fever, or from children with uncomplicated streptococcal pharyngitis, bacteriocine production was uncommon. In these types both bacteriocine-positive and bacteriocine-negative strains occurred. The number of bacteriocine-negative strains, however, outnumbered those producing bacteriocines.

Bacteriocine production by non–Group A streptococci was also studied (Table II). Many strains of these groups inhibited the growth of Group A strains used as indicators to detect bacteriocine production by Group A strains. Non–Group A strains, therefore, appear to produce inhibitory substances similar to those produced by Group A streptococci. In most of the groups of non–Group A streptococci, bacteriocine-positive and bacteriocine-negative strains occurred with approximately equal frequency.

SUMMARY

Colicinelike substances, bacteriocines, are produced by many strains of Group A and non–Group A streptococci. Bacteriocines were detected by their inhibitory action on the growth of 2 Group A strains which served as indicators.

Streptococcal bacteriocines were demonstrable on agar plates only if the bacteriocine-producing strain and the indicator lawn were alive and actively growing. The inhibitory substances diffused slowly into the agar during the course of growth. Bacteriocines were not replicated in the zones of inhibition in which the microorganisms of the indicator strains were killed. Attempts by various methods to separate bacteriocines from living bacteria were unsuccessful.

In contrast to the many strains which produced bacteriocines, only a few strains of Group A streptococci were suitable to serve as indicators for bacteriocine production. Strains of Group A streptococci of the nephritogenic types 12, 4, and 49 produced bacteriocines most consistently. Strains of other types, isolated from patients with acute rheumatic fever or from children with uncomplicated streptococcal pharyngitis, produced bacteriocines infrequently. Among non–Group A streptococci bacteriocine-positive and bacteriocine-negative strains occurred in approximately equal numbers.

It was shown by Wilson and by Bernheimer that the majority of the strains of the nephritogenic types were leukotoxic and produced DPNase. Bacteriocine production is another common characteristic property of these special types.

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