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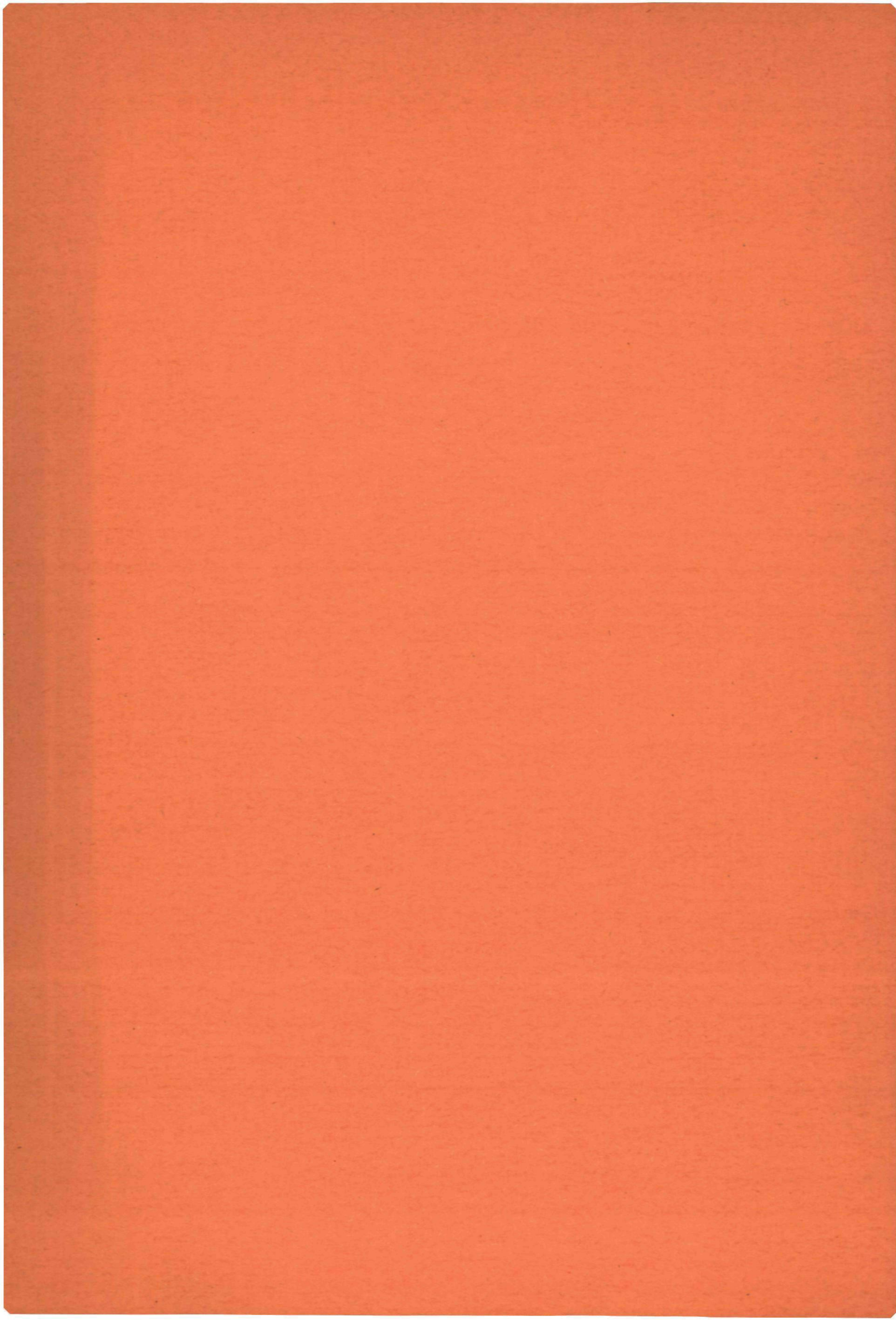
BACTERIOCINS OF STAPHYLOCOCCI

ANTONIUS MARINUS JETTEN



BACTERIOCINS OF STAPHYLOCOCCI

PROMOTOR: DR. IR. G. D. VOGELS

BACTERIOCINS OF STAPHYLOCOCCI

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
DE RECTOR MAGNIFICUS PROF MR F J F M DUYNSTEE
VOLGENS HET BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 8 JUNI 1973
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AAN MIJN OUDERS,
AAN ELS

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CHAPTER I

INTRODUCTION

General consideration

The inhibition of growth of one bacterial strain by another was observed for the first time in 1877 by Pasteur and Joubert (1) Later on many investigators (2) observed antagonism between bacterial strains of many species Jacob *et al* (3) defined the group of antagonistic, bactericidal substances of high-molecular weight, produced by bacteria and active against bacteria, as bacteriocins These substances are distinct from lytic enzymes and phages, and were classified on basis of the specificity of their action (4, 5, 6) Extensive studies on bacteriocins, especially those produced by *Escherichia coli*, have revealed many intriguing features, which may contribute to the elucidation of various biochemical processes such as active transport, protein synthesis, membrane synthesis and DNA-replication

The present introduction will focus on two aspects of bacteriocin studies, namely, mode of action and genetics Various aspects of bacteriocins are reviewed (7, 8, 9, 10) and monographed (11, 12) previously

MODE OF ACTION

The mode of action of a bacteriocin, namely colicin E1, which is produced by *Escherichia coli* ML, was studied for the first time by Jacob *et al* (13) Later on this study was extended by Nomura (14, 15) and Luria (16) Since that time studies on the mode of action of various bacteriocins have revealed that quite different biochemical effects are exerted on sensitive bacteria In Table I the general effects exerted by the three main types of bacteriocins are summarized

What is the primary target of the bacteriocins?

Bacteriocins of type I The general characteristics of their mode of action given in Table I indicate that bacteriocins of type I affect probably the energy metabolism and/or transport phenomena How are these processes influenced by these bacteriocins?

An effect on the energy metabolism was obvious from the observed decrease of the cellular ATP level which was supposed to be caused by an activation of membrane-bound ATPase by colicin E1 (43) However in cells treated with colicin E1 in the presence of dicyclohexylcarbodiimide, an inhibitor of this ATPase, the ATP level did not decrease, while the inhibition of macromolecular syntheses and leakage of potassium ions still occurred (43) So, the fall of the ATP level in colicin E1 treated cells seems to be a secondary effect

Since cells of *Escherichia coli* are resistant to colicins E1 and K under strict anaerobic conditions (16, 19), it was suggested that the electron transport chain and/or oxydative phosphorylation are primarily affected This assumption can explain why the transports of amino acids and of methylthio ß-D-galactoside are inhibited by these bacteriocins, but not that of α-methyl-D-glucoside (18, 44) The latter is mediated by a phosphoenolpyruvate-dependent phosphotransferase system in *Escherichia coli* while recent studies (45) show that the former transport systems may be energized by a proton gradient and/or electrical potential built up by the electron transport chain or internal ATP These bacteriocins may act like some uncouplers, such as 1,2-dinitrophenol, by fostering proton permeability what results in the collapse of the proton gradient However, colicins A and E1 do not promote proton permeability (22, 43) Alternatively, these bacteriocins cause dissipation of the electrical potential as a result of the development of ionpermeabilities other than that of protons However, the knowledge of the effect on ion-permeabilities is rather scarce The type I bacteriocins cause a rapid efflux of potassium or rubidium ions accumulated in the cells (46, 47) and recently Lusk and Nelson (48) showed that colicins E1 and K cause also an

Table 1 General effects exerted by the three main types of bacteriocins

Type	Bacteriocin	Producing strain	Ref	General characteristics of mode of action
I		Gram negative		
	Colicin E1	*Escherichia coli* ML	13, 14, 15, 16, 17, 18, 19	a) Simultaneous inhibition of DNA, RNA and protein syntheses b) No extensive degradation of nucleic acids c) Inhibition of active transport d) No direct cell lysis e) No or limited effect on respiration f) Decrease of cellular ATP level g) Inhibition of motility h) Phage replication inhibited
	Colicin A	*Citrobacter freundii* CA31	20, 21, 22, 23	
	Colicin B	*Salmonella typhimurium* LT2	24	
	Colicin Ia	*Escherichia coli* CA53	25	
	Colicin Ib	*Escherichia coli*	25	
	Colicin K	*Escherichia coli* K235	14, 17, 18, 19, 26	
	Colicin S8	*Shigella sonnei*	27	
	Marcescin JF 246	*Serratia marcescens*	28	
	Pyocin	*Pseudomonas aeruginosa*	29, 30	
		Gram-positive		
	Megacin Cx	*Bacillus megaterium* KM	31	
	Staphylococcin 1580	*Staphylococcus epidermidis* 1580	22, 23, 32	
II		Gram-negative		
	Colicin E2	*Escherichia coli* K12	14,15,17,33,34,35,36	a) Rapid inhibition of DNA and RNA syntheses b) Inhibition of protein synthesis after a lag period c) Extensive degradation of DNA but limited effect on RNA d) Induction of prophages in lysogenic strains e) No effect on oxydative phosphorylation, active transport and respiration
	Pesticin P1	*Pasteurella pestis*	37	
	Vibriocin	*Vibrio comma*	38	
		Gram-positive		
	Megacin C	*Bacillus megaterium*	39,40	
III		Gram-negative		
	Colicin E3	*Escherichia coli* K12	14,15,17	a) Immediate inhibition of protein synthesis b) DNA and RNA syntheses continue for a long time c) No effect on respiration and active transport
	Colicin D	*Escherichia coli* K12	41	
	Cloacin DF 13	*Enterobacter cloacae* DF13	42	

increased efflux of magnesium ions but these actions seem to be secondary ones

Although type I bacteriocins uncouple active transport from energy supply, the exact mechanism of action is yet unknown. A direct interaction between components of the membrane and the bacteriocins may induce conformational changes (49) and/or alterations of membrane composition (50) and affect the transport systems in such a way.

Bacteriocins of type II. Colicin E2 appears to affect primarily chromosomal DNA causing its extensive degradation (Table I). This degradation can be distinguished into three stages involving single-strand cleavage of the DNA-duplex, degradation of DNA into double-stranded fragments and further degradation of these fragments by exonucleolytic action (51, 52, 53, 54).

Since colicin E2 itself has no nucleolytic activity (55, 56) two mechanisms are proposed to explain DNA degradation. Either colicin E2 activates directly one or more nucleases or it interferes with DNA or the DNA-membrane complex rendering them susceptible to nucleases present in the cell (52). Evidence for the first possibility was brought about by Almendinger and Hager (55) who showed that colicin E2 initiates the movement of periplasmic endonuclease I to the membrane or into the cytoplasm. Otherwise, Beppu and Arima showed that colicin E2 causes dissociation of DNA-membrane complexes (57, 58) without degrading DNA (59). Furthermore, addition of colicin E2 to DNA resulted in a decrease of DNA melting temperature (60). All results may be interpreted as a direct physicochemical reaction of colicin E2 with DNA causing distortion of the DNA molecule (59, 60). However, the interaction of colicin E2 with DNA may also be an aspecific effect like the action of ribonuclease on DNA (61).

Both mechanisms mentioned above suppose the involvement of nucleases in the DNA degradation. Obinata and Mizuno (51) concluded that endonuclease I was not involved, since colicin E2-induced breakdown proceeded normally in an endonuclease I-negative mutant in contrast to the results of Saxe and LURIA (62) and Almendinger and Hager (55). The involvement of endonuclease III was also suggested (52) since no degradation occurs in the presence of DNP, however, in endonuclease III-negative mutants degradation proceeded normally (53).

Recent results with a new class of mutants, which lost their colony-forming ability upon colicin E2 treatment without degrading DNA or inhibition of DNA synthesis, revealed that colicin E2 probably affects primarily cell division and not DNA synthesis (63).

Bacteriocins of type III. The general characteristics of the mode of action indicate that colicin E3 inhibits specifically protein synthesis (Table I). How does colicin E3 affect protein synthesis? Treatment of sensitive cells of *Escherichia coli* renders inactive ribosomes which still can bind mRNA but have a markedly reduced binding of tRNA (64, 65, 66). Experiments with hybrid ribosomes, reconstituted of subunits obtained from treated and untreated cells, revealed that the defect resides in the 30 S subunit. Polyacrylamide gel electrophoresis and microdensitometer profiles of proteins from untreated and colicin E3-treated ribosomal subunits were identical (67, 68), and reconstitution of the 30 S subunits from 16 S rRNA's and ribosomal proteins indicated that the defect is located in the 16 S rRNA. This defect was also demonstrated by a reduced sedimentation coefficient of the 16 S rRNA from ribosomes of treated cells (designated as E3-16 S rRNA) with respect to the control 16 S rRNA (67). Upon polyacrylamide gel electrophoresis the E3-16 S rRNA moved faster than the control 16 S rRNA (68). Furthermore, a small RNA fragment (designated as E3-fragment rRNA) could be detected that was absent in the untreated preparation. These results indicate that colicin E3 causes cleavage of the 16 S rRNA. Fingerprint analyses of the 16 S, E3-16 S, and E3-fragment rRNA revealed that the E3-fragment is detached from the 16 S rRNA at a position near the 3'-terminus and contains about 50 nucleotides (67, 68).

Colicin E3 can cleave the 16 S rRNA only when this rRNA is an integral part of the 70 S ribosome. This suggests that this bacteriocin requires a specific ribosome conformation in order to be active (69, 70, 71, 72). Three mechanisms were proposed (73) to explain the cleavage reaction, a) colicin E3 activates a ribonuclease which is bound to the ribosome, b) colicin E3 itself is a ribonuclease, c) colicin E3 becomes a ribonuclease in conjunction with ribosomal proteins. Recent studies of Meyhack et al. (73) showed that RNAses I and II are not involved in colicin E3 action and they concluded that colicin E3 itself is or becomes a ribonuclease when attached to ribosomes. This interpretation may explain the low specificity of colicin E3 action since ribosomes of an *Azotobacter* species and

of *Bacillus stearothermophilus* are also inactivated by colicin E3 (74)

Like colicin E3, cloacin DF 13 inhibits protein synthesis specifically Ribosomes from cloacin-treated cells were unable to support protein synthesis in a cell-free system under direction of **phage MS_2-RNA** but appear unable to bind fMet-**$tRNA^{fMet}$ (75).**

Adsorption and penetration

After several authors (76, 77, 78, 79) had shown that bacteriocins, like bacteriophages, are adsorbed to specific receptor sites on the cell surface of bacteria, the question arose whether bacteriocins penetrate into the cytoplasm after adsorption or remain at the receptor site Until recently it was generally believed that bacteriocins do not penetrate into the cytoplasm This idea was mainly based on the observation that trypsin can rescue cells long after they had adsorbed bacteriocins This holds for colicins K (26), E1 (80, 81) and colicins E2 and E3 (56, 80, 82) According to a general model derived from these results (14, 15, 56), the bacteriocin stays at the receptor site and affects the target indirectly by a specific stimulus mediated by a specific transmission system probably located in the membrane Changeux and Theiry (83) proposed that the transmission system involves conformational transitions of the membrane, which are induced by bacteriocins

Recent studies indicate that some bacteriocins penetrate into the cytoplasm Colicin E3 enters the cell after adsorption and affects ribosomes (69, 70) Ringrose (60) concluded that colicin E2 penetrates into the cytoplasm and binds to DNA The bacteriocins of type I, which act directly on the membrane, may not enter the cell, but no compelling evidence exists for or against it

Identity of the receptor. Several authors (84, 85, 86, 87) suggested that the receptor sites reside in the lipopolysaccharide layer (LPS layer) of the cell envelope, since LPS isolated from sensitive cells inhibited the activity of colicins E1, K, B and E2 However, no correlation was observed between colicinsensitivity or -resistance of cells and the colicin-binding activity of the LPS layer isolated from these cells, so, this binding activity seems to be an aspecific one On the basis of their studies Weltzien and Jesaitis (88), and Sabet and Schnaitman (89) located the receptor in the cell wall rather than in the cytoplasmic membrane or in the LPS layer However, various other studies indicate that the receptor is located in the cytoplasmic membrane Arguments in favor hereof are that colicin E1 (44) inhibits amino acid uptake into membrane vesicles and colicin E2 dissociates isolated DNA-membrane complexes (57) Furthermore, Smarda showed that stable L-forms are often more sensitive to colicins E1, E2 and E3 than complete cells (90, 91, 92) These results indicate that receptors located in the cell wall are not essential for action of these bacteriocins but may be auxiliary in the movement of the bacteriocin to the membrane or into the cytoplasm Recently Takagaki *et al* (93) isolated mutants, which were devoid of cell wall receptors but were nevertheless sensitive to colicin K The knowledge on the nature of the receptors of type I bacteriocins is very scarce These bacteriocins act directly on the membrane and the receptor may be simultaneously the biochemical target In case of bacteriocins, which penetrate into the cytoplasm, the receptor may be involved in the transport of the bacteriocin through the membrane

Resistance and tolerance. Nomura (56) discovered two kinds of colicin-insensitive mutants Mutants, which can no longer adsorb bacteriocins to receptor sites, were called resistant mutants, whereas mutants which still adsorb bacteriocins but were insensitive to its action were called tolerant mutants by Nomura (94) and Nagel de Zwaig (95) or refractory mutants by Hill and Holland (96) Later on Bhattacharyya *et al* (44) called the colicin-insensitive mutants resistant and tolerant, when their spheroplasts were colicin-sensitive and -insensitive, respectively On the basis of their tolerance to colicins E1, E2, E3, K and A the tolerant mutants of *Escherichia coli* were divided into eight major groups called Tol I to Tol VIII by Nomura (94) and Nagel de Zwaig (95) and Ref I to Ref VIII by Hill and Holland (96) Further classification was based on the map location of the mutation and the physiological properties of the mutants The main tolerant and resistant mutants are given in Table II

The nature of tolerance and resistance. Many tolerant mutants exhibited a broad spectrum of phenotypic changes as a consequence of one single mutation, such as high sensitivity to deoxycholate, ethylenediaminetetraacetate, sodium dodecyl sulfate, vancomycin bacitracin and novobiocin, indicating that the cell envelope is altered (94, 95 96, 98, 108) This was confirmed

Tabel II Some properties of the main to lerant and resistant mutans of Escherichia coli.

Class	Sensitivity to [a]							Map [b] location near	Termination[b]	Ref.
	E1	E2	E3	K	A	BF 23	T_6			
Tolerant										
Tol I	s	s	s	t				gal		94, 93
Ref III	s	s	s*	t		s				96
Tol II	t	t	t	t	t	s	s	gal	Tol A	94, 95, 97
Tol IIa	pt	pt	pt	pt	pt	s	s	gal	Tol P	95, 97, 98
Ref VI	s*	t	t	t	t	s		gal		96
Tol III	s	t	t	t	t	s	s	gal	Tol B	94, 95, 98
Ref V	s	t	t	t				his; gal		96
Ref VII	s	t	s*	t		s		gal		96
Tol IV	s	t	t	s				gal		94
Tol IV	s	s*	s*	s				—		94
Ref IV	s	t	t	s		s		his-arg		96
Tol III/IV	s	t	t					pyr. D	Tol D	99
Tol V	t	t	t	s				gal		94
Tol VI	t	t	s	s				—		94
Tol VII	s	t*	s	s				thr		94
Ref II	s	t*	s	s		s		thr	Cet B; Cet C	96, 100, 101, 102, 103, 104
Tol VIII	s*	s	s	s	s	s	s	thy	Tol C	95, 97, 105
Ref I	t	s	s	s		s		thy		96, 105
Resistant										
T_6^r Col K^r	s	s	s	r	s		r			93
Bfe^r	r	r	r			r		arg-thi	bfe [c]	106, 107

[a] s, r, t and pt means sensitive, resistant, tolerant and partially tolerant, respectively, to colicins or phages (BF 23, T_6) at 30 C and 40 C; s* means sensitive at 30 C but tolerant at 40 C, and t* means tolerant at 30 C but sensitive at 40 C.

[b] Gives the designation of the locus of the muta tion on the map of *Escherichia coli* K 12.

[c] Refers to mutants resistant to phage BF 23 and colicins E1, E2 and E3.

by Samson and Holland (109), who observed changes in the envelope protein composition of Tol VII mutants, and Rolfe and Onodera (110), who observed an alteration of membrane protein composition of Tol VIII mutants, probably due to loss of a protein.

Difference between tolerance and resistance is not quite explicit. Recent studies of Takagaki et al. (93) showed that spheroplasts of Tol I mutants were sensitive to colicin K; probably tolerance of this mutant is due to a barrier which permits adsorption to the cell wall receptors but prevents colicin K to reach the cytoplasmic membrane.

The resistant mutants had often lost concomitantly the ability to adsorb specific phages. Resistant mutants of *Escherichia coli* K 12 were isolated which were unable to adsorb both phage BF 23 and colicins E1, E2 and E3 (106, 107): colicin K-resistant mutants had lost simultaneously the ability to adsorb phage T_6 (93). Resistance involves a mutation of the lipopolysaccharides or cell wall components, in such a way that the bacteriocin can no longer adsorb to the cytoplasmic membrane and the cell wall receptor (44, 93).

Immunity

Bacteria are generally not susceptible to the bacteriocin they produce, although they retain the ability to adsorb it (40, 82). This phenomenon, called immunity, is a consequence of the presence of a bacteriocinogenic factor, a plasmid. This can be transferred to nonbacteriocinogenic strains rendering them immune (13, 111). Recent studies of Bowman *et al.* (70) and Boon (71) showed that the immunity against colicin E3 was due to the production of immunity substances. These compounds seem to be no simple proteases and do not inactivate the bacteriocin irreversibly. Washed ribosomes from immune cells are just as sensitive to colicin E3 action as ribosomes from sensitive cells (69), but ribosomes in crude extracts (S-30 lysates) obtained from immune cells were much less sensitive, indicating that the immunity substance

Tabel III Characteristics of various Col-factors.

Col-factor[a]	Synonym	Integration into chromosome	Self-transfer	Transfer of other DNA	Pilus > type	Curable by acridine orange	Repression of fertility	Compatible with F	Ref.
V-K94	V2	+	+	+	F		—	—	114, 115, 116, 117
V-K30	V3	+	+	+	F	+	—	—	117, 118
VB-K260		+	+	+	F	+			119, 120
B-CA18	B1	+	+	+	F				121
B-K77	B2	+	+	+	F		+	+	122, 123
B-K166	B3	+	+	+	F			+	123
B-K98	B4	+	+	+	F		+	+	121, 123, 124,
Ib-P9		—	+	+	I	—	+	+	125, 126, 127
Ia-CA53			+	+	I	+	+	+	128
E1a			+	+	I		+	+	129, 130
V-CA7	V1		—	—				—	117, 130, 131
E1-K30	E1	—	—	—		—		+	114, 119, 132, 133
E2-P9	E2	—	—	—		—		+	129, 133
E2-K317			—	—		—			128, 129
E3-CA38	E3	—	—	—		—		+	133
K-K49			—	—		—		+	129
K-K235			—	—		—		+	129
A-CA31						—			
Ref.		134, 135, 136, 137	138, 139	138, 139	140, 141	142	143, 144	124	

[a] The Col-factors are named to the bacterial strain and the produced colicin.

is a cytoplasmic factor and probably binds to colicin E3 to form an inactive complex (71) It is known that colicinogenic cells are sensitive to high concentrations of the respective colicin (25, 111) This phenomenon, called immunity breakdown, may be explained by the adsorption or penetration of more colicin E3 molecules than can be bound by the amount of immunity substance present

GENETICS

The potentiality of a bacterial strain to produce a bacteriocin is a heritable character and due to genetic determinants called bacteriocinogenic factors, in case of colicin production called C- or Col-factors The Col-factors appear to be extrachromosomal elements of which some could be integrated into the chromosome and were called episomes, while other could not be integrated and were called plasmids (112, 113) Further distinction of Col-factors could be made on basis of the fertility Some Col-factors are able to initiate conjugation by the formation of sex pili and to promote its own transfer and in some instances transfer of chromosomal markers and other plasmid DNA in the absence of the F-factor They are called transmissible factors, and can be divided on basis of the sex pili induced by them The colicin production and fertility expressed by some Col-factors are sometimes repressed by a cytoplasmic repressor

Some plasmids cannot stably maintained together in the same cell On the basis of this phenomenon, called incompatibility, the Col-factors can be further distinguished

In Table III some characteristics of various Col-factors are summarized

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CHARACTERIZATION AND EXTRACHROMOSOMAL CONTROL OF BACTERIOCIN PRODUCTION IN *STAPHYLOCOCCUS AUREUS*

About 6% of the tested strains of *Staphylococcus aureus* produced antagonistic substances against staphylococcal indicator strains The production was low in liquid cultures and could not be induced by ultraviolet irradiation or by treatment with mitomycin C The antagonistic substances could be classified into at least five groups on basis of their properties and cross-resistance pattern One group consisted of lytic enzymes, the four others of staphylococcins of which one type was active against *S aureus* only and the three other types had a broader inhibitory spectrum against gram-positive organisms but not against gram-negative bacteria A relationship was found between some groups of producing strains and their phage type The ability to produce staphylococcins was eliminated spontaneously upon storage and more rapidly by treatment with ethidium bromide, acriflavine, acridine orange, sodium dodecyl sulfate, and growth at 42 C The resistance to several inorganic salts was co-eliminated No co-elimination of penicillinase production was observed Selective effects during elimination were ruled out and the results suggest that the genes for staphylococcin production are plasmid borne determinants

Antagonism between staphylococci was first observed by Cornil and Babes (5) Later many authors demonstrated various antagonistic actions among staphylococci (7, 13, 15, 21) The bactericidal substances, produced by certain bacterial strains and active against the same or related species, are named bacteriocins Analogically, Frederique (12), Hamon and Peron (16), and Lachowicz (22) defined staphylococcins as bactericidal substances of staphylococcal origin, inhibitory in the growth of staphylococci and other gram-positive bacteria, and with a high molecular weight with respect to the "classical" antibiotics Most staphylococcins exert a much broader inhibitory spectrum than bacteriocins from gram negative bacteria Recent studies on the mode of action of two staphylococcins (6, 19, 20) showed, that the membrane plays an important role in its mode of action

Bacteriocin production and sensitivity patterns have become useful criteria in differentiation of *Pseudomonas aeruginosa* (10), *Serratia marcescens* (9), and *Escherichia coli* (34) strains Some bacteriocins have found application as therapeutic agents staphylococcin A in the treatment of staphylococcal infections (31), and pyocin 78-C 2 in the treatment of *Pseudomonas aeruginosa* infections in mice (25)

Little is known about the genetic regulation of staphylococcin production The genes for staphylococcin production have been identified as unstable determinants (7, 13 22, 30) Barber (2) observed that penicillinase production was spontaneously and irreversibly lost and it has been established that the genes controlling the synthesis of this enzyme are born on a plasmid in most strains of *S aureus* (27, 28)

This report deals with the properties of some bacteriocins produced by a number of 270 freshly isolated staphylococci and the genetic regulation of the production

MATERIALS AND METHODS

Bacterial strains Strains tested for production of antagonistic substances were isolated by Dr G van der Ploeg (Department of Medical Micro-

biology, University of Nijmegen) from various staphylococcal infections *Staphylococcus aureus* strains NCTC 9752, Oxford 209 P, NCTC 8507 were used as indicator strains Stable L-forms of *S aureus* 502 A were obtained from Dr B Kagan (Cedars - Sinai Hospital, Los Angeles, Calif), and were maintained on 3 8% brain heart infusion, 5% NaCl, and 1 5% agar supplemented with 20% unheated horse serum *Bifidobacterium bifidum var pennsylvanicus* and its cell wall preparation were obtained from Dr J Veerkamp (Department of Biochemistry, University of Nijmegen) All strains were subcultured once a week on Trypticase soy agar (TSA) obtained from BBL

Demonstration of antagonistic activity The spontaneous production of antagonistic substances was demonstrated according to Halpert *et al* (15) The strains were inoculated as a spot on agar and after 48 hr at 37 C, the indicator strain was sprayed over it The production of antagonistic substances was obvious by inhibition zones around the colonies

Cell wall lytic activity Cell walls of *Bifidobacterium bifidum var pennsylvanicus* were suspended in 0 05 M potassium phosphate buffer (pH 7 0) at a final concentration of 5 mg/ml and incubated at 37 C with and without the antagonistic substances to be tested for lytic activity (100 A U /ml) The optical density at 600 nm was followed and the number of reducing and free amino groups was determined according to Ghuysen and Strominger (14)

Production. From overnight cultures of the producing strains one ml was added to 200 ml Trypticase soy broth (TSB) or proteose peptone no 3 (PP) medium Incubation was at 37 C in a gyratory shaker (New Brunswick) At various time intervals samples were withdrawn and centrifuged for 10 min at 10,000 x g The supernatants were sterilized by filtration on membrane filters (0 45 μ pore size, Millipore Corp), by addition of chloroform or heat-treatment at 55 C for 20 min and assayed for activity No effect of the sterilization procedure was observed

Induction. Induction of the production of antagonistic substances by ultraviolet irradiation and by treatment with mitomycin C was performed under the same conditions as described previously (21)

Assay. The assay of the antagonistic substances was described previously (21) The activity was expressed as arbitrary units (A U) per ml (21)

Elimination by chemical agents. From an overnight culture 0 1 ml was added to 25 ml nutrient broth (pH 7 6) containing per ml 1 to 5 μg acriflavine, 0 5 to 2 0 μg ethidium bromide or 5 to 20 μg acridine orange, or to 25 ml TSB containing per ml 5 to 50 μg sodium dodecyl sulfate After 36 hr at 37 C suitably diluted amounts of 0 2 ml were spread onto TSB- or PP-agar and single colonies were tested for the production of antagonistic substance and phage type Metal ion resistance of the colonies was tested by replicative scoring on TSA containing **7×10^{-5}** M $CdSO_4$ or 10^{-3} M Na_2HAsO_4 Penicillin resistance was tested by replicative scoring on TSA containing 1 unit of penicillin per ml

High temperature elimination. The producing strains were grown for 18 hr at 37 C in nutrient broth The cultures were diluted 500-fold with nutrient broth and then aerated for 1 hr at 37 C Samples of 25 ml were incubated further at 37 C or 42 C After 18 hr of growth suitably diluted amounts of 0 2 ml were spread on PP-agar and single colonies were tested for bacteriocin production, phage type, metal-ion resistance and penicillinase production

Phage-typing. The phage-typing was carried out by the National Institute of Health (Utrecht, The Netherlands) according to Blair and Williams (4) with the International Basic Set of typing phages Single colonies of freshly cultured bacteria were used

Extracellular products. Lecithinase (egg yolk factor) and caseinase were tested according to Mc Clung and Toabe (24), deoxyribonuclease according to Jeffries *et al* (18), and gelatinase according to Bentley *et al* (3) Coagulase was tested by addition of bacterial cultures to human plasma Hemolysis was tested on sheep blood agar, and catalase by flooding 3% hydrogen peroxide on colonies

Penicillinase production. Penicillinase was preinduced with 0 5 μg of methicillin per ml in a fresh culture with an optical density of 0 05 at 600 nm At an optical density interval of 0 5 to 0 7 the penicillinase activity was measured by the micro-iodometric assay (26)

Carbohydrate utilization. An inoculum of 10^5 bacteria per ml was seeded onto Bacto Sanders Enrichment agar (Difco) The production of acid from sugars was determined with differentiation disks (Difco) Production of acid from glucose was determined by anaerobic incubation in 1 0% tryptone, 0 1% yeast extract, 1 0 % glucose, 0 004% bromocresol purple, and 0 2% agar covered with liquid paraffin

Antibiotic sensitivity test. A standardized inoculum of 10^5 bacteria per ml was seeded onto Diagnostic Sensitivity Test agar (Oxoid). The antibiotic sensitivity was determined with the multodisks (Oxoid).

Resistance to inorganic salts. The resistance to sodium arsenate, cadmium sulfate, zinc sulfate, mercuric nitrate and lead nitrate was determined according to Novick and Roth (29).

Chemicals. Mitomycin C and ethidium bromide were purchased from Sigma Chemical Co., St. Louis, Mo. Acridine orange was obtained from Merck, Darmstadt, Germany. Acriflavine was purchased from Fluka, Buchs, Switzerland. Diaflo ultrafiltration membranes PM 10 were purchased from Amicon Co., Lexington, Mass.

RESULTS

Production. The production of antagonistic substances was demonstrated by a modified procedure of Halpert *et al.* described in Materials and Methods. The strains reacting positively in this test exhibited an inhibition zone (Fig. 1), and are designated S^+-strains in this study. About 270 strains of *Staphylococcus aureus* were tested against three arbitrarily chosen indicator strains: *S. aureus* NCTC 9752, Oxford 209 P, and NCTC 8507. Seventeen strains (6.3%) from various origins produced an inhibition zone (Table 1). The size of the inhibition zone depended on the indicator strain chosen and the medium used, especially for strains 289, D-14, 80, 104, and 5612. The production of antagonistic substances by all strains, except 289 and D-14, in fluid proteose peptone no. 3, nutrient broth, Trypticase soy broth, and brain heart infusion media was very low and not reproducible. No activity could be detected in the pellets and supernatants from cultures of strain 704, while the culture supernatants from strain 289 and D-14 contained an activity of 5 to 10 arbitrary units (A.U.) per ml and from all other strains 0 to 5 A.U./ml. In none of the S^+-strains the production could be induced by ultraviolet irradiation or by mitomycin C treatment at the various conditions and concentrations tested.

Characteristics of the antagonistic substances. The antagonistic substances obtained from liquid cultures were examined (Table 1). All were sensitive to 15 min incubation with trypsin (200 µg/ml) at 37 C and pH 7.5 and were not dialyzable through a membrane PM 10 (Amicon) indicating that the molecular weights of the substances

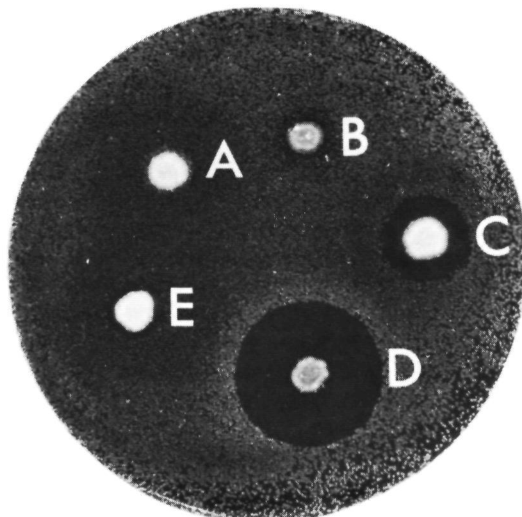

Fig. 1. *Demonstration of the spontaneous production of antagonistic substances by S. aureus strains. The tested strains were inoculated as a spot. After 48 hr at 37 C the indicator strain S. aureus NCTC 8507 was sprayed over it. A. S. aureus Oxford 209 P; B. S. aureus 704; C. S. aureus 89; D. S. aureus 289; E. S. aureus 97.*

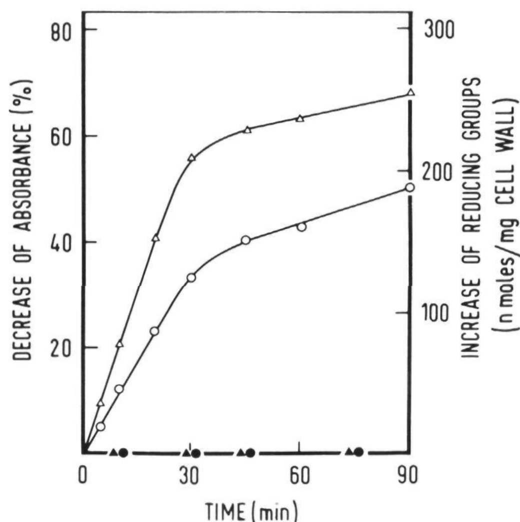

Fig. 2. *Hydrolysis of cell walls of Bifidobacterium bifidum var. pennsylvanicus by the lytic enzyme produced by S. aureus 289. The cell walls (5 mg/ml) were suspended in 0.05 M phosphate buffer (pH 7.0) and incubated at 37 C with (open symbols) and without lytic enzyme of strain 289 (closed symbols). The decrease of absorbance (triangles), and the increase of reducing groups (circles) were determined as a function of time.*

Table 1 Characteristics of the various antagonistic substances

S^+-strain	S^+-origin	Inhibition zone (mm)					Lytic[a] activity	Heat[b] stability	Inhibition[c] L-forms	Group
		NCTC 9752			NCTC 8507	Oxford 209 P				
		Blood agar	PP agar	TSA	PP agar	PP agar				
289	Throat	1	15	0	15	7	+	+	—	A
D-14	Skin	1	17	0	14	8	+	+	—	A
80	Throat	6	11	0	7	3	—	+	+	B
104	Pus	6	10	0	6	3	—	+	+	B
5612	Nose	7	11	0	6	2	—	+	+	B
97	Pus	6	4	5	3	3	—	—	+	C
37	Pus	7	4	4	2	3	—	—	+	C
34	Bladder-fistula	6	4	5	3	2	—	—	+	C
55	Pus	5	5	5	2	3	—	—	+	C
61	Nose	6	4	4	3	3	—	—	+	C
82	Jaw-absces	7	6	11	6	6	—	+	+	D
89	Skin	6	5	10	7	7	—	+	+	D
32	Pus	6	6	10	6	6	—	+	+	D
96	Jaw-absces	7	6	11	6	7	—	+	+	D
238	Skin	6	6	11	6		—	+	+	D
918	Throat	7	5	11	7	6	—	+	+	D
704	Pus	1	2	0	1	1	N D	N D	N D	E

[a] + refers to substances exposing lytic activity against cells of *S aureus* NCTC 9752 The cells were incubated with the substance and the optical density at 600 nm was followed.
[b] + refers to substances stable at 80 C for 15 min
[c] + refers to substances inhibiting stable L-forms of *S aureus* 502A
N D = Not determined

Table 2 Phage-type and Phage-type and drugresistance of the S^+-strains

S^+-group	S^+-strain	Phage-group	Phage type [a] pattern	Drugresistance: Penicillin (10 μg)	Tetracycline [b] (10 μg)	Erythromycin [c] (10 μg)
A	289	I, III	29, 52, 52A, 79, 80, 53, B5, 77 Ad, 88, 77	S	S	S
A	D-14	I, III	29, 52, 52A, 79, 80, 47, 54, 83A, 77Ad, 42E, 42D, B5	S	S	S
B	80	I, III	29, 52, 52A, 80, 81, N, 7, 47, 53, 54, 88, 83A, 75, 77, 77Ad, 42E	S	S	S
B	104	I, III	29, 52, 52A, 80, 81, 88, 83A, 77, 42E	S	S	S
B	5612	III	88, 87	S	S	S
C	37	II	3A, 3B, 3C, 55, 71	S	S	S
C	34	II	3A, 3B, 3C, 55	S	S	S
C	97	I	29, 52	S	S	S
C	55	I, III	81, N, 6, 7, 47, 54, 88, 83A, 42E, 42B, B5	R	R	R
C	61	I, III	81, N, 6, 7, 47, 54, 88, 83A, 42E, 42B, B5	R	R	S
D	82	II	3A, 3B, 3C, 55, 71	R	S	S
D	89	II	71	R	S	S
D	32	II	3A, 3B, 3C, 55, 71	R	S	S
D	96	II	3A, 3B, 3C, 55, 71	R	S	S
D	238	II	3A, 3B, 3C, 55, 71	R	S	S
D	918	II	3B, 3C, 55, 71	R	S	S
E	704	II	3A, 3C, 55	S	S	S

[a] Tested at 1000 x routine test dilution
[b] Similar for streptomycin (25 μg), kanamycin (30μg), methicillin (10 μg), and ampicillin (10 μg)
[c] Similar for chloramphenicol (10 μg)
The signs S and R mean sensitive and resistant, respectively

are higher than 10,000 All substances, except those from strains 97, 37, 34, 55 and 61, were heat-stable Strains 289 and D-14 produced a substance which caused lysis of cells of *S aureus* NCTC 9752 These compounds produced protoplasts from *Bifidobacterium bifidum var pennsylvanicus* and reduced the optical density of a cell wall preparation of these bacteria The number of reducing groups increased simultaneously, but no free amino groups were formed as a result of the action of this lytic material (Fig 2) All other antagonistic substances did not lyse bacterial cells and were active against stable staphylococcal L-forms, in contrast to the lytic enzymes On basis of these results, given in Table 1, the S -strains can be divided into at least five groups, A to E

Characterization of S^+-strains. The possible relationship of the production of the various antagonistic substances to other known staphylococcal characteristics was examined All S^+-strains produced coagulase, catalase, deoxyribonuclease, caseinase, gelatinase, and exposed a positive egg-yolk reaction Moreover, all strains produced acid from glucose under anaerobic conditions and fermented mannitol, dextrose, maltose, sucrose, and trehalose, while none of the S^+-strains fermented arabinose, dulcitol, inositol, inuline, raffinose, salicine, sorbitol and xylose All strains fermented galactose and lactose except strains 80 and 104, which did not ferment galactose and lactose, and strains 96, 918 and 704, which did not ferment lactose All strains produced hemolysin and pigments except

Table 3 Cross resistance pattern of the various groups of S^+-strains

Group of indicator strains	Inhibition zone (mm) Group of S^+-strains: A	B	C	D	E
A	0	8	5	5	1
B	8	0	4	5	1
C	9	5	0	0	1
D	9	5	0	0	1
E	9	2	2	2	0

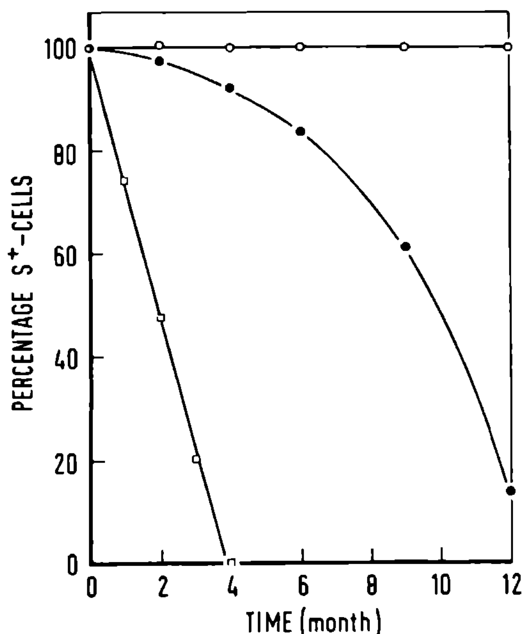

Fig. 3. *Spontaneous elimination of staphylococcin production upon storage. The S⁺-strains were stored on TSA and subcultured each fortnight and the number of staphylococcin producing cells was determined. o, S. aureus 289; •, S. aureus 89; □, S. aureus 55.*

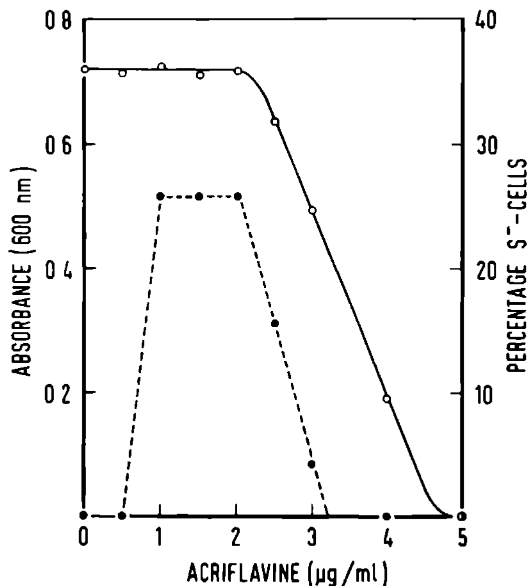

Fig. 4. *Dependence of the elimination of the staphylococcin production of S. aureus 89 cells on the concentration of acriflavine. Cells of an overnight culture were inoculated in nutrient broth (pH 7.6) containing various concentrations of acriflavine. After 36 hr incubation at 37 C in the dark the percentage of S⁻-cells (•) and the optical density at 600 nm (o) was determined.*

Table 4. Inhibitory spectrum of the various types of antagonistic substances

Microorganisms tested	No. of tested strains	No. of strains senitive to A Group	B Group	C and D Group	E Group
Gram-positive					
Staphylococcus aureus	32	30	26	23	20
Staphylococcus epidermidis	9	8	6	6	0
Streptococcus faecalis	2	0	2	2	0
Streptococcus allantoicus	3	3	3	3	0
Streptococcus mutans	3	0	3	3	0
Bacillus cereus	2	2	2	2	0
Bacillus megaterium	1	1	1	1	0
Bacillus subtilis	2	2	2	2	0
Bacillus pumilis	1	1	1	1	0
Corynebacterium diphteriae	1	1	1	1	0
Corynebacterium pseudodiphtheriticum	1	1	1	1	0
Bifidobacteruim bifidum var. pennsylvanicus	1	1	1	1	0
Gram-negative					
Escherichia coli	7	0	0	0	0
Citrobacter freundii	1	0	0	0	0
Klebsiella pneumoniae	1	0	0	0	0
Proteus morganii	2	0	0	0	0
Serratia marcescens	1	0	0	0	0
Salmonella typhimurium	2	0	0	0	0
Pasteurella pseudotuberculosis	1	0	0	0	0
Agrobacterium tumefaciens	1	0	0	0	0
Pseudomonas aeruginosa	6	0	0	0	0

5612, 97 and 704 Phage-type and drug-resistance are given in Table 2 Among the S⁺-strains 47% was penicillin resistant and 1, 9, 1 and 6 strains belonged to groups I, II, III, I and III, respectively

Inhibitory spectrum. On basis of the differences in the cross-resistance pattern (Table 3), the division of the 17 S⁺-strains into groups A to E could be further established No difference was observed between group C and D None of the S⁺-strains was sensitive to the antagonistic substance of its own group Moreover, *S aureus* 704 was only active against *S aureus* strains All other substances had a broader inhibitory spectrum, and many other gram-positive bacteria were sensitive, but none of the tested gram-negative bacteria (Table 4) No correlation was found between phage-type and susceptibility to the antagonistic substances of the various groups Except compound 704, which was not tested, all antagonistic substances had no effect on red blood cells

Spontaneous elimination. Most S⁺-strains lost spontaneously their ability to produce the antagonistic substances upon subcultivation on TSA slabs each fortnight, the inhibition zones on agar plates and the percentages of S -cells decreased (Fig 3) Such elimination was observed with all strains except strains 704, 289 and D-14 The elimination was not limited to a special phage type

Inductive elimination. Various physical and chemical treatments have been used to increase the rate of elimination of plasmid-borne determinants in *S aureus* (23, 27, 32, 33) We tested *S aureus* 89 S in more detail Growth of this strain in the presence of acriflavine caused an increase of the elimination rate which appeared to be concentration dependent (Fig 4) 89 S⁺ cells surviving acriflavine treatment at high concentrations (above 2 μg/ml) were more resistant

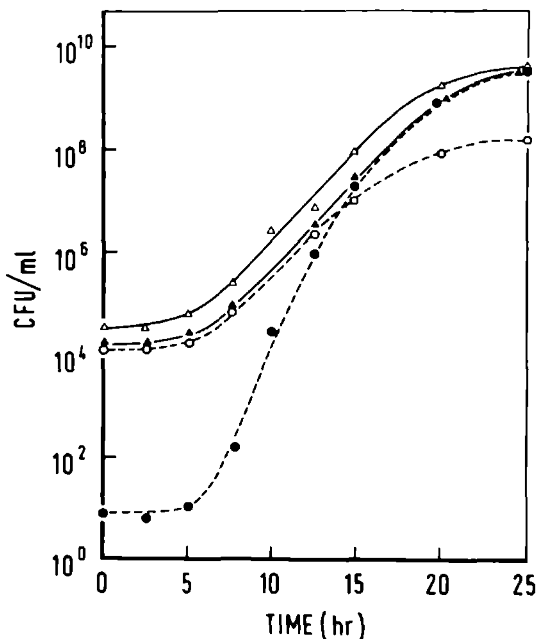

Fig 5 Growth of S aureus 89 S⁺ in nutrient broth (pH 7 6) containing 1 2 μg of ethidium bromide per ml at 37 C At the time intervals indicated the number of colony-forming units (CFU) of total cells (▲), S⁺ (●) and S (o) cells derived from 89 S+ were determined The growth curve of strain 89 S−(Δ) in the presence of ethidium bromide is also given

Table 5 Elimination by various treatments of the production of antagonistic substances from S aureus 89 S⁺

Treatment [a]	Total number of colonies tested	Number S	Elimination (%)
Control	988	0	0 1
Ethidium bromide	245	230	94 0
Acridine orange	191	23	12 1
Acriflavine	260	65	25 0
Sodium dodecyl sulfate	129	129	100
Growth at 42 C	283	279	98 6

[a] The experiments were performed as described in Materials and Methods Concentrations optimal for elimination were used for ethidium bromide (1 25 μg ml), acridine orange (15 0 μg ml), acriflavine (2 μg/ml), and sodium dodecyl sulfate (30 μg/ml)

to acriflavine and showed a 50 to 200-fold lower elimination rate upon a second acriflavine curing Acridine orange, ethidium bromide and sodium dodecyl sulfate (SDS) exposed a similar effect as acriflavine (Table 5) Growth at 43 to 44 C results in a high elimination of the penicillinase plasmid (I 23) Growth of the 89 S strain at 42 C caused a very rapid elimination, while at temperatures higher than 43 C the cells were killed (Table 5)

To exclude selection of the S⁻-cells during curing conditions, cells of strain 89 containing 0 1% S -cells were incubated in 0 05 M phosphate buffer (pH 7 6) containing various concentrations of acriflavine No increase in the percentage of S -cells was observed among the surviving cells indicating that 89 S and 89 S -cells are equally sensitive to acriflavine Furthermore, the growth kinetics of 89 S cells in the presence of ethidium bromide showed that during the initial lag-phase the number of S⁻-cells remained constant, but increased very rapidly when cell multiplication started (Fig 5) The growth curves of the 89 S and 89 S⁻ strains were identical in the presence of ethidium bromide (Fig 5) or sodium dodecyl sulfate (30 µg/ml)

No elimination was observed in strains 289, D-14 and 704 under all conditions used for strain 89 S , but all other S strains yielded results similar to strain 89 S However, the elimination rates were markedly lower at elevated temperature and on treatment with sodium dodecyl sulfate for strains 5612, 80 104 and 97

Penicillin and metal-ion resistance. *S aureus* strain 89 S is resistant to penicillin and various metal-ions These characteristics are located on a plasmid in many strains of *S aureus*, and can be eliminated under various curing conditions (27, 28) No penicillinase-negative cells were detected in strain 89 under various curing conditions It appeared that the ability to produce antagonistic substance and the resistance to the inorganic salts was always eliminated concomitantly When 10^9 cells of strain 89 S⁻ were plated on TSA containing 7x10 ⁵M cadmium sulfate, a few resistant colonies arose after a few days of incubation These colonies were only resistant to cadmium and did not produce antagonistic substances Similar results were found for all strains of group D

Characterization of the S -strains. All S⁻-strains remained resistant to the antagonistic compound, the original S -strain produced Furthermore, the coagulase, catalase hemolysin, deoxyribonuclease caseinase and gelatinase production the fermentation of the various sugars the pigmentation, and the sensitivity to antibiotics were identical for all the S and corresponding S strains The induced penicillinase activities of 89 S and S strains, and 32 S and S strains were identical

The phage susceptibility of several S and S⁻ strains were compared (Table 6) The S strains tested were obtained by various curing conditions which revealed similar results All 80 S strains became more susceptible to phage types 29, 52 52 A, and 80, while the 97 S⁻ and 89 S⁻ strains were all as sensitive as their parent S strains Two types of 32 S strains were obtained, one with the same sensitivity as 32 S

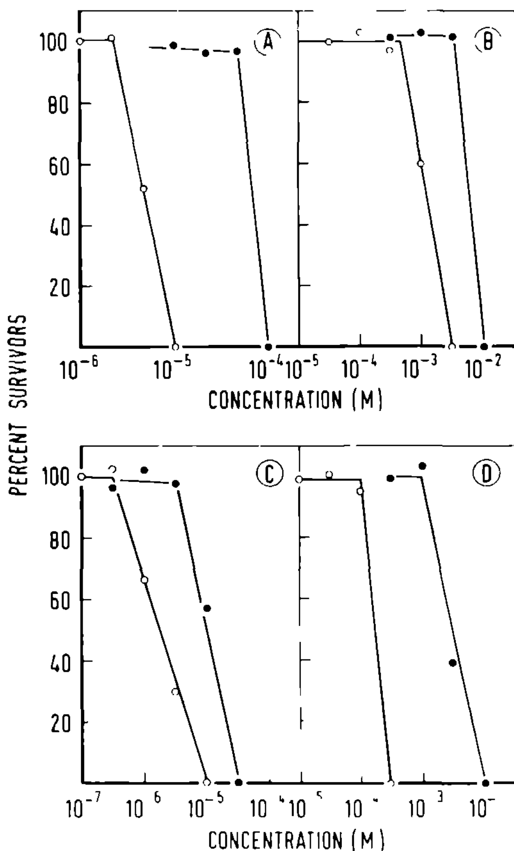

Fig 6 *Survival of S aureus 89 S⁺ (o) and 89 S (•) as a function of the concentration of various inorganic salts About 10^4 colony-forming units per ml were spread on TSA containing various concentrations of inorganic salts After 30 hr incubation the percentage of survivors was determined A Cadmium sulfate B Zinc sulfate C Mercuric nitrate, D Sodium arsenate*

Table 6. Comparison of the susceptibility of S^+ and S^- strains to various phages

Strain	Number of plaque forming units															
	29	52	52A	80	81	N	7	47	53	54	88	83A	75	77	77Ad	42E
							(units x 10^8)									
97 S^+	15	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
97 S^-	14	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
80 S^+	0.04	0.2	0.2	0.2	1.0	0.2	5.4	17	0.3	13	1.2	1.2	24	5.4	5.4	15
80 S^-	5	2.8	4.2	3.0	1.0	0.2	4.5	19	0.3	15	1.2	1.9	19	6.6	4.8	13

Strain	3A	3B	3C	55	71
	(units x 10^6)				
89 S^+	0	0	0	0	94
89 S^-	0	0	0	0	88
32 S^+	0.12	3.1	37	25	81
32 S_1^-	0.01	3.1	38	29	80
32 S_2^-	0.10	3.1	38	34	87

strains and one with a lower sensitivity to phage type 3 A. Strain 89 S^- was not sensitive to phage preparations obtained from strain 89 S^+ by ultraviolet irradiation.

The 89 S^+ and 89 S^- strains were compared for resistance to various inorganic salts (Fig. 6). The resistance index or RI values, defined as the ratio between the concentrations at 50% survival for positive and negative strains, were 13, 4.5, 5.6, 16 and 4.1 for cadmium sulfate, zinc sulfate, mercuric nitrate, sodium arsenate, and lead nitrate, respectively. Similar results were found for all strains of group D.

DISCUSSION

Seventeen (6.3%) out of 270 tested strains of *Staphylococcus aureus* produced antagonistic substances against the three indicator strains used. The producing strains (S^+ strains) belouged to the phage group I (1 strain), phage group II (9 strains), phage group III (1 strain), and to phage groups I and III (6 strains) and 8 out of 17 were penicillin resistant. In accordance with Lachowicz (22) and Fink and Ortel (11) most S^+ strains belonged to the phage group II.

On basis of their properties and inhibitory spectrum the antagonistic compounds reported here could be divided into at least five groups (A to E). The substances of group A caused lysis of cells and hydrolysis of a cell wall preparation and were defined as lytic enzymes. The compounds of groups B, C and D inhibited stable staphylococcal L-forms without causing lysis and exhibited molecular weights of higher than 10.000. So they are distinct from epidermidins (17) and cell wall lytic enzymes and may be defined as staphylococcins on basis of the definition given in the introduction. The two strains of group A exposed a similar phage type pattern as did the strains of groups B and D, and the S^+-strains within the groups seemed closely related. No differences were observed in the inhibitory spectrum of groups C and D, but the size of inhibition zones against several indicator strains and the heat-stability were different. All S^+-strains belonging to group D are penicillin resistant, belong to phage group II and seem to be similar to S^+-strains of phage type 71 decribed by Parker and Simmons (30), Dajani et al (7.(AND Lachowicz (2).

These authors demonstrated loss of the ability to produce staphylococcin in several strains upon storage. However, no detailed studies were made to elucidate this phenomenon. We found that the genes involved in staphylococcin production and resistance to inorganic salts are rather unstable in S^+-strains of group D, while the genes involved in penicillinase production are stable characters. The loss might be explained as the elimination of a prophage and coelimination of the adjacent chromosomal genes for staphylococcin production and resistance to inorganic salts. Alternatively, the presence of a prophage in *S. aureus* may suppress or induce one or more characteristics (8). However, no differences were observed in the phage type pattern of strains 89 S^+ and 89 S^-, and strains 89 S^- were not susceptible to the phage lysates obtained from strains 89 S^+ by ultraviolet irradia-

tion Therefore, the involvement of a prophage is unlikely Otherwise the elimination may be explained by the loss of a plasmid harboring the genes for staphylococcin production and metal-ion resistance Elimination of these genes was enhanced by growth of the bacteria at 42 C Similar results were obtained for the penicillinase plasmid (1, 2, 23) This elimination could be due to heat-induced mutations (35), but is improbable since no selecting conditions for S^- cells were applied and it would require an extraordinarily high mutation rate Ethidium bromide, acriflavine, and acridine orange increased the elimination rate without changing the phage type pattern A similar result was found for penicillinase plasmids (27, 32) The dyes do not expose selective advantages for S^- cells under curing conditions because S^- and S^+ cells growed equally well and no increase in the percentage of S^- cells was obtained in buffered dye solutions The kinetics of curing with ethidium bromide are similar to those of the penicillinase plasmid (32) Also sodium dodecyl sulfate, a known curing agent for several plasmids (27, 33) increased the elimination rate strongly

These results suggest that the S^+ strains of type D carry a plasmid harboring the genes involved in staphylococcin production and in metal-ion resistance The dyes may intercalate with the adjacent base pairs of the plasmid, inhibiting its replication and causing loss of the plasmid Sodium dodecyl sulfate is supposed to interfere with the initiation site on the membrane causing a block in replication of the plasmid and subsequently an elimination (27, 32, 33) Though the S strains of group D are penicillin resistant, the ability to produce penicillinase seems to be chromosomal determinants, because no elimination was observed

Transduction studies on these phenomena are further in progress

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CHAPTER III

PRODUCTION AND PURIFICATION OF A *STAPHYLOCOCCUS EPIDERMIDIS* BACTERIOCIN

Liquid cultures of *Staphylococcus epidermidis* 1580 contained rather small amounts of a bacteriocin, staphylococcin 1580, which was found both in the supernatant fluid and in the cell pellet It could be extracted from the cells with 5% NaCl solution The staphylococcin production could not be induced by ultraviolet irradiation or treatment with mitomycin C Bacteria grown on semi-solid medium produced a much larger amount of the compound with a high specific activity The staphylococcin was purified by ammonium sulfate precipitation, ultracentrifugation, and chromatography on Sephadex columns The purified material was homogeneous on polyacrylamide gel electrophoresis The molecular weight was between 150,000 and 400,000 The bacteriocin was com posed of protein, carbohydrate, and lipid and consisted of subunits exhibiting a molecular weight of about 20,000

Bacteriocins are high molecular-weight bactericidal substances produced by various spe cies of bacteria and are active against the same and related species The specificity of their action and their nature distinguish them from most of the "classical" antibiotics Many bac teriocins have been described and classified (23, 32, 35), but only a few of them have been studied in detail The best studied of the bacteriocins are the colicins, bacteriocins produced by certain strains of *Enterobacteriaceae*

Investigations of the chemical nature of bac teriocins have shown them to be a heterogeneous group of substances ranging from simple proteins (7, 13, 17, 20) and proteins complexed with carbohydrates and lipids (22, 24, 26) to particles resembling phages (18 25, 30, 40)

The lethal action of a bacteriocin on sensi tive bacterial cells seems to occur in two phases, namely, initial combination of the bacteriocin with specific receptor sites located on the cell surface followed by effect on an intracellular biochemical target via the mediation of a specific transmission system (19, 31)

The ability to synthesize bacteriocin de pends upon the presence of a bacteriocinogenic factor Certain bacteriocinogenic factors have been identified as extrachromosomal elements (plasmids) (3, 38)

The staphylococcins, bacteriocins produced by staphylococci, were first described by Frederique in 1946 (9) He distinguished several different staphylococcins on the basis of their inhibition spectrum Probably because of the difficulty of producing (1 2, 10) and isolating (21, 27, 34) many staphylococcins in large amounts, little is known about their nature, genetics, and mode of action (6, 28) Recent studies of Dajani et al (4, 5) and Gagliano and Hinsdill (10) described the isolation and purification of two different staphylococcins They have shown that, unlike many other bacteriocins, the production of staphylococcin seems not to be induced by ultraviolet irradiation or treatment with mitomycin C

This study deals with the production and purification of a bacteriocin produced by *Staphylococcus epidermidis* This bacteriocin is different from the two staphylococcins just mentioned

MATERIALS AND METHODS

Microorganisms. The staphylococcin producing strain *S epidermidis* 1580 was obtained from T Lachowicz of the Institute of Hygiene and Epidemiology, Krakow, Poland The strain is coagulase negative, does not ferment mannitol, and is not hemolytic *S aureus* Oxford 209 P was used as indicator strain Both strains were grown on Trypticase soy agar and were subcultured once a week

Bacteriocin assay. Staphylococcin was determined by the method of Reeves (35) with slight modifications One milliliter of serial dilutions of the bacteriocin was mixed in sterile tubes with 1 ml of Trypticase soy broth (TSB) and 10^7 bacteria from an exponential phase culture of the indicator strain Subsequently, the tubes were incubated at 37 C for 4

hr, and bacterial growth was determined by measurement of the optical density at 600 nm (Fig 1) The increase in optical density corresponding to 100% survival was determined in a control tube containing no staphylococcin The reciprocal of the dilution yielding 50% increase was taken to be the activity in arbitrary units per milliliter (A U /ml) Specific activities are represented as arbitrary units per milligram of protein (A U /mg)

Protein assay. The protein was determined by the method of Lowry (29) with bovine serum albumin as reference

Induction. *S epidermidis* 1580 was inoculated and grown overnight in 2% proteose peptone no 3 (Difco) The overnight culture was diluted to about 10^8 cells/ml with the same medium The diluted culture was transferred to a New Brunswick gyratory shaker at 37 C When the culture reached a concentration of 6×10^8 cells/ml, mitomycin C was added in a final concentration of 0 1, 0 5, or 1 μg per ml After 30 min the cells were harvested by centrifugation and incubated in proteose peptone medium for an additional 4 hr

Induction by ultraviolet irradiation was performed with an ultraviolet lamp (Mineralight R51) at a distance of 25 cm for 5, 10, 15, 20, and 30 sec (about 800 ergs per cm² per sec) A 10-ml bacterial suspension (10^8 cells/ml) in a 10 cm petri dish was used

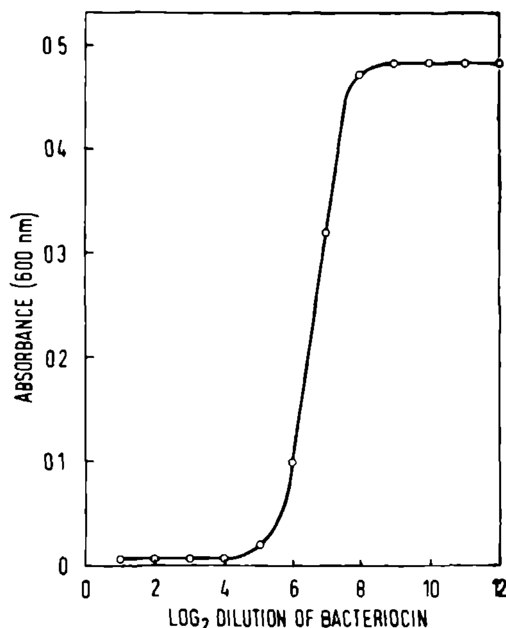

Fig 1 *Quantitative determination of the staphylococcin activity Serial dilutions (1 ml) of the bacteriocin were mixed with 1 ml of exponential-phase culture of the indicator strain (10^7 cells/ml) After incubation, bacterial growth was determined by measurement of the absorbance at 600 nm The reciprocal of the dilution yielding 50% increase in absorbance with respect to the control (containing no staphylococcin) was taken to be the activity in arbitrary units per milliliter*

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed according to the methods described by Ornstein (33) and Weber (39) Gels (3 and 10%) in 0 05 M phosphate buffer (pH 7 0) with or without 0 1% sodium dodecyl sulfate (SDS) were used, 5 mA was applied per tube (diameter 6 mm) at room temperature The proteins were stained with Coomassie brillant blue R 250 (12), and the carbohydrates were located with the periodic acid-Schiff reaction (12) The staphylococcin activity and lipid compounds could be detected after removal of the SDS by soaking the gels in 0 01 M tris(hydroxymethyl)aminomethane - hydrochloride buffer (pH 7 0) for 18 hr To detect lipid compounds the gels were stained with Oilred O (12) The staphylococcin activity could be located by embedding the gel in 1 3% agar plus 3% TSB and by spraying the indicator strain over it After one night at 37 C the inhibition zone locates the staphylococcin 1580

Continuous flow dialysis. Continuous flow dialysis was performed in an ultrafiltration cell model 52 from Amicon Co , Lexington, Mass

Chemicals. Mitomycin C and Antifoam B were purchased from Sigma Chemical Co , St Louis, Mo. Sephadex G-200 and G 50 were obtained from Pharmacia, Uppsala, Sweden Trypticase soy broth was purchased from BBL, Cockeysville, Md Acrylamide, *N,N,N',N'*-tetramethylethylenediamine and methylenebisacrylamide were purchased from Fluka A G , Buchs. Switzerland Proteose peptone no 3, nutrient broth, and brain heart infusion were purchased from Difco Laboratories, Detroit, Mich

RESULTS

Demonstration of bacteriocin production. *S epidermidis* 1580 produced a substance with antagonistic activity against various staphylococci and other gram-positive bacteria This was demonstrated first by using a technique described by Gratia (14) *S. epidermidis* 1580 was inoculated as a spot on a solid medium and incubated at 37 C for 18 hr. Then the indicator strain *S. aureus* Oxford 209 P was sprayed over it After another 18-hr incubation an inhibition zone could be detected around the inoculum This inhibition zone was not due to the production of acid or base or phages but to a bacteriocin called staphylococcin 1580 in this study No phages had been produced since material extracted with water from the inhibition zone did not produce plaques when incubated at 37 C for 24 hr in soft brain heart infusion agar containing 10^6 cells of *S. aureus* Oxford 209 P

Production of staphylococcin 1580. *S. epidermidis* 1580 produced the bacteriocin in well aerated liquid cultures in which foaming was restricted by Antifoam B (Sigma) No staphylococcin activity could be detected in the supernatant fluid or in the pellet obtained by

centrifugation of a *S epidermidis* 1580 culture grown under anaerobic conditions Addition of antifoam was necessary since this staphylococcin was very sensitive to mechanical agitation as was shown by a rapid inactivation upon bubbling air through an active preparation This inactivation was not due to oxidation since mechanical agitation in a pure nitrogen atmosphere caused the same effect

Various media, including proteose peptone no 3, Trypticase soy broth (TSB), brain heart infusion, a synthetic medium as described by Richmond (36), and nutrient broth were compared for staphylococcin production (Table 1) The largest production and the highest specific activity were obtained in the TSB medium No detectable amounts of the bacteriocin were produced in the synthetic medium Activity was always found both in the supernatant fluid (about 80%) and the pellet (about 20%) The bacteriocin activity of all supernatant fractions was greatly enhanced by dialysis Possibly, the cultures contain a small molecular-weight inhibitor either produced by *S epidermidis* 1580 or present in the original media This inhibiting substance needs further study The staphylococcin can be obtained from the bacterial pellet by extraction with 5% NaCl in 0 05 M phosphate buffer (*p*H 7 0) In a similar way colicins were previously isolated by Herschman and Helinski (17)

The optimal temperature and *p*H range for staphylococcin production were between 35 and 38 C and between *p*H 6 5 and 8 0, respectively

The amount of staphylococcin 1580 present in liquid cultures was too small to allow an efficient purification procedure Moreover, staphylococcin production was not induced by ultraviolet irradiation or treatment with mitomycin C (Fig 2) In the presence of 0 5 μg of mitomycin C per ml or with ultraviolet irradiation of 15 sec the highest phage titer of about 10^6 plaque-forming units per ml was measured Upon growth of the cells on a semisolid medium, a crude staphylococcin preparation was obtained with an activity 20 times higher than that in liquid cultures of the same volume

Extraction of staphylococcin 1580. *S epidermidis* 1580 was grown in petri dishes containing a semisolid medium consisting of 0 4% agar and dialyzed TSB at 37 C for 48 hr Dialyzed TSB was the outer fluid obtained on dialysis of 60 g of TSB in 100 ml of water against 2 liters of water at 4 C for one night A semisolid medium on the basis of dialyzed TSB was chosen to facilitate the further purification steps by the absence of high-molecular-weight substances After the incubation

TABLE 1 *Comparison of staphylococcin production in various media*[a]

Medium	Activity in supernatant		Activity in pellet (A U)	Specific activity	
	Before dialysis (A U)[c]	After dialysis (A U)		Supernatant[b] (A U/mg)	Pellet (A U/mg)
Proteose peptone no 3	0	90	10	0 27	0 1
Brain heart infusion	0	180	40	0 51	0 2
Nutrient broth	0	110	20	0 62	0 15
Synthetic medium	0	0	0	0 0	0 0
Trypticase soy broth	50	400	80	1 0	0 45
Dialyzed Trypticase soy broth	50	400	80	93 0	0 45

[a] The pellet and supernatant of a 100 ml culture was tested

[b] Determined after dialysis

[c] A U Arbitrary units, see Materials and Methods

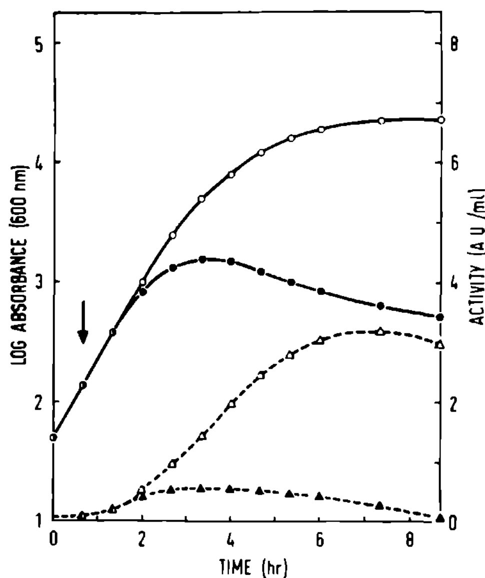

FIG 2 *The effect of mitomycin C on the production of staphylococcin 1580 To a culture of Staphylococcus epidermidis 1580 in the exponential phase mitomycin C (0 5 µg/ml) was added at the moment indicated by the arrow Samples withdrawn at the times indicated were assayed for staphylococcin activity (▲) and absorbance at 600 nm (●) Samples of an untreated culture were assayed in the same way (open symbols)*

period the petri dishes were placed at −20 C for 4 hr Thereafter, the medium was thawed The contents of the petri dishes were pooled and the resulting slurry was centrifuged at 18,000 × *g* for 10 min in a Sorvall RC 2B centrifuge The supernatant fluid obtained was designated as the crude staphylococcin preparation

Purification of staphylococcin 1580. A scheme of the followed purification procedure is given in Fig 3 A saturated solution of ammonium sulfate (*p*H 7 0) was added slowly to the crude preparation of staphylococcin under constant stirring at 4 C The majority of the compound was obtained in the fraction precipitated between 0 and 55% saturation (Fig 4) The 55% saturated suspension was further stirred for 30 min and centrifuged at 18,000 × *g* for 10 min The pellet was dissolved in 0 05 M phosphate buffer (*p*H 7 0), and the clear solution was dialyzed for 18 hr against the same buffer During dialysis an inactive precipitate was formed which was removed by centrifugation at 18,000 × *g* for 10 min This procedure yielded a high purification (Table 2), because all the low molecular-weight substances had been removed

The next steps of the purification procedure are based on the high molecular weight of the staphylococcin and the dissociation of it into subunits in the presence of 6 M urea The bacteriocin preparation was centrifuged at 150,000 × *g* in a Spinco L-50 ultracentrifuge for one night The pellet containing all the activity was dissolved in 0 05 M phosphate buffer (*p*H 7 0) Upon centrifugation in 0 1% SDS or in 6 M urea the activity could be detected only in the supernatant fraction After removal of the SDS or urea by dialysis and centrifugation of the staphylococcin preparation at 150,000 × *g*,

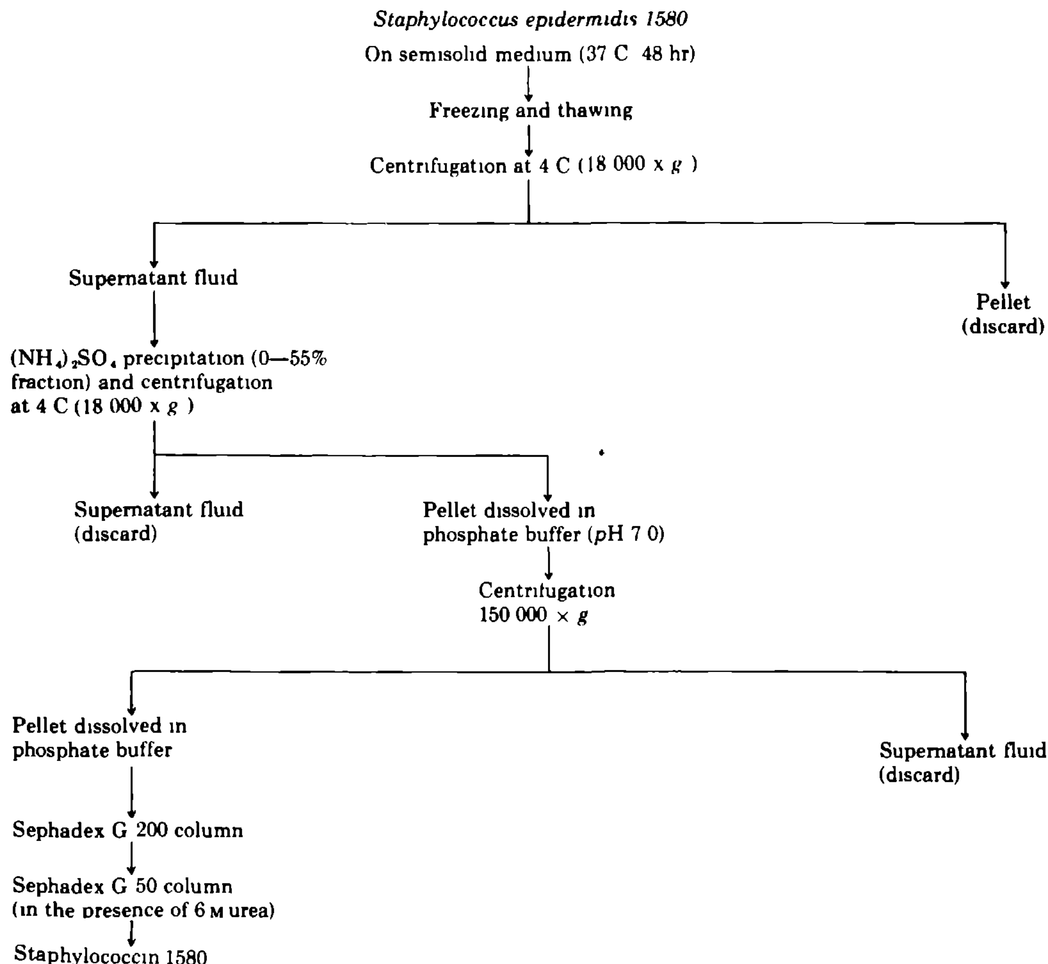

FIG 3 *Isolation and purification of staphylococcin 1580*

the activity could be detected again only in the pellet Therefore, it is likely that the staphylococcin consists of subunits The dissolved pellet was applied to a Sephadex G-200 column (2 8 by 44 cm) prepared in 0 05 M phosphate buffer (pH 7 0) The activity was eluted from the column in the first peak at the void volume (Fig 5) The active fractions were

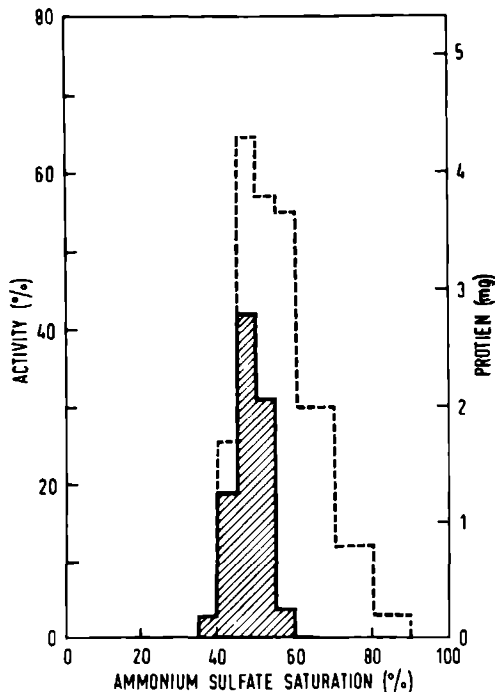

FIG 4 *Distribution of the staphylococcin activity and protein in the fractions precipitated by ammonium sulfate A crude staphylococcin preparation (100 ml) was fractionated by addition of a saturated ammonium sulfate solution The precipitates formed were dissolved in 0 02 M phosphate buffer (pH 7 0) and after dialyzation the total staphylococcin activity (solid line) and protein (dashed line) was determined*

pooled and concentrated in an ultrafiltration cell model 52 (Amicon) This preparation was applied to a Sephadex G-50 column (2 8 by 40 cm) prepared in 0 2 M phosphate buffer (pH 7 0) containing 6 M urea (Fig 6) Now, the activity was eluted from the column in the second peak The active fractions were pooled, dialyzed in a continuous-flow dialyzer model 52 (Amicon), and lyophilized This substance is designated as the purified staphylococcin 1580 Lyophilization of purified staphylococcin 1580 resulted in a total loss of the activity which could be prevented by addition of 0 5% bovine serum albumin to the pure bacteriocin

The results of this purification are summarized in Table 2 Several other attempts were made to purify the staphylococcin further Gradient elution from diethylaminoethyl cellulose columns with a variety of sodium chloride and pH gradients, differential adsorption, and elution from calcium phosphate gels failed to yield preparations with increased specific activities Upon precipitation with alcohol or acetone, a precipitate was formed which contained no activity Staphylococcin 1580 was applied to a Sepharose 4B column (2 0 by 26 cm) prepared in 0 05 M phosphate buffer (pH 7 0) The staphylococcin was eluted as a single peak in the region of the low molecular-weight substances This means that the molecular weight of staphylococcin 1580 was smaller than 400,000

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis at pH 7 0 or 8 9 in the presence or absence of 0 1% SDS was used as a criterion of homogeneity of the purified staphylococcin 1580 In the absence of SDS it appeared as a large diffuse zone on 3% gels upon staining for protein, lipid, or carbohydrate, whereas in 10% gels the bacteriocin did not migrate from the origin and the activity could be detected at the top of the gel In the presence of 0 1% SDS, dissociation into subunits occurred and the staphylococcin appeared as a single band coincident with the

TABLE 2 *Purification of staphylococcin 1580*[a]

Purification step	Vol (ml)	Arbitrary units (A U)	Protein (mg)	Specific activity (A U /mg)	Recovery (%)	Times purified
Crude preparation	1,200	100 000[b]	5,250	19	100	1
Ammonium sulfate precipitate	60	95,000	12 0	7,900	95	415
150,000 × g pellet	10	95,000	4 0	23,800	95	1,250
Sephadex G 200 gel filtration	25	90,000	2 2	40,800	90	2,150
Sephadex G 50 gel filtration	50	82,000	0 73	112,500	82	5,900

[a] Eighty petri dishes (1,600 ml) were used
[b] Determined after dialysis of the crude preparation

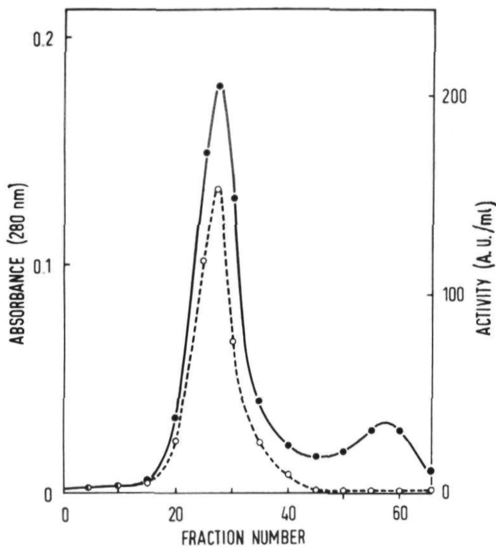

FIG. 5. *Sephadex G-200 gel filtration of staphylococcin 1580. The elution occurred in 0.05 M phosphate buffer (pH 7.0). Each 5-ml fraction was assayed for staphylococcin activity (○) and protein (●). Tubes 21 through 34 were pooled yielding a 95% recovery of the total activity applied to the column.*

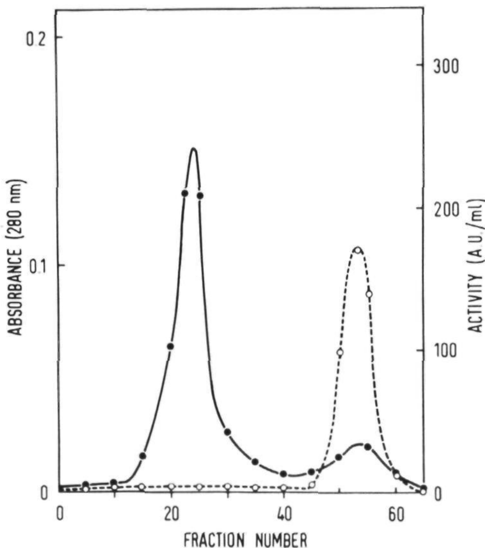

FIG. 6. *Sephadex G-50 gel filtration of staphylococcin 1580. The elution occurred with 0.2 M phosphate buffer (pH 7.0) containing 6 M urea. Each 5-ml fraction was assayed for staphylococcin activity (○) and protein (●). The tubes 45 through 57 were pooled yielding a 90% recovery of the total activity applied to the column.*

zone which stained for protein, lipid, and carbohydrate (Fig. 7). Comparison with the position of molecules with known molecular weight showed that the subunit must have a molecular weight between 10,000 and 25,000. Controls were run in order to detect interference of SDS in the determination of the lipid and bacteriocin activity band.

DISCUSSION

Bacteria grown on semisolid Trypticase soy agar yielded the largest extracellular staphylococcin production and the highest specific activity. The production was optimal under the same conditions which are optimal for the production of extracellular proteins (37). In contrast to the staphylococcins isolated by Dajani et al. (5) and Gagliano and Hinsdill (10), staphylococcin 1580 could be obtained from the cells by extraction with 5% NaCl solution. Colicin E2 and colicin E3 can also be obtained by extraction with a NaCl solution (17). In contrast to many other bacteriocins, the staphylococcin 1580 production could not be in-

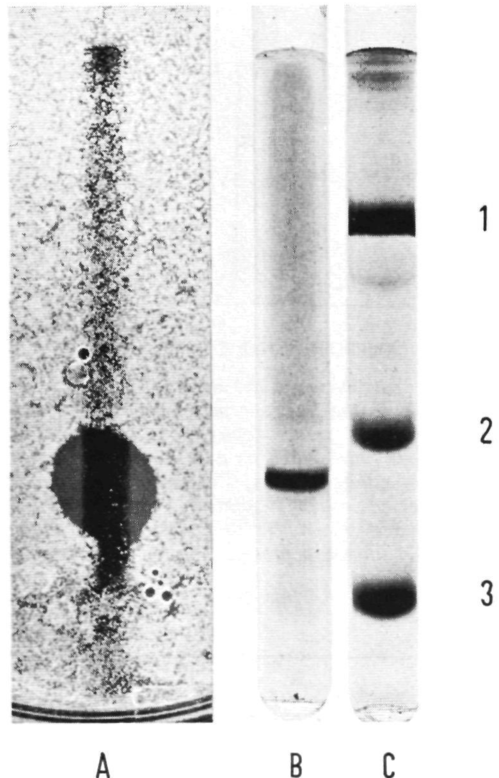

FIG. 7. *Polyacrylamide gel electrophoresis of purified staphylococcin 1580 on 10% gels with 0.1% sodium dodecyl sulfate. The gels were tested on staphylococcin activity (gel A) and stained for protein (gel B). Serum albumin (1), α-chymotrypsinogen (2), and cytochrome C (3) were used as references (gel C). About 100 μg of protein was applied on the gel.*

duced by ultraviolet irradiation or treatment with mitomycin C. This is in agreement with the results of Gagliano and Hinsdill (10) and Dajani et al. (4).

Purification of staphylococcin 1580 was achieved by a combination of ammonium sulfate precipitation, dialysis, ultracentrifugation, and gel filtration on Sephadex columns.

Many bacteriocins have been purified in recent years. Bacteriocins from certain *Escherichia coli* strains (30) as well as from *Pseudomonas aeruginosa* (18), *Listeria monocytogenes* (15), *Vibrio comma* (40), and *Lactobacillus acidophilus* (25) resemble elements of bacteriophages or whole phages. Megacin C, a bacteriocin produced by *Bacillus megaterium* (20), colicin E2 and E3 (17), and cloacin DF 13, a bacteriocin produced by *Enterobacter cloacae* DF 13 (13), are simple proteins with molecular weights of approximately 60,000.

Different from these bacteriocins are those which have been identified as lipo-polysaccharide-protein complexes. Colicin V-K 357 (22) and colicin I, produced by *Salmonella strasbourg* (24), and a bacteriocin produced by *Lactobacillus fermenti* strain 466 (26) have been shown to be lipo-polysaccharide-protein complexes. Staphylococcin 1580 seems to belong to this class of bacteriocins, whereas the bacteriocin produced by *Staphylococcus aureus* type 71, isolated by Dajani et al. (5), is only proteinaceous of nature. Staphylococcin 1580 resembles the bacteriocin isolated by Gagliano and Hinsdill (10) as to the chemical composition but differs as to the behavior during production and isolation. Staphylococcin 1580 is totally different from the staphylococcin recently isolated by Dobardzic et al. (8) which appeared to be a phage-like particle. Comparison with other staphylococcins is not possible because the lack of data (2, 11, 21, 28).

Urea and SDS split staphylococcin 1580 into smaller units, which migrated as one band on polyacrylamide gel electrophoresis and consisted of protein, lipid, and carbohydrate. The dissociation and association of the staphylococcin into subunits was a reversible process. Gel filtration indicated that the molecular weight of the subunits is between 10,000 and 25,000. However, this molecular weight cannot be calculated exactly from the position on the polyacrylamide gel with respect to the position of the references (39), since the subunits consist of protein, lipid, and carbohydrate and the references consist only of protein. Protein generally exposes a much more compact structure than carbohydrate.

A subsequent paper deals with a further study of the chemical nature and some properties of staphylococcin 1580.

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NATURE AND PROPERTIES OF A *STAPHYLOCOCCUS EPIDERMIDIS* BACTERIOCIN

Staphylococcin 1580, produced by *Staphylococcus epidermidis* 1580, consisted of 41 8% protein, 34% carbohydrate, and 21 9% lipid In the protein fraction, the acidic amino acids, glutamic and aspartic acid, and the neutral amino acids, glycine and alanine, predominated Neutral sugars consisted of glucose, galactose, and fucose in a molar ratio of 6 3 1 The purified bacteriocin was not inactivated by heating for 15 min at 120 C in the presence of 0 5% serum albumin and was stable in the *p*H range from 3 5 to 8 5 The compound was sensitive to the action of the proteolytic enzymes trypsin, Pronase, and chymotrypsin All gram-negative bacteria tested were resistant, a large number of gram-positive bacteria were sensitive to staphylococcin 1580 action Growth of stable staphylococcal L forms was inhibited by the bacteriocin to the same extent as their parent strains The staphylococcin was adsorbed to cell walls, cell membranes, and resistant cells The effect of staphylococcin 1580 appeared to be bactericidal but not bacteriolytic

A previous paper (23) described the production and purification of a bacteriocin named staphylococcin 1580 and obtained from *Staphylococcus epidermidis* 1580 grown on a semisolid medium. The bacteriocin was homogeneous upon polyacrylamide gel electrophoresis, exhibited a molecular weight between 150,000 and 400,000, and consisted of protein, carbohydrate, and lipid. In 6 M urea or in 0 1% sodium dodecyl sulfate, staphylococcin 1580 was split into subunits with a molecular weight between 10,000 and 25,000 This paper deals with the nature and some properties of this staphylococcin

MATERIALS AND METHODS

Microorganisms. The staphylococcin was produced by *S epidermidis* 1580 *S aureus* Oxford 209 P was used as indicator strain Stable staphylococcal L-forms and their parent strains, *S aureus* 212 and 6538, were obtained from B Kagan, Cedars-Sinai Hospital, Los Angeles, Calif The other strains of the several bacterial species were isolated from various sources in the hospital or were obtained from NCTC All bacterial strains were maintained on Trypticase soy agar (BBL) Staphylococcal L-forms were maintained on brain heart infusion agar (BHI agar, Difco) supplemented with 5% NaCl and 20% unheated horse serum

Isolation, purification, and assay of staphylococcin 1580. Staphylococcin 1580 was isolated, purified, and tested according to the methods described previously (23)

Chemical analysis. The protein content was calculated from the amino acid composition or determined by the method of Lowry et al (29) by using bovine serum albumin as reference Total nitrogen was determined by the micro-Kjeldahl method (2) Total phosphate was measured according to Fiske and Subbarow (13) Deoxyribonucleic acid and ribonucleic acid were determined according to Burton (7) and Webb (41), respectively

Amino acid analysis. Samples of staphylococcin 1580 dissolved in 6 N HCl were heated at 110 C for 24 hr in sealed, evacuated tubes The amino acid composition and the hexosamine content were determined with a Beckman automatic analyzer model 120 The data were corrected according to the method of Starbuck et al (38) The tryptophan content was estimated according to Beaven and Holiday (4)

Identification of carbohydrates. A sample of the bacteriocin was hydrolyzed in 2 N H_2SO_4 at 100 C for 2 hr After neutralization with $Ba(OH)_2$ and centrifugation, the hydrolysate was applied to a Dowex 50W-X8 (H^+ form) column (1 5 by 20 cm) prepared in water Neutral carbohydrates were eluted with water Hexosamines were eluted with 2 N HCl and quantitatively determined according to Boas (6) In order to identify the neutral carbohydrates, thin-layer chromatography was done for 9 hr on cellulose plates (Merck) with a mixture of pyridine, 1-butanol, and water (3 5 3, v/v/v) as solvent Samples of D-glucose, D-galactose, D-mannose, L-fucose, L-rhamnose, and D-xylose were used as references The chromatograms were dried and sprayed with a freshly prepared solution of 2% *p*-anisidine in a mixture of 1-butanol, ethanol, and 2 N HCl (4 1 1, v/v/v) con-

taining a trace of stannous chloride. After a few minutes at 110 C the hexoses yielded brown spots, the pentoses red spots, and the methylpentoses yellow spots (21). The total content of neutral sugars was determined according to the anthrone method (28). The determination of the relative amounts of the neutral sugars was performed after development of a 2-cm band on Whatman no. 3 paper by descending chromatography for 18 hr with a mixture of ethyl acetate, acetic acid, and water (3:2:2; v/v/v) as solvent. The chromatograms were dried and cut into strips (0.5 by 4 cm). The sugars were eluted with 5 ml of water and determined according to the anthrone method. In order to confirm the identity of the sugars, they were characterized by the Dische cysteine reactions (12).

Estimation of lipids. The lipid content was determined by the methods described by Smit et al. (37).

Determination of the action spectrum. *S. epidermidis* 1580 was streaked diametrically across the surface of a Trypticase soy agar or blood agar plate. The plate was incubated at 37 C for 30 hr. Cultures from indicator strains were streaked in duplicate on the surface of the plate at right angles to the line of the original inoculum. The plate was incubated at 37 C for another 18 hr. Strains, of which the growth was inhibited, were sensitive to staphylococcin 1580.

Isolation of cell walls. Cell walls were prepared according to Chatterjee (8), but modified as indicated below. Late exponential-phase cells of *S. aureus* Oxford 209 P were mechanically disrupted with glass beads in a Braun disintegrator at 4 C. After removal of the glass beads, the suspension was treated with deoxyribonuclease and ribonuclease (3 μg/ml each) for 60 min. Whole cells were removed by successive centrifugations at 2,000 $\times$ *g* for 10 min. The cell walls were sedimented at 15,000 $\times$ *g*, suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.6), and heated at 90 C for 10 min. After centrifugation at 15,000 $\times$ *g* and resuspension in the same buffer, the cell walls were treated with trypsin (200 μg/ml) at 37 C for 1 hr. After centrifugation at 15,000 $\times$ *g* the pellet was washed several times with water and finally suspended in water. This cell wall preparation was checked for the presence of whole cells with a phase-contrast microscope. Cell walls of *Escherichia coli* NCTC 10418 were prepared in a similar way.

Isolation of cell membranes. Cells of *S. aureus* Oxford 209 P, growing in the exponential phase, were centrifuged and suspended in 0.05 M Tris-hydrochloride buffer (*p*H 7.2) containing 25% NaCl. After treatment with lysostaphin (50 μg/ml) at 37 C for 1 hr, the suspension was centrifuged at 10,000 $\times$ *g* for 20 min. The spheroplasts were suspended in 0.05 M Tris-hydrochloride buffer (*p*H 7.2) and treated with deoxyribonuclease and ribonuclease (3 μg/ml each) at 37 C for 1 hr. Whole cells were centrifuged at 2,000 $\times$ *g* for 10 min and the cell membranes at 35,000 $\times$ *g* for 20 min. The cell membranes were treated once more with lysostaphin. After centrifugation at 35,000 $\times$ *g* and suspension in water, the final membrane preparation was obtained. Isolation and preparation of spheroplasts and cell membranes of *E. coli* NCTC 10418 was performed in a similar way according to Kaback (24).

Viable count. The viable count, expressing the total number of colony-forming units (CFU), was determined by plating a suitable amount of the sample on proteose peptone no. 3 agar (Difco). Colonies were counted after incubation at 37 C for 18 hr.

Chemicals. Bovine serum albumin, lysozyme, trypsin, chymotrypsin, deoxyribonuclease, ribonuclease, and the various sugars were purchased from Sigma Chemical Co., St. Louis, Mo. Pronase P was purchased from Serva, Heidelberg, Germany. Thin-layer chromatography was done on cellulose TLC aluminum sheets from Merck, Darmstadt, Germany. Lysostaphin was purchased from Schwarz/Mann, Orangeburg, N.Y. Horse serum was purchased from Oxoid Limited, London.

RESULTS

Chemical analysis. The chemical composition of staphylococcin 1580 is shown in Table 1. Staphylococcin 1580 consisted of protein (41.8%), carbohydrate (34%), and lipid (21.9%). The sum of the amounts of these compounds did not quite equal the total dry weight. The ratio between the percentage of protein calculated from the amino acid analysis and that of total nitrogen indicated that almost all nitrogen was originated from the protein. The protein determination according to Lowry et al. (29) revealed an amount 20% higher than that expected on basis of the amino acid analysis and the nitrogen content. The staphylococcin was free from nucleic acids and its phosphate content was low.

The protein and carbohydrate fractions were studied further by determination of the amino acid and sugar composition, respectively. The amino acid composition and hexosamine content are given in Table 2. The acidic amino acids, aspartic and glutamic acid, and the neutral amino acids, glycine and alanine, predom-

TABLE 1. *Chemical composition of staphylococcin 1580*

Component	Percentage
Dry weight	100
Protein	41.8[a]
Neutral carbohydrates	33.6
Hexosamines	0.4[b]
Lipid	21.9
Nitrogen	6.47
Phosphate	0.15
Deoxyribonucleic acid	0.0
Ribonucleic acid	0.0

[a] Calculated from the amino acid analysis.

[b] Determined according to Boas (6).

inated in staphylococcin 1580; histidine, arginine, methionine, and tyrosine occurred only in small amounts. The presence of tryptophan was questionable since a tyrosine-tryptophan ratio of about 23 was estimated as described by Beaven and Holiday (4) from the absorption spectrum in 0.1 N NaOH (Fig. 1). The hexosamine content determined in the amino acid analysis was 0.95% and corresponded to the amount determined according to Boas (6).

Neutral sugars could be identified in the acid hydrolysate of the staphylococcin by thin-layer chromatography using a mixture of pyridine, 1-butanol, and water as solvent (Fig. 2). Two brown spots and one yellow one could be detected having R_F values identical to those of galactose, glucose, and fucose, respectively. Development of chromatograms with mixtures of ethyl acetate, pyridine, and water (8:2:1; v/v/v), benzene, 1-butanol, pyridine, and water (1:5:3:3; v/v/v/v), and ethyl acetate, acetic acid, and water (3:3:1; v/v/v) as solvents and using also fructose and arabinose as references led to the same conclusion. The relative amounts of the neutral sugars were determined after elution from paper chromatograms (Fig. 3). Three distinct peaks could be observed. The sugar contents calculated from the areas of the peaks were 59% glucose, 31% galactose, and 10% fucose. In order to confirm the identity of glucose, galactose, and fucose, the sugars were analyzed by the Dische cysteine reactions (12). The absorption spectra were

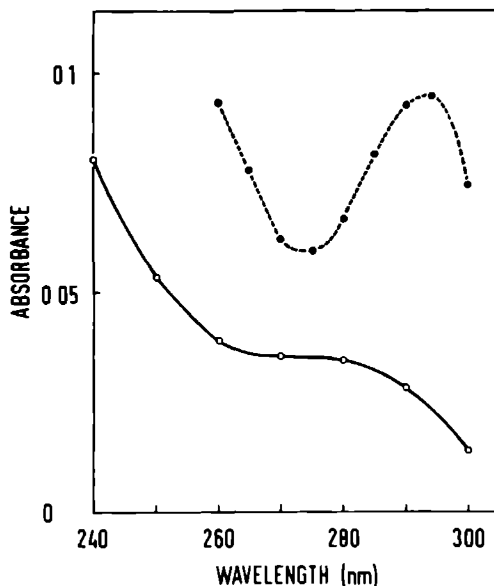

FIG. 1. *Absorption spectra of staphylococcin 1580. Staphylococcin was dissolved in water and the absorbance at the wavelengths indicated was measured (O). The same was done with staphylococcin 1580 dissolved in 0.1 N NaOH (●).*

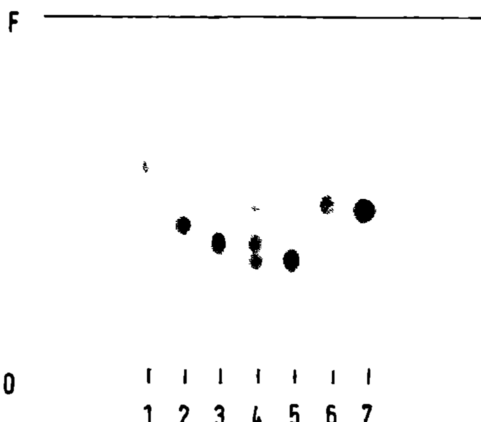

FIG. 2. *Chromatography of neutral sugars in the acid hydrolysate of staphylococcin 1580. Thin-layer chromatography was performed on cellulose plates with a mixture of pyridine, 1-butanol, and water as solvent. A sample of the hydrolysate was applied as a spot in the middle (4) Samples of rhamnose (1), mannose (2), glucose (3), galactose (5), fucose (6), and xylose (7) were used as references After development, the components were detected upon spraying with a p-anisidine solution. O, Origin. F, Front line of the solvent.*

TABLE 2 *Amino acid composition and hexosamine content of staphylococcin 1580*

Amino acid	Moles/100 moles
Aspartic acid	10 26
Threonine	5 49
Serine	6.98
Glutamic acid	10 59
Proline	6.37
Glycine	9 98
Alanine	10 10
Valine	6 66
Methionine	1.01
Isoleucine	7.20
Leucine	6 34
Tyrosine	1.92
Phenylalanine	6 72
Lysine	6 56
Histidine	2.19
Arginine	1.50
Cysteine	0 00
Tryptophan	0.08[a]
Hexosamine	0.95[b]
Ammonia	12.46

[a] Calculated according to Beaven and Holiday (4) using the date of Fig. 1.

[b] Corrected for loss during hydrolysis.

identical to those of the authentic substances (Fig. 4).

Stability of the bacteriocin activity. Crude preparations of the staphylococcin were stable upon heating for 15 min at 120 C. Purified staphylococcin was fully destroyed under these conditions but was stable in the presence of 0.5% bovine serum albumin (Table 3). The staphylococcin 1580 was very sensitive to mechanical agitation: a rapid decrease of activity was observed upon bubbling nitrogen through an active preparation. Lyophilization and storage at 4 C for a long period strongly reduced the activity. This effect could not be prevented by addition of β-mercaptoethanol (0.06 M) or glycerol (10 or 30%). The decrease in activity was even larger in the presence of glycerol. However, in the presence of 0.5% bovine serum albumin, staphylococcin 1580 could be lyophilized and stored for a long period without loss in activity.

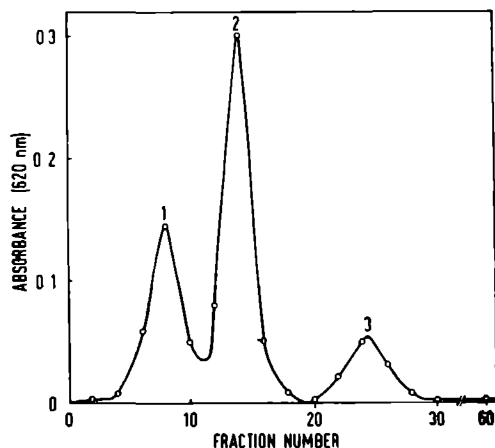

FIG. 3. *Paper chromatography of neutral sugars in the acid hydrolysate obtained from staphylococcin 1580. The hydrolysate was applied as a 2-cm band on Whatman no. 3 paper. After development, the chromatogram was cut into strips and the sugars were eluted from these strips with 5 ml of water. In the eluted fractions the sugar was determined according to the anthrone method. The peaks corresponded to galactose (1), glucose (2) and fucose (3). The molar ratio of the sugars could be calculated out of the area of the peaks. Fractions 1 and 55 correspond to the application place and the front of the solvent, respectively.*

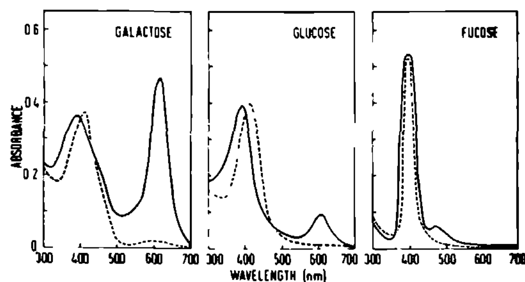

FIG. 4. *Characterization of the neutral sugars present in the acid hydrolysate of staphylococcin 1580. Paper chromatography was performed on Whatman no. 3 paper with a mixture of ethyl acetate, acetic acid, and water (3·2·2, v/v/v) as solvent. After development the sugars were eluted from the paper and characterized by the primary (broken line) and secondary (solid line) cysteine reaction.*

TABLE 3. *Effect of various treatments on the activity of purified staphylococcin 1580*[a]

Addition	Conditions	Percentage residual activity
None	2 weeks, 4 C	60
None	2 months, 4 C	5
BSA[b]	2 weeks, 4 C	100
BSA[b]	2 months, 4 C	100
None	15 min, 120 C	0
BSA[b]	15 min, 120 C	100
Glycerol 10%	2 weeks, 4 C	5
Glycerol 30%	2 weeks, 4 C	5
β-Mercaptoethanol[c]	2 weeks, 4 C	60
Trypsin[d]	30 min, 37 C	0
Pronase[d]	30 min, 37 C	0
Chymotrypsin[d]	30 min, 37 C	20
Lysozyme[d]	60 min, 37 C	100
Lysostaphin[e]	60 min, 37 C	100
Phenol	Extraction	0
Hexane	Extraction	0

[a] Five micrograms of purified staphylococcin, equivalent to 560 arbitrary units, present per milliliter of incubation mixture.

[b] 0.5% Bovine serum albumin.

[c] 0.06 M β-Mercaptoethanol.

[d] Trypsin, Pronase, chymotrypsin, or lysozyme (200 μg/ml) was incubated with the staphylococcin preparation at pH 7.5.

[e] Lysostaphin (10 μg/ml) was incubated with the staphylococcin preparation at pH 7.0.

The staphylococcin was sensitive to the action of trypsin, Pronase, and chymotrypsin. Lysozyme and lysostaphin had no effect on the activity of the staphylococcin. Separation of the protein moiety by treatment with an equal volume of 90% phenol at 4 C resulted in an inactive protein preparation. Extraction with an equal volume of hexane destroyed the activity. The activity was not destroyed by a 2-hr incubation period at pH values between 3.5 and 8.5 at 37 C (Fig. 5).

Action spectrum. The action spectrum was determined according to the method described in Materials and Methods. This method proved to be very reproducible. The growth of bacteria which were sensitive to staphylococcin

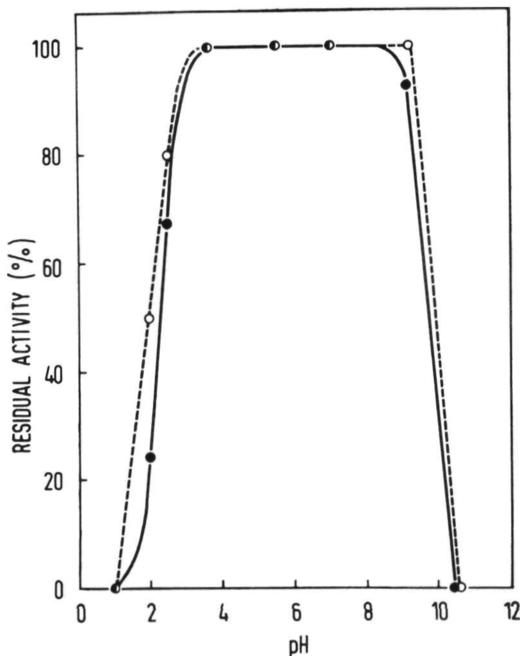

FIG. 5. *Stability of staphylococcin 1580 at various pH values. Samples of staphylococcin 1580 (3,500 arbitrary units) were placed at various pH values for 15 min (○) and 2 hr (●) at 37 C. pH 5.5 and 7.0 were adjusted with 0.1 M acetate and 0.1 M phosphate buffer, respectively. The other pH values were adjusted with 2 N HCl or 1 N NaOH. After this incubation the pH was brought back to pH 7.0 with HCl or NaOH and the staphylococcin activity of the several samples was determined.*

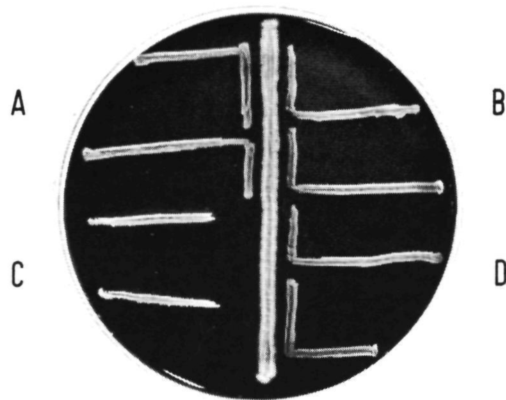

FIG. 6. *Demonstration of sensitivity of various bacteria to staphylococcin 1580. Staphylococcus epidermidis 1580 was streaked diametrically across the surface of a blood agar plate. After incubation the test strains were streaked in duplicate as indicated. A, Staphylococcus aureus NCTC 6571. B, Staphylococcus epidermidis 1580. C, Staphylococcus aureus Oxford 209 P. D, Streptococcus faecalis NCTC 9790. The sensitive strain Staphylococcus aureus Oxford 209 P showed inhibition.*

1580 was inhibited, whereas resistant strains were not (Fig. 6). All gram-negative bacteria tested were resistant (Table 4). Most of the gram-positive bacteria were sensitive to the action of the bacteriocin. Eighty different strains of *S. aureus*, isolated from patients in the hospital, were tested for sensitivity, and about 28% of the strains were sensitive. These strains represented various phage types and no correlation between susceptibility and phage type was noticeable. The staphylococcin-producing strain *S. epidermidis* 1580 was resistant to the action of its own bacteriocin.

Among the sensitive bacteria, a variation of susceptibility was found not only among various species but also among different strains of the same species (Table 5).

Stable staphylococcal L-forms, growing in 3.8% BHI supplemented with 5% NaCl and 20% unheated horse serum were equally as sensitive to staphylococcin 1580 as their parent strains (Table 5). Spheroplasts of *E. coli* NCTC 10418, growing in 3.0% TSB supplemented with 3% NaCl, 5% unheated horse serum, and 50 μg of lysozyme/ml, were still as resistant as cells of their parent strain.

Adsorption. Staphylococcin 1580 was incubated with bacterial cells, cell walls, and cell membranes at 37 C for 1 hr. After centrifugation the staphylococcin activity was determined in the supernatant fluid (Table 6). Staphylococcin 1580 was adsorbed both to cell walls and cell membranes of *S. aureus* Oxford 209 P and *E. coli* NCTC 10418. Whole cells of *S. epidermidis* 1580, *S. aureus* NCTC 6571, *E. coli* NCTC 10418, and *S. faecalis* NCTC 9790, which were resistant to the action of the bacteriocin, adsorbed staphylococcin 1580.

Bactericidal effect of staphylococcin 1580. Treatment of *S. aureus* Oxford 209 P grown in the exponential phase with staphylococcin 1580 caused a rapid decrease in the number of CFU whereas the optical density at 600 nm remained constant (Fig. 7). These results indicated that the effect of staphylococcin 1580 on sensitive bacteria was bactericidal rather than bacteriolytic.

DISCUSSION

Investigations of the chemical nature of bacteriocins have shown them to be a very heterogeneous group of substances ranging from simple proteins and proteins complexed with lipids and carbohydrates to particles resembling phages.

TABLE 4. *Inhibitory spectrum of staphylococcin 1580*

Microorganisms tested	No of strains tested	No of strains sensitive
Gram-positive		
Staphylococcus aureus	80	23
Staphylococcus epidermidis	11	7
Streptococcus faecalis	3	0
Streptococcus allantoicus	16	16
Streptococcus hemolyticus	3	3
Lactobacillus casei	1	0
Bacillus cereus	3	0
Bacillus megaterium	2	2
Bacillus subtilis	3	3
Bacillus pumilis	1	1
Corynebacterium diphtheriae	1	1
Corynebacterium pseudodiphtheriticum	1	1
Arthrobacter simplex	1	1
Arthrobacter globiformis	1	1
Clostridium perfringens	1	0
Listeria monocytogenes	1	1
Gram-negative		
Escherichia coli	9	0
Klebsiella pneumoniae	1	0
Aerobacter aerogenes	2	0
Proteus rettgeri	2	0
Proteus morganii	2	0
Erwinia aroideae	1	0
Serratia marcescens	1	0
Salmonella orion	1	0
Salmonella typhimurium	2	0
Pasteurella pseudotuberculosis	1	0
Agrobacterium tumefaciens	1	0
Pseudomonas aeruginosa	11	0
Pseudomonas fluorescens	2	0
Pseudomonas stutzeri	1	0

TABLE 5. *Susceptibility of various bacterial strains to staphylococcin 1580*

Microorganisms tested	N_0[a] (10^5 CFU/ml)	N_T[b] (10^5 CFU/ml)	N_0/N_T
Staphylococcus aureus NCTC 6571	771	770	1
S. aureus Oxford 209 P	793	0.33	2,410
S. aureus NCTC 9752	789	1.21	650
S. aureus 212	83	0.071	1,160
S. aureus 212 stable L-form	81	0.079	1,030
S. aureus 6538	69	0.043	1,610
S. aureus 6538 stable L-form	71	0.038	1,860
Bacillus subtilis NCTC 8236	791	0.79	1,040
B. subtilis 60015	798	1.11	720
Streptococcus allantoicus V 4014	790	0.47	1,680

[a] N_0 is the original number of colony-forming units (CFU) per ml.

[b] N_T is the number of CFU per ml after 5 min incubation at 37 C with 1,000 arbitrary units of staphylococcin 1580 per ml.

TABLE 6. *Adsorption of staphylococcin 1580 to cells and cell fractions*

Cell or cell fraction	Residual activity in supernatant (%)
Control without bacteria	100
Staphylococcus aureus Oxford 209 P[a]	0
S. aureus NCTC 6571[a]	0
Staphylococcus epidermidis 1580[a]	0
Streptococcus faecalis NCTC 9790[a]	0
Escherichia coli NCTC 10418[a]	0
Cell walls, *S. aureus* Oxford 209 P	0
Cell membranes, *S. aureus* Oxford 209 P	0
Cell walls, *E. coli* NCTC 10418	0
Cell membranes, *E. coli* NCTC 10418	0
L-forms, *S. aureus* 6538	0

[a] About 10^9 cells were incubated with 100 arbitrary units of staphylococcin 1580 in 1 ml for 60 min at 37 C.

Staphylococcin 1580 consisted of protein (41.8%), lipid (21.9%), and carbohydrate (34%).

Comparison of the amino acid composition of staphylococcin 1580 and several other bacteriocins (11, 19, 25) reveals many similarities. In all these bacteriocins, the acidic amino acids, glutamic and aspartic acid, and the neutral amino acids, glycine and alanine, were predominant. In contrast to most of the other bacteriocins, the phenylalanine content was high in staphylococcin 1580.

Comparison of the amino acid compositions and eventually the sugar contents of staphylococcin 1580 and other staphylococcins is not possible because no data are available (10, 15, 27).

The purified staphylococcin 1580 was inactivated by lyophilization, mechanical agitation, storage at 4 C over a long period, and heat treatment at 120 C for 15 min. Addition of bovine serum albumin had a stabilizing effect on the bacteriocin. Similar results were found in studies on colicin E2 (30).

The protein fraction of the staphylococcin 1580 seems to be very important in its activity since proteolytic enzymes destroyed the activity immediately. Goebel and Barry (17) were able to isolate an active protein from colicin K after extraction with phenol. Phenol and hexane extraction of staphylococcin 1580 yielded an inactive protein preparation. The presence of carbohydrates and lipids in the most purified and homogeneous preparation of

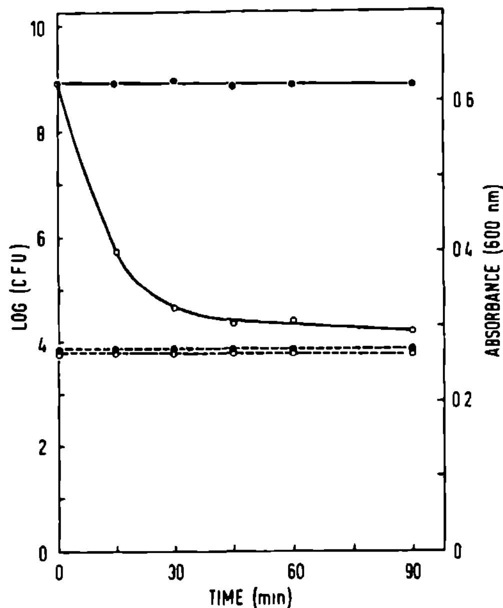

FIG. 7. *Bactericidal effect of staphylococcin 1580. Staphylococcus aureus Oxford 209 P was grown in Trypticase soy broth. Cells from the exponential growth phase were centrifuged, washed, and resuspended in 0.02 M phosphate buffer (pH 7.0). Samples of this suspension were treated with staphylococcin 1580 (O) or with phosphate buffer (●) and were aerobically incubated at 37 C. At suitable time intervals the number of colony forming units (CFU, solid lines) and the absorbance at 600 nm (broken lines) were determined.*

the staphylococcin indicates that these compounds form an integral part of the staphylococcin.

The inhibitory effect of the staphylococcin 1580 is not restricted to staphylococci, since a number of gram-positive organisms were sensitive to it. None of the gram-negative bacteria were found to be inhibited. Frederique (14) and Hamon and Peron (18) demonstrated that bacteriocins produced by gram-positive organisms had a much wider action spectrum than bacteriocins produced by gram-negative organisms. Also, staphylococcins recently isolated by Gagliano and Hinsdill (15) and Dajani et al. (9) showed a broad action spectrum. Some bacteriocin-producing cells are sensitive to their own bacteriocin (34). A similar result was found by Gagliano and Hinsdill for staphylococcin 414. However, *S. epidermidis* 1580 was resistant to its bacteriocin.

Reports in literature concerning the attachment of bacteriocins to indicator cells proposed that the receptor site of bacteriocins was situated on the cell membrane (5, 33), since stable L-forms still adsorbed bacteriocins. However, there is some discrepancy about the sensitivity of stable L-forms to the lethal action of bacteriocins (32, 36). It is not elucidated that the cell wall plays a role by the correct orientation of the bacteriocin to the cell membrane (20, 31).

Stable staphylococcal L-forms were inhibited by staphylococcin 1580 to the same extent as their parent strains. Spheroplasts of *E. coli* NCTC 10418 are as resistant as their parent strain. Staphylococcin 1580 was adsorbed to whole cells, cell walls, and cell membranes of resistant and sensitive strains. One may conclude that, on the one hand, the bacteriocin can be adsorbed to a specific receptor site on the cell membrane resulting in the death of the cell, but that, on the other hand, the bacteriocin can be adsorbed to various aspecific receptor sites on the cell wall and cell membrane, thus explaining the attachment even to *E. coli* cells. Further studies on adsorption are in progress.

Staphylococci produce many inhibitory substances, which show a bacteriolytic (1, 35) or a bactericidal (3, 16, 22) effect on other staphylococci. In contrast to the bactericidal substances, the knowledge of bacteriolytic enzymes is well established (39, 40). However, the latter compounds are not related to bacteriocins. Staphylococcin 1580 is bactericidal to sensitive cells like the staphylococcins isolated by Gagliano and Hinsdill (15), Dajani et al. (10), and Lachowicz (26).

Studies on the mode of action are in progress.

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CHARACTERISTICS OF THE KILLING EFFECT OF A *STAPHYLOCOCCUS EPIDERMIDIS* BACTERIOCIN

Staphylococcin 1580 killed cells of *Staphylococcus aureus* Oxford 209P At low concentrations of staphylococcin the decrease of the viable count, the rubidium efflux, and inhibition of amino acid uptake exposed a linear relationship to the amount of the bacteriocin used The killing action could not be reversed by trypsin treatment, was optimal at pH 7 6 to 7 8 and was dependent on the incubation temperature Cells pregrown at 37 C were much more sensitive to the staphylococcin than cells pregrown at 20 C

The nature of the resistance of various bacteria to staphylococcin may be due to the protection against the bacteriocin by their cell wall

Staphylococcin 1580, a bacteriocin produced by *Staphylococcus epidermidis*, inhibits macromolecular syntheses and transport of various compounds (1) Like colicins E1, K, and A (2,3), the staphylococcin exerts its action directly on the membrane since amino acid uptake into membrane vesicles is inhibited too (4,5) This action may be exerted by adsorption to specific receptor sites on the cytoplasmic membrane and subsequent changes in composition, conformation, and/or physicochemical characteristics of this membrane The cell wall does not play an essential role, but can block the penetration of the bacteriocin

This study deals with some characteristics of the killing effect of staphylococcin 1580

MATERIALS AND METHODS

Microorganisms. *S epidermidis* 1580 was the staphylococcin producing strain and *S aureus* Oxford 209P was used as indicator strain The staphylococcin-resistant *S aureus* Cowan and its sensitive mutant Cowan VI were kindly supplied by A Forsgren, Department of Microbiology, University of Uppsala, Uppsala *S aureus* 502A and its stable L-form were kindly supplied by B Kagan, Cedars-Sinai Hospital, Los Angeles, Calif , and *Bacillus caldolyticus* by W Heinen, Department of Exobiology, University of Nijmegen, Nijmegen All staphylococci were subcultured at 37C in Trypticase soy broth (TSB from BBL), *B caldolyticus* at 70 C in TSB containing 1% casein, and the stable staphylococcal L-forms on 3 8% brain heart infusion (Oxoid) supplemented with 5% NaCl and 20% unheated horse serum (Oxoid)

Preparation and assay of staphylococcin Staphylococcin 1580 was prepared and assayed as described previously (6) The activity was expressed as arbitrary units (A U) per ml (6)

Viable count. The viable count expressing the total number of colony forming units (C F U), was determined by plating suitably diluted amounts on Trypticase soy agar (TSA) The R_t-value was defined as the ratio between the viable counts of treated and untreated bacteria

Induction of spheroplasts. The spheroplasts of *S aureus* were induced according to the method described by Dienes (7)

Transport ^{86}Rb-efflux from cells and uptake of ^{14}C-glutamate into membrane vesicles of *S aureus* Oxford 209P were determined as described previously (1,5)

RESULTS AND DISCUSSION

Killing effect on sensitive cells. Treatment of cells of *S aureus* Oxford 209P with staphylococcin 1580 resulted in a rapid decrease of the viable count, as shown in Fig 1 At the lowest bacteriocin concentration the killing effect levelled, probably due to exhaustion of free bacteriocin by adsorption, since no residual activity could be detected in the supernatant obtained by centrifugation The effect of staphylococcin on the viable count was identical for cells grown in TSB, nutrient broth, proteose peptone No 3, and brain heart infusion

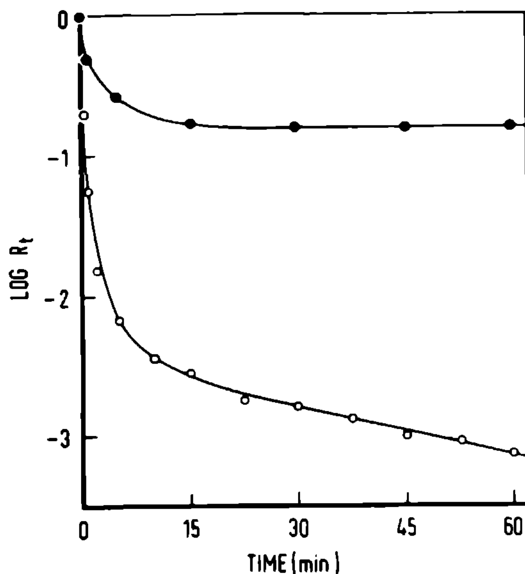

Fig. 1. Decrease of the viable count of S. aureus Oxford 209P cells caused by treatment with staphylococcin 1580. Cells were grown in the exponential phase in TSB and centrifuged. After resuspension in 0.05 M phosphate buffer (pH 7.6) at a concentration of 6x 10^8 cells/ml, and staphylococcin was added at a final concentration of 40 A.U./ml (•) or 200 A.U./ml (o). The viable count was measured at the time intervals indicated and the log R_t-value plotted against the time.

Trypsin is known to reverse the killing action of several bacteriocins (8), either in the absence or presence of 1,2-dinitrophenol or chloramphenicol. Though staphylococcin 1580 is sensitive to trypsin (200 μg/ml at pH 7.5 for 15 min), no reversal of its action was observed upon trypsin treatment (5 mg/ml at pH 7.5 for 20 min) either in the absence or presence of 1,2-dinitrophenol (10^{-3} M) or chloramphenicol (100 μg/ml). Probably the action of staphylococcin is rapid andirreversible or the adsorbed staphylococcin is resistant to trypsin.

Concentration dependence. The killing of *S. aureus* 209P cells, the efflux of rubidium ions, and inhibition of the uptake of glutamate was linearly proportional to the amount of staphylococcins at low concentrations (Fig. 2A-C). The concentration and survivor plots may indicate single hit kinetics (9, 10).

Temperature dependence. The killing effect of staphylococcin 1580 on cells of *S. aureus* Oxford 209P grown at 37 C decreased on lowering the temperatures of incubation (Fig. 3A). When cells were previously grown at 20 C, the killing effect was markedly reduced and hardly dependent on the incubation temperature (Fig. 3B). Differences in the membrane compositions of cells grown at 20 and 37 C may cause the different sensitivity (11). Staphylococcin 1580 is also active at 70

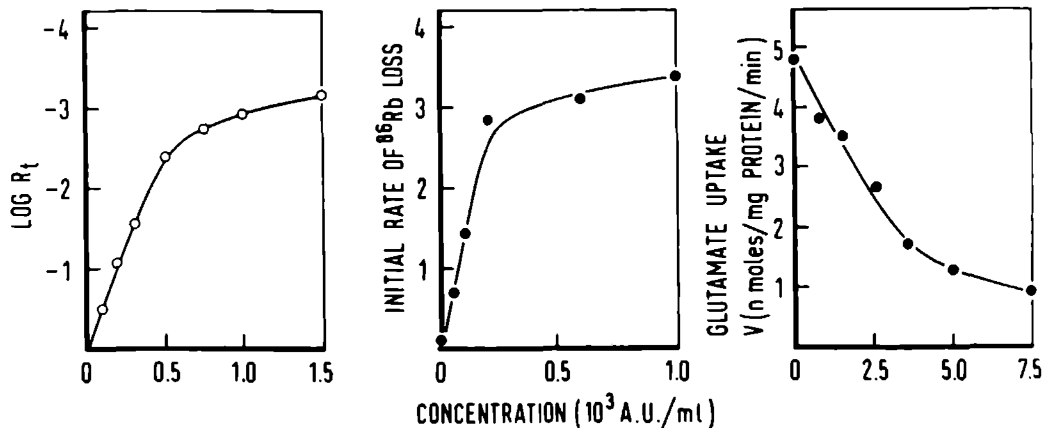

Fig. 2. Concentration dependence of the initial rate of staphylococcin 1580-induced decrease of the viable count, rubidium loss, and glutamate uptake. The experiments were carried out as described in Materials and Methods. A. 3x 10^9 cells of S. aureus Oxford 209P per ml in 0.05 M phosphate buffer (pH 7.6) were incubated with various staphylococcin concentrations and after 1 min incubations the viable count was determined. The log R_t-value is plotted against the staphylococcin concentration. B. The logarithm of the initial rate of ^{86}Rb loss, is plotted as a function of staphylococcin concentration; the initial rate was defined as the percentage of rubidium efflux. C. The initial rate of glutamate uptake is plotted as a function of the staphyloccoccin concentration.

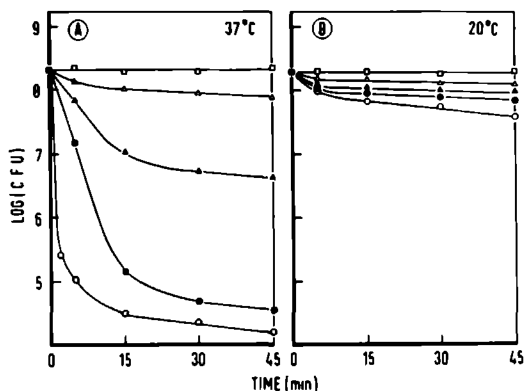

Fig 3 Temperature dependence of staphylococcin 1580 action Cells of S aureus Oxford 209P were grown at 37 C (A) in TSB centrifuged and resuspended in 0 05 M phosphate buffer (pH 7 6) at a final concentration of 2 5 x 10^8 cells/ml The cell suspension was incubated without (□) and with staphylococcin 1580 (750 A U /ml) at 37 C (o) 20 C (●), 10 C (▲) and 0 C (△) A similar procedure was followed for cells pregrown at 20 C (B) At various time intervals the viable count was determined

C, since growth of *Bacillus caldolyticus* was inhibited at this temperature

pH dependence. The effect of staphylococcin 1580 on cells of *S aureus* Oxford 209P was pH dependent (Fig 4) An optimal killing effect was observed in the region of pH 7 8 in tris (hydroxymethyl) aminomethane buffer and of pH 7 6 in phosphate buffer

Resistant, tolerant and sensitive mutants. As previously shown, staphylococcin-resistant mutants can be obtained from the staphylococcin-sensitive *S aureus* Oxford 209P, and staphylococcin-tolerant L-forms from the staphylococcin sensitive *S aureus* 502A L-forms (5) The latter L-forms were obtained from the staphylococcin-resistant *S aureus* 502A The wild strain *S aureus* Cowan is resistant to staphylococcin 1580 while its spheroplasts are sensitive A mutant of this strain, Cowan VI, was obtained from A Forsgren (12), the mutant lacks protein A in the cell wall and its phage type could not be determined, in contrast to the wild strain which produces protein A and exhibits the phage type pattern 52/52 A/80/81 The mutant is staphylococcin-sensitive which indicates that some wild strains are protected against staphylococcin by their cell wall and that sometimes sensitive mutants can be obtained from such strains, probably due to mutations in the cell wall composition Recently, a colicin K-sensitive mutant was obtained from a resistant strain of *Escherichia coli* (13)

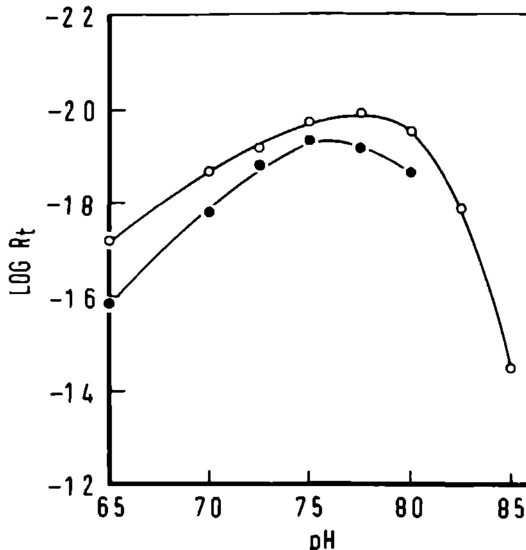

Fig 4 pH dependence of staphylococcin 1580 action Cells of S aureus Oxford 209P were grown in the exponential phase centrifuged and resuspended (10^8 cells/ml) in phosphate buffer (●) or in tris (hydroxymethyl) aminomethane (o) of different pH s and of constant ionic strength adjusted with KCl After 1 min incubation with staphylococcin (500 A U /ml) the viable count was determined

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MODE OF ACTION OF A *STAPHYLOCOCCUS EPIDERMIDIS* BACTERIOCIN

Staphylococcin 1580, a bacteriocin produced by *Staphylococcus epidermidis* 1580, is bactericidal to sensitive cells of many gram positive bacteria and stable staphylococcal L-forms The bacteriocin inhibited simultaneously the syntheses of deoxyribonucleic acid, ribonucleic acid, and protein, and caused neither degradation of deoxyribonucleic acid nor induction of phages in lysogenic, sensitive cells After 1 hr of treatment, extensive degradation of ribonucleic acid occurred, which was accompanied by leakage of ultraviolet absorbing material out of the cell The incorporation of glucose in acid precipitable and glycogenlike material was inhibited Furthermore, the staphylococcin inhibited the transport of glucose, glutamic acid, rubidium ions, and *o*-nitrophenyl-β-galactoside The uptake of oxygen was only gradually affected, but the intracellular adenosine triphosphate level fell rapidly to 15% of the control value The motility of sensitive *Bacillus subtilis* cells was markedly reduced on treatment Staphylococcin 1580 exhibited no phospholipase activity The phenomena are interpreted as resulting from an altered conformation and composition of the membrane, from an inhibition of transport through the membrane, or from a combination of these effects

Adsorption of bacteriocins to specific receptor sites on the cell membrane induces striking alterations of cellular functions and loss of viability The various bacteriocins exert their lethal action by affecting different biochemical targets such as deoxyribonucleic acid (DNA), ribosomes, and the electron transport chain, all of which have physical connection with the bacterial membrane Thus, it is quite possible that the bacteriocins act on these targets by way of the mediation of changes in the cell membrane

Colicin E2 causes degradation of DNA as a result of the activation of one or more nucleases (2, 33) Colicin E3 (6, 25) and cloacin DF 13 (20) affect protein synthesis The former causes cleavage of 16*S* ribosomal ribonucleic acid (rRNA) at a specific position near the 3′ terminus, which leads to the inactivation of the 30*S* subunits (25) Recent results of Boon (5) and Bowman (7) show that colicin E3 inactivates ribosomes in vitro and suggest that the killing of bacteria by this bacteriocin involves penetration into the cell Treatment of cells with cloacin DF 13 results in an inhibition of binding fMet transfer RNA^{fMet} to the messenger RNA ribosome complex (20) Colicins E1, K, A, Ia, and Ib (27, 29, 30) all inhibit energy dependent processes such as macromolecular synthesis and active transport

Little is known about the mode of action of staphylococcins Dajani et al (13) isolated a bacteriocin from phage type 71 *Staphylococcus aureus* This substance causes immediate cessation of protein and DNA synthesis as well as degradation of newly and previously formed RNA (14) Like colicins G, Q, and E1 (8), it causes extensive structural changes in susceptible bacteria (12)

Previously we have shown (21, 22) that the nature of staphylococcin 1580, a bacteriocin produced by *S epidermidis* 1580, is different from that of the bacteriocin described by Dajani et al (13) This paper deals with the mode of action of staphylococcin 1580 Its effect on macromolecular syntheses and transport phenomena will be described and compared with that of other bacteriocins

MATERIALS AND METHODS

Microorganisms. The bacteriocin producing strain was *S epidermidis* 1580 *S aureus* Oxford 209P and *S aureus* NCTC 6571 were used as bacteriocin sensitive and resistant strains, respectively *Bacillus cereus* V 5003 and *B subtilis* NCTC 60015 were used as indicator strains in the motility experiments *S aureus* strains 34 and NCTC 8319 were used in the phage induction experiments The strains were subcultured once a week on Trypticase soy broth agar (BBL) Stable L forms of *S aureus* 502 A were ob

tained from B Kagan, Cedars-Sinai Hospital, Los Angeles, Calif The L-forms were maintained on medium composed of 1 3% agar, 3 8% brain heart infusion, and 5% NaCl, and containing 20% unheated horse serum

Production, purification, and assay of staphylococcin 1580 The production and purification of staphylococcin 1580 and the assay of its activity have been described previously (22) The activity was expressed as arbitrary units (AU) per milliliter, as defined before (22)

Medium. Medium AJ1, containing 1% casein hydrolysate (enzymatically hydrolyzed, Difco), 0 8% glucose, 2×10^{-2} M potassium phosphate (*p*H 7 0), 10^{-3} M $MgSO_4 \cdot 7H_2O$, 1 ml of the oligodynamic solution described by Pollock and Kramer (32) per liter, 2×10^{-5} M thiamine, 2×10^{-3} M cysteine, 2×10^{-3} M histidine, and 1 μg of thymidine, uracil, adenine, and xanthine per ml, was used as the basic semisynthetic medium The casein hydrolysate contained 0 495 μmole of glutamic acid mg (Difco)

Incorporation of radioactive substrates. DNA, RNA, and protein synthesis and glucose uptake in *S aureus* Oxford 209P cells were studied by measurement of the incorporation of the radioactive precursors ^3H-thymidine, ^3H uracil, ^{14}C-glutamic acid, and ^{14}C-glucose, respectively In determinations of the incorporation of ^3H thymidine and ^3H-uracil, the cells were grown in AJ1 medium in the absence of the unlabeled compounds In the studies on the incorporation of ^{14}C glutamic acid or ^{14}C-glucose, cells were grown in AJ1 medium or AJ1 medium containing 1 mg of glucose per ml, respectively The incubation media were aerated at 37 C The incorporation of radioactivity was measured by addition of 2 ml samples to 2 ml of 10% trichloroacetic acid containing 50 μg of the unlabeled substrate per ml After 30 min at 0 C, the acid precipitated material was collected on membrane filters (0 45 μm pore size, Millipore Corp , Bedford, Mass) and washed with five volumes of cold 5% trichloroacetic acid solution supplemented with 50 μg of the unlabeled substrate per ml The filters were dried, and the radioactivity was measured in a Packard liquid scintillation counter, model 544, by use of a scintillation fluid consisting of 4 g of 2,5-diphenyloxazole and 0 1 g of 1,4 di-2(5 phenyloxazolyl)benzene per liter of toluene

Incorporation of ^{14}C glucose into glycogenlike material was measured according to Abraham and Hassid (1) Total ^{14}C-glucose uptake was measured by filtering the samples through membrane filters and washing with 0 02 M phosphate buffer (*p*H 7 0) containing 20 μg of glucose per ml

Transport of glutamic acid. The transport of glutamic acid was determined by the method of Nagel de Zwaig (29)

Measurement of $^{86}Rb^+$ efflux. The intra- and extracellular $^{86}Rb^+$ contents were determined by collecting the cells by filtration through membrane filters which were washed with 5 ml of 0 3 M sucrose solution buffered with 0 01 M tris(hydroxymethyl)aminomethane hydrochloride (*p*H 7 0) The radioactivity on the filter and in the filtrate was measured in a liquid scintillation counter and in a Philips gamma scintillation counter, respectively

Respiration. Oxygen uptake was measured in a Warburg type respirometer (Becker, Delft, The Netherlands) with standard manometric techniques described by Umbreit (36).

Measurement of ATP. Adenosine triphosphate (ATP) was assayed according to Stanley (35) Cells were grown under aeration in AJ1 medium at 37 C In the exponential phase, samples of 2 ml were removed and added to 1 ml of 30% (w/v) $HClO_4$ at 0 C, neutralized after 15 min with 2 M KOH and 0 5 M potassium phosphate (*p*H 7 4), and centrifuged For the assay, 0 4 ml of the supernatant fluid was added to 0 8 ml of 0 2 M glycylglycine buffer (*p*H 7 4) and 6 6 ml of water The reaction was initiated by addition of 0 2 ml of firefly lantern extract (10 mg/ml, in 0 05 M K_3AsO_4 and 0 02 M $MgSO_4$, *p*H 7 4) The photon emission was counted after exactly 10 sec in a Packard liquid scintillation counter operating with the photomultipliers out of coincidence, at 75% amplification and at settings of 65 to 85 divisions.

β-Galactosidase assay. The hydrolysis of *o* nitrophenyl β galactoside (ONPG) was determined by the method of Kennedy and Scarborough (24)

Motility. The cells were grown at 37 C in AJ2 medium containing 0 1% casein hydrolysate, 1% glucose, 2×10^{-2} M potassium phosphate (*p*H 7 0), 10^{-3} M $MgSO_4 \cdot 7H_2O$, 2×10^{-2} M $(NH_4)_2SO_4$, 2×10^{-3} M L-tryptophan, 2×10^{-3} M ammonium citrate, and 0 1% Tween SD 80 In the early exponentional phase, the motility was determined according to Shoesmith (34) and by observation of small hanging drops under a phase contrast microscope

Phage induction. To determine phage induction by staphylococcin 1580, cells of the sensitive and lysogenic strain *S aureus* 34, growing in AJ1 medium, were incubated in the presence and absence of staphylococcin The number of plaques forming units in the samples was determined against indicator strain *S aureus* NCTC 8319

Phospholipase activity. Phosphatidyl glycerol, diphosphatidyl glycerol, and lysylphosphatidyl glycerol were extracted from ^{32}P-labeled cells of *S aureus* Oxford 209P by the method of Bligh and Dyer (4) The mixture of phospholipids was suspended in 0 1 M phosphate buffer (*p*H 7 5) containing 20 mM MgCl and 0 4% Triton X-100, and an equal volume of an aqueous solution of staphylococcin 1580 or water was added After 3 hr of incubation at 37 C, the phospholipids were extracted with a mixture of chloroform and methanol (3:1) and were separated by thin layer chromatography on silica gel plates with a mixture of chloroform, methanol, and water (65:25:4) as solvent An autoradiogram was made to detect the ^{32}P-labeled compounds, which were quantitatively determined in a liquid scintillation counter A similar procedure was followed for unlabeled lecithin, after thin layer chromatography lecithin and its hydrolysis products were detected with iodine vapor

Chemicals. The firefly lantern extract, ATP, and ONPG were purchased from Sigma Chemical Co ^{14}C L glutamic acid (260 mCi/mmole), ^3H uracil (1 Ci mmole), ^3H thymidine (5 Ci mmole), ^{14}C glucose, uniformly labeled (3 mCi/mmole), $^{86}RbCl$ (2 to 10 mCi/mg), and sodium dihydrogen ^{32}P-orthophosphate (10 mCi mmole) were purchased from the

Radiochemical Centre, Amersham, England. Tween SD 80 was purchased from Atlas-Goldschmidt GMBH, Essen, Germany.

RESULTS

Macromolecular syntheses. Treatment of cells of *S. aureus* Oxford 209P with staphylococcin 1580 resulted in a rapid inhibition of the incorporation of labeled thymidine, uracil, and glutamic acid into acid-insoluble material (Fig. 1). The synthesis of macromolecules was not affected when trypsin-degraded staphylococcin was used or when the staphylococcin (1,000 AU/ml) was added to resistant *S. aureus* (NCTC 6571) cells. The inhibition occurred immediately, as was observed for colicins K and E1 (30). In contrast, the effects of colicins E2 and E3 manifest themselves slowly and at high bacteriocin concentrations (30). Staphylococcin affected the synthesis of DNA, RNA, and protein simultaneously and independently. No extensive degradation of DNA occurred because no decrease in trichloroacetic acid-insoluble thymidine-labeled material was noticed even after 2 hr of incubation with staphylococcin. Moreover, the staphylococcin did not induce vegetative replication of phages in lysogenic sensitive strains, an effect observed with colicin E2 and due to the degradation of DNA (30). After prolonged incubation of cells with staphylococcin, the RNA was extensively degraded (Fig. 2). Furthermore, after a lag period of 1 hr, material optimally absorbing at 260 nm leaked from staphylococcal cells (10^9 cells per ml) treated with 4,000 AU of staphylococcin per ml. However, the action of staphylococcin 1580 is bactericidal rather than bacteriolytic (21). Stable L-forms of *S. aureus* 502 A were also sensitive to staphylococcin and did not lyse on treatment.

No incorporation of labeled glutamic acid, uracil, and thymidine into trichloroacetic acid-insoluble material of staphylococcin 1580-pretreated cells was observed.

Glucose incorporation. Staphylococcin 1580 inhibited immediately the incorporation of ^{14}C-glucose into acid-insoluble material (Fig. 3). Moreover, the total uptake of ^{14}C-glucose and its incorporation into glycogenlike material were 90% inhibited in cells pretreated for 5 min with staphylococcin (2,000 AU/ml) and incubated for 20 min in the presence of ^{14}C-glucose (0.5 μCi/ml).

Glutamic acid transport. The uptake of glutamic acid was blocked in cells which were preincubated with staphylococcin and chloramphenicol prior to the addition of labeled glutamic acid (Fig. 4A). The addition of bacteriocin to chloramphenicol-treated cells resulted in a rapid release of pre-accumulated ^{14}C-glutamic acid (Fig. 4B).

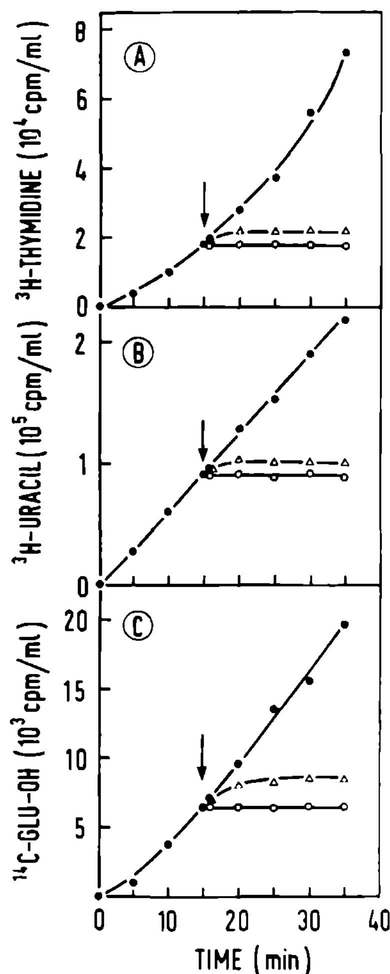

FIG. 1. *Effect of staphylococcin 1580 on macromolecular syntheses. Cells of Staphylococcus aureus Oxford 209P were grown in AJ1 medium at 37 C to a concentration of 10^8 cells per ml. (A) Effect of staphylococcin on ^{3}H-thymidine incorporation. The experiment was started by addition of 0.5 μCi of ^{3}H-thymidine per ml; after 15 min at 37 C, the culture was divided into three equal parts: two received different amounts of the bacteriocin in 0.01 M phosphate buffer (pH 7.0), and one received only buffer to serve as the control. At various time intervals, the ^{3}H-thymidine incorporation into acid-precipitable material in 2-ml samples was measured. (B) Effect of staphylococcin on ^{3}H-uracil incorporation. The procedure was the same as under A but 0.5 μCi of ^{3}H-uracil was added per ml. (C) Effect of staphylococcin on ^{14}C-glutamic acid incorporation into hot-acid-precipitable material. The procedure was the same as under A, but 0.5 μCi of ^{14}C-glutamic acid was added per ml. Symbols: ●, control; △, 100 AU of staphylococcin per ml of incubation mixture; and ○, 1,000 AU per ml.*

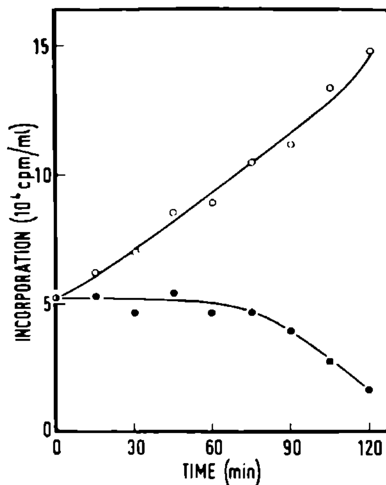

FIG. 2. *Degradation of RNA induced by staphylococcin 1580. The same conditions were used as described in Fig. 1B. Symbols:* ○, *without staphylococcin;* ●, *3,000 AU of staphylococcin per ml of incubation mixture.*

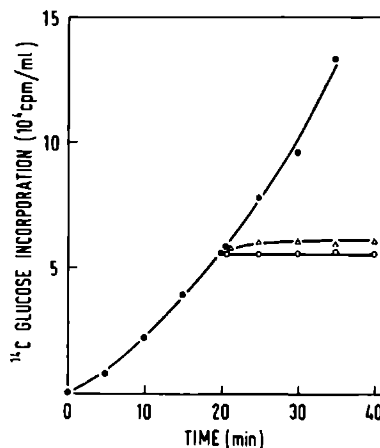

FIG. 3. *Effect of staphylococcin 1580 on the incorporation of ^{14}C-glucose. Cells of Staphylococcus aureus Oxford 209P were harvested in the exponential growth phase and incubated at 37 C in AJ1 medium supplemented with 1 μCi of ^{14}C-glucose per ml. After 20 min, the cell suspension was divided into three equal parts: two received staphylococcin in 0.01 M phosphate buffer (pH 7.0), and the third received only buffer. At various time intervals, samples were withdrawn, and the ^{14}C-glucose incorporation into acid-precipitable material was determined. Symbols:* ●, *control;* △, *100 AU of staphylococcin per ml of incubation mixture;* ○, *1,000 AU per ml.*

Rubidium efflux. A rapid efflux of preaccumulated rubidium was observed, resulting in a marked depletion of cellular rubidium (Fig. 5). More than 90% of the intracellular rubidium was lost within 4 min.

Respiration. The oxygen consumption of cells growing on glucose was inhibited by staphylococcin 1580 (Fig. 6). However, this effect increased on prolonged incubation and was less drastic than as was observed for macromolecular syntheses. Therefore, the effect on respiration does not seem to be the primary effect of the bacteriocin.

ATP level. The ATP level was determined according to the firefly assay. The assay was linearly on a full logarithmic scale from 10 to 500 pmoles of ATP per ml. In cells treated with staphylococcin, the ATP content decreased rapidly (Fig. 7), and reached 15% of the original value within 10 min.

ONPG transport. No hydrolysis of ONPG could be detected in whole cells, which were preinduced for this enzyme and transport system immediately after addition of staphylococcin (Fig. 8). No effect of the bacteriocin (3,400 AU/ml) was observed on the β-galactosidase in cell-free extracts, obtained by disintegration in a Braun homogenizer. No β-galactosidase leaked out of the cells; most probably the permeation of ONPG or the product of the enzymatic reaction, or both, through the membrane was markedly reduced.

Motility. Cells of staphylococcin-resistant *B. cereus* V5003 and -sensitive *B. subtilis* NCTC 60015 were used. The cells are actively motile in the early exponentional phase, when grown in AJ2 medium. The effect of staphylococcin 1580 on motility was quantitatively determined by the method of Shoesmith (34) and by observation of small hanging drops of control and staphylococcin-treated cells (1,000 AU of staphylococcin per ml) under a phase-contrast microscope. Control cells remained vigorously motile during the tests, whereas the motility of treated sensitive cells slowed down (Fig. 9). The motility of cells of *B. cereus* was not affected by the staphylococcin.

Phospholipase activity. In view of the action of megacin A, which has been identified as phospholipase A (31), the phospholipase activity was investigated. A mixture of ^{32}P-phosphatidyl glycerol, ^{32}P-diphosphatidyl glycerol, and ^{32}P-lysylphosphatidyl glycerol was incubated at 37 C in the presence and absence of staphylococcin 1580 (2,000 AU per ml) for 3 hr. No evidence of hydrolysis of the phospholipids was found. In both cases, only three spots could be observed on the autoradiogram, and the percentages of phosphatidyl glycerol (69%), diphosphatidyl glycerol

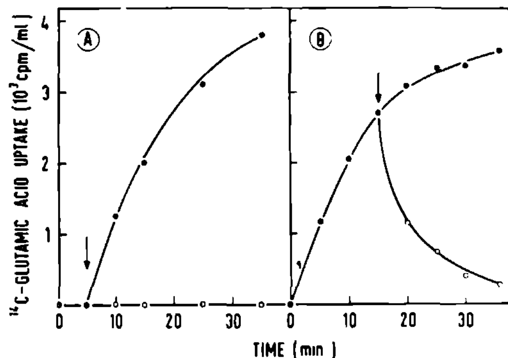

FIG 4. *Effect of staphylococcin 1580 on amino acid transport Cells of Staphylococcus aureus Oxford 209P were grown in AJ1 medium. When the culture reached an optical density of 0 5 at 600 nm, chloramphenicol (100 μg/ml) was added (A) After 15 min at 37 C, the culture was divided into two parts· one received staphylococcin 1580 in 0 01 M phosphate buffer (pH 7 0) and the other received only the buffer. After 5 min, 0 1 μCi of ^{14}C-glutamic acid was added per ml At various time intervals, samples were removed and the uptake of ^{14}C-glutamic acid was measured (B) To the chloramphenicol pretreated cell suspension 0 1 μCi of ^{14}C-glutamic acid was added per ml After 15 min, the suspension was split out into two flasks, one containing staphylococcin 1580 in 0 01 M phosphate buffer (pH 7 0) and the other containing only the buffer Samples were taken at the times indicated, and intracellular ^{14}C-glutamic acid was measured Symbols· ●, control, ○, 1,000 AU of staphylococcin per ml of incubation mixture*

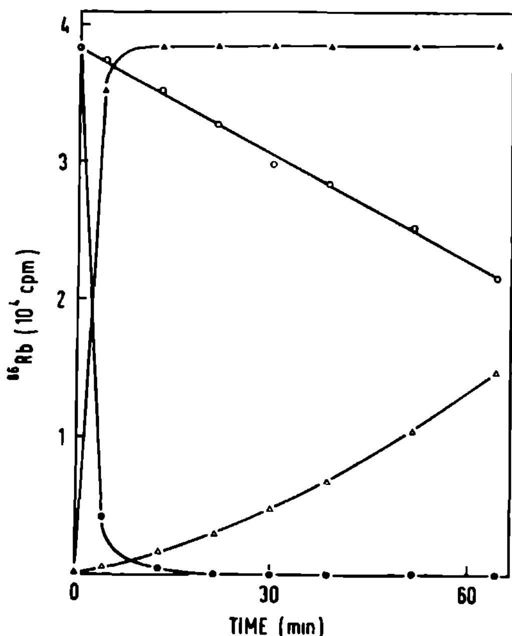

FIG 5 *Effect of staphylococcin 1580 on rubidium transport Cells of Staphylococcus aureus Oxford 209P were grown for 4 hr at 37 C in 100 ml of nutrient broth supplemented with 0 1 ml of ^{86}RbCl (2 to 10 mCi/ml). When the culture reached an optical density of 0 5 at 600 nm, the cells were centrifuged, washed, and resuspended in 0 3 M sucrose solution buffered with 0 01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7 0) One part was treated at 37 C with staphylococcin 1580 (final concentration of 1,000 AU per ml of incubation mixture) in buffered sucrose solution (closed symbols), and the control received only buffered sucrose solution (open symbols) At the time intervals indicated, intracellular (circles) and extracellular (triangles) radioactivity of 2-ml samples was determined*

(12%), and lysylphosphatidyl glycerol (19%) remained unaltered in the presence of staphylococcin In a separate test with lecithin we observed no degradation products after incubation in the presence of staphylococcin

DISCUSSION

Staphylococcin 1580 is bactericidal to sensitive staphylococci, many other gram-positive bacteria, and stable staphylococcal L-forms

The effects of staphylococcin 1580 on the various biochemical processes in the cell are very similar to those observed for colicins Ia, Ib (27), A (29), E1, and K (30), and resemble in many aspects the mode of action of the staphylococcin of the phage type 71 staphylococci (14) Like these bacteriocins, staphylococcin 1580 inhibited DNA, RNA, and protein synthesis The inhibition of the three processes seems to occur simultaneously and independently After 1 hr of treatment with staphylococcin, extensive degradation of RNA was initiated, but no extensive degradation of DNA was observed Therefore, degradation of RNA seems not to be the primary effect of the staphylococcin but may be due to structural changes induced by the observed leakage of monovalent cations, rendering ribosomes susceptible to the attack of nucleases, as suggested for colicin K (25) Like megacin A (31), colicins E1, K, G, A, and Q (8–10), and the staphylococcin of phage-type 71 staphylococci (12), staphylococcin 1580 caused leakage of ultraviolet-absorbing material which most probably contains degradation products of RNA

Furthermore, staphylococcin 1580 exerted a marked effect on the transport of various compounds, e g , glutamic acid, glucose, Rb ions, and ONPG

These effects on biochemical processes indicate that at least three mechanisms of primary action may be involved, namely, inhibition of the energy supply, an alteration of the membrane perme-

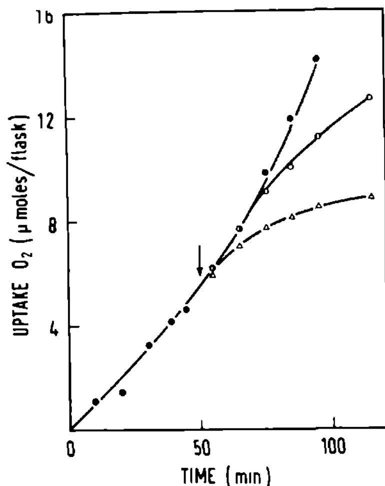

FIG 6 *Effect of staphylococcin 1580 on the uptake of oxygen Cells of Staphylococcus aureus Oxford 209P were harvested in the exponential phase and resuspended in 2 ml of AJ1 medium to an optical density of 0 6 at 600 nm The uptake of oxygen was measured in a Warburg apparatus After 50 min (arrow), staphylococcin in 0 01 M phosphate buffer (pH 7 0) or phosphate buffer was added from a side arm of the Warburg flask Symbols* ●, *control,* ○, *800 AU of staphylococcin per ml of incubation mixture,* △, *1,500 AU per ml*

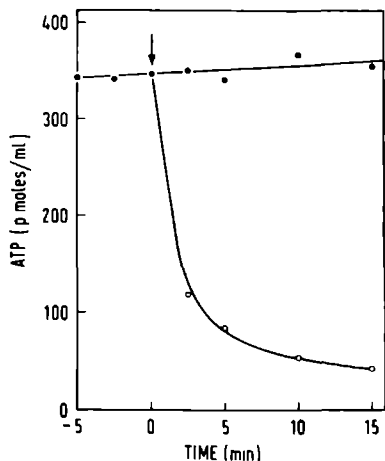

FIG 7 *Effect of staphylococcin 1580 on the intracellular ATP level Cells of Staphylococcus aureus Oxford 209P were grown in AJ2 medium at 37 C At time zero, the cell suspension was divided into two parts one received staphylococcin 1580 (final concentration, 1,500 AU per ml of incubation mixture) in 0 01 M phosphate buffer, pH 7 0* (○), *the other received only buffer* (●) *At the time intervals indicated, samples were removed and assayed for ATP*

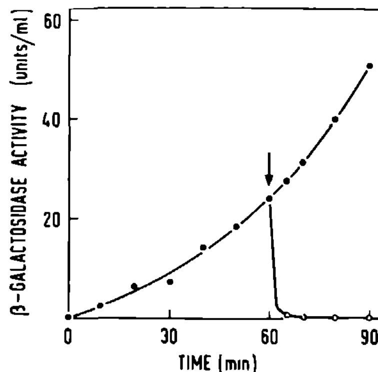

FIG 8 *Effect of staphylococcin 1580 on β-galactosidase activity of whole cells β Galactosidase was induced by galactose in Staphylococcus aureus Oxford 209P cells After 60 min (arrow), the cell suspension was divided into two parts one was treated with staphylococcin 1580 (final concentration of 1,000 AU per ml of incubation mixture) in 0 01 M phosphate buffer, pH 7 0* (○), *and the other received only buffer* (●) *Samples were removed, and β galactosidase activity was assayed in whole cells*

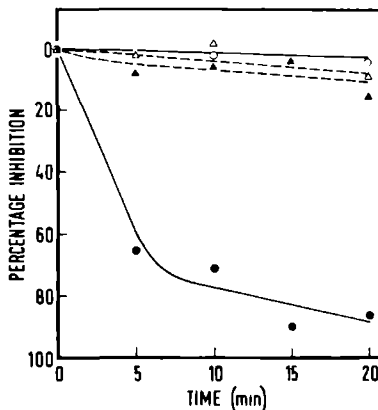

FIG 9 *Effect of staphylococcin on motility Bacillus cereus V 5003 (broken lines) and Bacillus subtilis NCTC 60015 (solid lines) were grown in AJ1 medium and treated in the exponential phase with staphylococcin 1580 (final concentration of 1,000 AU per ml of incubation mixture) in 0 01 M phosphate buffer, pH 7 0 (closed symbols), or with buffer (open symbols) Samples were taken at the times indicated, and the motility was assayed quantitatively according to Shoesmith (34)*

ability, or drastic changes in the membrane structure These possibilities are not mutually exclusive

The effects caused by colicins E1 and K (17, 18), Ia and Ib (27), and A (29) were interpreted as an interference with the supply of energy in the af

fected *Escherichia coli* cells. This interference may be a selective inhibition of the oxidative phosphorylation or an activation of membrane-bound adenosine triphosphatase. Results of Feingold (16) indicated that the inhibition of active transport and macromolecular syntheses was not caused by cessation of the ATP production. Like the above-mentioned colicins, staphylococcin 1580 gradually inhibited the oxygen uptake of sensitive cells. The ATP level was reduced rapidly by staphylococcin to a level 15% of the original one. This effect may explain the inhibition of motility of the sensitive *B. subtilis* cells, but seems not to be the primary action of the staphylococcin, since a distinct amount of ATP remains available to the cells. Moreover, the effect on the various transport phenomena reported here cannot be explained on basis of ATP depletion, since ATP is not directly involved in them.

An effect of a bacteriocin on transport phenomena was previously shown for colicin E1 (3) and colicin A (29). The transport of amino acids and some β-galactosides in *E. coli* cells under aerobic conditions is tightly coupled to a membrane-bound D-lactate dehydrogenase and to a smaller extent to a succinate dehydrogenase, which both are coupled to the electron transport chain (23). Stable high-energy phosphate compounds are not involved in this transport. Recently, it was demonstrated that colicin E1 and K uncouple both D-lactate and succinate dehydrogenases from proline transport in isolated membrane vesicles (J. P. Kabat and S. E. Luria, Bacteriol. Proc., p. 62, 1970).

The transport of glutamic acid and various other amino acids, measured in isolated membrane vesicles of *S. aureus*, seems to be almost exclusively coupled to a membrane-bound α-glycerolphosphate dehydrogenase, which is coupled to the electron transport chain (26). Since oxygen uptake is only gradually inhibited, the staphylococcin, like colicin E1, may uncouple the electron transport chain from amino acid transport rather than block the electron transport chain itself. In accordance with this view, streptococci and hemin-negative mutants of *E. coli*, both of which lack cytochromes, are still sensitive to staphylococcin 1580 (21) and colicin E1 (17), respectively. Alternatively, the staphylococcin interferes directly with potassium, rubidium, or proton transport like valinomycin or 2,4-dinitrophenol, both of which abolish aspartate uptake in *S. aureus* (19). However, the inhibition of the transport of ONPG, which is mediated by a phosphoenolpyruvate phosphotransferase system in *S. aureus* (24), is not in accordance with both alternatives.

It must be emphasized also that the effect of staphylococcin on the synthesis of DNA, RNA, protein, and glycogenlike substances can be merely a result of inhibition of the transport of the radioactive precursors used in our experiments.

Various membranous and transport activities are dependent on lipid-protein interactions in the membrane (28). The stimulation of aspartate uptake by several lipids may be interpreted as a physical change in membrane structure (19). Inhibition of transport and other processes caused by staphylococcin 1580 could be due to alterations of lipid composition and lipid-protein interactions. This hypothesis can explain most of the effects caused by the staphylococcin. The bacteriocin may adsorb to the cell membrane, inducing conformational changes in the proteins or lipids, or both, of the membrane. These changes may be allosteric in nature, and a model has been suggested by Changeux and Thiéry (11) to explain how such alterations are spread throughout the membrane. Alternatively, the staphylococcin interferes directly with phospholipid metabolism, causing an altered lipid composition, followed by conformational changes over the membrane. Preliminary experiments presented here show that staphylococcin 1580 exposes no phospholipase activity on phospholipids in vitro; neither did colicin E1, K, and A, though the latter cause changes in phospholipid composition of the membrane (9). In this aspect these bacteriocins differ from megacin A, which acts like phospholipase A on the cell membrane and several phospholipids (31).

Theories similar to those proposed here for the mode of action of colicins and staphylococcin 1580 have been suggested for the mode of action of phage ghosts (15) and several morphine derivatives (19).

Studies on the effect of staphylococcin 1580 on phospholipid metabolism and transport through membrane vesicles are in progress and may further elucidate the primary action.

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EFFECTS OF COLICIN A AND STAPHYLOCOCCIN 1580 ON AMINO ACID UPTAKE INTO MEMBRANE VESICLES OF *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

Staphylococcin 1580 increased the relative amount of diphosphatidylglycerol and decreased the amount of phosphatidylglycerol in cells of *Staphylococcus aureus*, while the amounts of lysylphosphatidylglycerol, phosphatidic acid and total phospholipid remained constant.

Treatment of cells of *Escherichia coli* and *S. aureus* with colicin A and staphylococcin 1580, respectively, did not affect proton impermeability but subsequent addition of carbonyl cyanide-m-chlorophenylhydrazone resulted in a rapid influx of protons into the cells.

Bacteriocin-resistant and- tolerant mutants of *E. coli* and *S. aureus* were isolated. The bacteriocins caused leakage of amino acids preaccumulated into membrane vesicles of resistant mutants and had no significant effect on membrane vesicles of tolerant mutants.

The uptake of amino acids into membrane vesicles was inhibited by both bacteriocins, irrespective of the electron donors applied. The bacteriocin inhibition was noncompetitively. The bacteriocins did not affect oxygen consumption and dehydrogenases in membrane vesicles.

Both bacteriocins suppressed the decrease in the fluorescence of 1-anilino-8-naphthalenesulfonate caused by D-lactate or α-glycerolphosphate when added to membrane vesicles.

It is concluded that the bacteriocins uncouple the carrier proteins from the electron transport system.

Bacteriocins are high-molecular, bactericidal antibiotics produced by certain strains of bacteria. Colicin A and staphylococcin 1580 are bacteriocins produced by *Citrobacter freundii* and *Staphylococcus epidermidis*, respectively. The former consists of a single protein molecule with a molecular weight of 60,000 (1) and the latter of a lipid-carbohydrate-protein complex (2,3).

Bacteriocins adsorb to specific receptor sites on the membrane of sensitive bacteria. Mutant strains no longer sensitive to bacteriocins can be distinguished into two classes, namely, bacteriocin-resistant mutants which have lost the ability to adsorb the bacteriocin onto the specific receptor site and bacteriocin-tolerant mutants which still adsorb bacteriocin to the receptor site but which are not killed (4).

Studies on the mode of action revealed quite different specific biochemical effects on sensitive bacteria. Colicin E2 (5), megacin C (6) and vibriocin (7) cause degradation of the bacterial DNA. Colicin E3 (8) and cloacin DF 13 (9) inhibit protein synthesis. It seems that colicin E2 (10) and E3 (11) molecules penetrate somehow into the cytoplasm after adsorption and then affect DNA and ribosomes, respectively. Colicins E1 and K (12), Ia and Ib (13), and A (14,15), and staphylococcin 1580 (16,17) inhibit macromolecular synthesis and transport of various compounds and seem to act directly on the membrane. The mechanism of action of these bacteriocins is yet unknown but it has been suggested previously (16,18), that conformational changes, alterations in phospholipid metabolism or in transport might be involved in it. The aim of this study was to evaluate these possibilities. Furthermore, the study on these bacteriocins may be of interest for the elucidation of transport phenomena and the possible role which cations and lipids may play in it.

MATERIALS AND METHODS

Bacterial strains The colicin A- and staphylococcin 1580-producing strains were *Citrobacter freundii* C31 and *Staphylococcus epidermidis* 1580, respectively The indicator strains used were *Escherichia coli* K12, *S aureus Oxford* 209P and *Bacillus subtilis* W23, which was kindly donated by W N Konings All strains were maintained on Trypticase soy agar (TSA) Stable L-forms of *S aureus* 502A were maintained on a medium containing 1 5% agar, 3 8% brain heart infusion, 5% NaCl and 20% unheated horse serum

Production and purification of bacteriocins Colicin A was obtained by a modified procedure according to Dandeu (1) An overnight culture of *C freundii* C31 cells in 3% Trypticase soy broth (TSB) was inoculated 1 100 into the same medium and grown under aeration at 37°C When the culture reached an optical density of 0 2 at 600 nm colicin A induction was achieved by addition of mitomycin C (0 5 mg/l) The culture was incubated further for 4 h and centrifuged for 10 min at 10,000 x g Colicin A was partially purified by ammonium sulfate precipitation and DEAE-cellulose chromatography Staphylococcin 1580 was produced and purified as described previously (2)

Assay of bacteriocin activity The bacteriocins were assayed as described previously (2) The activity was expressed in arbitrary units (A U) per ml

Isolation of mutants Cells of *S aureus* Oxford 209P were irradiated for 15 s with ultraviolet light (900 ergs/cm^2/s) Staphylococcin-resistant mutants were enriched by growth in the presence of staphylococcin 1580 (2000 A U /ml) and selected by growth of suitably diluted amounts on TSA containing staphylococcin 1580 (2000 A U /ml) A similar procedure was followed to isolate staphylococcin-tolerant mutants of stable L-forms of *S aureus* 502A Colicin A-resistant and - tolerant mutants of *E coli* K12 were isolated as growing colonies after spreading of 10^8 cells on TSA containing colicin A (5000 A U /ml) In order to distinguish between resistant and tolerant mutants D-cycloserine- or penicillin-induced spheroplasts (19) were tested on bacteriocin resistance In the experiments *E coli* strains K12 R and K12 T were used as colicin-resistant and - tolerant strains, and *S aureus* strains Oxford 209 PR (V4231) and 502 AT as staphylococcin-resistant and - tolerant strains, respectively

Extraction and analysis of phospholipids Cells of *S aureus* Oxford 209P were exponentially grown at 37°C in 500 ml of TSB, supplemented with 0 5 mC $^{32}P_i$ Phosphatidylglycerol, diphosphatidylglycerol, phosphatidic acid and lysylphosphatidylglycerol were extracted according to Bligh and Dyer (20) The phospholipids were separated by thin-layer chromatography on silicagel with a mixture of chloroform, methanol and water (65 25 4, by vol) as solvent Percentages of the different phospholipids were determined by counting the radioactivity of the different spots

pH-measurement Cells exponentially growing in TSB were gathered on membrane filters (Millipore, 0 45 μ pore size, 5 cm diameter) and washed with 0 3 mM potassium phosphate (pH 6 6) and suspended in the same buffer at a concentration of 10^9 cells per ml The pH was measured with a Radiometer Digital pH-meter (PHM52) and a combined electrode (Radiometer GK 2302C), and monitored with a Servogor recorder (Goerz Electro, Vienna)

Preparation of membrane vesicles Membrane vesicles were prepared by modified methods according to Kaback (21) *S aureus* cells were grown in the medium described by Short and White (22) *E coli* was grown in Medium A (21) containing 0 5% sodium succinate and 0 2% casein hydrolysate, and *B subtilis* in 0 8% Bacto-Tryptone containing 0 5% NaCl The cells were harvested in the late exponential phase and washed twice with 10 mM Tris-HCl (pH 8 0) Cells of *E coli* and *B subtilis* were resuspended in 30 mM Tris-HCl (pH 8 0) containing 20% sucrose Subsequently ethylenediaminetetraacetate (EDTA) and lysozyme were added to final concentrations of 10 mM and 0 5 μg/ml, respectively Alternatively, cells of *S aureus* were resuspended in 50 mM potassium phosphate (pH 7 3) containing 0 2 M NaCl and 20% sucrose, and lysostaphin (50 μg/ml) was added After 45 min at 30°C the spheroplasts were centrifuged resuspended in 0 1 M potassium phosphate (pH 6 8)

containing 20% sucrose and 20 mM $MgSO_4$ and lysed at 37°C by 500-fold dilution in 50 mM potassium phosphate (pH 6 8) containing 10 μg/ml of each deoxyribonuclease and ribonuclease Subsequently, EDTA (10 mM final concentration) was added, followed by $MgSO_4$ (15 mM final concentration), and after each addition the mixture was incubated for 15 min The suspension was centrifuged for 20 min at 16,000 x g The pellet was resuspended in 50 mM potassium phosphate (pH 6 8) and after differential centrifugations at 800 x g and 45,000 x g the membrane vesicles were stored at -20°C in the same buffer

Measurement of transport The incubation mixtures contained final concentrations of 50 mM potassium phosphate (pH 6 8), 10 mM $MgSO_4$ and 1 to 2 mg of membrane protein per ml and were preincubated for 15 min at 25°C (^{14}C) glutamic acid (10 μM, final concentration) and electron donors (20 mM, final concentration) were rapidly added and the mixtures (0 1 ml final volume) were incubated at 25°C under special oxygenation (23) The reaction was terminated by addition of 2 ml 0,1 M LiCl and the mixtures were filtered on membrane filters (Millipore, 0,45 μ pore size) The filters were washed with 2 ml 0 1 M LiCl, dried and counted in a liquid scintillation counter

Measurement of oxygen consumption The rates of oxygen uptake were measured by use of a Clark electrode with the Oxygraph (Gilson Medical Electronics, Middleton, Wisconsin) The assay mixture (1 0 ml) contained 50 mM potassium phosphate (pH 6 8), 10 mM $MgSO_4$ and 50 to 500 μg of membrane protein The reaction was initiated by injecting 20 μl of the substrate (20 mM final concentration, 5 mM for NADH) The measurements were performed at 30°C

Assay of dehydrogenase activity Dehydrogenase activities were assayed by use of 2,6-dichlorophenolindophenol (DCI) and in case of NADH dehydrogenase potassium ferricyanide as electron acceptor Incubation mixtures contained 0 2 M potassium phosphate (pH 7 3), 0 1 to 0 5 mg of membrane protein per ml, 0 002% DCI or 0 1 M potassium ferricyanide and 20 mM lithium D-lactate disodium succinate, sodium α-glycerolphosphate or 10 mM NADH The optical density at 620 nm (DCI) or 425 nm (ferricyanide) was followed

Fluorescense measurements Fluorescence was measured with an Aminco Bowman spectrofluorometer The slit arrangement was 3 mm for positions 1, 3, 4 and 6, and 2 mm for positions 2, 5 and 7, the sensitivity was 45 The excitation and emission wavelengths were 285 and 475 nm, respectively The reaction mixture (2 ml) contained approximately 0 2 mg of membrane protein per ml, 50 mM potassium phosphate (pH 6 8), 10 mM $MgSO_4$ and 50 μM 1-anilino-8-naphthalene sulfonic acid (ANS) and was well aerated The measurements were performed at 30°C and the fluorescence monitored

Chemicals L (U-^{14}C) glutamic acid (260 mC /mmol), L- (U-^{14}C) proline (165 mC/mmol) and sodium dihydrogen (^{32}P)-orthophosphate (10 mC/mmol) were purchased from the Radiochemical Centre, Amersham Lysostaphin was purchased from Schwarz-Mann Orangeburg N Y , lysozyme from Boehringer Mannheim and N-methylphenazonium methosulfate from Schuchardt, Munchen 2,6-dichlorophenol-indophenol was obtained from Merck, Darmstadt and 1-anikino-8-naphthalene sulfonic acid from Sigma Cemical Co , St Louis Trypticase soy broth was purchased from BBL, Cockeysville

RESULTS

Isolation of mutants Two kinds of mutants of *E coli* K12 were isolated namely, colicin A-resistant strains (K12 R), of which spheroplasts were sensitive to colicin A, and colicin A-tolerant strains (K12 T), of which protoplasts were still resistant to colicin A The tolerant mutants isolated exhibited a higher sensitivity to deoxycholate (DOC) and EDTA than the wild and resistant strains since the growth of the tolerant mutants was inhibited completely at concentrations of 1 0% DOC or 1 mM EDTA, whereas concentrations ten times higher were required to inhibit the growth of the wild and resistant strains From *S aureus* Oxford 209 P staphylococcin 1580 resistant strains (209 PR) could be isolated of which spheroplasts were sensitive to the bacteriocin Staphylococcin 1580 tolerant L-forms (502 A I) were isolated as mutants of stable L-forms of *S aureus* 502 A These tolerant mutants were equally sensitive to DOC as their parent L-forms

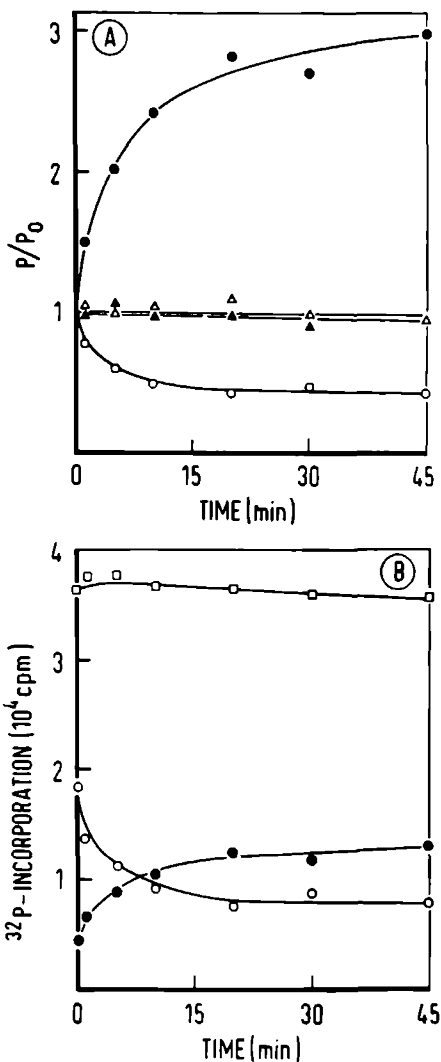


Fig. 1. *Effect of staphylococcin 1580 on the phospholipid composition of S. aureus Oxford 209 P cells. Cells were grown exponentially in a medium containing $^{32}P_i$. When the culture reached the absorbance of 0.5 at 600 nm staphylococcin 1580 (1000 A.U./ml) was added. At various time intervals samples (100 ml) were removed and the ^{32}P-contents of the various phospholipids were determined. A. Ratio between the percentages of ^{32}P of various phospholipids in treated (P) and untreated (P_0) bacteria as a function of time. B. Absolute amount of ^{32}P in phospholipids from 100 ml sample of treated cells as a function of time.*
●, diphosphatidylglycerol; o, phosphatidylglycerol; △, phosphatidic acid; ▲, lysylphosphatidylglycerol; □, total phospholipid.

Effect on phospholipid metabolism. Treatment of *E. coli* cells with colicins A, E1 and K caused an increase of the percentages of diphosphatidylglycerol and lysophosphatidylethanolamine and a decrease of phosphatidylglycerol (24). Like these colicins staphylococcin 1580 caused an increase of the relative amount of diphosphatidylglycerol and a decrease of phosphatidylglycerol in *S. aureus* Oxford 209 P cells but had no significant effect on phosphatidic acid and lysylphosphatidylglycerol (Fig. 1A). The total amount of ^{32}P-labeled phospholipids decreased very slowly and the absolute alterations of diphosphatidylglycerol and phosphatidylglycerol were nearly equal (Fig. 1B).

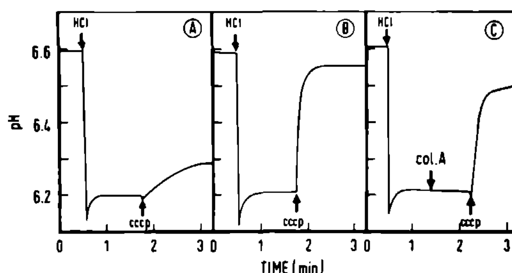


Fig. 2. *Effect of colicin A on proton permeability of E. coli K12 cells. The cell suspension (10^9 cells/ml) was prepared as described in Materials and Methods. A. About 40 µl of 0.01 N HCl was added to a 10 ml suspension of untreated cells, followed by addition of cccp (2 µM final concentration). B. About 40 µl of 0.01 N HCl was added to a 10 ml suspension of colicin A-pretreated (1000 A.U./ml for 10 min) cells, followed by addition of cccp. C. About 40 µl of 0.01 N HCl was added to a 10 ml suspension of untreated cells, followed by addition of colicin A (col A, 5000 A.U./ml) and cccp.*

Effect on protonpermeability. The pH of a cell suspension of *E. coli* K12 was lowered from pH 6.6 to 6.2 by addition of HCl. Subsequent addition of carbonyl cyanide m-chlorophenylhydrazone (cccp) caused a rather slow and gradual increase of pH (Fig. 2A). A similar behavior was observed with colicin A-pretreated and untreated cells of *E. coli* K12 R and K12 T. Upon addition of HCl and cccp to a suspension of colicin A-pretreated cells of *E. coli* K12, the pH raised very sharply (Fig. 2B). Addition of colicin A after the pH was lowered and subsequent incubation with cccp caused a sharp rise of pH (Fig. 2C). Comparable results were obtained in tests with *S. aureus*

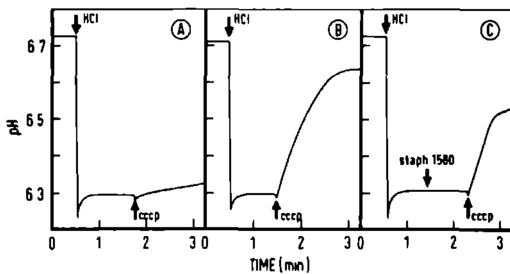


Fig. 3. Effect of staphylococcin 1580 on proton permeability of S. aureus Oxford 209 P cells. The cell suspensions (10^9 cells/ml) were prepared as described in Materials and Methods. A. About 40 µl of 0.01 N HCl was added to a 10 ml suspension of untreated cells followed by addition of cccp (2 µM final concentration). B. About 40 µl of 0.01 N HCl was added to a 10 ml suspension of staphylococcin pretreated (2000 A.U./ml for 15 min) cells, followed by addition of cccp. C. 40 µl of 0.01 N HCl was added to a 10 ml suspension of untreated cells, followed by addition of staphylococcin 1580 (staph. 1580, 4000 A.U./ml) and cccp.

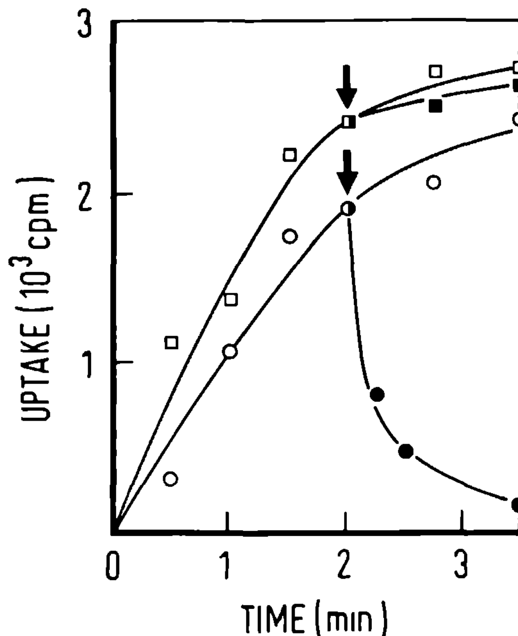


Fig. 4. Effect of colicin A on (^{14}C) glutamate accumulation by membrane vesicles of E. coli K12 (circles) and K12 T (squares). The uptake was measured in the presence of ascorbate (20 mM) and PMS (100 µM). Open symbols: control. Closed symbols: colicin A (2000 A.U./ml) added after 2 min.

Oxford 209 P and Oxford 209 PR cells and staphylococcin 1580, although the effect of cccp was somewhat less (Fig. 3A-C). E. coli K12 and S. aureus Oxford 209 P cells pretreated for more than 30 min with colicin A and staphylococcin 1580, respectively, required more HCl to lower the pH to 6.2 and the pH rise upon cccp addition became less drastically. The results described here were not influenced by the presence of 50 mM KCl in the cell suspension.

Effect on amino acid transport. Colicin A caused a rapid leakage of (^{14}C) glutamate accumulated in membrane vesicles of *E. coli* K12 and K12 R, but no significant effect was observed with membrane vesicles of *E. coli* K12 T (Fig. 4). Also staphylococcin 1580 caused a rapid leakage of (^{14}C) glutamate from membrane vesicles of *S. aureus* Oxford 209 P and its resistant mutant 209 PR (Fig. 5A). Membrane vesicles of *S. aureus* 502 A behaved in a similar way, while little effect was observed on membrane vesicles of its tolerant mutant strain 502 AT (Fig. 5B).

The amino acid uptake by membrane vesicles prepared from *E. coli* cells can be stimulated by the following energy donors: ascorbate plus phenazine methosulfate (PMS), D-lactate, succinate and NADH, given here in order of decreasing effectiveness (25, 26). Irrespective of the electron donor applied, the uptake of glutamate was inhibited 85 to 95% by 7500 A.U. of colicin A per ml (Fig. 6A-B). Parallel experiments with heat-inactivated (30 min at 120°C) colicin A or

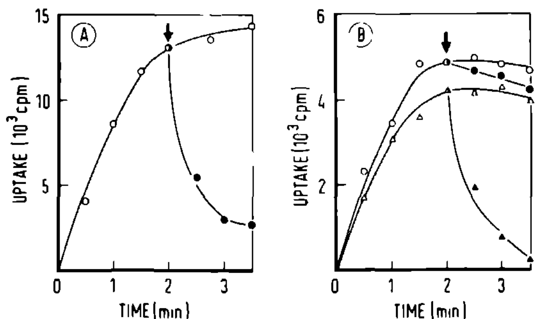
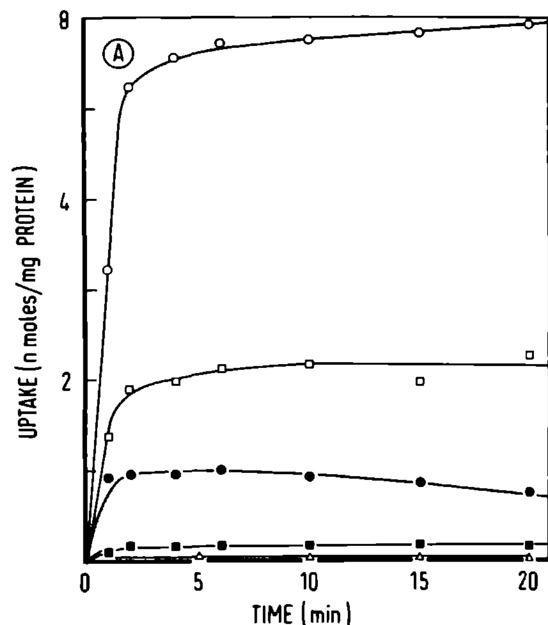


Fig. 5. Effect of staphylococcin 1580 on (^{14}C) glutamate accumulation by membrane vesicles of S. aureus. The uptake was measured in the presence of ascorbate (20 mM) and PMS (100 µM). Open symbols: control. Closed symbols: staphylococcin 1580 (2500 A.U./ml) added after 2 min. A. Membrane vesicles of S. aureus Oxford 209 P. B. Membrane vesicles of S. aureus 502 A (triangles) and 502 AT (circles).



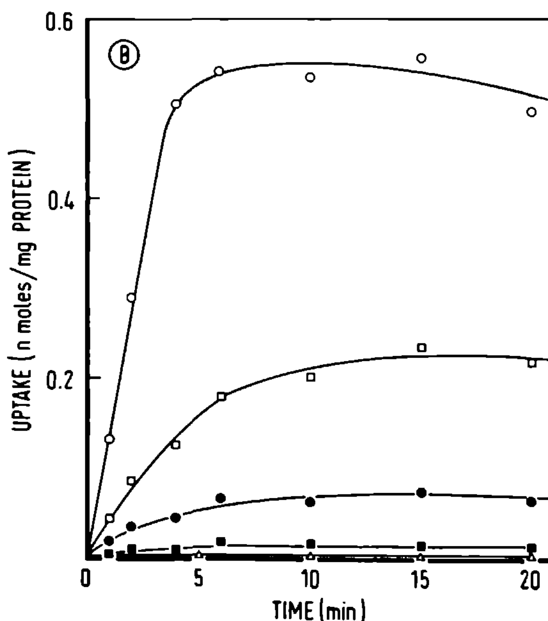


Fig. 6. *Effect of colicin A on (*[14]*C) glutamate uptake into membrane vesicles of E. coli K12. The uptake was measured in the absence (open symbols) or presence of colicin A (7500 A.U./ml, closed symbols). Electron donors applied: A. △, none; o, ascorbate (20 mM) plus PMS (100 μM); □, D-lactate (20 mM). B. △, none; o, succinate (20 mM); □, NADH (20 mM).*

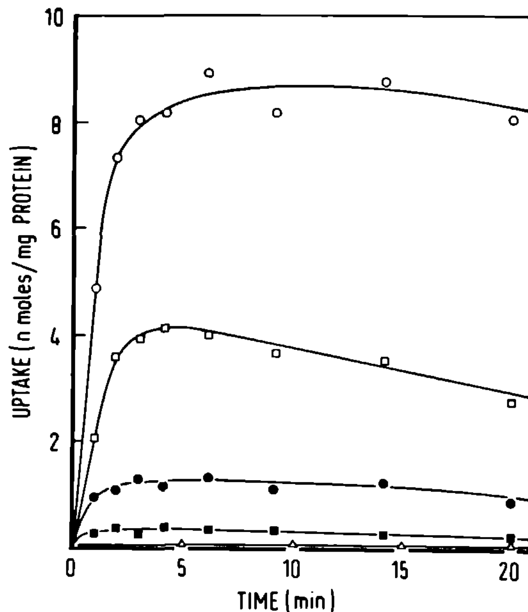


Fig. 7. *Effect of staphylococcin 1580 on (*[14]*C) glutamate uptake into membrane vesicles of S. aureus Oxford 209 P. The uptake was measured in the absence (open symbols) or presence of staphylococcin 1580 (7500 A.U./ml, closed symbols). Electron donors applied: △, none; o, ascorbate (20 mM) plus PMS (100 μM); □, α-glycerolphosphate (20 mM).*

with staphylococcin 1580 (5000 A.U./ml) did not show any inhibition. The transport of amino acids in membrane vesicles of *S. aureus* could be stimulated by α-glycerolphosphate and by ascorbate plus PMS (27) and was 85 to 95% inhibited in both cases by 7500 A.U. of staphylococcin 1580 per ml (Fig. 7). The uptake of amino acids in membrane vesicles of *B. subtilis* W23 was sensitive to staphylococcin 1580. Colicin A (7500 A.U./ml) and heat-inactivated (30 min at 120°C) staphylococcin 1580 did not affect the uptake in membrane vesicles of *B. subtilis* W23 or *S. aureus* Oxford 209 P. The inhibiting effect of both bacteriocins was not restricted to glutamate uptake but affected proline uptake as well.

Both bacteriocins inhibited noncompetitively glutamate uptake energized by ascorbate plus PMS (Fig. 8-9). The K_m- and V-values of glutamate uptake in *E. coli* K12 (*S. aureus* Oxford 209 P) membrane vesicles were 4.4 μM (40 μM) and 5.8 nmoles/mg/min (25 nmoles/mg/min), respectively. The K_i- values were in the range of 10 nM and 100 nM for colicin A and staphylococcin 1580, respectively. It was assumed that the

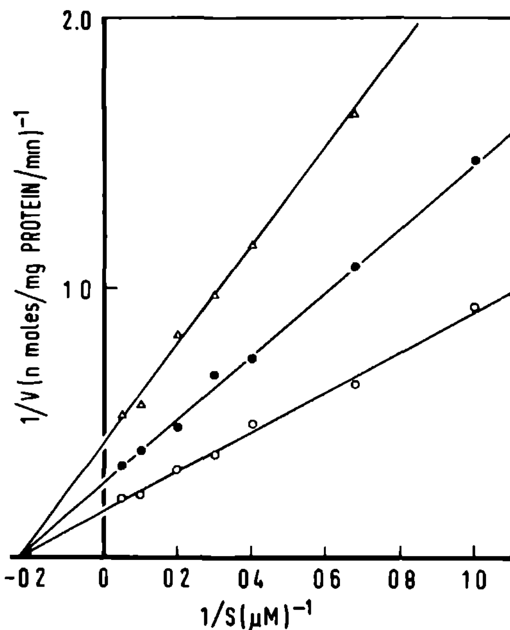


Fig 8. *Colicin A as a noncompetitive inhibitor of (^14C) glutamate uptake into membrane vesicles of E coli K12. The amount taken up in 1 min at 25°C in the presence of 20 mM ascorbate plus 100 μM PMS was measured o, control, •, 1000 A U of colicin A per ml; Δ, 2000 A U./ml*

molecular weights of staphylococcin 1580 and colicin A were 250,000 and 60,000, respectively, and that 1 A.U. of staphylococcin = 820 pmoles and 1 A.U. of colicin A = 840 pmoles.

Effect on oxygen consumption and dehydrogenase activities. To determine whether the inhibition of amino acid uptake was caused by an inhibition of the dehydrogenases or of the electron transport chain, the effect of the bacteriocins on oxygen consumption was determined Table I shows that both bacteriocins have no or little effect on the oxygen consumption with various electron donors The inhibition by staphylococcin 1580 in the presence of NADH could not be enhanced further by higher staphylococcin concentrations For comparison also the effects of KCN and 2,4-dinitrophenol were determined Furthermore, both bacteriocins had no effect on the NADH, D-lactate, α-glycerolphosphate or succinate dehydrogenase activity in membrane vesicles with DCI or ferricyanide as electron acceptor.

1-Anilino-8-naphthalene sulfonate fluorescence. Changes in the fluorescence of 1-anilino-8-naphthalene sulfonate (ANS) show a striking correlation with the effects exerted on dehydrogenase-coupled transport system in membrane vesicles and seem to be associated with structural transitions in components of the membrane (28) or/and alterations of the membrane potential (29, 30). Addition of D-lactate to membrane vesicles of *E. coli* K12, K12 R and K12 T in the presence of ANS resulted in a rapid decrease of the fluorescence (Fig 10A). A similar effect was observed for membrane vesicles of *E coli* K12 T pretreated with colicin A, but not for colicin A-pretreated vesicles of *E coli* K12 and K12 R cells (Fig 10B) Colicin A reversed the decrease of fluorescence when added after D-lactate (Fig. 10C). A similar result was obtained with KCN and amino acids, in accordance with the results of Reeves et al (28). The ANS fluorescence also decreased when α-glycerolphosphate was added to membrane vesicles of *S. aureus* Oxford 209P and 209 PR and

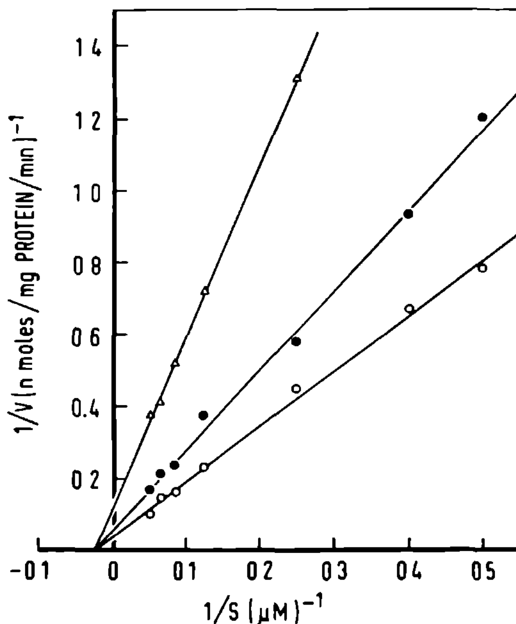


Fig 9 *Staphylococcin 1580 as a noncompetitive inhibitor of (^14C) glutamate uptake into membrane vesicles of S aureus Oxford 209 P The amount taken up in 1 min at 25°C in the presence of 20 mM ascorbate plus 100 μM PMS was measured. o control, •, 750 A U of staphylococcin 1580 per ml; Δ 3500 A.U /ml*

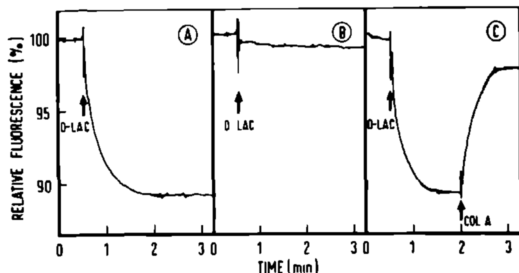


Fig 10 *Effect of colicin A on D-lactate-induced fluorescence changes The experiments were carried out with E coli K12 membrane vesicles as described in Materials and Methods A D-lactate (D-Lac , 20 mM) was added to untreated membrane vesicles B D-lactate (20 mM) was added to membrane vesicles pretreated with 6000 A U of colicin A per ml for 10 min C D-lactate (20 mM) was added to untreated vesicles, followed by addition of colicin A (col A, 6000 A U /ml)*

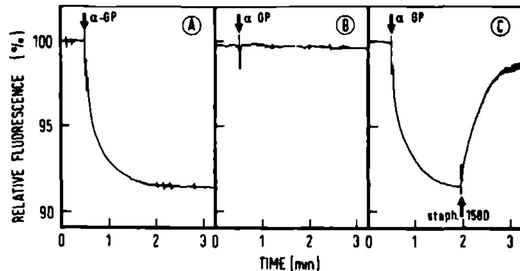


Fig 11 *Effect of staphylococcin 1580 on α-glycerolphosphate (α-GP)-induced fluorescence changes in membrane vesicles of S aureus Oxford 209 P The experiments were carried out as described in Materials and Methods A α-GP (20 mM) was added to untreated membrane vesicles B α-GP (20 mM) was added to vesicles pretreated with 7000 A U of staphylococcin per ml for 15 min C α-GP (20 mM) was added to untreated vesicles followed by addition of staphylococcin (staph 1580, 7000 A U /ml)*

staphylococcin pretreated vesicles of strain 502 AT (Fig 11A) This decrease was prevented by staphylococcin 1580 added before α-glycerolphosphate and was abolished when it was added after α-glycerolphosphate (Fig 11B-C) Both bacteriocins caused a small increase (1 to 2%) of fluorescence when added to membrane vesicles in the presence of ANS

DISCUSSION

Two classes of colicin A-insensitive mutants were obtained from *E coli* K12, namely, colicin A-resistant mutants, whose spheroplasts were sensitive to colicin A, and colicin A-tolerant mutants, whose spheroplasts were still resistant to colicin A Staphylococcin 1580 - resistant and-tolerant mutants were isolated from *S aureus* Oxford 209P and stable L-forms of *S aureus* 502A, respectively The cell wall of the resistant mutants might be altered in such a way that the bacteriocins could no longer attach to the receptor site, whereas in tolerant mutants the biochemical target appeared resistant to bacteriocin action (31) The higher sensitivity of colicin A-tolerant mutants to deoxycholate and EDTA indicates that the membrane of the mutants may be altered

Amino acid accumulation into membrane vesicles of wild and resistant strains of *E coli* K12 and *S aureus* was sensitive to the respective bacteriocins, while the vesicles of tolerant strains were unaffected by them Similar results were obtained for colicin E1 (31) These results

Table 1 Effect of colicin A, staphylococcin 1580, potassium cyanide and 2,4-dinitrophenol (DNP) on the oxidation system of membrane vesicles

Membrane vesicles	Electron donor (20 mM)	Relative rate of oxygen consumption (%) Control	Staphylococcin 1580 (5000AU/ml)	Colicin A (5000AU/ml)	KCN (2 mM)	DNP (1 mM)
E coli K 12	Ascorbate PMS	100	101	98	11	100
	D lactate	100	95	101	9	98
	Succinate	100	99	97	3	97
	NADH	100	102	105	1	95
S aureus Oxford 209P	Ascorbate-PMS	100	103	101	9	101
	α-Glycerol-phosphate	100	96	97	6	97
	NADH	100	81	102	2	96

Membrane vesicles were pretreated with bactesriocins, KCN or DNP for 10 min Then the substrate was added and oxygen consumption was measured as described in Materials and methodes

show that this group of bacteriocins affect primarily a biochemical process in the membrane or the membrane itself

The inhibition of amino acid uptake into membrane vesicles of *E coli* K12 and *S aureus* Oxford 209P by the respective bacteriocins was noncompetitive and the extent did not depend on the amino acid or electron donor applied According to Kaback (32,33) amino acid transport is exclusively coupled via one or more dehydrogenases and transport carriers to the electron transport chain The results presented here reveal that inhibition of amino acid uptake was not caused by a blockade in the electron transport chain or dehydrogenase activities, as exerted by cyanide and oxamate, respectively Hence, the transport carriers appear no obligatory electron transfer intermediates as was already concluded from the action of uncoupling agents

A proton gradient and electrical potential was suggested to provide the immediate driving force for the active transport of a variety of compounds in bacterial cells (34,35) The proton gradient may be generated by an oxydative input under aerobic conditions and a phosphorylative input under anaerobic conditions in intact cells, but in membrane vesicles only the first action is operative (36,37), possibly because of the loss of coupling factors involved in the phosphorylative input (38)

Transport inhibition could result from the development of proton permeability causing a collapse of the proton gradient, the action of DNP and cccp may be explained in this way (39) Colicin A and staphylococcin 1580 did not promote proton permeability However, addition of cccp to colicin- or staphylococcin-treated cells resulted in a rapid loss of the proton gradient Since previous experiments (16) showed that staphylococcin 1580 caused a rapid leakage of rubidium ions out of the cell, counter-ion movement is probably required to manifest the effect of cccp maximally A similar interpretation was offered for the action of cccp in the presence of colicin El or valinomycin plus potassium ions (40)

A decrease of ANS-fluorescense was observed when membrane vesicles of *E coli* K12 or *S aureus* Oxford 209P were energized by D-lactate or α-glycerolphosphate, respectively, and may result from a decreased binding of ANS caused either by conformational changes (28) of the membrane or by an alteration of the potential (29, 30, 38) across it The effects of colicin A and staphylococcin 1580 on ANS-fluorescence may be explained to be due to a reduction of the energized state of the membrane

Colicin A and staphylococcin 1580 inhibit the uptake of amino acids both in intact cells (14,16) and in membrane vesicles The mechanism by which the uptake is coupled to the energized state of the membrane is unknown The results presented here may be interpreted as an uncoupling of transport by these bacteriocins either by dissipation of the energized state or by an interaction with proteins or phospholipids

The mechanism by which these group of bacteriocins affect this coupling is not yet clear Colicin A (23) and staphylococcin 1580 cause an increase of diphosphatidylglycerol and a decrease of phosphatidylglycerol These changes may induce conformational changes and affect transport, otherwise, they are a consequence of transport-inhibition

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SUMMARY

This thesis deals with a study on staphylococcins, bacteriocins produced by staphylococci.

Bacteriocins are bactericidal substances of high-molecular weight, produced by bacteria and active against bacteria. In contrast to bacteriocins produced by gram-negative bacteria (e.g. *Escherichia coli*), the bacteriocins from gram-positive organisms are scarcely investigated. The introduction surveys the current knowledge on the mode of action of various bacteriocins and special attention is given to the general characteristics, the biochemical process which is primarily affected, and roll of the cell surface in the mediation of the bacteriocin action.

About 6.3% out of 270 tested strains of *Staphylococcus aureus* produced substances antagonistic against three reference strains. On the basis of their properties and inhibitory spectrum a division was made into five classes. One class consisted of lytic enzymes, the four others of staphylococcins. The ability to produce staphylococcins was eliminated spontaneously upon storage and more rapidly on treatment with various curing agents and growth at 42 C. The resistance to inorganic salts was co-eliminated. These results suggest that the genes for staphylococcin production and for resistance to inorganic salts are plasmid-borne determinants.

The production of staphylococcin 1580 by *Staphylococcus epidermidis* 1580 was optimal when cells were grown on semi-solid medium and could not be enhanced by ultraviolet irradiation or treatment with mitomycin C. The staphylococcin was purified 5 900 times and the material obtained was homogeneous. Staphylococcin 1580 was built up from subunits and consisted of protein (41.8%), carbohydrates (34%), and lipids (21.9%).

Bacteriocins of gram-negative bacteria exhibit a smaller inhibitory spectrum than those of gram-positive bacteria. Staphylococcin 1580 is bactericidal to several gram-positive bacteria but gram-negative organisms are insensitive.

Staphylococcin 1580 and colicin A were adsorbed to cell walls as well as to cell membranes. The binding to the cell wall appeared not essential for the action of these bacteriocins, since also L-forms and isolated membrane vesicles were sensitive to them. Tolerant and resistant mutants were isolated whose spheroplasts were insensitive and sensitive to the bacteriocins, respectively. Though staphylococcin 1580 caused alteration of phospholipid composition of the membranes, it appeared not to act like a phospholipase.

The staphylococcin inhibited simultaneously the syntheses of deoxyribonucleic acid, ribonucleic acid, and protein. This may be explained as a general effect of the bacteriocin on the energy supply or on the transport of the precursors of these macromolecules. The staphylococcin inhibited the transport of glucose and o-nitrophenyl-ß-galactoside, which are transported by a phosphoenolpyruvate phosphotransferase system, and of amino acids, which are transported by a system coupled to the electron transport chain. Both systems are not directly dependent on ATP supply which indicates that the observed reduction of ATP level in the cell was not responsible for the inhibition of these transports. The inhibition of two different transport systems suggests that the staphylococcin affects the membrane.

Staphylococcin 1580 and colicin A inhibited amino acid uptake into membrane vesicles. This uptake is coupled via transport carriers to dehydrogenases and the electron transport chain. The bacteriocins did not block the electron transport chain and did not inhibit dehydrogenase activities indicating that the transport carriers are no obligatory parts of the electron transfer chain. This transport system may be energized by a proton or electron gradient across the membrane, or by conformational changes, all generated by the electron transport chain. 1-Anilino-8-naphthalene sulfonic acid fluorescence studies, presented here, proved that the bacteriocins prevent the energized state of the membrane. The bacteriocins did not promote proton permeability but enhanced the effect of uncoupling agents on proton permeability.

Further studies on this subject may reveal fascinating information on membrane action and function.

SAMENVATTING

Dit proefschrift beschrijft een onderzoek naar stafylococcines, bacteriocines, die geproduceerd worden door stafylococcen

Bacteriocines zijn bactericidale stoffen met een hoog molecuulgewicht, die geproduceerd worden door en werkzaam zijn tegen bacterien In tegenstelling tot de bacteriocines van gram-negatieve bacterien (b v *Escherichia coli*) zijn de bacteriocines van gram-positieve bacterien slechts weinig onderzocht De inleiding geeft een overzicht van de huidige kennis van de werkingswijze van verscheidene bacteriocines en bijzondere aandacht wordt geschonken aan de algemene kenmerken, het biochemische proces dat primair beinvloed wordt en de rol die de celwand en het celmembraan daarbij spelen

Ongeveer 6,3% van de 270 geteste *Staphylococcus aureus* stammen produceerden een antagonistische stof tegen drie referentie stammen Op grond van de eigenschappen en het remmingsspectrum van deze antagonistische stoffen kon een indeling in tenminste vijf groepen gemaakt worden Een groep bestond uit lytische enzymen, de vier andere uit stafylococcines Tijdens het bewaren van de stammen ging het vermogen om stafylococcine te produceren verloren, dit werd versneld door behandeling met een aantal stoffen en groei bij 42 C De resistentie tegen een aantal anorganische zouten ging gelijktijdig verloren Deze resultaten wijzen erop dat de genen, die coderen voor stafylococcine produktie en voor resistentie tegen anorganische zouten, op een plasmid liggen

De productie van stafylococcine 1580 door *Staphylococcus epidermidis* 1580 verliep optimaal bij groei van de cellen op een halfzacht medium, en kon niet vergroot worden door bestraling met ultraviolet licht of door behandeling met mitomycine C Het stafylococcine werd een zuivering bereikt van 5 900 maal en het verkregen materiaal was homogeen Stafylococcine 1580 is opgebouwd uit subeenheden en bestaat uit eiwit (41,8%), suikers (34%), en lipiden (21,9%)

Bacteriocines van gram-positieve bacterien hebben een breder remmingsspectrum dan die van gram-negatieve bacterien Stafylococcine 1580 doodt vele gram-positieve bacterien maar gram-negatieve zijn ongevoelig

Stafylococcine 1580 en colicine A adsorberen zowel aan celwanden als aan celmembranen De binding aan celwanden blijkt niet essentieel te zijn voor de werking van deze bacteriocines, omdat ook L-vormen en geïsoleerde membraanblaasjes gevoelig zijn Zowel tolerante als resistente mutanten werden geïsoleerd waarvan de sferoplasten respectievelijk ongevoelig en gevoelig voor het bacteriocine zijn Hoewel het stafylococcine 1580 de fosfolipide samenstelling van het membraan verandert, blijkt het geen fosfolipase te zijn

Het stafylococcine remt simultaan de syntheses van desoxyribonucleinezuur, ribonucleinezuur en eiwit Dit kan een gevolg zijn van een algemeen effect van het bacteriocine op de energievoorziening of op het transport van bouwstenen van deze macromoleculen Het stafylococcine remt het glucose- en o-nitrofenyl-ß-galactosidetransport, dat via een fosfoenolpyruvaat afhankelijk fosfotransferase systeem verloopt, en het aminozuurtransport, dat verloopt via een systeem dat gekoppeld is aan de electronentransportketen Beide systemen zijn niet direct afhankelijk van de ATP-voorziening, wat erop wijst dat de remming van deze transportsystemen niet veroorzaakt wordt door de waargenomen daling van het ATP niveau De remming van twee verschillende transportsystemen, suggereert dat het stafylococcine op het membraan inwerkt

Stafylococcine 1580 en colicine A remmen de opname van aminozuren in membraanblaasjes noncompetitief Deze opname is via transportcarriers gekoppeld aan dehydrogenases en aan de electronentransportketen De bacteriocines hebben geen invloed op de electronentransportketen of dehydrogenase activiteiten, wat erop wijst dat de transportcarriers geen noodzakelijke intermediairen in de electronentransportketen zijn De energie voor het transportsysteem wordt mogelijk geleverd door een protonen- of electronengradient over het membraan, of door conformatieveranderingen in het membraan, die alle teweeg gebracht worden door de electronentransportketen Fluorescentie studies met 1-anilino-8-naftaleensulfonzuur bewezen dat de bacteriocines het optreden van de energierijke toestand van het membraan verhinderen De bacteriocines veroorzaken geen permeabiliteit van protonen maar versterken het effect van ontkoppelaars

Verdere studies over dit onderwerp kunnen belangrijke gegevens verstrekken over de werking en functie van het membraan

CURRICULUM VITAE

De schrijver van dit proefschrift is geboren op 26 juni 1946 te Nijmegen. In 1963 heeft hij het eindexamen HBSb afgelegd aan de Gemeentelijke HBS te Nijmegen en studeerde daarna chemie aan de Katholieke Universiteit te Nijmegen. Na zijn doctoraalexamen in maart 1969 was hij werkzaam als wetenschappelijk medewerker op de afdeling biochemie en sinds september 1971 op de afdeling chemische microbiologie van deze Universiteit.

STELLINGEN

1.

Simoni en Shallenberger stellen op grond van de door hen beschreven experimenten met D-lactaatdehydrogenase-negatieve mutanten ten onrechte dat de physiologische betekenis van het D-lactaat effect in *Escherichia coli* twijfelachtig is.

Simoni, R. D., and M. K. Shallenberger. 1972. Proc. Nat. Acad. Sci. USA **69**: 2663-2667.

2.

De werking van colicine A en staphylococcine 1580 bevestigt dat de aminozuurtransportcarriers geen noodzakelijke intermediairen in de electronentransportketen zijn.

3.

De mening van Burke *et al.*, dat stabiel mRNA een rol speelt bij de vorming van zoosporangiën aan hyfetoppen van *Allomyces*, vindt onvoldoende steun in de door hen beschreven experimenten.

Burke, D. J., Seale, T. W., and B. J. McCarthy. 1972. J. Bacteriol. **110**: 1065-1072.

4.

De correlatie tussen bindingseiwitten en actief transport is onvoldoende aangetoond.

Kerwar, G. K., Gordon, A. S., and H. R. Kaback. 1972. J. Biol. Chem. **247**: 291-297.

5.

Vele effecten, die toegeschreven worden aan sulfhydryl-reagentia, zijn te wijten aan niet-specifieke, structurele veranderingen in het membraan.

Carter, J. R. 1973. Biochem. **12**: 171-176.

6.

De conclusie, dat de remming van de catalasesynthese in hepatoma cellen wordt veroorzaakt door de interactie van een inhibitor met het mRNA, is onvoldoende aangetoond.

Uenoyama, K., and T. Ono. 1973. J. Mol. Biol. **74**: 453-466.

7.

Voor het optimaal functioneren van veel membraangebonden activiteiten is de 'liquid crystal phase' van de lipiden in de membraan een noodzakelijke voorwaarde.

Taniguchi, K., and S. Iida. 1972. Biochim. Biophys. Acta **274**: 536-541.

8.

Het is aan te bevelen de werkzaamheden van o.a. huisarts, maatschappelijk werker, apotheker, en tandarts in een wijkcentrum te concentreren.

9.

De onsterfelijkheid van bacteriën hangt ontologisch samen met hun onvrijheid.

A. M. Jetten
8 juni 1973