THE NATURE OF THE COLICIN K RECEPTOR OF ESCHERICHIA COLI CULLEN*

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Colicins differ from other antibiotics in that they are proteins endowed with an extremely narrow range of bactericidal activity (1, 17). As a rule they are toxic only for those bacteria which belong to the same or to a closely related species as the colicinogenic microorganism. Moreover, bacteria which are sensitive to one type of colicin can be fully resistant to another (1). To account for this it was postulated by Fredericq as early as 1946 that bacteria which are sensitive to a certain colicin contain a specific receptor substance on their surface which serves as the site of attachment for the bacteriocin (2). It was also observed that microorganisms which are sensitive to colicins M, K, or E were always attacked by bacteriophage T1, T6, or BF23, respectively, and that mutants which had lost their sensitivity to one of the colicins were resistant to the corresponding phage. It was suggested therefore that in each of these instances the receptor for the bacteriocins and virus was identical (3, 4). In the ensuing years the receptor hypothesis was substantiated by the work of others. Thus, Bordet and Beumer have shown that extracts of colicin-sensitive bacteria inhibit bacteriocins in vitro (5). Mayr-Harting has demonstrated that colicin E2-sensitive microorganisms absorb the bacteriocin from solution and that their ability to do so is destroyed by heating or by chemical treatment (6). More recently, Guterman and Luria found that colicin B is inactivated by lipopolysaccharides of *Escherichia coli* strains which are sensitive to the bacteriocin (7). The location of the receptors in the bacterial cell has been studied by Nomura and his coworkers. They have shown that bacteria which were exposed to colicins can be rescued by treating them with trypsin and therefore they suggested that the bacteriocin is adsorbed to the surface of the cell (8). This observation was corroborated by the finding that approximately 90% of radioactively labeled colicin E2 is absorbed to a cellular fraction containing the bacterial envelopes (9).

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In order to learn more of the chemical composition of the bacterial substances which react with bacteriocins we have undertaken a study of the colicin K receptors of *E. coli* strains B and Cullen (10, 11). Since these bacteria are also susceptible to phage T6, we also attempted to ascertain whether the receptors for the colicin and phage are identical. In the following account it will be shown that the receptors for the colicin and virus form part of the bacterial cell wall. It will also be seen that the two receptors differ in their sensitivity to enzymes and to chemical reagents and hence that they must be of different chemical nature. Evidence will also be presented that peptides containing tryptophan are essential constituents of the colicin K receptor.

Materials and Methods

Microorganisms.—*E. coli* strains B and Cullen used in these studies were originally obtained from Dr. Mark H. Adams of The New York University as were the bacteriophages T1–T7 and C16. Stocks of the viruses were prepared by growing on *E. coli* B in nutrient broth. Colicin K-tolerant mutants (12, 13) of *E. coli* strains B and Cullen were obtained by growing the sensitive strains in nutrient broth containing 1 mg of colicin K/ml. Colicin-resistant mutants of the two bacteria (i.e. bacteria free of colicin K receptors) could be isolated only when the tolerant strains were grown in the presence of phage T6 (10^{10} phage (p)/ml). They are referred to as *E. coli* B/K,T6 and *E. coli* Cullen/K,T6.

Bacteriocins.—The colicin K used in this study was given us by Dr. Walther F. Goebel. This substance was derived from mitomycin C-induced *E. coli* K235 L^+OC^+ and was partially purified by precipitation with ammonium sulfate (14). The activity of the preparation was 32,000 AU of colicin K or 5×10^{12} lethal units/mg.

Antisera.—Antisera to the cell walls of *E. coli* Cullen and *E. coli* Cullen/K,T6 were obtained by injecting rabbits intravenously on alternate days over a period of 2 wk with graded doses of cell wall suspensions in saline. Quantities of 20–1000 μg of material/dose were used. Three courses of injections were given and the animals were bled a week after the last injection by cardiac puncture. Antisera to *E. coli* Cullen were prepared by injecting rabbits, first intradermally with 10^{10} $CHCl_3$-killed bacterial cells, then intravenously with graded doses of a bacterial vaccine (10^8–10^{10} cells/injection) as described above.

Cultivation of Microorganisms.—15 liter cultures of the colicin-sensitive or colicin-resistant strains of *E. coli* B and Cullen bacilli were grown to a concentration of 5×10^9 cells/ml in casamino acid–glucose medium at pH 7.0 (15). The bacterial growth was terminated by adding 100 ml of chloroform and the cultures were stored overnight at 4°C. The bacterial cells were collected in a Sharples centrifuge (Sharples Centrifuges, Pennwalt Corporation, Warminster, Pa.), resuspended in water, and then either frozen or lyophilized.

Preparation of Bacterial Cell Walls.—Cell walls were prepared by a modified procedure of Weidel et al. (16). 100 g of wet cells were suspended in 500 ml of water. Under vigorous stirring the pH of the suspension was adjusted to 9.8 by the addition of 0.1 N NaOH and immediately thereafter was readjusted to pH 7.0 with CO_2. The suspension was centrifuged for 20 min at 10,000 *g*, the sediment resuspended in water, and the procedure then repeated. The final sediment was resuspended in 0.01 M tris (hydroxymethyl)aminomethane (Tris) buffer at pH 7.5 (total volume 200 ml) and then mixed with a solution containing 1 mg of DNase and 1 mg of RNase. To this was added 1 ml of 0.1 M $MgCl_2$ and the mixture was incubated for 30 min at 37°C. The cells were then disrupted by sonicating the digested mixture twice for 3 min at 0°C (Sonifier, Branson Instruments, Stamford, Conn.) Coarse bacterial debris was removed by centrifugation at 4000 *g* and the cell walls were collected by centrifugation at

50,000 *g* for 1 hr. The sediment was washed three times, resuspended in 150 ml of water, and precipitated with 450 ml of 95% ethanol at 0°C. The precipitate was collected by centrifugation at 10,000 *g*, resuspended, and dialyzed. The cell walls were then collected by centrifugation for 60 min at 50,000 *g*, resuspended in water, and lyophilized. The yield was 2–3 g of dry cell walls. Chemical analysis of the cell walls was performed as previously described (17).

Bacteriocin and Phage Assays.—

Colicin K assay: The quantitative determinations of colicin K were performed by depositing droplets of serial dilutions of the bacteriocin on plates seeded with *E. coli* B as described earlier (method B in reference 15). 1 unit of colicin is defined as the minimum amount of the bacteriocin contained in 1 ml of a solution which completely inhibits the growth of the indicator bacteria.

Colicin inhibition tests: The ability of a bacterial substance to inhibit the activity of colicin K was determined as follows. A sample of the material to be tested was suspended in nutrient broth at a concentration of 2 mg/ml and sterilized by heating at 60°C for 30 min. 1 ml portions of this suspension were added to 3 ml portions of 0.7% agar seeded with 5×10^7 cells of *E. coli* B and the mixtures were poured on nutrient agar plates. Plates containing only the indicator bacteria served as controls. 2-fold serial dilutions of colicin K, containing from 500 to 0.015 μg of bacteriocin/ml, were made in nutrient broth, and 0.02 ml droplets of the appropriate dilutions were then deposited on the test and control plates. The latter were incubated for 6 hr at 37°C and the highest dilutions of colicin which still completely inhibited the growth of the indicator bacteria on the test plates (dilution A) and on the control plates (dilution B) were determined. The concentration of bacteriocin in dilution A (units per milliliter) was calculated by multiplying the ratio of dilution factors of A and B by the concentration of colicin in dilution B, which by definition is 1 unit per milliliter. The figure so obtained approximates the maximum number of colicin units per milliliter which were inhibited on plates containing 2 mg of the material tested. This value is used in our study as a measure of the colicin-inhibiting activity of the various bacterial substances under investigation. In those instances in which the colicin concentrations in dilutions A and B are identical, the inhibiting activity of the material tested is designated as zero. Since colicin K is bound only by the cell walls of the colicin-sensitive microorganisms containing the specific receptor for the bacteriocin (Table III), it is apparent that the colicin-inhibiting activity is a measure of the quantity of colicin receptor present in the material.

Phage-inhibition tests: The ability of bacterial substances to inactivate phage was determined in the following manner. 0.5 ml portions of phage dilution (4×10^5 p/ml) were mixed with an equal volume of a sterile suspension of the materials to be tested in broth containing 2000, 200, 20, and 2 μg/ml. A mixture of equal volumes of phage and broth served as control. Nutrient broth used for these experiments contained 0.85% NaCl. The solutions were incubated for 3 hr at 37°C and a portion of each was diluted a 100-fold in broth. 0.1 ml portions of the dilutions were added to 3.0 ml portions of soft agar seeded with *E. coli* B and the mixtures were then plated. After incubation for 18 hr at 37°C, the number of phage plaques on each plate was counted and the percentage of inhibited phage then calculated (27). For the phage typing of the various bacterial strains, suspensions of the different phages containing 10^7 p/ml were used (18).

Serological Tests:—

Agglutination tests: Performed as follows. 2 ml portions of a suspension of cell walls in nutrient broth (1 mg/ml) were distributed in a series of tubes. Equal volumes of appropriate serial dilutions in broth of an antiserum were added to each. The mixtures were incubated for 1 hr at 37°C and then overnight at 4°C. A mixture of equal volumes of cell wall suspension and nutrient broth served as a control. The content of the tubes was then stirred and the absorbance of the suspensions was measured at 600 mμ and compared with that of the con-

trol. The reciprocal of the highest dilution of antiserum still causing an increase in the absorbance equal to 0.02 was considered to be the agglutination titer.

Receptor-neutralization tests: Carried out in the following manner. 1 ml portions of a sterile suspension of cell walls in nutrient broth (2 mg/ml) were mixed with an equal volume of appropriate serial dilutions of an antiserum in the same medium. A suspension of cell walls in nutrient broth (1 mg/ml) served as control. The mixtures were incubated for 1 hr at 37°C and allowed to stand overnight at 4°C. 1 ml portions of the suspensions were then used for colicin-inhibition tests. The reciprocal of the highest dilution of the antiserum which decreased the inhibiting activity of the cell walls by 50% is termed the receptor-neutralization titer.

Chemical Treatments.—

Digestion of Bacterial Cell Walls with Enzymes.—20–100 mg samples of bacterial cell walls were suspended in an appropriate buffer at a concentration of 10 mg/ml and mixed with a solution containing 2–10 mg of the enzyme to be tested. The digestion mixture was dialyzed against 20 volumes of the same buffer at 37°C. Sterility was maintained by saturating the buffer with chloroform. The buffer was changed three times at 12-hr intervals. The suspension of digested cell walls was then spun for 60 min at 80,000 *g*, the sediment washed twice with water to remove the enzymes, and finally lyophilized. The materials so obtained were used for colicin- and phage-inhibition assays.

Digestion with trypsin, chymotrypsin, *Aspergillus* lipase, and phosopholipases A, C, and D were carried out in 0.01 M Tris buffer at pH 7.5 containing 0.001 M $CaCl_2$. Treatment with lysozyme was performed in 0.01 M Tris buffer at pH 7.5 in presence of 0.001 M ethylenediaminetetraacetic acid. 0.01 M HCl at pH 2.0 and 0.03 M sodium phosphate buffer at pH 7.5 were used for the digestions with pepsin and pronase, respectively. Treatment with papain and ficin was carried out in solution containing 0.01 M NaCN and 0.005 M cysteine-HCl at pH 7.0. The action of cellulase, amylase, and β-glucosidase was tested in 0.05 M sodium acetate buffers at pH 4.0, 4.7, and 5.0, respectively.

Treatment of Cell Walls with Amino Acid Reagents.—50–100 mg samples of trypsinized cell walls of *E. coli* Cullen were suspended in an appropriate solvent at a concentration of 5 mg/ml. To this was added the required amount of the reagent to be tested and the mixture was allowed to react for the prescribed interval. When the reaction was completed, the cell walls were sedimented for 1 hr at 80,000 *g*, the pellet was washed with solvent to remove excess reagent, and then resuspended in water. The suspension was dialyzed, lyophilized, and the inhibiting properties of the material assayed. In each instance it was ascertained that the solvent used in the experiment had no measurable effect upon the inhibiting properties of the cell walls. The condition of each reaction and the literature references are given in Table VII.

EXPERIMENTAL

Properties of E. Coli Strains B and Cullen.—Various strains of colicin K-sensitive bacteria differ in their response to bacteriophage T6. Certain bacteria support the growth of the virus, whereas others are killed by it without producing phage particles (19). Since one of the goals of this study was to determine the relationship between the receptors for bacteriocin and virus, we have chosen both types of bacteria for investigation. One of these was *E. coli* strain B, a standard indicator for colicin K and the eight phages listed in Table I. The other strain was *E. coli* Cullen, a bacterium used by Adams for the study of phage C16 (20). This bacillus is sensitive to colicin K and to phages T1, T3, T5, T7, and C16. It appears to be resistant to the T2, T4, and T6 viruses when di-

lute phage stocks (10^7 p/ml) are used for typing. However, when more concentrated viral stocks (10^{10} p/ml) are used, T2 and T6 phages inhibit bacterial growth, whereas T4 is without effect. Moreover, upon infecting the cultures of this bacterium in nutrient broth with T2 or T6 phage, the bacteria are killed and there is a pronounced decrease in the titer of the viruses. Thus, the Cullen bacillus is sensitive to the T2 and T6 phages, yet is unable to support their growth.

In order to compare the properties of the materials derived from receptor-containing and receptor-free bacteria, colicin K-resistant mutants of *E. coli* strains B and Cullen were isolated as described in Materials and Methods. From the phage-sensitivity patterns in Table I it can be seen that both colicin-resistant mutants are fully resistant to phage T6 and C16. Apparently the genes

TABLE I

Sensitivity of E. Coli B and E. Coli Cullen Strains to Colicin K and Bacteriophages

Bacterial strain	Colicin K	Bacteriophage							
		T1	T2	T3	T4	T5	T6	T7	C16
E. coli B	+	+	+	+	+	+	+	+	+
E. coli Cullen	+	+	±	+	○	+	±	+	+
E. coli B/K,T6	○	+	+	+	+	+	○	+	○
E. coli Cullen/K,T6	○	+	±	+	○	+	○	+	○

+ Bacterial growth completely inhibited by 1 unit of colicin K/ml or by 10^7 phage/ml.

± Bacterial growth partially inhibited by 10^{10} phage/ml.

○ Bacterial growth unaffected by 1000 units of colicin K/ml or 10^{10} phage/ml.

Droplets of colicin or phage solutions were deposited on agar plates seeded with 5×10^7 cells of the bacterial strain tested.

controlling the production of the receptors for these three agents are closely linked.

Properties of Bacterial Cell Walls.—When bacterial cells are brought in contact with radioactive colicin and then ruptured and fractionated by differential centrifugation, the major portion of the bacteriocin is found to be in those fractions which contain the bacterial walls and membranes (9). It was also reported that L-forms of *E. coli* B are sentitive to colicin K and resistant to phage T6 and other viruses (21, 22). Because of this it has been suggested that the receptors for the bacteriocin and the phage are different substances and that the colicin receptor is located in the cytoplasmic membrane of the bacterium (22).

In order to gain more evidence concerning the sites of these receptors, we isolated the cell walls of *E. coli* strains B and Cullen by the modified procedure of Weidel (16) as described in Materials and Methods. The chemical properties of the materials so obtained are listed in Table II. It may be seen that cell walls of the two bacteria have similar nitrogen and phosphorus contents. They consisted

of approximately 80% protein, 10% lipid, and 4–6% carbohydrate. Their monosaccharide components were hexoses, heptoses, hexosamines, and ketodeoxyoctonic acid. Upon centrifugation in sucrose gradients (20–45%), the cell walls sedimented without forming a distinct boundary and hence were polydisperse. Their biological properties are summarized in Table III. As may be

TABLE II

Chemical Composition of Cell Walls of E. Coli Strains B and Cullen

	E. coli B	*E. coli* Cullen
	%	%
Nitrogen	13.6	13.2
Phosphorus	0.7	0.7
Protein*	85.0	79.0
Lipid	7.8	10.5
Carbohydrate‡	4.5	5.6

* Determined by means of the biuret reaction using bovine serum albumin as standard.
‡ Sum of hexose, hexoseamine, and ketodeoxyoctonic acid contents.

TABLE III

Inhibition of Colicin K and Bacteriophages by Cell Walls of Different E. Coli Strains

Cells walls	Inhibiting activity for			
	Colicin K	T2	T6	C16
E. coli B	256*	2	2	1‡
E. coli Cullen	1024	3	3	3
E. coli B/K,T6	0	2	0	0
E. coli Cullen/K,T6	0	3	0	0

* Units of colicin K/ml inhibited.
‡ Figures 4 to 0 indicate the phage-inhibiting activity of the materials:
4 = 50% or more of phage inhibited by 1 μg/ml
3 = " " " " " " " 10 μg/ml
2 = " " " " " " " 100 μg/ml
1 = " " " " " " " 1000 μg/ml
0 = less than 50% of phage inhibited by 1000 μg/ml

seen, the cell walls of colicin-sensitive strains readily inhibited the bacteriocin as well as the T2, T6, and C16 phages in viro. Those derived from *E. coli* Cullen were more potent inhibitors than the walls of *E. coli* B. On the other hand, the cell walls of the mutants which are resistant to colicin K and phage T6 had no effect upon the bacteriocin. They still inhibited phage T2, however, but failed to inactivate the T6 and C16 viruses. Thus, the cell walls of each bacterium contained receptor substances specific for those biological agents which attack the microorganism in question. Furthermore, it is evident that the mutation of

a bacterium from sensitivity to resistance is accompanied by a loss of the corresponding receptor.

Properties of Membrane Fractions Derived from Spheroplasts.—The bacterial cell walls obtained by the procedure employed might still contain fragments of cytoplasmic membranes. To ascertain whether the latter might harbor receptor substances, we prepared spheroplast membranes of the *E. coli* strains B and Cullen and fractionated them into cytoplasmic and outer membranes by the procedure of Miura and Mizushima (23, 24). Biological properties of the membranes are shown in Table IV. Here it is evident that in both instances the outer membranes, which banded in sucrose solution having the specific density of 1.21, contained the receptors for colicin K and phage T6. On the other hand, the cytoplasmic membranes, which banded at a lower density, were free of T6 receptor and exhibited only traces of anti-colicin activity. This suggests that the receptors for colicin K and phage T6 are located in the outer layers of the cell wall and not in the cytoplasmic membrane.

TABLE IV

Properties of Membrane Fractions from E. Coli B and Cullen Spheroplasts

Material	Specific density*	Inhibiting activity‡ for	
		Colicin K	T6
E. coli B:			
Outer membranes	1.21	1024	3
Cytoplasmic membranes	1.15	8	0
E. coli Cullen:			
Outer membranes	1.21	2048	3
Cytoplasmic membranes	1.15	16	0

* Specific density of sucrose solution containing the material in question after equilibrium centrifugation.

‡ See Table III for explanation of figures.

Attempts were also made to obtain the receptor substances in soluble form, i.e. as a material of low molecular weight, by extracting lyophilized cells with various solvents. However, extraction with trichloroacetic acid and sodium carbonate (5), with pyridine and 7 M urea (25), or with aqueous phenol at 65°C (26) yielded materials which either had a very low anti-colicin activity or which were entirely inactive. Considerable amounts of a fairly active substance were obtained by extracting cells with water at 60°C and then precipitating the extracts at pH 4.0 (27). On centrifugation most of the activity was sedimented at 80,000 *g*, a fact which indicated that the active material was particulate. Since the biological and chemical properties of the sedimentable fraction were similar to those of the cell walls (Tables II and III), it is not unlikely that this material consisted of cell wall fragments.

Serological Properties of the Cell Walls of E. Coli Cullen.—From the foregoing it is apparent that the cell walls of colicin-sensitive and resistant Cullen bacilli differ in their anti-bacteriocin and antiphage activities. To determine whether these materials also differ serologically, rabbits were immunized with *E. coli* Cullen bacteria, with the cell walls of this bacillus, and with the cell walls of the colicin-resistant mutant, *E. coli* Cullen/K, T6. As shown in Table V, the three antisera readily agglutinated the cell walls of the colicin-sensitive *E. coli* Cullen and neutralized their ability to inhibit colicin K. Evidently, the antisera to the cell walls of both colicin-sensitive and resistant bacteria contained not only the agglutinins but also the antibodies which prevented the colicin receptor-containing cell walls to bind the bacteriocin. Moreover, the antiserum to the colicin-

TABLE V

Serological Reactions of Bacterial Cell Walls in Various Antisera

Antisera tested	*E. coli* Cullen cell walls		*E. coli* B cell walls	
	Agglutination	Neutralization	Agglutination	Neutralization
E. coli Cullen anti-cell wall	256*	128‡	32	0
E. coli Cullen/K,T6 anti-cell wall	256	128	32	0
E. coli Cullen antibacterial	512	256	32	0
E. coli Cullen antibacterial absorbed with *E. coli* Cullen/K,T6 cell walls	16	0	0	0
E. coli Cullen antibacterial absorbed with *E. coli* B cell walls	256	128	0	0

* Agglutination titer.

‡ Receptor-neutralization titer.

sensitive Cullen bacterium, which has been absorbed with cell walls of the colicin-resistant mutant, failed to neutralize the anti-colicin activity of the cell walls of the sensitive bacterium and agglutinated them but slightly. Thus, it became apparent that the cell walls of colicin-sensitive and resistant variants of the Cullen bacillus have almost identical antigenic composition.

This was surprising since the cell walls of the sensitive bacterium contain the bacteriocin receptor, and those of the resisrant variant do not. It is possible, therefore, that the colicin-resistant mutant contains a substance which has the same serological specificity as the colicin receptor, but which is incapable of binding the bacteriocin. It is equally possible that the colicin K receptor of the sensitive bacteria is not antigenic and that the neutralization of the colicin-binding capacity of the cell walls is caused by a nonspecific blocking of the receptor sites by an antibody directed against a substance which is present in both colicin-resistant and sensitive bacteria.

To ascertain which alternative is the more likely, we studied the serological

reactions of the cell walls of *E. coli* B with antisera elicited by *E. coli* Cullen. It may be seen in Table V that all antisera agglutinated this material but failed to neutralize its anti-colicin activity. Moreover, when the antiserum to *E. coli* Cullen was exhaustively absorbed with the cell walls of *E. coli* B, it still agglutinated and neutralized the cell walls of the Cullen bacterium. Thus, the cell walls of these two bacteria are serologically different despite the fact that both contain the receptor for colicin K. Since the *E. coli* Cullen antiserum does not neutralize the colicin receptors of the cell walls of *E. coli* B, and since the absorption of this antiserum with the latter does not diminish the ability of the serum to neutralize the cell wall receptors of the Cullen bacillus, one must conclude that the antiserum does not contain antibodies which react specifically with the colicin K receptor. The receptor substance seems to be either not antigenic or a very poor antigen. The neutralization of the colicin-inhibiting properties of the cell walls of *E. coli* Cullen must therefore be attributed to a nonspecific blocking of the receptor sites by an antibody directed against an antigen which is present in *E. coli* Cullen and absent in *E. coli* B.

A similar conclusion was reached previously by Mayr-Harting who investigated the serological properties of the colicin E2-sensitive strains of *E. coli* C6 and *Shigella sonnei* II. Antisera to these microorganisms protected the homologous bacterium against the action of the colicin and failed to confer the protection upon the heterologous strain. Hence it was inferred that the colicin E2 receptor is not antigenic and that the attachment of the colicin to its receptor is prevented by steric hindrance produced when the antigen–antibody reaction on the bacterial surface covers up the colicin receptor (6).

Treatment of Cell Walls with Enzymes.—Since the receptors for colicin K and phages T2, T6, and C16 proved to be firmly attached to other cell wall constituents, we studied their chemical nature *in situ*. First, we investigated the effects of various enzymes upon the inhibiting properties of the cell walls. For this purpose the latter were digested as described in Materials and Methods, and the biological properties of the materials so obtained were tested. As may be seen in Table VI, trypsin-treated cell walls of *E. coli* Cullen and B strains inhibited colicin K and phages T2, T6, and C16 more vigorously than did the undigested material. Most likely this increase in activity is due to the removal of inert protein which blocks the receptor sites for these agents. Subsequent treatment of the trypsinized cell walls with chymotrypsin or lysozyme had no effect upon their inhibiting capacity. The receptors were resistant to the action of the three enzymes. On the other hand, papain and ficin selectively destroyed the receptor for T2 phage, whereas pronase and pepsin diminished considerably the capacity of the cell walls to inhibit colicin K and T2 virus. Either proteins or peptides are apparently constituents of the receptors for colicin K and for phage T2. Moreover, the receptors for the bacteriocin, the T2 phage and the T6 and C16 viruses exhibit differences in sensitivity to proteolytic enzymes, a fact which indicates

that the groupings which determine their specificity must be chemically different. Trypsinized cell walls were also treated with phospholipases A, C, and D, *Aspergillus* lipase, cellulase, amylase, and β-glucosidase, but these were all without effect upon the ability of the cell walls to inactivate colicin K. Most likely chemical groupings containing linkages which are sensitive to these enzymes are either inaccessible or absent in the receptor.

Effects of Amino Acid Reagents on the Biological Activity of Bacterial Cell Walls.—The modification of an amino acid residue in the active center of an enzyme frequently results in a loss of its catalytic activity (28). Since peptides or proteins are constituents of colicin K and phage T2 receptors, one might expect

TABLE VI

Effect of Enzymes on Inhibiting Activity of Bacterial Cell Walls

Cell walls	Enzyme	Inhibiting activity* for			
		Colicin K	T2	T6	C16
E. coli Cullen	Untreated	1024	3	3	3
	Trypsin	2048	3	4	4
E. coli Cullen (trypsinized)	Chymotrypsin	2048	3	4	3
	Lysozyme	2048	3	4	3
	Papain	2048	0	4	4
	Ficin	2048	1	4	3
	Pepsin	256	0	3	3
	Pronase	128	0	4	4
E. coli B	Untreated	256	2	2	1
	Trypsin	1024	4	3	2

* See Table III for the explanation of figures.

that treatment of bacterial cell walls with amino acid-specific reagents might destroy their ability to inactivate the bacteriocin or phage. Moreover, the nature of the reagent which causes inactivation might reveal which amino acids are essential for the biological activity of the receptor. We therefore treated trypsinized cell walls of *E. coli* Cullen with various reagents as described in Materials and Methods and examined the biological activities of the altered materials. The results of these experiments are presented in Table VII.

Two different preparations of cell walls were used, one of which inhibited approximately 8000 units of colicin K, the other 2000. It can be seen from the table that those chemicals which react with sulfhydryl or thioether groups such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetate or hydrogen peroxide had no effect on the biological activity of the cell walls (29–31). Acylation of the walls with acetic or succinic anhydrides, treatment with 1-fluoro-2,4-dinitrobenzene or nitrous acid, or nitration with tetranitromethane did not

change their ability to inhibit the bacteriocin or phages to any significant degree (32–36). Thus, cysteine, methionine, and amino acids containing free amino or hydroxyl groups, including tyrosine, are apparently not involved in the reaction of the walls with colicin or phages. In contrast, those chemical reagents which combine with tryptophan or oxidize it invariably destroy the receptor for colicin K. Thus, the tryptophan-specific 2-hydroxy-5-nitrobenzylbromide (HNBB)[1] specifically inactivated only the colicin K receptor (37). This indi-

TABLE VII

Effects of Amino Acid Reagents on Inhibiting Activity of Trypsinized Cell Walls of E. Coli Cullen

Cell walls treated with	Reactive groupings or amino acid residues	Inhibiting activity* for				Reaction conditions and references	
		Colicin K	T2	T6	C16		
Untreated (preparation I)	—	8192	4	4	4	—	
p-Chloromercuribenzoate	SH	8192	4	4	4	10^{-4} M, pH 7.0, 25°C	(29)
N-Ethylmaleimide	SH	8192	3	4	4	10^{-4} M, pH 7.0, 25°C	(29)
Iodoacetate	SH, Met	8192	3	4	3	$3 \cdot 10^{-3}$ M, urea, pH 7	(30)
Hydrogen peroxide	Met	8192	4	4	4	10^{-3} M, pH 2.7	(31)
Acetic anhydride	NH_2	8192	3	3	3	1.3 M Na-acetate 0°C	(32)
Succinic anhydride	NH_2, OH	8192	3	4	3	Water, pH 7.0	(33)
Fluorodinitrobenzene	NH_2, SH, phenol	8192	3	4	3	$5 \cdot 10^{-4}$ M, water, pH 7.8	(34)
Untreated (preparation II)	—	2048	3	4	3	—	
Nitrous acid	NH_2	1024	3	3	3	0.4 M $NaNO_2$, pH 4.0, 0°C	(35)
Tetranitromethane	Tyr	2048	3	4	3	$8 \cdot 10^{-3}$ M, pH 7.8, 25°C	(36)
2-Hydroxy-5-nitrobenzylbromide	Try, SH	16	3	3	3	10^{-2} M, 20% acetone	(37)
2-Nitrophenylsulfenyl chloride	Try, SH, SS	2	0	0	0	10^{-2} M in DMF‡	(38)
N-Bromosuccinimide	Try, Tyr, SH, His	0	0	0	0	$3 \cdot 10^{-3}$ M, urea, pH 4	(41)
Bromine	" " " "	2	0	0	0	" " "	
Iodine	Tyr, SH, His, Try	2048	0	4	3	Excess I_2 in urea, pH 4	(42)
Lithium periodate	Cis-diol, Try, Tyr, SH	1024	2	3	2	10^{-3} M, 2 hr, 0°C	(43)
Lithium periodate	" " " " "	64	2	3	2	0.1 M, 18 hr, 25°C	(43)

* See Table III for explanation of figures.

‡ DMF = Dimethylformamide.

cates that tryptophan is an essential constituent of this receptor. On the other hand, 2-nitrophenylsulphenyl chloride (NPSC) which reacts with tryptophan, cysteine, and perhaps with disulfide linkages (38–40), destroyed all the receptors. It was observed, however, that upon treatment of the cell walls with this reagent in dimethylformamide[2] about 50% of the material became soluble. This

[1] *Abbreviations used in this paper:* HNBB, 2-hydroxy-5-nitrobenzylbromide; KDO, ketodeoxyoctonic acid; NBS, N-bromosuccinimide; NPSC, 2-nitrophenylsulphenyl chloride.

[2] This solvent was used as the reaction medium instead of 50% acetic acid (38), because the latter inactivates the colicin receptor (Table VIII).

fraction was devoid of biological activity, and hence, the inactivation of the receptors of NPSC may be attributed not only to the blocking of the tryptophan residues, but also to the disaggregation of the cell wall structure. *N*-bromosuccinimide (NBS) and bromine also destroyed the receptors for bacteriocin and phages. These reagents, however, react not only with tryptophan and cysteine, but with histidine, tyrosine, methionine, and probably other components of the bacterial cell wall as well. In this instance, the loss of biological activity may be therefore attributed to the oxidation of different functional groupings in each of the four receptors. Iodine, on the other hand, appears to be more specific in its action, as it destroyed only the T2 receptor. Since this halogen combines with tyrosyl and histidyl residues and reacts with tryptophan weakly (28), it is possible that formation of iodo-compounds of the first two amino acids causes the inactivation of the receptor.

Cell walls were also oxidized with periodate. When the reaction was performed under mild conditions (1 mM $LiIO_4$, 2 hr at 0°C) their anti-colicin activity decreased to 50%, whereas the antiviral activity was diminished by about 90%. When the walls were oxidized more vigorously, however, their ability to bind colicin decreased to 3% of the original value, yet the antiviral activity was not diminished further. The interpretation of these results is uncertain. It is known that periodate oxidizes cis-glycols and one might deduce from this that carbohydrates are constituents of the colicin K receptor. On the other hand, one must not loose sight of the fact that tryptophan, tyrosine, and cysteine are also oxidized by this reagent (43). Since we have already demonstrated that treatment of the cell walls with various tryptophan-specific reagents results in a loss of their colicin-binding capacity, it is possible that the inactivation of the receptor by periodate can be attributed to an oxidation of the tryptophan component. The fact that the phage receptors are not fully destroyed even after prolonged periodate treatment indicates that periodate-sensitive carbohydrates alone cannot be responsible for the specific adsorption of the three phages. In contrast, the sensitivity of the viral receptors to NBS or NPSC which react specifically with amino acids, rather suggests that peptides or proteins are essential components of the receptor substances.

Effects of Solvents on Colicin and Phage Receptors.—To determine whether bacterial cell walls contain other constituents which are essential for their biological activities, the effects of various solvents on the inhibiting properties of trypsinized cell walls of *E. coli* Cullen were studied. These experiments are summarized in Table VIII. As one can see, the colicin receptor is thermostable at 60°C and pH 7.5, but is inactivated at 80°C. The receptors for T2, T6, and C16 phages, on the other hand, are stable at this temperature. Dilute hydrochloric acid at 37°C has but little effect on bacteriocin and phage receptors, yet weak alkali (0.1 M Na_2CO_3 at pH 11.0) destroys the colicin receptor without damaging the viral receptors. Stronger alkali (0.1 M NaOH at pH 13.0) inacti-

vates all the receptors. Treatment of the cell walls with 50% acetic acid also inactivates selectively the colicin receptor, but exposure to formic acid results in complete destruction of their biological activity. Thus, the colicin receptor is a relatively thermolabile substance which is susceptible to both weak alkali and acids. Apparently readily hydrolyzable groupings are essential for its biological activity. The viral receptors differ from the bacteriocin receptor in that they are more resistant to heat and to hydrolytic reagents.

TABLE VIII

Effects of Solvents on Inhibiting Activity of Trypsinized Cell Walls of E. Coli Cullen

Solvent	Inhibiting activity* for				Duration and temperature of treatment
	Colicin K	T2	T6	C16	
Control (untreated)	2048	4	4	4	—
0.01 M Tris buffer, pH 7.5	2048	4	4	4	1 hr, 60°C
" " " " "	32	4	4	4	1 hr, 80°C
0.01 M HCl	2048	3	4	4	1 hr, 37°C
0.1 M Na_2CO_3, pH 11.0	4	3	3	3	1 hr, 37°C
0.1 M NaOH, pH 13.0	0	0	0	0	1 hr, 37°C
Acetic acid, 50%	4	3	3	3	1 hr, 25°C
Formic acid, 99%	0	0	0	0	1 hr, 25°C
Lipid solvents‡	2048	3	4	3	4°C or 25°C
Chloroform methanol (2:1)	512	2	2	1	4°C
Phenol, 88%:					30 min, 25°C
Fraction I	0	0	0	0	
Fraction II	2	0	0	0	
Fraction III	0	0	0	0	
E. coli Cullen lipopolysaccharide	0	0	0	0	
Sodium dodecylsulfate, 1%:					30 min, 25°C
Sedimentable fraction	32	1	2	1	
Soluble fraction	0	0	0	0	

* See Table III for explanation of figures.

‡ Solvents used: ethanol, methanol, acetone, ether, formamide, dimethylformamide, and dimethylsulfoxide.

Upon extraction of the cell walls with lipid solvents such as ethanol, methanol, ether, acetone, formamide, or dimethylformamide, they retain their inhibiting properties. Apparently, loosely bound lipids are not necessary for the activity of the colicin and viral receptors. However, the extraction with chloroform or with chloroform–methanol mixture decreases significantly the inhibiting activity of the cell walls. Since these solvents inactivate the receptors only partially, regardless of the number of extractions, it is conceivable that this inactivation is caused by partial denaturation of their protein or lipoprotein constituent rather than by the removal of soluble lipids.

Solvents which dissociate the cell walls into their components also destroy

their receptor activity. Thus, when the cell walls were stirred with 88% phenol at 25°C, approximately 75% of the material dissolved. The insoluble fraction, recovered by centrifugation at 50,000 *g* (fraction I), was found to be biologically inactive. On dialysis of the phenol solution the protein-rich material was precipitated (fraction II) whereas a small quantity of carbohydrate-rich material remained in solution (fraction III). Both the precipitated and the soluble materials were devoid of receptor activity. Moreover, the lipopolysaccharide of *E. coli* Cullen, obtained from bacterial cells by the phenol procedure (26), did not inhibit the colicin or any of the three phages. A solution of sodium dodecyl sulfate also dissolved about 80% of the cell wall material. In this instance the insoluble material exhibited only traces of the receptor activities an the soluble fraction was totally inactive. Other dissociating reagents such as 7 M urea, 1% sodium deoxycholate, or 1% Nonidet P 40 (nonionic detergent, Shell Chemical Corp., New York) failed however to dissolve the cell walls and were without effect upon their biological activity.

The reason as to why phenol and dodecylsulfate inactivate the receptors is not fully understood. The inactivating effect of phenol might be attributed to the denaturation of the protein component of the receptor, for the lipopolysaccharides of *Sh. sonnei* and *E. coli*, obtained by procedures employing this solvent, still inhibit T4 phage and colicin B, respectively (27, 7). It is more difficult to account for the inactivation of the receptors by dodecylsulfate as this surfactant is known to unfold the protein molecules (44) and to dissociate the lipopolysaccharide micelles into subunits as well (45). However, since deoxycholate, which also dissociates the lipopolysaccharides into their subunits (46), fails to destroy the biological activity of the cell walls, it is possible that the inactivation of the receptors by dodecylsulfate is caused by the denaturation of their protein components.

DISCUSSION

When bacteriocins or bacteriophages are brought in contact with a sensitive microorganism they combine with specific receptors present on the surface of the bacterial cell (1, 6, 9). The chemical nature of the substances or cell wall structures which specifically adsorb or inactivate the biological agent in question is poorly understood. In some instances the specific bacterial receptors which react with phage have been purified and their chemical composition has been ascertained. Thus, Miller and Goebel (25) have shown that the somatic antigen of *Shigella sonnei* phase II inhibits in vitro T3, T4, and T7 phages. It was subsequently found that by extracting phenol-killed bacteria with water at 60°C one could obtain a substance which inhibited those viruses and the T2 and T6 phages as well. Digestion of this material with pancreatin and dissociation with phenol yielded a lipopolysaccharide which was a potent inhibitor of T3, T4, and T7 but which lost its activity against phages T2 and T6 (27).

These results were subsequently confirmed by Weidel who studied the receptors of *E. coli* B (47). Moreover, the sugars which are present in the lipopolysaccharides derived from the T3, T4, T7-sensitive *Sonnei* or colon bacilli differed from those in the lipopolysaccharides of phage-resistant mutants of these two bacteria (47, 48). Weidel also isolated the T5 receptor from *E. coli* B. This material was particulate and had a chemical composition similar to that of the bacterial cell wall (49). The chemical and serological properties of the T5 receptor were essentially the same as those of particulate material obtained from a T5-resistant mutant of *E. coli* B (49, 50). More recently, Beumer and his coworkers investigated the viral receptors of *Sh. flexneri*. They found that lipopolysaccharides of the F6S strain of this microorganism contain receptors for the Lisbonne phages H-F6S, H^+, and V. Moreover, they showed that extracts and cell walls of this bacillus inactivate also the T2 and T6 phages and that the inhibiting activity of the cell walls is destroyed by phenol. From this they inferred that the receptors for the two viruses are located in the lipoprotein layer of the bacterial cell walls (51).

The phage receptors of Gram-positive bacteria were first studied by Krause who showed that the group-specific polysaccharide derived from hemolytic. streptococcus Group C inactivated the viruses which attack this microorganism The group A-specific polysaccharide, however, was without effect on the phages which lyse Group A Streptococci (52). Vidaver and Brock isolated from spheroplasts of *Streptococcus faecium* a microgranular material which inactivated phage P3 (53). As in the case of the T5 receptor, the chemical composition of this material resembled that of the whole cell wall of the bacterium (53). In other instances, phage receptors appeared to be firmly bound to the bacterial wall. Thus, Morse has demonstrated that the phage receptors of *Staphylococcus aureus* could not be separated from the cell walls of the organism (54). Coyette and Ghuysen confirmed this and showed that *N*-acetyl-D-glucosaminyl-D-ribitol units of teichoic acid, linked to the peptidoglycan layer of the cell wall, serve as receptor sites for the staphylococcal phages (55).

Much less is known about the nature of bacteriocin receptors. Bordet and Beumer were the first to show that bacteriocin produced by *E. coli* V is inactivated in vitro by extracts of microorganisms which are sensitive to this colicin (5). Mayr-Harting investigated the colicin E2 receptors of *E. coli* C6 and *Sh. sonnei* II. She found that bacterial cells killed with 2% phenol, 0.1% formol, or by heating at 60°C readily adsorbed the colicin, and that those which were treated with 80% ethanol or heated at 100°C failed to do so. She concluded therefore that properties of colicin E2 receptor are similar to those of flagellar antigens (6). More recently Guterman and Luria reported that colicin B is inhibited by lipopolysaccharides derived from *E. coli* K12 strains which are sensitive or tolerant to this bacteriocin (7). In addition Marotel-Schirmann and Barbu found that lipopolysaccharides of certain rough strains of *Salmonella minnesota* which are resistant to colicins A, E1, E2, and K inactivate these bac-

teriocins in vitro; the lipopolysaccharides of smooth strains of this microorganism have no inhibitory effect (56). These observations led the authors to the conclusion that the ketodeoxyoctonic acid (KDO)-lipid A complex might play a role in the binding of the four colicins. However, it was not established whether this complex does indeed serve as a receptor for the bacteriocins in colicin-sensitive bacteria. It may be noted that a preparation of the somatic antigen of *Sh. sonnei* phase II/3,4,7 (48), which contains only KDO and hexosamine as its sugar components, had no inhibitory effect on colicin K to which this microorganism is susceptible (unpublished observation).

The study which has been reported here has revealed that the cell walls of colicin K-sensitive strains of *E. coli* B and Cullen are potent inhibitors of this bacteriocin. The inhibition is specific, for the cell walls of resistant mutants do not inactivate colicin K. Upon separation of spheroplasts of these bacteria into cytoplasmic and outer membranes, the receptor activity is found only in the latter fraction. This suggests that the colicin K receptor is a constituent of the bacterial cell wall. The anti-colicin activity of the cell walls is destroyed by heat, alkali, acetic acid, certain proteolytic enzymes, and by reagents which combine with tryptophan. We have concluded from this that proteins or peptides are essential constituents of the colicin K receptor, and that tryptophan is involved in determining its specificity.

Whether substances other than protein form part of the colicin receptor is not yet certain. Soluble lipids have been excluded, for the extraction of cell walls with lipid solvents was without effect. However, it is possible that protein-bound lipid is a component of the receptor as the latter is partially inactivated by chloroform. It is more difficult to ascertain whether carbohydrates participate in the specific adsorption of colicin K. Certain evidence suggests that this is not the case. First, it is known that the T4 receptor of *Sh. sonnei* is a lipopolysaccharide, the biological activity of which is unaffected by exposure to phenol (27). The colicin K receptor of *E. coli* Cullen, on the other hand, is readily destroyed when the cell walls are dissociated in this solvent, a fact which suggests that polysaccharides are not functional constituents of the receptor. Secondly, the somatic antigens of Gram-negative bacteria are potent immunogens and their serological specificity is determined by the sugar components of their lipopolysaccharide moieties. In view of the fact that the cell walls of colicin-sensitive bacteria do not elicit an antibody specifically directed against the colicin K receptor, it seems justifiable to conclude that the polysaccharide component of the cell wall does not determine the specificity of the receptor.

Our study has included an investigation of the chemical nature of the receptors for T2, T6, and C16 phages. We have found that the T2 receptor is sensitive to a number of proteolytic enzymes and hence must be a protein-containing material. It differs from the colicin K receptor in that it is inactivated by papain, ficin, and iodine, though not by 2-hydroxy-5-nitrobenzyl-bromide. The proper-

ties of the T6 and C16 receptors were found to be very similar to one another. Not a single reagent which inactivated one of the receptors was without effect upon the other. The chemical groupings which determine the specificity of the two receptors must therefore be very similar. Most likely, proteins are the constituents of the two receptors as they are inactivated by certain amino acid-specific reagents and by protein-denaturing solvents. The receptors for T6 and C16 differ from those for colicin K and phage T2 as they are resistant to all enzymes and chemicals which specifically inactivate the receptors for the bacteriocin and phage T2. Thus, the specificity of the receptors for colicin K, phage T2, and the T6 and C16 viruses is determined by chemical groupings which are obviously different. Whether the groupings which react with the bacteriocin or with each of the phages are located in different molecules or whether they form part of the same macromolecule is a question which cannot be answered with certainty on the basis of the data on hand. However, the fact that mutants of *E. coli* which are resistant to colicin K and phages T6 and C16 still contain the T2 receptor suggests that the latter may be a separate substance. The observation that the somatic antigen of *Sh. sonnei* II/3,4,7 is a potent inhibitor of the T2 virus and weakly inactivates the T6 phage (48) confirms this viewpoint. On the other hand, the fact that colicin K-resistant mutants of the colon bacteria are always resistant to T6 and C16 phages suggests that the specific groupings which react with the bacteriocin and those which combine with the two viruses might form part of the same receptor macromolecule.

SUMMARY

Cell walls of *E. coli* strains B and Cullen contain specific receptors for colicin K and for the T2, T6, and C16 phages. The receptors for the bacteriocin and the T6 virus are located in the outer layers of the cell wall of these microorganisms and are absent in their cytoplasmic membrane.

The receptors for colicin K, phage T2, and the T6 and C16 viruses differ in their stability toward enzymes and chemical reagents. Their specificity must therefore be determined by different chemical groupings.

The colicin K receptor is inactivated by certain proteolytic enzymes and by reagents which combine with tryptophan. It is concluded therefore that proteins or peptides containing this amino acid are essential for biological activity of the receptor.

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