The Isolation of Colicine V and a Study of Its Immunological Properties

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ABSTRACT Colicine V has been obtained from the culture medium in which the colicinogenic bacillus *E. coli* K357 L_T is grown. The material is electrophoretically homogeneous and proves to be a lipocarbohydrate protein complex identical with the type-specific O antigen of the parent bacillus. Colicine V is toxic both for mice and for rabbits and readily stimulates the elaboration of precipitins and bacterial agglutinins, as well as antibodies which neutralize the antibacterial activity of the colicine itself. The colicine is also toxic for certain strains of Enterobacteriaceae. Although colicine V and colicine K, previously described in this laboratory, have many properties in common, they exhibit no cross-serological relationship whatsoever.

A study of the bacteriophages has been one of Dr. Northrop's many interests over the years. It is therefore not inappropriate that a volume of scientific papers dedicated to him should contain an account of the colicines. Phages and colicines bear a remarkable resemblance to one another (1). Both agents apparently initiate their attack upon the susceptible host by attaching themselves through identical receptor sites. Furthermore, it is known that certain microorganisms which are susceptible to both phage and colicines yield variants which are resistant to both agents (2). Finally, it will be recalled, that in many instances the physical and chemical agents which induce lysogenic bacteria to liberate phage will frequently induce the colicinogenic organism to produce colicine (3).

Striking as these resemblances are it is questionable whether these relationships are other than fortuitous. Strong and irrefutable support of this hypothesis is found in an observation made some years ago in the writers' laboratory (4). It was shown that colicine K and phage T6, both of which exhibit certain of the similarities which have been discussed (5), are entirely unrelated immunologically.

Nearly a decade ago our laboratory undertook the formidable task of

isolating, purifying, and characterizing the colicines (6). A perusal of the literature did nothing to make easy the problem, for it was obvious that the chemical efforts which had been made by others toward this end were both fragmentary and contradictory. Gratia was the first to describe in detail an antibacterial agent which resembled bacteriophages (7). This substance, which he named "principle V," inhibited the growth of several sensitive bacterial strains. Unlike bacteriophages, however, his agent could not be transmitted on serial passage. Gratia characterized this agent, now known as colicine V, as a filterable entity which was heat-resistant, dialyzable, and precipitable by acetone.

Following these initial observations the question of the chemical nature of the colicines remained dormant until 1946 when Heatley and Florey (8) initiated a chemical study of the colicine produced by a strain of *E. coli* known as CF1. These investigators sent their colicinogenic strain to Gratia who stated at the time that it appeared to be identical in all respects with his coli V strain which produced the "V principle." Heatley and Florey isolated a substance from the culture medium of their microorganism which was precipitable by alcohol and which resembled the colicine of Gratia in that it too was "freely dialyzable," heat-resistant, and sensitive to proteolytic enzymes. On the basis of these observations, they concluded that their colicine, like that of Gratia, was in all probability either a protein or a peptide.

Colicines which appeared to be of high molecular weight, because they failed to dialyze through semipermeable membranes, were first isolated by Halbert (9) and by Gardner. One of these, studied by Gardner, was identified by Frédéricq (10) as colicine V. This substance, supposedly the same as that of Gratia, was not dialyzable; it was inactivated by proteolytic enzymes and was precipitated by both trichloroacetic and picric acids. The author came to the conclusion that this substance was probably a protein. Little attempt was made by these investigators to establish the purity of their products, nor did they characterize them by chemical analyses. This, in brief, was the state of affairs when our laboratory initiated its studies on colicine K in 1952 (6).

In sum let it be said that from the culture medium in which a colicine K–producing organism known as *E. coli* K235 was grown, there was obtained a thermostable, non-dialyzable substance which we believed to be identical with colicine K (11). The substance was highly toxic, both for bacteria and mammals. It was a potent antigen, and elicited both precipitating and neutralizing antibodies when injected in minute quantities into rabbits.

Highly purified colicine K proved to be a lipocarbohydrate-protein complex containing some 5 per cent of nitrogen, 1.5 per cent of phosphorus, 20 per cent of lipid, and yielded 50 per cent of reducing sugar upon hydrolysis with dilute mineral acid. Its antibacterial activity was destroyed by a variety

of proteolytic enzymes. Despite this, colicine K cannot be called a protein. Nevertheless the molecular complex can be readily dissociated into its protein and lipocarbohydrate components (12). In so doing it is found that all of the antibacterial activity accompanies the so-called protein component, whereas the lipocarbohydrate is the bearer of the mammalian toxin (11). In our opinion colicine K is identical with the somatic O antigen of the microorganism from which it is derived.

Observations similar to ours have been made by Nüske and his coworkers (13). In 1957 these investigators purified a colicine from a bacillus known as *E. coli* SG-710. Their colicine preparations contained 45 to 55 per cent of protein, 20 to 25 per cent of polysaccharide, 15 to 20 per cent of lipid, and 5 to 10 per cent of inorganic constituents and gave but a single broad band after paper electrophoresis. Both trypsin and chymotrypsin destroyed the antibacterial activity of their product. From the analysis this colicine too appeared to be a lipocarbohydrate-protein complex.

This paper will describe the isolation and properties of colicine V. It will be shown that this substance, like colicine K, is a lipocarbohydrate-protein complex endowed with potent toxic properties for bacteria and mammals. It will be demonstrated that, although the chemical properties of colicine V resemble those of colicine K, the two bear no immunological relationship to one another.

MATERIALS AND METHODS

Strains of Microorganisms The original colicinogenic strain used in these studies was kindly sent us by Dr. Pierre Frédéricq of the University of Liège. This bacillus, designated as *E. coli* K357, produced an antibacterial agent which he identified as colicine V. When the microorganism which we received was first streaked on neopeptone agar we observed two colony types. The first of these was shiny, sticky, and opaque. When examined by India ink stain, the bacillus was encapsulated. It was later shown to possess a heat-labile surface or K antigen. In accordance with the Kaufmann nomenclature (14) it was designated as an L_+ strain. Since the colonies were opaque, the strain was termed *E. coli* K357 L_+O, in accordance with our previous nomenclature (4). The second variant produced dull, grainy, translucent colonies on neopeptone agar and did not possess the L antigen; consequently it was characterized as *E. coli* K357 L_-T.

The colicine K–producing bacillus used in this investigation is a variant of the strain *E. coli* K235 sent to us in 1952 by Frédéricq. This microorganism exists in at least four different forms. Two of these are encapsulated and elaborate an L antigen. Of these two, one grows on neopeptone agar as translucent colonies, and the other as opaque colonies. The strains are designated as *E. coli* K235 L_+T and L_+O respectively. Two non-encapsulated or L_-variants have also been isolated and these too grow as translucent (K235 L_-T) and opaque colonies (K235 L_-O). It is the

encapsulated and opaque variant which was used in these studies; *i.e.*, *E. coli* K235 L_+O.

In 1958 we sent these four strains to the Statens Seruminstitut in Copenhagen where they were typed. The variant *E. coli* K235 L_+O used here was found to have the serotype 01:K1 and to be devoid of an H antigen. We wish to express our great appreciation to Dr. Fritz Ørskov for his many kindnesses throughout our work.

In order to assay the activity of colicine V preparations obtained during the course of purification, the sensitive strain *E. coli* φ, originally used by Gratia to detect his V principle and sent to us by Frédéricq, was employed. The strain *E. coli* B was used when colicine K assays were performed.

Culture Medium The culture medium used for growing *E. coli* K357 L_-T was prepared so as to contain only dialyzable substances. To 15 liters of 1 per cent casamino acid medium containing 0.03 M phosphate at pH 7.0, was added the sterile, concentrated dialysate derived from 4 pounds of beef heart infusion (15). In addition, the sterile dialysate of 25 gm of Difco yeast extract was added. As an energy source, 300 ml of a neutralized 30 per cent solution of sodium lactate and 450 ml of 50 per cent dextrose were employed.

Colicine Assay The quantitative estimation of colicine V activity was made by a ring test previously described (16). One unit of colicine V activity has been defined as that amount of substance in γ/ml which, when placed as a droplet (0.02 ml) on the surface of nutrient agar contained in a Petri dish and seeded with 5×10^7 cells of *E. coli* ϕ, completely inhibits growth of the bacilli encompassed by the area of the droplet.

Antisera Four different antisera were used in this study. The sera of rabbits immunized with the living colicine K–producing bacillus *E. coli* K235 L_+O were obtained as previously described (4). Sera to the colicine V–producing bacillus *E. coli* K357 L_+O were prepared in a similar manner. Antisera to highly purified colicine K and colicine V were likewise prepared by a procedure described in an earlier publication (11), using as antigens electrophoretically homogeneous preparations of the two colicines.

Electrophoretic and Chemical Methods Electrophoretic analyses of colicine V preparations were made in the Tiselius apparatus using a 2 ml cell (17). The microanalytical methods for the estimation of nitrogen, phosphorus, protein, carbohydrate, etc. are the same as those used in our studies on colicine K (11).

Agar Diffusion Reactions Agar diffusion tests were carried out by a modification of the procedure described by Ouchterlony (18). A piece of 1 inch adhesive tape was placed across one end of a microscope slide (5×7.5 cm). 5 ml of molten 1.5 per cent agar dissolved in 0.1 M borate buffer at pH 7.6 was placed on the slide. After solidification, a well pattern was cut using a lucite mold and a stainless steel cutter. 6 mm holes were cut and the pattern was so arranged that the holes were separated symmetrically and in all directions by a distance of 8 mm, center to center. 2 drops of the various solutions to be tested were placed in the wells; the slides were incubated at room temperature for 48 hours in a humidified atmosphere and then photographed.

Colicine Neutralization Tests In order to test the ability of colicine K and colicine V antisera to neutralize colicine activity, 0.3 ml of the appropriate colicine antiserum was added to 2.5 ml of 0.7 per cent nutrient agar at 50°C and the tube seeded with the test organism (5×10^7 B/ml). The soft agar was then poured on a Petri plate containing 18 ml of sterile nutrient broth–agar (1.5 per cent). After standing for 15 minutes at 4°C, 0.02 ml samples of varying concentrations of colicine were placed upon the surface of the agar. The plate was permitted to stand until the droplets had dried and was then incubated for 6 hours at 37°C, and read. The end point was considered to be the highest dilution of colicine which caused complete inhibition of growth of the test organism.

Toxicity Tests A sterile solution (5 mg/ml) of the colicine to be tested was prepared in 0.9 per cent saline. A series of 1:1 dilutions was made and 1 ml of each was injected intraperitoneally into each of 3 mice, using 20 to 22 gm animals 4 to 5 weeks old. The Collins-Nelson-Rockefeller Institute strain of Swiss mice was employed. The animals were observed for 5 days and their death or survival recorded.

Agglutination and Precipitin Tests These tests were carried out in the usual manner. In conducting the agglutination tests, serial dilutions of the antisera, starting at 1:50, were incubated with a suspension of the microorganism to be tested, containing about 1×10^9 B/ml. Either living cells or cells which had been heated for 1 hour at 100°C, in order to reveal the somatic O antigen and to destroy the capsular L antigen, were employed. The tubes containing the serum and cells were incubated at 37°C for 2 hours and the agglutination reactions were read after 18 hours at 4°C.

In conducting the precipitin tests, 0.5 ml of a dilution of antiserum (2 parts of serum and 3 parts of 0.9 per cent NaCl solution) was mixed with 0.5 ml of appropriate dilutions of the antigen to be tested. After 2 hours of incubation at 37°C and 18 hours at 4°C, the tubes were read.

RESULTS

Preparation of Colicine V 15 liters of culture medium maintained at 37°C and at pH 7.0, was seeded with 1×10^5 cells of *E. coli* K357 L_T growing in the logarithmic phase. The culture was aerated at the rate of 4 liters per minute and was maintained at pH 7.0 by automatic titration with 2 M Na_2CO_3 (16). 18 hours later growth of the organism ceased abruptly. The culture was now maintained at pH 7.0 for 6 additional hours by automatic titration with 2 N HCl. During this interval there is a maximal release of colicine. The bacilli were killed by stirring with 100 ml of chloroform for 5 minutes. 150 ml of glacial acetic acid was then added and the bacilli were removed by centrifugation. The centrifugate, containing some 400 units of colicine per ml, was reduced *in vacuo* at 18°C to a volume of 1 liter. The solution was now dialyzed against distilled water at 4°C for 24 hours. The material was reconcentrated, redialyzed, and then dried from the frozen state. 3.4 gm of a gray, friable powder was recovered. This substance was

designated "crude colicine V." The activity of the preparation is, as a rule, very high. 0.02 ml of a solution, containing 1.5 to 3 γ/ml, completely inhibited the growth of the test organism *E. coli* φ when using the ring test (16).

The crude colicine V was further purified by fractional precipitation with ethyl alcohol. Cold ethanol was added to a 1 per cent solution of the colicine in 0.02 M sodium acetate at 0°C to bring the final concentration of alcohol to 30 per cent. After standing for 1 hour at 0°C a small amount of precipitate was centrifuged off and discarded. Sufficient ethanol was now added to bring the final concentration to 60 per cent and the precipitated colicine was separated by centrifugation. Approximately 75 per cent of the starting material was recovered. The inactive pigmented supernatant was discarded. The precipitate was now dissolved in water and dialyzed overnight at 4°C and then freeze-dried. This material, "purified colicine V," was obtained as a crumbly, slightly grayish white product. Alcohol precipitation serves to remove most of the pigment from the preparations, but the procedure must be carried out with dispatch and at low temperature to avoid a significant loss in activity.

Although the colicine obtained at this juncture is electrophoretically and serologically homogeneous, it is still pigmented, possibly because of a contaminant from the culture medium. This can be successfully eliminated in the following manner.

4 gm of 200 mesh DEAE cellulose was washed on a sintered glass funnel four times with 200 ml of 0.3 M tris buffer, containing 0.5 M NaCl at pH 7.2. The material was then washed thoroughly with 0.05 M tris at pH 8.0. A slurry was made in the 0.05 M buffer and the cellulose was poured into a column 1.9 cm in diameter. The material was finally compressed to form a column 10 cm in height. 1.65 gm of the alcohol-precipitated colicine V was dissolved in 100 ml of 0.05 M tris at pH 8.0. The solution was placed on the column, and permitted to flow through by gravity. The column was washed with 200 ml of the same buffer in order to wash through the colicine. A small pigmented band of material about 2 to 3 mm thick remained adherent to the cellulose at the very top of the column. After dialysis at 4°C the colicine was recovered from the solution by freeze-drying. 1.31 gm was recovered. This procedure resulted in no loss in specific activity.

Properties of Colicine V Crude colicine V and the purified substance show only slight differences in their physical and chemical properties. The procedure employed for the purification of the colicine results primarily in the elimination of pigment. The purified product is soluble in water and gives an opalescent solution. Its biological activity is such that 0.02 ml of a solution containing 1 to 2 γ/ml completely inhibits the growth of *E. coli* φ when spotted on a colicine assay plate seeded with 5×10^7 bacilli. This corresponds to an activity of 500 to 1000 units per mg.

Chemical Properties Colicine V appears to be typical of the lipocarbohydrate-protein complexes, or O antigens, found in many enteric bacilli. The substance contains 3.8 per cent of nitrogen and 2.0 per cent of phosphorus, and on acid hydrolysis (1 N H_2SO_4 at 100°C for 5 hours) it yields some 43 per cent of reducing sugars calculated as glucose. An insoluble residue appears

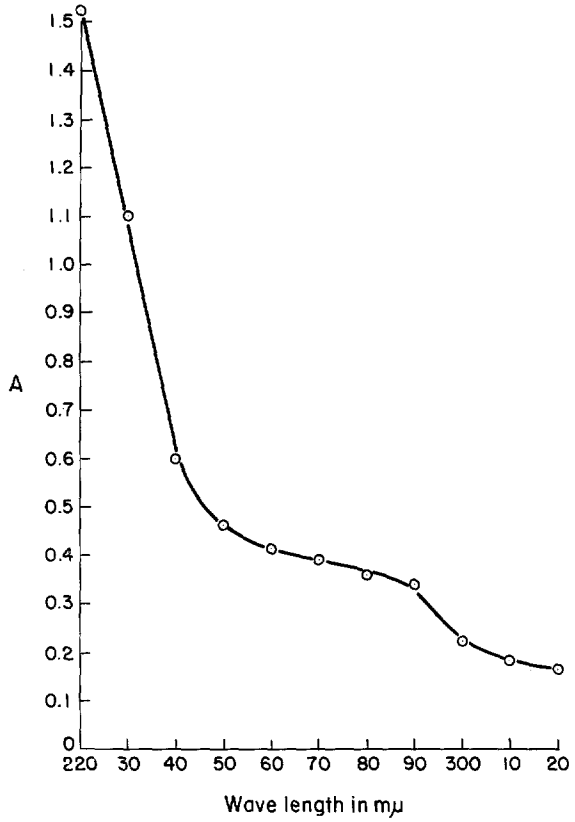

FIGURE 1. Absorption curve of colicine V. A = absorbance of a solution of colicine V containing 0.5 mg per ml in tris buffer at pH 8.0.

on hydrolysis,—a lipid which is soluble in chloroform and which comprises some 11.3 per cent by weight of the original lipocarbohydrate-protein complex. Colicine V gives both strong biuret and Folin tests, as well as a strong positive anthrone test. The Folin-positive material (calculated as serum albumin) amounted to 10.4 per cent. The anthrone-positive material (calculated as glucose) was 20.6 per cent. Colicine V shows absorption in the ultraviolet region, and a typical spectrum is given in Fig. 1, where it is seen that the colicine is not contaminated with any appreciable quantity of nucleic acid. Colicine V is not precipitated either by picric or trichloroacetic acids nor by the salts of heavy metals.

We have not attempted to dissociate the complex with phenol by the procedure of Morgan (12), nor have any attempts been made to characterize the carbohydrate or the other components of the colicine. As with colicine K, the antibacterial activity of colicine V is readily destroyed by crystalline trypsin. Purified colicine V, obtained by the procedure described above, is electrophoretically homogeneous. A solution of the colicine in 0.1 M sodium borate at pH 9.20 showed but a single symmetrical peak after 10,300 seconds of electrophoresis as shown in Fig. 2. The calculated mobility was minus 4.16 $\times$ 10^{-5} cm^2/volt sec.

Toxicity of Colicine V Colicine V exhibits marked toxicity both for mice and for rabbits. 1 ml of a sterile solution of colicine V, containing 1.25

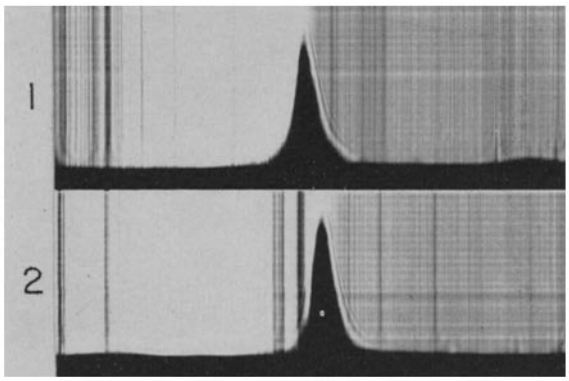

FIGURE 2. Electrophoretic pattern of colicine V. 1, ascending boundary of a solution of colicine V in 0.1 M borate buffer at pH 9.2 (concentration 5 mg/ml) after 10,300 seconds. 2, descending boundary.

mg/ml, when injected intraperitoneally into young 4 to 5 week old mice of the Collins-Nelson-Rockefeller Institute strain of Swiss mice, killed all animals as can be seen in Table I.

When rabbits were given an intradermal dose of 1 mg of the colicine dissolved in 0.2 ml of saline, a marked local and systemic reaction occurred within 24 hours. The animals suffered malaise, a marked rise in temperature, and a severe reaction at the site of injection, which was accompanied by edema and an area of central necrosis.

The Immunological Properties of the Colicinogenic Bacilli E. coli K235 and K357 The colicine V–producing bacillus *E. coli* K357 is an exceedingly labile organism from the point of view of its progeny and their antigenic makeup. We have isolated at least six different colonial forms from the strain which was originally sent us and we have ascertained their ability to produce colicine V. Of the six, two were set aside for further study. One of these produced large, mucoid, and opaque colonies when plated on neopeptone agar. This

variant was encapsulated when examined by an India ink stain and was therefore designated as *E. coli* K357 L_+O, because the colonies were opaque (O) and because the bacillus had a thermolabile (L) antigen.

The other colicinogenic variant grew as flat, rough, and translucent colonies, which agglutinated spontaneously in the antiserum of rabbits immunized with the L_+O bacillus. The unencapsulated bacillus was designated *E. coli* K357 L_-T. Both variants elaborated appreciable quantities of colicine V when grown in liquid culture (400 to 800 units/ml). It will be recalled (16) that the colicine K–producing bacillus *E. coli* K235, sent us some years ago by Frédéricq, also yielded a variety of colonial forms.

Our work on colicine K, and now that on colicine V, has revealed that the two highly purified colicines which we have isolated are in all probability

TABLE I

TOXICITY OF COLICINE V IN MICE

Colicine injected, *mg.*			
5.0	2.5	1.25	0.62
D_{24} D_{24} D_{24}	D_{24} D_{24} D_{24}	D_{24} D_{24} D_{48}	S S S

D = death (subscript denotes hours survival after injection of material).
S = survival.

identical with the type-specific O antigens of the respective organisms. Since these substances are both lipocarbohydrate-protein complexes and since they are so similar in chemical properties, it was of interest to ascertain whether the two strains of bacilli themselves might not be serologically related.

For the purpose of this study sera of rabbits immunized with the encapsulated L_+O forms of the two colicine-producing bacilli were used. In addition, the sera of rabbits which had been injected with the two highly purified and electrophoretically homogeneous colicines themselves were also studied. The results of the agglutination tests in the antibacterial sera are given in Table II. It may be seen that, at the dilutions tested, the encapsulated colicine V–producing bacillus failed to agglutinate in its homologous antiserum, as one might expect. Only after the microorganism had been heated at 100°C for an hour in order to destroy its capsular or L antigen, did it indeed agglutinate. This agglutination is, of course, due to the interaction of the type-specific O antibody and the bacterial O antigen, which is revealed only after the thermolabile L antigen is removed by the boiling process. The non-encapsulated L_-T variant, however, agglutinated spontaneously. It should be noted that neither the unheated nor the heated colicine K–producing bacillus *E. coli* K235 L_+O agglutinated in the immune serum elicited by the colicine V microorganism.

A reciprocal relationship is seen to exist with respect to the colicine K–producing bacillus *E. coli* K235 L_+O and its antiserum. Here the microorganism showed some slight agglutination in homologous antiserum due to the interaction of the capsular L antigen and its antibody. After heating, the organism agglutinated readily. There is no cross-reaction, however, of the colicine V–producing organism in the K antiserum. Thus, a study of the agglutination reactions of these two colicine-producing bacilli indicates

TABLE II

AGGLUTINATION OF *E. COLI* K357 L_+O AND L_-T (COLICINE V) AND OF *E. COLI* K235 L_+O (COLICINE K) IN ANTIBACTERIAL SERA

Serum No.	Animal immunized with *E. Coli*	Test antigen *E. Coli*	Final dilution of antiserum						
			1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
28	K357 L_+O (colicine V)	K357 L_+O	0	0	0	0	0	0	0
		K357 L_+O boiled	4	4	3	2	1	½	0
		K357 L_-T	4	4	4	4	4	1	½
		K235 L_+O	0	0	0	0	0	0	0
		K235 L_+O boiled	0	0	0	0	0	0	0
131	K235 L_+O (colicine K)	K357 L_+O	0	0	0	0	0	0	0
		K357 L_+O boiled	0	0	0	0	0	0	0
		K357 L_-T	0	0	0	0	0	0	0
		K235 L_+O	2	1	0	0	0	0	0
		K235 L_+O boiled	3	4	4	3	2	1	0

E. coli K235 L_+O = encapsulated (L_+) form of the colicine K–producing bacillus.
E. coli K357 L_+O = encapsulated (L_+) form of the colicine V–producing bacillus.
E. coli K357 L_-T = non-encapsulated (L_+) form of the colicine V–producing bacillus.
4 = complete agglutination with clear supernate.
1 = slight agglutination.
0 = no agglutination.

clearly and unequivocally that neither the capsular L nor the somatic O antigens (*i.e.* the colicines) of the two bacteria are serologically related.

If one examines the agglutination reactions of the two colicinogenic bacilli in antisera elicited by the two colicines, it is again clearly evident (Table III) that the antibodies evoked by the two purified preparations are also highly specific. Although colicine V antiserum agglutinates both the heated encapsulated form of *E. coli* K357 and the unencapsulated L_-T variant it is without effect upon the colicine K–producing microorganism, *E. coli* K235. Similarly, the colicine K antiserum agglutinates only the homologous bacillus, and does not cross-react with the heterologous colicine V–producing strain. These experiments reveal that colicine V and colicine K bear no serological relation-

ship to one another and that the two colicines derived from the two different strains of *E. coli* are also serologically unrelated.

Precipitation Reactions of Colicines V and K in Antisera The specificity of the agglutination reactions of the two different strains of colicinogenic bacilli is paralleled by the specificity of precipitation reactions of the two chemically purified colicines V and K. When these substances are tested in their own antisera and in the sera of rabbits immunized with the two colicinogenic bacilli, it will again be seen that there is no serological crossing (Table IV).

TABLE III

AGGLUTINATION OF *E. COLI* K357 L_+O AND L_-T (COLICINE V) AND OF *E. COLI* K235 L_+O (COLICINE K) IN COLICINE ANTISERA

Serum No.	Rabbit immunized with	Test antigen *E. Coli*	Final dilution of antiserum 1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
14	Colicine V	K357 L_+O	0	0	0	0	0	0	0
		K357 L_+O boiled	4	3	3	3	3	2	1
		K357 L_-T	4	4	4	4	4	2	1
		K235 L_+O	0	0	0	0	0	0	0
		K235 L_+O boiled	0	0	0	0	0	0	0
17	Colicine K	K357 L_+O	0	0	0	0	0	0	0
		K357 L_+O boiled	0	0	0	0	0	0	0
		K357 L_-T	0	0	0	0	0	0	0
		K235 L_+O	0	0	0	0	0	0	0
		K235 L_+O boiled	3	4	4	3	2	1	0

From the table it is evident that colicine V precipitates only in the antiserum of rabbits immunized with the colicine V–producing bacillus or with the purified colicine V itself. It does not cross-react in the heterologous K antisera. Similarly, colicine K reacts only in those antisera in which the colicine K–producing organism served as the antigen or in which colicine K itself was used as the immunizing agent. Thus it may be concluded that colicine K and colicine V are immunologically unrelated as are the two strains of bacilli from which the colicines were derived. When the two colicines were tested by the agar diffusion technique using antibacterial sera (Fig. 3), it could be seen that each colicine produced but a single band and reacted only with its homologous antibody. Here again are no discernible cross-reactions.

Neutralization of Colicines V and K by Antiserum Our previous work has shown that the sera of animals immunized with purified colicine K contain

antibodies which both precipitate and neutralize the antibacterial properties of the colicine (4). In order to determine whether antisera to colicine V behave similarly, or whether the two different colicine antisera neutralize the activity of the heterologous colicine, the following experiment was performed.

0.3 ml of colicine K antiserum was added to a tube containing 2.5 ml of nutrient broth and 0.5 per cent agar at 50°C. To a second tube was added 0.3 ml of colicine V antiserum and to a third control tube was added 0.3 ml of

TABLE IV

PRECIPITATION REACTIONS OF COLICINES V AND K IN ANTIBACTERIAL SERA AND IN COLICINE ANTISERA

Serum No.	Rabbit immunized with	Test antigen	Final serum dilution 1:2,000	1:10,000	1:50,000	1:250,000
28	*E. coli* K357 L_+O	Colicine V	++++	+++	++	+
		Colicine K	0	0	0	0
14	Colicine V	Colicine V	++++	++++	++±	+
		Colicine K	0	0	0	0
131	*E. coli* K235 L_+O	Colicine V	0	0	0	0
		Colicine K	++±	+++	+++	+
17	Colicine K	Colicine V	0	0	0	0
		Colicine K	++	+++	++	+

++++ = heavy disk-like precipitate.
+ = slight precipitate.
0 = no precipitate.

sterile saline. The tubes were now seeded with 5×10^7 bacilli of a culture of *E. coli* B. Three similar tubes were prepared and seeded with 5×10^7 bacilli of a culture of *E. coli* φ. The six tubes were now poured upon nutrient broth-agar (1.5 per cent) plates. After standing at 4°C for 15 minutes the first three plates were spotted with 0.02 ml samples of solutions of decreasing concentrations of colicine K starting at 20 γ/ml. The remaining three plates, those seeded with *E. coli* φ, were spotted with solutions of decreasing concentrations of colicine V. After standing for 30 minutes at room temperature, the plates were incubated at 37°C for 6 hours. They were then removed and observed.

The results of this experiment are presented in their entirety in Table V. A photograph of this experiment, without the control plates, is also presented in Fig. 4. From the table it will be seen that the inhibition of growth of the test organism *E. coli* B by colicine K is marked indeed (control tube) and that

0.02 ml of a solution containing 1.25 γ still caused complete inhibition of growth. On the plate on which colicine K antiserum had been incorporated there was no inhibitory action of the colicine (Table V and Fig. 4A). The latter had been completely neutralized by the antiserum and hence there was uniform growth of the test organism over the entire area of the plate. The plate in which colicine V antiserum had been incorporated, however, showed marked inhibition of growth of the test organism by the colicine K (Fig. 4B). This fact indicates that colicine V antiserum was without effect upon colicine K; *i.e.*, the colicine is not neutralized by the heterologous colicine V antiserum.

An identical pattern was observed when colicine V was tested on plates

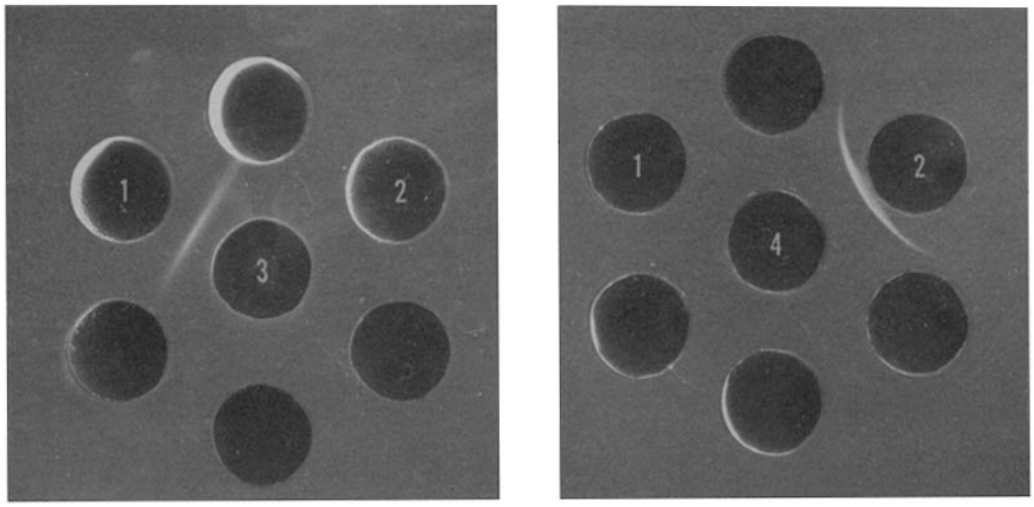

FIGURE 3. Agar diffusion reactions of colicines V and K. 1, colicine K 1 mg/ml. 2, colicine V 1 mg/ml. 3, antiserum to colicine K–producing organism *E. coli* K235 L_+O. 4, antiserum to colicine V–producing organism *E. coli* K357 L_+O.

seeded with *E. coli* φ. The colicine itself (control tube) inhibited the growth of the test bacillus again at a concentration of 1.25 γ/ml (Table V). Colicine V antiserum, on the other hand, completely neutralized the antibacterial activity of the colicine (Fig. 4C), whereas colicine K antiserum was without effect (Fig. 4D). This experiment reveals the fact that the antisera to colicine V and to colicine K both contain neutralizing antibodies. These antibodies are highly specific, however, and they neutralize only the homologous colicine. A photograph of this experiment, not including control plates, is presented in Fig. 4.

DISCUSSION

At the time we began our work on colicine V we were fortunate in procuring from Dr. Frédéricq some seven different colicinogenic strains of *Escherichia*

coli, all of which elaborated the colicine in which we were interested. In addition, we had on hand the original colicine V–producing strain of Gratia, sent us some years ago by Dr. Frédéricq. All these strains were carefully screened for their ability to elaborate colicine V in the culture medium used in these studies, but only one strain, *E. coli* K357, did so in any promising quantity. From this bacillus were obtained six variants which differed in colonial form and of these, two elaborated some 400 to 800 units of colicine V when grown under rigorously controlled conditions of pH and in the medium described. The L_T variant was selected for study rather than

TABLE V

NEUTRALIZATION OF ANTIBACTERIAL ACTIVITY OF COLICINES V AND K BY HOMOLOGOUS ANTISERA

Plate No.	Antibacterial serum added	Colicine tested	Colicine used in test, γ					
			20	10	5	2.5	1.25	0.6
1	Colicine K		0	0	0	0	0	0
2	Colicine V	Colicine K	4	4	4	4	4	2½
3	None (control)		4	4	4	4	4	2
4	Colicine V		0	0	0	0	0	0
5	Colicine K	Colicine V	4	4	4	4	4	3
6	None (control)		4	4	4	4	4	2½

Plates 1, 2, 3 seeded with test organism *E. coli* B.
Plates 4, 5, 6 seeded with test organism *E. coli* ϕ.
4 = complete inhibition of growth of test organism.
2 = partial inhibition of growth of test organism.
0 = no inhibition of growth of test organism indicating complete neutralization of colicine activity.

the L_+O bacillus, largely because the use of this strain would obviate the necessity of separating any L antigen from the colicine during the process of purification. That this was a happy choice is obvious, because the ultimate isolation of a highly purified preparation of colicine V presented no untoward difficulty. The product which was eventually obtained exhibited both electrophoretic and serological homogeneity. Its chemical analysis, which resembled that of colicine K, characterized the substance unequivocally as a lipocarbohydrate-protein complex which exhibited toxicity both for mammals and for bacteria. Our studies on colicine V have not yet included an investigation of the components of the complex. Yet there is little reason to believe that such a study would not reveal that this O antigen, like others of the *Enterobacteriaceae*, would yield its so-called protein and lipocarbohydrate constituents upon dissociation with phenol.

Colicine V is a potent antigen as our investigations have shown, for not

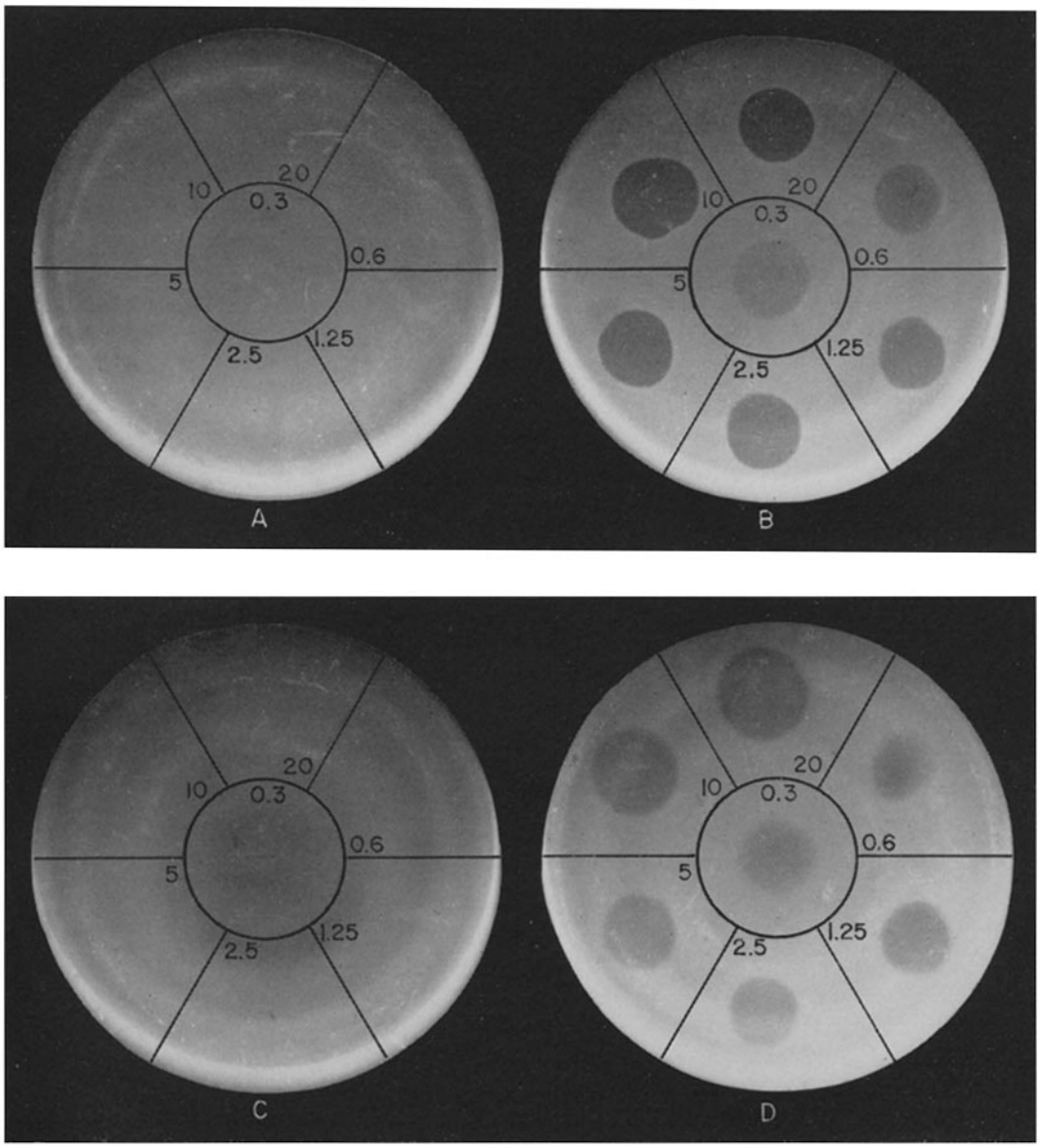

FIGURE 4. Inhibition of antibacterial activity of colicines V and K by homologous antisera. A and B, plates seeded with *E. coli* B and spotted with colicine K. C and D, plates seeded with *E. coli* ϕ and spotted with colicine V.

The soft agar of plates A and D contains 0.3 ml of colicine K antibacterial serum. The soft agar of plates B and C contains 0.3 ml of colicine V antibacterial serum. Note that the activity of the two colicines on their respective hosts is inhibited only by the homologous antibacterial serum.

only does it give rise to specific bacterial agglutinins and precipitins in the sera of immunized rabbits, but, like colicine K, it also stimulates antibodies which neutralize the antibacterial properties of the colicine. We find it of considerable interest that the immune sera elicited by both the colicinogenic bacillus *E. coli* K357 and the purified colicine itself show no serological cross-reactions with the microorganism from which colicine K is obtained, or with the purified colicine K.

There is one observation which we have not been able to reconcile with those of earlier investigators, namely the fact that colicine V was found by them to be readily diffusible through semipermeable membranes and we did not. We have no evidence whatsoever that the colicine V which we have prepared will dialyze through cellophane membranes, manufactured by the Visking Co. (Chicago), be they "seamless cellulose tubing, thin walled," or for "dialysis." It is not impossible that the porosity of the cellophane which was used in Europe two or three decades ago differed from that of the cellophane which is manufactured today. All this is unfortunately a discrepancy for which we have no explanation. The fact remains that colicine V is now the third of the colicines to be well characterized, and, like colicine K and the colicine of Nüske, colicine V appears to be a high molecular weight lipocarbohydrate-protein complex which is identical with the O antigen of the bacillus from which it was obtained.

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