COLICINE K

II. The Preparation and Properties of a Substance Having Colicine K Activity

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(Received for publication, September 27, 1957)

Bacteriocines are substances of unknown nature which are elaborated by certain bacteria and which have the ability to kill specifically and selectively still other strains of microorganisms. The evidence which links bacteriocines with the bacteriophages is persuasive indeed, so much so that a number of investigators have, to all intent, accepted as fact that the bacteriocines must be regarded as precursors of bacterial viruses or as being intimately related to them (1). The rationale is ingenious. For example, since those agents which induce phage formation in lysogenic bacilli also bring about liberation of colicine from colicinogenic microorganisms, phage and colicine must be related (2). Or, since phage-resistant variants of some enteric bacilli are frequently colicine-resistant, phage and colicine must share the same host receptor site and hence must bear some structural relationship to one another (3). These and other reasons have served to link bacteriocines with phage. Persuasive as these arguments may be, one should not lose sight of the fact that they are presumptive; no one has yet obtained a purified colicine nor has it been possible to compare its chemical, physical, and immunological properties with those of purified phage. Because the matter is of some moment, this laboratory has concerned itself during the past three years with the problem in question.

Materials and Methods

Strains of Microorganisms.—The original colicine K—producing strain of *Escherichia coli* known as K_{235}, was kindly sent us by Dr. Pierre Fredericq of the University of Liége. Three variants of this microorganism have since been obtained in this laboratory and were described in a previous publication (4). In this study the variant known as *E. coli* K_{235} L_+0 was used for the production of colicine K.

Culture Medium.—150 gm. of Difco technical casamino acids was placed in cellophane tubing and dialyzed for 3 days against 16 liters of tap water at 4°C. To 15 liters of the dialysate was added 18 gm. of Na_2HPO_4 and 7.5 gm. of KH_2PO_4. The pH of the solution was adjusted to 7.0 by the addition of approximately 5 ml. of 1 N NaOH. The solution was boiled for 15 minutes, filtered, and autoclaved in a 5 gallon pyrex bottle for 1 hour at 10 pounds' pressure. Prior to seeding with *E. coli* K_{235}, 450 ml. of a sterile solution of 50 per cent glucose was added as well as the filtered, sterile, dialysate of 75 gm. of Difco yeast extract.

Antisera.—Antisera to purified colicine K and to enzymatically degraded colicine were obtained as follows. Sterile solutions of the two materials were prepared by wetting a weighed sample of each in a sterile test tube with a 0.5 ml. of 70 per cent ethanol and dissolving them in sterile 0.9 per cent saline. Two groups of rabbits were injected intradermally with 0.2 ml. of the solution containing 5.0 mg. per ml. One week later each group received a course of intravenous injections given on alternate days of 100, 200, and 500 μg. of each antigen respectively. After a week's rest a second course of injections was given in amounts of 0.5 and 1 mg. of antigen which was followed 1 week later by a final course of injections in similar amount. The animals were finally bled 1 week after the last injection.

Colicine Assay.—The determination of the colicine activity of the various fractions studied was made by means of the ring test described in Paper I of this series (4). The activity of a given fraction is expressed as units per milligram. One unit of colicine K activity is that amount

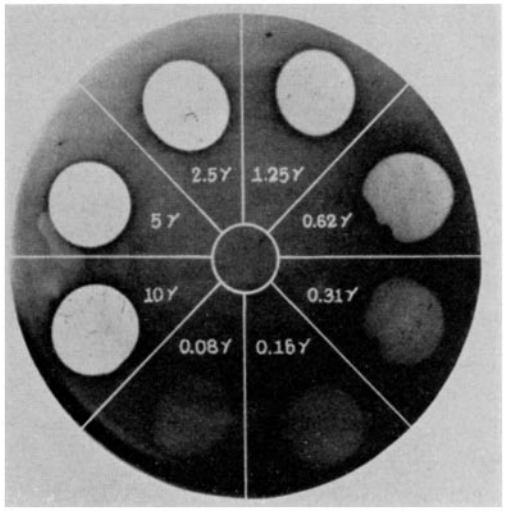

FIG. 1. Colicine K ring test.

of substance per milliliter which gives complete inhibition of growth of *E. coli* B (4). A typical test is shown in Fig. 1.

Electrophoresis.—Electrophoretic analyses of colicine K fractions were carried out in borate buffer of pH 9.2 and 0.05 ionic strength at 0.4°C. in the Tiselius apparatus using the schlieren scanning method (5). 7.0 volt/cm.[2] was employed.[1] Mobilities were calculated from the descending patterns by the method of Tiselius and Kabat (6).

Analytical Methods.—Throughout this investigation continued recourse was made to various analytical procedures in order to determine the chemical constants of the various materials obtained. Nitrogen analyses were performed by the method of Koch and McMeekin (7), phosphorus by the procedure of Allen (8), and protein analyses by the modified procedure of Folin and Ciocalteu as described in reference 9. The absorption spectra of the various materials studied were measured in a model DU Beckman spectrophotometer. Turbidimetric quantitative precipitin reactions were performed by the technique of Libby (10) and precip-

[1] The electrophoretic analyses reported in this communication were performed by Mrs. Elizabeth Miller Smidth. The authors wish to acknowledge her assistance in this important aspect of the work.

itable antibody nitrogen by the method of Kabat and Bezer (11). The percentage of lipides was determined by weighing chloroform-soluble material after hydrolysis for 6 hours at 100°C in 1 N HCl. Glucosamine was determined by the method of Sørensen (12).

The carbohydrate content of the various fractions under investigation was determined as follows: 1 ml. samples of an aqueous solution of substance containing approximately 500 μg. were placed in test tubes. 0.2 ml. of a freshly prepared solution of recrystallized anthrone (2.5 per cent) dissolved in ethylacetate, was added. 2.5 ml. of concentrated H_2SO_4 was next added, the tubes were shaken, and heated at 100°C. for 3 minutes. The absorbance of the color which developed was read in a Beckman spectrophotometer at 620 mμ. The per cent carbohydrate was calculated, using a reference curve in which glucose served as standard. The values for the carbohydrate content of the various fractions are expressed in terms of per cent glucose.

Zone Electrophoreses.—Purified preparations of colicine K were studied by means of zone electrophoresis using polyvinylchloride as the supporting medium. These experiments were performed at several pH values with solutions of the material in 0.05 M borate, and 0.1 M acetate buffers. The procedure described by Kunkel was employed (13).

EXPERIMENTAL

Preparation of a Fraction from E. coli K_{235} Having Colicine K Activity.—15 liters of 1 per cent dialyzed casamino acid medium containing 1.5 per cent glucose and the dialysate from 75 gm. of Difco yeast extract was seeded with 5×10^4 cells of *E. coli* B K_{235} L_+0, growing in the logarithmic phase. The medium was at 37°C. and at pH 7.0. It was aerated (4 liters per minute) and maintained at pH 7.0 ± 0.05 by an electronic device which has been previously described (4). 17½ hours later growth of the organism ceased abruptly. The culture was then treated with 100 ml. of chloroform and the mixture stirred for 5 minutes. 35 ml. of glacial acetic acid was added to adjust the pH to approximately 5.0 and the bacilli were removed by centrifugation. The supernate, containing as a rule 1600 to 6400 units of colicine K per ml., was concentrated *in vacuo* to 1 liter.

The manner in which the distillation is performed is important. If the evaporation is too slow or if the temperature of the water bath is too high there is considerable loss of colicine activity. The distillation was therefore carried out in a 22 liter flask fitted with a multitubed copper condenser and a 12 liter receiving flask. The flask was heated in a water bath maintained at 73–75°C. and tap water at 8°C. was run through the condenser at a rapid rate. The vacuum was maintained at 11 to 12 mm. The distillation proceeded at an average rate of 3 l. per hour and the temperature of the solution within the distilling flask never exceeded 20°C.

The concentrated medium containing the colicine was now dialyzed against distilled water for 24 hours at 4°C. in cellophane tubing. The solution, at a volume of 4 liters, was reconcentrated *in vacuo*, filtered through a Berkefeld candle, and redialyzed at 4°C. for 24 hours. The solution was finally concentrated *in vacuo* to 300 ml. and then dried from the frozen state. The residue (6.5 gm.), containing only non-dialyzable products of bacterial origin, had a high colicine activity, approximately 500 U/mg. The end product was pale yellow in color and was readily soluble in water. This material will be referred to as "crude colicine."

Further purification was accomplished by precipitating the material at 0°C. and at low ionic strength with ethanol. 6.5 gm. of substance was dissolved in 0.02 M sodium acetate solution (300 ml.) at 0°C. 900 ml. of absolute ethanol at −10°C. was slowly added with stirring. After 1 hour at −10°C. the precipitate, containing all the colicine activity, was centrifuged off at −10°C. The pigmented supernate was discarded and the precipitate was dissolved in 200 ml. of cold water (4°C.). The solution was then dialyzed at 4°C. for 24 hours. The material was isolated by freeze drying. 5.1 gm. was recovered.

Properties of the Fraction Containing Colicine K.—Crude colicine K is a fluffy amorphous product which gave a highly viscous yellowish brown solu-

tion. Neither prolonged dialysis nor repeated precipitation with alcohol eliminated the pigment. The substance contained 4.8 per cent of nitrogen and 1.5 per cent phosphorus. It gave strong positive biuret and anthrone tests, as well as a test for glucosamine and a positive test with Ehrlich's reagent. The substance was serologically active and precipitated at concentrations as low as 1 μg./ml. in the sera of animals which had been immunized with *E. coli* $K_{235}L_4O$ or with purified colicine K itself. The most remarkable property of this substance, however, was its ability to inhibit the growth of *E. coli* B. Thus, the material after alcoholic precipitation contained approximately 750 U/ml. Although alcohol precipitation eliminated some 20 per cent of inert substance the active material was not a single entity, as will be seen from that which follows.

Further Purification of Colicine K.—5.1 gm. of alcohol-precipitated colicine K was dissolved in 300 ml. of 0.01 molar phosphate buffer at pH 5.6. 197.1 gm. of ammonium sulfate was added to the solution in order to bring the concentration to 90 per cent saturation. After standing for an hour at room temperature the precipitate, containing the serologically active type-specific lipocarbohydrate-protein complex together with all colicine activity, precipitated and was separated by centrifugation. The supernate, containing colominic acid, (14) was set aside. The precipitate was now redissolved in water and was twice reprecipitated with ammonium sulfate at 90 per cent saturation. The solution was finally dialyzed at 4°C. until free of sulfate ion, concentrated *in vacuo* and then dried from the frozen state. 3.5 gm. of material was recovered.

This substance, which contained practically all of the original colicine activity, had essentially the same nitrogen content as did crude colicine. As a rule the fraction isolated at this step contained 4.5 to 5.2 per cent nitrogen, and 1.2–1.5 per cent phosphorus. It had an activity of approximately 1,000 U/mg. Precipitation with ammonium sulfate was an important step in the purification of the colicine because it separated the latter from colominic acid. The colicine-containing fraction, however, still contained small amounts of colominic acid as well as a serologically active polysaccharide which was separated as follows.

The substance (3.5 gm.) was dissolved in 200 ml. of 0.02 M acetate buffer at pH 5.0 and cooled to 0°C. The solution was emulsified at 0°C. in a Waring blender with 75 ml. of chloroform–octyl alcohol mixture (3:1) at 1400 R.P.M for 15 minutes. After 1 hour of centrifugation (32,000 *g* and at 4°C.) the supernate which separated from the chloroform-octyl alcohol emulsion layer was drawn off. The supernate was emulsified twice more with chloroform–octyl alcohol and separated as before. The combined emulsion cakes were washed twice with 35 ml. portions of the acetate buffer. The washings were emulsified with chloroform–octyl alcohol mixture and, after centrifugation, the emulsion cake was combined with the main lot.

The combined washed emulsion cakes, containing the major portion of the colicine, were next treated with 10 volumes of absolute ethanol at 0°C. The colicine-containing precipitate was centrifuged and washed with cold ethanol several times. The precipitate was dissolved in water, and dialyzed for 18 hours at 4°C. The material was finally centrifuged at high speed to remove inert particulate matter and then freeze-dried. 2.5 gm. of substance was obtained, the

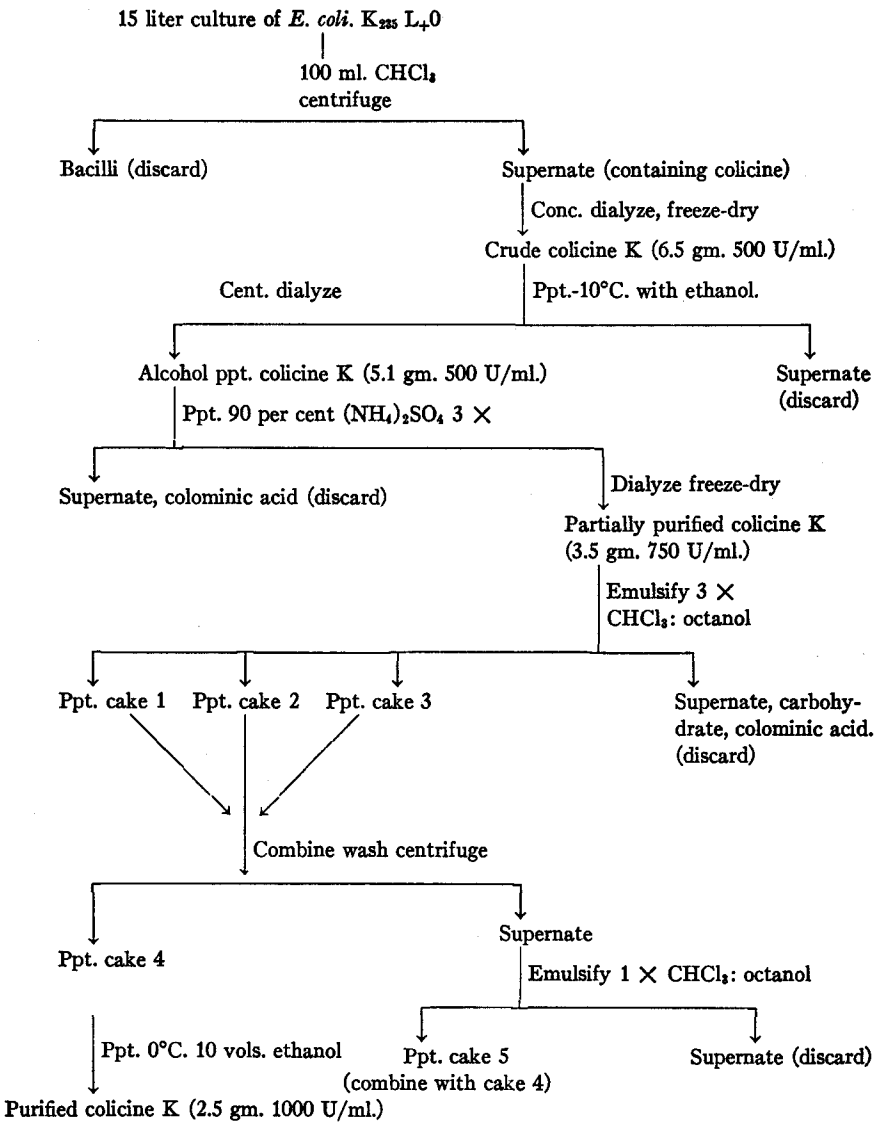

FIG. 2. Fractionation procedure for purification of colicine K.

activity of which was approximately 1000 U/mg. This material will be referred to as purified colicine K. A summary of the fractionation procedure is given in Fig. 2.

It should be stated that there were occasions when, despite all precautions, the biological activity of the colicine was ephemeral,—and for reasons not understood. In such instances, which fortunately were infrequent, the product

obtained had a chemical analysis identical with that of a fully active preparation.

Properties of Purified Colicine K.—Purified colicine K is a yellowish water-soluble amorphous product which yields somewhat pigmented viscous solutions when dissolved in water. Different preparations contain 4.8 to 5.2 per cent nitrogen, 1.2 to 1.5 per cent phosphorus, and yield both lipide and reducing sugar on acid hydrolysis. They give a strong positive biuret test as well as tests for certain amino acids. Purified colicine K is not precipitated from solution by the salts of heavy metals or by trichloracetic or picric acids. The substance has marked toxic properties when injected into rabbits and it is a potent antigen. In short, the material is typical of many of the antigenic and toxic lipocarbohydrate-protein complexes, or so called O antigens derived from Gram-negative bacilli which have been studied in this and other laboratories (15). It differs, however, in one important aspect. It has the capacity to kill certain other enteric bacilli and in particular the test strain used in these studies, *E. coli* B. Whether the colicine activity of this material is an inherent property of the O antigen of *E. coli* K_{235}, or whether it is a substance which accompanies the O antigen and is merely difficult to separate, was the question now faced and toward which subsequent experimental work was directed.

Purified colicine K is remarkably stable over a wide range of pH values. Solutions of the material between pH 2.8 and 9.6 show no loss in activity on standing at room temperature for several hours. Prolonged standing, however, results in a gradual diminution in activity. Solutions of colicine K can be boiled for 10 minutes at neutral or near neutral pH without demonstrable loss in activity. Upon boiling for 20 minutes, however, some 80 per cent of the activity is lost and prolonged heating completely destroys activity. The activity is also destroyed by formaldehyde. Thus, a solution of colicine K containing 1 per cent formalin loses some 90 per cent of its antibacterial activity within 2½ hours. The substance is remarkably stable, however, in urea solution. A 0.1 per cent solution in 7 M urea at 37°C. loses no activity whatsoever within 6 hours, and only 50 per cent is lost within 27 hours. Activity is readily destroyed, however, by proteolytic enzymes. At pH 8.0 both crystalline trypsin and chymotrypsin bring about complete inactivation of the antibacterial activity of colicine K within a few hours. Likewise, pepsin at pH 2.4 and ficin at pH 7.0 destroy the activity.

Electrophoretic Properties of Fractions Obtained during the Purification of Colicine K.—It was shown that crude colicine K could be separated from the culture medium of *E. coli* K_{235} after dialysis by fractional precipitation with alcohol and that the material could be partially purified by precipitation with ammonium sulfate. The substance was then obtained free of colominic acid

(14) and a serologically active carbohydrate by shaking with octyl alcohol–chloroform.

Electrophoretic analyses were made of the various fractions obtained during the purification procedure in 0.1 molar sodium borate at pH 9.2. The patterns which were observed are shown in Fig. 3, and the mobilities of the various components are listed in Table I. From Fig. 3a it will be seen that alcohol-precipitated colicine K contained at least two components and that a fast moving component predominated. When the active component was precipitated with 90 per cent ammonium sulfate, the material recovered after dialysis and freeze-drying showed, upon electrophoresis, a marked increase in the amount of fast moving component (Fig. 3 b). The material recovered from the ammonium sulfate supernate (Fig. 3 d) had a mobility almost identical with that of colominic acid (Fig. 3 e) and it will be shown in a later communication that this fraction is indeed rich in this material. When a solution of the partially purified (ammonium sulfate–precipitated) colicine was emulsified with chloroform–octyl alcohol the substance recovered from the emulsion layer, containing all of the colicine activity, showed one major slow moving component and a very small quantity of the fast moving constituent (Fig. 3 c). The latter was also believed to be a contaminant of colominic acid.

Although the electrophoresis patterns are not shown, it was observed that further precipitation of purified colicine K with ammonium sulfate and then with alcohol failed to alter materially the electrophoretic pattern of the colicine-containing fraction. The very small amount of colominic acid which accompanied the colicine could not be eliminated by these procedures. It is of considerable interest that even after 3½ hours of electrophoresis the pattern of the colicine-containing material still showed but a single though somewhat asymmetric peak. Finally it should be stated that upon electrophoresis of purified colicine K in veronal buffer at pH 8.6 or in acetate buffer at pH 4.5, only a single boundary was formed.

The results of the electrophoresis experiments clearly indicate that colicine K activity is intimately and tenaciously associated with the slow moving lipocarbohydrate-protein component. On the basis of these experiments one must conclude either that colicine K activity is an inherent property of the O antigen of *E. coli* K_{235} or that the antigenic complex and the colicine are a mixture comprised of two components having very similar electrophoretic mobilities.

Zone Electrophoresis of Colicine K.—Although it was not possible to separate a colicine-active component from the lipocarbohydrate-protein complex of *E. coli* K_{235} by free electrophoresis, attempts were made to achieve this by zone electrophoresis. Preliminary experiments, using starch and cellulose as the supporting media, revealed that neither was satisfactory. Polyvinylchloride, however, served as an excellent medium. The electrophoresis was carried out in

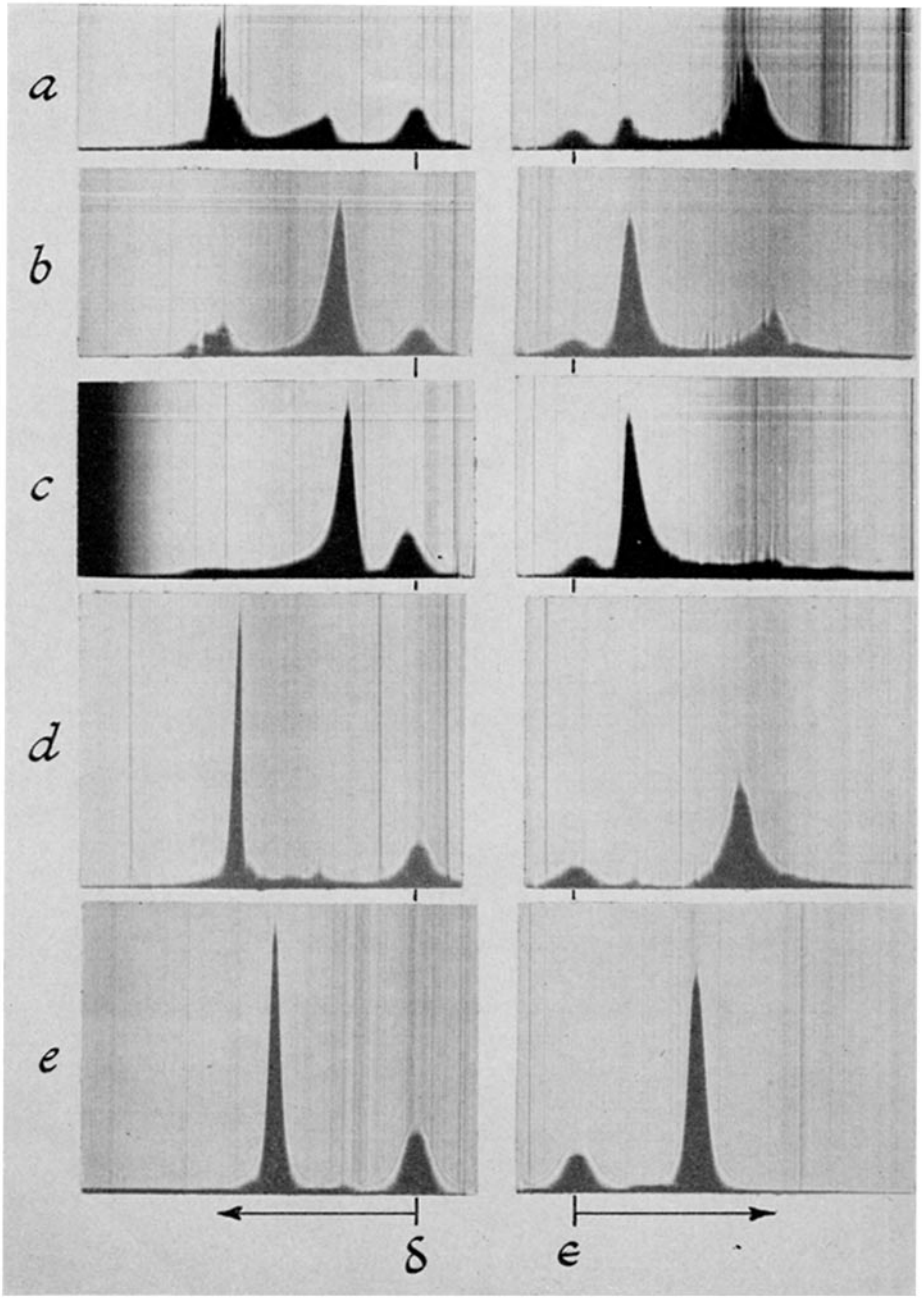

FIG. 3. Electrophoretic patterns of fractions obtained during purification of colicine K. (*a*) Ethanol-precipitated colicine K (3200 seconds). (*b*) $(NH_4)_2SO_4$ precipitated colicine K (3600 seconds). (*c*) Purified colicine K (3600 seconds). (*d*) Material in $(NH_4)_2SO_4$ supernate (3000 seconds). (*e*) Colominic acid (2400 seconds).

0.05 M borate buffer at pH 9.2 and in 0.1 M acetate buffer at pH 4.6. In both instances the fractions obtained by elution of the polyvinyl segments were analyzed for carbohydrate, for protein, and for colicine activity. The experimental procedure was as follows:—

300 gm. of polyvinylchloride (B. F. Goodrich Co., New York, "geon" type resin 400 × 65) was suspended in distilled water, filtered by suction, and repeatedly washed. The resin was then washed three times with 500 ml. portion of 0.05 M sodium borate (pH 9.2) and was finally suspended in enough of the buffer to give a thick cream. A box-like mold was constructed having a glass plate 35 × 10 cm. as a base and two removable lucite strips as sides. The latter were the same length as the plate and extended 1 cm. above it. The open-ended box was now lined with a sheet of thin polyethylene, 35 × 26 cm., and the ends of the box were closed with pieces of folded photographic blotting paper. The slurry was now poured into the box, and after the excess fluid had been absorbed by the blotting paper, the end and side pieces of the

TABLE I

Electrophoretic Mobilities of Fractions Obtained during Purification of Colicine K

Fraction tested	Mobility × 10^{-5} cm.²/volt sec.	
	Slow component	Fast component
Ethanol ppt. colicine K	−4.03	−12.4
$(NH_4)_2SO_4$ ppt. colicine K	−3.37	−11.5
Purified colicine K	−3.82	−11.9*
Trypsin degraded colicine K	−3.34	−12.2*
Colominic acid	—	−11.2

* This fraction comprised but a very small per cent of the total material and its mobility is approximate.

mold were removed. The block was moistened with a few milliliters of buffer, and a slit 7 cm. long was cut crosswise in the center of the block. A solution of colicine (15 mg. in 2 ml. of buffer) was now carefully pipetted into the slit and the latter was closed by applying gentle pressure on either side. The block was covered with the two flaps of polyethylene, after first placing cotton cloth wicks, (9 × 12 cm.) saturated with buffer and extending 2 cm. upon the polyvinyl resin. The ends of the plate were rested on the rim of two glass vessels each filled with 1 liter of buffer. The wicks were left dangling in the buffer. The two vessels supporting the block were bridged by means of U tubes 2 cm. in diameter to two similar vessels, one at either end, and filled with the same buffer. Two platinum foil electrodes, 2 cm. square, were then placed in the two outer vessels. The latter were connected by a siphon, previously filled with buffer, in order to eliminate any inequality in hydrostatic pressure.

A current of 15 volts/cm. was applied for 12 hours while maintaining the experiment at 4°C. The block was now removed and cut into 12 half-inch segments beginning ¼ inch on either side of the origin. The segments were transferred one at a time to a sintered glass funnel, sucked dry, and washed with 3 ml. of buffer. The 25 filtrates were analyzed for carbohydrate, protein, and for biological activity. An identical experiment was also performed using acetate buffer of the same ionic strength at pH 4.6. The results of both experiments are presented in Fig. 4.

It is apparent from the results of these two experiments that the material under examination migrated as a cation at pH 9.2 a distance of approximately 5 cm. and at pH 4.6 about 1.5 cm. It will be recalled that this same substance, by free electrophoresis, migrated as an anion. In the present experiments its migration toward the cathode must be attributed to electroendosmotic flow. The experiments revealed that, regardless of pH, only one peak was observed. Analyses of the eluates showed that no separation of substances having differences in carbohydrate and protein content could be effected and that biological

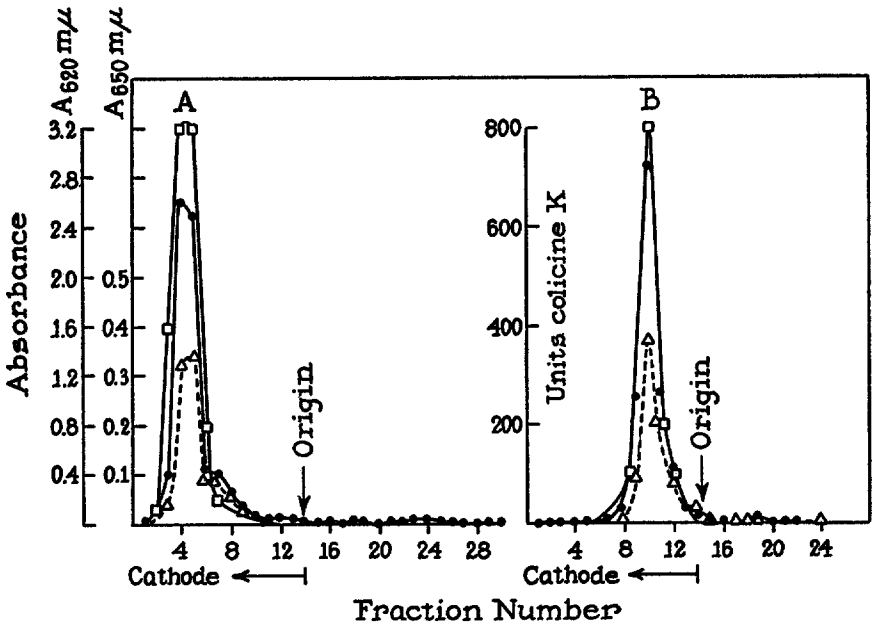

FIG. 4. Zone electrophoresis of purified colicine K. □ Colicine K activity U/ml. ● Total absorbance of anthrone-reactive material in eluate (λ=620). △ Total absorbance of Folin reactive material in eluate (λ = 650).

activity remained associated with the substance responsible for the electrophoretic peak,—the lipocarbohydrate-protein complex.

Degradation of Colicine K by Trypsin.—Early in the course of this investigation it was observed that the antibacterial activity of colicine K could be destroyed by digestion with crystalline trypsin. Tests revealed that the colicine activity was lost within the first 3 hours, but digestion and dialysis were permitted to continue for the interval described. The non-dialyzable material was recovered after dialysis. This substance, a partially degraded lipocarbohydrate-protein complex, was still antigenic in rabbits and still functioned as a potent precipitinogen. It reacted specifically in antisera evoked by *E. coli* K_{235} and in sera elicited by the undegraded colicine K complex itself.

200 mg. of purified colicine K was dissolved in 20 ml. of 0.02 M phosphate buffer at pH 8.0. 1 mg. of crystalline trypsin was added to the solution together with a few drops of chloroform. The material was dialyzed in a cellophane bag for 18 hours at 37°C. against 1 liter of the same buffer, saturated with toluene. Dialysis was continued against distilled water for 48 hours and the non-dialyzable residue was finally isolated by freeze-drying. 190 mg. was recovered.

The undigested original colicine K had a specific activity of 1000 U/mg. whereas the digested material was completely inactive. The enzymatically degraded colicine contained 3.8 per cent N, the intact substance 4.5 per cent N. Thus, some 15 per cent of the nitrogen of the parent complex was rendered dialyzable by treatment with crystalline trypsin. The diffusible nitrogenous components are presumably amino acids or peptides derived in part, at least, from the protein portion of the antigenic complex. The properties of the enzymatically degraded colicine K were for the most part similar to those of enzy-

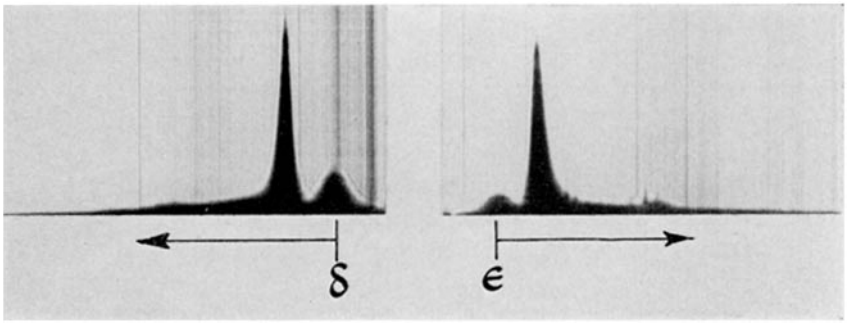

FIG. 5. Electrophoretic patterns of trypsin degraded colicine K (2800 seconds).

matically degraded O antigens derived from the Flexner group of Gram-negative organisms (15) and will not be elaborated upon here.

When a 1 per cent solution of the material was subjected to electrophoretic analysis in 0.1 M sodium borate at pH 9.2, the pattern observed was very similar to that of untreated colicine. A single slightly asymmetric peak was obtained (Fig. 5). It is noteworthy that the mobility of the degraded colicine preparation coincided closely with that of the undegraded material.

The enzymatic degradation of colicine K resulted not only in a loss of antibacterial properties but destroyed for the most part, but not entirely, its capacity to incite specific colicine K neutralizing antibodies. This phenomenon will be dealt with more fully in a later communication. In conclusion, it should be pointed out that although the enzyme brought about a complete loss in the capacity of the colicine to inhibit growth of *E. coli* B it scarcely affected its ability to precipitate in the antiserum of a rabbit immunized with purified undegraded colicine K.

Dissociation of Colicine K with Phenol.—Morgan (16) first described the dissociation of lipocarbohydrate-protein complexes derived from Gram-negative

bacilli by means of liquid phenol. This procedure subsequently proved effective for the breakdown of a number of antigenic lipocarbohydrate-protein complexes derived from a variety of Gram-negative bacilli (15). It was found that phenol could also be used to dissociate the lipocarbohydrate-protein complex of *E. coli* K_{235} to yield a protein-like constituent bearing the colicine K activity.

3.0 gm. of purified colicine K was stirred for 1 hour at room temperature with 100 ml. of 90 per cent phenol. A small amount of insoluble material (0.12 gm.) was removed by centrifugation and discarded, and the supernate was dialyzed free of phenol at 4°C. A precipitate (Fr. 1) separated which was removed by centrifugation. It was washed with water, resuspended, and freeze-dried. The supernate from the dialysis (Fr. 2), containing the lipocarbohydrate component, was concentrated *in vacuo* and freeze-dried. Fr. 1 yielded 0.31 gm. of material. Fr. 2 yielded 2.5 gm. of a water-soluble substance.

The original undissociated complex contained 4.85 per cent nitrogen. Fr. 1 contained 13.0 per cent nitrogen and gave tests typical of a protein. Fr. 2 contained 2.16 per cent nitrogen and gave tests typical of a lipocarbohydrate.

Properties of the Protein and Lipocarbohydrate Components.—Fr. 1, the protein component, is a water-insoluble yellow amorphous granular powder. It dissolves, however, in 50 per cent acetic acid, and in dilute Na_2CO_3 and NaOH. It is also soluble in 7 M urea, and if such a solution is dialyzed in cellophane against 0.001 M sodium borate at pH 9.2, a clear pale yellow solution remains in the dialysis bag. When the pH of this solution is gradually lowered to 8.5 it becomes slightly opalescent. At pH 6.0 about 25 per cent of the substance can then be centrifuged out. At pH 5.0 about 70 per cent is precipitated, and at pH 4.1 some 90 per cent is precipitated. If the solution is now further acidified the precipitate redissolves, and at pH 1.5 it is completely soluble. Salts of heavy metals, trichloracetic, picric, and phosphotungstic acids all precipitate the material from solution. This substance still contains a small amount of carbohydrate, however, for it gives a weak anthrone test. Because of this and because of its somewhat low nitrogen content (13.0 per cent), Fr. 1 cannot be considered as a pure protein.

Fr. 1 has very potent antibacterial properties, for its activity is approximately 10,000 U/mg. This is some ten times as great as that of the substance from which it was derived. The material gives a precipitin reaction in the sera of rabbits which have been immunized with purified colicine K and in addition it is itself fully antigenic for it evokes both precipitating and colicine K neutralizing antibodies in rabbits.

Fr. 2, the lipocarbohydrate component, is a water-soluble snow white amorphous powder. When the material is boiled with dilute mineral acid it yields reducing sugars, as well as a chloroform-soluble lipide. The lipocarbohydrate component contains a small amount of a protein-like material, as evidenced by the fact that it gives weak positive Folin and biuret tests.

Fr. 2 is also serologically active for it gives a potent precipitin reaction in

colicine K antisera. In addition, it evokes a weak antibody response when injected into rabbits. The immunological properties of these two fractions will be dealt with in a later communication.

In sum, it can be stated that purified colicine K can be dissociated by means of 90 per cent phenol to yield two fractions, one rich in protein and representing 10 per cent by weight of the original complex, possesses potent colicine K activity; the other, rich in lipocarbohydrate, comprises 80 per cent of the complex and is without activity.

Attempts to Purify Colicine K by Counter-Current Distribution.—Because fractional precipitation and zone electrophoresis both failed to bring about separation of colicine K activity from the lipocarbohydrate-protein complex of *E. coli* K_{235}, attempts were made to achieve this by counter-current distribution. Consequently, the distribution constants of purified colicine K were determined in a number of different two phase solvent systems. Their pH was varied between 2 and 10 and a number of different salt and solvent combinations were tested. Colicine K was found to be relatively insoluble in most of the systems and in only a few was the solubility and distribution constant suitable to warrant further investigation. The distribution constants were calculated from the ratio of the amounts of material distributed between equal volumes of each phase. Since colicine K is intimately associated with the lipocarbohydrate-protein complex of *E. coli* K_{235}, the concentration of these two components (*i.e.* protein and carbohydrate) was measured by means of the anthrone and Folin reactions. The biological activity was measured by means of the ring test.

The most promising system for the distribution of colicine K proved to be *n*-propyl alcohol containing 5 per cent cyclohexylamine as the upper phase, and 0.8 M KH_2PO_4 as the lower phase. The pH of this system was 7.2 and its settling time was rapid. The solvent system employed was prepared by mixing 720 ml. of 0.8 M KH_2PO_4, 36 ml. of redistilled cyclohexylamine, and 684 ml. of redistilled *n*-propanol. This mixture gave 1,440 ml. of the two phases of which 204 ml. settled in the lower phase and the remainder in the upper phase.

The disproportionate distribution of the volumes of the two phases made it desirable to analyze them in order that they might be prepared separately. Nitrogen and potassium analyses were performed on aliquots of each phase. Propanol concentration was determined on the distillates of each phase by measuring their specific gravities. Thus it was found that 100 ml. of upper phase solvent could be prepared by mixing, in the order given, 1.49 gm. of KOH, 45.0 ml. of H_2O, 1.63 ml. of 85 per cent H_3PO_4, 52.1 ml. of *n*-propanol, and 2.15 ml. of cyclohexylamine. Similarly, lower phase solvent could be prepared by mixing 7.26 gm. of KOH, 72.0 ml. of H_2O, 6.0 ml. of 85 per cent H_3PO_4, 17.0 ml. of *n*-propanol, and 2.70 ml. of cyclohexylamine. If equal volumes of the two synthetic phases were mixed, no change in volume occurred.

The distribution constants of anthrone-positive and Folin-positive substances present in colicine K rich fractions obtained during the purification procedure, and in purified colicine K itself are given in Table II. It is apparent that there

were wide variations in the distribution constants of the carbohydrate and protein constituents in these preparations. This fact indicated that several components were present in the various fractions under investigation and that they could be separated in the solvent system by employing the counter-current technique.

Several preliminary 8 and 10 stage counter-current distribution experiments were performed using different preparations of purified colicine K. In each experiment it was observed that approximately 90 per cent or more of the biological activity was lost and that at each stage of the distribution a very small amount of precipitate accumulated at the interface of the two phases. This precipitate accounted for some 10 per cent of the weight of the starting material. The substance was insoluble in water but dissolved readily in dilute

TABLE II

Distribution Constants of Fractions Obtained during Purification of Colicine K in Propanol-Cyclohexylamine-Potassium Phosphate System

Fraction tested	Concentration	K_1	K_2	K_2/K_1
	mg./ml.			
Crude colicine K	1	0.69	9.00	13.1
Ethanol ppt. colicine K	1	0.39	4.55	10.0
$(NH_4)_2SO_4$ ppt. colicine K	1	0.95	4.35	4.7
Purified colicine K	1	1.09	5.00	4.8
	10	0.38	1.27	3.3
$(NH_4)_2SO_4$-soluble material	1	0.12	4.00	33.0
	10	0.13	0.52	4.0

K_1, distribution constant of anthrone-positive material.
K_2, distribution constant of Folin-positive material.

sodium carbonate and in 50 per cent acetic acid. When tested for its ability to inhibit the growth of *E. coli* B it was found that virtually all of the colicine K activity was present in this fraction.

Dissociation of Colicine K with Lower Phase Solvent.—The fact that some 90 per cent of the biological activity of colicine K was found in the material at the interface between the upper and lower solvent phases, suggested that it might be possible to precipitate the active component by adding one of the two phases to an aqueous solution of purified colicine K. It was decided to employ the lower phase solvent for this purpose because the component responsible for the biological activity of the colicine was less soluble in this solvent than was the carbohydrate with which it was associated (*cf.* Table II).

3.5 gm. of purified colicine K was dissolved in 85 ml. of water and the mixture was stirred for 2 hours at room temperature to assure complete solution. 300 ml. of artificially prepared lower phase solvent was slowly added followed by 7.5 ml. of *n*-propanol. The opalescent solu-

tion was stirred for an additional 2 hours and then stored overnight at 0°C. The precipitate (Fr. A) was collected by centrifugation and the opalescent supernatant fluid (Fr. B) was decanted. The precipitate (Fr. A) was washed with 30 ml. of fresh lower phase solvent followed by recentrifugation. The supernatant fluid (Fr. C) was decanted, and the precipitate (Fr. A) suspended in 30 ml. of water and dialyzed at 4°C. against 15 liters of distilled water. Frs. B and C were dialyzed separately. All of the fractions were dialyzed free of phosphate. Fr. A, insoluble in distilled water, was collected by centrifugation; it was resuspended in 20 ml. of water and lyophilized. Frs. B and C were concentrated to approximately 75 and 25 ml. respectively, filtered, and lyophilized. The combined weight of all fractions was 3.3 gm., a recovery of 94 per cent of the original material. Fr. A weighed 266.7 mg. and represented 7.6 per cent of the original weight. Fr. B weighed 2.39 gm. and Fr. C, 94.5 mg.

In Table III are recorded the biological activities of purified colicine K and of the fractions obtained after its dissociation with lower phase solvent. The results clearly indicate that Fr. A had an activity some 8 to 16 times

TABLE III

Activity of Purified Colicine K and of Fractions Obtained after Dissociation with Lower Phase Solvent

Fraction tested	Concentration, μg./ml.														
	50	25	12	6	3	1.5	0.75	0.37	0.18	0.09	0.04	0.02	0.01	0.005	0.002
Purified colicine K....	4	4	4	4	4	4	[4]	3	2½	1½	1	±	0	0	—
A.....	4	4	4	4	4	4	4	4	4	4	[4]	3	2½	2	1
B.....	2	1½	1	±	0	0	0	0	—	—	—	—	—	—	—
C.....	3	2	1½	1	±	0	0	0	0	0	—	—	—	—	—

4, complete inhibition of growth of *E. coli* B.

0, no inhibition of growth of *E. coli* B.

[4], end point.

greater than that of the original material and that the carbohydrate fractions were almost inactive. The analyses of the fractions obtained from several dissociation experiments are recorded in Table IV. It will be seen that Fr. A had a high nitrogen content and contained but little carbohydrate or phosphorous. Fr. B, on the other hand, had a low nitrogen content and contained large amounts of carbohydrate and phosphorous. Fr. A gave an intense Folin reaction, but Fr. B did not. It may be concluded from these results that the component responsible for colicine K activity is probably protein in nature and that the carbohydrate and phosphorous-containing components with which it is associated are inactive. Fr. A. is soluble in 7 M urea, in dilute sodium carbonate, and in 50 per cent acetic acid. In addition, it is partially soluble in 8 M lithium bromide solution at 50°C.

It will be noted from Table IV that Frs. A and B showed some variation in their nitrogen, carbohydrate, and lipide content. The reason for this is not

known but it is believed that these discrepancies arise from slight variations in experimental technique. Thus, the concentration of starting material or its time of exposure to lower phase solvent might possibly influence the degree to which its undergoes dissociation.

Both fractions A and B were serologically active for they gave precipitin reactions in colicine K antiserum. In addition, they elicited antibodies in rabbits. These properties will be discussed in a subsequent communication.

Electrophoretic Analysis of Protein Fr. A and of Lipocarbohydrate Fr. B.—Electrophoretic analyses were performed on fractions A and B in order to ascertain their relative purity and electrophoretic mobilities. The experiments were carried out in the Tiselius apparatus on 1 per cent solutions of the materials dissolved in 0.05 M borate buffer at pH 9.2.

TABLE IV

Summary of Analyses of Purified Colicine K Preparations and of Fractions Obtained after Dissociation with Lower Phase

Substance analyzed	N	P	Lipide	Carbohydrate*	Ratio Folin/anthrone	U/mg.
	per cent	*per cent*	*per cent*	*per cent*		
Purified colicine K	4.4–6.2	1.4–1.8	18–20	25–27	0.4–0.5	500–1000
Fr. A	13.3–14.7	0.19–0.65	4.6–8.0	2.0–4.0	18.5–36.0	5000–10,000
Fr. B	2.6–2.7	1.2–1.5	10–13	28–30	0.13–0.20	10

* Determined by anthrone reaction and calculated as glucose.

In Fig. 6 are shown the electrophoretic patterns of the two fractions. Here it can be seen that the protein (Fr. A) moves as a single asymmetric peak in both channels of the electrophoresis cell. The mobility of Fr. A was calculated to be -5.46×10^{-5} cm.^{2}/volt sec. The lipocarbohydrate (Fr. B) on the other hand, failed to give a sharp electrophoretic pattern. Disturbances in the solution in the descending channel of the apparatus made it difficult to obtain satisfactory analyses. It will be noted, however, from Fig. 6 that the lipocarbohydrate showed only a single asymmetric peak. Its mobility was calculated to be -3.75×10^{-5} cm.^{2}/volt sec.

Identification of the Monosaccharides Present in Purified Colicine K.—In order to characterize the monosaccharide components of the lipocarbohydrate moiety of colicine K, Fr. B was hydrolyzed with dilute mineral acid and the reducing sugars separated by paper chromatography. Hexosamine analyses, however, were performed directly on the hydrolysate itself.

200 mg. of Fr. B was hydrolyzed with 100 ml. of 1 N H_2SO_4 for 1 hour at 100°C. A small amount of precipitate was removed and the supernate reheated for an additional 5 hours. The acid was neutralized with barium hydroxide to pH 6.8 and the precipitated barium sulfate was filtered and washed. The combined filtrates were concentrated *in vacuo* to dryness,

and the residue dissolved in 1.6 ml. of water. A small amount of insoluble material was removed by centrifugation leaving a clear amber colored supernate. The reducing saccharides present in the hydrolysate were separated as follows.

A base line was drawn across a sheet of washed Whatman No. 1 filter paper (17 × 17 inches) 3 inches from one edge. The line was divided into 10 equal segments. At segments 5, 7, and 9 were deposited respectively 17, 8, and 4 μl. of hydrolysate. Samples containing 100 μg. of glucose, galactose, fucose, and rhamnose solutions were deposited on the remaining segments (Fig. 7). The chromatogram was developed for 16 hours at room temperature by the ascending method, using pyridine-butanol-water (4:6:3) mixture. The paper was dried, sprayed with aniline phthalate reagent (17), and finally heated at 100°C. for 5 minutes.

It can be seen from Fig. 7 that the hydrolysate gave spots having R_f values which coincided with those of fucose, rhamnose, galactose, and glucose. In

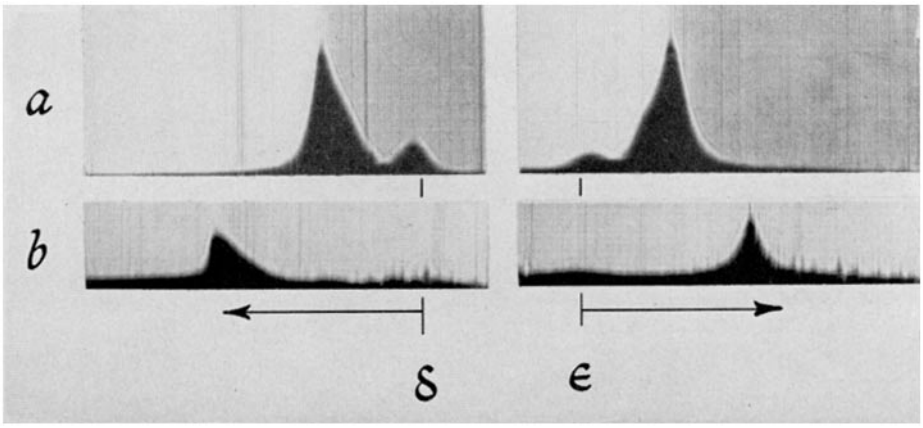

FIG. 6. Electrophoretic patterns of fractions obtained from colicine K after dissociation with lower phase. (*a*) Fr. A protein fraction (3600 seconds). (*b*) Fr. B lipocarbohydrate fraction (10,800 seconds).

order to confirm the identity of the monosaccharides present in the hydrolysate, a second chromatogram was prepared, and the sugars were eluted and characterized by the Dische cysteine reaction (18).

300 μl. of the hydrolysate of Fr. B was distributed on the base line of a sheet of washed Whatman No. 1 paper (17 × 17 inches) in 18 spots ¾ inch apart. The chromatogram was developed for 16 hours, and then air-dried. A vertical strip was cut from each edge and from the center of the paper. Each strip was sprayed with the aniline phthalate reagent and heated as before in order to determine the positions of the various sugars. The strips were refitted into the original chromatogram and pencil lines were drawn horizontally across the sheet to mark off the boundaries of each sugar (Fig. 8). The strips, numbered 1 to 6, were cut out from the unstained portion of the chromatogram and each was eluted with two 20 ml. portions of water. Strips 1 and 6 served as controls in the experiment. The combined extracts were filtered and concentrated *in vacuo* to 1 ml. The sugars in the eluates were now characterized by means of the Dische cysteine reaction.

The absorption spectra of the saccharides present in each eluate and in those of the four known sugars are shown in Fig. 9. Here it is seen that fractions 3, 4, and 5 have absorption bands similar to those of glucose, fucose, and rhamnose respectively. However, fraction 2 has adsorption maxima at 395 and at 605 mμ. The intense absorption at the latter wave length is typical of galactose. It is possible to calculate the absorbance contributed by this sugar present in fraction 2. If this value is subtracted from the total absorbance one obtains an absorption

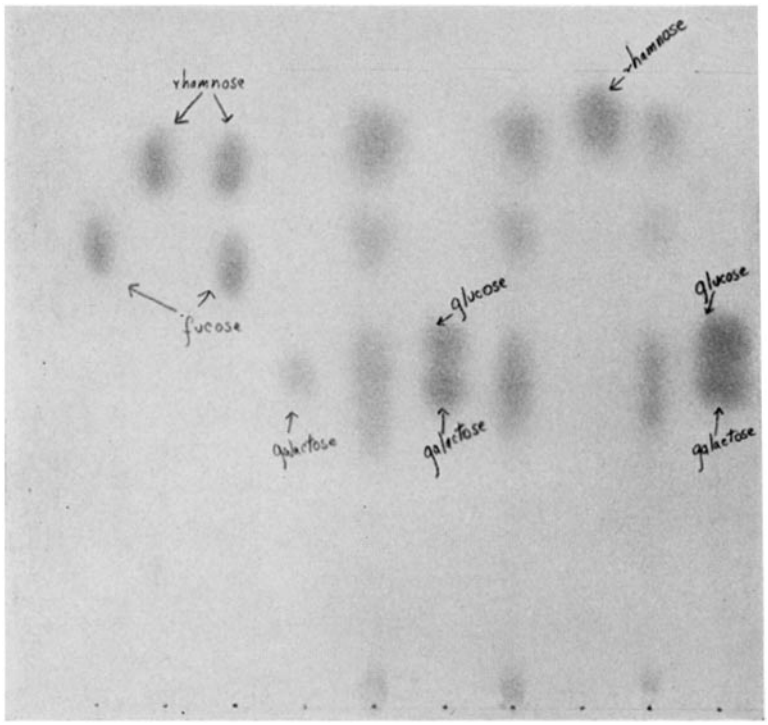

FIG. 7. Chromatogram of reducing sugars in hydrolysate of lipocarbohydrate obtained from colicine K.

curve typical of a methyl pentose (*cf.* rhamnose and fucose in Fig. 9). Subsequent experimental work has revealed that this residual band can be attributed to a disaccharide constituted of rhamnose and fucose. Finally, it should be stated that extracts of the control strips 1 and 6 did not show any color-producing substance when tested by the Dische cysteine reaction.

The quantities of the sugars present in the hydrolysate could not be determined precisely. If, however, the assumption is made that identical amounts of each sugar are lost on hydrolysis and subsequent manipulation, then it can be calculated from the absorption data that the reducing sugars which consti-

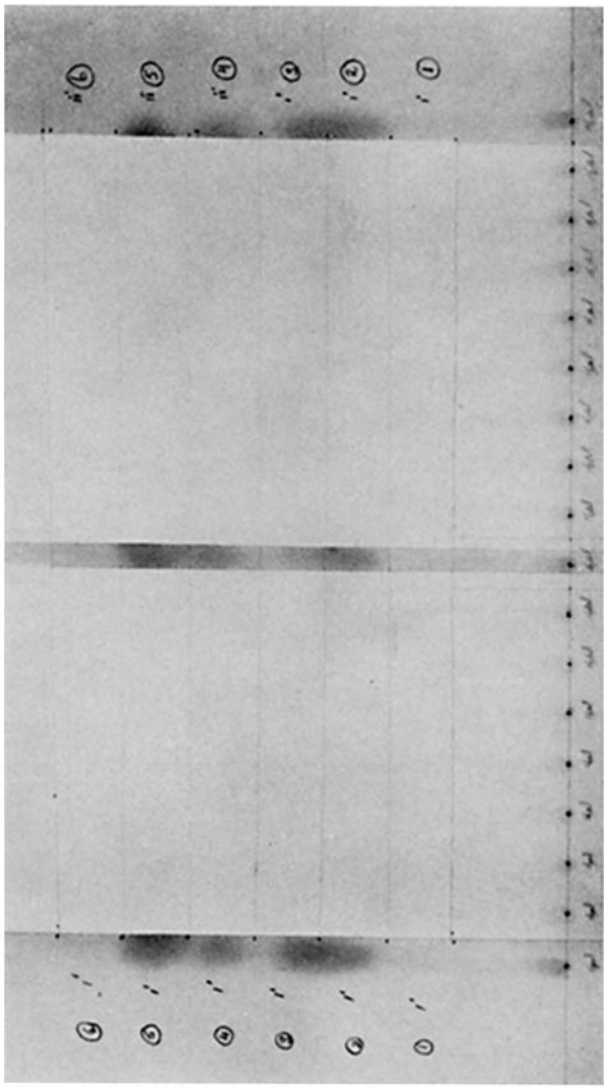

FIG. 8. Chromatogram for isolating reducing sugars from lipocarbohydrate of colicine K.

tute fraction B are present in the following molar proportions: galactose 9, glucose 6, fucose 10, and rhamnose 24. Thus, some 60 per cent of the reducing sugars which comprise fraction B are of the methylpentose type. In addition, analyses of the hydrolysate of Fr. B revealed that some 5 per cent of hexosamines were present. In conclusion, it should be emphasized that the presence

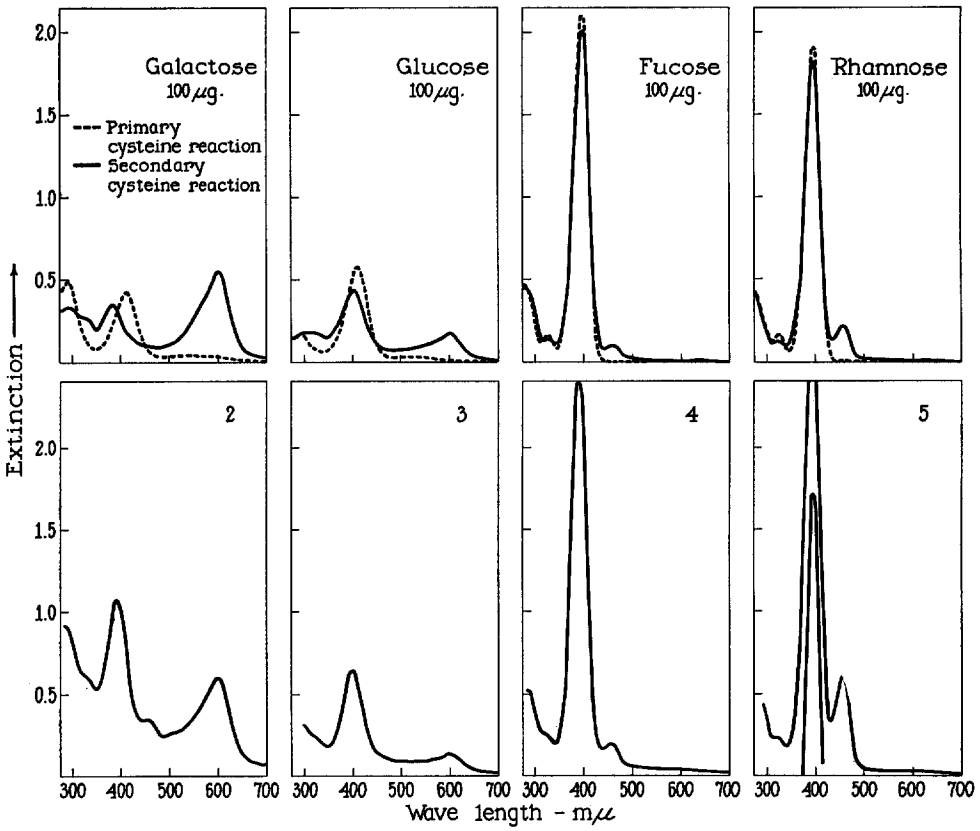

FIG. 9. Cysteine absorption spectra of reducing sugars present in lipocarbohydrate of colicine K.

of fucose in the carbohydrate fraction is of interest because so few *Escherichia coli* strains have been shown to contain this saccharide.

Analysis of the Amino Acid Composition of Fraction A.—In order to characterize more fully the chemical nature of the material responsible for colicine K activity, the amino acid composition of the protein Fr. A was investigated. It is conceivable that this fraction, having potent colicine activity, might be composed of only a few different amino acids instead of the many usually encountered in complex proteins.

5 mg. of Fr. A was hydrolyzed in a vacuum sealed tube with 1 ml. of 6 N HCl for 24 hours at 110°C. The acid was removed by evaporation *in vacuo*, and the residue dissolved in 0.5 ml. of water to give a concentration of 10 μg. per μl. 20 μl. was placed at one corner of a washed Whatman No. 1 filter paper (17 by 17 inches) at a point 3 inches from either edge. The material on the paper was then subjected to two dimensional chromatography (19). The first development was carried out by the descending technique using a basic solvent composed of 52.5 ml. of pyridine, 52.5 ml. of 3 methyl-butanol and 45.0 ml. of water. The second development was performed by the ascending technique, using a freshly prepared acidic solvent composed of 15 ml. of 88 per cent formic acid, 75 ml. of 2-butanol, and 10 ml. of water. The chromatogram was developed for 24 hours in each solvent system. After drying in air the paper was sprayed with a 0.2 per cent solution of ninhydrin dissolved in absolute alcohol, and heated at 50°C. for 30 minutes. Pink, blue, and yellow spots appeared on the paper.

The chromatogram was compared with one in which a mixture of nearly all known amino acids had been subjected to the same treatment. It was observed that some 19 different amino acids were present in Fr. A. Among them were cysteine, lysine, arginine, histidine, serine, glycine, threonine, alanine, proline, hydroxyproline, tyrosine, tryptophane, methionine, leucine, isoleucine, phenylalanine, valine, aspartic and glutamic acids. From the intensity of the color produced by the spots for valine and alanine it would appear that these amino acids are present in large amount. This experiment reveals that Fr. A is composed of a large number of amino acids, and that the biological activity of the fraction, if it is indeed a single substance, cannot be ascribed to a material composed of only a few amino acids.

Properties of Colicine K Protein Prepared by Dissociating Purified Colicine K with Lower Phase Solvent and with Phenol.—Two methods have been described for the dissociation of purified colicine K. It is of interest therefore to compare the biological and chemical properties of the fractions obtained by these two procedures.

Thus, two samples (630 mg.) of the same lot of purified colicine K were dissociated by means of liquid phenol and by lower phase solvent. The protein and lipocarbohydrate fractions so obtained were isolated, analyzed, and their biological activities studied.

The biological activities of the various fractions are recorded in Table V where it can be seen that the protein fractions obtained by the two procedures possessed an identical and potent colicine K activity. The lipocarbohydrate fractions, on the other hand, had very low activities.

Chemical analyses of the two protein fractions, performed on different lots of material, have invariably shown them to contain between 13 and 15 per cent of nitrogen and from 1 to 4 per cent of carbohydrate. Both methylpentose and hexosamine were present in the carbohydrate fractions and the ratio of total sugar to methylpentose or of methylpentose to hexosamine was in each case identical. Thus it would appear that the dissociation of the lipocarbohydrate-protein complex by the two procedures resulted in the same end products. The technique employing lower phase solvent appeared to have a decided advantage

over the use of phenol, because the pH of the mixture was at neutrality (pH 7.2) and the handling of the solvent was easier.

It cannot be stated with certainty at the present time whether dissociation with lower phase solvent is a procedure generally applicable to the lipocarbohydrate-protein complexes derived from other Gram-negative bacilli. Preliminary work employing the O antigen of Type Z *Sh. flexneri* has indicated that it too can be separated into two fractions, one having a higher and the other a lower nitrogen content than the starting material.

The experiments reported here have revealed that colicine K activity is closely associated with the O antigen of the organism *E. coli* K_{235} and can be

TABLE V

Comparison of Biological Activities of Fractions Obtained from Purified Colicine K after Dissociation with Lower Phase Solvent and with Phenol

Fraction tested	Concentration, μg./ml.										
	12.5	6.25	3.1	1.5	0.75	0.37	0.19	0.09	0.04	0.02	0.01
Purified colicine K	4	4	4	4	[4]	3	1	—	—	—	—
A (lower phase protein)	4	4	4	4	4	4	4	4	[4]	3½	2
B (lower phase lipocarbohydrate)	2	1½	1	0	0	0	0	—	—	—	—
C (phenol protein)	4	4	4	4	4	4	4	4	[4]	3½	2
D (phenol lipocarbohydrate)	3	2	1½	0	0	0	0	—	—	—	—

4, complete inhibition of growth of *E. coli* B.
0, no inhibition of growth of *E. coli* B.
[4], end point.

separated from it only by employing procedures which bring about degradation of the antigenic complex. It has been repeatedly shown that the lipocarbohydrate moiety of the O antigens of Gram-negative bacilli are toxic for mammals (15 *c*). In contrast to this it has now been found that the antibacterial activity of colicine K is associated with a protein-rich material and not a lipocarbohydrate. Thus, it may be concluded that if colicine K activity can indeed be attributed to the O antigen of *E. coli* K_{235}, its toxicity for mammals and for bacteria must originate from different moieties of the same macromolecule.

DISCUSSION

The nature of bacteriocines has been a challenging problem to microbiologists ever since their discovery some three decades ago (20). There is reason enough to presume that these substances are related to the bacteriophages,

largely from evidence based upon similarities in their biological activities. Striking as this biological relationship may be, there is almost no chemical evidence to support it. Recently it has been suggested that bacteriocines might be proteins which are related to the protein of the phage tail (1), yet here again there is no direct experimental proof to substantiate such an attractive hypothesis. The fact is that the true nature of the colicines remains enigmatic.

The present study has contributed in some measure to our understanding of the chemical nature of these agents. That colicine K must be a reasonably large molecule is evident for it does not dialyze through semipermeable membranes. Furthermore, it is a first rate antigen. Yet none of these properties have revealed whether colicine K can be regarded as a protein or some other type of macromolecular substance.

Colicine K is thermostable, a fact which at first sight might argue against its protein-like nature, yet it is readily destroyed by proteolytic enzymes and by formalin as well. In this respect it should be pointed out that other colicines have been shown to be susceptible to the action of proteolytic enzymes,—a fact which serves as the best available chemical evidence in support of the numerous contentions that they are proteins (21). It is fortunate that colicine K is elaborated into the culture medium during growth of the microorganism, for this fact facilitates enormously its chemical fractionation. The chief non-dialyzable products of biosynthesis of this colicine-producing strain are three in number,—colominic acid, an immunologically active carbohydrate, believed to be identical with the hapten portion of the third component, the O antigen. It is the latter which bears colicine K activity and which has resisted all attempts at further fractionation. Colicine K must be regarded therefore either as an integral part of this complex or as being bound so firmly to it that it cannot be separated by the fractionation procedures employed. In this respect it should be recalled that Cluff (22) considers the Type Z antigen of *Sh. flexneri* to be a mixture of a single lipocarbohydrate in combination with several different protein moieties. Although the lipocarbohydrate has but one immunological specificity, the protein moieties show differences. By zone electrophoresis he showed that the Type Z antigen, as he prepared it, could be separated into components which showed different serological specificities. This is not the case, however, with the O antigen of *E. coli* K_{235}, for when it is subjected to zone electrophoresis on a polyvinyl chloride resin, only one chemically defined component is to be found.

When purified colicine K is treated with reagents which bring about the dissociation of O antigens, there is obtained a non-dialyzable component high in nitrogen content and constituted from a large variety of amino acids. This material has potent colicine K activity, and if further study reveals that it is indeed protein, the first step in the elucidation of the nature of the colicine will have been achieved.

Finally, it should be stated that this study has not included data concerning

the immunological properties of colicine K. This will follow. Let it suffice to say that by chemical fractionation it has been possible to obtain from the culture medium of a colicine K producing organism a substance having potent antibacterial activity. This substance, protein-like in nature, is intimately associated with the O antigen of the colicine K producing microorganism.

SUMMARY

By chemical fractionation a substance having colicine K activity has been obtained from the culture medium of *E. coli* K_{235} L_4O.

Colicine K activity was found associated with the O antigen of this microorganism.

When the O antigen was dissociated, colicine K activity remained with the protein component of the antigen.

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